Focal Adhesion Kinase Overexpression Enhances Ras-dependent Integrin Signaling to ERK2/Mitogen-activated Protein Kinase through Interactions with and Activation of c-Src*

(Received for publication, January 17, 1997, and in revised form, March 11, 1997)

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Cell adhesion to extracellular matrix proteins such as fibronectin (FN) triggers a number of intracellular signaling events including the increased tyrosine phosphorylation of the cytoplasmic focal adhesion protein-tyrosine kinase (PTK) and also the stimulation of the mitogen-activated protein kinase ERK2. Focal adhesion kinase (FAK) associates with integrin receptors, and FN-stimulated phosphorylation of FAK at Tyr-397 and Tyr-925 promotes the binding of Src family PTKs and Grb2, respectively. To investigate the mechanisms by which FAK, c-Src, and Grb2 function in FN-stimulated signaling events to ERK2, we expressed wild type and mutant forms of FAK in human 293 epithelial cells by transient transfection. FAK overexpression enhanced FN-stimulated activation of ERK2 ~4-fold. This was blocked by co-expression of the dominant negative Asn-17 mutant Ras, indicating that FN stimulation of ERK2 was Ras-dependent. FN-stimulated c-Src PTK activity was enhanced by wild type FAK expression, whereas FN-stimulated activation of ERK2 was blocked by expression of the c-Src binding site Phe-397 mutant of FAK. Expression of the Grb2 binding site Phe-925 mutant of FAK enhanced activation of ERK2, whereas a kinase-inactive Arg-454 mutant FAK did not. Expression of wild type and Phe-925 FAK, but not Phe-397 FAK, enhanced p130Cas association with FAK, Sha tyrosine phosphorylation, and Grb2 binding to She after FN stimulation. FN-induced Grb2-She association is another pathway leading to activation of ERK2 via Ras. The inhibitory effects of Tyr-397 FAK expression show that FAK-mediated association and activation of c-Src is essential for maximal signaling to ERK2. Moreover, multiple signaling pathways are activated upon the formation of an FAK-c-Src complex, and several of these can lead to Ras-dependent ERK2 mitogen-activated protein kinase activation.

The family of transmembrane integrin receptors mediate cell adhesion to the extracellular matrix and also trigger intracellular signaling events such as the stimulation of the mitogen-activated protein kinase ERK2 (1–5). Recent evidence suggests that these integrin-initiated signals may act with other mito-

* This work was supported in part by Public Health Services Grants CA14195 and CA59780. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: PTK, protein-tyrosine kinase; RPTK, growth factor receptor PTK; FN, fibronectin; WT, wild type; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; CAS, p130CAS; MBP, myelin basic protein; IP, immunoprecipitate; mAb, monoclonal antibody; FAK, focal adhesion kinase; Pipes, 1,4-piperazinediethanesulfonic acid; HA, hemagglutinin; RIPA, radioimmune precipitation buffer; Pyk2, proline-rich tyrosine kinase 2; SH2, Src homology 2; SH3, Src homology 3.
Althought integrin-initiated signaling to ERK2 is dependent on the integrity of the cytoskeleton and may also involve the activation of the Rho family of small GTPases (5, 35), the important signaling proteins and pathways downstream of integrin receptors have not been clearly defined. Ras GTP-loading (3, 36) and both Raf-1 and ERK2/mitogen-activated protein kinases of the Ras cascade are activated by integrin stimulation (4). However, there are conflicting reports as to whether Ras is essential for ERK/mitogen-activated protein kinase activation. In two studies the dominant negative Asn-17 mutant of Ras was found to block FN-mediated ERK2 activation in NIH3T3 cells (3, 37), whereas it had a minimal effect on integrin signaling to ERK2 in NIH3T3 cells in another study (4). In addition, there may be more than one signaling pathway upstream of Ras, since antibody-mediated clustering of integrins in suspended cells can generate signals to ERK2 in the absence of FAK tyrosine phosphorylation (37), whereas presentation of fibroblasts to an insoluble FN matrix stimulates FAK tyrosine phosphorylation, transient c-Src association, and Grb2 binding in a time course that parallels ERK2 activation.2

In this study we tested the role of FAK in FN-stimulated signaling events to ERK2. In human 293 epithelial cells, FAK overexpression enhanced c-Src kinase activity and FN-stimulated signaling to ERK2, whereas dominant negative Ras expression blocked ERK2 activation without affecting FAK tyrosine phosphorylation or c-Src activity. Expression of Phe-397 inactivation (4) of Ras was found to block FN-mediated ERK2 activation in NIH3T3 cells (37), whereas presentation of fibroblasts to an insoluble FN matrix stimulates FAK tyrosine phosphorylation, transient c-Src association, and Grb2 binding in a time course that parallels ERK2 activation.2

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EXPERIMENTAL PROCEDURES

Antibodies—Monoclonal antibodies to c-Src (mAb 2–17) and to the hemagglutinin epitope tag (anti-HA, mAb 12CA5) were kindly provided by J. Meisenhelder (The Salk Institute) as mouse ascites fluid. Polyclonal antisera to p130Cas (anti-Cas2) and to Shc were generously provided by J. Meisenhelder (The Salk Institute) as mouse ascites fluid. Polyclonal antisera to p130Cas (anti-Cas2) and to Shc were generously provided by J. Meisenhelder (The Salk Institute) as mouse ascites fluid. Polyclonal antisera to p130Cas (anti-Cas2) and to Shc were generously provided by J. Meisenhelder (The Salk Institute) as mouse ascites fluid. Polyclonal antisera to p130Cas (anti-Cas2) and to Shc were generously provided by J. Meisenhelder (The Salk Institute) as mouse ascites fluid. Polyclonal antisera to p130Cas (anti-Cas2) and to Shc were generously provided by J. Meisenhelder (The Salk Institute) as mouse ascites fluid.

DNA Constructs and Cell Transfections—The mouse FAK cDNA containing a triple-HA epitope tag at the FAK C terminus was kindly provided by Steve Hanks (Vanderbilt University). The various FAK constructs used were prepared by site-directed mutagenesis and subcloned into the pCDNA3 eukaryotic expression vector as described (27). The Shc SH2 domain as a glutathione S-transferase fusion protein was obtained from the laboratory of Tony Pawson. HA-tagged p42 ERK2 in pLNC was a generous gift from M. Weber (University of Virginia). pLNC was a generous gift from M. Weber (University of Virginia). pLNC was a generous gift from M. Weber (University of Virginia). pLNC was a generous gift from M. Weber (University of Virginia).

RESULTS

FAK Overexpression Enhances but Dominant Negative Ras (Asn-17) Inhibits ERK2 Activation by FN Stimulation—To test the role of FAK in adhesion-mediated signal transduction events to ERK2, site-directed mutagenesis was used to create single-site phenylalanine replacements of FAK tyrosine residues (397, 407, 861, and 925) that have been shown to be phosphorylated in vitro (23, 24, 27). Mutagenesis was also used to produce a kinase-inactive FAK Arg-454 mutant (24, 26) and to create an N-terminally truncated form, Δ1–100 FAK (Fig. 1). HA-tagged wild type (WT) or mutant FAK constructs, HA-
tagged ERK2, and a dominant negative Asn-17 mutant of Ras were transiently expressed from cytomegalovirus promoter-driven eukaryotic expression vectors in human 293 epithelial cells that were either serum-starved (Fig. 2, lanes 1–6) or serum-starved and FN-stimulated (Fig. 2, lanes 7–12).

Since both the FAK and ERK2 constructs were HA-tagged, FAK expression and ERK2 gel shifts were monitored simultaneously by immunoblotting whole cell lysates with the 12CA5 mAb (Fig. 2A). In serum-starved cells, there was a low basal level of ERK2 activity that was stimulated ~5-fold by expression of the Δ1–100 FAK construct, which is highly tyrosine-phosphorylated and exhibits elevated association with c-Src (27). WT FAK overexpression enhanced ERK2 activity ~3-fold (Fig. 2B), and the extent of FAK-induced ERK2 activity was correlated with the extent of FAK Tyr(P) levels (data not shown). Co-expression of Asn-17 Ras had no effect on FAK Tyr(P) levels (data not shown), but its expression inhibited expression of Asn-17 Ras had no effect on FAK Tyr(P) levels (data not shown) leading to inhibition of FN and FAK-stimulated signaling to ERK2 (Fig. 2). Our studies are consistent with the reported Ras dependence of integrin signaling to ERK2 (3). FN-stimulated ERK2 activation (data not shown). By placing the Asn-17 Ras construct into a vector with a cytomegalovirus promoter (pCLXSN), transient Asn-17 Ras expression was significantly elevated compared with pZIPneoAsn-17 Ras (data not shown) leading to inhibition of FN and FAK-stimulated signaling to ERK2 (Fig. 2). Our studies are consistent with the reported Ras dependence of integrin signaling to ERK2 (3). May explain why studies using pZIPneoAsn-17 Ras did not detect a dominant negative Ras effect on integrin signaling to ERK2 (4).

Enhancement of c-Src Kinase Activity by FAK—Since previous studies have shown that integrin-activated FAK transiently associates with Src family PTKs (2, 27, 34), the effects of FAK overexpression on endogenous 293 cell c-Src kinase activity were evaluated (Fig. 3). Src kinase activity as measured by in vitro phosphorylation of acid-denatured enolase was low in lysates from serum-starved 293 cells and was increased 2-fold by the expression of Δ1–100 FAK (Fig. 3, lane 2), and this stimulation was not significantly affected by the expression of Asn-17 Ras (Fig. 3, lane 3). Overexpression of WT FAK in serum-starved 293 cells did not lead to measurable increases in c-Src kinase activity (data not shown). We speculate that the differences between WT and Δ1–100 FAK may be related to the fact that Δ1–100 FAK exhibits enhanced tyrosine phosphorylation and association with c-Src in serum-starved cells (27).
Previous studies have shown that c-Src activity is transiently increased after FN stimulation of rodent fibroblasts (61). We found that FN stimulation of control-transfected 293 cells enhanced c-Src kinase activity 3-fold above the serum-starved level (Fig. 3, lane 4), and this stimulation was not significantly affected by the expression of Asn-17 Ras (Fig. 3, lane 5). FN stimulation and overexpression of WT, Phe-407, Phe-925, and Δ1–100 FAK resulted in up to a 6-fold increase in total c-Src kinase activity compared with the serum-starved level, and these FAK-stimulated increases in c-Src kinase activity were independent of Ras (Fig. 3). Controls were performed with WT FAK IPs (made from Triton-only cell lysates) and vector control transfected cells that were either serum-starved (S) or serum-starved then FN-replated for 30 min (F). Endogenous c-Src kinase activity was measured by in vitro kinase (IVK) assays in c-Src IPs from RIPA lysates using acid-denatured enolase as a substrate. The 32P-labeled enolase (top panel) was quantitated by Cerenkov counting. Average values from three separate experiments are shown with error bars indicating the standard deviation.

Expression of Phe-397 or kinase-inactive Arg-454 FAK did not enhance endogenous c-Src kinase activity above the FN-stimulated levels nor did the expression of these constructs result in a reduction in c-Src kinase activity (Fig. 3, lanes 7 and 9). The failure to detect a significant reduction in c-Src kinase activity in cells expressing Phe-397 and Arg-454 FAK may be due to the fact that these assays were measuring total c-Src rather than FAK-associated c-Src activity, and that these mutants were expressed in less than 50% of the cells based on the efficiency of calcium phosphate-mediated transfection. In addition, c-Src activation after FN stimulation may be partially independent of FAK as a result of separate signaling events potentially involving protein kinase C and dephosphorylation of c-Src Tyr-527 (40). Nevertheless, all the kinase-active FAK constructs that contained an intact Tyr-397 binding site promoted the enhancement of c-Src kinase activity, and these results support the hypothesis that this FAK-c-Src complex is important for FN-stimulated signaling events.

Mutation of the c-Src but Not the Grb2 Binding Site on FAK Blocks FN-initiated Signaling to ERK2—FAK is phosphorylated at a number of different tyrosine sites both in vitro (23, 24, 27), and Tyr-397 and Tyr-925 create binding sites for the SH2 domains of Src family PTKs and Grb2, respectively. However, the role of other phosphorylation sites such as Tyr-407 and Tyr-861 in signaling events is not known. To investigate the roles of tyrosine phosphorylation at specific sites, the various FAK site-directed mutants were transiently expressed, and their effects on ERK2 activation were evaluated in FN-stimulated 293 cells (Fig. 4). Compared with the endogenous integrin signaling response as determined with vector control transfections (Fig. 4, lanes 1 and 2), expression of WT and Δ1–100 FAK enhanced the activity of co-transfected ERK2—4-fold after FN stimulation (Fig. 4, lanes 3 and 10). FAK overexpression coupled with FN stimulation elevated ERK2 activity ~10-fold above the basal level as determined in the presence of Asn-17 Ras (Fig. 4, lane 3), and these results are consistent with those presented in Fig. 2. Expression of FAK Phe-407, Phe-861, or Phe-925 in combination with FN stimulation all resulted in ~6-fold enhancement of ERK2 activity compared with the basal level of transfected ERK2 activity (Fig. 4).

The activity of transfected ERK2 was not increased by FN stimulation in cells expressing the c-Src binding site mutant of FAK (Phe-397). Rather, FAK Phe-397 expression reduced ERK2 activation to a level below the FN-stimulated control and...
equivalent to that found with Asn-17 Ras (Fig. 4). This result indicates that FAK is essential for integrin signaling to ERK2 and is consistent with results obtained with Pyk2 where expression of the c-Src binding site Pyk2 mutant blocked lysophosphatidic acid- and bradykinin-stimulated signaling to ERK2 (29). We speculate that Phe-397 FAK may act in a dominant negative fashion by displacing endogenous FAK at sites of integrin clustering. This model is consistent with the fact that overexpression of the FAK C-terminal domain, which localizes to focal contacts but lacks kinase activity or a c-Src binding site, results in reduced endogenous FAK Tyr(P) levels and delays in FN-stimulated cell spreading events (41, 42). Phe-397 FAK localization with integrins would prevent the formation of a productive signaling complex with adaptor proteins such as Grb2 (27) due to its inability to associate with and activate Src family PTKs.

Expression of Arg-454 kinase-inactive FAK did not enhance but also did not result in the inhibition of transfected ERK2 after FN stimulation (Fig. 4, lane 7). This result was unexpected and differs from results obtained with Pyk2, where expression of a kinase-inactive Pyk2 disrupts calcium-mediated (18) or bradykinin-stimulated (29) ERK2 activation. The failure of Arg-454 FAK to block integrin signaling to ERK2 may be because it becomes weakly tyrosine-phosphorylated and associated with c-Src after FN stimulation of 293 cells (27). Therefore, any inhibitory effect of Arg-454 FAK expression may be counterbalanced by slight increases in c-Src activity through binding interactions at FAK Tyr-397. The combined results with Phe-397 and Arg-454 FAK suggest that at a minimum FAK kinase activity is needed to phosphorylate Tyr-397 to promote signal transduction events.

Surprisingly, expression of the Grb2 binding site mutant of FAK (Phe-925) resulted in enhanced activation of ERK2 (Fig. 4, lane 9). This result suggests that direct Grb2 binding to FAK may not be essential for ERK2 signaling or that FAK phosphorylation at sites other than Tyr-925 can compensate for the loss of Grb2 binding. In addition, since Phe-925 FAK still activated c-Src kinase activity after FN stimulation (Fig. 3, lane 10), it is possible that FAK promotes signaling events through other Src family PTKs or that FAK itself phosphorylates other target proteins leading to ERK2 activation. Results from Src-deficient cell studies support this latter idea, since expression of a potentially dominant negative fragment of c-Src (1–298) in Src fibroblasts constitutively associated with FAK and prevented integrin-stimulated Grb2 binding to FAK but did not block signaling to ERK2 (34). Instead, Src (1–298) expression promoted FAK tyrosine phosphorylation of p130Cas and low level signaling to ERK2. p130Cas may weakly signal to ERK2 through the binding of either the Crk (43) or Nck (34) adaptor proteins.

FAK Association with c-Src Promotes Multi-protein Signaling Complex Formation Involving p130Cas, Grb2, and Shc—To investigate whether FAK coordinates signaling events through multiple pathways either WT, Phe-397, or Phe-925 FAK constructs were expressed in human 293 cells, and combination IP/immunoblot analyses were performed after FN stimulation (Fig. 5, A–D, lanes 1–4). Analysis of HA-tag (12CA5 mAb) IPs revealed that the FAK constructs were highly expressed (Fig. 5C) and that WT and Phe-925 FAK exhibited higher Tyr(P) levels than Phe-397 FAK (Fig. 5B). As expected, endogenous human c-Src was associated with WT and Phe-925 FAK but not with Phe-397 FAK (Fig. 5D). Interestingly, increased association of p130Cas (CAS) was detected in the WT and Phe-925 FAK IPs compared with Phe-397 FAK (Fig. 5A). Previous studies have shown that the SH3 domain of CAS can bind directly to a proline-rich region in the FAK C-terminal domain (44, 45). Our own work has shown that CAS association with FAK may also be indirect and may be mediated through c-Src binding to both CAS and FAK (34). It is possible that the CAS associated with Phe-397 FAK represents a direct binding interaction, whereas the increased level of CAS associated with WT and Phe-925 FAK could be mediated by associated c-Src.

Evidence for the increased stability of CAS-c-Src-FAK complexes was obtained by blotting analyses of c-Src IPs (Fig. 5, A–D, lanes 5–8). Expression levels of endogenous human c-Src protein were quite high in lysates of 293 cells (Fig. 5D), and CAS was associated with c-Src after FN stimulation (Fig. 5A, lane 5) but not after poly-L-lysine control replating (data not shown). Previous studies have shown that both c-Src SH2- and SH3-mediated binding interactions can facilitate associations with CAS (46). Both WT and Phe-925 FAK were found to be associated with endogenous c-Src (Fig. 5C), and under these conditions enhanced levels of CAS were also detected in the c-Src IPs (Fig. 5A, lanes 6 and 8). Phe-397 FAK did not significantly associate with c-Src (Fig. 5C, lane 7), and its expression did not promote increased CAS association with c-Src (Fig. 5A, lane 7). From these results we conclude that CAS association with FAK or c-Src may be mediated by direct and indirect interactions. A complex of c-Src stably bound via its SH2 domain with phosphorylated FAK Tyr-397 may act as a template for CAS SH3-mediated interactions with FAK or c-Src SH3-mediated interactions with CAS.

Increased CAS association with Phe-925 but not Phe-397 FAK may promote downstream signaling events, since the SH2-mediated binding of both Crk (43) and Nck (34) adaptor proteins to CAS has been shown to occur after FN stimulation.
Because CAS was tyrosine-phosphorylated (data not shown) and associated with c-Src and FAK in cells expressing Phe-397 FAK (Fig. 5A, lanes 3 and 7), CAS-mediated signaling events may not efficiently stimulate ERK2 as was also previously demonstrated by studies with Src-deficient fibroblasts (34). To determine whether Phe-925 FAK expression enhances FN-stimulated signaling to ERK2 through pathways other than CAS, the proteins associated with endogenous Grb2 were characterized in FN-stimulated 293 cells expressing WT, Phe-397, and Phe-925 FAK (Fig. 5, E–H, lanes 1–4). As expected, Grb2 was associated with WT but not with Phe-397 or Phe-925 FAK (Fig. 5E). Interestingly, in cells expressing WT or Phe-925 FAK, an enhanced association of a ~52-kDa Tyr(P)-containing protein with Grb2 was detected (Fig. 5F). This Grb2-associated 52-kDa protein cross-reacted with antibodies to Shc (Fig. 5G).

WT and Phe-925 FAK but not Phe-397 FAK expression enhanced Shc tyrosine phosphorylation (Fig. 5F) and Grb2 association with Shc after FN stimulation (Fig. 5H) but not poly-l-lysine stimulation (data not shown). These results are consistent with a recent report showing that integrin antibody cross-linking promotes Shc tyrosine phosphorylation and Grb2 binding (37). Shc tyrosine phosphorylation events could have been mediated through FAK stimulation of c-Src kinase activity (Fig. 3), since Shc is known to be a good substrate for activated Src (47). Another candidate is FAK itself, since, unexpectedly, both WT and Phe-925 FAK were present in Shc IPs after FN stimulation (Fig. 5E). FAK does not contain a consensus Shc phosphotyrosine-binding motif, but a glutathione S-transferase-Shc SH2 domain fusion protein bound to tyrosine phosphorylated WT and Phe-925 but not Phe-397 FAK in vitro (data not shown). Interestingly, the residues surrounding FAK Tyr-397 (Tyr(P)-Ala-Glu-Ile) match the consensus recognition sequence of the Shc SH2 domain (Tyr(P)-Ile-X-Ile) (48).

**DISCUSSION**

Our results provide evidence that FN-stimulated signaling to ERK2 involves multiple pathways and a series of sequential phosphorylation events. Contrary to previous studies (37), we provide evidence that FAK is involved in FN-initiated signaling events to ERK2. Specifically, we found that FAK overexpression in 293 human epithelial cells enhanced FN-stimulated c-Src activation and signaling to ERK2. Co-expression of dominant negative Ras did not affect FAK or c-Src activation events, but its expression inhibited FN-stimulated activation of ERK2, which supports previous conclusions that integrin-mediated ERK2 activation is Ras-dependent (3, 37). Expression of the c-Src binding site mutant of FAK (Phe-397) did not enhance FN-stimulated c-Src kinase activity and resulted in the inhibition of downstream signal transduction events to ERK2. This result suggests that FAK association and activation of c-Src is essential for maximal signaling to ERK2. With the recent elucidation of the c-Src crystal structure, we speculate that FAK overexpression and exposure of the Tyr(P)-containing Tyr-397 FAK motif may compete with c-Src Tyr-527 for binding to the Src SH2 domain, thereby disrupting the overall inactive c-Src structure and promoting kinase activation (49).

What are the pathways through which an FAK/c-Src complex could promote signals to ERK2? We found that FAK overexpression and association with c-Src also facilitated and stabilized direct and indirect associations with CAS. Since our previous results showed that FAK phosphorylation of CAS may only provide a weak signal to ERK2 (34) and since CAS was associated with Phe-397 FAK, we speculate that the predominant signaling routes to ERK2 after integrin stimulation involve Grb2 binding to either tyrosine-phosphorylated FAK or Shc. Interestingly, expression of the Grb2 binding site mutant of FAK (Phe-925) resulted in enhanced FN-stimulated ERK2 activation, and this is consistent with previous results showing that there are additional FN-stimulated pathways to ERK2 that do not involve direct Grb2 binding to FAK (34). The fact that expression of Phe-925 but not Phe-397 FAK enhanced Shc tyrosine phosphorylation, Grb2 binding to Shc, and ERK2 activation after FN stimulation suggests that Shc is an important target for both FAK and c-Src-mediated signaling events, although we have not yet tested this directly.

Although signaling events potentiated as a result of FAK overexpression may not accurately reflect stimulated events in primary cells, FAK overexpression has been correlated with the metastatic phenotype of many human tumors (50), and our studies may provide insights into the molecular mechanisms promoting tumor cell growth and migration. Our results are also in accord with recent reports showing that Grb2 binding to Shc is necessary and sufficient for the activation of the ERK2 pathway in response to integrin stimulation (37), and that signals from the ERK2 pathway may feed back to regulate integrin activation (51).

Because signals from integrin receptors have been shown to synergize with signals from growth factor receptor PTKs (RPTKs) in promoting biological processes such as anchorage-dependent cell growth (6, 52) and the control of cell cycle progression (37, 53, 54), it is possible that Shc may be a common target for both integrin and RPTK signaling events. Although recent studies have shown that integrin aggregation can promote growth factor-independent RPTK clustering and enhance growth factor-stimulated ERK2 activation in suspended cells (8), Shc binding to RPTKs through phosphotyrosine binding domain interactions (55) and to integrin-stimulated FAK-c-Src complexes through SH2 domain binding interactions may provide another point of linkage between RPTK and integrin signaling pathways.

In addition to signals that promote cell growth, a recent report has shown that overexpression of WT but not Phe-397 FAK in Chinese hamster ovary cells enhances FN-stimulated cell migration events (56). Our studies have shown that WT but not Phe-397 FAK can activate ERK2. Interestingly, a recent report documents that myosin light chain kinase is a substrate for activated ERK2, and that integrin or growth factor-stimulated signals that promote ERK2 activation lead to enhanced cell migration events (58). Future studies will be directed toward elucidating more precise roles of FAK, Src family PTKs, and ERK2 activation as they relate to cell proliferation and migration.

**Acknowledgments**—We thank Jill Meisenhelder and Helen Mondala for lab support, Steve Hanks for generosity in providing the HA-tagged FAK cDNA, and Hisamara Hirai and Peter van der Geer for p130Cas for lab support. We thank Jill Meisenhelder and Helen Mondala for lab support, Steve Hanks for generosity in providing the HA-tagged FAK cDNA, and Hisamara Hirai and Peter van der Geer for p130Cas and Shc antisera, respectively. We also thank Richard Klemke and David Cheresh for sharing results prior to publication.

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