Stimulation of a number of cell surface receptors, including integrins and G protein-coupled receptors, results in the activation of a non-receptor tyrosine kinase known as focal adhesion kinase (FAK). In turn, this kinase is believed to play a critical role in signaling to intracellular kinase cascades controlling gene expression such as extracellular signal-regulated kinases (ERKs), by a yet poorly defined mechanism. Furthermore, whether this tyrosine kinase also mediates the activation of other mitogen-activated protein kinase family members, such as c-Jun NH2-terminal kinases (JNKs), is still unclear. We show here that the activation of FAK by anchoring to the cell membrane is itself sufficient to stimulate potently both ERK and JNK. These effects were found to be phosphatidylinositol 3-kinase-independent, as FAK effectively stimulated Akt, and wortmannin suppressed Akt but not ERK or JNK activation. As previously reported by others, activation of ERK correlated with the ability of FAK to induce tyrosine phosphorylation of Shc. Surprisingly, however, stimulation of JNK was not dependent on the kinase activity of FAK or on the ability to induce tyrosine phosphorylation of FAK substrates. Instead, we provide evidence that FAK may stimulate JNK through a novel pathway involving the recruitment of paxillin to the plasma membrane and the subsequent activation of a biochemical route dependent on small GTP-binding proteins of the Rho family.

Stimulation of a variety of cell surface receptors causes the rapid elevation of the enzymatic activity of a family of closely related proline-directed serine-threonine protein kinases, known as mitogen-activated protein kinases (MAPKs)1 (1). The function of MAPKs is to convert extracellular stimuli to intracellular signals, which, in turn, control the expression of genes that are essential for many cellular processes, including cell growth and differentiation (2). At present, 10 mammalian MAPKs have been identified, which can be broadly divided into three families: extracellular signal-regulated kinases (ERKs), c-Jun NH2-terminal kinases (JNKs), also termed stress-activated protein kinases (SAPK), and p38 (also known as CSBP, RK, and SAPK2a) (3). ERKs phosphorylate and regulate the activity of certain enzymes, including phospholipase A2, and nuclear proteins, such as the ternary complex factor p62Tcf or Elk-1 (4). The latter represents a critical event in controlling the expression of several genes, including c-fos (5). In the case of JNKs, they have been shown to phosphorylate the transactivating domain of c-Jun and ATF2 (6), thereby increasing their transcriptional activity.

A prototypical model of the pathway linking cell surface receptors to MAPKs of the ERK family has been proposed from studies on the tyrosine kinase class of growth factor receptors (7). For example, binding of epidermal growth factor to its cognate tyrosine-kinase receptor leads to the rapid phosphorylation of the receptor itself on tyrosine residues, which provides binding sites for the adaptor molecules Shc and Grb2, thus resulting in the recruitment to the plasma membrane of the guanine-nucleotide exchange factor Sos and the activation of Ras (reviewed in Ref. 7). GTP-bound forms of Ras then recruit and activate the serine-threonine kinase Raf, leading to the activation of a kinase cascade composed of Raf, MEKs, and ERKs (1). Tyrosine kinases have been also shown to play an important role in signaling from G protein-coupled receptors (GPCRs) and integrins to ERKs. For GPCRs, they include tyrosine kinases of the Src family (8, 9), Syk and Btk (10, 11), and the recently identified Pyk2 (12), as well as the engagement of tyrosine kinases of the receptor class (13). Similarly, focal adhesion kinase (FAK)-Src complexes also play an important role in mediating the signal from integrins to ERKs through the recruitment of Grb2 and Shc (14), in addition to the proposed association of certain integrins with Shc (15).

In contrast, the mechanism of activation of JNKs is still poorly understood. JNKs were shown to be activated by a variety of stimuli distinct from those that elevate the enzymatic activity of ERKs, including protein synthesis inhibitors, heat shock, changes in osmolarity, and ultraviolet irradiation (6, 16). JNKs can be also activated by agents acting on cell surface receptors, such as tumor necrosis factor-α, IL-1, or epidermal growth factor (16). Furthermore, we have recently shown that JNK activity can be elevated upon stimulation of GPCRs (17, 18) and integrin aggregation (19). In these cases, the involvement of tyrosine kinases in the signaling pathway from GPCR and integrins to JNK is less clear. One group has provided evidence that Pyk2 (also known as CAKB, RAFTK, and CADTK) (12, 20), a new member of the FAK family of tyrosine kinases with restricted tissue distribution, may play a role in signaling from MAPKs to JNK. Conversely, it has...
been established that FAK is activated by cell binding to extracellular matrix components such as fibronectin (21) and by several agonists acting on GPCRs (22–25).

Based on these observations, it can be hypothesized that FAK may participate in a biochemical route linking cell surface receptors to MAPK cascades. Here, we explored the ability of FAK to stimulate signaling events leading to the activation of ERK and JNK, using the expression of an activated form of FAK as an experimental approach. Interestingly, expression of an activated FAK upon targeting this molecule to the cell membrane was sufficient to stimulate potently ERK and JNK activity. The activation of ERK correlated with the ability of FAK to induce tyrosine phosphorylation of Shc. In contrast, however, stimulation of JNK was not dependent on the kinase activity of FAK, or on the ability to induce tyrosine phosphorylation of FAK substrates or to activate PI3K. Instead, we show that recruitment of paxillin to the plasma membrane is sufficient to stimulate a biochemical route depending on small GTP-binding proteins of the Rho family, thereby providing a likely mechanism by which FAK can stimulate JNK.

MATERIALS AND METHODS

Reagents—Lyso-phosphatidic acid and anisomycin were obtained from Sigma. Human plasma fibronectin and poly-D-lysine were purchased from Roche Molecular Biochemicals.

Cell Lines and Transfection—Human kidney 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Tissue culture plates were treated with phosphate buffered saline (PBS) containing 20 μg/ml poly-D-lysine for 15 min before seeding the cells, to prevent them from detaching from the plates in serum-free conditions. For the stimulation by fibronectin, cells were seeded on the plates coated with human plasma fibronectin (10 μg/ml in PBS). Cells were serum starved for 2 h in Dulbecco’s modified Eagle’s medium containing 10% HEPES before lysis for kinase assays, or analyses of tyrosine phosphorylation. Cells were transfected by the calcium-phosphate precipitation method adjusting total amount of DNA to 5–10 μg/plate with the appropriate vector control.

DNA Constructs—Plasmids expressing epitope-tagged ERK, JNK, and Shc (pcDNA3 HA-ERK2, pcDNA3 HA-JNK1, and pcDNA3 HA-Shc, respectively) and the dominant negative mutants of the small GTP-binding proteins Rac1, and Cdc42 have been described (26–28). For expression of wild type and membrane-targeted forms of FAK, the cDNA was subcloned into pcDNA3 (Invitrogen) and pCEFL myr (a HA-tagged FAK construct, Molecular Dynamics) upstream of poly-CAS.I sites (pCMV IL2R-paxillin). The exons were amplified from chicken c-Src; (27), respectively, which includes the c-Src amino acids of chicken c-Src; (27), respectively, which includes the c-Src binding proteins RhoA, Rac1, and Cdc42 have been described (26–28).

Flow Cytometry—Transfected 293T cells were harvested with 5 mM EDTA, washed in PBS and PBS containing 3% BSA, respectively, prior to incubation with 100 μl of fluorescein isothiocyanate-conjugated anti-CD25 (Serotec), at 10°C for 30 min. After washing in PBS containing 1% BSA and PBS, the cells were fixed in ice-cold 70% ethanol at 50 °C for 30 min. Prior to re-probing with different primary antibody, stripped membranes were washed extensively in TN buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) and placed in blocking buffer (TN containing 4% bovine serum albumin). Antibodies against FAK (Coverslips were prepared from Santa Cruz Biotechnology) and against paxillin and p130Cas antibodies were obtained from Transduction Laboratories. Monoclonal anti-phosphotyrosine (4G10) was purchased from Upstate Biotechnology.

RESULTS

Engagement of integrins and stimulation of GPCRs lead to the activation of FAK and of many intracellular signaling path-
ways, including those controlling the activity of members of the MAPK superfamily of serine-threonine kinases (19, 31). Furthermore, recent reports suggest that FAK can participate in biochemical routes resulting in increased ERK activity (32). As an experimental approach to investigate whether FAK can alone enhance the activity of MAPKs, we overexpressed in 293T cells a wild type and a membrane-targeted form FAK (myr-FAK). The latter was constructed by fusing the amino-terminal myristoylation signal from c-Src with FAK (27), a modification that has been shown to render FAK constitutively active (33). As seen in Fig. 1A, similar levels of the two proteins were observed in 293T cells after transfection, as judged by Western blot analysis with an antibody to FAK. Endogenous FAK was detected after prolonged exposure (data not shown) or after immunoprecipitation with a FAK-specific antibody (Fig. 1A, right panel). However, the mobility of myr-FAK in SDS-polyacrylamide gel electrophoresis was slower, most likely due to the addition of the amino-terminal myristoylation signal from c-Src, as demonstrated by immunoblotting with an anti-Src antibody. As expected, subcellular fractionation revealed that myr-FAK accumulates in the membrane-containing cellular compartment (Fig. 1B), in contrast to FAK, which is exclusively cytosolic. Interestingly, the myristoylated form of FAK was found to be heavily tyrosine-phosphorylated (Fig. 1A, right panel), further supporting that the membrane-targeted form of FAK is constitutively active (33).

We next asked whether the membrane-anchored form of FAK could activate biochemical routes enhancing the activity of an epitope-tagged form of ERK2 (HA-ERK) and JNK1 (HA-JNK) when transiently expressed in 293T cells. As shown in Fig. 2, overexpression of FAK had only a limited effect on ERK and JNK activity, respectively, but in contrast, expression of myr-FAK potently stimulated the phosphorylating activity of the HA-ERK and HA-JNK. These results indicate that the recruitment of FAK to the plasma membrane is sufficient to evoke downstream signaling event(s), including those leading to ERK and JNK activation. These findings prompted us to explore further the nature of the signaling pathway connecting FAK to ERK and JNK.

Recent data have shown a role for PI3K in signaling ERK and JNK activation in a number of cellular systems (28, 34). Furthermore, GPCR activation and cell attachment to extracellular matrix enhances PI3K activity, resulting in Akt activation (30, 35, 36). Thus, as PI3K is reported to bind FAK (37), it is reasonable to postulate that activation of FAK by integrins and GPCRs may up-regulate PI3K activity, which in turn might mediate the activation of ERK and JNK. To address this possibility, we asked whether the constitutively active form of FAK could stimulate Akt activity, whose enzymatic activity is enhanced in response to PI3K activation (38). As demonstrated in Fig. 3, expression of myr-FAK potently stimulated the histone kinase activity of a co-expressed epitope-tagged Akt (HA-Akt), and this activation was abolished by pretreatment of cells with increasing concentrations (25–100 nM) of a PI3K blocker, wortmannin (39). In contrast, however, treatment with wortmannin demonstrated no effect on the ability of myr-FAK to stimulate ERK and JNK (Fig. 3). Taken together, these data suggest that FAK can directly signal Akt activation, likely through PI3K. However, PI3Ks appear not to be involved in the biochemical route(s) linking FAK to ERK and JNK in this cellular system.

The tyrosine phosphorylation of Shc is proposed to be a downstream event of FAK, leading to ERK activation (32). In addition, two cellular proteins, paxillin and p130Cas, are direct substrates of FAK, although the functional consequences of paxillin and p130Cas tyrosine phosphorylation are still not fully understood (40, 41). Thus, we next explored whether these molecules participate in JNK and ERK activation by FAK. We initially engineered epitope-tagged forms of Shc, paxillin, and p130Cas (HA-Shc, HA-paxillin, and HA-p130Cas, respectively) and confirmed their expression in total lysates and anti-HA immunoprecipitates from transiently transfected 293T cells (Fig. 4A and data not shown). We next examined the status of tyrosine phosphorylation of these molecules when co-transfected with a plasmid control, or together with wild type or myr-FAK. As
seen in Fig. 4B, overexpression of wild type FAK was sufficient to cause the accumulation of tyrosine-phosphorylated species of paxillin and p130cas, which became more prominent when each molecule was co-expressed with myr-FAK. By contrast, Shc was only poorly tyrosine-phosphorylated when co-expressed with wild type FAK, but myr-FAK induced a dramatic increase in the level of Shc phosphorylation detectable by anti-phospho-tyrosine antibodies (Fig. 4B). Thus, the ability to induce the tyrosine phosphorylation of Shc, but not of paxillin and p130cas, appears to correlate with ERK and JNK activation.

To analyze further whether the tyrosine kinase activity of FAK contributes to its ability to induce the activation of these MAPKs, we generated a kinase-deficient mutant of myr-FAK, by replacing a critical lysine residue in position 454 for arginine (designated myr-FAK KR). In addition, considering the possibility that the binding of Grb2 to FAK may participate in FAK-induced activation of MAPKs, we generated an additional mutant of myr-FAK, replacing tyrosine 925, the Grb2 binding site (42), by phenylalanine (designated myr-FAK YF). The former is expected to encode a kinase-inactive form of FAK, and the latter would be expected to have lost the ability to bind Grb2 (42).

When transfected into 293T cells, all myr-FAK mutants were expressed at similar levels, as judged by Western blot analysis with anti-Src antibody (Fig. 5A, where wild type myr-FAK is designated as myr-FAK WT for clarity). However, as expected, the level of tyrosine phosphorylation of myr-FAK KR was significantly reduced when compared with that of wild type myr-FAK and myr-FAK YF (Fig. 5A). Under these experimental conditions, myr-FAK YF retained the ability to phosphorylate paxillin, p130cas, and Shc to an extent similar to that of wild type FAK. However, myr-FAK KR failed to phosphorylate HA-paxillin and Shc, although it caused a weak increase in the tyrosine phosphorylation of HA-p130cas by a still unclear mechanism. We next explored the effects of these mutated forms of myr-FAK on ERK and JNK activity (Fig. 5C). Whereas myr-FAK YF stimulated ERK activity to a degree comparable to that observed for wild type myr-FAK, myr-FAK KR failed to activate ERK. These data provide further support to the proposed function of the tyrosine kinase activity of FAK in signaling ERK activation (32). In contrast, to our surprise, all myr-FAK proteins potently stimulated JNK to a very similar extent.

These results suggested that the recruitment of FAK to the plasma membrane, rather than its catalytic activity, might be the critical factor determining its ability to stimulate JNK activation.

Paxillin and p130cas have been reported to form a stable complex with FAK (43–45), thus suggesting that they may be recruited to plasma membrane upon expression of myr-FAK. To test this possibility, we investigated whether HA-paxillin and HA-p130cas would co-immunoprecipitate with wild type or the mutated myr-FAKs. As shown in Fig. 6, all myr-FAK constructs were detected in anti-HA immunoprecipitates from co-transfected 293T cells, but not in control transfected cells (data not shown). These data demonstrate that FAK can form stable molecular complexes with paxillin and p130cas, and that complex formation does not require the tyrosine kinase activity of FAK or the tyrosine phosphorylation of these FAK substrates (see above).

This observation raised the possibility that the recruitment of paxillin or p130cas to the plasma membrane may be by itself sufficient to activate JNK. To test this possibility, we examined the biochemical consequences of targeting paxillin and p130cas to the membrane. For p130cas, we engineered a myristoylated form (myr-p130cas). Because the myristoylated form of paxillin could not be expressed successfully, the coding sequence of paxillin was fused in frame to a membrane anchor consisting of the extracellular and transmembrane domain of the interleukin 2-receptor α subunit (IL2R-paxillin). Both IL2R-paxillin and myr-p130cas were efficiently expressed, as judged by Western blot analysis with the appropriate antibodies, or by fluo-
rescence-activated cell sorting analysis using an anti-IL2Rα antibody (Fig. 7A). Endogenously expressed paxillin was also detected, but that required longer exposure of the immunoblots (data not shown). As shown in Fig. 7B, whereas myr-p130Cas stimulated JNK only poorly, expression of IL2R-paxillin resulted in a remarkable increase (6–9-fold) in HA-JNK activity. In contrast, no activation of co-transfected ERK was detected in myr-p130Cas and IL2R-paxillin expressing cells (data not shown). Thus, we concluded that recruitment of paxillin to the plasma membrane is by itself sufficient to signal JNK activation, and that the pathway linking FAK to JNK diverges from that communicating FAK to ERK.

We next explored whether tyrosine phosphorylation was required for the activation of JNK by the membrane-targeted paxillin. As observed in Fig. 7C, immunoprecipitated myr-p130Cas was tyrosine-phosphorylated to a level comparable to that of the endogenous p130Cas, but, unexpectedly, we could not detect any tyrosine phosphorylation of IL2R-paxillin even after prolonged exposure of the anti-phosphotyrosine Western blots, although tyrosine phosphorylation of endogenous paxillin was detected. Taken together, we conclude that recruitment of paxillin to the plasma membrane triggers JNK activation without the need for its tyrosine phosphorylation, which is consistent with the observation that the membrane-bound form of FAK induces JNK activation irrespective of its tyrosine kinase activity. Thus, the interaction of FAK and paxillin leading to the recruitment of paxillin to plasma membrane, rather than the tyrosine phosphorylation of paxillin, might play an important role in JNK activation.

As activation of FAK might affect integrin function, we next asked whether activation of JNK by the membrane-targeted forms of FAK and paxillin was a consequence of cellular stress caused by diminished attachment to the plates. For these experiments, we transfected 293T cells with expression plasmids for FAK and paxillin together with a plasmid for β-galactosidase. Two days later, cells were suspended by pipetting, washed, and allowed to attach to fibronectin-coated plates for different periods of time. β-Galactosidase activity was then determined in attached cells, and normalized for the total enzymatic activity in each cellular sample. Under these conditions, myr-FAK-expressing cells attached to the plates poorly, as shown in Fig. 8A for a 15 min time point. In contrast, wild type FAK, and wild type and IL2R-paxillin did not affect the ability of cells to attach to the plates. Similarly, only myr-FAK caused cell rounding, as judged by the morphology of fluorescent cells upon co-transfection with a GFP expression vector (Fig. 8B). Thus, whereas myr-FAK may affect integrin function as suggested by the decreased attachment to fibronectin-coated plates and cell rounding, this does not appear to be the case for IL2R-paxillin. Furthermore, we did not observe any apoptotic or growth inhibitory effect in cells expressing IL2R-paxillin (data not shown), thus suggesting that cellular stress is not responsible.
for the activation of JNK by this membrane-localized form of paxillin.

In this regard, as small GTP-binding proteins of the Rho family have been suggested to represent integral components of many signaling pathways regulating JNK activity (27, 46), we next asked whether these GTPases participate in signaling from paxillin to JNK using their corresponding dominant-negative mutants (Rho N19, Rac N17, and Cdc42 N17) as an approach. As shown in Fig. 9, co-expression of these mutants diminished the activation of JNK by IL2R-paxillin, without displaying any demonstrable effect on the JNK response to anisomycin when used as a control. Of interest, the dominant-negative mutant of Rho was the most effective, which is consistent with the finding that, in 293T cells, Rho can play a role regulating JNK (47). Taken together, these data suggest that the recruitment of paxillin to the plasma membrane does not

Fig. 7. A membrane-targeted form of paxillin, but not of p130Cas, potently activates JNK. A, 293T cells were transfected with the indicated expression plasmids (pcDNA3 paxillin, pCMV IL2R-paxillin, and pCEFL myr-p130Cas, 2 μg/plate). Lysates containing 50 μg of total protein from transfectants were subjected to Western blot (WB) analysis with the indicated antibodies. To confirm the expression of IL2Rα in IL2R-paxillin transfected cells, fluorescence-activated cell sorting analysis was performed using fluorescein isothiocyanate-conjugated anti-CD25 antibody (upper right panel). B, 293T cells were transfected with pcDNA3 HA-JNK1 (2 μg/plate) together with vector expressing extracellular and transmembrane domains of interleukin-2 receptor α (IL2R) or NH2-terminal 21 amino acids of chicken c-Src (myr) or the indicated amount of expression vectors carrying cDNAs for IL2R-paxillin or myr-p130Cas. Kinase reactions were performed in anti-HA immunoprecipitates from the corresponding cellular lysates. Labeled substrates are indicated. The autoradiograms correspond to a representative JNK assay. Immunoblot analysis was performed with anti-HA antibodies using total cell lysate to confirm similar expression levels of HA-JNK. Data are expressed as -fold increase with respect to control vector-transfected cells and represent the mean ± S.E. of three independent experiments. C, expression vectors for the indicated membrane-targeted proteins were transfected into 293T cells. Cells were lysed, and the tyrosine phosphorylation status of these molecules was determined in the corresponding immunoprecipitates (IP) by Western blot (WB) using anti-phosphotyrosine (PY) antibodies. The same membranes were stripped and re-blotted with the indicated antibodies (right panel). Identical results were obtained in three independent experiments.
cause cellular stress, but may initiate the activation of a pathway involving small GTPases of the Rho family, which, ultimately, leads to JNK stimulation.

DISCUSSION

FAK is regarded as a merging point of the GPCR and integrin-initiated signaling pathways (21, 25). However, the nature of the biochemical routes regulated by FAK is still poorly understood. With the aim of investigating signaling events mediated by FAK, we took advantage of the observation that the membrane-bound form of FAK can behave as a constitutive active mutant (33). As such, the expression of myr-FAK potently activated an epitope tagged-ERK, which has been previously reported to be a downstream target of FAK function (32). This activated FAK was also able to induce a remarkable increase in the activity of a co-expressed epitope-tagged JNK, which was consistent with our recent observations that both integrin and GPCR stimulation enhances JNK activity, albeit by a yet poorly understood biochemical route (17–19).

**FIG. 8.** Effect of myr-FAK and IL2R-paxillin overexpression on the attachment of cells to fibronectin and cell morphology. A, 293T cells were transfected with the indicated expression plasmids (1.0 μg/plate) together with pcDNAIII-βgal (0.5 μg/plate), and their abilities...
data therefore suggested that FAK might participate in JNK stimulation, and prompted us to explore the mechanism whereby this ubiquitously expressed tyrosine kinase activates JNK.

Initially, we asked whether PI3K, which has been shown to bind FAK (37), could participate in MAPK and JNK activation by FAK by using a potent PI3K inhibitor, wortmannin, as an experimental approach. As a control, we assessed the ability of the activated FAK to stimulate Akt, a known downstream target for PI3K (38). We observed that FAK was able to activate Akt, which was abolished by the treatment with wortmannin. This finding, initially meant to be used as a control, might have important implications regarding the in vivo function of FAK, as Akt is known to act as a key component of cell survival pathways (38), and recent observations suggest that FAK can prevent cell death induced by deprivation of cell attachment to substrates, a phenomenon known as anoikis (48). Whether Akt participates in the anti-apoptotic response to FAK is under current investigation. Interestingly, in contrast to Akt, the activation of ERK and JNK by FAK was found to be insensitive to wortmannin, thus suggesting that, whereas FAK can activate Akt through PI3K, the pathway linking FAK to ERK and JNK does not require PI3K function.

We next explored whether ERK and JNK stimulation correlated with the tyrosine phosphorylation of a common set of molecules believed to act downstream of FAK, focusing our attention on Shc, p130Cas, and paxillin. We observed that overexpression of wild type FAK was sufficient to induce the appearance of tyrosine-phosphorylated species of p130Cas and paxillin, but without ERK or JNK activation. Thus, we concluded that the phosphorylation of p130Cas and paxillin on tyrosine residues was not by itself sufficient to activate these MAPK cascades. In contrast, myr-FAK caused a remarkable tyrosine phosphorylation of Shc and ERK activation, and a kinase-defective mutant of FAK that failed to induce Shc phosphorylation also failed to enhance ERK activity. Thus, the ability to induce ERK activation by FAK was parallel to its ability to cause Shc tyrosine phosphorylation, which provides further support for the recently proposed model of Schlaepfer et al. (32), who suggested that FAK and Src activate ERKs though tyrosine phosphorylation of Shc on integrin stimulation. Unexpectedly, however, the tyrosine kinase-deficient mutant of myr-FAK was still able to stimulate JNK to a similar level as that caused by the wild type myr-FAK form. These data indicated that Shc phosphorylation is not required for JNK activation, and that the pathway connecting FAK to JNK is distinct from that linking FAK to ERKs.

Our initial expectation was that FAK might activate JNK through tyrosine phosphorylation of paxillin and/or p130Cas, as these two proteins bind to the adaptor protein Crk in a tyrosine phosphorylation-dependent manner (40, 49–51), and the Crk/C3G complex is proposed to be involved in JNK activation (52, 53). However, this was not found to be the case, because we observed that the membrane-bound form of the kinase-deficient mutant of FAK stimulated JNK even without tyrosine phosphorylation of p130Cas or paxillin. In this regard, however, it has been shown that FAK forms stable complexes with paxillin and p130Cas, and that complex formation is independent of the protein-tyrosine kinase activity of FAK (43, 45). Thus, we speculated that the recruitment of p130Cas or paxillin to the plasma membrane might participate in JNK activation. Indeed, we observed that, by targeting paxillin to the membrane, JNK was activated even without detectable tyrosine phosphorylation. These results suggested that the ability of FAK to recruit paxillin to the membrane might be sufficient to stimulate biochemical routes leading to JNK activation. However, whether recruitment of paxillin is strictly required for JNK activation by FAK is still unknown. Although the carboxy-terminal domain of FAK has been shown to bind paxillin with high affinity (43, 54), we observed that a COOH-terminal truncated form of myr-FAK, including amino acids 1–840, could still associate in vivo with paxillin-containing complexes and activates JNK, and overexpression of the COOH-terminal domain of FAK (designated FRNK, amino acids 693–1052; Ref. 55) was not sufficient to prevent the association of myr-FAK with paxillin or its ability to stimulate JNK.2 These observations raise the possibility that FAK might possess additional binding sites for paxillin besides its carboxy-terminal region, or that additional molecules participate in the indirect association of paxillin with FAK. These, as well as other possibilities, are under current investigation.

Our findings strongly suggest that recruitment of paxillin to the plasma membrane is sufficient to cause JNK activation. So far, enzymatic activity has not been demonstrated in paxillin. Instead, this molecule exhibits a number of structural domains suggestive of a role in signal transduction, including four LIM domains, five repeats of a leucine-rich sequence named LD motif, an Src homology 3 binding site, and tyrosine phosphorylation-dependent Src homology 2 binding sites (56–58). Thus, these structural features provide many potential mechanisms by which paxillin-binding molecules can be activated upon recruitment to the plasma membrane. Interestingly, while this study was under revision, Turner et al. (59) demonstrated that a new 95-kDa protein, termed p95PKL (paxillin-kinase linker), binds directly to the LD4 domain of paxillin. Furthermore, they have shown that this novel protein also binds a guanine nucleotide exchange factor for Rho-like proteins, PIX, which in turn recruits to the paxillin-containing complex the protein-kinase PAK, an upstream component of the JNK pathway (60, 61). Taken together, these findings and our present results strongly suggest that paxillin can interact with the large family of Dbl-related guanine-nucleotide exchange factors for small GTP-binding proteins of the Rho family (62) and with signal transducing kinases, thus providing a direct link between paxillin, the activation of Rho proteins, and the stimulation of the JNK pathway.

In conclusion, our data demonstrate that FAK can stimulate the activity of divergent signaling pathways acting on each MAPK cascade. Several lines of evidence suggest that cell surface receptors, including GPCRs and integrins, provoke the activation and tyrosine phosphorylation of FAK, paxillin, and p130Cas (22, 25, 63). FAK, in turn, stimulates Akt through PI3K, and activates ERKs through the tyrosine phosphorylation of the adapter molecule Shc. Independently, FAK initiates the activation of a distinct biochemical route resulting in JNK activation. In this regard, the recruitment of paxillin to the membrane may initiate the activation of a signal transducing pathway dependent on GTP-binding proteins of the Rho family, thereby providing a novel mechanism by which cell surface receptors and FAK may signal to the nucleus through JNK.

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