Integrin-dependent Activation of the p70 Ribosomal S6 Kinase Signaling Pathway*

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Rajesh K. Malik‡§ and J. Thomas Parsons¶¶
From the Departments of Pediatrics and ‡Microbiology, Health Sciences Center, University of Virginia, Charlottesville, Virginia 22908

Interaction of the cell surface integrin receptors with extracellular matrix proteins results in the activation of intracellular signaling pathways, including activation of the p42/p44 mitogen-activated protein kinases. The protein tyrosine kinase focal adhesion kinase, or FAK, is linked to integrin signaling and interacts with several molecules involved in signal transduction. Here we report that exposure of fibroblast cells to extracellular matrix proteins activates the p70/p85 ribosomal S6 kinase (S6K) pathway in a ligand dependent manner. Treatment of cells with inhibitors of phosphatidylinositol 3-kinase, or FRAP (FKBP 12/rapamycin-associated protein) blocks integrin-mediated activation of S6K. In contrast to the integrin-directed activation of the mitogen-activated protein kinases, cytochalasin D treatment does not inhibit S6K activation. Treatment with the protein tyrosine kinase inhibitors herbimycin A and genistein completely blocks S6K activation, indicating a requirement for tyrosine kinase activity. Overexpression of the COOH-terminal noncatalytic domain of FAK, FRNK (FAK-related non-kinase) in chick embryo cells results in a significant reduction in the integrin-mediated activation of S6K and a concomitant reduction in FAK tyrosine phosphorylation. These results indicate at least a partial requirement for FAK in the S6K activation pathway.

Ubiquitously expressed integrin receptors are involved in the regulation of a variety of important biological functions, including, embryonic development, wound repair, hemostasis, and prevention of programmed cell death (1–7). They are also implicated in abnormal pathological states, e.g. tumor directed angiogenesis, tumor cell growth, and metastasis (8–10). A major role of these heterodimeric receptors is to bridge the cytoplasmic actin-cytoskeleton with proteins present in the extracellular matrix (ECM) and/or on adjacent cells (1, 11). Clustering of the integrin receptors on the cell surface leads to regions of close contact with the ECM, called focal adhesions or focal contacts. Within focal adhesions, cytoplasmic domains of integrins are linked to actin stress fibers via interactions with focal adhesion proteins, thus contributing to the structural integrity of the cell (12, 13). Engagement of integrins with the ECM also leads to activation of signal transduction pathways and regulation of gene expression (1, 14, 15). Previous studies have shown that integrin engagement with the ECM leads to the activation and increased tyrosine phosphorylation of a focal adhesion-associated protein tyrosine kinase, Focal Adhesion Kinase, pp125FAK or FAK (16), indicating that FAK is linked to integrin signaling (17, 18). Several cell types also express a 41/43-kDa protein identical in sequence with the carboxy-terminal domain of FAK, termed FRNK, FAK-related non-kinase (19). FRNK has been shown to regulate FAK functional activity and is encoded by an independently transcribed mRNA (20).

In v-Src transformed cells, a large portion of FAK is associated with v-Src whereas in normal adherent cells FAK associates with c-Src and c-Fyn (21–23). The Src family kinases interact with FAK via their Src homology 2 (SH2) domain binding to the autophosphorylation site of FAK (21). FAK also associates with phosphatidylinositol 3-kinase (PI-3K) via both SH2 and SH3 mediated binding of the p85 regulatory subunit of PI-3K with the carboxyl terminus of FAK (24, 25). Integrin engagement also activates the mitogen-activated protein kinase pathway (23, 26, 27). In cells plated on fibronectin, Grb2 and mSOS1 were found to associate with FAK, thus potentially linking integrin-mediated FAK activation with the Ras signaling pathway (23). The p70 ribosomal S6 kinase (S6K) is a mitogen-activated Ser/Thr kinase that phosphorylates the 40 S ribosomal subunit protein S6 (28, 29). S6K appears to regulate progression from G1 to S in response to serum treatment (30, 31). In addition, S6 phosphorylation correlates with increased translation of mRNAs containing a polypyrimidine tract at their 5' end (32), including mRNAs for the elongation factors eEF-1 and eEF-2, and insulin-like growth factor II (33).

The requirement of PI-3K activity for the activation of S6K by platelet-derived growth factor and insulin treatment was demonstrated by utilizing the selective PI-3K inhibitors wortmannin and LY294002 (34, 35). In addition, mutation of the platelet-derived growth factor receptor tyrosine residues required for binding of the p85 regulatory subunit of PI-3K to the platelet-derived growth factor receptor prevents S6K activation upon platelet-derived growth factor treatment (34). In contrast to the mitogen-activated protein kinases, expression of dominant-negative mutants of p21ras or p74raf do not block S6K activation by epidermal growth factor or phorbol ester treatment, indicating that S6K is activated by a p21ras-independent pathway (36). Serum induced S6K activation can be completely blocked by treatment of cells with rapamycin, an effect that is mediated by interaction of rapamycin with the recently identi-
fied FRAP (FKBP 12/rapamycin associated protein) or RAFT (rapamycin and FKBP 12 target) protein (28, 29, 37–39). The phorbol ester induced activation of S6K is not blocked by Wortmannin while it is completely inhibited by rapamycin treatment, indicating that FRAP is downstream of PI-3K in the S6K activation pathway (35). Since FRAP does not directly associate with S6K, additional protein(s) may be required for S6K activation (40).

In this report we demonstrate the activation of p70 S6K upon integrin-dependent attachment and spreading of cells on ECM proteins. Treatment of cells with inhibitors of PI-3K and FRAP block the integrin-mediated activation of S6K. Treatment with cytochalasin D does not inhibit S6K activity. However, the S6K activity in cytochalasin D-treated cells can be inhibited by Wortmannin treatment, indicating that PI-3K appears to be largely responsible for activating S6K in the presence of cytochalasin D. Treatment of cells with the tyrosine kinase inhibitors herbimycin A and genistein completely inhibits S6K activity indicating the requirement for tyrosine kinase(s) in the S6K activation pathway. Finally, we show that reduction of FAK tyrosine phosphorylation in cells that overexpress FRNK is associated with a 50% decrease in the fibronectin-dependent activation of S6K, indicating that FAK is at least partially required for the integrin-mediated S6K activation.

MATERIALS AND METHODS

Cell Growth and Protein Expression—REF 52 cells were obtained from ATCC and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (Life Technologies, Inc.), 10 μg/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). Chick embryo (CE) cells were isolated and grown as described previously (41). Other reagents included cytochalasin D (Sigma), Wortmannin (Sigma), rapamycin (Calbiochem), GRGDSP and GRGESP peptides (Bachem), herbimycin A (Calbiochem), and genistein (Life Technologies, Inc.). For the expression of FRNK protein, the FRNK cDNA sequence was cloned into the Csl I site of the RCAS A vector as described previously (19). 1 μg of plasmid DNA was transfected into CE cells by calcium phosphate DNA precipitation, and cells were used for experiments 7–10 days after transfection.

Cell Lysates, Kinase Assays, and Immunoblotting—Confluent cells were serum starved overnight, briefly trypsinized by incubation with 0.025% trypsin (Life Technologies, Inc.), 1 mM EDTA, washed twice with phosphate-buffered saline containing 0.5 mg/ml soybean trypsin inhibitor (Sigma), and placed in suspension for 1 h at 37°C. 4.5 × 10⁵ cells were plated under serum free conditions onto Petri dishes that were coated with either 40 μg/ml fibronectin (Sigma), 0.2 mg/ml poly-L-lysine (Sigma), or 20 μg/ml vitronectin (Sigma), or 20 μg/ml laminin (kindly provided by Dr. R. Ogle, University of Virginia) in phosphate-buffered saline at 37°C for 1 h, and blocked for 1 h with 2 mg/ml heat-denatured bovine serum albumin (Sigma) in phosphate-buffered saline at 37°C. Whole cell lysates were prepared using HO lysis buffer consisting of 50 mM Hepes (pH 7.5), 150 mM sodium chloride, 1% Nonidet P-40, 2 mM EDTA, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 50 μM leupeptin (Boehringer Mannheim), 0.5% azoprotein (Sigma), 1 mM phenylmethylsulfonfluoride (Sigma), and 3 mM benzamidine (Sigma). The lysates were clarified by centrifugation at 15,000 × g for 15 min and stored at −70°C until use. S6K activity was assessed by performing immune complex kinase assays (42). Briefly, S6K was immunoprecipitated with an amino-terminal polyclonal S6K antibody (kindly provided by Dr. J. Blenis, Harvard Medical School). The immune complexes were incubated for 30 min at 30°C with kinase buffer consisting of 50 mM Hepes (pH 7.5), 10 mM MgCl₂, 50 μM ATP, 1 mM dithiothreitol, 10 mM β-glycerophosphate, 5 μCi of [γ-³²P]ATP, and 10–20 μg of 40 S ribosomes isolated from Artemia (kindly provided by Drs. K. Coker and T. Sturgill). The samples were solubilized in sample buffer and resolved by 9% SDS-polyacrylamide gel electrophoresis and the gel was cut at the 44-kDa molecular mass marker. The portion of the gel containing proteins of 44 kDa or smaller was dried and the phosphorylated ribosomal proteins were visualized by autoradiography. The proteins in the upper portion of the gel were transferred electrophoretically onto a nitrocellulose membrane and immunoblotted with a carboxyl-terminal S6K antibody (also provided by Dr. J. Blenis) to determine the amount of S6K present in the immunoprecipitates from the different samples. The kinase activity was quantitated by either using a PhosphorImager or by excising the radiolabeled bands and determining radioactivity.

Analysis of FAK from REF 52 cells was carried out by immunoprecipitation with the monoclonal anti-FAK antibody 2A7, and from CE cells with the polyclonal antibody BC3, as described previously (16). Presence of phosphorysotyrosine was determined with the antiphosphotyrosine antibody 4G10 (UBI). Paxillin was immunoprecipitated with a monoclonal anti-paxillin antibody (Transduction Labs) and the presence of phosphorysotyrosine determined by immunoblotting with 4G10.

RESULTS

Activation of S6K Is Integrin-dependent—To determine whether ECM proteins could activate S6K in the absence of serum, serum-starved cells that had been removed from tissue culture dishes by brief trypsinization were replated onto dishes coated with either fibronectin or poly-l-lysine. Cells plated on poly-l-lysine-coated dishes attach due to electrostatic charge but stay rounded, whereas cells plated onto fibronectin-coated dishes spread and form focal adhesions due to integrin engagement (43). As shown in Fig. 1A, a fibronectin-dependent increase in S6K phosphorylation of the 40 S ribosomal protein S6 was detected 20 min after replating cells and S6K activity reached a maximal level at 40–60 min. The maximal stimulation in S6K activity in cells plated on fibronectin compared with poly-l-lysine was approximately 3.2 ± 0.5 (n = 15)-fold compared with a 5.5-fold increase that occurred following serum treatment (n = 6). Fig. 1B shows that equal amounts of S6K was present in all the immune complexes.

To determine whether S6K activation was integrin-dependent, cells in suspension were incubated for 30 min with peptides containing the RGD or RGE sequence and replated onto fibronectin-coated dishes for 60 min in the presence of the appropriate peptide. The RGD peptide sequence is present in many ECM proteins and is a major β1 integrin receptor binding sequence (44, 45). Cell spreading was inhibited upon incubation with the GRGDSP peptide whereas the control peptide, GRGESP, failed to block cell spreading (data not shown). Incubation of cells with GRGDSP blocked the fibronectin-dependent increase in S6K activity (Fig. 1C), indicating that the S6K activation was dependent upon ligand binding. To ascertain whether different β integrin heterodimers could activate S6K, cells were plated on vitronectin (αvβ3)- or laminin (αβ1)-coated dishes. Plating cells on vitronectin and laminin yielded an activation of S6K comparable to that observed when cells were plated on fibronectin (Fig. 2), indicating that S6K could be activated by several different integrin receptors.

Integrin-dependent S6K Activation Pathway—To determine whether PI-3K was required for the integrin-dependent activation of S6K, cells were treated with 0.1% Me₂SO, 10 nM, or 100 nM wortmannin for 15 min prior to trypsinization. The cells were maintained in the drug during trypsinization, washing, suspension, and upon replating onto poly-l-lysine- or fibronectin-coated dishes for 30 and 60 min. Treatment with 100 nM wortmannin decreased the fibronectin-dependent S6K activation by approximately 70% (Fig. 3, A and C) but had no effect on cell spreading (data not shown). As expected, wortmannin treatment also caused a similar inhibition in the serum activation of S6K (Fig. 3A). To ascertain whether activity of FRAP was also required for the integrin-dependent S6K activation, cells were treated with 1 and 10 nM rapamycin and were plated onto fibronectin-coated dishes in the presence of rapamycin. The rate of cell spreading was not altered by rapamycin treatment (data not shown), however, the integrin-dependent S6K activation was completely inhibited (Fig. 4A). Rapamycin treatment also completely inhibited the serum activation of S6K (Fig. 4A). These data indicate that both integrin- and mitogen-mediated S6K activation utilize similar wortmannin and rapamycin sensitive intermediates.
S6K Activation Is Not Affected by Cytochalasin D Treatment—Maintenance of an intact actin cytoskeleton is often required for efficient integrin mediated signaling (26, 27). To determine whether an intact actin cytoskeleton was required for S6K activation, cells were treated with cytochalasin D to disrupt actin stress fiber formation. Treatment of cells with 0.1 μM cytochalasin D partially impaired cell spreading, whereas 1 μM treatment completely inhibited cell spreading on fibronectin (data not shown). However, S6K activation was not significantly affected by cytochalasin D treatment at either concentration (Fig. 5A). In parallel control experiments, FAK and paxillin were immunoprecipitated and analyzed by anti-phosphotyrosine immunoblotting to examine the effect of cytochalasin D treatment on their phosphotyrosine content. Treatment of cells with 1 μM cytochalasin D decreased the tyrosine phosphorylation of both FAK and paxillin by approximately 70–80%, thereby reducing the phosphotyrosine content of both proteins to levels present in cells maintained in suspension (Fig. 5, C and E). The addition of sodium orthovanadate to the cytochalasin D-treated cells did not alter the phosphotyrosine content of FAK and paxillin (Fig. 5, C and E), indicating that the reduction in tyrosine phosphorylation was not due to increased phosphatase activity withinthecytochalasin D-treated cells. Incubation of cytochalasin D-treated cells with wortmannin inhibited S6K activity to a level similar to that in cells treated with wortmannin alone, indicating that a wortmannin-sensitive intermediate continued to be necessary for the activation of S6K under these conditions (Fig. 6).

S6K Activation Requires Tyrosine Kinase Activity—To explore the requirement for tyrosine kinase activity in the integrin-mediated activation of S6K, we utilized the tyrosine kinase inhibitors herbimycin A and genistein. Herbimycin A treatment inhibits the tyrosine phosphorylation of FAK and paxillin upon plating cells on a matrix substrate (47). Treatment of cells with either herbimycin A overnight or genistein for 15 min, followed by plating cells on fibronectin in the presence of either inhibitor for 60 min, resulted in the complete inhibition of the integrin-dependent activation of S6K (Fig. 7A). Exposure to either inhibitor also blocked cell spreading (data not shown). In addition, herbimycin A treatment completely inhibited the tyrosine phosphorylation of FAK and paxillin. In contrast, exposure to genistein resulted in a modest decrease in the tyrosine phosphorylation of FAK and paxillin (Fig. 7, C and E), while completely inhibiting S6K activity. These data indicate that the integrin-dependent S6K activation is exquisitely sensitive to the requirement of tyrosine kinase(s).

Involvement of FAK in S6K Activation—To examine the role of FAK in the activation of S6K, we utilized CE cells that were transfected with the replication competent avian retroviral
vector RCAS A containing the cDNA coding for FRNK, the autonomously expressed COOH-terminal domain of FAK. The overexpression of FRNK in CE cells results in a significant reduction in the tyrosine phosphorylation of FAK and a reduced rate of cell spreading compared to mock-transfected cells (20). Thus, overexpression of FRNK appears to function as a negative inhibitor of FAK function. Cells overexpressing FRNK exhibited an approximately 50% reduction in the fibronectin-dependent activation of S6K following plating on fibronectin or poly-L-lysine for 3 h (Fig. 8A). Cells overexpressing FRNK also demonstrated a significant reduction in FAK tyrosine phosphorylation compared to mock transfected controls (Fig. 8, C and E). These data indicate that the FRNK dependent inhibition of S6K activity correlates with a significant reduction in FAK activation.

DISCUSSION

In this report we provide evidence that engagement of β1 and β3 containing integrin receptors activates the S6K pathway. The fibronectin-dependent S6K activation was inhibited by prior incubation of cells with a peptide containing the RGD sequence, establishing that the S6K activation is integrin-dependent. The efficient inhibition of S6K activation by wortmannin and rapamycin indicate that integrins and growth factor receptors utilize a similar pathway to signal to S6K. This pathway presumably involves the activation of a wortmannin-sensitive PI-3K as well as FRAP/RAFT (28, 29, 34, 35, 37–39). We have no indication that the integrin stimulated pathway differs significantly from pathways activated by various mitogens. Comparison of the two pathways does reveal that the extent of the integrin-mediated S6K activation is approximately 50% of the level detected upon serum stimulation of cells. This reduced activation of S6K by the integrin receptors may reflect an intrinsically inefficient activation of the S6K pathway upon interaction with matrix components. Alternatively, when cells are replated on fibronectin, cells settle, attach, and spread at different rates thus yielding an asynchronous activation of integrin signals. This is in contrast to the more synchronous signal generated upon mitogen treatment of a cell monolayer. In addition, activation of S6K by the integrin pathway may be inefficient or restricted, either by compartmentalization of the signal or by fewer active signaling complexes available to couple with integrin receptors.

Is FAK Required for S6K Activation?—Since PI-3K is required for the mitogen activation of S6K, and has been demonstrated to interact with FAK (24, 25), we examined its role in the activation of S6K by the integrins. Treatment of cells with nanomolar concentrations of wortmannin significantly reduced S6K activity. Under these conditions wortmannin has been demonstrated to be a specific inhibitor of PI-3K (46). Furthermore, another protein involved in the mitogen-mediated activation of S6K is FRAP/RAFT. Inhibition of FRAP activity by treating cells with rapamycin also completely inhibits the integrin-mediated activation of S6K. These data suggest that both mitogen and integrin signaling pathways may use similar intermediates for S6K activation.

Surprisingly, cytochalasin D treatment of cells failed to block
the integrin-dependent activation of S6K while significantly reducing the tyrosine phosphorylation of FAK and paxillin and cell spreading. This result suggests that S6K activation is not dependent on cell spreading per se. Indeed, blocking S6K activation with wortmannin or rapamycin does not inhibit cell spreading, suggesting that activation of the S6K pathway is independent of pathways that regulate formation of focal adhesions. In contrast, cytochalasin D has been reported to inhibit mitogen-activated protein kinase activation by integrin engagement (26, 27). There are several possible mechanisms to explain the activation of S6K in cytochalasin D-treated cells in the absence of a robust activation of FAK tyrosine phosphorylation. First, residual integrin-mediated activation of FAK present in cytochalasin D-treated cells might be sufficient to trigger downstream signaling, for example, by activating PI-3K. Second, activation of S6K might occur via a FAK-independent pathway. Third, the activation of PI-3K might be dependent on the recruitment of FAK to the integrin receptors but independent of FAK kinase activity. With respect to the latter possibilities, recent reports suggest that structures resembling focal adhesions might be present in cytochalasin D-treated cells. Nobes and Hall (48), demonstrated that focal adhesions were present in cytochalasin D-treated cells that were microinjected with the small GTPase protein Rho, even though the formation of actin stress fibers was inhibited. Under these conditions, they also detected FAK and paxillin within the focal adhesions. Miyamoto et al. (49), reported that the association of FAK with beads coated with either ECM proteins or integrin antibody was not affected by cytochalasin D treatment of cells. These studies support the idea that clustering of FAK (and perhaps limited FAK mediated signaling) can occur even in the presence of cytochalasin D.

The complete inhibition of S6K in cells treated with the tyrosine kinase inhibitors herbinycin A and genistein argues strongly for the requirement of tyrosine kinase activity in the

FIG. 5. Effect of cytochalasin D treatment on the activation of S6K. Serum-starved REF 52 cells in suspension were treated for 15 min with 0.1 or 1 μM cytochalasin D either in the presence or absence of 100 μM Na3VO4. The cells were then plated on fibronectin-coated dishes in the presence of the appropriate agent, or maintained in suspension. A, S6K activity was determined by immune complex kinase assay. B, immunoblot analysis using a COOH-terminal S6K antibody. C, FAK was immunoprecipitated from the lysates with the anti-FAK monoclonal antibody 2A7, and the phosphotyrosine content of FAK was determined by immunoblot analysis with the antiphosphotyrosine antibody 4G10. D, immunoblot analysis of the immunoprecipitated FAK was performed with the polyclonal antibody HUB3. E, paxillin was immunoprecipitated from the lysates with a monoclonal anti-paxillin antibody and its phosphotyrosine content was determined by immunoblot analysis with the antiphosphotyrosine antibody 4G10.

FIG. 6. Effect of cytochalasin D and wortmannin treatment on the activation of S6K. Serum-starved REF 52 cells in suspension were treated for 15 min with 0.1 or 1 μM cytochalasin D either in the presence or absence of 200 nM wortmannin (Wt), or with wortmannin alone. The wortmannin-treated cells were exposed to wortmannin for 15 min before trypsinization. The cells were then plated on fibronectin-coated dishes for 60 min (F60) in the presence of the appropriate agent. A, the S6K activity was determined by immune complex kinase assay. B, immunoblot analysis using a COOH-terminal S6K antibody.

FIG. 7. Effect of tyrosine kinase inhibitors on the activation of S6K. Serum-starved REF 52 cells were treated overnight with herbimycin A (875 nM) or carrier solution (0.1% Me2SO). In parallel, additional cells were treated with genistein (100 μg/ml) or carrier solution (1% Me2SO) for 15 min while held in suspension. All cells were plated onto fibronectin-coated dishes for 1 h in the presence of the appropriate drug. A, S6K kinase assay was performed as described in the legend to Fig. 5. B, immunoblot analysis using a COOH-terminal S6K antibody. C, FAK was immunoprecipitated from the lysates and the phosphotyrosine content of FAK and immunoblot analysis of the immunoprecipitated FAK was performed as described in the legend to Fig. 5. D, FAK was immunoprecipitated and its phosphotyrosine content and paxillin immunoblot analysis (panel F) was performed as described in Fig. 5.
S6K activation pathway. Is this tyrosine kinase FAK? S6K was completely inhibited upon genistein treatment, however, under these conditions, tyrosine phosphorylation of FAK and paxillin was not significantly reduced and the activity of c-Src was largely unaffected (data not shown). These data argue that a genistein-sensitive tyrosine kinase may be required for the efficient integrin-dependent activation of S6K. This tyrosine kinase could either be downstream of the FAK-Src complex, or be activated independently of FAK/Src kinase activity. A role for FAK in the integrin-mediated activation of S6K is indicated by the reduced activation of S6K in cells overexpressing FRNK, a negative regulator of FAK function. Overexpression of FRNK resulted in a 50% reduction in S6K activity, indicating that FAK is at least partially required for S6K activation. It is possible that FAK plays a role in recruiting PI-3K to the integrin signaling complex. Since the site of interaction of FAK and PI-3K has been mapped to the COOH-terminal domain of FAK (24, 25), overexpression of FRNK may suffice to block the efficient recruitment of PI3K, thus inhibiting S6K activity.

Role of Integrin-mediated S6K Activation—Since there is little evidence that S6K is required for the process of cell adhesion and spreading, what role does S6K play in adhesion signaling? One possibility is that matrix directed signaling might serve a “homeostatic” function by keeping the protein translation machinery of the cell primed to respond to the acute signals generated upon exposure to mitogens. This would allow the cell to progress through the cell cycle upon mitogen exposure. In this regard, the integrins have been demonstrated to be important for cell cycle progression (50). In addition to the homeostatic function, S6K activation may provide a cell survival signal. Recent evidence shows that the ECM can serve as a survival factor and that the integrins transduce this signal (5, 6), although the nature of the signal is not known. Since S6K activity is required for both protein translation and cell cycle progression, it is possible that activation of S6K by the integrins might be important for continued cell survival. Finally, recent reports suggest that β-actin and potentially other mRNAs can be translated in cytoplasmic ribosomal particles, resulting in a spatially localized increase in protein concentration (51). The integrin-dependent activation of S6K, presumably at or close to the focal adhesions, could potentially mediate this increased local translation of initiation factors and, possibly, actin. Thus, it might be important in assisting directional cell motility.

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FIG. 8. Effect of exogenous FRNK expression on S6K activation. CE cells were transfected by calcium phosphate DNA precipitation with 1 μg of vector alone (Mock) or 1 μg of RCAS vector containing the FRNK coding sequence. Ten days later a cell spreading assay was performed on cells that had been serum starved overnight. A, S6K activity was determined by immunocomplex kinase assay. B, immunoblot analysis with anti-S6K antibody. C, FAK was immunoprecipitated from the lysates and the phosphotyrosine content of FAK and immunoblot analysis for FAK (panel D) were performed as described in the legend to Fig. 5. E, FRNK was immunoprecipitated with the polyclonal antibody BC3, and FRNK was visualized by immunoblot analysis with the polyclonal anti-FAK antibody BC3.
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