The β1 integrin, functioning as a mechanoreceptor, senses a mechanical stimulus generated during collagen matrix contraction and down-regulates the phosphatidylinositol 3-kinase (PI3K)/Akt survival signal triggering apoptosis. The identities of integrin-associated signal molecules in the focal adhesion complex that are responsible for propagating β1 integrin viability signals in response to collagen matrix contraction are not known. Here we show that in response to collagen contraction focal adhesion kinase (FAK) is dephosphorylated. In contrast, enforced activation of β1 integrin by anti-β1 integrin antibody, which protects fibroblasts from apoptosis, preserves FAK phosphorylation. We demonstrate that ligation of β1 integrin by type I collagen or by enforced activation of β1 integrin by antibody promotes phosphorylation of FAK, p85 subunit of PI3K, and serine 473 of Akt. Wortmannin inhibited Akt but not FAK phosphorylation in response to enforced activation of β1 integrin by antibody. Blocking FAK by pharmacologic inhibition or by dominant negative FAK attenuated phosphorylation of p85 subunit of PI3K and Akt. Dominant negative FAK augmented fibroblast apoptosis during collagen contraction, and this was associated with diminished Akt activity. Constitutively active FAK augmented levels of p85 subunit of PI3K and Akt phosphorylation, and fibroblasts were protected from apoptosis. Our data identify a novel role for FAK, functioning upstream of PI3K/Akt, in transducing a β1 integrin viability signal in collagen matrices.

Integrins are cell surface adhesion receptors that regulate cell viability in response to cues derived from the extracellular matrix (1, 2). The nature of these extracellular cues that regulate cell viability are diverse. In the case of polarized epithelial and endothelial cells, whose basal surface is attached to the basement membrane, direct loss of integrin-matrix interaction can trigger anoikis (3–5). In the case of mesenchymal cells, which exist encompassed by the extracellular matrix, matrix-derived mechanical stimuli can regulate viability. In this latter scenario, integrins function as mechanoreceptors that detect mechanical stimuli originating from the extracellular matrix and convert them to chemical signals that regulate cell viability pathways (6–12).

For example, fibroblast survival in type I collagen matrices is regulated by integrin-extracellular matrix interactions (6). In response to contraction of type I collagen matrices fibroblasts undergo apoptosis (6, 13, 14). During the process of collagen contraction Akt becomes dephosphorylated (6). This down-regulation of Akt activity during collagen contraction is mediated by β1 integrin. Up-regulation of Akt activity, either by enforced activation of β1 integrin by anti-β1 integrin antibody or by ectopic expression of constitutively active phosphatidylinositol 3-kinase (PI3K), protects fibroblasts from collagen gel contraction-induced apoptosis (6). Therefore, functioning as a mechanoreceptor, the β1 integrin is capable of sensing a matrix-derived mechanical stimulus and regulates fibroblast survival by modulating a PI3K/Akt survival pathway. Matrix-derived mechanical stimuli can be transmitted through the direct or indirect interaction of integrins with associated lipid or protein signaling molecules in the focal adhesion complex (15, 16). Currently the identities of integrin-associated signaling molecules that are responsible for propagating the β1 integrin viability signal via PI3K/Akt in response to collagen matrix-derived mechanical signals are unclear. Focal adhesion kinase (FAK), a potential candidate signaling molecule, has been shown to be capable of regulating integrin-mediated survival signaling (17–22). However, the downstream signaling pathways that mediate integrin-FAK survival signaling are diverse, and the factors determining which pathway is utilized remain obscure. Downstream signal pathways implicated in FAK viability signaling include a c-Jun N-terminal kinase survival pathway that inactivates the tumor suppressor protein p53-regulated cell death pathway (23), death-associated protein kinase (24), and the PI3K/Akt pathway regulating epithelial and endothelial cell viability (22, 25). Data supporting a role for FAK in the β1 integrin/PI3K/Akt viability pathway include the finding that integrin-extracellular matrix interaction recruits FAK to the focal adhesion complex and activates it. Integrin ligation and clustering activates FAK by autophosphorylation of tyrosine 397. This creates a potential binding site for the SH2 domains of the p85 subunit of PI3K (26–28). Phosphorylation of the p85 subunit of PI3K by FAK may activate the p110 catalytic subunit of PI3K and the

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PI3K/Akt signal pathway. Although it is plausible that FAK regulates fibroblast survival within a three-dimensional collagen matrix via the \( \beta_1 \) integrin/PI3K/Akt signal pathway, the role of FAK in this process has not been examined.

In this study we show that in response to collagen matrix contraction FAK is dephosphorylated. In contrast, enforced activation of \( \beta_1 \) integrin by anti-\( \beta_1 \) integrin antibody, which protects the fibroblasts from collagen-contraction-induced apoptosis, preserves FAK phosphorylation. We demonstrate that ligand of \( \beta_1 \) integrin with type I collagen or enforced activation of \( \beta_1 \) integrin by anti-\( \beta_1 \) integrin antibody promotes the phosphorylation of FAK, the p85 subunit of PI3K, and serine 473 of Akt. Blocking FAK function by pharmacologic inhibition or by dominant negative FAK inhibits both the phosphorylation of the p85 subunit of PI3K and serine 473 of Akt. Furthermore, dominant negative FAK promotes fibroblast apoptosis in both anoikis and collagen gel assays. Conversely constitutively active FAK augments p85 phosphorylation and Akt activity and protects fibroblasts from collagen gel contraction-induced apoptosis. Our data identify a novel role for FAK functioning upstream of PI3K and Akt in mediating \( \beta_1 \) integrin viability signaling of fibroblasts in collagen matrices.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human lung fibroblasts (CCL-210, American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% heat-inactivated fetal calf serum and used between passages 9 and 11.

**Antibodies and Reagents**—Mouse monoclonal antibody P5D2 (raised against the human \( \beta_1 \) integrin subunit) was provided by Dr. Leo Furcht (University of Minnesota). Polyclonal anti-Akt antibody and anti-phosphorylated Akt antibody were purchased from Cell Signaling Technology Inc. (Beverly, MA). Polyclonal anti-FAK was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-FAK antibodies were directed to tyrosine 397, 407, 576, 577, 861, or 925 were from BIOSOURCE (Camarillo, CA). Anti-PI3K-p85 subunit antibody and anti-phosphorylated tyrosine antibody P20 were from BD Transduction Laboratories. Wortmannin was from Sigma, and P2P and PFP were from Calbiochem.

**Dominant Negative FAK cDNA Construct**—Dominant negative FAK (FRNK) was generated by amplification of the cDNA FRNK encoding sequence of FAK obtained from human lung fibroblasts by reverse transcription-PCR (29). The primers used to generate dominant negative FAK were: 5′-CAG CAC AAT ATC GAT CAG CAA GAG CAC-3′ and 5′-GGC CAC ATT TGG CAT CTT GGA TTT-3′. A Clai restriction site was introduced at the beginning of 5′-FRNK. Both IRES-GFP (pIRES2-EGFP) vector from Chao Chen and FRNK were cloned into the pBluescript II KS′ vector. FRNK-IRES-GFP was then inserted into the Swal site in vector pAxCaWt (Takara, Tokyo, Japan) to generate recombinant adenovirus. Recombinant adenoviruses with FRNK-GFP and IRES-GFP (Ad-FRNK and Ad-GFP) were made according to the company’s instruction. Adenovirus with IRES-GFP only (Ad-GFP) served as a control. Adenovirus titers were analyzed by the agarose plaque method.

**Constitutively Active FAK Construct**—Constitutively active FAK (CD2-FAK) in the pCDM8 vector was a gift from Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA. A unique NotI restriction site was introduced at the beginning of 5′-CD2-FAK in the pCDM8 vector by PCR. The primers used to generate restriction site were 5′-TCA CAG GTC AGG AGT GTG TT-3′ and 5′-ATC GCC GCC GCT TCT AGA GAT CCC TCG-3′. The CD2-FAK fragment from the pCDM8 vector and IRES-GFP from the pIRES2-EGFP vector were subcloned into the NotI-SalI site in the pBluescript II KS′ vector. Next the CD2-FAK-IRES-GFP fragment cut by NotI and SalI from the pBluescript II KS′ vector was inserted into the pCI-neo and SgHi and SalI sites in the amphotropic retrovirus expression vector MIGR1.str. The resulting plasmid MIGR1-CD2-FAK and retrovirus genomic plasmid PCL were cotransfected into H-293 cells using a calcium phosphate precipitation method to produce the replication-defective recombinant retrovirus, retrovirus-CD2-FAK-GFP/RV-CD2-FAK. Retrovirus-GFP (RV-GFP) serves as a control. In addition, the fragment of CD2-FAK and IRES-GFP was cloned into the Swal site in the vector pAxCaWt to generate the recombinant adenovirus Ad-Cd2-FAK according to the manufacturer’s instruction.

**Constitutively Active p110 Subunit of PI3K Construct**—Dr. Julian Downward provided the constitutively active p110 subunit of PI3K.

CA-p110 was subcloned into the retroviral vector MIGR1.str containing IRES-GFP, creating the bicistronic construct CA-p110/ IRES-GFP as described previously (6). A population of CA-p110/GFP-positive cells was obtained by cell sorting using fluorescence-activated cell sorter analysis.

**Adenoviral and Retroviral Infection**—Human lung fibroblasts were plated in tissue culture dishes and infected with Ad-GFP, Ad-FRNK, and Ad-CD2-FAK at a multiplicity of infection of 1:50 for 12 h with RV-GFP and RV-CD2FAK for 48 h in Dulbecco’s modified Eagle’s medium + 10% fetal calf serum. After infection the medium was changed to serum-free Dulbecco’s modified Eagle’s medium, and the cells were serum-starved for 48 h prior to performing the individual experiments. After 48 h of serum starvation the cells were ready to be used for different integrin treatments with anti-\( \beta_1 \) integrin antibody treatments at 6 μg/ml or various reagents (wortmannin, 200 nM; PF2 or PF3, 10 μM).

**Immunoprecipitation and Western Analysis**—Fibroblasts were lysed in lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 0.5% sodium deoxycholate, 1% Nonidet P-40, 2.5 mM Na3PO4, 1 mM glyceral phosphate, 1 mM Na2VO4, 1% protease inhibitor mixture, and 1 mM phenylmethylsulfonyl fluoride. Lysates were preclarified for 60 min at 4°C with protein G-coupled agarose beads and immunoprecipitated overnight (16 h) at 4°C with the appropriate primary antibody. Western analysis of human lung fibroblasts was performed as described previously (6). Briefly equal amounts of protein from cell lysates were subjected to 8–10% SDS-PAGE and transferred (45 min, 24 V) to nitrocellulose membrane. Membranes were blocked with 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.05% Tween 2 containing 6% nonfat dry milk; incubated with the primary antibody; washed; and incubated with horseradish peroxidase-conjugated secondary antibody. The membranes were developed using the ECL method (Amersham Biosciences).

**Anoikis Assay**—Anoikis assays were performed as described by Frisch and Francis (4). Tissue culture plates were coated twice with poly-HEMA (10 mg/ml in ethanol, Sigma) and rinsed extensively with phosphate-buffered saline. Fibroblasts resuspended in Dulbecco’s modified Eagle’s medium were plated on the poly-HEMA plates. At the indicated times, the cells in suspension were recovered and analyzed by fluorescence TUNEL assay.

**TUNEL Assay**—Fibroblasts recovered from anoikis assays and type I collagen gels were analyzed for apoptosis using an in situ cell death detection kit (fluorescence TUNEL assay, Roche Applied Science) according to the manufacturer’s instructions. Briefly recovered cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 60 min at room temperature and permeabilized with 0.1% Triton X-100 for 2 min at 4°C. The cells were resuspended in TUNEL reaction mixture, incubated for 60 min at 37°C, washed, and analyzed by fluorescence microscopy (30).

**Collagen Gel Assay**—Collagen gels were prepared as described previously (6). Human lung fibroblasts (1.2 × 105) were resuspended in 2.0 ml of 1.5% Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Vitrogen (3 mg/ml; Cohesion, Palo Alto, CA) was added to the cell suspension to achieve a final concentration of 0.5 mg/ml. The cell/collagen solution was incubated in a water bath for 10 min at 37°C, poured into 3.5-cm uncoated plastic dishes, and placed in a cell culture incubator at 10% CO2 and 37°C where the gels polymerized in ~30–60 min.

**Statistical Analysis**—Data are expressed as the mean ± S.D. Experiments were performed three times. For assessment of the percentage of apoptotic cells within collagen gels or on dishes coated with poly-HEMA using the TUNEL assay, microscopic analysis of at least 200 cells/slide were performed. Paired t tests were performed to determine the restriction site used to generate the recombinant constructs. The significance of each experiment, and significance was determined by Student’s t test. The significance level was set at \( p < 0.05 \).

**RESULTS**

**Collagen Matrix Contraction Is Associated with FAK Dephosphorylation**—Regulation of fibroblast viability in collagen matrices is mediated by the \( \beta_1 \) integrin and involves a PI3K/Akt signaling pathway. In response to type I collagen matrix contraction, Akt becomes dephosphorylated triggering fibroblast apoptosis. Previous studies have implicated FAK with \( \beta_1 \) integrin viability signaling; therefore we were interested in determining the effect of collagen matrix contraction on FAK activity. Phosphorylation of tyrosine 397 of FAK has been used as a marker of FAK activity. We examined phosphorylation of tyrosine 397 of FAK as a function of time in contractile collagen
Collagen fibroblasts were incorporated into contractile type I collagen gels. The cells were allowed to attach and spread for 2 h. After 2 h P5D2 anti-β1 integrin antibody (6 μg/ml) or isotype control was added to the gels, and the gels were allowed to contract. At the designated times, lysis buffer was added directly to the gels, FAK was immunoprecipitated with polyclonal anti-FAK antibody, and SDS-PAGE was performed. The level of phosphorylation of tyrosine 397 of FAK was determined using an anti-phospho-FAK tyrosine 397 antibody. Total FAK was measured by polyclonal anti-FAK antibody in the same membrane. IP, immunoprecipitation; WB, Western blot.

Gels. During the first 2–4 h after fibroblasts have been incorporated into type I collagen matrices but before contraction begins, the cells attach and spread. During this time period FAK became phosphorylated (Fig. 1). However, as matrix contraction progresses the cells become increasingly round in appearance (6). By 24 h the gels have contracted, and many of the cells are undergoing apoptosis (6). At the 24-h time point we found that FAK was dephosphorylated. Therefore, in conjunction with our previous finding that Akt becomes dephosphorylated during matrix contraction, these data suggested a link between FAK and Akt activity and regulation of fibroblast viability in collagen matrices.

We have found previously that enforced activation of β1 integrin by P5D2 anti-β1 integrin antibody protects fibroblasts from collagen contraction-induced apoptosis by augmenting Akt phosphorylation/activity (6). Therefore, we assessed the effect of P5D2 antibody treatment on FAK phosphorylation during collagen matrix contraction. Fibroblasts were incorporated into contractile collagen gels. The cells were allowed to attach and spread for 2 h in the gels after which time P5D2 antibody was added to the collagen gels as described previously (6). The effect on FAK phosphorylation was assessed at various times. At the 4- and 6-h time points, phosphorylation of tyrosine 397 of FAK in contractile collagen gels treated with P5D2 antibody was similar to that found in control contractile gels. It should be noted that at these time points little collagen contraction had occurred. However, at the 24-h time point when the gels have largely completed contraction, the level of FAK phosphorylation was preserved in contractile collagen gels treated with P5D2 antibody compared with control contractile gels (Fig. 1). This suggests that the protection of fibroblasts from collagen contraction-induced apoptosis in response to enforced activation of β1 integrin by antibody involves FAK.

FAK Phosphorylation and Akt Activity Increase as a Function of Ligation of β1 Integrin with Type I Collagen or by Enforced Activation of β1 Integrin by Anti-β1 Integrin Antibody—To further examine whether FAK plays a role in integrin-mediated survival signaling in collagen matrices, we evaluated the effect of culturing fibroblasts on collagen-coated plates on FAK phosphorylation and Akt activity. Human lung fibroblasts were serum-starved for 48 h and then plated on tissue culture plastic dishes coated with type I collagen. Phosphorylation of serine 473 of Akt was used to assess Akt activity (31). Akt phosphorylation increased as a function of time as the fibroblasts were allowed to adhere and spread on type I collagen (Fig. 2A). To examine whether the increase in Akt activity was dependent upon β1 integrin interaction with type I collagen, we examined the effect of blocking β1 integrin-type I collagen interaction on Akt activity using anti-β1 integrin antibody. When cells in suspension are preincubated with P5D2 antibody prior to plating on type I collagen, the anti-β1 integrin antibody functions as an inhibitor, blocking integrin-collagen interaction (6). Preincubation of fibroblasts with antibody prior to plating on type I collagen abrogated the increase in Akt activity (Fig. 2A). We next examined whether the β1 integrin-mediated increase in Akt activity associated with ligation of type I collagen was dependent upon P13K. Serum-starved fibroblasts were pretreated with wortmannin prior to plating on type I collagen-coated dishes. Inhibition of P13K by wortmannin attenuated the increase in Akt activity associated with β1 integrin-type I collagen interaction (data not shown). These data indicate that β1 integrin-type I collagen interaction promotes Akt activity in a P13K-dependent manner.

The effect of plating fibroblasts on collagen-coated dishes on FAK phosphorylation was also examined. Human lung fibroblasts were serum-starved for 48 h and then plated on type I collagen-coated dishes. Similar to Akt activity, FAK phosphorylation increased as a function of time as the fibroblasts were allowed to adhere and spread on type I collagen (Fig. 2B). To examine whether the increase in FAK phosphorylation was dependent upon β1 integrin-type I collagen interaction, we examined the effect of preincubating fibroblasts with anti-β1 integrin antibody on FAK phosphorylation. Inhibition of β1 integrin-type I collagen interaction by anti-β1 integrin antibody abrogated the increase in FAK phosphorylation associated with plating the cells on type I collagen (Fig. 2B).

We have shown previously that enforced activation of β1 integrin by adding P5D2 β1 integrin monoclonal antibody to adherent fibroblasts increases Akt activity in a P13K-dependent fashion (Ref. 6; see also Fig. 3C). Under these culture conditions, the P5D2 anti-β1 integrin antibody functions in an agonist fashion. Therefore, to perform these experiments fibroblasts were plated on tissue culture dishes and serum-starved prior to addition of anti-β1 integrin antibody. Consistent with the results obtained when integrin was ligated by type I collagen, we found that enforced activation of β1 integrin by P5D2 antibody also increased FAK phosphorylation in serum-starved fibroblasts adhered to tissue culture dishes (Fig. 3A). We next
assessed which FAK phosphorylation sites were phosphorylated upon ligation of $\beta_1$ integrin with P5D2 antibody by immunoblotting lysates of P5D2 antibody-treated cells with a panel of phosphotyrosine-specific antibodies. These antibodies recognize the tyrosine-phosphorylated state of amino acid residues 397, 407, 576, 577, 861, or 925 of FAK. Identification of FAK tyrosine residues phosphorylated in response to enforced activation of $\beta_1$ integrin by antibody was assessed using a panel of phosphotyrosine-specific antibodies. B, to assess the phosphorylation level of the p85 subunit of PI3K, cell lysates were immunoprecipitated with anti-PI3K-p85 antibody (PI3K-p85). The phosphorylation level of the p85 subunit of PI3K was assessed by Western analysis using an anti-phosphotyrosine (PY20) antibody. Total p85 is shown as a loading control. C, the level of phosphorylated ($p$-Akt-ser473) and total Akt in response to enforced activation of $\beta_1$ integrin by antibody was assessed by Western analysis. All results were obtained from three independent experiments. $p$-, phosphorylated; IP, immunoprecipitation; WB, Western blot.

Because ligation of $\beta_1$ integrin with type I collagen or P5D2 anti-$\beta_1$ integrin antibody phosphorylates FAK, we sought to determine whether ligation of $\beta_1$ integrin promotes the association of FAK with $\beta_1$ integrin. Lysates of serum-starved fibroblasts treated with P5D2 antibody were immunoprecipitated using anti-$\beta_1$ integrin antibody and probed for the presence of FAK or vice versa. A direct physical association between integrin and FAK could not be demonstrated in response to enforced activation of $\beta_1$ integrin by anti-$\beta_1$ integrin antibody (data not shown).

Phosphorylation of tyrosine 397 of FAK may provide a binding site for the Src homology 2 domains of the p85 subunit of PI3K (31). Since enforced activation of $\beta_1$ integrin by anti-$\beta_1$ integrin antibody promotes the phosphorylation of tyrosine 397 of FAK, we sought to determine whether ligation of $\beta_1$ integrin by antibody also promoted the association of FAK with PI3K. Immunoprecipitation of FAK and immunoblotting for the p85 subunit of PI3K or vice versa did not demonstrate a direct physical association of FAK with the p85 subunit of PI3K. However, we found that enforced activation of $\beta_1$ integrin with anti-$\beta_1$ integrin antibody promoted phosphorylation of the p85 subunit of PI3K in a time-dependent manner (Fig. 3A). For these experiments, serum-starved adherent fibroblasts were treated with P5D2 antibody for various times. Cell lysates were immunoprecipitated with anti-PI3K-p85 antibody. Phosphorylation of p85 subunit was assessed using an anti-phosphotyrosine antibody and Western analysis. Although we were unable to demonstrate a direct physical association of integrin with FAK or FAK with PI3K, our results suggest a link between ligation of $\beta_1$ integrin and activation of FAK, PI3K, and Akt. These data support the concept that FAK may play a role in $\beta_1$ integrin/PI3K/Akt survival signaling on collagen matrices.

The above data indicate that ligation of $\beta_1$ integrin by either type I collagen or by enforced activation of $\beta_1$ integrin by antibody increases FAK, PI3K, and Akt activity. We were also interested in determining what effect blocking integrin-mediated FAK phosphorylation would have on PI3K and Akt activity. To begin to approach this issue we used PP2, a selective Src kinase family inhibitor. By virtue of its ability to inhibit Src kinases, PP2 has been shown to block integrin-induced FAK phosphorylation (32). We found that PP2 effectively abolished the phosphorylation of tyrosine 397 of FAK, the p85 subunit of PI3K, and serine 473 of Akt (Fig. 4) that occurs upon enforced activation of $\beta_1$ integrin by anti-$\beta_1$ integrin antibody. In contrast, PP3, an inactive analog of PP2, had no effect. These results suggest a possible link between integrin-induced FAK phosphorylation and activation of PI3K and Akt.

Down-regulation of FAK Activity Inhibits the Phosphorylation of FAK, the p85 Subunit of PI3K, and Akt—These studies strongly suggested to us that FAK activity might be involved in regulating PI3K/Akt survival signaling in response to collagen matrix contraction. To investigate this further, we examined the effect of FRNK on phosphorylation of Akt to determine whether FAK is required for the activation of Akt in response to ligation of $\beta_1$ integrin by collagen and anti-$\beta_1$ integrin anti-
body. We first examined whether dominant negative FAK would inhibit FAK phosphorylation induced by β1 integrin-type I collagen interaction and by enforced activation of β1 integrin by P5D2 antibody. Human lung fibroblasts were infected with either Ad-FRNK or Ad-GFP (empty vector control). The cells were then serum-starved for 48 h and then either plated on tissue culture dishes coated with type I collagen or treated with anti-β1 integrin antibody. Compared with cells infected with empty vector control, cells infected with dominant negative FAK were less well spread and more rounded in appearance (Fig. 5A). Dominant negative FAK (Ad-FRNK) effectively blocked FAK phosphorylation in response to adhesion to type I collagen (Fig. 5B). Shown in Fig. 5B are the results for enforced activation of β1 integrin by antibody. Similar results were obtained by ligation of β1 integrin with type I collagen (data not shown). In contrast, in fibroblasts infected with control vector (Ad-GFP), FAK phosphorylation increased as a function of time after plating on type I collagen or ligation of β1 integrin with P5D2 antibody. We next examined the effect of dominant negative FAK on phosphorylation of the p85 subunit of PI3K in response to enforced activation of β1 integrin by anti-β1 integrin antibody. Dominant negative FAK inhibited phosphorylation of p85 in response to P5D2 antibody treatment. However, p85 phosphorylation in response to antibody treatment was preserved in fibroblasts infected with empty vector control (Ad-GFP) (Fig. 5C). To determine the effect of down-regulation of FAK on Akt activity, phosphorylation of serine 473 of Akt in response to adherence to type I collagen or enforced activation of β1 integrin by antibody was examined in human lung fibroblasts infected with either Ad-FRNK or Ad-GFP. Dominant negative FAK (Ad-FRNK) decreased Akt phosphorylation in fibroblasts treated with anti-β1 integrin antibody or plated on type I collagen-coated dishes. In contrast, Akt phosphorylation was preserved in fibroblasts infected with empty vector (Ad-GFP) (Fig. 5D). These data confirm that inhibition of FAK abrogates both the phosphorylation of the p85 subunit of PI3K and activation of Akt that occur in response to ligation of β1 integrin by type I collagen or anti-β1 integrin antibody.

**Down-regulation of FAK Augments Fibroblast Apoptosis in Type I Collagen Matrices**—We have demonstrated previously that fibroblasts undergo apoptosis during type I collagen matrix contraction (6). In response to collagen contraction Akt becomes dephosphorylated. Up-regulation of Akt activity by enforced activation of β1 integrin by anti-β1 integrin antibody or by ectopic expression of constitutively active PI3K protects fibroblasts from contraction-induced apoptosis. To directly examine the role of FAK in regulating fibroblast viability in collagen matrices, we assessed the effect of dominant negative FAK on fibroblast viability in contractile collagen matrices.
Fibroblasts from Apoptosis—To further assess the role of FAK in regulating fibroblast viability via the β1 integrin/PI3K/Akt signaling pathway, we up-regulated FAK using the constitutively active CD2-FAK fusion protein as described previously (17). CD2-FAK was overexpressed in fibroblasts by infection with the retroviral vector RV-CD2-FAK. Infection of fibroblasts with RV-CD2-FAK promoted FAK phosphorylation (Fig. 7A).

We next sought to examine the effect of up-regulation of FAK on PI3K and Akt activity in response to ligation of β1 integrin by type I collagen or enforced activation of β1 integrin by anti-β1 integrin antibody. CD2-FAK augmented the phosphorylation of the p85 subunit of PI3K in response to ligation of β1 integrin by either collagen or antibody compared with control (RV-GFP) (Fig. 7B). Results are shown for antibody-treated cells. Similar results were obtained for cells plated on type I collagen. Importantly CD2-FAK also augmented the level of Akt phosphorylation in response to ligation of β1 integrin by collagen or antibody compared with control (Fig. 7C).

Having determined that CD2-FAK augments Akt activity, we next sought to determine the effect of up-regulation of FAK on fibroblast viability using the anoikis assay. Fibroblasts were infected with either RV-CD2-FAK or RV-GFP for 48 h. The infected fibroblasts were then plated onto poly-HEME-coated dishes in the absence of serum. After 36 h the cells were harvested, and the level of apoptosis was assessed by TUNEL assay. At 36 h in suspension culture, 63% of fibroblasts infected with the empty vector control (RV-GFP) were apoptotic (Fig. 7D). In contrast, CD2-FAK fibroblasts were protected from anoikis. At 36 h in suspension culture only 29% of CD2-FAK fibroblasts were TUNEL-positive.

We also examined the effect of up-regulation of FAK on fibroblast survival within contractile collagen matrices. Normal lung fibroblasts were incorporated into 0.5 mg/ml collagen gels. The cells were allowed to attach and spread within the gels for 4 h after which time the cells were infected with either adenovirus GFP (control) or adenovirus CD2-FAK or were not infected. The gels were allowed to contract. After infection the gels were digested with collagenase, and the recovered cells were analyzed by TUNEL. At 36 h after infection, 63% of control cells (adenoviral GFP) were apoptotic versus 18% of adenoviral CD2-FAK cells (Fig. 7E). 41% of non-infected fibroblasts were apoptotic at this time point. Collectively these data indicate that up-regulation of FAK augments the β1 integrin/PI3K/Akt survival signal and protects fibroblasts from apoptosis.

FAK Functions Upstream of PI3K in Regulating Fibroblast Survival via Akt—The above studies establish a role for FAK in regulating the PI3K/Akt survival signal in response to collagen matrix contraction. Next we were interested in determining the position of FAK in the β1 integrin/PI3K/Akt viability pathway. Our studies in which we used dominant negative FAK to down-regulate FAK activity resulted in the down-regulation of phosphorylation of the p85 subunit of PI3K and serine 473 of Akt. Dominant negative FAK also promoted fibroblast apoptosis. These data suggested that FAK was upstream of PI3K in activating Akt. To extend and confirm these results, we next analyzed the effect of up-regulation of PI3K using constitutively active p110 subunit of PI3K on the survival of fibroblasts expressing dominant negative FAK. We have shown previously that up-regulation of PI3K by ectopic expression of constitutively active p110 subunit of PI3K protects fibroblasts from apoptosis (6). To perform these experiments, fibroblasts were infected with dominant negative FAK (adenoviral FRNK) combined with either control (RV-GFP) or the constitutively active p110 subunit of PI3K (retroviral CA-p110). As a control fibroblasts were infected with the retroviral GFP and adenoviral GFP control vectors. To begin these experiments we first con-

Fig. 6. Dominant negative FAK augments dephosphorylation of Akt and fibroblast apoptosis in contractile collagen matrices. A, to assess the effect of dominant negative FAK on fibroblast viability in contractile collagen matrices, fibroblasts were first incorporated into 0.5 mg/ml type I collagen gels, and the cells were allowed to attach and spread for 4 h. The cells were infected by adding Ad-GFP or Ad-FRNK to the gels for 12 h. The collagen gels were allowed to contract for 24-36 h. The cells were then harvested from the gels by collagenase treatment, and the level of apoptosis in the recovered cells was quantified by TUNEL assay. Shown are the percentages of apoptotic cells in contractile collagen gels treated with dominant negative FAK (Ad-FRNK) and empty vector control (Ad-GFP) at the 36-h time point. The data are presented as the percentage of recovered cells that were TUNEL-positive. *, p < 0.002 versus control. B, the level of phosphorylation of serine 473 of Akt in fibroblasts infected with Ad-FRNK or empty vector control (Ad-GFP) in contractile collagen gels at the 24-h time point was assessed by Western analysis. p-, phosphorylated.
firmed that infection of fibroblasts with constitutively active p110 subunit of PI3K increases the level of phosphorylation of serine 473 of Akt as we have shown previously (6). Constitutively active PI3K augmented phosphorylation of serine 473 of Akt (Fig. 8A). Fig. 8B demonstrates that dominant negative FAK down-regulates phosphorylation of serine 473 of Akt. Next we examined the effect of dominant negative FAK, combined with either control (RV-GFP) or constitutively active PI3K (CA-p110), on the level of Akt phosphorylation. Interestingly we found that phosphorylation of serine 473 of Akt was aug-
Constitutively active PI3K overrides the effect of dominant negative FAK on promoting Akt dephosphorylation and fibroblast apoptosis. Western analysis of control fibroblasts (RV-GFP) and fibroblasts expressing constitutively active p110 subunit of PI3K (RV-p110) (A) or control fibroblasts (RV-GFP + Ad-GFP), dominant negative FAK fibroblasts (Ad-FRNK + RV-GFP), and fibroblasts co-expressing dominant negative FAK and constitutively active p110 subunit of PI3K (RV-p110 + Ad-FRNK) (B). The infected cells were lysed, and equal amounts of protein were subjected to 10% SDS-PAGE. The levels of Akt phosphorylation (serine 473) and total Akt were analyzed by Western analysis using the appropriate primary antibody. Total Akt is shown as a loading control. C, control fibroblasts (RV-GFP), dominant negative FAK fibroblasts (Ad-FRNK & RV-GFP), and fibroblasts co-expressing dominant negative FAK and constitutively active p110 subunit of PI3K (RV-p110 & Ad-FRNK) were maintained in suspension cultures (poly-HEME-coated dishes) for 36 h. Following 36 h in suspension culture the cells were analyzed for anoikis using a fluorescent TUNEL assay kit (bottom panels), and the nuclei were identified using a fluorescent 4,6-diamidino-2-phenylindole stain (top panels). Note that the majority of FRNK fibroblasts are TUNEL-positive, whereas the number of TUNEL-positive cells expressing both dominant negative FAK and constitutively active p110 subunit of PI3K is reduced. D, shown are the percentages of control fibroblasts (RV-GFP + Ad-GFP), dominant negative FAK fibroblasts (Ad-FRNK + RV-GFP), and fibroblasts co-expressing dominant negative FAK and constitutively active PI3K (Ad-FRNK + RV-p110) undergoing apoptosis at 36 h in suspension culture. The data are presented as the percentage of recovered cells that were TUNEL-positive. *, p < 0.002 versus control.
crease in Akt activity brought about by enforced activation of adenoviral GFP cells were apoptotic, whereas 31% of fibroblasts expressing constitutively active FAK (CD2-FAK) were apoptotic (Fig. 9C). Moreover, inhibition of PI3K with wortmannin abrogated the protective effect of CD2-FAK. 82% of CD2-FAK fibroblasts treated with wortmannin were apoptotic at 36 h. These results are consistent with our previous findings and indicate that FAK is upstream of PI3K/Akt in regulating fibroblast viability.

**DISCUSSION**

A β1 integrin/PI3K/Akt viability pathway regulates fibroblast survival in response to mechanical stimuli generated by contraction of three-dimensional collagen matrices. However, the identities of integrin-linked signal transduction molecules that are responsible for propagating this β1 integrin viability signal are unknown. In the current study, we investigated the role of FAK in mediating the β1 integrin/PI3K/Akt survival signal in collagen matrices. Although FAK is a potential candidate signaling molecule, the downstream pathways that mediate integrin-FAK survival signaling are diverse, and the circumstances under which a particular pathway is utilized is obscure. In this regard, survival signals emanating from fibronectin-integrin interaction are transduced by FAK and involve activation of e-c-Jun N-terminal kinase survival pathway (23) but not PI3K/Akt. Activation of e-c-Jun N-terminal kinase then inactivates a p53-regulated apoptotic pathway. Other work suggests that integrin-FAK survival signaling may be modulated by death-associated protein kinase, which also involves a p53-dependent apoptotic pathway (24). Alternatively, studies on epithelial and endothelial cell viability have linked FAK survival signaling to activation of the PI3K/Akt pathway (25, 33). These cells are polarized, and activation of FAK by attachment of their basal surface to the underlying basement membrane is required for their survival. Although the precise mechanism by which integrins activate FAK is unclear, integrin ligation and clustering phosphorylate FAK. FAK contains tyrosine residues in motifs for binding SH2 domains. Phosphorylated tyrosine 397 of FAK has been shown to serve as a binding site for the SH2 domains of the p85 subunit of PI3K (28). Thus integrin-ECM ligation may activate FAK and promote FAK association or activation of PI3K. Within a three-dimensional collagen matrix, we have found that the β1 integrin, functioning as a mechanoreceptor, is capable of sensing a mechanical stimulus generated during collagen matrix contraction and regulates the PI3K/Akt survival signal and cell viability (6). Therefore we sought to determine whether FAK was involved in transducing the β1 integrin viability signal, which regulates fibroblast survival in collagen matrices. Here we provide data indicating that FAK functions upstream of PI3K/Akt in propagating the β1 integrin viability signal in collagen matrices.

**Role of FAK in Regulating the β1 Integrin PI3K/Akt Viability Signal**—Previously we have found that in response to collagen contraction Akt becomes dephosphorylated triggering apoptosis (6). In the current study we demonstrate that collagen matrix contraction is also associated with dephosphorylation of FAK, and this correlates with the onset of fibroblast apoptosis. This suggested a link between down-regulation of PI3K/Akt and Akt activity and loss of a survival signal in response to collagen matrix contraction. Furthermore we have demonstrated previously that enforced activation of β1 integrin by anti-β1 integrin antibody protects fibroblasts from collagen matrix contraction-induced apoptosis by preserving Akt activity. Consistent with a role for FAK in regulating fibroblast viability we found that enforced activation of β1 integrin by antibody also preserved FAK phosphorylation during matrix contraction. Together these data support the hypothesis that FAK is responsible for propagating the β1 integrin viability signal via PI3K/Akt in...
response to collagen matrix-derived mechanical signals.

Plating fibroblasts on type I collagen induces FAK phosphorylation. Ligation of β1 integrin by collagen is responsible for FAK activation. This is demonstrated by experiments in which preincubation of fibroblasts with β1 integrin antibody prior to plating on type I collagen abrogates FAK activation. The mechanism of FAK activation by integrins is incompletely understood. Mutational analyses involving integrin cytoplasmic domains suggest that direct binding of FAK to integrin alone is unlikely to be responsible for FAK activation (26, 27). Our data are consistent with this concept. We did not find a direct physical association of FAK with β1 integrin upon enforced activation of β1 integrin by anti-β1 integrin antibody, a process that promotes the PI3K/Akt survival signal. However, we have found that enforced activation of β1 integrin promotes the phosphorylation of the two N-terminal tyrosine kinase residues in FAK, tyrosines 397 and 407. Integrins may activate FAK by their association with other signaling molecules, such as the Src family of kinases that in turn activate FAK. Furthermore, FAK contains tyrosine residues in motifs for binding SH2 domains. Phosphorylated tyrosine 397 of FAK has been shown to serve as a binding site for the SH2 domains of the p85 subunit of PI3K (28). Phosphorylation of the p85 subunit is required for activation of the p110 catalytic subunit of PI3K. Thus, FAK may either directly or indirectly activate PI3K. Additionally, wortmannin blocked Akt but not FAK phosphorylation (28). Phosphorylation of the p85 subunit is required for PI3K activity. Therefore, in our system, integrin activation results in the indirect activation of FAK likely through Src kinase activation. Activation of FAK in turn promotes PI3K activation.

Down-regulation of FAK activity, by blocking Src kinases using the PP2 inhibitor or by expression of dominant negative FAK, decreased Akt activation in response to enforced activation of β1 integrin by anti-β1 integrin antibody. Interestingly, the selective Src family kinase inhibitor PP2, which by virtue of its ability to block Src kinases also inhibits FAK, completely abolished FAK, p85 subunit of PI3K, and Akt phosphorylation in response to enforced activation of β1 integrin by antibody. However, dominant negative FAK, despite effectively blocking FAK phosphorylation, did not completely abolish p85 or Akt phosphorylation. This suggests that although FAK is involved in regulating PI3K and Akt activity in the β1 integrin viability pathway, other signaling molecules/pathways such as Src kinases may be involved. In this regard, recent work indicates that the Src family of protein-tyrosine kinases reduces the ability of PTEN to dephosphorylate phosphatidylinositol-4,5-bisphosphate (34). PTEN is a phosphatase that inhibits both lipid and tyrosine kinases (35, 36). Therefore, by virtue of its ability to block Src kinases, PP2, in addition to inhibiting integrin-induced FAK phosphorylation, may also up-regulate PTEN function. Increased PTEN activity could promote FAK dephosphorylation. Alternatively, Src can directly bind the p85 subunit of PI3K promoting p85 subunit phosphorylation and p110 catalytic activity. This relieves the inhibitory effect of p85 on p110 resulting in activation of the PI3K/Akt pathway (37). Therefore, PP2 may also inhibit PI3K activation in this manner. Thus the mechanism by which PP2 completely inhibits FAK, PI3K, and Akt phosphorylation in response to enforced activation of β1 integrin by anti-β1 integrin antibody is likely complex. The combination of the inhibitory effects of PP2 on Src activity might be expected to more fully abolish PI3K/Akt activity compared with the inhibitory effects of dominant negative FAK on FAK alone.

To confirm the role of FAK in regulating Akt activity and fibroblast survival in the β1 integrin viability pathway, we used FAK gain and loss of function studies using dominant negative and constitutively active FAK. Expression of dominant negative FAK augmented fibroblast apoptosis in response to collagen matrix contraction. Furthermore, the level of Akt phosphorylation in gels treated with dominant negative FAK was decreased compared with control, confirming that inhibition of FAK blocks Akt activity thereby augmenting the level of apoptosis in response to matrix contraction. Consistent with our previous findings that up-regulation of PI3K/Akt activity protects fibroblasts from apoptosis, constitutively active FAK also protected fibroblasts from apoptosis. Supporting the concept that FAK regulates the β1 integrin viability signal and fibroblast survival via PI3K/Akt, we found that up-regulation of FAK by expression of constitutively active FAK promoted both the phosphorylation of the p85 subunit of PI3K and of serine 473 of Akt. Collectively our up- and down-regulation studies indicated that FAK plays an important role in propagating the β1 integrin viability signal regulating fibroblast survival in response to matrix-derived mechanical signals.

**Position of FAK in the β1 Integrin/PI3K/Akt Viability Pathway**—The downstream pathways by which FAK can regulate cell survival are diverse. Therefore, we performed multiple studies that confirmed that FAK operates upstream of PI3K in regulating fibroblast survival in our model system. First, we found that up-regulation of FAK by constitutively active FAK increased both PI3K and Akt activity. Second, we then examined the effect of blocking PI3K on the ability of constitutively active FAK to promote cell survival. Wortmannin effectively blocked constitutively active FAK-induced cell survival. In addition, wortmannin blocked Akt but not FAK phosphorylation in response to enforced activation of β1 integrin by anti-β1 integrin antibody.
integrin antibody. Third, we found that down-regulation of FAK by dominant negative FAK decreased PI3K and Akt activation in response to enforced activation of β1 integrin by anti-β1 integrin antibody. It also augmented fibroblast apoptosis in contractile collagen gels. Fourth, confirmation of these results was provided by experiments in which we co-expressed constitutively active PI3K (p110 subunit of PI3K) with dominant negative FAK. Constitutively active PI3K was able to override the apoptosis-promoting effect of dominant negative FAK further indicating that FAK is capable of functioning upstream of PI3K in regulating fibroblast survival.

Together our data demonstrate a novel role for FAK in functioning as an integrin-associated signaling molecule capable of transducing cell viability signals generated by matrix contraction that are sensed and integrated by the β1 integrin receptor. The β1 integrin FAK/PI3K/Akt viability pathway regulating fibroblast viability in type I collagen matrices is illustrated in Fig. 10. Our data leave open the possibility that other integrin-associated signaling molecules/pathways, such as Src kinases, may collaborate with FAK in regulating PI3K/Akt activity and fibroblast viability in collagen matrices. Further elucidation of the signal molecules/pathways that regulate fibroblast viability in collagen matrices may provide insight into the control of mesenchymal cell fate during tissue repair after injury.

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Focal Adhesion Kinase Is Upstream of Phosphatidylinositol 3-Kinase/Akt in Regulating Fibroblast Survival in Response to Contraction of Type I Collagen Matrices via a β1 Integrin Viability Signaling Pathway
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