Inhibition of pp125^{FAK} in Cultured Fibroblasts Results in Apoptosis

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Abstract. The tyrosine kinase called pp125^{FAK} is believed to play an important role in integrin-mediated signal transduction. pp125^{FAK} is associated both functionally and spatially with integrins, which are the cell surface receptors for extracellular matrix components. Although the precise function of pp125^{FAK} is not known, two possibilities have been proposed: pp125^{FAK} may regulate the assembly of focal adhesions in spreading or migrating cells, or pp125^{FAK} may participate in a signal transduction cascade to inform the nucleus that the cell is anchored. To test these models in living cells, a peptide representing the focal adhesion kinase (FAK)–binding site of the β_1 tail was coupled to carrier protein and injected into cultured cells to competitively inhibit the binding of pp125^{FAK} to endogenous integrin, thus inhibiting activation of pp125^{FAK} on a cell-by-cell basis. In addition, an antibody directed against an epitope adjacent to the focal adhesion targeting sequence on pp125^{FAK} was microinjected, as an alternative means of inhibiting pp125^{FAK} activation. It was observed that when rounded cells were injected with either the integrin peptide or the anti-FAK antibody, the cells rapidly began to apoptose, within 4 h after injection. These results indicate that pp125^{FAK} may play a critical role in suppressing apoptosis in fibroblasts.

THE focal adhesions of cultured cells have been known for decades as sites of tight structural attachment of the cell membrane to the underlying extracellular matrix (ECM).[1] In addition, the focal adhesion has become recognized as an important site of signal transduction (for review see Damsky and Werb, 1992; Schwartz, 1992; Juliano and Haskill, 1993; Lo and Chen, 1994; Clark and Brugge, 1995). Information that is conveyed from the outside to the inside of the cell at the focal adhesion can affect complex cell behaviors such as migration, proliferation, differentiation, and cell survival.

Integrins are the transmembrane receptors for the ECM, and, as such, they play a critical role in both the cell attachment and the signal transduction functions of the focal adhesions. Integrins are requisite heterodimers, and considerable effort has been focused on assigning functional roles to the integrin α and β subunits. Current evidence strongly suggests that the β subunit is principally responsible for targeting integrins to the focal adhesion (for review see Sastry and Horwitz, 1993). However, both of the integrin subunits appear to play a role in integrin-mediated signal transduction. Binding of integrins to the ECM results in the activation of a number of biochemical pathways that could convey information to the inside of the cell. These include increased intracellular pH, transient Ca^{++} influx, and increased activity of specific kinases, including the tyrosine kinase pp125^{FAK} (for review see Clark and Brugge, 1995).

pp125^{FAK} is associated both functionally and spatially with integrins (for review see Otey, 1996). The tyrosine phosphorylation and the kinase activity of pp125^{FAK} are up-regulated when integrins bind to the extracellular matrix (Guan and Shalloway, 1992; Burridge et al., 1992; Kornberg et al., 1992), but the activation of pp125^{FAK} is dependent upon clustering of the integrins (Guan et al., 1991; Kornberg et al., 1991; Pelletier et al., 1995), not simply occupation of the ligand-binding site. This suggests that a conformational change in integrin is involved in activating pp125^{FAK}. The precise downstream function of pp125^{FAK} is not known, but two possibilities have been proposed: pp125^{FAK} may regulate the assembly of focal adhesions in spreading or migrating cells, or pp125^{FAK} may participate in a signal transduction cascade to inform the nucleus that the cell is anchored to the extracellular matrix, thus suppressing apoptosis.

Recently, the integrin β subunit was shown to bind directly to pp125^{FAK} in vitro (Schaller et al., 1995). The mapping of a binding site for pp125^{FAK} within the β-integrin cytoplasmic tail has suggested a novel way in which to investigate the function of pp125^{FAK} in living cells. A peptide corresponding to this sequence was generated for in-

1. *Abbreviations used in this paper:* ECM, extracellular matrix; CEF, chicken embryo fibroblast; FAK, focal adhesion kinase.

jection into living fibroblasts. We reasoned that the short integrin peptide would occupy the integrin-binding site on pp125^{FAK}, but it would not convey the conformationally dependent activation signal to pp125^{FAK}. Thus, through competition with endogenous integrin, the peptide would specifically interfere with the integrin-dependent activation of pp125^{FAK}. In this paper we report that rounded cells injected with this peptide rapidly begin to apoptose within 4 h after injection. These results indicate that pp125^{FAK} plays a critical role in suppressing apoptosis in fibroblasts.

Materials and Methods

Cell Culture

Primary cultures of fibroblasts were isolated from 10-d-old chicken embryos. Briefly, the head, wings, limbs, and internal viscera were removed, and the remaining embryonic tissue was incubated in trypsin-EDTA solution (GIBCO BRL, Gaithersburg, MD) for 15 min at 37°C. Tissue was dispersed by trituration, and then diluted with complete medium (DME supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin) to inhibit further digestion. The isolated cells were collected by centrifugation, resuspended in complete medium, transferred to tissue-culture dishes, and maintained in complete medium at 37°C. Chicken embryo fibroblasts (CEFs) from the second to twelfth passage were used in all experiments.

Microinjection and Immunofluorescence

Synthetic peptides designated SP1 (CKLLMIIHDRREFA), SP2 (CFAK-FEKEKMNAKW), SP3 (CKWDTGENPIYKSA), SP4 (CAVTTVVNP-KYEGK), and SCR (CDFIMEKHARRIL) were either obtained from the Protein Chemistry Laboratory (University of North Carolina at Chapel Hill) or purchased from Quality Controlled Biochemicals (Hopkinton, MA). Methods of synthesis and purification were described previously (Otey et al., 1993). Peptides were synthesized with an NH$_2$-terminal cysteine for use in coupling to carrier protein and were conjugated to BSA at a ratio of 70–100 mol of peptide per mol of BSA using a heterobifunctional coupling agent (sulfo-MBS; Pierce Chemical Co., Rockford, IL). The peptide–BSA conjugates were dialyzed into injection buffer (75 mM KCl, 10 mM potassium phosphate buffer, pH 7.5) and concentrated to ~2 mg/ml using a Centricon-30 (Amicon Corp., Danvers, MA) apparatus. An mAb to pp125^{FAK}, 2A7 (a gift of Dr. J. Thomas Parsons, University of Virginia), and an isotype-matched mAb to an unrelated chromosomal protein, 3F3 (gift of Michael Campbell and Dr. Gary Gorbsky, University of Virginia), were purified from ascites fluid on a recombinant protein G column or on a protein A–Sepharose column, concentrated to 2 mg/ml, and dialyzed into microinjection buffer. Immediately before injection, the peptides and antibodies were centrifuged and filter sterilized. Fab fragments of the 2A7 monoclonal were generated from column-purified antibody on a ficin column, using the Immunopure IgG$_1$ Fab and F(ab')$_2$ preparation kit (Pierce Chemical Co.) and following the protocol provided by the manufacturer. Purified Fab fragments were concentrated and dialyzed as described above.

CELLocate coverslips (Eppendorf, Madison, WI), which are etched with a lettered-grid and used to map the injected cells, were coated with 50 μg/ml human plasma fibronectin (Sigma Chemical Co., St. Louis, MO). Cultured CEFs were trypsinized, resuspended in complete media, washed twice in serum-free media, and plated onto the coated coverslips in serum-free medium. The CEFs were injected with synthetic peptides or antibodies within 15 min after plating, so that they were attached but still round at the time of injection. Injections were performed using either an Eppendorf microinjector 5246 connected to a micromanipulator and inverted IM 35 microscope (Carl Zeiss Inc., Thornwood, NY), or an Eppendorf Transjector 5246 and Micromanipulator 5171 connected to a Zeiss Axiovert 135 microscope. After injection, the cells were either returned to the incubator for 4–6 h, or one field was videotaped continuously for 6 h, while the cells were maintained at 37°C on a heated microscope stage. The cells were then fixed (4% formaldehyde in PBS) and labeled as described below. For the SP1 and SCR peptides, double-blind injections were performed.

For experiments on spread cells, CEFs were plated onto fibronectin-

coated, CELLocate coverslips in complete media and allowed to spread. After 2 h, the complete medium was replaced with serum-free medium, and the cells were injected with either the peptide or mAb. Injected cells were returned to the incubator for 4–6 h, and then fixed (4% formaldehyde) and labeled (see below).

To analyze the stress fiber formation in injected cells, cells were fixed and labeled with rhodamine-conjugated phalloidin (Sigma Chemical Co.). Apoptosis of injected cells was detected with the ApopTag in situ detection kit according to the protocols described by the manufacturer (Oncor, Gaithersburg, MD). Labeled cells were observed on a microscope (E. Leitz, Inc., Rockleigh, NJ) equipped with differential interference contrast and epifluorescence optics.

Scanning EM

CEFs were injected with the SP1 or SCR peptide, as described above. At 2–4 h after injection, the cells were fixed in glutaraldehyde, critical point dried, shadowed with gold palladium, and observed in a scanning electron microscope (6400; JEOL USA, Peabody, MA).

Flow Cytometry

Single-cell suspensions of ~10^7 CEFs were incubated in either complete medium or serum-free medium at 37°C. Aliquots of 10^6 cells from each experimental condition were removed at hourly intervals, washed in cold PBS, and fixed for 15 min in 1% formaldehyde in PBS. The cells were washed in cold PBS, postfixed in 70% ethanol at −20°C, washed again in PBS, and stained with the Apoptag in situ detection kit, following the directions provided by the manufacturer (Oncor). Flow cytometry analysis was performed on a FACScan® flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Data from 10^4 cells per sample were collected and analyzed using LYSIS II software (Becton Dickinson & Co.).

Results

Synthetic peptides representing slightly overlapping regions of the β$_1$-integrin cytoplasmic tail were generated (Table I). In vitro coprecipitation assays have shown that pp125^{FAK} binds directly to the β$_1$ cytoplasmic tail in the region represented by the SP1 peptide (Schaller et al., 1995). A randomly ordered version of SP1, designated SCR, was generated for use as a control. The synthetic peptides were coupled to carrier protein and used for microinjection of single cells to determine if activation of pp125^{FAK} is required for cells to spread and to assemble focal adhesions and stress fibers.

Cells were plated onto fibronectin and injected while they were still rounded (i.e., within 15 min after plating). Cells were maintained in serum-free medium during the course of the microinjection experiment to prevent the activation of pp125^{FAK} by serum factors. As shown in Fig. 1, cells that were injected with SP1 while rounded failed to spread by 4 h after injection (see *arrows* in *b*) and did not form stress fibers (*c*) or focal adhesions (data not shown). In contrast, neighboring uninjected cells on the same coverslip had fully developed stress fiber arrays by 4 h after injection. Cells that were injected while rounded with the control peptide spread normally (Fig. 1 *e*) and assembled both stress fibers (Fig. 1 *f*) and focal adhesions (data not shown) by 4 h after injection.

In comparing the SP1-injected cells and their uninjected neighbors, it was noted that the nuclear morphology of the injected cells was condensed and lobular, which is characteristic of cells undergoing apoptosis. To determine if the SP1-injected cells were indeed becoming apoptotic, we used the criterion of DNA fragmentation, assayed with the

Table I. Sequences of Short Peptides Derived from β₁-Integrin Cytoplasmic Tail

752	762	772	782	792

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β₁ - KLLMIIHDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK
     ★
SP1 - CKLLMIIHDRREFA
                   ★
          SP2 - CFAFEKEKMNAKW
                             ★
                    SP3 - CKWDTGENPIYKSA
                                        ★
                             SP4 - CAVTTVVNPKYEGK
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Four synthetic peptides (*SP1–SP4*) were generated from the amino acid sequence of the full-length, β₁-integrin cytoplasmic domain (shown with residue numbers listed above). Each peptide was synthesized with an additional NH₂-terminal cysteine residue (★) to facilitate coupling to a carrier protein.

ApopTag in situ detection kit. ApopTag reagents fluorescently label free DNA ends so that apoptotic nuclei are intensely stained. In addition, we were able to evaluate nuclear morphology by phase microscopy for all of our injected and noninjected cells. As shown in Fig. 2, cells that had been injected with either SP1 or SCR peptide while still rounded were allowed to recover for 4 h, and then fixed and stained with the ApopTag kit. Injection of SP1 into rounded cells resulted in both nuclear condensation (Fig. 2 *b*) and strong ApopTag labeling (Fig. 2, *a–c*), while cells injected with SCR had normal nuclei that failed to stain with ApopTag (Fig. 2, *d–f*). Necrotic cells, such as the rounded cell located at the top of the field in Fig. 2 *b*, did not label with ApopTag reagents.

To investigate more thoroughly the specificity of this effect, cells were also injected with peptides SP2, SP3, and SP4. As shown in Table I, these peptides represent the membrane-distal regions of the β₁ cytoplasmic tail. Previously, these peptides were shown to have no binding activity for pp125^{FAK} in peptide–bead precipitation assays (Schaller et al., 1995). These three peptides were coupled to carrier protein, injected into rounded CEFs, and stained with the Apoptag kit. As shown in Fig. 3, only the SP1 peptide induced a significant amount of apoptosis in the injected cells.

A second criterion for identifying apoptotic cells is extensive blebbing of the plasma membrane. Scanning EM was used to examine the membrane morphology of peptide-injected cells. Fig. 4 *a* shows an SP1-injected cell, which displays many small plasma membrane blebbs. Fig. 4 *b* shows control injected cells, which had the same smooth, fully spread morphology at 4 h after injection as the uninjected cells in neighboring areas of the coverslip.
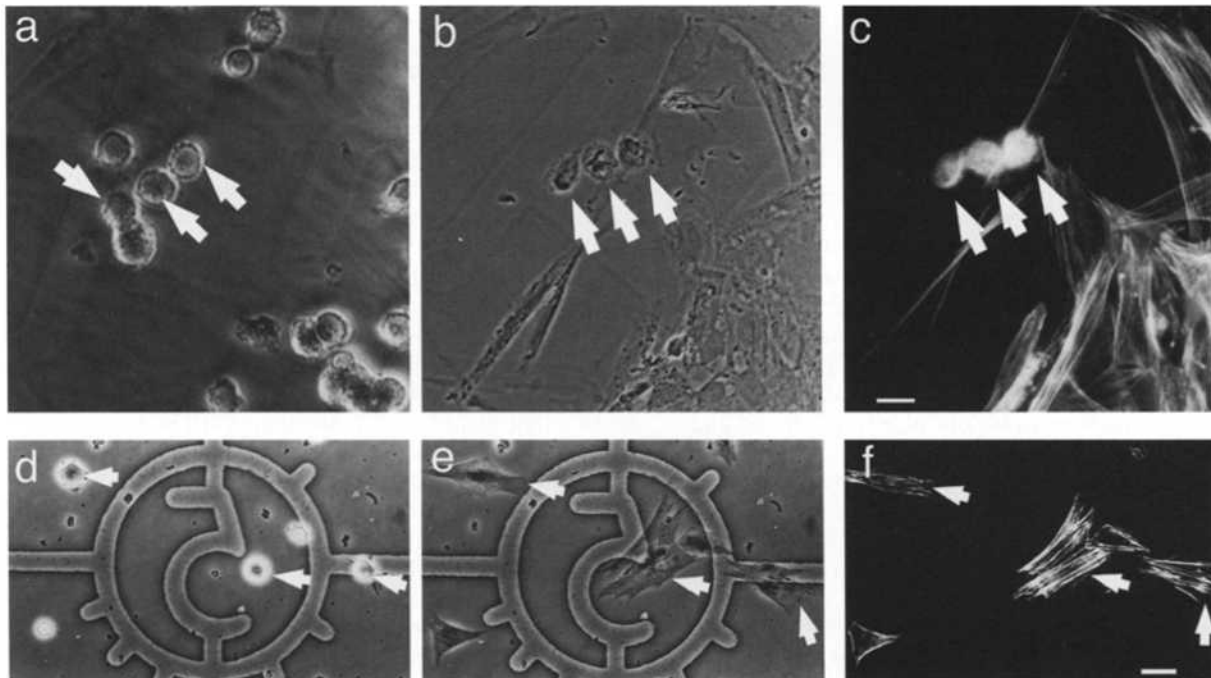

Figure 1. Cultured chick embryo fibroblasts were injected while rounded with either the SP1 peptide (*a–c*) or the randomly scrambled, SCR peptide (*d–f*). In *a*, the cells marked by the arrows were injected with SP1. At 4 h after injection, these cells were still rounded (*b*, *arrows*) and did not contain actin stress fibers, as shown by rhodamine-phalloidin labeling (*c*, *arrows*). Neighboring uninjected cells spread normally and contained fully developed stress fibers. Cells injected with the SCR peptide (*d*, *arrows*) spread normally by 4 h after injection (*e*, *arrows*) and contained a typical array of actin stress fibers (*f*, *arrows*). Bars: (*a–c*) 25 μm; (*d–f*) 50 μm.
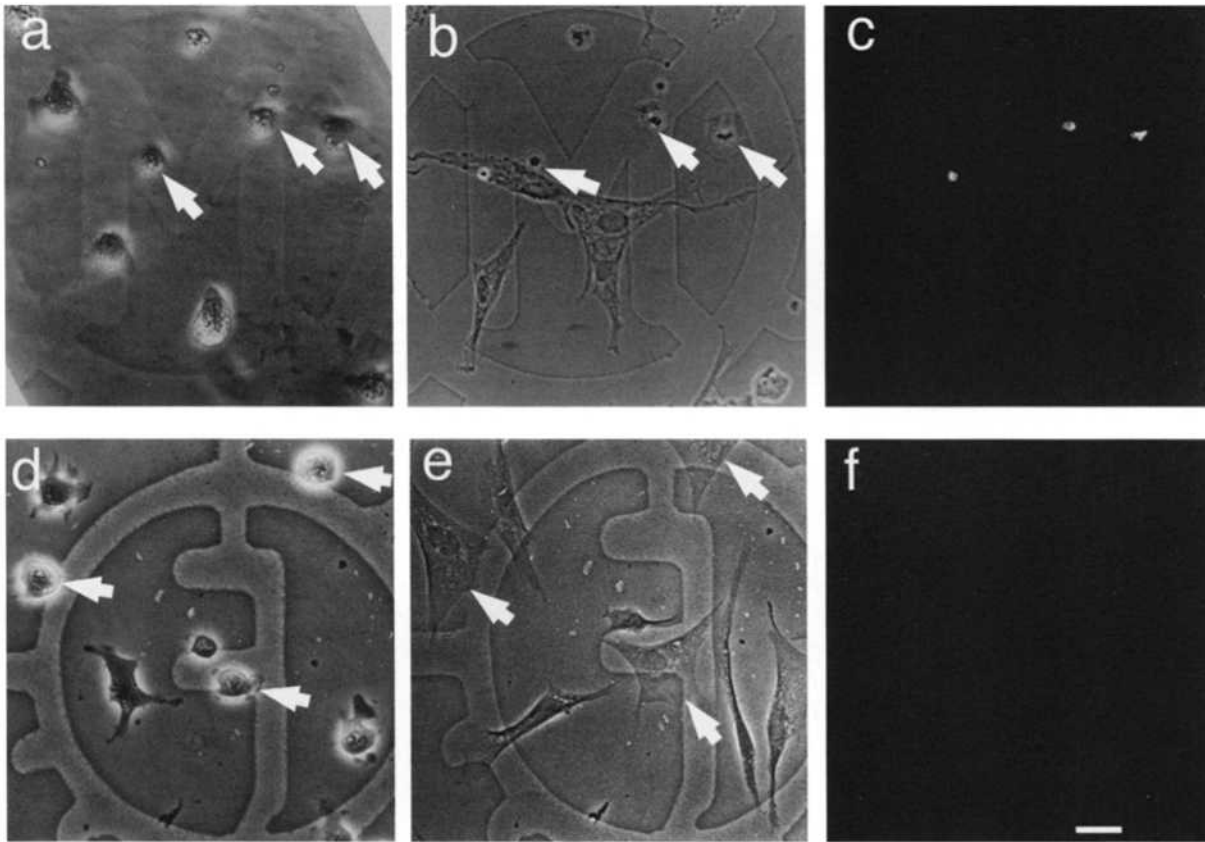
Figure 2. Cells injected with SP1 while rounded became apoptotic. The cells marked by arrows in *a* were injected with SP1, fixed at 2 h after injection (*b, arrows*), and labeled with the ApopTag in situ detection kit. As shown in *c*, apoptotic nuclei are strongly fluorescent and condensed. In contrast, cells injected with control peptide (*d, arrows*) were spread at 2 h after injection, and their nuclei failed to label with ApopTag (*f*). Bar, 25 μm.

As an alternative method of inhibiting pp125^{FAK} activation, cells were microinjected with an mAb to pp125^{FAK} called 2A7. This antibody binds to the COOH-terminal region of pp125^{FAK}, which contains the targeting sequence that is required for efficient recruitment of pp125^{FAK} to the focal adhesions. By injecting rounded cells with the 2A7 antibody, we expect to interfere with pp125^{FAK} localization to sites that contain integrins, thus interfering with integrin-mediated activation of pp125^{FAK}. The results of the 2A7 injections were similar to the SP1 peptide experiments: cells injected with 2A7 while rounded exhibited the morphology that is typical of apoptotic fibroblasts (Brancolini et al., 1995; Kulkarni and McCulloch, 1994) and had Apoptag-positive nuclei by 6 h after injection (Fig. 5, *a* and *b*), while cells injected with a control antibody to an unrelated chromosomal protein spread normally and did not apoptose (Fig. 5, *c* and *d*). The same result was obtained when the cells were injected with Fab fragments generated from the 2A7 antibody (data not shown).

We also asked if injection of the SP1 peptide or the 2A7 anti-FAK antibody would cause fully spread cells to lose their adhesions and become rounded. CEFs were plated onto fibronectin, allowed to spread for 2 h, and injected with either the SP1 peptide or 2A7 antibody. As shown in Fig. 6, cells injected with SP1 after they were fully spread exhibited normal nuclear morphology (Fig. 6 *a*), maintained normal actin stress fiber arrays (Fig. 6 *b*), and ex-

hibited no detectable loss of adhesion. When these cells were labeled with ApopTag reagents, no signs of nuclear fragmentation were apparent (data not shown). Similarly, injection of 2A7 into spread cells had no detectable effect on cellular morphology or adhesion (data not shown).

Our results suggest that activation of pp125^{FAK} via integrins is required for unspread fibroblasts to suppress the default pathway of apoptotic cell death. As there has been some question regarding the anchorage dependency of cultured fibroblasts, we used flow cytometry in combination with Apoptag staining to determine if CEFs become apoptotic when held in suspension for periods of up to 13 h. These experiments were performed both in the presence or the absence of serum, to determine if serum factors might protect the cells from apoptosis. As shown in Fig. 7, 44% of the fibroblasts became apoptotic by 13 h in the presence of serum, and 77% were apoptotic after 13 h in suspension in the absence of serum. These results indicate that primary chick embryo fibroblasts do undergo programmed cell death when deprived of contact with the extracellular matrix, and that factors present in serum may serve to protect the cells from the onset of apoptosis.

Discussion

Since the initial discovery and characterization of pp125^{FAK} (Guan et al., 1991; Schaller et al., 1992; Hanks et al.,
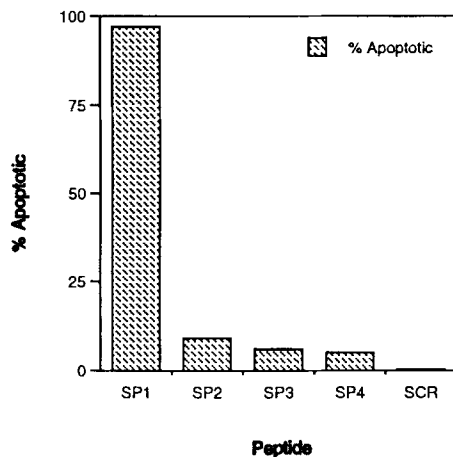
Figure 3. The effect of peptide SP1 is specific. Synthetic peptides SP1, SP2, SP3, and SP4 were coupled to carrier protein, injected into rounded CEFs, and analyzed with the ApopTag in situ detection kit. After injection of at least 100 CEFs with each peptide, only SP1 was shown to induce a significant amount of apoptosis (97%) in injected cells.

1992), a number of different functions have been proposed for this unusual tyrosine kinase. The observation that newly spreading cells contained elevated levels of phosphotyrosine led to the suggestion that $pp125^{FAK}$ might play a role in focal adhesion assembly during cell spreading. However, in one cell type (mouse aortic smooth muscle cells), $pp125^{FAK}$ does not appear to be kinase-active when the cells are spreading and beginning to assemble focal adhesions (Wilson et al., 1995). This argues that focal adhesion assembly is not strictly dependent upon $pp125^{FAK}$ activation. In addition, fibroblast-like cells grown from explants of a focal adhesion kinase (FAK)(−) mouse were able to spread in culture and to form focal adhesions and stress fibers (Ilic et al., 1995). Together, these data suggest that a role in focal adhesion assembly is not a universal or essential function of $pp125^{FAK}$.

Recently, a potential role for $pp125^{FAK}$ in the suppression of apoptosis has been suggested. Epithelial and endothelial cells, both in vitro and in vivo, were observed to have an absolute requirement for attachment to the ECM (Meredith et al., 1993; Brooks et al., 1994; Frisch and Francis, 1994; Re et al., 1994; Boudreau et al., 1995; Coucouvanis

and Martin, 1995; Pullan et al., 1996). When integrin–ECM interactions are inhibited, these cells undergo apoptosis. This type of anchorage dependency has been named "anoikis" by Frisch and Francis (1994). Several lines of evidence suggest that signal transduction events downstream of integrin–ligand engagement are involved in suppressing apoptosis in anchored cells. For example, attachment of mammary epithelium to the ECM via integrins was shown to regulate expression of interleukin-1β converting enzyme, a protein associated with apoptotic cell death (Boudreau et al., 1995). In addition, Meredith et al. (1993) have shown that the addition of vanadate, an inhibitor of protein tyrosine phosphatases, suppresses apoptosis in detached endothelial cells. Since activation of $pp125^{FAK}$ is known to contribute to the phosphotyrosine activity associated with integrin-mediated attachment, Ruoslahti and Reed (1994) have suggested that $pp125^{FAK}$ may be a key player in suppression of anoikis. This model gained support from the recent results of Frisch et al. (1996), who showed that constitutive activation of $pp125^{FAK}$ was sufficient to rescue MDCK cells from anoikis. Our data support the hypothesis that activation of $pp125^{FAK}$ via integrin engagement plays a critical role in the suppression of apoptosis.

Injection into rounded cells of the SP1 peptide, but not SP2–SP4, resulted in the rapid onset of apoptosis. We cannot rule out the possibility that other molecules may be affected by SP1 injection, since a surprisingly large number of proteins have been shown to colocalize with integrins in living cells (Miyamoto et al., 1995). To date, however, $pp125^{FAK}$ and paxillin are the only proteins that are believed to have a direct physical interaction with the region of the β_1 cytoplasmic tail represented by the SP1 peptide (Schaller et al., 1995). As an alternative approach for inhibiting the activation of $pp125^{FAK}$ in rounded CEFs, we also injected the cells with an anti-FAK mAb called 2A7. The 2A7 epitope has been mapped to a region of the $pp125^{FAK}$ COOH terminus that is adjacent to the focal adhesion targeting sequence. Thus, injection of 2A7 into unspread cells should specifically interfere with the efficient recruitment of $pp125^{FAK}$ into nascent focal adhesions. Although the SP1 peptide and the 2A7 antibody interfere with $pp125^{FAK}$ activation through different mechanisms, injection of either reagent gave the same result: cells became apoptotic within hours.
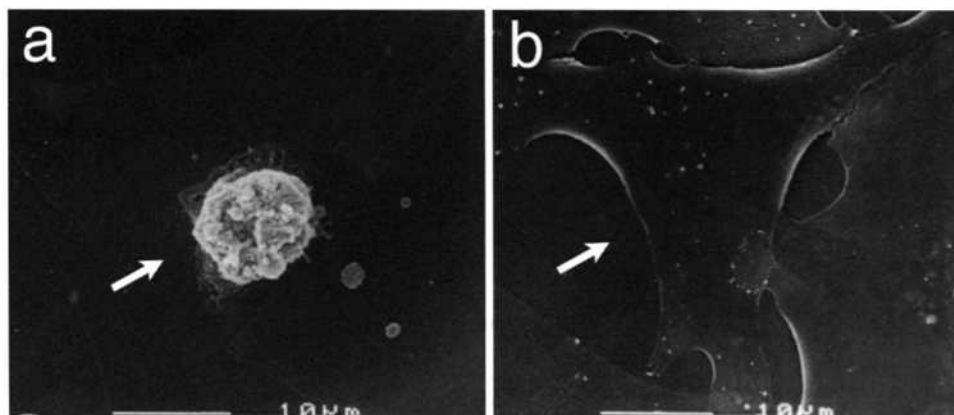

Figure 4. Surface morphology of injected cells was examined as an alternative means for assaying apoptosis. By scanning EM, SP1-injected cells were seen to display many small surface blebbs (g, arrow), while control injected cells had a normal, smooth surface morphology (h, arrow). Bar, 10 μm.
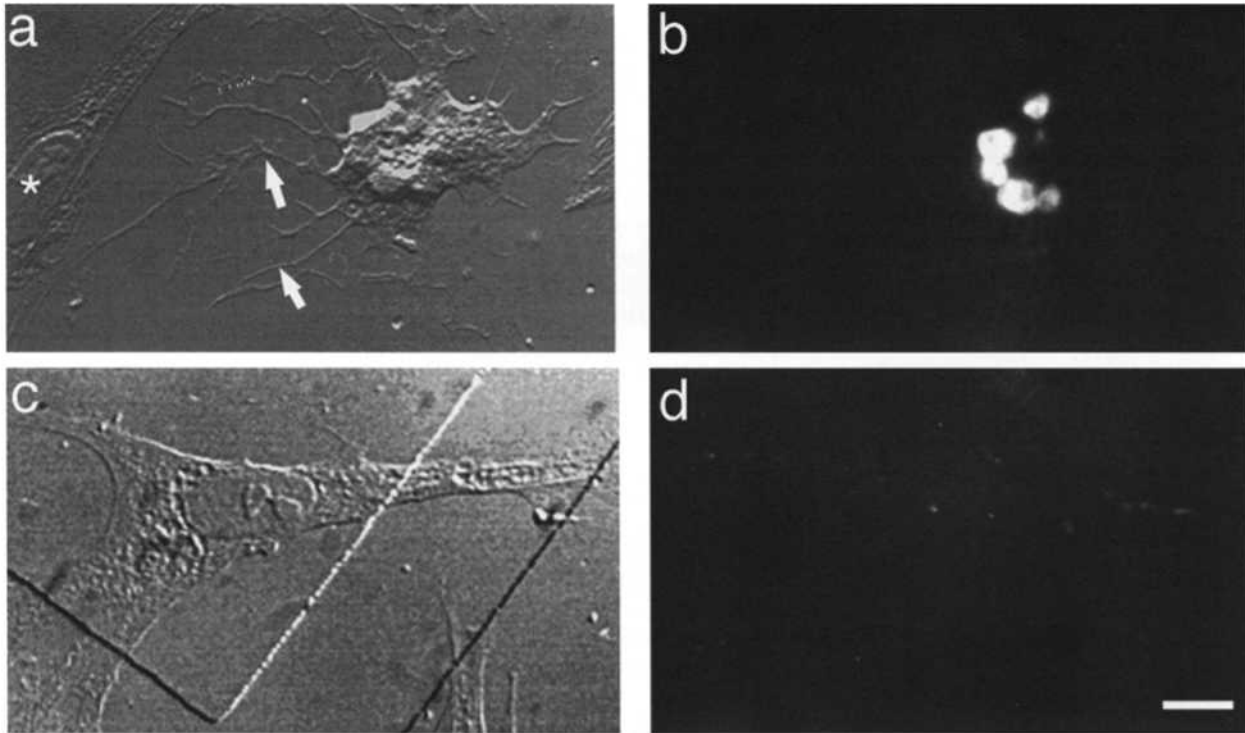
Figure 5. Cultured chick embryo fibroblasts were injected with an mAb to pp125^{FAK} (2A7; *a* and *b*) or with a control antibody to an unrelated protein (*c* and *d*). Cells injected while rounded with 2A7 were apoptotic by 6 h after injection (*a* and *b*). Some 2A7-injected cells were observed to extend many fine processes, as shown by the differential interference contrast image in *a* (*arrows*). Cells injected while rounded with the control antibody spread normally, exhibited typical nuclear morphology (differential interference contrast image; *c*), and did not become apoptotic (*d*). Bar, 10 μm.

The majority of cells injected with either the SP1 peptide or 2A7 antibody remained round and became apoptotic in 4–6 h. These results can be interpreted in two ways: (*a*) inactivation of pp125^{FAK} causes the cells to initiate an apoptotic program such that normal cell spreading is prevented; or (*b*) inactivation of pp125^{FAK} inhibits the cells from spreading, causing apoptosis. While it is not possible for us to distinguish between these mechanisms based upon results presented here, we have observed a subset of injected cells in which spreading occurs temporarily and is followed by rerounding and apoptosis (Hungerford, J.,

and C. Otey, unpublished observations). Future experiments in the laboratory will be directed toward understanding why this occurs in only a subset of injected cells.

Taken together, our results argue strongly that FAK plays a critical role in suppressing apoptosis. It appears that if signal transduction via pp125^{FAK} activation is prevented, either by interfering with recruitment of pp125^{FAK} to sites where integrins are localized or by inhibiting the binding of pp125^{FAK} to endogenous integrins through competition with a peptide, the default pathway is apoptotic death. When cells were allowed to spread fully before
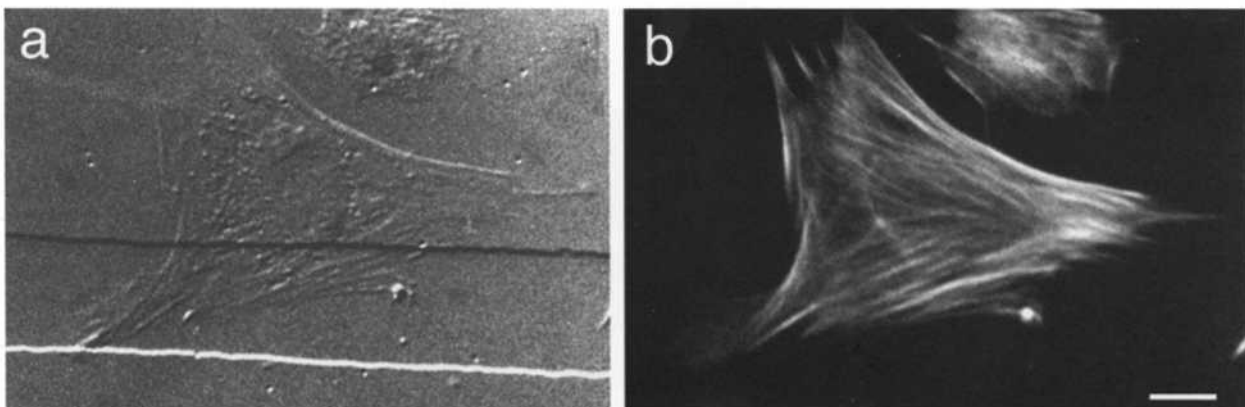

Figure 6. CEFs were allowed to spread completely and were then injected with either SP1 peptide or 2A7 mAb. Cells injected with SP1 remained spread after 4 h (*a*), exhibited normal nuclear morphology (*a*), and maintained actin stress fibers, as labeled by FITC-conjugated phalloidin (*b*). Bar, 10 μm.
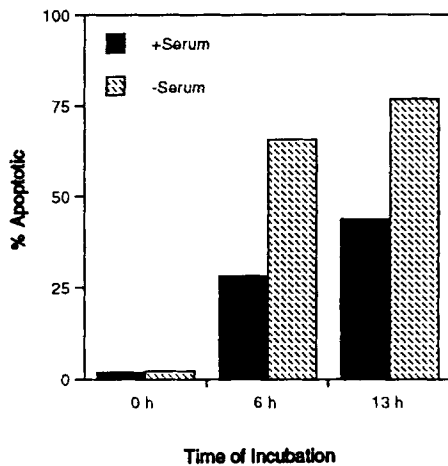
Time of Incubation

Figure 7. To analyze anchorage dependency in CEFs, cells were maintained in suspension in the presence or absence of serum. Aliquots of cells from each experimental condition were analyzed for the presence of apoptotic nuclei by flow cytometry of Apoptag-stained cells. CEFs underwent rapid apoptosis when deprived of contact with the ECM, and the rate of apoptosis was increased in the absence of serum.

injection with either the SP1 peptide or 2A7 antibody, they remained spread and did not become apoptotic. This result suggests that once $pp125^{FAK}$ has been incorporated into the focal adhesion complex, the molecule may no longer be accessible to bind either the integrin peptide or the anti-FAK antibody. Alternatively, additional signaling pathways may be functioning in a fully spread cell to inhibit cell death.

Two recent studies investigating potential mechanisms for the cellular regulation of FAK activity have focused on the COOH-terminal domain of FAK. Richardson and Parsons (1996) overexpressed in chicken fibroblasts a truncated isoform of FAK ($pp41/43^{FRNK}$), which is identical to the COOH-terminal domain of full-length FAK. They observed that the tyrosine phosphorylation of FAK was reduced by FRNK overexpression, suggesting that FRNK might act as a competitive regulator of FAK. In cells overexpressing FRNK, a delay in cell spreading was also observed. Gilmore and Romer (1996) used single-cell microinjection to introduce the COOH-terminal domain of FAK into cultured human umbilical vein endothelial cells (HUVEC) and observed a decrease in cell migration. Both of these results support a model in which FAK plays a role in the assembly of new focal adhesions. Neither study directly tested a role for FAK in suppressing apoptosis, although Gilmore and Romer (1996) observed that in some of their experiments up to 40% of the microinjected cells detached or died within 24 h.

Our results were surprising in that other researchers have reported that fibroblasts are not strongly anchorage dependent (Meredith et al., 1995; Frisch and Francis, 1994). In addition, when antisense oligonucleotides were used to attenuate the expression of $pp125^{FAK}$ in tumor cells, these cells lost their matrix attachment and became apoptotic, but no effect was observed in normal fibroblasts (Xu et al., 1996). However, it has been demonstrated that fibroblasts do apoptose under certain conditions such as

serum deprivation (Kulkaini and McCulloch, 1994; Ishizaki et al., 1995). Our data and that of others (Malik, R., and J.T. Parsons, manuscript in preparation) suggest that primary embryonic fibroblasts are very susceptible to apoptosis (anoikis) when held in suspension. Indeed, embryonic fibroblasts are similar to epithelial cells and endothelial cells in that apoptosis is a default pathway: active suppression of apoptosis is essential for cell survival (Ishizaki et al., 1995). Our flow cytometry data demonstrate that, in primary chick fibroblasts, anchorage to a substrate is an essential requirement for survival, but serum may help to protect unattached cells from undergoing apoptosis for periods of several hours.

In conclusion, our results suggest that events downstream from the activation of $pp125^{FAK}$ are required for anchorage-dependent cells such as fibroblasts to suppress apoptosis. The current challenge is to understand the steps that occur between the integrin-mediated activation of $pp125^{FAK}$ and the downstream effects in the nucleus.

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