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The Focal Adhesion Kinase Suppresses Transformation-associated, Anchorage-independent Apoptosis in Human Breast Cancer Cells

INVOLVEMENT OF DEATH RECEPTOR-RELATED SIGNALING PATHWAYS*

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The focal adhesion kinase (FAK) is a mediator of cell-extracellular matrix signaling events and is overexpressed in tumor cells. In order to rapidly down-regulate FAK function in normal and transformed mammary cells, we have used adenoviral gene transduction of the carboxyl-terminal domain of FAK (FAK-CD). Transduction of adenovirus containing FAK-CD in breast cancer cells caused loss of adhesion, degradation of $p125^{FAK}$, and induced apoptosis. Furthermore, breast tumor cells that were viable without matrix attachment also underwent apoptosis upon interruption of FAK function, demonstrating that FAK is a survival signal in breast tumor cells even in the absence of matrix signaling. In addition, both anchorage-dependent and anchorage-independent apoptotic signaling required Fas-associated death domain and caspase-8, suggesting that a death receptor-mediated apoptotic pathway is involved. Finally, FAK-CD had no effect on adhesion or viability in normal mammary cells, despite the loss of tyrosine phosphorylation of $p125^{FAK}$. These results indicate that FAK-mediated signaling is required for both cell adhesion and anchorage-independent survival and the disruption of FAK function involves the Fas-associated death domain and caspase-8 apoptotic pathway.

As normal epithelial cells become transformed and develop the capacity for invasion and metastasis, they must acquire the property of anchorage-independent growth. This is essential for tumor cells to survive the apoptotic stimuli associated with the loss of adhesion, proteolysis, and migration through their extracellular matrix (ECM)[1] (1, 2). Oncogenic transformation has been shown to suppress apoptosis as a means of enhancing tumor cell survival (3–6). Cells from human tumors have been shown to be more resistant to apoptotic stimuli than normal cells (7). Normal cells undergo apoptosis when they lose ECM adhesion, and this phenomenon has been termed "anoikis" (8, 9). Tumor cells have been thought to be resistant to anoikis, thus allowing them to grow in an anchorage-independent fashion. One of the critical signaling molecules involved in both cell-ECM interactions and anoikis is the focal adhesion kinase (FAK) (10), a tyrosine kinase that localizes to focal adhesions (11, 12).

Previous studies have shown that FAK is overexpressed in breast, colon, and thyroid cancers (13–17), whereas normal tissues express little detectable FAK. The overexpression of FAK in tumors is likely to affect three functions as follows: motility, adhesion, and survival. FAK is thought to play a role in adhesion-mediated survival because overexpression of a constitutively activated form of FAK in Madin-Darby canine kidney cells has been shown to confer resistance to apoptosis following loss of adherence (10). FAK overexpression in Chinese hamster ovary (CHO) cells caused an increase in migration (18), suggesting that FAK may play a role in motility of CHO cells. Although these experiments were performed in normal cells, they raise the possibility that tumor cells up-regulate FAK expression in order to leave their tissue of origin, invade their surrounding stroma, and migrate into new environments. However, FAK overexpression has also been demonstrated in preinvasive tumors (13), suggesting a role for FAK in tumors that occurs before development of anchorage-independent growth potential.

Earlier work from this laboratory (19) raised the possibility of an additional function for FAK in tumor cells, that FAK might act as an inhibitor of apoptosis. Attenuation of FAK expression by antisense oligonucleotides led to loss of adhesion and subsequent apoptotic cell death of a variety of tumor cell types, suggesting that FAK plays a role in tumor cell survival (19). This resembles the work of Frisch *et al.* (10), who demonstrated that an activated form of FAK leads to resistance to anoikis in the absence of adhesion. Finally, Ilic *et al.* (20) have also proposed that FAK acts as a survival signal in murine FAK-deficient ES cells deprived of ECM attachment.

The carboxyl-terminal domain of FAK (FAK-CD) is analogous to the FRNK molecule (FAK-related non-kinase) that is expressed as a separate transcript in chick embryo fibroblasts (21), which is initiated from an alternative promoter within an intron (22). Avian FRNK decreases phosphorylation of $p125^{FAK}$ and the protein paxillin and slows the spreading of avian fibroblasts (23). Our group has shown (24) that inducible ectopic FAK-CD expression resulted in decreased $p125^{FAK}$ tyrosine

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[1] The abbreviations used are: ECM, extracellular matrix; FAK, focal adhesion kinase; FAK-CD, FAK COOH-terminal domain (human homologue of FRNK); AdFAK-CD, adenovirus containing FAK-CD; FRNK, FAK-related non-kinase; FADD, Fas-associated death domain; ΔFADD, dominant-negative version of FADD; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; BPE, bovine pituitary extract; poly-HEMA, poly(2-hydroxyethylmethacrylate); BrdUrd, 5-bromodeoxyuridine; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; TUNEL, TdT-mediated dUTP nick end labeling; PARP, poly-(ADP-ribose) polymerase; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; HME, human mammary epithelial.

phosphorylation, loss of adhesion, and cell death in human breast cancer cells. However, each of these approaches was limited by various factors, including the cell type of origin, the efficiency of gene expression, or the means of inducing FAK-CD expression. Thus, the mechanisms that are involved in the cell death by expression of FAK-CD remained largely unknown.

In this report, we have utilized an adenoviral gene transduction system to interrupt rapidly FAK function, and we demonstrated that adenoviral transduction of FAK-CD caused loss of adhesion and apoptotic cell death in tumor cells and corresponded with loss of endogenous $p125^{FAK}$ from focal adhesions. Apoptosis occurred independently of cell adhesion and ECM signaling but required FADD, caspase-8, and caspase-3, suggesting an important role of the FAK signaling pathway in inhibiting death receptor or death receptor-related apoptosis. In contrast, loss of endogenous $p125^{FAK}$ from the focal adhesions of normal mammary epithelial cells had no effect on adhesion or viability. Thus, we have shown that FAK has two distinct functions in tumor cells, promoting adhesion to ECM and providing survival signals independent of matrix attachment.

EXPERIMENTAL PROCEDURES

Cell Culture—BT474 and MCF-7 human breast carcinoma cells were purchased from ATCC, and the MCF-10A human mammary epithelial cells (kindly provided by Dr. Channing Der) were cultured as described (24–26). Human mammary epithelial cells, isolated from normal human mammary tissue, were obtained from Clonetics (San Diego, CA) and maintained in mammary epithelial cell growth medium (Clonetics) supplemented with 10 ng/ml recombinant human epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 50 μg/ml gentamicin, 50 ng/ml amphotericin-B, and 52 μg/ml bovine pituitary extract. 293 human primary embryonal kidney cells were cultured in DMEM containing 10% fetal bovine serum. All of the cell lines were incubated at 37 °C in 5% CO_2.

Construction and Preparation of Recombinant Adenoviruses—FAK-CD, amino acids 693–1052 of FAK fused to the HA epitope tag, was cloned into the pCRII vector (Invitrogen) as described previously (24). pCRII-FAK-CD was digested with *Kpn*I and inserted into the *Kpn*I site of the adenoviral shuttle vector pACCMV.PLPASR(+). We named this plasmid pACCMV-FAK-CD. Recombinant virus was generated by transfection of 293 human embryonic kidney cells with 10 μg of pACCMV-FAK-CD and 10 μg of adenovirus type 5 dl309 plasmid (27), digested with *Xba*I and *Cla*I. After transfection, cells were overlaid with DMEM containing 10% fetal bovine serum and 2% low melt agarose and then were fed every 3 days. After 11 days, plaques were picked using a Pasteur pipette and inoculated into 0.5 ml of media. 100 μl of the media was used to amplify plaques by infecting confluent 293 cells. The recombinant virus containing FAK-CD, called AdFAK-CD, was confirmed by amplifying the FAK-CD-pACCMV junction sequence by polymerase chain reaction and sequencing the polymerase chain reaction fragment at the University of North Carolina Automated Sequencing Facility. HA-tagged expression of FAK-CD was also confirmed by Western blot analysis of infected 293 cells using an anti-HA monoclonal antibody (12CA5, Roche Molecular Biochemicals).

Large amounts of AdFAK-CD were generated by subsequent infection of 293 cells with AdFAK-CD for 48 h in DMEM containing 2% fetal bovine serum. Cells were then harvested, resuspended in PBS, and lysed by freezing and thawing three times. Cell debris was removed by centrifugation, and the supernatant containing AdFAK-CD was purified by two cesium chloride density centrifugations (27). The number of virions per ml was estimated by measuring absorbance at 260 nM (A_{260}), where an A_{260} unit is approximately 10^{12} virions. Concentrated virus was dialyzed, aliquoted, and stored at −70 °C. The adenovirus type 5, containing an AU-1 tagged dominant-negative version of FADD (ΔFADD or NFD4), has been described elsewhere (28, 29). Adenovirus carrying the *lacZ* gene, which expresses β-galactosidase protein, was provided by Dr. J. Samulski (University of North Carolina, Chapel Hill) and served as a control. The number of virions was confirmed as described above for the AdFAK-CD virus.

Adenoviral Infections—Cells were plated at 1.5×10^6 per 100-mm culture plates and allowed to attach for 24 h and then infected with AdFAK-CD or Ad*lacZ* for various time points at an optimal concentration of virus in complete media (Table I). Optimal concentrations of virus were determined by infection of cells with the β-galactosidase-expressing virus (Ad*lacZ*) at different doses and subsequent staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). We used titers that caused expression of β-galactosidase in 100% of the cells without visible toxic effect. For AdFAK-CD, cells were similarly transduced with a range of viral concentrations. We typically infected cells with 2×10^3 virions per cell and detected HA-tagged FAK-CD expression in greater than 90% of the cells using an anti-HA monoclonal antibody (HA11, Babco, Richmond, CA) by fluorescence microscopy. For co-infection experiments, cells were infected with AdFAK-CD or Ad*lacZ* together with ΔFADD adenoviruses at various virions per cell for 24 h. Expression of FAK-CD or ΔFADD was verified by Western blotting using anti-HA or anti-AU-1 (Babco) monoclonal antibodies.

Poly-HEMA-coated Tissue Culture Dishes and Suspension Cell Culture—Tissue culture dishes were coated with a film of poly-HEMA (Aldrich) as described by Folkman and Moscona (30). Briefly, a 12% solution of polyhydroxyethylmethacrylate polymer (poly-HEMA) in 95% ethanol was mixed overnight, centrifuged at 2,500 rpm to remove undissolved particles, and diluted 1:10 with 95% ethanol. 100-mm dishes were coated with 4 ml of poly-HEMA solution and left to dry at room temperature. Dishes were washed twice with PBS and once with HBSS before use.

BT474 cells were typsinized, resuspended, and added to the poly-HEMA coated dishes at a density of 1.5×10^6 per dish in serum-containing medium. Cells were maintained in suspension culture for various time points and assayed for proliferation, including growth rate, BrdUrd incorporation, or cell survival, such as TUNEL and MTT assay as described below.

For adenoviral infection of BT474 cells grown in suspension culture, 1.5×10^6 cells were added to poly-HEMA coated tissue culture dishes and then immediately infected with AdFAK-CD or Ad*lacZ* at a concentration of 2×10^3 virions per cell and incubated for 24 h. For co-infection experiments, cells were infected with AdFAK-CD or Ad*lacZ* together with various concentrations of adenovirus containing ΔFADD and incubated for 24 h. Cells were then harvested and subjected to analyses.

Treatment with Caspase Inhibitors—A total of 1.5×10^6 BT474 cells were plated on 100-mm uncoated tissue culture plates or plates coated with poly-HEMA and treated with Ac-DEVD-CHO, Ac-YVAD-CHO (Babco), or benzyloxycarbonyl-IETD-fluoromethyl ketone (Enzyme Systems Products, Livermore, CA) at various concentrations for 6 h. Cells were subsequently infected with AdFAK-CD or Ad*lacZ* for 24 h and then harvested and assayed for apoptosis using the ApopTag kit (Intergen, Purchase, NY) as described below.

BrdUrd Labeling and Detection—Cells were maintained in suspension culture for 18, 42, or 66 h in serum-containing medium, and then labeled with 30 μM BrdUrd (5-bromodeoxyuridine) for an additional 6 h. Cells were then harvested and fixed in 70% ethanol for 30 min. Subsequently, cells were treated with 2 N HCl containing 0.5% Triton X-100 for 30 min at room temperature followed by 0.1 M $Na_2B_4O_7$ for 5 min. After washing with PBS, the cells were blocked with 0.5% bovine serum albumin for 30 min at room temperature. Anti-BrdUrd monoclonal antibody (Ab2, Calbiochem) was added at a concentration of 1 μg/ml to the cells and incubated for 1 h followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG. BrdUrd incorporation was determined by the number of positive cells *versus* the number of total cells using a Zeiss fluorescence microscope.

Assays of Cell Viability—Detection of apoptosis was performed by TUNEL assay using the ApopTag kit (Intergen, Purchase, NY) according to manufacturer's protocol. Cell survival was assayed by measuring mitochondrial dehydrogenase activity, conversion of soluble MTT into an insoluble formazan product as described by Mosmant (31). Briefly, BT474 cells grown in poly-HEMA-coated or uncoated tissue culture dishes for 24 h were plated into a 96-well tissue culture plate at a density of 2,500 cells per well in 100 μl of medium and allowed to attach overnight at 37 °C. 10 μl of MTT solution (5 mg/ml in PBS, Sigma) was added to each well and incubated for 1.5 h at 37 °C. The reaction was stopped by removal of the supernatant followed by addition of 100 μl of Me_2SO. The plate was left at room temperature for 15 min with gently rocking, and the absorbance at 595 nm was measured using a kinetic microplate reader (V_{max}, Molecular Devices). The percentage of survival was calculated as cells grown in poly-HEMA experiment/cells grown in monolayer culture × 100.

Immunofluorescence, Western Blotting, and Immunoprecipitation—Immunofluorescent staining for HA, $p125^{FAK}$, or paxillin using the anti-HA (HA11, Babco), anti-FAK, and anti-paxillin (Transduction Laboratories) monoclonal antibodies has been described previously (24). Tyrosine phosphorylation of $p125^{FAK}$ was analyzed by immunoprecipitation using the anti-FAK 4.47 (Upstate Biotechnology, Inc.) monoclonal antibody or C20 (Santa Cruz Biotechnology) polyclonal antibody,

followed by Western blotting with an anti-phosphotyrosine monoclonal antibody (4G10, Upstate Biotechnology, Inc.) as described (24). The expression of FAK-CD, $p125^{FAK}$, vinculin, or poly-(ADP-ribose) polymerase (PARP) was analyzed by Western blot using the anti-HA (12CA5, Roche Molecular Biochemicals), anti-FAK (C20, Santa Cruz Biotechnology, or clone 77, Transduction Laboratories), anti-vinculin (Sigma), and anti-PARP (Roche Molecular Biochemicals) antibodies.

RESULTS

Down-regulation of FAK Causes Loss of Cellular Adhesion and Induces Apoptosis in Breast Cancer Cells—To interrupt FAK signaling function, we generated an adenoviral FAK-CD construct containing amino acids 693–1052 of the FAK protein, fused in-frame at the amino-terminal end with a single copy of the HA epitope tag. Human BT474 breast cancer cells were infected with AdFAK-CD for 4, 8, 16, and 24 h. The expression of FAK-CD was detected as early as 4 h post-infection and reached the highest level between 8 and 24 h after infection (Fig. 1*A, top panel*). By 16 h following infection, FAK-CD led to loss of 125^{FAK} expression (Fig. 1*A, middle panel*). This loss of $p125^{FAK}$ expression was not due to a general degradation of cellular proteins, because the focal adhesion protein vinculin was stable throughout the experiment (Fig. 1*A, bottom panel*). Preceding this loss of $p125^{FAK}$ expression, the level of $p125^{FAK}$ tyrosine phosphorylation had dramatically decreased, and by 8 h, tyrosine-phosphorylated $p125^{FAK}$ was no longer detectable (Fig. 1*B*).

The expression of FAK-CD was also analyzed by immunofluorescence microscopy. Following 4 h of transduction, FAK-CD expression was detected at the focal adhesions as well as the cytoplasm (Fig. 2*A*). At 6 h some focal adhesion staining was still visible, but by 8 h, the cells had become rounded (Fig. 2*A*). By 16 h, greater than 90% of the cells expressing FAK-CD had lost adhesion and become suspended.

To elucidate whether FAK-CD expression led to loss of endogenous $p125^{FAK}$ from the focal adhesions, dual immunofluorescence microscopy was performed to co-localize $p125^{FAK}$ and FAK-CD, as well as $p125^{FAK}$ and paxillin. Before AdFAK-CD was transduced, endogenous $p125^{FAK}$ was detected in focal adhesions of BT474 cells, using an antibody to the kinase domain of FAK that does not recognize FAK-CD (data not shown). Following 6 h of transduction, $p125^{FAK}$ was no longer detectable at the focal adhesions of the cells that expressed FAK-CD (Fig. 2*B, panels a* and *b*). However, at this time point, the focal adhesions were still intact as demonstrated by paxillin staining (Fig. 2*B, panels* c and *d*). Cells infected with control *lacZ* virus maintained $p125^{FAK}$ and paxillin expression at the focal adhesions (Fig. 2*B, panels e–h*). These results demonstrate that expression of FAK-CD was associated with loss of the endogenous $p125^{FAK}$ from focal adhesions, resulting in a loss of cellular adhesion.

To determine the fate of the detached cells, we analyzed

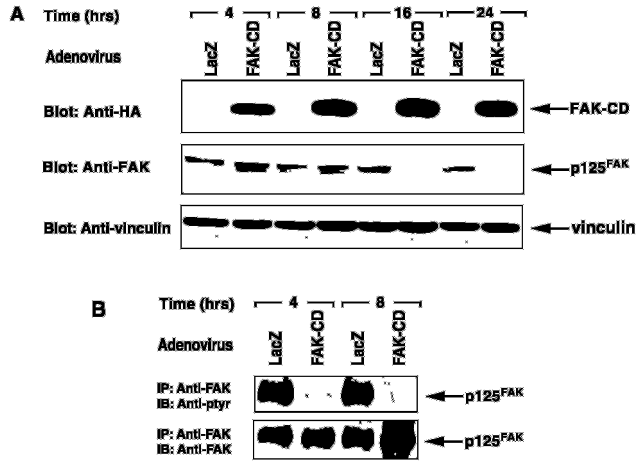

FIG. 1. **Western blot analysis of FAK-CD expression and tyrosine phosphorylation of $p125^{FAK}$ in BT474 cells.** *A,* expression of FAK-CD, $p125^{FAK}$, and vinculin following AdFAK-CD transduction in BT474 cells. Human BT474 breast cancer cells were infected with AdFAK-CD or a control *lacZ* adenovirus for 4, 8, 16, or 24 h and then lysed in Nonidet P-40 buffer. The expression of FAK-CD, $p125^{FAK}$, or vinculin was analyzed by Western blot using an anti-HA (12CA5, Roche Molecular Biochemicals) (*top panel*), anti-FAK (Transduction Laboratories), or anti-vinculin (Sigma) monoclonal antibodies. *B,* tyrosine phosphorylation of $p125^{FAK}$ following AdFAK-CD transduction in BT474 cells. Tyrosine phosphorylation and expression of $p125^{FAK}$ was analyzed by immunoprecipitating (*IP*) $p125^{FAK}$ with the anti-FAK monoclonal antibody (4.47, Upstate Biotechnology, Inc.) and then immunoblotting (*IB*) with either anti-phosphotyrosine antibody (4G10, Upstate Biotechnology, Inc.) or the anti-FAK (4.47) antibody.

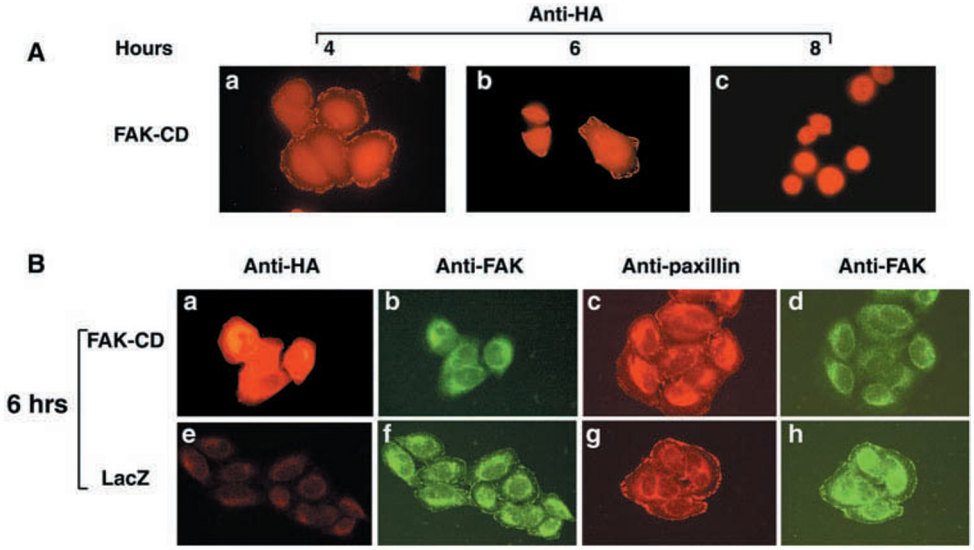

FIG. 2. **Expression of FAK-CD in the breast tumor cells led to loss of endogenous $p125^{FAK}$ from the focal adhesions, cellular rounding, and loss of adhesion.** *A,* BT474 cells were infected with AdFAK-CD for 4, 6, or 8 h and analyzed for FAK-CD expression by immunofluorescence microscopy using an anti-HA antibody. FAK-CD localized to the focal adhesions as well as the cytoplasm of the cells (*panels a* and *b*). By 8 h, the cells had become rounded (*panel c*). *B,* BT474 cells were transduced with AdFAK-CD for 6 h, and dual immunofluorescence microscopy was performed to co-localize endogenous $p125^{FAK}$ and FAK-CD, as well as $p125^{FAK}$ and paxillin, using the anti-HA (HA11, Babco), anti-FAK kinase domain (clone 77, Transduction Laboratories), or anti-paxillin (clone 349, Transduction Laboratories) monoclonal antibodies.

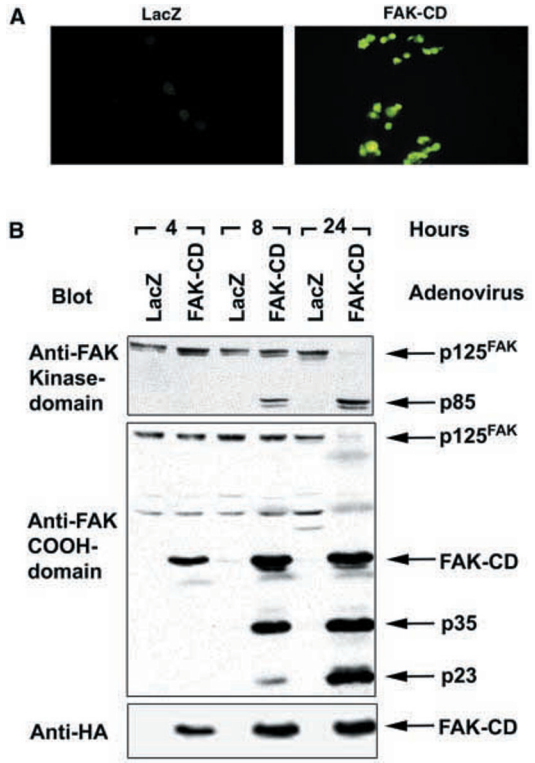

FIG. 3. **Induction of apoptosis and degradation of p125^{FAK} in BT474 cells by FAK-CD.** *A,* BT474 cells were infected with Ad-FAK-CD or Ad*lacZ* for 16 h, and apoptosis was detected by the TUNEL assay using the ApopTag kit (Intergen). The percentage of apoptotic cells was determined by counting a total of 100 cells in 3 random fields (40 times) and dividing the number of apoptotic cells by the number of total cells. *B,* BT474 cells were infected with AdFAK-CD or a control *lacZ* adenovirus for 4, 8, or 24 h. The expression and degradation of p125^{FAK} were analyzed by Western blot using the anti-FAK kinase domain monoclonal antibody (clone 77, Transduction Laboratories) (*upper panel*), anti-FAK COOH-terminal domain polyclonal antibody (C20, Santa Cruz Biotechnology) (*middle panel*), or anti-HA (12CA5) monoclonal antibody (*lower panel*).

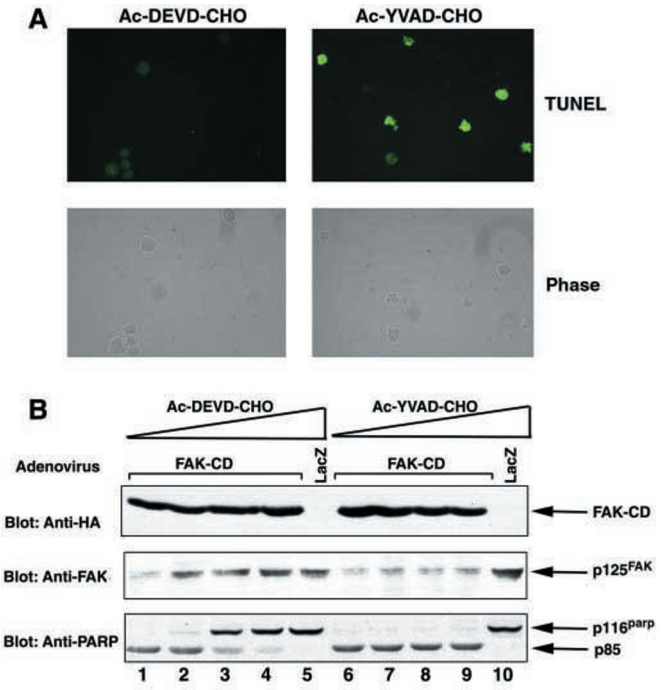

FIG. 4. **Pretreatment of BT474 cells with caspase-3 inhibitor followed by the transduction of AdFAK-CD blocked apoptosis and p125^{FAK} degradation.** *A,* BT474 cells were treated with 50 μM Ac-DEVD-CHO or Ac-YVAD-CHO for 6 h and then transduced with AdFAK-CD or Ad*lacZ* for 16 h. Apoptosis was analyzed by TUNEL assay using the ApopTag kit. *B,* BT474 cells were treated with Ac-DEVD-CHO (*lanes 1–5*) or Ac-YVAD-CHO peptide (*lanes 6–10*) at concentrations of 1 (*lanes 1* and *6*), 5 (*lanes 2* and *7*), 25 (*lanes 3* and *8*), or 50 (*lanes 4, 5, 9,* and *10*) μM for 6 h and then transduced with Ad-FAK-CD or Ad*lacZ* for 16 h. Expression of FAK-CD or p125^{FAK} was analyzed by Western blot using an anti-HA monoclonal antibody (12CA5, *upper panel*) or the anti-FAK polyclonal antibody (C20, *middle panel*). The inhibition of caspase-3 was demonstrated by Western blot using an anti-PARP polyclonal antibody (Roche Molecular Biochemicals) (*lower panels*).

TABLE I
Induction of loss of adhesion and apoptosis by transduction with Ad-FAK-CD

Cell line	Virions/cell	Loss of adhesion[a]	Apoptosis[b]
		%	
BT474	2×10^3	99.3 ± 1.6	86.1 ± 5.3
MCF-7	1×10^4	92.7 ± 2.3	45.0 ± 2.4
MCF-10A	1×10^4	0.1 ± 0.1	0
HME cells	1×10^4	1.1 ± 0.5	0

[a] The percentage of loss of adhesion was determined by dividing the number of detached cells *versus* the number of total cells following 24 h of AdFAK-CD transduction. This result was observed using two different preparations of adenovirus in more than three separate experiments for each preparation. In contrast, cells treated with an adenovirus containing the *lacZ* gene showed no significant loss of cellular adhesion.

[b] Detection of apoptosis was performed by TUNEL assay. The percentage of apoptosis was determined by dividing the number of apoptotic cells *versus* the number of total cells in ten × 40 microscopic fields with a fluorescence microscope. Data are presented as mean ± S.D. of three independent experiments.

them for apoptosis by TUNEL assay 16 h after FAK-CD transduction (Fig. 3*A*), and we showed an average of 86% of these cells underwent apoptosis (Table I). Furthermore, we could not detect the expression of endogenous p125^{FAK}, but instead we detected its degradation products of 85- (Fig. 3*B, upper panel*), 35-, and 23-kDa proteins (Fig. 3*B, middle panel*) as the cells underwent apoptosis. We cannot exclude the possibility that the two smaller proteins represent degradation of FAK-CD. This is consistent with the observation of Wen *et al*. (32) that p125^{FAK} is degraded during apoptosis. In contrast, cells infected with equivalent amounts of an adenovirus containing the *lacZ* gene remained adherent to the tissue culture dish, and these cells failed to show signs of apoptosis (Fig. 3*A*). To test whether other breast cancer cell lines were sensitive to FAK-CD induced apoptosis, we infected the MCF-7 breast cancer cell line and obtained similar results. More than 90% of the cells lost adhesion, and 45% of these cells displayed morphological signs of apoptosis (Table I). Thus, the induction of loss of adhesion and apoptosis by FAK-CD is not restricted to the BT474 cell line.

FAK Function Has a Direct Effect on Tumor Cell Adhesion to the ECM—Our initial experiments did not distinguish whether interruption of FAK function disrupted adhesion, leading to apoptosis, or whether FAK-CD directly induced apoptosis, with subsequent loss of adhesion. To address this, BT474 cells were pretreated with increasing doses of the peptide Ac-DEVD-CHO, a caspase-3 inhibitor, and then infected with AdFAK-CD. After 16 h of FAK-CD transduction, greater than 90% of cells lost adhesion, but these cells did not undergo apoptosis (Fig. 4*A, left panels*) and the degradation of endogenous p125^{FAK} was inhibited in a dose-dependent fashion (Fig. 4*B, middle panel*). In contrast, treating cells with a control peptide Ac-YVAD-CHO, the interleukin-converting enzyme inhibitor, followed by FAK-CD transduction did not block loss of adhesion, apoptosis (Fig. 4*A, right panels*), or p125^{FAK} degradation (Fig.

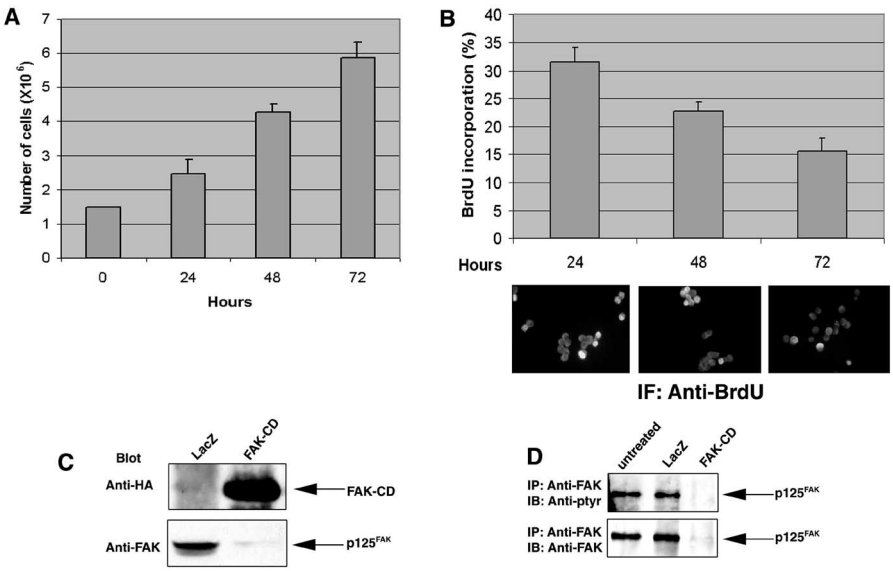

FIG. 5. **Transduction of FAK-CD caused dephosphorylation and degradation of p125^{FAK} in BT474 cells grown in suspension culture.** *A,* a total of 1.5×10^6 BT474 cells were added to 100-mm poly-HEMA-coated tissue culture dishes in medium containing 10% fetal bovine serum. Cells were harvested and stained with trypan blue, and the trypan-blue exclusive cells were counted at 24, 48, or 72 h. *B,* BT474 cells were grown in poly-HEMA-coated dishes in serum-containing medium for 18, 42, or 66 h and then labeled with BrdUrd for an additional 6 h. Cells were then harvested, fixed, and stained with anti-BrdUrd monoclonal antibody (Ab2, Calbiochem) as described under "Experimental Procedures." BrdUrd-positive cells were counted and photographed using a Zeiss fluorescence microscope. *C,* 1.5×10^6 BT474 cells were added on 100-mm tissue culture plates coated with poly-HEMA and then transduced with either Ad*lacZ* control or with AdFAK-CD for 24 h. The expression of FAK-CD and p125^{FAK} was analyzed by Western blotting using an anti-HA (12CA5) or anti-FAK (Transduction Laboratories) monoclonal antibodies. *D,* 1.5×10^6 BT474 cells were added in poly-HEMA-coated tissue culture dishes and then left untreated or treated with AdFAK-CD or control *lacZ* adenovirus for 24 h. Tyrosine phosphorylation and expression of p125^{FAK} were analyzed by immunoprecipitating p125^{FAK} with the anti-FAK polyclonal antibody (C20) and then immunoblotting with either anti-phosphotyrosine antibody (4G10) or the anti-FAK antibody (C20).

4*B*). Treatment of BT474 cells with the Ac-DEVD-CHO peptide inhibited caspase-3 compared with cells treated with Ac-YVAD-CHO (Fig. 4 *B*). A dose-dependent inhibition of caspase-3 was also confirmed by a decrease in cleavage of a caspase-3 substrate, PARP in treated cells (Fig. 4*B, bottom panel*). This demonstrates that FAK-CD causes a loss of adhesion and that subsequent p125^{FAK} degradation and apoptosis occur through a caspase-3-dependent mechanism. Therefore, we propose that expression of FAK-CD caused loss of adhesion, degradation of p125^{FAK}, and finally caspase-3-dependent apoptosis in tumor cells.

FAK Function Is Essential for the Survival of Breast Tumor Cells Grown without Matrix Attachment—The above experiments suggested that FAK prevented death in tumor cells by aiding in adhesion to the cellular substratum. However, FAK may also have a role as a survival signal that is independent of adhesion. To test this hypothesis, BT474 cells were maintained in poly-HEMA-coated tissue culture plates which allows for anchorage-independent growth. These cells grew predominantly as a single cell suspension culture and remained viable as determined by mitochondrial dehydrogenase activity using the MTT assay (data not shown). In addition, these cells proliferated (Fig. 5*A*) and incorporated BrdUrd (Fig. 5*B*) up to 72 h after they were subjected to suspension culture. By 72 h reduction of BrdUrd uptake correlated with a reduced rate of cellular growth. Surprisingly, p125^{FAK} remained tyrosine-phosphorylated even in the absence of ECM signaling (Fig. 5*D*), suggesting that these cells harbor a mechanism for constitutively activating p125^{FAK} that is independent of adhesion. However, when BT474 cells grown in suspension were transduced with AdFAK-CD, the endogenous p125^{FAK} was degraded (Fig. 5, *C* and *D*), and an average of 82% of these cells underwent apoptosis after 24 h of infection. In contrast, cells infected with the control adenovirus showed no detectable apoptosis. This result demonstrates that FAK-CD does not induce apoptosis solely by interrupting cellular adhesion and suggests that FAK plays an additional role as a survival signal in tumor cells that is independent of matrix signaling.

The Apoptotic Pathway Triggered by Down-regulation of FAK Requires FADD and Caspase-8 Activation—Having shown that expression of FAK-CD caused activation of caspase-3 and induced apoptosis in BT474 cells, we wished to identify the upstream components that were involved in the apoptotic pathway. One of these upstream molecules is caspase-8, an initiator caspase, which has been implicated in diverse forms of apoptosis (33). Thus, we examined caspase-8 activation in apoptotic cells treated with AdFAK-CD. The expression of FAK-CD resulted in a strong activation of caspase-8 as measured by Western blotting using an antibody that detects the pro-form of caspase-8 (Fig. 6*B, 2nd* and *7th lanes*). To test whether caspase-8 activation is required for FAK-CD-induced apoptosis, cells were pretreated with increasing concentrations of a caspase-8 specific inhibitor, benzyloxycarbonyl-IETD-fmk, for 6 h followed by infection with AdFAK-CD or control virus. These results showed that inhibition of caspase-8 prevented degradation of p125^{FAK} (Fig. 6*A, lower panel*) and inhibited FAK-CD-induced apoptosis in a dose-dependent manner (Fig. 6*A, upper panel*). Blockage of apoptosis by the caspase-8 inhibitor is independent of matrix adhesion, since the cells grown in suspension resembled cells grown in monolayer culture (Fig. 6*A*).

Caspase-8 is thought to be recruited and activated by Fas-associated death domain (FADD) protein in death receptor-mediated apoptosis (34, 35), raising the possibility that FAK-CD might trigger a FADD-dependent apoptotic pathway. To test this, caspase-8 activation was blocked by expressing a dominant-negative version of FADD (ΔFADD) that lacks the death effector domain (amino acids 1–79) (28). AU1-tagged ΔFADD was introduced into the cells by adenovirus transduction, and high levels of ΔFADD were detected in cells treated at all concentrations of adenovirus (data not shown). Expression of ΔFADD inhibited caspase-8 activation as demonstrated by

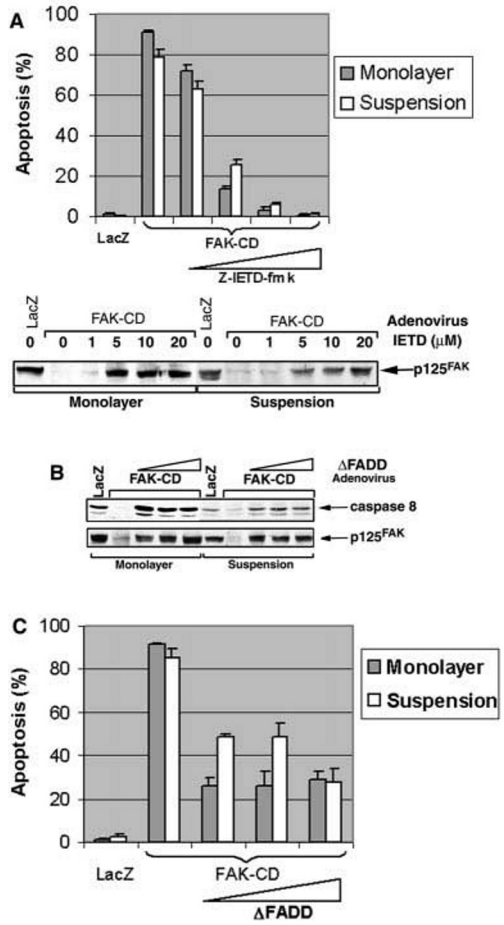

FIG. 6. **Inhibition of caspase-8 or ectopic expression of the dominant-negative version of FADD (ΔFADD) blocked apoptosis and p125^{FAK} degradation.** *A,* inhibition of caspase-8 prevented degradation of p125^{FAK} and blocked apoptosis induced by expression of FAK-CD. BT474 cells that were grown in monolayer or suspension culture were pretreated with a specific caspase-8 inhibitor, benzyloxycarbonyl-IETD-fluoromethyl ketone, at a final concentration of 0, 1, 5, 10, or 20 μM for 6 h and subsequently infected with AdFAK-CD or Ad*lacZ* for 24 h in the presence of caspase-8 inhibitor. Apoptotic cells were detected by TUNEL assay and quantitated as described above. Expression and degradation of p125^{FAK} were analyzed by Western blotting using the anti-FAK (C20) polyclonal antibody (Santa Cruz Biotechnology) (*lower panel*). *B* and *C,* ectopic expression of AU-1-tagged dominant-negative version of FADD (Δ*FADD*) inhibited caspase-8 activation and blocked apoptosis induced by FAK-CD. BT474 cells grown in monolayer or suspension culture were co-infected with AdFAK-CD or Ad*lacZ* together with adenovirus containing AU-1-tagged ΔFADD at a concentration of 0, 200, 500, or 1000 virions per cell for 24 h. *B,* the inhibition of caspase-8 and degradation p125^{FAK} were analyzed by Western blotting using the anti-proform of caspase-8 monoclonal antibody or anti-FAK (C20) polyclonal antibody (Santa Cruz Biotechnology). *C,* apoptosis was demonstrated by TUNEL assay, and the percentage of apoptosis was determined as described above.

the presence of the pro-form enzyme (Fig. 6*B, 3rd* to *5th* and *8th to 10th lanes*) and prevented p125^{FAK} degradation as well (Fig. 6*B*). In addition, FAK-CD-induced apoptosis was significantly blocked by ΔFADD even at a low concentration of adenovirus, both in monolayer and suspension cultures (Fig. 6*B*). Taken together, these results show the importance of death receptor-related death domain proteins in the apoptotic pathway triggered by down-regulation of FAK.

Normal Mammary Epithelial Cells Are Resistant to Loss of p125^{FAK} Function—Since FAK function had a direct effect on both the adhesion and survival of breast cancer cells, we next compared the interruption of FAK function in breast cancer cells and in normal mammary epithelial cells. In these experiments, we treated human human mammary epithelial (HME) cells with AdFAK-CD for 24 h. Following AdFAK-CD transduction, essentially all of the cells in the population expressed high levels of FAK-CD protein by immunofluorescence and Western blot with the anti-HA antibody (Fig. 7, *A* and *B*). Although there was some focal adhesion staining for FAK-CD, there was predominantly cytoplasmic staining in these cells (Fig. 7*A, panel a*). Thus, we cannot determine whether FAK-CD has directly caused loss of FAK from the focal adhesions or whether some other mechanism is operative. Unlike the tumor cells, however, the HME cells remained adherent**,** with no detectable apoptosis. When these cells were trypsinized and replated, approximately 60% were capable of readhering to the tissue culture plates, with no difference between AdFAK-CD and Ad*lacZ*-treated cells (data not shown).

Furthermore, the endogenous p125^{FAK} was no longer detected at the focal adhesions (Fig. 7*A, panels b* and *d*) in FAK-CD-treated cells as compared with *lacZ*-treated cells (Fig. 7*A, panels f* and *h*), in a manner similar to tumor cells, but paxillin did remain at the focal adhesions (Fig. 7*A, panel c*). Even though endogenous p125^{FAK} was no longer localized to the focal adhesions and its tyrosine phosphorylation was completely abrogated (Fig. 7*B, middle panel*), it did not become degraded with expression of FAK-CD (Fig. 7*B, bottom panel*). We also tested another normal mammary cell line, MCF-10A, and found that expression of FAK-CD did not cause cellular rounding, loss of adhesion, or apoptosis (Table I). Thus, under these conditions, p125^{FAK} was stable when removed from focal adhesions in non-transformed cells, but was quickly degraded in tumor cells.

DISCUSSION

These results suggest that in breast tumor cells, FAK has two separate functions, one promoting the adhesive interactions between tumor cells and their ECM, and the other acting as a survival signal that is independent of cellular adhesion. The effects of FAK-CD in BT474 cells were similar to anoikis (8, 10), whereby loss of adhesion was followed by a signal to degrade p125^{FAK} and to undergo apoptosis. However, this effect was not simply due to loss of matrix signals through the interruption of FAK function by FAK-CD, as breast tumor cells that were viable in suspension culture rapidly underwent apoptosis when transduced with FAK-CD. Additionally, this effect was not peculiar to BT474 cells since the different breast tumor cell lines that we tested were highly sensitive to the loss of adhesion induced by AdFAK-CD transduction but demonstrated varying rates of apoptosis (data not shown).

These studies also suggest that degradation of p125^{FAK} is essential for tumor cells to undergo apoptosis. Inhibition of caspase-3 prevented degradation of p125^{FAK} and apoptosis but did not have an effect on cellular adhesion. The degradation of p125^{FAK} in tumor cells by a caspase 3-based mechanism is in agreement with other investigators who have shown that p125^{FAK} is cleaved by this cysteine protease (32, 36) as cells undergo apoptosis. Furthermore, the blockage of apoptosis by caspase-8 inhibition and by expression of ΔFADD strongly suggests that a death receptor-induced signaling or death receptor-related death domain proteins are involved in the apoptotic process trigged by expression of FAK-CD. Activation of caspase-8 has been shown to initiate a caspase cascade that results in apoptosis through death receptor-mediated cell death (37–39). In addition, other investigators have linked detachment-induced apoptosis to FAK and caspase-8 (40, 41). However, our studies suggest the FAK signaling pathway is inhibiting death receptor-related apoptosis, independent of matrix signaling.

In contrast, these studies demonstrate a different require-

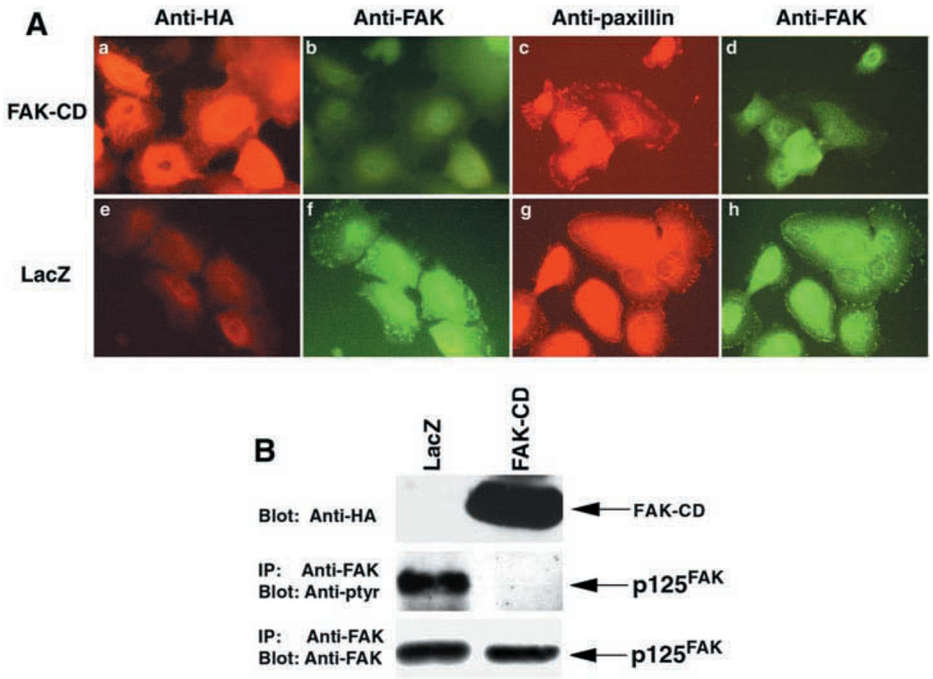

FIG. 7. **Loss of endogenous p125^{FAK} from the focal adhesions of HME cells is not accompanied by cellular rounding or loss of adhesion.** AdFAK-CD was transduced into normal human mammary epithelial cells and did not cause loss of adhesion, apoptosis, or degradation of p125^{FAK} in these cells. *A,* dual immunofluorescent analysis of FAK-CD and p125^{FAK} or paxillin and p125^{FAK} in HME cells. HME cells were transduced with AdFAK-CD or a control *lacZ* virus for 24 h. The expression of FAK-CD, p125^{FAK}, or paxillin was analyzed by immunofluorescence microscopy using an anti-HA, anti-FAK kinase domain, or anti-paxillin monoclonal antibodies. *B,* Western blot of FAK-CD expression following AdFAK-CD transduction in HME cells using an anti-HA monoclonal antibody (12CA5) (*top panel*). Tyrosine phosphorylation and expression of endogenous p125^{FAK} was analyzed by immunoprecipitating p125^{FAK} followed by immunoblotting with an anti-phosphotyrosine monoclonal antibody (4G10, Upstate Biotechnology, Inc.) (*middle panel*) or anti-FAK polyclonal antibody (C20, Santa Cruz Biotechnology) (*bottom panel*).

ment for FAK expression between breast cancer cells and normal mammary epithelial cells. Loss of p125^{FAK} from focal adhesions of normal breast epithelial cells resulted in the loss of FAK tyrosine phosphorylation but did not cause loss of adhesion or apoptosis, indicating that the function of FAK at the focal adhesions was not essential for normal mammary epithelial cell survival. It is unclear why down-regulation of FAK in breast tumor cells leads to activation of a caspase cascade and induces apoptosis, whereas down-regulation of FAK from normal mammary cells does not. We hypothesize that normal cells have other ways of compensating for the loss of FAK function caused by AdFAK-CD transduction, perhaps through other kinases such as the FAK homologue, CADTK. This resistance of normal cells to FAK down-regulation by FAK-CD is similar to findings by Xiong and Parsons (42) and Ilic *et al.* (20). However, these findings differ from those of Hungerford *et al.* (43) who detected apoptosis following injection of anti-FAK antibodies into chicken embryo fibroblasts. However, these investigators only detected apoptosis when cells were not fully spread.

Our findings are consistent with those reported by Frisch *et al.* (10) who showed that in Madin-Darby canine kidney cells, expression of a constitutively active, membrane-bound form of FAK was sufficient to transform the cells by anchorage-independent growth criteria and tumor formation in nude mice. In addition, the constitutively active FAK seen in breast tumor cells grown in suspension is similar to the results of Renshaw *et al.* (44) who showed that constitutively active FAK enhanced the anchorage-independent growth of *ras*-transformed fibroblasts. Furthermore, our findings suggest that the function for the high levels of FAK expressed in human tumor cells is to suppress apoptosis, although the precise biochemical pathway is not yet known. Richardson and *et al*. (45) have speculated that FAK acts as a "switchable adapter," bringing the Src tyrosine kinase into close proximity to its substrate paxillin. FAK-CD could bind to one or more of these carboxyl-terminal partners that associate with the FAK·Src complex, such as paxillin, and disrupt the stoichiometry of the FAK signaling complex. However, Src expression was not detectable in our BT474 cells (data not shown), suggesting that FAK-CD may disrupt other functions of FAK or be linked to other Src family kinases, such as Fyn. In addition, the role of paxillin phosphorylation in the FAK-mediated survival signal, if any, is not known. Indeed, our results suggest that the functions of FAK in breast cancer cells may be distinct, with some promoting adhesion and others acting as part of a survival signal pathway for the suppression of apoptosis.

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