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

IN 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 p125FAK, 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 p125FAK. 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 therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ 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-HMA, poly(2-hydroxyethylmethacrylate); BrdUrd, 5-bromo-deoxyuridine; 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.
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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 cells and corresponded with loss of endogenous p125FAK 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 p125FAK 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 50 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% CO2.

**Construction and Preparation of Recombinant Adenoviruses**—FAK-CD, amino acids 693–1052 of FAK fused to the HA epitope tag, was expressed in COS-7 cells and served as a control. The number of virions was confirmed as 10^12 virions. Concentrated virus was dialyzed, aliquoted, and stored at −80°C.

**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 (Bachem), or benzoyloxycarbonyl-IETD-fluoromethyl ketone (Enzyme Systems Products, Livermore, CA) at various concentrations for 6 h. Cells were subsequently infected with AdFAK-CD or AdlacZ for 24 h and then harvested and assayed for apoptosis using the ApoTag kit (Intergen, Purchase, NY) as described below.

**BrdUrd Labeling and Detection**—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.

**Assays of Cell Viability**—Detection of apoptosis was performed by TUNEL assay using the ApoTag 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 Mosman (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 DMSO. 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 medium control × 100.

**Immunofluorescence, Western Blotting, and Immunoprecipitation**—Immunofluorescent staining for HA, p125FAK, 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 p125FAK 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, p125FAK, 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. 1A, top panel). By 16 h following infection, FAK-CD led to loss of p125FAK expression (Fig. 1A, middle panel). This loss of p125FAK expression was not due to a general degradation of cellular proteins, because the focal adhesion protein vinculin was stable throughout the experiment (Fig. 1A, bottom panel). Preceding this loss of p125FAK expression, the level of p125FAK tyrosine phosphorylation had dramatically decreased, and by 8 h, tyrosine-phosphorylated p125FAK was no longer detectable (Fig. 1B).

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. 2A). At 6 h some focal adhesion staining was still visible, but by 8 h, the cells had become rounded (Fig. 2A). 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 p125FAK from the focal adhesions, dual immunofluorescence microscopy was performed to co-localize p125FAK and FAK-CD, as well as p125FAK and paxillin. Before AdFAK-CD was transduced, endogenous p125FAK 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, p125FAK was no longer detectable at the focal adhesions of the cells that expressed FAK-CD (Fig. 2B, panels a and b). However, at this time point, the focal adhesions were still intact as demonstrated by paxillin staining (Fig. 2B, panels c and d). Cells infected with control lacZ virus maintained p125FAK and paxillin expression at the focal adhesions (Fig. 2B, panels e–h). These results demonstrate that expression of FAK-CD was associated with loss of the endogenous p125FAK from focal adhesions, resulting in a loss of cellular adhesion.

To determine the fate of the detached cells, we analyzed
three independent experiments. lacZ p125FAK 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).

**TABLE I**

| Cell line | Virions/cell | Loss of adhesiona | Apoptosisb |
|-----------|-------------|-------------------|------------|
| BT474     | $2 \times 10^4$ | 99.3 ± 1.6        | 86.1 ± 5.2 |
| MCF-7     | $1 \times 10^4$ | 92.7 ± 2.3        | 45.0 ± 2.4 |
| MCF-10A   | $1 \times 10^4$ | 0.1 ± 0.1         | 0          |
| HME cells | $1 \times 10^4$ | 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 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<sup>FAK</sup> 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).

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, 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.

The two smaller proteins represent degradation of FAK-CD. This is consistent with the observation of Wen et al. (32) that p125<sup>FAK</sup> 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. 3A). 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.

**FIG. 3.** Induction of apoptosis and degradation of p125<sup>FAK</sup> in BT474 cells by FAK-CD. A, BT474 cells were infected with AdFAK-CD or AdlacZ 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<sup>FAK</sup> were 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 analysis of endogenous p125<sup>FAK</sup> degradation (Fig. 3B, upper panel), anti-FAK COOH-terminal domain polyclonal antibody (C20, 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<sup>FAK</sup> 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 AdlacZ 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 AdFAK-CD or AdlacZ for 16 h. Expression of FAK-CD or p125<sup>FAK</sup> 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).
BrdUrd-positive cells were counted and photographed using a Zeiss fluorescence microscope. Then harvested, fixed, and stained with anti-BrdUrd monoclonal antibody (Ab2, Calbiochem) as described under “Experimental Procedures.”

 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 AdlacZ control or with AdFAK-CD for 24 h. The expression of FAK-CD and p125FAK was analyzed by Western blotting using an 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 p125FAK were analyzed by immunoprecipitating p125FAK with the anti-FAK polyclonal antibody (C20) and then immunoblotting with either anti-phosphotyrosine antibody (4G10) or the anti-FAK antibody (C20).

Transduction of FAK-CD caused dephosphorylation and degradation of p125FAK 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 AdlacZ control or with AdFAK-CD for 24 h. The expression of FAK-CD and p125FAK was analyzed by Western blotting using an 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 p125FAK were analyzed by immunoprecipitating p125FAK with the anti-FAK polyclonal antibody (C20) and then immunoblotting with either anti-phosphotyrosine antibody (4G10) or the anti-FAK antibody (C20).

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the presence of the pro-form enzyme (Fig. 6B, 3rd to 5th and 8th to 10th lanes) and prevented p125FAK degradation as well (Fig. 6B). 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. 6B). 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 p125FAK 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 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. 7A, panel a). Although there was some focal adhesion staining for FAK-CD, there was predominantly cytoplasmic staining in these cells (Fig. 7A, 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 AdlacZ-treated cells (data not shown).

Furthermore, the endogenous p125FAK was no longer detected at the focal adhesions (Fig. 7A, panels b and d) in FAK-CD-treated cells as compared with lacZ-treated cells (Fig. 7A, panels f and h), in a manner similar to tumor cells, but paxillin did remain at the focal adhesions (Fig. 7A, panel c). Even though endogenous p125FAK was no longer localized to the focal adhesions and its tyrosine phosphorylation was completely abrogated (Fig. 7B, middle panel), it did not become degraded with expression of FAK-CD (Fig. 7B, 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, p125FAK 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 p125FAK 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 p125FAK is essential for tumor cells to undergo apoptosis. Inhibition of caspase-3 prevented degradation of p125FAK and apoptosis but did not have an effect on cellular adhesion. The degradation of p125FAK in tumor cells by a caspase-3-based mechanism is in agreement with other investigators who have shown that p125FAK 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 in the apoptotic process triggered 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-
ment for FAK expression between breast cancer cells and normal mammary epithelial cells. Loss of p125\textsuperscript{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 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. 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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