The COOH-Terminal Domain of the Focal Adhesion Kinase Induces Loss of Adhesion and Cell Death in Human Tumor Cells

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Abstract
Focal adhesion kinase (FAK) is a tyrosine kinase that is linked to signaling pathways between cells and their extracellular matrix. An alternate transcript of the COOH-terminal region of the FAK gene, called FAK-related nonkinase, has been shown to act as an inhibitor of FAK in chicken embryo fibroblasts. We have designed an analogous segment of human FAK, FAK COOH-terminal domain (FAK-CD), and transfected this construct into human tumor cells. Expression of FAK-CD inhibited cell growth in BT474 human breast cancer cells and C8161 human melanoma cells. To characterize the nature of growth inhibition, we developed an inducible system of FAK-CD expression and demonstrated that the induced FAK-CD protein localized to focal adhesions, causing cellular rounding, an irreversible loss of adhesion, and subsequent cell death. In addition, expression of FAK-CD reduced tyrosine phosphorylation of FAK, suggesting that FAK-CD may be a potent inhibitor of FAK in human tumor cells.

Introduction
FAK\(^2\) was originally identified in chicken embryo fibroblasts through its tyrosine phosphorylation by the transforming gene v-src \(1\). This intracellular tyrosine kinase is localized to focal adhesions, which are the contact points between a cell and its substratum. FAK is the major tyrosine-phosphorylated protein in focal adhesions and is activated by a number of signals, including integrin aggregation and stimulation by mitogens \(2-4\). We originally identified human FAK in a screen for tyrosine kinases expressed in human high-grade sarcoma \(5\). We subsequently found that FAK was overexpressed in human invasive and metastatic tumors at the mRNA level \(6\), as well as the protein level \(7,\,8\). These findings have been extended by investigators in other human tumor systems \(9,\,10\).

The elevated expression of FAK in tumor cells suggested that FAK might have a role in growth regulation or viability of tumor cells. To test this hypothesis, we attenuated FAK expression with antisense oligonucleotides and found that cells lost adherence and underwent apoptosis following antisense treatment \(11\). However, the utility of antisense oligonucleotides is limited by the potential nonspecific inhibition of message stability and other cellular effects. For this reason, we have sought other means of blocking the activity of FAK. An alternate transcript of the FAK gene, called FRNK \(12\), has been described in the avian system that is encoded by the COOH-terminal 360 amino acids of FAK. Overexpression of FRNK in chicken embryo fibroblasts inhibited cell spreading on fibronectin, blocked focal adhesion formation, and reduced tyrosine phosphorylation of FAK \(13\).

Here, we have developed an expression system, which allows induction of the COOH-terminal domain of the human FAK gene (FAK-CD) that is analogous to avian FRNK. We have shown that induction of FAK-CD expression in tumor cells causes an irreversible loss of adhesion and cell death. Using an inducible vector, we have demonstrated that FAK-CD is expressed at the focal adhesions, leading to a rounded morphology of cells and loss of viability. These effects accompanied a decrease in tyrosine phosphorylation of FAK and suggest that FAK-CD may be a potent inhibitor of FAK function in human tumor cells.

Results
FAK-CD Inhibits Growth of Tumor Cells. Because our previous studies suggested that FAK might play an important role in human cancer, we wished to determine whether interruption of FAK function might inhibit the growth of tumor cells. In chicken embryo fibroblasts, FRNK has been identified as an alternate transcript of FAK, consisting of 537 nucleotides of unique 5' sequence spliced to the 3' 2234 nucleotides of the avian FAK cDNA \(12\). The human homologue of FRNK has not been cloned. However, the sequences surrounding the initiating methionine of the avian FRNK sequence are identical to the human FAK sequence, and we have identified, in MDA-MB-435 human breast cancer cells, a Mr 41,000 protein that comigrates with chicken FRNK using an antibody to the COOH terminus of FAK, which strongly suggests that human FRNK is expressed under some conditions (data not shown). Although this pu-
Inhibitory human FRNK was expressed in MDA-MB-435 breast cancer cells, it did not affect the expression of FAK. In addition, it was not expressed to a high degree in any other tumor cell lines that we analyzed, including the BT474 and C8161 cell lines that we used in this study.

Because FRNK appears to be a potential inhibitor of FAK, we expressed the COOH-terminal 360 amino acids of human FAK and have termed this fragment FAK-CD (Fig. 1). We have subsequently analyzed the effects of FAK-CD on tumor cell growth. To distinguish this exogenous FAK-CD sequence from the endogenous FAK protein, FAK-CD was cloned in-frame with the HA epitope tag sequence at the NH\textsubscript{2} terminus into the pLXSN vector.

We transfected pLXSN-FAK-CD into BT474 human breast cancer cells and C8161 human melanoma cells because these cell lines were previously shown to express high levels of FAK (7). In each of these tumor cell lines, expression of FAK-CD caused inhibition of colony formation compared to pLXSN vector control (Table 1). Colonies that emerged after 2 weeks of G418 selection did not express detectable levels of FAK-CD (data not shown), suggesting that FAK-CD expression is counterselected. In contrast, the growth of nontransformed NIH 3T3 mouse fibroblasts was not as strongly inhibited by FAK-CD as the tumor cell lines (Table 1), and we were able to detect stable FAK-CD expression in transfected cells (data not shown).

Induction of FAK-CD Expression Causes an Irreversible Loss of Adhesion and Viability. Because FAK-CD inhibited colony formation in tumor cells, we wished to further study the nature of this growth inhibition. HA-FAK-CD was cloned into a metallothionein-inducible expression vector, pSAR-MT (14), and transfected into BT474 and C8161 cells. After G418 selection, stable clones of BT474 and C8161 cells transfected with pSAR-MT-FAK-CD were isolated and evaluated for inducible expression of FAK-CD. We screened clones from each cell line by Western blots probed with a monoclonal antibody directed to the HA epitope tag and selected one clone from BT474 and one clone from C8161 that had the highest expression of FAK-CD after 24 h of induction for further study. In each cell line, FAK-CD expression reached the highest levels between 10 and 24 h and then decreased at 48 h following zinc addition (Fig. 2). In contrast, cells transfected with vector alone did not express detectable levels of FAK-CD after zinc treatment (Fig. 2).

Next, we sought to determine whether FAK-CD induction caused a phenotypic change in these tumor cells. Cells were incubated in the presence or absence of zinc and analyzed by immunofluorescence with the anti-HA antibody. After 6 h of induction, staining for the FAK-CD protein paralleled staining for paxillin, suggesting that FAK-CD had localized to focal adhesions (Fig. 3, A and C, top). By 24 h, cells that expressed FAK-CD had shown a rounded morphology (Fig. 3, B and D, top left). Costaining these cells for the focal adhesion protein, paxillin, using the polyclonal antipaxillin antibody demonstrated the disruption of focal adhesions in FAK-CD-expressing cells (Fig. 3, B and D, top right). As a control, HA immunofluorescence was only minimally detectable in uninduced cells (data not shown), and was not expressed in cells containing the pSAR-MT plasmid (Fig. 3, B and D, bottom left), and these cells remained adherent to their culture plates. At the 24-h time point following zinc induction, an average of 42% of the BT474 cells containing the pSAR-MT-FAK-CD vector expressed FAK-CD in three separate experiments. However, we determined that an average of 35% of cells had already detached from the dish by 24 h. Thus, induction of FAK-CD may vary between cells during the course of zinc treatment, making it difficult to precisely assign the number of cells expressing FAK-CD during the entire course of the experiment. By 48 h after zinc treatment, the majority of cells containing the pSAR-MT-FAK-CD vector had detached from the dish (data not shown). We also examined the phenotype resulting from zinc induction of two other clones that expressed FAK-CD, but to a lesser degree than the isolate that we chose for final analysis. In each case, induction of FAK-CD expression caused a rounded morphology and loss of adhesion, suggesting that this was a specific effect of FAK-CD in these cells (data not shown). Similarly, in C8161 melanoma cells containing pSAR-MT-FAK-CD vector, 42% of cells expressed FAK-CD by 24 h after zinc induction, although 40% of cells had already detached from the dish. Thus, these results suggested that FAK-CD localized to the focal adhesion contacts following induction and that this expression interrupted cellular adhesion and, thus, ultimately led to a rounded morphology and loss of adhesion.

We next determined whether the cells that lost adhesion upon FAK-CD induction still remained viable. As noted above, zinc induction of BT474 cells containing pSAR-MT-FAK-CD vector resulted in loss of adhesion of 35% of cells by 24 h. In comparison, 12% of zinc treated cells containing the pSAR-MT vector detached from the dish, perhaps due to interference of ion-bound molecules by zinc. Equal numbers of the suspended cells following zinc treatment from either the vector control or the FAK-CD-expressing cells were replated into six-well plates and allowed to adhere and grow for 24 h. Suspected cells that were not capable of adhering were removed, and attached cells were photographed using an inverted light microscope (Fig. 4A) and then harvested and counted. In the vector control cells, 42% of BT474 cells gained adherence 24 h after replating. This was probably due to a reversible loss of adhesion from the zinc treatment itself (Fig. 4B). In contrast, only 14% of the FAK-CD-expressing BT474 cells regained adherence in three separate experiments (Fig. 4B). These results suggested that induction of FAK-CD expression caused an irreversible loss of adhesion and viability. We found similar results using the melanoma
Table 1. Expression of FAK-CD inhibits cell growth

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Experiment no.</th>
<th>No. of colonies</th>
<th>Colony formation (% of vector control)</th>
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<tr>
<td></td>
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<td>FAK-CD</td>
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<tr>
<td>BT474</td>
<td>1</td>
<td>2305</td>
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<tr>
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<td>2</td>
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<td></td>
<td>3</td>
<td>465</td>
<td>331</td>
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<tr>
<td></td>
<td>Average</td>
<td>404 ± 87</td>
<td>288 ± 57</td>
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</tbody>
</table>

* Cells (5 × 10^5) were plated in selective medium after transfection with FAK-CD or vector. After 2 weeks of selection, cells were fixed and stained with 1% crystal violet. The numbers of colonies were counted in FAK-CD- or vector-transfected cells. Percentage of growth inhibition was determined based on the number of colonies in FAK-CD-transfected cells versus that of vector-transfected cells. Data are presented as mean ± SD of three independent experiments.

Fig. 2. Western blot analysis of inducible expression of FAK-CD in BT474 and C8161 cells. Cell lines that were stably transfected with either pSAR-MT vector or pSAR-MT-FAK-CD were induced with zinc, and HA-FAK-CD expression was measured over a 48-h induction period.

FAK-CD interrupts FAK function in tumor cells. We hypothesized that FAK-CD induced the loss of viability in these tumor cells by interrupting FAK function. We analyzed the level of FAK phosphorylation following FAK-CD induction by immunoprecipitating FAK and analyzing phosphoryrosine content. After 24 h of FAK-CD induction in BT474 cells, the tyrosine phosphorylation of FAK was decreased (Fig. 5A), whereas the levels of p125FAK remained unchanged, demonstrating that induction of FAK-CD resulted in decreased p125FAK activity. The presence of zinc did not have an effect on endogenous FAK phosphorylation when analyzed in the vector control cells (Fig. 5A).

To exclude the possibility that this increase in tyrosine phosphorylation was a secondary effect of the cells’ loss of viability, we transiently coexpressed both FAK and FAK-CD in Cos-7 cells. In these experiments, we found that FAK-CD reduced both the tyrosine phosphorylation (Fig. 5B) of p125FAK and its autophosphorylation activity (Fig. 5C). Densitometric analyses of these experiments demonstrated that the expression of FAK decreased 3-fold, whereas FAK activity declined 7-fold and tyrosine phosphorylation was minimally detectable. Thus, we conclude that FAK-CD expression led to an attenuation of FAK activity.

Discussion

In these studies, we have demonstrated that exogenous expression of FAK-CD led to loss of adhesion and viability in human breast cancer and human melanoma cells. This phenotype, caused by FAK-CD induction in the tumor cells, was similar to that seen when FAK expression was attenuated by antisense oligonucleotides (11). In each system, interruption of FAK signaling led to a rapid rounding of the tumor cells and an irreversible loss of adherence and viability. These series of cellular events are similar to the phenomenon of anoikis, whereby cells that are detached from their substratum undergo apoptosis (15). Furthermore, FAK has been shown to play a role in the suppression of anoikis, which, by itself, might be sufficient for a cell to acquire the transformed phenotype (16). However, in contrast to the results in our antisense system, we did not see the same rapid induction of apoptosis in the tumor cells that expressed FAK-CD, although this loss of adhesion was not reversible. It is probable that some cells ultimately underwent apoptosis, but other mechanisms of cellular necrosis and death were also operative. It is also possible that zinc prevented the nonadherent cells from undergoing apoptosis because it has been shown that zinc is a potent inhibitor of the apoptotic protease, caspase-3 (17).

This interruption of tumor cell growth correlated with a decrease in FAK tyrosine phosphorylation, suggesting that FAK-CD expression interrupted the FAK signaling pathway in the tumor cells. This effect of FAK-CD expression in human cells agrees with the previous studies of FRNK in chicken embryo cells (19), in which FRNK expression inhibited
p125FAK tyrosine phosphorylation. However, the phenotypic effects of FAK-CD expression in the BT474 and C8161 tumor cells were markedly different from the effects reported using nontransformed cells. In chicken embryo fibroblasts, expression of FRNK did not cause loss of adhesion; it only slowed cellular spreading and delayed the appearance of focal complexes (13). Furthermore, in fully spread chicken embryo fibroblasts, inhibition of FAK function by other methods had no effect on cellular adhesion or rounding (18).

Other related experiments have similarly suggested that nontransformed cells do not require FAK for growth. First, mice carrying a homozygous deletion of the FAK gene die prenatally, but fibroblasts from these embryos can be maintained in culture (19). Second, when FAK signaling was inhibited in human umbilical vein endothelial cells, the cells had reduced cell motility and proliferation but did not die (20). Finally, Xiong and Parsons (21) have expressed avian FRNK in rat-1 cells and did not observe apoptosis. Our results in untransformed mouse fibroblasts also support this hypothesis because the expression of FAK-CD in NIH 3T3 cells caused much less growth inhibition than expression in BT474 or C8161 tumor cells. This suggests that normal cells may have a different requirement for p125FAK function and are, thus, less sensitive to the effects of FAK-CD expression.

In contrast, the transformed cells in our studies appeared to be quite sensitive to FAK-CD expression, rapidly developing an irreversible loss of adhesion and viability. We have previously shown that malignant human tumors exhibit a significant increase in p125FAK expression, compared to their normal counterparts (6–8). This implies that FAK expression is selected for in transformed cells and that ablation of FAK activity has more severe consequences. The other possibility that should be considered is that epithelial cells, from which our tumor cell lines were derived, are, in general, more sensitive to the loss of FAK function. However, we previously determined that mesenchymally derived rhabdomyosarcoma cells displayed an equal sensitivity to FAK antisense oligonucleotides as epithelially derived tumor cells (11).

To explain these results, it is possible that FAK-CD may induce effects in different pathways in tumor cells or that FAK has a critical function in maintaining viability in tumor cells that are subject to constant proliferative and apoptotic sig-
Fig. 4. Replating assay of BT474 and C8161 cells containing an inducible FAK-CD expression vector. Equal numbers of cells in suspension from induced FAK-CD-expressing or vector control cells were replated in the absence of zinc and allowed to adhere and grow for 24 h. A, Suspended cells were removed, and attached cells were photographed using an inverted light microscope. B, attached cells were then harvested and counted, and the percentage of replated cells was calculated by the number of reattached cells versus the number of cells plated. Columns, means of three independent experiments; bars, SD. □ control; □ FAK-CD.

Materials and Methods

Cell Lines and Cell Culture Conditions

The BT474 human breast ductal carcinoma cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 with 10% FBS, 10 μg/ml insulin, and 300 mg/ml L-glutamine. The C8161 human melanoma cell line (kindly provided by Dr. Bernard E. Weissman, University of North Carolina at Chapel Hill) was maintained in RPMI 1640 supplemented with 10% FBS. NIH 3T3 mouse fibroblasts and Cos-7 cells purchased from American Type Culture Collection were cultured in DMEM supplemented with 10% FBS. All of the cell lines were incubated at 37°C in 5% CO₂ in air.

Vector Construction

FAK Vector Construction. A FAK cDNA clone was isolated from the HT29 human colon cancer cell line⁴ and subcloned into the pcDNA3 (Invitrogen) expression vector, incorporating an in-frame sequence for the expression of the HA epitope containing amino acids of YPYDVPDYA at the NH₂ terminus of the protein.

FAK-CD Vector Construction. For pCRII-FAK-CD, FAK-CD, tagged at the NH2 terminus with HA, was PCR amplified using human FAK cDNA as a template. The primers were as follows: 5'-CGGGTGACGTCCGCGCCACCATGGACTACCCCTATGATGTGCCCGA1TACGCTGAGTCC-3' and 5'-ATTAACCTCTAATAAG. PCR-amplified HA-FAK-CD was cloned into pCRII vector (Invitrogen). For pLX-FAK-CD, pCRII-FAK-CD was cloned into the pLXSN expression vector (23) using standard molecular biology techniques. For pSAR-FAK-CD, pCRII-FAK-CD was cloned into pBlueScript SK vector and then subcloned into the pSAR-MT vector (kindly provided by Dr. Bert Vogelstein, Johns Hopkins University) containing a metallothionein-inducible promoter. For pCMV-FAK-CD, pCRII-FAK-CD was cloned into the pCMV expression vector, which contains a cytomegalovirus promoter.

Growth Inhibition Assay
pLX-FAK-CD or pLXSN vector was transfected into BT474, C8161, and NIH 3T3 cells and assayed for growth-inhibitory activity. Briefly, 10 μg of pLX-FAK-CD construct or pLXSN vector was mixed with 8 μg/ml Lipofectamine (Life Technologies, Inc.) in serum-free medium, left 30 min, added to cells, and incubated for 6–8 h at 37°C. Cells were grown in medium containing 10% FBS for 48 h at 37°C. A total of 5 × 10⁴ cells were then plated in complete medium containing 500 μg/ml genetin (G418; Life Technologies, Inc.) for BT474 and NIH 3T3 cells and 100 μg/ml for C8161 cells. After 2 weeks of selection in G418, cells were fixed with 3:1 methanol/acetic acid and stained with 1% crystal violet. Percentage of growth inhibition was determined by comparing the number of colonies in each plate.

Induction of FAK-CD Expression
Ten μg of pSAR-MT-FAK-CD or pSAR-MT vector and 1 μg of neomycin-resistant vector pSV2neo were mixed with 8 μg/ml Lipofectamine (Life Technologies, Inc.) in serum-free medium, transfected into BT474 and C8161 cells, and selected with G418. Ten colonies from each cell line were isolated, expanded, and tested for the ability to induce FAK-CD expression with ZnSO4. The concentrations of ZnSO4 for induction of FAK-CD expression were 75 and 50 μM for BT474 and C8161 cells, respectively. One clone that expressed FAK-CD following treatment with ZnSO4 was expanded from each cell line for further analysis.

Replating Assay
A total of 1.5 × 10⁶ cells were plated into 100-mm tissue culture plates and incubated for 24 h at 37°C. Cells were then washed with PBS and treated with ZnSO4 for 24 h. Suspended cells were harvested, stained with trypan blue, and counted. A total of 1 × 10⁶ trypan blue-excluding cells were replated into six-well plates in complete medium. The percentage of viable cells that were capable of replating was determined by counting the number of attached cells after a 24-h incubation at 37°C.

Immunofluorescence
BT474 or C8161 cells containing the pSAR-MT-FAK-CD or pSAR-MT plasmids were plated onto coverslips in six-well culture plates and treated with ZnSO4 for 6 or 24 h. Inducible expression of FAK-CD was analyzed by immunofluorescence using anti-HA monoclonal antibody (HA11 and BabC0) and then followed by goat antirabbit IgG conjugated with rhodamine. Expression of paxillin was also analyzed by immunofluorescence using antipaxillin polyclonal antibody (kindly provided by Drs. Jeffrey Thomas and Michael Schaller, University of North Carolina at Chapel Hill) followed by goat antirabbit IgG conjugated with FITC. Positive cells were visualized and photographed with a Zeiss fluorescence microscope (24).

Transfection of Cos-7 Cells
Subconfluent Cos-7 cells were cotransfected with 4 μg of pCMV4-HA-FAK-CD and 2 μg of pcDNA3-HA-FAK mixed with 20 μl of Lipofectamine (Life Technologies, Inc.) in serum-free DMEM and incubated overnight at 37°C. After a subsequent incubation in complete medium for 24 h at 37°C, the cells were lysed in NP40 lysis buffer and analyzed for FAK expression, tyrosine phosphorylation, and kinase activity as described below.

Western Blotting, Immunoprecipitations, and Kinase Assay
Whole-cell lysates were prepared as described (24), and 50 μg of cell lysate were analyzed for FAK-CD expression by Western blotting using anti-HA monoclonal antibody (12CA5; Boehringer Mannheim). For immunoprecipitation, 250 μg of cell lysate were incubated with 1 μg of anti-FAK polyclonal antibody (C20; Santa Cruz Biotechnology) or 10 μg of anti-HA monoclonal antibody (12CA5) in the presence of protein A/G-agarose (Calbiochem). The precipitated proteins were analyzed by Western blot using antiphosphotyrosine monoclonal antibody (4G10; Upstate Biotechnology) or anti-FAK antibody (C20). Proteins were visualized using the ECL detection system (Amersham).

For the FAK assay, anti-HA immunoprecipitates from transfected Cos-7 cells were incubated with 10 μCi of [γ-32P]ATP for 15 min at 30°C, stopped with SDS-PAGE sample loading buffer, and analyzed by SDS-PAGE.
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References


