Control of Adhesion-dependent Cell Survival by Focal Adhesion Kinase

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Abstract. The interactions of integrins with extracellular matrix proteins can activate focal adhesion kinase (FAK) and suppress apoptosis in normal epithelial and endothelial cells; this subset of apoptosis has been termed “anoikis.” Here, we demonstrate that FAK plays a role in the suppression of anoikis. Constitutively activated forms of FAK rescued two established epithelial cell lines from anoikis. Both the major autophosphorylation site (Y397) and a site critical to the kinase activity (K454) of FAK were required for this effect.

Activated FAK also transformed MDCK cells, by the criteria of anchorage-independent growth and tumor formation in nude mice. We provide evidence that this transformation resulted primarily from the cells' resistance to anoikis rather than from the activation of growth factor response pathways. These results indicate that FAK can regulate anoikis and that the conferral of anoikis resistance may suffice to transform certain epithelial cells.

Materials and Methods

Antibodies and Immunologic Detection Methods

For immunofluorescence, cells were fixed in 2% formaldehyde for 10 min, 3. Abbreviations used in this paper: FAK, focal adhesion kinase; MPB, myelin basin protein.

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permeabilized in cold acetone for 5 min, and reacted with the primary antibodies, followed by FITC-labeled goat anti-mouse (1:100; Boehringer Mannheim Biochemicals, Indianapolis, IN), and photographed using a microscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY) (final magnification, 450).

For experiments comparing phosphorylated proteins in attached vs. suspended cells, MDCK cells expressing CD2-FAK (wild-type), CD2-FAK (K454R), or CD2-FAK (397F) were detached by trypsinization followed by washing with DME/10% FCS and DME/0.5% BSA. Cells were then either washed once with PBS and lysed in modified RIPA buffer (Schlaepfer et al., 1994) (“suspended cells”), or plated on type I collagen (20 μg/ml)-coated dishes for 40 min at 37°C and lysed in modified RIPA buffer (“adherent cells”). Antibodies were added to lysates containing equal amount of protein, and samples were rotated at 4°C for 1 h. Gammabind Sepharose (Pharmacia, Uppsala, Sweden) was added to the lysates to precipitate the antibody-antigen complexes, and rotation was continued for 2 h. The immunoprecipitates were then washed three times in wash buffer (RIPA buffer without SDS or deoxycholate), resuspended in SDS-PAGE sample buffer, boiled, electrophoresed on 4–12% Precast SDS-PAGE gels (NOVEX, Encinitas, CA), and electrophotographed onto Immobilon-PVDF membranes. The blots were reacted with primary antibodies as indicated followed by HRP-labeled anti-mouse (Bio-Rad Laboratories, Hercules, CA), and visualized by enhanced chemiluminescence detection (Amersham Corp., Arlington Heights, IL).

For analysis of MAP kinase activation, MDCK cells, MDCK cells expressing CD2-FAK (wild-type), and rat embryonic fibroblast (REF-52) cells were serum starved in DME/0.5% FCS for 18 h and detached by trypsinization followed by washing with DME/0.5% FCS and DME/0.5% BSA. Cells were then either immediately lysed in modified RIPA buffer (time zero), or kept in suspension or plated on type I collagen (20 μg/ml)-coated dishes for 15–120 min and lysed in modified RIPA buffer. Cells stimulated with 500 nM PMA for 15 min were used as a positive control for MAPK activation. MAPK activity was assayed essentially as described (Chen et al., 1994). Briefly, MAP kinases were immunoprecipitated from total cell lysates (200 μg/sample) with polyclonal anti-MAP kinase antibody C-16 (Santa Cruz Biotechnology, Santa Cruz, CA) and Gammabind Sepharose as described above. The immunoprecipitates were washed twice with 250 mM Tris, pH 7.5, and once with 100 mM NaCl, 50 mM Hepes, pH 8.0. The immunoprecipitated MAP kinases were incubated in a mixture containing 1 μCi of [γ-32P]ATP, 50 μM ATP, 10 mM MgCl2, 1 mM DTT, 1 mM benzamidine, 0.3 mg/ml myelin basic protein (MBP; Life Technologies, Inc., Grand Island, NY) and 25 mM Hepes, pH 8.0, at 30°C for 20 min. The samples were electrophoresed on a 15% polyacrylamide gel. After staining with Coomassie brilliant blue, the gel was dried and analyzed by autoradiography. Quantitative analysis of the MBP bands was carried out by using the Bio-Rad GS PhosphorImager. Equal immunoprecipitation and loading of MAP kinases was assessed by immunoblotting samples of immunoprecipitated MAP kinases with a monoclonal anti-MAP kinase antibody (clone B3B9).

Antibodies were obtained from the following sources: CD2: clone RP2A2.10 (PharMingen, San Diego, CA); CD8: clone 2.43 (Sarmiento et al., 1980; gift of B. Buehler, Burnham Institute); src: clone 237 (obtained from Dr. J. Wetten, Syntex, Palo Alto, CA); FAK: clone 77 (Transduction Laboratories, Lexington, KY); FAK: clone 11 (Transduction Laboratories); paxillin: clone 349 (Transduction Laboratories); vinculin: (polyclonal, Sigma Chemical Co., St. Louis, MO).

Results

Expression and Localization of CD2-FAK

A chimeric protein created by the fusion of the CD2 antigen ectodomain to full-length FAK activates FAK such that it retains tyrosine 397 autophosphorylation and full tyrosine kinase activity in suspended cells (Chan et al., 1994). MDCK cells or the immortalized keratinocyte cell line HaCat were transfected with expression plasmids bearing the CD2-FAK chimeras in which the FAK sequences were wild-type or mutated to disable the src-SH2 target sequence (Y397F) or the kinase activity (K454R). Western blotting of the cell lines revealed expressed proteins of the predicted molecular weights that were reactive with anti-CD2 antibodies (Fig. 1 A). Western blotting with anti-FAK antibodies indicated that less CD2-FAK was expressed than endogenous FAK (Fig. 1 A).

Immunofluorescence analysis showed punctate, pericellular localization of CD2-FAK in MDCK and HaCat cells (Fig. 1 B). Because focal contacts of these cell lines were not well-formed under these experimental conditions, localization of CD2-FAK to these structures was confirmed in rat embryo fibroblasts that were transiently transfected with the CD2-FAK (wild-type or mutant) expression constructs (Fig. 1 B). The CD2 ectodomain protein expressed alone localized diffusely over the entire cell.

CD2-FAK Is Constitutively Activated in MDCK Cells

In contrast with wild-type FAK, CD2-FAK, expressed transiently in transfected COS cells, remains active in suspended cells (Chan et al., 1994). We used this property of CD2-FAK to examine the cellular effects of FAK activation.

First, we performed several experiments to determine whether CD2-FAK behaved as a constitutively activated FAK in MDCK cells. Tyrosine phosphorylation of the endogenous FAK was undetectable in suspended cells and increased rapidly in cells adhering to collagen (Fig. 2, top). This occurred whether the coexpressed CD2-FAK was in its wild-type or mutated form. However, the CD2-FAK (wild-type) was highly phosphorylated in suspended cells, and even more phosphorylated in attached cells. This phosphorylation was abolished by mutation of tyrosine 397 and dramatically reduced by mutation of lysine 454. The levels of the FAK or CD2-FAK proteins themselves were affected neither by the cells' attachment status nor by these point mutations.

The cytoskeletal protein paxillin is normally phosphorylated on tyrosine in response to cell adhesion, possibly by FAK (Schaller and Parsons, 1995). Suspended cells that expressed the wild-type CD2-FAK had hyperphosphorylated paxillin (Fig. 2, top) compared with those that expressed the CD2-FAK mutants. This result and the high phosphorylation level indicate that CD2-FAK has constitutive signaling activity in the MDCK cells.

FAK that is phosphorylated on tyrosine 397 complexes
Figure 1. Expression and focal contact localization of CD2-FAK in MDCK cells. The CD2-FAK (wild-type), CD2-FAK (397* or 454*), or CD2 plasmids (Chan et al., 1994) were cotransfected with a plasmid bearing the neomycin phosphotransferase gene into a homogeneous subclone of MDCK or HaCat cells. The Western blot probed with anti-CD2 (A, left) confirms expression of the ~175-kD CD2-FAK or ~50-kD CD2 proteins, while the blot probed with anti-FAK antibody (A, right) shows the relative expression levels of endogenous FAK (closed arrow) and CD2-FAK (open arrow) proteins. (B) CD2-FAK localizes to focal contacts. Control MDCK cells (b), control HaCat cells (d), or their respective CD2-FAK-expressing derivatives (a and c) were fixed and stained for CD2 (e-g) or vinculin (h).
Tyrosine phosphorylation of wild-type and mutant forms of CD2-FAK, endogenous FAK, and paxillin in suspended and adherent MDCK cell variants; in vivo association of CD2-FAK (wild-type) with c-src. Lysates prepared from suspended (S) or adherent (A) MDCK cells expressing CD2-FAK (wild-type), CD2-FAK(454*), or CD2-FAK(397*) were immunoprecipitated with anti-CD2, anti-FAK, anti-paxillin, or anti-src antibodies. Top left: anti-phosphotyrosine immunoblot of the immunoprecipitates; top right: the same blot stripped and reprobed with anti-CD2, anti-FAK, and anti-paxillin antibodies to confirm equal loading. Bottom left: anti-CD2 immunoblot of anti-src immunoprecipitates; bottom right: the same blot stripped and reprobed with anti-src antibodies.

CD2-FAK accelerates the spreading of MDCK cells on collagen. 2 × 10^6 MDCK (control), CD2-FAK (wild-type)/MDCK, or CD2-FAK (397*)/MDCK cells were plated on collagen-coated, BSA-blocked coverslips in serum-free medium containing 0.5 mg/ml BSA. After 8 min at 37°C, unattached cells were washed off, and the attached cells were photographed at the indicated times. Spread and unspread cells were scored by examination of the photographs (~400 total cells per time point) and the fractions of unspread cells were plotted as a function of time. —■— control; —○— CD2-FAK; —▲— CD2-FAK(397*).

CD2-FAK Confers Anoikis Resistance

MDCK cell lines that stably expressed the constructs described above were assayed for anoikis by the criteria of internucleosomal DNA cleavage and subgenomic DNA content in flow cytometric analyses. A large fraction (21.2%) of the cells expressing the unfused CD2 ectodomain alone underwent apoptosis when detached from matrix (Fig. 4). These results were similar to those reported previously for untransfected MDCK cells (Frisch and Francis, 1994). In contrast, MDCK cells expressing CD2-FAK (wild-type) showed ~10-fold less apoptosis than the other transfectants. Cells expressing either the kinase domain or SH2 target domain mutants of CD2-FAK remained fully sensitive to anoikis, demonstrating the dependence of anoikis rescue upon these activities of FAK. CD2-FAK similarly rendered HaCat cells anoikis resistant, showing that the effect occurs in at least two different epithelial cell types. The expression of a CD8-FAK fusion protein in MDCK cells conferred anoikis resistance as well, ruling out the in vivo with c-src or the src-related kinase, c-fyn (Cobb et al., 1994; Schlaepfer et al., 1994). To test whether CD2-FAK was capable of this interaction, proteins from CD2-FAK/MDCK cells were immunoprecipitated with anti-src antibodies and the immunoprecipitates were analyzed for the presence of CD2-FAK on Western blots (Fig. 2, bottom). CD2-FAK (wild-type) coprecipitated with c-src and this association was insensitive to the adhesion status of the cells. By contrast, CD2-FAK (397* or 454*) failed to associate with c-src. The combined results suggested that CD2-FAK localizes normally in attached cells, but is active in both suspended and attached cells. The MDCK cells that expressed CD2-FAK were found to spread on collagen-coated glass faster than control cells or cells expressing CD2-FAK (397*) (Fig. 3). This indicates that the activation of FAK accelerated the normal cellular response to matrix adhesion, and is consistent with the reported deficiency of cell spreading in FAK knockout cells (Ilic et al., 1995). Despite these effects, the morphology of the cells was not discernibly affected by CD2-FAK (data not shown).
Table I. CD2-FAK Confers Anchorage Independence in MDCK Cells

| Cell line                  | Soft agar plating efficiency (%) |
|----------------------------|-----------------------------------|
| CD2                        | 0                                 |
| CD2-FAK (397*) clone 4     | 0                                 |
| CD2-FAK (397*) clone 10    | 0                                 |
| CD2-FAK (wild-type) clone 9| 12.8 ± 1.2                         |
| CD2-FAK (wild-type) clone 30| 12.5 ± 1.2                         |
| bcl2/mdck clone 21         | 9.5 ± 1.6                          |
| bcl2/mdck clone 32         | 7.1 ± 1.3                          |
| src/mdck                   | 18.8 ± 3.4                         |
| ras/mdck                   | 0.8** ± 0                          |

3 × 10⁴ cells of the indicated cell lines were plated on soft agar as described previously (Frisch, 1991) incubated for 16 d and photographed; colonies greater than eight cells were counted on duplicate plates and averaged.

(NB: each colony was ~10-fold larger compared with the other cell lines).

possibility of spurious signaling effects due to the CD2 ectodomain.

These results suggested that FAK plays an important role in integrin-mediated control of cell survival and that the adhesion-independent activation of FAK can bypass this control. Moreover, the point mutants suggest that FAK kinase activity and interactions with SH2 domain proteins, such as src-family proteins, are both required for this effect.

**CD2-FAK Confers Anchorage Independence and Tumorigenicity upon MDCK Cells.**

To test whether the activated FAK would allow cells to grow independently of anchorage, soft agar colony assays were performed (Table I). CD2-FAK (wild-type), but not CD2-FAK (397*), endowed MDCK cells with anchorage independence. However, the HaCat keratinocytes, which normally undergo both terminal differentiation (Breitkreutz et al., 1993) and apoptosis in suspension, did not become anchorage independent; this implies that CD2-FAK may affect anoikis but not terminal differentiation, although the latter has not been tested directly.

CD2-FAK-expressing cells were also tumorigenic in nude mice (100% tumor incidence; Table II), although the CD2-FAK/MDCK cells produced smaller tumors (41.0 mg ±/− 16) than did v-Ha-ras-transformed MDCK cells (270 mg ±/− 150). The CD2-FAK (397*)-expressing cells were nontumorigenic.

**CD2-FAK Controls Anoikis But Not Growth Factor Responsiveness**

To assess the role of apoptosis in the oncogenic potential of the CD2-FAK-expressing cells, MDCK cells that overexpressed the bcl-2 protein—and were therefore resistant to anoikis (Frisch and Francis, 1994)—were tested for anchorage-independent growth (Table I) and tumorigenic potential (Table II). Surprisingly, the bcl-2-transfected cells scored positively in both assays. This result suggested that the acquisition of anoikis resistance was sufficient to confer the transformed phenotype in MDCK cells.

To investigate whether CD2-FAK affected growth factor pathways, we first examined MAPK activity. MAPK is stimulated by cell adhesion in fibroblasts in a process thought to involve FAK (Schlaepfer et al., 1994). As a control, we first studied the effect of cell adhesion to collagen on MAP kinase activity in rat embryonic fibroblast REF-52 cells. Activation of MAP kinase enzymatic activity was studied by an immunocomplex kinase assay using myelin basic protein (MBP) as a substrate. Consistent with the results reported by others (Chen et al., 1994), MAP kinase activity was transiently increased in REF-52 cells plated on an ECM substratum (Fig. 5). The maximal activity of MAP kinases, four to fivefold induction (normalized to the amount of MAPK) compared to detached cells, was seen at 15–30 min after plating, whereafter the kinase activity declined. When cells were kept in suspension, MAP kinase activity remained unchanged relative to constitutively growing controls (not shown). In contrast

Figure 5. CD2-FAK does not augment adhesion-stimulated MAP kinase activity. Serum-starved REF-52 cells (filled boxes), CD2-FAK/MDCK cells (filled circles), and MDCK cells (open circles) detached from plastic were replated on collagen for 15, 30, 60 or 120 min. Whole cell lysates (200 μg total protein) were subjected to MAP kinase immunocomplex assays using MBP as a substrate; phosphorylated MBP was separated by a 15% SDS gel and autoradiographed. MAP kinase activity is expressed as arbitrary units based on the quantitative analysis of the MBP bands by phosphorlager.

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Table II. CD2-FAK Confers Tumorigenicity in MDCK Cells

| Cell line                  | Cell number | Incubation time | Tumor Incidence |
|----------------------------|-------------|-----------------|-----------------|
| CD2-FAK (397*) clone 4     | 5 × 10⁶     | 56              | 0/8             |
| CD2-FAK (397*) clone 10    | 5 × 10⁶     | 56              | 0/8             |
| CD2-FAK (wild-type) clone 9| 5 × 10⁶     | 56              | 8/8             |
| CD2-FAK (wild-type) clone 30| 5 × 10⁶     | 56              | 8/8             |
| ras/mdck                   | 1 × 10⁶     | 30              | 8/8             |
| bcl2/mdck clone 21         | 5 × 10⁶     | 56              | 8/8             |

Cells in 0.3 ml of serum-free medium were injected at two sites per mouse into four female, 4–5-wk-old, nu/nu mice (Harlan Sprague-Dawley). Tumors were dissected and weighed after the incubation times indicated.
with the results we obtained with fibroblasts, MAP kinase activity was only slightly stimulated (1.2-fold) by adhesion in normal MDCK cells and in CD2-FAK-expressing cells; no quantitative or qualitative differences were found in the MAP kinase activation between the CD2-FAK-expressing and parental MDCK cells (Fig. 5). By contrast, the MAP kinase activity was only slightly stimulated (1.2-fold) by adhesion with the results we obtained with fibroblasts, MAP kinase activity was not affected by CD2-FAK (data not shown).

Finally, because it was still possible that novel targeting of c-src or other molecules might stimulate growth factor responsiveness in the CD2-FAK-expressing cell lines, the cell's dependence on serum concentration for growth was measured. Serum dependence was greatly reduced by the expression of v-src, but was not affected by CD2-FAK (Table III).

**Discussion**

In this report, we have shown that the activation of an integrin signal transducer, FAK, confers resistance to anoikis. In the case of MDCK cells, this was accompanied by anchorage-independent growth and significant tumorigenic potential.

Analogous to previous studies with such molecules as raf-1 kinase (Stokoe et al., 1994) and sos (Aronheim et al., 1994), the membrane targeting of FAK via either CD2 or CD8 served to activate it, by mechanisms that are not clear. Speculatively, the targeting may alleviate FAK's requirement for upstream activating signals, such as the proposed interaction of FAK with integrin β-subunits (Schaller et al., 1995). The possibility that CD2-FAK's effects might arise from spurious protein interactions rather than the preservation of FAK in an active state in suspended cells was considered; it was unlikely for several reasons. First, the CD2-FAK protein localized to focal contacts, suggesting that its carboxy-terminal focal adhesion targeting sequence (FAT; Hildebrand et al., 1993) is functional and that the CD2 transmembrane domain does not drive it to specific sites on the membrane. Second, the kinase and SH2 target sequences of the FAK moiety were required for the observed effects. Third, the substitution of CD2 extracellular and transmembrane domains with those of CD8 was without consequence. Finally, CD2-FAK stimulated cell spreading, an activity attributed to normal FAK (Ilic et al., 1995); artifactual interactions of CD2-FAK with novel cellular substrates would be difficult to reconcile with this effect. In recent complementary experiments, the perturbation of FAK in vivo using inhibitory peptides or antibodies was found to cause apoptosis (Hungerford, J., M. Compton, M. Matter, B. Hoffstrom, and C. Otey, manuscript submitted for publication). While this supports our conclusions, the interference with FAK causes cell rounding, which might suffice to cause apoptosis, confounding the interpretation.

The results reported herein suggest that FAK may contribute to epithelial carcinogenesis. In this connection, FAK is often overexpressed human carcinomas (Owens et al., 1995). However, the role of FAK in transformation is apparently unusual for a signal transducer: FAK controls anoikis without detectably altering growth factor response pathways. This conclusion was obtained through the use of a cell line (MDCK) in which the alleviation of anoikis sufficed to produce anchorage-independent growth, and in turn, tumor formation in vivo. That MDCK cells had this property was supported by results with bcl-2 expression. Bcl-2 is an apoptosis suppressor that is apparently devoid of growth-stimulatory effects and in fact inhibits the growth of several tumor cell lines (Pietenpol et al., 1994). We reported previously that bcl-2 conferred anoikis resistance in MDCK cells (Frisch and Francis, 1994). Surprisingly, we found herein that bcl-2 also transformed MDCK cells, demonstrating that the conversion of MDCK cells to an anoikis-resistant state suffices to transform them.

That the effect of CD2-FAK was primarily on anoikis rather than growth control was also supported by several revealing differences between cells transformed by CD2-FAK and those transformed by ras or src. First, in contrast with CD2-FAK, activated ras or v-src caused a dramatic relief of growth factor dependence and an increased maximal proliferation rate. Second, transformation by ras or src inhibited MDCK cell spreading on collagen (data not shown), while CD2-FAK stimulated it. Third, CD2-FAK expression neither morphologically transforms nor confers anchorage-independent growth in NIH3T3 fibroblasts (Schwartz, M., Scripps Research Institute, personal communication), a cell type that is normally resistant to anoikis but can be transformed by oncogenes that stimulate mitogenesis, such as ras. Finally, although cell adhesion transiently stimulates the (mitogenic) MAPK pathway in fibroblasts, we observed no such stimulation in MDCK cells. The basis for this discrepancy is unclear, but it may be that MAPK is stimulated by focal adhesion plaque formation or stress fiber formation, which are more extensive in fibroblasts than in epithelial cells, rather than by integrin–matrix interactions alone. In this connection, cytochalasin treatment prevents adhesion-dependent MAPK activation in fibroblasts (Chen et al., 1994).

In summary, the acquisition of resistance to anoikis is a potentially crucial step in the acquisition of a fully transformed phenotype by epithelial cells. Certain cells (such as MDCK) appear to fall short of the transformed state by this step alone, but it can be transgressed by the activation of FAK, or, potentially, other integrin signal transducers.

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**Table III. CD2-FAK Does Not Affect Serum Dependence**

| Cell line          | Maximal fold increase | Percent of maximum in 0.5% serum | Percent of maximum in 0.2% serum |
|--------------------|-----------------------|----------------------------------|----------------------------------|
| Control            | 25.3 ± 2              | 9.1 ± 2                          | 3.2 ± 1                          |
| CD2-FAK wtc 9      | 25.3 ± 2              | 9.1 ± 2                          | 3.2 ± 1                          |
| CD2-FAK wtc 30     | 21.3 ± 3              | 10.3 ± 3                         | 5.2 ± 1                          |
| src/mdck           | 17.7 ± 4              | 61.4 ± 6                         | 45.6 ± 9                         |

Cells (5 x 10⁶) were plated in 35-mm diameter wells and changed to medium containing the indicated content of FBS after 6 h. Cells were counted in triplicate at time zero, at 6 h postplating (time zero) and 96 h postplating. The maximal fold increases in cell number occurred in 10% serum.

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