Integrin α2-mediated ERK and Calpain Activation Play a Critical Role in Cell Adhesion and Motility via Focal Adhesion Kinase Signaling

IDENTIFICATION OF A NOVEL SIGNALING PATHWAY*

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Higher levels of focal adhesion kinase (FAK) are expressed in colon metastatic carcinomas. However, the signaling pathways and their mechanisms that control cell adhesion and motility, important components of cancer metastasis, are not well understood. We sought to identify the integrin-mediated mechanism of FAK cleavage and downstream signaling as well as its role in motility in human colon cancer GEO cells. Our results demonstrate that phosphorylated FAK (tyrosine 397) is cleaved at distinct sites by integrin signaling when cells attach to collagen IV. Specific blocking antibodies (clone P1E6) to integrin α2 inhibited FAK activation and cell motility (micromotion). Ectopic expression of the FAK C-terminal domain FRNK attenuated FAK and ERK phosphorylation and micromotion. Calpain inhibitor N-acetyl-leucyl-leucyl-norleucinal blocked FAK cleavage, cell adhesion, and micromotion. Antisense approaches established an important role for μ-calpain in cell motility. Expression of wild type μ-calpain increased cell micromotion, whereas its point mutant reversed the effect. Further, cytochalasin D inhibited FAK phosphorylation and cleavage, cell adhesion, locomotion, and ERK phosphorylation, thus showing FAK activation downstream of actin assembly. We also found a pivotal role for FAK Tyr661 phosphorylation in cell motility and ERK activation. Our results reveal a novel functional connection between integrin α2 engagement, FAK, ERK, and μ-calpain activation in cell motility and a direct link between FAK cleavage and enhanced cell motility. The data suggest that blocking the integrin α2/FAK/ERK/μ-calpain pathway may be an important strategy to reduce cancer progression.

Cell adhesion is of fundamental importance in that it affects cell motility, cell differentiation, signal transduction, and cell invasion (1). Changes in cell adhesion are facilitated by the integrin family of cell surface receptors of extracellular matrix (ECM) proteins. The integrins link cell surface cytoskeletal proteins like focal adhesion kinase (FAK) to actin as well as other cytoskeletal proteins. Significantly, it was shown in the past decade that integrins can directly activate intracellular signaling processes and are thus important signal transduction receptors for biological function. The mammalian integrin receptor family is composed of at least 18 distinct α-subunits and 9 β-subunits, thereby generating 28 distinct integrins through various modes of association (1, 2). GEO colon cancer cells express integrin α2β1, which forms by a noncovalent association of the α2 subunit as a monogamous partner to the promiscuous β1 subunit. Expression of integrin α2 is regulated during normal cell differentiation and is altered during tumorigenesis (3). The expression of integrin α2 has been correlated with metastatic behavior in breast cancer, hepatocarcinoma, and rhabdomyosarcoma (4, 5). Our results further support this linkage in colon carcinoma as well (6–8).

At present, the mechanisms underlying bidirectional signaling triggered by integrins are poorly understood. Ligand binding to integrins is generally regulated to reflect the activation state of the cell. Inside-out regulation of integrin affinity protects the host from pathological integrin-mediated adhesion such as in thrombosis, inflammation, and infectious diseases (1, 11, 12). The phosphorylation of cytosolic proteins plays an important role in inside-out signaling. Inside-out and outside-in signaling are associated with distinct conformational changes in the integrin extracellular domain (13, 14). Our experiments were aimed at determining the mechanism by which human colon cancer GEO cells activate FAK by “outside-in signaling” upon attachment to CN IV, which in turn controls cell adhesion and motility (inside-out signaling).

One of the earliest insights about integrin signaling was the observation that integrin-mediated adhesion and/or clustering led to enhanced tyrosine phosphorylation of a nonreceptor tyrosine kinase now called FAK (15–17). The N-terminal domain of FAK, including its erythrocyte membrane band 4.1-ezrin-radixin-moesin (FERM) region, directs interactions with other molecules such as integrins, c-Src, and phospholipase y. This domain contains a major tyrosine autophosphorylation site at residue 397. Its C-terminal noncatalytic domain includes FAK-related nonkinase (FRNK), a domain for multiple protein-protein interactions, as well as a focal adhesion target region. Both FRNK and focal adhesion target domains act as dominant negatives for cell adhesion functions (18). Recently, compelling evidence has indicated a role by FAK in the pathology of human cancer (19). High levels of FAK expression have been associated with increased invasiveness in malignant tumors. Increased levels of FAK in 17 of 20 invasive colon tumors and in all 15 metastatic tumors were reported by Weiner (20), whereas FAK gene expression...
Novel Integrin α2 Motility Signaling

was not detected in normal colon tissue. Recently, it has been suggested that up-regulation of FAK occurs at an early stage of tumorigenesis (21). A progressive increase in FAK mRNA levels was observed as tumor invaded and metastasized (22). The phenotype of FAK-deficient mice is embryonic lethal due to delayed embryonic migration. This phenotype is also reminiscent of fibronectin or integrin α5-deficient mice, further supporting the concept that extracellular matrix (ECM), integrins and FAK are closely linked (23). FAK−/− cells exhibit reduced migration, whereas cells overexpressing FAK display increased migration on fibronectin (24, 25). However, the mechanisms of FAK activation, cleavage, and deregulation of motility, a critical aspect of tumor progression, are not clear, and no clear model has emerged as to how FAK signaling functions in combination with integrin α2, ERK, and calpain to promote cell motility.

FAK also regulates integrin signaling by recruiting cytoskeletal and signaling proteins via multiple adaptor domains. The absence of cytoskeletal proteins like FAK or paxillin or mutations in the tyrosine residues of these components result in dramatic effects on cell adhesion and motility (26). The translocation of these proteins via the actin cytoskeleton is induced independently of growth factor receptor kinase activity (27). Gaps remain in understanding the sequential events that occur to assemble a focal contact upon cell adhesion. Recently, it was reported that FAK Tyr861 was crucial for H-Ras-induced transformation in fibroblasts through association of FAK with p130CAS (28). The mechanism(s) by which FAK tyrosine sites promote cell motility is less clear.

Cell motility requires a critical balance between cell attachment and detachment, and it requires the complex integration of motility-promoting and motility-inhibiting signals. Calpain has been shown to be a critical regulator of cell motility (29). Calpain is a cysteine protease activated by increased intracellular Ca2+ that localizes to focal adhesions, potentially causing cleavage of focal adhesion proteins. The calpain family includes at least 13 known members, of which two calpain isozymes, μ-calpain and m-calpain, are implicated in cell adhesion, spreading, and migration (30–32). Tumors that have metastasized have been shown to have higher levels of calpain than those that are not metastatic (33). The μ- and m-calpains are phosphorylated, thus enabling their role in signal transduction. However, examination of the precise role of calpains in signal transduction has just begun. It is still obscure as to how the calpains are activated by clustering of integrins. Whereas linkage of calpains with several pathological conditions has been reported, their role in cancer is poorly understood (32). It appears that the function of calpains in cell adhesion and motility may be limited their ability to cleave components of focal complexes in a limited fashion (remodeling), which in turn may increase adhesion turnover. The molecular mechanism of such events is not known and may be deregulated in cancer. Our data demonstrate a direct link between FAK cleavage and enhanced cell motility in colon cancer cells.

Recently, we showed that in human colon carcinoma HCT116 cells, ERK plays an important role mediating endogenous cellular control of integrin α2 expression, cell adhesion, and motility (7). Previously, Miyamoto et al. (34) have shown that integrin aggregation can initiate activation of the ERK signal transduction pathway. Aplin et al. (35) have shown that integrin-mediated ERK activation was via FAK activation, whereas growth factors responded via Shc signaling in NIH3T3 fibroblast cells, suggesting important differences in the activation of ERK/mitogen-activated protein kinase signaling by different ligands. These studies did not address the role of ERK/mitogen-activated protein kinase signaling in cell adhesion and motility. Recent experiments suggest that integrin activation of mitogen-activated protein kinase may be independent of FAK or Ras, through the use of alternative or parallel routes (25). Here, we identify a role for ERK/mitogen-activated protein kinase signaling in colon cancer cell motility via a novel integrin α2/FAK/MEK/calpain pathway.

EXPERIMENTAL PROCEDURES

Materials—Methylthiazole tetrazolium (MTT), CN IV, MeSO4, BSA, cytochalasin D, soy bean trypsin inhibitor, and polyclonal anti-actin antibody were purchased from Sigma. Monoclonal blocking antibodies P1E6 (α2), P1B5 (α3), and P1D6 (α5) and antibodies specific for integrin α2 and α3 subunits, μ-calpain, and m-calpain were purchased from Chemicon International (Temecula, CA), whereas the mouse isotype control IgG1 was from R & D Systems (Minneapolis, MN). Antibodies specific for integrin α5 and β1 (clone 18) were obtained from BD Biosciences. Calbiochem provided the calpain inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN) and the proteasome inhibitor lactacystin. The MEK inhibitor U0126 was obtained through Promega (Madison, WI). Antisense phosphothiorate-linked nucleotides for μ-calpain and m-calpain were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The polyclonal anti-FAK phosphospecific antibodies were provided by BIOSOURCE (Camarillo, CA), whereas the rabbit anti-human C-terminal FAK (SC-558), anti-human N-terminal FAK (SC-557), ERK phosphorylated ERK1/2, and cyclin E antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Microarrays of gold film-coated electrodes as well as the electric cell-substrate impedance-sensing (ECIS) software (model 1600R) used for cell motility (micromotion) experiments were purchased from Applied Biophysics (Troy, NY). Mouse and rabbit peroxide-conjugated AffiniPure goat IgG (H + L) secondary antibodies were from Jackson Laboratories (West Grove, PA). The reagents for cell transfection, Oligofectamine and Lipofectamine, were purchased from Invitrogen, whereas FuGENE 6 was purchased from Roche Applied Science.

Cell Culture—Trypsinization, Replating Assays, and Immunoblotting.

GEO cells were cultured in a humidified incubator at 37 °C for 3–4 days with 5% CO2 in a chemically defined serum-free medium. At about 80% confluence, the medium was changed to supplemented McCoy’s (SM) medium (8). Cells were harvested with trypsin (0.125%) at 37 °C and pelleted by gentle centrifugation at 800 × g in a clinical centrifuge. The pellet was resuspended in SM medium, soy bean trypsin inhibitor (0.5 mg/ml) was added, and further recovery of the cells was allowed for 1 h at 37 °C in a shaker incubator. Cells were then kept either in suspension or replated to attach for the indicated time periods to dishes pre-coated with CN IV (5 μg/ml). The precoating of culture dishes with CN IV was performed by incubating dishes for 2 h at 37 °C. Subsequently, nonspecific sites were blocked with 3% BSA for 3 h at room temperature and finally washed with 10 ml of cold PBS. After cell attachment at 37 °C, dishes were placed on ice and washed gently with cold PBS. The cells were lysed either in suspension or after attachment to precoated CN IV dishes. The lysis buffer A contained 50 mM Tris, pH 7.5, 150 mM NaCl, and 1% Nonidet P-40 along with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 50 mM β-glycerophosphate, 1 mM benzamidine). The cells attached to CN IV were harvested with 75 μl of lysis buffer/100-mm dish, whereas those that were in suspension were lysed with 50 μl of lysis buffer. The lysates were centrifuged in a microcentrifuge at 4 °C for 20 min at 16,000 × g. The supernatants were used for protein determination by the Bio-Rad protocol, and equal amounts of protein aliquots were separated by SDS-PAGE and analyzed by Western blotting.

Immunoprecipitation—GEO cells attached to CN IV were lysed as described above. The lysates (100 μg of protein) were precleared with normal rabbit serum and protein A/G-agarose beads (Calbiochem/Onco- gene) at 4 °C for 1 h. The supernatants were incubated with rabbit
anti-FAK antibody (N-terminal) overnight at 4 °C with rotation. Protein-antibody complexes were incubated with 35 μl of protein A/G-agarose beads for 2 h at 4 °C. After extensive washing with lysis buffer, the beads were boiled with 2× Laemmli buffer and analyzed for FAK by immunoblotting with anti-FAK antibody (N-terminal).

**Biotinylation**—To determine cell surface expression of integrins, GEO cells were biotinylated as described previously (6–8). Briefly, subconfluent GEO monolayers were treated with joklik’s EDTA for 8 min at room temperature, and cells were then scraped and pelleted by centrifugation. The pellet was washed twice with cold PBS, and cells were biotinylated with NHS-LC-Biotin (Pierce), 0.1 mg/ml in Me₂SO at room temperature for 1 h. The biotinylated cells were lysed in buffer A, sheared through a 26-gauge needle, and centrifuged at 16,000 × g for 20 min at 4 °C. Cell lysates were incubated with streptavidin agarose for 90 min at 4 °C, and beads were washed five times with lysis buffer. The beads were boiled in 2× Laemmli buffer for 7 min, supernatant was filtered through Bio-Rad columns, and proteins were separated by SDS-PAGE. The integrins were analyzed by immunoblotting using specific antibodies against α and β1 subunits.

**Short-term Cell Adhesion Assays**—For adhesion assays, 96-well tissue culture plates were coated for 2 h at 37 °C with CN IV at the indicated concentrations, blocked with 3% bovine serum albumin for 3 h, and then rinsed once with PBS. Subsequently, the MTT procedure was followed as described previously (6, 8). After trypsinization (0.125% trypsin in EDTA), GEO cells were preincubated at 37 °C with or without inhibitors for 2 h to determine cell adhesion. Cells were plated at 6 × 10⁴ cells/well on precoated CN IV plates and incubated for 90 min in the absence or presence of inhibitors. Nonadherent cells were removed by washing with SM medium. The relative number of attached cells was determined by the MTT method (36).

**Synthesis of Calpain Antisense Oligonucleotides**—Based on the published effective sequences (37), antisense phosphorothioate-linked nucleotides for μ-calpain (calpain I) and for m-calpain (calpain II) were synthesized by Integrated DNA Technologies (Corvalle, IA). The nucleotides were dissolved in sterile water and stored at −20 °C before use. The effects of the oligonucleotides were validated by analyzing the lysates from antisense oligonucleotide-treated cells for endogenous calpain levels by Western blotting.

**Plasmid Constructs and GEO Cell Transfection**—The FAK and kinase-dead FAK (kinase-defective FAK with a Lys⁶⁴ → Arg mutation in the ATP binding site) constructs were provided by Drs. Jun-Lin Guan (Cornell University, Ithaca, NY), and the FRNK construct was a gift of Dr. Schlaeferp (The Scripps Research Institute, La Jolla, CA). The HA-tagged dominant negative mutant Y861F and WT FAK were gifts of Drs. Eok-So Oh (Seoul, Korea) (28). The hemagglutinin-tagged construct of μ-calpain and its mutant (His⁷２ → Ala) subcloned into the mammalian expression vector pcDNA3 were described previously (38) (provided by Drs. J. Fox and S. Kulkarni; Cleveland Foundation). GEO cells were grown in serum-free medium containing 4 μg/ml transferrin and 20 μg/ml insulin at 37 °C with 5% CO₂. For transient transfection experiments, cells were grown to 50–60% confluence, medium was changed to SM, and transfections were carried out using FUGENE 6 according to our previous report (8). The ratio of FUGENE 6 to DNA was maintained at 10 μl of FUGENE 6 to 2.5 μg of DNA. The DNAs were mixed with the FUGENE 6 and set at room temperature for 45 min. The mixture was then added dropwise to the cells. The cells were harvested at 48 h post-transfection.

**Cell Motility (Micromotion) Measurements by the ECIS Technique**—Cell micromotion was measured using the ECIS technique previously reported (6–8, 39, 40). In the current system (model 1600R), cells were seeded at 1 × 10⁵ cells/well on small gold electrodes (diameter 250 μm) at the bottom of tissue culture wells (area 0.5 cm²). The gold electrodes were preincubated with 200 μl of supplemental McCoy’s medium for at least 1 h. A constant current source applied a noninvasive 1 μA, 4000 Hz AC signal between the small electrode and a much larger counter electrode (0.15 cm²). Any variation of current due to cell movement was recorded. The ECIS software (Applied BioPhysics, Troy, NY) calculated the resistance and capacitance values of the electrode over a period of time. Attachment and movement of the cells on the electrode change the flow of the current, resulting in fluctuations in the electrode resistance and capacitance. These cellular movements are called micromotion (39) and are a measure of the motile ability of the cell being measured. As the cells move on the electrode, the sensitive nature of the lock-in amplifier detects the fluctuations in the resistance and capacitance values (41). These fluctuations were analyzed statistically using ECIS software to reveal the percentage variation in resistance, which in turn is a reflection of cellular micromotion on the electrode. Since the measurements are electrical, they are quantitative and generate data that can be analyzed readily to provide sensitive measurements of changes in cell behavior. This technique detects both translational (xy plane) and vertical (z direction) movement of cells (39).

**RESULTS**

**GEO Cells Predominantly Express Integrin α2B1**—To determine the expression of various integrin receptors, GEO cells were cultured, harvested with joklik’s EDTA, biotinylated, and lysed as described previously (6–8). The lysates were analyzed by immunoblotting for protein expression of integrin α2B1, α3B1, and α5B1 using specific antibodies against the subunits. Fig. 1.4 shows that GEO cells predominantly express integrin α2B1, a lower extent of integrin α3B1, and very little if any integrin α5 subunit.

**GEO Cells Attach to CN IV (and Not to CN I) in a Concentration-dependent Fashion**—There are several reports that integrin α2 is a receptor of CN I in different cell types (42, 43). To determine whether that is the case with GEO cells, we tested attachment to CN I (1–5 μg/ml) as compared with BSA. To our surprise, GEO cells that predominately express integrin α2 did not significantly attach to CN I. Instead, under identical experimental conditions, GEO cells attached to CN IV in a concentration-dependent manner (Fig. 1B). These results show that the ligand for integrin α2 receptor is cell type-specific.

**Adhesion of GEO Cells to CN IV Is Mediated Predominantly by Integrin α2**—The specificity of integrin α2 in mediating adhesion of GEO cells to CN IV was further determined by treatment with specific functional blocking antibodies to inhibit binding to CN IV. Monoclonal anti-integrin α2 antibody (clone PIE6) was highly effective in preventing GEO cell adhesion to CN IV, and inhibition was concentration-dependent (Fig. 1C). Inhibitory levels ranged from 70 to 20% at antibody dilutions ranging from 1:50 to 1:500. Blocking antibody to the integrin α5 subunit had no effect on GEO cell adhesion to CN IV. Anti-integrin α3 subunit had much less inhibitory effect (6–30% at 1:50 dilution) on GEO cell adhesion to CN IV. Integrin subunits α3 and α5 are the predominant cell adhesion receptors for laminin and fibronectin, respectively.

**Plating on CN IV Enhances Tyrosine Phosphorylation of Proteins in GEO Cells**—In an effort to identify proteins that are activated by cell adhesion to CN IV, GEO cells were allowed to attach to CN IV-coated dishes for 20 or 60 min in SM medium. Cells kept in suspension served as a control for nonintegrin-mediated signaling. Antiphosphotyrosine immunoblot analyses of cell lysates using 4G10 primary antibody revealed several phosphorylated proteins. In contrast to cell suspensions (5), cell adhesion (A) to CN IV resulted in significantly enhanced
Expression of integrin α2β1, α3β1, and α5β1 (A), comparison of cell adhesion on CN I and CN IV (B), and inhibition of cell adhesion to CN IV by antibodies to integrin receptors (C). GEO cells were harvested at near confluence, biotinylated, and lysed by integrin lysis buffer. The lysates were analyzed by 7.5% polyacrylamide gel electrophoresis and immunoblotting with antibodies against human integrin α subunits as detailed under “Experimental Procedures.” The membranes were stripped at 50 °C for 30 min in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 μM Tris-HCl, pH 6.8), blocked with 5% nonfat dry milk for 1 h, and immunoblotted either for integrin β1 or for actin using specific antibodies. 96-well tissue culture plates were coated with different concentrations of CN I or CN IV as indicated. GEO cells were seeded at 6 × 10^4 cells/well onto coated plates and incubated for 90 min at 37 °C. The relative number of attached cells was determined by MTT assay as described under “Experimental Procedures” (B). 96-well tissue culture plates were coated with CN IV (5 μg/ml), and blocking monoclonal antibodies to integrin α2, α3, and α5 subunits were added at different dilutions as indicated. The control IgG1 was used at 1:50 dilution. Lane 1, without antibody; lanes 2–4, integrin α2 antibody; lanes 5–7, integrin α3 antibody; lane 8, integrin α5 antibody (C). Adhesion assays were performed as detailed under “Experimental Procedures.”

Tyrosine phosphorylation of ∼190-, 125-, 90-, 70-, and 40-kDa molecular mass proteins (Fig. 2A).

Cell Attachment to CN IV Leads to FAK Activation and Cleavage—To identify proteins in Fig. 2A, equal amounts of total protein from cell suspension and cell attachment lysates were fractionated by SDS-PAGE. Western blot analysis using specific phosphoantibodies showed that in contrast to suspended cells, attachment to CN IV enhanced tyrosine phosphorylation and cleaves phosphorylated FAK (Tyr397) to a 90-kDa fragment (Fig. 2B). Cells in suspension showed either little or no FAK cleavage. GEO cells attached to CN IV exhibited FAK cleavage at both 20 and 60 min. At 60 min, the cleavage of FAK was greater than at the 20-min period. The 90-kDa fragment in total FAK is detected by the anti-human N-terminal FAK (Fig. 2B, bottom).

Integrin α2 Mediates FAK Phosphorylation—We hypothesized that integrin α2 plays a pivotal role in FAK activation. To test this hypothesis, GEO cells were incubated with specific integrin α2-blocking antibodies (P1E6) for 30 min at 37 °C prior to replating on precoated CN IV dishes. The phosphorylation of FAK was significantly blocked by P1E6 in GEO cells (Fig. 2C).

Cell Attachment to CN IV Activates ERK1 and ERK2—In addition to FAK activation and cleavage, the same membrane was stripped and analyzed by specific phosphorylation antibodies against extracellular signal-regulated kinase (ERK) activation. Western blot analysis using a specific phospho–Tyr 204 antibody showed that the kinetics of ERK activation were similar to FAK activation and cleavage (Fig. 2D). These results suggested that integrin α2-mediated FAK activation was regulating downstream ERK signaling.

Cytochalasin D Inhibits FAK Activation, Cell Adhesion, and Haptotactic Micromotion in a Parallel Fashion—Actin polymerization is critical in formation of focal adhesions when cells attach to extracellular matrix protein. To determine the relationship between actin stress fiber organization and FAK activation and cleavage, GEO cells were treated with cytochalasin D (0.50 μM), an agent that binds to the barbed ends of F-actin filaments and prevents actin polymerization. Fig. 3A shows that cyto D inhibits integrin α2-mediated activation and cleavage of FAK. These results indicate that the integrity of the endogenous actin cytoskeleton is essential for FAK activation and cleavage. These results support the hypothesis that integrin α2-stimulated FAK activation is mediated by actin cytoskeletal association.

Actin polymerization is critical in cell spreading and motility; therefore, to evaluate the significance of intact actin, we performed adhesion assays and cell motility experiments using cyto D as the inhibitor. As shown in Fig. 3B, we observed 45% inhibition of GEO cell adhesion to CN IV with cyto D (0.25 μM) as compared with Me2SO-treated control cells. These results confirm that actin organization is important in GEO cell adhesion to CN IV.

Next, to determine the effect of CN IV on cell motility, we evaluated haptotactic cell micromotion using CN IV as a substrate. The ECIS technique was used to quantitate cell micromotion in GEO cells. Fig. 3C shows that in the uncoated electrode well (control), the percentage var-

**Figure 1.** Expression of integrin α2β1, α3β1, and α5β1 (A), comparison of cell adhesion on CN I and CN IV (B), and inhibition of cell adhesion to CN IV by antibodies to integrin receptors (C). GEO cells were harvested at near confluence, biotinylated, and lysed by integrin lysis buffer. The lysates were analyzed by 7.5% polyacrylamide gel electrophoresis and immunoblotting with antibodies against human integrin α subunits as detailed under “Experimental Procedures.” The membranes were stripped at 50 °C for 30 min in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 μM Tris-HCl, pH 6.8), blocked with 5% nonfat dry milk for 1 h, and immunoblotted either for integrin β1 or for actin using specific antibodies. 96-well tissue culture plates were coated with different concentrations of CN I or CN IV as indicated. GEO cells were seeded at 6 × 10^4 cells/well onto coated plates and incubated for 90 min at 37 °C. The relative number of attached cells was determined by MTT assay as described under “Experimental Procedures.” The membranes were stripped at 50 °C for 30 min in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 μM Tris-HCl, pH 6.8), blocked with 5% nonfat dry milk for 1 h, and immunoblotted either for integrin β1 or for actin using specific antibodies. 96-well tissue culture plates were coated with different concentrations of CN I or CN IV as indicated. GEO cells were seeded at 6 × 10^4 cells/well onto coated plates and incubated for 90 min at 37 °C. The relative number of attached cells was determined by MTT assay as described under “Experimental Procedures.”

**Figure 2.** (A) Expression of integrin α2, α3, and α5 (left). 96-well tissue culture plates were coated with different concentrations of CN I or CN IV as indicated. GEO cells were seeded at 6 × 10^4 cells/well onto coated plates and incubated for 90 min at 37 °C. The relative number of attached cells was determined by MTT assay as described under “Experimental Procedures.” The membranes were stripped at 50 °C for 30 min in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 μM Tris-HCl, pH 6.8), blocked with 5% nonfat dry milk for 1 h, and immunoblotted either for integrin β1 or for actin using specific antibodies. 96-well tissue culture plates were coated with different concentrations of CN I or CN IV as indicated. GEO cells were seeded at 6 × 10^4 cells/well onto coated plates and incubated for 90 min at 37 °C. The relative number of attached cells was determined by MTT assay as described under “Experimental Procedures.” The membranes were stripped at 50 °C for 30 min in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 μM Tris-HCl, pH 6.8), blocked with 5% nonfat dry milk for 1 h, and immunoblotted either for integrin β1 or for actin using specific antibodies. 96-well tissue culture plates were coated with different concentrations of CN I or CN IV as indicated. GEO cells were seeded at 6 × 10^4 cells/well onto coated plates and incubated for 90 min at 37 °C. The relative number of attached cells was determined by MTT assay as described under “Experimental Procedures.”

**Figure 3.** (A) Expression of integrin α2, α3, and α5 (left). 96-well tissue culture plates were coated with different concentrations of CN I or CN IV as indicated. GEO cells were seeded at 6 × 10^4 cells/well onto coated plates and incubated for 90 min at 37 °C. The relative number of attached cells was determined by MTT assay as described under “Experimental Procedures.” The membranes were stripped at 50 °C for 30 min in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 μM Tris-HCl, pH 6.8), blocked with 5% nonfat dry milk for 1 h, and immunoblotted either for integrin β1 or for actin using specific antibodies. 96-well tissue culture plates were coated with different concentrations of CN I or CN IV as indicated. GEO cells were seeded at 6 × 10^4 cells/well onto coated plates and incubated for 90 min at 37 °C. The relative number of attached cells was determined by MTT assay as described under “Experimental Procedures.” The membranes were stripped at 50 °C for 30 min in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 μM Tris-HCl, pH 6.8), blocked with 5% nonfat dry milk for 1 h, and immunoblotted either for integrin β1 or for actin using specific antibodies. 96-well tissue culture plates were coated with different concentrations of CN I or CN IV as indicated. GEO cells were seeded at 6 × 10^4 cells/well onto coated plates and incubated for 90 min at 37 °C. The relative number of attached cells was determined by MTT assay as described under “Experimental Procedures.”
methylation in resistance measured over a period of 70 min was 0.8467% (top) as compared with the percentage variation 2.147% of precoated CN IV electrode well (bottom). The results show that GEO cells attached to CN IV had an increase in motion of 154%.

Micromotion measured by the ECIS technique is directly related to conventional cell motility (44). The ECIS technique has been used to detect cell morphology, cell motility, and cell-ECM interactions in different systems (45, 46). To determine the role of actin polymerization in cell locomotion, cells (6 × 10⁴) were plated on electrodes precoated with CN IV. The subconfluent cultures were treated with 0.25 and 0.50 μM concentrations of cyto D, cells were allowed to attach for 3 h, and then micromotion was recorded. Fig. 3D shows that in the control GEO cells, the percentage variation in resistance was 2.309% (DMSO; top). Treatment of the cells with cyto D (0.25 μM; middle) decreased the percentage variation in resistance to 1.241%, indicating a decrease (46.25%) in cell micromotion. GEO cells were treated with higher concentrations of cyto D (0.50 μM; bottom), we observed a further decrease (69.64%) in cell micromotion (percentage variation in resistance was 0.702%), thus demonstrating that the inhibitory effect of cyto D on cell micromotion was concentration-dependent. These results show that actin polymerization plays a pivotal role in GEO cell micromotion on CN IV. Taken together, the data demonstrate that actin polymerization plays a critical role in FAK phosphorylation and cleavage, cell adhesion, and locomotion on CN IV.

Functional Blocking mAb to Integrin α2 Attenuates Cell Micromotion—To establish that cell micromotion on CN IV was specifically integrin α2-mediated, GEO cells were preincubated either with mouse IgG or with functional blocking mAb (clone PIE6) before recording micromotion by the ECIS technique. The percentage variations in resistance observed were 2.363% (control IgG) and 0.504% (PIE6). As shown in Table 1, the suppression in cell micromotion (resistance) of GEO cells by PIE6 was 78.7%, as compared with control IgG-treated cells.

ALLN Inhibits FAK Cleavage, Cell Adhesion, and Cell Micromotion in a Parallel Fashion—To explore the possibility that integrin α2-mediated signaling may activate calpains, which in turn cleave focal adhesion kinase, GEO cells were incubated in the presence of acetyl-leucyl-leucyl-norleucinal (ALLN), a cell-permeable calpain inhibitor (47), for 3 h at 37 °C prior to trypsinization and replating of cells precoated CN IV dishes. Cells lysates were analyzed by Western blot using an antibody specific to FAK Tyr397. There was a dramatic decrease in FAK cleavage in ALLN-treated lysates as compared with control (Me2SO-) treated lysates (Fig. 4A). These results show a critical role of calpain(s) in the cleavage of FAK in GEO cells.

To determine the function of FAK cleavage by calpain(s) in cell adhesion, assays were performed on CN IV-coated wells. Fig. 4B shows that integrin α2-mediated cell adhesion was inhibited by ALLN (46–85%) in a manner that was dependent on the concentration of CN IV.

To further define the role of calpain(s) in cell motility, we used the real-time ECIS technique. The electrode wells were precoated with CN IV, and GEO cells (1 × 10⁴) were inoculated in SM medium. Cells were allowed to attach for 3 h, and then micromotion was measured under the same experimental conditions in the presence of either Me₂SO or ALLN. Table 1 shows that in the control Me₂SO-treated cells, the percentage variation in resistance was recorded as 1.496%. Treatment of the cells with ALLN
FIGURE 3. Effect of cyto D on FAK phosphorylation (A) and on cell adhesion to CN IV (B). The ligand CN IV enhances cell micromotion (C), whereas cyto D attenuates micromotion in a concentration-dependent fashion (D). GEO cells were treated either with Me2SO (control) or with cyto D for 24 h. After trypsinization, cells were transferred to CN IV-precoated dishes and incubated at 37 °C in the presence of Me2SO or cyto D for 1 h. Lysates were collected and analyzed by Western blot using an antibody specific to phospho-FAK (Tyr397) (A). GEO cells were trypsinized after treatment with Me2SO or with cyto D for 24 h. Cells were then inoculated at 6 × 10^4 cells/well into BSA- or CN IV-coated 96 well plates and incubated at 37 °C for 90 min in the presence of Me2SO (DMSO) or cyto D as indicated. Nonadherent cells were washed off, and adherent cells were determined by MTT assay as described under “Experimental Procedures” (B). GEO cells in SM medium were seeded (6 × 10^4) in microarray wells either uncoated (top) or coated with CN IV (bottom). After 3 h of attachment, micromotion was recorded (C). Precoated CN IV (5 μg/ml) electrode arrays were used in these experiments. GEO cells were monitored for cell micromotion either in the absence (top) or presence of cyto D (0.25 μM (middle), 0.5 μM (bottom)) (D).
decreased the fluctuations such that the percentage variation in resistance was now 0.433%, indicating a 71% decrease in cell micromotion by ALLN. These results demonstrate that inhibition in FAK cleavage by calpain(s) attenuates cell motility. Alternatively, the results may be interpreted as showing that cleavage of FAK enhances cell motility.

**Lactacystin Does Not Attenuate FAK Cleavage**—Although ALLN is widely known as a selective calpain inhibitor, it may inhibit the 26 S proteasome pathway (48). To confirm that the effect of ALLN was selective in inhibiting FAK cleavage and did not inhibit cleavage via the proteasome pathway, GEO cells were treated with different concentrations of lactacystin, a selective proteasome inhibitor. The cell lysates were analyzed for cyclin E, as a positive control, by immunoblotting (49). The results showed that lactacystin treatment slightly enhanced cyclin E accumulation at 10 μM concentration, whereas 20 μM lactacystin dramatically increased the accumulation of cyclin E (Fig. 4, top).

**TABLE 1**
The percentage variation in resistance of micromotion

| Control            | Treatment | Variation with treatment | Percentage increase or decrease |
|--------------------|-----------|--------------------------|---------------------------------|
| Control (IgG)      |           |                          |                                 |
| Control (Me2SO)    |           | 2.363                    | 0.504                           |
| Control (empty vector pcDNA 3.1) |           | 1.496                    | 0.433                           |
| Control (empty vector pRC/CMV) |           | 2.297                    | 0.905                           |
| Control (empty vector pKH3) |           | 2.701                    | 4.537                           |
| Control (empty vector pK2H) |           | 2.007                    | 0.969                           |

Under the same conditions, cells treated either with Me2SO or with 20 μM lactacystin were replated on CN IV-coated dishes for 1 h in the presence of Me2SO or lactacystin (20 μM). The lysates were analyzed for pFAK (Tyr397) and total FAK. GEO cells treated with lactacystin did not show inhibition in FAK cleavage as compared with control (Me2SO) cells (Fig. 4C, bottom). These results show that the 26 S proteasome does not play a significant role in the cleavage of FAK in GEO cells, thus supporting the notion that inhibition of FAK cleavage by ALLN is calpain-dependent.

**Critical Role of μ-Calpain in Cell Adhesion and Micromotion**—Cell motility is dependent on cell-substrate attachment at the leading edge of the cell in coordination with cell-substrate detachment at the rear of the cell. The attachment at the leading edge of the cell is associated with the formation of the focal adhesion complexes, whereas detachment at the rear of the cell is associated with the disassembly of the focal adhesion complexes and the proteolytic cleavage of the proteins that make up the focal adhesion complexes (47, 50, 51). The major calpains expressed by GEO cells are μ- and m-calpains. To determine whether one or both forms of calpains contribute to cell micromotion, we used two approaches. First, specific antisense nucleotides for both calpains, anti-μ-calpain (5′-ACTCTCTGTCATCCTGGGG-3′) and anti-m-calpain (5′-TGCCCCGCCCAGTGGATACGTAC-3′) were synthesized (37). The antisense nucleotides inhibited endogenous levels of respective calpains as determined by the Western blot analyses using specific antibodies (Fig. 5A). The micromotion was recorded in the absence or presence of these antisense nucleotides. The percentage variation in resistance of control cells was 5.537% (Fig. 5B, top), whereas in the presence of μ-antisense nucleotide, the percentage variation in resistance decreased to 2.674% (middle), thus showing a 52% inhibition in cell mobility.
micromotion. Simultaneous recording of micromotion of GEO cells in the presence of \(\mu\)-calpain antisense nucleotides showed a percentage variation of 4.857% in resistance (Fig. 5B, bottom), indicating a small decrease in micromotion (12%). Each set of nucleotides functioned as a control for the other. These results provide evidence for the major role of \(\mu\)-calpain in micromotion.

To further confirm the role of \(\mu\)-calpain in cell adhesion and motility, we used another approach, which included transfection of cells with \(\mu\)-calpain plasmid. GEO cells were transiently transfected either with empty vector (pcDNA 3) or with WT HA-\(\mu\)-calpain expression vector. First we examined the effect of overexpression of \(\mu\)-calpain on cell adhesion to CN IV by an MTT assay (Fig. 5C). The results showed 69% inhibition in cell adhesion by WT \(\mu\)-calpain-transfected cells as compared with control empty vector transfectants. Since ectopic WT \(\mu\)-calpain expression inhibits cell adhesion, therefore, a mutant of \(\mu\)-calpain in which histidine 272 has been mutated to alanine would be expected to reverse the inhibitory effect of WT \(\mu\)-calpain. Under same experimental conditions, we observed only 32% inhibition in cell adhesion to CN IV by the mutant (as compared with 69% inhibition by WT \(\mu\)-calpain). These experiments strongly support the role of \(\mu\)-calpain in integrin \(\alpha_2\)-mediated GEO cell adhesion.

Next, we demonstrated a cause and effect relationship between calpain-mediated adhesion and motility triggered by integrin \(\alpha_2\) signaling. GEO cells were transfected either with empty vector or with the construct WT \(\mu\)-calpain or its mutant. The transfectants were seeded at a density of \(1 \times 10^5\) cells/well on CN IV-precoated microarray wells. Cells were allowed to attach for 3 h, and then micromotion was monitored by ECIS (Fig. 5D). The percentage variation in resistance of WT \(\mu\)-calpain expression inhibits cell adhesion, therefore, a mutant of \(\mu\)-calpain in which histidine 272 has been mutated to alanine would be expected to reverse the inhibitory effect of WT \(\mu\)-calpain. Under same experimental conditions, we observed only 32% inhibition in cell adhesion to CN IV by the mutant (as compared with 69% inhibition by WT \(\mu\)-calpain). These experiments strongly support the role of \(\mu\)-calpain in integrin \(\alpha_2\)-mediated GEO cell adhesion.
pain plasmid-transfected cells (2.224%; Fig. 5D, middle) was 167.3% higher than empty vector-transfected cells (percentage variation 0.832%; Fig. 5D, top). The increase in micromotion by μ-calpain transfectant was blocked by the μ-calpain mutant where histidine 272 was mutated to alanine (percentage variation 0.839%; Fig. 5D, bottom).

**FRNK Inhibits FAK Activation and Cell Micromotion, whereas Kinase-dead Mutant Increases Cell Micromotion**—The mechanism(s) of FAK phosphorylation and its cleavage in cell motility is not well understood. To determine the role of FAK in cell motility, GEO cells were transiently transfected either with empty vector (pcDNA 3.1) or with HA-tagged FRNK (gift of Dr. Schlaepfer), which acts as a dominant negative mutant of FAK and interferes with FAK signaling. The validity of FRNK function is demonstrated by Western blot analysis as shown in Fig. 6. The effect of FRNK transfection on cell micromotion (Table 1) shows that the percentage variation in resistance (2.297%) of empty vector-transfected cells was reduced by 60.62% in FRNK-transfected cells (percentage variation in resistance 0.905%). These results show that the carboxyl domain of FAK contributes to increased cell motility.

In contrast to the inhibitory effect of FRNK on cell micromotion, the FAK kinase-dead plasmid transfectants (Table 1; percentage variation in resistance 4.537%) showed enhanced micromotion (68%) on CN IV as compared with control empty vector-transfected GEO cells (percentage variation in resistance 2.701%). These results indicate that in GEO cells, the kinase domain of FAK is at least partly dispensable for micromotion on CN IV.

**Dominant Negative Mutant Y861F Inhibits Cell Adhesion and Haptotactic Cell Micromotion**—The tyrosine 861 site is located in between the proline 1- and proline 2-rich regions of the FAK carboxyl-terminal domain. The contribution of Tyr861 to cell motility is poorly understood. First, to determine whether the Tyr861 site is activated by integrin α2-mediated signaling, GEO cells were either kept in suspension or
replaced on CN IV-precoated dishes. We observed enhanced Tyr(S61)
phosphorylation with attachment of cells to CN IV (Fig. 7A). Therefore,
we examined the role of Tyr(S61) phosphorylation in cell adhesion
and motility. GEO cells were transfected either with empty vector (pRC/CMV)
or with the mutant Y861F (where Tyr(S61) has been mutated to a
phenylalanine residue). Adhesion assays showed that cell attachment
to CN IV by the mutant was suppressed by 52–57%, depending on the
concentration of CN IV as compared with empty vector transfectants
(Fig. 7B). The effect of the mutant on cell micromotion was similar to
that of cell adhesion. We observed that the percentage variation in
resistance of control cells was 2.007%, whereas the mutant Y861F-transfected GEO cells recorded percentage variation in resistance 0.969%
(Table 1). These values indicate attenuation of cell micromotion by
51.7% by the mutant. These results show that phosphorylation of the
Tyr(S61) site promotes cell motility in GEO cells, thereby playing an
important role in cell adhesion. Western blot analysis (Fig. 7C) shows
that overexpression of WT FAK enhances Tyr(S61) phosphorylation, as
compared with control empty vector transfectants, whereas cotransfec-
tion with dominant negative mutant Y861F blocks phosphorylation.

Inhibition of ERK Activation and Cell Micromotion by U0126—Since GEO cells enhance ERK phosphorylation upon attachment to CN IV
(Fig. 2D), we examined the role of downstream ERK/mitogen-activated
protein kinase signaling in motility. GEO cells were allowed to attach
collagen IV-precoated electrode wells in the absence or presence of
U0126, a well known MEK inhibitor (7). Those cells treated with MEK
inhibitor showed about 48% attenuation in micromotion (Fig. 8A; right;
percentage variation in resistance 0.676%) as compared with those in
the Me2SO-treated control cells (Fig. 8A; left; percentage variation in
resistance 1.311%). It is noteworthy that inhibition in micromotion was
directly linked to inhibition in ERK activation (Fig. 8D) by U0126 as
determined by Western blotting (Fig. 8B, top). To further illustrate the
mechanism of integrin α2-mediated ERK phosphorylation, we
performed the following experiments.

Effect of Cyto D, Dominant Negative FRNK, FAK Y861F Mutant, and
µ-Calpain Mutant on ERK Phosphorylation—Our results show that
cyto D dephosphorylates activated FAK (Fig. 3A). To determine the
functional link between FAK and downstream ERK phosphorylation,
GEO cells were treated with cyto D, trypsinized, and replaced on CN
IV-coated dishes for 1 h. The cell lysates were analyzed for ERK phos-
phorylation by Western blot using specific antibodies. The inhibitory
effect of cyto D on ERK phosphorylation showed that in GEO cells,
intact actin is important for both FAK and downstream ERK activation
(Figs. 3A and 8C).

To confirm the role of FAK in ERK activation, GEO cells were tran-
siently transfected either with empty vector (pcDNA 3.1) or with dom-
inant negative FRNK plasmid. After transfection, cells were trypsinized
and replaced on CN IV for 1 h. The cell lysates were analyzed for ERK
phosphorylation by Western blotting using specific antibodies. Fig. 8D
shows that DN FRNK diminished activation of ERK. The results con-
firm that downstream ERK activation, in response to integrin α2 stim-
ulation, is mediated by FAK signaling.

The experiments described above showed that FRNK attenuates ERK
activation. Therefore, to determine the role of FAK Tyr(S61) (located in
the FRNK domain) in ERK activation, GEO cells were transfected either
with empty vector pRC/CMV or with the mutant Y861F. Cell lysates
were analyzed for ERK by Western blotting with a specific phosphoryl-
tion antibody (Fig. 8E, lane 2) or with ERK antibody (Fig. 8E, lane 3),
inhibitory effect of cyto D on ERK phosphorylation showed that in GEO cells, intact actin is important for both FAK and downstream ERK activation
(Figs. 3A and 8C).

To confirm the link between µ-calpain activation and ERK signaling,
GEO cells were transfected either with empty vector or with the dom-
inant negative calpain mutant in which histidine 272 has been mutated
to alanine. Immunoblotting with specific antibodies (Fig. 8E, lane 3)
showed that the mutant attenuated ERK phosphorylation (Fig. 8F). These
results show that µ-calpain regulates downstream ERK phosphoryla-
tion. These experiments establish a direct link between FAK signaling
and downstream ERK activation in GEO cells.

DISCUSSION

To define the mechanism of integrin-mediated signaling (outside-in
signaling) in cell motility (inside-out signaling), we first determined the
expression of integrins by human colon cancer GEO cells. Western blot

![FIGURE 7. Role of FAK Tyr(S61) in cell adhesion and micromotion. GEO cells cultured in SM medium were trypsinized, incubated with soy bean trypsin inhibitor, and kept either in suspension or replated on precoated CN IV dishes. Cell lysates were analyzed by Western blot analysis using site-specific phospho-FAK (Tyr(S61)) antibody (top) or FAK antibody for equal loading control (bottom) (A). GEO cells were transfected either with empty vector (pRC/CMV) or with the mutant Y861F. The transfectants were seeded in a 96-well plate precoated with CN IV as indicated and analyzed by the MTT methodology as described under “Experimental Procedures” (B). GEO cells were transfected with empty vector (C; lane 1), WT FAK (lane 2), or cotransfected with WT FAK and mutant Y861F (lane 3) as described under “Experimental Procedures.” Cell lysates were analyzed by Western blotting for Tyr861 using specific phosphoantibodies. Actin was used as a loading control. For micromotion experiments, data are presented in Table 1, and experiments were performed as described under “Experimental Procedures.”]
analysis showed that the major integrin expressed by GEO cells was integrin α2β1. GEO cells also express integrin α3β1 to a smaller extent and do not express integrin α5 protein. To determine the ligand for integrin α2, adhesion assays were performed using CN I or CN IV as substrates. The results showed that GEO cells attached to CN IV in a concentration-dependent fashion, whereas under the same conditions, they did not significantly attach to CN I. Previously, CN I was reported as a substrate for integrin α2 in various types of cells (42, 43). Our experiments indicate that the ligand for integrin α2 receptor is a function of cellular context. Integrins are believed to recognize their ligands on the basis of the amino acid sequence within an inserted domain (I domain) at the N terminus of the α subunit of integrin (52). In GEO cells, the specificity of integrin α2 in mediating cell adhesion to CN IV was confirmed by treating cells with functional blocking antibodies to integrin α2 (clone P1E6). The inhibition of cell adhesion to CN IV was dependent on the concentration of P1E6.

Next we set out to determine the nature of proteins that are phosphorylated when GEO cells attach to CN IV, a major component of the basement membrane collagen. Western blot analysis using phosphotyrosine specific antibody (4G10) showed that relative to cell suspension lysates, cells attached to CN IV showed enhanced phosphorylation of FAK (Tyr397) and its major cleaved fragment (90 kDa). The 90-kDa fragment further cleaves into 50- and 40-kDa products (53). Under similar experimental conditions, the cleavage of activated FAK was either not observed or observed to a small extent in cell lysates prepared from cell suspensions. Previous investigators using human Caco-2 intestinal epithelial cells did not observe cleavage of FAK upon cell attachment to CN IV (54). Our results indicate that the limited cleavage of FAK is cell type-specific. Limited FAK cleavage may have a physiological function for cytoskeletal remodeling and may contribute to signal transduction as an alternative mechanism to phosphorylation or dephosphorylation events. The cell motility consequences of FAK cleavage have not been directly examined previously.

To determine the mechanism of phosphorylated FAK cleavage, we first investigated the role of integrin α2 signaling. GEO cells were preincubated with functional blocking monoclonal antibody P1E6 before attachment to precoated CN IV dishes. Western blot analysis of the cell lysates showed that the activation of FAK was significantly reduced by P1E6. To determine the factor contributing to FAK cleavage, we studied the role of calpains, which play an important role in cell adhesion. In initial experiments, we used ALLN, a well characterized inhibitor of calpains (55). GEO cells were pretreated with ALLN and subsequently allowed to attach on CN IV-coated dishes. ALLN blocked FAK cleavage (Fig. 4A). These results indicated that integrin α2-mediated signaling activates calpain(s), which in turn cleave FAK in a limited fashion. The possibility of proteasome-dependent cleavage being responsible for FAK cleavage was determined by using lactacystin as a proteasome-specific inhibitor. We followed the well characterized cyclin E protein stability as an independent positive marker of proteasome inhibition (49). In GEO cells, we observed that cyclin E reaches high levels when treated with 20 μM lactacystin, indicating that the proteasome system

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**FIGURE 8.** Effect of U0126, cyto D, and dominant negative mutants on ERK activation. GEO cells were treated either with Me2SO or with MEK inhibitor, U0126, for 24 h in SM medium. After trypsinization, cells were seeded on CN IV-coated microarray wells. Cells were allowed to attach for 3 h, and then micromotion was recorded by the ECIS technique. Results show the inhibitory effect of U0126 on micromotion (right) as compared with control (left) (A). B shows the inhibitory effect of U0126 on ERK phosphorylation as determined by Western blot analysis using either phosphospecific antibody (Y204; top) or ERK antibody (bottom). In C, GEO cells were treated either with Me2SO (control) or with cyto D for 24 h. After trypsinization, cells were transferred to CN IV precoated dishes and incubated at 37 °C in the presence of Me2SO or cyto D for 1 h. Lysates were collected and analyzed by Western blot using an antibody specific to phospho-ERK or total ERK, as indicated. In D–F, GEO cells were transfected either with empty vector or with the dominant negative FRNK, Y861F, or μ-calpain mutant for 48 h, respectively. The cell lysates were analyzed by using specific antibodies for phospho-ERK (top) or ERK (bottom).
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can be significantly blocked with this concentration of lactacystin. Under the same conditions, we did not observe attenuation of FAK cleavage by lactacystin. Together, these experiments exclude involvement of the proteasome in the calpain-mediated limited cleavage of FAK in GEO cells. These results provide further support for calpain as an important candidate for modulation of cell motility through its capacity to regulate focal adhesion dynamics.

Next we investigated the significance of FAK cleavage in biological functions such as cell motility. The mechanism and function of FAK cleavage in cell motility have not been clear. Cell motility is a multistep process that requires a critical balance between cell attachment and cell detachment on the dynamic ECM. These events are largely mediated by ECM receptors, integrins, and other proteins that form focal adhesions. Integrin clustering results in increased protein-tyrosine phosphorylation of FAK. The attachment of the leading edge of the cell is associated with the formation of focal adhesion complexes, whereas detachment at the rear of the cell is associated with the disassembly of focal adhesion complexes and the cleavage of the proteins that make up the focal adhesion complexes. The cleavage of FAK has been assumed to be associated with the disassembly of focal adhesions (47); however, a direct relationship of FAK cleavage with cell motility has not been previously demonstrated. To determine the potential role of FAK activation and its limited cleavage in cancer cell motility, we used an objective real time and quantitative ECIS technique. First, to determine whether CN IV affects cell micromotion, we compared cell micromotion on CN IV-coated and -uncoated gold electrodes, which are deposited in tissue culture wells. Fig. 3C shows that the percentage variation in resistance on the CN IV-coated electrode (2.147%) was higher than on the uncoated electrode (percentage variation in resistance 0.847%). These results show that FAK activation and cleavage on CN IV enhanced cell micromotion by 154%. To establish that cell micromotion on CN IV was specifically integrin α2-mediated, GEO cells were preincubated either with mouse IgG (control) or with integrin α2 blocking monoclonal antibody P1E6 before recording micromotion. Based on the percentage variation in resistance, we found that the reduction in cell locomotion of GEO cells by P1E6 was 79% as compared with control IgG-treated cells (Table 1). These results again support the concept that integrin α2-mediated FAK cleavage enhances cell motility. To explore other factors that may contribute to FAK cleavage and motility, we examined the hypothesis that calpains are activated in adherent cells plated on ECM and that calpains may be involved in integrin α2-induced remodeling of the cytoskeleton. For these experiments, we used the small molecule calpain-specific inhibitor ALLN (55) which reduced GEO cell adhesion to CN IV (46–85%) (Fig. 4B). To further define the role of calpains in cell motility, we measured micromotion under identical conditions, treating GEO cells either with Me2SO (control) or with ALLN. Treatment of the cells with ALLN decreased micromotion by 71% (Table 1). This is probably due to the blockade of integrin α2-CN IV detachment, which appears to be dependent on the breakdown of the focal adhesion complexes following FAK cleavage by calpains that is substantially reduced by ALLN. These results are consistent with a mechanism involving calpains in a pivotal role for control of GEO cell motility and suggest that inhibiting calpain-mediated cleavage of FAK may be a potential therapeutic approach to control pathological cell motility, such as cancer metastasis.

Further in unraveling the signaling pathways critically important to cell motility, we asked whether both typical isoforms of calpain, µ-calpain (calpain I) and m-calpain (calpain II), expressed by GEO cells are required for integrin α2-mediated cell micromotion. To determine whether one or both calpain isoforms contribute to cell micromotion, we used two approaches. First, specific antisense nucleotides to µ-calpain and m-calpain were synthesized according to the known sequences (37). The micromotion was monitored simultaneously in the presence or absence of either µ-calpain or m-calpain antisense nucleotides. The percentage variations in resistance observed were 5.54, 2.67, and 4.86%, respectively. Fig. 5B shows that, relative to control, about 52% inhibition in micromotion was observed in µ-calpain antisense-treated cells, whereas only 12% inhibition in cell micromotion was observed in cells treated with m-calpain antisense nucleotides. These results show that although GEO cells express both forms of calpain, it is µ-calpain that primarily contributes to enhanced cell micromotion. The above results demonstrate for the first time a physiological role for calpains in modulating integrin α2-mediated FAK cleavage, cell adhesion, and micromotion in colon cancer cells. As an additional approach to test the idea that the action of antisense calpain nucleotides resulted directly from inhibition of a specific type of calpain, GEO cells were transfected either with empty vector or with wild type µ-calpain expression vector. Fig. 5D shows an increase (167%) in cell micromotion in µ-calpain-transfected cells as compared with controls. These results were further supported by transfecting cells with a µ-calpain mutant in which histidine 272 was mutated to alanine. Fig. 5D (bottom) shows that the increase in locomotion by the WT µ-calpain construct was blocked by the mutant. The mutant inhibited ERK phosphorylation as well (Fig. 8F).

New adhesions form preferentially at the front of the cell. Studies indicate that organized integrin first appears in small aggregates at the leading edge (56). The rear of the cell (heel) is functionally and structurally distinct from the front (toe), generating an adhesive asymmetry. Extension of the leading edge is the first step of cell motility. The motile cell phenotype requires dynamic cytoskeletal reorganization, and actin is an important component of the cytoskeleton. Cyto D, which selectively disrupts the network of actin filaments, inhibited FAK phosphorylation and cleavage, demonstrating a relationship between FAK activation, cleavage, and upstream of this, the actin cytoskeleton. Results indicating that intact actin is required for FAK activation and its limited cleavage were generated. In addition, since actin polymerization is critical in cell spreading and motility, we performed adhesion assays and cell micromotion experiments. The results showed that cyto D inhibited cell adhesion to CN IV (45%), suggesting that actin polymerization is important in GEO cell adhesion to CN IV (Fig. 3B). Similarly, treatment of cells with cyto D in micromotion experiments showed concentration-dependent inhibition (46–70%). The interruption of the actin network inhibits the ability of the cell to undergo movement; therefore, there is less necessity for the activation and cleavage of FAK. These results show that linkage between FAK phosphorylation, cleavage, and actin polymerization plays an important role in cell micromotion.

Ectopic expression of the FAK dominant negative FRNK in GEO cells inhibited cell micromotion, suggesting that FAK is an important regulator of motility in colon carcinogenesis. Our observation that kinase-defective FAK enhances motility is consistent with previous reports (24). This phenomenon may be due to the transphosphorylation of the kinase-defective FAK by endogenous FAK on Tyr397, allowing its binding to c-Src and leading to increased micromotion on CN IV. However, Lim et al. (28) have reported decreased migration in kinase-defective FAK transfected NIH3T3 fibroblasts. The difference in results from normal and cancer cells suggests that the motility function of GEO cells is dysregulated.

Signaling events downstream of FAK activation and cleavage may contribute to motility of human colon carcinoma cells. There are different schools of thought concerning the mechanism by which integrin-mediated signaling activates ERK. Earlier investigators used fibroblasts, endothelial cells, and 293T cells with fibronectin activation of integr
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