Collagen IV-dependent ERK Activation in Human Caco-2 Intestinal Epithelial Cells Requires Focal Adhesion Kinase*

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Integrin-initiated extracellular signal-regulated kinase (ERK) activation by matrix adhesion may require focal adhesion kinase (FAK) or be FAK-independent via caveolin and Shc. This remains controversial for fibroblast and endothelial cell adhesion to fibronectin and is less understood for other matrix proteins and cells. We investigated Caco-2 intestinal epithelial cell ERK activation by collagen I and IV, laminin, and fibronectin. Collagens or laminin, but not fibronectin, stimulated tyrosine phosphorylation of FAK, paxillin, and p130Cas and activated ERK1/2. Shc, tyrosine-phosphorylated by matrix adhesion in many cells, was not phosphorylated in Caco-2 cells in response to any matrix. Caveolin expression did not affect Caco-2 Shc phosphorylation in response to fibronectin. FAK, ERK, and p130Cas tyrosine phosphorylation were activated after 10-min adhesion to collagen IV. FAK activity increased for 45 min after collagen IV adhesion and persisted for 2 h, while p130Cas phosphorylation increased only slightly after 10 min. ERK activity peaked at 10 min, declined after 30 min, and returned to base line after 1 h. Transfection with FAK-related nonkinase, but not substrate domain deleted p130Cas strongly inhibited ERK2 activation in response to collagen IV, indicating Caco-2 ERK activation is at least partly regulated by FAK.

Cell-matrix interactions activate extracellular signal-regulated kinases (ERKs)* in several cell types (1–5). Integrins, the principal cellular receptors for the extracellular matrix, have been theorized to initiate this activation by at least two different pathways. In one proposed mechanism, $\alpha_\beta$ integrin activation activates ERK1 and -2 via association with Src tyrosine-phosphorylated focal adhesion kinase (FAK) and subsequent activation of the Ras/Raf/MAP kinase kinase (MEK) pathway (6, 7). In contrast, a FAK-independent mechanism has been described for $\alpha_\beta_1$, $\alpha_\beta_1$, and $\alpha_\beta_3$ integrins. In this pathway, extracellular matrix activates ERK via association of these integrins with the integral membrane protein caveolin and subsequent tyrosine phosphorylation of the adaptor protein Shc by the tyrosine kinase Fyn in some cell types (8, 9). Other studies have also indicated Ras and FAK-independent activation of ERK in response NIH 3T3 cell adhesion to fibronectin (10, 11). Much of this work has focused on fibroblast and endothelial cell lines adherent to fibronectin. Less is known about mechanisms of ERK activation in other cell types or initiated by other matrix proteins such as collagens or laminin.

Intestinal epithelial cells in vivo exist on a basement membrane rich in laminin and type IV collagen, while type I collagen and tissue fibronectin are present in the interstitial matrix below the basement membrane. Several investigators, including our own group, have described regulation of intestinal epithelial cell adhesion, spreading, migration, and brush border enzyme activity on these matrix proteins (Refs. 12–18; reviewed in Refs. 19–23). While in vivo and in vitro studies suggest that tyrosine kinase signaling initiated by intestinal epithelial cell-matrix interactions is likely to play an essential role in maintenance of the normal differentiated phenotype and in wound healing, the mechanisms of integrin signaling in intestinal epithelial cells have not been studied in as much detail. Thus, an understanding of tyrosine kinase signaling pathways, including ERK activation, initiated by cell-matrix interactions in intestinal epithelial cells is likely to be essential for delineating how the extracellular matrix regulates cellular differentiation in the intestinal mucosa.

The purpose of the present work was to characterize the mechanism of ERK activation initiated by matrix proteins present in the extracellular matrix of the intestinal epithelium. For modeling integrin-mediated intestinal epithelial cell-matrix interactions, we chose to study human intestinal epithelial Caco-2 cells. These cells are highly differentiated, possess an integrin complement similar to that of cells in the normal gut mucosa, and have been widely used as a model system (24–26). We characterized ERK activation in response to collagen I, collagen IV, laminin, and fibronectin adhesion and compared this to tyrosine phosphorylation of FAK, paxillin, and p130Cas. In addition, since a FAK-independent pathway involving caveolin and the adaptor protein Shc has been proposed for ERK activation in response to fibronectin adhesion, we evaluated the role these proteins might play in Caco-2 cells. We monitored the time course of ERK activation in response to collagen IV adhesion and compared this to FAK activation and the tyrosine phosphorylation of p130Cas and paxillin. Finally, we evaluated the effects of expression of dominant negative forms of p130Cas and FAK on ERK activation in response to collagen IV adhesion.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium, fetal bovine serum, LipofectAMINE Plus reagent, epidermal growth factor (EGF), and monoclonal antibodies to $\alpha_\alpha$, $\alpha_\beta$, and $\beta_\beta$ integrin were obtained from Life Technologies, Inc. Transferrin and monoclonal antibody 12CA5 to the...
hemagglutinin (HA) tag were obtained from Roche Molecular Biochemicals, while HA tag monoclonal antibody HA.11 and polyclonal HA antibody were purchased from BabCO (Richmond, CA). Trypsin, soybean trypsin inhibitor, lamin (Engelbreth-Holm-Swaggern), collagen I, collagen IV, plasma fibrinectin, poly-(L-lysine) (PLL; M, 70,000), 0.1% fetal bovine serum, and phosphatase and phosphomonoesterase (RC20) and the polyclonal antibody to caveolin (for monitoring expression of caveolin in caveolin-transfected Caco-2 cell lines) were obtained from Transduction Laboratories (Lexington, KY). Polyclonal antibodies to FAK, Fyn, and monoclonal alpha integrin antibodies to VLA4, 5, 6, and 7 were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal antibodies to FAK Tyr(P)397 and FAK Tyr(P)576 were obtained from BIOSOURCE International (Camarillo, CA). Monoclonal antibody to active ERKs and polyclonal antibody to ERK protein were obtained from New England Biolabs. Monoclonal alpha integrin antibodies TS2/37 and FB12 were obtained from, respectively, American Type Culture Collection (Rockville, MD) and Chemicon International, Inc. (Temecula, CA). Horseradish peroxidase-conjugated donkey anti-rabbit IgG, protein A-Sepharose CL4B, electrophoresis equipment, transfer apparatus, and Hyperfilm MP were obtained from Amersham Pharmacia Biotech. [gamma-32P]ATP (3000 Ci/mmol) was obtained from PerkinElmer Life Sciences. Protein molecular weight standards were marker-Rad, pDNA, and Invitrogen (La Jolla, CA). Western blot stripping reagent was obtained from Chemicon. Caveolin-transfected Caco-2 cell lines 33-4, 50-1, and 50-2 were generously provided by Dr. Bo van Deurs (University of Copenhagen, Denmark). Expression vectors with HA-tagged FAK-related nonkinase (FRNK) and HA-tagged FAK were a generous gift from Dr. David Slaippe (The Scripps Research Institute, La Jolla, CA). Expression vector with HA-tagged ERK2 was a generous gift from Dr. Michael Weber (University of Virginia, Charlottesville, VA). Expression vectors with HA-tagged p130Cas, HA-tagged p56lck, and the control vector pSHEA were a generous gift from Dr. Tetsuya Nakamoto and Dr. Hisamaru Hirai (University of Tokyo).

Caco-2 Cell ERK Activation by Collagen IV Requires FAK

RESULTS

Cell Culture—Passage 45–65 Caco-2 cells were maintained at 37 °C with 5% CO2 in Dulbecco’s modified Eagle’s medium (10 mM HEPES, pH 7.4, 3.7 g/liter NaHCO3 and supplemented with 10% fetal bovine serum. Caco-2 cells were then incubated for 4 h at 4 °C with protein A-Sepharose and the monoclonal antibody 12CA5. Cells were then immunoprecipitated a second time with protein A-Sepharose and HA monoclonal antibody for 4 h at 4 °C. A similar double immunoprecipitation method was used for cells cotransfected with HA-FAK and HA-FAK/FRNK or HA-p130Cas and HA-SD-p130Cas in experiments measuring inhibition of FAK phosphorylation or p130Cas phosphorylation. The two immunoprecipitations were pooled and then incubated with cell lysis buffer and twice with kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2). 20 µl of kinase buffer containing 25 µM ATP, 1.0 µCi of [gamma-32P]ATP, and 2 µM of myelin basic protein (Upstate Biotechnology, Inc.) were then added, and the kinase reaction was initiated by incubating tubes at 30 °C for 20 min. Tubes were gently agitated during the course of the assay to prevent sedimentation. Tubes were placed on ice to stop the kinase assays. 10 µl of 2X loading buffer was then added, and samples were boiled and separated on a 15% SDS-polyacrylamide gel. Gels were then dried using a Bio-Rad model 583 gel dryer and exposed to film.

Immunoprecipitation and Western Blotting—Protein-matched samples were diluted to equal volumes with immunoprecipitation buffer (modified radioimmunoprecipitation buffer without SDS or sodium deoxycholate), and immunoprecipitations with monoclonal antibodies to FAK, paxillin, or p130Cas or polyclonal antibody to Phd were performed overnight at 4 °C. Rabbit anti-mouse IgG (for monoclonal antibodies) and protein A-Sepharose CL4B were then added, and immunoprecipitations were continued for another 2 h at 4 °C. Immunoprecipitations were rinsed three times with immunoprecipitation buffer, and then 2× gel-loading buffer (1× gel-loading buffer: 2% SDS, 10% glycerol, 100 mM Tris, pH 6.8) was added, and samples were boiled and resolved on SDS-polyacrylamide gels with 3.5% stacking gels. Gels were transferred to Hybond ECL nitrocellulose or Immobilon P (Millipore, Bedford, MA). Fifteen to 20 µg protein-matched aliquots were used for Western blots of cell lysates. Blots were detected by the ECL method (Amersham Pharmacia Biotech).

Statistical Analysis—Where indicated, results were compared using Student’s t test and considered statistically significant when p < 0.05.
Lysates of Caco-2 cells in suspension (S) or adherent to poly-L-lysine (PLL), collagen I (Col I), collagen IV (Col IV), fibronectin (Fn), or laminin (Ln) for 30 min were separated on a 4–20% gradient gel and analyzed by phosphotyrosine blotting using antibody RC20. Regions of increased phosphorylation are indicated with brackets or arrows. Experimental procedures for lysis and adhesion were as described under “Experimental Procedures.” One of three similar experiments is shown.

Adhesion to Collagens and Laminin Increases Tyrosine Phosphorylation of FAK, p130Cas, and Paxillin (68 kDa) following adhesion to extracellular matrix has been reported in several cell types including NIH 3T3 fibroblasts, rat embryo fibroblasts, and mouse Balb/c3T3 cells (7, 31, 33). Since the molecular weights of the first three of these proteins correspond to regions of increased tyrosine phosphorylation in blots of crude cell lysates after adhesion to matrix, we assessed whether tyrosine phosphorylation of these proteins is stimulated by matrix adhesion in Caco-2 cells. Lysates of cells adherent to matrix-coated dishes were analyzed by immunoprecipitation followed by adhesion (10 min EGF stimulation). Procedures for lysis and immunoprecipitation were as described under “Experimental Procedures.” One of at least three similar experiments is shown.

Adhesion to Collagans and Laminin Increases Tyrosine Phosphorylation of FAK, p130Cas, and Paxillin and ERK Activation—Increased tyrosine phosphorylation of focal adhesion kinase (125 kDa), p130Cas, and Paxillin (68 kDa) following adhesion to extracellular matrix has been reported in several cell types including NIH 3T3 fibroblasts, rat embryo fibroblasts, and mouse Balb/c3T3 cells (7, 31, 33). Since the molecular weights of the first three of these proteins correspond to regions of increased tyrosine phosphorylation in blots of crude cell lysates after adhesion to matrix, we assessed whether tyrosine phosphorylation of these proteins is stimulated by matrix adhesion in Caco-2 cells. Lysates of cells adherent to matrix-coated dishes were analyzed by immunoprecipitation followed by phosphotyrosine blotting (Fig. 2A). Tyrosine phosphorylation of each protein was increased following adhesion to collagen I, collagen IV, or laminin compared with PLL. Densitometric analysis indicated that tyrosine phosphorylation of FAK and paxillin was increased to the greatest extent following adhesion to collagen I, followed by adhesion to collagen IV and then adhesion to laminin. Tyrosine phosphorylation of p130Cas was increased to a similar extent on each of these matrix proteins. Adhesion to fibronectin did not increase tyrosine phosphorylation of any of these proteins compared with PLL adhesion.

In other cell types, adhesion of cells to matrix stimulates activation of ERK1 and 2 (1–5). We investigated activation of ERK1 and 2 using an antibody that recognizes the activated forms of the kinases. Densitometric analysis indicated that the rank order of ERK stimulation following matrix adhesion was similar to that seen for stimulation of tyrosine phosphorylation of FAK and paxillin (Fig. 2A), with the strongest activation observed following adhesion to collagens I and IV. While a slight increase in ERK activation following fibronectin adhesion compared with collagen I was noted, ERK activation was not significantly increased over PLL control. It is notable that FAK and paxillin were highly phosphorylated in the absence of matrix adhesion, as was ERK1 and 2 activation (Fig. 2A), indicating that membrane expression of these proteins by the stationary Caco-2 cells is sufficient for the basal level activation seen on phosphotyrosine blotting (Fig. 2).

Increased tyrosine phosphorylation of focal adhesion kinase, p130Cas and Paxillin and ERK Activation Following Adhesion to Matrix—Increased tyrosine phosphorylation of proteins in the 35–45 kDa region was observed following adhesion to collagen I, collagen IV, and laminin. Densitometric analysis indicated that this increase was not statistically significant compared with PLL adhesion (1.20 ± 0.19 compared with PLL, n = 5, p = 0.36).

**Shc**—The adaptor protein Shc is tyrosine-phosphorylated in response to fibronectin adhesion in human umbilical vein endothelial cells (8), immortalized 3T3 cells (9), 293T cells (6, 9), and NIH 3T3 cells (7). Although Western blots of Caco-2 cell lysates demonstrated expression of the 46-, 52-, and 66-kDa forms of Shc independently of the matrix substrate (Fig. 2B). This indicates that the Shc in Caco-2 cells is capable of undergoing tyrosine phosphorylation but that such phosphorylation is neither directly triggered nor potentiated by adhesion to matrix.

**Matrix Receptors**—Six integrins (α1β1, α2β1, α3β1, α4β1, α10β1, α11β1) have been observed to bind collagens (34–39). β1 integrin antibody immunoprecipitation of cell lysates from surface-biotinylated Caco-2 cells revealed two major bands at 120 kDa, corresponding to the apparent molecular mass of the β1 integrin subunit, and 140 kDa, which corresponds to the approximate molecular mass of the αc integrin subunit, was also visible in longer exposures. Immunoprecipitation with αc integrin antibody revealed the same two major bands in similar proportions to β1 integrin antibody immunoprecipitation, suggesting that the major collagen-related interactions occurring between Caco-2 cells and the extracellular matrix are mediated through the αcβ1 integrin receptor. In contrast, 30 ng/ml EGF treatment of Caco-2 cells adherent to laminin or PLL strongly increased tyrosine phosphorylation of the 46- and 52-kDa forms of Shc independently of the matrix substrate (Fig. 2B). This indicates that the Shc in Caco-2 cells is capable of undergoing tyrosine phosphorylation but that such phosphorylation is neither directly triggered nor potentiated by adhesion to matrix.
FIG. 3. Integrin immunoprecipitants. Caco-2 cells were biotinylated, and integrins were immunoprecipitated with β1, α2, or α5 integrin antibodies as described under “Experimental Procedures.” One of three similar experiments is shown.

As fibronectin adhesion failed to appreciably stimulate ERK activity (Fig. 2A), biotinylated Caco-2 cells were also immunoprecipitated with α5 integrin antibody to determine if this integrin subunit is expressed. This indicated that α2β1 integrin is present in Caco-2 cells, although at lower amounts than α5β1 integrin (Fig. 3). The presence of α2β1 integrin in Caco-2 cells was also confirmed by flow cytometry (data not shown).

Effect of Caveolin Expression on Shc Tyrosine Phosphorylation—One proposed mechanism of α2β1 integrin-initiated signal transduction involves a multiprotein complex containing caveolin and involves tyrosine phosphorylation of Shc. In this pathway, Shc is phosphorylated by the tyrosine kinase Fyn, and this phosphorylation is dependent on the presence of caveolin (9). While there is some controversy in the literature over whether Caco-2 cells contain caveolin (27, 41, 42), which has been detected in intestinal epithelium in vivo (41), Western blotting analysis failed to detect caveolin in the Caco-2 cells used in these experiments (not shown). Since Western blotting analysis indicated that Fyn (not shown), Shc, and α2β1 integrin (Fig. 3) are present in Caco-2 cells, we determined whether the failure of fibronectin adhesion to stimulate tyrosine phosphorylation of Shc was related to the absence of caveolin. In three caveolin-transfected Caco-2 cell lines, however, Shc was not phosphorylated in response to fibronectin adhesion (Fig. 4).

Mechanism of ERK Activation in Response to Collagen IV Adhesion—We further investigated the mechanism by which ERK is activated following adhesion to collagen IV, a major component of the intestinal epithelial basement membrane. ERK activation peaked at 10 min of adhesion to collagen IV, remained at this level until 30 min of adhesion, and then declined (Fig. 5A). Following 1 h of adhesion to collagen IV, activity was similar to that of cells adherent to the control substrate PLL. FAK activity, as measured by phosphotyrosine blotting and an antibody that is specific for FAK Tyr(P)397 (the FAK autophosphorylation site), indicated that FAK activity was slower in onset and reached maximal levels following 30–45 min of adhesion to collagen IV. Activity then remained at this level until 2 h of adhesion, the final time point examined (Fig. 5, B and C). Stimulation of FAK 576 tyrosine phosphorylation, one of the major Src phosphorylation sites in FAK (43), was also observed in response to collagen IV adhesion (Fig. 5D) and followed a similar pattern. Phosphorylation of paxillin (Fig. 5E) followed a profile similar to that of FAK, while p130Cas (Fig. 5F) was rapidly phosphorylated with only a slight increase in phosphorylation after the initial 10 min. No increase in Shc phosphorylation was observed during the 2-h time course (not shown).

In order to determine the role of FAK in the activation of ERK, we used FRNK, the C-terminal region of FAK that localizes to focal adhesions but does not contain the FAK kinase domain (44, 45), to block activation of FAK in response to collagen IV adhesion. Cotransfection of HA-tagged FRNK and HA-tagged FAK indicated that expression of FRNK blocked tyrosine phosphorylation of FAK by at least 80% following adhesion to collagen IV (Fig. 6, A and B). Cotransfection of HA-tagged FRNK and HA-tagged ERK2 also indicated that FRNK expression blocked ERK activation (Fig. 6C). Since p130Cas has also been observed to play a role in activation of ERK and c-Jun N-terminal kinase in some systems (46–48) and phosphorylation of this protein was rapidly stimulated following adhesion to collagen IV (Fig. 5F), we used ∆SD-p130Cas to examine the role of p130Cas in ERK activation. ∆SD-p130Cas lacks the tyrosine phosphorylation sites that mediate association of the adaptor protein Crk with p130Cas (49) but still localizes to focal adhesions (50). While expression of ∆SD-p130Cas blocked the increase in tyrosine phosphorylation of p130Cas in response to collagen IV adhesion (Fig. 6D), it did not inhibit ERK activation (Fig. 6E). This indicates that ERK activation in response to collagen IV adhesion is mediated at least in part via a signal transduction pathway proceeding through FAK but that tyrosine phosphorylation of p130Cas is not essential.

DISCUSSION

Controversy exists in the literature over the mechanism by which integrin-mediated adhesion stimulates ERK1 and -2. This has been most commonly studied in fibroblasts, endothelial cells, and 293T cells using fibronectin activation of α5β1 integrin as a model system. In addition to the controversy over whether FAK is involved in fibronectin activation of ERK, it is by no means clear that the same mechanisms would be operational for other cell types or other matrix proteins. Indeed, this study suggests that the mechanism of collagen IV activation of ERK1 and -2 in human Caco-2 intestinal epithelial cells may exhibit some significant differences from integrin stimulation of ERK activity for other matrix proteins and cell types. In particular, FAK activation seems to be a critical regulator (although perhaps not the only one) of ERK activation in response to adhesion to collagen IV in these cells, and this appears independent of Shc tyrosine phosphorylation. In contrast, ERK activation was not observed in response to...
fibronectin adhesion. A previous report has described FAK and paxillin tyrosine phosphorylation and ERK activation in Caco-2 cells in response to fibronectin adhesion as well as collagen IV and laminin (51). Since there are several different subclones of the original Caco-2 cell line, it is possible that the Caco-2 cells used in this work may differ from those used in the previous study.

The observation that adhesion to collagen IV activates ERK1 and -2 in these cells is not necessarily predictable from the previous literature, since they do not express the α1 integrin subunit at detectable levels. Collagen I and collagen IV initiate ERK activation in normal mice dermal fibroblasts that express α1β1, α2β1, and α3β1 integrins, but not in dermal fibroblasts derived from α1 integrin-deficient mice (40). Since Takeuchi et al. (32) also observed α2β1 activation of ERK in osteoblasts following adhesion to collagen I (32), this clearly indicates that mechanisms of integrin activation of ERK1 and -2 may vary from one cell type to another.

Initial comparisons of the time courses over which FAK and ERK are activated in response to Caco-2 adhesion to type IV collagen reveal potentially important similarities and differences between FAK and ERK activation. Both signals were

![Time course of protein phosphorylation of cells adherent to collagen IV.](image-url)
Caco-2 Cell ERK Activation by Collagen IV Requires FAK

Mechanism of ERK Activation—At least two pathways have been proposed for ERK activation in response to integrin-mediated cell adhesion. According to one proposed pathway, Src binds to activated FAK Tyr(D)577 following association of FAK with focal adhesions. Src then phosphorylates FAK on Tyr925 and Shc on multiple sites. The adaptor protein Grb2 then binds to Tyr(P)525 FAK and to phosphorylated Shc, leading to activation of Ras via Son of Sevenless and then ERK activation via Raf and MEK. Evidence for this pathway includes the finding that in human embryonic kidney 293T cells expression of FAK Y397F inhibits fibronectin-stimulated signaling to ERK2, that FAK overexpression enhances signaling to ERK2 and enhances Shc tyrosine phosphorylation and association of Grb2 with Shc, and that expression of dominant negative Ras interferes with fibronectin-stimulated ERK2 activity (6).

Our observation that expression of FRNK inhibits activation of ERK is consistent with the essential role for FAK proposed in this mechanism. The observation of increased FAK 576 phosphorylation also indicates that Src is activated and is consist-

Interestingly, both our time course observations and those described above differ from those observed in osteoblasts adhering to collagen I. In these cells, FAK tyrosine phosphorylation is not observable until 20 min of adhesion, while ERK activation begins after 20 min and reaches maximal levels at 120 min of adhesion (32). The differences observed in kinetics of FAK and ERK activation in these various studies might reflect differences between cell types, differences in lysis and detection methods (7), or differences in matrix substrates studied. Certainly, the observation of synchronous early and rapid FAK and ERK activation in Caco-2 cells adhering to collagen IV would not be incompatible with a role for FAK in ERK activation. However, the delayed decrease in ERK activation in the face of persistent FAK activity suggests that some other factor may intervene to down-regulate ERK activity in this setting.

The kinetics of early activation of both FAK and ERK in this study was observed to correlate with the number of integrin-fibronectin bonds (52). These authors also described disparities in late FAK and ERK activation similar to our present observations. In NIH 3T3 cells, a similar early activation of FAK and ERK was observed in response to fibronectin adhesion (10). These authors found, however, that FAK was not measurably activated until after initial ERK activation following adhesion to dishes coated with a fibronectin fragment containing type III repeats 7–12.

the gel was transferred and detected with polyclonal ERK antibody. The membrane was then stripped and reprobed with polyclonal HA antibody to verify HA-ΔSD-p130 expression. One of three similar experiments is shown.
ent with previous work in Caco-2 cells growing on tissue culture plastic showing coimmunoprecipitation of Src and FAK (51). Our present result is also consistent with the observation that reduction of osteoblast FAK expression by antisense FAK inhibits activation of ERK in response to collagen I adhesion (32). However, we failed to observe a significant increase in Shc phosphorylation throughout the 2-h time course, although Shc is present and capable of undergoing tyrosine phosphorylation in response to EGF, suggesting a somewhat different mechanism from the one described for αβ1 integrin. Since the major collagen receptor present in these Caco-2 cells is the αβ1 integrin, our failure to observe Shc phosphorylation is consistent with the observation that Shc was not phosphorylated in response to αβ1 and αβ3 integrin cross-linking in MG-63 osteosarcoma cells (8). Since it has been previously demonstrated that Grb2 associates with FAK Tyr(P)925 in response to fibronectin adhesion (4), it is possible that Grb2 association with FAK Tyr(P)925 might play a role in the ERK activation observed in Caco-2 cells in response to collagen IV adhesion. It should also be noted that in another study tyrosine phosphorylation of Shc was not observed in NIH 3T3 fibroblasts in response to fibronectin adhesion (53) and that Shc Y317F did not interfere with ERK activation in response to fibronectin adhesion in transfected human embryonic kidney 293T cells (7). This indicates that under some circumstances Shc may not be essential for ERK activation in response to integrin-mediated cell adhesion. Additionally, other signaling molecules such as phosphatidylinositol 3-kinase (54) and Grb7 (55) have been shown to associate with FAK Tyr(P)925 in response to fibronectin adhesion, and phosphatidylinositol 3-kinase has been observed to coimmunoprecipitate with FAK in Caco-2 cells growing on tissue culture plastic (51). The potential role of these signaling molecules in the ERK2 activation observed here awaits further investigation.

A FAK-independent mechanism has been proposed for αβ1, αβ3, and integrin activation of ERK involving association of caveolin with the α integrin cytoplasmic domain, activation of Fyn by association with caveolin, and then phosphorylation of Shc by Fyn (8, 9). Evidence for this pathway includes the finding that fibronectin-stimulated ERK activation and Shc phosphorylation is restored by caveolin expression in caveolin-deficient Fischer rat thyroid cells, that ERK activation in human embryonic kidney 293T cells is inhibited by Shc Y317F or dominant negative Fyn expression, and that ERK activation in these cells is not inhibited by dominant negative FAK expression (9). Additionally, Shc phosphorylation was observed in wild type NIH 3T3 fibroblasts but not in immortalized 3T3 fibroblasts derived from Fyn knockout mice (9). Also in support of FAK-independent ERK activation, it has been observed that maximal ERK2 activation preceded FAK tyrosine phosphorylation following adhesion to fibronectin type III repeats 7–12, as discussed above, and that FRNK expression did not affect fibronectin-stimulated activation of ERK2 in NIH 3T3 cells (10).

Since the Caco-2 cells used in this study did not contain detectable amounts of αβ1 integrin, the mechanism of collagen IV activation is not relevant to the FAK-independent mechanism of ERK activation described for αβ1 integrin (9). However, the absence of Shc phosphorylation and other signals in response to fibronectin adhesion is not explained in this model. Of the components proposed to be involved in this signal transduction pathway, the one apparently missing in these Caco-2 cells is caveolin. However, Shc tyrosine phosphorylation in response to fibronectin adhesion was not affected in three Caco-2 cell lines stably transfected with caveolin, suggesting that this was not responsible for the failure to observe Shc phosphorylation and ERK activation. It is possible that some additional unidentified component not present in these Caco-2 cells is necessary for the phosphorylation of Shc in response to fibronectin adhesion.

In several systems, p130
\(^{ab}
\) has been observed to play a role in signaling to ERK and c-Jun N-terminal kinase by integrin activation or by activation of proteins implicated to play a role in integrin signaling. Expression of ΔSd-p130
\(^{ab}
\) inhibits c-Jun N-terminal kinase activation by fibronectin adhesion in COS-7 cells (47) and by expression of the FAK-related kinase Pyk2 in transfected human embryonic kidney 293T cells (48). Our observation that expression of ΔSd-p130
\(^{ab}
\) does not inhibit ERK activation following collagen IV adhesion (Fig. 6E) suggests that tyrosine phosphorylation of the p130
\(^{ab}
\) substrate domain and, presumably, the resulting association with Crk is not essential for ERK activation under these conditions. This is consistent with observations that ΔSd-p130
\(^{ab}
\) does not inhibit ERK activation in other systems, including ERK activation by expression of Pyk2 in human embryonic kidney 293T cells (48) and activation of the serum response element by v-Src via ERK in COS-7 cells (46). Since p130
\(^{ab}
\) contains a number of additional domains that mediate interactions with other proteins (reviewed in Ref. 56), the possibility that other regions of p130
\(^{ab}
\) might play a role in collagen IV signaling to ERK remains to be determined.

The urokinase receptor has also been found to interact with caveolin (57) and play a role in αβ1 ERK activation in response to fibronectin adhesion (57, 58). Disruption of urokinase receptor-caveolin interactions reduced fibronectin-dependent ERK activation in vascular smooth muscle cells (57), and disruption of urokinase receptor-αβ1 integrin interactions in carcinoma cells expressing high levels of urokinase receptor also interfered with ERK1/2 activation (58). While the urokinase receptor has been reported to be present in Caco-2 cells (59), the role it might play in integrin activation of ERK in Caco-2 cells remains to be determined.

In conclusion, we have observed that collagen IV, a major component of the intestinal epithelial basement membrane, stimulates ERK activity in Caco-2 cells by a mechanism in part dependent on FAK, although appearing to differ in some aspects from previously described models for other matrix proteins and cell types. While the adhesion model used here in other studies may be relevant only to cells at the leading edge of the migrating front in a healing wound, our results, in conjunction with those of Yu et al. (30), nevertheless suggest that FAK may play an important role in intestinal epithelial cell biology and matrix-initiated signal transduction.

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\(^{ab}
\), and HA-ASD-p130
\(^{ab}
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