p130cas but Not Paxillin Is Essential for Caco-2 Intestinal Epithelial Cell Spreading and Migration on Collagen IV*

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We have previously observed that collagen IV regulates Caco-2 intestinal epithelial cell spreading and migration via Src kinase and stimulates Src-dependent tyrosine phosphorylation of p130cas. We observed that collagen IV also stimulates Src-dependent phosphorylation of both paxillin Tyr31 and paxillin Tyr118. Caco-2 transfection with paxillin or p130cas siRNAs inhibited expression of these proteins by more than 90% for at least 5 days after transfection. Although p130cas siRNA inhibited cell spreading on collagen IV by 33%, three different paxillin siRNAs did not inhibit cell spreading. p130cas siRNA did not affect Src Tyr416 or Src Tyr527 phosphorylation, FAK Tyr925 phosphorylation, or Src-dependent phosphorylation of FAK Tyr397, suggesting that p130cas did not inhibit cell spreading by altering FAK or Src activity. Rat p130cas expression after siRNA knock-out of endogenous human p130cas in Caco-2 cells reduced cell spreading inhibition by 71%. In contrast, expression of rat p130cas from which the Src-phosphorylated substrate domain was deleted did not rescue siRNA inhibition of cell spreading. Combined treatment with siRNAs to Crk and CrkL, which bind to the p130cas substrate domain, inhibited cell spreading by 54%. Both p130cas siRNA and the combined Crk/CrkL siRNAs strongly inhibited (52 and 46% inhibition, respectively) Caco-2 sheet migration on collagen IV and noticeably inhibited lamellipodial extension, whereas paxillin siRNA only inhibited migration by 18% and did not noticeably affect lamellipodial extension. These results suggest that Src may regulate Caco-2 migration on collagen IV via both p130cas and paxillin but that Src phosphorylation of p130cas is more important for this process.

Intestinal epithelial cells exist in vivo on a basement membrane that is rich in type IV collagen and laminins and also contains fibronectin. The functional unit of the intestinal epithelium, the crypt-villus axis, includes two main distinct cell populations, the proliferating, poorly differentiated crypt cells and the mature enterocytes of the villus. Intestinal epithelial populations, the proliferating, poorly differentiated crypt cells

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The abbreviations used are: ERK, extracellular-signal regulated kinase; FAK, focal adhesion kinase; NT1 siRNA, nontargeting control siRNA 1; siRNA, small interfering RNA; SH, Src homology.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium, Oligofectamine, Lipofectamine, and Plus Reagent were obtained from Invitrogen. Western blot stripping reagent was obtained from Chemicon International (Temecula, CA). Human transferrin was obtained from Roche Applied Science. Trypsin, soybean trypsin inhibitor, collagen IV, poly-L-lysine (Mw = 70,000–150,000), and horseradish peroxidase-conjugated rabbit anti-mouse IgG were obtained from Sigma. Paxillin, Crk, and p130cas monoclonal antibodies were obtained from Transduction Laboratories (Lexington, KY). Phosphospecific polyclonal antibodies to focal adhesion kinase (FAK) Tyr(P)397 or FAK Tyr(P)118 were obtained from respectively, BIOSOURCE International (Camarillo, CA) or Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific polyclonal antibodies to paxillin Tyr(P)165 or paxillin Tyr(P)118 were obtained from respectively, BIOSOURCE International. CrkL monoclonal antibody and phosphospecific polyclonal antibodies to focal adhesion kinase were obtained from Cell Signaling Technology (Beverly, MA). α-Tubulin monoclonal antibody was obtained from Calbiochem. Horseradish peroxidase-conjugated donkey anti-rabbit IgG was obtained from Amersham Biosciences. Double-stranded siRNAs targeting human forms of p130cas, Crk, CrkL, and paxillin and control nontargeting siRNA 1 (NT1 siRNA) were purchased
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Fig. 1. Effect of Src inhibition on paxillin phosphorylation. Prior to adhesion cells were treated with either 10 μM PP2 or PP3 as described under ‘Experimental Procedures.’ Lysates of cells adherent to the control substrate poly-1-lysine (PLL) or collagen IV (Col) were immunoblotted with phospo-specific antibodies to paxillin Tyr(P)31 or paxillin Tyr(P)116. The paxillin Tyr(P)116 blot was stripped and reprobed for total paxillin protein. Results from one of three similar experiments are shown.

medium on collagen IV-coated Petri dishes. Cells were maintained in reduced serum medium for 2 days until reaching complete confluence, and migration assays were then performed in serum-free medium as described previously (11). Migration assays were stopped 20 h after the start of migration. Migration area was determined from the area of five or more randomly chosen fields for each condition in each experiment.

RESULTS

Paxillin Tyr31 and Tyr116 Phosphorylation Is Regulated by Src—We have previously observed that collagen IV-stimulated tyrosine phosphorylation of p130cas is strongly inhibited by the Src inhibitor PP2 (11). Immunoblottting with phospo-specific antibodies to paxillin Tyr(P)31 and paxillin Tyr(P)116 indicated that phosphorylation of both sites was stimulated in Caco-2 cells following adhesion to collagen IV and that this phosphorylation was also strongly inhibited by PP2 treatment. Treatment with the inactive structurally similar compound PP3 did not affect paxillin tyrosine phosphorylation (Fig. 1).

Transfection with p130cas but Not paxillin siRNA Inhibits Spreading of Caco-2 Cells on Collagen IV—Transfection with paxillin or p130cas siRNAs inhibited expression of each of these proteins by more than 90% in Caco-2 cells (Fig. 2A). p130cas siRNA transfaction significantly inhibited cell spreading on collagen IV as indicated by both mean and median cell area (Fig. 2B). Transfection with paxillin siRNA, however, did not significantly inhibit either mean or median cell area (Fig. 2C). Two additional paxillin siRNAs that were equally effective in inhibiting paxillin expression also did not affect Caco-2 cell spreading (data not shown). In a subset of the experiments summarized in Fig. 2, B and C, we also examined the combined effect of p130cas and paxillin siRNA transfection on cell spreading. Combined transfection with both siRNAs resulted in significantly more inhibition of mean cell area compared with transfection with only p130cas siRNA (36.6 ± 2.7 versus 25.0 ± 2.5% inhibition, n = 4, p < 0.05). Combined treatment with these siRNAs also increased inhibition of median cell area compared with transfection with p130cas siRNA alone, but this increase did not achieve statistical significance (26.7 ± 1.1 versus 21.2 ± 1.7% inhibition, n = 4, p = 0.056). Taken together, these results suggest that p130cas has a more important role than paxillin in regulation of Caco-2 cell spreading on collagen IV.

siRNA Knock-out of p130cas Expression Does Not Affect Src or FAK Phosphorylation—In parallel with the spreading studies described above, cells transfected with p130cas siRNA were allowed to adhere to collagen IV and were lysed. p130cas can bind to Src via the Src SH2 and SH3 domains, and Src activity in C3H10T1/2-5H murine fibroblasts is modulated by p130cas protein levels (24). Because we have previously observed that Src regulates Caco-2 spreading and migration on collagen IV...
(11), we examined whether siRNA knock-out of p130cas may have inhibited cell spreading through an effect on Src activity. Additionally, because p130cas binds to FAK in several cell types (25, 26) and our previous work suggests a role for FAK in Caco-2 cell migration (10), we also examined whether FAK Tyr397 autophosphorylation and Src-dependent phosphorylation of FAK Tyr925 (11) were inhibited by siRNA knock-out of p130cas. Phosphorylation of Src (phosphorylated Tyr416 and nonphosphorylated Tyr527) and FAK following Caco-2 cell adhesion to collagen IV was not significantly affected by siRNA knock-out of p130cas expression (Fig. 3). This suggests that the effects of p130cas siRNA on cell spreading were not exerted through an effect on Src or FAK activity but through other proteins that interact with p130cas.

Src-phosphorylated Substrate Domain of p130cas Is Required for Cell Spreading—We examined whether the Src-phosphorylated substrate domain of p130cas is required for cell spreading by expressing rat p130cas in Caco-2 cells after siRNA knock-out of endogenous human p130cas. The region of human p130cas targeted by the p130cas siRNA used in these studies differs from rat p130cas at 6 of 19 nucleotides and thus would not be expected to inhibit rat p130cas expression. Expression of full-length rat p130cas rescued 71% of the inhibition of cell spread-
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The differential expression of integrins in intestinal epithelial cells (reviewed in Ref. 2) and basement membrane extracellular matrix proteins (reviewed in Ref. 30) along the intestinal epithelial crypt-villus axis in vivo suggests a potential role for cell-matrix interactions in regulating intestinal epithelial cell differentiation and migration. Additionally, the extracellular matrix influences restitution of mucosal wounds not only as a physical substrate but also by modulating the function of the relevant intracellular proteins and the receptors for extracellular soluble factors, which influence cell migration (reviewed in Refs. 3, 31, and 32). The effects of physical forces such as repetitive deformation on intestinal epithelial cells also appear to be matrix- and integrin-mediated (33). Our previous work (11) indicated that Src is an important regulator of Caco-2 spreading and migration on collagen IV and that Src regulates collagen IV-initiated tyrosine phosphorylation of p130\(^{\text{cas}}\). In this previous work we observed a small but not statistically significant inhibition of Caco-2 cell spreading on collagen IV by substrate domain-deleted p130\(^{\text{cas}}\) expression as indicated by the percent of cells that were spread (11) rather than measurements of cell area as were done in the current study. Overexpression of dominant negative forms of multifunctional adaptor proteins with mutations or deletions of Src-phosphorylated tyrosines, however, may up-regulate other functions of these proteins. Additionally, the low transfection efficiency of plasmid DNA in Caco-2 cells makes this method inappropriate for examining protein function in more physiologically relevant sheet migration assays. We therefore used siRNA to reexamine the role of p130\(^{\text{cas}}\) in Caco-2 cell spreading and migration on collagen IV and also to characterize the role of the adaptor protein paxillin in these processes.

p130\(^{\text{cas}}\)—Although previous studies indicate that p130\(^{\text{cas}}\) regulates cell spreading and haptotactic migration (reviewed in Refs. 34 and 35), the role of p130\(^{\text{cas}}\) in epithelial cell sheet migration has not been examined in as much detail. Yano et al. (19) recently observed that siRNA knock-out of p130\(^{\text{cas}}\) expression in HeLa cells did not affect sheet migration of HeLa cells on collagen I. In contrast, our data indicated an important role for p130\(^{\text{cas}}\) in both spreading and sheet migration of Caco-2 cells on collagen IV (Figs. 2, 4, and 5). Our results from Fig. 4 also suggested that the tyrosine-phosphorylated substrate domain of p130\(^{\text{cas}}\) plays an important role in Caco-2 spreading. The adaptor proteins Crk, CrkL, Nck, and the phosphatase SHIP2 have been reported to interact with the phosphotyrosines in the p130\(^{\text{cas}}\) substrate domain via their SH2 domains (reviewed in Refs. 34 and 35). Of these proteins our data indicated an important role for Crk and CrkL in regulation of Caco-2 cell spreading and migration on collagen IV (Figs. 4C and 5, B and C). Determining the role of other p130\(^{\text{cas}}\) substrate-domain-binding proteins in regulating p130\(^{\text{cas}}\)-dependent spreading and migration of Caco-2 cells will be an interesting topic for future studies.

Several phosphatases, kinases, and adaptor proteins also interact with p130\(^{\text{cas}}\) via its SH3 domain, its proline-rich region, or its C-terminal region (reviewed in Refs. 34 and 35; see also Refs. 36 and 37). Among those proteins that associate with p130\(^{\text{cas}}\) under various conditions we have previously observed a role for Src in the regulation of Caco-2 spreading and migration on collagen IV (11) and for FAK in the regulation of Caco-2 migration on collagen I (10). A role for p130\(^{\text{cas}}\) in regulating Src activity has been reported in other cell types. For example, p130\(^{\text{cas}}\) overexpression enhances Src phosphorylation of cortactin, paxillin, and FAK in murine C3H10T1/2 fibroblasts that overexpress Src. This was not dependent on p130\(^{\text{cas}}\) tyrosine phosphorylation, because a C-terminal fragment of p130\(^{\text{cas}}\) lacking the tyrosine-phosphorylated substrate domain was sufficient to stimulate Src activity (24). In addition, p130\(^{\text{cas}}\) overexpression enhances Src Tyr\(^{416}\) phosphorylation in HEK 293 cells (38). Our results in Fig. 3, however, suggested that siRNA knock-out of p130\(^{\text{cas}}\) did not inhibit cell spreading on collagen IV through an inhibition of Src activation, because Src Tyr\(^{416}\) and Tyr\(^{527}\) phosphorylation and Src-dependent phosphorylation of FAK Tyr\(^{925}\) were not affected by p130\(^{\text{cas}}\) siRNA. Furthermore, FAK Tyr\(^{575}\) autophosphorylation, which is not regulated by Src activity in this experimental system (11), was also unaffected by knock-out of p130\(^{\text{cas}}\), suggesting that p130\(^{\text{cas}}\) did not affect cell spreading through an effect on FAK autophosphorylation.

Paxillin—Paxillin phosphorylation at Tyr\(^{31}\) and Tyr\(^{118}\) creates potential binding sites for the Crk and CrkL adaptor proteins (39, 40), which we observed to play an important role in regulation of Caco-2 cell spreading and migration on collagen IV (Figs. 4C and 5, B and C). In addition, C-terminal Src tyrosine kinase, which negatively regulates Src activity through phosphorylation of Src Tyr\(^{527}\) (41), also associates with tyrosine-phosphorylated paxillin (42). Tyr\(^{118}\) phosphorylation of paxillin promotes random migration of NBII-T bladder epithelial cells on collagen I, although Tyr\(^{31}\) phosphorylation does not affect cell spreading on this matrix protein (14). In another

To confirm a role for the p130\(^{\text{cas}}\) substrate domain in regulation of Caco-2 cell spreading on collagen IV, we examined the effect of siRNAs targeting Crk and CrkL on cell spreading. These related adaptor proteins bind via their SH2 domains to the phosphorylated substrate domain of p130\(^{\text{cas}}\) (27, 28) and are important for cell migration in many cell types (reviewed in Ref. 29). As observed with p130\(^{\text{cas}}\) siRNA, transfection with Crk siRNA significantly inhibited Caco-2 cell spreading on collagen IV, whereas combined transfection with Crk and CrkL siRNAs more strongly inhibited cell spreading (Fig. 4C).
**FIG. 4.** Full-length rat p130 \(^{as}\) but not substrate domain-deleted rat p130 \(^{as}\) rescues cell spreading on collagen IV. Cells transfected with either NT1 or p130 \(^{as}\) siRNA were transfected with either SSR-\(\alpha\) control plasmid or rat p130 \(^{as}\) expression plasmids as described under "Experimental Procedures." Cells were cotransfected with a lacZ expression plasmid to indicate transfected cells. A, remaining cells not used for spreading studies were lysed and immunoblotted for p130 \(^{as}\). Note that because the p130 \(^{as}\) monoclonal antibody was raised against rat p130 \(^{as}\), it may bind more strongly to the transfected rat p130 \(^{as}\) than endogenous human p130 \(^{as}\). Therefore, the difference in intensity of bands of human p130 \(^{as}\) in NT1 control siRNA-transfected cells and of rat p130 \(^{as}\) in cells transfected with rat p130 \(^{as}\) expression vectors may not be indicative of relative expression. ΔSD p130Cas, rat p130 \(^{as}\) with deletion of tyrosine-phosphorylated substrate domain. B, measurements of cell area were based on three independent experiments. In each experiment the size of at least 150 lacZ-positive cells for each condition was measured. hCas, human p130 \(^{as}\); rCas, rat p130 \(^{as}\); \(\times\), \(p < 0.05\) compared with cells transfected with p130 \(^{as}\) siRNA and pSSR \(\alpha\) plasmid DNA. C, Caco-2 cells transfected with NTI (200 nM), NTI (100 nM) + Crk (100 nM), NTI (100 nM) + CrkL (100 nM), or Crk (100 nM) + CrkL (100 nM) siRNAs were allowed to spread on collagen IV for 1 h after an initial 15-min adhesion as described under "Experimental Procedures." Results are based on three independent experiments. The Crk siRNA targeted a sequence common to both CrkI and CrkII. The CrkII band is shown in the immunoblot. In each experiment the cell area was determined from measurements of at least 150 cells for each condition. **, \(p < 0.01\) compared with NT1 control siRNA; ##, \(p < 0.01\) compared with all other siRNA treatments.
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In conclusion, our data suggest that p130\textsuperscript{CAS} is an important regulator of Caco-2 intestinal epithelial cell spreading and migration on collagen IV, a major component of the intestinal epithelial basement membrane. Contrary to observations in some other cell types, however, our data suggest that paxillin is not essential for these processes. Our results also suggest that the Src-phosphorylated substrate domain of p130\textsuperscript{CAS} and the Crk and CrkL adaptor proteins that bind to this region are important for Caco-2 cell spreading and migration on collagen IV. Taken together with our previous observations, the results described in this manuscript suggest that Src-dependent phosphorylation of p130\textsuperscript{CAS} initiated by intestinal epithelial basement membrane matrix proteins such as type IV collagen may regulate intestinal epithelial cell migration.

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FIG. 5. Effect of p130\textsuperscript{CAS}, combined Crk and CrkL, or paxillin siRNA transfection on Caco-2 sheet migration on collagen IV. A. Representative immunoblot demonstrating persistent siRNA inhibition of p130\textsuperscript{CAS} or paxillin expression at the end of migration assay 4 days after transfection. B. Representative sheet migration of control NT1, p130\textsuperscript{CAS}, and combined Crk and CrkL siRNA-transfected Caco-2 cells on collagen IV. Arrows indicate lamellipodial extensions present in NT1 siRNA-transfected cells but greatly reduced in both p130\textsuperscript{CAS} and combined Crk and CrkL siRNA-transfected cells. Migration studies were performed as described under “Experimental Procedures.” C. Measurement of collagen IV sheet migration of Caco-2 cells transfected with NT1 control, p130\textsuperscript{CAS}, combined Crk and CrkL, or paxillin (Pax) siRNAs. Measurements of migration area are based on at least five randomly chosen fields for each condition in each experiment. **, p < 0.01 compared with NT1 siRNA-transfected cells; *, p < 0.05 compared with NT1 siRNA-transfected cells.

study, however, overexpression of paxillin but not a tyrosine phosphorylation site mutant of paxillin inhibits haptotactic migration of mouse mammary epithelial NMuMG cells, COS7 cells, and MM1 cells toward type I collagen (18). siRNA knock-out of paxillin also enhances spreading and movement of HeLa cells on type I collagen, interferes with the formation of N-cadherin-mediated cell-cell contacts, and enhances activation of Rac (19). Both paxillin Tyr\textsuperscript{394} and Tyr\textsuperscript{118} undergo Src-dependent phosphorylation in collagen IV adherent Caco-2 cells (Fig. 1). Inhibition of paxillin expression by three different siRNAs, however, did not significantly affect cell spreading on collagen IV (Fig. 2) and only slightly inhibited cell migration (Fig. 5C). Although this observation would suggest that Src phosphorylation of paxillin is not important in cell spreading and migration under these conditions, it is possible that knocking out potential binding sites for both the C-terminal Src kinase and Crk could exert opposing effects on cell spreading and migration. The strong inhibition of cell migration by p130\textsuperscript{CAS} siRNA, however, suggests that Crk binding to paxillin cannot compensate for the loss of Crk binding to p130\textsuperscript{CAS}. Although we obtained greater than 90% reduction of paxillin expression by each of the paxillin siRNAs used in the current
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