Calpain-mediated Proteolysis of Paxillin Negatively Regulates Focal Adhesion Dynamics and Cell Migration*†

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The dynamic turnover of integrin-mediated adhesions is important for cell migration. Paxillin is an adaptor protein that localizes to focal adhesions and has been implicated in cell motility. We previously reported that calpain-mediated proteolysis of talin1 and focal adhesion kinase mediates adhesion disassembly in motile cells. To determine whether calpain-mediated paxillin proteolysis regulates focal adhesion dynamics and cell motility, we mapped the preferred calpain proteolytic site in paxillin. The cleavage site is between the paxillin LD1 and LD2 motifs and generates a C-terminal fragment that is similar in size to the alternative product paxillin delta. The calpain-generated proteolytic fragment, like paxillin delta, functions as a paxillin antagonist and impairs focal adhesion disassembly and migration. We generated mutant paxillin with a point mutation (S95G) that renders it partially resistant to calpain proteolysis. Paxillin-deficient cells that express paxillin S95G display increased turnover of zyxin-containing adhesions using time-lapse microscopy and also show increased migration. Moreover, cancer-associated somatic mutations in paxillin are common in the N-terminal region between the LD1 and LD2 motifs and confer partial calpain resistance. Taken together, these findings suggest a novel role for calpain-mediated proteolysis of paxillin as a negative regulator of focal adhesion dynamics and migration that may function to limit cancer cell invasion.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Fibronectin was purified from human plasma by affinity chromatography as described previously (10). Mouse anti-paxillin (clone 349) antibody was purchased from BD Transduction Laboratories. Mouse anti-GFP antibody was purchased from Abcam (Cambridge, MA). Rabbit anti-phospho-paxillin (phospho-Tyr-118) and anti-phospho-paxillin (phospho-Tyr-31) were purchased from BIOSOURCE (Camarillo, CA). Anti-phospho-tyrosine antibody (4G10) was obtained from Upstate Biotechnology (Lake Placid, NY). Mouse anti-FLAG (clone M2) and anti-vinculin (clone h-VIN1) antibodies were purchased from Sigma. Rabbit anti-tubulin antibody was purchased from Cell Signaling Technology (Danver, MA). Rabbit anti-GFP antibody, Rhodamine Red-X goat anti-mouse IgG and Alexa Fluor 680 goat-anti-mouse IgG were purchased from Invitrogen. IRRye 800CW goat anti-rabbit IgG was obtained from Rockland Immunocchemicals (Gilbertsville, PA). Rabbit anti-mAb1 antibody has been described previously (11). Calpain inhibitor N-acetyl-Leu-Leu-Met (ALLM)

The abbreviations used are: FAK, focal adhesion kinase; ALLM, N-acetyl-Leu-Leu-Met; ANOVA, analysis of variance; RFP, red fluorescent protein; shRNA, short hairpin RNA.

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was purchased from Calbiochem. Ionomycin was purchased from Sigma.

**DNA Constructs**—Paxillin was subcloned with the BglII and EcoRI sites into linearized pEGFP-C1 vector (Clontech, Mountain View, CA) and was a gift from A. R. Horwitz (University of Virginia School of Medicine). pcDNA 3.1 FLAG was made by annealing a FLAG tag oligo containing the PstI and XhoI sites into the linearized pcDNA 3.1 vector (Invitrogen). Paxillin was subcloned into the linearized pcDNA 3.1-FLAG vector with the Nhel and XhoI restriction sites. pcDNA3.1-paxillin-FLAG with a BglII-containing primer: forward, 5’TGGGAAGATCTTACCGCTCCTTGG; and an EcoRI-containing primer: reverse, 5’CGGCGCAATTCTCAACAGAAGAGTTGAG; followed by ligation into pEGFP-C1 vector (Invitrogen). The paxillin fragment corresponding to the calpain cleavage product was amplified from pcDNA 3.1-paxillin-FLAG with a BglII-containing primer: forward, 5’AACCAAGATCTGCACCAGCATGCGCTTGGTCC; and a Kpnl-containing primer: reverse, 5’AAAAAGTACCTTTACACAGAAAGATCTGCTCCTTGGTCC; and subsequently ligated into pEGFP-C1 vector. Mutations were introduced into the paxillin calpain cleavage site by site-directed mutagenesis of pEGFP-C1-paxillin or pcDNA3.1-paxillin-FLAG: 5’CTAGTGCAAAACCGCGAGGATGCCTCGTTGTTGCC; and 5’CAGTGCCCTGTTGGAAGAGTCCGCTCAG (P100G primer). pENTR21-human paxillin (variant 2) mRNA was purchased from Invitrogen, amplified with a forward primer containing an AccIII site and a reverse primer containing a HindIII site, followed by ligase into pEGFP-C1 vector. Mutations were introduced into the paxillin calpain cleavage product was amplified from pcDNA 3.1-paxillin-FLAG with a BglII-containing primer: forward, 5’TGCAGAAGAGTTT and subsequently ligated into pEGFP-C1 vector. Mutations were introduced into the paxillin calpain cleavage site by site-directed mutagenesis of pEGFP-C1-paxillin or pcDNA3.1-paxillin-FLAG: 5’TGCAGAAGAGTTT and subsequently ligated into pEGFP-C1 vector.

**Cell Culture and Transfection**—HEK 293, H522, A431, and HeLa cells were purchased from the ATCC. MDA-231 cells were provided by Dr. Alan Raepraeger (University of Wisconsin-Madison). HEK, A431, and HeLa cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, nonessential amino acids, and antibiotics. H522 cells were maintained in RPMI medium supplemented with 10% heat-inactivated FBS and antibiotics. HEK transfections were performed on ice using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocols. Where indicated, HeLa cells were serum-starved for 16–18 h in DMEM containing 2% FBS.

**siRNA**—HeLa control and paxillin siRNA cells were generated by retroviral infection with pSUPER.retro.puro (Oligoengine, Seattle, WA) containing shRNA against non-targeting sequence or human paxillin: 5’TTCCTCCGAACCTTGACGTG’ (control); 5’GGCCTTCACCAATAGTGT3’ (target sequence for paxillin si-A); and 5’TGGTGACGTCACACACGAC3’ (target sequence for paxillin si-B). All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA).

**Cleavage Site Mapping**—Paxillin-FLAG was expressed in HEK 293 cells. 24–48 h later, cells were plated on dishes coated with 10 μg/ml fibronectin and incubated at 37 °C under 5% CO₂ for 1 h. Media was exchanged with complete media containing a vehicle control, 50 μg/ml ALLM or 1 μM iodonium, and 10 μM CaCl₂, and cells were incubated for 10 min at 37 °C under 5% CO₂. Cells lysates were incubated with anti-FGL M2-agarose beads (Sigma) for 2 h, which were washed extensively with lysis buffer. Beads were boiled in non-reducing sample buffer to elute protein and then separated on SDS-PAGE gels. Protein was transferred to polyvinylidene difluoride membrane (Bio-Rad), stained with Coomassie Blue, and an individual band was subjected to N-terminal sequencing (performed at the Baylor College of Medicine Protein Chemistry/Proteomics core).

**Immunocytochemistry**—Cells were plated on coverslips coated with 10 μg/ml fibronectin in complete media for 6 h. Cells were then fixed, permeabilized, and stained as described previously (13).

**Transwell Migration Assay**—Transwell assays were performed with 8-μm porous chambers (Fisher) and coated with 10 μg/ml fibronectin on the top and bottom according to the manufacturer’s recommendations. 10% FBS was used as chemotactant in the lower compartment. Cells were allowed to migrate through the membrane for 6 h, 48 h after transient expression of the indicated constructs. Only experiments with ~70% transient transfection efficiency were used for the migration assays. Expression was confirmed by Western immunoblotting for GFP. The cells that migrated to the lower surface of the membrane were then fixed and stained using a HEMA Stain 3 kit (Fisher).

**Immunoblot Analysis**—Cells were scraped into lysis buffer (50 mM Tris (pH 7.6), 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 0.5 mM MgCl₂, 0.2 mM PMSF, 1 μg/ml pepstatin, 2 μg/ml aprotinin, 1 μg/ml leupeptin) on ice and clarified by centrifugation. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce). Cells were then fixed, permeabilized, and stained as described previously (13). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Equal amounts of total protein were loaded on 6–20% gradient SDS-PAGE gels and transferred to nitrocellulose. Western blots were imaged with an Odyssey infrared imaging system (LI-COR Biosciences, Omaha, NE).

**Live Imaging and Adhesion Dynamics**—Live fluorescence microscopy and quantification of adhesion dynamics was performed 1–2 h after cells were plated on fibronectin-coated glass-bottom dishes to analyze the dynamics of early adhesions. Fluorescence imaging of live cells was performed using a 60× objective on a Nikon inverted microscope housed in a 37 °C closed system. Glass-bottom dishes were acid-washed and coated with 10 μg/ml fibronectin. Cells were plated in DMEM containing 10% FBS and nonessential amino acids and allowed to adhere for 30 min, after which time the media was replaced with Ham’s F-12 medium supplemented with 10% FBS, nonessential amino acids, and 20 mM HEPES (pH 7.4). Fluorescent images were then captured every 2 min for 2 h using MetaVue imaging software for cells with equivalent GFP expression (Molecular Devices, Downington, PA). Quantification of adhesion dynamics was performed as described previously (3, 14).
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Measurements were made from 10–15 adhesions from at least six individual cells per condition over three separate days.

Statistical Analysis—For statistical comparison, the two-tailed, paired Student’s t test or one-way analysis of variance (ANOVA) with Tukey’s post hoc test was used, with p values of <0.05 considered significant.

RESULTS

Mapping the Calpain Proteolytic Site of Paxillin—Previous studies have demonstrated that paxillin is a calpain substrate (6, 7). However, how calpain-mediated proteolysis of paxillin affects adhesion dynamics and cell migration has not been previously addressed. To determine how calpain proteolysis of paxillin regulates adhesion turnover and migration, we mapped the calpain proteolytic site in paxillin. HEK 293 cells that express paxillin-FLAG were cultured in the presence or absence of the calcium ionophore, ionomycin, to increase calpain activity, calpain inhibitor (ALLM), or ALLM + IONO (A−f). Cells were lysed, and cell lysates were analyzed by immunoblotting and probed with anti-FLAG antibody. The arrow indicates the calpain proteolytic fragment. B, cell lysates from A were incubated with anti-FLAG beads, and protein complexes were transferred to a PVDF membrane and stained with Coomassie. The arrow indicates the FLAG-tagged, calpain-dependent 55-kDa fragment that was analyzed by N-terminal sequencing. C, schematic of paxillin denoting the site of calpain cleavage after serine 96, between the LD1 and LD2 domains. D, schematic comparing the paxillin fragment generated by calpain proteolysis to the alternative translation product, paxillin delta.

The Calpain-generated Paxillin Fragment Impairs Cell Migration—Previous work demonstrated that expression of paxillin delta is inhibitory to cell migration (5). To determine whether calpain proteolysis of paxillin also generates an inhibitory fragment of paxillin, we characterized the effects of paxillin delta or the calpain-mediated C-terminal fragment on cell migration of HeLa cells. Paxillin delta and the cleavage fragment were expressed at approximately equal levels in HeLa cells confirmed by immunoblotting (Fig. 2), and both localized to focal adhesions (Fig. 3). Transient expression of either paxillin delta or the calpain proteolytic fragment of paxillin impaired HeLa (Fig. 2) and HEK 293 cell migration (data not shown) using a transwell assay. The inhibitory effects of the calpain-mediated proteolytic fragment on migration were modest but statistically significant. The findings suggest that expression of the calpain-generated paxillin fragment, as previously reported for paxillin delta (5), impairs cell migration.

The Calpain-generated Paxillin Fragment Impairs Focal Adhesion Turnover—Previous studies have demonstrated that calpain-mediated proteolysis of either FAK or talin regulates focal adhesion disassembly (9, 14). To determine whether the calpain proteolytic fragment of paxillin affects focal adhesion dynamics, live time-lapse imaging was performed on RFP-zyxin-containing focal adhesions in cells that express wild-type 1B. The predominant proteolytic site is in the N-terminal region of paxillin after Ser-96 between the LD1 and LD2 motifs (Fig. 1C). The calpain proteolytic site is in close proximity to the alternative start site that generates the inhibitory fragment paxillin delta at Met-133 (5) (Fig. 1D). Taken together, our findings indicate that calpain-mediated proteolysis of paxillin generates a fragment that is similar in size to the alternative product paxillin delta.

FIGURE 2. The calpain-generated paxillin fragment and paxillin delta impair cell migration. HeLa cells were transiently transfected with GFP alone, GFP-tagged wild-type paxillin (WT), the calpain cleavage fragment of paxillin (Cleaved), or paxillin delta (Delta), and assayed for their ability to migrate by transwell assay. A portion of the cells used for transwell analysis were lysed and analyzed by immunoblotting with anti-GFP antibody and anti-tubulin antibody as a loading control. Migration was quantified and is shown relative to GFP expressing cells. *, p < 0.05 (one-way ANOVA, Tukey’s post hoc test) compared with GFP control cells.
GFP-paxillin, GFP-paxillin delta, or the GFP-paxillin cleavage fragment (Fig. 3 and supplemental movies 1–3). The duration of RFP-zyxin-containing adhesions was increased 2-fold in cells expressing paxillin delta or the cleaved form of paxillin. Moreover, focal adhesion disassembly rates were significantly impaired in cells that express either paxillin delta or the paxillin fragment compared with full-length paxillin (Fig. 3). Taken together, the findings suggest that the calpain-mediated paxillin fragment, like paxillin delta, impairs focal adhesion turnover.

**Generation of a Calpain-resistant Mutant of Paxillin**—To further address the effects of calpain proteolysis of paxillin on adhesion dynamics, we generated a calpain-resistant mutant of paxillin through site-directed mutagenesis at several residues near the cleavage site (Fig. 4A). Point mutations at serine 95 or proline 100 (S95G or P100G) impaired calpain-mediated proteolysis of GFP-paxillin (Fig. 4B) or paxillin-FLAG (data not shown). Treatment of HEK cells that express wild-type GFP-paxillin with ionomycin induced the generation of a 35-kDa calpain-mediated proteolytic fragment compared with vehicle alone (data not shown). Quantification of susceptibility to calpain-mediated proteolysis of the paxillin mutants demonstrated that the S95G mutation rendered paxillin 50% resistant to calpain proteolysis compared with wild-type paxillin in the presence of ionomycin (Fig. 4B). Several other mutations near the cleavage site, including deletion mutations, also rendered paxillin only partially resistant to calpain proteolysis (data not shown), suggesting that there is flexibility in the precise site of calpain proteolysis. This is in contrast to other focal adhesion proteins, talin and FAK, where point mutations rendered the substrates almost completely calpain-resistant (9, 14). Calpain-resistant GFP-paxillin retained several properties of wild-type paxillin, including phosphorylation at tyrosine 31 and 118 (Fig. 4C) and interactions with specific binding proteins, including vinculin (data not shown).

**FIGURE 3.** The calpain-generated paxillin fragment and paxillin delta inhibit focal adhesion turnover. HeLa cells were transiently co-transfected with wild-type GFP-paxillin, GFP-paxillin delta, or GFP-paxillin cleavage fragment and RFP-zyxin. Cells were plated on fibronectin-coated glass-bottom dishes and analyzed by time-lapse fluorescent microscopy. Images on the left show localization of wild-type GFP-paxillin, paxillin delta, and cleaved paxillin at focal adhesions. Time-lapse montages show representative images of RFP-zyxin dynamics in cells co-expressing RFP-zyxin and wild-type GFP-paxillin (WT), paxillin delta, or cleaved paxillin over a period of 80 min. Scale bar = 10 μm. See corresponding supplemental movies 1–3. Duration was measured as the time elapsed between the appearance and dissolution of an observed adhesion. Rate constants for net adhesion assembly and disassembly were calculated from plots of fluorescence intensities of RFP-zyxin over time, as described under “Experimental Procedures.” Data for each condition are mean ± S.E. At least 50 adhesions were measured for each condition, from at least eight different cells, on three independent days. *, p < 0.05 (one-way ANOVA, Tukey’s post hoc test) compared with wild-type GFP-paxillin-expressing cells.
To determine whether the calpain-resistant paxillin affects cell migration, we depleted endogenous paxillin in HeLa cells using siRNA and expressed either wild-type GFP-paxillin or calpain-resistant GFP-paxillin (Fig. 5A). Paxillin levels were decreased by ~70% in paxillin-deficient cells compared with control cells (Fig. 5A). Paxillin-deficient HeLa cells (target B) were transiently transfected with wild-type or calpain-resistant (S95G) GFP-paxillin and assayed for their ability to migrate by transwell assay (Fig. 5B). We observed increased migration with ectopic expression of calpain-resistant paxillin compared with wild-type paxillin despite approximately equal expression levels by immunoblotting (Fig. 5B). These findings suggest that calpain proteolysis of paxillin may function to limit cell migration.

Paxillin Proteolysis by Calpain Regulates Adhesion Dynamics of RFP-Zyxin—To determine whether paxillin proteolysis affects focal adhesion dynamics, we examined the dynamics of RFP-zyxin to quantify focal adhesion dynamics in paxillin-deficient cells expressing either wild-type GFP-paxillin or calpain-resistant paxillin (S95G) (Fig. 5C and supplemental movies 4 and 5). Both wild-type and calpain-resistant GFP-paxillin localized to focal adhesions (Fig. 5C). The duration of RFP-zyxin-containing adhesions was significantly reduced by ~10 min in cells expressing calpain-resistant paxillin compared with wild-type paxillin (Fig. 5C). There was also a slight but not statistically significant increase in assembly and disassembly rates of RFP-zyxin in paxillin-deficient cells expressing calpain-resistant paxillin compared with wild-type paxillin (Fig. 5C). Taken together, our findings indicate that calpain-mediated proteolysis of paxillin impairs focal adhesion turnover.

Paxillin Is Cleaved in Cancer Cell Lines, and Cancer-associated Somatic Mutations in Paxillin Render Paxillin Partially Resistant to Calpain Proteolysis—Previous studies have reported that calpain-mediated proteolysis of substrates is enhanced in Src-transformed cells (15) and that calpain expression is increased in several cancer cell lines (16–18). To determine whether endogenous paxillin is cleaved in breast and lung cancer, we screened a series of breast and lung cancer cell lines for paxillin proteolysis. We observed detectable levels of endogenous calpain-mediated paxillin proteolysis in several invasive cancer cell lines, including MDA-231 and A431 breast cancer cells and H522 lung cancer cells, even in the absence of ionomycin stimulation (Fig. 6), suggesting that paxillin is an endogenous calpain substrate in cancer cell lines.

Interestingly, several somatic mutations of paxillin have been identified from human lung cancer specimens that reside in the N-terminal region of paxillin close to the calpain proteolytic site. The most common mutation, A127T, results in a more invasive phenotype with enhanced cancer cell migration and invasion (19). It is intriguing that several of these mutations span a region between the LD1 and LD2 motifs near the calpain proteolytic site (Fig. 7A). To determine whether these mutations alter calpain-mediated proteolysis of paxillin, we generated GFP-tagged versions of wild-type human paxillin and two of the most frequent somatic mutations, G105A and A127T (Fig. 7A). HEK 293 cells expressing wild-type, G105A, or A127T GFP-paxillin were treated with ionomycin to stimulate calpain activity (Fig. 7B). Both the G105A and A127T GFP-paxillin were treated with ionomycin to stimulate calpain activity (Fig. 7B). Both the G105A and A127T mutations were resistant to calpain-mediated proteolysis compared with wild-type GFP-paxillin (Fig. 7B). In particular, the most common
mutation, A127T, was ~50% resistant to calpain-mediated proteolysis (Fig. 7B). Paxillin-deficient cells expressing either cancer-associated mutation exhibited enhanced transwell migration compared with wild-type paxillin (Fig. 7C).

To determine whether the A127T mutation modulates focal adhesion dynamics, we performed live imaging of RFP-zyxin containing focal adhesions in paxillin-deficient HeLa cells expressing wild-type human GFP-paxillin or calpain-resistant GFP-paxillin A127T (Fig. 8 and supplemental movies 6 and 7). The duration of RFP-zyxin containing adhesions was significantly reduced by ~10 min in cells expressing the A127T mutation compared with wild-type paxillin (Fig. 8). This reduction in focal adhesion duration was similar to that observed in cells expressing the calpain-resistant paxillin S95G (Fig. 5C).

**DISCUSSION**

Previous studies have demonstrated that calpain is necessary for efficient cancer cell migration and invasion (13, 18, 20) and that proteolysis of specific substrates, talin and FAK, increases adhesion turnover in motile cells (9, 14). Here, we have identified a novel inhibitory role for calpain-mediated proteolysis of paxillin in adhesion dynamics and cell migration. Calpain proteolysis of paxillin occurs between the LD1 and LD2 motifs and generates a C-terminal fragment that is similar in size to the alternative product paxillin delta. The calpain-proteolytic fragment of paxillin, like paxillin delta, functions as a paxillin antagonist that impairs adhesion turnover and cell migration in HeLa cells. In contrast, expression of calpain-resistant paxillin is associated with increased cell migration and enhanced turnover of zyxin-containing focal adhesions compared with wild-type paxillin. Collectively, our findings identify a novel inhibitory role for calpain-mediated proteolysis of paxillin in regulating focal adhesion dynamics and cell migration.

Calpains target many adhesion and cytoskeletal proteins to regulate adhesion turnover and cell migration. Calpain-mediated proteolysis of the cytoskeletal proteins talin (14) and FAK...
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![Image](image1.png)

FIGURE 6. Paxillin is an endogenous calpain substrate in breast and lung cancer cell lines. MDA-231 (top), A431 (center), or H522 (bottom) cells were treated with vehicle, with calpain inhibitor (ALLM), 1 μM ionomycin + 10 mM CaCl₂ (IONO) to stimulate calpain activity, or ALLM + IONO (A+I) and lysed. Cells lysates were analyzed by immunoblotting and probed with anti-paxillin antibody. The arrow indicates the calpain-generated proteolytic fragment.

(9) is important for efficient adhesion dynamics. Moreover, calpain-mediated proteolysis of the actin-associated protein cortactin has been shown to positively regulate membrane protrusion (21) and invadopodia dynamics (13). Calpains have also been implicated in regulating cell spreading through proteolysis of the β-integrin cytoplasmic tail (22–24) and α-actinin (25). Calpain-mediated proteolysis of adhesion and cytoskeletal proteins is generally associated with increased cell migration and invasion, consistent with the observation that calpains are upregulated in invasive cancers (16–18) and have been suggested as a potential therapeutic target (20). It is intriguing that, in contrast to other substrates, we find that calpain proteolysis of paxillin is inhibitory to cell migration and adhesion dynamics, suggesting that calpains may be either positive or negative regulators of cell migration and invasion, depending on the substrate that is targeted (Fig. 9).

It is interesting that calpain proteolysis of paxillin generates a fragment similar to paxillin delta (5) that also antagonizes the function of endogenous paxillin in adhesion dynamics and cell migration. Calpain proteolysis generates an inhibitory paxillin fragment locally, providing a means to temporally and spatially regulate paxillin function in motile cells. It is possible that generation of cleaved paxillin may compensate for a lack of paxillin delta expression. This idea is consistent with the observation that cleavage of endogenous paxillin is a minor event in most cell types (data not shown), although endogenous cleavage is observed in many different cancer cell lines. Interestingly, calpain proteolysis of FAK generates a fragment similar in size to the alternative transcript known as FRNK; however, the direct consequence of the calpain-generated FAK fragment has not been tested (9). Calpain-mediated proteolysis of protein kinase C also generates a fragment similar in size to an alternative protein kinase C gene transcript (26). This raises the interesting possibility that calpain proteolysis may serve to generate protein fragments with redundant functions to alternative transcripts and translated products that may mediate crosstalk between transcriptional and posttranslational mechanisms of cell signaling.

Recent evidence has shown that single amino acid mutations between the LD1 and LD2 motifs of paxillin are correlated with enhanced invasive potential of cancer cells (19). We demonstrated that two of the paxillin mutations previously identified in cancer cells affect susceptibility to calpain proteolysis (Fig. 6C). These findings raise the interesting possibility that somatic mutations in paxillin induced during cell transformation may result in enhanced invasive potential, at least in part by rendering paxillin resistant to calpain-mediated proteolysis, preventing the generation of an inhibitory paxillin proteolytic fragment.

A future challenge will be to determine the mechanism by which the calpain proteolysis of paxillin impairs cell motility. The mechanism by which paxillin delta inhibits cell migration is still largely unknown (5). It is likely that paxillin delta and the paxillin calpain fragment inhibit adhesion turnover and migration by similar mechanisms and likely involves changes in binding interactions. Several proteins preferentially bind to the LD1 domain of paxillin, including actopaxin (27) and integrin-linked kinase (28), which link paxillin to the actin cytoskeleton and regulate cell adhesion and spreading. Association with paxillin is required for actopaxin and integrin-linked kinase targeting to focal adhesions (29) and promotes adhesion formation (27). Recent structural studies indicate that separation of the LD1 domain from paxillin would alter interactions with key players such as actopaxin and integrin-linked kinase and may modulate adhesion dynamics (30, 31). In accordance, we observed altered adhesion dynamics upon expression of paxillin delta and the paxillin calpain cleavage fragment, which lack the LD1 domain (Fig. 3). It is likely that the abrogation of these interactions upon calpain proteolysis of paxillin plays some role in the altered adhesion dynamics and migration in cells expressing paxillin delta and the paxillin cleavage fragment.

Alternatively, tyrosine phosphorylation of paxillin at Tyr-31 and Tyr-118 promotes association with Crk and is necessary for efficient cell migration (32). It has been proposed that the absence of these phosphorylation sites in paxillin delta prevents Crk association, thereby inhibiting cell migration (5). However, an important difference between paxillin delta and the calpain-generated fragment of paxillin is that the cleavage product lacks the Tyr-31 site but still contains the Tyr-118 site. If the calpain-generated fragment and paxillin delta function similarly to
inhibit cell migration, this would suggest that the mechanism does not involve phosphorylation at Tyr-118.

In summary, our findings support a novel role for calpain-mediated proteolysis of paxillin in limiting cell migration, at least in part by affecting focal adhesion dynamics and maturation. Future mechanistic studies are necessary to determine the relationship between paxillin delta and calpain-mediated proteolysis of paxillin in cells. A challenge for future investigation

FIGURE 7. Cancer-associated somatic mutations in paxillin are partially resistant to calpain proteolysis and mediate enhanced cell migration. A, schematic of human GFP-paxillin showing previously identified cancer-associated somatic mutations (G105A and A127T) that were introduced between the LD1 and LD2 domain of human paxillin. B, HEK 293 cells were transiently transfected with GFP vector control, wild-type human GFP-paxillin, GFP-paxillin-G105A, or GFP-paxillin-A127T. Cells were treated with 1 μM ionomycin + 10 mM CaCl₂ to stimulate calpain activity and lysed. Cell lysates were analyzed by immunoblotting and probed with anti-GFP antibody. The arrow indicates the calpain proteolytic fragment. Quantification of relative cleavage is shown as mean ± S.E. from four independent experiments. *, p < 0.05 (one-way ANOVA, Tukey’s post hoc test) compared with wild-type GFP-paxillin. C, HeLa cells stably expressing paxillin siRNA (target B) were transiently transfected with wild-type GFP-paxillin, GFP-paxillin-A127T, or GFP-paxillin-G105A and assayed for their ability to migrate by transwell assay. A portion of the cells used for transwell analysis were lysed and analyzed by immunoblotting with anti-GFP antibody and anti-vinculin antibody as a loading control. Migration was quantified and is shown relative to wild-type paxillin-expressing cells. *, p < 0.05 (one-way ANOVA, Tukey post-test) compared with wild-type GFP-paxillin-expressing cells.

FIGURE 8. The A127T paxillin mutation enhances focal adhesion turnover relative to wild-type paxillin. HeLa cells stably expressing paxillin siRNA were transiently transfected with wild-type GFP-paxillin or GFP-paxillin-A127T and co-transfected with RFP-zyxin. Cells were plated on fibronectin-coated glass-bottom dishes and analyzed by time-lapse fluorescent microscopy. Images on the left show wild-type GFP-paxillin and A127T localization at focal adhesions. Time-lapse montages demonstrate representative images of RFP-zyxin dynamics in cells co-expressing RFP-zyxin and wild-type GFP-paxillin or GFP-paxillin A127T over a period of 80 min. Scale bar = 10 μm. See corresponding supplemental movies 6 and 7. Duration was measured as the time elapsed between the appearance and dissolution of an observed adhesion. Rate constants for net adhesion assembly and disassembly were calculated from plots of fluorescence intensities of RFP-zyxin over time, as described under “Experimental Procedures.” Data for each condition are mean ± S.E. At least 50 adhesions were measured for each condition, from at least six different cells, on three independent days. *, p < 0.05 (paired Student’s t test) compared with wild-type GFP-paxillin-expressing cells.
will also be to determine the physiological relevance of somatic mutations in paxillin that render paxillin partially resistant to calpain-mediated proteolysis.

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