Degraded Collagen Fragments Promote Rapid Disassembly of Smooth Muscle Focal Adhesions That Correlates with Cleavage of pp125^FAK, Paxillin, and Talin

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Abstract. Active matrix metalloproteinases and degraded collagen are observed in disease states, such as atherosclerosis. To examine whether degraded collagen fragments have distinct effects on vascular smooth muscle cells (SMC), collagenase-digested type I collagen was added to cultured human arterial SMC. After addition of collagen fragments, SMC lose their focal adhesion structures and round up. A nalysis of components of the focal adhesion complex demonstrates rapid cleavage of the focal adhesion kinase (pp125^FAK), paxillin, and talin. Cleavage is suppressed by inhibitors of the proteolytic enzyme, calpain I. In vitro translated pp125^FAK is a substrate for both calpain I- and II-mediated processing. Mapping of the proteolytic cleavage fragments of pp125^FAK predicts a dissociation of the focal adhesion targeting (FAT) sequence and second proline-rich domain from the tyrosine kinase domain and integrin-binding sequence. Coimmunoprecipitation studies confirm that the ability of pp125^FAK to associate with paxillin, vinculin, and p130cas is significantly reduced in SMC treated with degraded collagen fragments. Further, there is a significant reduction in the association of intact pp125^FAK with the cytoskeletal fraction, while pp125^FAK cleavage fragments appear in the cytoplasm in SMC treated with degraded collagen fragments. Integrin-blocking studies indicate that integrin-mediated signals are involved in degraded collagen induction of pp125^FAK cleavage. Thus, collagen fragments induce distinct integrin signals that lead to initiation of calpain-mediated cleavage of pp125^FAK, paxillin, and talin and dissolution of the focal adhesion complex.

Keywords: extracellular matrix • matrix metalloproteinase • calpain • focal adhesion kinase • pp125^FAK

Extracellular (ECM) molecules regulate cell function by acting as both structural support and signaling mediators (Hay, 1989; Hynes, 1992). Integration of the intracellular signaling and structural responses initiated by ECM-integrin engagement may be mediated through the focal adhesion complex (Burridge et al., 1988). Focal adhesions are localized to sites of integrin-ECM adhesion (Burridge and Chrzanowska-Wodnicka, 1996). They consist of numerous, tightly associated, structural and signaling proteins and the cytoplasmic tail of β integrins, which provide intracellular signal transduction machinery but also serve as a physical link between the ECM and the cytoskeleton (Sastry and Horwitz, 1993).

Focal adhesion kinase (pp125^FAK) is a cytoplasmic tyrosine kinase that localizes to focal adhesions and may play an important role in the integration of signals from integrins and growth factors (Hanks and Polte, 1996; Zachary and Rozengurt, 1992). The pp125^FAK protein can be subdivided into three functional domains: an NH2-terminal integrin-binding domain necessary for association with the cytoplasmic domain of β1 integrin subunits (Schaller et al., 1995), an internal kinase domain, and a COOH-terminal domain consisting of two proline-rich motifs and a focal adhesion targeting sequence required for association with other focal adhesion proteins, including p130cas, paxillin, and talin (Chen et al., 1995; Hildebrand et al., 1995; Polte and Hanks, 1995). pp125^FAK also contains a number of sites of tyrosine phosphorylation involved in binding to the SH2 domains of signaling proteins such as P-I3 kinase, pp60^src and Grb2 (Schaller et al., 1994; Schlaepfer et al., 1994; Bachelot et al., 1996). Through these protein-bind-
ing domains, pp125FAK plays a central role in the recruitment of signaling molecules to the focal adhesion complex and in the transduction of downstream signals derived from integrin-ECM interactions (reviewed in Guan, 1997). Previous studies have implicated tyrosine phosphorylation of pp125FAK as the primary mechanism whereby integrin-ECM interactions regulate pp125FAK activity and recruitment of signaling molecules (Schaller and Parsons, 1995). However, a recent study carried out on platelets proposed a novel mechanism of pp125FAK regulation, in which the calcium-activated proteolytic enzyme calpain I induces cleavage of pp125FAK, leading to downregulation of pp125FAK autokinase activity and altered intracellular localization (Cooray et al., 1996).

ECM-integrin engagement may affect numerous biological processes in vivo, including cell participation in the development of lesions of atherosclerosis (Thyberg et al., 1990). Smooth muscle cells within the medial layer of normal arteries are surrounded by a dense network of interstitial type I and III collagen fibers (Barnes, 1985). However, disruption of collagen fibers during atherogenesis is thought to occur as a result of increased activation of matrix metalloproteinases derived from either infiltrating macrophages or activated SMC (Bendeck et al., 1994; Galis et al., 1994). SMC are arrested at the G1 stage of the cell cycle in vitro on polymerized type I collagen fibrils, whereas mononuclear collagen supports SMC proliferation (Koyama et al., 1996).

From these studies, we hypothesized that the ECM surrounding SMC in the normal media may be nonpermissive for SMC proliferation. Degradation of the ECM during different stages of lesion development may release SMC from this nonpermissive state and promote SMC migration and proliferation. Previous studies have indicated that denaturation or proteolysis of the type I collagen triple helix may reveal cryptic RGD integrin-binding motifs (Davies, 1992; Montgomery et al., 1994). Recent studies also demonstrate that processing of the ECM protein laminin 5 by matrix metalloproteinases generates fragments that have different cell signaling properties from intact laminin 5 (Gianelli et al., 1997). These studies raise the possibility that fragments of degraded collagen generated within the atherosclerotic lesion may modulate SMC behavior by possessing specific integrin signaling properties, which are distinct from those of intact native collagen found in normal arteries.

In this study, we describe the rapid induction of calpain-mediated cleavage of pp125FAK, paxillin, and talin in response to treatment of SMC with collagenase-degraded collagen fragments. The cleavage of pp125FAK, paxillin, and talin in adherent SMC occurs in parallel with functional and molecular focal adhesion disassembly and cell rounding. We propose that calpain-mediated cleavage of pp125FAK represents a dynamic mechanism for regulating pp125FAK activity and together with paxillin and talin cleavage promotes focal adhesion disassembly in response to matrix degradation.

**Materials and Methods**

**Cell Culture and Collagen Matrix Preparation**

Human newborn arterial SMC were isolated from the thoracic aorta as described previously (Bornfeldt et al., 1994). SMC between passages 5 and 9 were cultured on the surface or in the presence of the following collagen type I preparation: polymerized collagen gels (1.0 mg/ml final concentration) prepared by neutralization of the collagen solution (Vitrogen 100, Collagen Corp.) with 1/6 volume of 7X DME concentration, dilution to a final 1X DME solution, and incubation at 37°C for 24 h. M nomon collagen-coated dishes were prepared by incubating with 0.1 mg/ml of collagen solution in 0.1 M acetic acid for 24 h. Monomer collagen-coated dishes were washed twice with DME before cell seeding. Degraded type I collagen was prepared by incubating polymerized collagen gels (prepared as above) with 2.5 mg/ml collagenase type 1 (Worthington Biochemical Corp.) for 30 min at 37°C. A filter digestion, collagenase activity was inhibited by the addition of an equal volume of 1X DME containing 2% plasma-derived serum (Raines and Ross, 1985). Fibronectin was purified from human plasma as previously described (Faull et al., 1994). Fibronectin-coated dishes (0.625 µg/cm²) were prepared by overnight incubation at room temperature.

**Inhibitors and Antibodies**

Calpain and caspase inhibition studies were performed with two inhibitors: calpain inhibitor 1 (ALLN: Calbiochem-Novobiochem Corp.) and the caspase inhibitor benzoyloxycarbonyl Val-Ala-Asp fluoromethyl ketone (ZVAD-fmk; Alexis Biochemicals). SMC were pretreated with ALLN or ZVAD (100 µM) for 3 h or overnight before treatment with degraded collagen in the presence of ALLN or ZVAD. To block integrin function, SMC were preincubated at 37°C for 30 min with nonblocking (P1H6) and blocking (P1H5) Fab antibody fragments against α2, blocking α3 Fab antibody (P1B8), blocking αvβ3 (LM 609; Chemicon) at 5 µg/ml 200,000 cells and αv blocking (cyclic penRGD) and control (RGE) peptide (100 µM) (GIBCO BRL). Monoclonal antibody supernatants for α2 and α3 were provided by W.G. Carter (Fred Hutchinson Cancer Research Center, Seattle, WA). Matrix metalloproteinase inhibition studies were performed with recombinant human tissue inhibitors of metalloproteinase (TIMP1 and TIMP2; 3 µg/ml; Oncogene research Products). Antibodies for Western blot detection, immunoprecipitation, and immunostaining included: 9N3 pp125FAK and 103/105C pp125FAK (Santa Cruz Biotechnology), 34A3 N pp125FAK (Transduction Laboratories), α-actinin (Sigma), p130cas, paxillin (Transduction Laboratories), talin-8d4 (Sigma), and vinculin (Calbiochem-Novobiochem Corp.). A nito-mouse and rabbit peroxidase-conjugated secondary antibodies were purchased from Vector Laboratories Inc.

**Immunocytochemistry**

SMC were plated on monomeric collagen- or fibronectin-coated plastic chamber slides for 24 h and either left untreated or treated with degraded collagen fragments for 1–5 min. Cells were fixed in 4% paraformaldehyde, permeabilized in 0.5% Triton X-100 in PBS and washed serially in PBS, chamber slides for 24 h and either left untreated or treated with degraded collagen in the presence of ALLN or ZVAD. To block integrin function, SMC were preincubated at 37°C for 30 min with nonblocking (P1H6) and blocking (P1H5) Fab antibody fragments against α2, blocking α3 Fab antibody (P1B8), blocking αvβ3 (LM 609; Chemicon) at 5 µg/ml 200,000 cells and αv blocking (cyclic penRGD) and control (RGE) peptide (100 µM) (GIBCO BRL). Monoclonal antibody supernatants for α2 and α3 were provided by W.G. Carter (Fred Hutchinson Cancer Research Center, Seattle, WA). Matrix metalloproteinase inhibition studies were performed with recombinant human tissue inhibitors of metalloproteinase (TIMP1 and TIMP2; 3 µg/ml; Oncogene research Products). Antibodies for Western blot detection, immunoprecipitation, and immunostaining included: 9N3 pp125FAK and 103/105C pp125FAK (Santa Cruz Biotechnology), 34A3 N pp125FAK (Transduction Laboratories), α-actinin (Sigma), p130cas, paxillin (Transduction Laboratories), talin-8d4 (Sigma), and vinculin (Calbiochem-Novobiochem Corp.). A nito-mouse and rabbit peroxidase-conjugated secondary antibodies were purchased from Vector Laboratories Inc.

**Cell Lysis, Immunoblotting, and Immunoprecipitations**

Cells were washed twice with PBS and lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 1 mM NaF, 0.1 mM Na3VO4, 10 mM β-mercaptoethanol, 0.5 mM PM SF, 10 µg/ml leupeptin, and 10 µg/ml aprotonin) for 30 min on ice. Cell lysates were clarified by centrifugation at 27,000 g for 20 min, and protein concentration was determined using the BCA protein assay (Pierce). Lysates were separated on 10% or 7.5% SDS-PAGE; proteins were transferred to Immobilon membrane (Millipore) and immunoblotted with specific antibodies against p130cas, paxillin, and vinculin. All immunoblots were visualized by enhanced chemiluminescence (ECL, A mersham Corp.). For pp125FAK coimmunoprecipitation studies, cell lysates were preclarified with protein A-agarose (Santa Cruz Biotech.) incubated with 2 µg of the 9N3 pp125FAK antibody, and immunoblotted with specific antibodies against p130cas, paxillin, and vinculin.

Triton X-100-soluble (cytoplasmic) and -insoluble (cytoskeletal) fractions were prepared as previously described (Jackson et al., 1994), with the exception of modification of Triton X-100 and radioligandprecipitation assay (RIPA) lysis buffers. In brief, SMC were lysed in Triton X-100
lysis buffer (20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 5 mM EDTA, 0.4 mM leupeptin, 0.2 mM Na<sub>2</sub>VO<sub>4</sub>, and 0.1 mM PM 5F) for 1 h at 4°C. Triton X-100 insoluble and soluble extracts were separated by centrifugation at 15,000 g for 5 min. The cytoskeletal pellet was washed twice with Triton-free lysis buffer, and proteins were extracted using RIPA buffer (10 mM Tris-HCl [pH 7.2], 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 1 mM PM SF, and 1 mM Na<sub>2</sub>VO<sub>4</sub>).

**In Vitro Cleavage of pp125FAK**

In vitro transcription and translation of pp125FAK were performed with the TNT<sup>®</sup>-coupled reticulocyte lysate system (Promega) and [35S]methionine (1,000 Ci/mmol, Amersham Corp.), according to the manufacturer’s instructions. The expression plasmid for chicken pp125FAK was given by J.T. Parsons (University of Virginia, Charlottesville, VA). Of the reaction, 1/25 was used as a substrate and incubated with a range of 0.25–2 activity units of purified calpain I and calpain II (Calbiochem-Novabiochem Corp.) for 30 min at 30°C in reaction buffer (50 mM Tris-HCl, pH 7.4, 10 mM CaCl<sub>2</sub>, 5 mM β-mercaptoethanol, and 30 mM NaCl) in the presence and absence of the human recombinant endogenous calpain inhibitor, calpstatin (30 μM; Calbiochem-Novabiochem Corp.). Reactions were terminated by the addition of 4× SDS sample buffer. Common molecular mass standards (Bio-Rad Laboratories) were used to determine Rf values and proteolytic fragment size for both in vitro and cellular pp125FAK cleavage analysis.

**Collagen Radiolabeling and Degradation Assay**

Vitrogen (Collagen Corp.) concentration was adjusted to 1 mg/ml and neutralized after dialysis against 10 mM borate, 0.2 M CaCl<sub>2</sub>, pH 8. The vitrogen solution was then radiolabeled by acetylation with [3H]acetic anhydride (NEN Life Science) as described previously (Mookhtiar et al., 1986). 3H-labeled collagen degradation was assayed by modification of a previously described procedure (Aimes and Quigley, 1995). In brief, an aliquot of radiolabeled vitrogen was used to generate polymerized fibrillar collagen gels, as described above. SM C were cultured on the labeled polymerized collagen, and at subsequent time points after cell seeding culture supernatants were analyzed for degraded 3H-labeled collagen fragments by liquid scintillation spectroscopy.

**Zymography**

Cell lysates were prepared in lysis buffer as described above and serum-free conditioned media was collected from SM C cultures. Samples were prepared in non-denaturing loading buffer and separated on 10% SDS-polyacrylamide gel impregnated with 1 mg/ml gelatin. A lter electrophoresis, gels were washed twice in 2.5% Triton X-100, briefly rinsed with water, and incubated for 24 h at 37°C in collagenase buffer (50 mM Tris-HCl buffer, pH 7.5, 200 mM NaCl, and 10 mM CaCl<sub>2</sub>). Gels were subsequently fixed and stained in Coomassie blue fixative solution (50% methanol and 10% acetic acid containing 0.25% Coomassie blue R 250).

**Results**

**Degraded Collagen Fragments Induce Cell Rounding and Promote Cleavage of pp125FAK**

Human arterial SM C cultured on polymerized type I collagen fibrils are arrested in the G1 phase of the cell cycle and do not respond to growth factor stimulation, whereas SM C on monomeric type I collagen proliferate in response to growth factors (Koyama et al., 1996). We hypothesized...
Degraded Collagen Promotes Calpain-mediated Cleavage of pp125FAK in Smooth Muscle Cells
Because degraded type I collagen reversibly alters cell spreading and focal adhesion formation, we asked whether it alters a major component of focal adhesions, focal adhesion kinase (pp125FAK). Incubation with degraded collagen (1 mg/ml concentration) rapidly induces pp125FAK processing, resulting in a significant reduction in the levels of native pp125FAK, as monitored by Western analysis with an antibody to the NH2 terminus of pp125FAK (Fig. 2 A). This reduction in native pp125FAK is accompanied by an increase in levels of 90-, 50-, and 42-kD bands. By immunoblotting the same lysates with an antibody to pp125FAK directed against residues 2–18 at the NH2 terminus (2–18N), we detect 90- and 40-kD fragments (Fig. 2 B). Using a polyclonal antibody against the COOH terminus of pp125FAK, we identify a cleaved 35-kD fragment (Fig. 2 C). This cleavage of pp125FAK is reversible, and native pp125FAK is restored to maximal levels within 3 h when the degraded collagen is removed and the SMC are replated on fresh monomeric collagen (data not shown).

pp125FAK cleavage mediated by caspases has been demonstrated in Jurkat T cells (Wen et al., 1997) and endothelial cells (Levkau et al., 1998). Cleavage of pp125FAK has also been observed in platelets, where it is mediated by the calcium-activated protease calpain I, which results in proteolytic fragments of 90, 45, and 40 kD (Cooray et al., 1996), similar in size to those observed when SMC are treated with degraded collagen. To determine whether calpain and/or caspases are responsible for pp125FAK processing, we treated SMC with degraded collagen in the presence of the calpain inhibitor ALLN (100 μM) or the broad caspase inhibitor ZVAD (100 μM). As shown in Figs. 3 and 4, inhibition of calpain partially reduces processing of native pp125FAK, whereas the caspase inhibitor (ZVAD) has no influence on pp125FAK processing.

To identify which calpain(s) is capable of cleaving pp125FAK, we incubated in vitro translated pp125FAK with purified calpains I or II. As shown in Fig. 4 A, both calpain I and II induce dose-dependent processing of pp125FAK to 90-, 50-, 40-, and 35-kD fragments. The proteolytic activity of either calpain I or II can be blocked by incubation with the specific endogenous calpain inhibitor calpastatin (30 μM). The size of the proteolytic pp125FAK fragments obtained in vitro correlates with the proteolytic cleavage of pp125FAK, observed when SMC are treated with degraded collagen fragments (Fig. 2).

Degraded Collagen Promotes Calpain-mediated Cleavage of pp125FAK in Smooth Muscle Cells

Figure 3. pp125FAK cleavage is blocked by calpain inhibition. (A) SMC in suspension were incubated, with collagenase degraded collagen (+ Deg. Col.) or degraded BSA (+ Deg. BSA) for 30 min. Cell lysates were separated by SDSPAGE transferred to an Immobilon membrane and immunoblotted with 21H–pp125FAK antibody. (B) SMC cultured on monomeric collagen (M) or in suspension (S) for 30 min in the presence or absence of inhibitors of calpains (ALLN, 100 μM) and caspases (ZVAD, 100 μM). Total cell lysates were prepared, separated by SDSPAGE, transferred to a membrane, and immunoblotted with 21H–pp125FAK antibody.
Integrins Interact with Degraded Collagen to Induce pp125FAK Cleavage

We asked whether the ability of degraded collagen to promote pp125FAK cleavage was mediated through ligation with cell surface integrin receptors. SMC cultured on fibronectin were preincubated with Fab fragments of blocking (P1H5) or nonblocking (P1H6) antibodies against the α2 integrin subunit, blocking α3 integrin subunit antibody (P1D6), blocking αvβ3 antibody (LM 609), or αv-blocking (cyclic RGD) or control (RGE) peptides, followed by 30 min incubation in the presence of degraded collagen fragments (0.25 mg/ml). Preincubation with nonblocking anti-α2 Fab fragments, a blocking anti-α3 Fab, blocking anti-αvβ3, or RGE peptide did not prevent pp125FAK cleavage. However, when degraded collagen was added to SMC preincubated with either blocking anti-α2 Fab, cyclic RGD peptide, or α2 Fab and cyclic RGD peptide in combination, the induction of pp125FAK cleavage was suppressed (Fig. 5 A). Changes in pp125FAK cleavage with integrin blockade were quantified by densitometric scanning of both native 125 kD pp125FAK and the 90-kD cleavage fragment. Densitometry values from three separate experiments were combined and are shown in Fig. 5 B. A asterisks indicate significant difference from control + degraded collagen (P < 0.05 by Student’s t test). A though suppression of pp125FAK cleavage by preincubation with αv-blocking cyclic RGD peptide is a consistent observation, statis-
Degraded Collagen Promotes Calpain-mediated Cleavage of Paxillin and Talin but Not α-Actinin

Three other focal adhesion proteins, paxillin, talin, and α-actinin, have been shown to be substrates for calpain (Yamaguchi et al., 1994; Selliah et al., 1996; Schoenwaelder et al., 1997). We asked whether these proteins are also cleaved in SMC exposed to degraded collagen. Native paxillin levels (68 kD) are slightly reduced in SMC cultured with degraded collagen, and a 55-kD band appears (Fig. 6 A). Inhibition of calpain (ALLN, 100 μM) prevents processing to the 55-kD paxillin fragment, whereas caspase inhibition with ZVAD (100 μM) has no effect. Similarly native talin (230 kD) also appears to be proteolytically cleaved giving rise to a 190-kD fragment in SMC treated with degraded collagen (Fig. 6 B). The generation of a 190-kD cleavage fragment of talin is identical in size to a previously identified calpain-mediated cleavage product of talin (Schoenwaelder et al., 1997). Inhibition of calpain but not caspase activity suppresses talin cleavage (Fig. 6 B). In contrast to paxillin, talin and pp125FAK, α-actinin is not processed in response to degraded collagen (Fig. 6 C), nor is actin, another known substrate of calpain (data not shown).

Degraded Collagen-induced Cleavage of pp125FAK Decreases Its Interaction with Components of the Focal Adhesion Complex and Alters Its Intracellular Localization

The COOH-terminal cleavage fragment of pp125FAK (35 kD) contains the focal adhesion targeting (FAT) sequence and second proline-rich domain (p2) of pp125FAK, sites involved in pp125FAK interaction with other focal adhesion proteins, such as p130cas, paxillin, and vinculin (Fig. 4 B). We asked whether the ability of pp125FAK to associate with other focal adhesion signaling molecules is altered in SMC after treatment with degraded collagen. Coimmunoprecipitation studies using the 2–18NH2-terminal pp125FAK antibody demonstrate a significant reduction in the association of paxillin, vinculin, and p130cas with pp125FAK in SMC cultured with degraded collagen compared with cells on monomeric collagen (Fig. 7). Control coimmunoprecipitation assays using a normal rabbit IgG were performed in parallel with pp125FAK immunoprecipitations, and rabbit IgG did not associate with paxillin, vinculin, or p130cas in either untreated or degraded collagen-treated SMC samples (results not shown).

The subcellular localization of pp125FAK between cytoplasmic and cytoskeletal fractions is also altered in SMC treated with degraded collagen (Fig. 8). Significantly less native pp125FAK is in the cytoskeletal fraction, and the NH2-terminal proteolytic fragments of pp125FAK are predominantly found in the cytoplasmic fraction. Levels of native pp125FAK in the cytoplasmic fraction are only slightly reduced in response to treatment with degraded collagen, suggesting that cytoskeleton-associated pp125FAK may be more sensitive to calpain-mediated processing.

Pretreatment with Calpain Inhibitor Suppresses Degraded Collagen-induced Cell Rounding and Disassembly of Actin Cytoskeleton

To directly test the role of calpain activity in focal adhesion disassembly and cell rounding induced by degraded collagen, SMC cultured on fibronectin were preincubated with or without calpain inhibitor 1 (ALLN, 100 μM) for 18 h before addition of degraded collagen (0.25 mg/ml). Immunostaining with antibodies against paxillin (Fig. 9, A–C) and FITC-labeled phalloidin (Fig. 9, D–F) demonstrate that, in the absence of calpain inhibition, SMC round up and retain limited numbers of focal adhesions one minute after the addition of degraded collagen (Fig. 9 A). In con-
Intracellular translocation of pp125\(^{F\text{AK}}\) cleavage fragments to the cytoplasm. SMC cultured on monomeric collagen in the presence (+) or absence (−) of degraded collagen (Deg. Col.) fragments were lysed with Triton X-100 lysis buffer and fractionated into Triton X-100-soluble (cytoplasmic) and −insoluble (cytoskeletal) extracts. Extracts were then separated by SDS-PAGE, transferred to a membrane, and immunoblotted with \(^{2\text{H}}\)-pp125\(^{F\text{AK}}\) antibodies.

We hypothesized that the pp125\(^{F\text{AK}}\) cleavage observed when SMC are cultured on polymerized collagen may be initiated as a result of generation of degraded collagen fragments from an induction of endogenous SMC MMP activity. Gelatin zymography demonstrates that pro and active forms of MMP2 are increased in SMC cultured on polymerized collagen as early as 6 h after plating (Fig. 10 D). Western blotting of cell lysates also demonstrates significant increases in intracellular levels of both MMP1 and MMP2 when SMC are cultured on polymerized collagen (data not shown). Addition of recombinant TIMP1 and TIMP2 has little effect on pp125\(^{F\text{AK}}\) cleavage, and treatment with the calpain inhibitor ALLN (100 \(\mu M\)) significantly suppresses the cleavage of pp125\(^{F\text{AK}}\) on polymerized collagen.

Figure 8. Intracellular translocation of pp125\(^{F\text{AK}}\) cleavage fragments to the cytoplasm. SMC cultured on monomeric collagen in the presence (+) or absence (−) of degraded collagen are shown.

**Figure 9.** Preincubation with calpain inhibitor suppresses loss of substrate attachment and cell rounding induced by degraded collagen. Human arterial SMC were cultured on fibronectin in the absence (A and D) or presence (B, C, E, and F) of 100 \(\mu M\) calpain inhibitor (ALLN) for 18 h. Degraded collagen was then added to these cultures. 1 and 5 min after the addition of degraded collagen, SMC were fixed and immunostained with antibodies to paxillin (A−C) or FITC-labeled phalloidin (D−F) and examined by confocal microscopy. Bars: 20 \(\mu M\).
Figure 10. Increased endogenous SMC MMP activity, generation of degraded collagen fragments, and pp125FAK cleavage in response to culture on polymerized collagen. (A) Time course of release of 3H-labeled collagen fragments into culture media of SMC plated on 3H-labeled polymerized collagen. (B) Time course of pp125FAK cleavage in response to culture on polymerized collagen. (C) Protein extracts from SMC plated on polymerized collagen for 24 h in the presence of the calpain inhibitor (ALLN, 100 μg/ml) or the caspase inhibitor (ZVAD, 100 μg/ml) were separated by SDS-PAGE, transferred to a membrane, and immunoblotted with antibodies to 2–18N-pp125FAK. (D) Gelatin zymography of serum-free conditioned media from SMC cultured on polymerized collagen at sequential time points after plating on polymerized collagen.

TIMP2 (3 μg/ml) to SMC cultured on polymerized collagen for 24 h significantly reduces both the induction of MMP2 activity as determined by gelatin zymography (Fig. 11 A) and subsequent degradation of 3H-labeled polymerized collagen (data not shown). Western blotting of total cell lysates demonstrates that inhibition of MMP activity and collagen degradation by TIMP1 and TIMP2 is accompanied by a significant reduction in the extent of pp125FAK cleavage (Fig. 11 B).

Discussion

Matrix Proteolysis Exposes Cryptic Sites with Distinct Activities

The possibility that structural changes in the ECM may be necessary for cell migration during tissue remodeling and tumor invasion is supported by two examples of matrix degradation products promoting distinct migratory responses. MMP2 digestion of laminin 5 confers unique signaling properties that promote cell migration, which are not possessed by native laminin 5 (Gianelli et al., 1997). MMP2 cleavage of the laminin 5 γ2 chain exposes a cryptic promigratory site on laminin 5. This altered form of laminin 5 is found in tumors and in tissues undergoing remodeling, but not in quiescent tissues. It has also been shown that thrombin cleavage of osteopontin enhances its haptotactic activity (Senger and Perruzzi, 1996), and the NH2-terminal cleavage fragment contains a cryptic adhesion sequence recognized by α5β1 integrin (Smith et al., 1996).

The studies presented in this report demonstrate unique signaling properties of degraded collagen primarily mediated through the integrin α2β1 that rapidly promotes focal adhesion disassembly and cell rounding. Previous work has proposed that denaturation or metalloproteinase degradation of collagen reveals cryptic RGD integrin-binding motifs (Davis, 1992; Montgomery et al., 1994). MMP degradation of collagen differentially affects α2β1 binding to the collagen fragments (M essent et al., 1998) and reveals a cryptic β3 integrin binding motif, which induces specific integrin-signaling events in SMC, resulting in increased tenasin-C expression (Jones et al., 1997). In our studies, the molecular changes mediated by the interaction with degraded collagen fragments are not mimicked by RGD peptides, nor mediated by αvβ3. The effects of degraded collagen are independent of the substrate (and integrins) involved in cell adhesion.

Rapid and Selective Cleavage of Focal Adhesion Components by Calpain after Stimulation by Degraded Collagen Fragments

Studies have postulated that limited proteolysis of components of the focal adhesion complex by proteolytic enzymes is a potential mediator for focal adhesion disassembly (Beckerle et al., 1987; Huttenlocher et al., 1997). Particularly interesting candidate enzymes are the calpains, a family of intracellular, calcium-activated cysteine proteases, consisting of two ubiquitously expressed isozymes, calpain I and II (reviewed in Molanari and Carafoli, 1997). Calpains are localized to sites of focal adhesions in several cell types (Beckerle et al., 1987), and have also been demonstrated to cleave a number of focal adhesion proteins in platelets or in vitro, including pp125FAK, pp60 src, paxillin, talin, actin, and α-actinin (Yoshida et al., 1984; Beckerle et al., 1987; Oda et al., 1993; Yamaguchi et al., 1994; Cooray et al., 1996; Selliah et al., 1996). However, a direct link between calpain-mediated proteolytic activity and disassembly of focal adhesion complexes in adherent cell populations has not been established.

In this study, we demonstrate that proteolytic cleavage...
of pp125FAK, paxillin, and talin is rapidly induced in adherent vascular SMC treated with degraded collagen fragments, and can be observed in longer term SMC cultures on polymerized collagen after MMP induction and the endogenous generation of degraded collagen fragments. Calpain inhibitor studies and in vitro cleavage assays indicate that pp125FAK, paxillin, and talin cleavage is mediated by calpain. Cleavage of other focal adhesion proteins, including known calpain substrates, such as α-actinin and actin is not observed, indicating that calpain proteolysis induced by degraded collagen is fairly selective for the focal adhesion components pp125FAK, paxillin, and talin. Our kinetic studies demonstrate that calpain proteolysis of pp125FAK, paxillin, and talin is observed within 5 min after addition of degraded collagen and occurs in parallel with focal adhesion disassembly and loss of cell attachment.

The mechanisms that regulate calpain proteolytic activity in vivo are still poorly understood. Studies have proposed that translocation of calpain to the plasma membrane and focal adhesion sites promotes activity in response to elevated calcium (Molanari and Carafoli, 1997). Integrin ligation with ECM ligands increases intracellular calcium levels (Jaconi et al., 1991), and rapid initiation of calpain-mediated cleavage of substrates is observed in response to integrin αIIbβ3 ligation on platelets (Fox et al., 1993; Inomata et al., 1996; Schoenwaelder et al., 1997). The rapid kinetics of calpain-mediated cleavage of pp125FAK, paxillin, and talin that we observe in SMC in response to exposure to degraded collagen fragments is consistent with the time course of integrin-mediated calpain activation observed previously (Cooray et al., 1996; Inomata et al., 1996). Our integrin-blocking studies further suggest that initiation of pp125FAK cleavage is at least partially dependent on the association of the collagen fragments with α2 and possibly also αv-containing integrins. This raises the possibility that degraded collagen fragments possess specific integrin-signaling properties, including initiation of calpain activation. However, the precise downstream signaling mechanisms responsible for initiating calpain cleavage of pp125FAK, paxillin, and talin remain to be elucidated.

pp125FAK, a Critical Regulator of Focal Adhesion Turnover, Is Functionally Modulated by Calpain Cleavage

Numerous studies have established pp125FAK phosphorylation as a key step in regulating focal adhesion turnover (Burridge et al., 1992; Schaller et al., 1992). A utrophosphorylation of pp125FAK on tyrosine 397 generates a high affinity binding site for the src family kinases (Schaller et al., 1994; Polte and Hanks, 1995). Formation of a pp125FAK/src signaling complex enhances the catalytic activity of both pp125FAK and src and recruitment of other focal adhesion proteins, further promoting assembly of the focal adhesion signaling complex (Schlaepfer et al., 1994; Polte and Hanks, 1997). Our studies suggested that calpain-mediated cleavage of pp125FAK (Fig. 4 B) should dissociate the COOH-terminal focal adhesion targeting sequence (FAT) and second proline-rich domain from the NH2-terminal catalytic kinase and integrin binding domains. Further, we demonstrate that calpain cleavage of pp125FAK induced by degraded collagen impairs the ability of pp125FAK to function as an adapter protein and to associate with paxillin, p130cas, and vinculin. pp125FAK cleavage also results in decreased levels of native pp125FAK associated with the cytoskeletal fraction and accumulation of NH2-terminal cleavage fragments in the cytoplasmic fraction. This translocation of NH2-terminal fragments to the cytoplasm is most likely a consequence of cleavage of the COOH-terminal FAT sequence and second proline-rich domain, which are required for localization of pp125FAK to focal adhesion sites (Hildebrand et al., 1993; Harte et al., 1996; Polte and Hanks, 1997). Thus, intracellular translocation of pp125FAK, combined with impaired ability to associate with and recruit other focal adhesion proteins, strongly suggests that calpain cleavage represents an important mechanism for regulating pp125FAK activity and focal adhesion disassembly.

Previous studies suggest that pp125FAK activity may also be regulated by expression of an alternatively spliced form called pp125FAK-related non-kinase (FRNK) (Schaller et al., 1993). FRNK is a truncated 41-kD protein identical to the COOH-terminal domain of pp125FAK. It is not catalytically active because it lacks the kinase domain, but localizes to focal adhesions through protein associations mediated by the COOH-terminal proline-rich domains and FAT sequence (Schaller et al., 1993). Previous studies suggest FRNK may regulate pp125FAK activity by acting as a competitive inhibitor (Richardson and Parsons, 1996). Our results demonstrate that calpain cleavage of pp125FAK generates a 35-kD COOH-terminal fragment consisting of the COOH-terminal FAT sequence and second proline-rich domain and thus is structurally similar to FRNK. Due to very low levels of the 35-kD COOH-terminal fragment, we have been unable to detect whether it localizes to the cytoskeletal fraction or is associated with other focal adhesion proteins, such as paxillin. The domain structure of the 35-kD COOH-terminal fragment indicates that it has the potential to act as a competitive inhibitor of pp125FAK, and further contribute to disassembly of focal adhesions that accompanies calpain cleavage of pp125FAK. However, its effectiveness in such a role would depend on the maintenance of this fragment at high levels within focal adhesion sites.

Tyrosine phosphorylation of pp125FAK is not required for focal adhesion assembly and cell spreading in response to SMC adhesion to a fibronectin substrate (Wilson et al., 1995). This is consistent with our studies, which demonstrate that, with adhesion of SMC to distinct collagen or fibronectin substrates, there is no significant regulation of pp125FAK tyrosine phosphorylation. We, therefore, propose that, under certain conditions, the primary mechanism for regulating pp125FAK activity and focal adhesion assembly in SMC is mediated through calpain-dependent proteolytic processing, rather than tyrosine phosphorylation. The calpain-mediated proteolysis impairs recruitment of other signaling proteins, decreases association with the cytoskeletal fraction, and generates fragments structurally similar to FRNK that may further contribute to focal adhesion disassembly by acting as competitive inhibitors of pp125FAK.

Our studies demonstrate that pretreatment of SMC with a calpain inhibitor before incubation with degraded col-
lagent fragments suppresses the cell rounding and loss of substrate attachment. The influence of calpain inhibition on focal adhesion disassembly is less clear, as many of the weakly attached SM C, after treatment with degraded collagen, are lost during immunostaining, and only the most adherent cells remain attached and are evaluated by confocal microscopy. However, results presented in this study indicate that pretreatment with calpain inhibitors before addition of degraded collagen appears to enhance the number of focal adhesion structures, compared with cells not pretreated with the calpain inhibitor. More significantly, calpain inhibition maintains substrate anchorage, a spread morphology, and organized actin stress fibers in all cells incubated with degraded collagen fragments. These results further implicate calpain cleavage of pp125FAK, paxillin, and talin in promoting focal adhesion disassembly and cell rounding induced by degraded collagen. Our data are also consistent with previous studies demonstrating that proteolytic cleavage of pp125FAK occurs in parallel with cell rounding and loss of substrate attachment (Fincham et al., 1995; Crouch et al., 1996).

Our studies have focused primarily on the influence of calpain cleavage on pp125FAK function and focal adhesion integrity. However, calpain cleavage of paxillin and talin is also likely to have significant consequences on focal adhesion complexes and may contribute to disassembly of focal adhesions observed when SM C are treated with degraded collagen fragments.

In Vivo Implications of Calpain Dissolution of SMC Focal Adhesions in Response to Increased MMP Activity and Collagen Degradation

Focal adhesions are dynamic structures that are assembled and disassembled at different stages in the cell cycle, indicating their possible involvement in regulation of cell proliferation (David-Pfeuty and Singer, 1980; Herman and Pledger, 1985). It has also been proposed that focal adhesion assembly and disassembly control cell migration by regulating attachment to the substratum at the leading edge with detachment from the posterior end of the cell (Huttenlocher et al., 1995). Cells derived from pp125FAK−/− embryos exhibit a reduced capacity for cell migration and have more focal adhesions than pp125FAK cells (Illic et al., 1995). Thus, pp125FAK may play a critical role in the regulation of focal adhesion turnover and cell migration.

During the development of atherosclerotic and restenotic lesions, the ECM surrounding SM C is modified by increased MMP activity derived from both vascular SM C and inflammatory monocytes (Bendeck et al., 1994; Galis et al., 1994). Modulation of the vascular ECM may contribute to transition of SM C to a “synthetic” phenotype, which results in enhanced SM C proliferation, migration, and lesion progression (Thyberg et al., 1990; Cheng et al., 1998). Our demonstration that degraded collagen fragments promote calpain-mediated disassembly of SM C focal adhesions suggests the possibility that, in localized sites of MMP expression, SM C anchorage to the surrounding ECM may be disrupted, thereby promoting their migration and invasion into the arterial intima. Loss of focal adhesion contacts with the surrounding ECM may contribute to enhanced SM C proliferation by releasing SM C from a nonpermissive matrix environment, such as polymerized collagen fibrils (Pauly et al., 1994; K oyama et al., 1996).

In conclusion, our results characterize an alternative to phosphorylation of pp125FAK for regulating pp125FAK activity and focal adhesion assembly in a viable adherent cell population, one dependent on calpain-mediated cleavage of pp125FAK, paxillin, and talin. We further demonstrate that degraded collagen fragments initiate this cleavage through integrin signals distinct from those of native collagen.

We would like to thank Bonnie A shieman and the W. M. Keck Center for A dvanced Studies in Neural Signaling for technical assistance with confocal microscopy. We would also like to thank Drs. W. G. Carter and J. T. Parsons for kindly providing reagents.

This study was supported by a grant from the National Heart, Lung, and Blood Institute, National Institutes of Health, HL 18645.

Submitted: 8 Apr 1999
Revised: 27 September 1999
Accepted: 27 September 1999

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