J. Biochem. **136**, 777–785 (2004)
DOI: 10.1093/jb/mvh187

Degradation of Fodrin by m-Calpain in Fibroblasts Adhering to Fibrillar Collagen I Gel

Kaori Sato[1,*], Shunji Hattori[2], Shinkichi Irie[2], Hiroyuki Sorimachi[3], Mitsushi Inomata[4] and Seiichi Kawashima[1]

[1]Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613; [2]Nippi Research Institute of Biomatrix and Institute of Leather Research, Nippi Co., Ltd., Tokyo 112-8601; [3]Department of Enzymatic Regulation for Cell Functions, Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613; and [4]Department of Protein Biochemistry, Tokyo Metropolitan Institute of Gerontology, Tokyo 173-0015,

Received May 21, 2004; accepted September 4, 2004

When skin fibroblasts were cultured on fibrillar collagen I gel, we observed rapid degradation of talin, fodrin and ezrin, which are well-known calpain substrates. The protease m-calpain was activated only in cells adhering to fibrillar collagen, whereas μ-calpain was activated in cells adhering to monomeric or fibrillar collagen at the same level. The calpain inhibitor Z-Leu-Leu-aldehyde inhibited degradation of fodrin, but not talin. Degradation of fodrin, α-actinin and ezrin was prevented by over-expression of dominant negative m-calpain. However, over-expression of calpastatin, an endogenous calpain inhibitor, had no effect the degradation of these three proteins. These results suggest that m-calpain is responsible for degradation of their membrane proteins *via* adhesion to fibrillar collagen I gel.

Key words: calpain, collagen, degradation, fodrin, talin.

Abbreviations: Ab; antibody; BSA, bovine serum albumin; DEVDal, acetyl-Asp-Glu-Val-Asp-aldehyde; LLal, benzyloxycarbonyl-Leu-Leu-aldehyde; DMEM, Dulbecco's modified Eagle's medium; DN-mCL-EGFP, dominant negative-m-calpain–enhanced green fluorescence protein; ERK, extracellular-signal regulated kinase; FAK, focal adhesion kinase; mAb, monoclonal antibody; pAb, polyclonal antibody; WAVE, Wiscott Aldrich syndrome protein-family verprotein homologous protein.

Adhesion to collagen I gel lattice induces selective proteolysis of focal adhesion complex proteins in various cell types (*1*). Integrin α2β1 is a major receptor for the fibril form of collagens (*2*, *3*). Interestingly, α2β1-triggered proteolysis has been found to occur in cells adhering to fibrillar gel but not in cells adhering to the coated fibril form or coated monomeric form of collagen (*1*). Collagen gel culture of skin fibroblasts is used as a model culture system for early wound healing in the dermis.

Membrane-lining proteins such as fodrin (non-erythrocyte-type spectrin), ankyrin and ERM (ezrin, radixin and moesin) stabilize cell-surface membranes. Fodrin is present in lamellipodia of cultured chicken fibroblasts (*4*). Cytoskeletal elements such as filamentous actin, microtubules and vimentin filaments also help maintain cell shape (*5*, *6*). The membrane-lining cytoskeleton is formed by binding of fodrin and ankyrin, Band 4.1 family proteins (ERM family and talin) or annexin in cells, and is connected to actin-based peripheral stress fibers (*7*).

Calpain, a cytoplasmic cysteine protease, can rapidly degrade fodrin and various other signaling molecules (*8*–*10*). The calpain family consists of two major ubiquitous isoforms (μ- and m-calpain) and calpains 3 to 14 (*11*). In platelets (*12*), neutrophils (*13*) and endothelial cells (*14*, *15*), calpains are involved in morphological changes including formation of lamellipodia and formation of phagocytic cups. Diminished calpain activity, due to calpain inhibitors (*12*) or in calpain4 knockout mouse-derived cells (*16*), can cause cessation of fodrin/talin degradation and platelet morphological changes (*12*). Spatial Ca^{2+} influx and calpain activation stabilize integrin-mediated adhesion sites, thus reducing filopodial motility and lamellipodial protrusion and promoting repulsive growth-cone turning (*17*). In addition, μ-calpain is activated by an adhesion signal mediated by integrin, and activates Rho GTPase (RhoA and Rac), which stimulates actin filament formation (*14*, *15*). These findings suggest that degradation of fodrin and talin by calpain is necessary for appropriate relocalization of integrin on the cell surface following filopodia/lamellipodia organization mediated by Rho GTPase.

Recently, we found that, when fibroblasts are cultured on fibrillar collagen gel, the major cell shape–organizing cytoskeletal component switches from filamentous actin to microtubules during dynamic shape changes in which the cells come to resemble dendritic cells (*18*). In the present study, we assayed for other changes in the membrane-lining cytoskeleton consisting of fodrin and associated proteins. We observed dramatic degradation of fodrin, ezrin and α-actinin after cell adhesion to fibrillar collagen gel, and found that m-calpain is the protease responsible for degradation of fodrin.

*To whom correspondence should be addressed at the present address: The present address is Department of Matrix Biology, Kennedy Institute of Rheumatology, Faculty of Medicine, Imperial College London. 1 Aspenlea Road, Hammersmith, London W6 8LH, UK, Fax: +44 (0)20-8383-4499, E-mail: ksq@mac.com

MATERIALS AND METHODS

Antibodies—Anti–α-actinin (C-20), anti-ezrin (C-19) and anti-talin (C-20) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti–α-fodrin mAb (clone AA6) was obtained from Biohit OY (Kajaani, Finland). Anti-paxillin mAb was obtained from BD Biosciences (San Jose, CA, USA). Anti–β-actin mAb was obtained from Chemicon International Inc. (Temecula, CA, USA). Rabbit polyclonal antibodies, anti–talin 47-kDa fragment (*19*, *20*), anti–fodrin 140-kDa fragment (*8*) and anti–post autolytic μ-calpain (*21*) were raised using synthetic peptides as antigens. Anti–m-calpain (1D4E8 and 4B1E1) and anti–μ-calpain (4B9F10) mAb were prepared as described elsewhere (*22*).

Cell Culture of Human Neonatal Foreskin Fibroblasts on Collagen I—Acetic acid-soluble type I collagen purified from neonatal bovine skin was digested with papain as described elsewhere (*23*). Papain-treated collagen induces more cell spike formation than acid-soluble collagen or pepsin-treated collagen (Sato et al., unpublished data).

Human neonatal foreskin fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Culture dishes (35 mm) were coated with monomeric collagen I (0.1 mg/ml in 5 mM acetic acid) from 1 h to overnight at room temperature. Human umbilical vein endothelial cells, human aortic smooth muscle cells, and EGM-2 and SmGM medium were obtained from Sanko Junyaku (Tokyo, Japan).

To prepare fibrillar collagen I gel, dishes were covered with papain-treated collagen I (0.18 mg/0.9 ml or indicated concentration) in DMEM containing 1 mg/ml bovine serum albumin (BSA) and kept at 37°C in a 5% CO_2 atmosphere from 1 h to overnight to polymerize collagen I. To obtain fibril-coated dishes, the collagen gel was mechanically removed from the edge of the culture dish using a plastic tip. Confluent skin fibroblasts were dispersed in PBS containing 0.05% bovine trypsin and 1 mM EDTA, re-suspended and washed twice with DMEM containing 1 mg/ml BSA, then plated on collagen. Human umbilical vein endothelial cells, human aortic smooth muscle cells, and HT-1080 cells were dispersed in EGM-2, SmGM, and DMEM containing 10% fetal bovine serum, respectively, and were then plated on collagen.

For inhibitor treatment, suspended cells were preincubated with 10 to 200 μM benzyloxycarbonyl-leucyl-leucinal (LLal, a calpain active site–specific inhibitor) (Peptide Institute, Inc., Osaka, Japan), 1 or 10 μM clastolactacystin β-lactone (lactacystin, a proteasome-specific inhibitor) (Boston Biochem, Cambridge, MA, USA), 200 μM acetyl-Asp-Glu-Val-Asp-aldehyde (DEVDal, a caspase3 inhibitor), 20 μM PD98059 (Calbiochem), 20 μM U0126 (Sigma, MEK1/2 (ERK kinase) inhibitor) or a vehicle (0.1% dimethylsulfoxide) for 30 min at room temperature. They were then plated at a density of 2 to 3 × 10^5 cells/35-mm dish, and cultured for the indicated periods. Cells were photographed under phase-contrast microscopy using a digital camera (Camedia C4040, Olympus, Tokyo, Japan).

Construction and Transfection of m-Calpain-EGFP and Calpastatin Genes—Dominant negative m-calpain was amplified from a plasmid vector containing the dominant negative m-calpain gene (*24*) by PCR using Ex-Taq polymerase (Takara Bio Inc., Shiga, Japan) and the primers 5′-AGTCAAGCTTCCTGCGGGCATCGCGGC-3′ and 5′-AGCACTGTCGACTGTTAGTTATAACTTCAAAGTAC-3′. PCR product was treated with *Sal*I and *Hin*dIII, ligated into EGFP-C3 vector (Invitrogen), and amplified in *E. coli*. DH5α. The plasmid vector was purified and transfected into HT-1080 fibrosarcoma using Fugene 6 transfection reagent (Roche) in RPMI1640 medium containing 8% fetal calf serum. After 24 h, medium was changed to G418 sulfate (1 mg/ml) containing RPMI1640 complete medium, and cells stably expressing mCL-DN were collected. For transient expression of dominant negative m-calpain EGFP (DN-mCL-E) to human foreskin fibroblasts, Lipofectamin 2000 was used as recommended by the manufacturer. Briefly, fibroblasts (2 × 10^5 cells in 1.5 ml of medium) were cultured for 6–18 h in a 35-mm culture dish, and medium was replaced with OptiMEM serum-free medium (Gibco) before transfection. Plasmid DNA (0.5 μg) was diluted with OptiMEM (0.25 ml), mixed with Lipofectamin 2000 (3 μl diluted with 0.25 ml of OptiMEM) and incubated for 30 min. The mixture was added to cells and incubated for 20 h, and transfectants were analyzed by F-actin staining or immunoblotting. F-actin staining was performed as previously described by Sato *et al.* (*18*).

Plasmid vector containing calpastatin cDNA was generously donated by Dr. Masatoshi Maki of Nagoya University (GenBank acc. No. D16217). The full-length pcDNA-I-hemagglutinin–tagged human calpastatin cDNA was cloned into the expression vector PMKIT-neo(+) (a gift from Dr. K. Maruyama, Tokyo Medical and Dental University, Tokyo, Japan). The plasmid DNA was transfected into HT-1080 fibrosarcoma using Fugene 6 transfection reagent, positive clones were selected with G418 sulfate (1 mg/ml), and the stable clone CS14 was maintained in RPMI1640 complete medium containing 0.5 mg/ml G418 sulfate.

Cell Fractionation and Immunoblotting—Cells were cultured on monomeric or fibrillar collagen gel for indicated times, washed once with PBS, lysed for 15 min in RIPA buffer (1% NP-40, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM NaF, 0.5 mM PMSF, and 20 μM leupeptin) at room temperature, and centrifuged at 15,000 rpm for 10 min. Soluble and residue fractions were then separated. The soluble fraction (cytosol and membrane fraction) and the residue (cell nuclear and cytoskeletal fraction) were dissolved in SDS sample buffer of the same volume with cell lysates.

SDS-PAGE and Immunoblotting Were Performed as Previously Described by Sato *et al.* (*18*)

RESULTS AND DISCUSSION

Selective Degradation of Fodrin and Talin in Fibroblasts Adhering to Fibrillar Collagen—On collagen gel, human foreskin fibroblasts exhibited a specific morphology resembling dendritic cells, and contacted the collagen gel surface via integrin α2β1, as described in previous reports (*1*, *18*). The localization and levels of membrane skeletal proteins were examined by immunoblot analysis (Fig. 1). A network composed of fodrin, a membrane skel-

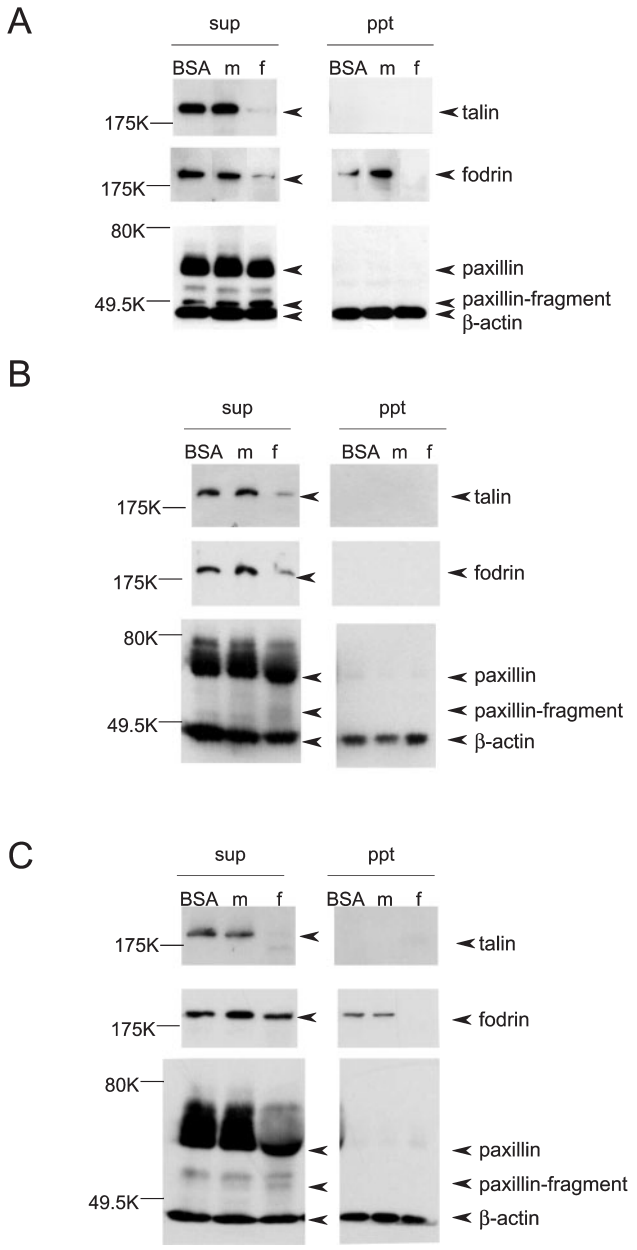

Fig. 1. **Translocation and degradation of membrane skeletal proteins in cells upon adherence to monomeric collagen and fibrillar collagen I gel.** Human foreskin fibroblasts (A), human umbilical vein endothelial cells (B), and human aortic smooth muscle cells (C) were plated onto dishes coated with albumin (BSA), monomeric collagen (m) or fibrillar collagen gel (f), and cultured for 90 min. Cells were lysed with RIPA buffer containing NP-40, harvested, and separated by centrifugation into supernatants (sup) and pellets (ppt). Both fractions were analyzed by SDS-PAGE on 7.5% gel, and talin (upper panel), fodrin (middle panel), paxillin and β-actin (lower panel) were detected by immunoblot analysis. Arrowheads indicate the positions of each protein at right.

etal protein, was detected in the cytoskeletal fraction (ppt; lysed with RIPA buffer containing 1% NP-40) of cells cultured for 90 min on monomeric collagen (Fig. 1, A–C, lane m). Free fodrin was detected in the cytosol fraction (sup), but not the cytoskeletal fraction, of cells adhering to control BSA-coated dishes (Fig. 1, A–C, lanes BSA). In neonatal normal skin fibroblasts, aortic smooth muscle cells and human umbilical vein endothelial cells, fodrin disappeared from both the cytoskeletal and cytosol fractions after adherence to fibrillar collagen gel (Fig. 1, A–C, lane f). The integrin-associated cytoskeletal proteins talin and paxillin were detected only in the NP-40–soluble fraction, and talin specifically decreased in cells that adhered to fibrillar collagen gel. There was a small decrease in the level of paxillin in cells that adhered to fibrillar collagen gel (0.2 mg/ml), and β-actin was used as an internal protein standard because the level of β-actin did not differ between the soluble and insoluble fraction (Fig. 1, A–C, lower panel).

Next, we examined the changes with time in levels of adhesion proteins. Talin was degraded much faster than fodrin. Fodrin decreased time-dependently over a period of 15 min after attachment to fibrillar collagen gel, and talin disappeared within 3 min after attachment to fibrillar collagen gel (Fig. 2A). We examined the involvement of the Ca^{2+}-dependent cysteine protease calpain in decreases of these proteins, because fodrin and talin are substrates of calpain. Immunoblot analysis was performed using rabbit pAbs against calpain-generated neoepitopes of the 140-kDa fodrin fragment and the 47-kDa talin head domain. The 140-kDa fodrin fragment was detected from 3 to 15 min after adherence, correlating with the disappearance of fodrin from cells that adhered to fibrillar collagen gel (Fig. 2A). The 140-kDa fodrin fragment was detected in the NP-40–soluble fraction, whereas the talin 47-kDa fragment was detected in the insoluble fraction (Fig. 2A). Previous reports also indicate that these talin fragments localize differently in activated platelets (*20*). The present results suggest that the decrease in levels of these proteins is the result of degradation, and that calpain degrades fodrin after cell adhesion to fibrillar collagen gel.

Next, we examined the effect of collagen concentration in the gel on protein degradation. The cytoskeletal proteins fodrin, α-actinin and ezrin were degraded in a collagen concentration–dependent manner in cells adhering to collagen gel at low concentration (0.1–0.5 mg/ml) and at high concentration (1 mg/ml). Talin was completely degraded in cells adhering to any concentration of collagen gel (Fig. 2B). This suggests that these cells regulate their amounts of cytoskeletal proteins based on circumstantial matrix concentration or rigidity.

Loss of talin may cause loss of ability to assemble focal adhesions (*25*). Various types of focal adhesions have recently been reported, including focal adhesions (usually observed in cells adhering to collagen or fibronectin-coated dishes), fibrillar adhesions (thin and long), and 3-D adhesions (*26*). Talin is an important connecting molecule between actin and the integrin α2β1 cytoplasmic tail, and it is thought that degradation of talin causes switching to different adhesion modes mediated by the 47-kDa talin fragment (*27*) or loss of the ability to assemble focal adhesions (*25*). m-Calpain has been observed at focal adhesion plaques in several cell types (*28*). It is unclear whether the loss of talin is sufficient to cause loss of focal adhesions and whether m-calpain binds to such adhesion complexes in skin fibroblasts adhering to fibrillar collagen gel.

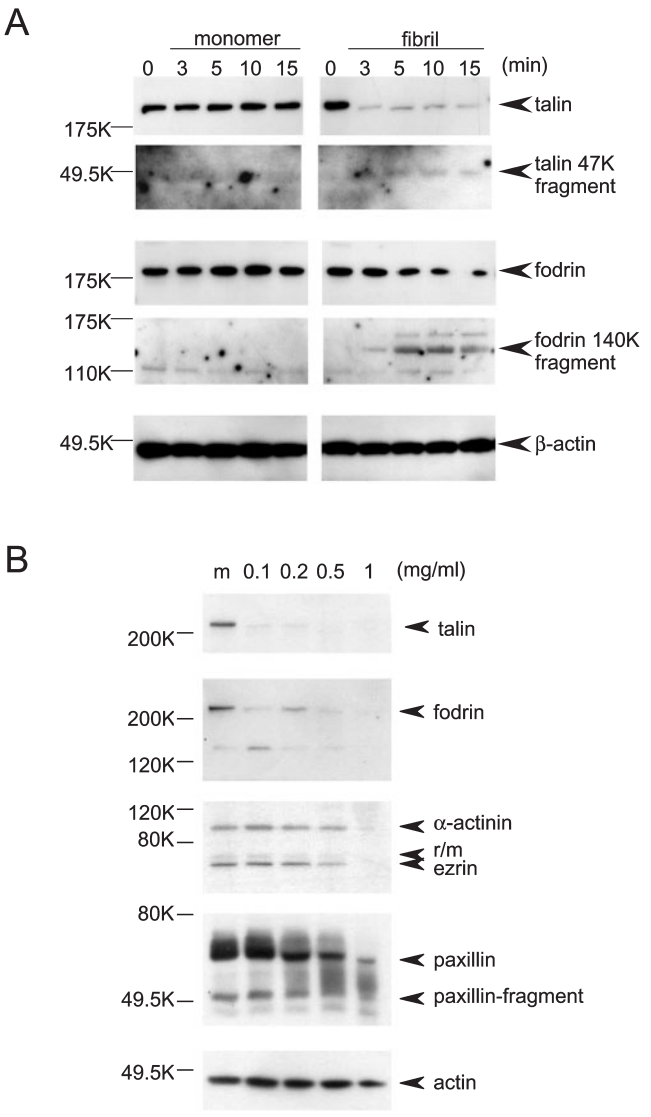

Fig. 2. **Time-dependent and collagen density-dependent degradation of membrane skeletal proteins in fibroblasts upon adherence to fibrillar collagen I gel.** (A) Human foreskin fibroblasts were plated on monomeric collagen (monomer) or fibrillar collagen gel (fibril), cultured for the indicated times, lysed with RIPA buffer containing NP-40, harvested, and separated by centrifugation into supernatants (sup) and pellets (ppt). Cell supernatants were subjected to SDS-PAGE, and talin, fodrin, fodrin 140-kDa fragment and β-actin were immunoblotted using appropriate antibodies, and cell pellets were used for detection of talin 47-kDa fragment. Arrowheads indicate the positions of each protein at right. (B) Collagen I was coated in monomeric form (m), or fibrillar gels were formed at indicated concentration (0.1 to 1 mg/ml). Cells were plated, cultured for 30 min, and removed with SDS sample buffer. The cytoskeletal proteins talin, fodrin, α-actinin, ezrin, paxillin and β-actin were detected by immunoblot analysis. The upper band of ezrin was expected to contain the homologous protein radixin.

Fodrin is a mesh protein that lines cytoplasmic membranes and stabilizes cellular organelles (*7*). The peripheral ruffle membrane is regulated by the WAVE2 pathway, and invasion by cell protrusions is regulated by both WAVE1 and WAVE2 (*29*). Fodrin is a membrane ruffle–localized protein, and disappearance of fodrin may cause switching from membrane ruffling (WAVE2 regulated) to invasion by cell protrusions (WAVE1/2-regulated).

Degraded collagen induces proteolysis of focal adhesion kinase (FAK) in smooth muscle cells (*30*), but degradation of FAK was not observed in the present cell culture system, suggesting a different mechanism of selection of proteolytic targets (*18*, data not shown).

Degradation of Fodrin Was Inhibited by Calpain Inhibitor—To identify the protease responsible for degradation of talin and fodrin, a protease inhibition experiment was performed using LLal (a calpain-active-site inhibitor), Lactacystin (a proteasome inhibitor), and DEVDal (a caspase3 inhibitor) in cultured fibroblasts adhering to 0.2 mg/ml fibrillar collagen gel. This concentration of collagen induced degradation of talin and fodrin, but degradation of ezrin and actinin was not observed in skin fibroblasts (Fig. 2); α-actinin and ezrin were therefore used as protein standards (Fig. 3A, left). Talin degradation was not affected by the three protease inhibitors. The inhibition of talin degradation by LLal was also not observed in NIH3T3 cells (data not shown). Fodrin degradation was significantly inhibited by LLal (200 μM), but not by Lactacystin or DEVDal (Fig. 3A). The effect of LLal (20 μM) was sufficient to abolish the activity of μ-calpain (Fig. 3C).

Caspase 3 has a proteolytic effect on fodrin (*31*), but the active form (20 kDa) of caspase 3 was not detected in cells adhering to fibrillar collagen gel, and the inhibitor DEVDal had no effect on fodrin degradation (data not shown). These results suggest that caspase3 is not involved in degradation of fodrin.

Other proteases that may degrade talin including cathepsins, and thus the effect of a cathepsin B inhibitor, CA074-Me, was examined. This inhibitor did not inhibit the talin degradation in cells adhering to fibrillar collagen (data not shown), suggesting that the cathepsin B is not involved in talin degradation. These results suggest that fodrin is degraded by calpain, but the protease responsible for degradation of talin was not identified in this experiment.

Calpain Activation in Cells Adhering to Monomeric or Fibrillar Collagen I—We examined calpain activation in cells adhering to monomeric or fibrillar collagen I. To detect calpain activation, two methods were tested: (*1*) detection of the autolytically cleaved N-terminus of μ- or m-calpain by immunobloting; (*2*) translocation of calpain to cell membranes (*9–10*, *32*). Activation of μ-calpain was detected by immunobloting using anti–μ-calpain N-terminal neo-epitope pAb (Fig. 3, B and C). We also performed this assay using anti–m-calpain N-terminal neo-epitope pAb, but no significant signal was detected (data not shown), and the N-terminal amino acid sequence analysis of purified m-calpain from the cell cultured on collagen-gel produced no apparent signal. These results mean that the N-terminal of m-calpain is blocked and no autolysis occurred. Therefore, we assayed translocation of m-calpain to NP-40–insoluble fractions, which is reportedly a sign of calpain activation (*32*).

Active μ-calpain was detected in cells adhering to monomeric or fibrillar collagen after 20 min of culture; monomeric and fibrillar collagens showed comparable levels of activation (Fig. 3B). The translocation rate of active μ-calpain was 28.6% (on monomer) and 22.7% (on collagen

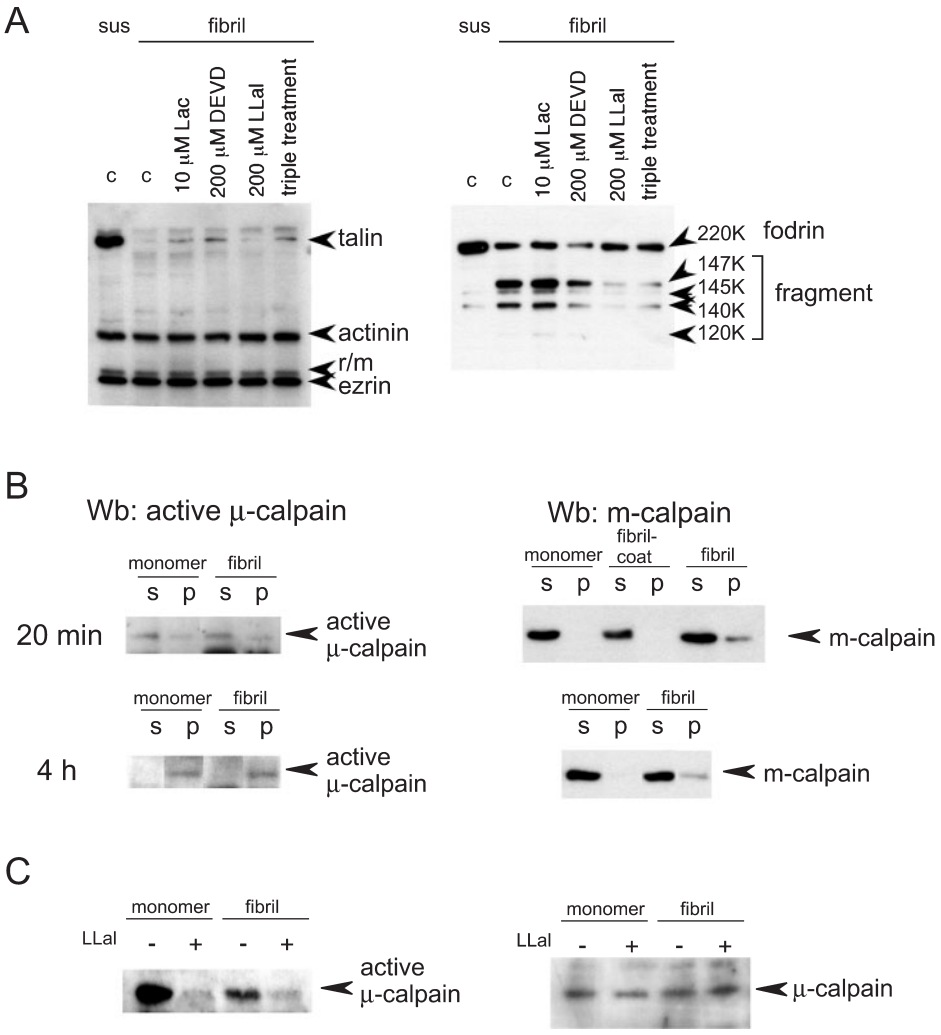

Fig. 3. **Calpain activation in cells adhering to collagen I and inhibition of fodrin degradation by LLal.** (A) Effects of LLal, Lactacystin and DEVDal on degradation of talin and fodrin were examined. Dispersed fibroblasts were incubated with or without (c) 200 μM LLal, 10 μM Lactacystin, 200 μM DEVDal or a mixture of these three inhibitors for 30 min, plated on fibrillar collagen I gel (f) (0.2 mg/ml), and cultured for 20 min. Cell lysates extracted with SDS sample buffer were subjected to SDS-PAGE, and blotted proteins were stained with anti-talin, anti-α-actinin, anti-ezrin pAb (left panel) or anti-fodrin mAb (right panel). (B) Dispersed fibroblasts were plated on monomeric collagen I (m), fibrillar collagen I gel (f) or coated fibrils (fibril-coat, see "MATERIALS AND METHODS"), and cultured for indicated times. Cell lysates (s) and pellets (p) separated by RIPA buffer were subjected to SDS-PAGE (7.5%), and active μ-calpain and m-calpain (1D4E8) was immunoblotted as in B. The membrane-translocated autolytic μ-calpain at 20 min in cells adhering to monomeric collagen and fibrillar collagen gel is 28.6% and 22.7%, respectively. Also, the proportions of membrane-translocated m-calpain in cells adhering to fibrillar collagen gel at 20 min and 4 h were 22.7% and 16.5%, respectively, which were detected by immunoblotting and calculated from the relative intensity of each band using NIH Image 1.62f. (C) Dispersed fibroblasts were incubated with or without 20 μM LLal for 30 min, plated on monomeric collagen I (m) or fibrillar collagen I gel (f), and cultured for 20 min. Cell lysates extracted with RIPA buffer were subjected to SDS-PAGE, and blotted proteins were stained with anti-active form of μ-calpain pAb (left panel) or anti–μ-calpain mAb (right panel).

gel) at 20 min, and increased to almost 100% at 4 h, in cells adhering to monomeric or fibrillar collagens by using anti–μ-calpain N-terminal neo-epitope pAb. These results indicate that the activation of μ-calpain involves autolysis of the N-terminus before membrane translocation. In contrast, m-calpain translocated to the NP-40-insoluble fraction (cytoskeletal/nuclear fraction) at 20 min to 4 h in cells adhering to fibrillar collagen, the rate being 22.7% and 16.5 %, respectively, but m-calpain did not translocate in cells adhering to monomers during 20 min to 4 h. This suggests that m-calpain was activated in cells adhering to fibrillar collagen I, but not in cells adhering to monomeric collagen. These findings suggest that m-calpain is rapidly activated and cleaves substrates such as fodrin and ezrin in the early phase of adhesion to fibrillar collagen (3–15 min). The μ-calpain also appeared to be active in the early phase in cells adhering to monomeric collagen as shown in Fig. 2. However, fodrin was not degraded. The reason why substrates were not degraded is not clear at present, but a possible explanation is that a conformational change of substrates is needed in addition to the activation of calpain to degrade the substrate in the cell.

Wang *et al.*, using cells including MDCK, TCCSUP, HeLa, 293, and NIH 3T3, found that talin degradation occurred in three-dimensional collagen gel culture but not in fibril-coated culture dishes (*1*). We assayed activation of m-calpain and found that it was not activated in cells attached to fibrillar collagen-coated dishes (Fig. 3B, lane fibril-coat). We did not observe degradation of fodrin or talin in fibroblasts adhering to fibrillar collagen–coated dishes (data not shown), as reported by Wang *et al.* (*1*). This suggests that the three-dimensional fibrillar collagen gel, but not coated fibrils, can activate m-calpain and fodrin/talin degradation.

Dominant Negative m-Calpain Inhibits Fodrin Degradation—Because specific m-calpain activation was observed in fibroblasts adhering to fibrillar collagen gel (Fig. 3), we examined whether m-calpain is responsible for degradation of fodrin, using the dominant negative form of m-calpain (DN-mCL), which is conjugated to EGFP at the N-terminus of m-calpain (DN-mCL-EGFP).

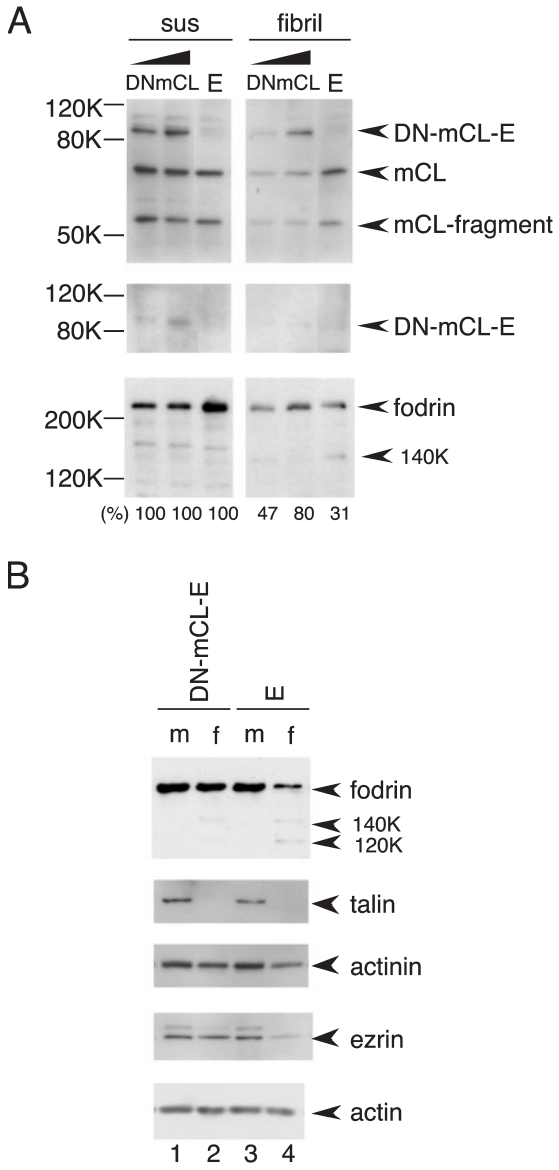

Fig. 4. **Dominant negative m-calpain-EGFP inhibits fodrin degradation.** (A) Transfectants were dispersed by tripsinization and lysed with SDS sample buffer or plated onto fibril collagen gel (0.2 mg/ml) for 30 min before lysing. The expression level of dominant negative–m-calpain-EGFP (DN-mCL-E) or EGFP (E) transfected into HT-1080 fibrosarcoma was assessed by immunoblot using anti-EGFP or m-calpain (1D4E8) antibodies. Fodrin immunoblot is shown in the lower panel, and remaining fodrin is shown in the bottom panel (%). Each appropriate band is indicated at right. (B) Inhibition of degradation of talin, fodrin, α-actinin and ezrin in DN-mCL-EGFP- or EGFP-transfected cells was examined. Transfectants were plated on monomeric collagen I (m) or fibrillar collagen I gel (f) and cultured for 30 min. The upper band of ezrin was expected to contain the homologous protein radixin. Each appropriate band is indicated at right.

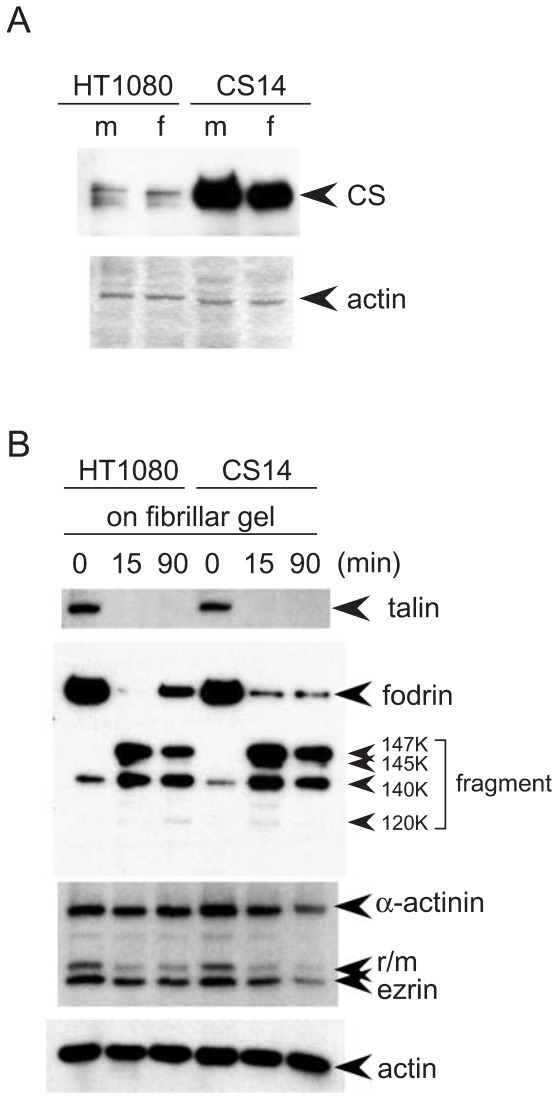

Fig. 5. **Calpastatin does not inhibit fodrin degradation.** (A) Parental HT-1080 cells and the stable clones CS14 were cultured on monomeric collagen or fibrillar collagen gel (0.2 mg/ml) for 90 min before lysing with SDS sample buffer. The whole-cell lysates were immunoblottted with antibodies against calpastatin, or the acrylamide gel was stained with Coomassie Brilliant Blue and the actin band (40 kDa) is indicated at right. (B) Parental HT-1080 cells and the stable clones CS14 were cultured on fibrillar collagen gel for 0, 15, or 90 min before lysing with SDS sample buffer. The whole cell lysates were immunoblottted with antibodies against fodrin, talin, α-actinin, ezrin and β-actin. Each appropriate band is indicated at right.

As in the case of skin fibroblast, degradation of talin, fodrin, α-actinin and ezrin was also detected in HT-1080 cells adhering to fibrillar collagen-gel (Figs. 4 and 5). DN-mCL-EGFP or control EGFP was transfected into HT-1080 fibrosarcoma cells, and stable clones were obtained by neomycin selection. The transfectants expressed endogenous m-calpain, with low (1:0.7) and high (1:1.2) expression of DN-mCL-EGFP (Fig. 4A, upper), which were detected with anti-m-calpain mAb and calculated from the relative intensity of each band using NIH Image 1.62f. DN-mCL-EGFP was also detected with anti-EGFP pAb at identical sites to those detected with anti-m-calpain mAb, and this suggests that the construction and expression of DN-mCL-EGFP gene was successful (Fig. 4A, middle). When the transfectants were attached to fibrillar collagen gel, degradation of fodrin was inhibited by DN-mCL-EGFP in dose-dependently, 31% of undegraded was increased to 47% and 80% by the low (1:0.7) and high (1:1.2) expression of DN-mCL-EGFP,

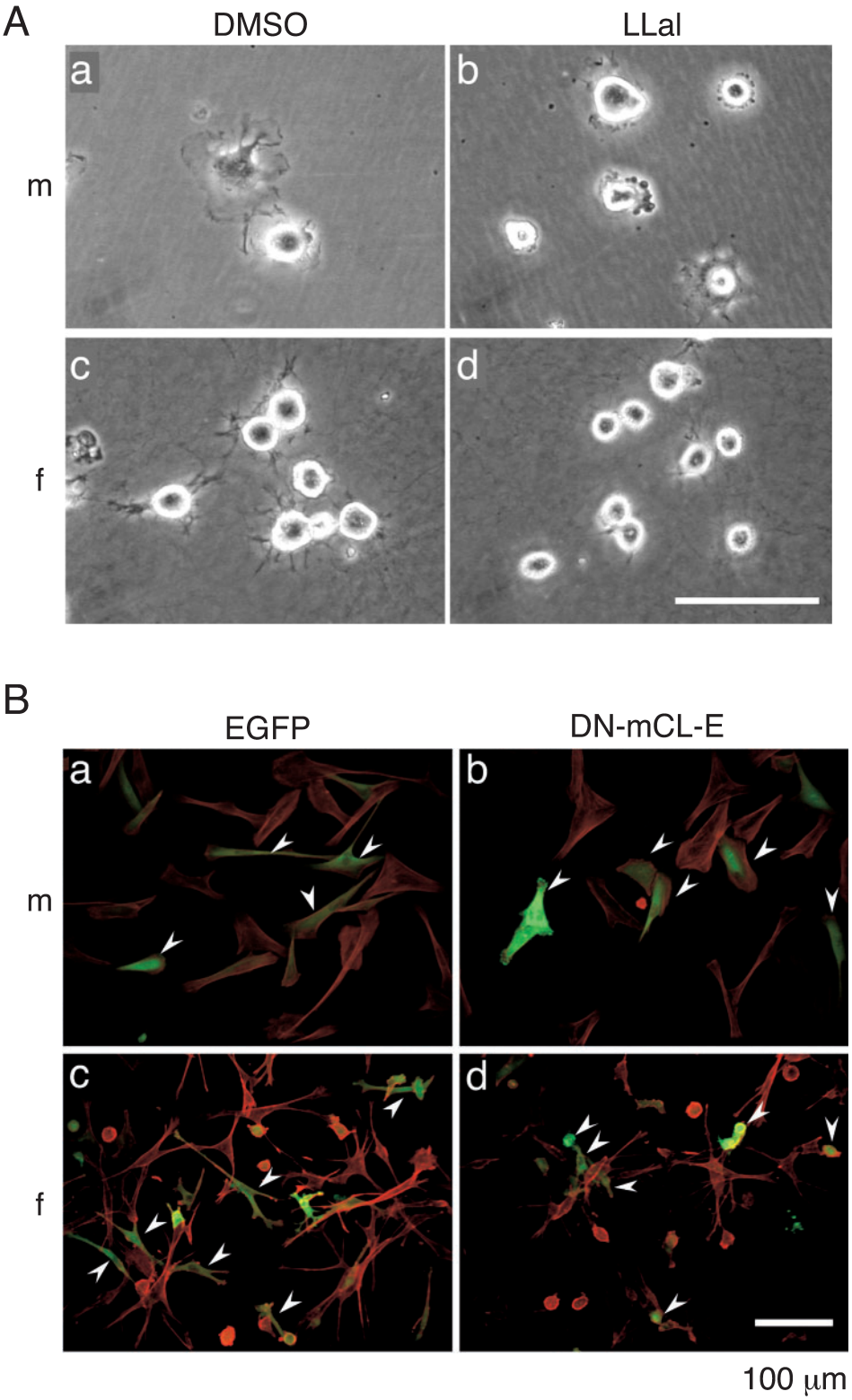

Fig. 6. **Calpain inhibitor LLal and DN-m-calpain inhibit cell spike formation.** (A) LLal inhibits human skin fibroblasts adhering to fibrillar collagen gel but not to monomeric collagen. Skin fibroblasts were pretreated with 200 μM LLal (b, d) or 0.1% DMSO (a, c) for 30 min, cultured for 30 min on monomeric collagen (a, b) or fibrillar collagen I gel (c, d), and photographed by phase-contrast microscopy. Bar, 100 μm. (B) The expression of DN-mCL-EGFP, but not EGFP inhibits cell spike formation. Skin fibroblasts were transiently transfected with DN-mCL-EGFP (b, d) or EGFP (a, c), and cultured for 3 h on monomeric collagen (a, b) or fibrillar collagen I gel (c, d) before rhodamin-conjugated phalloidin staining. Images of DN-mCL-EGFP (green), EGFP (green) and F-actin (red) were observed by immunofluorescense microscopy. DN-mCL-EGFP- or EGFP-expressing cells are indicated by white arrowheads. Bar, 100 μm.

respectively (Fig. 4A, lower). Also, the production of the fodrin 140-kD fragment was strongly decreased by DN-mCL-EGFP (Fig. 4A, lower and 4B, upper). Levels of both DN-mCL-EGFP and endogenous m-calpain were decreased in DN-mCL-EGFP–transfected HT-1080 cells after adhesion to fibrillar collagen gel (Fig. 4A, upper and middle). Presumably, DN-mCL-EGFP was autolysed by endogenous m-calpain. Degradation of fodrin and ezrin was strongly inhibited by DN-mCL-EGFP, and degradation of actinin was significantly inhibited, but degradation of talin was not inhibited (Fig. 4B). Talin is the protein degraded most readily by m-calpain *in vitro* (*28*) and probably *in vivo*. Inhibition of degradation of talin may require a higher level of DN-mCL expression or DN-

μ-calpain expression. This inhibition preference of substrates is consistent with the results of the pharmacological inhibition assay using LLal (Fig. 3A). These results suggest that m-calpain cleaves the fodrin, ezrin, α-actinin but not talin in cells adhering to fibrillar collagen gel.

Calpastatin Did Not Inhibit Degradation of Fodrin, Talin, Actinin and Ezrin—We also transfected plasmid DNA containing the hemagglutinin-tagged full-length human calpastatin gene into HT-1080 cells. Calpastatin is an endogenous inhibitor of μ- and m-calpain. The result was quite different from what we had expected. Immunoblot analysis showed no inhibition of degradation of fodrin, α-actinin and ezrin (Fig. 5B), whereas persistently high levels of calpastatin (greater than 5-fold) were expressed and remained in CS14 transfectant (Fig. 5A). The relative expression level of calpastatin in CS14 adhering to fibrillar collagen gel was retained 76.0 ± 4.6% comparing to that of calpastatin in CS14 adhering to monomeric collagen (Fig. 5A). Endogenous calpastatin was not degraded in HT-1080 parental cells (Fig. 5A), and a similar result was obtained in human skin fibroblasts (data not shown). These results suggest that calpastatin does not inhibit fodrin/talin degradation in cells adhering to fibrillar collagen gel. The reason is not clear, but it is related to the separate localizations of active–m-calpain and calpastatin.

Effect of Calpain Inhibitor and Dominant Negative m-Calpain on Cell-Spike Formation—Human foreskin fibroblasts formed cell spikes from the cell body adhering to fibrillar collagen gel (*18*), and it is an important question whether inhibition of fodrin/talin degradation affects the cell morphological change including F-actin formation by LLal or expression of DN-m-calpain. Human skin fibroblasts pretreated with LLal formed slightly diminished lamellipodia on monomeric collagen-coated dishes (Fig. 6A, b) compared to non-treated fibroblasts (Fig. 6A, a). Cell-spike formation in cells adhering to fibrillar collagen gel was abolished by the presence of LLal (Fig. 6A, d). For transfection of DN-m-calpain-EGFP, we used Lipofectamin 2000 for skin fibroblasts, and their transfection efficiency was 24% in 20 h. Similar to LLal-treated cells, DN-m-calpain-EGFP transfected fibroblasts could spread on monomeric collagen-coated dishes (Fig. 6B, b), as could control EGFP-transfected fibroblasts (Fig. 6B, a), but they did not form cell spikes when adhering to fibrillar collagen gel (Fig. 6B, d), unlike control EGFP-transfected fibroblasts (Fig. 6B, c). These results strongly suggested that m-calpain activity is essential to form cell spikes in three-dimensional collagen gel culture but not in collagen-coated dish culture. The spike formation is induced perhaps *via* degradation of fodrin and Rho GTPase family activation of F-actin/microtubule rearrangements.

Activation of m-calpain by extracellular-signal-regulated kinase (ERK) and inactivation by protein kinase A have recently been reported (*33*, *34*). If so, ERK activation must be observed and the ERK inhibitor must block the calpain cascades. However, ERK1/2 were activated transiently in the adherent fibroblasts at around 30 min of culture, and early transient ERK activation (within 1–3 min) was not observed (data not shown). Also, MEK inhibitors U0126 and PD98059, which inhibit ERK1/2 phosphorylation, had no inhibitory activity on degradation of protein such as talin, actinin and ezrin, or on m-calpain translocation, which is a hallmark of activation (data not shown). These results suggest that the phosphorylation of calpain by ERK is not involved in this cell system.

In conclusion, cytoskeletal protein degradation in cells cultured on collagen gel is apparently not limited to specific cell lines, but rather is a phenomenon common to various cell types. Such degradation may be essential for cell functions such as cell-spike formation and invasion. The present findings indicate the following: (*1*) fodrin, α-actinin and ezrin are degraded in cells attached to fibrillar collagen gel; (*2*) the calpains are responsible for degradation of these adhesion-related proteins; (*3*) m-calpain is responsible for degradation of fodrin, α-actinin and ezrin, but not talin; (*4*) calpastatin does not interfere with the degradation; (*5*) loss of m-calpain activity causes inhibition of cell-spike formation. Clarification of calpain function is important for understanding the essential processes in formation of cell spikes and invasion into the collagen lattice, which involves much signaling between integrin and Rho family proteins on the cell membrane. The hypothesis that degradation of adhesion proteins by calpains is necessary for cell-spike formation and invasion into three-dimensional matrix apparently involves the degradation of intracellular proteins by m-calpain, in addition the previously indicated function on rear detachment during cell migration (*9*).

We are grateful to Dr. Masatoshi Maki of Graduate School of Bioagricultural Sciences, Nagoya University for donating the plasmid vector containing hemagglutinin-tagged full-length human calpastatin cDNA.

REFERENCES

1. Wang, Y.K., Wang, Y.H., Wang, C.Z., Sung, J.M., Chiu, W.T., Lin, S.H., Chang, Y.H., and Tang, M.J. (2003) Rigidity of collagen fibrils controls collagen gel-induced down-regulation of focal adhesion complex proteins mediated by α2β1 integrin. *J. Biol. Chem.* **278**, 21886–21892
2. Heino, J. (2000) The collagen receptor integrins have distinct ligand recognition and signaling functions. *Matrix Biol.* **19**, 319–323
3. Langholz, O., Rockel, D., Mauch, C., Kozlowska, E., Bank, I., Krieg, T., and Eckes, B. (1995) Collagen and collagenase gene expression in three-dimensional collagen lattices are differently regulated by α1β1 and α2β1 integrins. *J. Cell Biol.* **31**, 1903–1915
4. Merilainen, J., Palovuori, R., Sormunen, R., Wasenius, V.-M., and Letho, V.-P. (1993) Binding of the α-fodrin SH3 domain to the leading lamellae of locomoting chicken fibroblasts. *J. Cell Sci.* **105**, 647–654
5. Ishizaki, T., Morishima, Y., Okamoto, M., Furuyashiki, T., Kato, T., and Narumiya, S. (2001) Coordination of microtubules and the actin cytoskeleton by the Rho effector mDia1. *Nat. Cell. Biol.* **3**, 8–14
6. Nakamura, Y., Hashimoto, R., Amano, M., Nagata, K., Matsumoto, N., Goto, H., Fukusho, E., Mori, H., Kashiwagi, Y., Kudo, T., Inagaki, M., and Takeda, M. (2000). Localized phosphorylation of vimentin by Rho-kinase in neuroblastoma N2a cells. *Genes Cells* **5**, 823–837
7. De Matteis, M.A. and Morrow, J.S. (2000) Spectrin tethers and mesh in the biosynthetic pathway. *J. Cell Sci.* **113**, 2331–2343
8. Saido, T.C., Yokota, M., Nagao, S., Yamaura, I., Tani, E., Tsuchiya, T., Suzuki, K., and Kawashima, S. (1993) Spatial resolu-

tion of fodrin proteolysis in postischemic brain. *J. Biol. Chem.* **268**, 25239–25243
9. Glading, A., Lauffenburger, D.A., and Wells, A. (2002) Cutting to the chase: calpain protease in cell motility. *Trends Cell Biol.* **12**, 46–54
10. Sato, K. and Kawashima, S. (2001) Calpain function in modulation of signal transduction molecules. *Biol. Chem.* **382**, 743–751
11. Sorimachi, H. and Suzuki, K. (2001) The structure of calpain. *J. Biochem.* **129**, 653–664
12. Croce, K., Flaumenhaft, R., Rivers, M., Furie, B., Furie, B.C., Herman, I.M., and Potter, D.A. (1999) Inhibition of calpain blocks platelet secretion, aggregation, and spreading. *J. Biol. Chem.* **274**, 36321–36327
13. Dewitt, S. and Hallett, M.B. (2002) Cytosolic free Ca^{2+} changes and calpain activation are required for beta integrin-accelerated phagocytosis by human neutrophils. *J. Cell Biol.* **159**, 181–189
14. Kulkarni, S., Saido, T.C., Suzuki, K., and Fox, J.E. (1999) Calpain mediates integrin-induced signaling at a point upstream of Rho family members. *J. Biol. Chem.* **274**, 21265–21275
15. Bialkowska, K., Kulkarni, S, Du, X., Goll, D.E., Saido, T.C., and Fox, J.E.B. (2000) Evidence that β3 integrin-mediated Rac activation involves the calpain-dependent formation of integrin clusters that are distinct from the focal complexes and focal adhesions that form as Rac and RhoA become active. *J. Cell Biol.* **151**, 685–695
16. Dourdin, N., Bhatt, A.K., Dutt, P., Greer, P.A., Arthur, J.S., Elce, J.S., and Huttenlocher, A. (2001) Reduced cell migration and disruption of the actin cytoskeleton in calpain-deficient embryonic fibroblasts. *J. Biol. Chem.* **276**, 48382–48388
17. Robles, E., Huttenlocher, A., and Gomez, T.M. (2003) Filopodial calcium transients regulate growth cone motility and guidance through local activation of calpain. *Neuron* **38**, 597–609
18. Sato, K., Hattori, S., Irie, S., and Kawashima, S. (2003) Spike formation by fibroblasts adhering to fibrillar collagen I gel. *Cell Struct. Funct.* **28**, 229–241
19. Inomata, M., Hayashi, M., Ohno-Iwashita, Y., Tsubuki, S., Saido, T.C., and Kawashima, S. (1996) Involvement of calpain in integrin-mediated signal transduction. *Arch. Biochem. Biophys.* **328**, 129–134
20. Hayashi, M., Suzuki, H., Kawashima, S., Saido, T.C., and Inomata, M. (1999) The behavior of calpain-generated N- and C-terminal fragments of talin in integrin-mediated signaling pathways. *Arch. Biochem. Biophys.* **371**, 133–141
21. Saido, T.C., Nagao, S., Shiramine, M., Tsukaguchi, M, Sorimachi, H., Murofushi, H., Tsuchiya, T., Ito, H., and Suzuki, K. (1992) Autolytic transition of μ-calpain upon activation as resolved by antibodies distinguishing between the pre- and post-autolysis forms. *J. Biochem.* **111**, 81–86
22. Kasai, Y., Inomata, M., Hayashi, M., Imahori, K., and Kawashima, S. (1986) Isolation and characterization of monoclonal antibodies against calcium-activated neutral protease with low calcium sensitivity. *J. Biochem.* **100**, 183–190
23. Sato, K., Ebihara, T., Adachi, E., Kawashima, S., Hattori, S., and Irie, S. (2000) Possible involvement of aminotelopeptide in self-assembly and thermal stability of collagen I as revealed by its removal with proteases. *J. Biol. Chem.* **275**, 25870–25875
24. Masumoto, H., Yoshizawa, T., Sorimachi, H., Nishino, T., Ishiura, S., and Suzuki, K. (1998) Overexpression, purification, and characterization of human m-calpain and its active site mutant, m-C105S-calpain, using a baculovirus expression system. *J. Biochem.* **124**, 957–961
25. Albiges-Rizo, C., Frachet, P., and Block, M.R. (1995) Down regulation of talin alters cell adhesion and the processing of the α5β1 integrin. *J. Cell Sci.* **108**, 3317–3329
26. Cukierman, E., Pankov, R., Stevens, D.R., and Yamada, K.M. (2001) Taking cell-matrix adhesions to the third dimension. *Science* **294**, 1708–1712
27. Yan, B., Calderwood, D.A., Yaspan, B., and Ginsberg, M.H. (2001) Calpain cleavage promotes talin binding to the β3 integrin cytoplasmic domain. *J. Biol. Chem.* **276**, 28164–28170
28. Beckerle, M.C., Burridge, K., DeMartino, G.N., and Croall, D.E. (1987) Colocalization of calcium-dependent protease II and one of its substrates at sites of cell adhesion. *Cell* **51**, 569–577
29. Suetsugu, S., Yamazaki, D., Kurisu, S., and Takenawa, T. (2003) Differential roles of WAVE1 and WAVE2 in dorsal and peripheral ruffle formation for fibroblast cell migration. *Dev. Cell* **5**, 595–609
30. Carragher, N.O., Levkau, B., Ross, R., and Raines, E.W. (1999) Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of pp125(FAK), paxillin, and talin. *J. Cell Biol.* **147**, 619–630
31. Wang, K.K.W. (2000) Calpain and caspase: can you tell the difference? *Trends Neurosci.*, **23**, 20–26
32. Rock, M.T., Brooks, W.H., and Roszman, T.L. (1997) Calcium-dependent signaling pathways in T cells. Potential role of calpain, protein tyrosine phosphatase 1b, and p130Cas in integrin-mediated signaling events. *J. Biol. Chem.* **272**, 33377–33383
33. Glading, A., Uberall, F., Keyse, S.M., Lauffenburger, D.A., and Wells, A. (2001) Membrane proximal ERK signaling is required for M-calpain activation downstream of epidermal growth factor receptor signaling. *J. Biol. Chem.* **276**, 23341–23348
34. Shiraha, H., Glading. A., Chou. J., Jia, Z., and Wells, A. (2002) Activation of m-calpain (calpain II) by epidermal growth factor is limited by protein kinase A phosphorylation of m-calpain. *Mol. Cell. Biol.* **22**, 2716–2727