Calpain Mediates Integrin-induced Signaling at a Point Upstream of Rho Family Members*

(Received for publication, February 8, 1999, and in revised form, March 31, 1999)

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Integrin-induced adhesion leads to cytoskeletal reorganizations, cell migration, spreading, proliferation, and differentiation. The details of the signaling events that induce these changes in cell behavior are not well understood but they appear to involve activation of Rho family members which activate signaling molecules such as tyrosine kinases, serine/threonine kinases, and lipid kinases. The result is the formation of focal complexes, focal adhesions, and bundles and networks of actin filaments that allow the cell to spread. The present study shows that μ-calpain is active in adherent cells, that it cleaves proteins known to be present in focal complexes and focal adhesions, and that overexpression of μ-calpain increased the cleavage of these proteins, induced an overspread morphology and induced an increased number of stress fibers and focal adhesions. Inhibition of calpain with membrane permeable inhibitors or by expression of a dominant negative form of μ-calpain resulted in an inability of cells to spread or to form focal adhesions, actin filament networks, or stress fibers. Cells expressing constitutively active Rac1 could still form focal complexes and actin filament networks (but not focal adhesions or stress fibers) in the presence of calpain inhibitors; cells expressing constitutively active RhoA could form focal adhesions and stress fibers. Taken together, these data indicate that calpain plays an important role in regulating the formation of focal adhesions and Rac- and Rho-induced cytoskeletal reorganizations and that it does so by acting at sites upstream of both Rac1 and RhoA.

Adhesion of cells on integrin substrates results in cell spreading, migration, differentiation, and proliferation and is essential for events such as inflammation, platelet clot formation, development, and wound healing (1–3). Recent evidence has shown that one of the early events following integrin-ligand interactions is the clustering of integrins into small complexes with signaling molecules (4, 5). The subsequent activation of Cdc42 and Rac1 induces the polymerization of new actin filaments that organize into bundles and submembranous networks, thus, causing the extension of filopodia and lamellipodia (4–8). The dynamic breakdown and formation of new focal complexes in the extending lamellipodia allows the cells to spread. RhoA then becomes activated and induces the formation of larger complexes of integrins, cytoskeletal proteins, and signaling molecules known as focal adhesions. RhoA also induces myosin to interact with actin filaments, causing the filaments to assemble into bundles known as stress fibers that terminate at the focal adhesions and allow tension to be generated on ligand-occupied integrin in the spreading cells (9–12).

By analogy to activation of Ras proteins by growth factor receptors, it is likely that Rho family members are activated by exchange factors that are recruited to sites of ligand-occupied integrin (13–19). However, little is known about the mechanisms inducing recruitment or activation of such factors. The ability of activated Rho family members to induce the formation of focal complexes and adhesions (collectively referred to as focal adhesion complexes) and to reorganize the cytoskeleton presumably results from activation of effector molecules by the Rho proteins. Signaling molecules that have been identified at sites of ligand-occupied integrin or shown to be activated as a consequence of integrin-ligand interactions include tyrosine kinases, serine/threonine kinases, and lipid kinases (1, 2). Several of these have been implicated as playing a role in mediating integrin-induced signaling but the molecular details of the way in which these regulate the formation of focal adhesions or the integrin-induced cytoskeletal reorganizations are, in general, not well understood.

Another signaling molecule that could conceivably be involved in mediating integrin-induced signaling is calpain (20). Calpains are intracellular, non-lysosomal, Ca2+-dependent cysteine proteases that are active at physiological pH (21). They are heterodimers containing a common regulatory subunit of 30 kDa and either of two genetically distinct, catalytic subunits of 80 kDa (22–24). One form of calpain requires micromolar Ca2+ (μ-calpain or calpain I) for half-maximal activation while the other requires millimolar concentrations (μ-calpain or calpain II) (25). Although both forms require higher calcium concentrations for activation than those thought to exist within cells under normal physiological conditions, it is becoming apparent that calpain activation may be regulated during normal signal transduction mechanisms (20, 26). One cell in which calpain has been shown to be activated during normal signaling is the platelet (20, 27, 28). In this cell, its activation occurs as a consequence of engagement of αIIbβ3, the major platelet integrin, by its ligand (20). Direct evidence that calpain is activated in adherent cells is lacking. However, the millimolar form of calpain has been detected in focal adhesions of cultured cells (29) and recent reports in which calpain was inhibited by membrane-permeable calpain inhibitors in CHO1

* This work was supported by Research Grants HL-30657 and HL-56264 (to J. E. B. F.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: CHO, Chinese hamster ovary; BAEC, bovine endothelial cells; Me2SO, dimethyl sulfoxide; TRITC, tetramethyl rhodamine isothiocyanate.
Calpain Regulates Integrin-induced Rho Protein Activation

| 45 min | Plus Calpeptin |
|--------|----------------|
| 1.5 h  |                |
| 6 h    |                |
| 16 h   |                |
| Recovery |             |

FIG. 1. Effect of calpeptin on the morphology of bovine aortic endothelial cells. BAE cells were serum-starved and allowed to spread on fibronectin-coated dishes in serum-free medium in the presence or absence of 70 μg/ml calpeptin. At the indicated times, cells were examined under a light microscope. The calpeptin-containing medium was then replaced with growth medium and allowed to recover for an additional 16 h. Bar, 151 μm.

Materials and Methods

Reagents—The membrane-permeable inhibitors of calpain used in this study were MDL (333) (Chz-Val-Phe-H, a gift from Dr. S. Mehdi, Merrell Dow, Cincinnati, OH), calpeptin (34) (Z-Leu-Nle-H, Novabiochem, San Diego, CA), and inhibitors specific for μ-calpain were Z-Leu-Abu-CONH-CH2-CHOHCH2F6 (compound 2) or m-calpain (Z-Leu-Abu-CONH-CH2-CHOHCH2Ph (compound 1) and Z-Leu-Abu-CONH-(CH2)54-morpholinyl (compound 3) kindly provided by Dr. James Powers, Georgia Institute of Technology, Atlanta, GA) (35, 36). All inhibitors were solubilized in dimethyl sulfoxide (Me2SO). Monoclonal antibodies against actin and vinculin were obtained from Sigma; monoclonal antibodies against talin were from Genosys Biotechnologies (The Woodlands, TX); monoclonal antibodies against phosphotyrosine and protein kinase C type-III were from UBI (Lake Placid, NY); those against α5β1 were from East Acres Biologicals (Southbridge, MA). Polyclonal antibodies against HA epitope were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and monoclonal antibodies were from Roche Molecular Biochemicals (Indianapolis, IN). Monoclonal antibodies against integrin β1-subunit were from Transduction Laboratories (Lexington, KY). Polyclonal antibodies specific for the 80-kDa subunit of μ-calpain were raised and characterized as described previously (37).

Cell Culture—BAE cells (provided by Dr. Paul Dicorleto, Cleveland Clinic Foundation) and human embryonic kidney 293 cells (XbaI site) were maintained in DMEM/F-12 (Dulbecco’s modified Eagle’s medium and Ham’s F-12, 1:1, Biowhittaker) medium with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY) containing penicillin, streptomycin, and glutamine (Life Technologies, Inc.) essentially as described in the manufacturer’s protocol. Briefly, 5 × 105 BAE cells were plated in 100-mm dishes overnight and washed with serum-free medium once before transfection. Transfection was carried out at 37°C in a total volume of 6.8 ml of serum-free medium containing 20 μl of LipofectAMINE and 10 μg of either vector DNA (pcDNA3) or HA-tagged wild-type μ-calpain cDNA per 100-mm dish. After 5 h, transfection media were replaced with growth media containing 10% serum. Cells were allowed to recover for 72 h and split as 1:3 into selection medium containing 200 μg/ml active G418 (Life Technologies, Inc.). After 15 days, clones were removed using cloning cylinders, cultured, and frozen.

Stable transfections were carried out using LipofectAMINE (Life Technologies, Inc.) as described in the manufacturer’s protocol. Briefly, 5 × 105 BAE cells were plated in 100-mm dishes overnight and washed with serum-free medium once before transfection. Transfection was carried out at 37°C in a total volume of 6.8 ml of serum-free medium containing 20 μl of LipofectAMINE and 10 μg of either vector DNA (pcDNA3) or HA-tagged wild-type μ-calpain cDNA per 100-mm dish. After 5 h, transfection media were replaced with growth media containing 10% serum. Cells were allowed to recover for 72 h and split as 1:3 into selection medium containing 200 μg/ml active G418 (Life Technologies, Inc.). After 15 days, clones were removed using cloning cylinders, cultured, and frozen.

Transient transfections with constitutively active and inactive mutants of Rho family members or catalytically inactive μ-calpain were carried out in BAE cells cultured on fibronectin-coated coverslips in 6-well plates. Cells were cultured overnight and transfected with 4 μg of each DNA mixed with LipofectAMINE plus reagent (6 μl of plus reagent and 4 μl of LipofectAMINE) in 1 ml of serum-free medium for 5 h at 37°C.

Immunofluorescence and Microscopy—Cells were cultured on fibronectin-coated coverslips (Becton Dickinson, San Jose, CA), fixed with 1.4% formaldehyde in TBS (Tris-buffered saline, 50 mM Tris-HCl, 0.15 mM NaCl, and 0.1% NaN3) for 10 min, permeabilized with 0.5% Triton X-100 for 5 min, then blocked with TBS containing 4% horse serum. Coverslips were incubated with appropriate dilutions of primary proteins and/or antibodies against actin, vinculin, talin, phosphotyrosine, c-Abl, and H-Ras in primary antibody were diluted 1:1000 and 1:500 for actin or vinculin, respectively, 1:200 for talin, 1:500 for phosphotyrosine, 1:1000 for H-Ras, and 1:200 for c-Abl. After incubation with primary antibody, coverslips were washed 3 times with TBS and incubated for 1 h at room temperature with 4% donkey serum and Texas Red-labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Coverslips were washed, mounted in a solution of 80% glycerol, 10% PBS, and 10% DABCO, and examined using a confocal microscope (Zeiss, Jena, Germany).
antibodies in TBS containing 4% horse serum. Unbound antibodies were removed by extensive washing and samples incubated with biotinylated secondary antibodies (Amersham International, Buckinghamshire, United Kingdom), followed by streptavidin conjugated to fluorescein isothiocyanate (1:500 in TBS). Actin was detected by staining with TRITC (tetramethyl rhodamine isothiocyanate) coupled to phalloidin (Sigma, 0.5 mg/ml).

Control samples were prepared using secondary antibodies alone or irrelevant primary antibodies. Images were collected using either a Zeiss immunofluorescence microscope or a Leica TCS-NT confocal laser-scanning microscope. Laser intensities were adjusted so that the excitation of the fluorochromes did not allow any cross-talk between the channels.

**Analysis of Cell Proteins by Western Blotting**—Cells were cultured on fibronectin-coated dishes (35–100 mm, Beckton Dickinson). Media were removed and cells solubilized in cold RIPA buffer containing Tris-HCl, pH 7.5 (20 mM), NaCl (150 mM), sodium deoxycholate (0.1%), Triton X-100 (1%), SDS (0.1%), and inhibitors phenylmethylsulfonyl fluoride (1 mM), aprotinin (10 μg/ml), leupeptin, (10 μg/ml), EDTA (2 mM), sodium fluoride (50 mM), and Na3VO4 (1 mM). In experiments analyzing extracts of cells overexpressing μ-calpain, calpeptin (75 μg/ml) was included in the preparation of extracts in addition to the above inhibitors. Total protein was estimated using bovine serum albumin as the standard protein in a colorimetric assay (Bio-Rad microassay kit, Hercules, CA). Proteins were denatured by addition of SDS sample buffer and electrophoresed through SDS gels containing 3.5% polyacrylamide in a stacking gel and 7.5% polyacrylamide in a resolving gel.

**RESULTS**

**Experiments to Determine Whether Calpain Inhibitors Affect the Cytoskeletal Organization or the Formation of Adhesion Complexes following Integrin-induced Signaling**—To determine whether calpain is required for the formation of integrin adhesion complexes or the cytoskeletal reorganizations that take place following integrin-induced signaling, cells were plated on fibronectin-coated coverslips in the absence of serum and in the presence or absence of the membrane-permeable inhibitors of calpain, MDL, and calpeptin. In the absence of inhibitors, the number of spread cells gradually increased with...
time reaching 60% or more by 6 h. In contrast, cell spreading was markedly inhibited in the presence of inhibitors (Fig. 1). The optimum concentration of calpeptin required to inhibit spreading was 50–100 μg/ml, that of MDL was 150–250 μM. Cells spread normally following removal of the inhibitors (Fig. 1) indicating that the concentrations of calpain inhibitors that inhibited spreading were not toxic to the cells. Furthermore, following removal of inhibitor, the viability of the cells (as determined by trypan blue exclusion) was approximately 85% for control cells and 70% for cells treated with 150 μM MDL for 16 h (data not shown). Similar inhibitory effects of calpain inhibitors were observed on the spreading of CHO cells and 293 cells (data not shown).

To gain insight into the possibility that calpain is involved in integrin-induced signaling pathways leading to the formation of submembranous actin filament networks and focal complexes at early stages of spreading, serum-starved BAE cells were plated on fibronectin-coated coverslips in serum-free medium in the presence or absence of 70 μg/ml calpeptin. After 6–24 h, cells were fixed and permeabilized. Actin filaments were detected with TRITC-labeled phalloidin, focal adhesions were detected with antibodies against phosphoryrosine or vinculin. Bar, 10 μm.

![Effect of calpeptin on the formation of stress fibers and focal adhesions.](image)

Fig. 3. Effect of calpeptin on the formation of stress fibers and focal adhesions. BAE cells were serum-starved and allowed to spread on fibronectin-coated coverslips in serum-free medium in the presence or absence of 70 μg/ml calpeptin. After 6–24 h, cells were fixed and permeabilized. Actin filaments were detected with TRITC-labeled phalloidin, focal adhesions were detected with antibodies against phosphoryrosine or vinculin. Bar, 10 μm.
respread, reassembled stress fibers, and reformed focal adhesions (bottom 2 panels of Fig. 4).

Identification of μ-Calpain as Being Involved in Integrin-induced Cytoskeletal Reorganizations and Integrin-induced Adhesion Complexes—The experiments described above suggested a requirement of calpain function during the formation of focal complexes, focal adhesions, stress fibers, and actin filament networks. As a more direct approach of determining whether calpain is involved in these integrin-induced events, we performed experiments in which calpain was overexpressed or in which cells were transfected with a dominant negative inhibitor for μ-calpain but was barely affected by two m-calpain selective inhibitors, even though the concentrations used were as much as 10,000 times in excess of the \( K_i \) of the inhibitors.

The use of the selective inhibitors suggested that the micromolar form of calpain is required for the integrin-induced cytoskeletal reorganizations. As one approach to directly test this, cells were transfected with the cDNA for HA-tagged catalytic subunit of μ-calpain or with vector DNA alone and selected for stable transfectants by G418 resistance. The amount of calpain expression was assessed on Western blots with an antibody specific for the catalytic subunit of μ-calpain (Fig. 5). This antibody detected equivalent amounts of μ-calpain in extracts of nontransfected cells and in extracts of cells transfected with vector alone (VT1). Increased expression of μ-calpain was detected in two clones transfected with the cDNA (ST1 and ST4) (Fig. 5). The expression levels were about 2-fold over the control for clone ST1 and 5-fold for clone ST4 as determined by quantitation of three independent gels by NIH image analysis using levels of actin in the same gel as an internal control.

Examination of the shape of transfected cells revealed that cells transfected with vector alone had a regular, cobblestone shaped morphology (Fig. 6, panel a). In contrast, cells overexpressing μ-calpain (clones ST1 and ST4) had a very large, overspread morphology (Fig. 6, panels b and c). Moreover, these

![Fig. 4. Effect of MDL on pre-existing focal adhesions in bovine aortic endothelial cells. BAE cells were allowed to spread on fibronectin-coated coverslips for 16 h. Me₂SO or 150 μM MDL were then added to the medium. After 16 or 24 h, cells were fixed, permeabilized, and stained with TRITC-labeled phalloidin to show the distribution of actin filaments and antibodies against vinculin to show focal adhesions. The bottom two panels show cells that were incubated with MDL for 16 h and then allowed to recover in growth medium for an additional 16 h. Bar, 11 μm.](image)

**Table I**

| Addition                | \( K_i (\mu M) \) | Membrane permeability | Spread cells |
|-------------------------|-------------------|-----------------------|--------------|
|                         | Calpain I         | Calpain II            |              |
| No addition             |                   |                       |              |
| Calpeptin               | 0.052\( ^a \)     | 0.034\( ^a \)         |              |
| Z-Leu-Nle-H             | 1.1\( ^b \)       | 0.015\( ^b \)         | 69\( ^b \)   |
| Compound 1              | 0.050\( ^b \)     | 0.2\( ^b \)           | ND\( ^c \)   |
| Compound 2              | 0.14\( ^b \)      | 0.041\( ^b \)         | 45\( ^b \)   |
| Compound 3              |                   |                       |              |
| Z-Leu-Abu-CONH-CH₂CH(OH)-C₆H₅ |             |                       |              |
| Z-Leu-Abu-CONH-CH₂CH(OH)-C₆H₅ |             |                       |              |
| Z-Leu-Abu-CONH-CH₂CH(OH)-C₆H₅ |             |                       |              |

\( ^a \) Obtained from Li et al. (35, 36).

\( ^b \) Obtained from Ref. 34.

\( ^c \) ND, not determined.

![Fig. 5. Western blot demonstrating μ-calpain in transfected bovine aortic endothelial cells. Nontransfected (NT) cells, clones VT1 (vector transfectant), and ST1 and ST4 (transfectants of μ-calpain) were cultured on fibronectin-coated dishes for 3 days and solubilized in an SDS-containing buffer. Aliquots were electrophoresed through SDS gels, transferred to nitrocellulose, and probed with an antibody specific for μ-calpain (top panel) or an antibody that recognized actin (lower panel). DMSO, Me₂SO.](image)
cells showed reduced [3H]thymidine incorporation suggesting inhibition of growth (data not shown). Examination of the cells at early stages of spreading showed that those expressing \( \mu \)-calpain contained many more focal complexes (as detected by complexes containing phosphotyrosine (A) or \( \alpha_\beta_1 \) (B)). In this figure, samples were not dual labeled, each panel shows an individual field. Bar, 8 \( \mu \)m.

As an additional approach to test the idea that the actions of the calpain inhibitors resulted directly from inhibition of \( \mu \)-calpain, BAE cells were transfected with the cDNA for a dominant negative \( \mu \)-calpain. Attempts to obtain stable transfectants expressing significant levels of the catalytically inactive subunit were unsuccessful. We reasoned that if \( \mu \)-calpain was essential for integrin-induced cell spreading, it would not be possible to obtain such cells, thus, in an alternative approach, cells that had already spread on fibronectin were transiently transfected with the cDNA for the inactive subunit of HA-tagged \( \mu \)-calpain. Transfected cells were identified by staining with anti-HA antibody and the organization of the cytoskeleton was examined by dual-immunofluorescence using TRITC-labeled phalloidin to detect actin stress fibers and antibodies against the fibronectin receptor \( \alpha_\beta_1 \) to detect focal adhesions. Bar, 16 \( \mu \)m.

Calpain Function Is Required Upstream of Rho GTPases—The experiments described so far demonstrate that calpain is required for the formation and maintenance of integrin-induced focal complexes and actin filament networks. Because Rac1 is required for the formation of these structures, we determined whether calpain acts upstream or downstream of Rac1. Moreover, the experiments indicate that calpain is also required for the formation of stress fibers and focal adhesions; because these structures are formed following activation of RhoA, independently of Rac1 activation (4), we also performed experiments to determine whether calpain acts upstream or downstream of RhoA. BAE cells were cultured on fibronectin, transiently transfected with constitutively active HA-tagged Rac1 or RhoA and then treated with the calpain inhibitor,
calpeptin. Transfected cells were identified by staining with anti-HA antibody. Actin filaments were identified by staining with fluorescently labeled phalloidin while focal complexes and adhesions were detected using antibodies against phosphotyrosine or the integrin α5β1. Fig. 10A shows that just like nontransfected cells, cells transfected with constitutively active Rac1 (RacQ61L) lost stress fibers when calpeptin was added. However, the cells remained spread and unlike nontransfected cells they contained submembranous actin filaments (Fig. 10A). Quantitation revealed that 95% of the cells transfected with active Rac1 were spread and contained submembranous actin networks (Table II). The use of antibodies against phosphotyrosine revealed that, the arrowhead shaped focal adhesions present in untreated cells (Fig. 10B) were disassembled when cells expressing constitutively active Rac1 were exposed to calpeptin. However, unlike the nontransfected cells, cells expressing constitutively active Rac1 contained numerous focal complexes, as detected by small punctate phosphotyrosine complexes (Fig. 10B). These results indicate that calpain function is required upstream of Rac1 during cell spreading and that provided active Rac1 is present, actin filament networks and focal complexes can form even if calpain is inhibited.

To determine whether calpain acts upstream or downstream of RhoA, cells were transfected with constitutively active RhoA and treated with calpeptin. In contrast to control cells, cells expressing constitutively active RhoA (RhoQ63L) did not disassemble actin stress fibers when exposed to calpeptin (Fig. 11A and Table II). In fact, in many cells there appeared to be more bundles of stress fibers than in nontransfected cells. Similarly, addition of calpeptin to cells expressing constitutively active RhoA did not appear to induce disassembly of focal adhesions, as detected by the presence of phosphotyrosine in large clusters around the cell periphery (Fig. 11B) or integrin α5β1 in arrow-shaped complexes (Fig. 12). As a control, cells were transfected with an inactive form of RhoA (RhoT19N). As shown in the lower two panels of Fig. 11, A and B, and quantitated in Table II, inactive RhoA did not prevent the inhibitory effects of calpeptin on the spread cells.

**Calpain Cleaves Components of Integrin Adhesion Complexes**—The experiments described so far indicate that calpain plays an important role in mediating the integrin-induced activation of Rac1 and RhoA. We reasoned that one way in which it might do this is by cleaving proteins present in focal complexes. To gain insight into the possibility that µ-calpain is active at these sites, we determined whether any of the known components of focal complexes was cleaved by calpain in adherent cells. One protein that is cleaved by calpain following signaling across αIIbβ3 in platelets (40) and is present at the sites of ligand-occupied integrin is talin. This cytoskeletal protein is present in both focal complexes (Fig. 2) and focal adhesions (data not shown) of BAE cells. Thus, BAE cells were cultured on fibronectin-coated dishes and Western blots of extracts were probed with an antibody against talin. In platelets, calpain cleaves talin into fragments of 200 and 47 kDa (20, 40). As shown in Fig. 13A, the 47-kDa fragment was present in extracts of endothelial cells (*lane 1*). When cells were cultured

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**Fig. 9.** Immunofluorescence images showing the disruption of stress fibers and rounding of cells in bovine aortic endothelial cells transiently transfected with a catalytically inactive form of µ-calpain. BAE cells were grown on fibronectin-coated coverslips for 16 h. Cells were transiently transfected with HA-tagged catalytically inactive µ-calpain. After 16 h, cells were fixed and permeabilized. Cells expressing the inactive form of µ-calpain were detected with anti-HA antibodies and the organization of actin filaments was detected using TRITC-labeled phalloidin. Bar, 8 μm.

**Fig. 10.** Immunofluorescence images showing the effect of constitutively active Rac1 on the organization of actin filaments and integrin adhesion complexes in bovine aortic endothelial cells exposed to calpeptin. BAE cells were grown on fibronectin-coated coverslips for 16 h. Control cells or cells transiently transfected with HA-tagged constitutively active Rac1 were exposed to 70 μg/ml calpeptin. After 16 h, cells were fixed and permeabilized. In A, cells expressing the active form of Rac1 were detected with anti-HA antibodies and the organization of actin filaments was detected using TRITC-labeled phalloidin. In B, cells expressing the active form of Rac1 were detected with anti-HA antibodies and the presence of integrin adhesion complexes was detected with phosphotyrosine antibodies. Arrows indicate transiently transfected cells. Bar, 10 μm.
in the presence of calpain inhibitor MDL, the talin fragment was no longer detectable (lanes 2 and 3) and in extracts of cells allowed to recover, the fragment reappeared (lane 4). Inhibition of calpain resulted in an accumulation of intact talin (lanes 2 and 3). There was no effect on the amount of actin (lower panel), a protein which is not known to be cleaved by calpain. The concentrations of calpeptin and MDL that were optimal in inhibiting the generation of the 47-kDa talin fragment were the same as those that were optimal in inhibiting the spreading of cells.

To determine whether talin was cleaved by μ-calpain, the effects of inhibitors selective for μ-calpain or m-calpain, were examined. Concentrations of μ-calpain inhibitor (128 μM) that were approximately 2,560 times in excess of the Kₚ for μ-calpain and 640 times in excess of the Kₚ for m-calpain completely inhibited the generation of the fragment of talin. In contrast, concentrations of two m-calpain inhibitors (153 and 169 μM for compounds 1 and 3, respectively) that were as much as 10,200 times in excess of the concentration needed to half-maximally inhibit m-calpain in vitro, had little effect on talin cleavage (data not shown). Another indication that the generation of the 47-kDa fragment of talin resulted from the activity of μ-calpain in the adherent cells came from examination of the amount of this fragment in cells overexpressing μ-calpain. As shown in Fig. 13B, the amount of the 47-kDa fragment in clones expressing calpain was greater than that in extracts of cells expressing vector alone. Comparison of Figs. 5 and 13 shows that cells expressing most μ-calpain contained most of the calpain-induced talin fragment.

One of the signaling molecules known to be present in integrin adhesion complexes that is also known to be cleaved by calpain following integrin-induced signaling in platelets (41) is protein kinase C. The known calpain-induced cleavage product has a molecular mass of 50 kDa. As shown in Fig. 14, the 50-kDa fragment was also detected in BAE cells and was present in decreased amounts in MDL-treated cells. Taken together, these data provide direct evidence that μ-calpain is active in BAE cells and that it cleaves cytoskeletal and signaling molecule(s) known to be present in integrin-induced adhesion complexes.

**DISCUSSION**

Movement of cells occurs as integrins in the cell membrane interact with adhesive ligands on a surface and transmit signals that induce cytoskeletal reorganizations. Work on platelets has shown that calpain is one of the signaling molecules that is activated following integrin-ligand interactions (20, 26). Because calpain is present in all cells, is regulated by altered Ca²⁺ concentrations, cleaves cytoskeletal proteins (26, 28, 40, 42–48) and signaling molecules (41, 49, 50), and has been detected in focal adhesions of cultured cells (29), it has been suggested that calpain could play an important role in mediating integrin-induced signal transduction in other cells. In the present study, we (1) provide direct evidence that calpain is active in adherent cultured cells and that it cleaves talin and protein kinase C, two proteins present in integrin signaling complexes in platelets and in adherent cells; (2) show that inhibition of calpain, with inhibitors specific for μ-calpain or by expression of an inactive form of μ-calpain, resulted in disassembly of stress fibers, loss of focal complexes and focal adhesions, and rounding up of the cells; (3) show that overexpression of μ-calpain led to the formation of increased focal adhesions and stress fiber formation and increased cell spreading; (4) show that calpain acts upstream of both Rac1 and RhoA. These findings are consistent with a model in which calpain is an early signaling molecule in inducing integrin-induced spreading and activating upstream of activation of both Rac1 and RhoA.

Based on the finding that it is the m-form of calpain that can be detected in focal adhesions (29), it might be expected that it would be this form that has a role in integrin-induced signaling. A recent study demonstrating decreased lamellipodia extension and mRNA levels for m-calpain in cells overexpressing calpastatin is consistent with a potential involvement of m-calpain in integrin signaling (30). In contrast, a study showing that CHO cells expressing low levels of μ-calpain showed decreased motility indicated an involvement of μ-calpain (31, 32). In the present study, we show that two inhibitors selective for m-calpain had little effect on the generation of talin fragment, formation of focal complexes and adhesions, or morphology of the cells, while an inhibitor that was selective for μ-calpain had marked inhibitory effects. Although we cannot exclude the possibility that the two inhibitors of m-calpain were unable to enter the cells, the similarity in structures of each of the inhibitors makes this unlikely. Furthermore, cells overexpressing μ-calpain showed increased cleavage of talin, increased focal adhesions and stress fibers, and an overspread morphology while cells transiently expressing the inactive protease lost focal adhesions and stress fibers and rounded up. While these findings point to a role of μ-calpain in the early stages of integrin-induced spreading and in maintenance of a spread morphology, they do not exclude the possibility that m-calpain has other functions not detected in this study.

Cell spreading is a very dynamic process. Thus, signaling molecules regulating spreading must include those for both assembling and disassembling integrin adhesion complexes and for constantly reorganizing actin and associated cytoskeletal proteins at specific locations within the cell. One group has shown that inhibition of calpain in CHO cells led to an inhibition of cell migration and a decreased retention of integrin on the matrix as cells migrated (31). These findings were interpreted as evidence that calpain disrupts focal adhesions at the rear of migrating cells. However, the findings in the present study indicate that μ-calpain is involved in the formation of focal complexes and adhesions. Perhaps the decreased retention of integrin on the matrix in the earlier studies (31) occurred because μ-calpain was only partially inhibited, thus formation of focal adhesions and migration were partially inhibited. Another possibility is that μ-calpain has multiple actions, perhaps being involved in the formation of focal com-
FIG. 11. Immunofluorescence images showing the effect of constitutively active RhoA on the organization of actin filaments and integrin adhesion complexes in bovine aortic endothelial cells exposed to calpeptin. BAE cells were grown on fibronectin-coated coverslips for 16 h. Control cells or cells transiently transfected with HA-tagged constitutively active or inactive RhoA were exposed to 70 μg/ml calpeptin. After 16 h, cells were fixed and permeabilized. In A, cells expressing the active or inactive forms of RhoA were detected with anti-HA antibodies and the organization of actin filaments was detected using TRITC-labeled phalloidin. In B, cells expressing the active or inactive forms of RhoA were detected with anti-HA antibodies and the presence of integrin adhesion complexes was detected with phosphotyrosine antibodies. Arrows indicate transiently transfected cells. Bar, 10 μm.
FIG. 12. Immunofluorescence images showing the effect of constitutively active RhoA on focal adhesions in bovine aortic endothelial cells exposed to calpeptin. BAE cells were grown on fibronectin-coated coverslips for 16 h. Control cells or cells transiently transfected with HA-tagged constitutively active RhoA were exposed to 70 μg/ml calpeptin. After 16 h, cells were fixed and permeabilized. Cells expressing the active form of RhoA were detected with anti-HA antibodies and the presence of focal adhesions was detected with antibodies against the fibronectin receptor α5β1. Bar, 8 μm.

FIG. 13. Western blots demonstrating the effect of calpain inhibition or overexpression on talin cleavage in BAE. In A, BAE cells were allowed to spread overnight on fibronectin-coated dishes. MDL was then added to the culture medium (150 μM in Me2SO). Control cells received an equal volume of Me2SO. Cells were solubilized in an SDS-containing buffer, samples electrophoresed through an SDS-containing gel, and proteins transferred to nitrocellulose. The Western blot was probed with a monoclonal antibody that recognize intact talin and the 47-kDa calpain-induced hydrolytic fragment of talin (top panel). The blot was reprobed with actin antibodies (lower panel). Lane 1, control cell extracts incubated with Me2SO for 16 h; lane 2, extracts of cells in the presence of MDL for 4 h; lane 3, extracts of cells in the presence of MDL for 16 h; lane 4, extracts of cells treated with MDL for 16 h and then allowed to recover for 16 h. In B, Clones VT1 (vector transfectant) (lane 1), ST1 and ST4 (transfectants of μ-calpain) (lanes 2 and 3, respectively) were cultured on fibronectin-coated dishes for 3 days and solubilized in an SDS-containing buffer. Samples were electrophoresed through SDS gels, transferred to nitrocellulose, and probed with the monoclonal antibody against talin (top panel) or an antibody that recognized actin (lower panel).

FIG. 14. Western blot demonstrating protein kinase C cleavage and its inhibition by MDL in adherent bovine endothelial cells. BAE cells were allowed to spread overnight on fibronectin-coated dishes. MDL was then added to the culture medium (150 μM in Me2SO). Control cells received an equal volume of Me2SO. Cells were solubilized in an SDS-containing buffer, samples electrophoresed through an SDS-containing gel, and proteins transferred to nitrocellulose. The Western blot was probed with an antibody that recognizes protein kinase C type-III and its 50-kDa hydrolytic product. Lane 1, control cell extracts incubated with Me2SO for 16 h; lane 2, extracts of cells in the presence of MDL for 4 h; lane 3, extracts of cells in the presence of MDL for 16 h. PKC, protein kinase C.

complexes and adhesions and also playing a role in the subsequent detachment of focal adhesions from the extracellular matrix in migrating cells. Activation of signaling molecules in focal adhesions regulates anchorage-dependent cell growth (51). In the present study, μ-calpain transfected cells were larger and divided more slowly than normal. While this may be an indirect consequence of the formation of increased numbers of focal adhesions, it could also result from additional unidentified actions of calpain within the focal adhesions. Further studies will be needed to investigate these possibilities.

Early steps in regulating integrin-induced cell spreading are activation of Rac1 and RhoA (4, 5). In the present study, expression of the constitutively active forms of both Rac1 and RhoA enabled the cells to assemble integrin adhesion complexes and cytoskeletal structures in the presence of calpain inhibitors suggesting that critical sites of action of calpain in terms of inducing the changes that allow normal cell spreading are upstream of Rac1 and RhoA activation. It is of interest to speculate how calpain activation could lead to activation of Rho family proteins. Integrin engagement appears to induce activation of Rac1 and RhoA by two independent mechanisms (4, 5). Steps implicated in activation of these proteins following signaling through other receptors include Pleckstrin homology domain or phosphotyrosine-dependent recruitment of exchange factors to submembranous locations (13–15, 17, 19) and activation of the exchange factors by D3-phosphoinositides (16). By analogy, it appears likely that activation of calpain at sites of ligand-occupied integrin may regulate Rac1 and RhoA by inducing events leading to the recruitment and/or activation of exchange factors at these sites. The physiological substrates for calpain have been characterized in most detail in platelets and include the cytoskeletal proteins actin-binding protein, spectrin, talin, and dystrophin-related protein (28, 40, 43), the cytoplasmic domain of the β2-integrin subunit (52), and several signaling molecules (41, 49, 50, 53, 54). Many of these proteins are known components of the complexes of integrin, cytoskeletal proteins, and signaling molecules that exist in unstimulated platelets (54), others are recruited to these complexes following integrin-ligand interactions. Many have been de-
tected in focal adhesions of spreading cells (11). Additional cytoskeletal proteins that have been reported to be substrates for calpain include protein 4.1 (44), ezrin (45), ankyrin (46), and α-actinin (47) all of which are known linkers between the cytoskeleton and the plasma membrane. Since most of the structural proteins are cleaved into a limited number of fragments, at least some of which remain in the integrin-signaling complexes (28, 40, 43, 55–57), while cleavage of several of the signaling molecules is known to induce altered activities (49, 53, 58), it appears likely that these calpain-induced cleavages would lead to reorganizations of the integrin signaling complexes that might in turn induce altered recruitment or activation of proteins required upstream of Rho protein activation. Future studies will be needed to identify the consequences of cleavage of each of calpain substrates, to determine the precise mechanisms by which they lead to remodeling of submembranous complexes, and to identify those that are involved in events leading to either Rac1 or RhoA activation.

Acknowledgment—We thank Gene Lazuta for graphics.

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