Calpain Regulates Actin Remodeling during Cell Spreading

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Abstract. Previous studies suggest that the Ca^{2+}-dependent proteases, calpains, participate in remodeling of the actin cytoskeleton during wound healing and are active during cell migration. To directly test the role that calpains play in cell spreading, several NIH-3T3–derived clonal cell lines were isolated that overexpress the biological inhibitor of calpains, calpastatin. These cells stably overexpress calpastatin two- to eightfold relative to controls and differ from both parental and control cell lines in morphology, spreading, cytoskeletal structure, and biochemical characteristics. Morphologic characteristics of the mutant cells include failure to extend lamellipodia, as well as abnormal filopodia, extensions, and retractions. Whereas wild-type cells extend lamellae within 30 min after plating, all of the calpastatin-overexpressing cell lines fail to spread and assemble actin-rich processes. The cells genetically altered to overexpress calpastatin display decreased calpain activity as measured in situ or in vitro. The ERM protein ezrin, but not radixin or moesin, is markedly increased due to calpain inhibition. To confirm that inhibition of calpain activity is related to the defect in spreading, pharmacological inhibitors of calpain were also analyzed. The cell permeant inhibitors calpeptin and MDL 28, 170 cause immediate inhibition of spreading. Failure of the intimately related processes of filopodia formation and lamellar extension indicate that calpain is intimately involved in actin remodeling and cell spreading.

Although it is well accepted that Ca^{2+} transients are associated with cell motility (Marks and Maxfield, 1990; Brundage et al., 1991; Hahn et al., 1992; Stossel, 1993; Janmey, 1994; Shuster and Herman, 1995), the role that Ca^{2+}-regulated proteolysis plays in coordinating cytoskeletal remodeling is undetermined. Interest in Ca^{2+} as a regulator of the cytoskeleton has been primarily focused on its potential roles in the severing and capping of actin filaments by members of the gelsolin family (for reviews see Stossel, 1993; Janmey, 1994). Recently, it has been suggested that calpain, the ubiquitous Ca^{2+}-activated protease, may regulate cell motility by cleaving actin-associated cytoskeletal proteins in a site-specific manner (Beckerle et al., 1987; Yao et al., 1993; Shuster and Herman, 1995; Huttenlocher et al., 1997). Actin-associated calpain substrates proposed to have roles in cell motility include the membrane-bridging protein talin, the cross-linking proteins α-actinin and actin binding protein (ABP-280), and the cortical proteins spectrin and ankyrin (for review see Croall and DeMartino, 1991). Recently, interest has been focused on the implications of calpain cleavage of the membrane-bridging protein ezrin in gastric parietal cells (Yao et al., 1993) and motile endothelial cells (Shuster and Herman, 1995). Integrin cleavage by calpain at the rear of the cell has also been indirectly implicated in motility via the disruption of cell–matrix interactions (Huttenlocher et al., 1997). In addition, it is possible that calpain cleavage of protein kinase C (PKC)[1] (Melloni et al., 1985) and focal adhesion kinase (p125 FAK) (Cooray et al., 1996) may regulate remodeling of the actin cytoskeleton (Vuori and Ruo-

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1. *Abbreviations used in this paper*: Δ3, exon 3–deleted; AMC, aminomethylcoumarin; CM, complete medium; DIC, differential interference microscopy; ERM, ezrin/radixin/moesin; FAK, focal adhesion kinase; PI, phosphatidylinositol; PKC, protein kinase C; suc-LLVY-AMC, succinyl-leucyl-leucyl-valyl-tyrosyl-7-amino-4-methylcoumarin; $ZLLYCHN_2$, benzyloxycarbonyl-$(leucyl)_2$-tyrosyl-diazomethane.

slahti, 1993; Lewis et al., 1996). Together, these observations point to Ca^{2+}-dependent proteolysis as a potential regulator of cytoskeletal protein–protein interactions during cell motility.

Despite the identification of many cytoskeletal substrates for calpain, there are few examples of physiological participation of calpain in cytoskeletal protein cleavage. A fundamental and incompletely understood process in cell motility is the spatial and temporal regulation of actin filament uncapping, postulated to simultaneously uncouple actin filaments from the membrane and permit their extension (Stossel, 1993; Lauffenberger and Horwitz, 1996; Welch et al., 1997). Recently, it has been suggested that calpain facilitates cytoskeletal reorganization during cell motility by cleaving ezrin molecules (Shuster and Herman, 1995) that form a bridge between the membrane and actin filaments (Algrain et al., 1993). Ezrin, a member of the ezrin/radixin/moesin (ERM) family of proteins, has been identified as binding specifically, but indirectly, to β-actin filaments via the newly identified β-actin–specific capping protein βcap73 (Shuster and Herman, 1995; Shuster et al., 1996). Presumably, ezrin is proteolyzed by calpain when cells are stimulated to crawl, thereby fostering βcap73 dissociation from the β-actin filaments located at the membrane (Shuster and Herman, 1995; Shuster et al., 1996). This is consistent with the finding that Ca^{2+} transients colocalize at the leading lamella of crawling cells, suggesting that calpain may regulate ezrin–βcap73–β-actin interactions. The recent observations that calpain inhibition impedes cell migration in a transwell assay and inhibits cleavage of integrins at the rear of the cell during migration (Huttenlocher et al., 1997) also lend credence to a model in which calpain activation is required for cell motility.

One approach to demonstrating a specific function for calpain in cell motility and actin dynamics is to exploit the specificity of the biological inhibitor of calpain, calpastatin. Calpastatin inhibits the two ubiquitous calpains, μ- and m-calpain, named for their respective micromolar or millimolar Ca^{2+} ion concentrations required for in vitro activity. Calpastatin has four internally repeated domains, each of which independently binds a Ca^{2+}-loaded calpain molecule with high affinity (Maki et al., 1990; Mellgren and Lane, 1990; Yang et al., 1994). Calpastatin is a highly specific inhibitor of calpain, having no other direct targets of regulation (Maki et al., 1990; Croall and DeMartino, 1991; Croall and McGrody, 1994). In addition to specificity of inhibition, there are several other reasons to test calpastatin as a dominant-negative inhibitor of calpain function. Calpains are long-lived enzymes with a half-life of ~5 d (Zhang et al., 1997), making antisense depletion difficult. Also, calpains are not substrate accessible until they are activated by Ca^{2+} (Croall and DeMartino, 1991), raising questions about the kinetics of inactivation by suicide substrate inhibitors in vivo. In addition, many pharmacological inhibitors lack specificity for calpain (Wang and Yuen, 1996). Finally, the ubiquitous calpains have not yet been targeted by gene disruption (Sorimachi et al., 1994), a process that may be complicated by their proposed involvement in cell division (Schollmeyer, 1988).

To study the potential role of calpain in cell motility, multiple clonal NIH-3T3 fibroblast cell lines that stably overexpress calpastatin were isolated. Overexpressed calpastatin downregulated both calpain activity and abundance in a dose-dependent fashion. With these cells in hand, it was asked whether cell spreading is a calpain-dependent process and, if so, whether the associated actin remodeling is calpain dependent. Quantification of cell spreading rates indicated that cell spreading is inhibited by 90% with two- to eightfold calpastatin overexpression. Actin cytoskeleton rearrangements that occur during spreading of control cells, including filopodial and lamella formation, are severely impaired in the calpastatin-overexpressing cell lines. The actin cytoskeleton of calpastatin-overexpressing cells is remarkable for the absence of lamellipodia, and an abundance of stress fibers, aberrant filopodia, and retraction fibers. These results were confirmed using the peptidyl cell–penetrating calpain inhibitors calpeptin and MDL (Mehdi et al., 1988; Tsujinaka et al., 1988), both of which cause immediate inhibition of spreading, as well as immediate inhibition of filopodial and lamellipodial protrusion formation. The less soluble tyrosine-containing peptidyl calpain inhibitor, benzyloxycarbonyl-$(leucyl)_2$-tyrosyl-diazomethane ($ZLLYCHN_2$) (Crawford et al., 1988; Anagli et al., 1991), also inhibits spreading after longer preincubation times. The ERM protein and calpain substrate, ezrin, is markedly increased in calpastatin-overexpressing cells and modestly increased in $ZLLYCHN_2$-treated cells, in contrast to its homologues radixin and moesin, suggesting that ezrin is specifically regulated by calpain. The immediacy of MDL/calpeptin inhibition of spreading combined with the specificity of calpastatin inhibition together make a strong argument that spreading, filopodial and lamella formation, and isoactin remodeling are calpain-dependent processes.

Materials and Methods

Cell Culture

NIH-3T3 fibroblasts, obtained from the American Type Culture Collection (Rockville, MD), were grown in DME with 10% heat-inactivated calf serum (GIBCO BRL, Gaithersburg, MD) and added L-glutamine, penicillin, and streptomycin (complete medium, CM) at 5% CO_2 (Revco Ultima Incubator; Revco Scientific, Inc. Asheville, NC). Clones stably transfected with the calpastatin construct pRC/CMV–Δ3CSN were grown in the above medium supplemented with 400 μg/ml of G418.

Transfection and Selection of Calpastatin-overexpressing Clonal Cell Lines

The full-length human calpastatin exon 3–deleted (Δ3) cDNA, one of the major calpastatin-splicing isoforms of fibroblasts, was inserted in the polylinker EcoRI site of the mammalian expression vector pRC/CMV (Invitrogen, Carlsbad, CA). The Δ3 cDNA was generated by fusing the overlapping cDNA inserts of λCS19, lacking exons 1–8 and 11, and λCS143, lacking exon 3 and domains 3 and 4 (internally repeated calpain inhibitory motifs) of calpastatin, at the SacI site in exon 14, resulting in a full-length cDNA lacking only exon 3 (Asada et al., 1989). NIH-3T3 fibroblasts were transfected using Lipofectin (GIBCO BRL) and then selected in the presence of 500 μg/ml of G418. G418-resistant clonal cell lines were isolated by ring cloning and then subsequent culture was done in the presence of 400 μg/ml of G418. The A3, A4, B5, B10, and B11 cell lines, transfected with pRC/CMV–Δ3CSN, were found to overexpress calpastatin more than twofold and were thus chosen for analysis. The E4 control cell line, transfected with pRC/CMV and selected under identical conditions, was indistinguishable from the wild-type NIH-3T3 cell line with respect to calpain and calpastatin levels, morphology, and spreading behavior. Multiple equivalent frozen stocks were made within passage 3–5 and then stored in liquid nitrogen. Passage of thawed cells was limited to 4–6 wk.

Quantitation of Calpastatin, m-Calpain, and Cytoskeletal Calpain Substrates in NIH-3T3 Clonal Cell Lines

Cytoplasmic extracts were made by Dounce homogenization and differential centrifugation, in the presence of protease inhibitors. Confluent monolayers were washed twice and then scraped in cold (4°C) PBS, 2 mM EDTA, and 2mM EGTA. All subsequent steps were performed at 4°C. Cell pellets were resuspended for 30 min in hypotonic lysis buffer A (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Hepes, pH 7.9, 1 mM DTT) (Dignam et al., 1983), containing 1 mM EDTA, 1 mM EGTA, 100 μM leupeptin, 2 μM pepstatin A, 2 μM chymostatin, 2 μM antipain, and 1 mM PMSF. Homogenization (tight pestle) was then performed by Dounce (40 strokes). Nuclei were pelleted by centrifugation for 10 s at 14,000 *g*. The crude cytoplasmic supernatant was combined with a one-tenth vol of buffer B (1.4 M KCl, 30 mM $MgCl_2$, 300 mM Hepes, pH 7.9, 1 mM DTT) (Dignam et al., 1983) with EDTA, EGTA, and protease inhibitors, as above, and then microfuged at 14,000 *g* for 70 min to separate the cytoplasmic extract (supernatant) from the membrane/cytoskeletal fraction (pellet).

Immunoblot analysis of cytoplasmic extracts was performed by standard techniques involving SDS-PAGE and electrophoretic transfer to 0.2-μm nitrocellulose filters. Blots were blocked (5% nonfat dry milk, 50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, and 1 mM DTT) overnight and subsequently probed with primary antibodies. The primary antibodies included the murine monoclonal anti-canine calpastatin antibody 8E11C (gift of R. Mellgren, Medical College of Ohio, Toledo, OH; Mellgren and Lane, 1990), the murine monoclonal anti-human calpastatin antibody CSF3-3 (Yokota et al., 1991), the rabbit anti–m-calpain peptide antiserum R389 (Croall et al., 1992), the rabbit anti-murine antisera #464, 457, and 454 raised to murine ezrin, radixin and moesin, respectively (gifts of F. Solomon, Massachusetts Institute of Technology, Cambridge, MA; Winckler et al., 1994), the rabbit anti–human talin antibody #N681 (gift of K. Burridge, University of North Carolina, Chapel Hill, NC), the rabbit anti-erythrocyte α-spectrin (gift of H. Hassoun, St. Elizabeth's Hospital, Brighton, MA), the murine monoclonal anti-human PKC hinge region (anti-α, -β) (MC5; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and the rabbit anti–human p125 FAK (C-20; Santa Cruz Biotechnology, Inc.), all diluted in PBS/3% BSA per 0.05% NaN_3. Secondary antisera were horseradish peroxidase-coupled goat anti–mouse IgG or goat anti–rabbit IgG (both from Bio-Rad Laboratories, Hercules, CA). Immunoblots were developed by a luminol chemiluminescent system and exposed to film. The relative levels of antigens were quantified by densitometry.

Determination of Calpain Activity in Clonal Calpastatin-overexpressing NIH-3T3–derived Cell Lines

The calpain assay of whole cell extracts measured Ca^{2+}-dependent acid-soluble radioactivity released by proteolysis of a ^{14}C-labeled methyl-casein substrate, as previously described (DeMartino et al., 1986; Croall and DeMartino, 1991). Because the assay Ca^{2+} concentration is 6.4 mM, both m- and μ-calpain activities are measured, although m-calpain, which has double the specific activity of μ-calpain for the methyl-casein substrate, is detected with greater sensitivity (Yoshimura et al., 1983). To determine the specific activity of calpain in whole cell extracts (Ca^{2+}-dependent acid soluble cpm/minute per microgram of extract protein), assays were performed in duplicate (at three different dilutions) and then the specific activities were measured and averaged. Calpain activity was determined by subtracting Ca^{2+}-independent from Ca^{2+}-dependent acid-soluble radioactivity (cpm) and then dividing by protein amount (μg) and the assay time (30 min).

Calpain activity in intact cells was determined by measuring Ca^{2+} ionophore-specific hydrolysis of the peptidyl 7-amino bond of the calpain substrate succinyl-leucyl-leucyl-valyl-tyrosyl-7-amino-4-methylcoumarin (suc-LLVY-AMC) (modification of the method of Bronk and Gores, 1993). suc-LLVY-AMC is cleaved by m- and μ-calpain with similar specific activities (Sasaki et al., 1984). Cells from confluent dishes were treated with 0.05% mM trypsin/0.53 mM EDTA. The trypsin was neutralized with CM containing 10% serum. Equal numbers of cells per assay (7.5×10^5) were washed twice in Hepes-buffered (10 mM Hepes–NaOH, pH 7.4) Hanks' balanced salt solution (without phenol red) in microfuge tubes. The cells were resuspended in Hepes-buffered Hanks' balanced salt solution (without phenol red) at 2.5×10^5 cells/ml and then held on ice until assay. To assay calpain activity, the cell suspension was prewarmed to 37°C for 10 min with stirring, in an SLM ALMINCO 8000 fluorimeter (Spectronic Instruments, Rochester, NY). At $t = -1$ min, ionomycin in DMSO (2.5 μM final concentration) or DMSO vehicle was added to the cells. At $t = 0$ min, the substrate suc-LLVY-AMC was added to 50 μM of concentration. The excitation wavelength was 360 ± 5 nm and the emission detection wavelength 460 ± 10 nm. The initial rate of substrate cleavage, which was linear, was measured at 2–3 min. Triplicate rate measurements were performed for each cell line. This assay measured the initial rate of Ca^{2+}-dependent substrate cleavage, determined by subtracting the ionomycin-independent rate from the ionomycin-dependent rate. Aminomethylcoumarin (AMC) standard solutions were used to determine pmoles of AMC generated from raw emission data. By trypan blue exclusion, the cells remained viable through the procedure.

Determination of Calpain and ERM mRNA Levels

Total cellular RNA was isolated from confluent monolayers of calpastatin-overexpressing and control cell lines by the Trizol method (GIBCO BRL; Chomczynski and Sacchi, 1987). Total cellular RNA (20 μg per lane) was electrophoresed on formaldehyde gels and transferred to Zeta-probe GT nylon membrane (Bio-Rad Laboratories). ^{32}P-labeled DNA hybridization probes were generated from the excised cDNA of either rat m-calpain (gift of J. Elce, Queen's University, Kingston, Ontario, Canada), human ezrin (gift of A. Bretscher, Cornell University, Ithaca, NY), or murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (gift of F. Young, University of Rochester, Rochester, NY) by the random primer method (Feinberg and Vogelstein, 1983). GAPDH mRNA levels, used for normalization of the blots, were not affected by calpastatin overexpression as determined by comparison of 4 pRC/CMV–transfected control cell lines and 5 pRC/CMV–Δ3CSN–transfected calpastatin-overexpressing cell lines.

Measurement of Spreading of Calpastatin-overexpressing Cells

The NIH-3T3 control and calpastatin-overexpressing cell lines used in spreading experiments were grown to near confluence. To prepare the cells for plating, they were trypsinized at confluence, treated with CM to neutralize trypsin, and then resuspended in fresh CM and placed on ice. This procedure resulted in a uniform population of round cells for each cell line. The cells were diluted in fresh, cold (4°C) CM to a density of 10^5 cells/ml just before plating on fibronectin-coated coverslips. At this density, >75% of the cells plated individually without touching adjacent cells.

Spreading experiments were performed on coverslips coated with 10 μg/ml of fibronectin, a condition chosen because NIH-3T3 cell adhesion to fibronectin at concentrations above 4 μg/ml was unaffected by calpastatin overexpression (our unpublished data). UV-irradiated coverslips (VWR Inc., St. Louis, MO) were coated with fibronectin at a concentration of 10 μg/ml in Tris-buffered saline, for 2.5 h at 37°C and then blocked with 3 mg/ml BSA, in PBS at room temperature. Blocking coverslips with the BSA solution inhibited glass-dependent spreading during the first 30 min of cell plating (control not shown).

Video microscopy of cells spreading on fibronectin-coated coverslips mounted on a temperature-controlled stage was recorded by a time-lapse S-VHS VCR from a low-light video camera mounted on a differential interference contrast (DIC) microscope (model IM35 inverted; Carl Zeiss Inc., Thornwood, NY, with 20× objective; Hoffman-La Roche, Nutley, NJ). Spreading experiments were begun by pipetting 80 μl of a cold (4°C) cell suspension onto a fibronectin-coated coverslip mounted in the stage assembly. The stage assembly was then mounted on the microscope. The stage temperature was maintained by a thermocouple-regulated air curtain blower. Stage temperature was recorded as the stage warmed from ambient temperature (22°–25°C) to 37°C. The $t = 0$ min time point was defined as the time at which the stage reached 37°C. The spreading process was recorded by time-lapse videotaping for at least 40 min. Still images of the videotape record were captured at 2–5-min time intervals allowing cell contours to be traced. Only nontouching individual cells were measured. Cells that subsequently touched neighbors were deleted from the quantitation of cell spreading. The number of measurable cells per video frame ranged between 15 and 25. Cell contours were scanned and saved as TIFF files. Cell areas were measured using NIH Image 1.55 (National Institutes of Health, Bethesda, MD).

Static images of the F-actin cytoskeleton of spreading cells were also obtained by fluorescein-phalloidin staining of cells fixed during the spreading process. Cells were spread at 37°C on fibronectin-coated coverslips prepared identically to those used for DIC microscopy. The cells

were plated in 80-μl volumes, as used for video microscopy, to allow similar rapid settling times and temperature equilibration. The cells on coverslips were then fixed with 3.7% formaldehyde in Pipes-buffered saline (PiBS) at various time points, permeabilized with 0.5% Triton X-100 in PiBS for 15 min and then stained with fluorescein phalloidin. A Zeiss (model Axioskop G42-110-e with 63× objective; Carl Zeiss, Inc.) microscope was used for fluorescence microscopy. Photomicrographs were taken with Kodak Tri-X film (Eastman-Kodak, Rochester, NY). Filopodia are defined in these studies as peripheral actin microspikes extending normally with respect to the cell surface. Lamellae are defined as convex F-actin–containing structures at the cell border, indicative of protrusion formation. Retraction fibers are defined in these studies as concave cortical actin fibers, indicative of cellular retraction.

Pharmacological Inhibition of Calpain

The reversible calpain inhibitors calpeptin and MDL were dissolved in DMSO at 50 mM concentration to make stock solutions. Inhibitors were added to NIH-3T3 cells and suspended in fresh CM at the indicated final concentrations. An equal volume of DMSO vehicle was added to NIH-3T3 cells in control experiments. The cells were incubated in the presence of inhibitor or vehicle for 10 min and then plated on fibronectin-coated coverslips. Spreading was observed by DIC video microscopy or by fluorescein-phalloidin fluorescence photomicrography, as described above. The concentrations of MDL (50 μM) and calpeptin (80 μM) used were minimal inhibitory concentrations for spreading, determined in pilot experiments. Cells incubated for 10 min in the presence of the most potent inhibitor, MDL, demonstrated a 50% reduction of calpain activity by caseinolysis assay of cytoplasmic extracts. Inhibition of calpain by calpeptin and E64d was not detectable under these conditions, probably due to the 10-fold dilution of these less potent calpain inhibitors, necessary for hypotonic lysis, during preparation of the extracts.

The suicide substrate inhibitor of calpain, benzyloxycarbonyl-(leucyl)$_2$-tyrosyl-diazomethane (ZLLYCHN$_2$) (Crawford et al., 1988; Anagli et al., 1991) (gift of H. Angliker, Friedrich Miescher Institut, Basel, Switzerland) was dissolved in DMSO at 50 mM concentration to make a stock solution. NIH-3T3 cells treated with ZLLYCHN$_2$ for 18–24 h were suspended in CM and then plated for spreading studies on fibronectin-coated coverslips in the presence or absence of additional ZLLYCHN$_2$ (50 μM). Spreading was studied by video microscopy and phalloidin staining as described above.

Results

m-Calpain Is Downregulated in Calpastatin-overexpressing Cells

Calpastatin inhibits μ- and m-calpain proteolysis of both protein and peptidyl substrates in vitro (for review see Maki et al., 1990; Croall and DeMartino, 1991) and was therefore expected to inhibit calpain activity in vivo. To determine whether calpastatin regulates calpain in vivo, the Δ3 isoform of calpastatin (Asada et al., 1989; Lee et al., 1992; Takano et al., 1993) was stably overexpressed in NIH-3T3 fibroblasts. 9 out of 20 selected clonal cell lines were analyzed for calpastatin overexpression. Five of the nine cell lines demonstrated greater than at least twofold calpastatin overexpression by immunoblot analysis of cytoplasmic extracts (A3, A4, B5, B10, and B11) and were chosen for further study. These cell lines have distinctive morphologies, such as prominent filopodia, extensions, and retractions not found in the parental and control cell lines (see Fig. 3). A control cell line, E4, was chosen from eight identically derived cell lines transfected with the empty vector, pRC/CMV. The E4 cell line and the parental NIH-3T3's have identical calpain and calpastatin levels, as well as identical morphologies (data not shown). Calpastatin is increased twofold in the A4, B5, and B11 cell lines (Fig. 1 *A* and Table I, *low overexpressors*) and eightfold in the A3 and B10 cell lines (Fig. 1 *A* and Table I, *high overexpressors*). The cell lines within each group were found to be indistinguishable from each other in subsequent cytoskeletal studies, although specific differences in morphology were found between the high and low overexpressor cell lines (see Fig. 3).

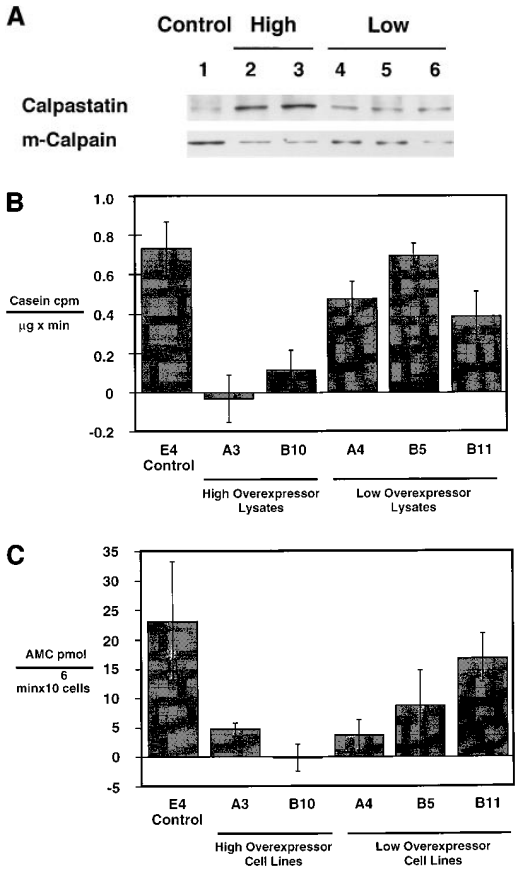

Figure 1. m-Calpain levels and activity are downregulated in calpastatin-overexpressing NIH-3T3 fibroblasts. (*A*) Calpastatin overexpressors were divided into two groups, the high overexpressors (eightfold overexpression) A3 and B10, in lanes *2* and *3*, respectively, and the low overexpressors (twofold overexpression) A4, B5, and B11, in lanes *4–6*, respectively. The vector control cell line, E4, is shown in lane *1*. Immunoblots of cell extracts were probed with antisera to the indicated antigens, as described in Materials and Methods. The m-calpain blot shows the 80-kD large subunit of m-calpain. The calpastatin blot shown was probed with the 8E11C monoclonal antibody. The identity of the molecular mass of the 100-kD mobility calpastatin band was confirmed with a second monoclonal antibody, CF3-3 (Yokota et al., 1991) (data not shown). Quantitation is presented in Table I. (*B*) Calpain activity was measured in whole cell lysates by proteolysis of ^{14}C-labeled methyl casein, as described in Materials and Methods. The mean and standard deviation of casein cpm released per minute, normalized to lysate protein is indicated for each cell line. (*C*) Calpain activity was measured in intact cells by monitoring the cleavage of the cell-penetrating substrate suc-LLVY-AMC by fluorimetry as described in Materials and Methods. The initial rate of substrate cleavage in the absence of ionomycin was subtracted from the initial rate in the presence of ionomycin to determine the rate of Ca^{2+}-dependent proteolysis of the substrate. The mean of triplicate measurements is given with the standard deviation. The E4 control cell line was measured in duplicate.

Table I. Quantitative Comparison of Steady-state Levels of Calpain and Calpain–associated Proteins in Calpastatin-overexpressing Cell Lines Compared to the Control Cell Line E4

Calpain-associated protein	High calpastatin overexpressor cell lines (normalized mean ± SD)	Low calpastatin overexpressor cell lines (normalized mean ± SD)
Calpastatin	7.8 ± 1.1	2.3 ± 0.25
m-Calpain	0.22 ± 0.035	0.37 ± 0.23
Ezrin	7.1 ± 3.7	27 ± 3.8
Radixin	2.0 ± 0.23	2.5 ± 0.48
Moesin	2.3 ± 0.035	1.7 ± 0.49
Talin	0.80 ± 0.22	0.53 ± 0.085
α-Spectrin BDP	1.3 ± 0.078	1.0 ± 0.35
Focal adhesion kinase	0.74 ± 0.30	1.1 ± 0.14
PKC α/β	0.86 ± 0.13	0.23 ± 0.14

Measurements, normalized to the E4 control, were determined by densitometry of Western blot analysis of protein levels. High calpastatin overexpressor cell lines were A3 and B10. Low overexpressor cell lines were A4, B5, and B11.

Table II. Quantitative Comparison of M-Calpain and ERM Protein mRNA Levels in Calpastatin-overexpressing Cell Lines Compared to the Control Line E4

Calpain-associated protein	High calpastatin overexpressor cell lines (normalized mean ± SD)	Low calpastatin overexpressor cell lines (normalized mean ± SD)
m-Calpain	0.70 ± 0.099	1.36 ± 0.24
Ezrin	1.15 ± 0.18	2.30 ± 0.72
Radixin/moesin	1.09 ± 0.086	1.21 ± 0.32

Measurements, normalized to the E4 control, were determined by densitometry of Northern blot analysis of mRNA levels. High calpastatin overexpressor cell lines were A3 and B10. Low overexpressor cell lines were A4, B5, and B11. The difference between the m-calplain mRNA levels of the high and low overexpressors was statistically significant ($P = 0.0058$ by Student's paired *t*-test). The difference between the ezrin mRNA levels of the high and low overexpressors was also statistically significant ($P = 0.028$ by Student's paired *t*-test).

Individual cell types have characteristic calpastatin/calpain ratios (for review see Croall and DeMartino, 1991) that can vary dramatically during cell growth and differentiation (Barnoy et al., 1996), suggesting that there is coordinated regulation of these proteins. It is expected that calpastatin is a critical regulator of calpain in vivo (Goll et al., 1992), although the exact mechanisms are unknown. Thus, the effect of overexpressed calpastatin on endogenous calpain levels could not be predicted and had to be determined. Because the major isoform of calpain in NIH-3T3 fibroblasts is m-calpain (data not shown), an antiserum specific for m-calpain (Croall et al., 1992) was used to determine m-calpain levels in the five calpastatin-overexpressing cell lines, as well as the control cell line (Fig. 1 *A* and Table I). The high overexpressors (A3 and B10) have the lowest levels of m-calpain, 22% of the E4 control (Fig. 1 *A*, Table I). The low overexpressors (A4, B5, and B11) have m-calpain levels that are 37% of the E4 control. The decrease in m-calpain is proportional to the degree of calpastatin overexpression, unexpectedly indicating that calpastatin regulates calpain abundance.

Because the downregulation of m-calpain abundance in calpastatin-overexpressing cells was unexpected, a Northern analysis was performed to determine whether there was a corresponding decrease of m-calpain mRNA. An alteration of mRNA levels could not be predicted at first, because the calpain–calpastatin system had not been implicated in the regulation of specific genes. The high overexpressors of calpastatin, A3 and B10, demonstrated a 30% reduction of m-calpain mRNA relative to the E4 control cell line, which can account for less than half of the 78% reduction in m-calpain protease. In contrast, the low overexpressors of calpastatin, A4, B5, and B11, demonstrated a 36% increase in m-calpain mRNA (Table II), which cannot account for the 63% decrease in m-calpain protease (Table I). The difference observed between the calpain mRNA levels of the low and high calpastatin overexpressors was statistically significant ($P = 0.0058$ by Student's paired *t* test). The observation that alteration of the calpastatin/calpain ratio results in alteration of m-calpain mRNA levels is novel and intriguing because it is the first demonstration of a role for the calpain–calpastatin system in the regulation of a specific gene. Nonetheless, despite the unexpected changes in m-calpain mRNA levels, it can be concluded that the reductions of m-calpain observed in either the high or low calpastatin overexpressor cells cannot be solely attributed to a reduction in m-calpain mRNA.

Calpain Activity Decreases with Increasing Calpastatin Overexpression

To determine whether calpastatin overexpression in NIH-3T3 fibroblasts reduces total cellular calpain activity, a casein hydrolysis assay was used to measure calpain in whole cell extracts. This assay demonstrated that calpastatin overexpression results in significant decreases in cellular calpain activity (Fig. 1 *B*) for four of the five cell lines. Furthermore, there is an inverse correlation between calpastatin overexpression and calpain activity. The high overexpressors, A3 and B10, demonstrated the lowest levels of calpain activity, 0 and 16% of control, respectively (Fig. 1 *B*). The low calpain activities of the high overexpressors are consistent with the low abundance of calpain measured by immunoblotting, 22% of control (Fig. 1 *A* and Table I). The low overexpressors, A4, B5, and B11, demonstrated calpain activities that were 64, 96, and 53% of control, respectively (Fig. 1 *B*). By this cell lysate assay, the calpain activities of the low overexpressors are somewhat greater than those expected by measured calpain levels (Fig. 1 *A* and Table I), but in general agreement with the expectation that they should be intermediate between the high overexpressors and controls.

Because the lysate assay allows free mixing of calpain and calpastatin and may not reflect calpain activity in vivo, calpain activity was measured in intact, living cells. Calpain activity was measured in situ by a fluorometric assay using the cell-penetrating calpain substrate suc-LLVY-AMC and was adapted to NIH-3T3 fibroblasts in culture (Bronk and Gores, 1993). By fluorometric assay, the high overexpressor cell lines, A3 and B10, demonstrated the lowest calpain activities, 20 and 0% of control, respectively (Fig. 1 *C*). By this same assay, the low overexpressor cell lines, A4, B5, and B11, demonstrated calpain activities that were 16, 38, and 73 of control, respectively (Fig. 1 *C*), indicating that calpain activity is inhibited in all of the calpastatin-overexpressing cell lines. The reductions of calpain activity measured for the high and low overexpressor cell lines were proportional to the reduction of calpain protease (Table I), since the high overexpressor cell lines

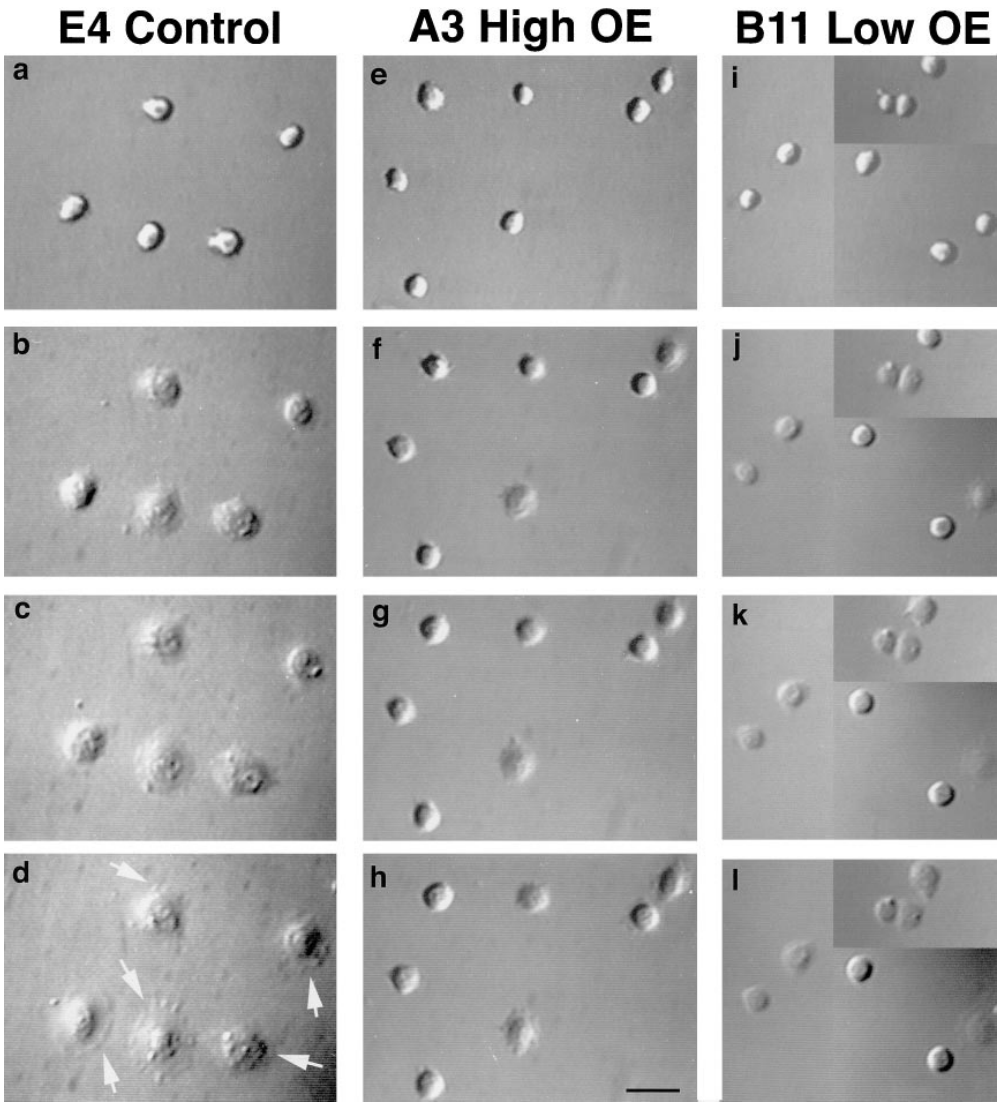

Figure 2. Spreading of calpastatin-overexpressing NIH-3T3 fibroblasts is severely impaired. (*a–l*) DIC video microscopy still frames of live, spreading high overexpressor cells of the A3 cell line, low overexpressor cells of the B11 cell line, and the control cell line, E4. Frames shown are: E4, $t = 0$, 10, 20, and 30 min (*a–d*); A3, $t = 0$, 10, 20, and 30 min (*e–h*); and B11, $t = 0$, 10, 20, and 30 min (*i–l*). Cells were trypsinized, washed, plated on fibronectin-coated coverslips, and then followed by DIC video microscopy at 37°C as described in Materials and Methods. The $t = 0$ min time point is the time at which the microscope stage reached 37°C. *Arrows*, lamellae observed at $t = 30$ min. Spreading data for the other calpastatin-overexpressing cell lines are presented in normalized form in Fig. 7. (*m*) Mean cell areas of calpastatin-overexpressing NIH-3T3 cells, represented in *a–l*, are shown as a function of time. The mean cell area is plotted for 15–25 measurements. Error bars indicate the standard error of the mean. One area unit is 1,150 μm^2. Bars, 25 μm.

had the lowest levels of calpain activity. The in situ assay, which measured calpain activity in living cells, agreed generally with the lysate assay and confirmed substantial reductions of calpain activity in calpastatin-overexpressing cells.

Cell Spreading and Spreading-related Rearrangements of the Actin Cytoskeleton Are Inhibited by Calpastatin Overexpression

To determine whether spreading is calpain dependent, calpastatin-overexpressing NIH-3T3 cell lines were spread on a fibronectin substratum and monitored by DIC video microscopy. Shape change of round, freshly plated E4 control cells begins about 2 min after warming to 37°C with loss of cellular refractility. >90% of the E4 cells display lamellae at 5 min, followed by radial spreading of a smooth-edged circumferential lamella (Fig. 2 *d*, *arrows*), resulting in a greater than fourfold increase in cell area by 30 min of spreading. The spreading rates of the E4 control and parental NIH-3T3 cell lines are equivalent (Table III),

Table III. Quantitative Comparison of Spreading Rates of Calpain-inhibited NIH-3T3 Cells

Cell type and conditions	Spreading rate	% of control rate
	$\mu m^2/min$	
E4 control	21.0	
A3 high overexpressor cell line	2.2	10.0
B11 low overexpressor cell line	1.3	6.2
NIH-3T3	20.0	
NIH-3T3, DMSO vehicle	18.0	
NIH-3T3, calpeptin (80 μM)	6.9	38.0
NIH-3T3, MDL (50 μM)	5.4	30.0
NIH-3T3, $ZLLYCHN_2$ (50 μM)	10.6	53.0

Spreading rates were measured by quantitation of cell area differences determined by DIC video microscopy during spreading (from t = either 4 or 5–30 min).

and both cell lines have equivalent mean cell areas initially and at 30 min. The most rapid rate of spreading of control fibroblasts occurs between 2 and 10 min (Fig. 2 *m*). In contrast, the cell lines overexpressing calpastatin at either high or low levels exhibit marked inhibition of spreading. The A3 and B11 cell lines, representative of the high and low overexpressors, respectively, have spreading rates that are 10 and 6.2% of control (Table III; Fig. 2 *m*). Lamella formation and loss of cellular refractility are significantly delayed for both the high and low overexpressor cell lines (compare Fig. 2 *d*, *arrows*, with Fig. 2 *h* and *l*). More than one-third of the calpastatin-overexpressing cells remain refractile even at 30 min (Fig. 2, *h* and *l*). The other high overexpressor line, B10, and the other low overexpressor lines, A4 and B5, demonstrate similar spreading defects (Fig. 7).

The actin cytoskeleton of spreading calpastatin-overexpressing fibroblasts differs markedly from that of control cells. Prominent filopodia and early lamellae, found at 4 min of spreading on the control cells, are nearly absent on cells of the high and low overexpressor cell lines (Fig. 3, *A3* and *B11*). At 60 min of spreading, the calpastatin-overexpressing cells are still markedly smaller in area. Calpastatin-overexpressing cells fail to form the continuous lightly staining lamellar border of F-actin found at the edges of control cells (Fig. 3, *E4*, *arrows*). At 60 min, the high overexpressors are remarkable for their small size and bristling aberrant filopodia, many tipped by pools of F-actin (Fig. 3; *A3*, *arrows*), whereas the low overexpressors are remarkable for their retraction fibers (Fig. 3; *B11*, *small arrows*) and prominent extensions (Fig. 3; *B11*, *large arrows*). Formation of stress fibers (Fig. 3; *A3* and *B11*) and focal contacts (by vinculin and talin stains, data not shown) did not appear to be affected by calpastatin overexpression.

Cell Spreading and Spreading-related Rearrangements of the Actin Cytoskeleton Are Inhibited by Pharmacological Calpain Inhibition

Although calpastatin-overexpressing fibroblasts were markedly impaired in spreading, this genetic approach, which inhibits calpain specifically, does not distinguish between a direct role for calpain in cytoskeletal remodeling, or an indirect role, perhaps through alteration of gene expression (refer to Table II). To address this important issue, it was

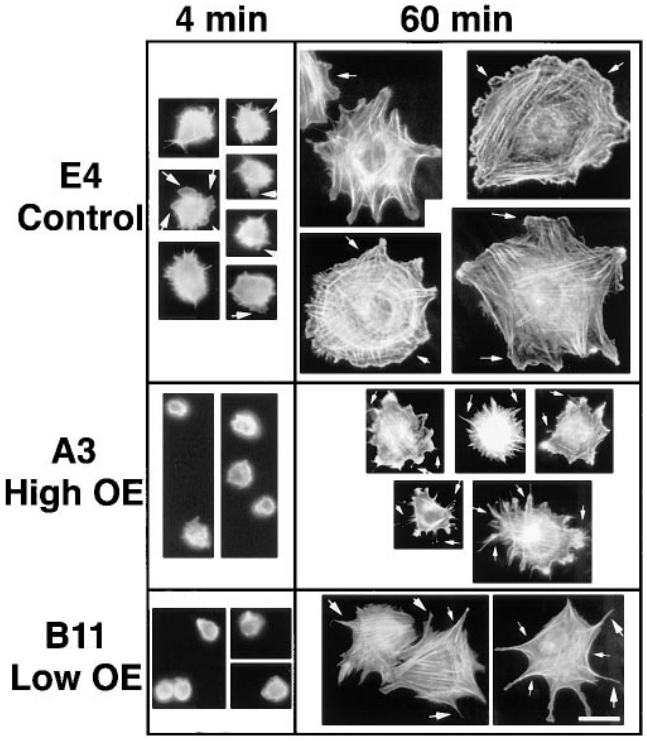

Figure 3. Filopodial and lamellipodial protrusion formation are severely impaired in calpastatin-overexpressing NIH-3T3 fibroblasts. The high overexpressor cell line, A3, the low overexpressor cell line, B11, and the control cell line, E4, were spread on fibronectin-coated coverslips, fixed at the indicated times, and then stained with fluorescein phalloidin as described in Materials and Methods. *Arrows*: early lamellae in E4, t = 4 min; cortical actin in lamellae in E4, t = 60 min; filamentous filopodia, many of which have enlarged ends, in A3, t = 60 min. *Small arrows*, B11, t = 60 min indicate retraction fibers; *large arrows,* filopodial-like protrusions. Bar, 25 μm.

determined whether pharmacological inhibition of calpain would result in immediate inhibition of spreading. In pilot studies, the cell permeant calpain inhibitors calpeptin, MDL, and E64d all inhibited spreading immediately, whereas $ZLLYCHN_2$ required overnight incubation for inhibition, probably due to limited solubility (data not shown). Poor intracellular penetration of $ZLLYCHN_2$ has been observed by others (Mellgren, 1997) with intracellular concentrations reaching only 1 μM after 16 h of exposure to culture medium containing 200 μm $ZLLYCHN_2$. Calpeptin and MDL were chosen for further study because they were the most potent immediate inhibitors of spreading, despite being reversible (refer to Table III).

Calpeptin and MDL potently inhibit spreading of NIH-3T3 cells (Fig. 4, *e–h* and *i–l*; *Control*, *a–d*; quantitation, *q*). Like calpastatin-overexpressing cells, the majority of the calpeptin- and MDL-treated cells fail to spread by 30 min (Fig. 4, *h* and *l*). Spreading rates of calpeptin- and MDL-treated cells were 38 and 30% of control, respectively (refer to Table III). Lamella formation and loss of cellular refractility are significantly delayed due to treatment with these peptidyl inhibitors (Fig. 4, compare *d* with *h* and *l*). More than one-third of the peptidyl inhibitor–treated cells remain refractile even at 30 min (Fig. 4, *h* and *l*). Analysis

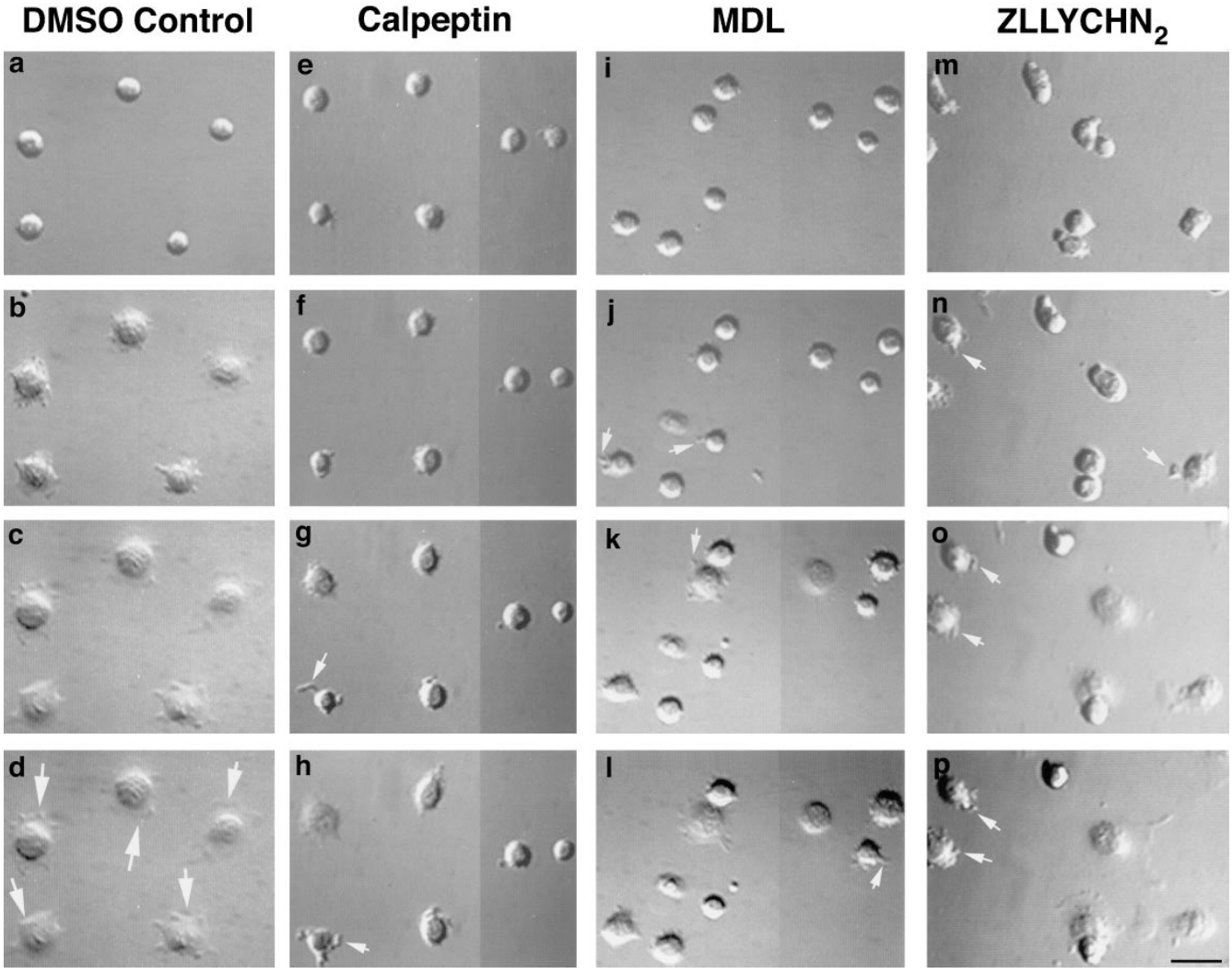

Figure 4. Spreading of NIH-3T3 fibroblasts is severely impaired by short-term incubation with the cell permeant calpain inhibitors calpeptin, MDL, and long-term incubation with $ZLLYCHN_2$. (*a–p*) DIC video microscopy still frames of live, spreading cells are shown. Cells were trypsinized, washed, plated on fibronectin-coated coverslips, and then followed by DIC video microscopy at 37°C, as described in Materials and Methods. The $t = 0$ min time point is the time at which the microscope stage reached 37°C. *Arrows*: (*d*) lamellae observed at $t = 30$ min; (*g–p*) abnormal extensions. The long-term incubation DMSO control for $ZLLYCHN_2$ treatment was indistinguishable from the short-term DMSO control shown (data not shown). (*q*) Inhibition of spreading by immediate calpain inhibition. Mean cell areas of MDL- and calpeptin-treated NIH-3T3 cells, represented in (*a–p*), are shown as a function of time. The mean cell area is plotted for 15–25 measurements. Error bars indicate the standard error of the mean. One area unit is 1,150 μm^2. Bar, 25 μm.

of the actin cytoskeleton of spreading calpeptin or MDL-treated fibroblasts revealed a marked delay in the formation of filopodia and lamellae (Fig. 5 *A*, *Calpeptin* and *MDL*, *4*, *10*, and *20 min*), similar to the calpastatin-overexpressor cell lines (refer to Fig. 3). In contrast, the control cells are well spread by 20 min and exhibit circumferential lamellae and stress fibers (Fig. 5 *A*, *DMSO*, *4*, *10,* and *20 min*). In contrast, many of the calpeptin- and MDL-treated cells exhibit aberrant, highly motile extensions (Fig. 4, *g–h* and *j–l* and Fig. 5 *A*, *small arrows*). Many of these extensions last only minutes and tend to move tangentially relative to the cell's radius of curvature. These extensions are bordered by a sheath of F-actin (Fig. 5 *A*, *arrows*) and are distinct from filopodia, which contain a central microspike of F-actin extending in the direction of the cell's radius of curvature. Similar extensions can be seen on $ZLLYCHN_2$-treated cells (see below and Fig. 6, *E arrow* and Fig. 4, *n–p*). Such extensions are also observed on spreading calpastatin-overexpressing cells, but are less frequent and shorter in length (data not shown).

Since all of the pharmacological calpain inhibitors tested in these studies also inhibit cathepsins, control studies

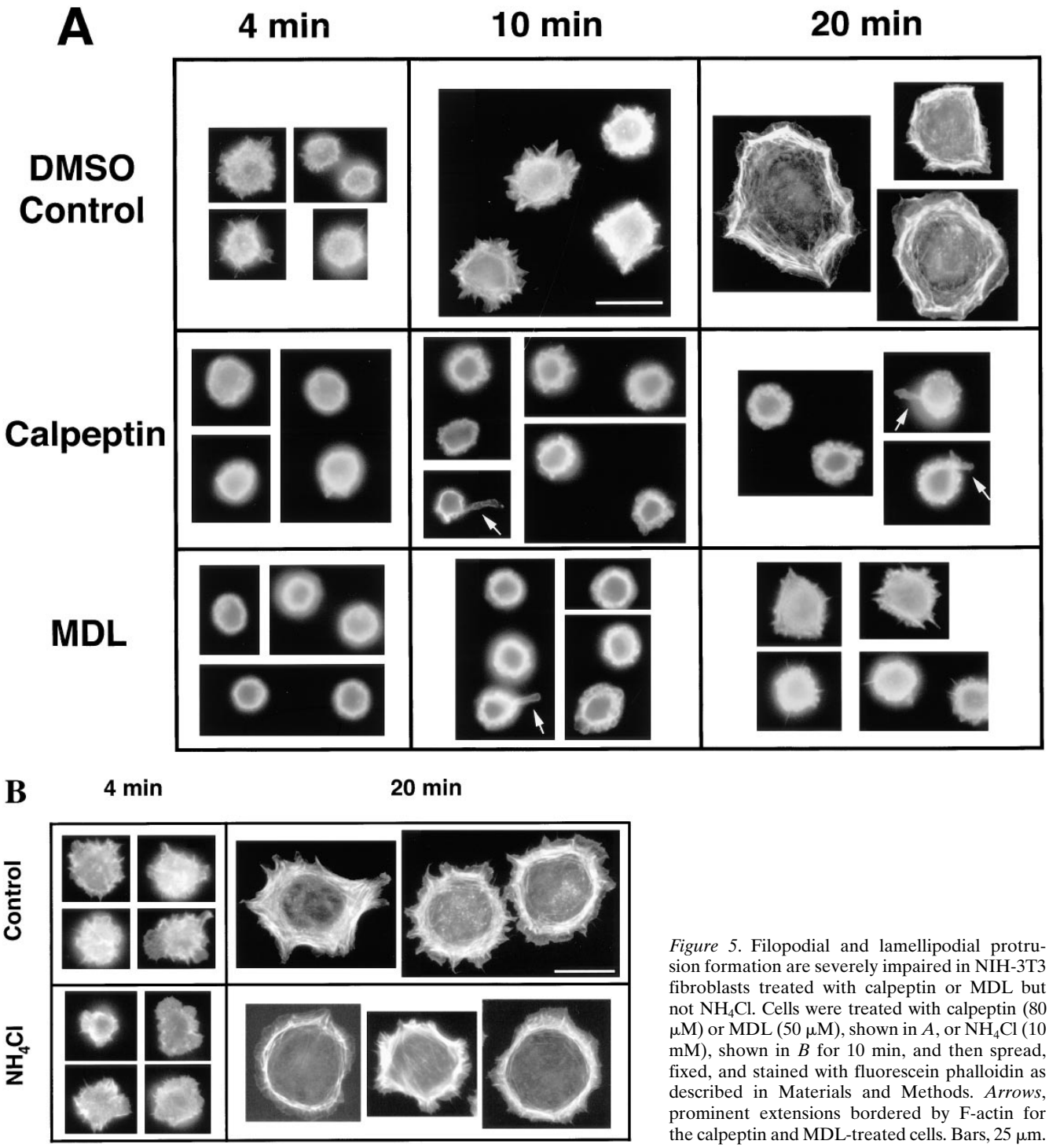

Figure 5. Filopodial and lamellipodial protrusion formation are severely impaired in NIH-3T3 fibroblasts treated with calpeptin or MDL but not NH_4Cl. Cells were treated with calpeptin (80 μM) or MDL (50 μM), shown in *A*, or NH_4Cl (10 mM), shown in *B* for 10 min, and then spread, fixed, and stained with fluorescein phalloidin as described in Materials and Methods. *Arrows*, prominent extensions bordered by F-actin for the calpeptin and MDL-treated cells. Bars, 25 μm.

were done to determine whether cathepsins play a role in cell spreading. To inhibit lysosomal function, NIH-3T3 cells were incubated with 10 mM NH_4Cl, a concentration that maximally inhibits cathepsin cleavage of cytosolic substrates (J.F. Dice, Jr., personal communication). Pretreatment of NIH-3T3 cells with 10 mM NH_4Cl does not affect spreading rates of NIH-3T3 cells (data not shown). In addition, spreading-associated remodeling of the actin cytoskeleton, including filopodia and lamella formation are not affected by NH_4Cl treatment (Fig. 5 *B*). This finding rules out the hypothesis that calpeptin, MDL, E64d, or $ZLLYCHN_2$ inhibit spreading by inhibiting cathepsins or lysosomal function. The above studies thus combine the immediacy of MDL- and calpeptin-dependent calpain inhibition with the specificity of calpastatin-dependent calpain inhibition to argue strongly that the actin remodeling of lamellipodial and filopodial protrusion formation is directly dependent on calpain.

To further confirm calpain's role in spreading, NIH-3T3 fibroblasts depleted of calpain activity by the slow-acting, but irreversible inhibitor, $ZLLYCHN_2$, were tested for spreading. $ZLLYCHN_2$ is specific for calpain, among cyto-

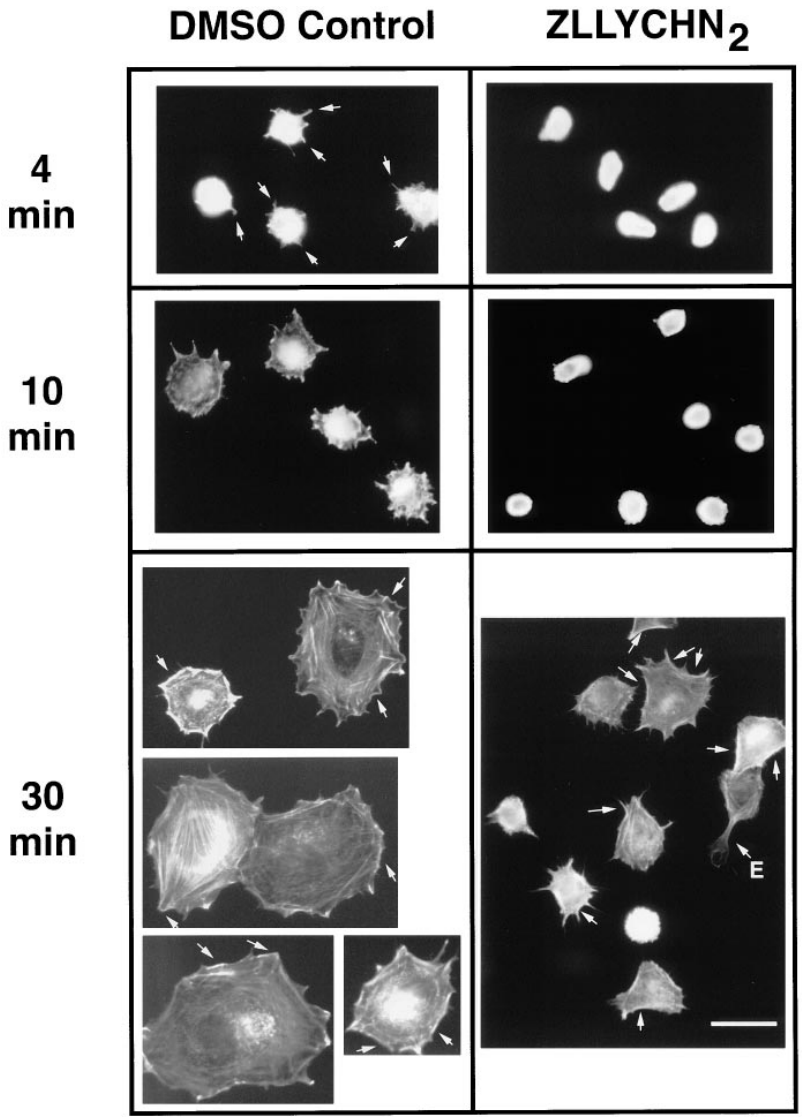

Figure 6. Filopodial and lamellipodial protrusion formation are severely impaired in $ZLLYCHN_2$-treated NIH-3T3 fibroblasts. Cells were cultured in the presence of 50 μM of $ZLLYCHN_2$ or DMSO vehicle, trypsinized, plated in the presence of $ZLLYCHN_2$, and then fixed and stained with fluorescein phalloidin at t = 4, 10, and 30 min as described in Materials and Methods. *Arrows*: filopodia in DMSO, t = 4 min; lamellar F-actin in DMSO, t = 30 min; retraction fibers in $ZLLYCHN_2$, t = 30 min. *E*, abnormal extension, having a border of F-actin similar to those seen with calpeptin and MDL treatment (refer to Fig. 5 *A*). Bar, 25 μm.

solic proteases, and preferentially inhibits calpain versus the proteasome (Mellgren et al., 1994; Mellgren, 1997). A novel approach had to be developed for the use of $ZLLYCHN_2$ in the study of spreading, since short-term (2-h) treatment of cells with the inhibitor did not result in significant calpain inhibition. Significantly lower solubility of $ZLLYCHN_2$ compared with calpeptin and MDL (data not shown), perhaps related to the tyrosine residue, likely resulted in slower cellular uptake and onset of calpain inhibition, relative to calpeptin and MDL. Because of this finding, and since long-term incubation of cells in the presence of $ZLLYCHN_2$ is known to deplete calpain activity (Mellgren et al., 1996), NIH-3T3 fibroblasts were incubated in the presence of $ZLLYCHN_2$ for 18–24 h. Cells treated as such with $ZLLYCHN_2$ were reduced in calpain activity by 36% relative to controls.

Similar to calpeptin and MDL treatment, $ZLLYCHN_2$ potently inhibited spreading of NIH-3T3 cells. (refer to Fig. 4, *m–p*; see Fig. 6). Spreading rates of cells treated with $ZLLYCHN_2$ were 53% of control (refer to Table III). Lamella formation and loss of cellular refractility are significantly delayed due to $ZLLYCHN_2$ treatment (refer to Fig. 4, compare *d* with *p*). Analysis of the actin cytoskeleton of spreading $ZLLYCHN_2$-treated cells revealed a marked decrease in the projection of filopodia and early lamellae at 4 min of spreading (Fig. 6), similar to cells treated with calpeptin and MDL (refer to Fig. 5 *A*), and calpastatin-overexpressing cells (refer to Fig. 3). At 4 min of spreading, the $ZLLYCHN_2$-treated cells demonstrate few filopodia, but instead display a smooth-edged amorphous F-actin network, similar to MDL- and calpeptin-treated cells, as well as calpastatin-overexpressing cells. At 10 min of spreading, the $ZLLYCHN_2$-treated cells still fail to form filopodia and lamellae, whereas the control cells form lamellae (Fig. 6). At 30 min, the $ZLLYCHN_2$-treated cells begin to spread, but are still much smaller in area than control cells. As observed with calpeptin and MDL treatment, $ZLLYCHN_2$-treated cells had prominent extensions displaying a sheath of F-actin (refer to Fig. 4, *n–p* and Fig. 6 *E*, *arrow*). Also as observed with calpastatin overexpression, the $ZLLYCHN_2$-treated cells display prominent retraction fibers (Fig. 6; *$ZLLYCHN_2$, 30 min*, *arrows*) and fail to demonstrate the continuous border of F-actin found at the lamellae of control cells (Fig. 6; *DMSO*, *30*

min, *arrows*). Spreading of $ZLLYCHN_2$-treated cells was impaired due to the 18–24-h pretreatment with $ZLLYCHN_2$ since the delay of spreading occurred whether or not $ZLLYCHN_2$ was present during spreading (compare Fig. 4 with Fig. 6). This observation is consistent with the notion that cell penetration by $ZLLYCHN_2$ is slow and indicates that the inhibition of spreading is due to the 18–24-h incubation with the inhibitor.

A Calpain-dependent Event Occurs Early in Cell Spreading

To determine whether there is a rate-limiting calpain-dependent event during spreading, cell spreading kinetics were compared for the calpain inhibition modalities used in these studies. Identification of such an event would help focus further studies of calpain's role in spreading. As such, the first goal of a kinetic analysis was to determine when maximal inhibition of spreading occurs. The second goal was to determine whether differences in initial cell size observed with certain modalities of calpain inhibition (see below) play any role in determining the kinetics of cell spreading. To these ends, the areas of calpastatin-overexpressing and calpain inhibitor-treated cells, normalized to the areas of control cells, were plotted as a function of spreading time. The time period during which slopes of the area–time curves, and thus spreading rates, were lowest was determined for each modality of calpain inhibition (Fig. 7, *A* and *B*).

The kinetic plots (Fig. 7, *A* and *B*) indicate that maximal inhibition of spreading occurs early, during the first 4–10 min of spreading, and that after the initial lag, the ratio of calpain-inhibited cell area to control remains relatively unchanged. Both biological and pharmacological inhibition of calpain resulted in the lowest (most negative) rates of cell spreading between 4 and 10 min (Fig. 7, *A* and *B*), indicating that calpain is involved in spreading almost immediately after cell contact with substratum. The second finding is that variations in the initial cell size due to different modalities of calpain inhibition does not affect the finding of an early delay in spreading. Although calpastatin-overexpressing cells are smaller than control cells, $ZLLYCHN_2$-treated cells are larger than control cells, and calpeptin or MDL-treated cells are the same size as control cells, the pattern of spreading delay is similar (Fig. 7, *A* and *B*).

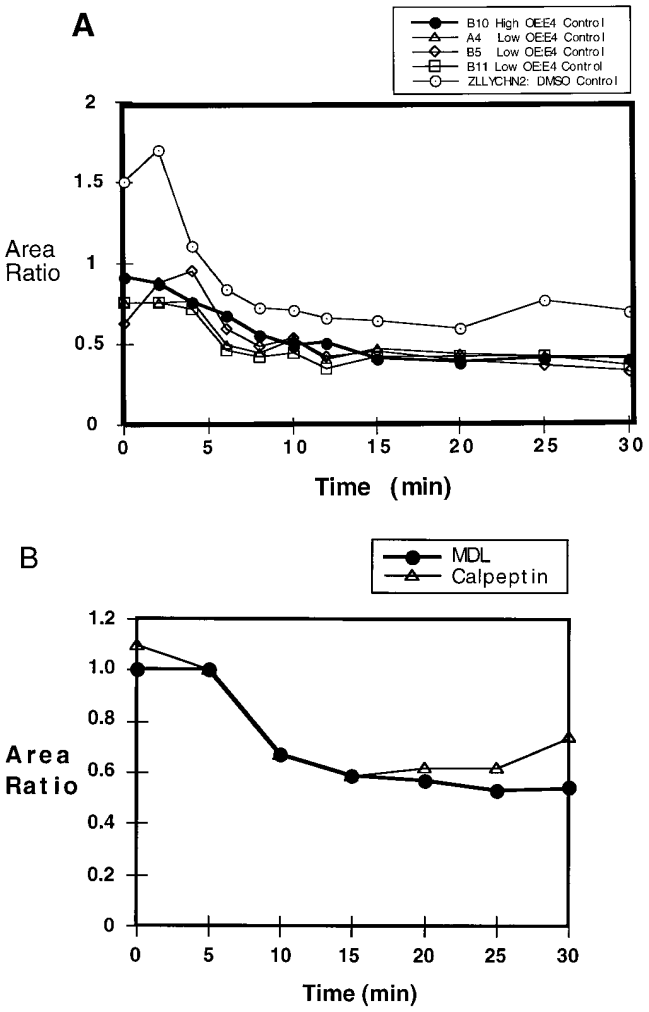

Figure 7. (*A*) The area ratio of calpain-inhibited to control NIH-3T3 fibroblasts plotted as a function of time during spreading on a fibronectin substratum. Cells were spread under the conditions described in Figs. 2 and 4, and their areas were measured as described in Materials and Methods. The spreading data are derived from the high overexpressor cell line B10 and the low overexpressor cell lines A4, B5, and B11. The measurements of $ZLLYCHN_2$-treated cells were derived from the experiment shown in Fig. 4. (*B*) The area ratio of calpain-inhibited to control NIH-3T3 fibroblasts plotted as a function of time during spreading on a fibronectin substratum is shown for calpeptin and MDL-treated fibroblasts (refer to Fig. 4).

Ezrin, a Calpain Substrate, Is Upregulated in Calpastatin-overexpressing Cells

Although numerous proteins involved in cytoskeletal structure and function are cleaved by calpain in vitro, there is limited information on calpain regulation of these substrates in vivo. Ezrin, located at the actin–membrane interface, is one calpain substrate that has been directly demonstrated to be a calpain target in vivo (Shuster and Herman, 1995). Based on this observation, it was predicted that calpain inhibition should result in an increase in steady-state ezrin levels, due to inhibition of basal cytoskeletal remodeling. Steady-state levels of ezrin were therefore determined in calpastatin-overexpressing cells, along with steady-state levels of the closely related proteins radixin and moesin and the more distantly related calpain target, talin. In addition, steady-state levels of calpain targets p125 FAK and PKC-α/-β, as well as the 150-kD breakdown (calpain and/or caspase proteolysis; Nath et al., 1996) product of α-spectrin (150-kD BDP) were determined. These steady-state measurements are important because the effects of the calpain–calpastatin system on the steady-state levels of calpain substrates in vivo are currently unknown. It was postulated that cytoskeletal proteins significantly altered in steady-state level by alteration of the calpastatin–calpain ratio would be potential physiological targets for calpain regulation in vivo.

Steady-state ezrin levels were measured in calpastatin-overexpressing cells. In contrast to the much smaller (two-fold) increases of radixin and moesin, immunoblot analysis

reveals that ezrin is increased sevenfold in the high overexpressor cell lines (refer to Table I). Ezrin levels are even higher in the low overexpressor cell lines, 27-fold above control (refer to Table I), which may be explained by an unexpected 2.3-fold increase in ezrin mRNA (refer to Table II). In contrast, the related proteins moesin and radixin were each increased only twofold in all of the calpastatin-overexpressing cell lines (refer to Table I) and their mRNA levels were not increased. These results are consistent with a 64% increase in ezrin levels ($P = 0.07$ by Student's paired t test) observed when NIH-3T3 cells were treated with $ZLLYCHN_2$ for 18 h, whereas moesin and radixin levels are not increased. The modest increase of ezrin is not unexpected, given the slow cell penetration of $ZLLYCHN_2$.

To determine whether the large increases of ezrin in calpastatin-overexpressing cells were posttranscriptional or due to increased steady-state ezrin mRNA levels, Northern blot analysis of total cellular RNA was done. Ezrin mRNA levels are not increased in the high overexpressor cell lines (refer to Table II), strongly suggesting that the sevenfold elevation of steady-state ezrin levels in these cell lines is posttranscriptional. In contrast, ezrin mRNA levels are increased 2.3-fold in the low overexpressor cell lines, ($P = 0.028$ by Student's paired t test; refer to Table II). Regardless of unexpected, but modest, ezrin mRNA elevations in the low overexpressors, these data collectively indicate that calpastatin overexpression results in an increase in ezrin through posttranscriptional mechanisms. This notion is supported by the modest increase in ezrin levels observed in NIH-3T3 cells treated with $ZLLYCHN_2$. Also of interest, radixin/moesin mRNA levels are not significantly increased in the calpastatin overexpressor cell lines (refer to Table II), suggesting that the increases of radixin and moesin observed in the calpastatin-overexpressing cell lines are due to posttranscriptional events. The increase of ezrin mRNA, in contrast to the lack of increase of radixin and moesin mRNA, in calpastatin overexpressing cells also suggests that, like m-calpain, ezrin gene expression is regulated by the calpain–calpastatin system.

The steady-state levels of other calpain substrates were examined to determine whether calpastatin overexpression raises the steady-state levels of other calpain substrates associated with cell spreading, perhaps due to inhibition of basal proteolysis. Such changes are of interest, since they could potentially affect spreading. In contrast to ezrin, steady-state levels of the ezrin-related calpain substrate talin, which has also been implicated in cell spreading/motility (Nuckolls et al., 1992), are decreased in the low overexpressor cell lines (53% of control) and near wild-type for the high overexpressors (refer to Table I). The PKC-α/-β and p125 FAK, which are calpain substrates likely to be important in cell spreading (Vuori and Ruoslahti, 1993; Lewis et al., 1996), are both near wild-type levels in the high overexpressor cell lines (refer to Table I). p125 FAK levels are also wild-type in the low overexpressors. Unexpectedly, the level of PKC-α/-β is decreased by ~80% in the low overexpressor cell lines (refer to Table I). Thus, talin, PKC-α/-β and p125 FAK steady-state levels are all near wild-type steady-state levels in the high overexpressors, making it unlikely that alteration of their levels is responsible for the inhibition of spreading in the high overexpressor cell lines. Although calpain cleavage products of ezrin, talin, PKC, and p125 FAK could be detected with the antisera used, none of these products were detected in the control or calpastatin-overexpressing cell lines. In contrast, the 150-kD α-spectrin BDP was present in the control cell line and all of the calpastatin-overexpressing cell lines, at equivalent levels (refer to Table I), indicating that regulatory processing of this calpain substrate by calpain and/or caspases (Nath et al., 1996) was unaffected by calpastatin overexpression. Together, the above observations indicate that calpain's regulation of its substrates is complex and that calpastatin overexpression has pleiotropic effects on calpain substrates that cannot be simply explained by inhibition of substrate turnover by calpain. Furthermore, among the spreading-associated calpain substrates examined, ezrin appears to be the protein most stabilized by calpain inhibition. These findings indicate that alteration of the calpain/calpastatin ratio alters ezrin levels.

Discussion

These studies demonstrate the Ca^{2+} and calpain dependence of cell spreading. Calpastatin overexpression and treatment with four distinct calpain inhibitors all inhibit spreading by suppressing the intimately related processes of early filopodial and lamellipodial protrusion formation. Calpain inhibition results in abnormal transient extensions containing a narrow rim of cortical F-actin. When spreading finally occurs in calpain-inhibited cells, the cells are significantly smaller than normal and have abnormal actin networks that lack true lamellipodia. In contrast, stress and retraction fiber formation do not appear to be affected by calpain inhibition. Kinetic analysis of spreading indicates that calpain is involved in spreading almost immediately after cell contact with the substratum. These results indicate that calpain plays a critical, rate-limiting role in the early stages of spreading via its effects on motility and actin network remodeling, perhaps through cleavage of actin-associated cytoskeletal proteins.

Our finding that calpain is involved in lamellipodial protrusion formation is consistent with the recent observation that cell migration is calpain dependent (Huttenlocher, et al., 1997). Although migration studies in CHO cells indicate that calpain inhibition stabilizes peripheral focal adhesions and decreases detachment rates at the rear of the cell, our results reveal the important role that calpain–cytoskeletal interactions play in forward protrusion formation as well. The CHO cell migration studies demonstrated no inhibition of lamellipodial projections and ruffling. In contrast, we find by multiple modalities of calpain inhibition that lamellipodial protrusion formation is a calpain-dependent process. In our hands, extended treatment of crawling NIH-3T3 cells with calpain inhibitors results in very small lamellae that would be difficult to quantitate by video microscopy because of their size and thinness. Thus, our studies point to the importance of calpain-dependent cytoskeletal remodeling, in both the polarized movement of crawling and the nonpolarized movement of spreading. It is therefore proposed that calpain activity is involved both in spreading and crawling, but in the latter case, both at the

rear of the cell and the forward lamella. Calpain may thus coordinately regulate forward protrusion formation and rearward retraction, providing a proteolytic tool to couple Ca^{2+} fluxes and cytoskeletal remodeling in crawling.

The methods of calpain inhibition chosen for these studies were chosen on the basis of their specificity. Calpastatin, which was overexpressed at two different levels in five independent cell lines, inhibits calpain specifically. No other protease has been found to be inhibited by calpastatin, including other sulfhydryl proteases such as cathepsins (Nishiura et al., 1978, 1979; Waxman and Krebs, 1978; for reviews see Murachi et al., 1980; Crawford, 1990; Croall and DeMartino, 1991). The high-affinity binding of calpain to calpastatin, having a dissociation constant of 3.1×10^{-9} M (Yang et al., 1994), is Ca^{2+} dependent (for review see Goll et al., 1992), adding a further level of specificity. Although artificial inhibitors also require Ca^{2+} to interact with calpain, they do not bind to the enzyme with high affinity. The high-affinity interaction between calpain and calpastatin can be used to affinity-purify calpain to homogeneity from crude cell fractions, again suggesting that its interaction with calpain is highly specific (Anagli et al., 1996). Nonetheless, the inhibition of calpain by calpastatin overexpression is not an immediate process and thus peptidyl inhibitors were used to confirm a direct role for calpain in cell spreading.

The peptidyl inhibitors used in these studies, $ZLLYCHN_2$, MDL, calpeptin, and E64d, were chosen on the basis of their specificities. All of these inhibitors inhibit only cysteine proteases. Calpeptin and MDL have similar specificities and inhibit both calpain and cathepsin B (Mehdi, 1991). Calpeptin has also been shown to inhibit cathepsin L (Angliker et al., 1992). E64 inhibits calpain and cathepsins B and L (for review see Wang and Yuen, 1994). $ZLLYCHN_2$, in contrast, inhibits calpain and cathepsin L, but inhibits cathepsin B poorly (Shaw, 1994). $ZLLYCHN_2$, although not immediately cell penetrating, has been found to be the most selective inhibitor, since radioiodinated $ZLLYCHN_2$ primarily labels two cellular proteins, calpain and to a lesser extent, cathepsin L (Anagli et al., 1991, 1993). Together, these data strongly suggest that a cysteine protease is required for spreading. The corresponding NH_4Cl control experiment, demonstrating that lysosomal inhibition does not affect spreading, greatly decreases the likelihood that this cysteine protease is a cathepsin. Finally, we found that MDL is the most potent inhibitor of calpain under the conditions used in our studies. It is well established that E64 is the least potent calpain inhibitor of the above group, with a 50% inhibitory concentration of 1.09 μM for m-calpain by caseinolysis assay (Saito and Nixon, 1993), ~20-fold higher than the corresponding 50% inhibitory concentration of calpeptin (Tsujinaka, 1988). The observation that MDL is the most potent inhibitor of spreading (minimal inhibitory concentration, 50 μM), among the immediately acting calpain inhibitors, whereas E64d is the least potent (minimal inhibitory concentration, 200 μM), indicates that the peptidyl calpain inhibitors inhibit spreading with minimal inhibitory concentrations corresponding to their potency of calpain inhibition. This observation strongly suggests that calpain is the biological target of the immediately acting inhibitors that is relevant to spreading.

These studies demonstrate that calpastatin regulates calpain levels and activity in vivo. The regulatory role of calpastatin on calpain has been studied extensively in vitro (for review see Maki et al., 1990; Mellgren and Lane, 1990; Croall and DeMartino, 1991). The biological importance of the calpain/calpastatin ratio in cells has been demonstrated for myoblast (Barnoy et al., 1996) and erythrocyte (Glaser and Kosower, 1986) fusion. Here, it is found that the reduction of calpain activity in NIH-3T3 fibroblasts induced by calpastatin overexpression can be accounted for mainly by the reduction of calpain itself, as demonstrated in five calpastatin-overexpressing cell lines. This observation is contrary to the simplest model for regulation of calpain activity, in which elevated calpastatin levels inhibit an unaltered level of calpain protease. The finding that the calpastatin/calpain ratio controls the abundance of m-calpain mRNA is also contrary to the simplest model for regulation of calpain gene expression, in which calpastatin should play no role. Our observations suggest that there is a calpastatin-dependent mechanism for turnover of calpain, since calpain downregulation was found to be proportional to calpastatin overexpression. Our observations also suggest that the level of calpain and/or the degree of calpastatin overexpression is connected to m-calpain gene expression. Steady-state levels of m-calpain mRNA are significantly elevated in the low overexpressors, but decreased in the high overexpressors. These unexpected results suggest that there is a calpain-dependent feedback loop that regulates m-calpain gene expression, since modest repression of calpain activity results in compensatory increases in m-calpain mRNA, whereas high repression of calpain activity results not only in loss of the compensatory response, but in a decrease of calpain mRNA. The increased levels of ezrin mRNA in the low overexpressors, but not the high overexpressors, also raises the question of whether the calpain–calpastatin system regulates ezrin gene expression in a similar fashion. Calpastatin overexpressing NIH-3T3 fibroblasts will be ideal for testing the mechanisms by which the calpastatin/calpain ratio is regulated in cells, both at the levels of gene expression and protein turnover.

Calpain inhibition, both biological and pharmacological, results in an increase of the steady-state levels of the actin-associated protein ezrin. These results are consistent with the observation that ezrin is a biological substrate of calpain during cell movement in wounded monolayers (Shuster and Herman, 1995). A role for calpain in the regulation of ezrin is also suggested by the increases in ezrin, in contrast to other ERM proteins, observed with both calpastatin overexpression and $ZLLYCHN_2$-treatment. The greater increase in ezrin protein levels in the low calpastatin overexpressors, compared to the high overexpressors, may be due to increased levels of ezrin mRNA. The mechanisms by which the calpain–calpastatin system regulates ezrin levels will be addressed in future studies.

Although it is possible that the spreading abnormalities of calpastatin-overexpressing cells could be related to increased ERM proteins, which might act in a dominant-negative fashion, this is unlikely. It is known that overexpression of wild-type ezrin, although enhancing adhesion, does not appear to affect cell morphology or spreading (Martin et al., 1995; Crepaldi et al., 1997). Similarly, overexpressed radixin does not appear to affect morphology or

spreading in NIH-3T3 fibroblasts (Henry et al., 1995). Another argument against a dominant-negative role for ezrin is the finding that the excess ERM proteins accumulating in the calpastatin-overexpressing cell lines are present primarily in soluble form in the cytosol, where they are likely to be self-associating and self-inhibitory, in terms of their interaction with either the plasma membrane or actin filaments (Gary and Bretscher, 1993, 1995). Finally, immediate inhibition of cell spreading with MDL and calpeptin is unlikely to alter the levels of cytoskeletal proteins.

Until recently, models of Ca^{2+} function at the lamellipodial protrusion have mainly focused on gelsolin rather than calpain. The Ca^{2+}-dependent actin-severing function of gelsolin provides a Ca^{2+}-regulated mechanism for coordinated uncoupling of the actin cytoskeleton from the cell membrane and simultaneous exposure of barbed ends (Cunningham et al., 1991; Stossel, 1993; Witke et al., 1995). The observations presented here now suggest a second pathway by which Ca^{2+}-transients can regulate the dynamics of the actin cytoskeleton and raises the question of whether some cell types have predominantly a gelsolin-mediated uncoupling and uncapping process whereas others have a predominantly calpain-dependent process. Since motility of NIH-3T3 cells and other fibroblasts is clearly gelsolin dependent (Cunningham et al., 1991; Witke et al., 1995) although NIH-3T3 cell spreading is calpain-dependent, the functions of calpain and gelsolin may be nonredundant. It is also possible that there are subtle differences in lamellipodial actin dynamics between spreading and crawling. The calpain and gelsolin systems may complement or supplement one another during spreading and/or crawling.

There are at least three mechanisms by which calpain could facilitate actin filament extension and thus cell spreading: (*a*) cleavage of ezrin linkages between capped β-actin filaments and the plasma membrane; (*b*) stabilization of phosphatidylinositols that uncap β-actin filaments; and (*c*) activation of the uncapping function of PKC. Supporting the first mechanism, ezrin indirectly binds barbed ends of β-actin filaments, is cleaved by calpain in motile cells, and colocalizes with β-actin and Ca^{2+} transients at the leading lamella of crawling cells (Shuster and Herman, 1995; Shuster et al., 1996). Furthermore, calpain cleavage of ezrin specifically disrupts its linkage with capped β-actin filaments. Calpain could also expose barbed ends for actin polymerization in a coordinated Ca^{2+}-dependent fashion, suggested by the observation that both ezrin and the novel β-actin barbed-end capping protein βcap73 are displaced from β-actin filaments by cytochalasin D (Shuster et al., 1996). A second mechanism by which calpain may function in spreading might be to elevate phosphatidylinositols (PIs), such as PI(4,5)P and PI(3,4)P, which can directly uncap β-actin filaments (Schafer et al., 1996). It has recently been demonstrated that PI-4-phosphatase is a calpain substrate, which can be inactivated by calpain cleavage (Norris et al., 1997). Inactivation of PI-4-phosphatase by calpain could thus elevate PtdIns(3,4)P and PtdIns(4,5)P levels and facilitate uncapping. A third mechanism by which reduction of calpain activity could affect spreading is through inhibition of PKC-dependent pathways of cell shape change. Calpain can cleave PKC to the constitutively active and diacylglycerol/Ca^{2+}-independent product PKM. PKC facilitates cell spreading on fibronectin, although its specific function in spreading remains to be determined (Vuori and Ruoslahti, 1993). One mechanism by which PKC could facilitate spreading is through its modest actin uncapping activity, which has been described in platelets (Hartwig et al., 1995). Nonetheless, given the observation that PKC-α/-β levels vary over a fivefold range between the low and high calpastatin overexpressors with no difference in spreading rates between them, and given the observation that the high calpastatin overexpressors and the control line exhibit no significant difference in the levels of PKC-α/-β, it is unlikely that PKC levels are rate-limiting for spreading in this system.

The demonstration that calpain activity is integral to lamella protrusion formation suggests a functional role for calpain in the regulation of β-actin dynamics at the leading lamella. The β-actin isoform is specifically localized to the lamellae of spreading and motile cells (Hoock et al., 1991; Herman, 1993; Latham et al., 1994; Shuster and Herman, 1995; Bassell et al., 1998). Furthermore, the localization of β-actin mRNA to the leading lamella is important for cell motility (Kislauskis et al., 1997). The localization of β-actin to the leading lamella may be due to local synthesis, since β-actin mRNA is specifically localized to the leading lamella of crawling cells (Hoock et al., 1991; Hill and Gunning, 1993; Kislauskis et al., 1993; Bassell et al., 1998). Lamellar localization of β-actin may also be due to the specific binding of β-actin to ezrin (Herman and Shuster, 1995; Yao et al., 1995, 1996) via βcap73 (Shuster et al., 1996). Electron microscopy has revealed that β-actin at the plasma membrane exists as short oligomers, 7–10 monomers in length (Shujath. J., and I. Herman, manuscript in preparation). Tethering of these β-actin oligomers to the plasma membrane by ezrin may provide a rich source of actin nuclei for the extension of free barbed ends, providing force and structure for lamellipodial extension. Because the binding of β-actin to ezrin has been shown to be indirect, involving the novel β-actin–specific barbed end capping protein, βcap73, it is proposed that calpain proteolysis of substrates, as yet unknown, lowers the affinity of βcap73 for barbed ends of β-actin nuclei or disrupts this interaction. One candidate substrate is ezrin, which has been demonstrated to be cleaved by calpain in motile cells (Shuster and Herman, 1995). The resulting β-actin nuclei would serve as sites for the elongation of barbed ends, facilitating the force pushing against the membrane, resulting in lamellipodial protrusion formation. Calpain proteolysis could thus be one of the mechanisms by which Ca^{2+}-transients that colocalize with ezrin and β-actin at the leading lamella uncap β-actin filaments to initiate lamellipodial protrusion formation (Shuster and Herman, 1995). Such a model would be consistent with the spreading defects seen when calpain is inhibited. Under these conditions, βcap73 would sequester β-actin and thus inhibit monomer addition and spreading. Identification of the biologically relevant targets of calpain, as well as molecular dissection of the interaction between βcap73, ezrin, and cortical β-actin filaments will likely be of critical importance to the understanding of how cytoskeletal remodeling occurs during cell motility and forward protrusion formation and why the β-actin isoform is specifically involved in these processes.

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