Published in final edited form as:
J Cell Sci. 2008 November 1; 121(Pt 21): 3581–3588. doi:10.1242/jcs.036152.

The calpain small subunit regulates cell-substrate mechanical interactions during fibroblast migration

Vishnu V. Undyala[1], **Micah Dembo**[2], **Katherine Cembrola**[3], **Benjamin J. Perrin**[4], **Anna Huttenlocher**[4], **John S. Elce**[5], **Peter A. Greer**[5,6], **Yu-li Wang**[3], and **Karen A. Beningo**[1,*]

[1]Department of Biological Sciences, Wayne State University, Detroit, MI 48202, USA

[2]Department of Biomedical Engineering, Boston University, Boston, MA 02215, USA

[3]Department of Physiology, University of Massachusetts Medical School, Worcester, MA 01605, USA

[4]Department of Pediatrics and Pharmacology, University of Wisconsin Medical School, Madison, WI 53706, USA

[5]Department of Biochemistry Cancer Research Institute, Queen's University, Kingston, Ontario, K7L 3N6 Canada

[6]Department of Pathology and Molecular Medicine, Cancer Research Institute, Queen's University, Kingston, Ontario, K7L 3N6 Canada

Summary

Cell migration involves the dynamic formation and release of cell-substrate adhesions, where the exertion and detection of mechanical forces take place. Members of the calpain family of calcium-dependent proteases are believed to have a central role in these processes, possibly through the regulation of focal adhesion dynamics. The ubiquitous calpains, calpain 1 (μ-calpain) and calpain 2 (m-calpain), are heterodimers consisting of large catalytic subunits encoded by the *Capn1* and *Capn2* genes, respectively, and the small regulatory subunit encoded by *Capn4*. We have examined the role of the calpain regulatory small subunit in traction force production and mechanosensing during cell migration. *Capn4*-deficient or rescued cells were plated on flexible polyacrylamide substrates, for both the detection of traction forces and the application of mechanical stimuli. The total force output of *Capn4*-deficient cells was ~75% lower than that of rescued cells and the forces were more randomly distributed and less dynamic in *Capn4*-deficient cells than in rescued cells. Furthermore, *Capn4*-deficient cells were less adhesive than wild-type cells and they also failed to respond to mechanical stimulations by pushing or pulling the flexible substrate, or by engaging dorsal receptors to the extracellular matrix. Surprisingly, fibroblasts deficient in calpain 1 or calpain 2 upon siRNA-mediated knockdown of *Capn1* or *Capn2*, respectively, did not show the same defects in force production or adhesion, although they also failed to respond to mechanical stimulation. Interestingly, stress fibers were aberrant and also contained fewer colocalised vinculin-containing adhesions in *Capn4*-deficient cells than *Capn1*- and *Capn2*-knockdown cells. Together, these results suggest that the calpain small subunit plays an important role in the production of mechanical forces and in mediating mechanosensing during fibroblast migration. Furthermore, the *Capn4* gene product might perform functions secondary to, or independent of, its role as a regulatory subunit for calpain 1 and calpain 2.

*Author for correspondence (beningo@wayne.edu).

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/121/21/3581/DC1

Keywords

Calpain; Migration; Focal adhesions; Mechanosensing; Traction force

Introduction

Cell migration is a physical event that requires coordination of a number of mechanical activities including protrusion, adhesion, contraction and retraction (Sheetz et al., 1999; Ridley et al., 2003). Mechanical forces of migrating, adhesive cells are transmitted through the adhesions onto the substrate, providing valuable insight into how cells interact physically with the environment to propel their migration (Bershadskey et al., 2003; Chen et al., 2004). Adherent cells also receive physical signals from the environment, such as pushing and pulling forces, changes in the compliance of substrate (Pelham and Wang, 1997; Lo et al., 2000) or changes in the topography of substrate anchorage (Grinnell, 2003; Beningo et al., 2004) (for a review, see Dalby et al., 2004), and show marked responses in the direction of migration and the organization of cytoskeleton (Ingber, 2002). However, little is known about how cells regulate the generation of traction forces or sense external mechanical or topographical signals. A key target site for the regulation of cell migration is at cell-matrix adhesions, where post-translational modifications of proteins such as phosphorylation and proteolysis are known to take place in response to external chemical and physical signals (Burridge and Chrzanowska-Wodnicka, 1996; Sastry and Burridge, 2000; Webb et al., 2002; Flevaris et al., 2007). Members of the calpain family of calcium-dependent cysteine proteases have been found at focal adhesions and are implicated in the turnover of adhesion structures (Beckerle et al., 1987; Bhatt et al., 2002; Franco et al., 2004a). Moreover, a number of proteins involved in motility and adhesion have been identified as potential physiological targets of calpains, including talin, paxillin, vinculin, spectrin and FAK (for a review, see Glading et al., 2002). The most heavily studied calpain isoforms are calpain 1 and calpain 2, also known as μ-calpain and m-calpain, respectively (for a review, see Goll et al., 2003). The holoenzyme consists of an 80 kDa large subunit encoded by the *Capn1* or *Capn2* genes in association with a common 28 kDa small subunit encoded by *Capn4*. Amino acid sequences show that calpain 1 and calpain 2 contain protease domains, glycine-rich domains, and six EF hand sequences. By contrast, the 28 kDa small subunit lacks the protease domain, and functions as a cofactor for the activation of calpains 1 and 2. Although both calpain holocomplexes are regulated by calcium and the specific endogenous inhibitor calpastatin, they differ in calcium sensitivity (for a review, see Goll et al., 2003).

The functions of calpains have been investigated by gene ablation and silencing. Knockout of *Capn1* results in viable offspring with an apparent defect in platelet functions (Azam et al., 2001), whereas ablation of both calpain 1 and calpain 2 activity by deletion of the C-terminal 25 amino acids of the *Capn4* gene product causes embryonic lethality (Arthur et al., 2000). Fibroblasts from these $Capn4^{-/-}$ mice show a reduction in migration rate and number of focal adhesions, with focal complexes localized predominantly at the periphery, suggestive of a defect in adhesion maturation and/or turnover (Dourdin et al., 2001). The observations with $Capn4^{-/-}$ fibroblasts are generally consistent with those using pharmaceuticals or overexpression of calpastatin to interfere with calpain activities (Huttenlocher et al., 1997; Potter et al., 1998; Bhatt et al., 2002). However, a more extensive deletion of the *Capn4* gene resulted in even earlier embryonic death and inability to isolate fibroblasts (Zimmerman et al., 2000; Tan et al., 2006). More recently, siRNA-mediated gene silencing has allowed studies of specific functions of calpain 1 and calpain 2 isoforms in fibroblast migration (Franco et al., 2004b). Although no obvious defect was observed with *Capn1* silencing, silencing of *Capn2* resulted not only in membrane protrusion defects, as

seen in *Capn4*$^{-/-}$ cells, but also in delays in focal adhesion disassembly dynamics (Franco et al., 2004a; Franco et al., 2004b).

Although these studies demonstrate the importance of calpains, they offer limited clues on the role of calpain in the specific events of cell migration. Treatment of migrating fibroblasts with chemical inhibitors of calpain causes elongation of cells under some conditions, suggesting that calpains might be involved in de-adhesion (Huttenlocher et al., 1997). Furthermore, calpain 2 is required for the incorporation of α-actinin (Bhatt et al., 2002) and the turnover of talin at focal adhesions (Franco et al., 2004a), suggesting a role in the maturation or disassembly of focal adhesions. Calpains might also regulate cell migration indirectly through interactions with protein kinases or growth factor receptors (Galding et al., 2000). Further complicating the picture is the relationship between the small and large subunits of calpain, as there are conflicting studies on the importance of the small subunit on the catalytic function of the large subunits (for a review, see Johnson and Guttman, 1997; Goll et al., 2003). Although fluorescence resonance energy transfer (FRET) studies support the interaction of small and large subunits during proteolysis, investigations of the effects of calpain are often complicated by the presence of calcium and endogenous calpastatin (Gil-Parrado et al., 2003).

Given the evidence implicating calpains in cell migration and adhesion, we have explored their role in the production of traction forces and in the response of cells to mechanical signals mediated by integrins. To our surprise we found that traction forces and substrate adhesion were inhibited by the disruption of *Capn4* expression, but not by the inhibition of *Capn1* or *Capn2* or the overexpression of calpastatin. Consistent with these observations, only *Capn4*-deficient cells showed abnormal stress fibers and a reduced number of stress-fiber-associated, vinculin-containing adhesions. However, both the small and the large calpain subunits are required for the response of cells to mechanical forces and substrate topography. Our results suggest that, in addition to the activation of calpain 1 and calpain 2, the small regulatory subunit might perform protease-independent functions in the regulation of traction forces, possibly through a mechanism of tension-induced reinforcement of stress fibers and adhesion.

Results

Capn4-deficient cells generate weaker and less dynamic traction forces than control cells

The functions of the calpain small subunit were investigated with a cell line in which the gene *Capn4* had been disrupted by deletion of the sequence coding for 25 amino acids at the C-terminus (Dourdin et al., 2001). Previous characterization of these *Capn4*$^{-/-}$ cells showed a reduction in migration speed and peripheral localization of focal adhesions. *Capn4*$^{-/-}$ cells stably or transiently re-expressing the 28 kDa rat small subunit were used as controls to ensure the same genetic background. To evaluate the effects of the calpain small subunit on the generation of traction forces, these cells were placed on flexible polyacrylamide substrates covalently coated with fibronectin, and traction stresses (forces per unit area) were calculated based on the deformation of the substrate (Dembo and Wang, 1999) (Fig. 1A,B).

Capn4$^{-/-}$ cells produced significantly less traction forces than rescued cells (0.17±0.079 dynes versus 0.73±0.16 dynes) (Fig. 1C). Moreover, although migrating rescued cells showed typical cycles of increasing and decreasing traction forces, *Capn4*$^{-/-}$ cells maintained a much more stable level of force output (Fig. 2A,B), as indicated by a much higher ratio between the mean and s.d. of integrated forces over the period of observation (Fig. 2C). These observations suggest a defect in the generation and regulation of traction forces.

We also observed that, although most rescued cells showed a defined anterior-posterior polarity in traction forces (Pelham and Wang, 1997), traction forces generated by *Capn4*$^{-/-}$ cells appeared to scatter randomly in several peripheral regions, probably reflecting the protrusion defects described previously (Dourdin et al., 2001; Franco et al., 2004b). The failure to concentrate active forces at a dominant leading edge, as seen in wild-type fibroblastic cells, might lead to the imbalance of forces and an even more severe reduction in energy stored in the substrate than the reduction of forces (Fig. 2D) (0.13×10^{-4} and 1.05×10^{-4} ergs for *Capn4*$^{-/-}$ and rescued cells, respectively). The energy output was obtained by integrating the product of the local traction stress and corresponding substrate displacement, and reflects how effectively the cell uses its traction forces for the deformation of flexible substrates. The lower energy output of *Capn4*$^{-/-}$ cells probably results from opposing forces in different regions of the cell as reflected in Fig. 2A,B,C.

Regulation of traction forces mediated by the calpain regulatory subunit is independent of calpain 1 and calpain 2

The *Capn4* gene product is known to serve as the regulatory subunit for the catalytic subunits calpain 1 and calpain 2 (for a review, see Goll et al., 2003). To determine whether the effects of the regulatory subunit on traction forces were mediated by calpain 1 or calpain 2, we used mouse embryonic fibroblasts (MEFs) and NIH3T3 cells in which *Capn1* or *Capn2* had been stably silenced by siRNA as previously described (Franco et al., 2004a).

Surprisingly, we found no statistically significant difference in the magnitude of traction forces exerted by the *Capn1*- or *Capn2*-silenced cells compared with control cells (Fig. 3A). Similar negative results on traction forces were obtained upon transient overexpression of hrEGFP-calpastatin in MEF cells, whose overexpression inhibits both calpain 1 and calpain 2 (Potter et al., 1998) (Fig. 3A,E), or in MEFs treated with chemical inhibitors of calpain 1 and calpain 2, including calpeptin and MDL (supplementary material Fig. S1). By contrast, similar inhibitory effects were observed upon inhibiting the regulatory subunit with either gene ablation in *Capn4*$^{-/-}$ cells and with siRNA treatment against *Capn4* in wild-type mouse embryonic fibroblasts (Fig. 3A). Effective silencing was confirmed by both immunofluorescence and RT-PCR where *Capn4* mRNA was reduced to 12% of the non-silenced control (Fig. 3B,C,D). Likewise, calpastatin overexpression was confirmed by western blot (Fig. 3E). Furthermore, Calpain activity was found to be equally inhibited in *Capn4*-silenced, *Capn4*$^{-/-}$ and calpastatin-overexpressing MEFs (supplementary material Fig. S2). These results suggest a unique function of the calpain small subunit, possibly independent of its regulatory role for calpain 1 and calpain 2.

Calpain-deficient cells fail to respond to mechanical and topographic signals mediated by the extracellular matrix

Various types of cell have been shown to respond to mechanical stimuli (Lo et al., 2000; Flanagan et al., 2002; Engler et al., 2004; Sieminski et al., 2004). The mechanism might involve calcium entry coupled to calcium-activated activities, such as stimulation of proteolysis by calpain (Lee et al., 1999; Munevar et al., 2004). We therefore tested various calpain-deficient cells for their ability to respond to mechanical signals.

As described in previous studies (Lo et al., 2000), 3T3 fibroblasts on flexible substrates responded to pushing or pulling forces applied by a blunted microneedle in front of the cell. A positive response is recorded when a cell reverses its direction in the case of pushing forces, or advances more rapidly in the case of pulling forces. A negative response is recorded if no change in behavior or migration direction is observed upon pushing or pulling. We discovered that, although all rescued *Capn4*$^{-/-}$ cells responded by advancing towards pulling forces or away from pushing forces (n=6) within 40 minutes, none of the

Capn4$^{-/-}$ cells responded (*n*=6) (Fig. 4). Unlike the experiments on traction forces, all *Capn1*-knockdown cells (*n*=6) and 85% of *Capn2* knockdown cells (6 of 7 cells) also failed to show a response, as did cells transfected with calpastatin, whereas all cells transfected with scrambled RNA responded normally (*n*=6) (supplementary material Movies 1–5).

We utilized a double-hydrogel substrate to test the responses of cells to the topography of extracellular matrix (ECM) engagement (Beningo et al., 2004; Beningo and Wang, 2006). Fibroblasts grown in a two-dimensional environment adopt a well spread morphology, in contrast to the elongated spindle shape found in a three-dimensional environment (Friedl and Brocker, 2000; Grinnell, 2003). We have recently demonstrated that this response is modulated by the engagement of dorsal ECM receptors (Beningo et al., 2004), by sandwiching fibroblasts between two ECM coated polyacrylamide substrates. We found that rescued *Capn4*$^{-/-}$ cells showed a similar elongation response to the presence of top substrate (Fig. 5C). By contrast, *Capn4*$^{-/-}$ cells maintained a similar shape in the presence or absence of the top substrate (compare Fig. 5B and 5D), as confirmed by the measurement of aspect ratio (Fig. 5E). As for the response to mechanical forces, *Capn1*- and *Capn2*-knockdown cells, calpastatin-transfected cells and cells treated with calpain inhibitors, also failed to respond to the top substrate. These results suggest that the regulatory subunit performs overlapping functions with calpain 1 and calpain 2 with regards to the detection of mechanical and topographic signals, possibly by functioning as a complex.

Cells deficient in *Capn4*, but not *Capn1* or *Capn2*, are defective in substrate adhesion and in stress fiber organization

The defect in traction forces unique to the *Capn4*-deficient cells may be due to defects in substrate adhesions that transmit forces. To determine whether substrate adhesion was weakened in calpain-deficient MEFs, *Capn4*$^{-/-}$ cells, *Capn1*- and *Capn2*-knockdown cells and MEFs overexpressing calpastatin were seeded onto fibronectin coated polyacrylamide substrates and allowed to adhere for 30 minutes before performing a centrifugation detachment assay. Compared with controls, only 14% of the *Capn4*$^{-/-}$ cells remained adherent whereas *Capn1*- and *Capn2*-knockdown cells showed only a slight reduction in adhesiveness (Fig. 6A). Greatly reduced adhesiveness was also observed when wild-type MEFs were treated with siRNA against *Capn4*.

Consistent with previous observations (Dourdin et al., 2001), we found that vinculin-containing adhesions in *Capn4*$^{-/-}$ cells were localized to the periphery of the cell and that actin stress fibers were not only fewer but also less prominent than in wild-type MEFs (Fig. 6B–E). This abnormal organization of adhesions and stress fibers was not observed by immunofluorescence in the calpain-1- and calpain-2-deficient cells or in MEFs overexpressing calpastatin (supplementary material Fig. S3). Furthermore, significantly fewer vinculin-containing adhesions colocalized with stress fibers in the anterior region of *Capn4*$^{-/-}$ cells than in the same region of control cells (Fig. 6F). Conversely, calpain-1- and calpain-2-deficient cells, and calpastatin-overexpressing cells showed only a slight difference in the number of adhesions colocalizing with stress fibers (Fig. 6F). This difference in stress-fiber-associated adhesions is probably responsible for the abnormal traction stress and migration, as well as the reduced adhesiveness observed uniquely in the *Capn4*-deficient cells.

Discussion

Active traction forces are generated near the leading edge of migrating adherent cells and are associated with the maturation of focal complexes into focal adhesions (Beningo et al., 2001). These forces are involved in a number of events during cell migration, including the detachment of adhesions, the retraction of cell body, and the regulation of adhesion

maturation. Moreover, traction forces might be involved in the communication of cells with the environment, by providing probing forces for the rigidity, sending mechanical signals to neighboring cells, and organizing the extracellular matrix. These signals probably play a role in such important processes as embryogenesis, angiogenesis and wound healing (Ingber, 2003).

At first glance, a process of calpain-mediated proteolysis may seem inefficient for controlling a prolonged function such as cell migration. However, owing to its irreversibility, proteolysis may represent the optimal mechanism for activating irreversible events, such as maturation of focal adhesions and detachment of adhesions. Previous studies have indeed implicated proteolytic activities by calpains in the de-adhesion process and in the turnover of proteins at focal adhesions (Huttenlocher et al., 1997). Experiments with *Capn4*$^{-/-}$ cells further demonstrate the essential role of calpains in maintaining the rate of migration and the organization of focal adhesions (Dourdin et al., 2001). Given these observations, we were curious to determine how calpains might influence the generation of traction forces. We initially chose to use *Capn4*$^{-/-}$ cells because they are known to be deficient in both calpain-1- and calpain-2-mediated proteolysis (Dourdin et al., 2001).

From the involvement of calpains in protein turnover at focal adhesions, one might expect an increase in traction force in *Capn4*$^{-/-}$ cells, as a result of stabilization of focal adhesions. However, we were surprised to find a decrease in traction forces for *Capn4*$^{-/-}$ and knockdown cells. In addition, traction forces in *Capn4*$^{-/-}$ were disorganized, resulting in a dramatic decrease in the amount of energy stored in the substrate. Reduction in forces may be explained at least partially by the abnormal organization of adhesions and stress fibers and the decrease in cell-substrate adhesive strength, as shown in the present study, which might inhibit the transmission of contractile forces to the substrate. Given that cellular tension is probably maintained by a feedback loop between the adhesion structures and stress fibers, the disruption in stress fiber organization may be due to either a defective linkage between actin fibers and the adhesion structures, or defects in the assembly process of the fiber itself. Interestingly, despite the weaker adhesions of *Capn4*$^{-/-}$ fibroblasts compared with those of *Capn1*- and *Capn2*-knockdown cells and cells overexpressing calpastatin, individual focal adhesions in these cells show a similar gross morphology (length and width), suggesting that the regulatory subunit regulates focal adhesions and traction forces not through the proteolysis of structural components but through a signaling event that triggers a contractility cascade. Therefore, a second possibility is that the regulatory subunit may be required for the activation of contractility or the transmission of forces to focal adhesions, which might in turn affect the strength of adhesions and the maturation of focal adhesions through `inside-out' signaling (Burridge and Chrzanowska-Wodnicka, 1996; Bershadsky et al., 2003).

As demonstrated in the present study, a significant, previously unknown, function of the calpain regulatory subunit is to mediate responses to the engagement of dorsal integrins and to integrin-mediated mechanical signals. Previous studies have shown that tensile forces stimulate lamellipodial extension, maturation of focal adhesions, and translocation of focal adhesions toward interior regions of the cell (Ridley et al., 2003). Although a detailed mechanism is unclear, one possible pathway involves stretch-activated ion channels (Lee et al., 1999). Mechanical signals stimulate calcium entry through these channels (Munevar et al., 2004), which may in turn activate calpain and other proteins, including calmodulin and myosin light chain kinase. Consistent with this idea, fibroblasts depleted of calcium failed to respond to topographical signals as for cells defective in calpain (Beningo et al., 2004). In addition, calpain is required for α-actinin localization to focal contacts, which is probably a critical event in the maturation of focal adhesions and transmission of mechanical signals (Bhatt et al., 2002).

One of the most intriguing findings is the difference between inhibition of the calpain regulatory subunit and inhibition of the catalytic subunits calpain 1 or calpain 2, either individually or simultaneously through the overexpression of calpastatin. If the sole function of the small subunit were to regulate calpain 1 and calpain 2, one would expect disruption of *Capn4* to generate identical or possibly a subset of phenotypes of the ablation of *Capn1* and *Capn2*. Instead, we showed that *Capn4* ablation inhibits both the generation of traction forces and responses to mechanical signals, whereas inhibition of calpain 1 and/or calpain 2 inhibits only mechanosensing. It is difficult to attribute these results to technical aspects of the experiments, because similar results were obtained with multiple approaches: we have inhibited *Capn4* with both gene ablation and siRNA-mediated gene silencing, and calpain 1 or calpain 2 with siRNA knockdown by both stable and transient approaches. In addition, calpain 1 and calpain 2 were inhibited simultaneously with pharmacological reagents and with overexpression of calpastatin.

It is possible that the differences between mechanosensing and traction forces of catalytic and regulatory subunits might be due to different extents of suppression or sensitivities in the mechanical assays. However, one argument against this possibility is that when comparing *Capn4*$^{-/-}$ cells, *Capn4*-silenced and calpastatinoverexpressing cells, all inhibited the digestion of the substrate ac-LLVY-AFC to the same extent (supplementary material Fig. S2), yet only the knockout cells were defective in the production of traction forces. The simplest explanation of our observations is that mechanosensing requires the holo-complexes of the regulatory subunit and catalytic subunits of both calpain 1 and calpain 2, whereas the generation of traction forces is regulated by the regulatory subunit, independently of its activation of the proteolytic activities of calpain 1 and calpain 2. Alternatively, the generation of traction forces might be mediated redundantly by either calpain 1 or calpain 2, whereas mechanosensing requires both isoforms, although again the results from overexpression of calpastatin would argue against this. Thus the processes of force generation and mechanosensing are likely to be temporally separated events, with the generation of forces occurring at an early stage of focal adhesion formation (Beningo et al., 2001; Zaidel-Bar et al., 2003) and the mechanosensitive maturation and translocation of focal adhesions to the interior region occurring subsequently or prior to adhesion formation. Although it is unclear how the calpain small subunit might perform a function without calpain 1 and calpain 2, its involvement in a broader range of functions than those of calpain 1 or calpain 2 is supported by the observation that *Capn4*$^{-/-}$ mice and *Capn2*$^{-/-}$ mice (Dutt et al., 2006) die at different stages of early fetal development whereas *Capn1*$^{-/-}$ mice show normal early development (Azam et al., 2001).

It is possible that the calpain small subunit functions in conjunction with another calpain such as calpain 9, which required the *Capn4* gene product to activate its proteolytic activity in vitro (Lee et al., 1998). Interestingly, deficiency of calpain 9 resulted in tumorigenesis and transformation of NIH3T3 cells (Liu et al., 2000). Several studies have also revealed novel binding partners of the *Capn4* gene product, such as the class II G-protein-coupled receptor and parathyroid hormone-related peptide receptor (PTH1R), whose stimulation by parathyroid stimulating hormone (PTH) increases the intracellular levels of cAMP, IP3, DAG and Ca^{2+} (Shimada et al., 2005). The calpain small subunit also binds to the αPIX, a Rho-GTPase guanine nucleotide exchange factor (GEF) thought to be involved in integrin-induced signaling (Rosenberger et al., 2005). These protein interactions could mediate functions independent of the proteolytic activities of calpain.

In summary, we have discovered previously unknown functions for the calpain small subunit as a regulator of traction forces and downstream events such as the strengthening of adhesions, independently of calpain 1 and calpain 2. In addition, the protein products of *Capn1*, *Capn2* and *Capn4* are all required for sensing environmental mechanical and

topographical signals, and the associated remodeling of the actin cytoskeleton and adhesions. Our results provide further insight into the functional diversity of calpains as well as their possible interactions with signaling pathways that are independent of proteolysis.

Materials and Methods

Cell culture, transfections and immunofluorescence staining

Mouse embryonic fibroblasts (MEFs) from embryos expressing a defective small calpain subunit have previously been described (Arthur et al., 2000; Dourdin et al., 2001), and will be referred to as *Capn4*$^{-/-}$ fibroblasts. Calpain activity was partially restored by stable transfection with the plasmid pSBC-r28kDa encoding the full-length rat calpain small subunit as described (Dourdin et al., 2001), or through transient cotransfection of this construct with EGFP-Capn4. The plasmid hrEGFP-calpastatin, as described previously (Bhatt et al., 2002), was transfected into MEF cells with an Amaxa nucleofector and kit R (Amaxa, Gaithersburg, MD). Cells brightly fluorescing with EGFP, suggestive of a high level of expression of calpastatin and strong suppression of calpain proteolytic activity (Potter et al., 1998), were used for data collection. Overexpression of calpastatin was confirmed by western blot using a 10% Tris-HEPES-SDS precast polyacrylamide gel system (Pierce, Rockford, IL) and transfer onto PVDF membranes. Blots were probed with a 1:200 dilution of polyclonal anti-calpastatin (Santa Cruz,), an HRP-conjugated anti-rabbit secondary (Amersham, UK) and developed with the Amersham ECL-plus chemiluminescence kit.

All cell cultures were maintained in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated at 37°C under 5% humidified CO_2. Cells were fixed for immunofluorescence in 4% paraformaldehyde and 0.1% Triton X-100 for 10 minutes, blocked for 1 hour with 5% BSA and incubated with 1:100 anti-vinculin (vin-11-5, Sigma, MO) and Alexa Fluor 488 anti-mouse secondary (Invitrogen, CA) and Alexa Fluor 546 phalloidin (Invitrogen, CA). A 1:200 Cy5 (650 nm) anti-mouse secondary antibody (Chemicon, MA) was used when EGFP-calpastatin was overexpressed. Collected images were pseudocolored and overlaid to identify areas of colocalization. To obtain the percentage of vinculin-containing adhesions attached to stress fibers in the anterior region, a straight line was first drawn perpendicular to the long axis of the cell along the front edge of the nucleus. The number of vinculin plaques that colocalize with actin fibers located between the line and the leading edge was then counted and divided by the total number of vinculin plaques in this region.

Gene silencing with siRNA

Wild-type MEFs were also used for selectively silencing *Capn4* via siRNA. The knockdown was generated through transient transfection of siRNA oligonucleotides using the SMARTpool system of Dharmacon. The efficiency of silencing is illustrated by the dramatic difference in immunofluorescence staining intensity between control cells and silenced cells, using an antibody against the N-terminus of the small subunit (Triple Point Biologics) and by real-time RT-PCR. For RT-PCR analysis, total RNA was extracted from control and silenced cells using the RnaEasy kit (Qiagen) and reverse transcribed using random hexamer primers and the Ready-to-go you-prime first-strand beads (Amersham Biosciences). The *Capn4* primers used were 5′-ACTATCGGTAGCCATGAACTCCCA-3′ and 5′-ATCCATGTTTCCGCTCTCATCTGC-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the control gene and the forward and reverse primers were respectively TCAACAGCAACTCCCACTCTTCCA and ACCCTGTTGCTGTAGCCGTATTCA. Amplification reactions were performed with 500 ng total cellular RNA and 0.3 μM primers in duplicate for each cell type. Reverse transcription was performed for 30 minutes at 60°C.

Reactions were heat denatured for 2 minutes at 95°C and 60 cycles of real time quantitative PCR were carried out using SYBR Green PCR Master Mix Reagents (ABGene) in a MX300P (Stratagene) machine. The results were calculated with the Stratagene system using the Comparative CT method for relative quantification. The average CT value of *Capn4*-silenced cells and control cells were calculated to be 18.52 and 15.55, respectively. Given the exponential nature of the amplification, the results are calculated as 2^n, where n=CT values between the test and control. The results indicate an 88% decrease in RNA levels in *Capn4*-silenced cells compared with the control cells. Stable suppression of *Capn1* and *Capn2* expression in MEF cells has been previously described (Franco et al., 2004a; Franco et al., 2004b).

Calpain activity assay

Calpain activity was quantified by using a commercially available calpain activity assay kit (Biovision, Mountain View, CA) as suggested by the manufacturer's instructions, with a modified lysis buffer containing 0.5% Triton X-100. Briefly, cell extracts (50 μg of protein) were mixed with the substrate Ac-LLY-AFC, incubated for 1 hour at 37°C in the dark. Reactions were measured at 400/505 nm with a SpectraMax M2 Microplate Reader (Molecular Devices, Sunnyvale, CA).

Preparation of polyacrylamide substrates

Flexible polyacrylamide substrates were prepared and coated with bovine plasma fibronectin (Sigma) at 5 $\mu g/cm^2$, as described previously (Beningo et al., 2002). All substrates used in this study were 5% acrylamide, and either 0.1% or 0.08% *N*,*N*-methylene-bis-acrylamide (bis) unless otherwise specified. The Young's modulus of the substrate was estimated to be 2.8×10^4 N/m^2 (0.1% bis) or 2.4×10^4 N/m^2 (0.08% bis), determined as described previously using a bead indentation method (Lo et al., 2000). Cells were seeded onto the substrates overnight before experiments. Traction data were collected at either single time points or over a time period at specified intervals.

Microscopy

All images were acquired with either a Zeiss Axiovert S100 or an Olympus IX81 ZDC inverted microscope, equipped with a 10×/0.25 NA CP-Achromat lens and a 40×/0.75 NA Plan-Neofluar lens for phase-contrast images, and a Nikon 60×/1.20 NA PlanApo water immersion lens with a Zeiss adaptor lens for fluorescence images. Live cells were imaged at 37°C on a microscope equipped with a custom stage incubator. All images were acquired with either a Roper Scientific (Trenton, NJ) 512BFT cooled CCD camera and a ST133 controller, driven by custom software or a Diagnostic Instruments (Sterling Heights, MI) Boost EM-CCD-BT2000 back-thinned camera driven by IPLab software (BD Biosciences). Dark counts were subtracted from the images before images were stored as tiffs.

Applications of mechanical stimulations

Mechanosensing experiments were performed essentially as described previously (Lo et al., 2000). Cells were plated onto substrates of 5% polyacrylamide, 0.1% bis coated with fibronectin and embedded with 0.2 μm fluorescent microbeads. After observing a cell for approximately 10 minutes to determine the trajectory of migration, a blunted microneedle was positioned onto the flexible substrate in front of the migrating cell. The microneedle was used to gently push the substrate toward the cell without touching the cell. This relaxes the tension on the substrate and causes retraction of the leading edge (Lo et al., 2000). Images were collected every 3 minutes for approximately 1 hour. Fluorescent images of the microbeads, taken before and after releasing the microneedle from the substrates, were used to judge the extent and direction of mechanical forces applied.

Analysis of traction forces and energy output

Data for the analysis of traction forces were collected as previously described (Beningo et al., 2002). Fluorescent microbeads embedded in the substrate were used to track substrate deformation. Images of null force were obtained by removing the cell with a microneedle. Traction stresses were calculated and rendered as described previously (Dembo and Wang, 1999; Marganski et al., 2003). The average integrated traction force magnitude was used directly for comparison. Energy stored in the substrate was calculated by integrating the product of traction force and corresponding deformation at each pixel over the entire cell.

Application of topographic stimulation by double-substrate cultures

The double-substrates culture system has been described previously (Beningo et al., 2004). To prepare the double substrate, cells were seeded onto the surface of a polyacrylamide substrate overnight. Prior to sandwiching, extraneous medium was removed by aspiration. The top polyacrylamide sheet, attached to a coverslip, was gently laid over the bottom substrate. A square piece of glass (20×20×5 mm, 4.4 g) was then placed on the coverslip attached to the top substrate to stabilize the sandwich. To reduce the distance between the top and bottom substrates, a weight of 30 g was applied for 30 seconds with a minimal amount of surrounding medium. The medium was then replenished and cells observed 24 hours after sandwiching. Distance between the substrates was determined using calibrated microscope focusing mechanism, moving from the top surface of the bottom substrate to the bottom surface of the top substrate as guided by the embedded fluorescent microbeads. Initial contact between the top substrate and the cell occurs over the nucleus (Beningo et al., 2004), only the cells actively engaging these receptors will transduce the signal to change their morphology.

Cell adhesion assay

A centrifugation assay was used to measure cell-substrate adhesiveness. This assay was a slight modification of that described by Guo et al. (Guo et al., 2006). Briefly 2.5×10^4 cells were seeded onto fibronectin-coated substrates contained within specially designed chambers and allowed to adhere for 30 minutes at 37°C. The chambers were then centrifuged in a Beckman (Fullerton, CA) TJ-6 centrifuge with a TH-4 rotor for 5 minutes at 1800***g***. Ten fields of cells were counted for each condition and expressed as a percentage of the control value. The experiments were performed in triplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Jim Tucker and Robert Thomas (Wayne State University) for assistance with RT-PCR. This project was supported by funds from Wayne State University (K.A.B.), NIH grants GM-32476 (Y.L.W.), T32GM07215-25 (B.J.P.), R01 CA85862-01 (A.H.), GM-61806 (M.D.) and a CIHR grant to P.A.G.

References

Arthur JS, Elce JS, Hegadorn C, Williams K, Greer PA. Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division. Mol. Cell. Biol. 2000; 20:4474–4481. [PubMed: 10825211]

Azam M, Andrabi SS, Sahr KE, Kamath L, Kuliopulos A, Chishti AH. Disruption of the mouse mu-calpain gene reveals an essential role in platelet function. Mol. Cell. Biol. 2001; 21:2213–2220. [PubMed: 11238954]

Beckerle MC, Burridge K, DeMartino GN, Croal DE. Co-localization of calcium-dependent protease II and one of its substrates at sites of cell adhesion. Cell. 1987; 51:569–577. [PubMed: 2824061]

Beningo KA, Wang Y-L. Double hydrogel substrate as a model system for three-dimensional cell culture. Methods Mol. Biol. 2006; 370:203–211. [PubMed: 17416996]

Beningo KA, Dembo M, Kaverina I, Small JV, Wang Y-L. Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts. J. Cell Biol. 2001; 153:881–887. [PubMed: 11352946]

Beningo KA, Lo C-M, Wang Y-L. Flexible polyacrylamide substrata for the analysis of mechanical interactions at cell-substratum adhesions. Methods Cell Biol. 2002; 69:325–339. [PubMed: 12071003]

Beningo KA, Dembo M, Wang Y-L. Responses of fibroblasts to anchorage of dorsal extracellular matrix receptors. Proc. Natl. Acad. Sci. USA. 2004; 101:18024–18029. [PubMed: 15601776]

Bershadsky AD, Balaban NQ, Geiger B. Adhesion dependent cell mechanosensitivity. Annu. Rev. Cell Dev. Biol. 2003; 19:677–695. [PubMed: 14570586]

Bhatt A, Kaverina I, Otey C, Huttenlocher A. Regulation of focal complex composition and disassembly by the calcium-dependent protease calpain. J. Cell Sci. 2002; 115:3415–3425. [PubMed: 12154072]

Burridge K, Chrzanowska-Wodnicka M. Focal adhesions, contractility, and signaling. Annu. Rev. Cell Dev. Biol. 1996; 12:463–518. [PubMed: 8970735]

Chen CS, Tan J, Tien J. Mechanotransduction at cell-matrix and cell-cell contacts. Annu. Rev. Biomed. Eng. 2004; 6:275–302. [PubMed: 15255771]

Dalby MJ, Riehle MO, Sutherland DS, Agheli H, Curtis AS. Use of nanotopography to study mechanotransduction in fibroblasts-methods and perspectives. Eur. J. Cell Biol. 2004; 83:159–169. [PubMed: 15260438]

Dembo M, Wang Y-L. Stresses at the cell-to-substrate interface during locomotion of fibroblasts. Biophys J. 1999; 76:2307–2316. [PubMed: 10096925]

Dourdin N, Bhatt AK, Dutt P, Greer P, Arthur JS, Elce JS, Huttenlocher A. Reduced cell migration and disruption of the actin cytoskeleton in calpain-deficient embryonic fibroblasts. J. Biol. Chem. 2001; 276:48382–48388. [PubMed: 11602605]

Dutt P, Croall DE, Arthur JS, Veyra TD, Williams K, Elce JS, Greer PA. m-Calpain is required for preimplantation embryonic development in mice. BMC Dev. Biol. 2006; 6:3. [PubMed: 16433929]

Engler AJ, Griffin MA, Sen S, Bonnemann CG, Sweeney HL, Discher DE. Myotubes differentiate optimally on substrates with tissue-like stiffness: pathological implications for soft or stiff microenvironments. J. Cell Biol. 2004; 166:877–887. [PubMed: 15364962]

Flanagan LA, Ju YE, Marg B, Osterfield M, Janmey PA. Neurite branching on deformable substrates. NeuroReport. 2002; 13:2411–2415. [PubMed: 12499839]

Flevaris P, Stojanovic A, Gong H, Chishti A, Welch E, Du X. A molecular switch that controls cell spreading and retraction. J. Cell Biol. 2007; 179:553–565. [PubMed: 17967945]

Franco SJ, Rodgers MA, Perrin BJ, Han J, Bennin DA, Critchley DR, Huttenlocher A. Calpain-mediated proteolysis of talin regulates adhesion dynamics. Nat. Cell Biol. 2004a; 6:977–983. [PubMed: 15448700]

Franco SJ, Perrin BJ, Huttenlocher A. Isoform specific function of calpain 2 in regulating membrane protrusion. Exp. Cell Res. 2004b; 299:179–187. [PubMed: 15302585]

Friedl P, Brocker EB. The biology of cell locomotion within three-dimensional extracellular matrix. Cell Mol. Life Sci. 2000; 57:41–64. [PubMed: 10949580]

Gil-Parrado S, Popp O, Knoch TA, Zahler S, Bestvater F, Felgenträger M, Holloschi A, Fernández-Montalván A, Auerswald EA, Fritz H, et al. Subcellular localization and in vivo subunit interactions of ubiquitous mu-calpain. J. Biol. Chem. 2003; 278:16336–16346. [PubMed: 12591934]

Glading A, Chang P, Lauffenburger DA, Wells A. Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. J. Biol. Chem. 2000; 275:2390–2398. [PubMed: 10644690]

Glading A, Lauffenburger DA, Wells A. Cutting to the chase: calpain proteases in cell motility. Trends Cell Biol. 2002; 12:46–54. [PubMed: 11854009]

Goll DE, Thompson VF, Li H, Wei W, Cong J. The calpain system. Physiol. Rev. 2003; 83:731–801. [PubMed: 12843408]

Grinnell F. Fibroblast biology in three-dimensional collagen matrices. Trends Cell Biol. 2003; 13:264–268. [PubMed: 12742170]

Guo W-H, Frey MT, Burnham NA, Wang Y-L. Substrate rigidity regulates the formation and maintenance of tissues. Biophys. J. 2006; 90:2213–2220. [PubMed: 16387786]

Huttenlocher A, Palecek SP, Lu Q, Zhang W, Mellgren RL, Lauffenburger DA, Ginsberg MH, Horwitz AF. Regulation of cell migration by the calcium-dependent protease calpain. J. Biol. Chem. 1997; 272:32719–32722. [PubMed: 9407041]

Ingber DE. Mechanical signaling. Ann. N. Y. Acad. Sci. 2002; 961:162–163. [PubMed: 12081890]

Ingber DE. Mechanobiology and diseases of mechanotransduction. Ann Med. 2003; 35:564–577. [PubMed: 14708967]

Johnson GVW, Guttman RP. Calpains: intact and active? BioEssays. 1997; 19:1011–1018. [PubMed: 9394623]

Lee H-J, Sorimachi H, Jeong SY, Ishiura S, Suzuki K. Molecular cloning and characterization of a novel tissue specific calpain predominantly expressed in the digestive tract. Biol. Chem. 1998; 379:175–183. [PubMed: 9524069]

Lee J, Ishihara A, Oxford G, Johnson B, Jacobson K. Regulation of cell movement is mediated by stretch-activated calcium channels. Nature. 1999; 400:382–386. [PubMed: 10432119]

Liu K, Li L, Cohen SN. Anti-sense RNA-mediated deficiency of the calpain protease, nCL-4, in NIH3T3 cells is associated with neoplastic transformation and tumorigenesis. J. Biol. Chem. 2000; 275:31093–31098. [PubMed: 10906334]

Lo C-M, Wang H-B, Dembo M, Wang Y-L. Cell movement is guided by the rigidity of the substrate. Biophys. J. 2000; 79:144–152. [PubMed: 10866943]

Marganski WA, Dembo M, Wang Y-L. Measurements of cell-generated deformations on flexible substrata using correlation-based optical flow. Meth. Enzymol. 2003; 361:197–121. [PubMed: 12624913]

Munevar S, Wang Y-L, Dembo M. Regulation of mechanical interactions between fibroblasts and the substratum by stretch-activated Ca^{2+} entry. J. Cell Sci. 2004; 117:85–92. [PubMed: 14627625]

Pelham RJ, Wang Y-L. Cell locomotion and focal adhesions are regulated by substrate flexibility. Proc. Natl. Acad. Sci. USA. 1997; 94:13661–13665. [PubMed: 9391082]

Potter DA, Trinauer JS, Janssen R, Croall DE, Hughes CN, Fiacco KA, Mier JW, Maki M, Herman IM. Calpain regulates actin remodeling during cell spreading. J. Cell Biol. 1998; 141:647–662. [PubMed: 9566966]

Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR. Cell migration: integrating signals from front to back. Science. 2003; 302:1704–1709. [PubMed: 14657486]

Rosenberger G, Gal A, Kutsche K. AlphaPIX associates with calpain 4, the small subunit of calpain, and has a dual role in integrin-mediated cell spreading. J. Biol. Chem. 2005; 280:6879–6889. [PubMed: 15611136]

Sastry SK, Burridge K. Focal adhesions: a nexus for intracellular signaling and cytoskeletal dynamics. Exp. Cell Res. 2000; 261:25–36. [PubMed: 11082272]

Sheetz MP, Felsenfeld D, Galbraith CG, Choquet D. Cell migration as a five-step cycle. Biochem. Soc. Symp. 1999; 65:233–243. [PubMed: 10320942]

Shimada M, Mahon MJ, Greer PA, Segre GV. The receptor for parathyroid hormone and parathyroid hormone-related peptide is hydrolyzed and its signaling properties are altered by directly binding the calpain small subunit. Endocrinology. 2005; 146:2336–2344. [PubMed: 15691895]

Sieminski AL, Hebbel RP, Gooch KJ. The relative magnitudes of endothelial force generation and matrix stiffness modulate capillary morphogenesis *in vitro*. Exp. Cell Res. 2004; 297:574–584. [PubMed: 15212957]

Tan Y, Dourdin N, Wu C, De Veyra T, Elce JS, Greer PA. Conditional disruption of ubiquitous calpains in the mouse. Genesis. 2006; 44:297–303. [PubMed: 16783822]

Webb DJ, Parsons JT, Horwitz AF. Adhesion assembly, disassembly and turnover in migrating cells: over and over and over again. Nat. Cell Biol. 2002; 4:E97–E100. [PubMed: 11944043]

Zaidel-Bar R, Ballestrem C, Kam Z, Geiger B. Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. J. Cell Sci. 2003; 116:4605–4613. [PubMed: 14576354]

Zimmerman UJ, Boring L, Pak JH, Mukerjee N, Wang KK. The calpain small subunit gene is essential: its inactivation results in embryonic lethality. IUBMB Life. 2000; 50:63–68. [PubMed: 11087123]

Fig. 1.

Inhibition of traction forces in *Capn4*$^{-/-}$ fibroblasts. (A,B) Vector plots of traction stress indicate magnitude and direction of traction stresses exerted by cells on the substrate, for *Capn4*$^{-/-}$ cells stably re-expressing the small subunit (A) and *Capn4*$^{-/-}$ cells (B). (C) Bar graph shows average integrated traction forces magnitude from each type of the cells. *Capn4*$^{-/-}$ cells show a significant reduction in traction forces compared with wild-type or rescued cells (Student's *t*-test *P*=0.0003).

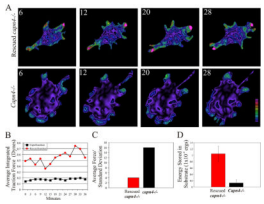


Fig. 2.

Defects in the dynamics of traction forces and energy output for *Capn4*$^{-/-}$ fibroblasts. (A) Color rendering of the magnitude of traction stress at 6, 12, 20 and 28 minutes in rescued *Capn4*$^{-/-}$ fibroblasts and in Capn*4*$^{-/-}$ cells shows weak, scattered traction forces for *Capn4*$^{-/-}$ fibroblasts, whereas control cells exert strong forces at the leading and trailing edges. Color scale represents a minimum magnitude of 1×10^3 dynes/cm^2 and a maximum of 2.9×10^5 dynes/cm^2. (B) Plots of average integrated traction forces against time show a much higher degree of dynamics for rescued *Capn4*$^{-/-}$ cells than *Capn4*$^{-/-}$ fibroblasts. (C) Dynamics of traction forces measured by taking a ratio between average integrated traction forces and the s.d. measured over a period of 45 minutes. Rescued *Capn4*$^{-/-}$ fibroblasts have a much smaller value than *Capn4*$^{-/-}$ cells (four cells, each recorded over 45 minutes), indicating a larger fluctuation in forces. The difference in the energy stored in the substrate is even more striking (Student's *t*-test *P*=0.00001), with a much lower output for *Capn4*$^{-/-}$ fibroblasts than rescued cells (*n*=13 and *n*=23, respectively).

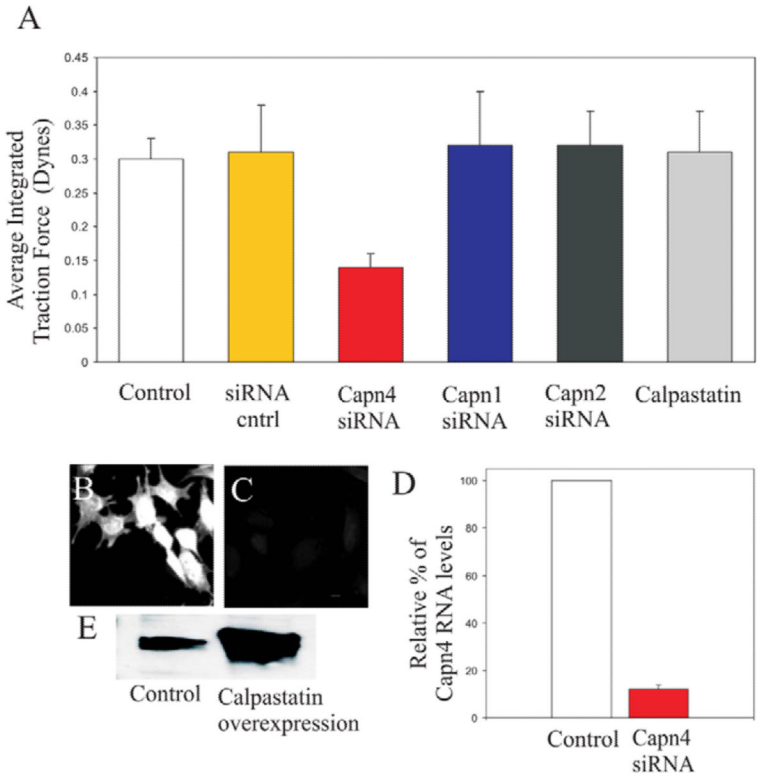
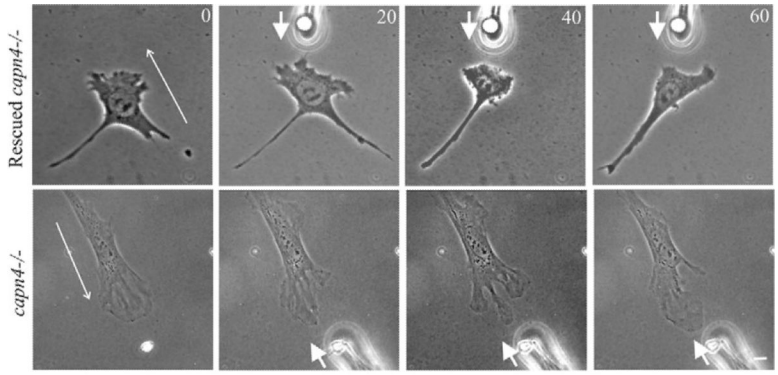


Fig. 3.

Generation of normal traction forces by cells defective in calpain 1 or calpain 2. (A) Average integrated traction forces generated by MEF cells treated with siRNA for either *Capn4*, *Capn1* or *Capn2* or cells overexpressing calpastatin. Neither silencing of calpain 1 or calpain 2, nor overexpression of calpastatin, causes a detectable reduction in traction forces. Data are compiled from measurements of a minimum of 15 cells for each cell type. Although wild-type MEF cells transfected with siRNA against *Capn4* show a significant reduction in the magnitude of traction stress (Student's *t*-test $P=0.0001$) ($n=19$). (B,C) Immunofluorescence of the calpain small subunit in MEFs transfected with control RNA (B), or with siRNA against *Capn4* (C) shows a striking reduction in the amount of small subunit upon siRNA-mediated gene silencing. (D) RT-PCR demonstrates an 88% reduction in the amount of Capn4 mRNAs. (E) Level of calpastatin protein expressed in wild-type MEF cells transfected with GFP plasmid alone and cells in which calpastatin-GFP has been overexpressed.



Response	MEF	Rescued *capn4-/-*	*Capn4-/-*	MEF ctrl siRNA	Capn1 siRNA	Capn2 siRNA	Calpastatin
	+	+	−	+	−	−	−

Fig. 4.

Involvement of calpain 1, calpain 2 and the calpain small subunit in cellular responses to mechanical forces. Images of *Capn4*$^{-/-}$ cells or rescued cells are recorded at 0 minutes, prior to micromanipulation, and at 20, 40, and 60 minutes after pushing on the substrate with a blunted microneedle against the direction of cell migration. Thin arrows indicate the direction of cell migration and thick arrowheads show the direction of pushing. Scale bar: 10 μm. The chart indicates a response (+) or a failure to respond (−) to stimulation under each of the cellular conditions.

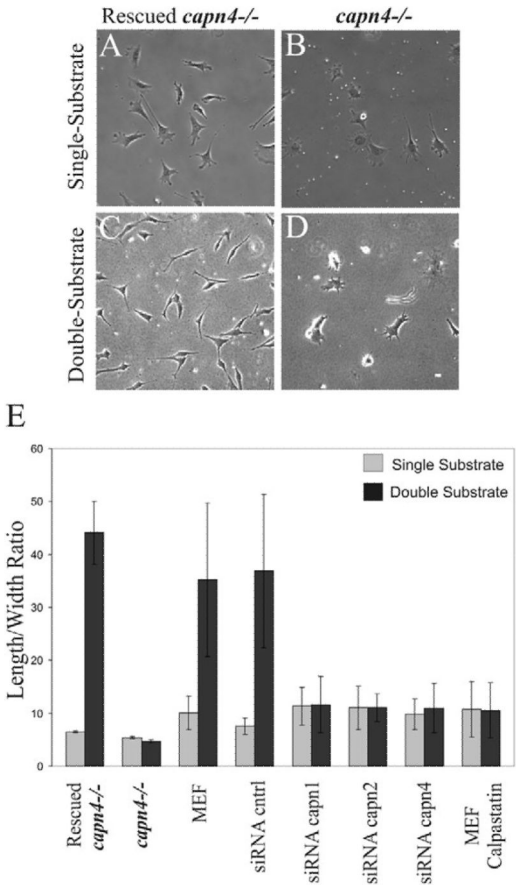


Fig. 5.

Failure of cells defective in calpain to respond to the engagement of dorsal integrins. *Capn4*$^{-/-}$ (B,D) or rescued (A,C) fibroblasts were cultured on the surface (A,B), or within two layers (C,D), of fibronectin-coated polyacrylamide substrates. Only rescued cells respond to the engagement of dorsal integrins by adopting an elongated shape. Scale bar: 10 μm. The response is quantified by taking the ratio of the length to the width of cells (E). In addition, similar results are obtained with siRNA-induced gene silencing using NIH3T3 cells. Each bar represents mean ± s.e.m. of 25 cells from three experiments.

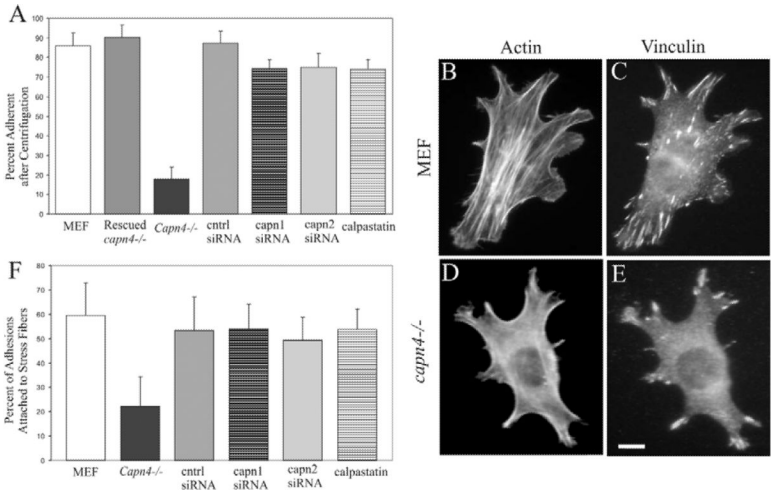


Fig. 6.

Capn4$^{-/-}$ fibroblasts are defective in adhesiveness and adhesion: stress fiber linkage. (A) Rescued *Capn4*$^{-/-}$ fibroblasts, Capn1- and Capn2-knockdown and calpastatin-overexpressing MEF cells, but not *Capn4*$^{-/-}$ cells, adhere strongly to the substrate in a centrifugation assay. Each bar represents mean ± s.e.m. results from three separate experiments, expressed as a percentage of control as defined by wild-type fibroblasts. Actin and vinculin immunofluorescence of representative control fibroblasts (B,C) and *Capn4*$^{-/-}$ fibroblasts (D,E) show the abnormal organization of these structures in the knockout cells. Scale bar: 10 μm.

(F) Colocalization analysis of actin and vinculin indicates a decreased association of prominent actin fibers with vinculin-containing adhesions in the *Capn4*$^{-/-}$ fibroblasts compared with the control and *Capn1*- and *Capn2*-silenced fibroblasts, as well as cells overexpressing calpastatin. The analysis involves counting the total number of vinculin-containing adhesions and those associated with actin stress fibers in the anterior region (*n*=15 cells).