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Calpain Is Required for the Rapid, Calcium-dependent Repair of Wounded Plasma Membrane*[S]

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Mammalian cells require extracellular calcium ion to undergo rapid plasma membrane repair seconds after mechanical damage. Utilizing transformed fibroblasts from calpain small subunit knock-out (*Capns1*$^{-/-}$) mouse embryos, we now show that the heterodimeric, typical subclass of calpains is required for calcium-mediated survival after plasma membrane damage caused by scraping a cell monolayer. Survival of scrape-damaged *Capns1*$^{-/-}$ cells was unaffected by calcium in the scraping medium, whereas more *Capns1*$^{+/+}$ cells survived when calcium was present. Calcium-mediated survival was increased when *Capns1*$^{-/-}$ cells were scraped in the presence of purified m- or μ-calpain. Survival rates of scraped *Capns1*$^{+/+}$, HFL-1, or Chinese hamster ovary cells were decreased by the calpain inhibitor, calpeptin, or the highly specific calpain inhibitor protein, calpastatin. *Capns1*$^{-/-}$ cells failed to reseal following laser-induced membrane disruption, demonstrating that their decreased survival after scraping resulted, at least in part, from failed membrane repair. Proteomic and immunologic analyses demonstrated that the known calpain substrates talin and vimentin were exposed at the cell surface and processed by calpain following cell scraping. Autoproteolytic activation of calpain at the scrape site was evident at the earliest time point analyzed and appeared to precede proteolysis of talin and vimentin. The results indicate that conventional calpains are required for calcium-facilitated survival after plasma membrane damage and may act by localized remodeling of the cortical cytoskeleton at the injury site.

Cells in multicellular organisms are constantly exposed to mechanical stresses and must repair plasma membrane breaks to survive (1, 2). Sustained breaks in the plasma membrane lead to loss of intracellular contents and necrotic cell death (3, 4). Calcium entry through a disruption triggers intracellular vesicles to fuse homotypically with each other, creating a "patch" (5). This multivesicle patch is then thought to rapidly fuse with the plasma membrane by a presently unknown mechanism to restore membrane integrity. It has been demonstrated that repair of breaks in skeletal muscle sarcolemma require the calcium and phospholipid-binding protein, dysferlin (6). Loss-of-function mutations of the latter are responsible for limb girdle muscular dystrophy type 2B (7). Inability of dysferlin-negative muscle fibers to undergo routine repair of sarcolemma disruption, it is hypothesized, leads to the muscle wasting observed in limb girdle muscular dystrophy type 2B patients. More recently, the calcium and phospholipid-binding protein annexin-A1 has been shown to accumulate at plasma membrane injury sites in BSC-1 and HeLa cells, where it may initiate fusion events (8). Given the importance of extracellular calcium in membrane repair, it is conceivable that other calcium and phospholipid-binding proteins, including calpain proteinases, could be involved in this process. In fact, previous studies have indicated that calpain activity is required for the calcium-stimulated healing of transected invertebrate giant axons (9) and neurites of differentiated PC12 cells (10). However, the slow resealing response in the neuronal processes (minutes) would not be likely to salvage a significant fraction of cells injured directly on the cell body and may differ mechanistically from the much faster response of fibroblasts and other cells (seconds) in resealing membrane disruptions.

Calpains are nonlysosomal, cysteine proteinases found in all animal cells. There are 14 calpain gene family members represented in the human genome (11). Dysfunction of calpain-3 in humans leads to limb girdle muscular dystrophy 2A (7, 12). The calpain gene family can be subdivided into typical calpains (containing domain IV, composed of multiple EF-hand calcium binding motifs) and atypical calpains. The most abundant calpains in most tissues are the typical μ- and m-calpains (also called calpain-1 and -2, respectively). Both are heterodimers composed of catalytic large subunits and a small, noncatalytic subunit that is required for expression of proteinase activity. The *Capns1* gene (also called Capn4) encodes the calpain small subunit. Both calpains require calcium ion for activity *in vitro*. However, their calcium affinities differ considerably. The μ-calpain isozyme requires ~5–50 μM calcium ion for half-maximum activity *in vitro*, whereas m-calpain requires ~200–800 μM calcium (11). Because of its high calcium requirement, it is difficult to envision an intracellular role for m-calpain. It has been reported that m-calpain is converted to a calcium-independent form by epidermal growth factor receptor-dependent phosphorylation (13). Exposure to extracellular

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calcium concentrations would potently activate m-calpain. However, there have been few published reports of its presence in the extracellular space (14–16).

Purified m- and μ-calpains catalyze proteolysis of many proteins. Physiologic substrates have been difficult to define, but cytoskeletal proteins have emerged over the years as likely targets for calpain in cells (17, 18). Recently, talin has been identified as a physiologic calpain substrate. Talin is an important regulator of integrin function and actin cytoskeleton remodeling during formation of attachment plaques (19). Its proteolytic modification by calpains to produce a 190-kDa fragment is important in regulating deadhesion from the substratum during fibroblast migration (20). The 190-kDa fragment was not detected in protein immunoblots of lysed primary fibroblasts from *Capns1*$^{-/-}$ mouse embryos (21), and these cells did not migrate as rapidly as wild-type fibroblasts. The defective deadhesion and migration are thought to derive from differences in cortical cytoskeletal remodeling, especially in formation and disassembly of adhesion plaques. It has been suggested that calpain-mediated cleavage of talin may be necessary for adhesion plaque disassembly (21).

Cytoskeletal remodeling is important in the mechanism for patching wounded plasma membrane (22), and exposure to extracellular calcium concentrations upon membrane disruption would favor activation of intracellular calpains, especially m-calpain. We here show, using loss of function (including *Capns1*-null cells) and gain of function models, that calcium-mediated membrane repair depends upon activation of the typical calpains. We have also begun to identify candidate proteolytic substrates of wound-activated calpains.

EXPERIMENTAL PROCEDURES

Materials—SV40T[2]-transformed mouse *Capns1*$^{-/-}$ and *Capns1*$^{+/+}$ fibroblasts were kindly provided by Drs. John Elce and Peter Greer (Queen's University, Kingston, Ontario, Canada). The thiol-reactive biotinylation reagent, maleimide-PEO_2-biotin (MPB) was obtained from Pierce (catalog number 21902). Commercial antibodies included 8d4 anti-talin mouse monoclonal and goat polyclonal anti-vimentin, obtained from Sigma. Anti-β1-integrin mouse monoclonal was purchased from Santa Cruz Biotechnology (catalog number sc-8978). Mouse monoclonal antibodies against bovine calpain small subunit and human calpastatin were prepared in house and have been previously described (23). Chicken antibody against rat m-calpain was a gift from Dr. Kevin Wang (University of Florida).

Cell Culture—SV40T-transformed mouse fibroblasts were routinely cultured in Dulbecco's modified Eagle's medium containing 10% FBS. Neonatal cardiac myocytes, isolated as previously described (24), were cultured in 4:1 Dulbecco's modified Eagle's medium/M199 medium without serum. Human skin fibroblasts (ATCC CRL 1782) and HFL1 human lung fibroblasts (ATCC CRL153) were cultured in Iscove's modified Dulbecco's medium containing 10% FBS. EcR-Chinese hamster ovary cells conditionally expressing full-length human large calpastatin (25) were routinely cultured in Ham's F-12 medium containing 10% FBS. Calpastatin expression was induced, where indicated, by 5 μM ponasterone A in the same medium. For the cell scraping protocol, cells were usually grown in 12-well plates to ~70–90% confluence. For biotinylation and isolation of cell surface proteins from scraped cells, fibroblasts were cultured in 150-mm dishes.

Laser Injury—Cells cultured in 35-mm dishes were subjected to laser wounding at 37 °C in the presence of FM1-43 dye. Dye uptake was monitored by time lapse image acquisition and image analysis as previously described (22).

Scrape Injury—Culture medium was removed by aspiration and replaced with 100 μl of Dulbecco's PBS containing 5.6 mM glucose and 0.33 mM pyruvate, pH 7.0 (scraping buffer), prewarmed to 37 °C. For most experiments, the scraping buffer contained either 2 mM calcium acetate (Ca^{2+}), or 2 mM calcium acetate and 5 mM EGTA (Ca^{2+}/EGTA). The latter buffer results in a free calcium ion concentration of about 10^{-8} to 10^{-7} M, approximating basal free calcium levels in most cells. Cells were immediately scraped with a sterile plastic cell scraper (Corning Costar catalog number 3010) for seven passes over a period of ~3 s. Further handling of the scraped cells depended on the experiment and will be discussed in the appropriate sections below. To assess the effect of the calpain inhibitor, calpeptin, on survival, the cells were preincubated with calpeptin for 30 min to 1 h before scraping, and calpeptin was also included in the scraping buffer.

Immunodetection of Vimentin on the Surface of Scratched, Resealed Cells—BSC-1 renal epithelial cells, a model cell type for investigating scrape injury of monolayers (26), were scratched in the presence of FDx as previously described (22). Briefly, the cells were plated on sterile coverslips and allowed to attach overnight. The cell monolayer was scratched at 37 °C with a sterile 18-gauge needle in the presence of FDx in scraping buffer and washed with ice-cold scraping buffer after 30 s to remove FDx. The cells were fixed without permeabilizing and immunostained with goat anti-human vimentin, followed by rhodamine isothiocyanate-labeled anti-goat second antibody. FDx retention in re-sealed cells, and vimentin surface labeling were determined by fluorescence microscopy.

Cell Viability—Routinely, viability after cell scraping was assessed by the spectrophotometric MTT reductase method, employing an absorption wavelength of 590 nm (27). Cells were scraped as described above and incubated in scraping buffer for various times at 37 °C. Dulbecco's modified Eagle's medium containing 10% FBS was added, and the cells were cultured for 4–12 h before MTT reductase measurement. In many different experiments, variation within this 4–12-h interval did not significantly affect the recovery of *Capns1*$^{+/+}$ or *Capns1*$^{-/-}$ fibroblast viability. Microscopic inspection of cultures prior to the MTT assay affirmed that the variation in color was associated with increase or decrease of cell number and not relative cellular content of MTT reductase. Nevertheless, to formally verify the suitability of the MTT assay to assess viability within the parameters of our studies, a clonal survival assay was carried out (Fig. 1*B*). The percentage of cell survival was calculated as

[2] The abbreviations used are: SV40T, simian virus 40 large T-antigen; FBS, fetal bovine serum; FDx, fluorescein-labeled dextran; MTT, methylthiotetrazole; MPB, maleimide-PEO_2-biotin; PBS, phosphate-buffered saline.

the A_{590} value of scraped cell assays divided by the A_{590} of nonscraped cells from the same culture plate multiplied by 100.

Biotinylation and Subsequent Affinity Isolation of Cell Surface Proteins—Fibroblasts grown in 150-mm dishes were scraped in 1.0 ml of scraping buffer with or without additions, using a large cell scraper (Corning Costar catalog number 3011). At various times thereafter, a freshly prepared aqueous solution of MPB obtained from Pierce was added to a concentration of 2 mM, and the samples were immediately placed on ice. The maleimide functional moiety of MPB is expected to react with the normally reduced Cys side chains of intracellular proteins exposed at membrane tears. Unless otherwise stated, all further steps were at 4–7 °C. After 60 min, dithiothreitol was added to 20 mM to quench unreacted MPB, followed by a further 10-min incubation. To remove cell debris, the cells were washed by centrifugation three times at 200 × *g* for 2 min in 1.5 ml of scraping buffer containing calcium. In some experiments, the first 200 × *g* supernatant was saved for preparation of a 200–21,000 × *g* pellet membrane fragment fraction. The washed cells were resuspended in 1 ml of PBS, SDS was added to 0.2%, and the samples were heated to 60 °C for 5 min. Triton X-100 was added to 1%, and the lysates were left refrigerated overnight. The lysates were centrifuged at 100,000 × *g* for 30 min, and 50 μl of UltraLink Neutravidin Plus® gel (Pierce) was added to the resultant supernatants (~1 ml). The gel slurries were rotated in the cold for 4 h to allow adsorption of biotinylated proteins. The gels were collected by centrifugation at 2000 × *g* for 1 min and washed with 1.5 ml of PBS containing 0.1% SDS and 1% Triton X-100. After four more washes with PBS containing 1% Triton X-100, the gel beads were resuspended in 150 μl of 3× diluted SDS-sample buffer (Invitrogen) and heated to 70 °C for 20 min. The gels were removed by centrifugation, and the proteins extracted in the SDS-sample buffer were employed in gel electrophoresis and protein immunoblotting studies. Nonscraped control cells were carried through all steps of the above protocol, except that they were not scraped before placing on ice. Instead, they were scraped in the cold 10 min after the addition of dithiothreitol to quench MPB.

Electrophoresis and Protein Immunoblotting—Samples were electrophoresed in Invitrogen precast 10% polyacrylamide gels and electrophoretically transferred to nitrocellulose. Except where otherwise indicated, a volume equivalent to 10% of the total sample was loaded on the gel. Therefore, each isolated cell fraction was normalized with respect to total cell number in the experiment.

An antibody was produced that recognizes vimentin only after cleavage by calpain. Rabbits were immunized against the short peptide SSVPGVC coupled through the carboxyl-terminal Cys to BSA. This peptide corresponds to the amino terminus of the major fragment of vimentin generated by cleavage with calpain (28) and is conserved in mouse and human vimentin. The antibody only recognized cleaved vimentin and detected the predicted 46-kDa fragment on immunoblot analysis (supplemental Fig. S1). Because it is not produced by cleavage after an aspartyl residue, the calpain-specific cleavage site should not be a good substrate for caspases, and indeed it is not a known primary caspase cleavage site in vimentin (29). Moreover, the antibody did not recognize vimentin fragments generated by proteasome, the other major cytosolic protease in animal cells, or by the model cysteine protease ficin (supplemental Fig. S1). A trace of the 46-kDa fragment was detectable after cleavage with clostripain, another cysteine protease, but only at the 5 min digestion time point. In contrast, calpain cleavage generated a relatively stable fragment that was evident after 50 min of digestion. Hence, the generation of the stable 46-kDa vimentin fragment appears to be a good marker for calpain-mediated degradation of vimentin.

Proteomic Analysis—Approximately 10 μg of biotinylated protein isolated from 1-min scraped *Capns1*$^{-/-}$ fibroblasts was subjected to SDS-PAGE and colloidal Coomassie Blue staining. Bands were cut from the gel and digested with sequencing grade, modified trypsin (Promega) overnight at 37 °C. Peptides were extracted with 60% acetonitrile, 0.1% trifluoroacetic acid and concentrated to ~15 μl using a vacuum centrifuge. A 2-μl aliquot of the sample was separated on a reverse phase column (75-μm inner diameter × 5 cm × 15 μm Aquasil C18 Picofrit column; New Objectives). The eluent was directly introduced into an ion trap mass spectrometer (LCQ-Deca XP Plus; Finnigan) equipped with a nanospray source. The mass spectrometer was operated on a double play mode where the instrument was set to acquire a full MS scan (400–2000 *m*/*z*) and a collision-induced dissociation spectrum on the most abundant ion from the full MS scan. Collision-induced dissociation spectra were either manually interpreted or searched against an appropriate nonredundant data base using the TurboSEQUEST program.

Scrape Loading Fibroblasts—Fibroblasts were cultured in 6-well plates until ~80% confluent. All procedures were carried out at 37 °C. Medium was removed, and the cells were washed twice with 2 ml of PBS. After the PBS was thoroughly removed by aspiration, 40 μg of purified bovine m-calpain, 40 μg of human erythrocyte μ-calpain, or 50 μg of human erythrocyte calpastatin, isolated as previously described (30), were added in 200 μl of scraping buffer containing 100 μM EGTA. Control samples contained buffer alone. The cells were scraped and incubated for 10 min to allow entry of the exogenous calpain or calpastatin. To allow membrane resealing, the volume was adjusted to 4 ml, and calcium was added to 2 mM. After 10 min, the samples were centrifuged at 200 × *g*, the supernatants were removed, and the cells were resuspended in 0.5 ml of Hanks' balanced salt solution containing 0.1% trypsin and 0.04% EDTA. After 15 min, the cells were centrifuged, and the trypsin solution was aspirated. The cells were resuspended in 8 ml of Dulbecco's modified Eagle's medium containing 10% FBS (calcium concentration, 2 mM) and plated in 12-well plates at 1 ml of cell suspension/well. Samples of nonscraped control cells were plated at the same density. MTT reductase activity was determined 4 or 12 h later, as indicated for the specific experiment.

Statistical Analysis—Where indicated, statistical comparisons utilized Student's unpaired, two-tailed *t* test, with graphic results expressed as mean ± S.D. A *p* value less than 0.05 was considered statistically significant.

RESULTS

Capns1$^{-/-}$ mouse embryos, which lack m- and μ-calpain activity, die *in utero* after embryonic day E10.5 (31). Whereas SV40T-transformed *Capns1*$^{-/-}$ embryonic fibroblasts differ little from their *Capns1*$^{+/+}$ counterparts in general growth properties (31), upon closer examination, they have a decreased clonal growth rate (25), compromised haptokinetic migration, and loss of central focal adhesions (21) compared with SV40T-transformed *Capns1*$^{+/+}$ fibroblasts. Cells in culture are subjected to plasma membrane wounding as they divide and migrate (32–34). To determine if cell membrane repair was compromised in *Capns1*$^{-/-}$ fibroblasts, we first applied a standard cell wounding method, scraping from the substratum, and measured survival. In eight independent experiments, scraping wild-type fibroblasts in the presence of calcium led to a highly significant increase in viability, relative to cells scraped in the presence of Ca^{2+}/EGTA (Fig. 1*A*). This observation is consistent with a large volume of previously published work demonstrating a calcium-dependent healing process in a variety of cell models (2). In contrast, the *Capns1*$^{-/-}$ fibroblasts displayed virtually no increased viability when scraped in the presence of calcium, as determined by the MTT reductase method. We utilized a clonal survival assay experiment to validate the results of the MTT studies (Fig. 1*B*).

If active calpain is required for calcium-enhanced survival after membrane disruption, then inhibition of calpain activity in *Capns1*$^{+/+}$ fibroblasts should eliminate the survival advantage. Exposure of the cells to 20 μM calpeptin immediately prior to and during scraping eliminated the calcium effect on cell viability (Fig. 1*C*). In contrast, calpeptin treatment did not further decrease *Capns1*$^{-/-}$ fibroblast survival. Importantly, 10 μM calpeptin produced half-maximal inhibition of calcium-dependent cell survival and ~70% inhibition of calpain activity in *Capns1*$^{+/+}$ cells (not shown). Although calpeptin is not strictly specific for calpains, the concentrations of calpeptin used in our studies were well below those required to inhibit the other identified calpeptin targets, proteasome (35) and phosphotyrosine phosphatases (36). In another experiment, *Capns1*$^{-/-}$ fibroblasts were scraped in the presence of purified m- or μ-calpain, allowing their entry at the site of plasma membrane disruption. Their introduction by this method significantly enhanced survival in medium containing calcium (Fig. 1*D*). Calcium enhancement of survival after membrane damage was rapid. The addition of excess EGTA only 5–10 s after scraping in the presence of calcium had little effect on survival (Fig. 1*E*). In summary, these studies indicate that the calcium-dependent activation of typical calpains rapidly imparts a survival advantage to *Capns1*$^{+/+}$ fibroblasts following plasma membrane damage.

Calpains may have specific effects in transformed cells that are not apparent in similar nontransformed cells (37). To address this issue, calcium- and calpain-dependent recovery of MTT activity after scraping was tested in two nontransformed cells: primary neonatal rat cardiomyocytes and normal human skin fibroblasts (Fig. 1*F*). In these experiments, the level of damage was greater than observed for scraped SV40T-transformed fibroblasts. Nevertheless, the increased recovery of MTT activity observed after

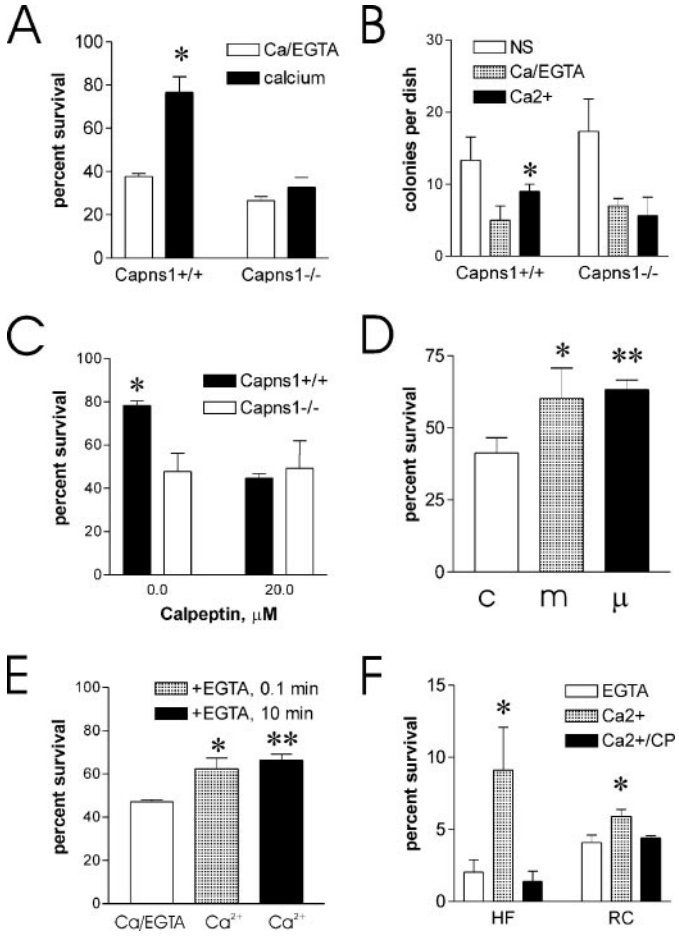

FIGURE 1. **Calcium-dependent survival of scraped SV40T-transformed mouse fibroblasts depends on calpain activity.** *A*, *Capns1*$^{+/+}$ and *Capns1*$^{-/-}$ fibroblasts were cultured to 70% confluence in 12-well culture plates and scraped in the presence of Ca^{2+}/EGTA or 2 mM calcium acetate. Cell viability was assessed by MTT reductase activity 12 h later. Results are representative of eight independent experiments. *, $p < 0.001$ *versus* all other experimental conditions ($n = 3$). *B*, clonal survival assay. Cells were scraped in the presence of calcium or Ca^{2+}/EGTA and incubated for 10 min at 37 °C. They were trypsinized for 15 min and plated in triplicate 60-mm culture dishes at 25 cells/dish, based on the cell density before scraping. Seven days later, colonies having at least 16 cells were scored. *NS*, not scraped. *, $p < 0.05$ *versus* *Capns1*$^{+/+}$ scraped in Ca^{2+}/EGTA ($n = 3$). *C*, fibroblasts were preincubated with or without 20 μM calpeptin for 1 h and then scraped in 100 μl of calcium-containing buffer with or without 20 μM calpeptin. After 10 min at 37 °C, 1.4 ml of culture medium was added, and MTT reductase activity was measured after 4 h. *, $p < 0.02$ *versus* all other experimental conditions ($n = 3$). *D*, *Capns1*$^{-/-}$ cells were scraped in the presence of purified bovine m-calpain (*m*) or human μ-calpain (μ) or without additions (*c*) as described under "Experimental Procedures." Twelve hours later, MTT reductase activity was measured. The percentage survival is relative to a nonscraped culture of identical density, diluted and replated at the same density as the scraped samples. *, $p < 0.02$ *versus* c ($n = 4$). **, $p < 0.001$ *versus* c ($n = 4$). *E*, *Capns1*$^{+/+}$ fibroblasts were scraped in the presence of buffer containing 2 mM calcium or Ca^{2+}/EGTA as indicated on the *x* axis. EGTA was added to 5 mM either 0.1 min or 10 min after scraping in calcium buffer, as indicated in the figure. Medium was added to the samples, and MTT reductase activity was measured 4 h later. *, $p < 0.01$ *versus* Ca^{2+}/EGTA ($n = 3$). **, $p < 0.005$ *versus* Ca^{2+}/EGTA. *F*, human skin fibroblasts (*HF*) or neonatal rat cardiomyocytes (*RC*) were scraped in 2 mM calcium, Ca^{2+}/EGTA, or 2 mM calcium plus 20 μM calpeptin (Ca^{2+}/CP). Medium was added after 10 min, and MTT reductase activity was measured 12 h after scraping. *, $p < 0.02$ *versus* Ca^{2+}/EGTA or Ca^{2+}/CP ($n = 4$).

scraping in the presence of calcium was completely abolished by including 20 μM calpeptin in the scraping buffer. In most cells, a highly specific inhibitor protein, calpastatin, regulates calpain activity. Calcium-dependent cell survival of HFL1 normal human

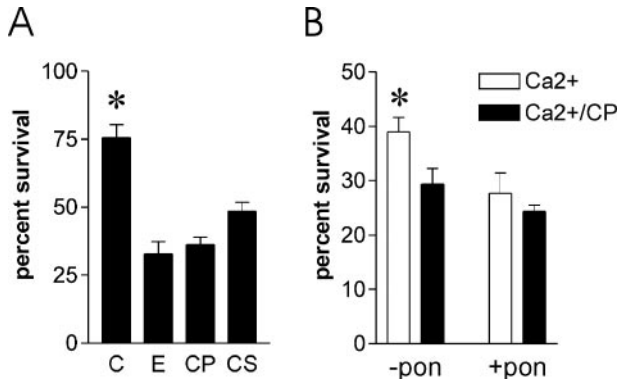

FIGURE 2. **Calpastatin decreases calcium-dependent survival after scraping.** *A*, HFL1 human lung fibroblasts were scraped in the presence of calcium alone (*C*), Ca^{2+}/EGTA (*E*), calcium plus 20 μM calpeptin (*CP*), or calcium plus 4 μM calpastatin (*CS*). MTT reductase activity was measured after 6 h. *, $p < 0.001$ *versus* all other experimental conditions ($n = 4$). *B*, Chinese hamster ovary cells stably transfected with calpastatin under control of the insect ecdysone receptor were cultured for 4 days with or without 5 μM ponasterone A to induce calpastatin expression. The cells were scraped in the presence of calcium with or without 20 μM calpeptin, and MTT reductase activity was measured after 6 h. *, $p < 0.02$ *versus* all other experimental conditions ($n = 3$).

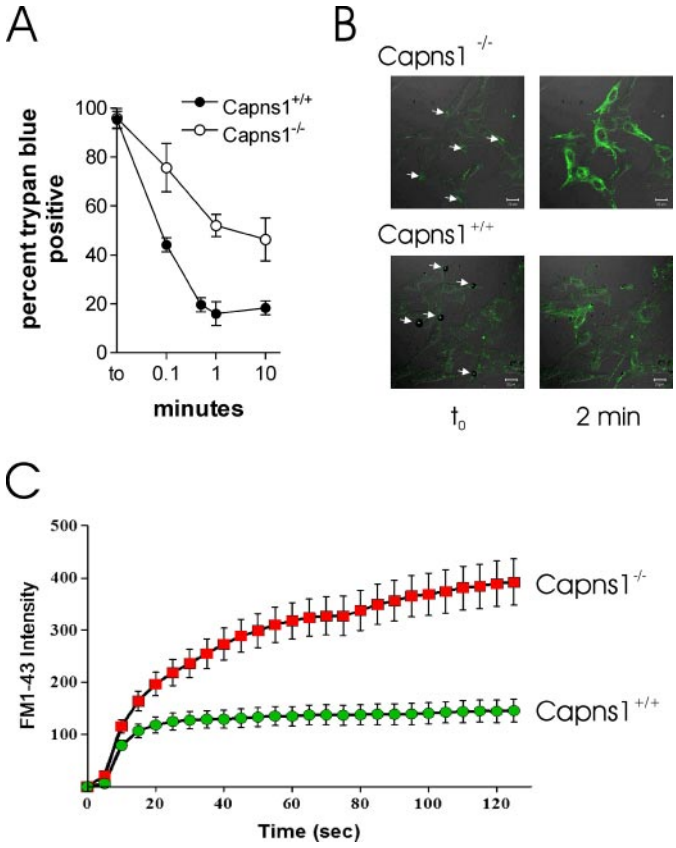

FIGURE 3. **Compromised plasma membrane repair in SV40T-transformed *Capns1*$^{-/-}$ fibroblasts.** *A*, *Capns1*$^{+/+}$ and *Capns1*$^{-/-}$ fibroblasts were scraped in buffer containing 2 mM calcium acetate or Ca^{2+}/EGTA. At the indicated times after scraping, trypan blue dye was added, and the samples were assessed microscopically for dye uptake. At least 100 cells were counted for each of triplicate data points. *B*, *Capns1*$^{+/+}$ and *Capns1*$^{-/-}$ fibroblasts in calcium-containing buffer were injured by a pulsed laser beam in the presence of cell-impermeant FM1-43 dye. The *arrows* indicate the sites of laser damage. The *Capns1*$^{-/-}$ fibroblasts did not reseal, as shown by increased FM1-43 uptake over a 2-min interval. *Bar*, 20 μm. *C*, time course of membrane resealing after laser injury.

lung fibroblasts was compromised by the addition of purified calpastatin in the scraping medium (Fig. 2*A*). Regulated expression of calpastatin in a transfected Chinese hamster ovary cell line also decreased survival (Fig. 2*B*). Thus, the intracellular balance of calpain and calpastatin levels may influence survival after plasma membrane damage.

To further investigate the failure of *Capns1*$^{-/-}$ cells to survive scraping injury, we measured the acute consequences of calpain loss of function on membrane resealing. It might be argued that the previously noted defects in cortical cytoskeletal organization (21) could result in a greater susceptibility of *Capns1*$^{-/-}$ fibroblasts to scrape damage. When scraped in the presence of trypan blue, >95% of *Capns1*$^{-/-}$ and *Capns1*$^{+/+}$ cells were positive for this cell-impermeant dye (Fig. 3*A*). Thus, although we cannot rule out a subtle effect of calpain loss of function on membrane fragility, the decreased survival of *Capns1*$^{-/-}$ fibroblasts after scrape damage did not simply reflect a greater fraction of cells, compared with wild type, undergoing gross membrane damage. *Capns1*$^{+/+}$ fibroblasts appeared to reestablish impermeance to trypan blue more rapidly than *Capns1*$^{-/-}$ fibroblasts (Fig. 3*A*), consistent with their increased survival. To visualize real time resealing in injured fibroblasts, we used a laser-based assay (5) to measure resealing kinetics. In this assay, staining of internal membranes, due to entry of an otherwise membrane-impermeant dye, FM1-43, is monitored microscopically over time after the laser disruption (2). Resealing blocks further dye entry through the disruption and hence halts cell staining. Measured whole cell fluorescence therefore plateaus upon the completion of resealing. Although the *Capns1*$^{+/+}$ fibroblasts resealed within 30 s after laser injury, as indicated by the plateau in fluorescent staining (Fig. 3, *B* and *C*; supplemental Video 1), *Capns1*$^{-/-}$ fibroblasts continued to take up FM1-43 dye for at least an additional 2 min (Fig. 3; supplemental video 2). These results indicate resealing failure in the *Capns1*$^{-/-}$ cells.

To identify cytoskeletal calpain substrates at the site of plasma membrane damage, fibroblasts were incubated with MPB at various times after scraping and placed on ice to prevent endosomal uptake of the reagent. Biotin-labeled surface proteins were then affinity-purified by adsorption to Neutravidin gel as described under "Experimental Procedures." As anticipated, given the lack of maleimide-reactive sulfhydryl groups on the undamaged cell surface, there was relatively little labeling of nonscraped cells (Fig. 4*A*, *NS lanes*). The major protein isolated from scraped cells was vimentin, as revealed by mass spectrophotometric analysis (not shown). High molecular mass proteins (~100–250 kDa) were also evident in the biotin-labeled fractions of both *Capns1*$^{+/+}$ and *Capns1*$^{-/-}$ fibroblasts, and they comprised a complex mixture of proteins that were not sufficiently resolved to permit analysis by mass spectrophotometry. We confirmed that vimentin and the 100–250-kDa proteins were biotinylated by probing protein blots with alkaline phosphatase-labeled streptavidin (not shown). Thus, they were not fortuitously co-purified with biotinylated surface proteins after cell lysis. Consistent with the membrane resealing and cell survival studies, much less protein was labeled in wild-type fibroblasts, indicating rapid repair of membrane damage. Scraping of wild-type cells in the presence of Ca^{2+}/EGTA or the addition of calpeptin to the calcium-containing

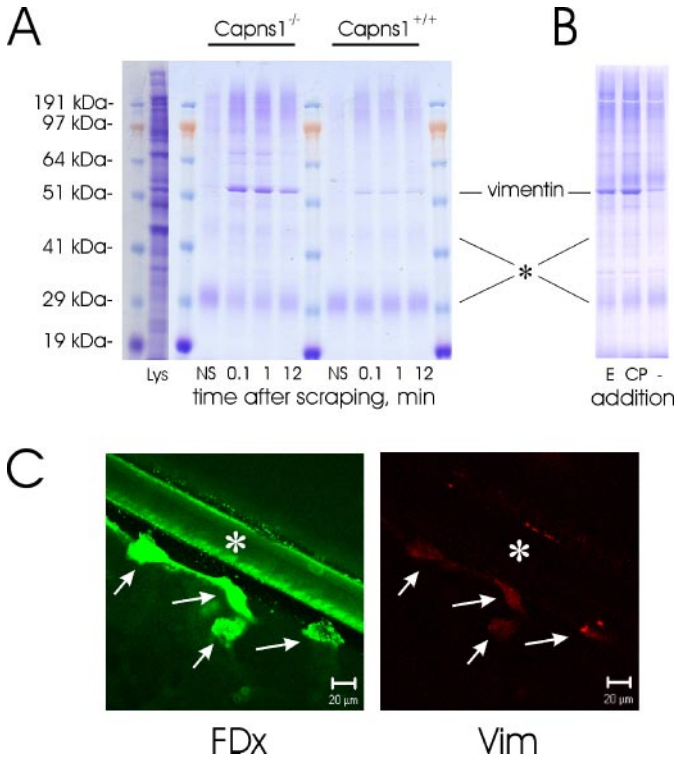

FIGURE 4. **Expression of cytoplasmic proteins at the cell surface after scrape damage.** *Capns1*$^{+/+}$ or *Capns1*$^{-/-}$ fibroblasts were scraped in the presence of calcium and labeled at various times thereafter with MPB, as described under "Experimental Procedures." Subsequently, cells were washed and lysed. Biotin-labeled proteins were affinity-purified by adsorption to Neutravidin gel, and one-tenth aliquots of the affinity-purified proteins were applied to SDS-PAGE and Coomassie-stained. *A*, time course for surface expression of *Capns1*$^{+/+}$ and *Capns1*$^{-/-}$ cytoplasmic proteins. *NS*, not scraped. *Lys*, 10 μg of total cell lysate protein from *Capns1*$^{+/+}$ fibroblasts. *Capns1*$^{-/-}$ fibroblast lysate gave essentially the same pattern (not shown). *, the position of protein bands that were detected with Neutravidin gel alone. *B*, *Capns1*$^{+/+}$ fibroblasts were scraped in the presence of Ca^{2+}/EGTA (*E*), 2 mM calcium plus 20 μM calpeptin (*CP*), or 2 mM calcium alone (−). After 12 min, MPB was added, and labeled proteins were subsequently isolated. In this experiment, the cell lysate was centrifuged at 22,000 × *g* instead of 100,000 × *g* (see "Experimental Procedures"). As a result, additional proteins were observed (*e.g.* immediately *above* the vimentin bands). *C*, immunodetection of vimentin (*Vim*) on the surface of resealed cells. Monolayer BSC-1 cells were scratched in the presence of FDx, as described under "Experimental Procedures," washed, and immunostained for vimentin without permeabilization. The *asterisk* indicates the scratched region of the coverslip. Cells that reseal after membrane damage immunofluoresce for trapped FDx and are indicated by *arrows*. Note the rhodamine isothiocyanate immunolabeling of vimentin on the same resealed cells in the *right panel*.

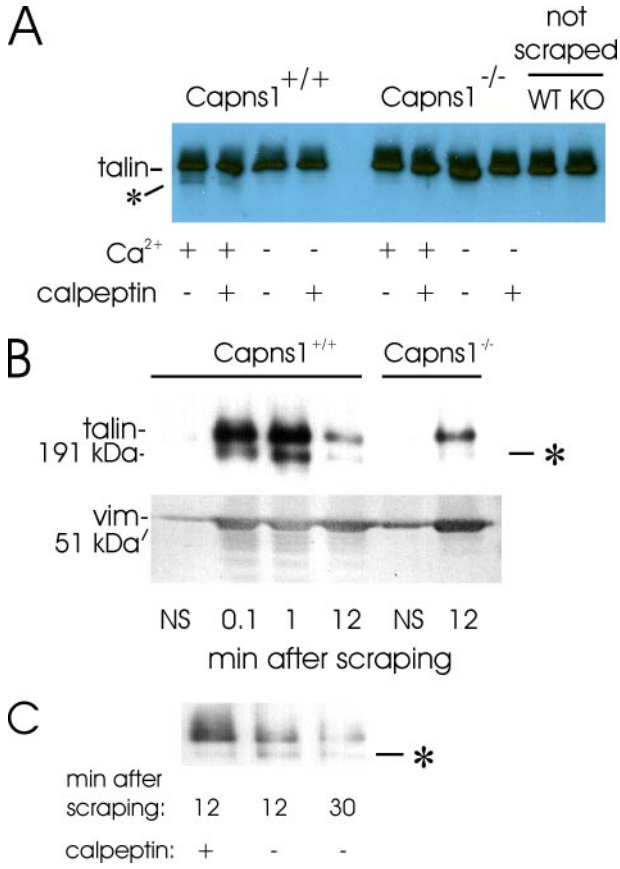

FIGURE 5. **Fragmentation of talin and vimentin after cell scraping.** Fibroblasts were scraped in the presence of Ca^{2+}/EGTA or 2 mM calcium. After 1 min, SDS-sample buffer was added, and talin cleavage was assessed by immunoblot analysis. *A*, whole cell lysates prepared after scraping with or without 20 μM calpeptin. *, trace amounts of talin fragment detected in the *Capns1*$^{+/+}$ fibroblasts scraped in the presence of calcium. Note that calpeptin attenuated talin cleavage in the *Capns1*$^{+/+}$ cells scraped in the presence of calcium. Little cleavage occurred in the absence of calcium, and little cleavage was noted in *Capns1*$^{-/-}$ cells in any of the conditions. Nonscraped (*NS*) control lysates were loaded on the *right side* of the gel. *B*, surface proteins were biotinylated at the indicated times after scraping and isolated by adsorption to Neutravidin gel. Note the increased proportion of fragmented talin (*asterisk*) in the *Capns1*$^{+/+}$ samples compared with the whole cell lysates in *A*. Vimentin (*vim*) was also cleaved in the *Capns1*$^{+/+}$ sample (*lower panel*). *C*, calpeptin inhibited the accumulation of talin fragment. *Capns1*$^{+/+}$ fibroblasts were scraped and biotinylated as in *B*, except that 20 μM calpeptin was included in the scraping buffer where indicated. Note that less talin was isolated 30 min after scraping compared with 12 min.

scraping buffer resulted in increased labeling of surface proteins to approximately the level observed in scraped *Capns1*$^{-/-}$ cells (Fig. 4*B*).

Trace amounts of cleaved talin were detected in whole cell lysates of *Capns1*$^{+/+}$ fibroblasts scraped in the presence of calcium (Fig. 5*A*). In contrast, talin fragmentation was not detected in *Capns1*$^{+/+}$ fibroblasts scraped in the presence of Ca^{2+}/EGTA and was decreased by scraping in the presence of 20 μM calpeptin. No talin fragmentation was detected in *Capns1*$^{-/-}$ cell lysates under any of the experimental conditions. Analysis of biotin-labeled and affinity-purified proteins demonstrated that talin was exposed after scraping (Fig. 5*B*). Unlike the results obtained with the whole cell lysates, much of the biotin-labeled talin in scraped *Capns1*$^{+/+}$ cells was present as the 190-kDa calpain-generated fragment. One minute after scraping, nearly one-half of the exposed talin was cleaved. By 12–30 min postscraping, the talin signal was reduced (Fig. 5, *B* and C), indicating that it had been cleared from the site of damage or masked by an ongoing cell surface repair process. There was no detectable increase in fragmentation of talin 12 min after scraping of *Capns1*$^{-/-}$ fibroblasts (Fig. 5*B*). As anticipated, inclusion of calpeptin prevented cleavage of exposed talin in scraped *Capns1*$^{+/+}$ fibroblasts (Fig. 5*C*). Vimentin was also processed in scraped *Capns1*$^{+/+}$ fibroblasts (Fig. 5*B*, *bottom*) but to a lesser extent than talin.

Because vimentin was the major intracellular protein labeled by MPB after cell scraping, we carried out further experiments to establish its expression on the outside cell surface after damage and its calpain-dependent processing. BSC-1 cells were scratch-loaded with FDx as described under "Experimental Procedures," allowed to reseal, and immunostained for vimen-

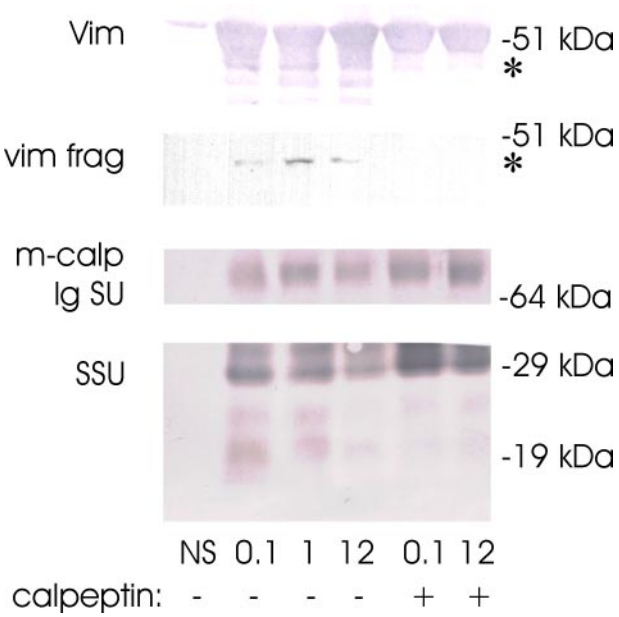

FIGURE 6. **Calpain accumulated at the scrape injury site and was activated prior to vimentin cleavage.** HFL1 human fibroblasts were scraped in the presence of calcium with or without the addition of 20 μM calpeptin and biotinylated with MPB at various times thereafter as described under "Experimental Procedures." After Neutravidin affinity gel purification, 10 μg of protein from each sample was electrophoresed, blotted, and immunostained with antibodies against vimentin (*Vim*), calpain-cleaved vimentin (*vim frag*), m-calpain large subunit, and calpain small subunit (*SSU*). *, relative migration of the 46-kDa calpain-generated vimentin fragment. *NS*, nonscraped.

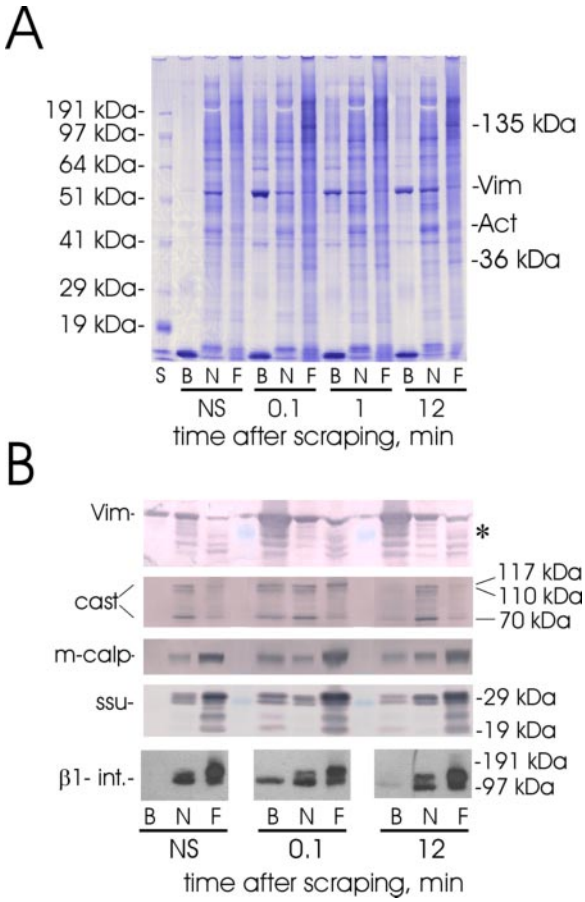

FIGURE 7. **Distribution of calpain and calpastatin to the cell surface and cell fragment fractions after scrape injury.** HFL1 cells were scraped and biotinylated at various times. The biotinylated proteins were affinity-purified on Neutravidin gel and subjected to SDS-PAGE in 10% polyacrylamide gels. The gels were either stained with Coomassie Blue (*A*) or electroblotted for immunologic studies (*B*). *Lanes B*, proteins purified by Neutravidin affinity chromatography. *Lanes marked n*, proteins that did not bind to the Neutravidin gel. *Lanes F*, cell fragments present in the scraping medium after the 60-min labeling period with MPB were obtained in the supernatant of the first centrifugation step at 200 $\times$ *g* (see "Experimental Procedures"). They were concentrated by sedimentation at 21,000 $\times$ *g* and heated in 60 μl of SDS sample buffer for electrophoretic analysis. Note that membrane fragments are observed in the nonscraped control fraction (*NS*). As stated under "Experimental Procedures," these cells were not scraped prior to the addition of MPB, but they were scraped after the 60-min incubation on ice. Sample loads were as follows. *A*, 10 μl/lane; *B*, 20 μl/lane for *lanes B* and 5 μl/lane for *lanes N* and *F*. *ssu*, small subunit.

tin without permeabilization. In this procedure, cells that successfully reseal retain FDx, whereas those that are irreversibly damaged lose FDx during the subsequent washout and do not fluoresce. Extracellular vimentin immunolabeling was detected at the edges of cell wounds that had resealed, as evidenced by FDx retention in the cells (Fig. 4*C*), indicating that vimentin was externalized upon damage and remained accessible to the antibody after successful plasma membrane repair. To show that calpain directly processed vimentin, we utilized a cleavage site-specific antibody (see "Experimental Procedures"). The 46-kDa calpain-specific vimentin fragment was detected in the biotinylated, surface-exposed fraction shortly after scraping HFL1 fibroblasts and was most evident at the 1-min time point (Fig. 6). Calpains rapidly accumulated at the scrape site, and calpain small subunit underwent very rapid autoproteolytic cleavage to 17–26-kDa fragments, a hallmark of calpain activation (11, 38). The extensive autoproteolysis only 0.1 min after scrape damage (Fig. 6, *SSU panel*) is consistent with calpain activation immediately prior to vimentin processing. As anticipated, calpeptin blocked vimentin cleavage and calpain autoproteolysis. It also increased the content of calpain associated with the damage site, as reflected in the increased intensity of calpain-immunoreactive bands on the *right side* of Fig. 6. Thus, inhibition by calpeptin did not prevent calpain accumulation at the site of membrane injury but probably prevented its turnover by autoproteolysis, allowing accumulation of native calpain.

If calpains rapidly process talin, vimentin, and perhaps other cytoskeletal proteins at the site of membrane damage, they should accumulate at nascent plasma membrane wounds and escape the inhibitory activity of endogenous calpastatin. To address this proposition, we compared relative amounts of vimentin, calpain, and calpastatin in the biotinylated and nonbiotinylated fractions of cells at various times after scraping, as described under "Experimental Procedures." In addition, we were interested in the composition of the cell fragments generated upon cell scraping that sedimented between 200 and 21,000 $\times$ *g*, and these were analyzed as well. Just as observed in the mouse cell studies, little of the total HFL1 protein was isolated by Neutravidin affinity purification (Fig. 7*A*, *B lanes*), and, again, vimentin was the major protein present in this fraction. The cellular proteins that did not bind to Neutravidin gel (*N* in Fig. 7*A*) did not appear to undergo gross proteolytic processing, consistent with a localized activation of calpains at the site of plasma membrane damage. Interestingly, the proteins in the 200–21,000 $\times$ *g* membrane fraction isolated from the post-scraping medium (Fig. 7*A*, *lanes F*) were enriched in actin and

had little vimentin (Fig. 7, *A* and *B*). Moreover, predominant 135- and 36-kDa proteins were evident, and proteins of this size are major components of the matrix vesicle particles released from fibroblasts and other cell types during migration in culture (32). Matrix vesicle particles sediment between 200 and 21,000 × *g*, are enriched in actin, and contain little vimentin. The 135-kDa protein in matrix vesicles has been identified as integrin-β_1 (32), and immunoblot analysis revealed its substantial enrichment in the cell fragment fraction (Fig. 7*B*).

Both calpain and calpastatin were present in the biotinylated fraction immediately after scraping (Fig. 7*B*, 0.1 min time point, *lane B*). However, both calpain small subunit (*ssu*) and m-calpain large subunit (*m-calp*) were more highly enriched in this fraction relative to calpastatin (*cast*); there was greater staining intensity of the two calpain subunits relative to nonbound calpain (compare *lanes B* and *N*), whereas calpastatin staining intensity in the Neutravidin-bound and nonbound lanes was approximately equivalent. Importantly, as noted in Fig. 6, the calpain small subunit underwent autoproteolysis, indicating that the amount of co-localized calpastatin was not sufficient to totally inhibit calpain activity. Although the content of surface-labeled calpain appeared to decrease slightly at 12 min postscraping, calpastatin immunoreactivity was nearly absent at this time. The distribution of calpain and calpastatin to the cell fragment fraction (Fig. 6*B*, *F lanes*) was even more striking. Calpain content was markedly elevated relative to the nonbound fraction. At the 0.1 min time point, the predominant form of calpastatin bound to the cell fragments was the 117-kDa species, which contains an amino-terminal L-domain responsible for binding to acidic phospholipids (39) and the cytoplasmic region of plasma membrane L-type calcium channels (40).

DISCUSSION

In the present study, we have shown that *Capns1* ablation prevented the calcium-dependent resealing of plasma membrane wounds in SV40T-transformed mouse fibroblasts. Previous studies had provided indirect evidence for a role of one or more of the calpains in the repair of transected axons and neurites (9, 10). However, the time course of membrane resealing in the cell body of a fibroblast or many other nonneuronal cells occurs on a seconds time scale (2) rather than on the order of minutes, as observed for neuronal processes (10). Based on this and other data, it has been proposed that resealing in neuronal processes is, in its early phases at least, mechanistically distinct from that occurring in the "body" of other cell types. In the neuron, but not other cells so far studied, patch formation awaits an endocytosis-derived intracellular membrane supply. Studies of neurons did not determine which step in the resealing response of the neuron requires calpain. The current investigation shows that calpain activation within the first few seconds following injury is sufficient to salvage cell viability (Fig. 1*E*). Its role, as discussed below, may be in the rapid cytoskeletal remodeling that accompanies the fusion events of plasma membrane resealing. This is the first investigation to establish a requirement for *Capns1* and probably the typical calpains, m- or μ-calpain, in fibroblast plasma membrane resealing. Expression of the other typical calpains appears to be restricted to specific tissues (41), and to our knowledge, they have not been found in fibroblasts. Calpains may be required for resealing membrane breaks in other mammalian cells as well. Preliminary studies suggest that cardiomyocytes require calpain for membrane repair (Fig. 1*F*).

Talin and vimentin fragmentation within seconds after injury is consistent with the notion that calpain activity is required for cytoskeletal remodeling immediately after membrane disruption. Previous studies have shown that removal of the actin cytoskeleton is a critical early event for resealing (22). Calpain-dependent disassembly of the damaged actin network may serve to prepare the wound area for subsequent events that initiate restoration of the cortical cytoskeleton. Indeed, it is well established in the amphibian oocyte system that wound closure requires an actomyosin contractile ring that forms *de novo* at the site of damage and recruits adjacent cortical actin fibers to fill in the wound (42). Contractile ring formation requires several min. If a similar mechanism exists in fibroblasts and other somatic cells, calpain-catalyzed cleavage of talin and vimentin would precede contractile ring formation (Fig. 4*A*), consistent with a role for calpain in clearing cytoskeleton remnants at the damage site prior to formation of a new cortical actin layer. Although it appears that talin and vimentin are processed by calpains immediately following injury, it is not presently clear which of these proteins, if either, must be cleaved to allow enhanced survival. Moreover, the extent of cytoskeletal remodeling that is required for increased survival is also unknown. Thus, although calpains appear to be activated within the first measurable time point following injury (Fig. 6, *SSU panel*, at 0.1 min), maximum vimentin fragment generation seems to take longer (1 min postscraping in Fig. 6). It is possible that much of this vimentin proteolysis occurred after the calpain-dependent survival event, which appeared to require only a brief exposure to calcium (Fig. 1*E*). Technical limitations, including the rapidity of calcium effects on survival, have prevented us from pinpointing proteolytic events that are required for survival, and alternative approaches will be necessary to address this important issue.

The balance of calpain and calpastatin content at the wound site is probably important for regulation of membrane repair (Fig. 2), and calpastatin association with the luminal surface of the plasma membrane may be especially critical. It has previously been suggested that calpastatin is localized at the plasma membrane to prevent spurious activity of calpains in the usual physiologic setting. For example, association of the 117-kDa form of calpastatin with L-type calcium channels on the plasma membrane may prevent their calpain-mediated cleavage during rapid calcium entry (43). Within the context of the current study, removal of localized 117-kDa membrane-associated calpastatin is brought about in part directly by mechanical damage, since this form is shed in the membrane fragment fraction (Fig. 7*B*, 0.1 min, *lane F*). This rapid, direct removal of localized calpastatin could facilitate the calpain-mediated processing of cortical cytoskeletal proteins during membrane repair. The membrane fragment fraction described in the present work is similar in composition to the naturally occurring matrix vesicles shed during cell migration, lending support to the concept that a similar calpain-mediated membrane resealing may

accompany cell movement. Further study is required to determine whether calpain-dependent clearing of residual calpastatin at the injury site is also important.

In summary, we herein provide the first evidence for a rapid, cell life-sustaining event requiring μ- or m-calpain activity. Moreover, the present findings suggest the need to reassess the rationale for application of calpain inhibitors as therapeutic agents to prevent pathologic activation of calpains. As we have shown here, calpains appear to have immediate protective effects following mechanical damage of the plasma membrane, and it may be beneficial to enhance this early function. For example, membrane-impermeant calpain activators might aid calpain-dependent repair of plasma membrane tears while avoiding enhancement of intracellular calpain functions, including their participation in apoptosis and necrosis. Development of such activators could provide valuable therapeutic agents for treating diseases associated with plasma membrane fragility, including Duchenne and other muscular dystrophies.

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REFERENCES

1. McNeil, P. L., and Kirchhausen, T. (2005) *Nat. Rev. Mol. Cell. Biol.* **6,** 499–505
2. McNeil, P. L., and Steinhardt, R. A. (2003) *Annu. Rev. Cell Dev. Biol.* **19,** 697–731
3. Walker, N. I., Harmon, B. V., Gobe, G. C., and Kerr, J. F. (1988) *Methods Achiev. Exp. Pathol.* **13,** 18–54
4. Uhlig, S. (2002) *Am. J. Physiol.* **282,** L892–L896
5. McNeil, P. L., Miyake, K., and Vogel, S. S. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 4592–4597
6. Bansal, D., and Campbell, K. P. (2004) *Trends Cell Biol.* **14,** 206–213
7. Richard, I., Broux, O., Allamand, V., Fougerousse, F., Chiannilkulchai, N., Bourg, N., Brenguier, L., Devaud, C., Pasturaud, P., Roudaut, C., Hillaire, D., Passos-Bueno, M. R., Zatz, M., Tischfield, J. A., Fardeau, M., Jackson, C. E., Cohen, D., and Beckmann, J. S. (1995) *Cell* **81,** 27–40
8. McNeil, A. K., Rescher, U., Gerke, V., and McNeil, P. L. (2006) *J. Biol. Chem.* **281,** 3502–35207
9. Godell, C. M., Smyers, M. E., Eddleman, C. S., Ballinger, M. L., Fishman, H. M., and Bittner, G. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 4751–4756
10. Yoo, S., Nguyen, M. P., Fukuda, M., Bittner, G. D., and Fishman, H. M. (2003) *J. Neurosci. Res.* **74,** 541–551
11. Goll, D. E., Thompson, V. F., Li, H., Wei, W., and Cong, J. (2003) *Physiol. Rev.* **83,** 731–801
12. Ono, Y., Shimada, H., Sorimachi, H., Richard, I., Saido, T. C., Beckmann, J. S., Ishiura, S., and Suzuki, K. (1998) *J. Biol. Chem.* **273,** 17073–17078
13. Glading, A., Bodnar, R. J., Reynolds, I. J., Shiraha, H., Satish, L., Potter, D. A., Blair, H. C., and Wells, A. (2004) *Mol. Cell. Biol.* **24,** 2499–2512
14. Fukui, I., Tanaka, K., and Murachi, T. (1989) *Biochem. Biophys. Res. Commun.* **162,** 559–566
15. Yamamoto, S., Shimizu, K., Suzuki, K., Nakagawa, Y., and Yamamuro, T. (1992) *Arthritis Rheum.* **35,** 1309–1317
16. Dourdin, N., Brustis, J. J., Balcerzak, D., Elamrani, N., Poussard, S., Cottin, P., and Ducastaing, A. (1997) *Exp. Cell Res.* **235,** 385–394
17. Dayton, W. R., Reville, W. J., Goll, D. E., and Stromer, M. H. (1976) *Biochemistry* **15,** 2159–2167
18. Wang, K. K. W., and Yuen, P.-W. (1999) in *Calpains: Pharmacology and Toxicology of Calcium-Dependent Protease* (Wang, K. K. W., and Yuen, P.-W., eds) pp. 77–101, Taylor and Francis, Philadelphia
19. Nayal, A., Webb, D. J., and Horwitz, A. F. (2004) *Curr. Opin. Cell Biol.* **16,** 94–98
20. Franco, S. J., Rodgers, M. A., Perrin, B. J., Han, J., Bennin, D. A., Critchley, D. R., and Huttenlocher, A. (2004) *Nat. Cell Biol.* **6,** 977–983
21. Dourdin, N., Bhatt, A. K., Dutt, P., Greer, P. A., Arthur, J. S., Elce, J. S., and Huttenlocher, A. (2001) *J. Biol. Chem.* **276,** 48382–48388
22. Miyake, K., McNeil, P. L., Suzuki, K., Tsunoda, R., and Sugai, N. (2001) *J. Cell Sci.* **114,** 3487–3494
23. Zhang, W., Lane, R. D., and Mellgren, R. L. (1996) *J. Biol. Chem.* **271,** 18825–18830
24. Peng, M., Huang, L., Xie, Z., Huang, W. H., and Askari, A. (1996) *J. Biol. Chem.* **271,** 10372–10378
25. Xu, Y., and Mellgren, R. L. (2002) *J. Biol. Chem.* **277,** 21474–21479
26. Pawar, S., Kartha, S., and Toback, F. G. (1995) *J. Cell. Physiol.* **165,** 556–565
27. Mosmann, T. (1983) *J. Immunol. Methods* **65,** 55–63
28. Fischer, S., Vandekerckhove, J., Ampe, C., Traub, P., and Weber, K. (1986) *Biol. Chem. Hoppe-Seyler* **367,** 1147–1152
29. Byun, Y., Chen, F., Chang, R., Trivedi, M., Green, K. J., and Cryns, V. L. (2001) *Cell Death Differ.* **8,** 443–450
30. Mellgren, R. L. (1991) *J. Biol. Chem.* **266,** 13920–13924
31. Arthur, J. S., Elce, J. S., Hegadorn, C., Williams, K., and Greer, P. A. (2000) *Mol. Cell. Biol.* **20,** 4474–4481
32. Lee, T. L., Lin, Y. C., Mochitate, K., and Grinnell, F. (1993) *J. Cell Sci.* **105,** 167–177
33. Palecek, S. P., Huttenlocher, A., Horwitz, A. F., and Lauffenburger, D. A. (1998) *J. Cell Sci.* **111,** 929–940
34. Mayer, C., Maaser, K., Daryab, N., Zanker, K. S., Brocker, E. B., and Friedl, P. (2004) *Eur. J. Cell Biol.* **83,** 709–715
35. Botion, L. M., Brasier, A. R., Tian, B., Udupi, V., and Green, A. (2001) *Endocrinology* **142,** 5069–5075
36. Schoenwaelder, S. M., and Burridge, K. (1999) *J. Biol. Chem.* **274,** 14359–14367
37. Carragher, N. O., Westhoff, M. A., Riley, D., Potter, D. A., Dutt, P., Elce, J. S., Greer, P. A., and Frame, M. C. (2002) *Mol. Cell. Biol.* **22,** 257–269
38. DeMartino, G. N., Huff, C. A., and Croall, D. E. (1986) *J. Biol. Chem.* **261,** 12047–12052
39. Mellgren, R. L., Lane, R. D., and Mericle, M. T. (1989) *Biochim. Biophys. Acta* **999,** 71–77
40. Hao, L. Y., Kameyama, A., Kuroki, S., Takano, J., Takano, E., Maki, M., and Kameyama, M. (2000) *Biochem. Biophys. Res. Commun.* **279,** 756–761
41. Huang, Y., and Wang, K. K. (2001) *Trends Mol. Med.* **7,** 355–362
42. Benink, H. A., and Bement, W. M. (2005) *J. Cell Biol.* **168,** 429–439
43. Mellgren, R. L. (1987) *FASEB J.* **1,** 110–115