

Fetuin A Stabilizes m-Calpain and Facilitates Plasma Membrane Repair*

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Yeast two-hybrid experiments identified α_2 -Heremans-Schmid glycoprotein (human fetuin A) as a binding partner for calpain domain III (DIII). The tandem DIIs of calpain-10 interacted under the most selective culture conditions, but DIIs of m-calpain, calpain-3, and calpain-5 also interacted under less stringent selection. DIIs of μ -calpain, calpain-6, and the tandem DIII-like domains of the *Dictyostelium* Cpl protein did not interact with α_2 -Heremans-Schmid glycoprotein in the yeast two-hybrid system. Bovine fetuin A stabilized proteolytic activity of purified m-calpain incubated in the presence of mM calcium chloride and prevented calcium-dependent m-calpain aggregation. Consistent with the yeast two-hybrid studies, fetuin A neither stabilized μ -calpain nor prevented its aggregation. Confocal immunofluorescence microscopy of scratch-damaged L6 myotubes demonstrated accumulation of m-calpain at the wound site in association with the membrane repair protein, dysferlin. m-Calpain also co-localized with fluorescein-labeled fetuin A at the wound site. The effect of fetuin A on calpain-mediated plasma membrane resealing was investigated using fibroblasts from *Capns1*^{-/-} and *Capns1*^{+/+} mouse embryos. *Capns1* encodes the small noncatalytic subunit that is required for the proteolytic function of m- and μ -calpains. Thus, *Capns1*^{-/-} fibroblasts do not express these calpains in active form. Fetuin A increased resealing of scrape-damaged wild-type fibroblasts but not *Capns1*^{-/-} fibroblasts. These studies identify fetuin A as a potential extracellular regulator of m-calpain at nascent sites of plasma membrane wounding.

Calpains, cytoplasmic cysteine proteinases found in all mammalian cells, appear to contribute to a variety of cell functions by producing limited, calcium-dependent proteolysis of key regulatory proteins (1, 2). The ubiquitous, typical calpains, μ - (micro) and m- (milli) calpains (calpains 1 and 2, respectively), are heterodimers comprised of unique large, catalytic subunits (*Capn1* and *Capn2*, respectively) combined with a small subunit (the *Capns1* gene product). Although the role of the small subunit has not been clearly established, it is thought to func-

tion as a regulatory, targeting, or stabilizing component for the catalytic subunit (1). Twelve other calpain genes are expressed in the human and mouse genomes. Some of these genes are ubiquitously expressed, but many have a tissue-selective expression pattern (1, 3). For example, *Capn3* is expressed predominantly in skeletal muscle, and inactivating mutations in human *Capn3* result in limb girdle muscular dystrophy type 2A (4, 5). Very little is currently known about the protein properties of most of the calpain gene products. Because the current work deals predominantly with μ - and m-calpain, we will refer to these generically as calpain hereafter, with the understanding that properties of the other calpain family members, when they come to be known, may differ substantially.

The calpain large subunit domain structure is well known from crystallographic studies (6–8). The amino-terminal domains I and II (DI and DII),³ contain the catalytic triad structure that assembles into an active site in the presence of calcium ion. DIII is similar in three-dimensional structure to C₂ calcium, phospholipid, and protein-binding domains previously identified in conventional protein kinase C isozymes, phospholipases, and several other proteins (9–11). DIV is a penta-EF hand domain that binds calcium and undergoes conformational alterations that facilitate calpain activation and its interaction with calpastatin, an endogenous calpain-specific inhibitor protein. Calpastatin, is a highly efficient regulator of the ubiquitous, typical calpains, producing complete inhibition at ~1:1 ratios of calpain to calpastatin inhibitory domain (12, 13). Depending on the splice variant produced in a particular cell, there are three or four functionally independent calpain inhibitory domains/calpastatin molecule (1, 14).

In addition to calpastatin, other factors are thought to tightly control calpain activity in healthy cells, consistent with the observation that uncontrolled proteolysis by calpains leads to cell death (15, 16). Of paramount importance is the near absence of calpain activity at basal free calcium concentrations in cells. Supraphysiologic intracellular calcium concentrations would be required for half-maximum proteolytic activity (~5–50 μ M for μ -calpain, and 200–1000 μ M for m-calpain). Moreover, the activation of calpains by calcium is a highly cooperative process (17), and neither isozyme would appear to have significant activity at cell volume-averaged levels of free calcium ion in healthy cells. Recent studies provide compelling

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³ The abbreviations used are: DI–DIV, calpain domains 1–4; AHS, α_2 -Heremans-Schmid glycoprotein; FBS, fetal bovine serum; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; MOPS, 4-morpholinepropane-sulfonic acid; DMEM, Dulbecco's modified Eagle's medium; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline.

evidence that μ -calpain is, however, active in cells (18–20). This is probably because transient free calcium gradients reach concentrations necessary for activation of this calpain isozyme at discrete subcellular locations. In other studies, m-calpain was found to be activated in cells following its growth factor-mediated phosphorylation (21), which may substantially decrease or abolish its requirement for calcium ion.

An obvious mechanism for calpain activation would be exposure to the extracellular space, where mM free calcium ion concentrations would allow nearly maximal activity of either μ - or m-calpain. Indeed, over the years there have been reports of extracellular calpain, for example in arthritic bone joints (22) and in the serum of thrombotic thrombocytopenic purpura patients (23, 24). There is evidence that calpain released from injured hepatocytes can produce extended damage of surrounding liver cells (25, 26). Recently, calpains have been associated with repair of mechanically damaged plasma membrane, functioning to remodel cortical cytoskeleton at the interface between the intracellular and extracellular environment (27). A catalytic function for extracellular calpains assumes that they will remain active for a significant period of time after their release from cells. In fact, *in vitro* studies with purified calpains raise doubts about this assumption. Calpains undergo rapid autoproteolytic inactivation (28) and also aggregate and precipitate within a few minutes (29, 30) at 37 °C in the presence of mM calcium ion. Neither autoproteolysis nor aggregation is influenced by the addition of large stoichiometric excesses of various other proteins, implying a marked tendency of calpains to self-associate in the presence of mM calcium ion. These properties would tend to rapidly limit the proteolytic activity of calpains that escape the intracellular environment.

Here we show that a major blood plasma protein, fetuin A, markedly stabilizes m-calpain while having no apparent effect on the stability or solubility of μ -calpain, in millimolar concentrations of calcium-containing buffer. Moreover, fetuin A facilitated calpain-mediated membrane repair of scrape-damaged fibroblasts. These findings represent to our knowledge the first example of a blood-borne factor enhancing plasma membrane repair. Extracellular m-calpain, stabilized by fetuin A, would have the potential to affect extracellular matrix and neighboring cells.

EXPERIMENTAL PROCEDURES

Materials—Fetuin A isolated from bovine serum was obtained from Sigma (product number F-3385). For immunofluorescence localization studies, it was labeled with FITC, using an established procedure (31), and purified by Sephadex G25 chromatography. BSA (product number A-6003) and Poloxamer 188 (Pluronic F-68[®], a product of BASF), were obtained from Sigma. Vitamin-free bovine casein, chicken egg lysozyme, and whale muscle myoglobin were purchased from Sigma. Human erythrocyte μ -calpain and bovine myocardial m-calpain were purified to near homogeneity by established procedures (32). They were stored at –20 °C as 50% glycerol solutions in 50 mM imidazole-HCl, 5 mM EGTA, 1 mM dithiothreitol, pH 7.4. Prior to use in experiments, they were diluted 5-fold in buffer A (20 mM MOPS, 150 mM NaCl, 0.2 mM EGTA, 1 mM dithiothreitol, pH 7.2), incubated at 37 °C for 10 min, and

then kept overnight on ice. Unless this procedure was followed, full recovery of calpain activity was not achieved.

Capns1-null mouse embryonic fibroblasts and wild-type fibroblasts from littermates, both transformed with SV40 large T-antigen, were kindly provided by Dr. Peter Greer (Queen's University, Kingston, Canada). HFL-1 normal human lung fibroblasts and L6 rat myoblasts were obtained from American Type Culture Collection. The cells were maintained at 37 °C in a humidified 5% CO₂ atmosphere, in DMEM (Invitrogen) supplemented with 10% FBS (Atlanta Biologicals). ECL extracellular matrix preparation was purchased from Upstate Biotechnology, Inc., Insulin-transferrin-selenium 100 \times stock solution was obtained from Invitrogen, and FM1–43FX, a fixable fluorescent membrane probe, was obtained from Molecular Probes.

The following first antibodies were used in confocal microscopic immunolocalization studies. Mouse anti-talin clone 8d4 (Sigma) at 40-fold dilution; mouse anti-dysferlin clone Ham1/7B6 (Vector) at 20-fold dilution; goat anti-annexin-A2 (Santa Cruz SC-1924) at 20-fold dilution; and a chicken IgY developed against rat m-calpain at 50-fold dilution. Second antibodies were: TRITC goat anti-mouse IgG (Sigma T-7782) at 200-fold dilution; Cy5 rabbit anti-mouse IgG (Zymed Laboratories Inc. 81-6716) at 100-fold dilution; TRITC rabbit anti-goat (Sigma T-7028) at 100-fold dilution; and Alexa 633 goat anti-chicken IgY (Molecular Probes A21103) at 200-fold dilution.

Yeast Two-hybrid Studies—Matchmaker[®] Two-Hybrid System 3 was utilized for our studies. In general, the methods recommended by the manufacturer (Clontech) were followed. For identification of calpain-10-interacting proteins, DIIX2 was cloned into the pGBKT7 bait vector. AH109 strain yeast bearing the bait vector was mated with yeast bearing the Matchmaker[®] fetal human liver cDNA library in the pGADT7 vector, and blue colonies were selected following plating on the highest stringency medium (SD/–Ade/–His/–Leu/–Trp/5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside). Plasmids from initially positive clones were rescued in *Escherichia coli*, and those shown to recapitulate interaction with DIIX2 in yeast were studied further. The following criteria were used to select positive clones from within this population: 1) failure to grow in highest stringency medium in the absence of the bait construct or when mated with yeast bearing negative control lamin C or p53 bait constructs; 2) the presence of multiple identical clones in the initial screen, as determined by Alu I digestion of plasmids; and 3) verification that the inserts were in-frame and represented at least part of the cDNA coding sequence. Four unique clones satisfied these criteria, out of the 3 \times 10⁶ copy library screen. One of them was the ~80% full-length AHSG clone that is the subject of this report.

To investigate the specificity of DIIX2 association with AHSG, the DIIXs of human μ -calpain (calpain-1), rat m-calpain (calpain-2), mouse calpain-3, mouse calpain-5, human calpain-6, and *Dictyostelium discoideum* Cpl were amplified from the corresponding cDNAs by PCR and inserted into the pGADT7 vector in-frame. Vectors bearing cDNAs for the individual DIIXa and DIIXb of mouse calpain-10 were also constructed. Yeast transformed with these vectors were mated with yeast bearing the prey vector containing the AHSG insert, and

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growth was assessed on SD/–His/–Leu/–Trp (low stringency) or SD/–Ade/–His/–Leu/–Trp (moderate stringency) plates. Mating efficiency was confirmed for all experiments by demonstrating vigorous growth on SD/–Leu/–Trp plates.

Calpain Stability and Aggregation in the Presence of Calcium—To study the effect of fetuin A on calpain stability, purified μ - or m-calpain were incubated with or without added fetuin A at 37 °C in 20 mM MOPS, 150 mM NaCl, 1.5 mM CaCl₂, pH 7.2. In different experiments, as indicated in the figure legends, calpain concentration varied between 25 and 100 μ g/ml. At various times, aliquots of reaction mixture were removed to ice and diluted 2-fold in buffer A containing 0.2 mg BSA/ml and 5 mM EGTA. When all of the samples were collected, they were assayed for calpain activity in the presence of 5 mM CaCl₂, using the standard caseinolysis assay and [¹⁴C]methylcasein as substrate (28). Maximum carry-over of fetuin A in the assay was 50 μ g/ml. At this concentration, fetuin A did not significantly affect the assay results.

Aggregation and precipitation of calpains was carried out in 20 mM MOPS, 1.5 mM CaCl₂, 1 mM dithiothreitol, 100 μ M leupeptin, pH 7.2, at 37 °C, with or without the addition of 1 mg/ml fetuin A. After 10 min, the samples were placed on ice and immediately centrifuged at 20,000 \times g for 5 min. The supernatants were removed, and the pellets were resuspended in an equivalent volume of MOPS buffer with 5 mM EGTA in place of CaCl₂. The samples were heated in SDS sample preparation buffer, and equivalent volumes were subjected to SDS-PAGE and Coomassie Blue staining. The same procedure was employed to investigate fetuin cleavage by calpains, except that leupeptin was omitted, and samples of incubation mixture were directly subjected to SDS-PAGE.

Calpain Assay and Kinetic Analysis—Calpain proteolytic activity was assayed using [¹⁴C]methylcasein as substrate. Inhibition by fetuin A was analyzed using the Woolf-Hanes kinetic plot (33). In this graphical representation, competitive inhibition is reflected by intersection of plots at a positive *Y* value when substrate (the *x* axis) is zero. In noncompetitive inhibition, plots intersect at the negative *x* axis ($-K_m$) when the *y* axis (substrate/velocity) is zero.

Confocal Immunofluorescence Microscopy—Confocal images were obtained using a Leica TCS SP5 multi-photon laser scanning confocal microscope housed in the Advanced Microscopy and Imaging Center, University of Toledo Health Science Campus. Except where noted, the microscope was focused through the middle of the cells. Emission and excitation wavelengths were chosen from the provided software for each fluorophore utilized in the studies, and we confirmed that settings used in co-localization studies did not produce overlapping fluorescent signals. Identical instrument sensitivity settings were used for all samples within a given experiment. Image manipulation using Corel PhotoPaint® was limited to alteration of intensity, contrast, and brightness settings, all of which were identical for data sets to be compared with each other.

L6 myoblasts were grown on acid-washed, ECL-coated microscope coverslips to confluence in DMEM containing 10% FBS and then switched to differentiation medium: DMEM without serum, supplemented with insulin, transferrin, and selenium from a commercial 100 \times stock solution (Invitrogen).

After 4 days, most of the myoblasts had differentiated, as determined by the presence of large, multinucleated myotubes that stained positive for desmin (not shown). Four-day-old myotubes were washed once with PBS and incubated in Dulbecco's PBS containing 1.5 mM CaCl₂, 2.8 mM D-glucose, and 0.33 mM sodium pyruvate at 37 °C. In some experiments 2 μ M FM1–43FX or 1 mg/ml FITC-fetuin A was included in the scraping buffer. The coverslips were scratched twice in a cross-pattern with a 23-gauge needle and aspirated after 1 min to remove buffer. The myotubes were then immediately fixed for 10 min with 0.5 ml of cold 4% paraformaldehyde in PBS. The samples were processed for immunofluorescence microscopy by a standard protocol (31) *without detergent permeabilization*. First antibodies were diluted in PBS containing 2% BSA, and second antibodies were diluted in PBS containing 10% nonimmune serum derived from the same species used to develop the antibody. Control immunostains using nonrelevant monoclonal first antibodies or without first antibody treatment were routinely employed and did not produce significant signals (data not shown).

In some experiments, relative fluorescence intensities of FM1–43FX in damaged myotubes were calculated using imagej software. The total fluorescence intensity was measured for all myotubes within the field of a 60 \times objective micrograph spanning both sides surrounding a needle-denuded zone of myotubes. All of the readings were corrected for background fluorescence determined from an area within the cell-free scratched zone.

Cell Damage Protocols—HFL-1 human lung fibroblasts or SV-40 large T-antigen transformed mouse embryonic fibroblasts were cultured in DMEM containing 10% FBS in 12-well plates until ~50% confluent. The cells were subjected to cell scrape damage as previously described (27). Briefly the cells were transferred to 37 °C Dulbecco's PBS containing 1.5 mM CaCl₂ and other additions where indicated. A cell scraper was used to produce membrane damage as previously described (27), with a minor modification when HFL-1 fibroblasts were employed. To minimize cell clumping after the scrape protocol, the HFL-1 cells were allowed to recover for 10 min, collected by centrifugation, and then trypsinized (Sigma #T-4174 trypsin-EDTA solution, diluted 2-fold with PBS). After 10 min, 10 volumes of growth medium was added, the cells were replated, and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reductase cell viability assay (34) was performed after 12 h of culture. The percentage of survival was determined by comparison with cells in wells of the same culture dish that were not scraped but carried through all other steps of the procedure.

Plasma membrane resealing was determined by assessing cellular uptake of trypan blue dye added 10 s after scrape-induced damage. Capns1^{-/-} or Capns1^{+/+} fibroblasts were scraped in the presence of 150 μ l Dulbecco's PBS containing 1.5 mM CaCl₂ and other additions where indicated. Ten seconds later, 150 μ l of 0.4% trypan blue dye in 0.85% saline (Invitrogen) was added. The cells were introduced into a hemacytometer and scored as trypan blue-positive or -negative within 2 min after the addition of dye. For zero time samples, trypan blue was included in the buffer before cell scraping. The percent of recovery of membrane impermeability 10 s after scraping was

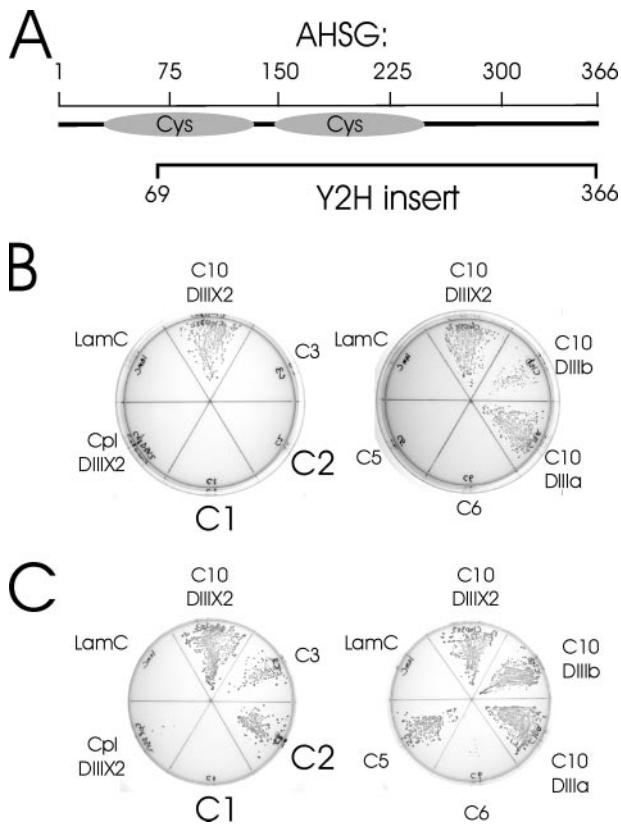


FIGURE 1. Yeast two-hybrid screening suggests an interaction between AHSG and DIIs present in several calpains. A shows the domain structure of AHSG and the partial clone identified in the yeast two-hybrid interaction studies. Cys, the tandem cystatin domains of AHSG. Yeast two-hybrid studies were carried out as described under "Experimental Procedures." Moderately selective culture conditions (SD/-Ade/-His/-Leu/-Trp) are depicted in B, whereas low stringency conditions (SD/-His/-Leu/-Trp) are represented in C. C1, μ -calpain DIII; C2, m-calpain DIII; C3, calpain-3 DIII; C5, calpain-5 DIII; C6, calpain-6 DIII; C10DIIIa, first DIII of calpain-10; C10DIIIb, second (carboxyl-terminal) DIII of calpain-10; C10DIIIc, tandem DIIs of calpain-10; Cpl DIII2, tandem DIII-like domains of *D. discoideum* Cpl protein; LamC, lamin C. Labels C1 and C2 are enlarged for ease of identification.

determined by subtracting the percentage of zero time impermeant (colorless) cells from the percentage of impermeant cells in the 10-s samples.

Statistical Analysis—Except where otherwise indicated, statistical analyses were performed using Student's unpaired *t* test. A *p* value of <0.05 was considered to reflect a statistically significant difference. All of the error bars in the graphs represent standard deviation.

RESULTS

Yeast Two-hybrid Studies Identify AHSG as a DIII-interacting Protein—Yeast two-hybrid screening of a human fetal liver library, as described under "Experimental Procedures," identified a clone expressing an insert comprising amino acids 69–366 of AHSG (human fetuin A) as a binding partner for the tandem DIIs of calpain-10 (DIII2) (Fig. 1A). Interaction of AHSG with various calpain DIIs was assessed using moderate stringency (Fig. 1B) or low stringency (Fig. 1C) plates. The amino-terminal DIIIa appeared to interact more strongly with AHSG than the carboxyl-terminal DIIIb (Fig. 1B). At less stringent selection, several other calpain DIIs appeared to interact with AHSG. In addition to the DIIs of calpain-10, DIIs of

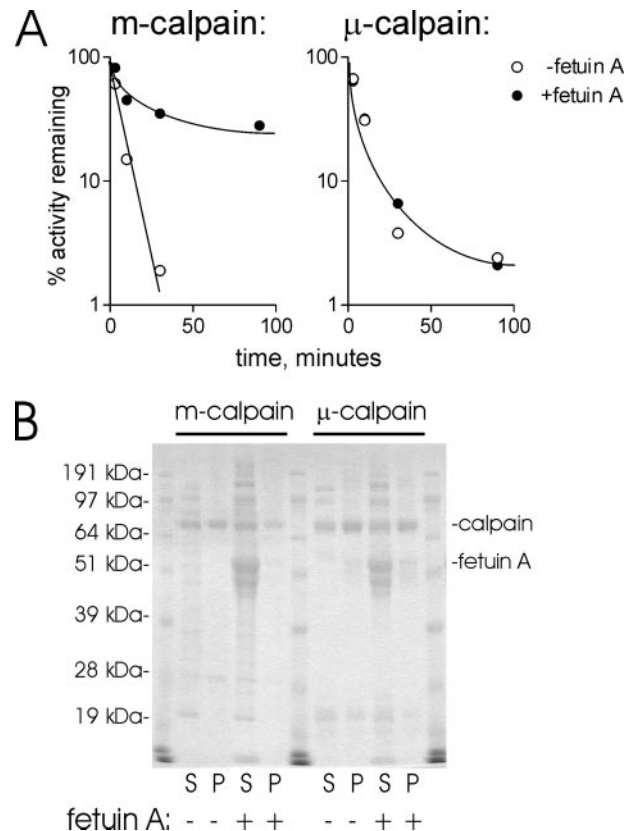


FIGURE 2. Fetuin A selectively stabilizes and solubilizes m-calpain in the presence of calcium ion. Calpains were incubated at 37 °C in the presence of 1.5 mM CaCl₂ plus or minus 1 mg/ml fetuin A and assessed for catalytic stability (A) or aggregation (B), as described under "Experimental Procedures." Calpain concentrations in both panels are 100 μ g/ml. B: Ten microliters of each fraction were loaded on SDS-PAGE gels. S, supernatant fraction; P, resuspended pellets.

m-calpain (C2 in Fig. 1C), calpain-3 (C3), and calpain-5 (C5) interacted with AHSG. The DIIs of μ -calpain (C1), and calpain-6 (C6) did not interact; neither did the tandem DIII-like domains of the *Dictyostelium* Cpl protein (35).

Fetuin A Stabilizes m-Calpain—Consistent with the weak interaction suggested by the yeast two-hybrid studies, co-precipitation studies, using either immobilized fetuin A or m-calpain as bait, failed to reproducibly demonstrate association of the two purified proteins (data not shown). However, because fetuin A is a major serum protein (36–38), it seemed likely that a relatively weak interaction with m-calpain could still have important physiologic consequences. Therefore, other studies were initiated to assess weak interactions between m-calpain and fetuin A.

We exploited the rapid autolysis of calpains to investigate their potential interaction with fetuin A. The caseinolytic activity of m-calpain decays with a half-life of only a few minutes when incubated at 37 °C in the presence of mM calcium ion (Ref. 28 and Fig. 2A, left panel, open circles). However, in at least three independent experiments, m-calpain was remarkably stable when incubated in the presence of 1.5 mM calcium chloride and 1 mg of fetuin A/ml (Fig. 2A, left panel, filled circles), whereas the stability of μ -calpain was not affected by fetuin A (Fig. 2A, right panel). Exposure to fetuin A also decreased calcium-mediated m-calpain aggregation and precipitation (Fig. 2B), whereas μ -calpain precipitation was not altered. Preserva-

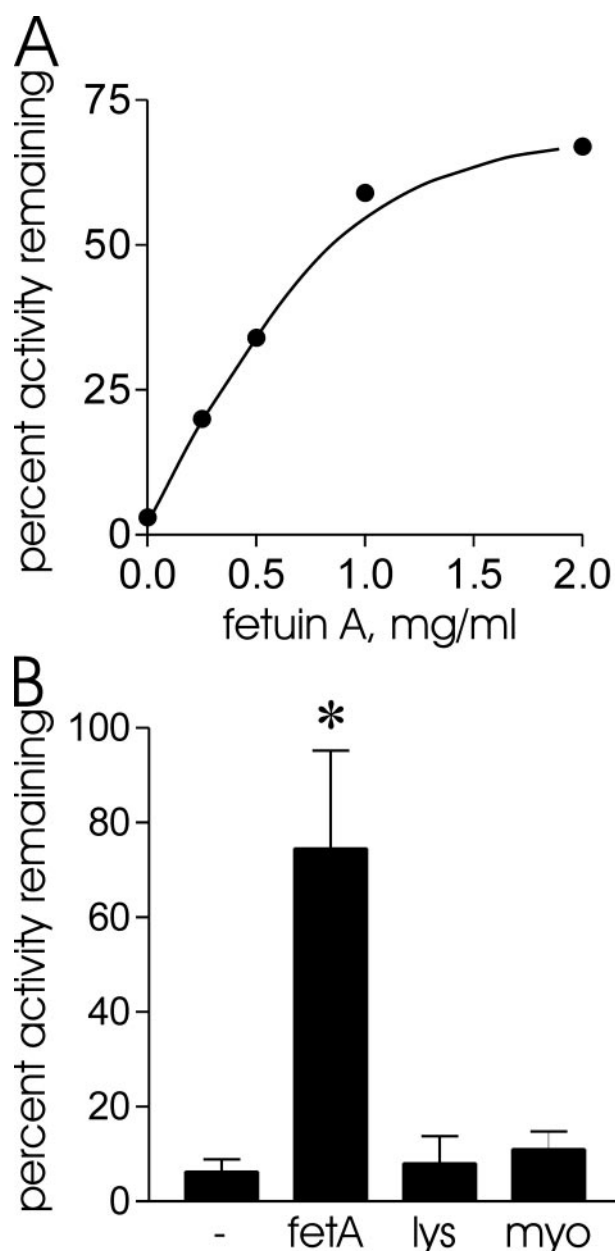


FIGURE 3. Effect of fetuin A concentration on m-calpain stabilization and specificity of the stabilizing effect for fetuin A. *A*, m-calpain at a concentration of 25 $\mu\text{g/ml}$ was incubated with the indicated concentration of fetuin A for 30 min at 37 °C, in the presence of buffer A containing 1.5 mM CaCl_2 . Activity remaining was assessed by comparison with untreated calpain in the same assay. *B*, m-calpain was incubated for 30 min at 37 °C in buffer A plus 1.5 mM CaCl_2 , and no addition, or 1 mg/ml fetuin A (*fetA*), lysozyme (*lys*), or myoglobin (*myo*). Asterisk, $p = 0.0006$ versus no addition ($n = 4$).

tion of m-calpain activity was dependent on fetuin A concentration, with significant protection observed at the average physiologic concentration range of fetuin A (AHSG) in human serum, 0.5–0.7 mg/ml (39) (Fig. 3A). Previous studies had shown that m-calpain autoproteolytic inactivation was not protected by the presence of added proteins (28). Accordingly, neither lysozyme nor myoglobin significantly protected m-calpain from calcium-mediated inactivation (Fig. 3B).

The studies depicted in Fig. 2B were carried out in the presence of 100 μM leupeptin to prevent possible proteolysis of fetuin A by the calpains. However, subsequent experiments car-

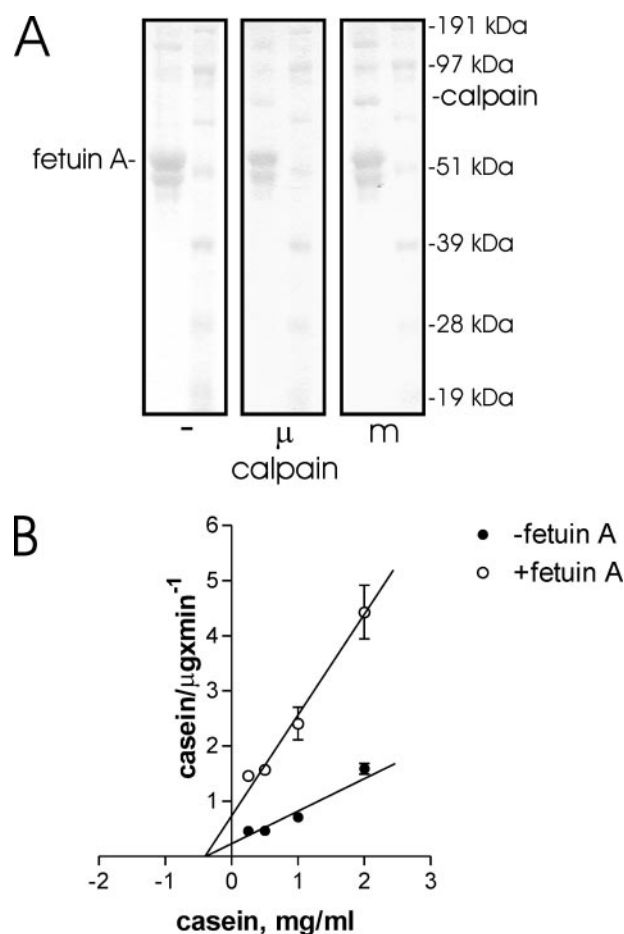


FIGURE 4. Fetuin A is not rapidly processed by calpains but is a weak noncompetitive inhibitor. *A*, one mg/ml samples of fetuin A were incubated at 37 °C for 20 min in buffer A containing 1.5 mM CaCl_2 alone, or in the presence of 100 $\mu\text{g/ml}$ μ -calpain (μ) or m-calpain (*m*). Ten-microliter samples were heated in SDS sample buffer, electrophoresed on a 10% polyacrylamide SDS-PAGE gel, and Coomassie-stained. The gel lanes depicted were from different locations of the same gel. In each case the right lane contains See Blue Plus 2[®] protein standards (Invitrogen). *B*, quadruplicate samples of m-calpain were assayed for caseinolytic activity at various casein concentrations plus or minus 1 mg/ml fetuin A. The data are plotted as described under “Experimental Procedures” by the method of Woolf and Hanes (33). Intersection of the graphs at the x axis at $y = 0$ indicates noncompetitive inhibition. The absence of error bars indicates that they lie within the data point.

ried out under similar conditions, but without leupeptin in the incubation buffer, showed that fetuin A was not rapidly processed upon incubation with μ - or m-calpain (Fig. 4A). Thus, whereas fetuin A stabilized m-calpain, it was not turned over within the time frames we have investigated. Fetuin A partially inhibited the caseinolytic activity of m-calpain, with half-maximal inhibition occurring at about 1 mg/ml. Importantly, inhibition appeared to be noncompetitive (Fig. 4B), consistent with fetuin A interaction with DIII (Fig. 1) instead of the calpain catalytic domains DI and DII.

These results show that the levels of fetuin A present in blood plasma stabilize and solubilize m-calpain in the presence of physiologic extracellular calcium ion. Consistent with its lack of interaction in the yeast two-hybrid studies, μ -calpain was neither stabilized nor solubilized by fetuin A.

Localization of Fetuin A with m-Calpain at Sites of Plasma Membrane Damage—Calpains are thought to be required for calcium-dependent repair of mechanically damaged nerve cell

axons and dendrites (40, 41), as well as mechanically damaged fibroblast plasma membrane (27). Because cytoplasmic proteins, including calpains, would be exposed to extracellular factors following plasma membrane rupture, we investigated whether fetuin A localizes at sites of membrane damage where it could potentially influence calpain-dependent repair. For these studies we utilized myotubes, which express dysferlin, a protein that has been shown to accumulate at sites of membrane injury and is a putative membrane repair protein (11, 42).

Previous studies demonstrated that calpain-null fibroblasts failed to reseal laser damage to the plasma membrane. These latter experiments measured intracellular uptake of the phospholipid-staining green fluorescent dye, FM1-43 (43), which is normally impermeant but accumulates in cells through damaged plasma membrane. We carried out preliminary studies with injured myotubes to confirm that they also resealed in a calpain-dependent fashion. Needle scratch damage of L6 myotubes, as described under "Experimental Procedures," allowed uptake of FM1-43FX, a fixable analog of FM1-43 (Fig. 5A). Preincubation with calpeptin to inactivate endogenous calpains resulted in increased accumulation of FM1-43FX (Fig. 5B, and middle rows in Fig. 5A), indicating failure to repair membrane damage in calpain-depleted myotubes.

Cell surface biotinylation studies indicated that talin and other cortical cytoskeletal proteins become associated with damaged plasma membrane of scraped fibroblasts (27). Because these proteins are cleaved by calpains after membrane damage, it has been proposed that their processing may facilitate cytoskeletal remodeling as part of the repair mechanism (27). Talin and dysferlin were exposed at the wound site of needle-scratched myotubes (Fig. 5A, top row and enlarged images in the bottom row), and calpeptin treatment decreased accumulation of talin at the injury site. Dysferlin still accumulated at the edge of the wound site in calpeptin-treated myotubes but was associated with regions that were highly labeled with FM1-43FX, indicating a failure to reseal (Fig. 5A, middle row). In contrast, dysferlin was present as discrete patches on FM1-43FX-labeled membranes in myotubes that were not pretreated with calpeptin (Fig. 5A, bottom row), similar to previously observed immunolocalization patterns at sarcolemma damage sites (11, 42). Our studies show that talin, like dysferlin, accumulates at sites of mechanical damage to myotubes. This observation is consistent with biotinylation studies showing talin accumulation at the cell surface after plasma membrane damage of fibroblasts (27).

Further experiments demonstrated accumulation of m-calpain in a pattern that overlapped dysferlin immunofluorescence (Fig. 6A). In contrast, annexin-A2, another protein thought to be involved in membrane repair (44), did not appear to co-localize with dysferlin (Fig. 6B). Previous studies had localized fetuin A labeling to cells adjacent to scrape zones of wounded monolayer cultures (45). However, its co-localization with membrane repair proteins was not investigated. We found that m-calpain co-localized with FITC-labeled fetuin A at wound sites in myotubes (Fig. 7A). In contrast, labeling of fetuin A and annexin-A2 was only partially overlapping. Fetuin was concentrated at the damaged ends of myotubes (asterisk in Fig. 7B), whereas the strongest signals for annexin-A2 were found at

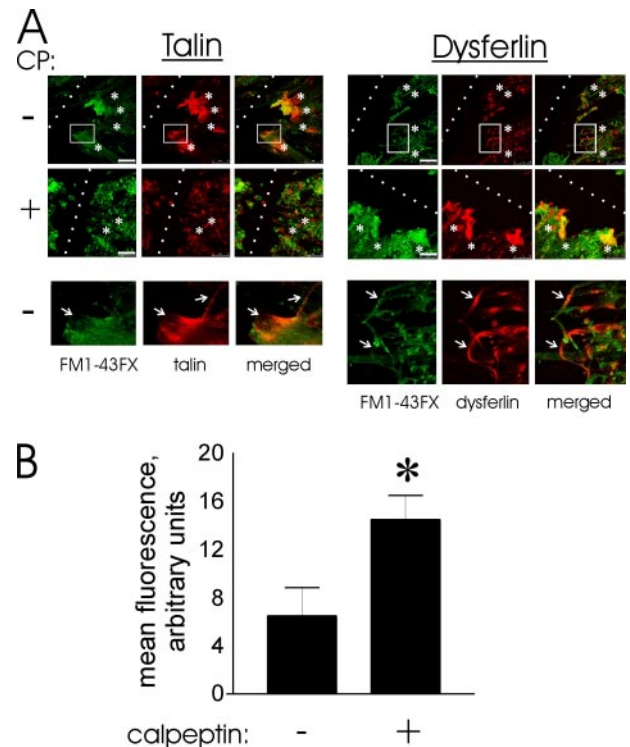


FIGURE 5. L6 myotubes undergo calpain-dependent resealing after needle scratch injury and express talin and dysferlin at the sites of damage.

A, 4-day differentiated rat L6 myotubes were injured by a needle scratch as described under "Experimental Procedures." Where indicated, the myotubes were preincubated for one h with 20 μM calpeptin (CP) to inactivate endogenous calpains. White dashed lines in the micrographs indicate the scratch site. The myotubes were scratched in the presence of 2 μM FM1-43FX dye and fixed in cold 4% paraformaldehyde 1 min after injury. The fixed, nonpermeabilized cells were immunostained for talin or dysferlin, as indicated in the figure, using TRITC-goat anti-mouse IgG as the second antibody. The bottom rows show magnified images of the boxed areas depicted in the top rows. All of the micrographs represent averaged fluorescent signals throughout all 10 z planes scanned through the cells. Asterisks indicate location of myotubes, and arrows show the presence of talin or dysferlin at the surface of damaged myotubes. Note that dysferlin appears as discrete patches continuous with green FM1-43FX fluorescence on cell membranes (arrows). Bars, 50 μm. **B**, mean fluorescence intensities of FM1-43FX in damaged myotubes that were preincubated with 20 μM calpeptin or vehicle (Me₂SO). Details of the analysis are provided under "Experimental Procedures." * $p = 0.015$ versus minus calpeptin samples ($n = 5$), Student's paired t test.

small punctate structures, sometimes coinciding with readily identifiable particles in phase contrast images (arrows in Fig. 7B, and upper arrowheads in Fig. 6B). These experiments demonstrate that fetuin A accumulates at damaged plasma membrane where it has the potential to interact with m-calpain.

Fetuin A Facilitates Plasma Membrane Resealing—To investigate the potential effect of fetuin A on plasma membrane repair, we subjected cell monolayers to scrape damage as previously described (27), with or without the addition of fetuin A in the scrape medium. About 30% of HFL1 human fibroblasts survived following scraping in standard calcium-containing buffer, under the conditions described under "Experimental Procedures" (Fig. 8, A and B). However, the addition of FBS, which contains high levels of fetuin A (37), significantly increased survival, as did 1 mg/ml or 5 mg/ml purified fetuin A. To determine whether the ubiquitous, typical calpains were required for enhanced survival imparted by fetuin A, Capns1^{-/-} and Capns1^{+/+} fibroblasts were scraped with or

Fetuin A Stabilizes m-Calpain

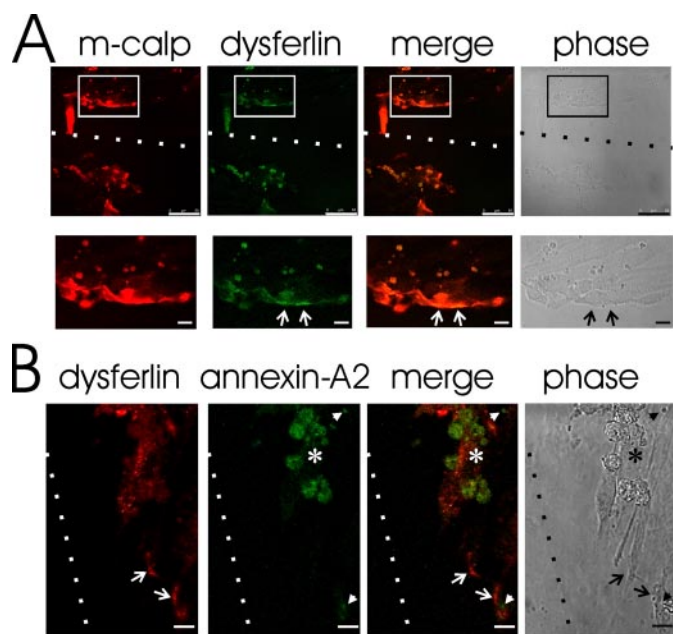


FIGURE 6. m-Calpain co-localizes with dysferlin at the sarcolemma of injured L6 myotubes. *A*, m-calpain (*m-calp*), stained with Alexa 633 goat anti-chicken, co-localized with dysferlin at membrane damage sites in needle-scratched L6 myotubes. Dysferlin was detected with TRITC-labeled anti-goat second antibody, false-colored green in the micrograph to distinguish it from the Alexa 633 signal. *Top row*, bar, 50 μm . *Bottom row*, enlarged image of boxed areas of the *top row*. Bar, 10 μm . The *arrows* show two signals for dysferlin localization, consistent with its presence in membrane repair patches derived from internal cell membranes (see Fig. 5). *B*, another putative membrane repair protein, annexin-A2, did not co-localize with dysferlin, but appeared to be associated with large cell fragments, perhaps dead cells (*asterisk*), and with smaller cell fragments (*arrowheads*). The *arrows* show dysferlin localization at the edge of the scratch zone. Bars, 10 μm .

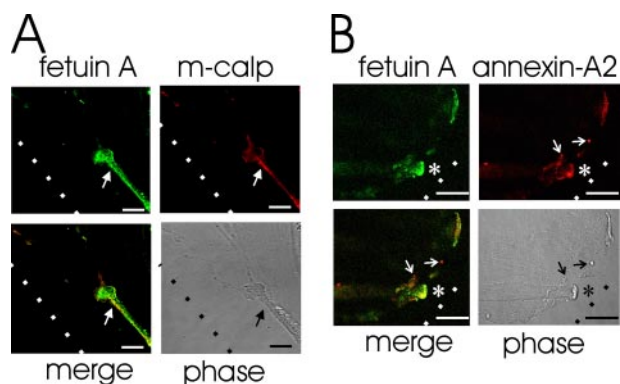


FIGURE 7. Fetuin A and m-calpain co-localize at the surface of injured myotubes. *A*, FITC-fetuin A and m-calpain were present at the injured surface of L6 myotubes adjacent to the scratch zone. Alexa 633 goat anti-chicken second antibody was used to detect m-calpain localization. Note the localization of m-calpain at the edge of the fetuin-labeled damage site (*arrow*), indicated by *yellow* coloration in the merged image. *B*, fetuin A and annexin-A2 did not extensively co-localize. Annexin was detected with TRITC rabbit anti-goat IgG second antibody. *Asterisk*, damaged end of a single large myotube. The *arrows* show annexin labeling of small cell fragments. Bars, 25 μm .

without fetuin A in the scrape medium (Fig. 8C). For comparison, poloxamer 188, which protects skeletal myocytes and other cells from mechanical damage (reviewed in Ref. 46), was also included in this study. Poloxamer significantly increased survival of both *Capns1*^{+/+} and *Capns1*^{-/-} fibroblasts. However, only *Capns1*^{+/+} fibroblasts appeared to be protected by fetuin A. When cells were scraped in the presence of both fetuin

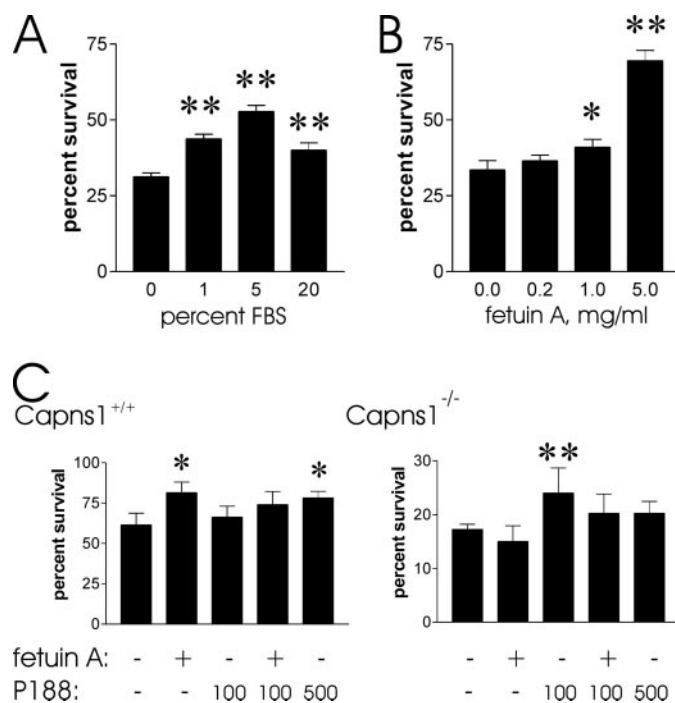


FIGURE 8. Fetuin A protects HFL-1 human lung fibroblasts and *Capns1*^{+/+} mouse fibroblasts from scrape damage-induced cell death. Quadruplicate wells were used for each condition. HFL-1 fibroblasts were scraped as described under "Experimental Procedures" in the presence of FBS (*A*) or purified fetuin A (*B*). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reductase cell survival assays were carried out 12 h later. *Asterisk*, $p = 0.01$ versus no fetuin; *double asterisk*, $p < 0.001$ versus no additions. *C*, *Capns1*^{-/-} and *Capns1*^{+/+} fibroblasts were scraped in the presence of fetuin A (5 mg/ml) or poloxamer 188 (100 or 500 μM) where indicated. *Asterisk*, $p = 0.007$ versus no additions; *double asterisk*, $p = 0.03$ versus no additions.

A and poloxamer 188, there was no additional increase in survival relative to treatment with either agent alone.

In previous studies, *Capns1*^{-/-} and *Capns1*^{+/+} fibroblasts were shown to take up trypan blue dye added at various times after scrape damage. The loss of trypan blue uptake with time after scraping correlated well with the time course of membrane resealing observed in laser-damaged *Capns1*^{+/+} fibroblasts (27). To determine whether fetuin A enhanced plasma membrane resealing in a calpain-dependent fashion, we added trypan blue to *Capns1*^{+/+} or *Capns1*^{-/-} fibroblasts 10 s after scraping, using the protocol described under "Experimental Procedures." At this time point, the fibroblasts were still undergoing resealing (27). Accordingly, both cell types incorporated trypan blue through damaged plasma membrane, indicating a failure to recover membrane impermeability (Fig. 9A). *Capns1*^{-/-} fibroblasts, which fail to reseal for at least 2 min after laser damage (27), were only 10–15% trypan blue-negative 10 s after scraping. *Capns1*^{+/+} fibroblasts were 38% trypan blue negative, consistent with their rapid recovery after laser injury. When *Capns1*^{+/+} fibroblasts were scraped in the presence of 1 or 5 mg fetuin A/ml, the number of trypan blue negative cells 10 s after scrape damage was significantly increased, indicating enhanced resealing of damaged plasma membrane. In contrast, 5 mg/ml BSA, the major protein constituent of adult bovine serum, did not significantly increase the rate of *Capns1*^{+/+} membrane resealing (Fig. 9B). The addition of 5 mg/ml fetuin A to *Capns1*^{-/-} scraping medium did not significantly affect

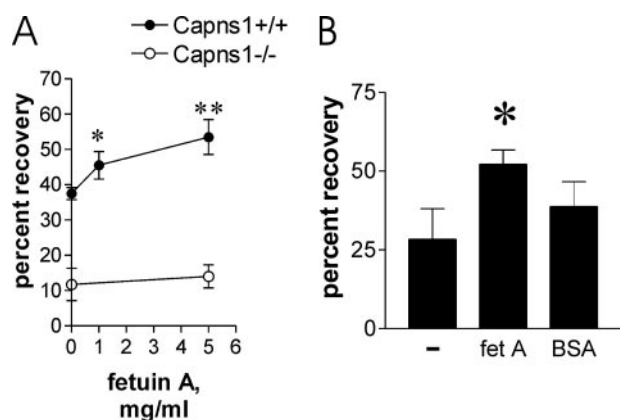


FIGURE 9. Fetuin A increased resealing of wounded Capns1^{+/+} fibroblasts. A, Capns1^{-/-} and Capns1^{+/+} fibroblasts were scrape-damaged in the presence of 1 mg or 5 mg fetuin A/ml, and membrane resealing was assessed by uptake of trypan blue 10 s later, as described under "Experimental Procedures." Asterisk, $p < 0.03$ versus no fetuin ($n = 4$); double asterisk, $p < 0.001$ versus no fetuin ($n = 4$). The results are representative of two other independent experiments. B, Capns1^{+/+} fibroblasts were scraped in the presence of no additions, 5 mg fetuin A/ml (fet A), or 5 mg BSA/ml. Uptake of trypan blue was assessed as in A. Asterisk, $p < 0.01$ versus either no additions or the plus BSA condition ($n = 5$ scraped wells for each condition).

trypan blue uptake 10 s after scrape damage (Fig. 9A). Similar results were obtained in two other independent experiments. In zero time control experiments, both Capns1^{-/-} and Capns1^{+/+} fibroblasts were >90% trypan blue-positive when scraped in the presence of the dye, in the absence or presence of 5 mg/ml fetuin A (not shown). Thus, fetuin A did not appear to decrease the number of cells damaged during the scraping process.

DISCUSSION

AHSG, human fetuin A, is most often associated with regulation of bone mineralization (47). By stabilizing hydroxyapatite constituents in the blood, fetuin A has been shown to prevent their deposition in soft tissues. Consistent with this role, fetuin A knock-out mice exhibit a modest degree of soft tissue calcification (48). AHSG may also have other functions, including acting as a negative regulator of insulin receptor signaling (49). It was this latter proposed role that was of interest when we initially identified AHSG as a binding partner for the tandem DIIIs of calpain-10, another diabetes-related protein, in yeast two-hybrid studies (Fig. 1). Because calpains are intracellular proteinases and AHSG/fetuin A is an extracellular protein secreted predominantly by the liver, their interaction under physiologic conditions was problematic, and this led us to explore hypotheses that would account for fetuin A and calpain interactions. For these studies, we chose μ - and m-calpains because they were readily available in highly purified form from natural sources and because much more is known about their enzymatic and physiologic properties compared with calpain-10 and other more recently described members of the calpain gene family. The apparent lack of interaction of μ -calpain DIII with AHSG in the yeast two-hybrid system (Fig. 1C) made this calpain isozyme a convenient negative control for exploring fetuin A interactions with m-calpain.

The studies presented in Figs. 2 and 3 show that fetuin A had specific stabilizing effects on m-calpain exposed to calcium

concentrations that would be encountered in the extracellular environment or at the interface between cytoplasm and extracellular space in damaged plasma membrane. Moreover, fetuin A did not appear to be a good calpain substrate (Fig. 4). Thus, it may function by interacting with calpain DIII domains (Fig. 1) without substantially blocking catalytic activity. The stabilization of m-calpain by fetuin A is reminiscent of recent studies demonstrating that fetuin A and other cystatin superfamily members can stabilize the gelatinolytic activities of the matrix metalloproteinase, MMP-9 (50), an important modifier of extracellular matrix architecture. The present investigation suggests that fetuin A could play a similar role for externalized m-calpain. Whether other cystatin family members influence calpain stability or activity remains to be established; however, it is intriguing that low and high molecular weight kininogens possess a potent calpain inhibitory domain (51).

The recent observation that calpains are required for repair of mechanically damaged plasma membrane (27), an event that necessarily exposes the intracellular environment to extracellular factors, made it possible to directly test the potential for fetuin A to facilitate repair. We first showed that m-calpain co-localized with dysferlin, a proposed plasma membrane repair protein (42), at the injured surface of damaged myotubes (Fig. 6). This placed calpain at the exterior surface of damaged sarcolemma, where it had the potential to interact with fetuin A. Further experiments utilizing FITC-labeled bovine fetuin A confirmed its accumulation at membrane damage sites. Importantly, m-calpain immunoreactivity co-localized with the FITC-fetuin A signal (Fig. 7A), confirming its potential to be influenced by interaction with fetuin A during membrane repair. In contrast, annexin-A2, a protein that is thought to participate in repair of damaged plasma membrane (44), was not closely associated with fetuin A at sites of membrane damage (Fig. 7B). Moreover, it did not co-localize with dysferlin (Fig. 6B). In contrast, earlier studies had demonstrated co-localization of dysferlin and annexin-A2 in damaged myotubes (44). The latter immunofluorescence experiments employed detergent-containing buffers in the first antibody solutions and would therefore have detected intracellular antigens. It is worth emphasizing that our cell studies employed fixed, but unpermeabilized, myotubes. Therefore, we believe that our localization studies identify cell surface antigens that remain externalized during membrane repair. Taken within this context, our observations suggest that externalized annexin-A2 is mainly localized at sites other than the patch complex containing dysferlin, m-calpain, and fetuin A. These results agree with the general concept that annexins and integrins are released from cells as major protein components of "matrix vesicles" (also called microparticles (52)) small plasma membrane-derived fragments released by blood platelets (53), migrating fibroblasts (54, 55), osteoblasts (56), and metastatic cancer cells (57). In previous studies similar fragments appeared to be released from scrape-damaged fibroblasts (27).

Fetuin A not only associated with m-calpain at membrane damage sites but also facilitated recovery after damage (Figs. 8 and 9). HFL-1 fibroblasts were more likely to remain viable when scraped in the presence of 1 or 5 mg/ml fetuin A (Fig. 8B). Not surprisingly, FBS, which contains ~20 mg fetuin A/ml (37),

also enhanced survival (Fig. 8A). However, the results cannot be interpreted strictly on the basis of fetuin content. 1% FBS significantly increased survival, and this would represent ~0.2 mg fetuin A/ml, a concentration that was not significantly protective on its own (Fig. 8B). Moreover, 20% FBS, though significantly protective, was not as effective as 5% FBS. We view it likely that serum contains other factors in addition to fetuin A that may either facilitate or inhibit plasma membrane wound repair.

Importantly, fetuin A appeared to protect Capns1^{+/+} fibroblasts from damage but not Capns1^{-/-} fibroblasts (Fig. 8C). Poloxamer 188 was capable of increasing survival of either Capns1^{+/+} or Capns1^{-/-} cells. Combining fetuin A and poloxamer 188 did not appear to increase survival compared with the addition of either agent alone (Fig. 8C), suggesting that both might work by the same general mechanism. The current hypothesis for calpain function in membrane resealing posits its role in removing cortical cytoskeleton around the injury site (27), thus eliminating resistance to spontaneous resealing of the torn lipid bilayer. Poloxamer 188 is also thought to increase plasma membrane fluidity at the repair site (46), but in this case by direct insertion of poloxamer into the injured lipid bilayer. The results presented in Fig. 9 confirm that fetuin A facilitated membrane resealing in a calpain-dependent fashion. Within the context of the present study, this result is most readily explained by a direct interaction between fetuin A and m-calpain at the wound site.

Association with fetuin A resolves two important issues that argued against a role of m-calpain outside the intracellular environment, its rapid self-aggregation, and its autoproteolytic inactivation. Our results suggest that fetuin A interacts with m-calpain at the site of membrane injury and facilitates its function, most likely the remodeling of cortical cytoskeleton following injury and preceding reparative membrane fusion (27). Whether stabilization of calpain by fetuin A plays a key role remains to be established. Previous studies indicated that calpain activity in the first few seconds after damage is sufficient to increase survival (27). Even without fetuin A, m-calpain should survive this limited exposure to mM calcium (Fig. 2A). Interaction with fetuin may perhaps serve to concentrate calpain at the site of injury. Clearly, further studies will be necessary to delineate the exact mechanism for calpain participation in membrane repair and to understand how its interaction with fetuin A facilitates this function.

The present investigation provides a rationale for investigating potential extracellular roles for m-calpain. Interaction with fetuin A allows substantial m-calpain activity to remain for at least 90 min after exposure to extracellular calcium concentrations (Fig. 2A). Therefore, it seems reasonable that this calpain, once released from a cell, could have a prolonged effect on tissue surrounding its cell of origin. One possible role would be proteolysis of fibronectin, an extracellular adhesion molecule and a proposed calpain substrate (58, 59). If distributed by the bloodstream, m-calpain would also have the potential to act at distant targets.

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