

# Characterization and Expression of Calpain 10

## A NOVEL UBIQUITOUS CALPAIN WITH NUCLEAR LOCALIZATION\*

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Hong Ma<sup>‡</sup>, Chiho Fukiage<sup>§</sup>, Yung Hae Kim<sup>¶</sup>, Melinda K. Duncan<sup>||</sup>, Nathan A. Reed<sup>||</sup>,  
Marjorie Shih<sup>‡</sup>, Mitsuyoshi Azuma<sup>‡</sup>, and Thomas R. Shearer<sup>‡\*\*</sup>

From the <sup>‡</sup>Departments of Oral Molecular Biology, Biochemistry and Molecular Biology, and Ophthalmology, Oregon Health & Science University, Portland, Oregon 97201, the <sup>§</sup>Laboratory of Biology, Senju Pharmaceutical Company, Ltd., 1-5-4 Murotani, Nishi-ku, Kobe 651-22, Japan, the <sup>¶</sup>Department of Animal Sciences, Oregon State University, Corvallis, Oregon 97331, and the <sup>||</sup>Department of Biological Sciences, Division of Molecular Biology and Genetics, University of Delaware, Newark, Delaware 19716-2590

Calpains are calcium-dependent intracellular nonlysosomal proteases that are believed to hydrolyze specific substrates important in calcium-regulated signaling pathways. Recently, an atypical member of the calpain family, calpain 10, was described, and genetic variation in this gene was associated with an increased risk of type II diabetes mellitus in humans. In the present report, a polyclonal antibody directed against rat calpain 10 was developed. This antibody was used to monitor the expression of calpain 10 protein in tissues from rats, mice, and humans. Calpain 10 protein was found to be present in all tissues examined by Western blotting including the lens, retina, brain, heart, and skeletal muscle. Although some calpain 10 was detectable in the water-soluble protein fraction of these tissues, it was preferentially found in the water-insoluble fraction. In the lens, immunohistochemistry revealed that calpain 10 was predominately located in the cytoplasm of epithelial and newly differentiating lens fibers at the transition zone. However, calpain 10 was found to be associated with the plasma membrane of differentiated lens fiber cells and the sarcolemma of skeletal muscle. In the lens epithelium-derived cell line,  $\alpha$ TN4-1, the calpain 10 protein was found in a punctate distribution in the cell nucleus as well as the cytoplasm. After the elevation of intracellular calcium levels with ionomycin, calpain 10 protein levels in the nucleus of  $\alpha$ TN4-1 cells increased markedly, whereas those in the cytoplasm decreased. In the lens, the elevation of intracellular calcium levels after selenite administration resulted in increased levels of calpain 10 RNA within 1 day and a loss of calpain 10 protein from the lens nucleus coincident with the onset of selenite cataract. In conclusion, calpain 10 seems to be a ubiquitous calpain, the expression level and subcellular distribution of which are dynamically influenced by calcium.

Calpains represent a superfamily of structurally related, intracellular, calcium-activated, nonlysosomal, cysteine proteases (1–2). The typical four-domain structure of the catalytic subunit is comprised of domain I (autolytic activation), domain II (cysteine catalytic site), domain III (“electrostatic switch”), and domain IV (calmodulin-like calcium binding sites) (3, 4). Calpains showing this structural format include the ubiquitous m- and  $\mu$ -calpains (5) and some tissue-preferred calpains such as calpain 3 (also termed p94) (6), stomach nCl-2 (7), digestive tubule nCl-4, lens Lp82 and Lp85 (8–9), and retina Rt88 (10). Recently, atypical calpains including calpains 5, 6, and 10 have been described, where certain domains have been replaced or deleted (11–14). Calpain 10 is especially interesting, because the calmodulin domain was replaced with a divergent T domain containing no calcium-binding EF-hand structures. TRA-3, an atypical calpain from *Caenorhabditis elegans* likewise has the T domain replacement, yet TRA-3 exhibited  $\text{Ca}^{2+}$ -dependent protease activity (15). Transcripts for calpain 10 were ubiquitously found in many human (16) and mouse (17) tissues examined. This raised the real possibility that most tissues contain a calpain that either does not need calcium for activity or uses an activation mechanism different from the traditional calpains. Furthermore, Horikawa *et al.* (16) recently used positional cloning to discover that genetic variation in the calpain 10 gene was associated with an increased risk for type 2 diabetes mellitus in certain human population groups. Alternative splicing may generate at least eight forms of calpain 10, some of which would also lack the protease domain. Altered expression of calpain 10 enzyme in pancreatic, muscle, and fat cells was postulated to lead to abnormal glucose homeostasis because of modification in an unknown calpain 10-dependent pathway.

To our knowledge, nothing is known about the protein expression or cellular localization of calpain 10 or whether calpain 10 is altered under conditions of elevated cellular calcium. Such data would be important for understanding the mechanism of action of calpain 10 in both normal and disease conditions. Thus, in the experiments reported below we developed and used an antibody to measure calpain 10 protein expression in normal human, rat, and mouse tissues as well as in cultured lens epithelial cells and experimental cataract, in which cellular calcium was elevated. Experimental cataract was used because it is a well documented example of excessive calpain activity leading to a pathologic condition (18).

EXPERIMENTAL PROCEDURES

*Animals, Tissue Collection, and RNA Isolation*—All animals were handled in compliance with National Institutes of Health guidelines (28). To produce nuclear cataract in rats, a single subcutaneous injection of sodium selenite at 30  $\mu\text{mol/kg}$  of body weight was administered to 12-day-old Harlan Sprague-Dawley rats (B & K International, Fremont, CA) as described previously (18). Lenses were subsequently removed by a posterior approach, and lens capsule/epithelium dissections were performed under a dissecting microscope ( $\times 16$ ) by removing the capsule with adhering epithelium. Ten preparations were pooled to

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\*\* To whom correspondence should be addressed: Oregon Health Sciences University, 611 SW Campus Dr., Portland, OR 97201. Tel.: 503-494-8770; Fax: 503-494-8918; E-mail: shearertr@ohsu.edu.

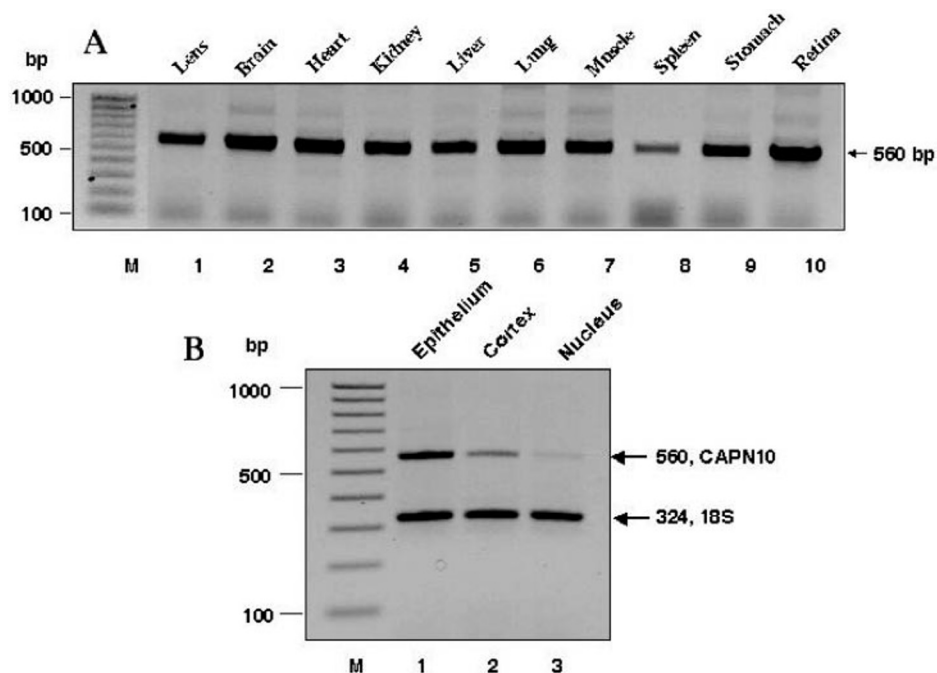


FIG. 1. **Calpain 10 gene transcripts.** A, ethidium bromide-stained gel (image inverted) showing RT-PCR for calpain 10 in various tissues from young rats. The arrow indicates the expected product for calpain 10 at 560 base pairs (560 bp). B, the relative RT-PCR for calpain 10 (CAPN10) in major regions of young rat lens with 18S ribosomal RNA internal standard.

obtain sufficient material. The interface between the cortex and nucleus of the remaining lens mass was determined by the appearance of cold cataract, and cortical and nuclear regions were dissected at ~50% of the lens radius. All lens and soft tissues were chilled throughout the dissection to prevent breakdown of RNA. After homogenization in TRIzol reagent (Life Technologies, Inc.) at 1 ml/100 mg of tissue, RNA was extracted according to manufacturer instructions. Total RNA was then treated with DNase to remove genomic DNA, and quantitation was performed spectrophotometrically.

**cDNA Cloning of Rat Calpain 10**—PCR<sup>1</sup> cloning was performed on rat lens total RNA. On the basis of the cDNA sequence for mouse calpain-like protease formerly named calpain 8 (17), the PCR primers used to amplify the partial rat calpain 10 cDNA were: upstream, 5'-GAA GGG GAG CCC AGG GCG CCG AAG ATG-3', and downstream, 5'-GCA GTG TTG CTG TAG GGT GAT ACG GAT G-3'. Five  $\mu$ g of lens RNA were reverse-transcribed using oligo(dT) primer and SuperScript II reverse transcriptase (Life Technologies, Inc.). Reverse transcription was performed for 40 min at 42 °C in a PTC-100 thermal cycler (MJ Research, Inc.). The mixture was then heated to 70 °C for 5 min. A 1- $\mu$ l RT reaction mixture was subsequently transferred to a PCR tube containing one Ready-To-Go PCR bead (Amersham Pharmacia Biotech) and 0.2  $\mu$ M each upstream and downstream primers. Water was then added up to a final volume of 25  $\mu$ l. For PCR, 35 cycles were executed as follows: 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 3 min. The PCR products (15  $\mu$ l) were electrophoresed on a 1% agarose gel with ethidium bromide staining (10 mg/ml) and subcloned by a TA cloning kit (Invitrogen). The resulting 1700-nucleotide PCR product was gel-purified and subcloned using the TA cloning kit. The inserts from three independent clones were sequenced and found to consist of a portion of the 5'-untranslated region 1677 bases of the coding sequence from calpain 10.

For cloning the 3' end of rat calpain 10 cDNA, 3' rapid amplification of cDNA ends PCR was performed with the 3' system for rapid amplification of cDNA ends according to manufacturer instructions (Life Technologies, Inc.). First-strand cDNA was synthesized at 42 °C for 50 min with 5  $\mu$ g of rat lens total RNA using an Adapter primer and SuperScript II reverse transcriptase. The next PCR amplification using this cDNA as a template and Ready-To-Go PCR bead was performed as follows: 94 °C for 2 min followed by 35 cycles of 94 °C for 1 min, 58 °C for 45 s, 72 °C for 1.5 min, and primer extension at 72 °C for 5 min with 3' rapid amplification of cDNA ends universal amplification primer and the target-specific primer based on the sequences of rat calpain 10. The resultant PCR products were subcloned into the pCR2.1 vector and sequenced according to manufacturer protocols (Invitrogen).

**RT-PCR Measurement of Calpain 10 mRNA**—For RT-PCR analysis,

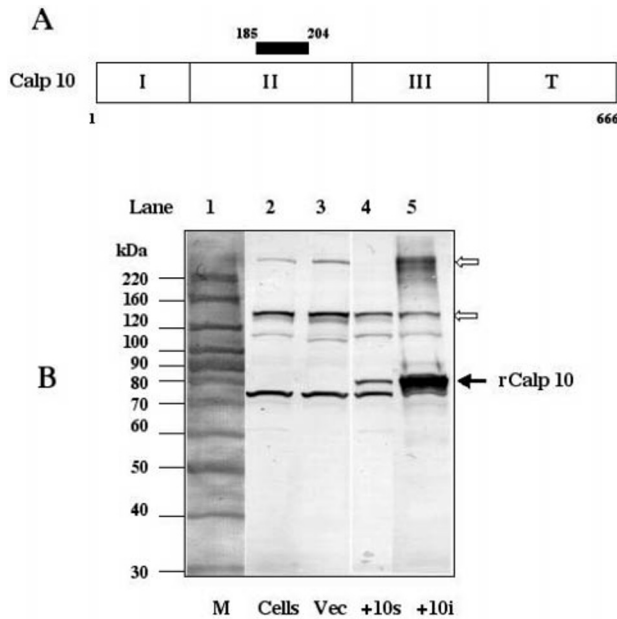
2.5  $\mu$ g of RNA from various rat tissues were used for the first-strand RT reaction using the oligo(dT) primer and SuperScript II reverse transcriptase. One-tenth of the resulting cDNA was subjected to 35 PCR cycles using the same downstream primer listed above and the upstream primer 5'-AAC CCA GCG AGG TGT GTG TGG CTG TT-3'. Ten  $\mu$ l of the amplified DNA samples were resolved in a 1.5% agarose gel. Gels were stained with ethidium bromide (10 mg/ml) and scanned by Gel Doc 2000 (Bio-Rad).

To allow direct comparison between multiple samples, QuantumRNA 18S internal standard was used as the internal control (Ambion). The PCR conditions of this relative RT-PCR for calpain 10 were 94 °C for 2 min followed by 33 cycles of 94 °C for 45 s, 63 °C for 45 s, 72 °C for 1 min, and primer extension at 72 °C for 5 min. The PCR products for both calpain 10 and 18S internal standard were evaluated during the exponential phase of the amplification process. Ten  $\mu$ l of amplified DNA samples were size-separated by electrophoresis on 1.5% agarose gels. The gels were stained with ethidium bromide (10 mg/ml) and scanned by Gel Doc 2000 (Bio-Rad). NIH Image 1.57 software was used to invert the image and measure the uncalibrated optical density of the bands corresponding to PCR products. To compare multiple samples, the ratio of densities for target/18S rRNA was calculated for each reaction.

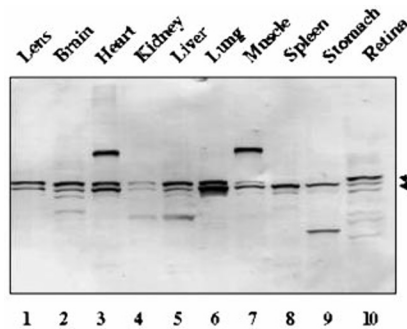
**Expression of Recombinant Calpain 10 in the Baculovirus System**—cDNA for the entire calpain 10 coding region from mouse was subcloned into the pFastBacHTb vector (Life Technologies, Inc.) containing N-terminal His tags and rTEV protease cleavage sites between *SalI* and *XbaI*. The calpain 10 plasmid was transformed into DH10 Bac-competent cells (Life Technologies, Inc.). High molecular weight DNA was prepared from selected *Escherichia coli* clones containing the calpain 10 bacmid; then this DNA was transfected to insect Sf9 cells. The cells were maintained in Sf-900 II SFM medium containing 50 units/ml penicillin and 50  $\mu$ g/ml streptomycin and were collected 5 days after transfection. The cells were washed with phosphate-buffered saline (pH 7.4) and homogenized on ice in lysis buffer containing 20 mM Tris (pH 7.5), 1 mM EGTA, 2 mM dithioerythritol, and protease inhibitor mixture (Roche Molecular Biochemicals).

**Preparation of Antibody for Calpain 10**—The antibody for calpain 10 for potential use with rat, mouse, and human samples was generated by first chemically synthesizing the peptide WSLKDIRKASGQDRPS-GGE, which corresponds to a 20-amino acid sequence in domain II of rat calpain 10 (Fig. 2A). The similarity of the rat immunogenic peptide to the sequence in mouse and human calpain 10 was 90 and 50%, respectively, with 100% similarity among all three species for a shorter amino acid sequence predicted to be surface epitopes (underlined in the peptide sequence above). The peptide was high pressure liquid chromatography-purified and conjugated to keyhole limpet hemocyanin by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide conjugation (TriplePoint Biologics, Portland, OR). The first immunization was with Freund's

<sup>1</sup> The abbreviations used are: PCR, polymerase chain reaction; RT, reverse transcription; BSA, bovine serum albumin.



**FIG. 2. Expression of recombinant calpain 10.** A, the domain structure of rat calpain 10 with 666 amino acids showing the location of a 20-amino acid peptide sequence (*heavy bar*, amino acid numbers) used to generate a polyclonal antibody. I, II (active site), III, and T are the major domains in calpain 10. B, an immunoblot analysis using the calpain 10 peptidic antibody against recombinant calpain 10 expressed in insect cells. Lane 2/*Cells*, noninfected cells; lane 3/*Vec*, cells infected with vector only; lane 4/*+10s*, soluble proteins from cells infected with vector containing the histidine-tagged cDNA for calpain 10; lane 5/*+10i*, calpain 10 in the insoluble pellet. Lane 1 contained molecular mass markers in kDa. The *solid arrow* indicates r-calpain (*rCalp*) 10 at the expected mass of 78.8 kDa caused by an extra 36 amino acids on the N terminus of the recombinant protein. The *open arrows* indicate unidentified immunoreactive bands.



**FIG. 3. Expression of calpain 10 protein in rat tissues.** An immunoblot for calpain 10 in the soluble proteins from various tissues from 12-day-old rats. The *arrows* indicate two major bands for calpain 10. The higher molecular mass bands in heart and muscle are unidentified.

complete adjuvant, and subsequent immunizations were at 4-week intervals with Freund's incomplete adjuvant. Rabbits were anesthetized and exsanguinated, and the antibody was purified from serum using the peptide bound to Aminolink resin (Pierce, Rockford, IL). The antibody gave positive staining after immunoblotting and/or immunohistochemistry against rat, mouse, and human tissues, an  $\alpha$ TN4-1 mouse cell line, and recombinant mouse calpain 10 overexpressed in insect cells (discussed below). The antibody did not cross-react with rat Lp82 calpain or m-calpain on immunoblots (data not shown), suggesting that the antibody possessed significant specificity for calpain 10.

**Preparation of Total Protein Extract**—Lenses and other soft tissues from 12-day-old and mother rats were homogenized in buffer A containing 20 mM Tris (pH 7.5), 1 mM EGTA, and 2 mM dithiothreitol. Human lenses from organ donors (Lions Eye Bank of Oregon) were homogenized in the same buffer A. Soluble proteins were obtained by centrifugation at  $13,000 \times g$  for 20 min at 4 °C. The supernatants were removed, and the pellets were washed in buffer A again. The remaining

pellets were resuspended in the same buffer for analysis. Protein concentrations were determined using the Bio-Rad dye-binding reagent with BSA as standard.

**Immunoblot Assays**—Proteins were separated on pre-cast 1.0-mm thick  $8 \times 8$ -cm 8% polyacrylamide mini-gels (Invitrogen). Immunoblotting was performed by electrotransferring the proteins from the mini-polyacrylamide gels to a polyvinylidene difluoride membrane at 30 volts (constant) for 90 min at 4 °C. The membranes were then incubated in Tris-buffered saline supplemented with 5% skim milk and 0.05% Tween 20 at room temperature for 1 h. The affinity-purified polyclonal antibody made against rat calpain 10 was used at 1:200 dilution, and immunoreactivity was visualized with alkaline phosphatase conjugated to anti-rabbit IgG secondary antibody and BCIP/NBT (Bio-Rad). Images of gels and immunoblots were digitized on a flat-bed scanner, and image analysis was performed by NIH Image 1.57 software.

**Immunohistochemistry**—For immunohistochemistry, unfixed lenses were embedded in tissue-freezing medium (Triangle Biomedical, Durham, NC), and 15- $\mu$ m frozen sections were prepared as described previously (19). Calpain proteins were detected with the antibodies described below. The primary antibody for calpain 10 was diluted 1:200 in PBS with 1% BSA. The bound nonfluoresceinated antibodies were visualized after incubation with the appropriate secondary antibody (Alexa Fluor 568 anti-rabbit IgG-conjugated, 1:50 dilution in PBS with 1% BSA; Molecular Probes, Eugene, OR), and the cell nuclei were detected by counterstaining with SYTO-13 (1:1000 dilution in PBS; Molecular Probes). The slides were coverslipped, and 50  $\mu$ l of mounting medium (9.25 mM *p*-phenylenediamine in 90% glycerol with PBS) were added to each slide. The slides were stored at -20 °C until confocal microscopy was performed on a Zeiss 510 LSM confocal microscope configured with an Argon/Krypton laser (488- and 568-nm excitation lines).

For immunofluorescence with cell lines, maintenance and preparation of the  $\alpha$ TN4-1 cells were followed as described previously (20).  $\alpha$ TN4-1 cells ( $1.0$ – $1.2 \times 10^5$ ) were inoculated on a chambered 1.5-mm cover-glassed tissue culture dish (Lab Tech II) for 20 h. The cells were treated with various concentrations of ionomycin for 30 min and then fixed with 4% paraformaldehyde in PBS for 10 min. The chambers were rinsed three times with PBS and blocked with 3% BSA in PBS for 75 min. The primary antibody was incubated at a 1:200 dilution in each chamber with 3% BSA in PBS overnight at 4 °C with shaking. The chambers were rinsed three times with 3% BSA in PBS for 10 min. The following steps were performed in a dimly lit work area. The secondary antibody was added at a 1:200 dilution in PBS with 3% BSA (Alexa Fluor 568 anti-rabbit IgG-conjugated, Molecular Probes), and the cell nuclei were detected by counterstaining with SYTO-13 at a 1:1000 dilution in PBS (Molecular Probes) for 60 min. The chambers were rinsed three times with 3% BSA in PBS and rinsed once with  $1 \times$  PBS. The PBS was removed, and two drops of SlowFade Component B (S-2828, Molecular Probes) was added just before scanning on a Zeiss 510 LSM confocal microscope configured with an argon/krypton laser (488 and 568 nm excitation lines).

## RESULTS

**Rat Calpain 10 Sequence**—The cDNA for calpain 10 from young rat lens total RNA was cloned and sequenced to provide the complete coding region along with portions of the 5'- and 3'-untranslated regions (GenBank accession no. AF227909). The nucleotide sequence was 70 and 91% identical with human and mouse calpain 10, respectively (16). The single long open reading frame in the cDNA for calpain 10 coded for 666 amino acids with a calculated molecular mass of 74,527 Da. The protein for rat calpain 10 could be divided into the canonical four-domain structure typical of calpains (Fig. 2A). Domain II contained the Cys, His, and Asn residues found in the active site of other calpain catalytic subunits (1–2). The divergent C-terminal domain T showed no significant similarity to the calmodulin-like Ca-binding domain IV of the traditional calpains. The overall similarity of rat calpain 10 with the catalytic subunit of ubiquitous m-calpain was only 15%, and the similarity with lens-specific calpain Lp82 was 18%.

**Expression of Calpain 10**—RT-PCR revealed that calpain 10 transcripts were ubiquitously expressed in all rat tissues examined (Fig. 1A). The highest level of expression in young rat was in brain, and the lowest was in spleen, whereas in the



FIG. 4. **Calpain 10 expression in human lens.** Immunoblot for calpain 10 in the soluble (S) and insoluble (I) proteins from samples of whole human lenses or cortex (c). The ages of the donors are indicated in weeks (w), days (d), or years (y) of gestation. A 10-kDa molecular mass ladder is shown on the left, and the arrow on the right indicates major protein bands for human calpain 10.

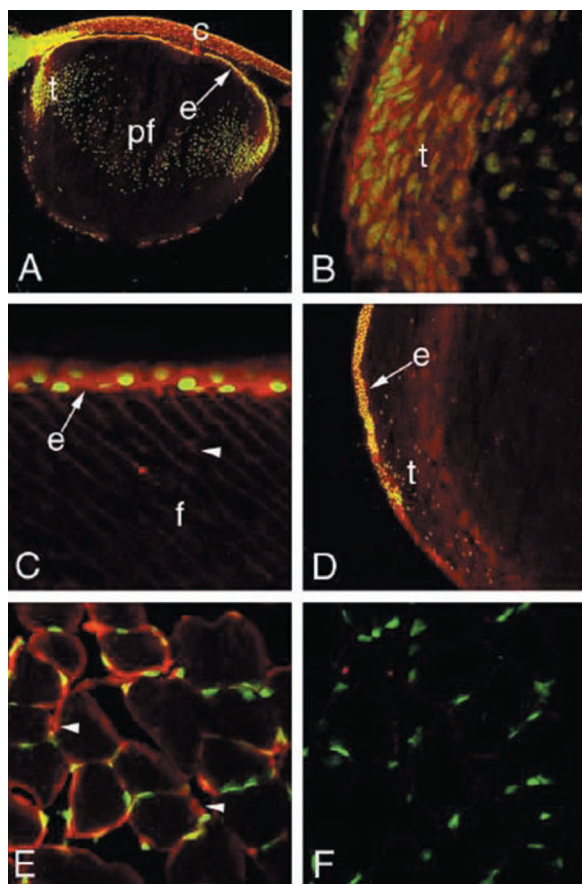
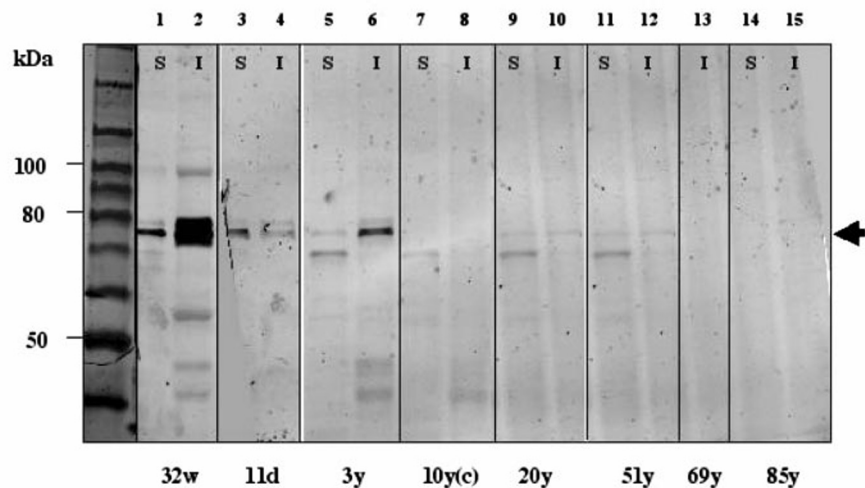


FIG. 5. **Immunohistochemical localization of calpain 10 in rat and human tissues.** Confocal micrographs showing immunohistochemical localization of calpain 10 (red) in newborn mouse lens (A), transition zone between epithelium and outer fiber cells in lens of newborn mouse (B), epithelium in 21-year-old human lens showing significant staining in fiber cell membranes (C), transition zone in 21-year-old human lens (D), quadriceps muscle in 4-week-old mouse showing calpain 10 staining in the sarcolemma around multinucleated fiber cell (E), and muscle negative control without primary antibody (F). Green, DNA staining in cell nuclei. Approximate magnifications: B, C, E, and F,  $\times 630$ ; A,  $\times 80$ ; and D,  $\times 100$ .

adult human, heart showed the highest level of expression (16). Lens was studied in detail in the present experiments as an example of a terminally differentiated tissue with very little protein turnover in the center (nucleus) during the lifetime of the host (21). mRNA for calpain 10 was detected in all lens regions with the most abundant in the outer region of

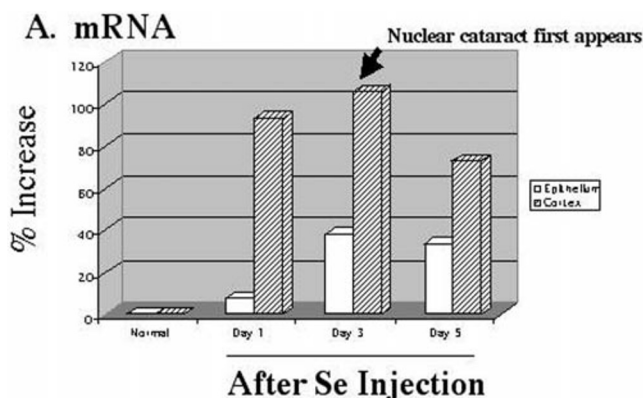
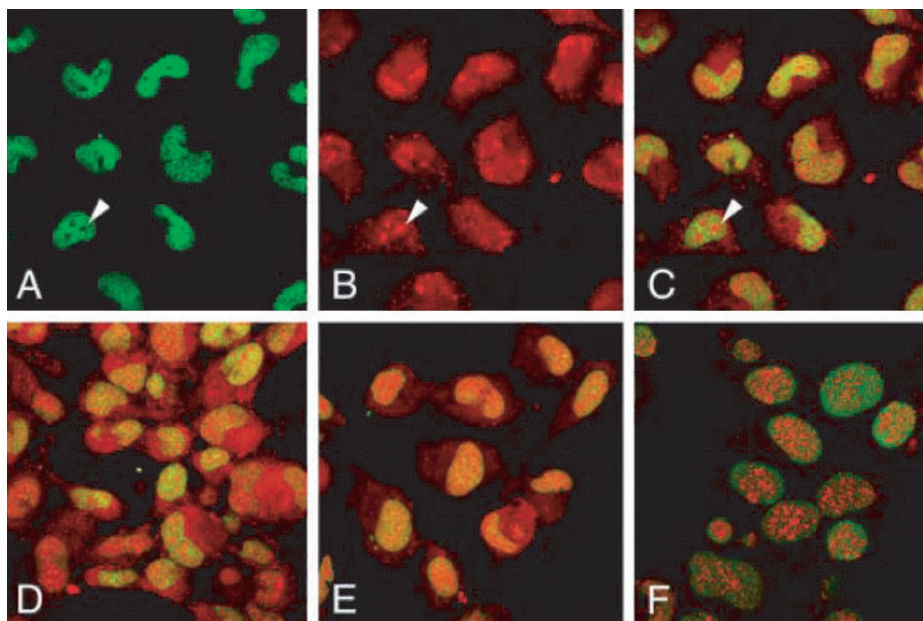
lens epithelium (Fig. 1B). The oldest portion of the lens, the inner nucleus region, showed the lowest level of mRNA for calpain 10. The cDNA sequence was shown to be a translatable message, because insect cells infected with the mouse cDNA overexpressed recombinant calpain 10 in the soluble and insoluble fractions at the expected 78.8 kDa position (extra 36 amino acids on the N terminus of the recombinant protein) (Fig. 2B, lanes 4 and 5, solid arrow). The lower immunoreactive band was presumed to be endogenous calpain 10, because it migrated at the expected size of 74.5 kDa for native calpain 10, and it was ubiquitously expressed in infected as well as noninfected cells (lanes 2 and 3). Immunoblotting also confirmed that calpain 10 transcripts were ubiquitously translated in tissues from young rats and that the protein was stable (Fig. 3, lanes 1–10). Two protein bands were present in most tissues near the expected size of 74.5 kDa (arrows). In these tissues, a large fraction of the calpain 10 protein was not readily soluble in aqueous buffers (data not shown). The full-length cDNA of mouse calpain 10 was also subcloned into the pcDNA3.1 vector for transient expression in COS-7 cells. Preliminary results showed two similar immunoreactive bands (data not shown).

Immunoblotting also identified calpain 10 orthologues in human lenses (Fig. 4). Young human lens showed immunopositive proteins near 74.5 kDa in both the soluble and insoluble lens proteins. Appreciable levels of immunostaining were found only in young lenses up to 3 years of age. Calpain 10 proteins were not detectable in aged human lenses (69–85 year old). These results suggested that calpain 10 protein expression was associated with developing lens and then lost with aging.

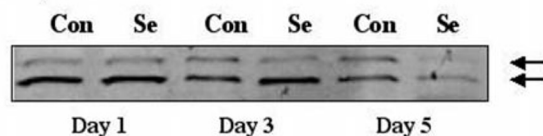
**Localization of Calpain 10**—Immunohistochemistry with the calpain 10 antibody revealed that calpain 10 was located mainly in the cytoplasm of the epithelium and transition zone of the newborn mouse lens (Fig. 5, A and B), whereas the primary and secondary fibers in the cortex and nucleus showed much lower levels. Calpain 10 in human lens was also concentrated in the lens epithelium (Fig. 5, C and D). However, in the outer fiber cells of human lens, calpain 10 was concentrated at the plasma membranes (Fig. 5C, arrow head). Similarly, calpain 10 immunoreactivity was predominantly found at the sarcolemma in skeletal muscle fibers from young mice (Fig. 5E, arrow head). Note that because not all of the immunopositive bands on immunoblots with calpain have been positively identified, we cannot exclude the possibility that the antibody may be labeling other proteins in addition to calpain 10.

Calpain 10 in cultured  $\alpha$ TN4–1 mouse lens epithelial cells

FIG. 6. **Subcellular localization of calpain 10 in  $\alpha$ TN4-1 cells.** A, green DNA channel showing cell nuclei; B, red calpain 10 channel; C, overlaid channels with the arrow indicating punctate accumulation of calpain 10 in the cell nucleus; D, cells pre-treated with 1  $\mu$ M ionomycin ( $\times 630$ ); E, 10  $\mu$ M ionomycin treatment showing a decrease of calpain 10 in the cytoplasm and an increase in the nucleus ( $\times 630$ ); F, same as described for E but at  $\times 1000$ .



#### B. Epithelium



#### C. Nucleus

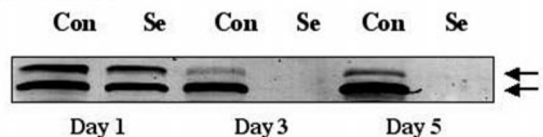


FIG. 7. **Changes in calpain 10 during formation of selenite cataracts.** A, the percentage of increase in mRNA for calpain 10 mRNA in the epithelium and cortex of lenses forming selenite cataract compared with control lenses as determined by RT-PCR. B, an immunoblot for calpain 10 in the epithelium. C, the nuclear regions of rat lenses after selenite injection. Con, control group; Se, selenite-treated group. The arrows indicate two immunoreactive bands for calpain 10.

was not only present in the cytosol but also in the nucleus in a punctate arrangement (Fig. 6B). Double staining of this region for both calpain 10 and DNA showed that calpain 10 was not associated with DNA in the nucleus (Fig. 6C). The nuclear localization of calpain 10 was confirmed further by a

Z-series analysis with the confocal microscope that showed that calpain 10 was partially localized in the nucleus in micro compartments (data not shown). These observations agree with the computer-aided PSORT analysis for nuclear localization signals. The predicted *calpain 10* gene product showed a characteristic nuclear localization signal of +0.46 located in domain III. This was in contrast to the nuclear localization signal score of -0.10 for m-calpain, which accumulated only in the cytoplasm of  $\alpha$ TN4-1 mouse cells (20). As noted above, some of the immunostaining may be caused by non-calpain proteins, but the observations suggested that the calpain 10 may function by association with nuclear proteins. Interestingly, the elevation of intracellular calcium with 10  $\mu$ M ionomycin caused decreased staining in the cytoplasm and increased staining in the nucleus (Fig. 6, E and F).

**Changes in Calpain 10 during Experimental Cataract Formation**—A selenite nuclear cataract model was used to further investigate the influence of massive increases in cellular calcium on calpain 10. The concentration of calcium in the nuclear region of lenses with selenite cataract is known to reach over 100  $\mu$ M free calcium compared with 0.2  $\mu$ M for the normal nucleus (18). mRNA levels for calpain 10 increased in the outer regions (epithelium and cortex) of lens during selenite cataract formation (Fig. 7A). Calpain 10 protein levels in rat lens in the epithelium (Fig. 7B) of selenite cataract decreased after day 3, whereas in nucleus (Fig. 7C) calpain 10 was already lost by day 3 when the cataract appeared. Thus, calpain 10 transcripts were rapidly up-regulated in the outer regions of the lens during nuclear cataract formation, but calpain 10 protein was degraded when cataract was present.

#### DISCUSSION

**Calpain 10 Protein in Tissues**—The data presented above provide the first evidence for the expression of the protein for calpain 10 in mouse, rat, and human tissues. This was important, because previous publications demonstrated transcription of the *calpain 10* gene (14, 16, 22), but no data have been provided until now that calpain 10 was actually translated in tissues. Our immunoblotting data demonstrated that calpain 10 protein was indeed ubiquitously expressed in all tissues examined. Calpain 10 is thus the third ubiquitous calpain in tissues along with m- and  $\mu$ -calpains. Most rat tissues showed at least two major protein bands near the expected 74.5-kDa predicted by the cDNA calpain 10. The



highest concentration of calpain 10 was in the water-insoluble fraction of tissues, suggesting that a significant portion of cellular calpain 10 may be insoluble in the cytosol or associated with membranes or cytoskeletal elements.

**Variability of Calpain 10 Expression Patterns in Normal Tissues**—Calpain 10 protein expression varied with the particular tissue. Young lens was dominated by two proteins, whereas retina showed at least four protein bands. This variability was interesting, because Horikawa *et al.* (16) hypothesized that polymorphism in the *calpain 10* gene contributed to the genetic susceptibility to diabetes type 2 in certain human population groups. In our studies, at least four factors may be responsible for variable calpain 10 expression patterns. 1) Protein degradation and autolysis: the traditional calpains are well known to undergo autolysis and degradation after calcium activation or aging (1–2). Recent studies also show that calpains are even substrates for each other after activation by calcium.<sup>2</sup> 2) Aging: advancing age was associated with major losses in calpain 10 protein levels. Very little calpain 10 was present in the lenses of mature human and rat lenses, and the oldest region of the rat lens, the nucleus, also contained the lowest concentration of calpain 10 protein. In contrast, the high level of calpain 10 observed in the outer regions of lens may be needed for proteolysis occurring in this region during cell remodeling for terminal differentiation of lens fiber cells. This extensive remodeling is characterized by conversion of the outer cuboidal epithelial cells to long fibers with concomitant loss of all organelles as the fibers are pushed into the interior of the lens. Our results suggested that calpain 10 protein expression was associated with developing lens cells and then lost with aging. 3) mRNA processing: eight splice variants of the *calpain 10* gene were identified in man (16), and potentially at least five of these could be recognized by our antibody. Furthermore, two lens-specific splice variants, Lp82 and Lp85 of calpain 3, were found in rodent lenses (8, 9). Thus, some of the multiple forms of calpain 10 observed in normal tissues may be splice variants generated by alternative exon usage. 4) Nonspecific immunostaining is also possible, and the various calpain 10 bands need to be sequenced. Future studies will be needed to determine whether calpain 10-specific proteins are associated with different functions or increased susceptibility to diseases.

**Response of Calpain 10 to Calcium**—To understand the function of calpain 10 in pathologic conditions, we also tested whether calpain 10 was responsive to increases in cellular calcium using two model systems. 1) Selenite cataract: selenite cataract is caused by a well documented mechanism involving calcium accumulation in the nuclear region of the lens and proteolysis of the structural proteins called crystallins by m-calpain and Lp82 (23–24). Like m-calpain (25), calpain 10 transcripts increased rapidly in the outer regions of young rat lens 24 h after selenite injection before the onset of nuclear cataract. This may be because of up-regulation in response to increased calpain 10 activity or another calpain 10-dependent process such as signal transduction. Apoptosis was also increased in selenite cataract (26), and calpain 10 may influence apoptosis by controlling I $\kappa$ B $\alpha$  turnover similar to that proposed for calpain 3 (27). Calpain 10 is degraded by Lp82 and m-calpain *in vitro*,<sup>3</sup> and these enzymes could also contribute to the loss of calpain 10 in selenite cataract. 2) Treatment of  $\alpha$ TN4–1 cells with ionomycin: the subcellular

distribution of calpain 10 was unusual among the calpains with partial localization to the cell nucleus but not in association with DNA. This nuclear localization was enhanced in  $\alpha$ TN4–1 cells by the elevation of intracellular calcium levels with 10  $\mu$ M ionomycin, suggesting binding of calpain 10 to nuclear components involved in signal transduction.

Thus, the two lines of evidence above clearly indicated that calpain 10 levels were influenced by changes in the calcium level within cells. At present it is not clear if this is a direct effect of calpain 10 activation or an indirect effect caused by other proteases. Calpain 10 lacks the typical calmodulin-like domain found in traditional calpains, and we have not yet been able to measure appreciable calcium-activated calpain 10 activity in animal tissues using zymography or fluorescence-labeled casein assays even when exogenous calmodulin was added. The acidic residues in domain III in m-calpain have also been proposed for targeting m-calpain to membranes (3), and membrane binding was a prominent feature of calpain 10 in the present studies. Caution must be exercised because of the low amino acid similarity of calpain 10 to m-calpain, but the issues of calcium activation and calpain 10 targeting are important. The current studies have shown that calpain 10 protein is ubiquitously expressed in tissues from insects cells to human, in which it could potentially affect a number of tissue processes ranging from lens differentiation to susceptibility to diabetes. For example, calpain 10 may have a role in normal lens development and fiber differentiation, and the uptake of calpain 10 in the nuclei of lens epithelial cells could also be part of the mechanism for experimental cataract formation.

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## REFERENCES

- Emori, Y., Kawasaki, H., Sugihara, H., Imajoh, S., Kawashima, S., and Suzuki, K. (1986) *J. Biol. Chem.* **261**, 9465–9471
- Croall, D. E., and Demartino, G. N. (1991) *Physiol. Rev.* **71**, 813–847
- Hosfield, C. M., Elce, J. S., Davies, P. L., and Jia, Z. (1999) *EMBO J.* **18**, 6880–6889
- Strobl, S., Fernandez-Catalan, C., Braun, M., Huber, R., Masumoto, H., Nakagawa, K., Irie, A., Sorimachi, H., Bourenkow, G., Bartunik, H., Suzuki, K., and Bode, W. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 588–592
- Deluca, C. I., Davies, P. L., Samis, J. A., and Elce, J. S. (1993) *Biochim. Biophys. Acta* **1216**, 81–93
- Sorimachi, H., Imajoh-Ohmi, S., Emori, Y., Kawasaki, H., Ohno, S., Minami, Y., and Suzuki, K. (1989) *J. Biol. Chem.* **264**, 20106–20111
- Sorimachi, H., Ishiura, S., and Suzuki, K. (1993) *J. Biol. Chem.* **268**, 19476–19482
- Ma, H., Fukiage, C., Azuma, M., and Shearer, T. R. (1998) *Investig. Ophthalmol. Vis. Sci.* **39**, 454–461
- Ma, H., Shih, M., Hata, I., Fukiage, C., Azuma, M., and Shearer, T. R. (2000) *Curr. Eye Res.* **20**, 182–189
- Azuma, M., Fukiage, C., Ma, H., and Shearer, T. R. (2000) *Curr. Eye Res.* **21**, 710–720
- Barnes, T. M., and Hodgkin, J. (1996) *EMBO J.* **15**, 4477–4484
- Dear, N., Matena, K., Vingron, M., and Boehm, T. (1997) *Genomics* **45**, 175–184
- Mugita, N., Kimura, Y., Ogawa, M., Saya, H., and Nakao, M. (1997) *Biochem. Biophys. Res. Commun.* **239**, 845–850
- Permutt, M. A., Bernal-Mizrachi, E., and Inoue, H. (2000) *J. Clin. Invest.* **106**, 819–821
- Sokol, S. B., and Kuwabara, P. E. (2000) *Genes Dev.* **14**, 901–906
- Horikawa, Y., Oda, N., Cox, N. J., Li, X., Orho-Melander, M., Hara, M., Hinokio, Y., Lindner, T. H., Mashima, H., Schwarz, P. E. H., Bosque-Plata, L. D., Horikawa, Y., Oda, Y., Yoshiuchi, I., Colilla, S., Polonsky, K. S., Wei, S., Concannon, P., Iwasaki, N., Schulze, J., Baier, L. J., Bogardus, C., Groop, L., Boerwinkle, E., Hanis, C. L., and Bell, G. I. (2000) *Nat. Genet.* **26**, 163–175
- Braun, C., Engel, M., Theisinger, B., Welter, C., and Seifert, M. (1999) *Biochem. Biophys. Res. Commun.* **260**, 671–675
- Shearer, T. R., Ma, H., Fukiage, C., and Azuma, M. (1997) *Mol. Vis.* **3**, 8 (abstr.)
- Reed, N. A., Dong-Jin, O., Czymmek, K. J., and Duncan, M. K. (2001) *J. Immunol. Methods* **253**, 243–252
- Ma, H., Shih, M., Fukiage, C., Azuma, M., Duncan, M., Reed, N., Richard, I., Beckmann, J. S., and Shearer, T. R. (2000) *Investig. Ophthalmol. Vis. Sci.* **41**, 4232–4239
- Harding, J. (1991) *Cataract: Biochemistry, Epidemiology, and Pharmacology*,

<sup>2</sup> H. Ma, C. Fukiage, Y. H. Kim, M. K. Duncan, N. A. Reed, M. Shih, M. Azuma, and T. R. Shearer, unpublished observations.

<sup>3</sup> C. Fukiage, Y. H. Kim, and T. R. Shearer; unpublished data.

- p. 21, Chapman & Hall, London
22. Baier, L. J., Permana, P. A., Yang, X., Pratley, R. E., Hanson, R. L., Shen, G. Q., Mott, D., Knowler, W. C., Cox, N. J., Horikawa, Y., Oda, N., Bell, G. I., and Bogardus, C. (2000) *J. Clin. Invest.* **106**, R69–R73
  23. David, L. L., and Shearer, T. R. (1984) *Investig. Ophthalmol. Vis. Sci.* **25**, 1275–1283
  24. Shearer, T. R., David, L. L., Anderson, R. S., and Azuma, M. (1992) *Curr. Eye Res.* **11**, 357–369
  25. Ma, H., Shih, M., Throneberg, D. B., David, L. L., and Shearer, T. R. (1997) *Exp. Eye Res.* **64**, 437–445
  26. Tanaka, Y., Fukiage, C., Nakamura, Y., Azuma, M., Kim, Y. H., and Shearer, T. R. (2000) *Biochem. Biophys. Res. Commun.* **275**, 300–306
  27. Baghdiguian, S., Martin, M., Richard, I., Pons, F., Astier, C., Bourg, N., Hay, R. T., Chemaly, Y., Halaby, G., Loiselet, J., Anderson, L. V. B., Munain, A. L. D., Fardeau, M., Mangeat, P., Beckmann, J. S., and Lefrans, G. (1999) *Nat. Med.* **5**, 503–511
  28. National Institutes of Health (1985) *Guiding Principles in Care and Use of Animals*, DHEW Publication no. 86-23