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Molecular cloning and characterization of rat and human calpain-5☆

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Abstract

Until today, 14 isoforms of mammalian calpains have been identified, including calpain-5. The *C. elegans* calpain-5 homologue tra-3 is reported to be essential for necrotic neuronal cell death. In this study, we cloned and characterized rat calpain-5, which is highly homologous to human and mouse sequences. The nucleotide sequence is 87% and 93% identical with human and mouse calpain-5, respectively. The protein sequence is well conserved, showing 96% identity in mouse and 92% in human. RT-PCR analysis revealed strong expression of calpain-5 in rat lungs, kidneys, and brain while week expression in heart, whereas in rat brain regions it is ubiquitously expressed. The mRNA expression in different human tissues showed equal expression. However, in human brain regions calpain-5 was strongly expressed in hypothalamus, thalamus, cerebellum, and frontal lobe. Western blot analysis on human neuroblastoma SH-SY5Y cells demonstrated calcium-dependent processing of calpain-5, despite the absence of calmodulin-like domain.

Keywords: Calpain; Proteases; Rat; Brain and cell death

Calpains are a family of calcium-dependent cysteine proteases involved in a wide variety of physiological processes and pathological disorders [1,2]. There are 14 known isoforms of the calpain family and are classified into two groups: ubiquitous and tissue-specific. The two ubiquitously expressed μ- and *m*-calpains have similar biochemical features, except for calcium concentration required for activation in vitro. Two tissue specific calpains identified are: nCL-1 (p94), expressed predominantly in skeletal muscle [3], and nCL-2, expressed predominantly in stomach [4].

Classical mammalian calpains (calpain-1 and calpain-2) are heterodimers, consisting of a large catalytic and a small regulatory subunit. The 80-kDa large subunit consists of four domains (I–IV). D-II contains the catalytic triad of Cys, His, and Asn. D-IV contains four calmodulin-like calcium binding EF-hand motifs, which confer calcium dependence on proteolytic activity. Domain-III has been suggested to bind to calcium via C2-like fold [2,5]. Moldoveanu et al. [6] also recently demonstrated the presence of calcium-binding sites within domain II that serve as calcium-switch in the

☆ *Abbreviations:* SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RT-PCR, real-time polymerized chain reaction; capn-5, calpain-5; MTX, maitotoxin.

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activation of classical calpain-1 and -2. Domain-I is cleaved off during initial activation by calcium. The small subunit contains calcium-binding domain similar to D-IV of the large subunit and is involved in regulation of enzymatic activities. Recently a new member of calpain family, human tissue-specific calpain, termed htra-3 or calpain-5 has been cloned and characterized which is highly homologous to the tra-3 gene of *C. elegans* [7,8]. The amino acid sequence of htra-3 was similar to other members of the family with an exception in domain IV. The calmodulin calcium-binding EF-hand structure was replaced by a unique sequence, termed domain T, which is homologous to *C. elegans* tra-3 [8,9]. Mouse calpain-5 has also been identified with structural similarity to human tra-3 [10].

Calpains have been implicated in a variety of processes including apoptosis, cell division, long-term potentiation in the central nervous system, and embryonic development [11–14]. Tra-3 in *C. elegans* has been known to be involved in sex determination and in necrotic cell death [8,15], however, the physiological role of calpain-5 its mammalian orthologue is unknown. Identification of the rat homologue of htra-3 may not only assist in elucidation of its function and evolutionary history, but also allow determining its functional role associated with neurological disorders in rats.

Materials and methods

Total RNA isolation. Total RNA was isolated from different rat tissues and brain regions using TRIzol reagent (Gibco-BRL, Rockville, MD) and suspended in 50–100 μL DEPC-treated water. RNA concentrations were quantified and stored at −75 °C for reverse transcription.

Cloning of full-length of rat calpain-5. Total RNA (3 μg) from rat brain tissue was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and oligo(dT). To clone full-length rat calpain-5, primers were designed from the highly conserved regions of human and mouse calpain-5 sequences. Two pairs of specific primers were designed with an overlapping region of 31 bases in order to correct any potential erroneousness and PCR amplified P1: 5′-GTC TAG CCT CCG CTC CAG TGC 3′ and P2: 5′-ACG CCC ATC TTC TCC CTC TCA 3′. P3: 5′-CGT CAG AGG AAT GGC AGA AAG 3′ and P4: 5′-ACA GTG GGT CAG TGG AAG AGT G 3′. PCR products were cloned into T/A cloning vector and sequenced using Dye Terminator Cycle Sequencing on CEQ 2000XL DNA Analysis System (Beckman–Coulter). DNA sequence was then confirmed by the local alignment search tool (BLAST) network service at the National Center for Biotechnology Information.

Real-time-PCR on different rat tissues. Total RNA (3 μg) was isolated from rat brain cortex, lung, kidney, liver, heart, and testes, and reverse transcribed as described above. Rat calpain-5 mRNA was amplified by real-time-PCR (RT-PCR) on Roche Light Cycler using 5′ GCT GCC TGC TCA TCA CTG GC 3′; and 5′ TCA CTC TTG CTC ACT TTC TG 3′ primers. The quantitative data were plotted on a bar graph.

RT-PCR on different rat brain regions. Total RNA was isolated from different brain regions: cortex, hippocampus, cerebrum, spinal cord, thalamus, substantia nigra, striatum, medulla, pons, frontal lobe, and temporal lobe. They were reverse transcribed and PCR-amplified using rat calpain-5 specific primers 5′ ACG GTG AGT TCT GGA TGA CC 3′ and 5′ GCC CAG AAA TTC ATC CTT CA 3′ and equal loading was determined by PCR amplification using GAPDH primers.

RT-PCR on different human tissues and brain regions. The calpain-5 mRNA expression was determined on the cDNA panel derived from different human tissues or brain regions (Human Rapid Scan panel or Human Brain Rapid Scan panel, OriGene Technologies). The1000× cDNA panel was PCR-amplified using 5′ CTGCTGGTTTGTGG CAGCTG 3′ and 5′ GCGGTCGTCCTGCACGGTCAC 3′ human calpain-5 specific primers. The β-actin primers provided in the kit were used to determine the equal sample loading.

Cell treatment and protein extraction. Human neuroblastoma SH-Sy5Y cells were grown to confluency on a 12-well plate in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml of Fungizone. At 80% confluency the cells were washed once with PBS without Ca^{2+} and Mg^{2+} and maintained in serum free MEM for 1 or 24 h containing one of the following: 0.3 nM maitotoxin (MTX) (calcium channel opener) or 2.5 μM A23187 (Ca^{2+} ionophore) [16]. MTX treated cell lysates were collected 1hr after incubation and A23187 treated cell lysates 24 h after treatment. Similar experiment was set up and the cells were pretreated for 1 h with different inhibitors: MDL-28170 (10 μM) (calpain inhibitors); ZD-DCB (50 μM) (caspase inhibitors); and *N*-ethylmaleimide (NEM, 50 μM) (thiol reactive agent) [16,17] followed by 1 h treatment with 0.3 nM MTX. The cell lysates were collected and used for Western blot analysis.

Western blot analysis. Total protein concentration of the above cell lysates was determined by BCA assay (Pierce). Fixed amount of proteins (20 μg) was run on a 4–20% SDS–PAGE gel and transferred onto PVDF membrane. The blots were probed with anti-human calpain-5 antibody (Cl1, Cedarlane, Canada); biotinylated secondary antibody followed by streptavidin conjugated alkaline phosphatase. The blots were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Results

Cloning of full-length of rat calpain-5

Total RNA from rat brain was PCR-amplified, sequenced, and cloned into T/A vector (Fig. 1A) (deposited as GenBank Accession No. AF484958). We identified 1876 bp cDNA which contained a single long open reading frame encoding a polypeptide of 640 amino acids with a calculated molecular mass of ~76 Da and an isoelectric point (p*I*) of 7.60. The nucleotide sequence was 87% and 93% identical with human and mouse calpain-5, respectively. The deduced amino acid sequence was very well conserved (Fig. 1B) with rather more similarity to the mouse (96% identity) than to the human (92% identity). We believe that our cDNA contains the entire functional region of the protein, because there was a single methionine residue in good Kozak consensus that also matches the human and mouse calpain-5. Similarity in each of the domains, I, II, III, and (T), was 79.2%, 92.2%, 94.4%, and 82.8%, respectively. The domain II was highly conserved particularly at the catalytical site of cysteine, histidine, and asparagine residues (Fig. 1B).

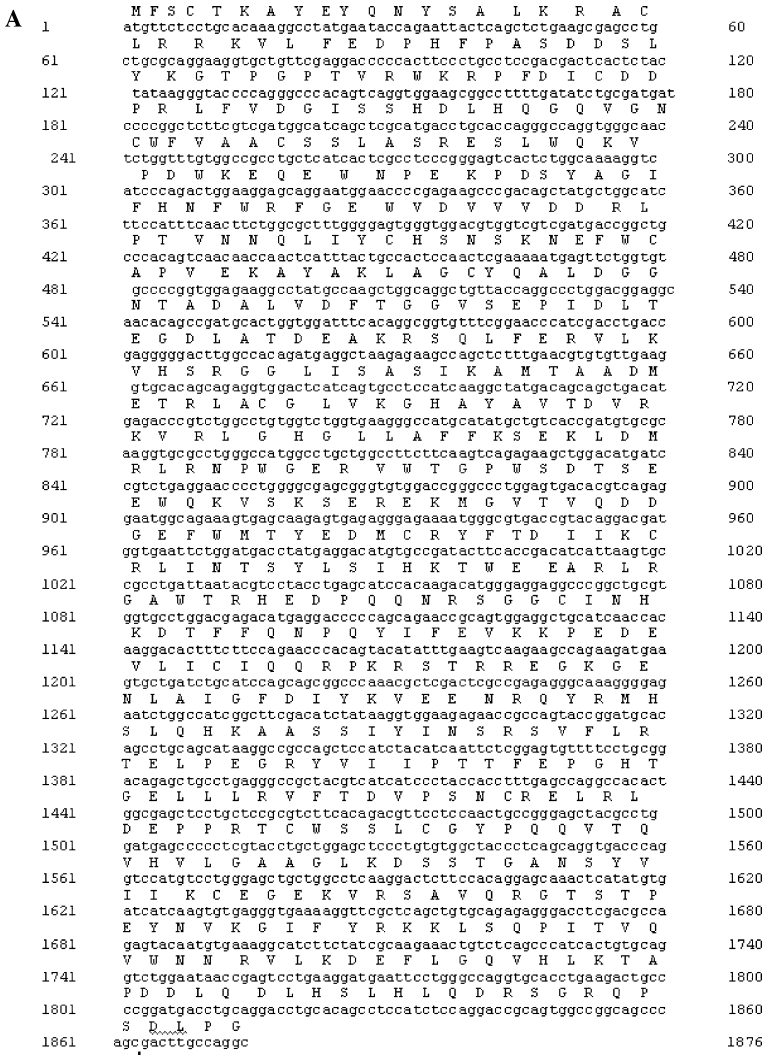

Fig. 1. Predicted cDNA and amino acid sequence of rat calpain-5. (A) Rat capn-5 sequence (GenBank Accession No. AF484958) consists of 1866 bp long cDNA with a single long open reading frame encoding a polypeptide of 640 amino acids. (B) Alignment of the predicted rat calpain-5 amino acid sequence with human and mouse calpain-5. All three sequences contained conserved domain structures indicated by relevant sequence segments and catalytic residues indicated by (▼) in domain II. Consensus sequence among the three proteins is indicated by (*).

mRNA expression of calpain-5 in different rat tissues

To determine the expression pattern of rat calpain-5 in different rat tissues, we performed real-time-PCR. The calpain-5 transcript was ubiquitously expressed in all rat tissues examined, with the highest level of expression being in lung and the lowest level in heart among tested tissues including heart, testes, brain cortex, liver, kidney, and lung (Fig. 2).

mRNA expression of calpain-5 in different rat brain regions

The expression pattern of rat calpain-5 in different rat brain regions was determined by RT-PCR analysis. The ~0.8-kb calpain-5 transcript was equally expressed in all the brain regions that were analyzed. The GAPDH PCR analysis showed equal loading (Fig. 3).

mRNA expression of calpain-5 in different human tissues

To determine the expression pattern of calpain-5 in different human tissues, we performed RT-PCR on the 1000× cDNA panel of different human tissues using human calpain-5 specific primers. The calpain-5 transcript (~700 bp) was equally expressed in all tissues (Fig. 4A). However, RT-PCR analysis on 250× cDNA panel showed expression only in colon, testes, and brain tissues (data not shown), suggesting that calpain-5 is strongly expressed in testis, brain, and colon, but at 1000×, its expression may be saturated in all tissues.

mRNA expression of calpain-5 in different human brain regions

The expression pattern of calpain-5 in different human brain regions was analyzed by RT-PCR on the

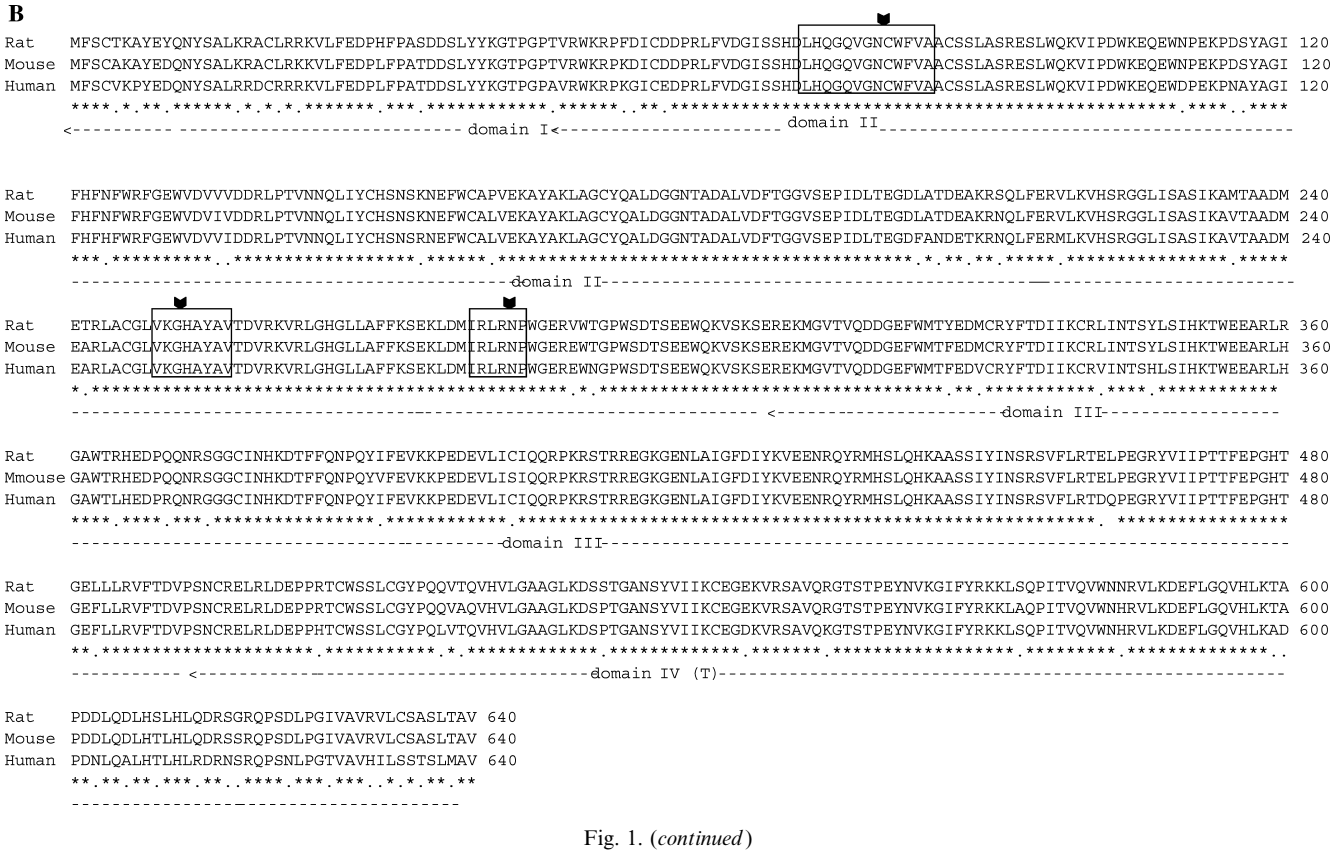

Fig. 1. (*continued*)

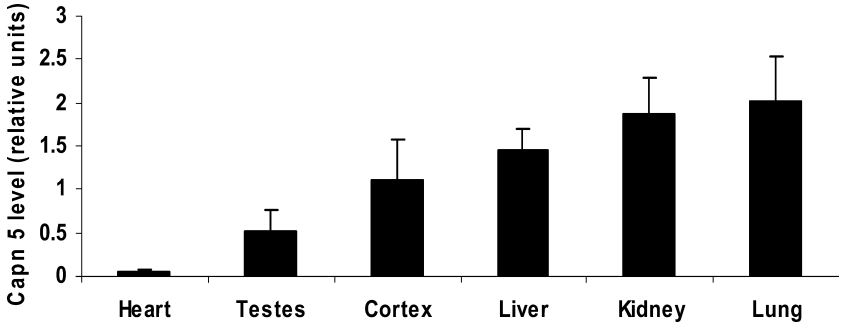

Fig. 2. Expression of calpain-5 transcript in different rat tissues. Total RNA was extracted and real-time PCR was performed on heart, testes, cortex, liver, kidney, and lung rat tissues using rat specific calpain-5 primers. GAPDH-specific primers were used for equal loading ($n = 3$).

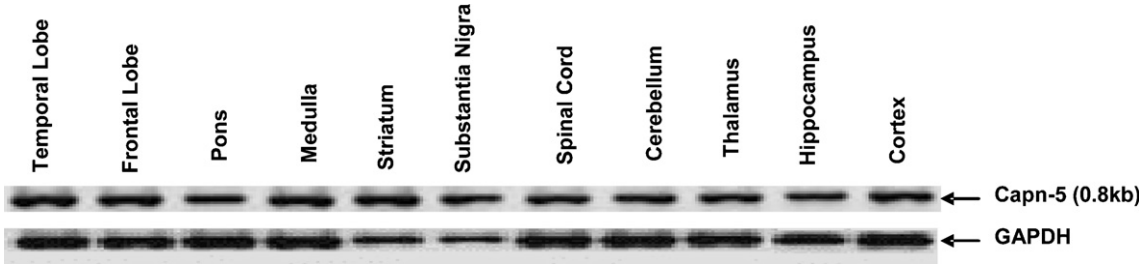

Fig. 3. Expression of calpain-5 transcript in different rat brain regions. Total RNA was extracted from different brain regions and RT-PCR analysis was performed using rat specific calpain-5 primers. GAPDH specific primers were used on the same set of samples for equal loading.

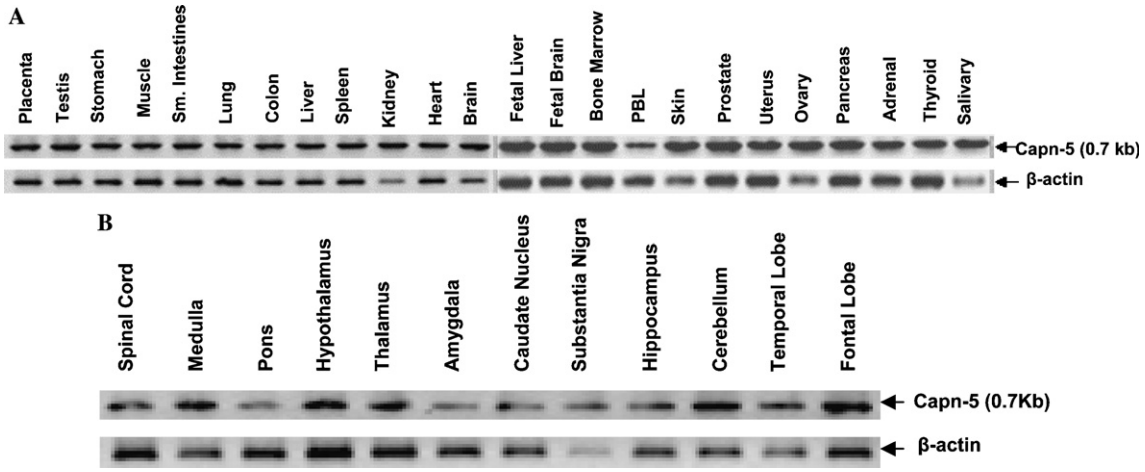

Fig. 4. Expression of calpain-5 transcript in human. Human specific calpain-5 primers were used for RT-PCR analysis on 1000× cDNA panel of (A). Tissue specific human rapid scan cDNA panel. (B) Human brain rapid scan cDNA panel. RT-PCR with β-actin specific primers was performed for equal loading.

1000× cDNA panel of different human brain regions using human calpain-5 specific primers. The human calpain-5 transcript (~700 bp) was strongly expressed in medulla, hypothalamus, thalamus, cerebellum, and frontal lobe (Fig. 4B).

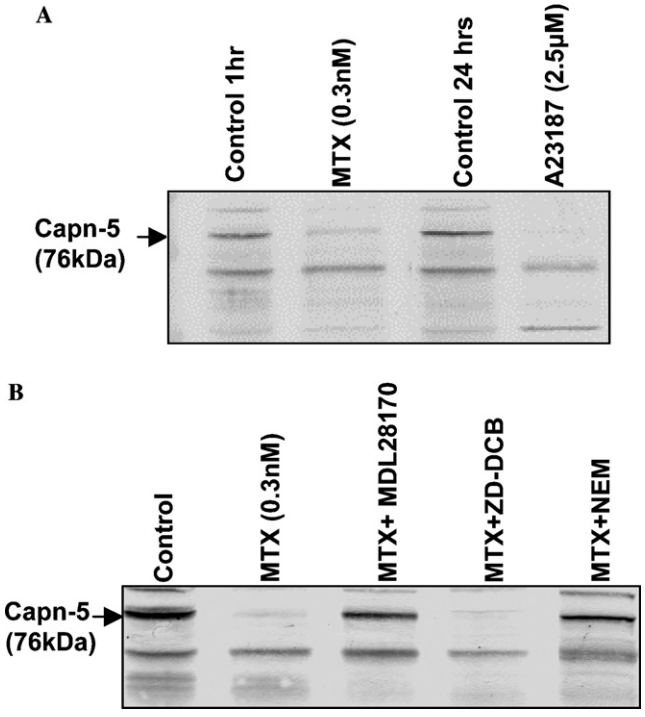

Fig. 5. Western blot analysis of the calpain-5 protein in SH-SY5Y. (A) Cell lysates from untreated (control) or treated with MTX (0.3 nM) for 1 h or with A23187 (2.5 μM) for 24 h and subjected to SDS–PAGE gel. The blots were probed with anti-human calpain-5 antibody (1:1000). The arrow indicates approximately 76 kDa full-length calpain-5 protein. (B) Cell lysates were preincubated with MDL28170 (10 μM), ZD-DCB (50 μM), or NEM (50 μM) for 1 h prior to treatment with MTX (0.3 nM). The samples were subjected to SDS–PAGE gel and probed with anti-human capn-5 antibody (1:1000). The arrow indicates approximately 76 kDa full-length calpain-5 protein.

Western blot analysis

To evaluate the ability of calcium-mediated activation of calpain-5, the maitotoxin (MTX, a potent calcium channel opener) (0.3 nM) or calcium ionophore A23187-(2.5 μM) treated SH-SY5Y cells were subjected to Western blot analysis and probed with anti-human calpain-5 antibody. The results show that in the absence of exogenous calcium, full-length calpain-5 band (~76 kDa) was detected (Fig. 5A, control). However, treatment with MTX or A23187 resulted in reduction or loss of the intact band, suggesting that calpain-5 is processed or autolyzed in the presence of calcium (Fig. 5A). Further, the SH-SY5Y cells were treated with calpain and caspase inhibitors prior to MTX treatment and observed that calpain-5 processing is blocked by calpain-1, -2 specific inhibitor (MDL-28170) and by thiol reactive agent, *N*-ethylmaleimide (NEM), but not by caspase inhibitor (ZD-DCB) (Fig. 5B). These results strongly suggest that other members of calpain family control the calcium mediated processing of calpain-5.

Discussion

Using oligonucleotide primers designed from human and mouse calpain-5 sequences and rat genomic DNA as a template, rat calpain-5 was identified and sequenced. Its nucleotide sequence was 87% and 93% identical to human and mouse calpain-5, respectively, and maintained the active triad sequence of Cys, His, and Asn in domain II. The single long open reading frame coded for 640 amino acids with a calculated molecular mass of ~76 kDa and an isoelectric point

(p*I*) of 7.60. The amino acid conservation for individual domains between rat, mouse, and human calpain-5 is: 79.2%, 92.2%, 94.4%, and 82.8%, respectively, for domains I, II, III, and T (Figs. 1A and B). Tissue distribution of capn-5 in rats has shown strong expression in lungs and kidneys while very week expression in heart. However, in different brain regions it is ubiquitously expressed in all tested regions (Figs. 2 and 3). In human capn-5 was equally expressed in all tissues may be due to saturation of the gene (Fig. 4A), because at lower cDNA concentration (250×) it was expressed only in colon, testes, and brain, similar to those observed by other studies [7,9] suggesting its role in these tissues. In addition, the distribution of capn-5 in different brain regions showed strong expression in medulla oblongata, hypothalamus, thalamus, cerebellum, and frontal lobe (Fig. 4B). However, Dear et al. [9] have shown no expression of calpain-5 in medulla oblongata, weak in thalamus, and the spinal cord expression pattern was similar to that observed by us. These differences could be due to difference in sample loading or the method of detection.

Domain IV, common to other mammalian members of calpain, contains a calcium-binding domain [6] and interacts with the small subunit and the endogenous inhibitor calpastatin [18,19]. Lack of domain IV may prevent capn-5 from interacting with calcium ions and the small subunit in the same manner as ubiquitous calpains. However, recent crystallization works clearly show that there are key calcium-bindings sites within domain II that serve as calcium-switch in the activation of classical calpain-1and -2 [6]. In addition, there is also the possibility of the conserved C2-like domain (D-III) that might serve as additional calcium sensor [2,5]. In SH-SY5Y cells we detected full-length calpain-5 (~76 kDa) in the absence of exogenous calcium however, addition of calcium showed reduction or loss of the full-length calpain-5 band (Fig. 5A). These results were similar to those observed previously, however our antibody could not detect the cleaved product (50 kDa) of calpain-5 as shown by others [9,20]. Further upon treatment with different calpain and caspase inhibitors prior to treatment with MTX, we observed recovery of the full-length capn-5 band after treatment with calpain inhibitor and with thiol reactive agent but not with caspase inhibitor (Fig. 5B), suggesting that calcium-mediated processing of calpain-5 is calpain- but not caspase-dependent. Previous reports suggest that interaction of small subunit (via domain VI) with the large subunit of classical calpain-1 or -2 (via domain IV) is important for calpain activation and heterodimer formation [21]. Due to lack of domain IV in calpain-5, it is highly likely that it is not associated with or regulated by a small calpain subunit. Another possibility is that domain T which is highly conserved may play an important role in regulating the activity of these proteins [8].

The physiological function of calpain-5 in mammals remains unclear, its high expression in testes as we and others have demonstrated [7,9] is supportive of sex-related function, but further studies are required for biological evidence. In addition, recent knowledge about various calpain family members that they contribute to neurodegeneration and neuronal loss [14], and the requirement of *C. elegans* tra-3 for necrotic cell death, and initiation of neuronal degeneration [15] suggest its possible role in neuronal loss. Identification of calpain-5 in mammalian brain suggests that similar mechanisms are conserved from nematodes to humans and they highlight the fact that calpain-5 may be a potential target for therapeutic intervention in an effort to battle neuronal loss and degeneration.

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