Rat brain contains multiple mRNAs for calpastatin

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Abstract This work was undertaken to establish the forms of the calpain inhibitor, calpastatin, expressed in the brain tissue. Five cDNA clones were obtained and the corresponding amino acid sequences were deduced. Three of these proteins contain an N-terminal domain (domain L) and four inhibitory repeats typical of the calpastatin molecule. The other two are truncated forms, containing the domain L, free or associated with a single inhibitory repeat. Other differences, due to exon skipping, produce calpastatin forms with different susceptibility to posttranslational modifications. The more represented mRNA form corresponds to a calpastatin molecule containing the four inhibitory domains. These results may be useful to understand the involvement of calpain in the onset of acute and degenerative disorders of the central nervous system.

Key words: Calpastatin; Calpain; Proteinase inhibitor; Ca^{2+}-dependent proteolysis; Rat brain

1. Introduction

All eukaryotic cells contain a calcium-dependent proteolytic system, consisting of one or more proteinases, named calpains. The two well studied calpain forms are heterodimeric proteins, distinguishable on the basis of calcium requirement and composed of different catalytic 80 kDa subunits and of identical small 30 kDa subunits [1–6]. These proteinases are regulated in the cell by the presence of an inhibitory protein, calpastatin, also localized in the cytosolic compartment [7]. Calpastatin interacts with the catalytic 80 kDa calpain subunit, preventing its conversion to the low calcium requiring autoproteolysed form, and consequently inhibiting the expression of its catalytic activity [8]. Phosphorylation of calpastatin has been proposed as a mechanism that changes the specificity of the inhibitor in isolated cells and in skeletal muscle [2,9]. Moreover, Mohan and Nixon [10] reported that multiple low molecular weight forms of calpastatin are present in bovine brain, probably produced by proteolysis of a larger precursor. The efficiency and the levels of calpastatin play an important role in preventing the so-called pathological function of calpains, which is triggered by degradation of the inhibitor protein, mediated by calpain [11,12].

Full-length or partial cDNA clones encoding calpastatin have been obtained previously from mRNAs of various mammalian tissues or cell lines [13–20]. In the N-terminal region of the protein sequences, deduced from human and rat cDNAs and corresponding to the domain L of unknown function, the presence of one or more peptide deletions, depending on the tissue analyzed, has been identified [14–16]. Human genomic DNA for calpastatin has also been sequenced and evidence has been obtained for the alternative splicing mechanisms producing deletion of one or more exons in domain L of rat and human calpastatin mRNAs [15]. The full-length rat liver cDNA for calpastatin, cloned previously [16], encoded a domain L carrying the deletion of three peptides, corresponding to exons 3, 4 and 6 [15], followed by four repetitive regions, each containing the inhibitory consensus sequence TIPPxYR [21]. Two other partial cDNA clones, obtained from rat fibroblasts, were found to encode calpastatins in which peptides corresponding to exon 3 or to exons 3 and 6, respectively, were deleted [14]. Considering these findings, we searched for mRNAs encoding calpastatin in rat brain. Since the intracellular level of calpastatin is directly correlated with the activation of calpain [22] and the brain tissue seems highly sensitive to calpain-induced intracellular damages [23–25], new information on the expression of calpastatin in brain is important to understand the 'pathological function' of calpastatin in this tissue.

2. Materials and methods

2.1. RNA isolation and cDNA synthesis

Total RNA was isolated from Sprague-Dawley rat brains by extraction with guanidinium thiocyanate [26] and cDNA was synthesized using MMLV reverse transcriptase (Promega) as described [27]. The primers for PCR were selected externally to the translated sequence of rat liver cDNA for calpastatin (accession number X56729). The sense primer was 5′-CTGGCACATCTCCCAGAATGAG-3′ (nucleotides 1–22); the antisense primer was 5′-TCAAAAGTCACCATCCACCAGC-3′ (complementary to nucleotides 1867–1888). PCR was carried out using AmpliTaq DNA polymerase (Perkin Elmer) and 30 cycles of amplification as previously described [27]. An aliquot (2 μl) of the reaction mixture was then used for cloning with the pGEM-T Vector System (Promega) as specified by the manufacturer.

2.2. DNA sequencing

Purified plasmid DNAs were sequenced with the dideoxy chain termination method [28] from both directions, using a Sequenase 2.0 kit (United States Biochemical Corp.). The presence of potential posttranslational modification sites was established on the deduced amino acid sequences with the PROSITE program [29] while the presence of good PEST sequences [30] was determined with the PEST-FIND program.

2.3. RNase protection assays

The plasmids containing the original cDNAs named RNCAST23 and RNCAST110 were used to synthesize antisense RNA probes. Subclones of RNCAST103, RNCAST104 and RNCAST107 clones were generated by PCR and cloned with the pGEM-T Vector System; the sense primer was the 1–22 oligonucleotide reported above, the antisense primer was 5′-GTGTCTATCAGGTCATCCAAAGC-3′

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Abbreviations: PKC, protein kinase C; RT, reverse transcription; PCR, polymerase chain reaction

The nucleotide sequences in this paper have been submitted to the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession numbers Y13587 RNCAST103; Y13588 RNCAST104; Y13589 RNCAST107; Y13590 RNCAST110; Y13591 RNCAST23.

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(complementary to nucleotides 267–289 of rat liver calpastatin cDNA; accession number X56729). The subclones were sequenced and then linearized with *Sac*I (clones RNCAST23 and RNCAST110) followed by transcription with T7 RNA polymerase or *Apa*I (subclones RNCAST103, RNCAST104 and RNCAST107) followed by transcription with SP6 RNA polymerase. Transcriptions were carried out using a MAXIscript Kit (Ambion, Inc.) using 62.5 pmol of [α-^{32}P]UTP (800 Ci/mmol) and 40 pmol of cold UTP in a volume of 20 μl. The hybridization mixtures (20 μl) contained 2 fmol of labeled specific probe, increasing amounts of rat brain RNA (0–20 μg) and a constant amount of total RNA (20 μg), obtained by addition of *Torulla* yeast RNA. After incubation at 50°C for 18 h, RNase digestion was performed at 37°C for 30 min with RPA II kit (Ambion, Inc.) by addition of RNase A (1.25 units) and RNase T_1 (50 units). The reaction was stopped with the RNase inactivation/precipitation mixture and the precipitates subjected to electrophoresis on a 5% polyacrylamide/urea gel. After autoradiography the protected fragments were cut from the gel and quantified as described [31].

3. Results

3.1. Isolation of rat brain calpastatin cDNAs

Rat brain mRNAs for calpastatin were identified by RT-PCR using two primers, based on the sequence reported previously for the 5′ and 3′ untranslated regions of rat liver calpastatin cDNA [16]. The amplified DNA fragments were separated by agarose gel electrophoresis and, as shown in Fig. 1, three bands with sizes of approximately 1900, 600 and 300 bp were obtained. To exclude that these bands were artifacts of the amplification procedure, the PCR products were cloned, sequenced and, among 50 randomly selected clones, all encoded calpastatin molecules with molecular masses corresponding to the sizes of the PCR products. Particularly, 22 clones showed a sequence (RNCAST107) of 1858 bp, 12 (RNCAST103) of 1927 bp, seven (RNCAST110) of 328 bp, five (RNCAST23) of 606 bp and four (RNCAST104) of 2041 bp. These sequences showed high homology with the rat liver cDNA for calpastatin [16], as demonstrated by their alignment, in both the translated and the 3′ untranslated regions (data not shown).

3.2. Structural analysis of calpastatin protein forms deduced from cDNA sequences

Starting from the first methionine codon of the five cDNA clones, an open reading frame encoded putative proteins of 616, 593, 654, 176 and 83 amino acid residues, respectively. The alignment of the sequences of these proteins, together with that of rat liver calpastatin described previously, revealed a high degree of similarity among all the primary structures (Fig. 2). In contrast, relevant differences were found in the N-terminal region of these calpastatin forms. In particular, we found that all the brain calpastatin forms contained a 13-residue peptide (residues 8–20), corresponding to exon 4 [15], absent in the liver form. In clones RNCAST104 and RNCAST110, a second peptide of 38 residues was also found (residues 39–76), corresponding to exon 6. In addition, the putative protein encoded by clone RNCAST110 was a truncated form of calpastatin, composed of 83 amino acids and corresponding to a domain L of the molecule, without any inhibitory domain. Furthermore, the 176-residue protein, encoded by clone RNCAST23, contained a domain L, lacking exon 6, associated with an almost complete inhibitory domain. The three calpastatins having four inhibitory domains also contained a peptide of seven residues, localized in the fourth inhibitory domain, showing a sequence different from that described for rat liver calpastatin. This change was due to a short frame shift of the cDNAs in which were deleted the nucleotide corresponding to T1603 of rat liver cDNA sequence (accession number X56729) followed by the insertion of nucleotide C (clone RNCAST103 and RNCAST107) or T (clone RNCAST104) between C1624 and T1625 in the cDNA of rat liver calpastatin (data not shown).

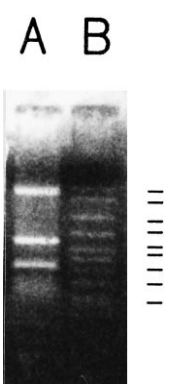

Fig. 1. Detection of rat brain calpastatin-related transcripts. Total rat brain RNA was reverse transcribed and subjected to PCR as described in Section 2. After 30 cycles of amplification, 10 μl of the reaction mixture was subjected to electrophoresis on a 1% agarose gel. Lane A, products of amplification visualized by ethidium bromide staining; lane B, AmpliSize DNA size standard 50–2000 bp ladder (Bio-Rad): from the top: 2.00, 1.50, 1.00, 0.70, 0.50, 0.40, 0.30, 0.20, 0.1 kb.

To evaluate the possible biochemical relevance of these multiple calpastatin forms in brain tissue, we analyzed on each protein molecule the presence of characteristic posttranslational modification sites which could be involved in the modulation of calpastatin function. All rat brain calpastatins, as compared to the liver form, contain an additional PKC target site (Ser^{16}) which increases the potential susceptibility of the protein to phosphorylation in the N-terminal region (Fig. 3). Those calpastatin forms having also the peptide corresponding to exon 6 possess a further site for PKC phosphorylation (Ser^{69}), together with a *N*-myristoylation site (Gly^{50}). Moreover, rat brain calpastatins encoded by RNCAST103, RNCAST104 and RNCAST107 clones show a PKC phosphorylation site at positions 546, 584 or 523 respectively. The truncated forms are lacking in good PEST sequences, proposed signals for protein degradation [30].

3.3. Levels of the mRNA forms for calpastatin in rat brain

The amount of the five calpastatin transcripts in rat brain was measured by the RNase protection assay method. Following the procedure employed [31], hybridization of increasing amounts of rat brain RNA samples with specific probes for each calpastatin mRNA yielded amounts of protected fragments, the localization of which is indicated by brackets in Fig. 3, correlated with the quantity of starting material. The results, reported in Table 1, indicated that RNCAST107 was the most abundant form, with a level of mRNA three times higher than that of RNCAST103. The levels of the other three calpastatin mRNAs were at least one order of magnitude lower than that of RNCAST107.

4. Discussion

Regulation of calpain is accomplished by the competitive

Fig. 2. Comparison of the deduced amino acid sequences of rat brain calpastatins with the rat liver calpastatin. The sequences were aligned using the CLUSTAL program. The differences between the primary structures of rat liver calpastatin (accession number P27321) and rat brain calpastatins are shown in bold characters. The exon structure has been assigned, based on human gene mapping [16].

inhibitor calpastatin able to control autoproteolysis, expression of catalytic activity as well as insertion of the enzyme into the plasma membrane, the preferred site for the activation of calpain [8,32]. The affinity of calpastatin for the autolysed calpain form is greater than that for the native enzyme [8], indicating that the structural and conformational changes occurring during autoproteolysis favor the formation of the enzyme-inhibitor complex. Regulation of calpain activity is fur-

Table 1
Level of calpastatin RNAs in rat brain

Clone	Level of calpastatin mRNA (amol/μg of total RNA)	Size of expected product (nucleotides)
RNCAST104	0.002 ± 0.001	442
RNCAST103	0.019 ± 0.004	328
RNCAST107	0.054 ± 0.008	259
RNCAST 23	0.002 ± 0.001	607
RNCAST110	0.007 ± 0.002	328

Samples of rat brain total RNA were hybridized with ^{32}P-labeled antisense RNA probes for different calpastatin transcripts as described in Section 2. After RNase digestion the presence of protected fragments was evaluated by electrophoresis on a polyacrylamide/urea gel followed by autoradiography and counting of the corresponding fragment of the gel. Each experiment was done in triplicate and the values, calculated as described in [31], are the means ± S.D.

ther complicated by the presence in the same cell compartment of two proteinase isozymes, showing different catalytic and functional properties [2–4]. Hence, regulation of calpain activity requires cellular mechanisms capable of modifying calpastatin inhibitory efficiency together with its enzyme selectivity. In vitro and in vivo, posttranslational modifications of calpastatin are promoted by a phosphorylation-dephosphorylation process which modifies calpastatin efficiency and specificity [2,9]. In addition, the levels of calpastatin may be decreased by a calpain-mediated proteolysis [33,34], producing cell conditions in which the proteinase is more susceptible to activation. The presence of multiple forms of calpastatin seems to be a crucial requirement for the regulation of the Ca^{2+}-dependent proteolytic system in many cell types [15]. We now report that the Ca^{2+}-dependent proteolytic system of rat brain contains five transcripts for calpastatin which encode proteins differing in amino acid sequence and molecular size. The rat brain calpastatins identified here are distinguishable from the reported rat liver form by the presence of peptides, corresponding to exons 4 and 6, in domain L. Moreover, clone RNCAST107 is characterized by the absence of a peptide corresponding to exon 8. Three of these protein forms, containing four inhibitory repeats, differ from the rat liver form also at the level of a seven-residue peptide present in the C-terminal end (fourth inhibitory repeat). These differences in the primary structure modify the susceptibility to posttranslational modifications of each rat brain calpastatin form. In particular, regions containing PKC phosphorylating sites or good PEST sequences can modulate calpastatin efficiency, specificity and digestibility. The presence of very different amounts of the transcripts encoding calpastatin, here identified in rat brain, suggests that some calpastatins are probably expressed only in specific brain regions. The identification of multiple mRNAs for calpastatin in rat brain and the availability of the corresponding cDNAs is particularly useful to produce recombinant calpastatin forms and hence to study their specificity, activity, binding site on calpain isozymes and susceptibility to proteolytic degradation, together with their in situ studies.

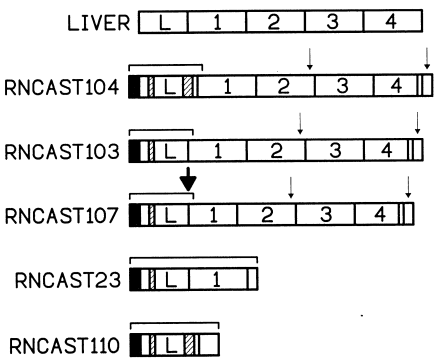

Fig. 3. Schematic representation of the main differences between rat brain calpastatins and rat liver calpastatin. The deduced amino acid sequences of rat brain and liver calpastatins have been aligned and the most relevant differences in the primary structures have been marked. L, domain L; 1–4, inhibitory repetitive domains; hatched boxes, peptides corresponding to exon 4 and exon 6 of the human gene for calpastatin; filled box, nucleotide sequence upstream of the translated start codon; open box, heptapeptide different from that of the liver form; thin arrow, good PEST sequence; bold arrow, peptide deletion with loss of a PKC phosphorylation site; bracket, position of the protected fragment in the RNase protection assay.

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