Correlation between bovine calpastatin mRNA transcripts and protein isoforms

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Received 28 April 2005, and in revised form 24 May 2005
Available online 20 June 2005

Abstract

Calpastatin is a specific calpain protease inhibitor: calpains are a family of calcium-activated neutral proteases, which have been implicated in various processes. Despite all the available data concerning calpastatin, little is known about how this gene is regulated, particularly in bovine. The existence of four types of transcripts differing at their 5′ ends (Type I, II, III, and IV) has been demonstrated. Here, we show that the Type I, II, and III transcripts are ubiquitous while Type IV is testis-specific. In addition, a Northern blot analysis revealed that the Type III transcript may have three different 3′ termini. Using specific anti-peptide anti-sera, a correspondence between a 145 and a 125 kDa isoforms, and Type I and/or II and III transcripts, respectively, has been established. Finally, we discuss the origin of a 70 kDa isoform, recognized by anti-sera directed against the N-terminal region.

Keywords: Calpastatin; Calpain; Bovine; mRNA expression; Isoforms; Proteolysis; Western blot; Northern blot

Calpastatin is a protease inhibitor that is specific for calpains, a family of calcium-activated neutral proteases. The calpain/calpastatin system has been implicated in various biological and pathological processes [1]. In striated muscle particularly, this system is implied in myoblast growth [2], migration [3], and fusion [4], in the limb-girdle muscular dystrophy type IIA [5] and in meat texture development [6]. Despite all the available information concerning calpastatin, little is known about how this gene is regulated at the transcriptional and translational level, especially in bovine. Because of the economic importance of the bovine species to the livestock industry, it appears clearly essential to clarify some of the factors related to calpastatin gene expression.

A single calpastatin gene exists in human [7], pig [8], mouse [9], and bovine [10]. The complete structure of the murine [9] and bovine [10] gene and a partial porcine structure [11] have been published so far. In bovine, the gene comprises 35 exons spanning at least 130 kb of genomic DNA. Four promoters direct the expression of four types of transcripts, named Type I, Type II, Type III, and Type IV, that differ at their 5′ ends in cattle [10], pig [11,12], and mouse [9,13]. Analysis of calpastatin amino acid sequences has revealed four homologous C-terminal inhibitory domains (I-IV) downstream of a non-inhibitory leader domain (L) of unknown function [14]. In addition, a N-terminal peptide sequence (XL) was identified in bovine [15]. Nevertheless, calpastatin isoforms isolated from different tissues are heterogeneous in
size, leading to their classification into two subtypes, a short erythrocyte type of 65–70 kDa and a larger tissue type with an apparent molecular weight in the range of 107–172 kDa. Full characterization has proved difficult because most isoforms exhibit aberrant electrophoretic mobility on sodium dodecyl sulphate (SDS)–PAGE [1,16]. Recently, a 145 kDa form detected in porcine heart and skeletal muscle has been assigned to the Type I mRNA translation product [12]. Besides that, no definitive correlation has been established between the different types of calpastatin mRNA transcripts and the various isoforms observed.

Here, we demonstrate that Type I, II, and III mRNAs are ubiquitous and Type IV is testis-specific in bovine. In addition, a Northern blot analysis has revealed an association between the Type III 5′ end with three different 3′ termini. Finally, using specific anti-peptide anti-sera, a correspondence between the Type I, II, and III mRNA transcripts and calpastatin isoforms has been established.

Materials and methods

Samples collection

Brain, heart, kidney, liver, spleen, testis, and various skeletal muscles (Diaphragm, Rectus Abdominis, Semi Tendinosus, Transversus Abdominis, and Longissimus Thoracis) were collected from three Charolais individuals. They were immediately cut into small pieces, frozen in liquid nitrogen, and stored at −80°C.

RNA preparation

RNA extraction was carried out using the RNasy Maxi Kit (Qiagen). Samples were homogenized using the ULTRA-TURRAX T25 basic IKA-WERKE in the buffer supplied in the kit. The extraction was then performed according to the manufacturer’s instructions. RNA samples were quantified using the RNA 6000 Nano kit and on Agilent 2100 Bioanalyzer (Agilent Biotechnologies).

RT-PCR of calpastatin transcripts with alternative 5′ ends

First-strand cDNA was synthesized from 1 μg of total RNA, in a total volume of 20 μl, using 0.5 μg oligo(dT) primer and SuperScript II RNase H− Reverse transcriptase (Invitrogen). The reaction was incubated for 50 min at 42°C and 15 min at 70°C. To amplify Type I, II, and III transcript 5′ ends, cDNAs were subjected to three separate nested touchdown PCRs. These were carried out in a final volume of 50 μl with two reverse primers, Ex9.R (5′-CTCTTCAAGTTCTCCTAAAGTG-3′) and Ex8.R (5′-CAGCTGTACGGCCGATG-3′) designed in exon 9 and 8, respectively, according to the structure of the bovine calpastatin gene previously described [10; Fig. 1A]. The specific forward primers Ex1xa.1F (5′-GCTTCGGGTCGCGGGC-3′) and Ex1xa.2F (5′-CATGTCCACGGCGGCC-3′), Ex1xb.1F (5′-GGCAGTGGAGTCCGAC-3′) and Ex1xb.2F (5′-GCCATGATCACATTGCAGT-3′), Ex1u.1F (5′-GGTCAGACCTCTGCATCACG-3′) and Ex1u.2F (5′-GAACCGCGGGCCGAG-3′) were designed in exons 1xa, 1xb, and 1u, respectively (Fig. 1A). To amplify Type IV 5′ UTR, a simple PCR was carried out using Ex14t.F (5′-GAGGACTCAGGGTGCCGATG-3′) and Ex29.R (5′-GGCTGGAAGTTT CCTCCT-3′) designed in exon 14T and 29, respectively (Fig. 1A). Touchdown PCR method was performed using the following parameters: a denaturation step for 3 min at 94°C; then 94°C for 30 s, 64°C for 30 s, and 72°C for 1 min for 37 cycles. The annealing temperature was decreased of one degree at each cycle for the first seven cycles and then maintained constant for the next 30 cycles. PCR products were then analyzed on 1.5% agarose gels.

Northern blot analysis of calpastatin mRNAs

Total RNA (20 μg) was fractionated on agarose–formaldehyde gel electrophoresis (1.2%) and transferred to a nylon membrane Hybond- XL (Amersham-Pharma
cia Biotech). RNA was cross-linked to the membrane using UVR (120 mJ). Membranes were probed with calpastatin cDNA corresponding either to exon 13–29 or to exon 1u. These probes were labelled with deoxyctydine 5′[32P]triphosphate by the random primer extension method [17], using a commercially available kit (Invitrogen) and then hybridized in ExpressHyb Hybridization Solution (BD Biosciences Clontech) at 65°C for 2 h. Membranes were washed successively in a 2×, 1×, and 0.1× SSC/0.1% SDS at 65°C.

Anti-calpastatin antibody and anti-sera

To reveal the various isoforms of calpastatin, one antibody and five anti-sera were used in Western blots. A commercial monoclonal antibody (Anti-DomIV) directed against the domain IV of human calpastatin (Calbiochem) was used at a 1:5000 dilution. The Anti- pep-DomIV serum (supplied by A. Ouali, INRA/Clermont-Ferrand Theix) recognized a human peptide (ELDDALDQLSDL) situated at the N-terminal end of the domain IV (1:1000 dilution). To detect specific calpastatin isoforms, four sera directed against porcine peptides (supplied by T. Parr, University of Nottingham) were used. Anti-Ex1xa [12] (1:2000), Anti-Ex1xb (1:300 or 1:500), Anti-DomXL (1:400), and Anti-Ex3 (1:400) anti-sera were raised in rabbit against peptideic sequences (MSQPQKPAASRP, MAFASWYK, TAP-
VTKVSPSSAST, and AIPVSKQLEGPHSP) from the translation of exons 1xa, 1xb, 1z, and 2 and 3, respectively.

Western blot analysis of calpastatin isoforms

Samples, stored at −80 °C, were homogenized using the FastPrep FP120 (BIO101, ThermoSavant) in an extraction buffer (10 mM Tris/HCl, pH 7.6, 1 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulphate, 200 mg/L ovomucoid, and 12 mg/L leupeptin). Homogenates were centrifuged at 13,000g for 15 min at 4 °C after which the supernatant was heated in a boiling water bath for 5 min and recentrifuged at 13,000g for 15 min at 4 °C. The protein content of the supernatant was determined by the Bradford method using BSA as standard [18]. Total proteins (30 µg) were analyzed by 7.5% SDS–PAGE. Gels were electroblotted onto nitrocellulose membranes, probed with an antisera or a monoclonal antibody against calpastatin (see previous paragraph for details about sera and antibodies), and visualised using BM chemiluminescence Blotting Substrate (Roche).

Results and discussion

Tissue distribution of calpastatin Type I, II, III, and IV transcripts

Four calpastatin transcripts (Type I, II, III, and IV), differing by their 5′ ends, were identified in cattle, as previously observed in mouse and pig [9,11]. These mRNAs are transcribed from four distinct promoters [10] located upstream of the first transcribed exons 1xa, 1xb, 1u, and 14T. To assess the expression pattern of these four different transcripts, a RT-PCR experiment was performed on equal quantities of total RNA from brain, heart, liver, kidney, spleen, testis, and five skeletal muscles. To distinguish between Type I, II, III, and IV, PCRs were carried out using specific forward primers in exons 1xa, 1xb, 1u, and 14T, respectively (Fig. 1A). The PCR predicted product sizes are 671, 634, 551, and 1259 bp for Type I, II, III, and IV, respectively. As a nested PCR method was used, just qualitative observations can be made. Only results obtained with RNA from heart, diaphragm, Rectus Abdominis, spleen, and testis are presented in Fig. 1B, but the RT-PCR analysis was done on 11 tissues.
and three different individuals (data not shown). Type I, II, and III were detected in all tissues tested whereas Type IV was only present in testis. These results confirm that Type I, II, and III are ubiquitous and that Type IV is a testis-specific transcript as it has been previously described in other species [9,11,19]. Doublets were observed for Type I, II, and III in various tissues (Fig. 1B and data not shown). By sequencing, we determined that they are due to the alternatively spliced exon 3. Both situations are observed for Type I, II, and III transcripts in most tissues. However, we failed to determine a regular rule for the exon 3 splicing. As other alternatively spliced exons have been previously described in various species [20–22], the situation in bovine may be more complex than it appears.

**Northern blot analysis of calpastatin mRNAs**

To confirm the expression pattern of calpastatin transcripts, Northern blotting was carried out with total RNA from various tissues. Membranes were probed with calpastatin cDNA corresponding either to exon 13–29 (Fig. 2A) or exon 1u (Fig. 2B). Using the region shared by all the transcripts as probe, four bands with estimated size of 4.5, 3.5, 2.6, and 1.6 kb were detected (Fig. 2A). The 1.6 kb mRNA is only present in testis while the others are present in all tissues tested. A similar pattern, with only three bands, is observed using the specific probe corresponding to exon 1u (Fig. 2B). We failed to detect any band with specific Type I and II probes corresponding to exon 1xa and 1xb even with 1 μg of poly(A)-rich RNA (data not shown), suggesting that the Type III transcript may have a higher expression. Quantitative RT-PCR experiments would be needed to resolve this issue. The fact that different sizes of transcripts are observed using the Type III specific probe can be explained by the existence of three different polyadenylation sites [15], which were indeed identified in exon 30. The existence of multiple polyadenylation sites has been previously described in the rabbit calpastatin gene [23]. Theoretical sizes of each transcript type associated with each 3‘ end are reported in Table 1. Regarding these sizes, each band of the pattern may correspond to at least three different transcripts. For example, the 2.6 kb may correspond to Type I, II, and III with the shorter 3′ UTR and also to Type IV with the medium one. Actually, this band is the weakest using the two probes (Fig. 2), which suggest that these 5′ and 3′ ends associations are not the most common. Indeed, as it is only present in testis, the 1.6 kb band may correspond to the Type IV mRNA associated with the shorter 3′ UTR. Northern blot analyses on bovine heart [15] and on Longissimus Thoracis [24], using probes recognizing all four transcripts, show a similar pattern with three bands of comparable sizes. In pig, a Northern blot analysis on porcine heart and skeletal muscle RNA displays 5.8, 3.2 kb and 5.4, 2.8 kb bands, respectively [11,25]. As calpastatin transcripts 5′ ends are very similar between pig.

![Fig. 2. Northern blot analysis of calpastatin mRNAs. Total RNA (20 μg) per lane were analyzed by Northern blotting. Membranes were then probed using either a region corresponding to exon 13–29 (A) or a specific one corresponding to exon 1u (B). Tissues are indicated as D for diaphragm, LT for Longissimus Thoracis, TA for Transversus Abdominis, RA for Rectus Abdominis, ST for Semi Tendinosus, T for testis, B for brain, H for heart, L for liver, S for spleen, and K for kidney. The RNA size markers and the sizes of the calpastatin mRNA bands are given.](image)

<table>
<thead>
<tr>
<th>Type</th>
<th>5′ end (bp)</th>
<th>3′ UTR (bp)</th>
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<tbody>
<tr>
<td>Type I</td>
<td>2744</td>
<td>2776</td>
</tr>
<tr>
<td>Type II</td>
<td>2701</td>
<td>3490</td>
</tr>
<tr>
<td>Type III</td>
<td>1584</td>
<td>2373</td>
</tr>
<tr>
<td>Type IV</td>
<td>1584</td>
<td>2373</td>
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Calculated sizes of the different combinations between 5′ and 3′ ends are indicated above. UTR, Untranslated region.
and bovine, the size differences may be due to the various 3′ UTR lengths. As only two bands appear with porcine RNA, there may be a missing polyadenylation site in pig. These data confirm that in addition to the four different 5′ ends, the calpastatin transcripts also display three 3′ termini differing by their sizes. This leads to multiple possibilities in the composition of calpastatin transcripts but at present their functional significance is unknown.

**Relationship between Type I, II, and III transcripts and calpastatin isoforms**

Multiple calpastatin isoforms have been described in various tissues and species in the last decade [1]. However, no correlation between the recently described four transcript types and all the different isoforms has been established. To assess this issue, we decided to use several antibodies specifically designed against different regions of the protein (Fig. 3). A commercial monoclonal antibody (Anti-Dom IV) directed against the domain IV of human calpastatin and a serum which recognized a human peptide situated at the N-terminal end of the domain IV (Antipep-Dom IV) were used to detect all the putative isoforms (Fig. 3). Using Antipep-Dom IV, the three different skeletal muscles examined display a three band pattern of approximately 145, 125 and 70 kDa, whereas an intense band around 145 kDa and a weak one of 70 kDa are identified in heart (Fig. 4A)

Sizes of the highest bands are similar to those previously described in bovine [15, 26]. The 145 and 125 kDa bands may correspond to the translation of Type I and/or II and Type III transcripts, respectively. The lowest band may be a C-terminal proteolytic product or the erythrocyte isoform. In fact, it has been demonstrated that the erythrocyte isoform contain domain II, III, and IV and migrate at 70 kDa on SDS–PAGE, whereas its calculated size is nearly 47 kDa [27]. The initiation of translation of the erythrocyte form seems to take place at a methionine residue situated at the boundary of domain I and II, in exon 14.

Because Antipep-Dom IV does not display any specific band with protein from testis, the commercial monoclonal antibody, Anti-Dom IV (Fig. 3), was used on a testis homogenate. This blot (Fig. 4A) shows a similar pattern of the one previously seen in heart with an extra 60 kDa band. The Type IV protein has a calculated size similar to that of the erythrocyte form, so it may also migrate at 70 kDa. However since the first 40 amino acids’ composition is different between the erythrocyte and Type IV proteins, their behaviour may be different on SDS–PAGE, suggesting that the 60 kDa band, only present in testis, may correspond to the Type IV protein.

To recognize specifically Type I and II proteins, Western blot analysis were performed using anti-peptide sera raised against the translation products of porcine exon 1xa (Anti-Ex1xa), 1xb (Anti-Ex1xb), and against domain XL (Anti-DomXL) (Fig. 3). In addition, an Anti-Ex3 serum was used to detect all isoforms containing the exon

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**Fig. 3.** Calpastatin isoforms and anti-calpastatin antibodies. Translation products of Type I, II, III, and IV and all the different antibody and anti-peptide anti-sera are presented here. Peptidic sequences corresponding to exons 1xa, 1xb, and 3 are shown. Names of the different regions of the calpastatin and amino acids numbers are indicated.
3 translation product (Fig. 3) including Type III protein. These anti-sera could be used because of the good similarity of these regions between bovine and pig calpastatin. Indeed, there are no differences between porcine and bovine peptide from the exon 1xb translation product. Five out of 14, and 1 out of 14 residues vary from bovine and pig in the peptides corresponding to domain XL and exon 1xa or exon 3, respectively.

Using Anti-Ex1xa, Anti-Ex1xb, and Anti-DomXL, a 145 kDa band was detected (Figs. 4B–D). As we expected, this form corresponds to the translation product of Type I and/or Type II transcripts. While Anti-Ex1xb detects a high molecular weight form in all tissues tested, Anti-Ex1xa only displays a 145 kDa band in heart (Figs. 4B and C). As we demonstrated by qualitative RT-PCR analysis that the two Type I and II transcripts are present together in these tissues (Fig. 1B), it can be suggested that, in skeletal muscles and testis, the amount of Type II mRNA transcripts is greater than the one of Type I, or that the translation of Type I mRNA may not be as efficient as for Type II. These hypotheses may explain why the 145 kDa band is not detected by Anti-Ex1xa in skeletal muscles and testis. As the Type III protein has no specific peptide sequence (Fig. 3), the only way to confirm the hypothesis that the 125 kDa form correspond to the translation product of the Type III mRNA, is to use an antibody which may recognize also others isoforms. Using Anti-Ex3 serum which should recognize Type I, II, and III protein, two bands of 145 and 125 kDa approximately are identified in diaphragm (Fig. 4E), in heart, and in the two other skeletal muscles (data not shown). These two bands may correspond to Type I and/or II and Type III protein, respectively. As a single band was observed by RT-PCR for Type III transcript in diaphragm (Fig. 1B), the presence of the alternatively spliced exon 3 in Type III mRNA was verified by specifically amplifying it using a forward specific primer in exon 1u and a reverse one in exon 3 (data not shown).

Western blots performed with Anti-Ex1xa, Anti-Ex1xb, and Anti-DomXL sera also display a 70 kDa band in all tissues tested (Figs. 4B–D). None of the sera used can recognize the erythrocyte form, which only con-
tains domain II, III, and IV. This form may have multiple origins; it may migrate at its calculated size or may be the result of post-translational modifications such as phosphorylation, glycosylation or proteolysis. Such a proteolytic cleavage may be specific and must take place towards the C terminus to produce these fragments with intact N-terminal epitopes. Considering calculated sizes of putative proteolytic products and a previously described ratio of 1.53 between the estimated size on SDS–PAGE and the theoretical one [16], the 70 kDa form may include neither domain III nor domain IV. In addition, a recent work trying to determine the sequential determinants of calpain cleavage, describe a consensus peptide sequence similar to that of calpastatin sub-domain B [28]. As calpains can degrade calpastatin [29], the proteolytic cleavage may occur in the sub-domain B of domain II. In addition, such a proteolytic cleavage would also lead to the formation of a C-terminal proteolytic product whose calculated size is similar to that of the N-terminal proteolytic product. Such a C-terminal product may correspond to the 70 kDa band recognized by Anti-DomIV and Antipep-DomIV (Fig. 4A). We also have to consider that if the 70 kDa band corresponds to a full length form, migrating at its calculated size, it will be recognized either by Antipep-DomIV and Anti-DomIV or by anti-sera from the N-terminal region. The description of a N-terminal proteolytic product of 70 kDa is not really new as a band with a similar size has been observed recently in porcine skeletal muscle with the same Anti-Ex1xa serum [12]. However, in a previous work [15], this 70 kDa band was not described in bovine. In the bovine tissue, the antiserum raised against the N-terminal XL region was used only on ammonium sulphate fraction where the 70 kDa may have not precipitated. Moreover, analysis of whole heart and liver homogenates was carried out using an antibody that recognizes an epitope in the C-terminal portion of the bovine calpastatin. This kind of antibody would not detect the N-terminal proteolytic product of 70 kDa but should recognize the C-terminal one. In fact, as it has been demonstrated that each of the calpastatin domain (I, II, III, and IV) can inhibit a single calpain molecule alone, the C-terminal product may be subjected to further specific proteolysis without altering inhibitor properties.

Surprisingly, no 70 kDa form is observed (Fig. 4E) using Anti-Ex3 serum. As the peptide corresponding to exon 3 is in the N-terminal part of the protein, this serum must recognize the putative N-terminal proteolytic product (Fig. 3). The absence of the 70 kDa band could be explained either by an inefficient Anti-Ex3 detection, regarding the 70kDa form or by the absence of the peptide sequence corresponding to the exon 3 in the 70kDa form, recognized by Anti-Ex1xa, Anti-Ex1xb, and Anti-DomXL (Figs. 4B–D). This absence may be due ’the exon skipping phenomenon observed with RT-PCR experiments. As the peptide sequence corresponding to exon 3 seems to be present in the entire protein forms only, it may protect against the specific proteolysis which lead to the formation of the 70kDa form. Indeed, the translation product of exon 3 has been suggested to be implied in the binding of calpastatin to membrane [21,30]. This particular localisation of calpastatin may be responsible for the putative protection by the peptide sequence corresponding to exon 3, from a specific proteolysis of calpastatin. To assess this new hypothesis, it appears clearly important to go further to establish whether and how exon 3 could protect some calpastatin isoforms from proteolysis.

**Conclusion**

Our observations demonstrate the complex expression of the bovine calpastatin gene. In addition of the existence of four promoters, this gene is subjected to multiple and differential levels of control (transcriptional, exon skipping, translational, and post-translational). This may have a significance for calpastatin’s role in inhibiting calpain-mediated proteolysis both physiologically and in meat texture development.

**Acknowledgments**

The authors thank Ahmed Ouali (INRA/Clermont-Ferrand, Theix) for providing Antipep-DomIV anti-sera, the slaughter house of the INRA centre (Clermont-Ferrand, Theix) for animal samples. Peggy Raynaud is supported by an INRA/Région Limousin grant.

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