Constant expression of mouse calpastatin isoforms during differentiation in myoblast cell line, C2C12*

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Abstract

C2C12 is a myoblast cell line which is used to study differentiation into multinucleated cells *in vitro*. Addition of calpain inhibitors, calpeptin or E-64d, to the culture medium prevented the myoblastic fusion of C2C12 cells. Immunoblot studies using affinity-purified antibody, revealed that the expressed levels of mouse calpastatin remained unaltered during C2C12 cell fusion. The detected calpastatin migrated as a protein of 130 kDa on SDS-polyacrylamide gel electrophoresis. The estimated molecular mass was somewhat greater than that in mouse liver and erythrocytes, and much greater than that reported in rat myoblasts. The 130 kDa isoform may contain an additional N-terminal region designated XL domain found in bovine calpastatin.

Abbreviations: CMV, cytomegalovirus; RT-PCR, reverse, transcription and polymerase chain reaction; FCS, fetal calf serum; HS, horse serum; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate buffered saline.

Introduction

Calpastatin is an endogenous inhibitor protein that acts specifically on calpain. Calpains (E.C.3.4.22.17), a family of Ca^{2+} -dependent cysteine protease, are suggested to be involved in limited proteolysis of certain enzymes, receptors, cytoskeletal proteins, transcription factors, etc. coupled with calcium mobilization (Sorimachi et al., 1997; Suzuki et al., 1998). Based on the Ca^{2+} requirement for their protease activity, two major ubiquitous isoforms of calpain have been identified: μ -calpain (μM -requiring form) and m-calpain (mM-requiring form). A calpastatin molecule contains four mutually similar regions of 140 amino acid residues (inhibitory domains 1–4) and a unique domain on the N-terminal side (L domain) (Takano et al., 1988). Regardless of the calculated molecular mass of less than 80 kDa, calpastatin migrates ab-

rantly at 100–120 kDa in SDS-PAGE probably due to the deviated amino acid composition: rich in Asp, Glu, Ser and Pro. The structure-function analyses of calpastatins and interactions with calpains have been performed (Ma et al., 1994; Yang et al., 1994; Hitomi et al., 1998a). The fact that each repetitive domain has inhibitory activity has been demonstrated by using recombinant proteins of pig and rabbit calpastatin domains expressed in *E. coli* (Maki et al., 1987a, b; Emori et al., 1988). Both calpain and calpastatin are known to be widely distributed in animal tissues and cells. The calpain-calpastatin system has been suggested to be involved in various physiological and pathological phenomena (Ostwald et al., 1993; Saito et al., 1993; Blomgren et al., 1995).

Skeletal muscle fibers are derived from multinucleated myotubes by fusion of mononucleated myoblasts (Wakelam, 1985; Schollmeyer, 1986). Myoblast fusion is a naturally occurring differentiation-process which has been used for the studies on the reorganization of cytoskeleton and the redistribution

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of membrane components. The fact that the fusion is Ca^{2+} -dependent and accompanies the intracellular proteolysis strongly suggests the involvement of calpain in the process.

Roles of calpains on myoblast fusion have been investigated using both primary cultures and established cell lines. Enhancement of m-calpain activity was observed during the burst of the cell fusion whereas the level of the μ -calpain did not change in rat myoblast cells (Balcerzak et al., 1998; Cottin et al., 1994; Dourdin et al., 1997; Kwak et al., 1993). Furthermore, antisense oligonucleotide against m-calpain was shown to inhibit myoblast fusion in rat primary culture (Balcerzak et al., 1995). Kosower et al. showed a marked decrease in the calpastatin levels during differentiation just prior to cell fusion in rat L8 cells (Barnoy et al., 1996, 1997). They also suggested the fusion-associated protein degradation by the relative increase in the calpain activity (Barnoy et al., 1998).

The C2C12 cells, originated from mouse muscle, undergo differentiation in low-nutrient medium and have been used for the study of muscle differentiation *in vitro*. During the differentiation, the cells begin to fuse into multinucleated cells, as in the cases of L6 and L8 cell lines, and rat primary cultured cells (Blau et al., 1985). In this study, the suppressive effects of calpain inhibitors on the C2C12 myoblastic cell fusion were shown. The levels of calpastatin expression were found to be rather constant during the periods of differentiation by immunoblot analysis using affinity-purified polyclonal antibody raised against recombinant calpastatin. Furthermore, the observed size of myoblast calpastatin was larger than that of the recombinant mouse calpastatin containing L domain and four inhibitory domains. The XL domain, an additional N-terminal region recently found in bovine calpastatin, was discussed.

Materials and methods

Cell culture

C2C12 cells were maintained at 37 °C in Dulbecco's Modified Eagle medium (DMEM) (Nissui, Japan) supplemented with 10% fetal calf serum (FCS) (Bioblab), 100 units ml^{-1} penicillin and 100 $\mu\text{g ml}^{-1}$ streptomycin under a humidified 5% CO_2 atmosphere throughout the culture periods. Glucose was contained at the final concentration of 4.5 mg ml^{-1} . For myoblastic differentiation, the medium was changed

to DMEM supplemented with 2% horse serum (HS) when the cells reached confluency.

Expression of human and mouse calpastatin in *E. coli*

Construction of pETCS, a plasmid containing human calpastatin cDNA in the pET-24d expression vector, was described previously (Hitomi et al., 1998b).

Mouse calpastatin cDNA encoding L domain and four inhibitory domains was obtained by RT-PCR method. Briefly, mouse liver total RNA was transcribed with RAV-2 reverse transcriptase using a specific primer (AATTTCTATTTCAGATACC) based on the EST database (AA109608). The reaction mixture was used as a template for PCR reaction. The 5'-primer (ATGGGTACCACAGAGACTAAG-GCTGTG) and 3'-primer (GAATTCATTAAGTGT-GTCTTGCATCTTC) were deduced from the previously isolated mouse partial cDNA clone (X62519) (Lee et al., 1992) and mouse cDNA sequence in the EST database (AA109608), respectively. A 2-kb DNA fragment of the PCR product was blunted and inserted into the *Sma*I site of enforcement cloning vector pKF3 (TAKARA). The *Nco*I-*Eco*RI fragment harboring mouse calpastatin cDNA was inserted into the pET-24d (pET-mCS) (Studier et al., 1990). *E. coli* strain BL21(DE3)pLysS was transformed and cultured in L-broth (tryptone, yeast extract, and NaCl, pH 7.4) containing 0.1% glucose and 30 $\mu\text{g ml}^{-1}$ kanamycin at 37 °C to the middle log-phase. Then, the induction was performed by the addition of IPTG to a final concentration of 1 mM. Incubation was continued for 3 h and the cells were harvested. Successful expression was confirmed by analyzing the total cell lysates by 7.5% SDS-PAGE. Purification procedure was described previously (Hitomi et al., 1998b).

Preparation of affinity-purified antibody against human and mouse calpastatin

The antiserum was prepared from a rabbit immunized with a mixture of purified human and mouse recombinant calpastatins. In order to obtain highly specific antibody, the antiserum was loaded onto an affinity column of mouse calpastatin which had been immobilized to a HiTrap NHS-activated column (1 ml, Pharmacia). After washing the column successively with TBS, washing buffer (20 mM Tris-HCl, pH 7.5, 1 M NaCl, 1% Triton-X 100), and 0.15 M NaCl, antibodies were eluted with 0.1 M glycine-HCl buffer, pH 2.5, and then the eluate was neutralized with Tris.

Western blot analysis

Cells ($1-5 \times 10^5$) grown on 60-mm dishes were washed with 5 ml of ice-cold PBS twice and 1 ml of 10% TCA solution was added directly. After incubation on ice for 30 min, cells were harvested with rubber policeman and centrifuged at 12 000 rpm for 5 min. The pelleted TCA-insoluble proteins were dissolved in 9 M Urea/1% DTT/0.1% Triton and mixed with SDS-dye buffer. Samples were neutralized with 1 M Tris and sonicated for 7.5% SDS-PAGE analysis.

After SDS-PAGE, proteins were transferred from gel to polyvinylidene difluoride membrane (Immobilon-P, Millipore) using a semi-dry blotter in the transfer buffer (24 mM Tris, 192 mM Glycine, 15% methanol, 0.01% SDS, pH 8.3). Rabbit polyclonal antibody, purified by affinity-purification as described above, was used for primary antibody (approximately $1 \mu\text{g ml}^{-1}$). Peroxidase-conjugated goat anti-rabbit IgG(H+L) purchased from Jackson ImmunoResearch Lab. (West Grove, PA) was used as a secondary antibody. Immuno-signals were detected by the color development method using diaminobenzidine (DAB).

Establishment of C2C12 cell line stably expressing human calpastatin

Mammalian expression vector (pCXN2) containing β -actin promoter was a kind gift from Dr Miyazaki (Niwa et al., 1991). A 2.5-kb human calpastatin cDNA fragment encoding L domain and four inhibitory domains was excised from pTicCS with EcoRI and then inserted into the vector at the EcoRI site.

Transfections were performed by the liposome method (Lipofectin, GIBCO BRL). C2C12 cells were transfected with $10 \mu\text{g}$ of the constructed plasmid (pCXNhCS) or pCXN2 as a control. The transfectants were selected in the medium containing G-418 at a final concentration of 0.4 mg ml^{-1} . Resistant cells were further cloned by the limiting dilution method. Finally, one of the established cell lines was designated as C-HCS1 and used for further experiments.

RT-PCR for detection of XL domain mRNA in C2C12 cells

Total RNA was prepared from growing C2C12 cells by the acid guanidine-thiocyanate phenol method. $2 \mu\text{g}$ RNA was reverse transcribed into DNA with RAV-2 reverse transcriptase (TAKARA Shuzo, Kyoto) and specific primer mCSRT2(5'-CTGTTTCATTTGATCTGGA-3') found in the EST

clone AI006681. PCR reaction was performed with the portion of reaction mixture as a template. The specific primers were $1 \mu\text{M}$ mCSXP1 (5'-CAAACCCTGAGCAGACAGTGTG-3') and $1 \mu\text{M}$ mCSXM1 (5'-GTTTTTCACAGCCTTAGTCTCTGTG-3') found in EST clone AA033475 and AI006681, respectively. The PCR product was analyzed by electrophoresis on 5% polyacrylamide gel.

Results and discussion

Inhibition of myoblastic fusion by calpain inhibitors

By replacing the growth medium for C2C12 cells with the differentiation medium, the cells began to align and fuse within 3–5 days (Figures 1A and B). To know the involvement of calpain in cell fusion, effects of two kinds of calpain inhibitors were examined. E-64d, a cell permeable inhibitor for thiol proteases, partially blocked the fusion (Figure 1C). Calpeptin, which was a specific inhibitor for calpain, prevented completely the myoblastic fusion of the cells (Figure 1D).

The level of calpastatin remained unaltered during the period of myoblastic differentiation of C2C12 cells

In order to investigate the level of the mouse calpastatin in differentiating C2C12 cells, total cell lysates were prepared and analyzed for Western blotting using affinity-purified polyclonal antibody (Figure 2). The calpastatin molecules were detected as the 130-kDa, 70-kDa and minor 60-kDa bands. Although myoblastic fusion started 48–72 h after the medium change, the levels of these bands in each cell extract were unchanged before and after cell fusion.

The involvement of calpain-calpastatin system in myoblastic cell fusion has been reported in rat primary cultured cells and cell lines. As in the cases of these cells, blocking of calpain activities resulted in the inhibition of the myoblastic fusion of mouse C2C12 cells (Figure 1). Although the calpain activities are necessary for the fusion event, the expression pattern of the calpastatin during differentiation was clearly different from those reported in rat myoblast cells.

First, the level of the expressed calpastatin remained unaltered upon induction of fusion event in C2C12 cells. In the reports for rat cells, calpastatin decreased just before the fusion, leading to the relative increase in the calpain activity (Balcerzak et al., 1998). Protein levels of both μ -calpain and m-calpain

Figure 1. Inhibitory effect of calpain inhibitors on C2C12 fusion. Phase-contrast micrographs of C2C12 myoblast cells. The cells normally growing in the 10% FCS/DMEM medium (A) differentiated by replacing the medium with fusion medium (DMEM containing 2% HS) (B). Cell fusion was prevented in the differentiation medium containing calpain inhibitors 10 μ M E-64d (C) or 20 μ M calpeptin (D).

Figure 2. Western blot analysis of mouse calpastatin in differentiating C2C12. The C2C12 cells differentiating under the fusion medium (DMEM containing 2% HS) were harvested at the indicated hours after changing to fusion medium at 0 h. Ten μ g proteins of total cell lysate in each lane were loaded on 7.5% gel for SDS-PAGE and transferred onto membrane. Western blot analysis was performed using anti-calpastatin polyclonal antibody.

were shown unchanged significantly either in fusing or non-fusing rat L8 myoblast cells (Barnoy et al., 1996, 1997). The increased levels of m-calpain activities are suggested to be responsible for the fusion in rat primary cells using antisense oligonucleotide method (Balcerzak et al., 1995). The increased calpain activities required for C2C12 cell fusion might be modulated not by down regulation of calpastatin protein level but by suppression of inhibitory activity, such as phosphorylation as described later.

Second, the size of the major calpastatin molecule (130 kDa) detected in C2C12 cells was quite different from that of rat 70-kDa molecule (Barnoy et al., 1997; Balcerzak et al., 1998). Although the 70-kDa molecule in addition to the higher molecular mass was also observed in the C2C12 cells, this molecule may be derived from 130-kDa form either by proteolytical processing *in vivo* or by degradation during preparation due to high sensitivity of calpastatin to proteases. It remains to be established whether rat myoblast calpastatins reported so far are processed products or generated by other mechanisms such as alternative splicing, translation initiation at internal sites and transcription initiation from downstream promoter.

Mouse calpastatin in myoblast revealed 130-kDa protein

Since the calpastatin in mouse myoblast cells was detected at an unexpectedly larger size (130 kDa) by polyclonal antibody in this study, extracted proteins from liver and erythrocytes were also analyzed. The calpastatins were detected at the molecular mass of 100 and 110 kDa in liver and erythrocytes, respectively (Figure 3A). A 100-kDa calpastatin band detected in liver extract migrated faster than the band of recombinant calpastatin (110 kDa), in agreement with the processing in L domain (Emori et al., 1987). Although human, pig and rabbit erythrocyte calpastatins were reported to migrate as 70-kDa products and lack domains L and I (Takano et al., 1982, 1986; Imajoh et al., 1987), a 110 kDa band was detected in mouse erythrocytes as reported for rat erythrocyte calpastatin (Inomata et al., 1993).

In order to investigate the effects of constitutive elevation for calpastatin activity on C2C12 cell fusion, we obtained the cell lines ectopically transfected with human calpastatin cDNA. One of the cell lines, designated as C-HCS1, expressing higher level of recombinant protein was selected. Upon differentiation,

the myoblastic fusion was significantly inhibited in C-HCS1 cells (data not shown).

The C-HCS1 cells also made it possible to compare the size of mouse myoblast calpastatin with that of human calpastatin of known molecular mass. The human calpastatin expressed in the C-HCS1 cells was detected as smaller size than the endogenous calpastatin (Figure 3B). Furthermore, the ectopically expressed protein was almost similar in size to the recombinant mouse and human proteins used as antigens, indicating no degradation in myoblast cells (Figure 3C). These results suggested that the mouse calpastatin of 130 kDa in myoblast cells is larger than the recombinant proteins containing L domain and inhibitory domains.

XL domain of mouse calpastatin in C2C12 cells

The size of the calpastatin band detected in C2C12 cells was somewhat larger than the recombinant human and mouse calpastatins, which start with L domains (Figures 3B, C). Translation initiation sites of mammalian calpastatins have been assigned according to the firstly cloned rabbit cDNA that contained an in-frame upstream stop codon (Emori et al., 1987). Recently, however, a new N-terminal region designated XL domain was found in bovine calpastatin (Cong et al., 1998). The detection of the unexpectedly larger size of mouse myoblast calpastatin led us to search the corresponding regions in human and mouse mRNAs. From the cDNA sequences of the EST database, putative XL domains were also found in both human (AI017990) and mouse (AA033475, AI006681) (Figure 4A). The first ATG is used as a translation initiation codon to produce the XL domain. In order to confirm the presence of mouse XL domain in the C2C12 cells, RT-PCR was performed using specific primers based on the sequence of EST clone. The DNA fragment was observed with the expected size (530 bp) in the PCR product (Figure 4B). Although the function of XL domain is unknown, significant homology among three species might be important for expression of calpastatin activity.

Four potential phosphorylation sites are found in mouse calpastatin XL domain: KKGS (41–44), SPR (11–13), SSK (35–37), TSK (78–80) (Figure 4A). Only KKGS is the potential protein kinase A phosphorylation site commonly found in all XL domains in the conserved region. Although phosphorylation of Ser-44 is not experimentally confirmed yet, modification of this residue may play roles in myoblastic cell

Figure 3. Detection of human and mouse calpastatin by Western blot analysis. Total cell lysates were analyzed for Western blot as described in the legend to Figure 2. (A) C2C12 cells (lane 1), mouse liver (lane 2), and mouse erythrocytes (lane 3). (B) C2C12 cells (lane 1) and stably transfected cells (C-HCS1) with human calpastatin cDNA containing domains L-4 (lane 2). (C) C-HCS1 cell extract (lane 1) compared with recombinant mouse (lane 2) and human (lane 3) calpastatin purified from *E. coli*. The open and closed triangles indicate the endogenous mouse myoblast calpastatin and the ectopically expressing human calpastatin, respectively.

Figure 4. Schema of mouse calpastatin proteins and the deduced amino acid sequences of calpastatin XL domain. (A) Highly conserved residues among all the four repetitive domains, domains 1–4, are clustered in three regions indicated. Predicted sizes of the mouse calpastatins from liver, erythrocytes, and myoblasts are indicated inferred from Figure 3A. The deduced amino acid sequences of XL domains of mouse, human, and bovine calpastatins are depicted. The human and mouse sequences are based on the EST database (human: AI017990; mouse: AA033475, AI006681). The N-terminal sequence of rabbit calpastatin is also aligned. The identical residues among the species are stippled. Potential protein kinase A and protein kinase C phosphorylation sites in mouse calpastatin found by the program with ScanProsite (<http://www.expasy.ch/sprot/prosite.html>) were indicated by the small closed and open boxes, respectively. (B) RT-PCR product was electrophoresed on a 5% polyacrylamide gel. Total RNA prepared from C2C12 cells was reverse-transcribed and subjected to PCR (lane 1). The size marker of ϕ X174-HincII digest was paralleled (lane 2).

fusion. Phosphorylated calpastatin isolated from rat skeletal muscle was reported to exert maximum inhib-

ition versus m-calpain, whereas an unphosphorylated form had greater inhibitory activity for μ -calpain

(Pontremoli et al., 1992; Salamino et al., 1994). On the other hand, Adachi et al. reported enhanced binding of phosphorylated form of calpastatin to membranes (Adachi et al., 1991). Since the expressed levels of calpastatin were found nearly constant during the mouse myoblast fusion process, phosphorylation or dephosphorylation might be involved in the inhibitory activity and subcellular localization. C-HCS1, a C2C12 transfectant cell line ectopically expressing human calpastatin, lost capacity to fuse under the differentiation condition. This result seemed reasonable because of the constitutive inhibition of calpain activity. Some of C2C12 subclones transfected with expression vector alone, however, did not show the cell fusion event as in the case of C-HCS1 either. Although the reason was not known, it was unexpected that the potential for myoblastic fusion of C2C12 cells was liable to decline by successive subculturing. Inducible and higher expression system might be necessary for conclusive analysis.

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