Stabilization of calpain large subunits by overexpression of truncated calpain small subunit in L8 myoblasts

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Abstract

The objectives were to investigate the function of the small subunit in the calpain system by expression of the autolytic form of this subunit in L8 myoblasts. Rat post-autolysis small subunit (21 kDa) cDNA expression plasmid was transfected into L8 myoblasts and selected by G418 containing medium. The concentrations of cytosolic \( \mu \)-calpain in transfected cells, SS2 and SS3, were found to be 15.7 and 17.3% higher than that in L8Neo control cells, and the concentrations of cytosolic m-calpain in SS2 and SS3 cells were 23.3 and 16.6% higher than that in control cells (L8Neo). The half-life of \( \mu \)-calpain in SS3 cells (36.5 h) was longer than that in L8Neo cells (32.4 h), while the half-life of m-calpain in SS3 cells (40.1 h) was longer than that in L8Neo cell (37.5 h). These results indicated that the expression of truncated small subunit increased the stability of \( \mu \)- and m-calpain large subunits in cytosol.

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1. Introduction

Calpains (EC 3.4.22.17) are \( \text{Ca}^{2+} \)-dependent cysteine proteases that are present in all animal cells studied to date (Sorimachi et al., 1997; Carafoli and Molinari, 1998). The completion of the human genome has revealed that there are at least 14 calpain genes (Sorimachi and Suzuki, 2001). There are two major forms of calpain, \( \mu \)-calpain and m-calpain, which differ in their requirements of \( \text{Ca}^{2+} \) for induction of activity in in vitro assays. So far, the function of calpains is not fully understood. However, it has been demonstrated that calpains are involved in many physiological and pathological events such as cell proliferation, differentiation, apoptosis, secretion, muscular dystrophy and Alzheimer’s disease (Cottin et al., 1994; Patel et al., 1994; Squeri et al., 1994; Karlsson et al., 1995; Murray et al., 1997; Ueyama et al., 1998). Biochemical studies revealed that isoforms of \( \mu \) and m are composed of a distinct large subunit (80 kDa) and a common small regulatory subunit (28 kDa; calpain 4) (Kawasaki et al., 1986). However, a novel human small subunit of calpain (css2) was cloned recently. It appears to be a tissue-specific, rather than ubiquitous, small subunit (Schad et al., 2002). The physiological function of this protein still remains to be determined.

The full length of rat calpain small subunit has 270 amino acid residues with a molecular weight close to 28 kDa (Sorimachi et al., 1996; Elce et al., 1997a). Calpain small subunit is composed of domain IV and domain V. Domain V is a hydrophobic region with glycine-rich residues and is suggested to function as the interaction site for phospholipids, which is an important cofactor activating calpains. The N-terminus of domain V undergoes autoproteolytic processing during activation (Imajoh et al., 1986; Saida et al., 1992). The domain IV of rat small subunit has a MW of 21 kDa which is a calmodulin-like domain. It is highly similar to the domain IV in calpain large subunit, and has 5 EF-hand motifs in one domain (Blanchard et al., 1997; Lin et al., 1997). Hydrophobic interaction between domain IV of large subunit and domain IV of small subunit has been demonstrated to be responsible for heterodimer formation (Nishimura and Goll, 1991; Crawford et al., 1993; Sorimachi and Suzuki, 2001).

In addition to lowering \( \text{Ca}^{2+} \) sensitivity of the large subunit, a chaperone-like effect of calpain small subunit is observed in \textit{E. coli} and in vitro system (Elce et al., 1997b). Furthermore, it is reported that calpain small subunit...
accompanies the renaturation of the isolated large subunit in vitro (Yoshizawa et al., 1995a). Recently, it is reported that homocryogenic disruption of murine small subunit eliminated both α- and m-calpain activities. The mutant embryos died at midgestation and showed defects in the cardiovascular system, hemorrhaging and accumulation of erythroid progenitors (Arthur et al., 2000). The exact mode of function of the small subunit toward the large subunit inside actual cells remains to be clarified. In this study, we overexpressed the autolytic calpain small subunit (21 kDa) in L8 myoblasts in order to investigate the possible role of the small subunit of domain IV in the calpain system.

2. Materials and methods

2.1. Materials

L8 rat myoblasts were obtained from Americans Type Culture Collection (Rockville, MD). Fetal calf serum (FCS), Dulbecco’s modified Eagle’s media (DMEM), penicillin/streptomycin solution and trypsin were purchased from Gibco (Grand Island, NY). Rainbow molecular weight marker, nitrocellulose membrane and the ECL Western blotting detection were purchased from Amersham (Arlington Heights, IL). Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG was purchased from Bio-Rad (Richmond, CA). All other chemicals were of molecular biology grade and purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Cloning of rat calpain small subunit cDNA and construction of expression vector

Total cellular RNA of rat skeletal muscle was isolated by single step guanidine thiocyanate procedure (Chomczynski and Sacchi, 1987). The final RNA pellet was dissolved in diethyl pyrocarbonate treated water and the RNA concentration was determined spectrophotometrically at 260 nm. The PCR reactions were performed with calpain small subunit specific primers (21K-1: 5′-GGGCGTCCATTCATTTCCAACATTGAGGC; 21K-2: 5′-GGCGCTCGAGTCAAGACTACGTCACTCGGACGCA) with initiation and stop codon and oligo-dT(15) was used as the 3′-end primer. A 650 bp band was identified in the positive clones.

Expression of calpain 21 kDa small subunit in transfected cells was identified by RT-PCR. The 21K-1 primer was used as the 5′-end primer and oligo-dT(15) was used as the 3′-end primer. One microgram total RNA from cells was used to perform RT-PCR. The condition for RT-PCR was followed according to the supplier’s instruction. The difference of PCR products in 3′ non-coding region was used to distinguish the endogenous from exogenous calpain small subunit mRNAs. RT-PCR products were electrophoresed on a 1% agarose gel. A 650 bp band was identified in the positive clones.

2.3. Cell culture and stable transfection

L8 myoblasts were cultured in basal media (DMEM, 100 unit penicillin/ml, 100 μg streptomycin/ml and 44 mM sodium bicarbonate, pH 7.4) supplemented with 10% FCS in a humidified atmosphere of 5% CO2 and 95% air at 37°C. The medium was changed every 2 days.

Transfection of L8 rat myoblasts was accomplished using Lipofectamine ( Gibco BRL) according to the supplier’s instructions with either the control pSV (vector without insert) or the vectors that contained rat 21 kDa calpain small subunit cDNA (pSV-SS). After 6-h transfection, the cells were cultured in non-selective medium for 24 h. G418 was then added at the concentration of 300 μg/ml. For isolation of single cell transfectants, a limited dilution method was used. L8Neo, from cells transfected with control plasmid, SS1, SS2 and SS3, from cells transfected with pSV-SS plasmid were selected for further expansion.

2.4. Identification of transfectants

A PCR method was used to identify the transfected clones. Specific primers for neomycin-resistant gene (Neo r-1: 5′-GGAGCAAGGTGAGATGACAGGAGAT; Neo r-2: 5′-GGGAGCAAGGTGAGATGACAGGAGAT) were synthesized according to cDNA sequence from GenBank (accession no. U02432). Genomic DNA from transfectant was extracted by DNA extraction kit (Bio-101, Inc.). One microgram genomic DNA was used to perform PCR. The PCR products were electrophoresed on a 1% agarose gel.

2.5. Western blot assay

Separation of cell cytosol and membrane fractions was conducted as described previously (Hong et al., 1995). Cytosol and membrane fractions were collected in homogenate buffer and subjected to 10% SDS-PAGE as described by Laemmli (1970). Proteins were transferred onto a nitrocellulose membrane in transfer buffer (25 mM Tris, 192 mM...
glycine and 20% methanol) at 4 °C according to method of Towbin et al. (1979). The 0.2% ponceau S red solution was routinely used to stain the membrane to ensure the loading of equal quantities of protein. Nitrocellulose membranes were incubated in blocking solution (5% non-fat dried milk in TTBS (0.05% Tween 20 in TBS; pH 7.5)) at 25 °C for 1 h, then incubated with rabbit anti-m-calpain polyclonal antibody or rabbit anti-μ-calpain polyclonal antibody at 25 °C for 1 h (Hong et al., 1995). Following three washes, membranes were incubated with HRP-conjugated goat anti-rabbit IgG antibody. Membranes were then washed four times with TTBS and the specific bonding of anti-calpain antibody onto membrane was detected by ECL detection system. The relative amounts of calpain presenting in various samples were estimated by densitometric scanning of the X-ray film using AlphaImage 2000 analysis system (Alpha Innotech Corporation).

2.6. Determination of half-life for calpain large subunits

Cells (L8Neo and SS3) were cultured in DMEM with 10% FBS. When cells were approximately 70% confluent, 5 μCi/ml 3H-tyrosine (NEN) were added to the cells in 4 ml fresh growth media for an additional 24 h. Afterward, the 3H-tyrosine-containing medium was discarded and replaced with 5 ml medium containing 2 mM tyrosine. Cells were collected at 0, 8, 16, 24, 48 and 76 h after incubation. One hundred micrograms of total proteins from each time point were incubated with rabbit anti-m- or m-calpain antibodies at room temperature for 1 h, then protein A conjugated with agarose (Sigma) was added to precipitate the calpain–antibody complex. The pellets were washed three times and the radioactivities of samples were determined by Beckman Model LS6000SE scintillation counter (Fullerton, CA).

2.7. Statistical analysis

All experiments were repeated at least three times with two or more replicates for each treatment. The data were examined for equal variance and normal distribution prior to statistical analysis. Mean values were compared by ANOVA with Fisher’s least-significant difference (LSD) method for comparing groups (Steel and Torrie, 1980). P values of less than 0.05 were taken as evidence of statistical significance. Linear regression was used to determine the half-life of calpain large subunits.

3. Results

3.1. Selection and identification of transfectants

L8 myoblasts were transfected with pSV and pSV-SS plasmids, respectively, and a single clone was selected by G418 (300μg/ml) containing medium. One clone from pSV-transfected cell (L8Neo) was selected and three clones from pSV-SS-transfected cells (SS1, SS2 and SS3) were
Fig. 3. Western blot analysis of calpain small subunit in transfected and control L8 cells. Transfected cells and control cells were collected in homogenate buffer and subjected to 10% SDS–PAGE. The blot was hybridized with polyclonal Ab against calpain small subunit. The ECL system was used to detect the specific signal of calpain small subunit.

selected for further analysis. PCR method was used to identify the clones of transfectants. Neomycin-resistant gene primers were used to amplify this gene. After PCR amplification, neomycin-resistant gene fragment (630 bp) could be observed in transfectants (L8Neo, SS1, SS2 and SS3). However, PCR product of the neomycin-resistant gene was not observed in wild type L8 cells (Fig. 1). This result indicated that the plasmid DNA was integrated into genomic DNA of selected transfectants.

3.2. Expression of 21 kDa calpain small subunit in L8 myoblasts

RT–PCR was used to distinguish the expression of both endogenous and exogenous calpain small subunits (28 and 21 kDa). Due to the lack of 3′ non-coding region of calpain small subunit in pSV-SS plasmid, a 570 bp of RT–PCR product would be amplified from the exogenous 21 kDa mRNA and a 1070 bp fragment would be amplified from the endogenous calpain small subunit mRNA. RT–PCR results showed that L8, L8Neo, SS1, SS2 and SS3 all expressed endogenous calpain small subunit mRNA, however, only SS2 and SS3 expressed exogenous calpain small subunit mRNA (Fig. 2A). RT–PCR products were collected from the gels and further confirmed by Hinfi restriction enzyme digestion. Fig. 2B showed that the 1070 bp fragment was digested to the 710 and 360 bp fragments, and the 570 bp fragment was digested to produce the 360 and 210 bp fragments. These results confirmed that the RT–PCR products were from calpain small subunit. In addition, polyclonal Ab against calpain small subunit was used in Western blot assay to confirm the expression of 21 kDa protein (Fig. 3). In control cells, most of calpain small subunit existed as the 28 kDa form and only a small amount of 21 kDa protein was detected in the cytosol. However, there was more truncated 21 kDa protein in transfected cells than that in control cells. The scanning densitometry ratios of 28 to 21 kDa bands were 0.136 and 0.495 in L8 and SS3, respectively.

3.3. Regulation of calpain large subunit protein steady state by calpain small subunit

To evaluate the possible role of 21 kDa calpain small subunit in L8 myoblasts, we determined the concentrations...
of μ- and m-calpain large subunit in cytosols and membranes. As shown by Western blot assay, the concentrations of μ-calpain large subunit concentrations in SS2 and SS3 cytosols were significantly higher than that in L8Neo cells (15.7 and 17.3%, respectively). However, SS1 cells, which did not express exogenous calpain small subunit, and control cell, L8Neo, did not show any change of calpain large subunit concentration in cytosols (Fig. 4). The concentrations of cytosolic m-calpain large subunit in SS2 and SS3 cells, but not SS1, were also higher than that in control cells (Fig. 5). These results indicated that the expression of 21 kDa calpain small subunit resulted in an increase in cytosolic calpain large subunit level. The amount of μ- and m-calpain large subunit in membrane fractions did not change among SS1, SS2 and control cells (data not shown). This result indicated that the lack of domain V causes the 21 kDa subunit to lose its ability to translocate to cell membrane and interact with membrane phospholipids.

3.4. Effect of calpain small subunit on the stability of calpain large subunits

To understand the mechanisms of enhancing cytosolic calpain concentration, radioimmunoprecipitation method (RIP) was performed to determine the calpain large subunit half-life in SS3 transfected cells and L8Neo control cells. The results showed that the half-life of μ-calpain large subunit was 36.5 h in SS3 cells and 32.4 h in L8Neo cells, respectively (Fig. 6). The half-life of m-calpain was 41 h in SS3 cells and 37.5 h in control cells, respectively (Fig. 7). These results suggest that the increase of cytosolic μ- and m-calpain large subunit in cytosol may be due to the increase of protein stability of calpain large subunit.
0, 8, 24, 48, and 67 h upon removal of the H-tyrosine. The cell lysate containing 2 mM non-radioactive tyrosine. The cells were collected after Ca²⁺ phospholipids in the membrane without a requirement for the importance of this domain is that it can interact with the null mice of calpain small subunit was lethal (Arthur et al., 2000). However, so far, the exact role of calpain small subunit exists intact in the cells. Expression of 21 kDa calpain small subunit increased the concentration of μ- and m-cytoesol calpain large subunits (Figs. 4 and 5). It is possible that the different integration site caused the change of the calpain large subunit expression. However, both SS2 and SS3 have the same effect on the concentrations of calpain large subunit. It is unlikely that both clones have the same integration sites. Elce et al. (1997a) reported that calpain small subunit contributed to the stability of active het- erodimer in vitro. And also, co-transfection of large and small subunit in insect cells would increase the expression of calpain large subunit. Furthermore, it is reported that homozygous disruption of murine small subunit eliminated both μ- and m-calpain activities (Arthur et al., 2000). To investigate the mechanism involved in the increase of μ- and m-calpain large subunits in transfected L8 cells, we examined the half-life of two calpain isoforms by radioimmuno- precipitation. The result showed that expression of 21 kDa calpain subunit in L8 myoblasts increased the half-life of μ-calpain large subunit from 32.4 to 36.5 h (Fig. 6) and the half-life of m-calpain large subunit from 37.5 to 41.0 h (Fig. 7). This indicated calpain large subunits were stabilized by 21 kDa small subunit in L8 myoblasts. Interestingly, it was reported that large subunits in both calpains have relatively short half-life. This indicates that the half-life of calpain may vary in different species and different tissues.

It is concluded that the expression of truncated small subunit increased the concentrations of μ- and m-calpain large subunits in muscle cytosol. These increases are postulated to be due to the enhanced stability of calpain large subunit by the small subunit.

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References


Fig. 7. Radioimmunoprecipitation analysis of m-calpain half-life in SS3 and L8Neo. The SS3 and L8Neo were pre-labeled with 5 Ci/ml [3H-tyrosine. The cells were collected after 0, 8, 24, 48, and 67 h upon removal of the H-tyrosine. The cell lysate was incubated with m-calpain antibody for 2 h at room temperature and then protein A/G agarose was added to precipitate m-calpain.


