Evidence for the Involvement of Cathepsin B in Skeletal Myoblast Differentiation

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Abstract   Our previous studies suggest that the cysteine protease cathepsin B (catB) is involved in skeletal myoblast differentiation (myogenesis). To test this hypothesis, we examined the effect of trapping one of the two catB alleles on the ability of C2C12 cells to differentiate. During differentiation, catB gene-trapped C2C12 mouse myoblasts (RT-27) demonstrated a similar pattern of intracellular catB activity and protein expression compared to that observed in control C2C12 myoblasts and myoblasts trapped in a gene other than catB. However, compared to control myoblast cell lines, levels of catB activity and protein at each stage of RT-27 differentiation were reduced. The reductions in levels of catB were associated with reductions in several myogenic phenotypes including reduced levels of creatine phosphokinase activity and myosin heavy chain protein, two late biochemical markers of myogenesis, and reduced myotube size and extent of myotube formation over time. Comparable reductions were not observed for myogenin protein, an early biochemical marker of myogenesis, or in myokinase activity and catB related cathepsin L-type activity, two non-specific proteins. Finally, both control and catB gene-trapped myoblasts secreted active catB at pH 7.0. However levels of active pericellular/secreted catB were 50% lower in catB gene-trapped myoblasts. Collectively, these results support a functional link between catB expression and skeletal myogenesis and suggest a role for active pericellular/secreted catB in myoblast fusion. J. Cell. Biochem. 84: 520–531, 2002.   © 2001 Wiley-Liss, Inc.

Key words: cathepsin B; gene-trapped; C2C12 myoblasts

Myogenesis is a dynamic process in which cycling, mononucleated skeletal myoblasts undergo biochemical and morphological differentiation to form functional, multinucleated muscle fibres [Yeoh and Holtzer, 1977; Olson, 1990; Stockdale, 1992]. Biochemical differentiation is characterized by highly regulated alterations in the expression of muscle-specific and non-specific genes. Morphological differentiation is characterized by the formation of non-cycling multinucleated myotubes. At the descriptive level, myotube formation involves recognition events that allow committed myoblasts to align and adhere after migration, and fusion events that allow adherent myoblasts to fuse with each other. The recognition events appear to be mediated by alterations in cell membrane glycoproteins [Knudsen and Horwitz, 1977], whereas the fusion events appear to be mediated by extensive reorganization of membrane components to form protein free areas of phospholipids [Thiery et al., 1982; Rieger et al., 1985]. There is increasing evidence that many cellular proteases may play a role in these events and that the genes for these proteases are strongly regulated during myogenesis [Kaur and Sanwal, 1981; Kirschke et al., 1983; Couch and Strittmatter, 1983; Colella et al., 1986; Schollmeyer, 1986; Bechet et al., 1991; Matsuishi et al., 1992; Brustis et al., 1994; Guerin and Holland, 1995; Dourdin et al., 1999]. Our research identifies the lysosomal cysteine protease cathepsin B (catB) as one of these proteases.

Using L6 rat skeletal myoblasts, we demonstrated a fusion-related increase in catB activity that was not observed in L6 variants incapable of forming myotubes, or in muscle fibroblasts [Jane and Dufresne, 1994]. These studies and the more recent ones reported by other researchers using L6 rat and C2C12 mouse myoblasts provide compelling evidence that catB is strongly regulated during myogenesis. In the current study, we use catB gene-trapped C2C12 myoblasts to examine the relationship between the regulated expression of catB and the expression of biochemical and morphological phenotypes associated with myoblast differentiation. Specifically, the effects of catB gene-trapping on intracellular catB activity in crude and fast protein liquid chromatographed (FPLC) cell-free fractions, and on pericellular and secreted activities in viable, whole cell preparations were determined using biochemical and "real-time" assays, respectively. Levels of catB protein were determined by Western blot analysis. The effects of gene-trapping on myotube formation and the expression of "early" and "late" muscle-specific genes were determined using microscopy, biochemical assays, and Western blot analysis. These results were compared to those obtained in control C2C12 myoblasts and control C2C12 myoblasts trapped in a gene other than catB. Collectively, the results in vitro support a functional link between catB expression and skeletal myogenesis in vivo, and suggest a role for active pericellular/secreted catB in myoblast–myoblast fusion.

MATERIALS AND METHODS

Superose 12 HR 10/30 FPLC columns were purchased from Pharmacia (Upsala, Sweden). The protein assay kit and nitrocellulose membranes were purchased from Bio-Rad (Mississauga, ON). Medium (alpha MEM), horse, and fetal bovine sera, gentamycin sulfate, trypsin, ethylenediaminetracetic acid (EDTA), and tissue culture plasticware were purchased from GIBCO (Burlington, ON). Bovine serum albumin (BSA), Tween, sodium selenite, Coomassie Brilliant Blue stain, Giemsa stain, E-64, 7-amino 4 methylcoumarin, creatine phosphate, Nicotinamide-adenine dinucleotide (reduced), Nicotinamide-adenine dinucleotide phosphate, glucose-6-phosphate dehydrogenase, hexokinase, creatine phosphokinase (CPK), lactate

dehydrogenase (LDH), myokinase (MK), catB, N-alpha-CBZ-argininyl-argininyl- 7-amido methylcoumarin. HCl (Z-arg-arg-NHMec), N-alpha-CBZ-phenylalanyl L-argininyl- 7-amido methylcoumarin. HCl (Z-phe-arg-NHMec) were purchased from Sigma Chemical Co. (St. Louis, MO). The catB selective inhibitor, CA074 was purchased from Peptides International, Inc (Louisville KY). Rabbit anti-human liver catB IgG was prepared as previously described [Moin et al., 1992]. Myosin heavy chain (MHC) antibody (MF20) was generously provided by Ilona Skerjanc (University of Western Ontario, Canada). The anti-rat myogenin, monoclonal antibody (F5D) developed by Wright W. was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA). The enhanced chemiluminescence Western blotting detection system was purchased from Amersham (Arlington Heights IL).

Cell Cultures

The control and gene-trapped C2C12 mouse skeletal myoblast cell lines used in this study are described in Gogos et al. [1996]. Two gene-trapped variant clones were used. The first clone, RT-27, was trapped in one of the two C2C12 myoblast catB gene alleles using a ROSAβgoe retroviral gene trap vector; the second clone, RT-11, was trapped in a gene other than catB using the same retroviral gene trap vector. RT-27 has been demonstrated to express reduced levels of catB protein relative to control myoblast cell lines [Gogos et al., 1996]. Cell cultures were maintained at 37°C in an atmosphere of 5% CO_2–95% air in alpha MEM supplemented with either 10% horse serum (differentiation conditions) or fetal bovine serum (nondifferentiating conditions) as previously described [Jane and Dufresne, 1994]. The formation of myotubes was quantified as percent fusion (i.e., fusion index) according to the established procedure of Morris and Cole [1972].

Preparation of Crude and FPLC Fractions

Cells were seeded at 2×10^5 cells/100-mm plate and incubated at 37°C. At each appropriate time point (i.e., phase of growth), attached cells were collected from each plate in 1 ml cold Phosphate buffered saline (PBS: 0.027 mM

KCl, 1.5 mM KH_2PO_4, 150 mM $Na_2HPO_4.7H_2O$, 140 mM NaCl, pH 7.6) using a rubber policeman and centrifuged at 1,000g for 5 min. After washing in PBS, cell pellets were resuspended in homogenization buffer (135 mM KCl, 0.1 mM EDTA, 0.1% Tween, and 15 mM KH_2PO_4, pH 6.0). Cell suspensions were homogenized on ice with a Brinkman Polytron PT-10. Crude homogenates were then clarified by micro-centrifugation for 5 min at 12,000g and used in enzyme assays before and after FPLC fractionation on a Superose 12 HR 10/30 FPLC column [Jane and Dufresne, 1994; Scaddan and Dufresne, 1995].

Measurement of Intracellular CatB and L-Type Activities

Intracellular catB and catL-type activity was measured using 0.02 M of the flourometric substrates, Z-arg-arg-NHMec and Z-phe-arg-NHMec, respectively [Jane and Dufresne, 1994]. A 500 µl aliquot homogenate sample (10.0 µg protein diluted in 0.1% Brij 35 solution) was preincubated for 5 min at 37°C with 250 µl of activation buffer (352 mM KH_2PO_4, 48 mM Na_2HPO_4, 4 mM disodium EDTA, and 8 mM dithiolthreitol, pH 6.0). The reaction was initiated with 250 µl of substrate in the presence or absence of the cysteine protease general inhibitor E-64 [Towatari et al., 1991]. Since both catB and catL hydrolyze the Z-phe-arg-NHMec substrate, catL-type activity was measured with Z-phe-arg-NHMec in the presence and absence of 10^{-6} M of the catB selective inhibitor CA074. The fluorescence of free aminomethylcoumarin in each reaction tube was measured at an excitation wavelength of 370 nm and an emission wavelength of 460 nm. One milliunit of proteolytic activity was defined as the quantity of enzyme releasing 1 nmol of aminomethylcoumarin per minute.

Measurement of Pericellular CatB Activity

Pericellular and secreted catB activity produced by cells growing on coverslips was measured using a continuous assay according to the established procedure of Linebaugh et al. [1999]. Cells growing on 9 mm × 9 mm glass coverslips were rinsed twice in PBS, placed in coverslip holders, and equilibrated in assay buffer (Hanks balanced-salt solution supplemented with 0.6 mM $CaCl_2$, 0.6 mM $MgCl_2$, 2mM L-cysteine, and 25 mM Pipes adjusted to pH 7.0) minus substrate at 37°C for 5 min. The rate of fluorescent product formation was recorded in a Shimadzu RF-450 spectrophotometer, set a 308 nm excitation and 460 nm emission wavelengths, equipped with a temperature controlled cuvette holder, microstirrer and a DR-3 data chart recorder. Recordings generally consisted of four readings: (a) a fluorescent baseline for the assay buffer containing 100 µM Z-arg-arg-NHMec substrate (5 min), (b) rate of fluorescent product formation due to the introduction of cells, i.e., pericellular catB activity, (c) rate of fluorescent product formation after the removal of cells from the cuvette, i.e., secreted catB activity (10 min), and (d) rate of fluorescent product formation after addition of CA074 (10 µM final concentration).

Measurement of CPK and MK Activities

The activities of the muscle-specific enzyme marker, CPK and the nonspecific enzyme, MK were measured according to established procedures [Dufresne et al., 1976].

Measurement of CatB, MHC, and Myogenin Protein

Cell homogenates were prepared from cells collected every 24 h on day 2 through day 6 of growth (Jane and Dufresne, 1994) with the following modifications. Cells were harvested in PBS supplemented with 0.5 µM ALLN, 1 mM PMSF, and 100 U/ml aprotinin. Clarified cell homogenates were snap-frozen in liquid nitrogen then stored at −80°C. The total protein concentration of each homogenate was measured using the Micro BCA protein assay reagent kit. Forty micrograms (for MHC or myogenin) or 10 µg (for catB) of lysate was separated on a 7.5%−12% SDS-polyacrylamide gel. Protein was transferred to nitrocellulose and probed with either anti-mouse MHC monoclonal antibody (MF20) at a dilution of 1:200 [Bader et al., 1982], anti-rat myogenin monoclonal antibody (F5D) at a dilution of 1:10 [Wright et al., 1991], or with anti-human liver catB polyclonal antibody at a dilution of 1:12,000 [Moin et al., 1992]. Human liver catB antibody has been shown to react with mouse catB [Moin et al., 1992; Ryan et al., 1995] and was used in our previous analysis of C2C12 mouse myoblasts [Gogos et al., 1996]. Either horseradish peroxidase-linked goat anti-mouse IgG at a dilution of 1:5,000 or horseradish peroxidase-linked goat anti-rabbit IgG at a dilution of 1:10,000 were used as secondary antibodies. Membranes were detected using an enhanced

chemiluminescence Western blotting detection system followed by exposure to Kodak film and analyzed by densitometry using a Fuji Imaging System.

Protein Determination

Protein levels were determined by the method of Bradford [1976] and by the Pierce Micro BCA Assay kit method.

DNA Assay

DNA levels were determined by the procedure of Downs and Wilfinger [1983] using bisbenzimide dye (H33258) and calf thymus DNA standard.

Degree of Reproducibility

Each result represents the mean of at least three separate experiments conducted in triplicate. Standard errors of the mean (S.E.M.) were computer generated using Microsoft Excel and are indicated on tables and figures. Calculations of Student's t-tests were computer generated using Sigmaplot.

RESULTS

Serum Affects the Growth of CatB Gene-Trapped RT-27 Myoblasts

The study of myoblast differentiation in cell culture routinely involves a transfer from medium supplemented with 10% fetal calf serum (i.e., nondifferentiating medium) to medium supplemented with horse serum (i.e., differentiating medium) [Gogos et al., 1996]. It has been our experience that both the transfer to and the concentration of horse serum in medium can affect the growth, and ultimately the differentiation phenotype, of the cells. To minimize the contribution of this confounding variable, we first established growth conditions that supported optimal growth and differentiation of the control and the catB gene-trapped C2C12 myoblast cell lines (Fig. 1). Cells were cultured in alpha MEM medium supplemented with either 10% horse serum (10HS), or 10% fetal calf serum then replaced after 48 h with medium supplemented with 2% (10FCS/2HS) or 10% (10FCS/10HS) horse serum. For both cell lines, the increase in cell number over time was similar in 10HS and in 10FCS/10HS growth conditions. Compared to these levels, the cell number in 10FCS/2HS growth conditions was reduced by 20%–30% at each time point after
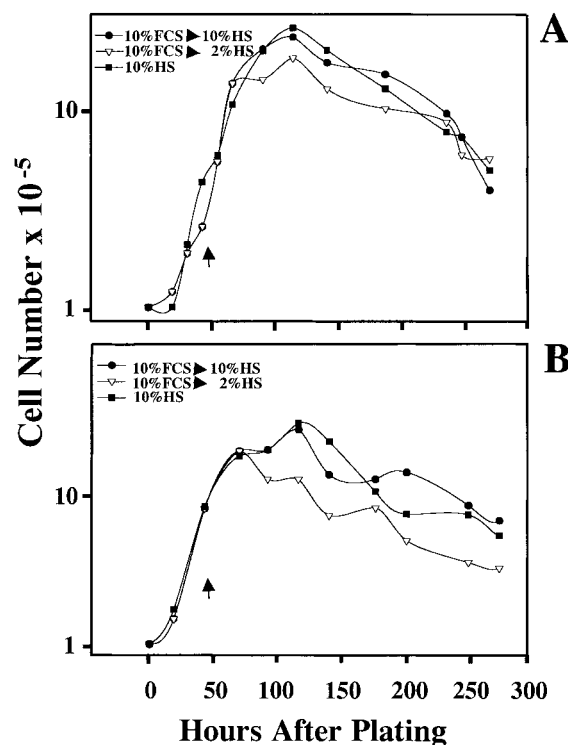

Fig. 1. Biology of cell lines. Growth and division of C2C12 and catB gene trapped RT-27 myoblasts in different serum containing medium. C2C12 (**A**) and RT-27 (**B**) myoblasts were seeded in 60 mm dishes at 1×10^5 cells/dish. For each panel, cells plated in 10% FCS containing media followed by a switch to 10% HS containing media after 48 h of growth are shown by solid circles. Cells plated in 10% FCS containing media followed by a switch to 2% HS containing media after 48 h of growth are shown by open triangles. Cells plated in 10% HS are shown by solid boxes. Every 12 h, cells from two plates for each condition were counted and the values were used to construct growth curves.

transfer to 2% HS; the greatest reduction was observed for catB gene-trapped RT-27 cells. Results for RT-11 cells, variants of C2C12 trapped in a gene other than catB, were comparable to those for control C2C12 cells (data not shown). In view of these results, all subsequent analyses were conducted in medium supplemented with 10% HS. Saturation densities for C2C12, RT-11 and RT-27 in 10HS were 8×10^4, 8×10^4, and 11×10^4 cells/cm^2, respectively; population doubling times were 17, 18, and 11 h, respectively.

During Differentiation, Levels of CatB Activity and Protein are Reduced in CatB Gene-Trapped RT-27 Myoblasts

Since differentiation is a progressive process, we determined the effects of catB gene trapping

on the expression of catB by measuring the levels of catB activity and protein on each day of myoblast differentiation. Cell homogenates were prepared from day 2 through day 7 cells, and assayed for catB activity using the fluorometric substrate, Z-arg-arg-NHMec (Fig. 2). To ensure the specificity of the substrate, assays were conducted in the presence and absence of the catB selective inhibitor, CAO74, and the general cysteine protease inhibitor, E-64. At each time point, levels of catB activity in catB gene-trapped RT-27 myoblasts were significantly lower than levels in C2C12 or RT-11 control cells ($P < 0.05$). Moreover, within each cell line, levels of catB activity increased during myoblast proliferation (days 2–4), decreased as myoblasts became post-mitotic (day 5), and then increased during myoblast fusion (days 6–7).

To confirm the catB activity results obtained in crude homogenates, and to examine molecular mass–enzyme activity associations, cell homogenates prepared from myoblast (day 2

through 5) and myotube (day 6 through 7), C2C12, RT-11, and RT-27 populations were fractionated on an FPLC-driven Superose 12 HR 10/30 column (Fig. 3). Column fractions were collected and measured for catB activity as previously described [Jane and Dufresne, 1994]. Two results were consistently obtained. First, peak levels of catB activity in the catB gene-trapped RT-27 fractions were approximately 50% lower than in C2C12 and RT-11 control fractions. Second, a single peak of catB activity corresponding to a molecular mass of 31 kDa was observed in myoblast and myotube populations of each cell line. This activity was completely eliminated when assays were conducted in the presence of the catB selective inhibitor, CAO74 (data not shown).

The relationship between levels of catB activity and protein in C2C12, RT-11, and RT-27 cell
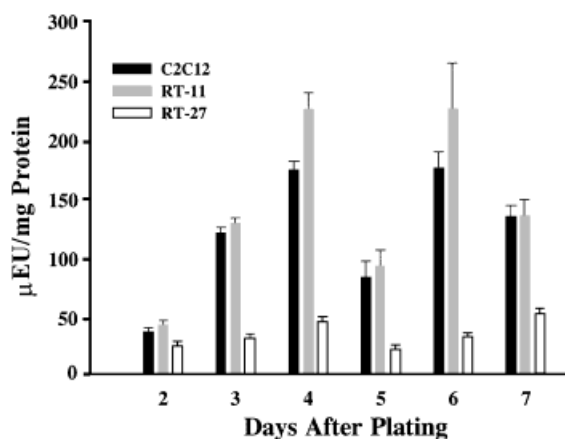

Fig. 2. Intracellular catB activity analyses. CatB activity was measured in cell homogenates prepared from presumptive (days 2 through 4), post-mitotic (day 5) and fused (day 6 and 7) control C2C12 (black bars), control gene-trapped RT-11 (grey bar) and catB gene-trapped RT-27 (open bars) cells using the substrate Z-arg-arg-NHMec. The selectivity of Z-arg-arg-NHMec for catB was confirmed using the selective catB inhibitor, CAO74. CatB activity is expressed as μEnzyme   U/mg of protein where one Unit is defined as the amount of protease that liberates 1 nmol of aminomethylcoumarin per min. Each value represents the mean of at least three measurements (N = 3) with error bars denoting standard error. Within C2C12 and RT-11, the differences in activities between successive days were significant ($P < 0.05$). For RT-27, the differences in levels of catB activities between days 4–5 (i.e., down) and days 5–6 (i.e., up) were significantly different ($P < 0.05$). Between C2C12 and RT-11, levels of catB activity for each day were not significantly different; however, these levels were significantly different from those for RT-27 ($P < 0.05$).
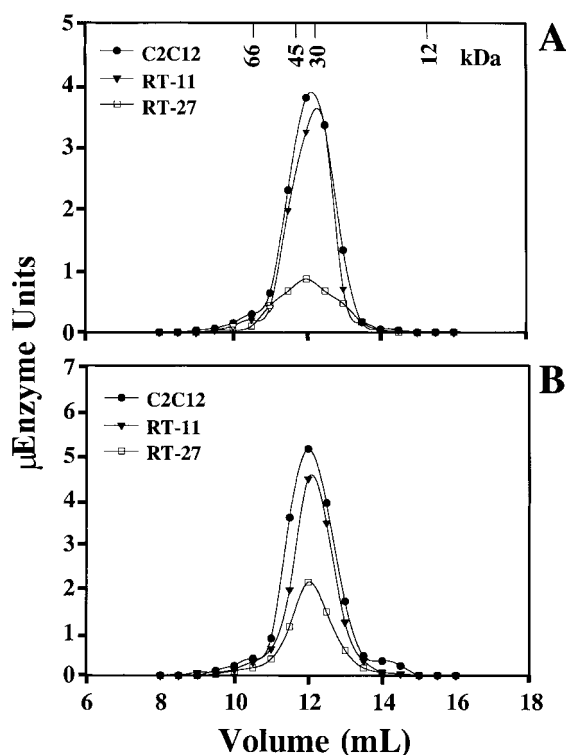

Fig. 3. Fast protein liquid chromatography (FPLC) analyses. Two hundred micrograms of protein from C2C12 (solid circle), RT-11 (solid triangle), and RT-27 (open box) myoblast (A) and myotube (B) homogenates were applied to a sepharose 12 HR 10/30 FPLC column and eluted at a flow rate of 1 ml/min. Five hundred microliter volumes were collected and measured for catB activity using the substrate Z-arg-arg-NHMec. The selectivity of the substrate was confirmed using the selective catB inhibitor, CAO74. CatB activity is expressed as μEnzyme U/mg protein where one Unit is defined as the quantity of protease that liberates 1 nmol aminomethylcoumarin/min.

homogenates, prepared from day 2 through day 7 cells, was examined using immunoblot analysis (Fig. 4). For each myoblast cell line, the pattern of catB protein levels paralleled that of catB activity. Specifically, levels of catB protein increased during myoblast proliferation (e.g., days 2–4), decreased in post-mitotic myoblasts just prior to myotube formation (day 5), then increased as myotubes formed (days 6–7). Moreover, densitometric analysis of blots for

Fig. 4. CatB Western blot analyses. For each of three independent experiments, eighty nanograms of protein from cell homogenates prepared from differentiating C2C12, RT-11, and RT-27 myoblasts (**A**) were used for immunoblot analyses using a monospecific rabbit anti-human liver catB antibody and enhanced chemiluminescence. Lane 1 was loaded with purified human liver catB. Densitometric analyses (**B**) of C2C12 (black bar), RT-11 (grey bar), and RT-27 (open bar) cells were performed on scanned images of Western blots exposed to hyperfilm for 30 s using a Fuji imaging system and are presented as relative optical density in arbitrary units per square millimeter (AU/mm^2). Three successive stages of differentiation are represented: (i) division of presumptive myoblasts (days 2–4), (ii) adherence of aligned, post-mitotic myoblasts (day 5), and (iii) fusion of non-cycling, post-mitotic myoblasts to form myotubes (days 6–7). To ensure equal loading, each gel was Coomassie stained after transfer, and band intensities in regions of constitutive expression were analyzed.

each cell line demonstrated that at each time point catB protein levels were lower in catB gene-trapped RT-27 myoblasts than in C2C12 and RT-11 control myoblasts (Fig. 4B). Despite this difference, the molecular mass of catB corresponded to the 31-kDa single chain, active form in all cell lines [Calkins et al., 1998; Gogos et al., 1996].

Levels of Pericellular and Secreted CatB Activities are Reduced in CatB Gene-Trapped RT-27 Myoblasts

A recently developed continuous assay of catB activity in "real time" has been used to provide evidence to support the hypothesis that the role of catB in tumor progression is mediated by its localization to and secretion from the cell surface of tumor cells [Linebaugh et al., 1999]. Since myotube formation is largely a membrane event, we have used the same assay to examine levels of pericellular and secreted catB in viable myoblast and myotube populations of catB gene-trapped and control cells (Fig. 5). First, it is important to note that both pericellular (i.e., cell surface + secreted) and secreted activities can be detected when the catB selective substrate Z-arg-arg-NHMec is added directly to myoblast and myotube cultures of catB gene trapped RT-27 and control C2C12 cell lines. In both cell lines, these activities are several-fold lower than the total activity determined after treatment of cells with Triton X-100. All of these activities are completely abolished in the presence of the catB selective inhibitor, CA074, and the cysteine protease general inhibitor, E-64 (data not shown). Finally, levels of total, pericellular, and secreted catB activities in catB gene-trapped RT-27 myoblast and myotube populations were approximately 50% lower than those measured in corresponding control C2C12 populations ($P < 0.01$).
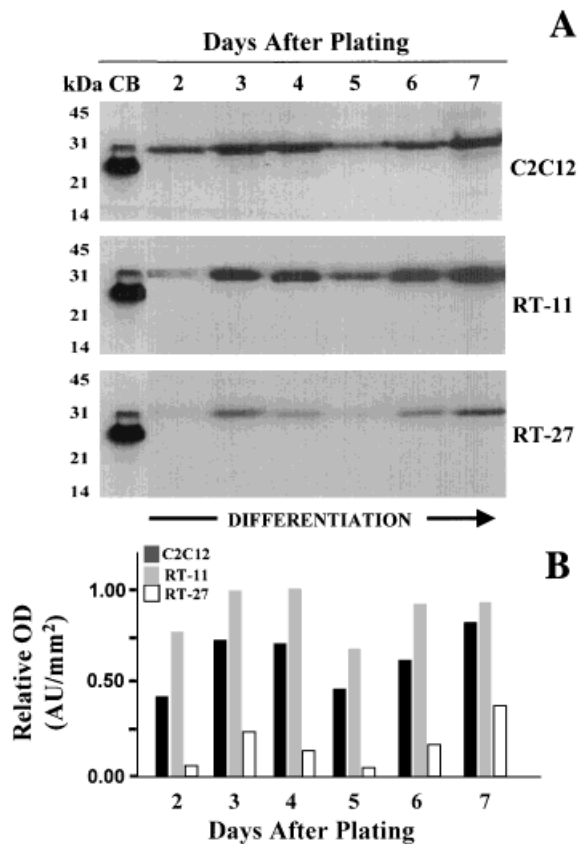
Reduced Levels of CatB Affect Morphological Differentiation of CatB Gene-Trapped RT-27 Myoblasts

The effects of reduced levels of catB activity within and secreted from catB gene-trapped RT-27 myoblasts on morphological differentiation was examined using microscopy of Giemsa stained cells at various stages of differentiation (Fig. 6). While the time of onset of myotube formation was similar among myoblast cell lines, the extent of myotube formation over time (i.e., percent fusion) was dramatically
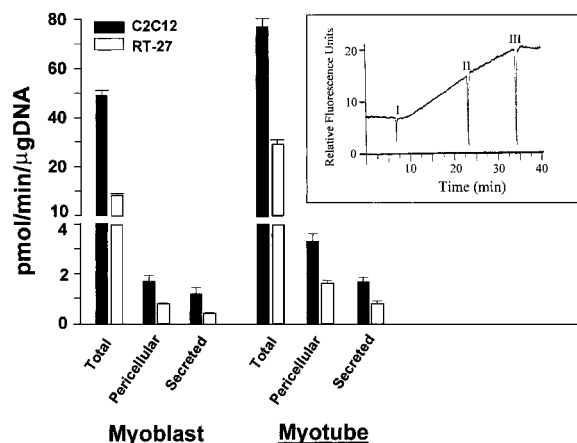
Fig. 5. Analyses of total, pericellular and secreted catB activities. After establishment of a baseline, three 9 mm × 9 mm coverslips containing monolayer cultures of myoblast or myotube populations of C2C12 (solid bar) and RT-27 (open bar) cells were added to assay buffer (I) containing Z-arg-arg-NHMec substrate, and pericellular catB activity was measured continuously for 10–15 min at 37°C. Cells were removed (II) and the soluble (secreted) catB activity remaining was monitored for 10 min at 37°C. Total catB activity was measured from one 9 mm × 9 mm coverslip by lysing the cells in the presence of 0.1% (v/v) triton X-100 and monitoring catB activity continuously for 10 min at 37°C. At the end of each reaction period (III) 10 μM CA074 was added and the activity was measured for an additional 5 min at 37°C. CatB activity was expressed as pmol per min by measuring the slope of the initial velocity curve and then expressed per μg of DNA. Each value represents the mean of at least 3 measurements (N = 3) with error bars denoting standard error. Between C2C12 and RT-27, levels of catB activity for total, pericellular, and secreted fractions were significantly different ($P < 0.01$).

reduced in catB gene-trapped RT-27, relative to C2C12 and RT-11 control myoblasts (Fig. 6A). During early differentiation (i.e., day 2–3), C2C12 (Fig. 6B) and RT-11 (Fig. 6D) control myoblasts were morphologically indistinguishable from RT-27 catB gene-trapped (Fig. 6F) myoblasts. However, as differentiated proceeded, (i.e., day 6), myotubes formed by the fusion of these myoblasts were generally smaller in RT-27 (Fig. 6G), containing fewer nuclei per myotube compared to C2C12 (Fig. 6C) and RT-11 (Fig. 6E) control myotube populations.

Reduced Levels of CatB Affect the Expression of Two Late Muscle-Specific Markers of Biochemical Differentiation

CPK activity and MHC, and myogenin are generally accepted as biochemical indicators to monitor the expression of late (i.e., CPK, MHC) and early (i.e., myogenin) muscle-specific genes during myogenesis [Dufresne et al., 1976;
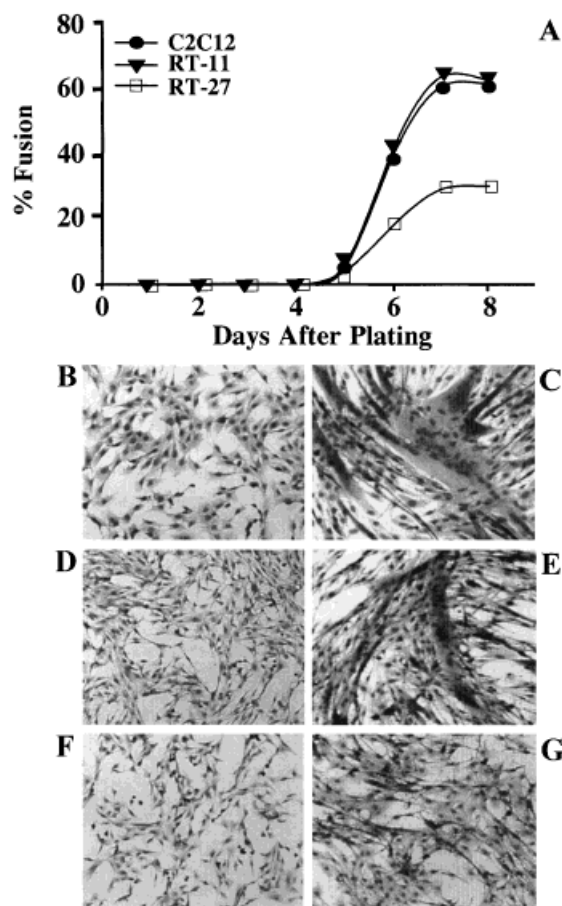

Fig. 6. Analyses of myotube formation. C2C12 (solid circles), RT-11 (solid triangles) and RT-27 (open boxes) myoblasts were plated in medium containing 10% HS to promote differentiation. Each day, cells were stained with Giemsa and analyzed for myotube formation (i.e., percent fusion) using an inverted microscope at 100 and 200× magnification (**A**). A cell was scored as a myotube, if it contained three or more nuclei within the same cell membrane. The range number of nuclei per myotube was 3–60 for C2C12 and RT-11, and 3–20 for RT-27. Geimsa stained day 2 presumptive myoblasts for C2C12 (**B**), RT-11 (**D**), and RT-27 (**F**); and Geimsa stained day 7 myotubes for C2C12 (**C**), RT-11 (**E**), and RT-27 (**G**) are represented.

Guttridge et al., 1997]. We measured the level of these markers in cell homogenates prepared from differentiating myoblasts of each cell line (Table I and Figs. 7 and 8). Within each cell line, levels of CPK activity were 2–3-fold higher in myotube compared to myoblast populations (Table I). However, among cell lines, absolute levels of CPK activity were 5–6-fold lower in homogenates prepared from catB gene-trapped RT-27 cells than levels in those prepared from C2C12 or gene-trapped RT-11 control cells. In contrast, levels of MK activity, a non-specific biochemical marker, were similar within the

TABLE I. Summary of Muscle-Specific and Non-Specific Enzyme Activities

Cell line	Population	CPK (U/mg)	MK (U/mg)	CatL-type (EU/mg)
C2C12	Myoblast	1.87 ± 0.07	44.8 ± 1.1	180.2 ± 10.1
	Myotube	3.75 ± 1.40	41.4 ± 0.8	51.7 ± 4.4
RT11	Myoblast	0.89 ± 0.37	45.7 ± 0.4	185.3 ± 12.6
	Myotube	2.20 ± 0.18	40.5 ± 1.4	68.1 ± 3.9
RT27	Myoblast	0.15 ± 0.05	39.4 ± 3.1	274.3 ± 18.5
	Myotube	0.51 ± 0.39	41.8 ± 3.3	252.9 ± 20.2

C2C12, RT-11, and RT-27 homogenates were prepared from cells maintained in alpha-MEM supplemented with 10% horse serum and assayed for creatine phosphokinase (CPK), myokinase (MK), and cathepsin L-type (catL-type) as described in Materials and Methods.
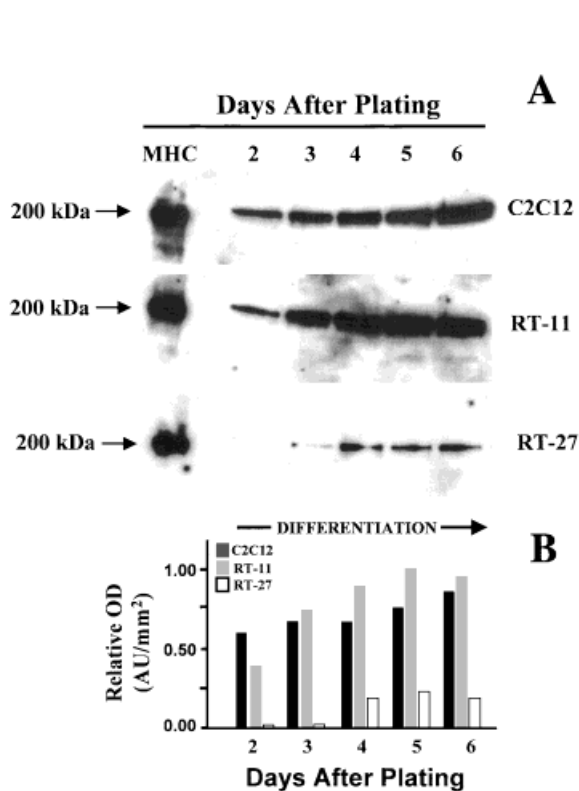Values represent the mean averaged activities $\pm$ S.D., where $N = 3$.

Fig. 7. MHC Western blot analyses. For each of three independent experiments, eighty nanograms of protein from cell homogenates prepared from differentiating C2C12, RT-11, and RT-27 myoblasts (**A**) were used for immunoblot analyses using a monospecific mouse anti-MHC antibody (MF20) and enhanced chemiluminescence. Lane 1 was loaded with purified rabbit muscle MHC. Densitometric analyses (**B**) of C2C12 (black bar), RT-11 (grey bar), and RT-27 (open bar) cells were performed on scanned images of Western blots exposed to hyperfilm for 30 s using a Fuji imaging system and are presented as relative optical density in arbitrary units per square millimeter (AU/mm^2). Three successive stages of differentiation are represented: (i) division of presumptive myoblasts (days 2–4), (ii) adherence of aligned, post-mitotic myoblasts (day 5), and (iii) fusion of non-cycling, post-mitotic myoblasts to form myotubes (days 6–7). To ensure equal loading, each gel was Coomassie stained after transfer, and band intensities in regions of constitutive expression were analyzed.

Fig. 8. Myogenin Western blot analyses. For each of three independent experiments, eighty nanograms of protein from cell homogenates prepared from differentiating C2C12, RT-11, and RT-27 myoblasts (**A**) were used for immunoblot analyses using a monospecific mouse anti-myogenin antibody (F5D) and enhanced chemiluminescence. Densitometric analyses (**B**) of C2C12 (black bar), RT-11 (grey bar), and RT-27 (open bar) cells were performed on scanned images of Western blots exposed to hyperfilm for 30 s using a Fuji imaging system and are presented as relative optical density in arbitrary units per square millimeter (AU/mm^2). Three successive stages of differentiation are represented: (i) division of presumptive myoblasts (day 2), (ii) adherence of aligned, post-mitotic myoblasts (day 5), and (iii) fusion of non-cycling, post-mitotic myoblasts to form myotubes (day 6). To ensure equal loading, each gel was Coomassie stained after transfer, and band intensities in regions of constitutive expression were analyzed.
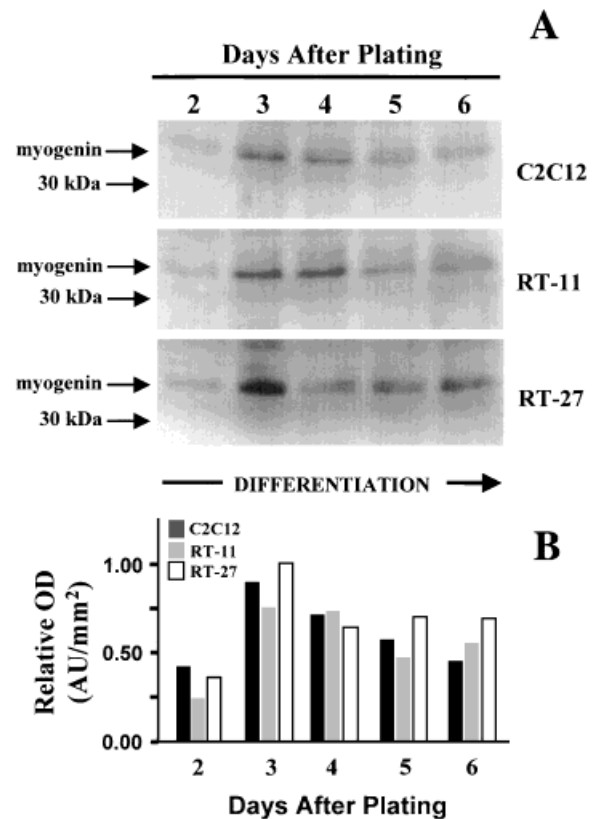
two populations of each cell line, and among cell lines (Table I). The expression of catL-type activity, a catB-related activity, is somewhat more complex (Table I). In C2C12 and RT-11 control cells, levels of catL-type activity were significantly greater in myoblast populations compared to myotube populations ($P < 0.01$). However, in RT-27 catB gene-trapped cells, levels of catL-type activity were similarly high in myoblast and myotube populations. Moreover, it is interesting to note that levels of catL-type activity in both RT-27 populations were significantly greater that those obtained in corresponding C2C12 and RT-11 control populations ($P < 0.01$).

Finally, immunoblot analysis was used to determine levels of MHC, a late muscle-specific, structural protein marker and of myogenin, an early muscle-specific, regulatory protein marker, in day 2 through day 6 cell homogenates. Within each cell line, MHC protein levels increased as differentiation proceeded, reaching maximum levels in terminally differentiated myotubes (Fig. 7). However, at each stage of differentiation MHC protein levels were dramatically lower in catB gene-trapped RT-27 myoblasts than in C2C12 and RT-11 control myoblasts. Myogenin protein levels also increased, but reached maximum levels prior to myotube formation (Fig. 8). Moreover, levels of myogenin protein were generally similar among the three myoblast cell lines.

DISCUSSION

Our past [Jane and Dufresne, 1994] and current results suggest that the lysosomal cysteine protease catB is strongly regulated during skeletal muscle cell differentiation. Unlike muscle-specific enzymes such as CPK, levels of catB activity were *high* in dividing presumptive C2C12 mouse myoblasts, but *low* in noncycling, aligned post-mitotic cells. Levels then *increased* in a fusion-related manner characteristic of muscle-specific enzymes such as CPK as myotubes formed [Dufresne et al., 1976; Ebisui et al., 1995]. This pattern of activity was observed in both crude and FPLC-purified fractions. FPLC effectively separates catB from its endogenous inhibitors [Jane and Dufresne, 1994; Scaddan and Dufresne, 1995]. Therefore, unlike proteases such as thrombin [Guttridge et al., 1997], changes in catB activity during C2C12 myoblast myogenesis do not appear to reflect alterations

in inhibitor levels. This interpretation is supported by the parallel patterns of catB activity and protein observed in differentiating C2C12 myoblasts, and is consistent with our previous results in L6 rat myoblasts [Jane and Dufresne, 1994]. Regardless of the mechanisms involved, the regulated expression of catB during myoblast differentiation is consistent with a more specific role for this protease during myoblast differentiation. Similar regulation/role relationships have been reported for catB in the generation of thyroid hormones, in bone resorption, and in the presentation and processing of antigenic proteins [Mort and Buttle, 1997].

The expression of catB activity during differentiation of RT-27 catB gene-trapped myoblasts was qualitatively similar to that observed during differentiation of C2C12 and RT-11 control myoblasts. However, levels of intracellular catB activity and protein in RT-27 at each stage of differentiation were at least 50% lower. This reduction was associated with alterations in some myogenic phenotypes. For example, fusion-related increases in CPK activity and MHC protein, two late muscle-specific biochemical markers of myoblast differentiation, were reduced by about 50% in catB gene-trapped myoblasts. In contrast, levels of myogenin, a muscle-specific transcription factor that transactivates muscle-specific genes [Guttridge et al., 1997] and serves as an early muscle-specific biochemical marker, were similar in catB gene-trapped and control myoblasts. Collectively, these data indicate that reduced expression of catB does not affect levels of all muscle-specific genes, and may preferentially affect late genes. Analysis of a greater number of late and early genes is required to substantiate this latter possibility.

Reduced expression of catB in RT-27 was also associated with alterations in a non-specific catB related protease, cathepsin L-type (catL-type) activity, but not in MK activity. However, in contrast to the reductions observed in muscle-specific CPK activity and HMC protein levels, catL-type activity was higher in catB gene-trapped myoblasts than in control myoblasts. A potential in vivo significance for increases in catL expression in catB compromised myoblasts is discussed later.

Our analyses of nondifferentiating-to-differentiating media transfer effects were primarily ones of experimental design. However, they also demonstrated that reduced levels of catB do not

negatively affect the viability of myoblasts in culture. RT-27 presumptive myoblasts were identical in appearance to control C2C12 and RT-11 myoblasts. Moreover, the population doubling time of catB gene-trapped myoblasts was actually lower than control populations (i.e., 11 h for RT-27 vs. 17 and 18 h for C2C12 and RT-11, respectively). This suggests that RT-27 myoblasts divided more rapidly then control myoblasts, at least during early stages of differentiation. These results are consistent with those published for the catB deficient mouse embryonic fibroblast cell line, MEFT−/− [Moin et al., 2000]. The viability of this cell line was not affected even though both catB alleles were "knocked out" by homologous recombination. Such observations are not really surprising given the large number of related proteases, for example catL, which could potentially compensate for catB's non-specific, catabolic functions. However, while cells may not require catB to grow and divide, our results indicate that myoblasts do require catB to fully differentiate.

Reduced levels of catB activity were associated with alterations in biochemical markers of myogenesis; however, they were most notably associated with alterations in the physiology of RT-27 myoblast differentiation. These alterations became apparent only after the onset of fusion. That is to say that both catB gene-trapped and control myoblasts began fusing at the same time, but the size of myotubes and the extent of myotube formation over time were dramatically reduced in RT-27 catB gene-trapped myoblasts. It is worth noting that the percent decrease in myotube formation in RT-27 corresponds with the percent decrease in levels of muscle-specific CPK activity and HMC. It has been known for some time that the expression of these late muscle-specific genes is directly related to myotube formation [Dufresne et al., 1976]. Therefore, it is reasonable to predict that reduced CPK activity and HMC protein levels in RT-27 reflect reductions in myotube formation which in-turn reflect reduced levels of catB activity. It follows that the regulated expression of catB activity may be required for the catabolic restructuring of myoblast membranes that precedes normal myotube formation [Knudsen and Horwitz, 1977; Thiery et al., 1982; Rieger et al., 1985].

A functional link between catB activity and the membrane of fusing myoblasts is supported, albeit indirectly, by several published and unpublished observations. First, confocal analysis demonstrated that catB protein is redistributed from the perinuclear regions of presumptive myoblasts towards the membrane of pre-fusion, post-mitotic myoblasts. Myoblast variants incapable of forming myotubes do not demonstrate a redistribution of catB protein or a fusion-related increase in catB activity [Jane and Dufresne, 1994]. Second, acid-activatable pro-catB is secreted from differentiating myoblasts in culture [Jane and Dufresne, 1994]. Third, exposure of differentiating myoblasts to the cell-penetrating catB selective inhibitor, CA074Me inhibits myotube formation, while exposure of primary myoblasts to the cell-penetrating cysteine protease general inhibitor, E-64d, negatively affects myotube formation and is associated with a build-up of β1-Integrin [Moncman and Wong, 1999]. Fourth, catB gene-trapped myoblasts transfected with sense pre-procatB cDNA form myotubes similar to those formed in control myoblasts, while control myoblasts transfected with antisense constructs form myotubes similar to those formed in catB gene-trapped myoblasts [Gogos et al., 1996].

Direct evidence for active catB at the plasma membrane or secreted from differentiating myoblasts (i.e., pericellular activity) has been difficult to obtain due to inactivation of the enzyme by inhibitors and oxidizing agents. Recently, Linebaugh et al. [1999] overcame this problem. They developed a continuous "real time" assay that combined with immunoprecipitation and immunoblotting allowed them to measure the secretion of a 31-kDa single chain active form of catB at pH 7.0 from numerous human, rat, and murine preneoplastic and malignant cells [Linebaugh et al., 1999; Hulkower et al., 2000]. The identification of pericellular activity has provided important insight into the functional significance of catB activity to tumor invasion and metastasis [Sameni et al., 2000]. In the current study, we provide the first direct evidence that intact, viable myoblasts secrete active catB activity at pH 7.0. Both C2C12 and catB gene-trapped RT-27 myoblasts demonstrated an increase in pericellular (secreted + membrane associated) and secreted Z-arg-arg-NHMec hydrolyzable activity during the fusing stage of myogenesis. However, levels of pericellular and secreted catB activity from catB gene-trapped myoblast and myotube populations were reduced by approximately 50% compared with control C2C12 cells. This

reduction is consistent with the reductions observed in the extent of myotube formation in catB gene-trapped compared to control myoblasts, suggesting a functional relationship between active pericellular/secreted catB at pH 7.0 and myotube formation. But exactly where and how does this function occur?

Exactly where and how active catB functions during myoblast fusion, remains unknown. However, it is interesting to note that catB protein in fusing myoblasts co-fractionates with caveolin-3, the muscle-specific structural component of membrane caveolae (unpublished observation). The functional significance of caveolae-associated catB in tumor progression has already been suggested in yeast two-hybrid studies [Mai et al., 2000]. Mai et al. [2000] hypothesize that catB interacts with proteins such as annexin II in the caveolae region of tumor cells and that these interactions initiate a proteolytic cascade involving remodeling proteases such as matrix metalloproteases, plasminogen, and tissue-type plasminogen activator. If a similar arrangement of protein and protein interactions exists in differentiating myoblasts, it is possible that active catB initiates a similar proteolytic cascade that facilitates intracellular and/or extracellular matrix remodeling necessary for myotube formation. While the concept of caveolae-associated catB interactions during myoblast fusion remains hypothetical, the evidence that active catB may work in concert with other proteases is substantial. First, the addition of selective inhibitors to matrix metalloprotease, an enzyme which appears to provide the insulin degrading enzyme activity required for the initiation of differentiation, inhibits myoblast differentiation [Kayalar and Wong, 1989]. Second, serine proteases and their inhibitors such as urokinase plasminogen activator and tissue-PA are localized to the surface of fusing myoblasts [Festoff et al., 1986]. Third, m-calpain increases in a fusion-related manner and exhibits a peripheral distribution in fusion-competent myoblasts [Kaur and Sanwal, 1981; Schollmeyer, 1986]. Fourth, calpain has been implicated in the cleavage of the fibronectin network and myoblast cytoskeletal components [Dourdin et al., 1999]. Fifth, heparin sulfate proteoglycans at the cell surface have been shown to bind and stabilize active catB and protect it from alkaline pH-induced inactivation [Almeida et al., 2001].

Finally, while our results suggest a direct and important link between catB and normal myotube formation in vitro, it is intriguing that knockout of catB [Deussing et al., 1998] or catL [Nakagawa et al., 1998] in stem cells in vivo does not affect skeletal muscle development in mice. This paradox could be explained if catB and catL compensate for each other in the in vivo stem cell knockout environment. There is some support for this possibility. For example, mice deficient in both catB and catL die shortly after birth [C. Peters, personal communication]. Moreover, we observed that increased levels of catL-type activity complement reduced levels of catB activity in catB gene-trapped RT-27 myoblasts. If a catL for catB compensatory mechanism operates in vivo, knockout of the catB gene in stem cells may not affect skeletal muscle development. While this possibility remains to be examined, the results of the current study and the demonstrated flexibility of catB to act as an endopeptidase, an exopeptidase, and an esterase [Bajkowski and Frankfatter, 1975] support an important role for cat B activity in the fusion of myoblasts during myogenesis.

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