Gene Trapping in Differentiating Cell Lines: Regulation of the Lysosomal Protease Cathepsin B in Skeletal Myoblast Growth and Fusion

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Abstract. To identify genes regulated during skeletal muscle differentiation, we have infected mouse C2C12 myoblasts with retroviral gene trap vectors, containing a promoterless marker gene with a 5' splice acceptor signal. Integration of the vector adjacent to an actively transcribed gene places the marker under the transcriptional control of the endogenous gene, while the adjacent vector sequences facilitate cloning. The vector insertionally mutates the trapped locus and may also form fusion proteins with the endogenous gene product. We have screened several hundred clones, each containing a trapping vector integrated into a different endogenous gene. In agreement with previous estimates based on hybridization kinetics, we find that a large proportion of all genes expressed in myoblasts are regulated during differentiation. Many of these genes undergo unique temporal patterns of activation or repression during cell growth and myotube formation, and some show specific patterns of subcellular localization. The first gene we have identified with this strategy is the lysosomal cysteine protease cathepsin B. Expression from the trapped allele is upregulated during early myoblast fusion and downregulated in myotubes. A direct role for cathepsin B in myoblast growth and fusion is suggested by the observation that the trapped cells deficient in cathepsin B activity have an unusual morphology and reduced survival in low-serum media and undergo differentiation with impaired cellular fusion. The phenotype is reproduced by antisense cathepsin B expression in parental C2C12 myoblasts. The cellular phenotype is similar to that observed in cultured myoblasts from patients with I cell disease, in which there is diminished accumulation of lysosomal enzymes. This suggests that a specific deficiency of cathepsin B could contribute to the myopathic component of this illness.

The expression of a myogenic basic helix-100p-helix (bHLH) transcription factor of the MyoD family is sufficient to convert a variety of cultured cells into skeletal muscle (for review see Minsterberg and Lassar, 1994). This initial switch is followed by an irreversible cascade of gene activation and repression events underlying the morphological differentiation. Earlier studies have analyzed global changes in sequence complexity and frequency distribution of messenger RNAs during muscle differentiation in vitro using DNA-RNA hybridization kinetics (Leibovitch et al., 1979). It was estimated that ~30,000 genes are expressed in skeletal myoblasts, and about two-thirds of those are regulated during the course of differentiation to myotubes.

Gene trap vectors provide an alternative way to study both global and gene-specific changes in transcription and mRNA accumulation (Wurst et al., 1995; Skarnes et al., 1995; DeGregori et al., 1994; Friedrich and Soriano, 1993). The vector that we used contains a promoterless marker gene with a 5' splice acceptor signal. Integration of the vector adjacent to an actively transcribed gene places the marker under the control of the endogenous transcription unit and facilitates its cloning. There are advantages compared to other approaches used to study gene induction or repression, such as subtractive hybridization or differential display. First, the trapping event is, in principle, independent of the abundance of the message, potentially allowing the identification of mRNA that exist in low numbers. Even differential display, the most sensitive of the hybridization methods, shows a strong bias towards high copy

1. Abbreviations used in this paper: bHLH, basic helix-100p-helix.

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number transcripts (Bertioli et al., 1995). Second, the gene trap vectors may generate fusion products between the reporter gene and part of the endogenous gene, which could include a subcellular localization signal, thereby providing information on the localization of the host encoded protein. Third, although integration of the gene trap is most likely to result in a recessive loss of function mutation, some genes may be vulnerable to haploinsufficiency, and some established cell lines are hypodiploid (Siminovich, 1976). Integrations into such genes could result in a mutant phenotype and provide additional information on the function of the gene.

We have therefore sought a strategy to identify and clone genes regulated during skeletal muscle differentiation employing retroviral gene traps, introduced into cultured mouse C2C12 mouse myoblasts. When C2C12 myoblasts, growing in serum rich media, are placed into media with low serum concentration, they undergo differentiation characterized by the formation of myotubes. We have been able to screen hundreds of genes differentially regulated during muscle maturation and selectively pursue the cloning of those in which mutations produce the most interesting phenotypes and/or patterns of expression with respect to temporal sequence and subcellular localization.

Among the first genes that we have identified in this manner is the lysosomal cysteine protease cathepsin B. We find that its expression is induced in myoblasts by serum starvation but downregulated in myotubes. Cells in which one cathepsin B allele is interrupted by the gene trap have a unique phenotype consisting of deficiency of myoblast fusion and an unusual growth morphology with decreased postmitotic survival. The phenotype is reproducible in parental C2C12 myoblasts with antisense cathepsin B expression. These results implicate cathepsin B in myoblast growth and fusion and suggest that a specific deficiency of cathepsin B could account for the myopathy of I cell disease, in which there is reduced localization of lysosomal enzymes.

Materials and Methods

Cell Culture and Retroviral Infection

Growth medium for C2C12 myoblasts and trapped subclones was Dulbecco's Modified Eagles Medium (DMEM) supplemented with 20% FCS. Differentiation was induced by culture for at least 48 hr in serum poor medium (DMEM supplemented with 2% heat-inactivated horse serum). 1% penicillin/streptomycin was present in all media. The GP+E86 ROSAgeo producer cell line was a gift from P. Soriano (FHCRC), and virus supernatant grown in medium (DMEM supplemented with 2% heat-inactivated horse serum) was used for transduction of the host cells. Differentiation was induced by culture for at least 48 hr in serum poor medium.

Sequence Confirmation of Cathepsin B Integration

In the clone trapped at the cathepsin B locus, endogenous flanking sequences were recovered through inverse PCR. Amplification products of ~400 bp in size were observed preferential amplification of the 5' product, the protocol was modified as follows: 3 μg of genomic DNA were digested to completion with 20 U of EcoRI in 1X PCR buffer (50 mM KCl, 10 mM Tris, pH 8.5, 2 mM MgCl2, 0.01% gelatin) in the presence of 0.01 μg RNAse A in a final volume of 100 μl. After heat inactivation of the enzyme, one third of the restriction digest was used for a subsequent self-ligation step, in a total volume of 100 μl adjusted with 1X PCR buffer, 1 μl of 10 mM ATP, and 1 μl of T4 DNA ligase (5 Weiss U/ml). Ligation was performed for 15 min at 37°C followed by an overnight incubation at room temperature. Ligase was inactivated for 10 min at 85°C, and 50 μl were digested with 20 U of XbaI for 60 min at 37°C. About one fifth of the restriction digest was used for a first round of PCR using two external primers, A (TCCATGCGCT-GCAAAAGATGG) and B (GGCAGGCCTCCGATACCTGCGCTTATT). First round of amplification was done in a 50-μl total volume containing 5 μl of 10× PCR buffer, 1 mM dNTPs, 1 μM concentration of each primer, and 2 μl of AmpliTag DNA polymerase (Perkin Elmer Corp., Norwalk, CT) using the following conditions: denaturation (94°C, 30 s), annealing (58°C, 45 s), and extension (72°C, 1 min) for 35 cycles. TaqStart antibody (Clontech, Palo Alto, CA) was used to facilitate "hot start" PCR. 5 μl from the first round product were cut separately with 5 μl of the following enzymes: Xhol or BamHI or PstI that cut within the amplified fragment originating from the gene trap vector. 1 μl of each one of these digests was then used in the second round of nested priming, using primers C (GGGCGTCGACCTTGCAACCTACAGGT) and D (CTC-GCTTCTGCTTCGCG). We also used a second primer that anneals to sequences on the 3' end of the trapping vector to the leader C of mouse cathepsin B and an internal forward primer that anneals to sequences downstream of the retroviral integration site from 3' of the trapping vector to the leader C of mouse cathepsin B. PCR products were sequenced.

X-Gal Staining

β-Galactosidase activity was detected by staining cells fixed to the plate with X-gal. Cells were fixed by incubation for 5 min in 4% paraformaldehyde in phosphate buffered saline (PBS). The cells were washed three times, and then incubated at 37°C for 1-24 h in PBS to which was added 5 mM K3Fe(CN)6, 5 mM K3Fe(CN)6, 1 mM MgCl2, and 1 mg/ml X-gal.

Immunofluorescent Staining

Immunofluorescent staining for β-galactosidase protein was performed by fixation of cells on plastic petri dishes for 3 min at room temperature in 20% formaldehyde, 50% acetic acid, and 30% methanol. Cells were permeabilized with 1μg/ml SB88 mouse monoclonal β-galactosidase antibody (Life Technologies, Grand Island, NY) in PBS for 1 h at room temperature, and secondary incubation with fluorescein-conjugated donkey α-mouse antibody (Jackson Labs, West Grove, PA) at 20 μg/ml in PBS for 30 min at room temperature. Immunofluorescent staining for the myosin heavy chain differentiation marker was performed by methanol/acetone fixation of cells growing in plastic dishes and primary incubation for 1 h at room temperature with 1:200 rabbit polyclonal antiserum (Sigma Chem. Co., St. Louis, MO) in PBS with secondary incubation with rhodamine-conjugated donkey α-rabbit antibody (Jackson Labs) at 20 μg/ml in PBS for 30 min at room temperature. Immunofluorescent detection of the myo-epitope tagged cathepsin B was performed by the same fixation method with primary incubation for 1 h at room temperature with 1:2 of mouse 9E10 hybridoma supernatant in PBS with secondary incubation with fluorescein-conjugated donkey α-mouse
antibody at 20 µg/ml in PBS for 30 min at room temperature and nuclear counterstaining with 0.5 µg/ml DAPI. The above staining was observed with a Zeiss photomicroscope III.

Specific cathepsin B antisera were raised in rabbits against the mature double chain form from human liver, and an IgG fraction was purified (Campo et al., 1994; Moin et al., 1992; Sloane et al., 1994a). This antibody recognizes procathepsin B (Sloane et al., 1994a) and single and double chain forms of the mature enzyme in immunoblots (Campo et al., 1994; Moin et al., 1992) and immunoprecipitates (Sloane et al., 1994c) mature and pro forms of the enzyme. Intracellular cathepsin B was localized (Sloane et al., 1994c) by growing cells to 60–80% confluence on glass coverslips and fixing with 3.7% formaldehyde in PBS at room temperature. After washing with PBS, cells were blocked with 2 mg/ml BSA in PBS. All subsequent antibody and wash solutions contained 0.1% saponin. Cells were incubated with primary antibody for 2 h and washed. In controls, preimmune rabbit serum was substituted for the primary antibody. After blocking with normal 5% donkey serum, cells were incubated for 60 min with Texas red-conjugated donkey anti-rabbit antibody (Jackson Labs) at 20 µg/ml. After washing the coverslips were mounted upside-down on slides with SlowFade (Molecular Probes, Eugene, OR) and observed with a Zeiss LSM 310 confocal microscope.

**Plasmids**

Mouse full-length preprocathepsin B cDNA (from pmCB58 (Chan et al., 1986), a gift of A. Frankfater (Loyola, Chicago, IL) was cloned as an EcoRI fragment in either sense of antisense orientation into pEMSVscribe (Davis et al., 1987). The carboxyl terminus myc epitope tagged cathepsin B construct was made by PCR of pmCB58 from bases 35 to 1047 (containing the entire coding sequence) with BamHI and ClaI sites incorporated into the upstream and downstream primers, respectively, and then ligated in frame into the corresponding sites of pCS2+(Myc epitope), (Turner and Weintraub, 1994; Rupp and Weintraub, 1994).

**DNA Transfection**

For stable transfection, 5 x 10⁵ cells/60-mm petri dish were cotransfected in HBS with 0.5 µg pEMSVscribe-preprocathepsin B and 10 µg pSV2PAC (containing a puromycin resistance gene). Individual clones were selected for 14-21 d in 2 µg/ml puromycin and then isolated and expanded. Subsequent experiments on growth and fusion were performed in the absence of puromycin.

**Reverse Transcription PCR**

To perform exon trapping from leader b of cathepsin B, RT PCR was performed using a primer specific to cathepsin B leader b from -2329 to -2299, following the numbering of Rhaissi et al., (1993), (CTGTGATCTTGGTGCACACA) and a primer specific to the β-galactosidase domain of the βgeo gene, nt 236 to 260 following the initiation codon (CCGTGCACTTGCCAGTTTGAGGGGA). A total of 200 ng of total RNA from the trapped cathepsin B clone was used. A control consisted of the same amount of total RNA from a different, arbitrarily chosen, clone trapped with ROSAβgeo. RT PCR was performed using the EZ rTh RNA PCR Kit (Perkin Elmer) following the manufacturer’s default instructions.

**FACS Analysis**

FACS analysis of isolated nuclei from subconfluent myoblasts (~10⁵ cells/100-mm petri dish) in growth medium, was performed with propidium iodide staining for DNA content as described (Vindelov et al., 1983).

**Immunoblotting**

10⁵ cells were resuspended in 100 µl of SDS-gel loading buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 1% bromophenol blue, 10% glycerol), heated to 100°C for 3 min and 10 µl were loaded and subject to 10% SDS-PAGE and electro-transfer to nitrocellulose. The filter was incubated with a 1:1,000 dilution of rabbit α-human cathepsin B antisera (Athens Research, Athens, GA) for 1 h. Secondary detection was by HRP-conjugated antibodies and ECL chemiluminescence (Amersham).

**Results**

**Identification of Genes Regulated during Differentiation**

We used the ROSAβgeo (Friedrich and Soriano, 1991) retroviral gene trap containing a splice acceptor 5' to a promoterless reporter gene encoding a fusion protein of β-galactosidase and neomycin phosphotransferase (βgeo).

When integrated into intronic sequences of an active gene, the vector may generate spliced fusion transcripts between the reporter and endogenous genes. The resulting gene product could be then, in some instances, a fusion of the endogenous protein with βgeo. Cells expressing βgeo can be selected with G418 as well as assayed by staining with X-gal. Insertions in the 5' untranslated region of a gene are also possible; in this case, the reporter gene is transcribed under the control of the regulatory elements of the host locus and is translated using its own initiation codon. Insertions of the gene trap vector may also result in mutation of one allele of the host gene. The insertion of the vector sequences facilitates the eventual cloning and identification of the trapped gene.

In pilot experiments, C2C12 myoblasts growing in high serum media were infected with pROSAβgeo at low multiplicity (to lessen the likelihood of more than one integration event per cell), and the cells were plated at clonal density with G418 selection. After allowing the clones to grow to ~1,000 cells/culture, the plates were stained with X-gal. Southern blots of six arbitrary clones probed with the vector confirmed random integration of the trap at a single locus in each clone (not shown). We examined several thousand trapped clones, each therefore corresponding to integration of the βgeo gene into a unique, active locus. A broad range of staining intensities, presumably corresponding to differing levels of expression of the endogenous transcript, was observed. We found that only ~32% of all G418 resistant clones show detectable X-gal staining. This results from the fact that G418 selection is more sensitive than X-gal staining (Friedrich and Soriano, 1991). However, the proportion of X-gal positive clones increases to 62% when the cells are stained after they have been induced to differentiate by culture for 3 d in low-serum media. This indicates that at least one third of active myoblast genes are transcriptionally upregulated upon differentiation.

A more detailed microscopic examination of individual clones showed that the upregulation of gene expression was either confined to the myotubes (such as in Fig. 1 A), or it was obvious in both the myotubes and undifferentiated myoblasts (not shown).

We also tested whether we can identify genes that are repressed upon differentiation. In these experiments we analyzed 130 individually isolated and expanded clones of cells trapped with ROSAβgeo. We characterized the pattern of X-gal staining both in growth media (high serum) and after 3 d in differentiation media (low serum). In agreement with the experiment described above, about one-third of the clones showed upregulation of X-gal staining upon differentiation. In addition to that, however, X-gal staining decreased in intensity in ~12% of the initially positive clones after differentiation. In some clones the myoblasts were X-gal positive in growth medium; after
incubation in differentiation media the residual myoblasts remained positive, but the myotubes were negative (an example shown in Fig. 1 B). In another two clones, the myoblasts were positive in growth medium, but after incubation in differentiation media both the residual myoblasts and the myotubes became negative (not shown).

Survey of Patterns of Trapped Genes during Differentiation

In some of the trapping events the reporter gene may form fusion proteins with the endogenous product (although this is enhanced when the initiation codon is removed from βgeo, Friedrich and Soriano, 1991). Therefore, the expression of the βgeo marker may reflect not only transcriptional control of the endogenous gene, but, in addition, the effects of translational regulation, posttranslational processing, and subcellular localization. For example, in one clone, immunofluorescent staining with β-galactosidase antibody revealed that βgeo fused to a protein capable of forming filaments (Fig. 1 C). Another clone shows X-gal staining only within the nuclei of myotubes, suggesting that the trapping forms a fusion to a nuclear-targeted, myotube-specific protein (Fig. 1 D). In a different clone there is intense cytoplasmic staining in doublets of cells completing mitosis, suggesting cell cycle regulation of the trapped locus (Fig. 1 E).

Gene Trap Integration in Cathepsin B

We recovered endogenous DNA sequences adjacent to the trapping vector integration site through a combination of 5’ RACE with βgeo primers, inverse PCR, and adapter ligation long range suppression PCR (see Materials and Methods). The sequences were searched against on-line databases. Sequences frequently were not found within the database, presumably representing novel genes and unsequenced intronic regions of known genes. With the

Figure 1. Survey of trapped clones. Cells were stained with X-gal (A, B, D, and E) or immunofluorescently stained with β-galactosidase antibody (C). Interesting expression patterns are shown. The trapped gene is myotube specific (A), myoblast specific (B), forms a filament (C), is nuclear myotube specific (D), or cell division upregulated (E).
advance of the genome project, a greater proportion of recovered sequences should become identifiable.

Among the first of previously described genes that we have identified with our strategy is the lysosomal cysteine protease cathepsin B (Rhaissi et al., 1993). In this case, the ROSAβgeo trap has integrated within an intron located between the two downstream (of its three proposed) transcription initiation sites (Fig. 2 A). As with the majority of gene trap events (Friedrich and Soriano, 1993), the vector has integrated into a 5' intron upstream of the endogenous initiation codon. An obvious potential exon trapping event would correspond to the exon initiating from leader b to splice at its 3' end to the 5' splice acceptor signal of the trapping vector. By performing RT PCR with an upstream primer contained within leader b and a downstream primer from β-geo, we confirm this interpretation by specifically amplifying an RNA product of the anticipated size (Fig. 2 B). No specific amplification occurs in the control of an arbitrarily chosen clone corresponding to gene trap integration at a different locus.

The pattern of X-gal staining in this clone indicates that βgeo expression from the cathepsin B locus is undetectable in myoblasts (Fig. 3 A), but induced in residual, unfused myoblasts (Fig. 3 B) following differentiation. Previous studies have found increases in cathepsin B enzymatic activity during myoblast differentiation and fusion (B'echet et al., 1991; Kirschke et al., 1983; Jane and Dufresne, 1994). X-gal staining of individual cells (as in Fig. 3 B) more precisely reveals the surprising absence of staining in mature myotubes. This probably represents downregula-

**Figure 3.** Expression of β-geo from cathepsin B locus. (A and B) X-gal staining. There is undetectable X-gal staining in myoblasts in growth medium (A), but staining is induced in residual, unfused myotubes after 2 d in differentiation medium (B).

**Figure 2.** Integration site of gene trap in cathepsin B promoter (A). The trap is inserted in an intron between the distal two (of three proposed) transcription initiation sites. The sequence follows the numbering of Rhaissi et al. (1993). Potential transcription factor binding sites are noted. The dashed line indicates the usual splicing, whereas the solid line indicates the predicted splicing from the 3' splice donor of leader b to the 5' splice acceptor of the gene trap. RT PCR (B), using an upstream primer contained within leader b and a downstream primer from β-geo, specifically amplifies a fragment of the size (410 bp) predicted for the generation of the cathepsin B/β-geo fusion transcript. Control RNA comes from a different, arbitrary trapped clone.
Growth and Fusion Phenotype of Trapped Clone

The trapped cell line displays several interesting phenotypes with respect to myoblast growth and fusion.

When the trapped cell line is plated in growth medium, we observed an unusual cellular morphology. The cells appear rounded and are frequently in globular clusters with pyknotic nuclei (Fig. 4). The morphology is most apparent at the growing edge of a colony when the cells are plated at clonal density.

We also observed that the trapped clone grew more slowly, in that the cells required passing at less frequent intervals than control C2C12 myoblasts. Time-lapse photomicroscopy of the trapped clone (not shown) suggests that many of the rounded cells fail to continue dividing. FACS analysis of myoblasts in growth medium (Fig. 5) reveals a substantial sub-G1 peak of cells in the trapped clone. Sub-G1 peaks correspond to necrotic and/or apoptotic cells (Qian et al., 1995; Tounekti et al., 1995; Schmid et al., 1994; Pellicciari et al., 1993; Ormerod et al., 1992). In support of this, we observed that there is obvious loss of cellular integrity evident as soon as 72 h following placement of the trapped clone in differentiation medium (Fig. 4), whereas wild-type C2C12 cells remain intact.

The phenotype most probably reflects an effect of haploinsufficiency due to interruption of one copy of the gene for cathepsin B. Immunofluorescent staining (Fig. 6, A and B) and immunoblotting (Fig. 6 C) with cathepsin B antibody reveals a reduction in cathepsin B protein. Note that for the immunoblotting, extracts were prepared from an equal number of cells; normalizing to total protein concentration or comparing to an arbitrary control protein is problematic in cells with a deficiency of lysosomal pro-
Expression and Reproduction of the Phenotypes in the Trapped Clone with Sense Cathepsin B Expression and Reproduction of the Phenotypes in the Trapped Clone with Sense Cathepsin B

The phenotypes are not complemented in control cotransfections with antisense cDNA (Table I, Figs. 4 and 7), and displayed similar phenotypes with respect to growth, fusion, and survival in low-serum media. (We speculate that the other seven subclones did not express sufficient quantities of the antisense construct to make the phenotype apparent.) This experiment provides confirmation that reduced levels of cathepsin B expression have an effect on myoblast growth and fusion.

**Effect of Overexpression of Cathepsin B in C2C12 Cells**

We next tested whether antisense expression of preprocathepsin B cDNA could reproduce the phenotype of the trapped clone. Three of ten tested subclones were stably cotransfected with the antisense cDNA (Table I, Figs. 4 and 7), and displayed similar phenotypes with respect to growth, fusion, and survival in low-serum media. (We speculate that the other seven subclones did not express sufficient quantities of the antisense construct to make the phenotype apparent.) This experiment provides confirmation that reduced levels of cathepsin B expression have an effect on myoblast growth and fusion.

**Discussion**

We have targeted gene trap integrations into genes active in myoblasts, and by staining with X-gal in growth and differentiation conditions, have identified genes whose expression is regulated upon differentiation in vitro. Consistent with earlier estimates based on hybridization kinetics, we observed that a large proportion of the genes expressed in myoblasts are regulated upon differentiation into myotubes. We have specifically identified the lysosomal cysteine protease cathepsin B (for review see Barrett and Kirschke, 1981; Sloane et al., 1994b) as being regulated during skeletal myoblast fusion and differentiation. The general promise of this approach is substantiated by our finding that, in a related screen for genes repressed by MyoD under high serum conditions (Gogos, J., and M. Horwitz, unpublished experiments), we have found integration of the trap into the first intron of the cell cycle control, RNA transport, and guanine nucleotide exchange factor RCC1. This intron also encodes the small nucleolar RNA U17, suggesting that these are among the earliest genes downregulated as MyoD induces differentiation.

There are three phenotypes in the trapped cells: unusual appearance, poor survival with prolonged incubation in...
differentiation media, and deficient myoblast fusion. We show by four independent experimental lines of evidence that these are attributable to haploinsufficiency of cathepsin B: (1) The trapped cell lines are unequivocally deficient in cathepsin B production (by both immunofluorescent staining and Western blotting). (2) All three phenotypes may be complemented in numerous subclones stably transfected with sense cathepsin B. (3) All three phenotypes may be reproduced in C2C12 cells by antisense cathepsin B. (4) Overexpression of cathepsin B in C2C12 myoblasts seems to produce the opposite effect of promoting fusion.

The tagged cathepsin B allele is upregulated in residual, unfused cells following serum starvation induced differentiation. Its expression is downregulated in mature myotubes. Examination of the promoter sequence indicates several potential myogenic bHLH responsive E boxes as well as a potential MEF2-binding site (Fig. 2 A). In preliminary unpublished studies, we have found, surprisingly, that MEF2A expression suppresses transcription from the trapped cathepsin B allele, potentially suggesting that MEF2A could act as an inhibitor of cathepsin B expression in myotubes. The timing of expression of cathepsin B from this and other studies has implicated it as a potential mediator of myoblast fusion and differentiation. B'echet et al. (1991) found an increase in cathepsin B activity with fetal bovine skeletal muscle differentiation. Kirschke et al. (1983) and Jane and Dufresne (1994) found an increase in cathepsin B activity to be temporally associated with fusion in cultured rat L6 myoblasts. Inhibitor studies have further suggested that proteases are required for myoblast fusion (Couch and Strittmatter, 1983). Support for this role comes from our observations that the trapped cells, with reduced cathepsin B expression because of the disruption of one allele, differentiate poorly, forming myotubes with few nuclei. Recently, the metalloprotease melt-
rin has been shown to promote skeletal myoblast fusion (Yagami-Hiromasa et al., 1995), and a deficiency of another cysteine protease, calpain 3, has been found as a cause of autosomal recessive limb girdle muscular dystrophy (Richard et al., 1995). Therefore, proteases do have a role in the normal maturation of myotubes.

Evidence that lysosomal enzymes, in particular, participate in myoblast fusion comes from observations in patients with I cell disease (for review see Kornfeld and Sly, 1995), an autosomal recessive mucolipidosis in which there is deficient targeting of lysosomal proteins, including cathepsin B (Kopitz et al., 1993). Among many problems, these patients have neuromuscular disability. Histopathologic skeletal myofibrillar disorganization has been interpreted to suggest that there is a defect in developing rather than mature muscle (Kula et al., 1984). In particular, cultured skeletal myoblasts from these patients poorly differentiate and form myotubes with few nuclei (Shanske et al., 1995).
Table I. Complementation of Growth and Fusion Phenotypes in Cathepsin B Trap Clone by Stable Transfection of Preprocathepsin B and Reproduction of Phenotypes in C2C12 Cells by Stable Transfection of Antisense Preprocathepsin B

|                         | “Rough edge” growth | Failure to form multinucleate myotubes | Failure to survive in low-serum media |
|-------------------------|---------------------|----------------------------------------|----------------------------------------|
| Cathepsin B trap clone/sense cathepsin B | 19/22               | 4/4                                    | 4/4                                    |
| Cathepsin B trap clone/antisense cathepsin B | 0/45               | 0/4                                    | 0/4                                    |
| C2C12/antisense cathepsin B               | 3/10               | 3/3                                    | 3/3                                    |
| C2C12/sense cathepsin B                  | 0/10               | 1/10                                   | 0/10                                   |

The numerator indicates the total number of transfected subclones displaying the phenotype of rough edge growth, failure to form multinucleate myotubes, and failure to survive in low serum. The denominator is the total number of transfected subclones that were tested. The criteria for multinucleate myotube formation was whether 75% or more of myotubes (in 20 random low power fields) contained three or more nuclei after 48 h in differentiation medium. The criteria for rough edge growth and survival after 72 h incubation in low-serum differentiation media were by subjective observation. (Representative results are illustrated in Figs. 4 and 7.) For the cathepsin B trap clone stably cotransfected with either sense or antisense cathepsin B, four of the clones were expanded and analyzed for the fusion and survival phenotypes. For the C2C12 cells, stably cotransfected with antisense cathepsin B, the three clones (from the 10 surveyed) that demonstrated a rough edge growth phenotype were also examined for the fusion and survival phenotypes. For the C2C12 cells, stably cotransfected with sense cathepsin B, 10 clones were examined for each of the three phenotypes.

1981). We speculate that a specific lysosomal deficiency of cathepsin B may be sufficient to account for the myopathy of I cell disease.

We find that the trapped cells have a distinctive growth defect, characterized by slow growth, clumping of cells (most prominent at the edge of a growing colony), and poor survival after prolonged incubation in low-serum differentiation medium. FACS analysis suggests a sizable portion of the population of myoblasts to correspond to a sub G1, and therefore presumably necrotic and/or apoptotic population. It is not clear why a deficiency of a lysosomal protease should have such an effect on cell growth. However, two clinical observations may suggest clues. First, in a variety of human tumors of differing tissue types, cathepsin B activity has been inversely correlated with prognosis as a result of increased anaplasticity, faster growth, and potential for local and metastatic spread (Barrett and Kirschke, 1981; Sloane et al., 1994a,b,c; Campo et al., 1994; Moin et al., 1992). While one interpretation of this data has been that proteases are required for local tissue invasion, our results would suggest a more direct effect of cathepsin B upon the control of cell growth. Second, diseases of lysosomal enzyme deficiency have been associated with decreased survival of postmitotic cells (for review see Neufeld, 1991). There is neuronal degeneration in the human storage diseases with deficiency of glycolipid degradation (Tay-Sachs, Neimann-Pick, and Gaucher disease) and the mucopolysaccharidoses (the Hurler, Hunter, Sanfilippo, and Morquio syndromes) in which there is a deficiency of lysosomal glycosaminoglycan degradation. Our results suggest that deficiencies of other hydrolytic lysosomal enzymes, including proteases, also may affect cell growth and survival. One possibility is that cathepsin B is required for the maturation of other lysosomal hydrolases, and that a deficiency of cathepsin B leads to general deficiency in the processing of other lysosomal proenzymes.

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