

Preferential MyoD Homodimer Formation Demonstrated by a General Method of Dominant Negative Mutation Employing Fusion with a Lysosomal Protease

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Abstract. We report on a general strategy for engineering dominant negative mutations that, in principle, requires neither extensive structural or functional knowledge of the targeted protein. The approach consists of fusing the lysosomal protease cathepsin B (CB) to a subunit of a multimeric protein. The CB fusion polypeptide can proteolytically digest the multimer and/or detour the multimer from its usual subcellular destination to the lysosome. We first demonstrate the general validity of the approach with CB fusion to *E. coli* lacZ, encoding tetrameric β -galactosidase. Cotransfection of NIH 3T3 cells with a vector expressing a CB-lacZ fusion inhibits the β -galactosidase activity produced by transfection of lacZ alone. We infer that the dominant negative inhibition results from both direct proteolysis of the β -galactosidase tetramer by the fu-

sion subunit and detour of the tetramer to the lysosome. In a specific application of this strategy, we have fused CB to the dimeric bHLH skeletal muscle transcription factor MyoD. The CB-MyoD fusion protein localizes to the cytoplasm, presumably the lysosome, demonstrating the dominance of lysosomal localization to nuclear localization. The CB-MyoD fusion appears to divert homodimerizing native MyoD from its usual nuclear destination, consequently inhibiting MyoD-mediated transactivation and in vitro differentiation of C2C12 myoblasts. Surprisingly, the CB-MyoD fusion fails to interact with the bHLH heterodimerization partners, E12 and E47, suggesting preferential MyoD homodimer formation, at least in the prenuclear cellular compartments.

THE selective suppression of gene expression is both an experimental tool for defining function and a potential means to medical therapy. One approach is antisense (for review see Wagner, 1995); another approach is to interfere with the protein product of the gene through "dominant negative mutation" of an associating protein (for review see Herskowitz, 1987). Designing a dominant negative mutation requires some understanding of the structure and function of that protein. In contrast, the advantage of antisense is apparent when structural and functional information for the gene product is limited.

We have sought a straightforward strategy for engineering dominant negative mutations that, like antisense, potentially requires minimal knowledge of the gene's structure and function. We reasoned that for a multimeric protein, fusion of one subunit to a lysosomal protease could dominantly inhibit the assembled multimer, and perhaps other associating proteins through a combination of

proteolysis of the native subunits and/or subcellular diversion of the multimer to the lysosome.

Experiments with the cellular CD4 receptor and its HIV envelope glycoprotein ligand bear on the possibility of achieving dominant negative inhibition by diverting a protein from its usual subcellular trafficking pattern. A CD4 molecule deleted in the transmembrane and cytoplasmic domains is a soluble molecule capable of competitively antagonizing ligand-receptor interaction (Smith et al., 1987). When an endoplasmic reticulum (ER) retention signal is fused to the gene encoding this soluble version of CD4, cells expressing the ER-CD4 fusion retain it in the lumen of the ER along with the associating envelope glycoprotein (Buonocore and Rose, 1990). An interesting derivative of this approach was developed by Tang and coworkers (Lin et al., 1993). When the gene encoding the soluble CD4 is combined with any of several genes encoding lysosomal proteins, HIV-infected cells expressing the fusion protein can divert, in trans, associating envelope glycoprotein to the lysosome, where it is degraded.

An open question of cell biology potentially prohibiting the adaptation of this strategy to other proteins relates to the sequence of events in lysosomal targeting. In general,

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proteins bound for the lysosome are synthesized on ER-bound ribosomes, posttranslationally modified with mannose-6-phosphate in the Golgi, recognized by specific receptors, and then incorporated into clathrin-coated vesicles eventually routed to the lysosome (for review see Kornfeld and Sly, 1995). The opportunities for a lysosomally directed protein to interact with proteins bound for other subcellular compartments or synthesized on free ribosomes might be limited. On the other hand, there is evidence for alternate pathways to the lysosome, and indications that lysosomal proteins can at times be found freely soluble in the cytoplasm or elsewhere in the cell (as discussed by Kornfeld and Sly, 1995). The frequency of encounters between proteins predominately localized to the lysosome and other subcellular destinations therefore remains unknown.

To test the possibility of using fusion with a lysosomal protease as a general means of effecting dominant negative inhibition, we have constructed gene fusions to the carboxyl terminus of the lysosomal cysteine protease preprocathepsin B (CB).¹ We first made a CB fusion with the *E. coli lacZ* gene encoding β -galactosidase, a homotetrameric enzyme, and found that its expression inhibits β -galactosidase activity when cotransfected into mammalian cells with native lacZ. Both lysosomal routing and an intact protease active site appear required for dominant negative inhibition of the β -galactosidase tetramer assembled from native and CB-fusion lacZ subunits. As a specific application of this strategy, we have constructed a dominant negative version of the skeletal muscle bHLH transcription factor MyoD. We find that a CB-fusion with MyoD inhibits myogenesis, but achieves the effect through homodimerization with MyoD rather than heterodimerization with E12 or E47.

Materials and Methods

Plasmids

pCS2+, a simian CMV promoter/enhancer expression vector, has been described (Turner and Weintraub, 1994; Rupp and Weintraub, 1994) and with the noted derivatives were gifts from D. Turner (Fred Hutchinson Cancer Research Center [FHCRC], Seattle, WA). pCS2+CB and pCS2+CB-myc₆ were made by PCR of the mouse preprocathepsin B cDNA of pmCB58 (Chan et al., 1986; a gift of A. Frankfater, Loyola, Chicago, IL) from bases 35 to 1047 (GenBank M14222) with HindIII and ClaI sites incorporated into the upstream and downstream primers, respectively, then ligated in frame, respectively, into the corresponding sites of pCS2+ and pCS2+myc₆, containing a hexameric myc epitope tag. pCS2+ β gal encoding *E. coli lacZ*, and pCS2+NLS- β gal (referred to as pCS2+NLS/lacZ in this paper, for consistency), encoding an amino terminus fusion of the SV40 T-antigen nuclear localization signal with lacZ, have been described (Turner and Weintraub, 1994; Rupp and Weintraub, 1994). pCS2+CB-lacZ was made by ligation of a HindIII-BsaBI fragment containing the CB coding sequence of pCS2+CB into the HindIII/Klenow filled-in BamHI sites of pCS2+ β gal. pCS2+CB-lacZ-HA₆ was constructed by ligation of hexameric influenza virus HA epitope tag from pCS2+HA₆ (a gift of R. Kopan, Washington University, St. Louis, MO) into pCS2+CB-lacZ. pCS2+NLS-lacZ-myc₆ was constructed by ligating a BamHI-XbaI fragment containing the hexameric myc epitope tag from pCS2+myc₆ into the BglII and XbaI sites of pCS2+NLS- β gal. pCS2+myc₆-MyoD, a vector containing myc₆ epitope-tagged full-length mouse MyoD cDNA has been described (Turner and Weintraub, 1994; Rupp and Weintraub, 1994). pCS2+CB**lacZ*, containing a frame shift mutation leading to termination codons between the CB and lacZ domains was con-

structed by ligation of a HindIII-ScaI fragment of CB into the HindIII/Klenow filled-in BamHI sites of pCS2+ β gal. pCS2+CB(C108S)-lacZ, containing a TGT to AGC substitution at codon 108 of the CB active site, resulting in replacement of cysteine by serine, was constructed by oligonucleotide cassette mutagenesis spanning from the BanII to BsmI site of the coding sequence of pCS2+CB. pCS2+CB-myc₆-MyoD was made by ligating the EcoRI-XbaI fragment of pCS2+myc₆-MyoD into the corresponding sites of pCS2+CB-myc₆. pCS2+CB*MyoD, a control plasmid that contains a stop codon and lacks the myc epitope tag between the two domains, was constructed by ligating the ClaI-XbaI fragment of pCS2+MyoD into pCS2+CB, identical to pCS2+CB-myc₆, except for the absence of the myc epitope tag. pCS2+CB-MyoD, also lacking the myc epitope tag but containing a continuous open reading frame of the fused domains, was constructed by removing the stop codon of pCS2+CB*MyoD with ClaI digestion, Klenow fill-in, and self-ligation. pCS2+CB-MyoD Δ HLH, pCS2+CB-myc₆-MyoD Δ HLH, pCS2+CB-MyoD Δ b, and pCS2+CB-myc₆-MyoD Δ b were constructed by PCR amplification of mouse MyoD (using primers corresponding to sequence 160-176 and 1116-1099 of GenBank M18779 and incorporating EcoRI and XbaI sites, respectively) from the plasmids pEMSV-MyoD Δ HLH(143-162), containing a deletion of part of the loop and helix 2, and pEMSV-MyoD Δ b(102-114), containing a deletion of part of the basic region, (Davis et al., 1990; gifts of the late H. Weintraub, Fred Hutchinson Cancer Research Center), respectively, with cloning into the corresponding sites of pCS2+CB and pCS2+CB-myc₆, respectively. pCS2+CB-MyoD Δ C was constructed by PCR amplification of mouse MyoD (using primers corresponding to sequence 160-176 and 657-640 and incorporating EcoRI and XbaI sites, respectively) from the plasmid pEMSV-MyoD(TM167), containing a termination codon substituted for residue 167 (Weintraub et al., 1991; a gift of H. Weintraub, Seattle, WA), with cloning into the corresponding sites of pCS2+CB. (pCS2+CB-MyoD Δ C(167) also contains myc₆ and HA₆ epitope tags, although these features were not used for the present studies.) pSV-myc₆-E12, an expression vector for human E12 cDNA was a gift of Y. Zhuang (Duke, Durham, NC). pCS2+CB-myc₆-E12 and pCS2+CB-myc₆-E47 were constructed by PCR amplification of human E12 and E47 from pEMSV-E12 and pEMSV-E47 (gifts of H. Weintraub) at positions 10-27 and 1336-1218 (with respect to the E12 sequence of Murre et al., 1989a) with the incorporation of EcoRI and XbaI sites, respectively, and cloning into the corresponding sites of pCS2+CB-myc₆. EMSV-E12-MyoD(BJ) (Davis and Weintraub, 1992), a construct containing a MyoD basic DNA-binding domain fused to the E12 HLH dimerization domain, was a gift of H. Weintraub. pD-4R- β -gal, a reporter vector containing 4 E-boxes and a minimal HSV TK promoter, is identical to the p4R-cat reporter described by Weintraub et al., (1990), except for replacement of the cat reporter gene with β -galactosidase, that which was a gift from S. Tapscott (Fred Hutchinson Cancer Research Center). pEMSVscribe expression vector for mouse MyoD cDNA (pEMSV-MyoD) has been described (Davis et al., 1987) and were gifts of H. Weintraub. pEMSV-CB-MyoD was constructed by ligating a Klenow filled-in fragment of CB-MyoD from pCS2+CB-MyoD into the SmaI site of pEMSVscribe. Plasmids were purified on columns (Qiagen, Chatsworth, CA).

Cell Culture and Transfection

Hybridoma supernatants were a gift of H. Weintraub and P.-F. Cheng (FHCRC). Mouse NIH 3T3 cells (Amer. Type Culture Collection [ATCC], Rockville, MD) were grown in DMEM with 10% bovine calf serum, and mouse C2C12 myoblasts (ATCC) were grown in DMEM with 15% FCS. Penicillin/streptomycin (1%) was added to the media. Myoblast differentiation was induced 24 h after transfection by placing cells in DMEM with 2% heat inactivated horse serum for 48 h. 5×10^5 cells were seeded onto 60-mm plates and transiently transfected \sim 12 h later by CaPO₄ precipitation using Hepes buffered saline (Kriegler, 1990) with 10 μ g DNA (unless stated otherwise). The CaPO₄/DNA precipitate remained on the cells for 17 h, when the cells were fed with fresh media. When used, NH₄Cl was added to the media at a concentration of 10 mM at the conclusion of transfection.

Immunofluorescent Staining and Photomicroscopy

48 h following transfection, cells were fixed for 3 min with 50% methanol/50% acetone, and incubated for 60 min at room temperature with the appropriate antibody at the indicated dilution in PBS: 1:3 of 9E10 anti-myc epitope mouse hybridoma supernatant (as referenced in Turner and Weintraub, 1994), undiluted 5.8 anti-MyoD mouse hybridoma superna-

1. *Abbreviation used in this paper:* CB, protease cathepsin B.

tant (specific for MyoD carboxyl terminus residues 170-209; Dias et al., 1992), 1:200 rabbit polyclonal anti-MyoD (Tapscott et al., 1988; a gift of H. Weintraub and L. Snider, FHCRC), 1:200 rabbit polyclonal anti-HA epitope (BABC0), and 1:200 rabbit polyclonal anti-skeletal myosin (Sigma Chemical Co., St. Louis, MO). Secondary detection was by incubation for 30 min at room temperature with 20 μ g/ml fluorescein- or rhodamine-conjugated, noncross-reactive, donkey anti-mouse or anti-rabbit, respectively, antibodies (Jackson Labs., West Grove, PA). Nuclei were counterstained by incubation for 3 min in 0.5 μ g/ml DAPI in PBS. Epifluorescence and phase contrast photomicroscopy was performed on a Zeiss Photomicroscope III with 45-s exposure on Kodak Ektachrome P1600 film and processed by E6 protocol with 1 stop push. Transparencies were scanned onto a Kodak Photo CD and composited with Adobe Photoshop (to adjust size, brightness, and contrast) and Adobe Illustrator software for the Macintosh with output to a dye sublimation printer.

Cell Extracts and β -Galactosidase Assay

Cells were harvested for extraction 48 h following transfection. Whole cell extracts were made by freeze-thaw lysis as described (Sambrook et al., 1989). Nuclear and cytoplasmic extracts were prepared as described (Schreiber et al., 1989): the cells were lysed with NP-40 and the nuclei salt-extracted in the presence of the protease inhibitors 1 mM PMSF, 1% (vol/vol) aprotinin, and 10 μ g/ml Mg-132 (Myogenics, Cambridge, MA). β -Galactosidase assay was performed on ONPG substrate with spectrophotometric quantification of activity as described (Sambrook et al., 1989). Each experiment was performed in duplicate or triplicate, repeated 3 to 5 times on different days, and a standard error of the mean calculated.

DNA Binding Assays

In vitro transcription and translation was performed using the TNT coupled reticulocyte lysate system with the T3 RNA polymerase following the manufacturer's instructions (Promega, Madison, WI). All gene fusions were moved into the pEMSV-scribe vector (Davis et al., 1987) as the source of the DNA programming the reaction. Translation was verified by parallel reactions performed with [³⁵S]methionine with size-resolution of the products by SDS-PAGE and autoradiography (not shown). DNA binding assays were performed as described by Davis et al. (1990) using the MCK "R" enhancer 25-bp double-stranded oligonucleotide with the following modifications: a total of 4 μ l of in vitro synthesized protein was used for each binding assay; proteins were incubated for 90 min at 30°C before adding the DNA binding cocktail; binding reactions were performed for 45 min at 25°C.

Results

CB-lacZ Associates with lacZ

E. coli lacZ transfected into mammalian cells produces β -galactosidase that normally is cytoplasmically soluble (Sambrook et al., 1989). To determine the subcellular localization and activity of a polypeptide encoded by a gene fusion of the lysosomal cysteine protease preprocathepsin B with lacZ (CB-lacZ), we first transfected an expression vector encoding this chimera into NIH 3T3 cells. Immunofluorescent staining with β -galactosidase antibodies reveals that CB-lacZ is cytoplasmically distributed, in a perinuclear granular pattern consistent with lysosomal localization (not shown, but see below). XGAL staining and an assay of β -galactosidase activity of crude cell extracts on ONPG substrate are both negative (not shown). In contrast, native lacZ gives diffuse cytoplasmic staining with β -galactosidase antibodies and confers positive XGAL staining and easily detectable activity in crude cell extracts (not shown and Sambrook et al., 1989). We conclude that CB-lacZ has an abnormal subcellular distribution and fails to produce detectable β -galactosidase activity.

To determine if CB-lacZ and native lacZ subunits could

physically associate, we tested the effect of simultaneous expression of the two polypeptides. Equal quantities of expression vectors encoding both sequences were cotransfected into NIH 3T3 cells. To differentiate the two polypeptides, CB-lacZ was epitope tagged with a carboxyl terminus hexameric influenza virus hemagglutinin tag (HA₆), to produce CB-lacZ-HA₆, while the native lacZ was epitope tagged with a hexameric myc epitope tag (myc₆) at its carboxyl terminus, to produce lacZ-myc₆. We were unable to discern a difference in staining pattern when cells transfected with both constructs were simultaneously immunofluorescently stained with antibodies directed against both epitope tags (not shown).

We judged that it would be difficult to discriminate between diffuse cytoplasmic staining and a lysosomal staining pattern. To therefore make any effects on subcellular redistribution more pronounced, we added an SV40 T-antigen nuclear localization signal (NLS) to the native lacZ subunit, to produce NLS-lacZ-myc₆. (NLS-lacZ-myc₆ results in nuclear localized β -galactosidase as judged by immunofluorescent staining with either myc epitope antibody or β -galactosidase antibodies or XGAL staining [Rupp and Weintraub, 1994; see the following].) Transient cotransfection of equal quantities of expression vectors for both constructs into NIH 3T3 cells with simultaneous immunofluorescent staining with mouse monoclonal myc epitope antibody (and secondary detection with fluorescein-conjugated goat anti-mouse serum) and rabbit polyclonal HA antibody (and secondary detection with rhodamine-conjugated goat anti-rabbit serum) reveals that both the CB-lacZ-HA₆ and the NLS-lacZ-myc₆ polypeptides are distributed in the cytoplasm in many of the cells (Fig. 1, top row). (A similar pattern was observed by staining with β -galactosidase antibody, not shown, confirming that the localization of the epitope tag is the same as the rest of the polypeptide.) In contrast, control cotransfection of vectors expressing NLS-lacZ-myc₆ with CB-HA₆ fails to result in the redistribution of nuclear-localized lacZ (Fig. 1, middle row). (Note that not all cells express both plasmids.) We conclude that CB-lacZ-HA₆ is able to effect the nuclear to cytoplasmic redistribution of NLS-lacZ-myc₆, presumably through the assembly of a β -galactosidase tetramer composed of both types of subunits.

CB-lacZ Inhibits β -Galactosidase Activity

We next tested whether cotransfection with CB-lacZ was capable of inhibiting the β -galactosidase activity that results from transfection with NLS-lacZ (Fig. 2A). A 2:1 mass ratio of plasmid DNA encoding CB-lacZ and NLS-lacZ, respectively, were transiently cotransfected into NIH 3T3 cells. Whole cell, nuclear, and cytoplasmic extracts were prepared and β -galactosidase activity was measured by spectrophotometric assay on ONPG substrate. Cotransfection with CB-lacZ, but not the control plasmid CB*lacZ containing a termination codon (*) between the two domains, inhibits about half of the β -galactosidase activity in the whole cell extracts as well as in the nuclear and cytoplasmic fractions. (Some β -galactosidase activity presumably "leaks" into the cytoplasm.) Similar results (not shown) were obtained with cotransfection of CB-lacZ and lacZ (without the NLS).

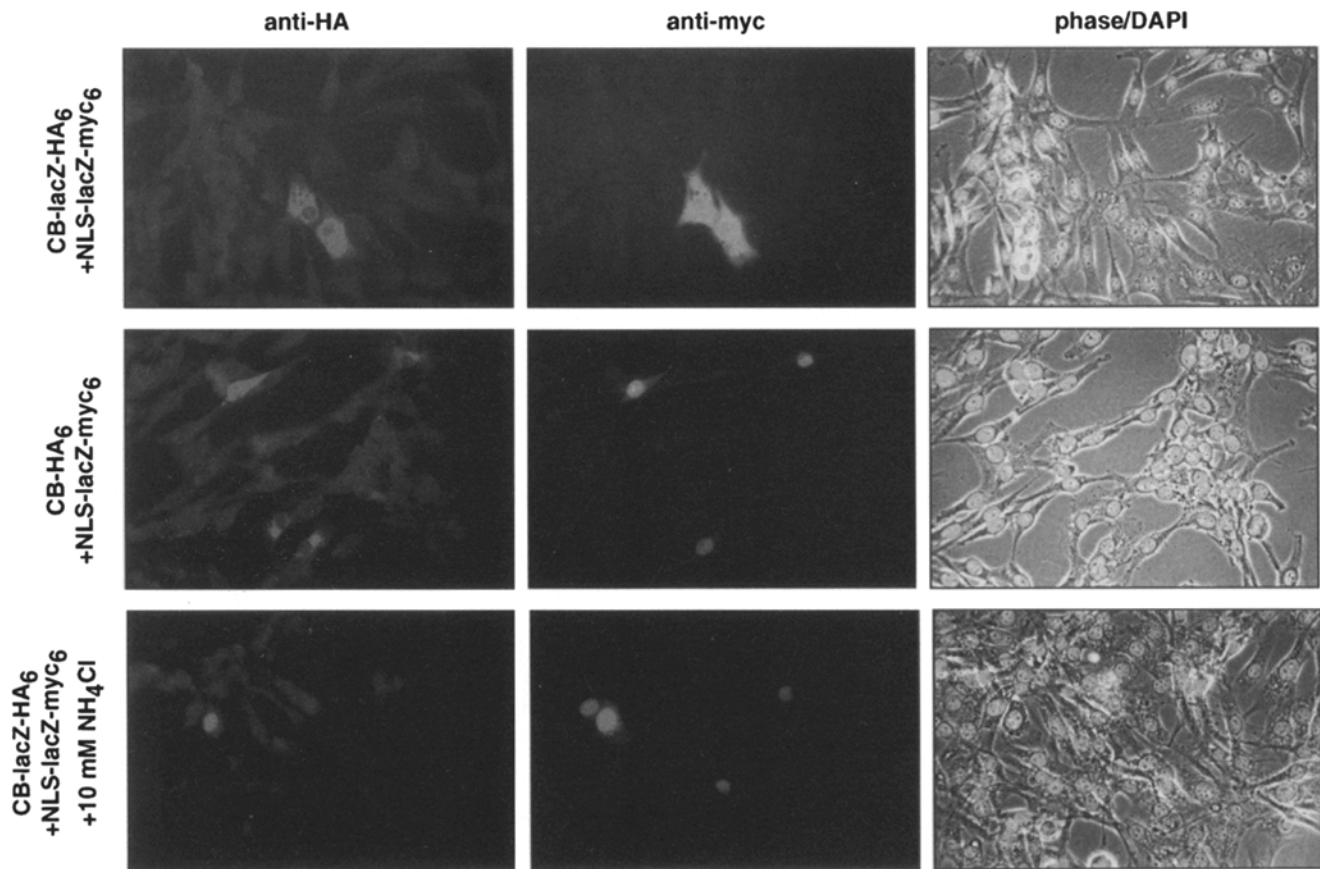


Figure 1. CB-lacZ-HA₆ redistributes NLS-lacZ-myc₆, but not in the presence of NH₄Cl. CB-lacZ-HA₆ or the control construct CB-HA₆ were transiently cotransfected with NLS-lacZ-myc₆ at a ratio of 5 μg:5 μg into NIH 3T3 cells, and simultaneously immunofluorescently stained with 9E10 mouse monoclonal anti-myc epitope with secondary fluorescein detection and rabbit polyclonal anti-HA epitope with secondary rhodamine detection in the absence or presence (for experiments with CB-lacZ-HA₆ only) of the lysosomal transport inhibitor NH₄Cl. Nuclei were DAPI counterstained.

Dominant Negative Inhibition Requires Lysosomal Routing

We sought to determine if lysosomal localization is required for the dominant negative inhibition. The most direct approach would be to delete the lysosomal localization signal from the CB coding sequence. A discrete lysosomal localization signal, however, has yet to be defined for CB (Sloane, B., personal communication). An alternative experiment is based on the observation that treatment of cells with NH₄Cl blocks lysosomal targeting by inhibition of the mannose-6-phosphate dependent pathway (as reviewed in Kornfeld and Sly, 1995). We repeated the above cotransfection experiments in the presence of NH₄Cl. Simultaneous immunofluorescent staining (Fig. 1, *bottom row*) shows that cotransfection of CB-lacZ-HA₆ with NLS-lacZ-myc₆ in the presence of NH₄Cl fails to result in the cytoplasmic redistribution of NLS-lacZ; in fact, the reverse situation occurs, and the CB-lacZ-HA₆ appears to now redistribute from the cytoplasm to the nucleus under these conditions. Assay of β-galactosidase activity confirms that CB-lacZ no longer achieves dominant negative inhibition in the presence of NH₄Cl (Fig. 2 *B*). We conclude that lysosomal localization of the CB fusion is required for dominant negative inhibition.

Dominant Negative Inhibition Requires CB Protease Activity

We next addressed if lysosomal localization alone is sufficient to achieve dominant negative inhibition, or whether the protease function of CB contributes to inhibition. CB protease activity requires an active site cysteine; mutation to serine results in an inactive enzyme (Rowan et al., 1992). We mutated the CB active site in CB-lacZ replacing the cysteine with a serine, to produce CB(C108S)-lacZ. The mutation has no effect on the subcellular distribution of the fusion protein, as determined by immunofluorescent staining with an epitope tagged derivative (not shown). The mutation did impair the ability of the fusion protein to inhibit β-galactosidase activity when cotransfected with NLS-lacZ in NIH 3T3 cells (Fig. 2 *C*). We conclude that proteolysis of the substituted tetramer by CB also contributes to the mechanism of dominant negative inhibition.

At Least Two Subunits of the β-Galactosidase Tetramer Must Be Substituted

We next asked how many subunits of the tetramer must be substituted with the CB fusion polypeptide in order to inactivate the holoenzyme. This was addressed by titrating the

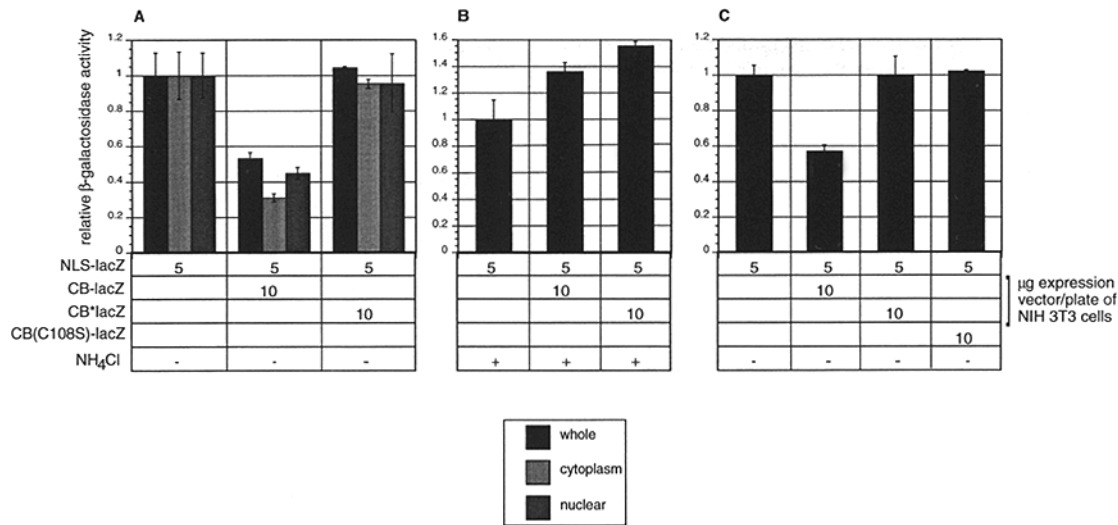


Figure 2. (A) Quantification of inhibition of β -galactosidase activity. NIH 3T3 cells were transiently transfected with the indicated quantities of vectors and whole cell, nuclear, and cytoplasmic extracts were prepared. β -Galactosidase activity was assayed spectrophotometrically on ONPG substrate and reported as normalized values. (B) No inhibition of β -galactosidase activity by CB-lacZ in the presence of the lysosomal transport inhibitor NH_4Cl . β -Galactosidase activity was assayed in whole cell extracts and normalized values are shown. (Overall β -galactosidase activity, even produced by transfection of NLS-lacZ alone, was reduced in the presence of NH_4Cl .) (C) No inhibition of β -galactosidase activity by CB-lacZ(C108S) in which the active site cysteine has been mutated. The conditions are as described above.

ratio of CB-lacZ to NLS-lacZ expression vector transfected into NIH 3T3 cells and determining the shape of the inhibition curve. The possible combinations and permutations of tetramers resulting from the two types of subunits represent a binomial expansion (Fig. 3 A). Assuming that the concentration of each subunit is proportionate to the amount transfected, that the probability of association of the two subunits is proportionate to their respective concentrations, and that β -galactosidase activity is proportionate to the concentration of active tetramer; then, the indicated equation (Fig. 3 B), derived from the binomial expansion, predicts resulting β -galactosidase activity for any value of r , where r is an integer equal to the number of CB-lacZ subunits tolerable in an active tetramer (Fig. 3 C). (Note that an additional, unproven assumption is that the stoichiometry of the β -galactosidase enzyme remains unchanged when CB-lacZ subunits are incorporated into the multimer.) Obviously, if $r = 4$ (that is, $(\text{CB-lacZ})_4$ retains activity), then β -galactosidase activity additively increases as more CB-lacZ is transfected into the cells (Fig. 3 D). Similarly, note that if $r = 3$ or $r = 2$ (that is, a tetramer substituted with three or two CB-lacZ subunits, respectively, retains activity), then inhibition also cannot be achieved. The results of the titration experiment are shown in Fig. 3 E. The best curve fit of the data was obtained using the indicated equation at values of $r = 1$ with a relative dissociation constant k for CB-lacZ of 0.59 ± 0.08 . We conclude that there is tolerance for one CB-lacZ subunit in an active tetramer, but that a tetramer substituted with two or more CB-lacZ subunits is inactive. We also conclude that steric hindrance from the CB domain in the CB-lacZ polypeptide contributes little to inhibition, as the calculated relative dissociation constant for CB-lacZ is near unity.

Cis Dominance of Lysosomal Routing to Nuclear Localization for MyoD

As a first application of this method of dominant negative mutation, we investigated the subcellular distribution and dimerization properties of MyoD. We first determined if the lysosomal targeting signal could function in dominance to nuclear localization signals (encoded in two motifs within the MyoD bHLH [Vandromme et al., 1995]) when present together on a single polypeptide. This is of importance to the premise of this strategy that CB fusion can be used as a dominant negative inhibitor in the absence of structural and functional knowledge of the gene to be studied. If the lysosomal localization signal does not act dominantly to other subcellular localization signals when present together on a single polypeptide, then these signals would first have to be identified and deleted for the method to work. We constructed an epitope tagged fusion of CB with the full-length MyoD protein coding sequence, CB-myc₆-MyoD. As shown by immunofluorescent staining for the myc epitope of transiently transfected mouse NIH 3T3 cells (lacking expression of endogenous MyoD) in Fig. 4, myc₆-MyoD (*top row*) appears nuclear, CB-myc₆ (*bottom row*) appears cytoplasmic (presumably lysosomal), and CB-myc₆-MyoD (*middle row*) is similarly cytoplasmic, indicating the *cis* dominance of lysosomal targeting. (Simultaneous staining with MyoD antibodies results in an identical pattern, not shown, indicating that the staining pattern is unlikely to be the result of a protein processing event that has caused the epitope tag to dissociate from the MyoD domain.) A corroborating result is that the SV40 T-antigen nuclear localization signal fused amino terminal to epitope tagged preprocathepsin B also produces a similar pattern of cytoplasmic staining (not shown). Although we cannot be certain that the CB-fusion pro-

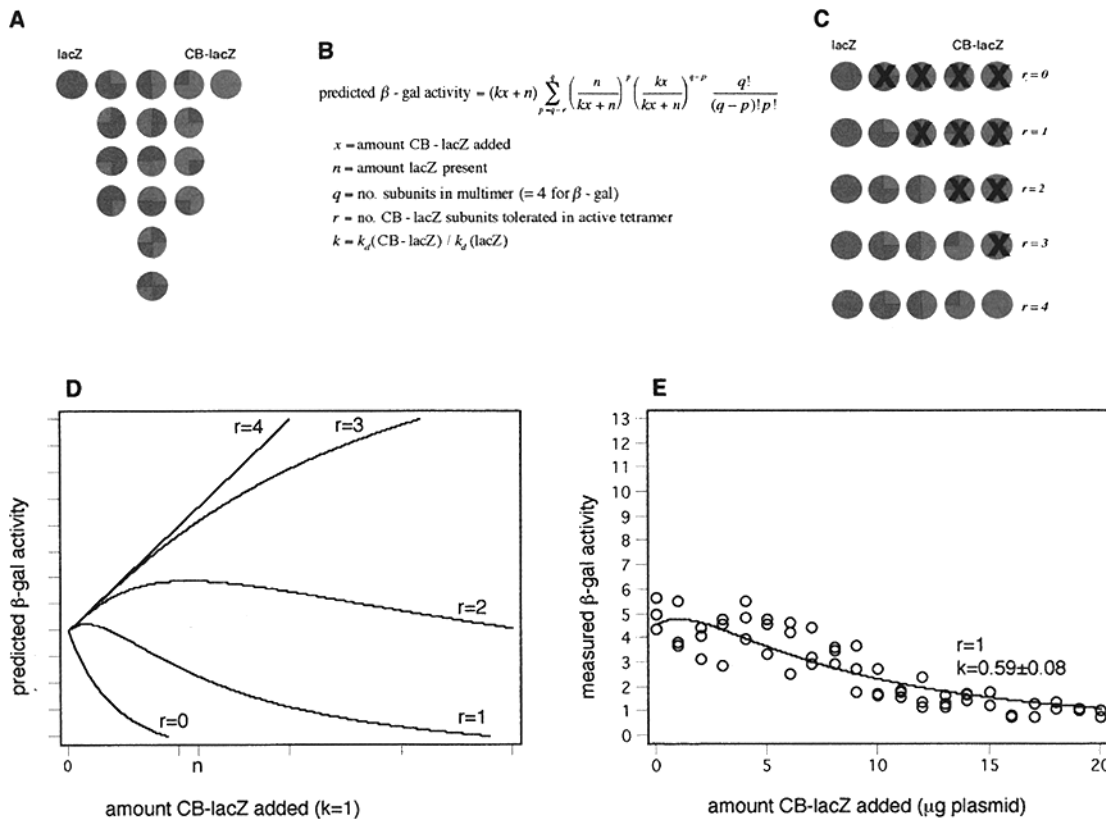


Figure 3. (A) Possible combinations and permutations of native and CB-fusion lacZ subunits in the tetramer. (B) Equation predicting β -galactosidase inhibition for differing number of CB-substituted subunits tolerable in the tetramer (r). (C) Graphical definition of r as the number of CB-substituted subunits tolerable in the tetramer. An "X" indicates an inactive tetramer. (D) Predicted shape of the titration curve for differing r values. (E) Titration experiments. 5 μg of NLS-lacZ expression vector were transiently transfected into NIH 3T3 cells with the indicated quantity of CB-lacZ expression vector (0–20 μg). β -Galactosidase activity was assayed from whole cell extracts and plotted relative to the quantity of vector added. Optimal values of r and k were determined by curve-fitting following the Davidson-Fletcher-Powell algorithm, using MacCurveFit software (Kevin Raner Software, Victoria, Australia). The correlation coefficient R^2 for quality of fit is 0.74. The experiment was performed once with integral quantities of CB-lacZ expression vector from 0 to 16 μg and repeated in duplicate on a different day for each of integral quantities of vector from 0 to 20 μg .

teins are located in the lysosome, the lysosomal localization signal, when present on the same polypeptide with a nuclear localization signal, does appear to result in at least the cytoplasmic redistribution of a nuclear protein.

Trans-Diversion of MyoD to the Cytoplasm

MyoD forms homodimers and also forms heterodimers with the ubiquitously expressed and alternately spliced products, E12 and E47, of the E2A gene (as reviewed by Münsterberg and Lassar, 1994; Edmondson and Olson, 1993). We next tested if CB-MyoD could divert homodimerizing native MyoD away from the nucleus (Fig. 5 A). Double immunofluorescent staining for the myc epitope tag and MyoD reveals that transient cotransfection of untagged CB-MyoD with myc₆-MyoD (Fig. 5 A, top row) results in the redistribution of some fraction of myc epitope-tagged MyoD to the cytoplasm in many of the cells. Cotransfection with the control construct CB*MyoD, identical except for a stop codon between the two domains, has no effect on MyoD nuclear localization (Fig. 5 A, second row). To verify that dimerization with CB-MyoD is required to redirect the native MyoD, we constructed CB-MyoD fusions

in which we deleted either the HLH dimerization domain or the basic DNA-binding region of MyoD. (Neither MyoD Δ HLH nor MyoD Δ b are active; MyoD Δ HLH does not form dimers while MyoD Δ b forms dimers but does not bind DNA [Davis et al., 1990].) Cotransfection with CB-MyoD Δ HLH, in which a portion of the HLH domain has been deleted, has no effect on MyoD nuclear localization (Fig. 5 A, third row) whereas CB-MyoD Δ b, in which part of the basic region has been deleted, retains the ability to redirect native MyoD to the cytoplasm (Fig. 5 A, bottom row). Surprisingly, myc epitope-tagged E12 remains exclusively nuclear in the presence of cotransfection with the untagged CB-MyoD chimera (Fig. 5 B, top row). Similar results were obtained with E47 (not shown). Conversely, cotransfection of CB-myc₆-E12 with MyoD also failed to divert MyoD from the nucleus (Fig. 5 B, bottom row).

In Vitro Dimer Formation by CB-MyoD with MyoD or E12

A potential explanation for why CB-MyoD fails to demonstrate intracellular interaction with E12 and E47 may be that the dimerization potential of the CB fusion protein is

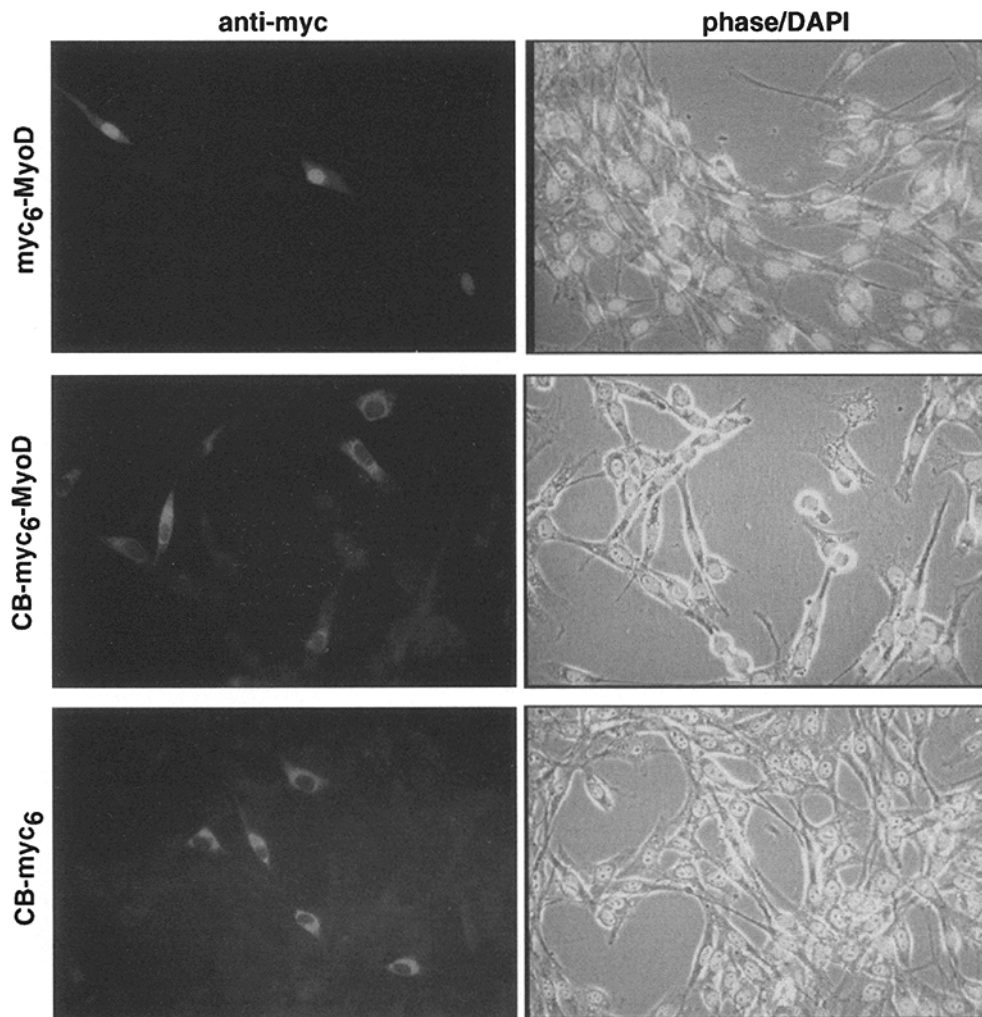


Figure 4. Dominance of lysosomal localization signal to MyoD nuclear localization. Plasmids expressing the indicated epitope-tagged proteins were transiently transfected into NIH 3T3 cells and immunofluorescently stained with mouse monoclonal 9E10 anti-myc epitope with secondary fluorescein detection. Nuclei were DAPI counterstained.

altered. To test this, we performed electrophoretic mobility shift assays using an E-box DNA probe and *in vitro* synthesized proteins. In agreement with prior observations (Davis et al., 1990), we find that MyoD (Fig. 6, lane 2) or E12 bind DNA (Fig. 6, lane 3) individually, but, when combined, bind DNA more efficiently (Fig. 6, lane 4). Because the shifted complex migrates to a position intermediate between that of either MyoD or E12 alone, these results have been taken as evidence that the preferential DNA-binding species is a MyoD/E12 heterodimer (Davis et al., 1990). In contrast with MyoD, we find that CB-MyoD fails to bind DNA by itself (Fig. 6, lane 1). When CB-MyoD is combined with E12 (Fig. 6, lane 5), DNA binding is increased, in comparison to E12 or MyoD alone, just as it is with native MyoD. Additionally, we note that the presumed CB-MyoD/E12 heterodimer migrates to a position between that of the E12 complex alone and the MyoD/E12 complex, confirming that the synergistic effect is achieved through heterodimerization with CB fusion protein. We conclude that, although CB-MyoD is incapable of binding DNA by itself, it retains the ability to form DNA-binding dimers with native E12. Therefore, the failure to observe *in vivo* interaction between CB-MyoD and E12 does not result from artifactual changes in dimerization capability.

Inhibition of C2C12 Myoblast Differentiation

We then determined whether the CB-myc₆-MyoD chimera could inhibit myoblast differentiation *in vitro*. Mouse C2C12 myoblasts, which endogenously express MyoD, were transfected with CB-myc₆-MyoD, CB-myc₆, or myc₆-MyoD, serum-starved to induce differentiation, and then doubly immunofluorescently stained with antibodies against the epitope and the differentiation marker skeletal myosin (Fig. 7 A). The transfected cells are distinguishable by their staining with the anti-epitope antibody. Differentiation is evident by myosin expression and the formation of multinucleated myotubes. After transfection with CB-myc₆ (Fig. 7 A, top row), the majority of myc epitope positive cells (72%, Fig. 7 B) have differentiated as judged by their formation of myosin positive, multinucleated myotubes. Differentiation is accelerated in cells transfected with myc₆-MyoD (Fig. 7 A, bottom row), as almost all the myc epitope positive cells were fused in myosin positive myotubes (92%, Fig. 7 B). Differentiation is reduced in cells transfected with CB-myc₆-MyoD (Fig. 7 A, middle row), as few myc epitope positive cells (22%, Fig. 7 B) were fused in myosin positive myotubes. As a definitive control, we also tested for an inhibitor effect of CB-MyoD-myc₆-ΔHLH (Fig. 7 C, top row) and CB-myc₆-MyoDΔb

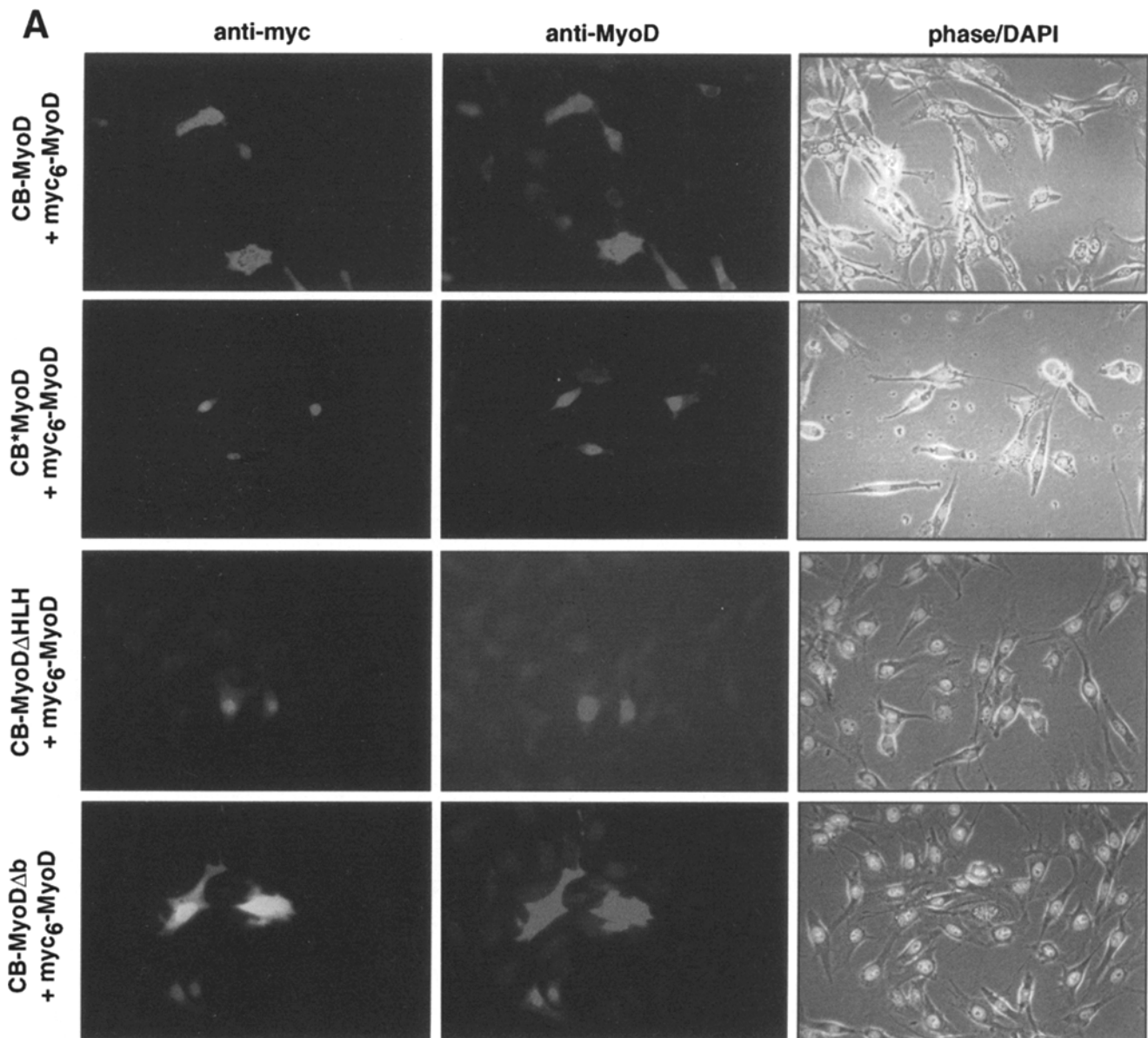


Figure 5. (A) CB-MyoD redistributes epitope-tagged MyoD to the cytoplasm. Plasmids expressing the indicated epitope-tagged proteins were transiently cotransfected with untagged CB-MyoD at a ratio of 5 μ g:5 μ g into NIH 3T3 cells, and simultaneously immunofluorescently stained with 9E10 mouse monoclonal anti-myc epitope with secondary fluorescein detection and rabbit polyclonal anti-MyoD with secondary rhodamine detection. Nuclei were DAPI counterstained. (B) CB-MyoD fails to redistribute epitope-tagged E12 and epitope-tagged E12-CB fails to redistribute untagged MyoD. The plasmids were transfected and cells stained as above. (Note that for the experiment of the bottom row, in contrast to those of the first five rows of the figure, the 9E10 mouse monoclonal anti-myc epitope detects the CB-fusion protein while the rabbit polyclonal anti-MyoD detects the target protein being tested for redistribution.)

(Fig. 7 C, bottom row). Of the two, only CB-myc₆-MyoD Δ b inhibited differentiation (52% differentiation vs 18% differentiation, respectively, Fig. 7 B), confirming that the dominant negative effect is mediated through HLH region dimerization but unaffected by the presence of the DNA-binding basic region. We also found that the CB-MyoD chimera inhibits myogenic conversion of NIH 3T3 cells when cotransfected with MyoD (not shown). In agreement with the previous results, neither CB-myc₆-E12 (Fig. 7 D, top row) nor CB-myc₆-E47 (Fig. 7 D, bottom row) inhibits differentiation, as the expression of these transfected chi-

meras is largely found in differentiated myotubes (68% and 76%, respectively, Fig. 7 B).

To confirm that the inhibition of differentiation by the CB-fusion results from direct inhibition of MyoD, we constructed CB-MyoD Δ C, in which the carboxyl terminus of MyoD has been deleted (beginning at amino acid 167). Removal of this portion of MyoD has little effect on activity (Weintraub et al., 1991), but the deleted carboxyl terminus does contain an epitope (spanning from amino acids 170-209) recognized by the MyoD monoclonal antibody 5.8 (Dias et al., 1992). In C2C12 myoblasts transfected

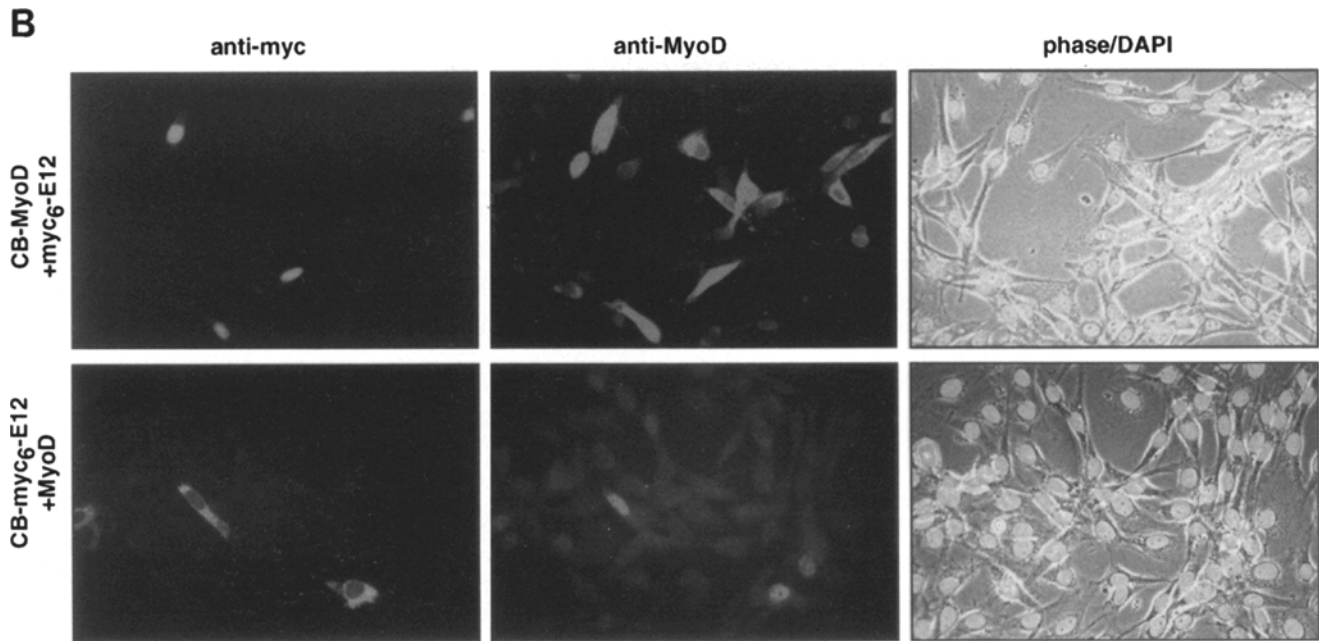


Figure 5.

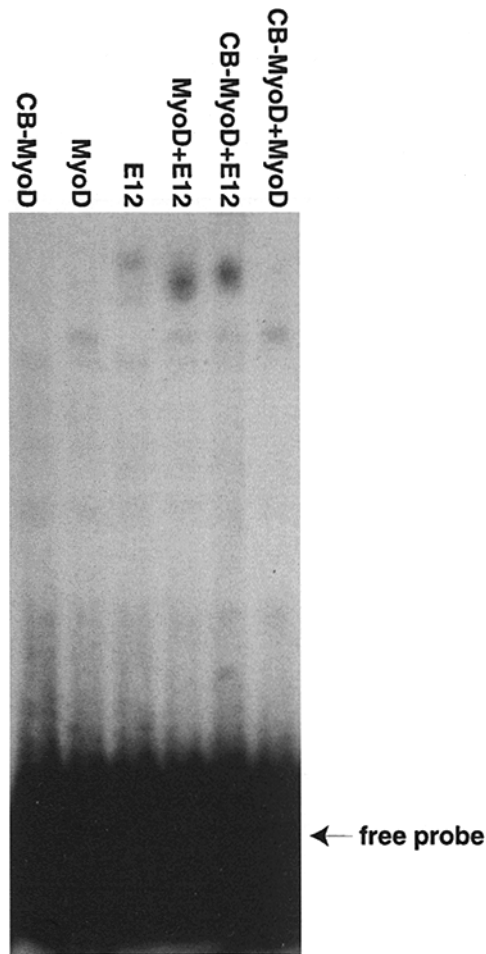


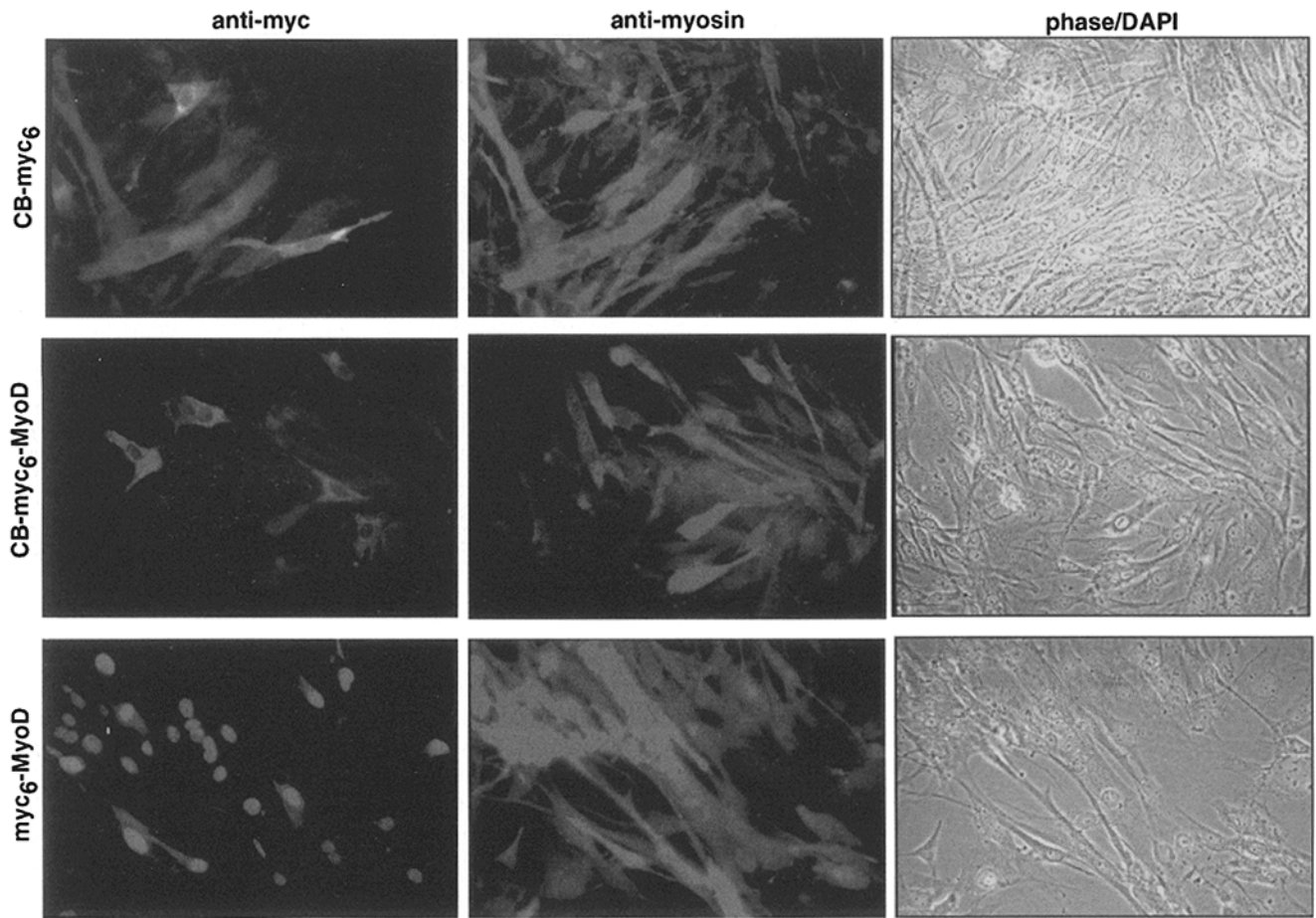
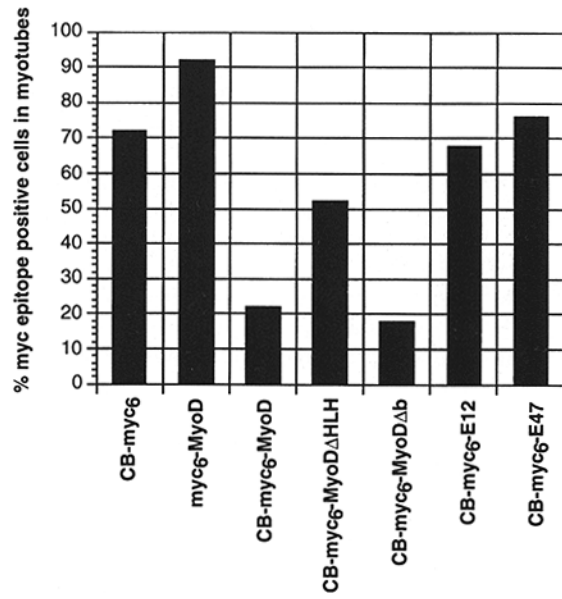
Figure 6. Electrophoretic mobility shift assay with CB-MyoD. The indicated proteins were synthesized in vitro and tested for their ability to shift an E box-containing ^{32}P -labeled double-stranded oligonucleotide probe, as shown in this autoradiogram.

with CB-MyoDAC, it should be possible by immunofluorescent staining to distinguish the endogenous MyoD (which is detectable both with monoclonal 5.8 and polyclonal anti-MyoD) from the CB-MyoDAC (which should be detectable with the polyclonal antibody, but not 5.8). Transfection of CB-MyoDAC into NIH 3T3 cells (Fig. 7 E, top row), which do not express endogenous MyoD, confirms that the fusion protein can be immunofluorescently stained with the polyclonal MyoD antibody (here detected secondarily with rhodamine), but not the 5.8 monoclonal antibody (here detected secondarily with fluorescein). CB-MyoDAC was transfected into C2C12 myoblasts, serum-starved to induce differentiation, then simultaneously stained with polyclonal antibody to MyoD and the 5.8 monoclonal antibody (with secondary rhodamine and fluorescein detection, respectively). As shown in Fig. 7 E (bottom row), the transfected cells are easily distinguishable using the polyclonal MyoD antibody on account of the bright, cytoplasmic staining pattern. Staining with monoclonal antibody 5.8 confirms that the endogenous MyoD is redistributed to the cytoplasm in the cells transfected with CB-MyoDAC.

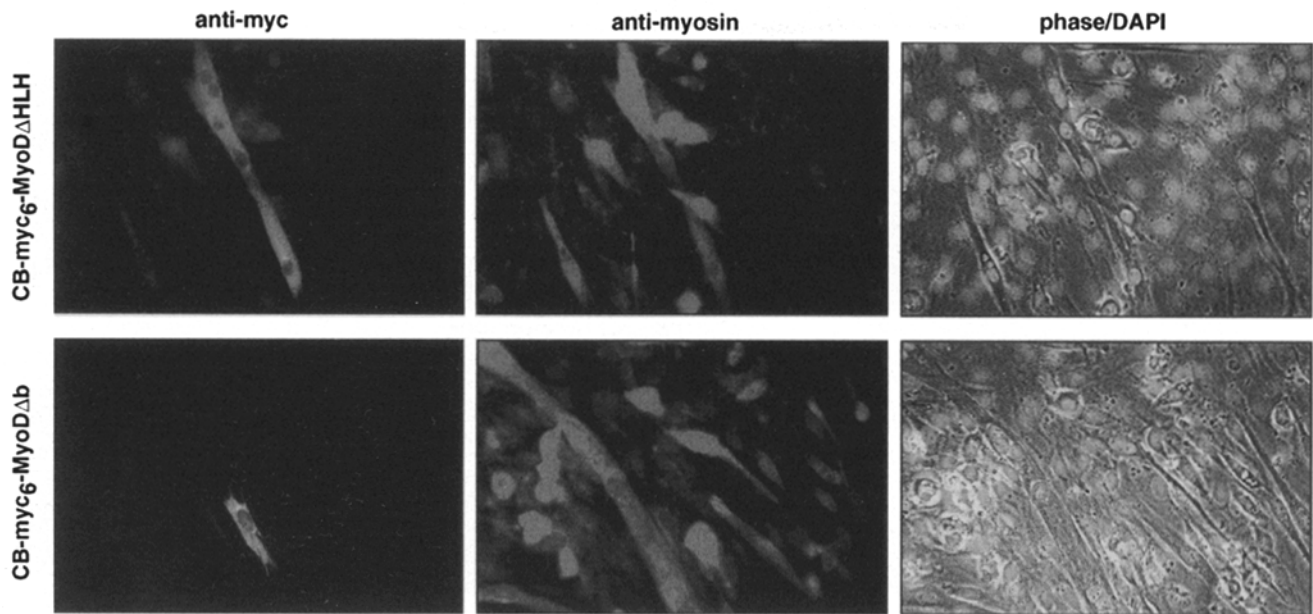
Furthermore, most of the cells transfected with CB-MyoDAC appear mononuclear ($\sim 80\%$), consistent with their failure to differentiate, presumably as a consequence of inhibition of MyoD.

Inhibition of MyoD trans-Activation of an E-box Containing Reporter

To confirm direct inhibition of MyoD *trans*-activation, we tested (Fig. 8) the ability of the CB-MyoD fusion to directly inhibit *trans*-activation of a reporter (containing the minimal "4R" enhancer (Weintraub et al., 1990), composed of four catenated MyoD E-box DNA sequences, and the thymidine kinase basal promoter). Under the transient assay conditions employed, cotransfection with MyoD results in about a threefold induction of β -galac-

A**B***Figure 7.*

C



D

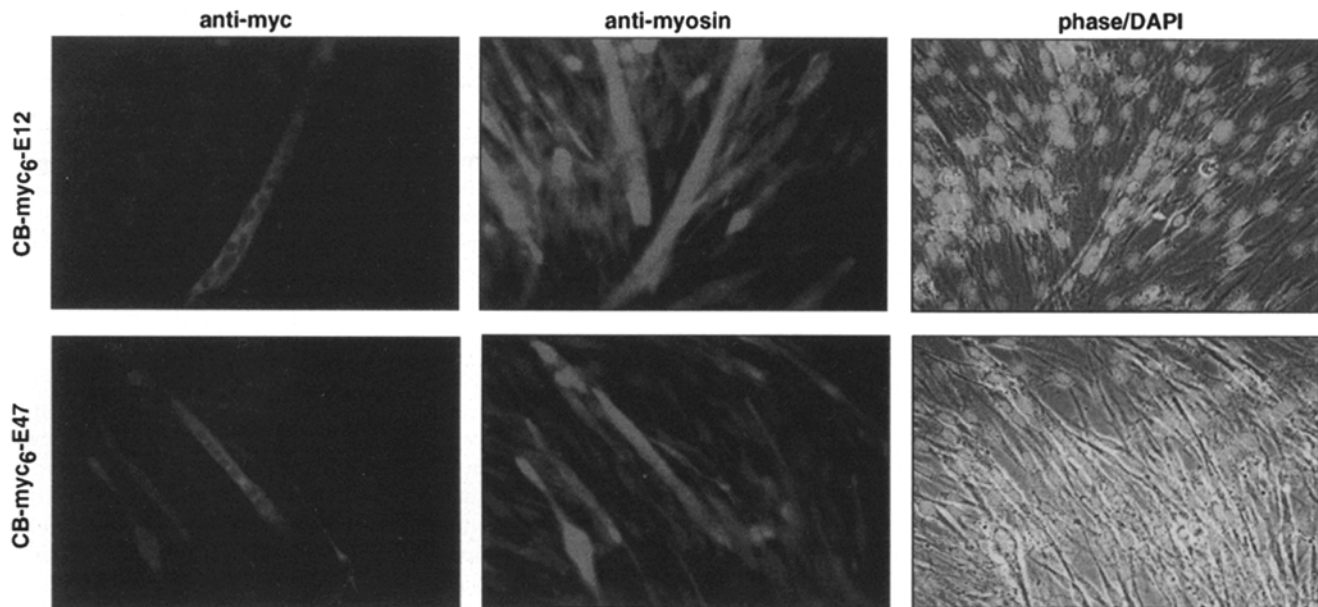


Figure 7.

tosidase activity (lane *D*) when normalized to control cotransfection with an empty expression vector (lane *A*). (Note that in order to accommodate an excess of the CB-MyoD expression vector, we have reduced the quantity of the MyoD expression vector compared to that typically used in assays producing greater levels of induction by MyoD (see for example, Weintraub et al., 1991).) MyoD activation of 4R is inhibited by the addition of CB-MyoD in a dose-dependent manner (lanes *E* and *F*), but it is not inhibited by the addition of the stop codon-containing control CB*MyoD (lanes *G* and *H*). Under the conditions of lane *F*, CB-MyoD abolishes *trans*-activation by MyoD.

Not only does CB-MyoD alone fail to function as an activator (lane *B*), but it also modestly suppresses basal activity of the reporter (compared to control lane *C*), conceivably through inactivation of interacting constitutive transcription factors. In agreement with the colocalization data of Fig. 2, the CB-MyoD chimera does not inhibit the activity of E12-MyoD(BJ) (Davis and Weintraub, 1992), a construct containing a MyoD basic DNA-binding domain fused to the E12 HLH dimerization domain, suggesting that the two do not interact (lanes *I*–*K*). Taken together with the previous results, we conclude that CB-MyoD inhibits myoblast differentiation by interfering with the tran-

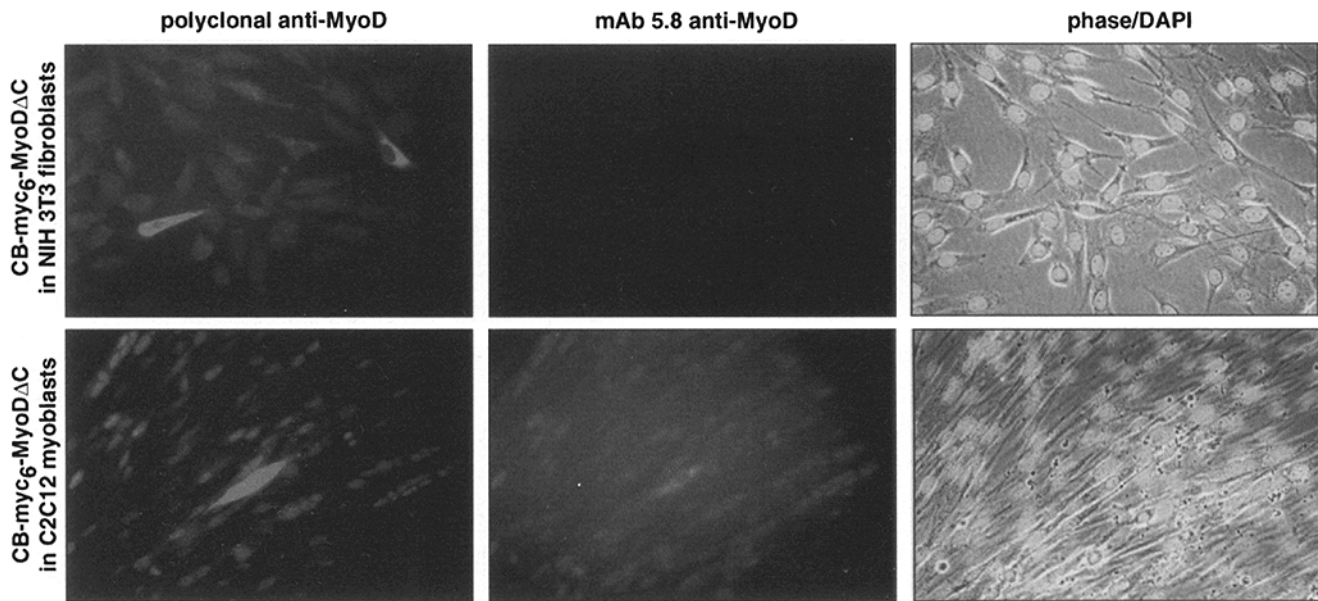
F

Figure 7. (A) CB-myc₆-MyoD inhibits myoblast differentiation. Plasmids expressing the indicated proteins were transiently transfected into mouse C2C12 myoblasts and serum starved to induce differentiation. The cells were then simultaneously immunofluorescently stained with 9E10 mouse monoclonal anti-myc epitope with secondary fluorescein detection, to identify transfected cells, and rabbit polyclonal anti-skeletal myosin with rhodamine detection, to distinguish differentiated myotubes. Nuclei were DAPI counterstained. Representative results are shown. (B) Quantification of differentiation for the experiments reported in this figure. 50 cells staining positively with anti-myc epitope were randomly examined; the percentage that are myosin positive myotubes with three or more nuclei is reported. (C) CB-myc₆-MyoD Δ H_{LH} does not inhibit differentiation while CB-myc₆-MyoD Δ b does. Transfection and staining were as above. (D) CB-myc₆-E12 and CB-myc₆-E47 do not inhibit differentiation. Transfection and staining were as above. (E) Redistribution of endogenous MyoD demonstrated with CB-MyoD Δ C, deleted in the epitope recognized by the 5.8 mouse monoclonal anti-MyoD. NIH 3T3 cells, which lack endogenous MyoD expression, and C2C12 myoblasts, which express endogenous MyoD, were transiently transfected with the construct and simultaneously stained with the rabbit polyclonal anti-MyoD with secondary rhodamine detection, which recognizes both endogenous MyoD and the transfected CB-fusion, and the 5.8 mouse monoclonal anti-MyoD with secondary fluorescein detection, which recognizes endogenous MyoD, but not the transfected CB-fusion. The C2C12 cells were serum-starved to induce differentiation following transfection. Nuclei were DAPI counterstained.

scriptional activation of native MyoD independently of association with E12 or E47.

Discussion

We demonstrate dominant negative inhibition of tetrameric β -galactosidase by a CB-lacZ fusion polypeptide and dimeric MyoD by a CB-MyoD fusion polypeptide. Substitution with two or more CB-fusion subunits of the tetrameric β -galactosidase inactivates enzymatic activity, as determined by titration experiments, and substitution of one subunit necessarily abrogates the transcriptional activity of dimeric MyoD. At least in the case of β -galactosidase, the inhibitor effect appears to result from a combination of subcellular redistribution to lysosomal pathways and direct proteolysis of the holoenzyme by the CB domain. Steric hindrance of multimer assembly by the CB domain appears negligible. It is perhaps surprising that a lysosomal protein should have the opportunity to associate with a protein localized in another subcellular compartment, such as the cytoplasm or the nucleus. There are several potential reasons why our method nevertheless succeeds. First, it is likely that lysosomal trafficking is a dynamic process and that proteins are continuously entering,

and, possibly, exiting the lysosome through at least several different routes. Support for this is found in the observations that some cell types have mannose-6-phosphate independent pathways to the lysosome (Owada and Neufeld, 1982; Waheed et al., 1982; Rijnboutt et al., 1991), and some proteins, such as acid phosphatase, are routed to the lysosome independently of phosphorylation (Waheed et al., 1988). Second, under some circumstances, CB is found diffusely localized in the cytoplasm (Amano et al., 1995; Terada et al., 1995; Campo et al., 1994; Visscher et al., 1994), and not invariably in the lysosome, potentially increasing the likelihood to interact with other proteins. Third, the gene fusions used in these experiments may result in proteins with unusual patterns of subcellular localization. For example, the CB-MyoD construct, which contains both nuclear localization and lysosomal localization signals is obviously cytoplasmic, but from the staining pattern the precise location with respect to the lysosome, Golgi, ER, and perinuclear region is unclear. Therefore, the CB-fusion protein could conceivably cross paths at different times with proteins trafficked to both the lysosome and the nucleus. Fourth, the transfection conditions here likely result in supraphysiologic levels of expression, and it is possible that the usual cellular compartments for CB-

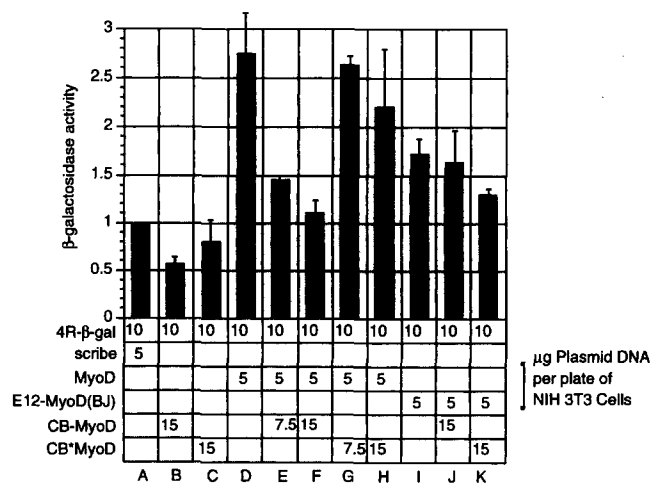


Figure 8. CB-MyoD inhibits *trans*-activation of a reporter by MyoD but not E12-MyoD(BJ). MyoD or E12-MyoD(BJ) fusion constructs encoded in EMSV-scribe expression vector (5 μ g) were transiently cotransfected into NIH 3T3 cells with plasmids expressing either CB-MyoD or the stop codon-containing CB*MyoD control (7.5 or 5 μ g of either) and 4R- β -gal reporter (10 μ g). Induction is normalized in each experiment by parallel cotransfection with 4R- β -gal reporter (10 μ g) and the empty scribe vector (5 μ g) to which the β -galactosidase activity is arbitrarily assigned a value of 1 (lane A). The reported β -galactosidase activity for lanes B–K is thus expressed as a ratio of the activity for the conditions in lane A.

lacZ, CB-MyoD, and NLS-lacZ (wherever they may be) have become saturated so that these polypeptides artifactually interact at sights within the cell where the cognate proteins are not normally found. While this suggests caution in the interpretation of the biological relevance of our results, the pertinence of our findings is underscored by the experiments reported in Fig. 7 E, which demonstrate that CB-MyoD Δ C is, in fact, able to associate with endogenous MyoD to inhibit differentiation in C2C12 myoblasts.

We propose this approach as a simple and potentially general strategy for engineering dominant negative mutation. Like antisense, it may be suitable even in the absence of extensive structural and functional knowledge of the protein to be studied, because the lysosomal localization signal in CB acts in *cis* dominance to nuclear localization signals. It is possible that a dominant negative inhibitor for any protein can be manufactured simply by fusing the coding sequence of the subject gene downstream from that of preprocathepsin B. A specific application may be the construction of fusion cDNA libraries that present all the cell's proteins as chimeras with CB. These libraries might allow for identification of genes by their ability to inhibit a particular activity. Although we have not yet tested the dominance of lysosomal routing to other subcellular localization signals, in preliminary experiments with cDNA fusion libraries, we find that after screening several hundred epitope-tagged random clones comprised of a cDNA fragment fused to CB, almost all direct localization of the encoded fusion protein is to the cytoplasm or, more specifically, the lysosome. This suggests that the lysosomal localization signal possibly acts in *cis* dominance to all

other subcellular localization signals. Another application may be in the development of "comtoxins" (Varshavsky, 1995), drugs composed of two moieties: in this case, a lysosomal targeting signal fused to an interaction domain. Some proteins, in addition to entering the lysosome cotranslationally within the cell, may enter the lysosome from the extracellular media through the cell surface via an endosomal route (as reviewed by Kornfeld and Sly, 1995). This observation identifies a potential means for therapeutic delivery of an engineered polypeptide or small molecule through a lysosomal cell surface receptor. This could be used, for example, to design a parenterally administrable drug targeting the destruction of a cellular molecule, such as a particular membrane-bound receptor, through lysosomal sequestration. A different approach for designing dominant negative inhibitors in the absence of extensive structural and functional knowledge of the targeted protein is known as "genetic suppressor elements" (Roninson, 1995); it consists of randomly fragmenting genes, expressing libraries of the sense strand, and selecting the resulting oligopeptides capable of acting as dominant negative inhibitors.

In our initial application of this strategy, we have investigated the dimerization properties of MyoD. We find that CB-MyoD is an effective inhibitor of *in vitro* myoblast differentiation, and that this results from the specific inhibition of MyoD's function as a transcriptional activator. We have previously reported that because MyoD is an auto-regulated protein, the steady-state level of MyoD is exquisitely sensitive to small fluctuations in the half-life of the protein (Horwitz, 1996). Thus, even a small and transient proteolytic destabilization of MyoD can impede differentiation by permanently switching off the MyoD auto-activation circuit and the cross-activation circuits with myogenin (as discussed in Horwitz, 1996).

Surprisingly, the inhibitor effect does not appear to be mediated through heterodimerization with E12 or E47. There is no evidence for association between CB-MyoD with E12 or CB-E12 or CB-E47 with MyoD: CB-MyoD fails to inhibit *trans*-activation by a chimeric E12 substituted with the MyoD basic region, and CB-E12 and CB-E47 fail to inhibit myoblast differentiation. Despite our results, irrefutable evidence does support heterodimerization of MyoD with E12 or E47 (Lassar et al., 1991; Murre et al., 1989b): they can be coimmunoprecipitated; they associate and synergistically bind DNA in gel shift experiments; and, they synergistically activate transcription of a reporter. Additionally, a covalently coupled heterodimer formed from a MyoD-E47 gene fusion is a potent activator, resistant to titration by the endogenous, dominant negative inhibitor Id (Neuhold and Wold, 1993).

In performing immunoprecipitation experiments, it is possible that subcellular compartments become homogenized, while a potentially unique advantage of our studies may be the opportunity to observe the associations of compartmentalized proteins in intact cells. Our data support the unanticipated possibility that, at least in some compartments of the cytoplasm, MyoD more avidly homodimerizes than heterodimerizes. A possible explanation for this preference could be that heterodimerization and nuclear transport are coupled, as has been proposed for the bHLH arylhydrocarbon receptor and arnt dimeriza-

tion partner, in particular (Reyes et al., 1992), and more generally for other bHLH heterodimers (Goldfarb and Lewandowska, 1994). There is also evidence that heterodimerization is catalytically facilitated (Thayer and Weintraub, 1993) in a process biochemically dependent upon phosphorylation (Lassar et al., 1991) or disulfide reduction (Benezra, 1994). Therefore, heterodimerization might be regulated through many means, including appropriate compartmentalization of the two factors before nuclear transport. A recent report suggests that one mechanism for accomplishing this may be through MyoD micelle formation (Laue et al., 1995). At least in solution, MyoD can form micelles composed of an average of ~120 MyoD molecules. Mixed micelles with E47 do not form, but the addition of E47 results in the stoichiometric formation of nonmicellar heterodimers. If micelle formation occurs intracellularly, then a single CB-MyoD monomer might have an opportunity to interact with hundreds of native MyoD monomers, while it is relatively unlikely to interact with an E47 or E12 monomer (and vice-versa for CB-E12 or CB-E47). Our experiments provide no direct evidence for micelle formation, but they are consistent with the physical segregation of MyoD from its heterodimerization partners before nuclear transit.

We caution that some of the unique properties of our experimental conditions may limit the application of this method for other proteins. The autoregulation of MyoD makes it exquisitely sensitive to small changes in its stability (Horwitz, 1996), and its possible potential to self-aggregate in large complexes could potentiate the dominant negative effect. More specifically, as demonstrated with β -galactosidase and detailed in Fig. 3, the method is best suited for multimers with a large number of subunits (high q) and a poor tolerance for CB substituted subunits (low r). Since complete inhibition would likely seldom be possible, the method also requires that a measurable phenotype result from moderate reductions in the level of the targeted protein.

We thank Joseph Gogos, Rafi Kopan, and Ralf Schreck for discussion and Steve Hauschka, Lawrence Loeb, and Stan Fields for manuscript criticism.

This work is supported by grants from the Markey Foundation and National Institute of Child Health and Human Development HD01080-03.

Received for publication 10 June 1996 and in revised form 10 September 1996.

References

- Amano, T., H. Nakanish, T. Kondo, T. Tanaka, M. Oka, and K. Yamamoto. 1995. Age-related changes in cellular localization and enzymatic activities of cathepsins B, L and D in the rat trigeminal ganglion neuron. *Mech. Ageing Dev.* 83:133-141.
- Benezra, R. 1994. An intermolecular disulfide bond stabilizes E2A homodimers and is required for DNA binding at physiological temperatures. *Cell* 79:1057-1067.
- Buonocore, L., and J.K. Rose. 1990. Prevention of HIV-1 glycoprotein transport by soluble CD4 retained in the endoplasmic reticulum. *Nature (Lond.)* 345:625-628.
- Campo, E., J. Munoz, R. Miquel, A. Palac'in, A. Cardesa, B.F. Sloane, and M.R. Emmert-Buck. 1994. Cathepsin B expression in colorectal carcinomas correlates with tumor progression and shortened patient survival. *Am. J. Pathol.* 145:301-309.
- Chan, S.J., B. San-Segundo, M.B. McCormick, and D.F. Steiner. 1986. Nucleotide and predicted amino acid sequences of cloned human and mouse procathepsin B cDNAs. *Proc. Natl. Acad. Sci. USA* 83:7721-7725.
- Davis, R.L., and H. Weintraub. 1992. Acquisition of myogenic specificity by replacement of three amino acid residues from MyoD into E12. *Science (Wash. DC)* 256:1027-1030.
- Davis, R.L., P.-F. Cheng, A.B. Lassar, and H. Weintraub. 1990. The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. *Cell* 60:733-746.
- Davis, R.L., H. Weintraub, and A.B. Lassar. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* 51:987-1000.
- Dias, P., D.M. Parham, D.N. Shapiro, S.J. Tapscott, and P.J. Houghton. 1992. Monoclonal antibodies to the myogenic regulatory protein MyoD1: epitope mapping and diagnostic utility. *Cancer Res.* 52:6431-6439.
- Edmondson, D.G., and E.N. Olson. 1993. Helix-loop-helix proteins as regulators of muscle-specific transcription. *J. Biol. Chem.* 268:755-758.
- Goldfarb, A.N., and K. Lewandowska. 1994. Nuclear redirection of a cytoplasmic helix-loop-helix protein via heterodimerization with a nuclear localizing partner. *Exp. Cell Res.* 214:481-485.
- Herskowitz, I. 1987. Functional inactivation of genes by dominant negative mutation. *Nature (Lond.)* 329:219-222.
- Horwitz, M. 1996. Hypermethylated myoblasts specifically deficient in MyoD autoactivation as a consequence of nuclear instability of MyoD. *Exp. Cell Res.* 226:170-182.
- Kornfeld, S., and W.S. Sly. 1995. I-cell disease and pseudo-Hurler polydystrophy: disorders of lysosomal enzyme phosphorylation and localization. In *The Metabolic and Molecular Bases of Inherited Disease*. Scriver, C.R., A.L. Beaudet, W.S. Sly, and D. Valle, editors. McGraw-Hill, New York, pp. 2495-2508.
- Kriegler, M. 1990. *Gene Transfer and Expression, A Laboratory Manual*. W.H. Freeman, New York, pp. 96-98.
- Lassar, A.B., R.L. Davis, W.E. Wright, T. Kadesch, C. Murre, A. Voronova, D. Baltimore, and H. Weintraub. 1991. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. *Cell* 66:305-315.
- Laue, T.M., M.A. Starovasknik, H. Weintraub, X.H. Sun, L. Snider, and R.E. Klevit. 1995. MyoD forms micelles which can dissociate to form heterodimers with E47: implications of micellization on function. *Proc. Natl. Acad. Sci. USA* 92:11824-11828.
- Lin, X., A. Dashti, R.F. Schinazi, and J. Tang. 1993. Intracellular diversion of glycoprotein GP160 of human immunodeficiency virus to lysosomes as a strategy of AIDS gene therapy. *FASEB (Fed. Am. Soc. Exp. Biochem.) J.* 7: 1070-1080.
- Münsterberg, A.E., and A.B. Lassar. 1994. Wiring diagrams: regulatory circuits and the control of skeletal myogenesis. *Curr. Opin. Cell Biol.* 6:651-660.
- Murre, C., P.S. McCaw, and D. Baltimore. 1989a. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell* 56:777-783.
- Murre, C., P.S. McCaw, H. Vaessin, M. Caudy, L.Y. Jan, Y.N. Jan, C.V. Cabrera, J.N. Buskin, S.D. Hauschka, A.B. Lassar, et al., 1989b. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* 58:537-544.
- Neuhold, L.A., and B. Wold. 1993. HLH forced dimers: tethering MyoD to E47 generates a dominant positive myogenic factor insulated from negative regulation by Id. *Cell* 74:1033-1042.
- Owada, M., and E.F. Neufeld. 1982. Is there a mechanism for introducing acid hydrolases into liver lysosomes that is independent of mannose 6-phosphate recognition? Evidence from I-Cell disease. *Biochem. Biophys. Res. Commun.* 105:814-820.
- Reyes, H., S. Reisz-Porszasz, and O. Hankinson. 1992. Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. *Science (Wash. DC)* 256:1193-1195.
- Rijnboutt, S., A.J. Kal, H.J. Geuze, H. Aerts, and G.J. Srou. 1991. Mannose 6-phosphate-independent targeting of cathepsin D to lysosomes in HepG2 cells. *J. Biol. Chem.* 266:23586-23592.
- Roninson, I.B., A.V. Gudkov, T.A. Holzmayer, D.J. Kirschling, A.R. Kazarov, C.R. Zelnick, I.A. Mazo, S. Axenovich, and R. Thimmapaya. 1995. Genetic suppressor elements: new tools for molecular oncology. *Cancer Res.* 55: 4023-4028.
- Rowan, A.D., P. Mason, and J.S. Mort. 1992. Rat procathepsin B. proteolytic processing to the mature form in vitro. *J. Biol. Chem.* 267(22):15993-15999.
- Rupp, R.A., and H. Weintraub. 1994. Xenopus embryos regulate the nuclear localization of XMyoD. *Genes Dev.* 8:1311-1323.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 18-34, 16.66-16.67.
- Schreiber, E., P. Matthias, M.M. Muller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids. Res.* 17:6419.
- Smith, D.H., R. Byrn, S.A. Marsters, T. Gregory, J.E. Groopman, and D.J. Capon. 1987. Blocking of HIV-1 infectivity by a soluble, secreted form of the CD4 antigen. *Science (Wash. DC)* 238:1704-1707.
- Tapscott, S.J., R. Davis, M.J. Thayer, P.F. Cheng, H. Weintraub, and A.B. Lassar. 1988. MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts. *Science (Wash. DC)* 242:405-411.
- Terada, T., T. Ohta, H. Minato, and Y. Nakanuma. 1995. Expression of pancreatic trypsinogen/trypsin and cathepsin B in human cholangiocarcinomas and hepatocellular carcinomas. *Hum. Pathol.* 26:746-752.
- Thayer, M.T., and H. Weintraub. 1993. A cellular factor stimulates the DNA-binding activity of MyoD and E47. *Proc. Natl. Acad. Sci. USA* 90:6483-6487.
- Turner, D.L., and H. Weintraub. 1994. Expression of achaete-scute homolog 3

- in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* 8:1434-1447.
- Vandromme, M., J.C. Cavadore, A. Bonnieu, A. Froeschl'e, N. Lamb, and A. Fernandez, A. 1995. Two nuclear localization signals present in the basic-helix 1 domains of MyoD promote its active nuclear translocation and can function independently. *Proc. Natl. Acad. Sci. USA.* 92:4646-4650.
- Varshavsky, A. 1995. Codominance and toxins: a path to drugs of nearly unlimited selectivity. *Proc. Natl. Acad. Sci. USA.* 92:3663-3667.
- Visser, D.W., B.F. Sloane, M. Sameni, J.W. Babiarz, J. Jacobson, and J.D. Crissman. 1994. Clinicopathologic significance of cathepsin B immunostaining in transitional neoplasia. *Mod. Pathol.* 7:76-81.
- Wagner, R.W. 1995. The state of the art in antisense research. *Nat. Med.* 1: 1116-1118.
- Waheed, A., S. Gottschalk, A. Hille, C. Krentler, R. Pohlmann, T. Bräulke, H. Hauser, H. Geuze, and K. von-Figura. 1988. Human lysosomal acid phosphatase is transported as a transmembrane protein to lysosomes in transfected baby hamster kidney cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2351-2358.
- Waheed, A., R. Pohlmann, A. Hasilik, K. von Figura, A. van Elsen, and J.G. Leroy. 1982. Deficiency of UDP-N-acetylglucosamine:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase in organs of I-cell patients. *Biochem. Biophys. Res. Commun.* 105:1052-1058.
- Weintraub, H., R. Davis, D. Lockshon, and A. Lassar. 1990. MyoD binds cooperatively to two sites in a target enhancer sequence: occupancy of two sites is required for activation. *Proc. Natl. Acad. Sci. USA.* 87:5623-5627.
- Weintraub, H., V.J. Dwarki, I. Verma, R. Davis, S. Hollenberg, L. Snider, A. Lassar, and S.J. Tapscott. 1991. Muscle-specific transcriptional activation by MyoD. *Genes Dev.* 5:1377-1286.