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Myostatin Dominant Negative Allele Products Interact Positively with Wild Type Monomers

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ABSTRACT

Myostatin is an extracellular negative regulator of muscle growth with an important role in bovine muscular hypertrophy. It belongs to the transforming growth factor β (TGFβ) superfamily, and has structural and functional characteristics similar to those of its other members. Based on these characteristics, we designed three gene constructs in order to create a series of dominant negative (DN) alleles for murine myostatin. As a first requirement for any DN strategy, we first showed that each of the three mutant DN monomers were able to interact with wild type mature myostatin (wt-Mstn), both in a pull-down and a mammalian two-hybrid assay. In addition, the degree of DN-Mstn/wt-Mstn interaction was similar to that of wt-Mstn/wt-Mstn. These results suggest that the three designed alleles are good candidates for use in a DN-based strategy for generating muscular hypertrophy in cattle.

Key Words: Mutagenesis; Dominant negative alleles; Myostatin; Muscular hypertrophy; Bovine.

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INTRODUCTION

Myostatin is a recently discovered member of the TGFβ superfamily. It is highly conserved across species and acts as a negative regulator of muscle cell growth. In myostatin-null mice it causes a dramatic increase in muscle mass via the hyperplasia and hypertrophy of muscle cells (1). Inactivating mutations of myostatin in different cattle breeds leads to the ''double-muscled'' phenotype, which confers enlarged musculature through hyperplasia of the muscle fibers (2–4), significantly increasing the economic value of these animals. Along with the other TGFβ family members, myostatin shares a conserved pattern of nine cysteine residues in its carboxy terminus following a proteolytic processing target (RSRR). After translation, intermolecular disulphide bridges link two monomers of TGFβ leading to dimeric precursor molecules. These are delivered to the Golgi apparatus where they are proteolytically cleaved by an endoprotease (5). Following this, the latent amino-terminal peptide (LAP) and the carboxy terminal structures remain attached until activated by further proteolytic cleavage, which releases the mature, active, homodimeric complex (6). The activated protein is then able to bind TGFβ type II receptors, starting a cascade of events that transmits a signal to the target genes in the nucleus (5). All of the described mutations leading to double muscling in cattle are recessive (4), indicating that crosses between a double-muscled and a wild type animal will always produce calves without the hypertrophied phenotype. In an agricultural context, dominant negative (DN) alleles may be of great importance in the production of double-muscled individuals, but DN alleles are not common in nature. The few examples known include collagen COL1A1 (7) and the interferon gamma receptor gene IFNγR1 (8). Several TGFβ superfamily members have already been modified, however, to introduce DN mutations, e.g., TGFβ-1 (9), activin (10), and BMP-4 and BMP-7 (11). The use of artificial DN alleles in cattle would allow the limitations of double muscle production to be overcome.

Different DN alleles were constructed to test the ability of their products to interact with wild type monomers and eventually block mature heterodimers through stearic hindrance. The ability of DN construct products to interact with wild type monomers is the first requirement of any DN strategy and is the focus of this article. We designed three DN constructs in mice, the expression products of which interacted with the wild type myostatin monomer to produce a heterodimer potentially unable to bind to the TGFβ type II receptor because of stearic hindrance. The ability of the three dominant negative monomers to interact with wild type myostatin monomers was examined in a pull-down assay and a mammalian two-hybrid assay.

MATERIALS AND METHODS

Mutagenesis and Cloning of Myostatin Variants (pSG5PL/DN Constructs: Stop, ProtSite, and C340) and Expression in a Reticulocyte Lysate

Stop Construct

The first and second Stop codons were modified to become tryptophan and lysine codons, respectively, using site-directed mutagenic overlapping PCR (12) with primer

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Table 1. Oligonucleotides used for mutagenesis of Stop and ProtSite constructions.

OL2Stop.for:	CCGCTGTGGGTGCTCATGGGCTTTGCATAAGCTTAGAAAT
OL2Prot.for:	CAAGAGGGCCCCGATAGACTTTGGGCTTGACTGCGATGAG

Note: Modified nucleotides are underlined.

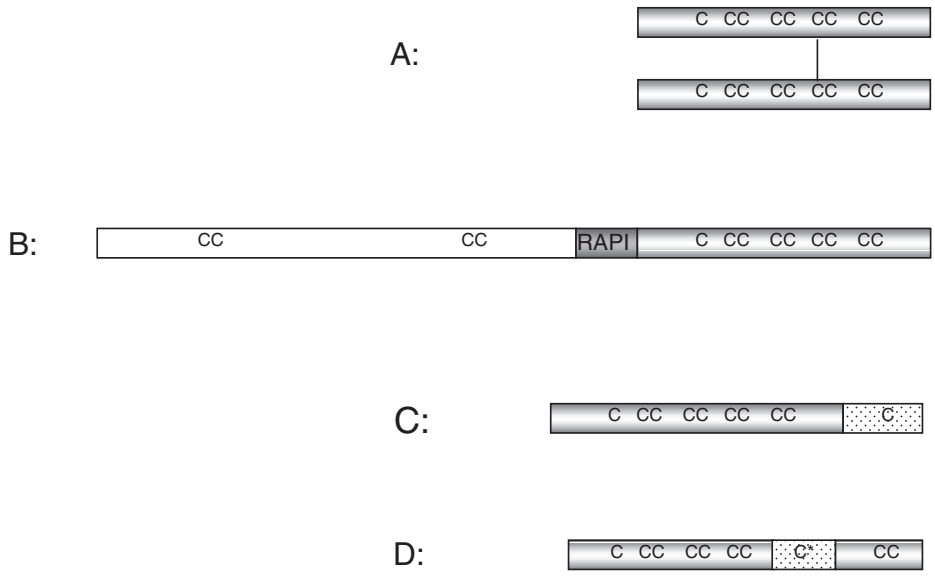

Figure 1. Structure of wild type and DN monomers of myostatin. (A) Carboxy terminal region of wild type myostatin, represented as a dimer. (B) ProtSite mutant; white region represents the latent peptide. (C) Stop mutant; dashed region represents the newly introduced fragment. (D) C340 mutant; dashed region represents the newly introduced fragment; C^* represents the new interchain cysteine.

OL2Stop.for (Table 1), which contains the mentioned modifications. The final overlapped PCR product was digested with *Kpn*I and *Bam*HI and cloned in the pSG5PL expression vector (a modified polylinker variant of pSG5; Stratagene). These modifications increase the length of the protein by 29 amino acids (27% of its original length). This was designed to cause stearic hindrance when linked to the wild type monomer, preventing the heterodimer from binding to the TGFβ receptor (Fig. 1).

ProtSite Construct

The normal cleavage site, amino acid sequence RSRR, was altered to RAPI using the overlapping technique (12). *Overlapping1* was generated using *murine pectoralis* muscle RNA and the Titan One-Tube RT-PCR System (Roche). Modifications were introduced with primer OL2Prot.for (Table 1). The final overlapped PCR products spanning amino acids 23–376 were cloned in pSG5PL.

This modification triples the length of the final protein, the hoped-for dominant negative effect being produced by stearic hindrance during binding to the TGFβ receptor (Fig. 1).

C340 Construct

A 22 amino acid–long fragment (330–351) containing a second inter-chain cysteine (C^{340}) residue was inserted between amino acids 349 and 350. Fragment C1 (amino acids 268–351) was digested with *Ava*I and *Kpn*I, fragment CC (amino acids 330–351) with *Kpn*I and *Xma*CI, and fragment C2 (amino acids 349–376) with *Xma*CI and *Bam*HI. The three fragments were cloned together in pSG5PL in the order C1-CC-C2. The inserted fragment provides the possibility of allowing heterotrimers to form through the duplication of the sequence responsible for monomer interaction. The DN condition is also produced by stearic hindrance (Fig. 1).

After screening for modified clones, one was chosen for each variant and completely sequenced (ABI 3100 Avant, Applied Biosystems) to confirm mutagenesis.

In vitro–translated, ^{35}S-labeled myostatin DN variants were prepared separately in a rabbit reticulocyte lysate (Promega) using L-[^{35}S]methionine (Amersham) according to the manufacturer's instructions. These were resolved by 15% SDS-PAGE under reducing conditions and visualized by autoradiography.

Expression and Purification of His-Tagged Wild Mature Myostatin Protein (His-wtMstn)

The mouse DNA myostatin coding sequence spanning amino acids 268–376 was amplified and cloned into the pRSET-A vector (Invitrogen) in frame with six histidine residues of the plasmid. A BL21(DE3)pLys *E. coli* strain (Novagen) was transformed with the resulting construct. An overnight culture harboring the recombinant wild myostatin expression vector was diluted and expression induced by adding 1 mM isopropyl thio-β-galactoside to 10 mL of the culture. Induction was allowed to proceed for 5 h. Cells were collected by centrifugation, resuspended in 400 μL of lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 8.0) and centrifuged. The supernatant was saved. To check for correct expression, samples were resolved by 15% SDS-PAGE and transferred to a PVDF membrane. Blocking of the membrane was achieved overnight in TBS 5% milk. Primary antibody mouse monoclonal anti-His_6 (Roche) and secondary anti-IgGmurine-AP antibody (Novagen) were used for immunodetection. Colorimetric reactions were performed using NBT/BCIP (Roche).

Hundred microliters of His-tagged wild type myostatin were bound to 25 μL of Ni-NTA Agarose (Qiagen) with 25 μL of a protease inhibitor cocktail (Complete Mini, Roche) in PBS, rotating for 1 h at room temperature. The product was washed three times with washing buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 6.3).

In Vitro Binding of Wild Type Myostatin to Stop, ProtSite, and C340

Fifty microliters of blocked Ni-NTA agarose-myostatin and 70 μL of PBS with protease inhibitors were incubated separately with 20 μL of each of the three reticulocyte lysate DN variants at 4°C overnight using a mixing wheel. Binding reactions were washed twice with PBS containing protease inhibitors to eliminate any unbound protein. Unbound Ni-NTA agarose was used in parallel as a negative control. Samples were boiled with loading buffer to release complexes of copurified proteins, resolved by 15% SDS-PAGE under reducing conditions, and visualized by autoradiography.

Mammalian Two-Hybrid Study

The TOPO® Tools Mammalian Two Hybrid Kit (Invitrogen, Carlsbad, CA) was used with some modifications to create ''bait'' (DNA binding domain) and ''prey'' (transcriptional activation domain) DNA constructs. The three elements to be joined to the gene of interest (Activation, Binding, and 3′ Element) were amplified by PCR and then digested. The P_{SV40}-GAL4 5′ binding domain element and P_{SV40}-VP16 5′ activation elements were digested with *EcoR*I and *BamH*I; the 3′ element was digested with *Not*I and *Pst*I. The murine myostatin DNA sequence spanning amino acids 268–376 was amplified by PCR and digested with *BamH*I and *Not*I to create wild type myostatin. Each of the three DN variants were amplified by PCR using pSG5PL/DN constructs as templates, and digested with *Bam*HI and *Not*I. The P_{SV40}-GAL4 5′ binding domain element, wild type myostatin, and 3′ element were ligated and cloned into pGEM®-3Zf(+) (Promega) to create BD-wtMstn. The P_{SV40}-VP16 5′ activation element, wild myostatin, and 3′ element were ligated and cloned into pGEM®-3Zf(+) to create AD-wtMstn. The P_{SV40}-VP16 5′ activation element, each of the three modified myostatins, and the 3′ element were also ligated and cloned into pGEM®-3Zf(+) to create AD-ProtSite, AD-Stop, and AD-C340, respectively.

CHO-K1 cells (ACC 110, DSMZ) were maintained in Ham's F12 medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 250 ng/mL of amphotericin B at 37°C in a humidified 5% CO_2 atmosphere. BD-wtMstn (150 ng) and 150 ng of each of the AD constructs were cotransfected with 120 ng of the reporter plasmid pGAL/lacZ into CHO-K1 cells using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfection was performed in quadruplicate. After 6 h the medium was changed to fresh maintenance medium. After 48 h, three wells for each tested interaction were assayed for β-galactosidase activity by monitoring hydrolysis of ortho-nitrophenyl-β-D-galactopyranoside (ONPG) (Invitrogen), according to the manufacturer's instructions. A further well was fixed for *in situ* staining for β-galactosidase activity. The absorbance (A_{420}) of three different volumes of cell lysate was measured for each of the wells to monitor the hydrolysis of ONPG. Results were normalized with respect to cell number and corrected by

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subtracting the background nontransfected control. A Student *t* test was performed to check for any significant difference in absorbance between wt-Mstn/wt-Mstn and DN-Mstn/wt-Mstn. For *in situ* staining, the medium was discarded from the wells and the cells fixed with 4% formaldehyde for 10 min at room temperature. The cells were then rinsed thoroughly with three washes of PBS and incubated for 4 h at 37°C with 1 mg/mL X-Gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 2 mM $MgCl_2$. Control transfections were carried out to ensure the specificity of interactions. For the background controls, each of the AD or BD constructs were cotransfected with the pGal/lacZ reporter plasmid.

RESULTS

Stop, ProtSite, and C340 Myostatin Variants Bind to Mature Wild Type Myostatin In Vitro

A pull-down assay was performed to check the interaction between each of the three myostatin DN variants and mature wild myostatin. After His-tagged wild myostatin was expressed in a BL21 *E. coli* culture (Fig. 2A), it was bound to a Ni-NTA agarose resin. ProtSite, Stop, and C340 were expressed in a reticulocyte lysate (Fig. 2B) and then incubated separately with His-tagged wild myostatin bound to Ni-NTA agarose. The *wt*-Mstn/DN-Mstn complexes were pelleted by centrifugation, resolved by SDS-PAGE under reducing conditions, and visualized by autoradiography. As shown in Fig. 2C, the incubation of each of the variants, ProtSite, Stop, and C340, with wild myostatin resulted in the formation of stable complexes that allowed the copurification of these dominant negative variants with wild myostatin.

Stop, ProtSite, and C340 Variants of Myostatin Interact with Wild Mature Myostatin in Mammalian Cells

Since mammalian proteins are more likely to retain their native conformation inside mammalian cells, a mammalian two-hybrid system was performed to validate the pull-down assay. Combinations consisting of one ''bait'' wt-myostatin fusion construct and one ''prey'' DN-myostatin construct were cotransfected with the lacZ reporter plasmid into CHO-K1 cells. Each of the three DN-myostatins specifically interacted with the wild type product, inducing the expression of the reporter gene lacZ, as observed in *in situ* staining for β-galactosidase (Figs. 3a–d). As a background control, each construct (bait or prey alone) was cotransfected with lacZ reporter to verify that each tested protein did not function as a transcriptional activator on its own. All of the tested constructs activated the reporter gene (Figs. 3e–i), confirming the specificity of the interaction. CHO-K1 cells were also assayed for β-galactosidase activity (by measuring the hydrolysis of ONPG) to compare the degree of interaction between wt-Mstn/wt-Mstn and wt-Mstn/DN-Mstn. This assay showed there to be no significant difference in the absorbance per microliter between the wt-Mstn/wt-Mstn interaction and any of the three

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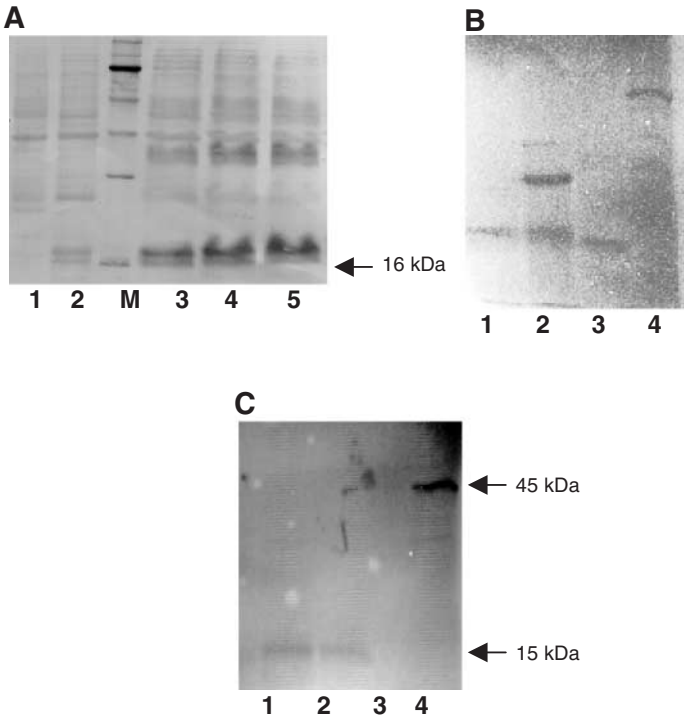

Figure 2. *In vitro* interaction of mature wt-myostatin with DN myostatin monomers. (A) Western blot of transformed BL21 with pRSET/wtMstn. Cell lysates were resolved by 15% SDS-PAGE under reducing conditions, blotted onto a PVDF membrane, and detected by monoclonal anti-His antibodies. Lane 1: Nontransformed BL21 cell lysate. Lane 2: Transformed BL21 cell lysate, $t = 0$. Lanes 3, 4, 5: Increasing quantities of BL21 cell lysate expressing His-wtMstn, $t = 5$. M: Molecular Protein Marker (Novagen). (B) Autoradiography of reticulocyte lysates. Samples were resolved by 15% SDS-PAGE under reducing conditions: 1. Stop, 2. Wt-myostatin, 3. C340, 4. ProtSite. (C) Autoradiography of His-wtMstn binding to DN-Mstn. Samples were resolved by 15% SDS-PAGE: 1. Pull-down of Stop, 2. Pull-down of C340, 3. Unbound Ni-NTA agarose interacted with a mixture of Stop, C340, and ProtSite reticulocyte lysate, 4. Pull-down of ProtSite.

DN-Mstn/wt-Mstn interactions (Fig. 4). This result suggests that the three DN-Mstn monomers can bind with wild type monomer with an efficiency similar to that of two wild type molecules.

DISCUSSION

Mutations in the myostatin gene are responsible for the double-muscled phenotype in cattle. Heterozygous animals have a normal phenotype due to the recessive pattern of inheritance of all the known mutated alleles responsible for bovine hypertrophy. With the aim of developing a tool for actively blocking wild

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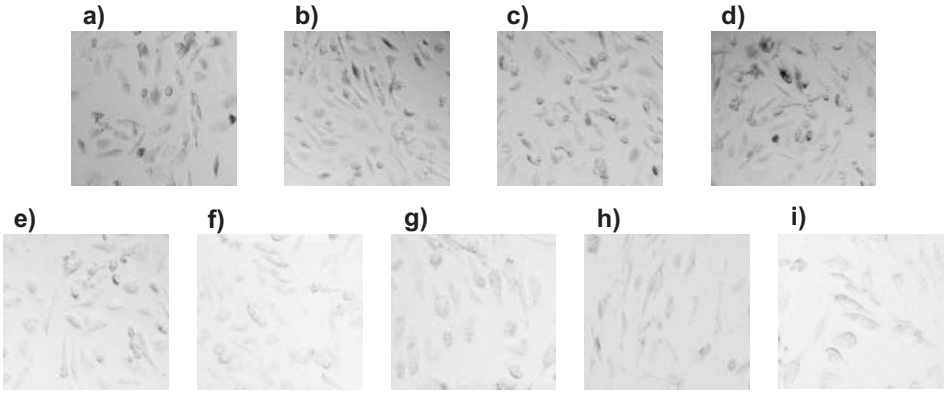

Figure 3. Mammalian two-hybrid assay with wt myostatin and the three synthetic DN alleles. The GAL4 DNA binding domain fused to wt-myostatin, the VP16 activation domain fused to each of the three dominant negative alleles, and the lacZ reporter plasmid were cotransfected into CHO-K1 cells which were assayed for β-galactosidase activity. (a) BD-wtMstn/AD-wtMstn, (b) BD-wtMstn/AD-ProtSite, (c) BD-wtMstn/AD-Stop, (d) BD-wtMstn/AD-C340, (e) AD-wtMstn, (f) BD-wtMstn, (g) AD-ProtSite, (h) AD-Stop, (i) AD-C340.

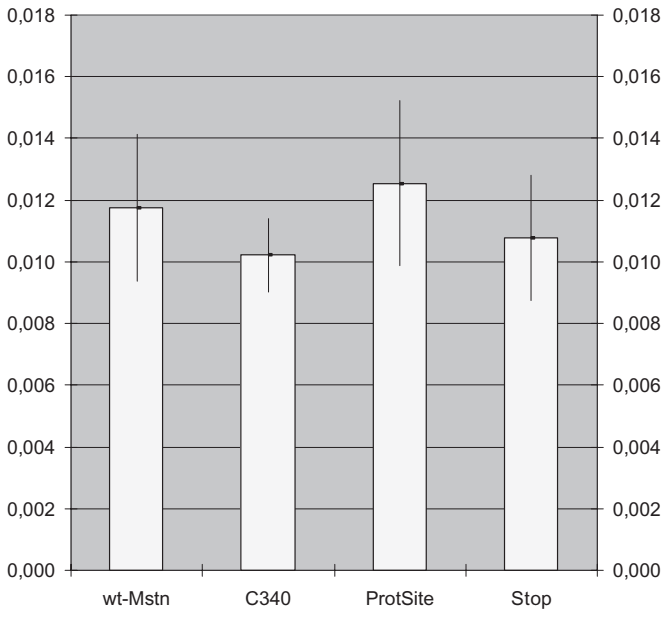

Figure 4. Comparison of wt-Mstn/wt-Mstn and wt-Mstn/DN-Mstn interactions in a β-galactosidase assay. The four interaction types were assayed for β-galactosidase activity by monitoring the hydrolysis of ONPG. Absorbance was measured at 420 nm nine times per cell lysate replicates. Results were normalized with respect to cell number and corrected by subtracting the nontransfected control background. Results are the mean and standard error of the nine results and are expressed as absorbance per microliter of cell lysate ($P > 0.8$).

type myostatin, we designed different dominant negative alleles on the basis of the known structural and functional characteristics of the TGFβ family of molecules. We then created three myostatin constructs based on the idea that, once dimerized with wild type myostatin monomer, the heterodimer would not bind TGFβ II receptors by stearic hindrance, knocking out the wild type function. In fact, there are several examples of mutations introduced into different TGFβ members leading to a DN phenotype (9–11).

This study focuses on the ability of new synthetic monomers to bind to wild type myostatin monomers. Here we show that wild type myostatin specifically interacts with three DN myostatin monomers, confirming this result in two independent observations. First, the three DN myostatin monomers from reticulocyte lysate were pulled down with mature wild type myostatin previously immobilized on Ni-NTA agarose (Fig. 2C). Second, wild type myostatin interacted specifically with all three DN-Mstn monomers in mammalian cells. In contrast to the pull-down assay, mammalian two-hybrid assays allow assessment of protein interaction in the physiological context of an intact cell (the reticulocyte lysate lacks the organelles and enzymes of a complete cell), providing the possibility of posttranslational modifications responsible for protein interactions (e.g., phosphorylation or fatty acylation) and proper protein folding (13). In addition, mammalian two-hybrid assays provide the possibility of quantitatively following interactions, allowing some assessment of efficiency and affinity. In this case, we compared the wt-Mstn/wt-Mstn interaction with each DN-Mstn/wt-Mstn interaction by measuring the absorbance (A_{420}) of the four types. After standardizing the data, statistical analysis showed there were no significant differences between wt-Mstn/wt-Mstn and DN-Mstn/wt-Mstn interactions for any of the mutated DN constructs. This means that the modifications made to produce the synthetic DN alleles do not alter the three-dimensional structure of the protein necessary, allowing interaction with wild type myostatin. Even the C340 product, which has a new fragment in the very middle of the mature protein, dimerizes with wild type myostatin to the same degree as two wild type myostatin monomers. In the case of the ProtSite monomer, the ability to interact with the wild type molecule and to exert a dominant negative effect was shown by Zhu et al. (14), who generated transgenic mice expressing DN myostatin mutated at its cleavage site. These mice showed an increase in skeletal muscle mass as a result of widespread muscle hypertrophy. This shows that stearic hindrance of TGFβ receptor II binding is a good strategy for creating new synthetic DN alleles.

In conclusion, we produced three potentially DN myostatin alleles and showed that the three monomers derived from these interacted with wild type myostatin in both an *in vitro* pull-down assay and a mammalian two-hybrid assay. Further experiments are necessary to assess their DN effect in cell systems.

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