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Single cysteine to tyrosine transition inactivates the growth inhibitory function of Piedmontese myostatin

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Berry, Carole, Mark Thomas, Brett Langley, Mridula Sharma, and Ravi Kambadur. Single cysteine to tyrosine transition inactivates the growth inhibitory function of Piedmontese myostatin. *Am J Physiol Cell Physiol* 283: C135–C141, 2002. First published March 6, 2002; 10.1152/ajpcell.00458.2001.—Myostatin, a member of the transforming growth factor-β superfamily, is a secreted growth factor that is proteolytically processed to give COOH-terminal mature myostatin and NH_2-terminal latency-associated peptide in myoblasts. Piedmontese cattle are a heavy-muscled breed that express a mutated form of myostatin in which cysteine (313) is substituted with tyrosine. Here we have characterized the biology of this mutated Piedmontese myostatin. Northern and Western analyses indicate that there is increased expression of myostatin mRNA and precursor myostatin protein in the skeletal muscle of Piedmontese cattle. In contrast, a decrease in mature myostatin was observed in Piedmontese skeletal muscle. However, there is no detectable change in the circulatory levels of mature myostatin in Piedmontese cattle. Myoblast proliferation assay performed with normal and Piedmontese myostatin indicated that mature wild-type myostatin protein inhibited the proliferation of C_2C_{12} myoblasts. Piedmontese myostatin, by contrast, failed to inhibit myoblast proliferation. In addition, when added in molar excess, Piedmontese myostatin acted as a potent "competitive inhibitor" molecule. These results indicate that, in Piedmontese myostatin, substitution of cysteine with tyrosine results in the distortion of the "cystine knot" structure and a loss of biological activity of the myostatin. This mutation also appears to affect either processing or stability of mature myostatin without altering the secretion of myostatin.

growth and differentiation factor 8; transforming growth factor-β

MYOSTATIN, ALSO KNOWN AS growth and differentiation factor 8 (GDF-8), is a member of the transforming growth factor (TGF)-β superfamily. The targeted deletion of the entire COOH-terminus of myostatin in mice (8) and several naturally occurring mutations in the *myostatin* gene in cattle (3) have been shown to cause heavy muscling (also referred to as double muscling), mainly resulting from hyperplasia. In the Belgian Blue cattle breed, for instance, there is an 11-bp deletion causing a frame shift and premature translational termination of myostatin (5). In another heavy-muscled cattle breed, Piedmontese, there is a guanine-to-adenine transition at position 938 (G938A) of *myostatin*, causing a substitution of a critical cysteine to a tyrosine in the signaling portion of myostatin (5). More recently, Grobet et al. (3) have identified seven DNA sequence polymorphisms of which five were predicted to disrupt the function of myostatin in various European breeds of double-muscled cattle. These studies have been used to clearly establish *myostatin* as a negative regulator of skeletal muscle growth in mammals.

Myostatin expression is first detected in the muscle precursor cells of the dermomyotome of somites, and the expression continues in the adult muscle (8). In addition to skeletal muscle, low levels of myostatin expression have also been detected in the mammary gland (4), heart (12), and brain (11). Despite the detection of myostatin in tissues other than skeletal muscle, the prominent phenotype observed in *myostatin*-null mice is one of increased skeletal muscle mass.

Function and structure studies of myostatin and other TGF-β family members have revealed insights into the biology of myostatin. Myostatin is synthesized in myoblasts as a 52-kDa propeptide that appears to be cleaved at RSRR (263–266), a furin protease site (9). This cleavage gives rise to a 26-kDa mature myostatin of 109 amino acids in length that is secreted to elicit its biological function. We have previously shown that a recombinant mature myostatin can regulate the proliferation of myoblasts (13). Mature myostatin has also recently been suggested to function by binding to the activin type II B receptor and possibly signals through the TGF-β type I receptor ALK5 (6).

Previous studies have also established that the mature portion of TGF-β members adopt a cystine knot structure (14), are secreted, and function as homodimers. Similarly, out of the nine cysteine residues in mature myostatin, six cysteine residues are positioned in a perfect spacing to form a cystine knot structure that is seen in TGF-β (Fig. 1). Like TGF-β, it

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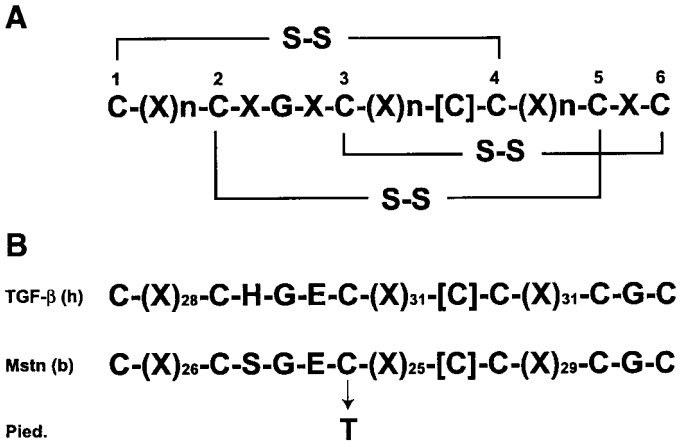

Fig. 1. *A*: schematic drawing of cystine knot structure. The 6 cysteine residues involved in knot formation are numbered consecutively, and their spacing is shown. S-S indicates disulfide bond. Cysteines 2 and 3 form disulfide bonds with cysteines 5 and 6, respectively. The third disulfide bond in the cystine knot is formed between cysteines 1 and 4. *B*: alignments of amino acid sequences that form the "cystine knot" in mature transforming growth factor (TGF)-β (human; h) and myostatin (Mstn; bovine; b). The cysteine-to-tyrosine transition seen in Piedmontese (Pied) myostatin is indicated.

is also established that mature myostatin forms a homodimer (6) and is secreted (2).

In the Piedmontese cattle, the third cysteine (C_3; amino acid 313) of myostatin is substituted with a tyrosine (Fig. 1). In this manuscript, we have characterized the biology of Piedmontese myostatin. Because cysteine mutations in TGF-β superfamily members can lead to aberrant signaling (14), we have characterized the synthesis, processing, secretion, and biological activity of Piedmontese myostatin. Here we report that, although the circulatory levels of myostatin in Piedmontese cattle are unaffected, mature Piedmontese myostatin fails to inhibit the growth of myoblasts in vitro. Given that C_3 is crucial for intramolecular disulfide linkage in the cystine knot structure, it is likely that the substitution of this cysteine to a tyrosine inactivates Piedmontese myostatin by distorting the cystine knot structure required for receptor binding.

MATERIALS AND METHODS

Expression and purification of mature myostatin. Expression and purification of wild-type mature bovine myostatin (amino acids 267–375) has been described previously (13).

The pET protein expression system (Novagen, Madison, WI) was used to express and purify recombinant Piedmontese myostatin. Piedmontese *myostatin* coding sequencing, spanning amino acids 267–375, was PCR amplified using genomic DNA and PCR primers as described previously (13). Amplicons were cloned into pET 16-B vector, in-frame with the 10-histidine tag according to the manufacturer's protocol (Novagen). An overnight BL 21 *Escherichia coli* culture transformed with the Piedmontese myostatin expression vector was diluted and grown to an optical density of 0.8 (600 nm) in 1 liter of Lennox L broth medium containing ampicillin (50 mg/l). Expression of the myostatin fusion protein was induced by adding 0.5 mM isopropyl thio-β-galactoside to the culture for 2 h. Bacteria were harvested by centrifugation, resuspended in 40 ml of lysis buffer (6 M guanidine hydrochloride; 20 mM Tris, pH 8.0; and 5 mM 2-mercaptoethanol), and sonicated. The lysate was centrifuged at 10,000 *g* for 30 min, and recombinant Piedmontese myostatin was purified from the supernatant by Ni-Agarose affinity chromatography (Qiagen), according to the manufacturer's protocol. The fractions containing soluble myostatin were pooled and dialyzed against two changes of 50 mM Tris·HCl (pH 8.0) containing 500 mM NaCl and 10% glycerol for 2 h.

C_2C_{12} myoblast proliferation assay. C_2C_{12} myoblasts (15) were grown, unless otherwise stated, in DMEM (Life Technologies, Grand Island, NY) buffered with 41.9 mM $NaHCO_3$ (Sigma Cell Culture, St. Louis, MO) and 6% gaseous CO_2, resulting in an initial media pH of ~7.4. Phenol red (7.22 nM; Sigma) was used as a pH indicator. Penicillin (1×10^5 IU/l; Sigma), 100 mg/l streptomycin (Sigma), and 10% FBS (Life Technologies) were routinely added to media.

Cell proliferation assays were carried out in uncoated 96-well Nunc microtitre plates (Nunc, Roskilde, Denmark). C_2C_{12} cultures were seeded at 1,000 cells/well. After a 16-h attachment period, myostatin test media (either recombinant wild-type myostatin or Piedmontese myostatin) was added, and cells were incubated for a further 72 h. The test wells in the plate were randomly assigned, and all tests were run in replicates of eight. Although such proliferation assays were repeated at the very least two times for reproducibility, the results presented in this paper are SE of eight replicates obtained from one experiment. After the incubation period, proliferation was assessed using a methylene blue photometric end-point assay, as previously described (10). In this assay, absorbance at 655 nm is directly proportional to final cell number. To test whether Piedmontese myostatin could compete with wild-type myostatin, myoblasts were incubated with either wild-type myostatin alone or with increasing molar excess concentrations of Piedmontese myostatin. Proliferation of myoblasts was assessed as described above.

Western analysis. Fifteen micrograms of muscle (musculus semitendinosus) extracts or plasma proteins were separated by SDS-PAGE (4–12% gradient gels) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in 50 mM Tris (pH 7.4), 100 mM NaCl, and 0.1% Tween 20 (TBST) and incubated with purified rabbit anti-myostatin antibodies (0.5 mg/ml; see Ref. 12) in 5% nonfat milk in TBST at room temperature for 3 h. The blot was washed (5×5 min) with TBST buffer and further incubated with a 1:2,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (HRP; DAKO, Carpinteria, CA) for 1 h at room temperature. The membrane was washed as before, and HRP activity was detected using an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) according to the manufacturer's protocol.

Northern analysis. Total RNA (15 μg) from musculus semitendinosus was separated on a 1% agarose-formaldehyde gel and transferred to a Hybond N^+ membrane (Amersham). The membrane was autocross-linked and prehybridized in 5× saline-sodium citrate (SSC), 50% formamide, 5× Denhardt's solution, 1% SDS, and 0.25 mg/ml salmon sperm DNA for 2 h. Hybridization was carried out in the same solution with bovine myostatin cDNA probe overnight at 42°C. The membrane was washed at 50°C for 15 min with 2× SSC and 0.5% SDS and then with 1× SSC and 0.5% SDS. The bovine myostatin cDNA was obtained as previously described (5) and radioactively labeled using [α-^{32}P]dCTP (Amersham Pharmacia Biotech) and a Rediprime II labeling kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

RESULTS

Expression of myostatin mRNA in normal and Piedmontese cattle. Piedmontese cattle, which are characterized by double muscling, express a mutant form of myostatin as a result of a guanine-to-adenine substitution at nucleotide position 938. To determine if this mutation leads to unstable myostatin mRNA and hence the double-muscling phenotype, we performed Northern blot analysis on the total RNA isolated from the skeletal muscle of normal and Piedmontese cattle. As shown in Fig. 2, the 2.9-kb myostatin mRNA is detected in the muscle of both normal and Piedmontese cattle. Interestingly, more myostatin mRNA is detected in the skeletal muscle of Piedmontese compared with normal cattle (Fig. 2*B*). This result suggests that myostatin may, in part, regulate its own expression by a "feedback inhibition" mechanism.

Processing of myostatin in cattle. Because the cysteine-to-tyrosine mutation may also affect the translation efficiency, stability, or cleavage of the protein, we next assessed the synthesis and processing of myostatin in Piedmontese cattle. Total protein was extracted from the musculus semitendinosus of normal and Piedmontese cattle and subjected to Western blot analysis. As shown in Fig. 3*A*, myostatin-specific antibodies recognized a 52-kDa precursor, a 40-kDa latency-associated peptide, and a 26-kDa processed mature myostatin in both normal and Piedmontese cattle muscle extracts. Densitometry analysis of the Western blot indicates that there is relatively more precursor myostatin protein in Piedmontese skeletal muscle, confirming that increased myostatin mRNA results in the increased translation of precursor myostatin (Fig. 3*C*). However, Western analysis also indicated that there was less mature myostatin protein detected in Piedmontese skeletal muscle (Fig. 3*C*). This result indicates that the cysteine-to-tyrosine mutation in Piedmontese myostatin decreases either the processing or stability of myostatin.

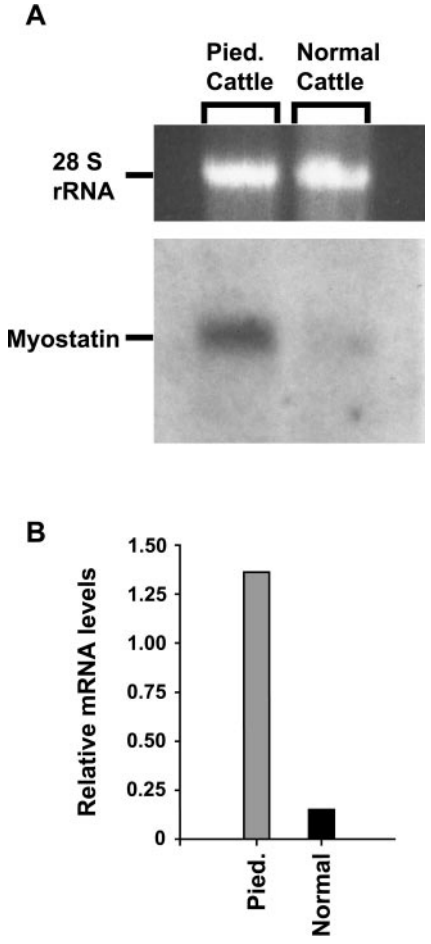

Fig. 2. *A*: Northern blot analysis to detect myostatin mRNA in musculus semitendinosus of normal and Piedmontese cattle. Ethidium bromide-stained 28S rRNA gel is also shown to indicate the equal loadings of total RNA. Myostatin mRNA is indicated. *B*: densitometry analysis of the Northern blot. Optical density values of relative myostatin mRNA levels of normal and Piedmontese cattle are shown.

Mature myostatin is secreted in Piedmontese cattle. Processed mature myostatin has been previously shown to be secreted (2) and biologically active (13). Because mutations in the mature TGF-β affecting the cysteine residues have been shown to affect secretion (1), we investigated if the double muscling seen in Piedmontese cattle is the result of abnormal myostatin secretion. To quantify the circulatory levels of myostatin in normal and Piedmontese cattle, we collected blood and performed Western blot analysis on plasma proteins. The Western analysis showed that the 26-kDa myostatin protein is detected in the plasma of both normal and Piedmontese cattle (Fig. 3*B*). Moreover, there appears to be no change in the circulatory levels of myostatin between normal and Piedmontese cattle (Fig. 3*D*).

Expression and purification of mutant myostatin protein. Because the cysteine-to-tyrosine mutation is predicted to disrupt the cystine knot structure of Piedmontese myostatin, the biological activity of Piedmontese mature myostatin was evaluated. To test the biological activity, we expressed both wild-type and Piedmontese mature myostatin as His-tagged proteins in *Escherichia coli*. The expressed histidine fusion proteins were purified on a Ni-Agarose column and separated by SDS-PAGE to check for purity. As shown on a Coomassie blue-stained gel (Fig. 4), a 15-kDa myostatin fusion protein was purified to a high degree in a single step. A typical yield of 6 mg of myostatin protein was purified per liter of induced bacterial culture. Furthermore, there appears to be no degradation of the recombinant myostatin during purification (Fig. 4).

Piedmontese myostatin does not inhibit myoblast proliferation. To test the biological activity of the normal and mutant myostatin, we incubated actively growing myoblast cultures with either wild-type or Piedmontese myostatin protein for 72 h and determined the myoblast number by methylene blue assay. As shown in Fig. 5*A*, increasing concentrations of wild-type myostatin inhibited the growth of myoblasts, with half-maximal inhibition occurring at a myostatin concentration of ~3.5 μg/ml. Moreover, myostatin treatment did not cause any morphological changes to the myo-

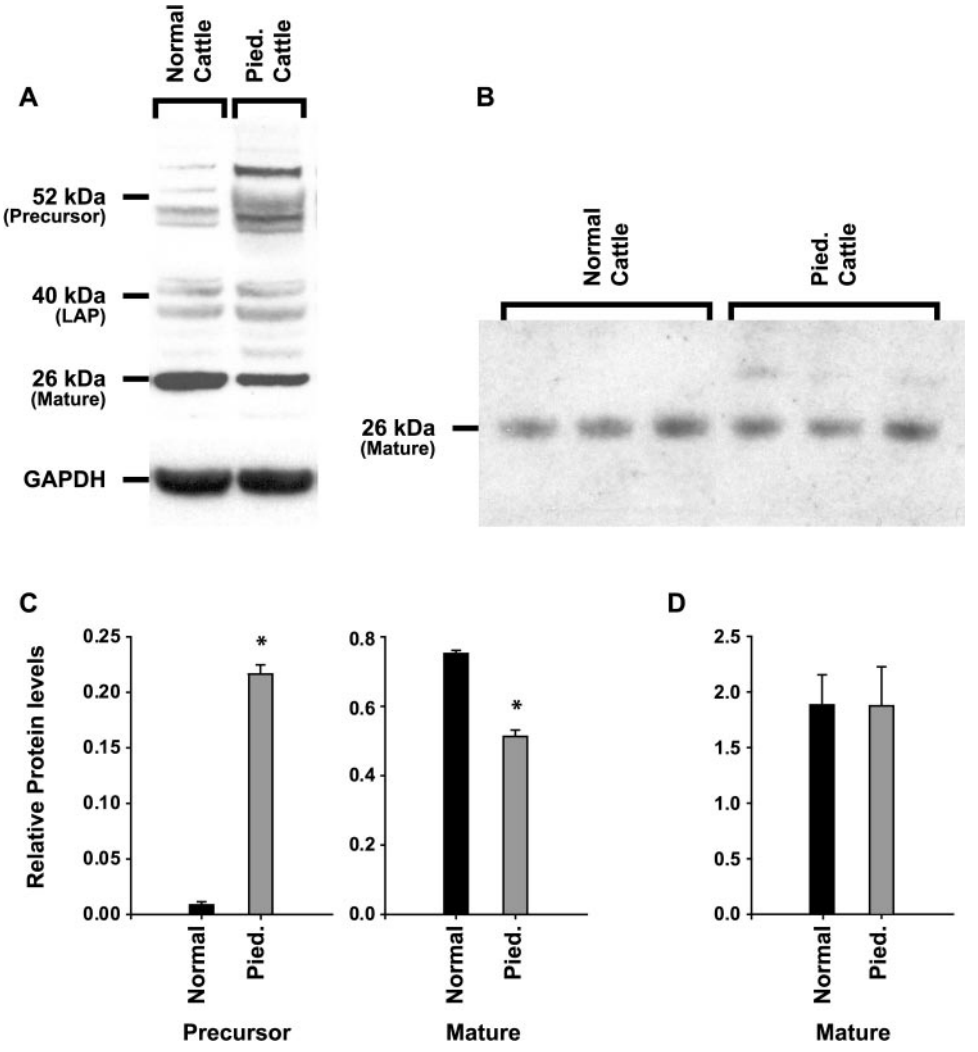

Fig. 3. Western blot analysis to detect myostatin in musculus semitendinosus protein extracts (*A*) and plasma (*B*) of normal and Piedmontese cattle. All three forms of myostatin [the precursor, the latency-associated peptide (LAP), and the mature peptide] are indicated, with their molecular masses in kDa. Densitometry analysis of the skeletal muscle (*C*) and plasma (*D*) Western blots. Optical density values of relative myostatin levels of normal and Piedmontese cattle are shown. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Bars represent means ± SE of data for 4 animals. *$P < 0.001$ compared with normal value.

blasts (Fig. 5*C*). This is consistent with our previous observation (13). In contrast to wild-type myostatin, the Piedmontese myostatin protein failed to inhibit the proliferation of myoblasts, even at the concentration of 10 μg/ml (Fig. 5*B*). Furthermore, not only did Piedmontese myostatin fail to inhibit myoblast proliferation, high concentrations significantly increased the proliferation of C_2C_{12} cells compared with nontreated control myoblasts (Fig. 5*B*). These results suggest the cysteine-to-tyrosine substitution at amino acid position 313 results in a biologically inactive myostatin molecule. In addition, the inactive Piedmontese myostatin appears to be acting as a "competitive inhibitor" protein over the wild-type myostatin already present in the media.

Piedmontese myostatin functions as a competitive inhibitor. To experimentally prove that the mutant myostatin can compete with the wild-type myostatin, myoblasts were incubated with either wild-type myostatin alone or with wild-type myostatin and increasing molar concentrations of Piedmontese myostatin. As shown in Fig. 6, a 1.5 μg/ml concentration of wild-type myostatin inhibited the growth of myoblasts ($P = 0.018$). Increased molar ratios of Piedmontese protein over the wild-type protein rescued the myoblasts from this growth inhibitory effect, with a one-to-one molar ratio of Piedmontese myostatin ablating the observed wild-type myostatin inhibition. These results are consistent with the Piedmontese myostatin competing with the wild-type myostatin for receptor binding, thereby neutralizing wild-type myostatin function.

DISCUSSION

Myostatin, a member of the TGF-β superfamily, is a key regulator of skeletal muscle growth. The targeted deletion of *myostatin* in mice (8) and natural mutations in bovine *myostatin* (5) lead to hyperplasia of skeletal muscle. Of the *myostatin* mutations that are described in double-muscled cattle, the Piedmontese allele is very interesting, since a single-point mutation (which results in replacement of cysteine 313 with tyrosine) causes heavy muscling (5). In this communication, we have characterized the molecular basis for the double-muscle phenotype caused by the Piedmontese myostatin. The results indicate that the cysteine-to-tyrosine transition does not seem to affect the circulatory levels of myostatin. Rather, the mutation appears to interfere

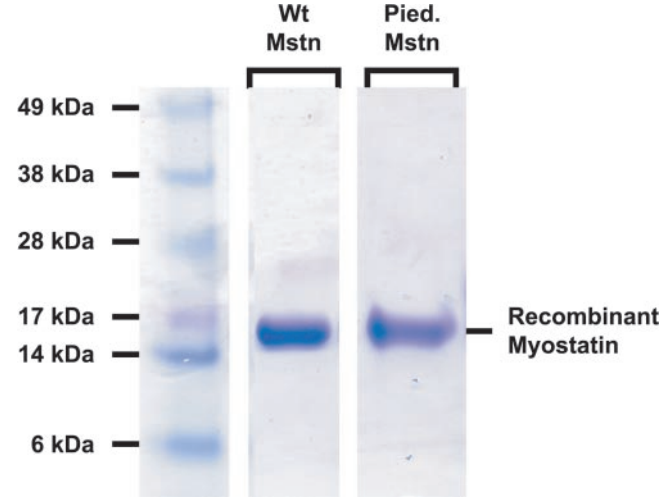

Fig. 4. Coomassie blue-stained gel showing 3 μg of purified wild-type (wt) and Piedmontese recombinant myostatin. Molecular mass standards in kDa are also shown.

with receptor binding ability, possibly through distortion of the cystine knot structure. Furthermore, we show that Piedmontese myostatin acts as a potent competitive inhibitor molecule.

TGF-β family member proteins are proteolytically processed at the site of synthesis, and mature biologically active peptides are secreted in blood (9). Therefore, the biological function of mature myostatin may be affected at the level of synthesis, processing, or secretion. To test if the Piedmontese mutation in *myostatin* affects mRNA transcription or stability, we performed Northern blot analysis on total RNA from skeletal muscle of Piedmontese and normal cattle. The results indicated that there is an increased expression of myostatin mRNA in Piedmontese skeletal muscle compared with the normal skeletal muscle (Fig. 2). The increased myostatin expression seen in double-muscled Piedmontese cattle may be attributed to lack of feedback inhibition of myostatin expression by nonfunctional myostatin. Indeed, unpublished results from our laboratory using the *myostatin* promoter and

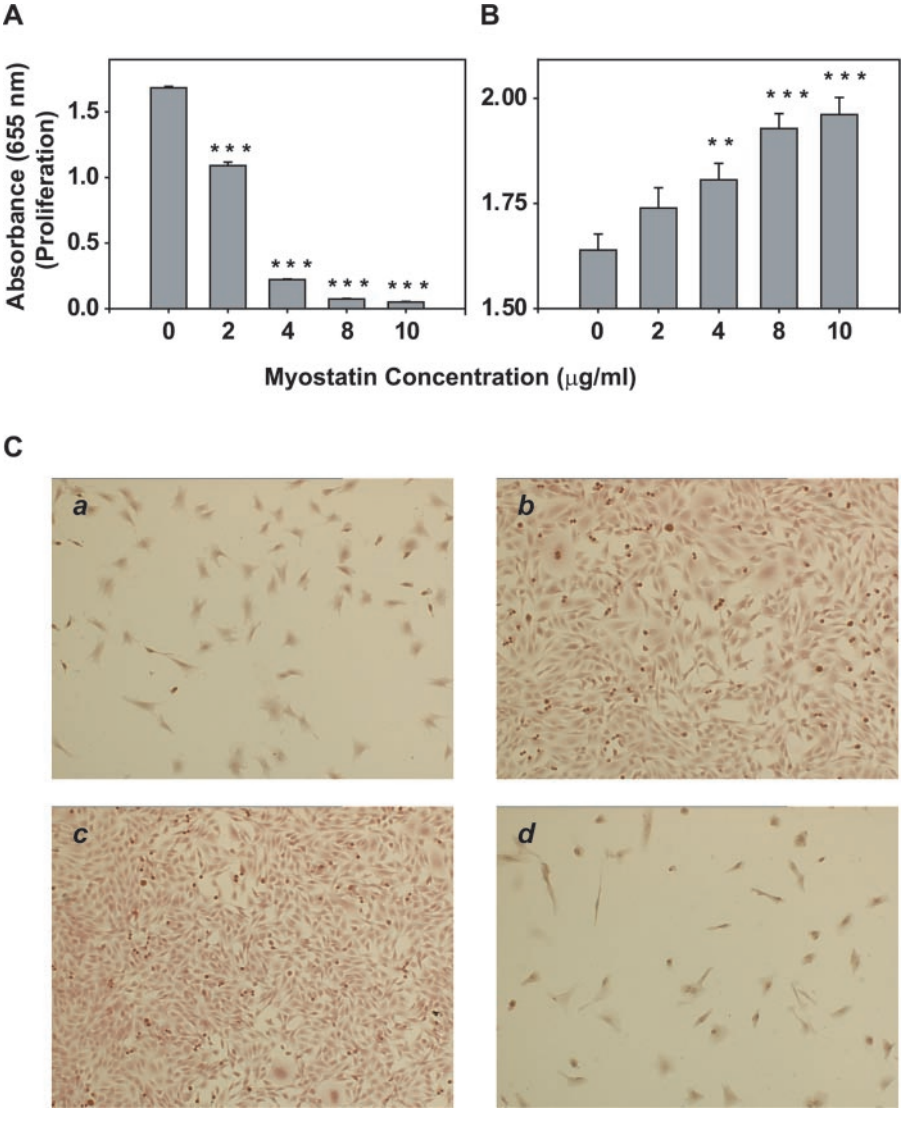

Fig. 5. Effect of myostatin on myoblast growth. C_2C_{12} myoblasts were grown in the presence of increasing concentrations of either wild-type or Piedmontese myostatin for 72 h, and proliferation was monitored by methylene blue assay. *A*: wild-type myostatin. *B*: Piedmontese myostatin. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with 0 μg/ml myostatin control. *C*: C_2C_{12} myoblasts were cultured for 0 and 72 h without myostatin (*a* and *b*, respectively) or with 10 μg/ml Piedmontese myostatin (*c*) or with 10 μg/ml wild-type myostatin (*d*) in growth media. Cells were fixed and stained with Gills hematoxylin and eosin.

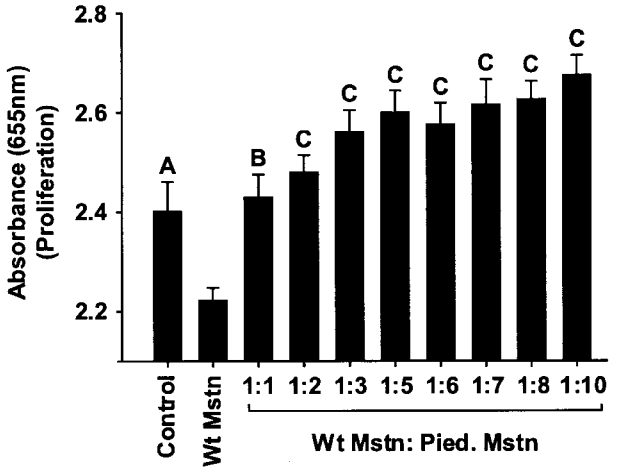

Fig. 6. Piedmontese myostatin functions as a competitive inhibitor. C_2C_{12} myoblasts were grown in the presence of wild-type myostatin (1.5 μg/ml) or with wild-type myostatin (1.5 μg/ml) in the presence of increasing molar ratios of Piedmontese myostatin, for 72 h, and proliferation was monitored by methylene blue assay. $^{A}P < 0.05$, $^{B}P < 0.01$, and $^{C}P < 0.001$ compared with wild-type myostatin (2-tailed Student's *t*-test).

luciferase reporter gene support this argument, since mature recombinant myostatin negatively regulates the myostatin promoter.

Experiments altering cysteine residues in the mature portion of TGF-β resulted in aberrant cleavage, suggesting that disulfide bonding may be required for proteolytic processing (1). To test if this may be the case for Piedmontese myostatin, we performed Western blot analysis to determine the processing of myostatin protein in Piedmontese cattle. In agreement with the Northern blot results, we observed a severalfold increase in the levels of Precursor myostatin in the Piedmontese relative to normal-muscled cattle. However, there appears to be a decrease in the levels of mature myostatin protein (Fig. 3, *A* and *C*). This paradox could be because of *1*) inefficient processing of the mutant myostatin protein or *2*) decreased stability of the mutant myostatin.

Systematic mutations of most cysteine residues within the mature TGF-β protein have also been shown to result in the loss of secretion (1). To test this possibility, we performed Western blot analysis on normal-muscled and Piedmontese cattle plasma to quantify the levels of mature myostatin in the circulation. Although there are decreased levels of mature myostatin at the site of synthesis, the circulatory levels of mature myostatin do not appear to be different between normal-muscled and Piedmontese cattle (Fig. 3, *B* and *C*).

To test the biological function of Piedmontese myostatin, we used the myoblast proliferation assay (13). We show that increasing concentrations of wild-type mature myostatin inhibited the proliferation of C_2C_{12} myoblasts in culture (Fig. 5*A*), which is consistent with the negative regulatory function of myostatin (13). By contrast, the Piedmontese myostatin could not inhibit the proliferation of actively growing C_2C_{12} cells, even at a very high concentration (Fig. 5*B*). TGF-β family members function by binding to their respective receptors, initiating a downstream signaling cascade (7). For receptor binding, mature TGF-β family members attain a cystine knot structure mediated by six cysteine residues in the mature portion of the molecule (14). These cysteine residues are thus highly conserved in TGF-β family members and are classified as 10-membered cystine knot proteins (14). Myostatin has nine cysteine residues within the mature portion of the peptide, six of which are appropriately spaced to form a cystine knot-forming domain identical to that in TGF-β (Fig. 1). It is also noteworthy that a recent scanning of a human cDNA sequence database for 10-membered cystine knot proteins has identified GDF-8 (myostatin) as one of the proteins that contains a potential cystine knot structure (14). The six cysteines involved in knot formation are spaced out with intervening amino acids. Cysteines 2 and 3 form intrachain disulfide bonds with cysteines 5 and 6, respectively, thus forming a ring. The third disulfide bond formed between cysteines 1 and 4 penetrates the ring. With Piedmontese myostatin, the cysteine at position 313 (which is C_3 in the cystine knot) is substituted with tyrosine and thus would not be able to form the required intrachain disulfide linkage, thereby distorting the cystine knot structure. This would probably affect receptor binding or receptor interaction. It has recently been confirmed that mature myostatin preferentially binds to activin type IIB receptor (6).

When a high concentration of Piedmontese myostatin was used in the bioassay, we observed that C_2C_{12} myoblasts actually proliferated significantly faster than the myoblasts incubated in the control media (Fig. 5*B*). This indicates that the mutant myostatin added to the actively growing media is acting as a "competitor" to the wild-type myostatin secreted in the media by the C_2C_{12} cells. Furthermore, in a competition experiment in which wild-type myostatin protein was incubated with increasing concentrations of Piedmontese protein, a decrease in the inhibitory effect of wild-type protein was seen (Fig. 6). This experiment confirmed that the Piedmontese myostatin indeed acts as a competitor over the wild-type myostatin. Two scenarios can be used to explain this neutralizing effect of Piedmontese myostatin. When added in excess, Piedmontese myostatin may bind to the receptor, albeit improperly, thereby outcompeting wild-type myostatin and attenuating its growth inhibitory effect. Alternatively, Piedmontese myostatin may be heterodimerizing with wild-type mature myostatin, resulting in a receptor binding-incompetent myostatin complex. The first scenario is the most plausible, since wild-type myostatin is secreted in a homodimerized form, which would be unlikely to disassociate, allowing Piedmontese myostatin to dimerize with it. However, because the cysteine mutation affects the required cystine knot structure, in either scenario high concentrations of Piedmontese myostatin would act as a myostatin mimetic and possibly disrupt the further downstream signaling by mature wild-type myostatin. Thus Pied-

montese myostatin presents itself as a potential inhibitor of myostatin. Experiments regarding the in vivo efficacy of Piedmontese myostatin to inhibit the wild-type myostatin function and myostatin-receptor cross-linking are underway.

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