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ORIGINAL ARTICLES

Endogenous Expression and Localization of Myostatin and Its Relation to Myosin Heavy Chain Distribution in C2C12 Skeletal Muscle Cells

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Myostatin is a negative regulator of skeletal muscle growth. We have previously reported that recombinant myostatin protein inhibits DNA and protein synthesis in C2C12 cells. Our objective was to assess if C2C12 cells express myostatin, determine its sub-cellular localization and the developmental stage of C2C12 cells in which myostatin mRNA and protein are expressed. To study the endogenous expression of myostatin, C2C12 myoblasts were allowed to progress to myotubes, and changes in the levels of endogenous myostatin mRNA expression were determined by RT-PCR. The myostatin protein and the two major myosin heavy chain (MHC) isoforms (MHC-I and -II) were determined by Western blot. Confirmation of the relative MHC expression patterns was obtained by a modified polyacrylamide gel electrophoretic (PAGE) procedure. Immunofluorescence staining was employed to localize the site of myostatin expression and the relative distribution of the MHC isoforms. Co-expression of these proteins was studied using a dual staining approach. Expression of myostatin mRNA was found in myotubes but not in myoblasts. Myostatin protein was seen in most but not all, of the nuclei of polynucleated fibers expressing MHC-II, and myostatin was detected in the cytoplasm of myotube. The localization of myostatin protein in myotube nuclei was confirmed by Western blot of isolated nuclear and cytoplasmic fractions. Incubation of C2C12 myotubes with graded doses of dexamethasone dose-dependently increased the intensity of nuclear myostatin immunostaining and also resulted in the appearance of cytoplasmic expression. In conclusion, myostatin was expressed mostly in C2C12 myotubes nuclei expressing MHC-II. Its predominant nuclear localization suggests that it may play a role in transcriptional regulation. *J. Cell. Physiol.* 190: 170–179, 2002. © 2002 Wiley-Liss, Inc.

Myostatin, a member of the TGF- β super family, has been proposed to be a negative regulator of skeletal muscle mass (McPherron et al., 1997; Gonzalez-Cadauid et al., 1998). Inactivating mutations of the myostatin gene in cattle (Kambadur et al., 1997; McPherron et al., 1997) and mice (McPherron and Lee, 1997; Szabo et al., 1998) are associated with hypermuscularity that may be due to skeletal muscle hypertrophy and possibly hyperplasia. Similarly, transgenic mice carrying dominant negative mutations of the myostatin gene have markedly increased muscle mass (Zhu et al., 2000). Increased levels of myostatin have been found in serum and in skeletal muscle in humans and experimental animals under conditions associated with loss of skeletal muscle such as severe HIV infection (Gonzalez-Cadauid et al., 1998), prolonged bed rest (Zachwieja et al., 1999; Wehling et al., 2000), aging, and exposure to microgravity during space flight (Lalani et al., 2000).

The mechanism by which myostatin may affect the maintenance of skeletal muscle mass in the adult animal is still unknown. This question has recently

been addressed utilizing the mouse C2C12 cells, an “in vitro” system widely used for the study of genes affecting muscle growth and differentiation. The addition of recombinant myostatin inhibits DNA and protein synthesis and cell replication but does not induce

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proteolysis or cell death (Bass et al., 1999; Taylor et al., 2001), suggesting that myostatin may act as an anti-anabolic rather than catabolic factor. These effects may be related to the modulation of gene expression by myostatin in C2C12 myoblasts and myotubes, as indicated by the DNA micro-array experiments (Taylor, personal communication). In addition the myostatin promoter in a luciferase expression vector are active upon transfection of C2C12 cells, and this expression can be modulated by glucocorticoids in the same fashion as "in vivo" in the rodent skeletal muscle (Ma et al., 2001).

Although myostatin is expressed predominantly in the skeletal muscle, it is also expressed in low abundance in ovine and rodent heart myocytes, particularly after myocardial infarction (Sharma et al., 1999), lactating pig mammary gland (Ji et al., 1998), and in brain and ovarian tissue (Roberts and Goetz, 2001; Rodgers et al., 2001). In rodents, myostatin mRNA expression has been detected only in fast type II fibers of the gastrocnemius muscle (Carlson et al., 1999). However, in the rat and humans, myostatin is localized to all muscle fiber types (Gonzalez-Cadavid et al., 1998; Kirk et al., 2000).

Although the C2C12 skeletal muscle cells have been widely used as an "in vitro" model of myoblasts/myotubes differentiation (Sakuma et al., 2000; Rios et al., 2001), and recently used to study the effects of myostatin on cell replication and apoptosis (Taylor et al., 2001; Bass et al., 1999), we do not know whether these cells express myostatin mRNA and protein, the subcellular localization of the myostatin protein and its role during myotube formation. Therefore we investigated whether C2C12 mouse skeletal muscle myoblasts and myotubes express myostatin mRNA and protein, its subcellular localization, and its association with MHC-I and -II fibers. Finally, we investigated the expression pattern of myostatin "in situ" when the C2C12 cells are challenged with dexamethasone, a known inducer of muscle loss.

MATERIALS AND METHODS

Cell culture

The C3H murine myoblast cell line C2C12 (CRL-1772, ATCC, Manassas, VA) was propagated in Dulbecco's modified Eagle's medium (containing 4 mM glutamine, 4.5 g/L of glucose, and antibiotics, Life Technologies, Rockville, MD) with 10% fetal bovine serum (Life Technologies) and incubated at 37°C and 5% CO₂ at 10–50% confluence (Taylor et al., 2001) on the appropriate plates for each assay (Lab-Tek chamber slide, eight- or six-well plates). For differentiation into myotubes, myoblasts were allowed to grow to approximately 90–100% confluence, after 2 days the medium was changed to DMEM plus 5% horse serum (Life Technologies). Myotubes began to form after 3–4 days, and multinucleated muscle fiber cultures were used after 7–10 days.

For studies on the effects of glucocorticoids on myotubes, we followed the same procedure except that after the initial 7–10 days of culture, the media was supplemented with 50, 100, 150, and 200 nM Dexamethasone (Sigma, St. Louis, MO), for 4–5 days, in triplicate.

RNA expression by RT-PCR

Total RNA was extracted from myotubes and myoblasts contained in six-well plates by applying TriZol reagent (Life Technologies). To check the quality of the RNA, sample aliquots were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Quantity of the RNA extracted was measured spectrophotometrically.

Aliquots containing 1 µg RNA were submitted to reverse transcription (RT) using a 16-mer oligo-dT primer, as previously described (Gonzalez-Cadavid et al., 1998).

The resultant cDNA, 1/10 of the RT reaction, was amplified using PCR in a total volume of 25 µl. Primers for myostatin (Ma et al., 2001) and the housekeeping gene GAPDH (Ercolani et al., 1988) were employed in a multiplex reaction. The thermal amplification of the 650 bp myostatin DNA fragment was conducted after a 3 min step of denature at 94°C, followed by 35 cycles of 94°C (35 sec), 58°C (45 sec), and 72°C (80 sec) and a final step at 72°C for 8 min.

RNA expression by Northern blot analysis

Twenty micrograms of samples of total RNA were separated using 1.2% agarose/formaldehyde denaturing gels, transferred overnight onto Hybond N+ membranes (Amersham Pharmacia Biotech, Piscataway, NJ) and fixed by exposure to ultraviolet light (Gonzalez-Cadavid et al., 1998). Blots were hybridized at 60°C, overnight in an ExpressHyb (Clontech, Palo Alto, CA) solution containing [³²P] dCTP-labeled random-primed myostatin. The membranes were then washed to a stringency of 0.2 × SSC/0.1% SDS at 60°C. Hybridization was visualized by autoradiography. The extent of RNA expression was normalized based on relative band intensities obtained by the successive hybridization of the same blot with a probe for the housekeeping gene GAPDH (Ercolani et al., 1988).

The 690 bp DNA fragment selected as a probe for myostatin was based on the published mouse myostatin sequence (nts 1–690). The region was specifically chosen to avoid possible cross hybridization with the closely related GDF11 gene (McPherron et al., 1999; Sakuma et al., 2000). The 1233 bp cDNA fragment used as a GAPDH probe was based on a region of the published rat sequence (Ercolani et al., 1988), which shares 100% homology with the corresponding mouse gene.

Protein expression

Protein was extracted from myoblasts and myotubes using a denaturing-reducing lysis buffer containing 1% SDS, Tris-HCl and 1/20 dilution of β-mercaptoethanol. Nuclei from both cell types were isolated from the cytoplasmic fraction using two separate procedures, NE-per (Pierce, Rockford, IL) and Promega cell culture lysis buffer. Protein concentrations were measured using the Micro BCA Protein assay reagent (Pierce), protein samples (30 µg) were heat denatured (95°C for 5min), electrophoretically separated using 12% Tris-glycine polyacrylamide gels (ReadyGel; Bio-Rad, Hercules, CA), and the proteins visualized using Coomassie brilliant blue staining. The electrophoretically

separated samples were transferred to a nitrocellulose membrane, and immunodetected using the previously described procedure (Gonzalez-Cadavid et al., 1998). The antibody employed for myostatin was a polyclonal antibody elicited against a synthetic peptide within the sequence of the 110 carboxy terminus aminoacids of human myostatin, which was named antibody B (Gonzalez-Cadavid et al., 1998). This antibody has been extensively validated by its ability to detect the recombinant 375 amino acids myostatin precursor and the processed 110 amino acids proteins (Lalani et al., 2000; Taylor et al., 2001) as well as a 30–32 kDa band in mouse, rat, and human skeletal extracts (Gonzalez-Cadavid et al., 1998) which is considered to be the glycosylated dimer of the 110 amino acid protein cleaved from the myostatin precursor. We also applied two monoclonal antibodies specific for either myosin heavy chain (MHC) type I (Franchi et al., 1990) (NCL-MHC₅, Novocastra Laboratories, Newcastle, UK) or type II (NCL-MHC_I). For myostatin, an anti-rabbit-IgG, and for MHC type I and II anti-mouse IgG secondary antibody, each linked to horseradish peroxidase (HRP), were used. In addition, a mouse monoclonal purified IgG against rabbit GAPDH from skeletal muscle (Chemicon international, Inc., Temecula, CA) was utilized for normalization (Kots et al., 1992). The degree of cross-contamination between the nuclear and cytoplasmic fractions was determined with this antibody as a cytoplasmic marker, as well as with a rabbit polyclonal IgG against the full length mouse MyoD protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), as a nuclear marker (Tamir and Bengal, 2000). Blots were developed with an enhanced chemiluminescent substrate for HRP and exposed to film (ECL Hyperfilm, Amersham Pharmacia Biotech).

MHC electrophoresis and analysis

MHC isoforms were separated, using a modification of the SDS–polyacrylamide gel electrophoretic procedure developed by Talmadge and Roy (1993) and Bamman et al. (1999). Briefly, cells extracts were homogenized on ice in 9 volumes of buffer (250 mM sucrose, 100 mM KCl, 5 mM EDTA, and 20 mM Tris, pH 6.8), and centrifuged at 10,000g for 10 min at 4°C. The pellet was washed (175 mM KCl, 2 mM EDTA, 0.5% Triton X, and 20 mM Tris, pH 6.8), centrifuged and resuspended in 100 µl of 150 mM KCl and 20 mM Tris pH 7. Protein content was estimated using the BCA method (see above). Samples were diluted in 2 × loading buffer (1% β mercaptoethanol, 4% SDS, 20% glycerol, 0.2% bromophenol blue, and 1 M Tris pH 6.8) to a final concentration of 1 µg/µl and reduced by boiling for 2 min. Gels were prepared (Stacking: 30% glycerol, 4% acrylamide:Bis (50:1), 70 mM Tris (pH 6.7), 4 mM EDTA, 0.4% SDS; Separating: 30% glycerol, 8% acrylamide:Bis (50:1), 0.2 M Tris (pH 8.8), 0.1 M glycine, 0.4% SDS) using the Bio-Rad Mini-Protean II system. Separate upper (0.1 M Tris, 150 mM glycine, 0.1% SDS) and lower (50 mM Tris, 75 mM glycine, 0.05% SDS) running buffers were used. The gels were run for 2,900 volt-hours at 4°C, fixed in 15% trichloroacetic acid, and the MHC isoforms visualized after staining with Coomassie brilliant blue.

Detection of myostatin and MHC by immunofluorescence

Myoblasts and myotubes plated on the eight-well chamber plates were fixed by immersion in 2% p-formaldehyde, and for myostatin assay, they were blocked with normal goat serum, and incubated with polyclonal antibody B against myostatin at a dilution of 1:250 (Gonzalez-Cadavid et al., 1998). For MHC type I and II, the blocking step was done with normal horse serum followed by incubation with monoclonal antibodies against MHC type I or II (Novocastra Laboratories). The detection of myostatin and MHC was followed by a 1/200 dilution of anti-rabbit biotinylated secondary antibody (Calbiochem, La Jolla, CA) or anti-mouse biotinylated secondary antibody, respectively, followed by streptavidin-FITC (13 µg/ml) (Vector Labs, Burlingame, CA). After several washes, the slides were counterstained with propidium iodide and mounted in “prolong fade” (Molecular Probes, Eugene, OR) and were examined under a fluorescence microscope equipped with the appropriate filter.

Double labeling immunodetection of myostatin and MHC

The double localization of myostatin and MHC was carried out on p-formaldehyde fixed C2C12 myoblasts and myotubes plated on eight-well plates (Lab-Tek, Chamber slide system, Nalge Nunc, IL). For myostatin, cells were blocked with normal goat serum, and incubated with polyclonal antibody B against myostatin (Gonzalez-Cadavid et al., 1998) (1/250), followed by a 1/200 dilution of anti-rabbit biotinylated secondary antibody (Calbiochem, La Jolla, CA). The subsequent reaction was carried out by incubating the cells in a 20 µg/ml solution of streptavidin-FITC (Vector Labs), followed by 10% normal horse serum and then a 1/250 dilution of anti-MHC type I or II monoclonal antibodies (Novocastra Laboratories). Fluorescence labeling was performed with secondary antibody-Texas Red (13 µg/ml; Vector Labs). Slides mounted in “prolong fade” were examined under a fluorescence microscope equipped with the appropriate filters.

Detection of myostatin expression by immunoperoxidase staining

Alternatively, fixed cells were quenched for endogenous peroxidase activity, then blocked with normal goat serum, and incubated with polyclonal antibody B (1/250). Detection was based on a secondary anti-rabbit biotinylated antibody (1/200) follow by the ABC complex (1/100) (Vectastain Elite ABC System, Novocastra Laboratories) and 3,3 diaminobenzidine (DAB) (Sigma), without counterstaining. In negative controls, we either omitted the first antibody, or replaced it with a rabbit IgG isotype antibody or by a rabbit anti-myostatin antibody B depleted IgG fraction, prepared by affinity chromatography (dil 1/250). Endogenous myostatin immunoreactivity was quantitated by computerized densitometric analysis using the Image-Pro plus 4.01 software (Media Cybernetics, Silver Spring, MD) coupled to an Olympus BH-2 microscope equipped with a SPOT RT color digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Results were expressed as

optical density (OD) per cell area (OD/area). In this algorithm, after images are properly calibrated for background lighting, OD/area results are proportional to the unweighted average optical density, which is used to determine the concentration of immunoreactive antigen.

Statistical analysis

Data were expressed as mean $\pm$ SEM. The normality of the distribution of the data was established using the Wilks-Shapiro test, and the outcome measures between two groups were compared by the Student's *t*-test. To compare multiple groups, one way analysis of variance (ANOVA) was used. If ANOVA revealed significant differences, then individual group comparisons were made by using the Newman-Keul's post-hoc test. The difference among groups was considered significant at $P < 0.05$.

RESULTS

Expression of myostatin in C2C12 muscle cells

Expression of myostatin mRNA by Northern blot analysis and RT-PCR. In order to determine whether myostatin is expressed in C2C12 cells, we looked for the presence of myostatin mRNA and protein in cultured myoblasts and myotubes. Northern blot analysis showed no evidence of detectable endogenous myostatin

mRNA expression in either total or poly A⁺-selected RNA from C2C12 myoblasts and myotube cultures (data not shown). Further examination of myoblasts and myotubes total RNA by RT-PCR amplification, using primers discriminating between myostatin and GDF-11 (Sakuma et al., 2000), found myostatin mRNA to be present only in myotube cultures, as indicated by the presence of the expected 0.65 kb band (Fig. 1A; left and middle panels) in comparison to the 0.15 kb band for the reference gene, GAPDH. Co-incubation with 100 nM dexamethasone, a glucocorticoid known to stimulate myostatin transcription (Ma et al., 2001) in C2C12 muscle cell cultures, failed to induce the expression of mRNA in myoblasts as determined by RT-PCR analysis (middle panel). In contrast, myostatin RNA expression in C2C12 myotubes was markedly increased by the same dexamethasone concentration (left panel), and this increase was dose-dependent (right panel).

Expression of myostatin protein by Western blot analysis. Further confirmation of the very low myostatin expression in myoblasts was provided by Western blot analysis, which showed only traces of myostatin protein as indicated by the faint 30 kDa band detected by our antibody against myostatin (antibody-B) (Fig. 1B), as compared to the expression of the 40 kDa reference protein, GAPDH. Conversely, myotube cultures were found to express the expected 30 kDa myostatin

Fig. 1. Endogenous expression of myostatin mRNA and protein in C2C12 cell line. **A:** Total RNA isolated from C2C12 myoblasts (MB), myotubes (MT), and MB and MT treated with dexamethasone (DEX) was used for RT-PCR using myostatin primers encompassing a 650 bp sequence, and GAPDH primers for a 153 bp reference sequence. Dexamethasone was used at 100 nM only (left and middle panels) or at 50, 100, and 200 nM in this order (right panel). MSM: mouse skeletal muscle RNA as positive control. **B:** Western immunoblot of

endogenous myostatin protein expressed in C2C12 myoblasts (MB) and myotubes (MT), using antibody B against myostatin, and an antibody against GAPDH as reference protein. MT were also incubated with DEX (100 nM). The myostatin immunoreactive band is seen at 30 kDa in all cell and tissue extracts, as well as in myotube-conditioned medium (MT-CM). The GAPDH 40 kDa band is included as reference. RSM: rat skeletal muscle extract as a positive control.

Fig. 3. Distribution of myostatin protein in sub-cellular fraction: C2C12 myoblasts (MB) and myotubes (MT) were subjected to cell membrane lysis by two different procedures (A: M-Per and B: Promega). The nuclear and post-nuclear fractions were subjected to

Western immunoblot analysis as described in the text, utilizing on separate nitrocellulose membranes antibodies against myostatin, the cytoplasmic marker GAPDH, and the nuclear marker MyoD. RSM, rat skeletal muscle; NF, nuclear fraction; CF, cytoplasmic fraction.

immunoreactive protein (Fig. 1B), and secrete it into the medium. In agreement with the RT-PCR results, incubation of myotubes with dexamethasone (100 nM) increased substantially the intensity of the 30 kDa myostatin band.

Expression of myostatin protein by immunocytochemical staining. Further confirmation of the above findings was provided by immunocytochemical analysis of myostatin expression in myotubes, by using a secondary fluorescent antibody, which showed marked myostatin protein expression in most of the polynucleated cells. Remarkably, the staining was specifically localized within the nuclei. Most, but not all of the nuclei visualized with propidium iodide demonstrated myostatin immunoreactivity (see arrows) in multinucleated myotubes cultures (Fig. 2). Although myostatin immunoreactivity was localized mostly in myotube nuclei, in some instances, myostatin expression was also found in late myoblasts cultures; however, the intensity of

staining in myoblasts was weak (data not shown). We do not know whether negative nuclei originate from myoblasts recently fused into pre-existing fibers, which at this stage do not yet express myostatin. Alternatively, the absence of myostatin staining in these nuclei may result from the persistence of mononucleated myoblasts in the culture.

Sub-cellular localization of myostatin immunoreactive protein in the nucleus

To verify whether myostatin is associated with the myotube nuclei, we separated nuclei from the cytoplasmic fraction by centrifugation through a sucrose layer, and submitted both fractions to Western blot analysis. Two techniques were employed to separate the protein fractions and in both cases the 30 kDa myostatin immunoreactive protein was only seen in the nuclear fraction of the myotubes (Fig. 3). Very little myostatin protein, if any, was observed in the myoblasts nuclei,

Fig. 4. Immunohistochemical analysis of dexamethasone-induced changes in the levels of myostatin protein in C2C12 myotubes. C2C12 myotubes were incubated with medium alone (B) or graded concentrations of dexamethasone, C: 50 nM dexamethasone, D: 100 nM dexamethasone, E: 150 nM dexamethasone, and F: 200 nM dexamethasone) for 4 days and then stained using antibody-B against myostatin, and biotin-streptavidin-diaminobenzidine (DAB) as chromogen. No counterstain was used. A: Negative control using rabbit IgG isotype as the first antibody. Similarly, no specific staining was observed when the primary antibody was omitted (data not shown). In

panel A, the arrows point to cells without specific myostatin staining. In panel B the arrows point to cells demonstrating histochemical staining for endogenous myostatin. In panel F, the arrows point to the increasing presence of myostatin in the cytoplasm at the highest dexamethasone concentration (200 nM). **Bottom:** Image analysis of immunostaining expressed as total optical density per unit area in the nucleus and the cytoplasm (left), and the ratio of the intensity of staining in the cytoplasm and the nucleus. Values are means $\pm$ SEM, *** $P < 0.001$ (nuclei); ### $P < 0.001$ (cytoplasm) for comparisons with the control without dexamethasone.

Fig. 2. Immunohistochemical detection of myostatin protein in C2C12 myotubes. Fluorescent micrography using an anti-myostatin, polyclonal antibody (antibody B) as the primary antibody and FITC-labeled secondary antibody. The arrows point to cells without specific myostatin staining. **A,C:** Myostatin. **B,D:** Counterstain with propidium iodide. Magnification in panels A and B is 400 $\times$, and in panels C and D 1000 $\times$.

Fig. 4

although a 50 kDa band was immunodetected in procedure B, and an equivalent very faint band was also observed in method A.

The distribution among both fractions of a cytosolic marker (GADPH) and a nuclear marker (MyoD), showed that in method B there was little, if any, cross-contamination. In method A, no nuclear proteins leaked to the cytosol, as shown by MyoD immunoblotting, but there was considerable entrapment of cytosolic proteins in this "nuclear" fraction. However, both fractionation procedures and the immunocytochemical detection below were in agreement that under basal conditions little or no myostatin protein was present in the cytosol.

Effects of dexamethasone on the sub-cellular localization of myostatin immunoreactive protein

Based on our previous findings that glucocorticoids up-regulate myostatin mRNA transcription, we incubated myotubes with increasing concentrations of this dexamethasone, and determined whether this increases the intensity of immunocytochemical staining for myostatin. To obtain a semi quantitative estimation of myostatin protein content, we applied a biotin-streptavidin-DAB procedure. Increasing concentrations of dexamethasone resulted in a dose dependent stimulation of nuclear staining with the anti-myostatin antibody (Fig. 4; top). At the higher dexamethasone concentrations (150 and 200 nM), the expression of myostatin was not limited to the nuclei but it appeared to be extended to the cytoplasm adjacent to the more darkly stained nuclei (Fig. 4E,F). The negative controls (no first antibody and IgG isotype) did not show any staining. When we used myostatin depleted IgG as a third negative control, we could observe mild non-specific staining both in the nucleus and the cytoplasm, but there was no difference in the intensity of stain between the nucleus and the cytoplasm (not shown). A final confirmation of the specificity of the up-regulation of myostatin staining exerted by dexamethasone was obtained in a separate experiment where C2C12 myotubes were incubated in the presence of 100 nM dexamethasone and the glucocorticoid receptor antagonist RU-486 (0, 1, 5, 10, 20 μ M). Myostatin immunoreactivity was up-regulated as expected by dexamethasone but the effect was completely obliterated by RU-486 at all concentrations, (data not shown).

The densitometric image analysis showed a statistically significant increase of myostatin immunoreactivity in both the nucleus and cytoplasm of myotubes incubated with 100–200 nM dexamethasone (Fig 4, bottom left). This was accompanied by a gradual translocation of myostatin from the nucleus to the cytoplasm, as shown by the increase of the ratio of the staining intensity in the cytoplasmic and the nuclear compartments (Fig 4, bottom right).

Stage-specific expression and co-localization of myostatin immunoreactive protein in C2C12 myotubes that express MHC type II isoform

The expression of the major MHC isoforms was investigated using three different techniques. First Coomassie brilliant blue staining of proteins, separated

on a prolonged PAGE gel procedure, indicated that myotubes strongly expressed MHC-II and had relatively small amount of MHC-I (Fig. 5A). No evidence of MHC-I was seen in myoblasts, (Fig. 5A). Western blot analysis and immunocytochemical staining confirmed the finding that myotubes expressed all three MHC isoforms (Fig. 5B,C). However, neither Western blot analysis (Fig. 5B) nor immunocytochemistry (data not shown) found any MHC form to be expressed by myoblasts. Another confirmation of the strong presence of MHC type II in C2C12 myotubes was provided by immunocytochemistry using a FITC labeling second antibody. Figure 6 panel A, shows MHC type II in myotubes at 100 $\times$ magnification. Panel B is a light microscopic picture of C2C12 myotubes cultures showing a typical fiber (100 $\times$).

In order to confirm the nuclear localization of myostatin in myotubes and to identify its relationship with MHC, a dual labeling immunocytochemistry procedure was employed in C2C12 cells at the myotube stage, using FITC-linked secondary antibody for myostatin (green filter) and Texas red-linked secondary antibody (red filter) for MHC-I and -II. Pictures were obtained separately with the respective filters. Myostatin was expressed in most of the nuclei of myotubes (panels C and E), with some fibers showing some cytoplasmic staining. These myotubes also had strong and well-defined expression of MHC type II (panel F), and a much fainter and diffuse MHC type I (panel D).

DISCUSSION

Our data demonstrate that C2C12 muscle cells express myostatin mRNA and protein predominantly in differentiated, polynucleated myotubes, that also express MHC-II isoform as a major marker of fiber formation, and to a much lesser degree, the MHC-I isoform. The C2C12 myotubes also secrete myostatin into the medium. We have also shown that myostatin is located essentially in the nucleus, and that when its synthesis is up-regulated in a dose-dependent and glucocorticoid receptor-specific manner by dexamethasone, a well known inducer of myostatin transcription, the protein is translocated to the cytoplasm. Its nuclear localization suggests that myostatin may have a role in C2C12 cells as a transcriptional factor, modulating the expression of genes important for muscle replication and/or differentiation, and that at high levels myostatin may be transferred to the cytoplasm in transit for its secretion to the medium.

The "in vitro" differentiation of C2C12 myoblasts into myotubes mimics to a certain extent the fusion of satellite cells into pre-existing myotubes in the skeletal muscle to increase fiber size (Seale et al., 2000). Both processes involve the sequential activation of genes in the MyoD family: myogenin and MRF4 or Myf6, in cooperation with MEF-2, and a series of other growth factors (Thai et al., 1998; Perry and Rudnick, 2000). Response elements for all these genes have been found in the myostatin promoter (Ma et al., 2001) and, therefore, it is likely that the expression of myostatin is controlled by constituents of the MyoD and MEF families during the myoblast-myotube transition. Our finding of the predominant expression of myostatin in

Fig. 5. Expression of MHC subtypes in C2C12. **A:** Relative MHC (type I, IIa, and IIb) expression patterns in C2C12 myotubes (MT) and myoblasts (MB), as assessed by a modified PAGE procedure and staining with coomassie brilliant blue dye. MM: muscle mix as a control. **B:** Western immunoblot of MHC (type I and II) protein

expressed in C2C12 myoblasts (MB) and myotubes (MT). **C:** Immunohistochemical detection of MHC Type II (left panel) and Type I (right panel) using monoclonal antibodies against MHC and Texas red-secondary antibody in C2C12 myotubes (100 $\times$).

the C2C12 myotubes, utilizing a variety of approaches, agrees with this interpretation. This also corroborates our previous results with the activation of constructs of the myostatin promoter in C2C12 myotubes (Ma et al., 2001), and with the expression of myostatin "in vivo" in the muscle fibers (Gonzalez-Cadavid et al., 1998; Lalani et al., 2000), and confirms the detection of myostatin mRNA in myotubes (Sakuma et al., 2000; Rios et al., 2001).

Our data indicate that myostatin mRNA and protein are expressed mostly in the differentiated myotubes. However very low levels of the 30 kDa myostatin protein were detected in myoblasts by Western blot, and in addition an immunoreactive 50 kDa band, of a size compatible with the myostatin precursor, was also found in the nuclear fraction of myoblasts, but its identity remains to be determined. The more sensitive fluorescence detection procedure confirmed the presence of some myostatin protein in the myoblasts. We have previously reported that the myostatin promoter is also activated in C2C12 myoblasts (Ma et al., 2001), although the transcriptional activity of the promoter is greater in differentiated myotubes than in myoblasts.

The co-localization of myostatin protein with the strongly expressed MHC type II, and also with the minor MHC-I isoform, provides further evidence that myostatin is produced in differentiated myotubes. We do not know whether myostatin plays a role in muscle fiber differentiation. Although myostatin has been postu-

lated to be associated "in vivo" in the skeletal muscle exclusively with MHCIIb (Perry and Rudnick, 2000), there are also reports of its association with both MHC-I and -II (Yamanouchi et al., 2000). Further work is needed to determine whether myostatin can activate the expression of MHC-II in C2C12 myotubes through its effect on muscle cell differentiation.

The nuclear location of myostatin is intriguing, but agrees with our previous results showing that recombinant myostatin protein inhibits muscle cell proliferation and modulates the expression of several cell cycle genes in both C2C12 myoblasts and myotubes (Taylor personal communication), including the down-regulation of genes critical for cell proliferation, such as cyclins, (Bass et al., 1999; Taylor personal communication), and for myogenic commitment, such as Myf5, and the up-regulation of several transcription and growth factors. Functional studies should be conducted to determine whether myostatin directly modulates the activation of promoter constructs for any of these genes, which would support a role for myostatin as a transcription factor during muscle differentiation, and fully explain the nuclear location of this protein.

The intensification of myostatin staining in the nuclei of C2C12 myotubes incubated with dexamethasone and its obliteration with the glucocorticoid antagonist RU-486, provides further evidence of the specificity of nuclear staining. These observations also confirm its putative role as a mediator of glucocorticoid action in

Fig. 6. Immunofluorescence detection of MHC Type II using monoclonal antibodies against MHC-II as a primary antibody and FITC-secondary antibody in C2C12 myotubes and co-expression of myostatin and MHC type I and II proteins using a dual staining approach. **A:** Immunofluorescence staining for MHC type II using FITC-labeled secondary antibody. **B:** Light microscopic photograph of C2C12 myotubes showing fibers. **C–F:** Immunofluorescence detection of myostatin (panels C and E) and either MHC Type I (panel D) and

MHC Type II (panel F), using a double-labeling technique with a polyclonal antibody against myostatin and a FITC-labeled secondary antibody (Panels C and E), followed by incubation with monoclonal antibodies against MHC I (panel D) and II (panel F), respectively, and Texas red-labeled secondary antibody. Myostatin staining was visualized by using a green filter, and MHC types I and II by using a rhodamine filter. Magnification: 100 $\times$.

muscle tissue both in vivo and in vitro, (Ma et al., 2001). It is possible that the appearance of myostatin in the cytoplasm of C2C12 myotubes when myostatin expression is considerably stimulated by high doses of dexamethasone, may be part of a process that ultimately results in secretion of this protein into the cytoplasm and finally into circulation. These observations are consistent with our previously reported findings that myostatin is secreted into the plasma (Gonzalez-Cadavid et al., 1998). The nuclear location of myostatin appears to be cell-type-specific, as we have evidence of a pure cytoplasmic location in vitro in other cell types.

In summary, our results demonstrate the endogenous expression of myostatin mRNA and protein during myotube formation and its nuclear location, opening up potential studies on its putative transcriptional role

in the regulation of muscle cell replication and/or differentiation.

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