Identification of a Novel Pool of Extracellular Pro-myostatin in Skeletal Muscle*

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Myostatin, a transforming growth factor-β superfamily ligand, negatively regulates skeletal muscle growth. Generation of the mature signaling peptide requires cleavage of pro-myostatin by a proprotein convertase, which is thought to occur constitutively in the Golgi apparatus. In serum, mature myostatin is found in an inactive, non-covalent complex with its prodomain. We find that in skeletal muscle, unlike serum, myostatin is present extracellularly as uncleaved pro-myostatin. In cultured cells, co-expression of pro-myostatin and latent transforming growth factor-β-binding protein-3 (LTBP-3) sequesters pro-myostatin in the extracellular matrix, and secreted pro-myostatin can be cleaved extracellularly by the proprotein convertase furin. Co-expression of LTBP-3 with myostatin reduces phosphorylation of Smad2, and ectopic expression of LTBP-3 in mature mouse skeletal muscle increases fiber area, consistent with reduction of myostatin activity. We propose that extracellular pro-myostatin constitutes the major pool of latent myostatin in muscle. Post-secretion activation of this pool by furin family proprotein convertases may therefore represent a major control point for activation of myostatin in skeletal muscle.

Regulation of skeletal muscle size is an essential feature of organism development and adult muscle homeostasis. Several circulating factors function to control muscle growth, including the transforming growth factor-β (TGF-β)2 superfamily ligand myostatin, which is a negative regulator of skeletal muscle growth (1). Loss of myostatin function, in knock-out mice or mice treated with inhibitors, results in up to a 2-fold increase in skeletal muscle mass due to an increased number of muscle fibers and increased muscle fiber size (1–4). In contrast, ectopic expression of myostatin in adult mice induces cachexia, a systemic wasting syndrome (5). Regulation of myostatin production and signaling is essential to achieve a balance between muscle growth and wasting.

In adults, myostatin activity is regulated at several levels. First, myostatin expression is limited to a few cell types, including skeletal muscle and, to a lesser extent, adipose and heart tissues (1, 6). Second, myostatin is synthesized as a precursor protein that remains inactive until it is modified by several post-translational events (7). Third, multiple extracellular inhibitors limit access of myostatin to cell surface receptors (3, 7).

The myostatin precursor, referred to as pro-myostatin, forms a disulfide-linked homodimer following synthesis and translocation in the endoplasmic reticulum (1, 3). Like other TGF-β superfamily ligands, pro-myostatin is cleaved into amino- and carboxyl-terminal fragments at a tetrabasic cleavage site by the furin family of proprotein convertases (PCs) (3, 8, 9). This cleavage is thought to occur primarily in the Golgi apparatus, and the COOH-terminal, disulfide-linked product of this cleavage is the mature myostatin ligand. The mature myostatin homodimer remains non-covalently associated with the prodomain in a latent complex that is abundant in mouse serum in vivo (1, 9, 10). The BMP-1/tolloid family of metalloproteinasises can activate this latent complex by proteolytic cleavage between Arg-75 and Asp-76 of the myostatin prodomain (11). Following this activation of the latent complex, mature myostatin activates the Smad2/Smad3 signal cascade (12, 13). Several additional myostatin inhibitors have been identified in serum, including follistatin, GASP-1, and FLRG. It is still unclear, however, to what extent these inhibitors act individually or in conjunction with the inhibitory prodomain (10, 14). Thus, the current model for myostatin activation suggests that the latent myostatin complex is constitutively secreted into circulation from myostatin-producing cells, and that the activity of this complex is regulated by BMP-1/tolloid metalloproteinasises and secreted inhibitors.

Like myostatin, canonical TGF-β ligands are retained in a latent complex containing a mature peptide homodimer and a non-covalently associated inhibitory prodomain. The latter is commonly referred to as latency associated peptide (LAP) (15). TGF-β latent complexes also covalently associate with latent TGF-β-binding proteins (LTBPs), which are required for efficient folding and secretion of the ligands (16). Four LTBPs have been identified and are designated LTBPs 1–4 (17–20). Of the four LTBPs identified, LTBPs 1, 3, and 4 form a disulfide linkage with cysteine 33 of the TGF-β LAP (15, 21, 22). The domain of LTBPs that forms this disulfide bond with LAP is an 8-Cys motif that is unique to the LTBPs (21, 23). Once secreted, the TGF-β/LTBPs complex, called large latent complex, covalently associates with the extracellular matrix (ECM) through LTBPs (24). TGF-β ligands must be released from this

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2 The abbreviations used are: TGF-β, transforming growth factor-β; ECM, extracellular matrix; LAP, latency associated peptide; LTBP, latent TGF-β-binding protein; PC, proprotein convertase; HA, hemagglutinin; GFP, green fluorescent protein; PNGase, peptide:N-glycosidase F; ConA, concanavalin A; NP-40, Nonidet P-40.

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latent complex to signal (25). The binding of latent TGF-β to LTBP's tethers the latent complex to the ECM, providing a mechanism for local activation of TGF-β signaling. In the case of latent myostatin in serum, however, how local activation in tissues might be regulated is not clear.

Despite our detailed understanding of how LTBP's interact with canonical TGF-β ligands, little is known about interactions between LTBP's and other ligands in the TGF-β superfamily. In Xenopus, LTBP-1 (xLTBP-1) has been shown to synergize with the TGF-β superfamily ligands activin and nodal to induce mesoderm, but the basis for this synergy is not known (26). To what extent LTBP's interact generally with TGF-β superfamily ligands, and whether these interactions share common mechanisms or functions with those reported for LTBP's and TGF-β, have not been elucidated.

The maturation of TGF-β superfamily ligands by furin-like proteases is thought to occur constitutively in the Golgi apparatus. In the case of the TGF-β superfamily ligand nodal, however, it has recently been demonstrated that the PCs furin and PACE4 act extracellularly to cleave secreted pro-nodal into mature ligand, thus localizing nodal activity near cells that secrete PCs (27). This provides an intriguing example of localization of ligand activity by extracellular localization of maturation activity, but whether this example has broader relevance to regulation of TGF-β superfamily ligands is not known.

In this article we demonstrate that the predominant form of myostatin detectable in muscle is the pro-form, that this pro-myostatin associates with LTBP's, and that the major LTBP expressed in skeletal muscle, LTBP-3, sequesters pro-myostatin in the ECM. This retention of pro-myostatin by LTBP-3 limits myostatin signaling, as demonstrated by LTBP-3-dependent reduction of myostatin-induced Smad2 activation. In addition, ectopic expression of LTBP-3 in adult mouse skeletal muscle increases fiber area, consistent with the local inhibition of myostatin activity. These observations point to local maturation of secreted pro-myostatin in skeletal muscle by furin-like PCs as a significant new point of regulation of myostatin function, and suggest new approaches to the therapeutic inhibition of myostatin function for the treatment of muscle-wasting diseases.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—pBS(KS+) mouse myostatin was a gift from Se-Jin Lee (John Hopkins University School of Medicine, Baltimore, MD). Mouse myostatin was subcloned into pCS4+ using the restriction sites SfiI and XhoI. FLAG myostatin and HA myostatin were constructed by inserting codons 23–376 of mouse myostatin into a pCS4+ vector containing the Xnr1 signal peptide (codons 1–12) followed by either 3-FLAG or 3-HA epitope tags. FLAG myostatin prodomain/mature domain was constructed with 3 FLAG epitope tags following the Xnr1 signal peptide, as above, followed by 3 FLAG epitope tags on codon 272 of the myostatin mature domain. FLAG myostatin prodomain was constructed by PCR-based subcloning. FLAG myostatin prodomain contains the Xnr1 signal peptide (codons 1–12), 3 FLAG epitope tags, and mouse myostatin prodomain (codons 23–266). FLAG myostatin ANAA was constructed by changing codons 39–42 from CNAC to ANAA by mutagenesis PCR using the template FLAG mouse myostatin and the mutagenesis primer 5′-GAGAGAGAAGAAAAATGTGGAAAAAGAC-GCCCTAGCTAATGCAGCGGGTGAACAAACACG-AGG-3′. HA epitope-tagged human LTBP-2 and mouse LTBP-3 were provided in pcDNA3 vectors by Daniel B. Rifkin (New York University School of Medicine, New York, NY). pCS4+ mouse LTBP-3ΔC HA was constructed by PCR-based subcloning. Mouse LTBP3-ΔC HA contains the mouse LTBP-3 signal peptide (codons 1–22), one HA epitope tag, and mouse LTBP-3 codons 41–883. pCS2+ 6Myc human Smad3 was provided by Jeffrey L. Wrana (Samuel Lunenfeld Research Institute, Toronto, Ontario).

pGEM7zf Furin:Flag was provided by Gary Thomas (Vollum Institute, Portland, OR). PCR-based subcloning was used to insert codons 1–802 of Furin:Flag into pCS4+ . pCS4+ Furin:FlagΔC was constructed by PCR-based subcloning of codons 1–723 of Furin:Flag into pCS4+.

**In Vitro Translation**—In vitro translation of pCS4+ FLAG myostatin was performed using the Promega TnT® SP6-coupled Reticulocyte Lysate System (L4600, Promega, Madison, WI).

**Tissue Culture**—293T human kidney epithelial cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (10-437-028, Invitrogen), 50 IU/ml penicillin-streptomycin (30-001-C1, Mediatech, Herndon, VA). Cells were transfected using 25-kDa linear polyethylenimine (23966, Polysciences, Warrington, PA) as described previously (28).

**Xenopus Ectoderm Explants**—Xenopus embryos were collected, fertilized, and cultured as previously described (29, 30). Each blastomere of 2-cell stage embryos was injected with synthetic mRNA that was transcribed using the SP6 mMessage mMACHINE™ Kit (Ambion, Austin, TX). 200 pg of HA myostatin and 1500 pg of LTBP-3 HA RNA were injected as indicated. Ectoderm explants were harvested as previously described (30).

**Transfection of Mouse Tibialis Anterior Muscle and Fiber Area Measurements**—Adult female CD1 mice weighing 34–39 g were used. All mice were housed in the Seeley G. Mudd Animal Facility at Harvard Medical School. Tibialis anterior muscles were transfected as described previously (31). A 150 mM NaCl solution containing 5 μg of pCS2+ GFP CAXA and 16 μg of pcDNA3 mouse LTBP-3 HA plasmid DNA was injected into the tibialis anterior muscle, as indicated. Electric pulses were applied to the muscle at 50 volts/cm, 5 pulses, 100-ms intervals. Muscles were harvested 7 days later and processed by cryosection. Sections were fixed with 4% paraformaldehyde. Pictures of muscle cross-sections were captured with a ×10 objective using a Zeiss Axio Imager.M1 with AxioVision Release 4.5 software. Images were layered and color was added using Adobe Photoshop Software (San Jose, CA). Muscle fiber area was determined for GFP positive fibers using IMAGE software (Scion, Frederick, MD). At least 725 fibers were counted for each condition in a total of four mice.

**Precipitation and Western Blotting**—Cultured 293T cells were rinsed three times in ice-cold phosphate-buffered saline.
then lysed in modified RIPA buffer (150 mM NaCl, 50 mM Tris (pH 8), 25 mM β-glycerophosphate, 100 mM sodium fluoride, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 2× Complete EDTA-free protease inhibitor mixture (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride) plus 2 mM EDTA, 1% Nonidet P-40 (NP-40). Lysates were centrifuged and supernatants collected. NP-40 insoluble cell fraction was obtained by adding modified RIPA buffer plus 2 mM EDTA, 1% SDS to cell pellets that remained after lysis. Xenopus ectoderm explants were homogenized in modified RIPA buffer, plus 2 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, as above. Skeletal muscle and liver samples were rinsed three times in ice-cold phosphate-buffered saline then lysed in modified RIPA buffer plus 0.5 mM EDTA, 0.5% NP-40, 0.1% SDS, 0.25% sodium deoxycholate. Samples were homogenized using a Polytron tissue homogenizer. Lysates were centrifuged and supernatants collected.

For concanavalin A (ConA) precipitation, samples were incubated in ConA-agarose (Sigma), 1 mM MnCl₂, 1 mM CaCl₂ for 4 h at 4 °C. Samples bound to ConA-agarose were washed twice with cold phosphate-buffered saline, 1% NP-40, 1 mM MnCl₂, 1 mM CaCl₂, once with cold phosphate-buffered saline, 1% NP-40, 300 mM NaCl, 1 mM MnCl₂, 1 mM CaCl₂ and once with cold Tris buffer (10 mM Tris (pH 8.0)), 1 mM MnCl₂, 1 mM CaCl₂. Protein was eluted from the ConA-agarose overnight at 4 °C with modified RIPA buffer plus 0.5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.75 M methyl α-D-mannopyrano-side (Sigma).

The following antibodies were used for immunoprecipitations. Anti-HA rat monoclonal antibody (clone 3F10; Roche), followed by incubation with protein A matrix preincubated in rat anti-rabbit IgG (Jackson Laboratories, West Grove, PA); anti-Biotin rabbit polyclonal antibody (Rockland Immunocchemicals, Gilbertsville, PA); anti-mouse GDF-8 propeptide sheep polyclonal antibody (AF1539, R&D, Minneapolis, MN), followed by incubation with protein A matrix preincubated in sheep anti-rabbit IgG (Jackson Laboratories); anti-mouse GDF-8 goat polyclonal antibody (AF788, R&D), followed by incubation with protein A matrix preincubated in goat anti-rabbit IgG (Jackson Laboratories).

The following antibodies were used for Western blotting. Anti-HA-peroxidase rat monoclonal peroxidase-conjugated antibody (clone 3F10; Roche); anti-FLAG-peroxidase mouse monoclonal peroxidase-conjugated antibody (Sigma); anti-Myc-peroxidase mouse monoclonal peroxidase-conjugated antibody (Roche); anti-actin mouse monoclonal antibody (A4700, Sigma); anti-P-Smad2 rabbit polyclonal antibody (Cell Signaling Technologies, Danvers, MA); anti-mouse GDF-8 propeptide sheep polyclonal antibody (AF1539, R&D); and anti-mouse GDF-8 goat polyclonal antibody (AF788, R&D).

Glycosidase Treatment—For PNGase treatment, immunoprecipitated proteins were treated with the glycosidase PNGase F (New England Biolabs, Beverly, MA) in 50 mM sodium phosphate (pH 7.5), 1% NP-40 for 2 h at 37 °C.

Sulfo-NHS-Biotin-LC Treatment—For 293T cells, 1 mg/ml EZ Link Sulfo-NHS-LC-Biotin (21335, Pierce) was added to 293T cells in HEPES-buffered saline (HBS) (pH 8.0). Cells were incubated for 30 min at room temperature, then sulfo-NHS-LC-Biotin was quenched with HBS, 100 mM glycine (pH 8.0).

For mouse skeletal muscle and liver, 1 mg/ml sulfo-NHS-LC-Biotin was added to dissected skeletal muscle or liver from adult female CD1 mice in Krebs bicarbonate ringer solution (Krebs buffer) (pH 8.0). Tissues were incubated on ice for 30 min, then sulfo-NHS-LC-Biotin was quenched with Krebs buffer, 100 mM glycine (pH 8.0).

RESULTS

Myostatin Interacts with Latent TGF-β-binding Proteins—LTBP3 assist in the secretion of canonical TGF-β ligands and their retention in the ECM (32). To determine whether LTBP3 play a role in the regulation of myostatin, we investigated whether myostatin interacts with LTBP3. We compared, in transfected 293T cells, the binding of myostatin to LTBP-3, the most highly expressed LTBP in skeletal muscle, and to LTBP-2, which is expressed at low levels in muscle cells (expression of ectopic LTBP-1 and LTBP-4 was very low in our hands and was not pursued) (18, 33). Pro-myostatin interacts with LTBP-2 and LTBP-3 in co-immunoprecipitations from 293T cells (Fig. 1A). The prodomain of myostatin alone interacts very poorly with LTBP-3 (Fig. 1, A and B), indicating that the mature region is important for stable interaction of pro-myostatin with LTBP-3.
Mature myostatin, however, is not immunoprecipitated by LTBP-3, indicating that myostatin does not interact with LTBP-3 following proteolytic cleavage (Fig. 1C), and that both the mature and pro-domains of myostatin are necessary for stable interaction with LTBP-3. We also observed (Fig. 1C) that LTBP-3 expression results in a decrease in the amount of mature myostatin in lysates, indicating that LTBP-3 can inhibit production of mature myostatin.

We noted that pro-myostatin is present in 293T cells as a doublet, and that each LTBP immunoprecipitated different forms of this pro-myostatin doublet; LTBP-2 predominantly precipitated the faster migrating form of pro-myostatin, whereas LTBP-3 almost exclusively precipitated the slower migrating form of pro-myostatin (Figs. 1A and 2A). The most likely basis for altered migration of pro-myostatin is the addition of N-linked glycosylations during passage through the secretory pathway (1, 34). To determine whether the pro-myostatin doublet we observed was a result of N-linked glycosylation, we compared the migration of pro-myostatin synthesized in vitro, in the absence of the secretory apparatus, to pro-myostatin isolated from cells and treated with or without the deglycosylating enzyme PNGase F (Fig. 2A). The faster migrating form of pro-myostatin corresponds in size to pro-myostatin expressed in vitro and to PNGase-treated pro-myostatin, indicating that the faster migrating form of pro-myostatin is the unglycosylated form. The slower migrating form of pro-myostatin is converted to the faster migrating form by PNGase F treatment, indicating that N-linked glycosylations are responsible for the difference in migration. When co-expressed myostatin and LTBP-3 were immunoprecipitated for the HA epitope tag in LTBP-3, PNGase F treatment shifted the LTBP-3-associated pro-myostatin from the slower migrating form to the faster migrating form (Fig. 2A). These data indicate that LTBP-2 preferentially associates with unglycosylated pro-myostatin, whereas LTBP-3 preferentially associates with glycosylated pro-myostatin. Because N-linked glycosylations are typically added as proteins move through the secretory apparatus, these data suggest that LTBP-2 binding is restricted to pro-myostatin that is retained in the endoplasmic reticulum in a form that is not competent for glycosylation or secretion, whereas LTBP-3 binds to pro-myostatin that can be secreted.

LTBPs are covalently linked to canonical TGF-βs by a disulfide bond between an 8-cysteine motif in the LTBPs and a cysteine near the N terminus of the TGF-β prodomain (15, 21–23, 35). The myostatin prodomain contains two cysteines at a similar position as the cysteine in TGF-β that links to LTBPs, raising the possibility that pro-myostatin also forms a disulfide linkage with LTBP. To determine whether myostatin interacts with LTBP-3 in a manner similar to the canonical TGF-β ligands, we created a myostatin in which the cysteines near the N terminus of the prodomain have been changed to alanines (Fig. 1B). Co-expression of pro-myostatin in which the prodomain cysteines have been mutated to alanine has no effect on LTBP-3 binding to myostatin (Fig. 1A), however, indicating that a disulfide linkage is not necessary for stable interaction between these proteins. Furthermore, an LTBP-3 construct that lacks the carboxyl-terminal region containing the TGF-β binding motif, LTBP-3ΔC (Fig. 1B), binds as effectively to pro-myostatin as does wild type LTBP-3 (Fig. 2B), indicating that pro-myostatin binds to a different region of LTBP-3 than does TGF-β. The pro-myostatin-LTBP-3 interaction is not retained during Laemmli gel electrophoresis under non-reducing conditions (not shown), further indicating that this interaction, unlike the binding of TGF-β to LTBPs, does not involve a disulfide linkage.

Pro-myostatin Is Retained in the Extracellular Matrix of Cells Expressing Latent TGF-β-binding Protein—3—LTBPs assist in the secretion of canonical TGF-β ligands (32). To determine whether LTBPs influence the secretion of myostatin, we monitored myostatin levels in NP-40-solubilized cell lysates, conditioned media, and the NP-40-insoluble cell fraction of cells expressing myostatin in the presence or absence of LTBP-3. In
contrast to expectations based on observations of the effect of LTBP on TGF-β secretion, co-expression of LTBP-3 dramatically reduced the amount of pro-myostatin in the conditioned media of 293T cells (Fig. 2B). Concomitant with this reduction of pro-myostatin in conditioned media by LTBP-3 expression, however, we observed an increase in pro-myostatin in the NP-40-insoluble cell pellet. This pellet could reflect either an intracellular NP-40-insoluble fraction (e.g. cytoskeleton) or the NP-40-insoluble ECM. To distinguish between these possibilities, we treated intact 293T cells with a cell impermeant labeling agent, sulfo-NHS-biotin (Fig. 2C). Sulfo-NHS-biotin exclusively labeled pro-myostatin that was co-expressed with LTBP-3, indicating that LTBP-3 increased the amount of pro-myostatin in the ECM (Fig. 2C). Sulfo-NHS-biotin did not detectably label a co-transfected intracellular protein, 6MyC-Smad3, confirming that labeling was not due to cell lysis or internalization of the labeling agent. Myostatin expressed in the absence of LTBP-3 is abundant in cell lysates but is not labeled by sulfo-NHS-biotin (Fig. 2C), providing an additional control that intracellular proteins are not labeled by sulfo-NHS-biotin. These data therefore demonstrate that LTBP-3 sequesters pro-myostatin in the ECM.

LTBPs are co-expressed with and bind to the canonical TGF-β ligands intracellularly (32), however, purified LTBP-1 can also regulate TGF-β activity (38). To determine whether co-expression of myostatin and LTBP-3 is required for the observed sequestration of pro-myostatin in the ECM, we either co-expressed myostatin and LTBP-3 in the same cells or mixed myostatin expressing cells with LTBP-3 expressing cells and then assayed the amount of myostatin in both the culture media and the NP-40-insoluble cell fraction (Fig. 3A). In contrast to what we observed in cells co-expressing myostatin and LTBP-3, we see no change in the levels of myostatin in the media or NP-40-insoluble fraction when cells expressing myostatin are mixed with cells expressing LTBP-3, indicating that LTBP-3 and myostatin must be expressed in the same cell for LTBP-3 to sequester myostatin in the ECM.

**Extracellular Pro-myostatin Is Cleaved by the Proprotein Convertase Furin**—If pro-myostatin is secreted prior to proteolytic processing, generation of the mature myostatin ligand from this pool of pro-myostatin requires extracellular cleavage by furin-like PCs. To test this prediction, we expressed myostatin and a soluble form of the PC furin in separate cell populations, then mixed these populations and asked if extracellular myostatin can be cleaved by extracellular furin. When myostatin expressing cells are mixed with cells expressing soluble furin, the amount of pro-myostatin in the conditioned media decreases, whereas the amount of myostatin prodomain and mature myostatin increases (Fig. 3B). These data are consistent with the possibility that extracellular pro-myostatin can be mobilized into active ligand by extracellular furin-like proteases.

**Pro-myostatin Predominates in Skeletal Muscle, and a Portion of This Pool Is Extracellular**—We find that ectopically expressed pro-myostatin is secreted into the ECM of 293T cells in the presence of LTBP-3. To examine whether endogenous pro-myostatin is present extracellularly in vivo, we first determined what forms of myostatin are present in mouse skeletal muscles and in mouse serum. Consistent with previous reports (5, 10), we found that proteolytically processed mature myostatin predominates in serum (Fig. 4A). In mouse hind-limb muscle, in contrast, pro-myostatin is the predominant form of myostatin detectable (Fig. 4A). To determine whether any of the pro-myostatin we observed in skeletal muscle was extracellular, we treated excised skeletal muscle with cell impermeant sulfo-NHS-biotin-LC. Biotin-labeled pro-myostatin was detectable (Fig. 4B). After 24 h, myostatin expressing cells were mixed with furin-LC expressing cells. Cells and conditioned media were harvested 24 h later.

**FIGURE 3. Sequestration of pro-myostatin in the ECM depends on co-expression of LTBP-3 and myostatin, and extracellular furin can cleave extracellular pro-myostatin.** A and B, 293T cells were transfected with myostatin (Flag Myo) containing 3 FLAG epitope tags in the prodomain, myostatin (Flag Myo Pro/Mat) containing 3 FLAG epitope tags in the prodomain, and 3 FLAG epitope tags in the mature domain and HA epitope-tagged LTBP-3 (L3 HA), as indicated. Conditioned media was harvested from cell cultures. Samples were immunoprecipitated (IP) and detected by immunoblot, as indicated. Transfection efficiency was controlled by normalizing for β-galactosidase values. A, sequestration of pro-myostatin in the ECM requires co-expression of LTBP-3 and myostatin. Cells were either co-transfected with myostatin and LTBP-3, as indicated, or transfected separately and then mixed 24 h after transfection (Mix). Cell lysates and media were harvested as described in the legend to Fig. 2. B, soluble furin increases the amount of mature myostatin in the conditioned media of adjacent cells. Cells were transfected with either myostatin or soluble furin (Furin ΔC). After 24 h, myostatin expressing cells were mixed with furin-LC expressing cells. Cells and conditioned media were harvested 24 h later.

**Latent TGF-β-binding Protein-3 Prevents Cleavage of Myostatin by Furin**—In the case of canonical TGF-β ligands, LTBPs are thought to localize latent TGF-β to the ECM, where a variety of stimuli can lead to local release of the ligand and activate TGF-β signaling (39). We have observed that LTBP-3 retains myostatin in the ECM, but the nature of the physiological


**Extracellular Pro-myostatin in Skeletal Muscle**

**FIGURE 4.** Pro-myostatin is the predominant form of myostatin in skeletal muscle, whereas cleaved myostatin predominates in serum. Mouse serum (S), skeletal muscle (M), and liver (L) were lysed and immunoblotted as indicated. Liver was used as a negative control for immunoblots. A, pro-myostatin predominates in skeletal muscle and the prodomain and mature myostatin predominate in serum. Samples were precipitated with concanavalin A, and then immunoprecipitated for the myostatin prodomain, as indicated. B, pro-myostatin is extracellular in skeletal muscle. Samples were treated with cell impermeant sulfo-NHS-Biotin-LC (S-NHS-Biotin). Samples were precipitated with concanavalin A, and then immunoprecipitated for the myostatin prodomain, as indicated. Samples were separated by non-reducing SDS-PAGE. C, LTBP-3 prevents furin cleavage of myostatin. 293T cells were transfected with myostatin (Flag Myo) containing 3 FLAG epitope tags in the prodomain, HA epitope-tagged LTBP-3 (L3 HA), and Furin, as indicated. Conditioned media was harvested from cell cultures. Samples were detected by immunoblot, as indicated. Transfection efficiency was controlled by normalizing for $\beta$-galactosidase values.

**FIGURE 5.** LTBP-3 increases skeletal muscle fiber area and reduces myostatin-induced signaling. A, LTBP-3 increases skeletal muscle fiber area. Adult mouse tibialis anterior muscles were transfected by electroporation with GFP and LTBP-3 as indicated. Transverse sections of muscles were analyzed for the cross-sectional area of GFP positive fibers. Histogram indicates the distribution of cross-sectional areas of fibers expressing GFP and LTBP-3 (red bars) and GFP alone (green bars). * Student’s $t$ test $p = 0.005$. Images depict GFP expressing fibers (green), GFP and LTBP-3 expressing fibers (red), and nuclei (blue). B, LTBP-3 reduces myostatin induced phosphorylation of Smad2. Xenopus embryos were injected with LTBP-3 and myostatin RNA, as indicated and kept at 23 °C. Animal caps were cut 3 h after RNA injection and harvested 4 h after dissection. Samples were detected by immunoblot, as indicated.

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stimuli that might lead to activation of this LTBP-3 bound pro-myostatin pool are not known. To examine whether binding to LTBP-3 increases or decreases accessibility of pro-myostatin to furin, we compared the accumulation of the cleaved myostatin prodomain in the presence of furin, with or without co-expressed LTBP-3 (Fig. 4C). LTBP-3 expression substantially decreased the cleavage of pro-myostatin to the free prodomain, indicating that under these conditions LTBP-3 masks access of furin to pro-myostatin. In addition, furin expression did not increase the amount of myostatin in the conditioned media, indicating that furin is not sufficient to release myostatin from retention in the ECM by LTBP-3. Whether an additional stimulus is necessary to render LTBP-3 bound pro-myostatin accessible to furin-like proteases, or whether a PC protease other than furin is responsible for the specific cleavage of pro-myostatin, will be interesting areas for future work.

**Expression of Latent TGF-β-binding Protein-3 Increases in Mouse Muscle Increases Fiber Size and Inhibits Myostatin Signaling**—In our *ex vivo* test system, LTBP-3 reduces pro-myostatin processing. These results would predict that in skeletal muscle *in vivo*, excess LTBP-3 might reduce or restrict endogenous myostatin activation and thus locally increase muscle fiber size. To test this prediction, we transfected mouse tibialis anterior skeletal muscle by electroporation (31). Muscles were transfected with GFP with or without LTBP-3, and the cross-sectional area of muscle fibers expressing ectopic LTBP-3 were compared with those that did not (Fig. 5A). LTBP-3 expression decreased the number of small fibers and increased the number of large fibers, and the average size of LTBP-3 expressing muscle fibers was significantly larger than fibers transfected with GFP alone ($p < 0.005$). These data are consistent with the hypothesis that ectopic LTBP-3 can reduce myostatin function.

The suppression of pro-myostatin cleavage by co-expressed LTBP-3, and the increase in muscle fiber size with LTBP-3 expression, suggest that LTBP-3 is likely to restrict myostatin signaling by directly inhibiting myostatin signals. Because expression of myostatin in cultured cells was not sufficient to activate Smad2 phosphorylation in these cells (not shown), we examined the effect of LTBP-3 on myostatin signaling in a system in which expression of myostatin directly induces phosphorylation of Smad2, the prospective ectoderm of the early *Xenopus* embryo (40). *Xenopus* prospective ectoderm (animal cap) was injected with mRNA encoding myostatin with or without LTBP-3 and the phosphorylation of Smad2 was examined.
As seen in Fig. 5B, expression of LTBP-3 decreases myostatin-induced Smad2 phosphorylation, indicating that LTBP-3 can inhibit myostatin signaling in addition to reducing myostatin processing and increases skeletal muscle fiber area.

**DISCUSSION**

The present studies have demonstrated the presence of a novel pool of extracellular pro-myostatin in skeletal muscle. In contrast to serum, where mature myostatin is predominantly found in a latent complex with its prodomain, we find that uncleaved pro-myostatin predominates in skeletal muscle, and that a portion of this pro-myostatin is extracellular. We suggest that this pool of inactive pro-myostatin is held in the ECM by LTBP-3 (Fig. 6). In cultured cells, LTBP-3 selectively retains fully glycosylated pro-myostatin in the ECM when LTBP-3 and myostatin are co-expressed. This extracellular pro-myostatin can be cleaved by soluble furin, and cleavage of pro-myostatin is inhibited by co-expression of LTBP-3. Co-expression of myostatin and LTBP-3 reduces myostatin-induced Smad2 phosphorylation, and ectopic expression of LTBP-3 in skeletal muscle cleaves mature myostatin in the extracellular space (27). Localization of expression of the PCs and the maturation proteases first encounter one another in the ER Golgi apparatus (Fig. 6) (16, 41). We have found that the pro-domain of myostatin to myostatin, a key negative regulator of muscle mass, by the furin family of proteases represents a novel control point for myostatin signaling in skeletal muscle.

LTBP-3 is the predominantly expressed LTBP family member in skeletal muscle, consistent with a role in regulating the mobilization of myostatin in this tissue (1, 19, 33, 42, 43). Genetic inactivation of LTBP-3 results in mice that are small in size, have high levels of corticosterone, and develop osteopetrosis (44–46). This phenotype does not correspond to what one would expect from generalized ectopic activation of myostatin (e.g. muscle wasting), but in our model of LTBP-3 regulation of myostatin, loss of LTBP-3 may only become apparent under specific physiological stresses required for local myostatin activation. It is also possible that other phenotypic effects of LTBP-3 inactivation mask local changes in myostatin regulation, or that other LTBPs (expressed at low but detectable levels in skeletal muscle) provide some compensation for the loss of LTBP-3.

Although extracellular maturation of TGF-β ligands is not believed to be the rule, several cases have been reported. For example, secretion of pro-TGF-β1,2 has been observed in human glioblastoma cells (47). In this case cleavage of pro-TGF-β1,2 is initiated by release of furin into tissue culture media. A more compelling example in which extracellular maturation may be critical for function in vivo involves the TGF-β superfamily ligand nodal, a key regulator of early embryonic patterning (48). The nodal gene is expressed in a different region of the embryo from the PCs (Furin and PACE4) required to generate the mature nodal ligand, and therefore pro-nodal and the maturation proteases first encounter one another in the extracellular space (27). Localization of expression of the PCs required for nodal activation is therefore a critical step in localized nodal activation and early embryo patterning. A similar mechanism has been elucidated for other signaling molecules, such as pro-neurotrophins (49). Which protease(s) are responsible for extracellular maturation of myostatin is unclear and difficult to predict. Whereas we find that ectopic furin is capa-

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**Extracellular Pro-myostatin in Skeletal Muscle**

![Diagram of myostatin and TGF-β ligand maturation and secretion](image)

**FIGURE 6. Model of myostatin and TGF-β ligand maturation and secretion.** Green arrows indicate the model proposed here.
ble of catalyzing this maturation, furin family proteases share overlapping substrate specificities. PACE4 and PC7/8 are highly expressed in a wide variety of tissues, whereas the other PCs have more limited expression profiles (8). Furin and PACE4 have been shown to act extracellularly (27), but an extracellular role for other PC proteases has not been ruled out.

The predominance of pro-myostatin that we observe in skeletal muscle contrasts with previous reports in which cleaved prodomain and mature myostatin are the major forms in which myostatin is extracted from muscle lysates (6, 50–52). We verified the specificity of our antibodies by multiple methods. First, we confirmed the size of expected bands on Western blots using both overexpressed and endogenous samples. As expected, under reducing conditions we observed mature myostatin and mature myostatin form covalent dimers under non-reducing conditions. Finally, we compared serum and skeletal muscle samples to lung samples that we did not expect to express myostatin. Several groups have used verified antibodies to visualize pro-myostatin in skeletal muscle, but none have contrasted this with relative levels of pro-myostatin, myostatin prodomain, and mature myostatin in both skeletal muscle and serum, and none have addressed whether this pool of pro-myostatin is extracellular (6, 53, 54).

Despite the clear importance of myostatin in restricting normal muscle growth, many aspects of myostatin function in muscle physiology and homeostasis remain obscure. Systemic myostatin inhibition has positive therapeutic effects in models of muscle atrophy such as muscular dystrophy and sarcopenia. However, the role of myostatin in local regulation of specific muscles or muscle groups has not been fully clarified. One possibility, suggested by the work of Greenspan and colleagues (11), is that serum myostatin, rendered latent by furin-like PC proteases to generate mature myostatin, might be activated locally by BMP-1/tolloid protease and BMP-1/tolloid protease. If maturation of ECM-deposited pro-myostatin is a major limiting step in endogenous myostatin activation, then development of inhibitors of extracellular furin family proteases provides a new avenue for therapeutic inhibition of myostatin activity. More generally, the identification of multiple instances (this work and Ref. 27) in which the maturation of TGF-β superfamily ligands takes place extracellularly rather than intracellularly suggests that the role of extracellular proteases in the maturation of TGF-β superfamily ligands during physiological regulation and development merits broader examination.

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