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Expression, purification, renaturation and activation of fish myostatin expressed in *Escherichia coli*: Facilitation of refolding and activity inhibition by myostatin prodomain

Bruria Funkenstein *, Yanai Rebhan

Department of Marine Biology and Biotechnology, Israel Oceanographic and Limnological Research, National Institute of Oceanography, P.O. Box 8030, Tel-Shikmona, Haifa 31080, Israel

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Abstract

Myostatin (growth and differentiation factor-8) is a member of the transforming growth factor-β superfamily, is expressed mainly in skeletal muscle and acts as a negative growth regulator. Mature myostatin (C-terminal) is a homodimer that is cleaved post-translationally from the precursor myostatin, also yielding the N-terminal prodomain. We expressed in *Escherichia coli* three forms of fish myostatin: precursor, prodomain and mature. The three forms were over-expressed as inclusion bodies. Highly purified inclusion bodies were solubilized in a solution containing guanidine hydrochloride and the reducing agent DTT. Refolding (indicated by a dimer formation) of precursor myostatin, mature myostatin or a mixture of prodomain and mature myostatin was compared under identical refolding conditions, performed in a solution containing sodium chloride, arginine, a low concentration of guanidine hydrochloride and reduced and oxidized glutathione at 4 °C for 14 days. While precursor myostatin formed a reversible disulfide bond with no apparent precipitation, mature myostatin precipitated in the same refolding solution, unless CHAPS was included, and only a small proportion formed a disulfide bond. The *trans* presence of the prodomain in the refolding solution prevented precipitation of mature myostatin but did not promote formation of a dimer. Proteolytic cleavage of purified, refolded precursor myostatin with furin yielded a monomeric prodomain and a disulfide-linked, homodimeric mature myostatin, which remained as a latent complex. Activation of the latent complex was achieved by acidic or thermal treatments. These results demonstrate that the *cis* presence of the prodomain is essential for the proper refolding of fish myostatin and that the cleaved mature dimer exists as a latent form.

Keywords: Active mature myostatin; Refolding; Prodomain; Purification; Furin cleavage; Latent complex; Latent complex activation; Reporter gene assay; Fish

Myostatin (MSTN[1], also known as growth and differentiation factor-8) is a member of the transforming growth factor-β (TGF-β) superfamily that acts as a negative regulator of skeletal muscle growth by suppressing proliferation and differentiation of myoblasts [1,2]. MSTN knockout mice have 2–3-fold greater muscle mass than their wild type littermates, mainly due to an increase in the number of muscle fibers (hyperplasia) and the thickness of the fibers (hypertrophy) [1]. Transgenic mice carrying a dominant negative MSTN showed up to a 35% increase in skeletal muscle mass and this increase was the result of muscle hypertrophy [3]. Similar increases in skeletal muscle mass are seen in certain cattle breeds like Belgian Blue, Piedmontese and South Devon that carry mutations in the MSTN coding sequence, a phenomenon known as "double muscling" [4–7]. The similarity in phenotypes of double-muscled cattle and MSTN-mutant mice suggests that MSTN plays a key role in controlling muscle mass during

* Corresponding author. Fax: +972 4 8511911.
E-mail address: bruria@ocean.org.il (B. Funkenstein).

[1] *Abbreviations used:* MSTN, myostatin; TGF-β, transforming growth factor-β; IB, inclusion bodies; Gdn-HCl, guanidinium hydrochloride; DTT, dithiothreitol; LARII, luciferase assay reagent II; CHO, Chinese hamster Ovary; NGF, nerve growth factor.

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development. Neutralization of MSTN by administrating anti-MSTN antibodies to adult mice significantly increased skeletal muscle mass [8]. By contrast, administration of MSTN induced significant muscle wasting in mice [9]. *In vitro* studies suggested that MSTN regulates muscle mass, at least in part, by controlling the proliferation of myoblasts, inducing cell cycle arrest in the G1 phase [10,11].

Like other members of the TGF-β superfamily, MSTN is synthesized as a precursor protein consisting of a signal peptide, an amino-terminal propeptide domain (or prodomain) and a carboxy-terminal mature (active) domain. The precursor undergoes two proteolytic processing events in order to generate the biologically active molecule. The first cleavage removes the signal peptide necessary for targeting the protein to the secretory pathway. The second cleavage occurs at a dibasic site (RXRR) by a furin-type protease to generate the amino-terminal propeptide and the carboxy-terminal mature protein. It is believed that the precursor protein forms a homodimer before the proteolytic processing [1,12,13]. The cleaved propeptide molecule remains non-covalently bound to the mature domain dimer, forming a latent complex and inhibiting its biological activity by inhibiting MSTN binding to its receptor [13]. Studies have shown that more than 70% of mature MSTN circulating in serum is bound to its propeptide as a latent complex [14], which can be activated by acid treatment [9]. The importance of MSTN prodomain for mature MSTN activity was further demonstrated in transgenic mice over-expressing the MSTN prodomain, which showed a dramatic muscling phenotype [15]. Various studies showed that both mature and precursor MSTN form a disulfide-linked dimer like many other members of the TGF-β superfamily [1,12,13].

While MSTN function is well documented in mammalian species [16], less is known about the role of MSTN in fish and especially it is still not clear if this growth factor plays a similar repressing role in muscle growth as it does in mammals. Few studies using the laboratory zebrafish model suggested that MSTN might act in fish as in mammals [17–19]. The key role of MSTN in muscle growth and its potential applications in animal husbandry has prompted the sequencing of MSTN cDNAs and genes from numerous fish species of commercial value to aquaculture [17,20–31]. These studies have shown that in contrast to mammals, fish express MSTN not only in the red and white muscle, but also in other tissues. Thus, fish MSTN might play a role not only in muscle growth but also in other, yet unidentified physiological processes. Mature MSTN in fish is 109 amino acids long and its sequence is highly conserved compared to other vertebrates and other fish species [6,31] suggesting that the protein is essential for maintaining a vital biological function. By contrast, MSTN prodomain varies between fish species in its length and its amino acid sequence [21,22,29].

Availability of large quantities of fish active MSTN is a first step for studies aimed at elucidating the physiological role of MSTN in fish. Although expression of recombinant peptides in *Escherichia coli* is the most convenient and economic method of choice, a major drawback is proper refolding of the proteins expressed in bacteria. Several reports have described the purification of biologically active mammalian MSTN protein produced as an isolated C-terminal fragment in bacteria [10,11]. However, these studies lack a detailed account on the yield, refolding and biochemical characterization, and as discussed recently [16], the concentration of bacterially produced MSTN protein required for demonstrating biological effects in these studies was quite high, suggesting that only a fraction of the purified preparation represented properly folded C-terminal dimer. By contrast, MSTN produced using the mammalian CHO cell line readily formed homodimer [12,13,32] and the purified homodimer was active in *in vitro* assays.

Production of recombinant fish mature MSTN was reported by only two groups. Zebrafish mature MSTN was expressed as a fusion protein in *E. coli* [33] but no attempts were made to refold the recombinant protein or show its biological activity. Zebrafish and tilapia MSTN were also expressed in a mammalian cell line [28,33], but again these studies failed to purify the peptide, show the characteristic disulfide-bond formation of the secreted peptide or test its biological activity. Using a variety of protocols suggested for refolding several members of the TGF-β superfamily, we were unsuccessful in our attempts to refold mature MSTN cloned from the marine fish *Sparus aurata* and produced in bacteria. A recent report demonstrated that MSTN prodomain was needed for the proper refolding of porcine MSTN produced in bacteria [34], however no biological activity was shown for this preparation. The MSTN prodomain of *S. aurata* is different from that of pig, including a track of 12 glutamine residues. In the current study, we undertook the production, in *E. coli*, of three forms of MSTN from *S. aurata*, namely, precursor, mature and prodomain. From the resultant inclusion bodies, the refolding of precursor MSTM, mature MSTN and a mixture of prodomain and mature MSTN was evaluated. Our results show that the presence of MSTN prodomain is essential not only for refolding fish MSTN *in vitro*, but also prevents its precipitation during the prolonged refolding process. Furthermore, our results show that following cleavage with the endopeptidase furin, the mature MSTN dimer remains as a latent form, which can be activated by acidic or thermal treatment to elicit biological activity.

Materials and methods

Chemicals

Oligonucleotides were prepared by Sigma (Israel). Restriction and modifying enzymes were purchased from New England Biolabs (Beverly, MA, USA) and Promega (Madison, WI, USA). Reagents for SDS–PAGE and molecular mass markers were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Glutathione (reduced and oxidized) was from Calbiochem (San Diego, CA, USA) and

Ni–NTA His-Bind Resin was from Novagen (Madison, WI, USA). All other chemicals were of analytical grade.

Amplification and cloning of S. aurata MSTN (saMSTN) cDNAs

Total RNA was extracted from *S. aurata* skeletal fast muscle or from a pool of 7d larvae by using TriReagent (Molecular Research Center, Cincinnati, OH, USA) or Trizol (Gibco-BRL, Rockville, MD, USA) commercial kits. The full-length saMSTN cDNA (1155 nt) was obtained by PCR using primers MSTN-1 and MSTN-2 (Table 1) and reverse-transcribed 7d larval RNA. The unprocessed precursor saMSTN (1092 nt, Ser-22–Ser-385) was obtained by PCR using primers MSTN-5 and MSTN-4 (Table 1) and reverse-transcribed muscle RNA. Mature saMSTN (327 nt, Asp-277–Ser-385) was obtained by PCR using primers MSTN-3 and MSTN-4 (Table 1) and the plasmid containing full-length saMSTN cDNA as template. Prodomain saMSTN (765 nt, Ser-22-Arg-276) was obtained by PCR using primers MSTN-5 and MSTN-8 and the plasmid containing precursor MSTN as template. All PCR fragments were gel-purified using QIAquick Gel Extraction Kit (Qiagen, GmbH, Hilden, Germany) and cloned in pGEM-Teasy vector (Promega, Madison, WI, USA) or pCR-Script Amp SK(+) vector (Stratagene, La Jolla, CA, USA), which were used to transform competent *E. coli* strain JM109 or XL10-Gold Kan ultracompetent cells, respectively, as recommended by the suppliers. Clones containing inserts were sequenced to verify that no errors were introduced. All primers (Table 1) were designed based on published sequence information [21] (GenBank Accession No. AF258448).

Construction of saMSTN expression vectors

Three constructs that contained the precursor, prodomain and mature saMSTNs were cloned in the expression vector pET-16b vector (Novagen, Madison, WI, USA). Primers MSTN-3 and MSTN-5 contained an *Nde*I restriction site. The ATG donated by this site was in the same reading frame as the saMSTN coding sequences. The downstream PCR primers MSTN-4 and MSTN-8 contained a stop codon immediately after the last codon, followed by a *Bam*HI restriction site. The inserts were cut out from the intermediate plasmids by double digestion with restriction enzymes *Nde*I and *Bam*HI and ligated into pET-16b vector, which was also digested with *Nde*I and *Bam*HI. The ligation products were used to transform *E. coli* strain JM109. The saMSTN (precursor, prodomain and mature) were placed in-frame with a His-Tag of ten histidine residues, located on the N-terminal end of the MSTN sequences. Plasmids carrying MSTN5-4/pET-16b, MSTN5-8/pET-16b and MSTN3-4/pET-16b were isolated, sequenced with T7 promoter primer and internal primers and then used to transform the host *E. coli* strain Origami (DE3)pLysS.

Expression of recombinant saMSTN

Transformed Origami (DE3)pLysS *E. coli* cells harboring saMSTN5-4/pET-16b (precursor MSTN) were grown in a starter culture of 5 or 50 ml LB (depending on the experiment) in the presence of ampicillin (100 μg/ml), kanamycin (15 μg/ml), chloramphenicol (34 μg/ml) and tetracycline (12.5 μg/ml), at 37 °C overnight. The starter culture was used to inoculate 100 or 500 ml LB, respectively, and cells were grown to an OD_{600} of 0.8. Similar starter cultures were used for mature and prodomain MSTNs. Expression of the recombinant MSTNs was induced by adding IPTG to a final concentration of 1 mM and cells were grown for an additional 4 h. Cells were harvested by centrifugation at 3000*g* for 5 min at 4 °C and stored at −20 °C.

Isolation and solubilization of inclusion bodies

For inclusion bodies (IB) purification, the frozen bacterial pellet from 2-L culture was thawed on ice and resuspended in 40 ml cold 0.1% Triton X-100 per 1 L. The cells were disrupted on ice by sonication for 3 min (1 s on, 1 s off) using Vibracell VC-130 Ultrasonic Cell Disruptor and centrifuged at 9000*g* for 15 min at 4 °C. The pellet, containing the IB, was resuspended once again in 40 ml cold 0.1% Triton X-100, sonicated and centrifuged as above. Subsequently, the pellet was washed and sonicated twice in distilled water. An aliquot of the resultant IB was checked for purity and amount by SDS–PAGE (see below) and the IB pellet was kept at −20 °C.

IB were solubilized using a protocol suggested previously for refolding BMP-2 [35] with some modifications. The solubilization solution contained 6 M guanidinium hydrochloride (Gdn-HCl), 0.1 M Tris–HCl, pH 8.5, 1 mM EDTA, pH 8.0, 0.1 mM dithiothreitol (DTT) and was carried out normally at a protein concentration of ~12 mg IB

Table 1
Primers used for cloning MSTN in expression vector

Name	Sequence	Used for cloning
MSTN1	5'-ATGCATCCGTCTCAGATTGT-3'	PreproMSTN
MSTN2	5'-AGAGCATCCACAACGGTCTAC-3'	PreproMSTN
MSTN3	5'-CGCGCATATGGACTCGGGCCTGGACTGT-3'	Mature MSTN
MSTN4	5'-GCGCGGATCCTCAAGAGCATCCACAACGGTC-3'	Mature & precursor MSTN
MSTN5	5'-CGCGCATATGAGCGAACAAGAGACGCAG-3'	Prodomain & precursor MSTN
MSTN8	5'-GGATCCTCATCTCCGGGACACGCTTGGG-3'	Prodomain MSTN

per 4 ml of solubilization solution, at room temperature (~20 °C) with a slow vertical rotation for 5 h. The solubilized fusion protein was separated from insoluble debris by centrifugation at 10,000*g* for 15 min at 4 °C.

In vitro refolding of recombinant MSTN

Refolding was performed immediately following the IB solubilization step, as suggested [35] with some modifications. The pH of the solubilized protein was adjusted to pH ~5.0 with few drops of concentrated HCl (32%) to keep the protein in a fully reduced condition and then dialyzed against 160 ml (~40 volumes) of a solution containing 6 M Gdn-HCl, 50 mM MES, pH 5.0, 1 mM EDTA, pH 8.0 (resulting in a pH ~5.0 of the solution) to remove the DTT and prevent its interference in the refolding buffer.

Dialysis was carried out at room temperature (~20 °C) for about 20 h. Subsequently, the solubilized protein was diluted in the refolding buffer to a final concentration of 245 μg/ml and 0.5 M Gnd-HCl. Refolding buffer consisted of 50 mM Tris–HCl, pH 8.5, 0.5 M L-arginine, 1 M NaCl, 5 mM EDTA, pH 8.0, 1 mM oxidized glutathione (GSSG), 5 mM reduced glutathione (GSH), pH 8.6. After the protein was added, the pH was adjusted to 8.5. The protein was left to refold at 4 °C for 14 days and checked occasionally for presence of precipitation. In some experiments the protein concentration in the refolding solution was increased to 800–1000 μg/ml, without obvious precipitation, or reduced to 100 μg/ml. In order to follow the kinetics of refolding, aliquots of 300 μl were removed at various times after refolding started; samples were dialyzed against 150 ml 20 mM Tris–HCl, pH 8.5, at 4 °C for ~20 h, concentrated by acetone and analyzed by SDS–PAGE as described below.

Purification of refolded saMSTN

Purification of the *in vitro* refolded saMSTN dimer was performed in two steps: Ni–NTA affinity chromatography, followed by size exclusion chromatography on Superose 12. Prior to binding to the Ni–NTA resin, the refolding solution was centrifuged at 10,000*g* for 15 min at 4 °C to remove any precipitated protein and the supernatant was dialyzed against 100 volumes of 20 mM Tris–HCl, pH 8.5, for ~20 h at 4 °C. After being filtered through Minisart 0.45 μm filter (Sartorius AG, Goettingen, Germany), the refolded recombinant saMSTN was purified by Ni–NTA chromatography. The protein was initially purified as a batch: Ni–NTA His-Bind Resin was washed in sterile water and then equilibrated in 20 mM Tris–HCl, pH 8.5. The protein solution was mixed with the resin (2 ml 100% resin per ~6 mg refolded protein) in a vertical rotator for 2 h at 4 °C to allow binding between the His-Tag and the Nickel ions. Subsequently, the protein solution was loaded onto a 0.8 × 4 cm Poly-Prep Chromatography Column (Bio-Rad). The column was washed sequentially with 20 ml of 50 mM Tris–HCl, pH 8.5, 0.3 M NaCl, 10 mM imidazole; 20 ml of 50 mM Tris–HCl, pH 8.5, 0.3 M NaCl, 30 mM imidazole; 20 ml of 50 mM Tris–HCl, pH 8.5, 0.3 M NaCl, 50 mM imidazole and then eluted (10 × 1 ml fractions) with the same solution containing 300 mM imidazole. This procedure was adopted after performing preliminary experiments in which the bound protein was eluted with increasing concentrations of imidazole: 100 mM, 150 mM, 200 mM, 500 mM, 1 M. Purification by Ni–NTA column was carried out at room temperature, but eluted fractions were kept at 4 °C. Purification and elution were monitored by SDS–PAGE under non-reducing conditions (see below). In the second step, fractions that contained refolded saMSTN (as determined by SDS–PAGE) were pooled and applied to a Superose 12 column (96 × 1.6 cm), using an AKTA FPLC (Amersham Biosciences, Piscataway, NJ), equilibrated with 20 mM HEPES, pH 7.6, 100 mM NaCl, 0.02% NaN_3. The protein was eluted with the same buffer at a flow rate of 0.5 ml/min and 1 ml fractions were collected and monitored by absorbance at 280 nm. Molecular mass was estimated from known molecular weight standards. Presence of precursor saMSTN dimer in peak fractions was also confirmed by SDS–PAGE under non-reducing conditions.

Furin proteolysis of refolded saMSTN

Fractions from the Superose 12 column that contained purified refolded saMSTN precursor dimer were cleaved by furin [an endopeptidase that cleaves at the minimal site RXXR but prefers the paired basic residues RX(K/R)R]. Proteolysis was carried out as recommended by the supplier. Purified refolded saMSTN precursor was incubated with furin (2 μg MSTN/0.5 U furin, New England Biolabs, Beverly, MA, USA) in a solution containing 100 mM HEPES, pH 7.5, 0.5% Triton X-100, 1 mM $CaCl_2$, 1 mM β-mercaptoethanol, for 70 h at 30 °C. The reaction products were analyzed by SDS–PAGE following acetone concentration (when a small amount of protein was digested). The time needed for complete proteolytic cleavage was determined in preliminary experiments. In cases where the protein was used subsequently for testing its biological activity, cleavage was performed without Triton X-100 and β-mercaptoethanol since these two components had an adverse effect on the cultured cells. In this case, the reaction was performed at a ratio of 2 μg MSTN/0.66 U furin. Complete cleavage was determined in preliminary experiments.

Activation of latent MSTN

For activation by acidification, the furin-cleaved MSTN was acidified to pH ~2.5 with 3.2% HCl for 1 h at 4 °C, followed by neutralization with 1 N NaOH to pH ~7.5 [36]. MSTN activity was determined in the A204 reporter gene assay [13]. For thermal activation, furin-cleaved MSTN

was heated in a PCR apparatus for 5 min at 80 °C [32] or for 1.5–10 min at 100 °C [36,37]. MSTN activity was determined in the A204 reporter gene assay [13].

pGL3-$(CAGA)_{12}$-luciferase reporter assay

The human rhabdomyosarcoma cell line A204 (ATCC HTB-82) was cultured in 75 cm^2 flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) until nearly complete confluence in McCoy's 5A medium (Biological Industries, Kibbutz Beit Haemek, Israel), supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma or Biological Industries), 1% (v/v) glutamine (200 mM) and 1% (v/v) antibiotics (stock solution: Pen-Strep-Nystatin: penicillin 10,000 U/ml, streptomycin 10 mg/ml; nystatin 1250 U/ml). One day before transfection, cells were trypsinized and plated at a density of 0.5–1 $\times 10^5$ cells per well in 24-well plates (Corning Costar Corporation, Cambridge, MA, USA) in the same medium but without antibiotics. After a 24-h attachment period, the cells were transiently transfected with 1 μg uncut pGL3$(CAGA)_{12}$-luciferase construct [13] using 3 μl Lipofectamine 2000 (Invitrogen), as recommended by the manufacturer. The ratio of plasmid DNA: Lipofectamine 2000 was determined in preliminary experiments. To account for differences resulting from transfection efficiency, cell viability and other sources of variability, such as differences in pipetting volumes, cell lysis efficiency and assay efficiency, cells were co-transfected with 0.1 μg DNA of the control vector pRL-RSV expressing *Renilla* luciferase (kindly provided by Dr. M. Walker) [38]. The results of the experimental reporter pGL3$(CAGA)_{12}$-luciferase were normalized to the control reporter. In addition, cells were transfected with pGL3-Control and pGL3-Basic vectors (Promega, Madison, WI, USA), which served as an additional control. After 23–26 h from transfection, growth medium was replaced with a minimal volume of the same medium (270 μl) but without FBS containing MSTN after various treatments (see above). Cells were collected 20 h after addition of MSTN. The medium was removed; cells were rinsed carefully with phosphate-buffered saline (pH 7.4) and lysed in 100 μl of 1× Passive Lysis Buffer (PLB, Promega). Lysed cells underwent one cycle of freeze-thaw at −70 °C and then were scraped and collected on ice. After a second round of freeze-thaw at −70 °C, the lysates were centrifuged at 20,800*g* for 30 s and supernatants were analyzed for luciferase reporter gene activity. Cell lysates were brought to room temperature and 20 μl were used to perform the luciferase assays by the Dual-Luciferase Reporter Assay System (Promega) as recommended by the manufacturer, using 50 μl Luciferase Assay Reagent II (LARII) and 50 μl Stop & Glo Reagent. The measurements were carried out using a 2 s pre-measurement delay followed by 10 s measurement period and sequential reading for firefly luciferase and *Renilla* luciferase were recorded. The reaction was performed at 25 °C in a Turner Designs Luminometer (model TD-20/20). The experiments were performed in 3 replicas in each experiment.

Determination of protein concentration

Protein concentration was determined by the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA), using BSA fraction V as standards.

Gel electrophoresis

SDS–PAGE (7.5%, 10%, 12% or 14% polyacrylamide gels) was performed according to a reported method [39]. Samples were mixed with loading buffer in the presence or absence of 3% β-mercaptoethanol and boiled for 5 min prior to loading on the gel. Gels were stained with Coomassie Brilliant Blue R-250.

Determination of N-terminal amino acid sequence of furin-cleaved mature MSTN dimer

Refolded and purified precursor saMSTN was cleaved with furin, separated by 14% SDS–PAGE under reduced conditions and transferred to Immun-Blot® PVDF membrane (Bio-Rad) as recommended by the manufacturer. The membrane was stained with Coomassie, destained and washed extensively in water. The N-terminal of the mature MSTN dimer was determined using ABI Procise 494 Protein Sequencer at The Weizmann Institute, Rehovot, Israel.

Results

Expression of recombinant saMSTN in E. coli

Expression of the precursor, prodomain and mature saMSTNs in IB is presented in Fig. 1. Induction of *E. coli* Origami (DE3) cells transformed with MSTN5-4/pET-16b (precursor), MSTN5-8/pET-16b (prodomain) or MSTN3-4/pET-16b (mature) with 1 mM IPTG resulted in the appearance of bands of the approximate molecular mass of 50 kDa, 37 kDa and 15 kDa proteins, which are the fusion proteins of precursor, prodomain and mature saMSTN, respectively (Fig. 1a). Presence of these bands in the pellet but not in the supernatant of bacteria, which were sonicated briefly, indicated that all three forms are produced as IB. The vector pET-16b without insert, which was induced and treated in exactly the same way, is included as control. The isolated IB of precursor, prodomain and mature MSTN are shown in Fig. 1b. The protocol employed in this study for preparation of IB resulted in relatively pure IB (more than 95%) with a very small amount of contaminating proteins as judged from SDS–PAGE analysis (Fig. 1b).

Refolding of mature and precursor saMSTN and purification by affinity chromatography

MSTN forms a disulfide dimer in its non-reduced native state, as other members of the TGF-β superfamily. Therefore, the appearance of MSTN dimers in SDS–PAGE gels under non-reducing conditions was used as an indication

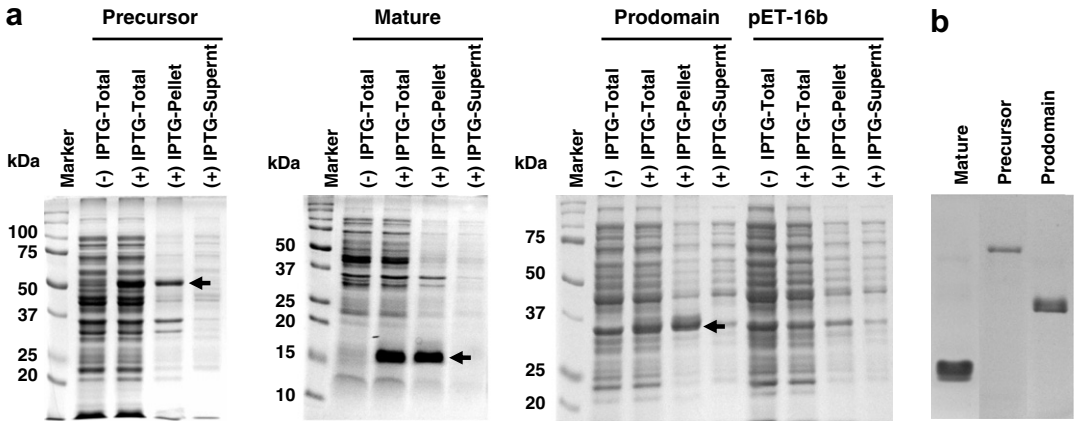

Fig. 1. Expression of recombinant saMSTNs and purification of MSTN inclusion bodies. (a) Total cellular proteins from *E. coli* cells uninduced (−IPTG) or induced with 1 mM IPTG (+IPTG) were analyzed by 10–12% SDS–PAGE under reducing conditions without sonication (total), or after a brief sonication and centrifugation to separate between pellet (pellet) and supernatant (supernt). (b) Purified inclusion bodies of precursor, prodomain and mature recombinant saMSTNs. Gels were stained with Coomassie brilliant blue R.

for proper refolding of the recombinant protein. To determine the time needed for refolding, samples were collected periodically (1 d, 2 d, 3 d, 6 d, 9 d and 14 d) during the incubation of solubilized IB in refolding buffer. After being dialyzed in order to remove the refolding constituents and concentrated by acetone, the samples were analyzed by SDS–PAGE under non-reducing conditions. As shown in Fig. 2, a 100 kDa unprocessed precursor MSTN dimer appeared under non-reducing condition already after 1 day, although the amounts of MSTN dimer was higher after 14 days, which was chosen as the optimal time in subsequent refolding experiments. In addition, bands of misfolded monomers were detected with a molecular mass around 50 kDa. After 14 days, the 50 kDa band (which was the strongest at the beginning of the refolding) weakened, while the 100 kDa band became more intense. Smaller bands than 50 kDa were detected as well. The high molecular weight band, which appears at the top of the gel, represents proba-

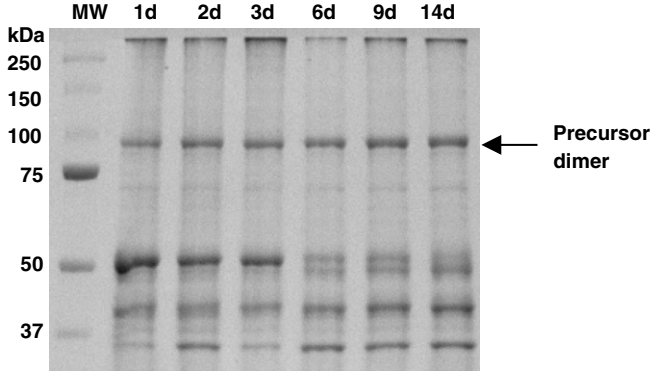

Fig. 2. Refolding of solubilized saMSTN precursor inclusion bodies. To determine the time required for refolding, samples were removed at different times during incubation (1 d, 2 d, 3 d, 6 d, 9 d and 14 d), concentrated by acetone and analyzed by SDS–PAGE (7.5%) under non-reducing conditions. Gels were stained with Coomassie brilliant blue R. A precursor dimer is visible already on day 1. The high molecular weight band is aggregates and smaller molecular weight bands represent misfolded monomers.

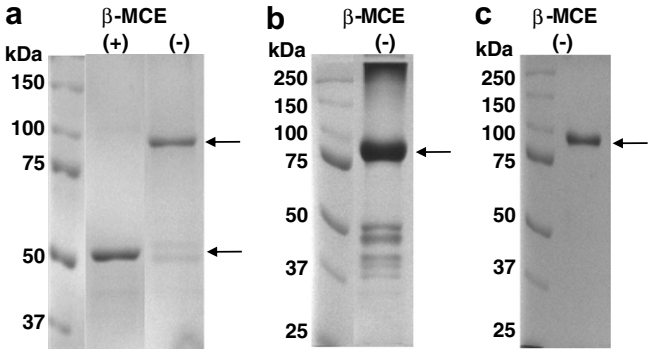

Fig. 3. Purification steps of refolded saMSTN precursor. (a) Purification of refolded saMSTN precursor by Ni–NTA affinity chromatography. Fractions containing MSTN that were eluted with 150 mM imidazole were analyzed by SDS–PAGE (7.5%) under non-reducing [(−)β-MCE] or reducing [(+)β-MCE] conditions. Protein concentration in the refolding solution was 100 μg/ml. A dimer of MSTN precursor is seen under non-reducing conditions (upper arrow) and a monomer of MSTN precursor is seen under reducing conditions (lower arrow). (b) Purification of refolded precursor saMSTN by Ni–NTA affinity chromatography. Fractions containing MSTN that were eluted with 300 mM imidazole were analyzed by SDS–PAGE (7.5%) under non-reducing [(−)β-MCE] conditions. Protein concentration in the refolding solution was 245 μg/ml. The proportion of the dimer MSTN precursor (arrow) is much higher than the lower molecular weight misfolded monomers or the aggregates. (c) Purification of refolded precursor saMSTN on Superose12 gel filtration chromatography. Fractions containing MSTN after purification by Ni–NTA column were pooled and purified on Superose12 column. Fractions containing MSTN as judged from absorbance at 280 nm were analyzed by SDS–PAGE under non-reducing conditions (see also Fig. 4). Shown is the peak tube containing pure refolded MSTN precursor (arrow). Gels were stained with Coomassie brilliant blue R.

bly aggregates formed during the folding reaction and were removed by purification on size-exclusion chromatography (see Fig. 4).

Purification of unprocessed refolded precursor MSTN by affinity chromatography on Ni–NTA column is shown in Fig. 3a. Analysis by SDS–PAGE under non-reducing conditions revealed the presence of a dimer of 100 kDa and in addition several faint bands of misfolded monomers,

all of which were reduced by 3% β-mercaptoethanol in the sample buffer to a single band of 50 kDa unprocessed MSTN monomer, indicating its purity. In this study, several concentrations of protein in the refolding reaction were tested, including a low (100 μg/ml) and a high (800–1000 μg/ml) concentration. The high concentration of 800–1000 μg/ml did not result in substantial precipitation that could be inspected visually. A dimer of 100 kDa unprocessed MSTN was obtained following purification on Ni–NTA column and elution with 300 mM imidazole as determined on SDS–PAGE (data not shown). An example of the results obtained using an intermediate concentration of protein in the refolding mixture (~245 μg/ml), followed by purification on Ni–NTA column and elution with 300 mM imidazole is shown in Fig. 3b. The proportion of 100 kDa dimer was high compared to the misfolded monomers or the high molecular weight aggregates.

Attempts to refold the recombinant mature MSTN under identical conditions resulted in precipitation of the protein (data not shown). Addition of 100 mM of the zwitterionic detergent CHAPS to the refolding solution prevented the precipitation but refolding was still very poor and most of the recombinant mature MSTN appeared as a monomer following its purification on Ni–NTA column and analysis under non-reducing conditions (data not shown).

We next tested the possibility to refold mature MSTN by the *trans* presence of MSTN prodomain in molar ratios of 1:1, 1:5 and 1:10 between mature and prodomain MSTN. Samples were removed at 1 d, 6 d and 9 d (or 10 d), concentrated by acetone and analyzed by SDS–PAGE under reducing and non-reducing conditions. Although no refolding of mature MSTN was obtained under these refolding conditions (data not shown), presence of the prodomain prevented the precipitation of mature MSTN, which otherwise occurred unless CHAPS was added.

Size-exclusion gel chromatography of refolded unprocessed saMSTN

To verify the molecular mass of the refolded unprocessed precursor MSTN and separate the precursor MSTN dimer from misfolded monomers and aggregates (see Fig. 2 and Fig. 3b), fractions from the Ni–NTA affinity chromatography (such as that shown in Fig. 3b) were pooled and subjected to gel filtration chromatography on Superose12. A representative elution profile is shown in Fig. 4. The first peak corresponds to aggregates while the second peak corresponds to the unprocessed precursor MSTN dimer (~100 kDa) as confirmed by analysis of the peak fractions by SDS–PAGE under non-reducing conditions (lower part in Fig. 4). The misfolded monomers eluted at the end of the peak. SDS–PAGE analysis under non-reducing conditions confirmed the purity of the peak fractions of the refolded precursor MSTN (Fig. 3c), which were used for subsequent experiments.

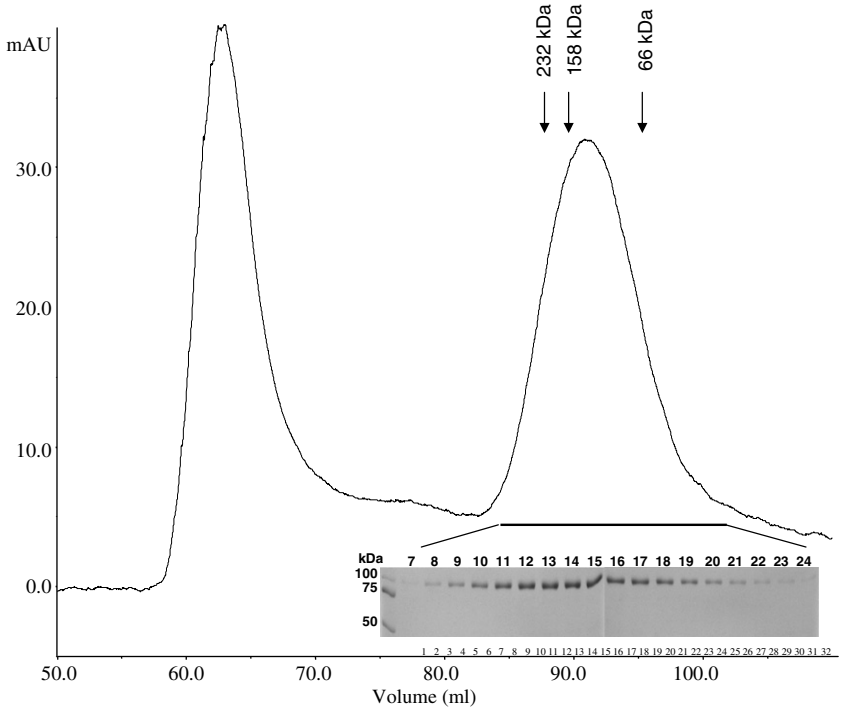

Fig. 4. Elution profile of refolded saMSTN precursor on Superose12 gel filtration chromatography. MSTN precursor was refolded for 14 days, purified by affinity chromatography on Ni–NTA column (see Fig. 3a and b) and then the fractions containing MSTN were pooled and subjected to Superose12 gel filtration with a buffer system of 20 mM HEPES, pH 7.6, 100 mM NaCl, 0.02% NaN_3. Protein was monitored by absorbance at 280 nm. The fractions under the peak (tubes 7–24) that are marked by a thick line were analyzed by SDS–PAGE (7.5%) under non-reducing conditions. Gel was stained with Coomassie brilliant blue R.

Furin proteolysis of refolded, unprocessed saMSTN

Activation of MSTN, as other members of the TGF-β superfamily, requires the removal of the N-terminal prodomain from the unprocessed MSTN by specific proteolysis at the paired basic RXRR site. Such paired basic residues are preferably recognized by furin, an endopeptidase involved in the conversion of a wide variety of precursor proteins into their mature forms. To determine that indeed the recombinant refolded precursor saMSTN can be cleaved at the predicted paired basic residues (RVRR in saMSTN), the refolded purified precursor saMSTN was incubated with furin. A time course of cleavage with furin followed by analysis on SDS–PAGE under non-reducing conditions was performed. As shown in Fig. 5a, incubation for 50 h (2 μg MSTN/0.5 U furin) resulted in cleavage of most of the precursor into two major proteins with the molecular weight of ~37 kDa and ~25 kDa, corresponding to the N-terminal prodomain and the C-terminal mature dimer MSTN, respectively. Analysis under reducing conditions revealed ~37 kDa and ~13–14 kDa peptides, corresponding to the N-terminal prodomain and the C-terminal mature MSTN, respectively (Fig. 5b). However, under non-reducing conditions the ~37 kDa remained, while the 13 kDa protein disappeared and a protein of ~25 kDa appeared (shown also in Fig. 5a), indicating the formation of a disulfide-linked homodimer of the mature MSTN but no disulfide-linked interactions between the prodomain and mature MSTN or between prodomain monomers.

Determination of the N-terminal of the mature MSTN dimer

Amino acid determination of the mature MSTN dimer obtained following cleavage of refolded purified precursor saMSTN with furin revealed the sequence DSGLD, in accordance with the predicted sequence of mature saMSTN as derived from the published cDNA sequence [21]. This data also confirmed that furin cleaved the precursor after the RVRR sequence, as expected.

Activation of latent saMSTN by acidic and thermal treatments

The biological activity of refolded, purified and furin-cleaved MSTN (as that shown in Fig. 5) was assessed by the A204 reporter gene assay in which the tested protein was added to A204 cells transfected with the pGL3-$(CAGA)_{12}$ plasmid. Luciferase activity was determined in cell extracts. No activity was found when non-cleaved, non-heated (2 μg/ml, Fig. 6a) or non-cleaved, heated precursor MSTN (~750 ng/ml, Table 2) was added to the A204 cells. Low activity was found in non-activated but furin-cleaved MSTN compared to the non-cleaved, non-heated or non-cleaved, heated precursor. Addition of cleaved MSTN that was heated for 5 min at 80 °C or acidified to pH 2.5 for 1 h resulted in 2–3-fold higher activities compared to non-activated, cleaved-MSTN (Fig. 6a). These results indicate that MSTN precursor needs to be cleaved by a furin-like endopeptidase but the resulting mature dimer and the prodomain are held together as a latent form that needs to be activated in order to elicit MSTN activity. The activation by heat is likely a separation between the prodomain and the mature dimer, as analysis on SDS–PAGE under non-reducing conditions revealed intact prodomain and mature dimer (data not shown). The residual activity found without activation is probably due to incomplete latent complex formation. Addition of increasing concentrations of furin-cleaved MSTN that was activated by heat treatment at 80 °C caused a dose-dependent increase in luciferase activity (Fig. 6b). These amounts were calculated based on the proportion that the mature dimer, released as a result of furin action, constituted of the total precursor amounts present in the Superose12 fraction used in the furin reaction. Since heating for 3 min at 100 °C was more effective

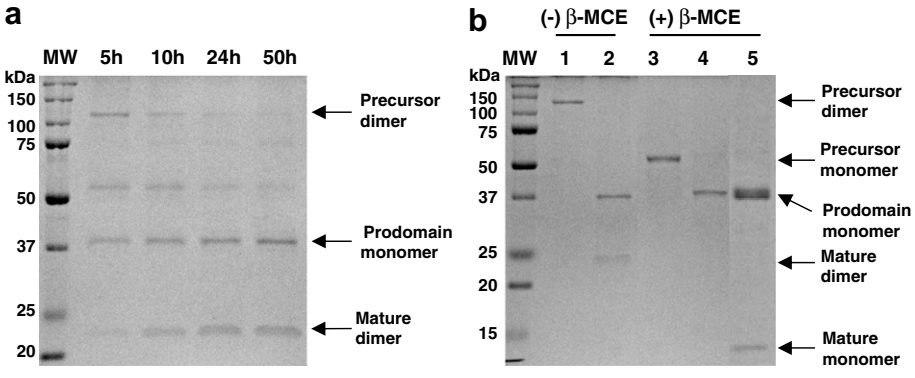

Fig. 5. Furin proteolysis of purified refolded MSTN precursor. (a) Refolded MSTN purified by Superose12 column was incubated for 5 h, 10 h, 24 h and 50 h at 30 °C with furin and analyzed by SDS–PAGE (12%) under non-reducing conditions. As MSTN precursor dimer disappeared gradually, more of the prodomain monomer and mature dimer appeared. Complete cleavage could be seen at 50 h. (b) Analysis of purified MSTN precursor cleaved with furin under non-reducing conditions [(−)β-MCE] and reducing conditions [(+)β-MCE]. Lanes 1 and 3, uncut pure MSTN precursor; lanes 2, 4 and 5, cleavage with furin. Lane 5 is similar to lane 4 but more protein was loaded onto the gel in order to see the mature monomer. Gels were stained with Coomassie brilliant blue R.

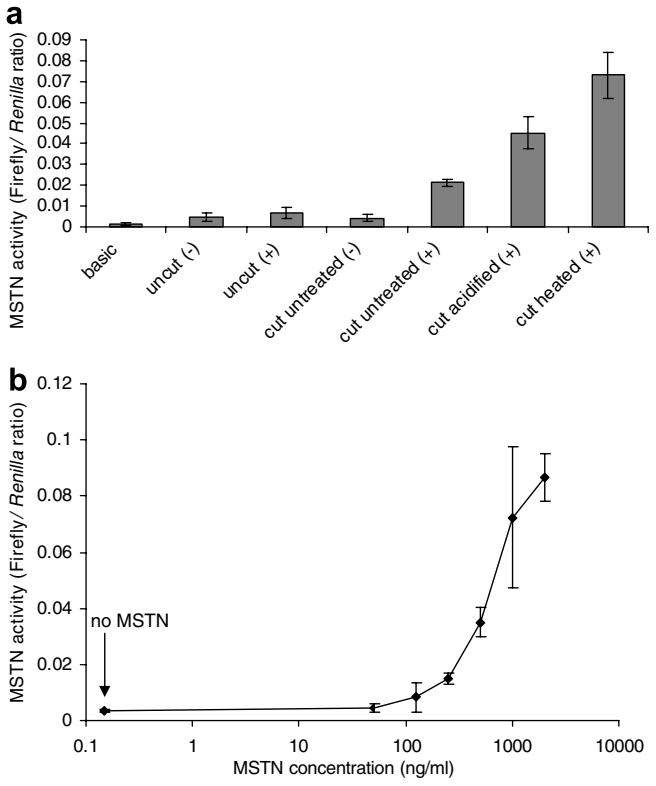

Fig. 6. Activation of latent recombinant fish MSTN. (a) Activation of recombinant MSTN prodomain/C-terminal dimer latent complex by heating at 80 °C for 5 min or acid treatment at 4 °C for 1 h at pH 2.5. Basic, pGL3-Basic; cut and uncut, with or without cleavage by furin; (−), no MSTN present, only the corresponding buffer; (+), MSTN present. Each well contained 2 μg/ml mature MSTN. (b) MSTN activity in the A204 reporter gene assay using increasing concentrations of activated recombinant MSTN prodomain/C-terminal dimer latent complex by heating at 80 °C for 5 min. Purified MSTN precursor (from Superose12 column) was cleaved with furin (2 U furin per ~6 μg MSTN) at 30 °C for 65–70 h. Activity in (a,b) was determined by the pGL3-$(CAGA)_{12}$-luciferase reporter gene assay in A204 cells and is expressed as the ratio between firefly and *Renilla* luciferase activities. Each point is the mean of three replicates.

Table 2
Effect of heating time at 100 °C on MSTN activity

Time (min)	MSTN activity (firefly/*Renilla* ratio)
1.5	0.142 ± 0.041
3	0.124 ± 0.043
4	0.124 ± 0.022
5	0.152 ± 0.031
7	0.156 ± 0.059
10	0.146 ± 0.032
3	0.004[a]
3	0.003[b]
3	0.006[c]

Activity was determined by the CAGA-reporter gene assay. A204 cells were treated with 750 ng/ml mature MSTN for 20 h.

[a] No MSTN, non-cleaved (fraction of the Superose12 column containing elution buffer but no MSTN).

[b] No MSTN, cleaved (same fraction, containing furin-cleaving buffer and furin).

[c] MSTN, non-cleaved (fraction of the Superose12 column with MSTN).

than 5 min at 80 °C in the reporter gene assay (0.111 ± 0.012 vs 0.086 ± 0.008), we next tested if increasing the time of heat treatment will result in increased activity. No significant difference was found between the activity seen after 1.5 min at 100 °C compared to 10 min at 100 °C (Table 2), suggesting that a prolong treatment at 100 °C did not damage the mature MSTN dimer assuming that all the mature dimer was separated from the prodomain already after 1.5 min. Alternatively, lack of difference between 1.5 min and 10 min could be the result of a balance between increasing amounts of mature MSTN being separated from the prodomain, as heating time is extended, and the amounts of mature MSTN being damaged. Since in other studies that used the A204 reporter assay [13] cells were treated with MSTN in the absence of serum in the culture medium and only for 6 h, we tested the effect of FBS and prolong exposure to the growth factor. Inclusion of 10% FBS in the culture medium of A204 cells tested for MSTN activity resulted in a lower activity compared to cells cultured without FBS (0.063 ± 0.014 vs 0.124 ± 0.043), suggesting the presence of binding/inhibiting factors in the serum. Therefore, all experiments described above were carried out in the absence of FBS. Similarly, MSTN activity in cells exposed to the activated MSTN for 20 h tended to be slightly higher than in cells exposed for 6 h (0.124 ± 0.043 vs 0.070 ± 0.014) and therefore in all the experiments reported above cells were exposed to MSTN for 20 h.

Discussion

Like other members of the TGF-β family, MSTN is synthesized as a precursor protein that undergoes two proteolytic cleavages. Removal of the signal sequence is followed by cleavage at a consensus furin motif, resulting in the N-terminal prodomain (propeptide) and the disulfide-linked C-terminal dimer, which is the biologically active molecule. The complex post-translational processing that MSTN must undergo in order to become an active molecule discouraged researchers from using the bacterial expression system for its production. Most reports to date have expressed and purified MSTN using a eukaryotic expression system like mammalian Chinese hamster ovarian (CHO) cells. In the present study we developed an efficient and consistent protocol for refolding *in vitro* fish unprocessed precursor MSTN, which is expressed in *E. coli* as IB. These experiments show that MSTN prodomain plays an important role in refolding MSTN produced in *E. coli*. Moreover, the prodomain also prevents the precipitation of misfolded intermediates when used in *trans* with the mature MSTN, perhaps through binding between the two forms, which prevents interactions that lead to precipitation.

The three forms of MSTN that were expressed in *E. coli* in this study: unprocessed precursor, prodomain and mature MSTNs were produced as fusion proteins with a histidine tag at the N-terminus. The IB containing these forms were purified (~95%) by a simple procedure of repeating washings and sonications in 0.1% Triton X-100

and distilled water. Production of MSTN in IB was reported also for porcine precursor MSTN [34] and human mature MSTN [10], both of which were expressed in bacteria.

The procedure of IB solubilization that was employed in the current study made use of 6 M Gnd-HCl in the presence of the reducing agent DTT, which ensured a complete reduction of all disulfide bonds. This method was used successfully for recombinant human bone morphogenetic protein-2 (rhBMP-2), another member of the TGF-β family [35]. In our initial studies, various protocols for solubilizing IB containing mature fish MSTN were tried such as acidic conditions (10% acetic acid) as suggested previously [40,41] and were found unsatisfactory. Other methods involved the use of 40 mM Trizma Base, 4.5 M urea and increasing the pH to 12, used in fish growth hormone purification [42], or 8 M urea. In the case of IB containing precursor MSTN, two methods were compared: 6 M Gnd-HCl and the procedure employed for porcine MSTN [34], in which the solution consisted of 50 mM CAPS, pH 11, 0.3% laurylsarcosine and 1 mM DTT. However, since the yield of solubilized IB was lower than that obtained using 6 M Gnd-HCl, the latter method was adopted for all our subsequent experiments.

The refolding mixture included several additives that are considered aggregation suppressors and promote renaturation: 0.5 M L-arginine and 0.5 M Gnd-HCl reviewed in [43]. In addition, the refolding buffer included the glutathione oxido-shuffling system: 1 mM oxidized glutathione (GSSG) and 5 mM reduced glutathione (GSH). The refolding was carried out at pH 8.5 at 4 °C. Previous studies of refolding rhBMP-2 demonstrated the effects that various pH, GSSG/GSH ratio and concentration, and Gnd-HCl concentrations may have on successful *in vitro* refolding [35]. Under the refolding conditions employed in the current study, no precipitation of MSTN occurred although some aggregates were formed in addition to the refolded precursor MSTN (Fig. 2). The refolding was carried out at a 25-fold higher protein concentration compared to that reported for the porcine MSTN [34] and for a prolonged time (14 days) although the appearance of a dimer (indicative of refolding) was noted already after one day. As noted also for hBMP-2 (Rinas, personal communication) and porcine MSTN [34], dimer formation is a slow process.

We attempted to refold mature fish MSTN using different combinations of parameters including zwitterionic agent like CHAPS or organic solvents like DMSO and DMF as suggested for TGF-β [44] with no success. Also the use of various ratios and concentrations of GSH/GSSG (as demonstrated for refolding rhBMP-2) [35,45] did not result in dimer formation. It is only when unprocessed precursor MSTN (which included the prodomain) was refolded that a dimer was formed, emphasizing the important role of the prodomain in proper refolding of MSTN *in vitro*. Similar results were reported for porcine MSTN [34]. The requirement of the prodomain for proper folding, disulfide bond formation and secretion of mature protein has been demonstrated *in vitro* for two members of the TGF-β family that contain the characteristic cystine knot (TGF-β1 and activin A) [46] as well as for human nerve growth factor (β-NGF) [47,48]. By contrast, a successful *in vitro* refolding of mature hBMP-2 was reported [35]. These differences in the requirements for the prodomain presence for a proper refolding *in vitro* illustrates that despite the fact that members of the TGF-β family share structural features and undergo a similar sequence of post-translational processing, the importance of the prodomain is not general and refolding of each protein of this family should be considered independently.

MSTN prodmain should be present in *cis* for proper refolding as no mature MSTN dimer was formed by the *trans* presence of MSTN prodomain. These findings are different from those reported for TGF-β1 and activin A [46], in which *trans* presence of the prodomain achieved by co-transfection of the pro-region and mature region into eukaryotic cells was effective in the secretion of a biologically active homodimer. Yet, a lower efficiency was seen compared to that found with *cis* presence of the prodomain. Our results are consistent, however, with those reported for hNGF in which the *trans* presence of the propeptide did not assist in refolding mature NGF [48]. It still remains to be seen if co-transfection into a eukaryotic cell expression system will result in dimer formation of mature MSTN in a similar way to that of TGF-β1 and activin A.

The procedure we described here for purification of refolded unprocessed precursor MSTM involved affinity chromatography followed by size-exclusion chromatography. This procedure removes the aggregates and misfolded monomers and results in a highly purified preparation as judged from SDS–PAGE under non-reducing conditions. *In vivo*, proteolytic maturation is needed in order to obtain an active MSTN. It is not known which endopeptidase of the proprotein convertases is responsible for this step. In order to mimic this requirement, we have used enzymatic digestion with furin, which is an endopeptidase that cleaves after the paired basic residues RX(K/R)R, resulting in two fragments of the approximate size of 37 kDa and 13 kDa under reducing conditions, corresponding to the prodomain and mature MSTN, respectively. Consistent with homodimer formation of mature MSTN [12,13,34], the 13 kDa fragment formed a disulfide-linked homodimer under non-reducing conditions following *in vitro* proteolytic cleavage by furin. By contrast, the prodomain remained as a 37 kDa fragment (unlike TGF-β prodomains that form disulfide-linked dimers [49–51]), confirming previous reports that MSTN prodomain does not form a disulfide-linked dimer [12,13,34,52]. Determination of the N-terminal amino acid sequence of furin-released mature MSTN confirmed unequivocally that the precursor was cleaved by furin after the RVRR sequence, as expected. Moreover, this analysis showed that the mature fish MSTN starts with the amino acid sequence of DSGLD, in accordance with the predicted sequence of mature saMSTN as derived from the published cDNA sequence [21]. Previous studies, in which mammalian CHO cells were engineered to

over express MSTN, have shown that MSTN is secreted as a latent complex of the N-terminal propeptide and a disulfide-linked dimer of the C-terminal fragment [12,13].

Correct refolding as evidenced by dimer formation should be supported by biological activity of the refolded peptide. Our attempts to separate between the mature dimer and the prodomain following furin cleavage yielded very small amounts of mature MSTN dimer. Therefore, we chose to use an alternative method to activate MSTN following furin cleavage of the precursor without prior separation between the two forms. Both acidic and thermal treatments were effective in activating furin-cleaved MSTN as determined *in vitro* using the A204 reporter gene assay [13]. Our findings emphasize that although the prodomain sequences of other members of the TGF-β superfamily like TGF-β1, -β2 and -β3 are different from fish MSTN prodomain, the latent complex between the prodomain and the mature dimer can be activated in a similar way, suggesting that it is held by similar forces [36,37]. Furthermore, our results demonstrate that the prodomain must be cleaved from the mature dimer before it can be activated. To our knowledge, this is the first report of a bacterially produced MSTN latent complex that had been activated *in vitro*. Acid and heat activation of the latent MSTN complex was shown previously only for MSTN produced and secreted by CHO cells [9,32]. Several reports have shown biological activity of mature mammalian MSTN that was produced in bacteria and was either purified by affinity chromatography and underwent a short dialysis [11] or was refolded in a buffer similar to one of the buffers that we have tried [10]. Yet, no convincing evidence was provided in these studies as to the formation of a disulfide-bond under these refolding conditions. Moreover, high concentrations of recombinant mature MSTN were used in those studies to demonstrate biological activity such as an inhibitory effect on myoblast proliferation, compared to studies which employed MSTN produced by CHO cells and used the CAGA reporter gene assay [13]. The concentrations of the cleaved mature MSTN which were active in the A204 assay in the study we report here are a little higher than those reported for the CHO-derived MSTN [13] but lower than those reported in the studies employing *E. coli*-derived mature MSTN [11]. The higher concentration needed in our study could have been due to some damage caused to the mature MSTN during heating or due to partial re-association of the latent complex.

In conclusion, the current study is the first to report proper refolding of recombinant MSTN expressed in *E. coli* as a precursor, which is cleaved by furin and can be activated *in vitro* to yield a biologically active MSTN in a mammalian CAGA assay. Furthermore, our findings also suggest that fish MSTN probably can signal through the same receptor and signaling pathway as mammalian MSTN. The approach we present here can be applied to refolding MSTN of both mammalian and non-mammalian vertebrates and will enable further studies on the role of the prodomain in MSTN post-translational processing, activation and stability.

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