Structural and functional characterizations of activin type 2B receptor (acvr2b) ortholog from the marine fish, gilthead sea bream, Sparus aurata: evidence for gene duplication of acvr2b in fish

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Abstract

Myostatin (MSTN), a negative regulator of muscle growth and a member of the transforming growth factor-β superfamily, can bind the two activin type 2 receptors (ACVR2). It has been previously shown that WT mice injected with ACVR2B extracellular domain (ACVR2B-ECD) had higher muscle mass. Likewise, fish larvae immersed in Pichia pastoris culture supernatant, containing goldfish Acvr2b-ECD, showed enhanced larval growth. However, it is not clear whether fish Mstn1 and Mstn2 signal through the same receptor and whether fish express more than one acvr2b gene. In the current study, three cDNAs encoding acvr2b (saacvr2b-1, saacvr2b-2a, and saacvr2b-2b) were cloned from gilthead sea bream. All three contain the short extracellular binding domain, a short transmembrane region, and a conserved catalytic domain of serine/threonine protein kinase. Bioinformatics analysis provided evidence for the existence of two acvr2b genes (acvr2b-1 and acvr2b-2) in several other fish species as well, probably as a result of gene or genome duplication. The two isoforms differ in their amino acid sequences. The direct inhibitory effect of Acvr2b-ECD on Mstn activity was tested in vitro. The saAcvr2b-1-ECD was expressed in the yeast P. pastoris. Evidence is provided for N-glycosylation of Acvr2b-1-ECD. The affinity-purified Acvr2b-1-ECD inhibited recombinant mouse/rat/human mature MSTN activity when determined in vitro using the CAGA-luciferase assay in A204 cells. A lower inhibitory activity was obtained when unprocessed purified, furin-digested, and activated saMstn1 was used. Results of this study demonstrate for the first time the existence of two acvr2b genes in fish. In addition, the study shows that bioactive fish Acvr2b-ECD can be produced from P. pastoris.

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Introduction

The transforming growth factor-β (TGFβ) superfamily of cytokines elicits diverse biological responses by interacting with two distinct but structurally related transmembrane receptors that are serine/threonine kinases (type 1 and type 2) (reviewed in de Caestecker 2004). The binding of dimeric ligands of the cytokines to the type 2 receptors is the first event in the transmembrane signaling. The characteristic structural feature of the TGFβ receptor superfamily is the three-finger toxin fold in the ligand binding extracellular domain (ECD), primarily dictated by disulfide bonds formed by eight conserved cysteines with a characteristic spacing, a single transmembrane domain (TMD), and an intracellular serine/threonine kinase domain (ICD; Greenwald et al. 1999).

Myostatin (MSTN) is a member of the TGFβ superfamily and is known as a negative regulator of skeletal muscle growth in mammals. Mutations in the Mstn gene result in increased muscle mass in cattle (Grobe et al. 1997, Kambadur et al. 1997, McPherron & Lee 1997, Smith et al. 2000), mice (McPherron et al. 1997, Szabó et al. 1998, Zhu et al. 2000), dogs (Mosher et al. 2007), and humans (Schuelke et al. 2004), suggesting the conservation of MSTN function in mammals. Neutralization of MSTN by anti-MSTN antibodies in adult mice significantly increased skeletal muscle mass (Whittemore et al. 2003), while systemic administration of MSTN (Zimmers et al. 2002) or over-expressing MSTN in transgenic mice (Reisz-Porszasz et al. 2003) decreased skeletal muscle mass. In chicken, in-ovo administration of anti-MSTN antibodies also improved post-hatch skeletal muscle growth (Kim et al. 2006, 2007). Similarly, offspring of female mice induced to produce MSTN antibody by immunization with synthetic MSTN peptide, before mating with male mice, showed higher growth performance than the controls (Liang et al. 2007).
Our knowledge on MSTN signaling derives almost exclusively from studies in mammals. These studies suggest that MSTN most likely signals through the two activin type 2 receptors (ACVR2A and ACVR2B; Lee & McPherron 2001, Rebbapragada et al. 2003). Ligand binding assays in transfected COS cells revealed binding of MSTN to ACVR2B and to a lesser extent to ACVR2A (Lee & McPherron 2001). Binding of MSTN to ACVR2B leads to the phosphorylation and activation of the activin type 1 receptor, which in turn initiates the intracellular signaling cascade by phosphorylation of the receptor-regulated proteins Smad2 and Smad3. Upon phosphorylation, Smads form heterodimer with a Co-Smad, Smad4. This complex translocates into the nucleus, binds to DNA, and finally modulates transcription of various target genes (Zhu et al. 2004, Joulia-Ekaza & Cabello 2006). ACVR2 can bind several other TGFβ family members in addition to MSTN (de Caestecker 2004, Lee et al. 2005, Sako et al. 2010). In their study to identify natural ligands to ACVR2, Souza et al. (2008) observed that both soluble ACVR2 and ACVR2B could block the inhibitory effect of GDF11/BMP11, activin A, B, and AB on the myoblast-to-myotube differentiation, implicating them as potential novel regulators of muscle growth in mammals in addition to MSTN.

One of the MSTN inhibiting strategies is to block the MSTN signaling induced by its interaction with the ACVR2B receptor. In vivo, transgenic mice overexpressing a truncated form of ACVR2B (lacking the kinase domain that results in loss of signal transduction), under the control of a muscle-specific promoter, exhibited a dramatic increase in muscle mass similar to MSTN knockout mice (Lee & McPherron 2001). Also, transgenic Mdx (Dystrophin-deficient) mice, carrying a dominant negative Acvr2b gene, had bigger muscles than Mdx mice with a normal Acvr2b gene (Benabdalhali et al. 2005). Injection of WT mice with a soluble form of ACVR2B (ACVR2B-ECD) protein resulted in an increased muscle mass. Moreover, treatment of MSTN knockout mice with the soluble ACVR2B-ECD produced a further increase in muscle mass compared with the muscle gain that resulted from MSTN deletion (Lee et al. 2005). Others provided evidence that administration of ACVR2B-ECD not only prevented muscle wasting but also restored prior muscle loss in various cancer cachexia models (Zhou et al. 2010). Using a different mode of administration, Morine et al. (2010) showed that systemic inhibition of ACVR2B signaling via adenoassociated virus-mediated gene transfer of a soluble form of ACVR2B, directed to the liver of Mdx mice, led to increased skeletal muscle mass. Taken together, these studies suggest that agents targeting MSTN signaling pathway are potentially useful therapeutic agents for not only preventing muscle wasting in humans but also increasing muscle mass in farm animals and may prove useful also in fish, thus improving aquaculture productivity.

Fish possess multiple mstn genes, as a result of a genome duplication event that occurred during early fish radiation, producing two distinct mstn clades, mstn1 and mstn2. A second duplication event within salmonids produced two subsequent divisions, one in each clade (Garikipati et al. 2007). In contrast to mammals, fish express the mstn gene in multiple tissues, in addition to red and white muscle, suggesting that Mstn’s functional role in fish is far more diverse than that in mammals.

Existing knowledge about the role of Acvr2 in fish is limited. Fish acvr2b cDNAs were cloned from several fish tissues: brain (Song et al. 2010), liver (Ostbye et al. 2007), ovary (Ge et al. 1997), and 6- to 72-h embryos (Garg et al. 1999). While considerable evidence exists with respect to the function of these receptors in fish reproduction (Ge 2000, 2005, Lau & Ge 2005) and during craniofacial development of zebrafish (Danio rerio, Albertson et al. 2005), relatively little is known regarding their involvement in signaling of Mstns in fish. The situation is complicated by the presence of two mstn genes. In a single report, immersion of fish larvae in Pichia pastoris culture supernatant containing recombinant goldfish (Carassius auratus) Acvr2b-ECD enhanced larval growth (Carpio et al. 2009). However, no direct evidence was provided that indeed Acvr2b-ECD binds MSTN and that the inhibition of MSTN activity is responsible for the growth enhancement. Likewise, it is not clear whether Mstn1 and Mstn2 signal through the same receptor and whether fish possess more than one acvr2b gene as expected from the fish-specific genome duplication that occurred in fish. Evidence is accumulating that during vertebrate evolution, entire genomes were duplicated through two rounds of duplication (the ‘one-to-two-to-four’ rule). Recent data suggested that the fish genome was duplicated a third time (Meyer & Schartl 1999, Christoffels et al. 2004, Hoegg et al. 2004, Dehal & Boore 2005). For many genes, two paralogous copies exist in ray-finned fish (Actinopterygii), whereas only one ortholog is present in tetrapods.

In this study, we report on the cloning of three acvr2b cDNAs from the marine fish gilthead sea bream (Sparus aurata), which we named saacvr2b-1, saacvr2b-2a, and saacvr2b-2b. Bioinformatics analysis provided evidence for the existence of two acvr2b genes in several other fish species as well, probably as a result of gene or genome duplication. The two paralogs differ substantially in their amino acid sequence. To test the direct inhibitory effect of Acr2b-ECD on Mstn activity in vitro, we cloned the ECD of saacvr2b-1 in an expression vector for production in the yeast P. pastoris. We provide evidence that saAcvr2b-1 is N-glycosylated. The affinity-purified saAcvr2b-1-ECD inhibited recombinant mouse/rat/human mature MSTN activity when
determined in vitro using the CAGA-luciferase assay in A204 cells. A lower inhibition was obtained when unprocessed purified, furin-digested, and activated saMstn (Funkenstein & Rebhan 2007) was used.

Materials and methods

Cloning of saacvr2b cDNAs

Cloning of full-length saacvr2b cDNAs was achieved by several steps. Initially, two primers, ActR-4 and ActR-5 (see Table 1 for primer sequences and scheme in Supplementary File 1, see section on supplementary data given at the end of this article, for location), were designed based on a Clustal analysis of four known fish acvr2b cDNAs (zebrafish (D. rerio), grass carp (Ctenopharyngodon idella), goldfish, Atlantic salmon (Salmo salar)), and one acvr2a (zebrafish) (see Supplementary File 2, see section on supplementary data given at the end of this article, for accession numbers). The forward primer, ActR-4, spans amino acids 37–43 and the reverse primer, ActR-5, spans amino acids 487–492. Amplification of reverse-transcribed RNA (325 ng) extracted from gilthead sea bream larvae aged 7 days post-hatching, using primers ActR-4 and ActR-5, resulted in a 1300–1400 bp fragment containing Acvr2b (59%) and with goldfish (57%). This analysis suggested that we have cloned the 5′-untranslated region, was cloned by 5′-RACE and 5′-end of the cDNA, including the signal peptide and 5′-untranslated region, was cloned by 5′-RACE and ActRIIB-1-ECD cloning in pPICZαA and cloning of full-length saMstn (Funkenstein & Rebhan 2007) was used.

BLAST analysis of predicted amino acid sequence derived from the ~1400 bp fragment revealed high similarity to Acvr2b from all fish species tested, with the highest score obtained for grass carp Acvr2b (92%).

The 5′-end of the cDNA, including the signal peptide and 5′-untranslated region, was cloned by 5′ RLM-RACE Protocol of The FirstChoice RLM-RACE kit (Ambion, Inc., Austin, TX, USA) as recommended by the manufacturer, using total RNA (10 μg) from gilthead sea bream 7-day larvae, 5′-RACE adaptor (Ambion), and random decamers for reverse transcription. Amplification by PCR was performed by two successive reactions. The first one used primers 5′-RACE outer (Ambion) and ActR-6; the second PCR used the primers 5′-RACE inner (Ambion) and ActR-4R (see Table 1 and Supplementary File 1). An amplified fragment of 400 bp was gel purified, cloned in pGEM-T Easy, and sequenced. BLAST analysis of the predicted amino acid sequence of this fragment revealed a 63% sequence similarity with several mammalian ACVR2B including chimpanzee (Pan troglodytes), human (Homo sapiens), bovine (Bos taurus), rat (Rattus norvegicus), and mouse (Mus musculus), and with grass carp and chicken (Gallus gallus). A lower degree of sequence similarity was found with predicted zebrafish Acvr2b (59%) and with goldfish (57%). This analysis suggested that we have cloned the 5′-end of acvr2b cDNA. However, attempts to clone the ECD using new primers based on the combined sequence of the 1400 bp (middle part) and the 5′-RACE product did not yield an amplification product. One reason could have been the existence of more than one acvr2b form. Therefore, we performed a 3′-RACE using two successive reactions. The first one used forward primer ActR-10 (located on the 5′-RACE fragment and designed for cloning the ECD in pcDNA expression vector) and 3′-RACE outer primer (Ambion). The second PCR used the primers ActR-8 (located also on the 5′-RACE fragment and designed for cloning the ECD in pPICZαA expression vector). Primers 5′- and 3′-RACE were designed using the CAGA-luciferase assay in A204 cells. A lower inhibition was obtained when unprocessed purified, furin-digested, and activated saMstn (Funkenstein & Rebhan 2007) was used.

Table 1 Names and sequences of primers used for cloning and for expression of S. aurata ActRIIB in P. pastoris

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>ActR-4</td>
<td>5′-GAGGTGGAAGAGACGACACCC-3′</td>
<td>Cloning</td>
</tr>
<tr>
<td>ActR-4R</td>
<td>5′-GGTTGGCTCTCCACCTCC-3′</td>
<td>5′-RACE</td>
</tr>
<tr>
<td>ActR-5</td>
<td>5′-CAGCAGCCCTGATGATT-3′</td>
<td>Cloning</td>
</tr>
<tr>
<td>ActR-6</td>
<td>5′-GGCCGGCTCATGAACTC-3′</td>
<td>5′-RACE and gene expression</td>
</tr>
<tr>
<td>ActR-8</td>
<td>5′-GAATCTAAGCAGGAGACACCGGAGT-3′</td>
<td>3′-RACE and ActRIIB-1-ECD cloning in pPICZαA</td>
</tr>
<tr>
<td>ActR-9b</td>
<td>5′-TCTAGATTTGAGACAGGG-3′</td>
<td>3′-RACE and cloning of full-length</td>
</tr>
<tr>
<td>ActR-10</td>
<td>5′-GATGTTGGATTTTGATACACATGCTCTTCTTCTCTTTACA-3′</td>
<td>Cloning of partial and full-length</td>
</tr>
<tr>
<td>ActR-12</td>
<td>5′-ATGTTGGATTTTGGATACACATGCTCTTCTTTACA-3′</td>
<td>Cloning of partial and full-length</td>
</tr>
<tr>
<td>ActR-14</td>
<td>5′-GATGCGCCCTGGTCGCTACTTTTG(T)12VN-3′</td>
<td>5′-RACE</td>
</tr>
<tr>
<td>5′-RACE outer</td>
<td>5′-GCTGATGCGCCCTGGTCGCTACTTTTG(G/C)-3′</td>
<td>5′-RACE</td>
</tr>
<tr>
<td>5′-RACE inner</td>
<td>5′-CGGCGATCCGAGGATCGGGTGGTCTGCTTGGATG-3′</td>
<td>5′-RACE</td>
</tr>
<tr>
<td>3′-RACE outer</td>
<td>5′-CGGCGAGCAGAATAATACAGCTACTTTGAGG(T)12VN-3′</td>
<td>3′-RACE</td>
</tr>
<tr>
<td>3′-RACE inner</td>
<td>5′-CGGCGAGCAGAATAATACAGCTACTTTGAGG(T)12VN-3′</td>
<td>3′-RACE</td>
</tr>
</tbody>
</table>

*Restriction sites are underlined.
fragment and designed to clone the ECD in pPICZαA expression vector) and 3'-RACE inner (Ambion). A fragment of ~2000 bp was amplified, gel purified, and cloned into pGEM-T Easy for sequencing. Alignment and comparison using Clustal of the fragment obtained originally with primers ActR-4 and ActR-5 and the new fragment revealed differences, suggesting that sea bream expresses, as we suspected, two forms of acvr2b. BLAST analysis of the predicted amino acid sequence of this 2 kb fragment showed high degree of sequence similarity with Acvr2b from grass carp (81%), with chicken (79%), and with human and horse (79%). A similar analysis of the assembled molecule (400 bp 5'-RACE fragment and the 2 kb 3'-RACE fragment) showed high sequence similarity with grass carp Acvr2b (81% identity, 417/513 aa), with chicken (79% identity, 407/513 aa), with human and chimpanzee (78% identity, 403/513 aa), with bovine and chimpanzee (79%), rat and mouse (78%, 402/513 aa), and 76% identity with a predicted zebrafish Acvr2b (395/517 aa). This cDNA was named saacvr2b-1 by us. Interestingly, the predicted amino terminus of saacvr2b-1-ECD is identical to mammalian ECD (GluAlaGlu). The second cDNA was named saacvr2b-1-ECD is identical to mammalian ECD.

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The ECD of saacvr2b-1 (amino acids 23–139) was obtained by PCR amplification of the appropriate clone and the primers ActR-8 and ActR-9b, introducing the restriction sites EcoRI and XbaI (see Table 1). The PCR fragment was gel purified and cloned first in pGEM-T Easy. The insert containing saacvr2b-1-ECD was cut out by double digestion with EcoRI and XbaI, gel purified, and ligated with EcoRI-XbaI double-digested vector. The recombinant plasmid was used to transform JM109 cells, grown on low salt LB plates containing 25 μg/ml Zeocin (InvivoGen, San Diego, CA, USA).

Nucleotide and amino acid sequence analyses and bioinformatics

The DNA sequences of saacvr2b clones were translated using Gene Runner, Version 3.05 (Hastings software, Inc. Hastings, NY, USA (http://www.generunner.com)). Similarity searches of the sequenced DNA fragments and predicted amino acid sequences were done by BLASTN and BLASTX using nr/nt database of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al. 1997). A multiple-sequence alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and Clustal X version 2.0.8 (Thompson et al. 1994, 1997) and BioEdit Sequence Alignment Editor Version 7.0.5.3 (Hall 1999). A phylogenetic tree was calculated using the neighbor-joining (NJ) method (Saitou & Nei 1987) with 1000 bootstrap (Felsenstein 1985) replicates using Clustal X 2.0.8 program and drawn using MEGA version 4.0 program (Tamura et al. 2007). The phylogenetic tree includes ACVR2B and ACVR2A from mammals, birds, amphibian, and fish that were retrieved from Genbank or from Ensembl database (Ensembl release 64 – Sep 2011; http://www.ensembl.org/index.html; Hubbard et al. 2009).


Preparation of saacvr2b-1-ECD expression plasmid construct

The P. pastoris host strain GS115 and the yeast expression vector pPICZαA were obtained from Invitrogen Ltd. The vector contains the α-factor signal sequence; therefore, the PCR fragments were constructed without the native secretion signal sequence of saacvr2b-1-ECD. The vector also contains P. pastoris alcohol oxidase (AOX1) promoter and transcription termination sequences separated by multiple cloning sites for insertion of the foreign gene of interest.

The ECD of saacvr2b-1 (amino acids 23–139) was obtained by PCR amplification of the appropriate clone and the primers ActR-8 and ActR-9b, introducing the restriction sites EcoRI and XbaI (see Table 1). The PCR fragment was gel purified and cloned first in pGEM-T Easy. The insert containing saacvr2b-1-ECD was cut out by double digestion with EcoRI and XbaI, gel purified, and ligated with EcoRI-XbaI double-digested vector. The recombinant plasmid was used to transform JM109 cells, grown on low salt LB plates containing 25 μg/ml Zeocin (InvivoGen, San Diego, CA, USA).

Transformation of P. pastoris and selection of transformants

The recombinant plasmid was linearized with SacI within the 5’-AOX1 region to direct the integration of the expression cassette into the AOX locus of P. pastoris genome, phenol-chloroform extracted, and precipitated. The linearized purified plasmid was then used to transform the GS115 strain of P. pastoris by electroporation. Zeocin-resistant transformants containing saacvr2b-1-ECD were selected on YPDS plates (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 1 M sorbitol, and 2% (w/v) agar), containing Zeocin (100 or 500 μg/ml) at 28 °C. Resistant colonies appeared after several days.
Screening for expression

The induction of protein expression was first tested in small-scale cultures. Selected transformants were inoculated into 5 ml BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 6-0, 1% (v/v) glycerol, 0-4 µg/ml biotin, and 1-34% (w/v) yeast nitrogen base with ammonium sulfate). Tubes were incubated overnight at 28°C in a shaker incubator (200–250 r.p.m.) to reach an OD A600 of ~2-0. Cells were harvested by centrifugation at 1750 g for 5 min in a bench-top centrifuge and resuspended in 3 ml BMMY medium (1% (w/v) yeast extract, 2% peptone (w/v), 100 mM potassium phosphate, pH 6-0, 0-5% (v/v) methanol, 0-4 µg/ml biotin, and 1-34% (w/v) yeast nitrogen base with ammonium sulfate) and incubation continued at 28°C in a shaker incubator (200–250 r.p.m.) for 48 h. Methanol was added to a final concentration of 0-5% (v/v) after 24 h to sustain the induction. To examine the presence of saAcvr2b-1-ECD in the supernatant, 300 µl were concentrated with cold acetone and analyzed by 16.5% Tris–Tricine SDS–PAGE (see below). Gels were stained with Coomassie Brilliant Blue R-250. An aliquot was taken from the yeast culture as control before initiation of induction with methanol, which was designated as T=0.

Expression and purification of Acvr2b-1-ECD

For biochemical analysis, a small-scale production was conducted. Clone #1 of saAcvr2b-1-ECD/pPICZaA was inoculated in a 250 ml Erlenmeyer flask containing 50 ml BMGY and incubated at 28–29°C in a shaker incubator (250 r.p.m.) to reach an OD A600 of ~2-0. Cells were collected (1750 g, 5 min) using a Heraeus Labofuge 400 bench-top centrifuge and resuspended in 50 ml BMMY. After 24 h, methanol was added to a final concentration of 0.5% (v/v) and incubation continued for an additional 24 h. Cells were centrifuged at 1750 g for 10 min at room temperature and supernatant was filtered through Minisart Syringe Filter, 0.2 µm (Sartorius Stedim Biotech GmbH, Goettingen, Germany). Purification of saAcvr2b-1-ECD was performed by Ni-NTA affinity chromatography (Ni-NTA His-Bind Resin was purchased from Novagen, Madison, WI, USA). Before binding to the Ni-NTA resin, the supernatant was dialyzed against 100 volumes of 20 mM Tris–HCl, pH 8.5, for ~20 h at 4°C. After being filtered again through Minisart Syringe Filter 0.2 µm, 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 (2×0.5 ml 100% resin) 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 two 0.8×4 cm Poly-Prep Chromatography Columns (Bio-Rad). The columns were washed sequentially with 5 ml of a solution containing 50 mM Tris–HCl, pH 8.5, 0.3 M NaCl, 10 mM imidazole; 5 ml of a solution containing 50 mM Tris–HCl, pH 8.5, 0.3 M NaCl, 30 mM imidazole, and then eluted (4×0.5 ml fractions) with the same solution containing 250 mM imidazole, 500 mM imidazole, and 1 M imidazole. 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 Tris–Tricine SDS–PAGE (16.5%) under reducing conditions (see below).

To obtain larger quantities of protein for biological activity assays, clone #1 saAcvr2b-1-ECD/pPICZaA was first inoculated in a 125 ml Erlenmeyer flask containing 15 ml BMGY and incubated at 28–29°C in a shaker incubator (250 r.p.m.) for 2 days to reach an OD A600 of ~2-5. The culture was transferred to a 21 Erlenmeyer flask containing 250 ml BMGY and incubation continued at 28–29°C with shaking (250 r.p.m.) for an additional 2 days to reach an OD A600 of ~2-366. Cells were collected (1750 g, 5 min) and resuspended in 250 ml BMMY. After 24 h, methanol was added to a final concentration of 0.5% (v/v) and incubation continued for an additional 24 h. Cells were centrifuged (1750 g, 10 min) at room temperature and supernatant was filtered through Acrodisc Syringe Filter with 0.45 µm Supor Membrane (Pall Corporation, Newquay, Cornwall, UK). The supernatant was loaded onto four 0.8×4 cm Poly-Prep Chromatography Columns (Bio-Rad) containing each 0.6 ml 20 mM Tris–HCl, pH 8.5, 0.3 M NaCl, 10 mM imidazole; 5 ml of a solution containing 250 mM Tris–HCl, pH 8.5, 0.3 M NaCl, and 10 mM imidazole. After loading, the columns were washed with a solution containing 50 mM Tris–HCl, pH 8.5, 0.3 M NaCl, and 20 mM imidazole until OD A280 of the washed solution was close to 0. The protein was eluted (4×0.5 ml fractions) with the same solution containing 250 mM imidazole, 500 mM imidazole, and 1 M imidazole. 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 Tris–Tricine SDS–PAGE (16.5%) under reducing conditions (see below).

SDS–PAGE

Reagents for SDS–PAGE and molecular mass markers were purchased from Bio-Rad Laboratories. Tris–Tricine SDS–PAGE (16.5%) was performed according to Schagger & Von Jagow (1987). Samples were mixed with loading buffer under reducing conditions. Proteins were stained with Coomassie Brilliant Blue R-250 for general proteins view or periodic acid/Schiff (PAS) for glycoproteins (see below).

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Deglycosylation of purified Acvr2b-1-ECD

The deglycosylation status of recombinant saAcvr2b-1-ECD was assessed using Peptide N-glycosidase F (PNGase F, New England Biolabs, Beverly, MA, USA) according to the manufacturer’s protocol. Purified saAcVR2B1-ECD in 1× glycoprotein denaturing buffer was heated for 10 min at 100 °C; then 2 μl PNGase F (500 U/μl) or H2O were added to the reaction, which contained in addition 1× G7 reaction buffer and 1% (v/v) NP-40. Incubation was carried out at 37 °C for 24 h. The digested product was analyzed by 16.5% Tris–Tricine SDS–PAGE under reducing conditions followed by Coomassie and PAS staining (see below).

PAS staining

PAS staining of the gels was carried out according to the protocol detailed in Thornton et al. (1996). Briefly, gels were washed in Solution C: 50% (v/v) ethanol for 30 min followed by washing in distilled water for 10 min and then incubation in Solution A: 1% (v/v) periodic acid in 3% (v/v) acetic acid, prepared freshly, for 30 min. Subsequently, the gels were washed in distilled water for at least six times for 5 min and left overnight in water. The next day, the gels were washed in Solution B: 0.1% (w/v) sodium metabisulfite in 10 mM HCl, prepared freshly, for two times for 10 min. Then, the gels were incubated in Schiff’s reagent for 1 h in the dark, immersed in Solution B for 1 h in the dark, and finally washed several times in Solution D: 0.5% (w/v) sodium metabisulfite in 10 mM HCl, prepared freshly, for a total of at least 2 h in the dark. Gels were stored in Solution E: 7.5% (v/v) acetic acid/5% (v/v) methanol in distilled water and photographed. All reagents for the PAS reaction were purchased from Sigma.

Silver staining

Staining was carried out using the Bio-Rad Silver Stain reagents according to the manufacturer’s protocol.

Biological activity

The ability of saAcvr2b-1-ECD to inhibit MSTN activity in vitro was measured using the pGL3-(CAGA)12 luciferase reporter assay in A204 rhabdomyosarcoma cells (ATCC, HTB-82). The CAGA boxes were found in the promoter of plasminogen activator inhibitor-type 1 and serve as binding sites to transcription factors Smad3/Smad4, participating in the signaling pathway of TGFβs (Dennler et al. 1998), including MSTN (Thies et al. 2001). A204 cells were cultured in 75 cm² flasks until nearly complete confluence in McCoy’s 5A medium (Biological Industries, Kibbutz Beit Haemek, Israel), supplemented with 10% (v/v) FBS, 1% (v/v) glutamine, and 1% (v/v) antibiotics. Cells were trypsinized and plated at a density of 10⁵ cells per well in 24-well plates in the same medium but without antibiotics. After a 48 h attachment period, cells were transiently co-transfected with 1 μg pGL3-(CAGA)12 luciferase construct (Thies et al. 2001) and 1 ng of the control vector pRL-RSV, expressing Renilla luciferase. Transfection was performed using 3 μl Lipofectamine 2000 (Invitrogen), as recommended by the manufacturer. Six hours after transfection, medium was replaced by fresh growth medium containing antibiotics and incubation continued for an additional 18 h. Cells were then washed with McCoy’s 5A medium to remove FBS and serum-free growth medium was added, containing 5 or 2.5 ng/ml recombinant mouse/rat/human GDF8/MSTN (R&D Systems, Minneapolis, MN, USA) with increasing amounts of purified saAcvr2b-1-ECD (which was dialyzed before the assay against PBS for ~24 h) or with PBS. Cells were collected 24 h later. Alternatively, transfected cells were cultured in serum-free growth medium containing purified refolded saMstn1 cleaved by furin that was activated by heat (100 °C, 3 min; Funkenstein & Rebhan 2007), with or without purified saAcvr2b-1-ECD. The saMstn1 and saAcvr2b-1-ECD were pre-incubated for 1 h at room temperature to allow binding between the soluble receptor and Mstn before their addition to the A204 cells. Cells were collected 24 h later. The medium was removed; cells were rinsed carefully with PBS (pH 7.4) and lysed in 100 μl of 1×Passive Lysis Buffer (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 and 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 results are expressed as the ratio of firefly to Renilla luciferase activities and represent the mean value of triplicates, with error bars depicting the S.E.M.

Results and discussion

Cloning and characterization of two acvr2b isoforms in the gilthead sea bream

Three cDNAs encoding acvr2b were cloned from 7-day larvae of the marine fish gilthead sea bream, which we
named saacvr2b-1, saacvr2b-2a, and saacvr2b-2b. The length of the cloned acvr2b-1 cDNA is 2295 bp, consisting of 194 bp 5'-untranslated region, 1542 bp open reading frame encoding a 514 amino acid peptide, and 557 bp 3'-untranslated region (GenBank accession no. JF906098). In addition, two closely related homologs of saacvr2b-2 were cloned: acvr2b-2a of 1524 bp, encoding a 508 amino acids peptide (GenBank accession no. JF906099) and acvr2b-2b of 1530 bp, encoding a 510 amino acids peptide (GenBank accession no. JF906100). Clustal analysis of the predicted amino acid sequences of the three acvr2b cDNAs indicated clearly that acvr2b-2a and -2b are similar to each other (score of 98) but substantially different from acvr2b-1 (score of 79) (Fig. 1 and Supplementary File 3, see section on supplementary data given at the end of this article). Clustal analysis of the nucleotide sequences of the two acvr2b-2 clones revealed a score of 95, suggesting that the two clones are probably not the result of gene duplication but rather point mutations, resulting in the potential existence of two alleles of the acvr2b-2 gene; however, further studies are needed to confirm this hypothesis using samples from a large population.

Amino acid sequence analysis showed that saAcvr2b contains the characteristic regions of TGFβ type 2 receptor superfamily including a short ECD, a hydrophobic TMD, and a larger ICD. Hydropathy analysis of saAcvr2b-1 using the method of Kyte & Doolittle (1982) and the TMpred – Prediction of Transmembrane Regions and Orientation program revealed two hydrophobic regions: a 19 amino acid stretch at the N-terminal assumed to be a signal peptide and a single putative 23 residue membrane-spanning region between residues 140 and 162 (Fig. 1). The signal peptide as predicted by SignalP prediction program (Petersen et al. 2011) is of 22 amino acids. The protein has a relatively short ECD (117 amino acids in saAcvr2b-1, 113 amino acids in saAcvr2b-2b, and 111 amino acids in saAcvr2b-2a) and a long C-terminal ICD (352 amino acids). The putative ligand binding ECD contains ten highly conserved cysteine residues (Fig. 1). Similar to other Acvr2bs, saAcvr2b also has two potential N-linked glycosylation sites at amino acid positions 42 and 65 (NR/QS, NSS). Two previous studies showed that seven amino residues (Tyr36, Val73, Trp78, Leu79, Phe82, Val99, and Phe101) in mouse ACVR2B are directly involved in binding to activin (Gray et al. 2000, Thompson et al. 2003). These residues are conserved in the ECD of saAcvr2b-1, -2a, and -2b in their position and spacing (Fig. 1 and Supplementary File 4, see section on supplementary data given at the end of this article). The TMD terminates on the intracellular side before a stretch of sequences rich in basic amino acids. In accordance with other type 2 receptors that contain proline-rich segments juxtaposed on both sides of the TMD, the TMD of saAcvr2b is also flanked by proline-rich sequences (Fig. 1). Analysis of the ICD of saAcvr2b-1, saAcvr2b-2a, and saAcvr2b-2b showed highly conserved sequences compared with mouse ACVR2B and slightly less to mouse ACVR2A, presenting the subdomains characteristic of protein kinases (Fig. 2). Furthermore, two of these subdomains (VIB and VIII) contain two serine kinase consensus sequences, DFKSKN and GTRRYMAPE, that are used to predict tyrosine vs serine/threonine substrate specificity (Hanks et al. 1988, Mathews & Vale 1991). The sequence of saAcvr2b-1, saAcvr2b-2a, and saAcvr2b-2b in both subdomains is conserved and characteristic of the serine/threonine kinases. Twelve kinase domain residues were recognized as conserved in the sequence alignment of multiple kinase domains of the eukaryotic protein kinase superfamily, indicating their functional importance (Hanks & Hunter 1995). Nine of these residues were identified in saAcvr2b-1, in addition to two well-conserved substitutions: G -> A and E -> D in saAcvr2b-1 and saAcvr2b-2a (Fig. 1). A crystal structure of this region in mouse ACVR2 revealed a three-finger toxin fold formed by four disulfide bridges, in addition to a fifth disulfide bond (Greenwald et al. 1999). A recent report of the crystal structure of ACVR2B kinase domain from human identified four kinase domain residues (Phe234, Leu245, Phe247, and Thr265) as playing a structural role in the active site of human ACVR2B (Han et al. 2007). These residues are highly conserved in saAcvr2b as well, except for Phe247, which is replaced by Tyr (Fig. 1).

Further analyses by BLAST and Clustal and construction of a NJ phylogenetic tree revealed that the two saAcvr2bs clustered separately. The saAcvr2b-1 clustered into a clade together with Acvr2b from green puffer (Tetraodon nigroviridis), Japanese puffer (Takifugu rubripes), one of two entries for three-spined stickleback (Gasterosteus aculeatus), medaka (Oryzias latipes), Atlantic cod (Gadus morhua), and zebrafish Acvr2b (Fig. 3; for details of sequences, accession numbers and species used for the comparison see Supplementary File 2). By contrast, saAcvr2b-2a and -2b clustered in a clade together with all known fish Acvr2b peptides, including a second entry for zebrafish, stickleback, cod, and medaka Acvr2b (Fig. 3), indicating for the first time that fish possess at least two genes for Acvr2b. The detailed Clustal analysis of all fish Acvr2b and representatives of amphibians, birds, and mammalian ACVR2B is shown in Supplementary Files 3 and 4. The comparison also includes ACVR2A from fish, birds, and mammals. All ACVR2A included in the analysis showed a score of 59–63 with both saAcvr2b-1 and -2, whereas scores of 77–95 were obtained in the comparison of saAcvr2b with all fish Acvr2b, substantiating our conclusion that the three cDNAs cloned by us are acvr2b and not acvr2a. The score between saAcvr2b-1 or
**Figure 1** Alignment of deduced amino acid sequences of gilthead sea bream (*Sparus aurata*) Acvr2b isoforms. Dots indicate amino acids identical to the first line, which corresponds to Acvr2b-2a. Dashes indicate gaps inserted to enhance sequence similarity. Predicted signal peptide is underlined and predicted transmembrane region is overlined. The ten conserved cysteine residues in the extracellular domain are indicated by an open triangle and the putative N-linked glycosylation sites are indicated by upper asterisks (**`). The conserved residues involved in binding of activin with Acvr2b (Thompson et al. 2003) are indicated by an arrow (\( \uparrow \)) and the conserved residues located at the active site of Acvr2b kinase domain (Han et al. 2007) are marked by black full circle. The kinase domain residues recognized as conserved in the sequence alignment of multiple kinase domains of the eukaryotic protein kinase superfamily (Hanks & Hunter 1995) are indicated by a black triangle and the two substitutions in sea bream Acvr2b are indicated by a black square. Proline-rich motifs juxtaposed to the transmembrane domain are boxed. The serine/threonine kinase-specific regions (Mathews & Vale 1991) are shaded gray.

<table>
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<td>G.A.TI.S...SPV.IO..E.....ST</td>
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Acvr2b-2a and -2b and that of mammalian ACVR2B are similar, in the range of 78–80 (Supplementary File 3), indicating that both isoforms of Acvr2b are highly conserved during vertebrate evolution. In order to further clarify the identification of the two saacvr2b cDNAs cloned by us, several individual Clustal analyses were performed. Analysis of saAcvr2b-1, green puffer, and Japanese puffer Acvr2b resulted in scores of 93 and 95 respectively (Supplementary File 5A, see section on supplementary data given at the end of this article), illustrating very high sequence similarity. A similar analysis of saAcvr2b-1 and the two entries for zebrafish Acvr2bs resulted in lower scores of 80 and 76 (Supplementary File 5B) whereas a similar comparison with saAcvr2b-2a or -2b resulted in scores of 88 and 75 (Supplementary File 5C). By contrast, a high similarity was found between saAcvr2b-2a and -2b and cyprinids (grass carp and goldfish) Acvr2b (scores of 91–92) (Supplementary File 5D).

Our novel conclusion of the existence of at least two genes for acvr2b in fish is further supported by gene annotation provided by Ensembl. Two acvr2b genes were identified in zebrafish: one localized to chromosome 2 and the second to chromosome 24. Two acvr2b were also identified in the stickleback: one localized to Scaffold 182 and the second to group III. Also medaka displayed two entries for acvr2b: one localized on ultracontig 199 and the second on chromosome 20 and cod possesses two acvr2b genes: one on Scaffold 2105 and the second on Scaffold 3681. Surprisingly, no second acvr2b gene could be found in the Ensembl-annotated genomes of green puffer and Japanese puffer fish. Conserved genes,

Figure 2 Comparison between mouse and sea bream ACVR2 kinase domains. Amino acid comparison between the kinase domains of mouse ACVR2A (accession no. NM007396) or ACVR2B (accession no. BC106189) and saAcvr2b-1 (accession no. JF906098), saAcvr2b-2a (accession no. JF906099), and saAcvr2b-2b (accession no. JF906100). Dots indicate amino acid residues identical to the first line. Kinase subdomains are indicated in roman numerals as described in Mathews & Vale (1991). At the bottom right, pairwise comparison of saAcvr2b-1, saAcvr2b-2a, and saAcvr2b-2b amino acid sequences with mouse ACVR2B and mouse ACVR2A and the scores given by the Clustal analysis.
Figure 3  Phylogenetic tree obtained by amino acid sequence comparison of different ACVR2B and ACVR2A. The phylogenetic tree was constructed from a single multiple alignment, using neighbor-joining method with Clustal X 2.0.8 and MEGA 4.0 tools. Numbers at the tree nodes represent bootstrap values after 1000 replicates. The saAcvr2b isoforms cloned in the current study are indicated by asterisks. For each sequence, NCBI or Ensembl accession number and species abbreviation are shown. Full scientific names of species abbreviations, common names, and their respective accession numbers are detailed in Supplementary File 2.
in addition to harboring similar coding sequences, often display conservation of synteny. To investigate the degree of locus conservation, we performed synteny analysis of the two \textit{acvr2b} isoforms in the six teleost genomes available to date using five genes that seem to be linked to \textit{acvr2b}. This analysis revealed a conserved synteny around the two loci of the two genes in zebrafish, but less clear in the case of other fish species, for which synteny conservation was found mainly for one locus. The chromosomal location of the different markers is shown in Fig. 4. Presence of duplicated marker genes in these regions in zebrafish, medaka, and stickleback supports the hypothesis that the two \textit{acvr2b} genes in these fish species are the result of chromosomal duplication of this region. In a few cases, the duplicated markers appear on a different linkage group suggesting translocation. Interestingly, green puffer and Japanese puffer fish lost the second \textit{acvr2b} gene, although the duplicated markers exist (Fig. 4). Incomplete annotation for the fish genomes (with the exception of zebrafish) might have resulted in the artificial loss of a particular duplicated gene.

Expression and characterization of saAcvr2b-1-ECD

The next step was to express saAcvr2b-1-ECD protein and test the ability of the ECD to block the interaction of MSTN with endogenous ACVR2B. This in turn is expected to induce skeletal muscle hyperplasia and/or hypertrophy, if MSTN mode of action in fish muscle growth is similar to that in mammals.

Transformants of \textit{P. pastoris} GS115 colonies with saAcvr2b-1-ECD were grown on YPDS plates containing 100 \( \mu \)g/ml Zeocin and were screened for the expression of the recombinant proteins in 5 ml cultures at 2 days after induction with methanol. As shown in Fig. 5, a faint band between 25 and 37 kDa could be seen for the ECD, when total secreted proteins were analyzed by Tris–Tricine SDS–PAGE (16.5%) gel.

This band was not present in aliquots of \textit{P. pastoris} culture taken at \( T=0 \), before initiation of induction with methanol.

Trix–Tricine SDS–PAGE analysis of the affinity purification of saAcvr2b-1-ECD produced from a 250 ml \textit{P. pastoris} culture is shown in Fig. 6A. Fractions eluted with 250 mM imidazole showed a band of \(~23\) kDa along with a broad smearing band above the 23 kDa band. The smearing pattern was also observed previously during human, pig, chicken, and goldfish ACVR2B-ECD expression in \textit{P. pastoris} (Daly & Hearn 2006, Carpio et al. 2009, Kim et al. 2012).

As mentioned earlier, saAcvr2b-1-ECD has two potential Asn-linked glycosylation sites at Asn\(^{12} \) and Asn\(^{65} \). To ascertain whether the recombinant saAcvr2b-1-ECD is
glycosylated and determine whether the broad band that was obtained following purification (Fig. 6A) is due to different levels of glycosylation of the same core protein, the purified saAcvr2b-1-ECD was digested with PNGase F. This enzyme hydrolyzes all types of polysaccharide chains linked to Asn but does not cleave O-linked oligosaccharides. The apparent molecular weight of purified saAcvr2b-1-ECD was reduced from a broad band extending from ~23 to 37 kDa to a strong, intense single band of ~17 kDa after N-deglycosylation (Fig. 6B, left panel). The molecular mass of the deglycosylated form is in good agreement with the calculated molecular mass of 16 642 Da of the fusion protein, including the 6xHis tag and myc epitope. Moreover, following treatment with PNGase F, the broad band of saAcvr2b-1-ECD became more intense and looked like a single band (Fig. 6B, left panel), suggesting that the recombinant saAcvr2b-1-ECD has different levels of N-glycosylation. The agreement between the observed molecular mass following N-deglycosylation and the calculated molecular mass suggests that saAcvr2b-1-ECD has only N-glycosylation. The presence of polysaccharides was also verified by PAS staining of the purified saAcvr2b-1-ECD following electrophoresis on Tris–Tricine gels (Fig. 6B, right panel). While the broad band of saAcvr2b-1-ECD stained pink with PAS, no staining was seen following treatment with PNGase F, indicating complete removal of glycoproteins.

Figure 6 Expression, purification, and deglycosylation of saAcvr2b-1-ECD. (A) Purification of saAcvr2b-1-ECD expressed in GS115 strain of Pichia pastoris by Ni-NTA affinity chromatography. Fractions containing saAcvr2b-1-ECD that were eluted with 250 mM imidazole were analyzed by Tris–Tricine SDS–PAGE (16.5%) under reducing conditions. Gels were stained with Coomassie Brilliant Blue R-250. (B) Deglycosylation and glycoprotein staining of saAcvr2b-1-ECD. Affinity-purified saAcvr2b-1-ECD was denatured and treated with or without PNGase F. The digested products were precipitated by adding five volumes of cold acetone, resuspended in loading buffer containing β-mercaptoethanol, and analyzed by Tris–Tricine SDS–PAGE (16.5%). Two gels were run in parallel; one stained for total proteins with Coomassie Brilliant Blue R-250 (left panel) and the second stained for glycoproteins with periodic acid/Schiff (PAS) (right panel). (C) Analysis of affinity-purified saAcvr2b-1-ECD after dialysis against PBS. Shown are aliquots (0.25, 0.625, 1.25, 2.5, and 5 µg) of dilutions used for biological assay (Fig. 8C and D). Undiluted Acvr2b-1-ECD (5 µg) was also analyzed under reducing (+β) and nonreducing (−β) conditions. Proteins were stained with Bio-Rad silver stain. Full colour version of this figure available via http://dx.doi.org/10.1530/JME-12-0075.
of carbohydrates. Essentially similar results regarding N-glycosylation were reported for the pig ACVR2B-ECD expressed in P. pastoris (Kim et al. 2012). By contrast, ACVR2B-ECD from goldfish, chicken, and human produced in P. pastoris (Daly & Hearn 2006, Carpio et al. 2009, Kim et al. 2012) and mouse ACVR2B-ECD produced in baculovirus-insect cell culture system (Donaldson et al. 1999) also seem to undergo O-linked glycosylation (on serine or threonine residues).

**Inhibition of MSTN activity by saAcvr2b-1-ECD**

The ability of affinity-purified saAcvr2b-1-ECD to inhibit the biological activity of MSTN was tested using the pGL3-(CAGA)12-luciferase reporter assay system. We first tested the inhibitory effect on saMstn1 biological activity. Addition of purified refolded, furin-digested, and activated saMstn1 (2000 ng/ml) to A204 cells, which are transiently transfected with the pGL3-(CAGA)12-luciferase plasmid, resulted in ~18-fold higher activity of the luciferase reporter gene than without Mstn (Fig. 7A), confirming our previous report (Funkenstein & Rebhan 2007). Addition of 6 µl of purified saAcvr2b-1-ECD after dialysis against PBS (equivalent to 1 µg or 3:37 µg/ml) did not inhibit saMstn1 activity (Fig. 7B). Reducing saMstn1 amounts to 400 ng/ml and adding increasing amounts of the same saAcvr2b-1-ECD batch resulted in a 17% inhibition of MSTN activity at the highest amounts (60 µl equivalent to ~10 µg or 33-6 µg/ml; Fig. 7D). In a third experiment, saMstn1 concentration was further reduced to 240 ng/ml and a new batch of saAcvr2b-1-ECD was tested. This low concentration of saMstn1 induced a 16:2-fold higher luciferase activity compared with no addition of MSTN. At a lowest dose (6 µl equivalent to 3:8 µg or 12-8 µg/ml), no inhibition could be seen (Fig. 7C). However, when saMstn1 concentration was reduced to 25 ng/ml and large amounts of saAcvr2b-1-ECD were added (20 and 60 µl equivalent to 12-8 and 38-5 µg or 33-6 and 77 µg/ml respectively), a 20% inhibition in saMstn1 activity was observed (Fig. 7E).

We next tested the inhibitory effect of saAcvr2b-1-ECD using commercial recombinant mouse/rat/human mature MSTN (m/r/hMSTN). Addition of large amounts (60 µl equivalent to 38-5 µg or 77 µg/ml) of purified saAcvr2b-1-ECD together with 5 ng/ml recombinant mouse/rat/human mature MSTN to A204 cells, which are transiently transfected with the pGL3-(CAGA)12-luciferase plasmid, resulted in 86-7% inhibition (Fig. 8A). The inhibition was dose dependent, as shown in Fig. 8B (5 ng/ml m/r/hMSTN and high doses of saAcvr2b-1-ECD) and Fig. 8C (2-5 ng/ml m/r/hMSTN and lower doses of saAcvr2b-1-ECD). The dialyzed and diluted saAcvr2b-1-ECD used in the experiment shown in Fig. 8C was analyzed by Tris–Tricine SDS–PAGE followed by silver staining (Fig. 6C). Analysis also included electrophoreses under reducing and non-reducing conditions, showing differences in electrophoretic mobility, indicative of the peptide being refolded. The highest
inhibitory effect (75% inhibition) was obtained with 60 µl saAcvr2b-1-ECD (equivalent to 38·5 µg protein based on BCA assay, or 77 µg/ml). Pre-incubation of the mature m/r/hMSTN (2·5 ng/ml) with saAcvr2b-1-ECD for 1 h at room temperature increased the inhibitory effect from 36·1 to 55·7% (Fig. 8D). The lowest amount of saAcvr2b-1-ECD, tested in these set of experiments, of 1 µl (equivalent to 0·65 µg or 1·3 µg/ml) elicited a minor inhibition of 4% (Fig. 8C). The ED$_{50}$ obtained in the absence of pre-incubations was about 22–23 µl (14–15 µg or 28–30 µg/ml; Fig. 8E and F).

The inhibitory effect of soluble ACVR2B-ECD on the luciferase activity in the CAGA assay indicates that it is an inhibitor of MSTN signaling, acting most likely by sequestering MSTN away from full-length membrane-bound ACVR2B. However, the saAcvr2b-1-ECD showed about fourfold lower inhibitory activity when purified, furin-cleaved, and activated saMstn1 was tested compared to that of the commercial mature m/r/hMSTN. One possibility for this difference is a lower affinity of the fish Mstn to the soluble fish receptor than to the endogenous receptor on the A204 cell membrane. Another possibility is that the
prodomain, which is removed from the mature MSTN by heat treatment and in this way the mature MSTN is being activated, has residual binding affinity and therefore higher amounts of the soluble receptor competitor are needed. In general, higher amounts of the soluble fish receptor were needed to achieve a comparable inhibition of MSTN activity to that obtained by chicken and pig ACVR2B-ECD produced in *P. pastoris* and tested by the CAGA-Luc assay in A204 cells (Kim et al. 2012). One explanation for the high amounts of soluble receptor that were needed to obtain MSTN activity inhibition, both the fish (homologous) or the mammalian (heterologous), is the presence of 6xHis tag and a myc epitope at the C-terminus of the fusion protein. Sako et al. (2010) showed that the C-terminal portion of ACVR2B-ECD plays a role in binding affinity between the receptor and activin A and GDF11. Amounts of soluble receptor that were used for the biological activity assay were calculated based on a BCA Protein Assay (Pierce, Rockford, IL, USA). The concentration as determined following dialysis against PBS was 762 ng/μl. SDS–PAGE followed by silver staining of aliquots of this batch (Fig. 6C) showed that the purified protein appeared essentially as in Fig. 6A and did not change following dialysis. Although the broad smearing of the protein (Fig. 6) may indicate that the protein is glycosylated to various degrees, it is unlikely that this is the reason for the need for large amounts in order to inhibit MSTN as it was shown that glycosylation of the ACVR2B-ECD is not essential for its activity (Kim et al. 2012). Yet another possibility is that differences exist between the affinity of Acvr2b-1 and -2 to MSTN1 and MSTN2. Interestingly, a 1000-fold excess of mouse ACVR2B-ECD to activin was needed in order to achieve about 50% inhibition of activin-induced FSH secretion from pituitary cells (Greenwald et al. 1998). This later study showed binding between the two by cross-linking experiments, using radiolabeled activin and mouse ACVR2-ECD expressed in *P. pastoris*. Furthermore, that study showed that glycosylation is not essential for high-affinity interaction between ACVR2-ECD and activin A. Subsequently, Donaldson et al. (1999) showed that soluble ACVR2-ECD expressed in insects also possess an intrinsic ability to bind ligands with high affinity, as evidenced in the cross-linking experiments with activin A and inhibit A. The study by Sako et al. (2010) showed, using a detailed kinetic characterization of soluble ACVR2B binding to several low and high affinity ligands, that both MSTN and GDF11 bind ACVR2B-ECD with affinities comparable to those of activin A. Conservation of the number and position of amino acid residues in saAcvr2b-1-ECD, shown earlier in mouse ACVR2B to be directly involved in binding to activin (Gray et al. 2000, Thompson et al. 2003), suggested that mature m/r/hMSTN, which was shown to bind human ACVR2B-ECD (Sako et al. 2010), can interact with the fish Acvr2b-ECD. This conclusion is based on our observation of the inhibitory effect of fish Acvr2b-ECD on m/r/hMSTN activity in the CAGA-luciferase assay, although high concentrations of the soluble receptor were needed. Previous studies have demonstrated that ACVR2B-ECD sequence is exceptionally conserved, with only one amino acid difference between mice and humans and ~90% identity between species as divergent as chickens and humans. As for saAcvr2b-ECD, although the residues important for activin binding are conserved between saAcvr2b-1-ECD, saAcvr2b-2a, and saAcvr2b-2b (Fig. 1), there are differences in the C-terminus of the ECD including an insertion of four residues, which may affect binding affinity to MSTN. In mice and humans, four different isoforms of ACVR2B were found (ACVR2B1, ACVR2B2, ACVR2B3, and ACVR2B4). The ECDs of ACVR2B1 and ACVR2B2 contain an insertion in the C-terminal portion of the ECD that is absent in isoforms ACVR2B3 and ACVR2B4. The biological significance of the different isoforms remains unclear. However, the longer isoforms ACVR2B1 and ACVR2B2 have been shown to have a three- to four-fold higher affinity for activin A than the shorter isoforms ACVR2B3 and ACVR2B4 (Attisano et al. 1992). Given that various forms of Acrv2b show different affinity to TGFβ ligands, future studies are needed to investigate the functional differences between the saAcvr2b-1 and -2. Interestingly, concentration-inhibitory curves with mammalian MSTN were biphasic, with a steep slope at the lower concentrations, followed by a gradual component at higher concentrations (Fig. 8F). These steep and gradual slopes may indicate heterogeneity in the binding affinities (high- and low-affinity binding sites between MSTN and ACVR). In conclusion, the current study shows for the first time that fish express two acvr2b paralog genes, probably as a result of gene duplication. The two paralogs differ substantially in their amino acid sequence. In addition, we showed that bioactive fish Acrv2b-1-ECD can be produced in *P. pastoris*, and the fish Acrv2b-1-ECD suppressed Mstn activity like other Acrv2b of mammalian and avian species.

**Note added in proof**

saacv2b-2a and saacv2b-2b homologs might differ in few additional amino acids to those shown in the paper in light of additional sequence data obtained for more clones of both homologs, which suggests that previously assumed sequence errors are in fact sequence differences.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-12-0075.
Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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