Characterization and identification of the inhibitory domain of GDF-8 propeptide

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Abstract

GDF-8 is a negative regulator of skeletal muscle mass. The mechanisms which regulate the biological activity of GDF-8 have not yet been elucidated. Analogous to the TGF-β system, GDF-8 propeptide binds to and inhibits the activity of GDF-8. In these studies, we define the critical domain of the GDF-8 propeptide necessary for inhibitory activity. Two molecules of GDF-8 propeptide monomer inhibit the biological activity of one molecule of GDF-8 homodimer. Although the propeptide contains N-linked glycosylation when synthesized in mammalian cells, this glycosylation is not necessary for the inhibition of GDF-8. Taking advantage of the bacterial expression system, we express and purify GDF-8 propeptide which retains full inhibitory activity. To define the functional regions of the propeptide, we express a series of truncated GST-propeptide fusion proteins and examined their inhibitory activity. We observe that fusion proteins containing the C-terminal region (amino acid residues 99–266) are very stable, but do not exhibit inhibitory activity; while fusion proteins containing the N-terminal region (amino acid residues 42–115) are labile but contain essential inhibitory activity. The data suggest that the C-terminal region may play a role in the stability of the GDF-8 propeptide and that the inhibitory domain is located in the region between amino acids 42 and 115.

Growth and differentiation factor-8 (GDF-8), also known as Myostatin, is a member of the transforming growth factor-beta (TGF-β) superfamily of structurally related growth factors, that plays an essential role in regulating skeletal muscle growth [1–4]. GDF-8 is a negative regulator of skeletal muscle mass [3,5–10]. Myostatin-null mice, generated by gene targeting, exhibit a marked hypertrophy and hyperplasia of skeletal muscle possessing twice the skeletal muscle mass of non-transgenic littermates [1]. Similar increases in skeletal muscle mass are known in naturally occurring mutations of the GDF-8 gene in cattle [1,2,11–15]. Both systemic administration of Myostatin and overexpression of Myostatin in transgenic mice resulted in the decrease of skeletal muscle mass [8,10]. In addition to increased skeletal muscle mass, Myostatin-null mice have a significant reduction in body fat [3,16]. In agouti lethal yellow (Ay) and obese (Lepob/ob) mice, two genetic models for obesity, the loss of the Myostatin function prevented fat accumulation in mice during the aging process and moderated clinical parameters associated with diabetes and obesity [16]. Thus, Myostatin has regulatory roles in skeletal muscle development and fat metabolism.

Like other members of the TGF-β superfamily, GDF-8 is synthesized as a precursor protein. The signal sequence which directs the protein to the cell membrane is cleaved during the secretion process. The amino-terminal propeptide is cleaved from the carboxy-terminal mature protein [1,17,18]. It is believed that the precursor protein forms a homodimer before proteolytic processing [17]. The cleaved propeptide molecules remain non-covalently bound to the mature domain dimer, forming a latent complex and inhibiting its biological activity. To interact with its receptor, the
GDF-8 ligand must be released from the latent complex [18]. Although the interaction of Myostatin and its propeptide is similar to TGF-β, the precise biological activation mechanism for latent GDF-8 complex to release mature GDF-8 is not well understood. Dominant-negative transgenic mouse studies have demonstrated that over expression of GDF-8 propeptide results in enhanced muscle development and increased skeletal muscle mass [18,19]. In this paper we present in vitro bioassay data demonstrating that the inhibitory activity of propeptide does not require either N-linked glycosylation or intermolecular disulfide bonds. We also show that propeptide expressed in bacteria is fully active. The in vitro bioassays indicate that the inhibitory activity of bacterial expressed propeptide is identical to that of propeptide expressed in CHO cells. Finally, we describe the characterization of the inhibitory domain of GDF-8 propeptide.

Materials and methods

Purification of human GDF-8 propeptide and mature protein from CHO cell conditioned media. Conditioned medium from a CHO cell line expressing Myostatin [17] was concentrated 15-fold and applied to a 2 x 20 cm Q-Sepharose FF (Amersham–Pharmacia Biotech) chromatography column previously equilibrated with wash buffer (100 mM NaCl in 20 mM Na₂HPO₄, pH 7.4). The column was washed with 20 column volumes of buffer; flow rate: 10 ml/min. The flowthrough and wash fractions were pooled and adjusted to 75 mM NaCl in 20 mM Na₂HPO₄, pH 5.0. The pooled fractions from Q-Sepharose were loaded onto a 2 cm x 20 cm SP-Sepharose FF (Amersham–Pharmacia Biotech) previously equilibrated with buffer (75 mM NaCl in 20 mM Na₂HPO₄, pH 5.0). The column was washed with 20 column volumes of buffer; flow rate: 10 ml/min. GDF-8 complex was eluted with 200 mM NaCl in 20 mM Na₂HPO₄, pH ~ 5.0, and concentrated 10-fold using CentriPlus 50K concentrator (Amicon). Concentrated GDF-8 complex was adjusted to 4 M guanidine–HCl, 0.1% TFA, and then filtered (Syringe Filters, Low Protein Binding, No. 4192; Pall Gelman Sciences). The sample was adjusted to 5% acetonitrile prior to reverse phase high performance liquid chromatography (Jupiter C4 300 Å, 250 x 10 mm. 5% acetonitrile, 0.1% TFA, flow rate: 5 ml/min, 1 min/fraction). Mature and propeptide proteins were eluted with a linear gradient of 5–70% acetonitrile.

Purification of the recombinant proteins from bacterial cell extracts. GST-fusion proteins were purified from bacterial cell extracts using a GST affinity column (Amersham–Pharmacia Biotech). The column was washed extensively with wash buffer (10 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, and 1 mM of 2-ME, pH 7.5) and the proteins were eluted with elution buffer [100 mM Tris–HCl, 150 mM NaCl, and 3 mg/ml reduced glutathione (CalBiochem), pH 7.5]. His-tagged propeptide was purified from bacterial cell extracts under native or denatured conditions using a Ni–NTA affinity column (Qiagen, manufacturer’s instructions). Proteins were eluted with 0.5 M imidazole. Proteins were eluted with 0.5 M imidazole.

Antibody. The anti-human GDF-8 (hGDF-8) propeptide polyclonal antibody was produced in rabbit (Spring Valley Laboratories) by immunization with purified GDF-8 complex.

Construction of expression vectors. The series of truncated human propeptide cDNAs (PCR amplified) were cloned onto XbaI and NotI sites of the pGEX4T3 vector (Amersham–Pharmacia Biotech) for the expression of the GST-propeptide fusion proteins. The XbaI site was previously introduced in pGEX4T3 vector immediately following the NotI site. The human GDF-8 propeptide cDNA (PCR generated 6-His tag in C-terminus) was cloned into NotI site of the pAAS07 vector, a derivative of pGH432 (Advanced Vectors). DTT-treated propeptide. Purified propeptide was treated with or without 1 mM DTT for 1 h (H₂O as mock treatment). The DTT was then removed by repeated centrifugation of the samples using a microfiltration spin column prior to assay for GDF-8 inhibitory activity.

Peptide N-glycosidase F treated CHO-expressed purified hGDF-8 propeptide. Purified hGDF-8 propeptide was treated with peptide N-glycosidase F (PNGase F) (New England Biolabs) at 37°C for 2 h in the presence of 1 x protease inhibitors P1 and P2 (1000 x, P1C1: 1 mg/ml leupeptin, 2 mg/ml antipain, and 10 mg/ml benzamidine, dissolved in 10,000 U/ml aprotinin and P2C2: 1 mg/ml chymostatin and 1 mg/ml pepstatin dissolved in dimethyl sulfoxide).

Luciferase bioassays. The luciferase reporter construct, pGL3-(CAGA)₁₂ [20] with the selectable marker gene, neo, was stably transfected into A204 rhabdomyosarcoma cells. Purified propeptide or GST-propeptide fusion proteins were pre-incubated with 1 mM Myostatin for 20 min at RT prior to adding to the A204 cells. Luciferase activity was measured as described previously [17].

Results

Monomeric propeptide inhibits GDF-8 and does not require N-linked glycosylation for activity

The latent complex of GDF-8 has been shown to consist of two propeptide molecules non-covalently bound to a disulfide-linked GDF-8 dimer [17,18]. Purified propeptide was treated with or without DTT for 1 h. The DTT was then removed by repeated centrifugation of the samples using a microfiltration spin column prior to assay for GDF-8 inhibitory activity. Both DTT-treated and mock-treated GDF-8 propeptide migrate the same distance, indicating that the GDF-8 propeptide does not form intermolecular disulfide bonds between two monomers (Fig. 1A, lanes 1 and 2). Interestingly, disulfide intermolecular bonding is not required for inhibitory activity by propeptide (Fig. 1B). This is different from the TGF-β propeptide, which interacts with TGF-β as a homodimer [21,22].

The difference between the apparent molecular weight (38 kDa) and the predicted molecular weight (28 kDa) of GDF-8 propeptide may be due to glycosylation since its amino acid sequence has a potential N-linked glycosylation site (Asn–X–Ser). In order to determine whether the propeptide contains N-linked glycosylation, both purified GDF-8 propeptide and mature protein were incubated with PNGase F, which specifically cleaves carbohydrate residues from proteins containing N-linked glycosylations. The PNGase-treated propeptide migrated faster than mock-treated propeptide (Fig. 1B). This is different from the TGF-β propeptide, which interacts with TGF-β as a homodimer [21,22].

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activity of propeptide, we expressed the propeptide in a bacterial host, which does not perform N-linked glycosylation. The cDNA encoding human propeptide (amino acid residues 24–266) was subcloned into a bacterial expression plasmid pAA507. The expressed His-tagged protein was purified using Ni–NTA chromatography. The in vitro bioassay (Fig. 3A) showed the bacterially expressed His-tagged human propeptide was functionally equivalent to propeptide expressed in CHO cells. The data indicate that the inhibitory activity of GDF-8 propeptide does not require N-linked glycosylation. In addition, when human propeptide was expressed in bacteria as a GST-propeptide fusion protein, the fusion protein was as active as propeptide isolated from a CHO expression system (Fig. 3B). The GST protein itself does not exhibit inhibitory activity.

Protease inhibitors can prevent GDF-8 propeptide breakdown and block the release of the mature GDF-8

In order to identify and characterize the inhibitory domain of GDF-8 propeptide, we purified propeptide from hGDF-8 secreting CHO cell culture conditioned medium. The GDF-8 latent complex was partially purified about 20-fold employing two ion exchange chromatography steps. Interestingly, we found that degradation of propeptide in the latent complex results in increased activity of GDF-8 measured by the A204 bioassay (Figs. 4A, lane 2 and B). These data suggest that a possible
mechanism for releasing mature GDF-8 from the latent protein complex involves proteolytic cleavage of propeptide. During the purification of human GDF-8 propeptide from CHO conditioned medium, a proteolytic fragment (~21 kDa) was isolated and subjected to N-terminal sequencing analysis. The N-terminus peptide sequence DDSSDG... (amino acid residues 99–105) revealed that this fragment was derived from a cleavage between the amino acid residues 98 and 99. Bioassay results showed that this fragment lacks inhibitory activity (data not shown). Subsequently, protease inhibitors were added to prevent the degradation of propeptide (Fig. 4A, lane 1) during the purification process. The activity of GDF-8 is not detectable when the propeptide in the latent complex is intact (Fig. 4B). In earlier experiments involving RD cells and QM7 cells, we had observed cleavage products derived from proteolytic cleavage between Arg98 and Asp99 of propeptide when we were isolating latent complex (personal communication, T. Ratovitski, data not shown). The consistency of these observations lead us to further explore the minimum essential sequence for inhibitory activity, and also suggested the possible relevance of cleavage at this site in the regulation of Myostatin activity in vivo.

Since our data showed that GST-propeptide fusion protein (amino acid residues 24–266) is functionally equivalent to that of the propeptide expressed in CHO cells, we made two GST fusion proteins, N-9 and C-19, which contained amino acid residues 24–98 and 99–266, of the propeptide, respectively (Fig. 5). GST-propeptide fusion construct C-19 (amino acids 99–266), containing the same C-terminal propeptide region as the 21 kDa fragment from CHO cells, has no inhibitory activity (Fig. 5). GST-propeptide fusion protein, N-9, containing the N-terminal half of propeptide (amino acids 24–98) also lacks inhibitory activity (Fig. 5). The data suggest that cleavage between amino acid residues 98 and 99 inactivates propeptide inhibitory activity.

**The inhibitory domain of propeptide is located in the N-terminus**

Since the GST-propeptide fusion protein expressed in bacteria had full inhibitory activity, a strategy was devised to identify the region critical for this activity. A series of truncated forms of the propeptide were expressed as GST-fusion proteins (Fig. 5). The proteins were purified using GST-affinity chromatography and assayed. The inhibitory activities of C-26 and NC-20 fusion proteins are virtually the same as that of full-length propeptide. Although the specific inhibitory activities of N-12, N-22, NC-10, and NC-8.5 fusion proteins are reduced in comparison with that of full-length propeptide, they still exhibit full inhibitory activity when added in higher molar ratios of fusion protein to mature protein. Both SDS–polyacrylamide gel and immunoblot analyses demonstrate that most propeptide fusion constructs, except for C-19 and C-21 (Fig. 6, lanes 2 and 6), were susceptible to proteolytic cleavage. N-9 appears especially labile and difficult to purify intact. Purified intact N-9 should migrate with a molecular weight of 37 kDa (arrow, Fig. 6). However, the major product purified from the N-9 fusion construct has an apparent molecular weight of 30 kDa (double arrow, Fig. 6). We believe these differences are due to degradation during the expression and isolation processes. Conversely, fusion propeptides containing only C-terminal regions (C-19 and C-21) appear very stable. Our data indicate that the C-terminal domain might play a role in mediating the protein stability. Truncated propeptides containing amino acids from 42 to 115 (N-12, N-22, C-26, NC-20, NC-10, and NC-8.5) exhibit full inhibitory activity (Fig. 5). Elimination of 10 amino acids from either N-terminal or C-terminus of NC-8.5 propeptide region (N-9, NC-8, NC-7.2, or NC-6.7) resulted in the loss of all inhibitory activity. Based on our truncation/deletion analysis of the
propeptide constructs, the region containing amino acid residues 42–115 is critical for inhibitory activity.

**Discussion**

Inactivation of the Myostatin gene, through conventional knockout techniques or a conditional knockout strategy, results in mice with increased skeletal muscle mass [23,24]. Similarly, transgenic mice overexpressing inhibitors (i.e., propeptide and follistatin) capable of binding to Myostatin exhibit increased skeletal muscle mass [18,19]. Administration of a monoclonal antibody against Myostatin to adult mice causes a dose dependent increase in skeletal muscle mass, reduces fat, and improves the efficiency of glucose utilization [25]. Administration of this antibody to **mdx** mice, a mouse model for Duchenne muscular dystrophy [26–28], results in the increase of skeletal muscle mass accompanied by an increase in muscle function [29]. The preponderance of evidence thus suggests that Myostatin plays a role in the regulation of post-natal skeletal muscle development and function. Myostatin has been detected in normal serum as part of a latent complex associated with either the Myostatin propeptide and/or follistatin-related gene (FRLG).
[30]. Recently GASP-1, a previously unknown protein, has been identified to bind not only to Myostatin but also to propeptide [31]. Although the interaction of Myostatin with its prodomain, FLRG, GASP-1, and follistatin, is all described, the details of the regulation of the interaction of Myostatin and its receptor(s) are as yet undefined.

Although it is still unclear how the TGF-β latent complex is activated in vivo, in vitro studies have shown that the propeptide in the latent complex can be cleaved by plasmin, thereby releasing the mature protein [32]. It has been shown that the Myostatin latent complex can be similarly affected by heating or extremes of pH [10]. In the present study, we observed activation of Myostatin in preparations of latent complex exhibiting partial degradation. This observation was consistent with previous observations in RD and QM7 cells. It was the appearance of Myostatin activity in such preparations that lead us to identify regions in the propeptide protein necessary for Myostatin inhibition. The results of this paper define the apparent minimum propeptide region necessary to prevent Myostatin interaction with its receptor(s). Although our analysis of the fragments does not distinguish binding from inhibiting activity, there is a presumption that binding is necessary for inhibition. It is possible that some of the peptides bind non-productively to Myostatin, i.e., bind but not inhibit biological activity. Nevertheless, our data indicate that a region of 73 amino acids near the amino terminus is essential for this inhibitory activity. The carboxy terminus of this region corresponds to the fragment identified from degraded propeptide, suggesting a causal relationship. Clearly, there are critical regions within the 73 amino acid peptide. The regions defined by residues 42–53 as well as the region defined by residues 98–115 must both be present in the same peptide in order to preserve inhibitory activity, since elimination of either of these regions completely abolishes inhibition of Myostatin.

Like TGF-β, Myostatin must be released from the latent complex to interact with its receptors. The factors that regulate or effect this release are presently unknown. It is tempting to speculate that release of Myostatin from the latent complex may be mediated by specific cleavage(s) of the propeptide, and that at least one of the sites responsible for this may be contained within the sequence from residue 98 to 115. This is based upon the data from the cloned fusion proteins, as well as the sequencing of fragments derived from this region in degrading latent complex preparations. The detailed mechanism of where (tissue specificity), and by what enzyme(s), this cleavage occurs remains unknown.

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References


