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Regulation of myostatin *in vivo* by GASP-1: a novel protein with protease inhibitor and follistatin domains

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ABSTRACT

Myostatin, a member of the TGF−β superfamily, is a potent and specific negative regulator of skeletal muscle mass. In serum, myostatin circulates as part of a latent complex containing myostatin propeptide and/or follistatin-related gene (FLRG). Here, we report the identification of an additional protein associated with endogenous myostatin in normal mouse and human serum, discovered by affinity purification and mass spectrometry. This protein, that we have named GDF-associated serum protein-1 (GASP-1), contains multiple domains associated with protease inhibitory proteins, including a WAP domain, a Kazal domain, two Kunitz domains, and a netrin domain. GASP-1 also contains a domain homologous to the 10-cysteine repeat found in follistatin, a protein that binds and inhibits activin, another member of the TGF−β superfamily. We have cloned mouse GASP-1 and shown that it inhibits the biological activity of mature myostatin, but not activin, in a luciferase reporter gene assay. Surprisingly, recombinant GASP-1 binds directly not only to mature myostatin, but also to the myostatin propeptide. Thus, GASP-1 represents a novel class of inhibitory TGF−β binding proteins.

INTRODUCTION

Myostatin (also called growth and differentiation factor-8, GDF-8) was recently identified as a member of the TGF−β superfamily that acts as a potent and specific negative regulator of skeletal muscle mass. Homozygous disruption of the myostatin gene in mice results in a significant increase in skeletal muscle mass and a reduction in body fat (1-3). Naturally occurring myostatin mutations in cattle display a similar double-muscle phenotype, confirming a role for myostatin in muscle development and regulation (4-6).

Myostatin appears to regulate muscle mass, at least in part, by controlling the proliferation of myoblasts, inducing cell cycle arrest in the G_1-phase (7, 8). Some studies have suggested that myostatin may also negatively regulate the differentiation of myoblasts into myotubes by reducing the expression of the muscle-specific transcription factor, MyoD (9, 10). The regulation of muscle by myostatin is not merely a developmental phenomenon. In adult mice, systemic overexpression of myostatin leads to muscle wasting (11). Furthermore, changes in muscle mass due to disease or misuse are often reflected by changes in myostatin expression (12-15).

Like other members of the TGF−β superfamily, myostatin is a secreted protein that is produced from a precursor protein by proteolytic processing. Proteolysis at an internal dibasic site by a furin-type protease results in two polypeptide products, the N-terminal myostatin propeptide and the C-terminal fragment that is the mature myostatin ligand (1, 16-18). Like TGF−β, dimers of the myostatin propeptide and mature myostatin remain non-covalently associated after cleavage, producing a latent complex in which the myostatin ligand is incapable of binding to its receptor (1, 18, 19). In fact, the majority of native myostatin circulating in

serum is associated with its propeptide, forming what is known as the small latent complex (20, 21).

To produce a signal *in vivo*, the small latent myostatin complex must be activated by removal of the propeptide moiety. In the case of TGF−β, which forms an analogous small latent complex, activation can occur via both physiochemical and proteolytic methods (reviewed in (21)). *In vitro*, the small latent TGF−β complex is activated by treatment with acid or heat, presumably reflecting a difference in the relative stability of mature TGF−β and the TGF−β propeptide (22, 23). Protease treatment with plasmin or cathepsin D also activates TGF−β by cleaving the TGF−β propeptide and unmasking mature active TGF−β (24, 25). Despite the prevalence of TGF−β research, the basis for TGF−β activation *in vivo* remains unclear and may occur by multiple mechanisms depending on the cellular context.

Myostatin is very highly related to another TGF−β superfamily member, bone morphogenetic protein-11 (BMP-11) (1, 26). Myostatin and BMP-11 differ by only 11 amino acids in the mature domain, but have different physiological roles, as highlighted by the phenotype of the BMP-11 knockout mouse which displays skeletal defects resulting from abnormal anterior-posterior patterning (27). Within the TGF−β superfamily, myostatin and BMP-11 are closely related to the activin/inhibin-beta subfamily, displaying approximately 40% identity. Unlike myostatin, activin does not bind to its propeptide, but is instead negatively regulated by a family of proteins that contain follistatin (FS)-domains (28-30). As the name implies, FS-domains were originally described as a 10-cysteine repeat found three times in follistatin, a protein that binds to activin and inhibits the activin-mediated release of follicle-stimulating hormone (31-33).

Like the myostatin propeptide, FS-domain proteins bind to certain TGF−β family members and prevent association with cellular receptors (18, 28, 34-36). FS-domain proteins have been categorized into two subfamilies, based on both sequence similarity and their ability to bind activin (37). The first subfamily contains follistatin and follistatin-related gene (FLRG), both of which bind and inhibit activin and myostatin (19, 20, 28, 29). Follistatin and FLRG show strong preferential binding for activin over other TGF−β family members, including TGF−β1, BMP-4, BMP-6, and BMP-7 (38, 39). However, the relative binding affinity of follistatin and FLRG for myostatin has not yet been determined. The second subfamily of FS-domain proteins contains proteins such as agrin, SPARC, hevin, and other proteins associated with the extracellular matrix. Members of this second subfamily have not yet been shown to bind activin or any other TGF−β family protein, despite having as many as nine distinct follistatin domains (33).

To gain insight into the mechanism of myostatin regulation *in vivo*, we set out to define the binding partners of endogenous circulating myostatin. To accomplish this, we have isolated native myostatin from normal serum and analyzed proteins that co-purified by mass spectrometry. Using this approach, we have previously reported that myostatin propeptide and FLRG bind and inhibit myostatin *in vivo* (20). Here, we report the identification and characterization of an additional myostatin binding protein identified in normal mouse and human serum. This protein, referred to here as GDF-associated serum protein-1 (GASP-1), is a novel member of the FS-domain protein family that also contains multiple protease inhibitor domains.

RESULTS

Myostatin binds to a novel protein in mouse and human serum

In order to characterize the major components of the circulating myostatin complex *in vivo*, we have isolated native myostatin and its associated proteins from wild-type mouse serum by affinity purification with an agarose-conjugated anti-myostatin monoclonal antibody, JA16. JA16-bound proteins were subjected to subsequent elution steps with PBS buffer alone (mock elution), a peptide that could compete with myostatin for JA16 binding, and SDS detergent to remove all remaining proteins. These samples were concentrated, run on a one-dimensional SDS-PAGE gel, and visualized by silver stain (Figure 1A). As previously reported, two bands unique to the JA16 purified samples are visible -- a 12 kDa band identified as myostatin, and a 36 kDa band containing both myostatin propeptide and FLRG (20).

To identify any additional myostatin binding proteins that are not visible by silver stain, the area of the silver-stained gel corresponding to molecular weights between 10 and 200 kDa was excised into 15 gel slices for both the mock and JA16 peptide-eluted samples (see Figure 1A). Each gel slice was then subjected to in-gel trypsin digestion and the resulting peptides were identified by nanoflow LC-MS/MS and database searching. These data were analyzed to provide a list of proteins that were found in each of the two samples, based on the presence of at least one sequenced peptide with a high quality MS/MS spectra. Many proteins were identified in both the negative control and JA16-purified samples, thus constituting background binding in the experiment. As expected, these proteins consisted of abundant serum proteins, such as albumin, immunoglobulins, and complement proteins.

We then looked for proteins that were found specifically in the JA16 immunopurified samples and not in the mock-purified negative control. Searches against the National Center for

Biotechnology Information non-redundant (NCBI nr) database from June 2001 identified myostatin, myostatin propeptide, and FLRG, as previously reported, but did not reveal any additional proteins specific to the JA16 samples. There was no evidence of other TGF–β superfamily members, including the highly related protein BMP-11/GDF-11, in the JA16 samples. Thus, the JA16 antibody specifically purified myostatin in these experiments.

These data were analyzed further by comparing the MS/MS spectra collected from the 15 samples to a database of theoretical proteins predicted from the Celera mouse genomic sequence (dated June 2001). This analysis identified a novel protein specific to the JA16-purified sample, hereby referred to as GDF-associated serum protein-1 (GASP-1). Since the initial identification of this protein, this sequence has been added to the NCBI nr database by the public genome sequencing effort as a predicted open reading frame under the accession number gi|20914039. Five peptides derived from the sequence of GASP-1 were identified on the basis of corresponding high-quality MS/MS spectra (Figure 1B, C). Table 1 shows the peptide sequence, charge state (z), and Sequest correlation coefficient (X_{corr}) for each identified peptide. Sequest X_{corr} scores above 2.3 are generally considered significant for 2^+ ions. Fortuitously, one of the peptides identified in our experiments (sequence = ECETDQECETYEK) spans the junction between the two exons that code for this protein, verifying the accuracy of the gene prediction algorithm in this instance. The GASP-1 peptides were found in the boxed region of Figure 1A, which contains proteins between 70 and 80 kDa. However, a specific band corresponding to this protein is not visible on the silver stained gel, probably due to the abundance of background immunoglobulins and albumin at this molecular mass. GASP-1 peptides were identified in the JA16 samples of 4 out of 4 repetitions of this experiment, but were never found in the negative control samples. No additional myostatin-binding proteins were identified in these samples.

GASP-1 contains a follistatin domain and multiple protease inhibitory domains

Based on the Celera predicted sequence, we used PCR to amplify GASP-1 from mouse heart cDNA and cloned the resulting product into a mammalian expression vector encoding a C-terminal V5-His tag. Sequencing confirmed that this clone matched the predicted Celera sequence, with the exception of some base substitutions in wobble codons that did not change the translated amino acid sequence. The cDNA and associated protein sequence of our mouse GASP-1 clone is shown in Figure 2A.

The GASP-1 cDNA encodes a 571 amino acid protein with a predicted molecular mass of 63 kDa. It has a putative signal sequence/cleavage site at its N-terminus and two possible sites for N-glycosylation at amino acids 314 and 514. Analysis of the GASP-1 protein sequence by Pfam and BLAST (40, 41) revealed that GASP-1 contains many conserved domains, including a WAP domain, a follistatin/Kazal domain, an immunoglobulin domain, two tandem Kunitz domains, and a netrin domain (Figure 2B). WAP domains, originally identified in whey acidic protein, contain 8 cysteines that form a four-disulfide core and are often found in proteins with anti-protease activity (42, 43). The C-terminal region of follistatin domains contains a similarity to Kazal serine protease inhibitor domains. Kunitz domains, originally identified in bovine pancreatic trypsin inhibitor, also inhibit serine proteases, thus establishing a likely role for GASP-1 in the regulation of this class of proteins. Furthermore, netrin domains have been implicated in the inhibition of metalloproteases (44, 45). Thus, based on the presence of these conserved regions, GASP-1 is likely to inhibit the activity of proteases.

BLAST searches against the mouse Celera transcript database revealed a protein that has >50% identity with GASP-1, referred to here as GASP-2. GASP-2 contains the same domain

structure as GASP-1, suggesting that these proteins define a two member family of multivalent protease inhibitors (Figure 2C). Interestingly, we only found peptides corresponding to GASP-1, not GASP-2, in our JA16 purified samples. This result suggests that GASP-1 and GASP-2 likely have different biological specificity. Alternatively, GASP-2 may not be present in serum. Both GASP-1 and GASP-2 are conserved in humans (>90% identity with mouse). The sequence for human GASP-1 is now available in the NCBI nr database (accession number gi|18652308). Although, the concentration of myostatin in human serum is considerably lower than that found in mouse serum (20), the sensitivity of mass spectrometric analysis of proteins allowed us to identify 3 peptides corresponding to the human homolog of GASP-1 from JA16 immunoprecipitations from human serum (Table 1). None of these peptides was found in the corresponding negative control. Again, there was no evidence of human GASP-2 in these experiments. Thus the interaction between GASP-1 and myostatin is present in both mouse and human.

Characterization of GASP-1

Myostatin RNA is produced nearly exclusively in skeletal muscle (1). In order to determine the tissue distribution of GASP-1 mRNA, a 551 bp fragment of GASP-1 was amplified from first-strand cDNA produced from a variety of mouse tissues and staged embryos (Figure 3). GASP-1 appears to be fairly widely-expressed, with particularly high expression in skeletal muscle and heart. Significant expression is also seen in brain, lung, and testis. In contrast, liver and kidney express relatively low levels of GASP-1 mRNA. Developmentally, the level of GASP-1 mRNA remains fairly constant, perhaps increasing slightly between day 7 and day 11 of mouse embryogenesis.

To determine the N-terminal sequence of the secreted GASP-1 protein, we transfected COS cells with a mammalian expression vector encoding GASP-1 with a C-terminal V5-His tag (GASP1-V5-His). Serum-free conditioned media was harvested 48 hours later and analyzed by Western blot analysis with an anti-V5 polyclonal antibody. A single band, running at approximately 80 kDa was seen, confirming that recombinant GASP-1 is secreted into the conditioned media (data not shown). Approximately 10 ml of this conditioned media was run over a His-affinity column and further purified by reverse phase chromatography. This purification scheme yielded a band corresponding to the expected size of full-length GASP-1 on a Coomassie stained SDS-PAGE gel. Edman sequencing of this band determined an N-terminal sequence of L-P-P-I-R-Y-S-H-A-G-I. Thus amino acids 1-29 of GASP-1 constitute the signal sequence that is removed during processing and secretion.

GASP-1 binds directly to both mature myostatin and myostatin propeptide

To confirm and further characterize the interaction between myostatin and GASP-1, we incubated purified recombinant myostatin and purified recombinant myostatin propeptide with conditioned media from COS1 cells transfected with either a vector control or GASP1-V5-His. We then immunoprecipitated myostatin with JA16-conjugated agarose beads and looked for co-purification of GASP-1 and myostatin propeptide using Western blots (Figure 4, left panel). Both GASP-1 (lane 3) and myostatin propeptide (lane 1) co-immunoprecipitated with myostatin, demonstrating that myostatin can interact with both of these proteins. Neither GASP-1 nor propeptide were detected in JA16 immunoprecipitates in the absence of myostatin (lane 4), eliminating the possibility of non-specific binding in these experiments. When all three proteins were present, both GASP-1 and myostatin propeptide were pulled down with myostatin,

suggesting the possibility that these proteins may form a tertiary complex (lane 5). However, this experiment does not eliminate the possibility that GASP-1 and propeptide are bound to the same epitope on separate myostatin molecules.

To further confirm the interaction between GASP-1 and myostatin, we performed the reverse immunoprecipitation by pulling down GASP-1 from conditioned media supplemented with myostatin and/or myostatin propeptide recombinant protein. To achieve this, we used an agarose-conjugated monoclonal antibody directed against the V5 epitope of the C-terminal V5-His tag on GASP-1. As expected, myostatin co-immunoprecipitated with GASP-1 (Figure 4, right panel, lanes 3 and 5), further confirming a direct interaction between these proteins. Surprisingly, myostatin propeptide also co-purified with GASP-1, even in the absence of myostatin (lane 4), suggesting that myostatin propeptide can bind directly to GASP-1. Thus GASP-1 binds to both myostatin and myostatin propeptide independently. Addition of both myostatin and propeptide consistently showed less propeptide binding to GASP-1 than when propeptide was added alone, suggesting the possibility that mature myostatin may compete with myostatin propeptide for GASP-1 binding. Since both propeptide and GASP-1 are pulled down by anti-myostatin antibodies, further experiments will necessary to determine whether GASP-1 binds effectively to the myostatin small latent complex.

GASP-1 inhibits the activity of myostatin and BMP-11, but not activin or TGF–β1

Two follistatin-domain proteins, follistatin and FLRG, have been previously shown to inhibit myostatin activity in a $(CAGA)_{12}$ luciferase transcriptional reporter assay (11, 20). Both of these proteins also inhibit the biological activity of the related proteins, activin and BMP-11

(20, 26, 28, 29). Since GASP-1 contains a follistatin domain, we tested the ability of GASP-1 to inhibit myostatin, BMP-11, activin, and TGF−β1 activity in the $(CAGA)_{12}$ reporter assay.

Various dilutions of conditioned media from COS cells transfected with V5-His tagged GASP-1 or a vector control were incubated with purified recombinant myostatin, BMP-11, activin, or TGF−β1 and assayed for growth factor activity in rhabdomyosarcoma cells expressing the $(CAGA)_{12}$ reporter construct. GASP-1 inhibited myostatin activity in a concentration dependent manner (Figure 5A). GASP-1 similarly inhibited the activity of BMP-11 in this assay (Figure 5B), as might be expected since mature myostatin and BMP-11 are highly conserved and differ by only 11 amino acids. Surprisingly, GASP-1 did not inhibit the activity of activin or TGF−β1 (Figure 5C, D), suggesting a level of specificity not yet seen in other follistatin-domain proteins. Thus, GASP-1 exhibits specificity in its inhibition of myostatin and BMP-11. To estimate the potency of GASP-1 inhibition of myostatin activity, we purified a small amount of GASP-1 and determined the IC50 for the inhibition of myostatin in the reporter gene assay. As can be seen in Figure 6, GASP-1 inhibits 20 ng/ml myostatin with an IC50 of approximately 3 nM.

DISCUSSION

In this paper, we have used affinity purification and mass spectrometry to identify a novel endogenous myostatin binding protein that we have named GDF-associated serum protein-1 (GASP-1). GASP-1 is associated with myostatin in normal mouse and human serum and can inhibit activity resulting from 20 ng/ml myostatin in a reporter gene assay with an IC50 of ~3 nM. The myostatin/GASP-1 interaction was identified in a pool of normal mouse and human serum, suggesting that this interaction occurs under physiological conditions. Previously, we

have shown that myostatin propeptide and FLRG also bind and inhibit myostatin in serum (20). Thus nature has provided three structurally distinct proteins to regulate circulating myostatin, emphasizing the biological importance of tight and specific regulation of this growth factor. These three proteins may form distinct multicomponent myostatin complexes that play unique roles in the regulation of skeletal muscle.

The possibility remains that additional proteins bind and regulate myostatin in serum. Although we did not identify any additional myostatin binding proteins in these experiments, we are limited to interacting proteins that do not mask the JA16 epitope, that are present in the protein databases, and that bind myostatin with a sufficiently low dissociation rate to withstand the washing steps. In addition, our experiments are biased against extremely high abundance serum proteins, since these proteins tend to be identified in the negative controls. Since mass spectrometry is not inherently quantitative, we are not able to determine whether a given protein is present at a higher level in the JA16 samples than in the control. Rather, we are limited to determining whether a protein is 'present' or 'absent'. Intriguingly, α2-macroglobulin has been shown to bind activin and inhibin, possibly playing a role in delivery or clearance (46, 47). In fact, we did identify α2-macroglobulin in both the mock-conjugated and JA16-conjugated peptide-eluted samples by mass spectrometry. However, since this protein was present in the negative control, we are unable to determine whether α2-macroglobulin binds myostatin by these experiments.

GASP-1 is a fairly widely expressed protein that contains a follistatin (FS)-domain and multiple protease inhibitory domains. The expression of GASP-1 is relatively high in skeletal muscle, suggesting that GASP-1 may bind to myostatin during, or shortly after secretion. However, whereas myostatin expression is generally limited to the skeletal muscle (1), GASP-1

is also produced in many other tissues and throughout development. This pool of GASP-1 may be released into circulation where it can bind to myostatin. Alternatively, GASP-1 may also act as an inhibitor of other growth factors including BMP-11. In fact, we have shown that BMP-11, which is known to play an important role in embryonic development (26, 27), can be inhibited by GASP-1.

The follistatin domain of GASP-1 may be responsible for mediating the interaction with myostatin. Two other FS-domain proteins, follistatin and FLRG, also bind and inhibit myostatin, presumably by preventing it from associating with its cellular receptor (11, 19, 20). FLRG has been shown to bind to circulating myostatin under physiological conditions, confirming a physiological role for FLRG in regulating myostatin activity (20).

FS and FLRG comprise a subfamily of FS-domain proteins that share a common N-terminal domain and high binding affinity for activin (37). Follistatin requires this 63 amino acid N-terminal domain, in particular some hydrophobic residues, to bind and inhibit activin (48, 49). Although GASP-1 has an N-terminal domain of the same size as follistatin, the two domains are not highly related, showing only 7% identity at the amino acid level. In contrast, the N-terminal domain of FLRG is over 30% identical to that of follistatin. Intriguingly, the N-terminal domain of FLRG does not appear to be required for activin binding, since a GST fusion protein containing the second FS-domain and the short acidic C-terminal tail is sufficient to bind activin (29). Since GASP-1 lacks significant similarity to follistatin and FLRG in this N-terminal domain, GASP-1 appears to fall within the second subfamily of FS-domain proteins. Members of this second subfamily are not generally thought to inhibit activin or other TGF–β family members (37). Thus, GASP-1 is the first example of a member of this second subfamily that can inhibit a TGF–β superfamily member. In addition, GASP-1 is the first example of an FS-domain

protein that can bind and inhibit a specific member of the TGF−β superfamily without also inhibiting activin. This selectivity may result from regions outside of the follistatin domain of GASP-1 that facilitate binding to myostatin. Alternatively, GASP-1 may bind to myostatin in a manner independent of its FS domain. It is interesting to note that GASP-1 shows the same selectivity as the myostatin propeptide, which does not contain an FS domain, but also inhibits myostatin and BMP-11, not activin (18).

Based on our *in vitro* binding results, GASP-1 may also regulate myostatin activity by a mechanism independent of the FS domain. Surprisingly, myostatin propeptide co-immunoprecipitates with GASP-1, even in the absence of mature myostatin. This is in contrast to the myostatin-FLRG interaction that is mediated entirely by mature myostatin (20). Furthermore, our data suggests that mature myostatin may compete with myostatin propeptide for binding to GASP-1. Thus, GASP-1 interacts independently with both mature myostatin and myostatin propeptide. We have shown that GASP-1 inhibits the biological activity of mature myostatin, but a role for GASP-1 in the regulation of myostatin propeptide is less clear.

Intriguingly, GASP-1 contains multiple protease inhibitory domains. Two tandem Kunitz domains show a very high degree of similarity to inter-alpha-trypsin inhibitor, indicating that these are almost certainly functional serine protease inhibitor domains. GASP-1 may also inhibit metalloproteases, based on the presence of a netrin domain at the C-terminus (44, 45). In addition, the FS-domain of GASP-1 is similar to Kazal serine protease inhibitory domains. All FS-domains contain two subdomains that can be distinguished based on their pattern of disulfide bond linkages and three dimensional structure (50). The N-terminal region of the FS domain encompasses the first 4 cysteine residues and displays structural similarity to epidermal growth factor (EGF). The C-terminal region, including cysteines 5-10, is tellingly similar to Kazal

serine protease inhibitory domains. Many FS-domain proteins, including the three FS domains in follistatin and the single FS domain in SPARC, contain a hydrophobic insertion into the region between cysteines 6 and 7, corresponding to the inhibitory loop of the Kazal protease inhibitor, ovomucoid (50). It has been proposed that this insertion may explain the lack of demonstrated protease activity in these proteins (50). Interestingly, GASP-1 does not contain an insertion in this region, opening the possibility that this domain may act as a functional Kazal-like serine protease inhibitor. Thus, based on the presence of conserved domains, GASP-1 may act as a multi-functional protease inhibitor with activity against both serine proteases and metalloproteases.

The biological significance of the protease inhibitory domains in GASP-1 remains unclear, but we can speculate that they may play a role in one of the regulatory processes thought to involve proteases: (1) the activation of latent myostatin complex, or (2) the internal processing of the myostatin proprotein to release myostatin propeptide and mature myostatin.

TGF−β is produced by proteolytic cleavage of a pro-protein at a furin cleavage site. This processing results in the production of two polypeptide chains, the TGF−β propeptide and the mature TGF−β ligand. After processing, these two protein products remain non-covalently associated and TGF−β is secreted as a small latent complex consisting of two mature TGF−β molecules and two TGF−β propeptide molecules. Although there is considerable interest in the regulation of TGF−β during development and homeostasis, it remains unclear how the small latent complex is activated *in vivo*. *In vitro*, the serine protease plasmin can cleave the propeptide of active TGF−β at an internal site, resulting in the release of the non-covalently bound propeptide moiety and the subsequent activation of the small latent complex (25). If the

small latent myostatin complex is also activated by proteolysis of the propeptide, GASP-1 may inhibit this process and serve to keep myostatin in a latent, inactive form.

Alternatively, GASP-1 could prevent the processing of the myostatin proprotein through inhibition of a furin-like protease, presumably resulting in an inactive unprocessed myostatin molecule. Treatment of C_2C_{12} myoblasts with hydroxamate-based chemical inhibitors of metalloproteases induces an increase in cell fusion leading to hypertrophy (51). This treatment also leads to an increase in unprocessed myostatin in C_2C_{12} lysates of these cells, suggesting that the hypertrophy may be the result of inhibiting the proteolytic processing of myostatin. In fact, we see evidence of unprocessed myostatin in our JA16 immunoprecipitations from normal serum (J.J. Hill, unpublished observations, see 48 kDa band in Figure 1), suggesting that regulation of secreted myostatin activity may occur partially through the regulation of furin-mediated myostatin processing.

In this paper, we have identified a novel protein, GASP-1, that interacts with myostatin in normal mouse and human serum. GASP-1 inhibits myostatin activity in a reporter gene assay, suggesting that it acts as a negative regulator of myostatin action. Surprisingly, GASP-1 binds independently to both mature myostatin and myostatin propeptide. This finding, in addition to the observation that GASP-1 contains numerous putative protease inhibitory domains, suggests that GASP-1 may regulate myostatin activity by an additional mechanism, not associated with the presence of a FS-domain and thus may constitute a novel class of inhibitory TGF−β binding proteins.

MATERIALS AND METHODS

Antibodies and conditioned media

JA16 monoclonal anti-myostatin antibody and the procedure used to produce JA-16 conjugated agarose beads have been previously described, as have the polyclonal anti-myostatin and anti-myostatin propeptide antibodies used for Western blots (20). Anti-V5 antibodies were purchased from Sigma. Immunoprecipitations were performed with clone V5-10 agarose conjugate and Western blots utilized an anti-V5 polyclonal rabbit antibody (catalog number V8137). Conditioned media was collected 48 hours after transfection of COS1 cells with GASP1-V5-His/pcDNA3.1D-V5-His-TOPO or empty vector using the FuGENE 6 reagent (Roche) in serum-free Dulbecco's modified Eagle's medium.

Immunoprecipitations and mass spectrometry

JA16 immunoprecipitations from mouse and human serum were performed essentially as described (20), but were scaled up approximately five-fold by adding 8 µl of 4x LDS sample buffer to the 100 µl eluant and reducing this volume to less than 30 µl by evaporation. All samples were supplemented with water up to a final volume of 35 µl and analyzed as previously described. Briefly, reduced and iodoacetamide-alkylated samples were separated by SDS-PAGE (NuPage, 10% Bis-Tris, Invitrogen) and silver stained. Each gel lane was sliced into 13-15 pieces and subjected to in-gel trypsin digestion. The resulting peptides were analyzed by nanoflow reverse phase LC-MS/MS on an LCQ Deca XP ion trap mass spectrometer as previously described (20). Peptide sequences were determined from MS/MS spectra by database searching using the Sequest algorithm and confirmed by manual inspection.

For immunoprecipitations with recombinant proteins, 400 µl conditioned media from vector- or GASP1-transfected cells was combined with 1.2 µg of recombinant purified myostatin and/or myostatin propeptide protein (18). JA16 (10 µl packed volume) or anti-V5 (30 µl) conjugated agarose beads were incubated with the supplemented conditioned media for two hours at 4 ^{o}C and washed twice in cold 1% Triton in phosphate-buffered saline (PBS) and twice in PBS. Beads were resuspended in 50 µl 1x LDS buffer with DTT. Western blots were performed as previously described (20).

Molecular biology

The GASP-1 coding sequence was amplified from mouse heart QUICKCLONE cDNA (Clontech) by PCR with PfuTurbo polymerase (Stratagene) (forward primer: 5' CACCATGTGTGCCCCAGGGTATCATCGGTTCTGG 3'; reverse primer: 5' TTGCAAGCCCAGGAAGTCCTTGAGGAC 3'). The PCR product from this reaction ran as a single major band of approximately 1700 base pairs on a 1 % agarose gel. The amplified cDNA was then cloned into the TOPO sites of the pcDNA3.1D/V5-His-TOPO vector (Invitrogen) so as to include an in-frame C-terminal V5-His tag according to manufacturers' recommendations. The full-length cDNA insert was sequenced on both strands to produce the sequence given in Figure 2.

Tissue expression profiling was performed using PCR to amplify a 551 bp fragment of GASP-1 from normalized mouse first strand cDNA panels (Clontech, Palo Alto CA) using the Advantage cDNA PCR Kit (Clontech) according to the manufacturer's recommendations (forward primer: 5' TTGGCCACTGCCACCACAATCTCAACCACTT 3'; reverse primer: 5' TCTCAGCATGGCCATGCCGCCGTCGA 3'). Aliquots of the PCR reaction were removed

after 23 cycles (G3PDH control only), 34, and 38 cycles (GASP-1 samples) and visualized by ethidium bromide staining of a 1% agarose gel.

GASP-1-His purification/N-terminal sequencing

GASP1-V5-His (10 mL) was purified from conditioned media using a cobalt affinity column (Clontech). Proteins were eluted with a linear gradient from 0 to 500 mM imidazole in 50 mM Tris, 1 M NaCl, pH 8.0, followed by reversed-phase HPLC on a 2 cm LC-304 C4 cartridge (Supelco) using an acetonitrile gradient in 0.1% trifluoroacetic acid. Fractions containing GASP-1 were concentrated by evaporation, separated by SDS-PAGE, transferred to a polyvinylidene fluoride membrane, and visualized with Coomassie blue. A major band corresponding to the expected size of full-length GASP-1 (~80 kDa) was excised and sequenced by Edman degradation on an ABI 492 sequencer (ABI, Foster City, CA). GASP1-V5-His protein utilized in the luciferase reporter gene assay (Figure 6), was purified from conditioned media on a cobalt affinity column and eluted as above. Fractions containing GASP-1 were further purified by size exclusion chromatography in PBS using a BioSepS3000 column (Phenomenex).

Reporter gene assay

A luciferase reporter construct, pGL3-$(CAGA)_{12}$ (52) was transiently transfected into A204 or RD rhabdomyosarcoma cells. Dilutions of conditioned media from vector or GASP-1 transfected cells were incubated for 30 minutes at 37 ^{o}C with 10 ng/ml myostatin, 10 ng/ml BMP-11, 10 ng/ml rh activin A (R&D Systems), or 0.5 ng/ml rh TGF−β1 (R&D Systems). Luciferase activity was measured as described previously (11, 18). In this assay, A204 cells

respond to myostatin, BMP-11, and activin, but do not respond well to TGF−β1. RD cells respond to both myostatin and TGF−β1. Thus, we used A204 cells to test for the ability of GASP-1 to inhibit myostatin, BMP-11, and activin and RD cells to monitor the activity of TGF−β and myostatin. A standard curve measuring the concentration dependence of the luciferase activity induced by each of these growth factors was generated for each experiment (data not shown). The growth factor concentrations used fall in the linear region of this curve such that small changes in growth factor concentration result in measurable changes in luciferase activity.

FIGURE LEGENDS

Figure 1 - A novel protein with multiple protease inhibitor domains is bound to myostatin in serum.

(A) Proteins were isolated from normal mouse serum using mock-conjugated (labeled '0') or JA16-conjugated agarose beads. Subsequent elutions with buffer ('mock elute'), a competing peptide, and SDS were separated by SDS-PAGE and visualized by silver stain. The gel was dissected into 15 slices from the peptide-eluted sample of both negative control and JA16 immunoprecipitates. The proteins in each slice were digested with trypsin and identified using nanoflow-LC-MS/MS. Proteins unique to the JA16 sample included mature myostatin (*lower arrow*), myostatin propeptide (*upper arrow*), FLRG (*upper arrow*), and a novel multivalent protease inhibitor named here as GDF-associated serum protein 1, GASP-1 (*boxed*).

(B, C) Representative MS/MS spectra from two GASP-1 peptides, showing both N-terminal (b) and C-terminal (y) fragment ions.

Figure 2 - GASP-1 is a multivalent protease inhibitor.

(A) cDNA and protein sequence of mouse GASP-1. The peptides identified by mass spectrometry in JA16 affinity purified samples are underlined.

(B) The domain structure of GASP-1. GASP-1 has a signal sequence/cleavage site after amino acid 29. In addition, GASP-1 contains two Kunitz/BPTI serine protease inhibitor domains, a follistatin domain (including a Kazal serine protease inhibitor motif) and a netrin domain.

(C) Phylogenetic tree of GASP-1 and -2 predicted from the mouse and human genomic sequences. Mouse and human GASP-1 are 90% identical. GASP-1 and GASP-2 are 54% identical.

Figure 3 - GASP-1 mRNA is expressed in many tissues and throughout development.

Tissue expression profiles of mouse GASP-1. A 551 bp fragment of GASP-1 was amplified from normalized first-strand cDNA panels from Clontech (Palo Alto, CA). A portion of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was amplified as a control. G3PDH expression is known to be high in skeletal muscle and low in testis. The cDNA panels were normalized against β-actin, phospholipase A2, and ribosomal protein S29, in addition to G3PDH.

Figure 4 - GASP-1 binds separately to both myostatin and myostatin propeptide.

Left panel: JA16 was used to immunoprecipitate myostatin from mock- or GASP-1-V5-His transfected COS cell conditioned media supplemented with recombinant purified myostatin and/or propeptide. Western blots with anti-V5 (top panel), anti-myostatin (middle panel), or anti-myostatin propeptide polyclonal antibodies were used to determine whether these proteins were present in the immunoprecipitate. *Right panel*: GASP-1 protein was immunoprecipited by anti-V5 tag antibodies from mock- or GASP-1-V5-His conditioned media supplemented with recombinant purified myostatin and/or propeptide. The immunoprecipitate was analyzed by Western blotting as above.

Figure 5- GASP-1 inhibits the biological activity of myostatin and the highly related BMP-11, but not activin or TGF-β. Various dilutions of conditioned media from mock (open circles) or GASP-1-V5-His (filled squares) were incubated with (A) 10 ng/ml myostatin, (B) 10 ng/ml BMP-11, (C) 10 ng/ml activin, or (D) 0.5 ng/ml TGF−β. These samples were then subjected to a luciferase reporter activity assay in A204 (A-C) or RD (D) cells to determine the activity of the added growth factors. Luciferase activity is shown in relative luciferase units (RLU). The activity resulting from each of the growth factors alone is shown by the filled diamonds and short dashed line. Without addition of any growth factor, the background activity in the assay is low, as shown by the long dashed line with error bars but no symbol.

Figure 6- GASP-1 inhibits myostatin with an IC50 of 3 nM. Purified GASP-1 was tested for its ability to inhibit 20 ng/ml myostatin in the $(CAGA)_{12}$ luciferase reporter assay in RD cells (filled squares). The activity resulting from myostatin alone is shown by the filled diamonds and short dashed line. The activity present when no growth factors are added is shown by the long dashed line.

TABLE

Table 1: GASP-1 peptides identified in JA16 immunoprecipitates from mouse & human serum

mouse serum	z	X_{corr}
(R)VSELTEEQDSGR	2+	3.88
(R)ECETDQECETYEK	2+	2.98
(R)EACEESCPFPR	2+	2.95
(R)SDFVILGR	2+	2.73
(R)ADFPLSVVR	2+	2.56
human serum	**z**	**X_{corr}**
(R)CYMDAEACSK	2+	2.69
(R)VSELTEEPDSGR	2+	2.44
(K)GITLAVVTCR	2+	2.42

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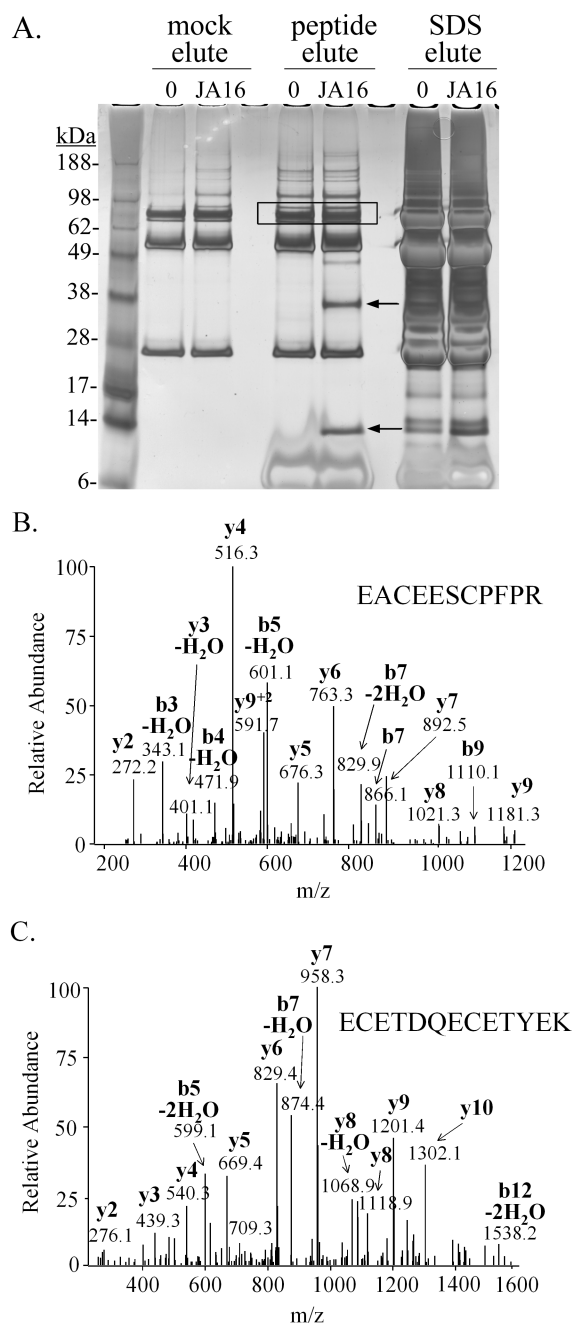
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591.7
y2
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y5
676.3
b7
829.9
b9
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471.9
866.1
y8
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y9
1181.3
401.1
Relative Abundance
m/z
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b7 -H2O
y6
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b5 -2H2O
599.1
874.4
y9
1201.4
y10
1302.1
y8 -H2O
y5
669.4
y8
1068.9
y4
540.3
1118.9
y3
439.3
b12 -2H2O
1538.2
y2
276.1
709.3
Relative Abundance
m/z

Figure 1

A.

```
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1   ATGTGTGCCCCAGGG TATCATCGGTTCTGG TTTCACTGGGGGCTG CTGTTGCTGCTGCTC CTCGAGGCTCCCCTT
                R  G  L  A  L   P  P  I  R  Y   S  H  A  G  I   C  P  N  D  M   N  P  N  L  W
76  CGAGGCCTAGCACTG CCACCCATCCGATAC TCCCATGCGGGCATC TGCCCCAACGACATG AACCCCAACCTCTGG
                V  D  A  Q  S   T  C  K  R  E   C  E  T  D  Q   E  C  E  T  Y   E  K  C  C  P
151 GTGGATGCCCAGAGC ACCTGCAAGCGAGAG TGTGAAACAGACCAG GAATGTGAGACCTAT GAGAAATGCTGCCCC
                N  V  C  G  T   K  S  C  V  A   A  R  Y  M  D   V  K  G  K  K   G  P  V  G  M
226 AATGTGTGTGGGACC AAGAGCTGTGTGGCA GCCCGCTACATGGAT GTGAAAGGGAAGAAG GGGCCTGTAGGCATG
                P  K  E  A  T   C  D  H  F  M   C  L  Q  Q  G   S  E  C  D  I   W  D  G  Q  P
301 CCCAAGGAGGCCACA TGTGACCATTTCATG TGCCTGCAGCAGGGC TCTGAGTGTGACATC TGGGACGGCCAGCCC
                V  C  K  C  K   D  R  C  E  K   E  P  S  F  T   C  A  S  D  G   L  T  Y  Y  N
376 GTGTGTAAGTGCAAA GATCGCTGTGAGAAG GAGCCCAGCTTCACC TGTGCCTCTGATGGC CTTACCTACTACAAC
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451 CGTTGCTTCATGGAC GCCGAAGCCTGCTCC AAGGGCATCACACTG TCTGTGGTCACCTGT CGTTATCACTTCACC
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526 TGGCCTAACACCAGC CCTCCACCGCCTGAG ACCACGGTGCATCCC ACCACCGCCTCTCCG GAGACTCTCGGGCTG
                D  M  A  A  P   A  L  L  N  H   P  V  H  Q  S   V  T  V  G  E   T  V  S  F  L
601 GACATGGCAGCCCCA GCCCTGCTCAACCAC CCTGTCCATCAGTCA GTCACCGTGGGTGAG ACTGTGAGTTTCCTC
                C  D  V  G  R   P  R  P  E  L   T  W  E  K  Q   L  E  D  R  E   N  V  V  M
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                R  P  N  H  V   R  G  N  V  V   V  T  N  I  A   Q  L  V  I  Y   N  V  Q  P  Q
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                D  A  G  I  Y   T  C  T  A  R   N  V  A  G  V   L  R  A  D  F   P  L  S  V  V
826 GATGCTGGCATATAC ACCTGTACAGCTCGA AATGTCGCTGGTGTC CTGAGGGCTGACTTC CCGTTGTCGGTGGTC
                R  G  G  Q  A   R  A  T  S  E   S  S  L  N  G   T  A  F  P  A   T  E  C  L  K
901 AGGGGTGGTCAGGCC AGGGCCACTTCAGAG AGCAGTCTCAATGGC ACAGCTTTTCCAGCA ACAGAGTGCCTGAAG
                P  P  D  S  E   D  C  G  E  E   Q  T  R  W  H   F  D  A  Q  A   N  N  C  L  T
976 CCCCCAGACAGTGAG GACTGTGGAGAGGAG CAGACACGCTGGCAC TTCGACGCCCAGGCT AACAACTGCCTCACT
                F  T  F  G  H   C  H  H  N  L   N  H  F  E  T   Y  E  A  C  M   L  A  C  M  S
1051 TTCACCTTTGGCCAC TGCCACCACAATCTC AACCACTTTGAGACC TACGAGGCCTGTATG CTGGCTTGTATGAGT
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1126 GGGCCATTGGCCACC TGCAGCCTGCCTGCC CTGCAAGGGCCTTGC AAAGCTTATGTCCCA CGCTGGGCCTACAAC
                S  Q  T  G  L   C  Q  S  F  V   Y  G  G  C  E   G  N  G  N  N   F  E  S  R  E
1201 AGCCAGACAGGCCTA TGCCAGTCCTTCGTC TATGGCGGCTGTGAG GGCAACGGTAACAAC TTTGAAAGCCGTGAG
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1426 GCCCTGGTGACCGTG GATGAGGTCTTAAAA GATGAGAAGATGGGC CTCAAGTTTCTGGGC CGGGAGCCTCTGGAA
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1576 GGGGAGGTGGACGGC GGCATGGCCATGCTG AGACCCGATAGCTTT GTGGGGGCATCGAGC ACACGGCGGGTCAGG
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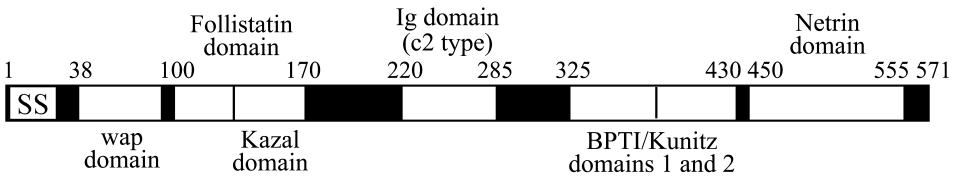
B.



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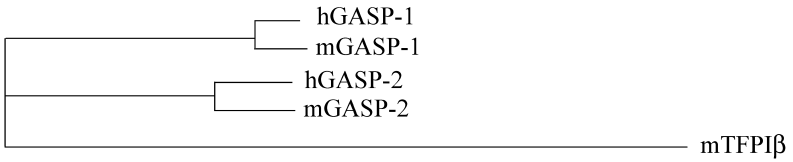
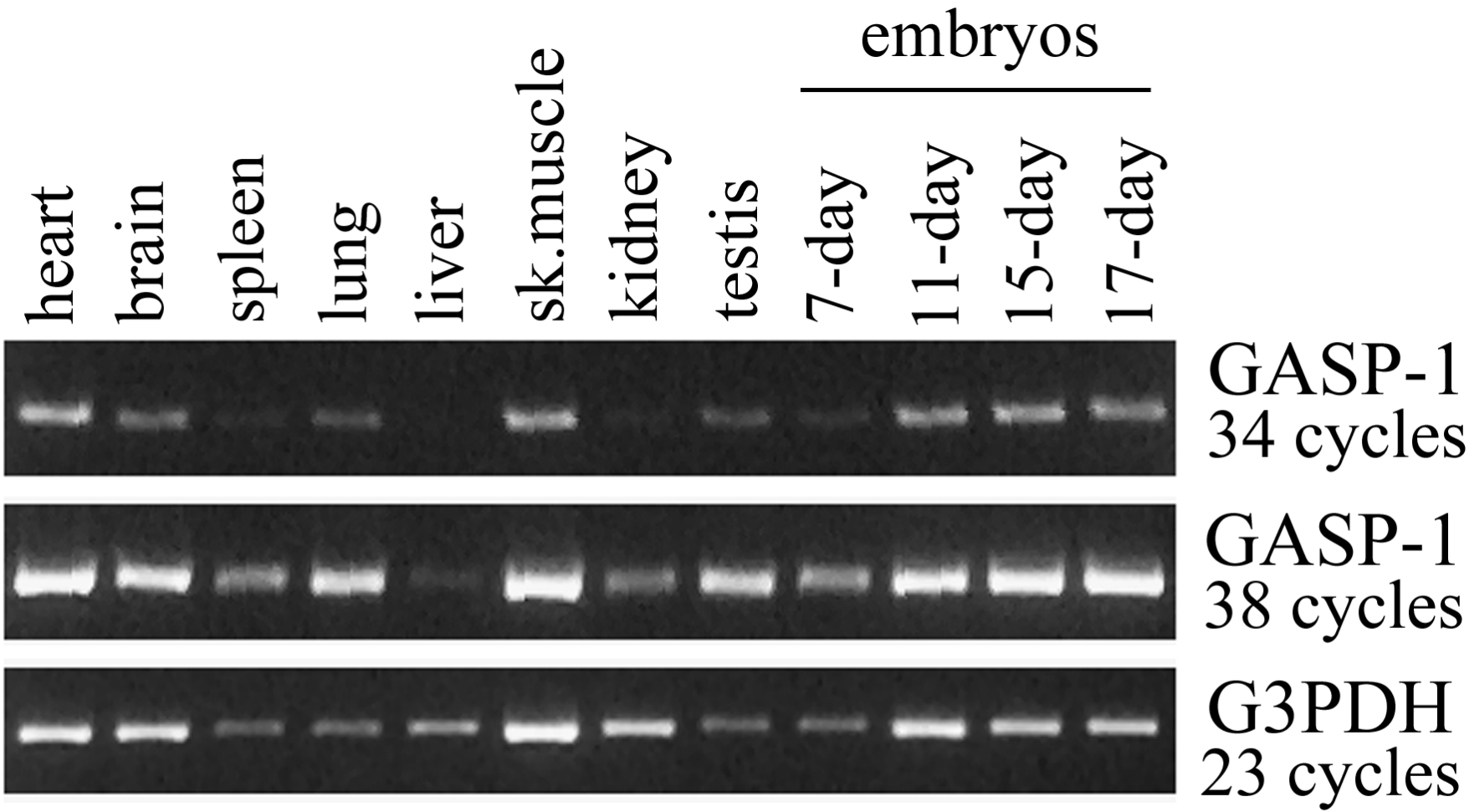


Figure 2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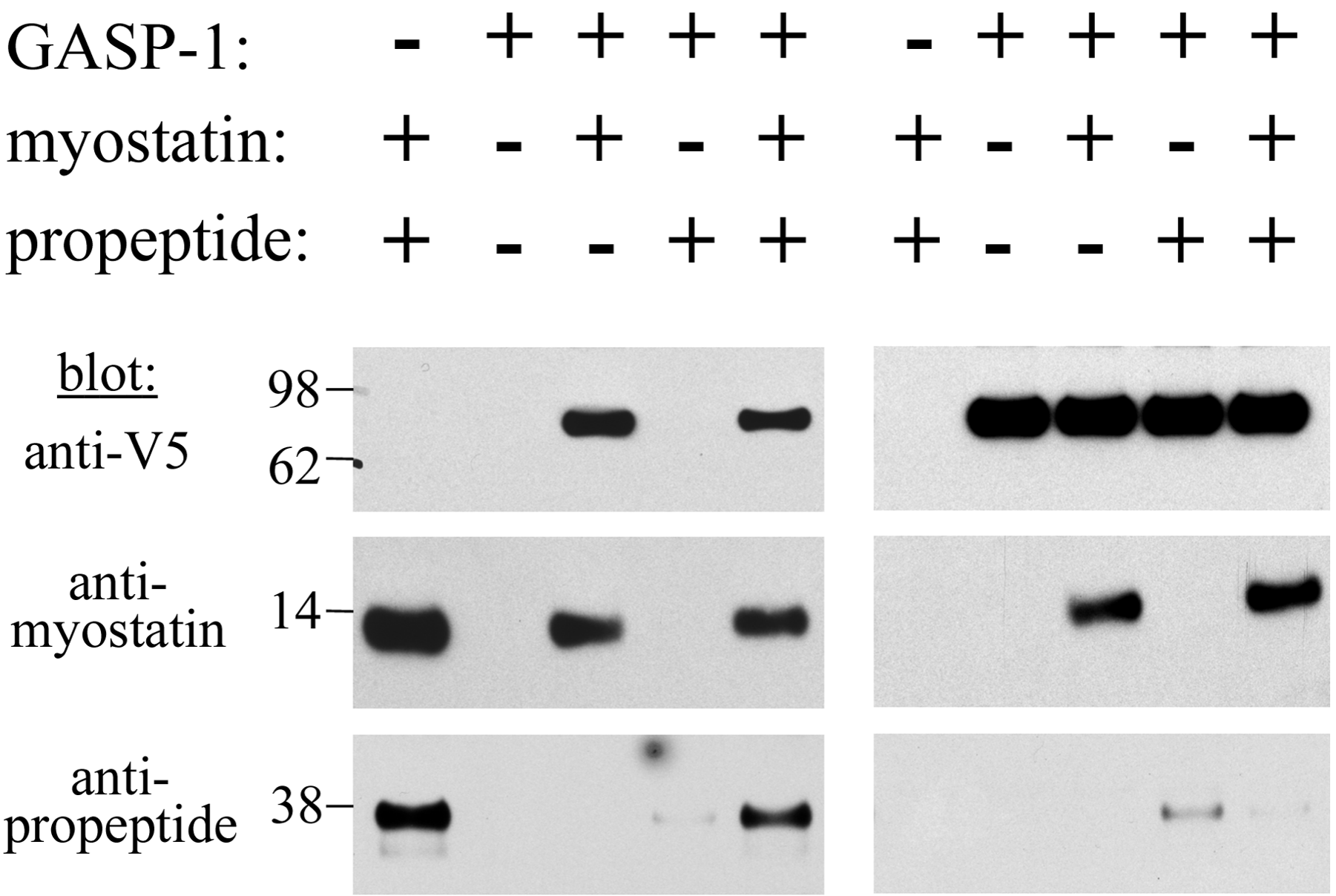
23 cycles

Figure 3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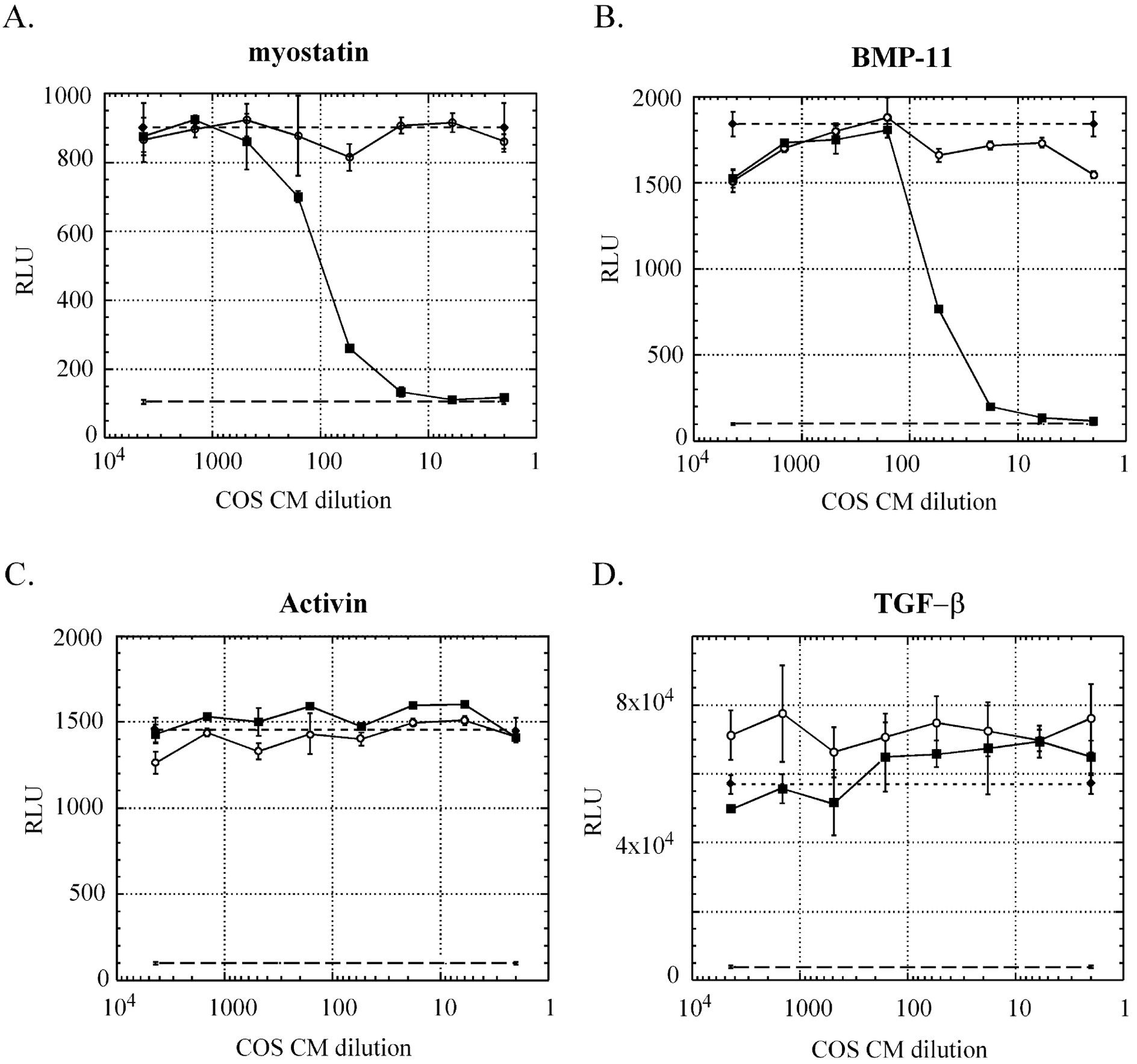
anti-propeptide
38

Figure 4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myostatin
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COS CM dilution
B.
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2000
1500
1000
500
0
RLU
10^4
1000
100
10
1
COS CM dilution
C.
Activin
2000
1500
1000
500
0
RLU
10^4
1000
100
10
1
COS CM dilution
D.
TGF–β
8x10^4
4x10^4
0
RLU
10^4
1000
100
10
1
COS CM dilution

Figure 5

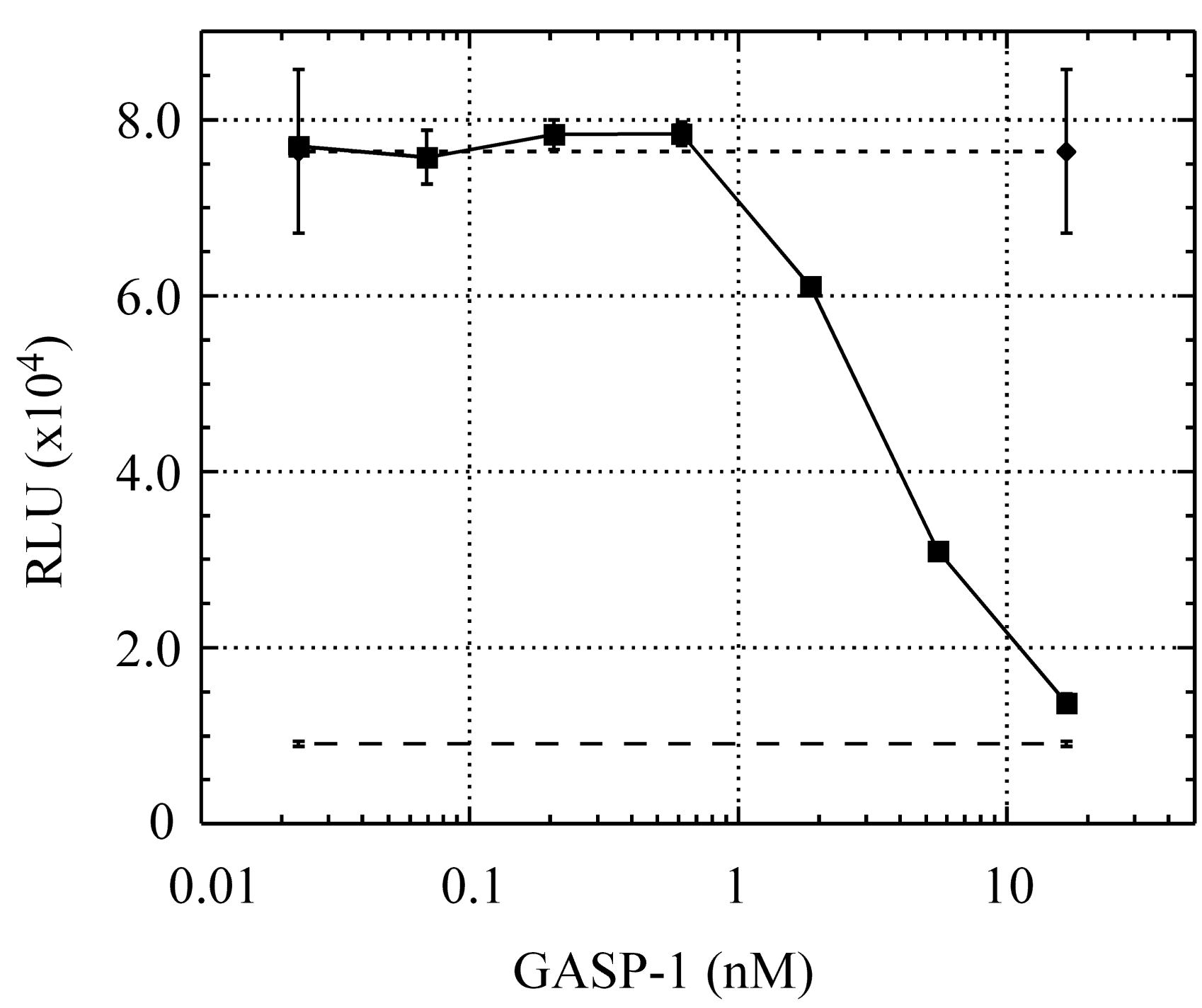

Figure 6