

Aprotinin, a Kunitz-type protease inhibitor, stimulates skeletal muscle differentiation

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SUMMARY

Aprotinin, a Kunitz-type inhibitor of serine proteases, stimulates myotube formation by mouse G8-1 and C₂C₁₂ skeletal muscle myoblasts. This stimulation of morphological differentiation is accompanied by accumulation of myogenin transcripts and production of muscle-specific proteins. In contrast, active TGF β prevents differentiation of G8-1 and C₂C₁₂ myoblasts. When active TGF β and aprotinin are both added to myoblast cultures, differentiation is inhibited, suggesting the active growth factor acts downstream of the protease inhibitor. TGF β is found in serum as a latent, dimeric propeptide that is cleaved by limited proteolysis to release the biologically active carboxy-terminal dimer. To address the possibility that

aprotinin may effect myogenesis by inhibiting proteolytic activation of latent TGF β , levels of the endogenous growth factor were measured in differentiating myoblast cultures. Latent TGF β is rapidly depleted from control cultures within 24 hours of plating, but remains relatively stable in aprotinin-treated cultures. Consistent with this, aprotinin-treated cultures have reduced levels of active TGF β . These data indicate that Kunitz-domain containing protease inhibitors may help orchestrate the onset of myogenesis, possibly by regulating the activity of TGF β -like molecules.

Key words: aprotinin, noggin, protease nexin-1, peptide growth factor signalling, myogenin, post-translational control

INTRODUCTION

During embryogenesis in vertebrates, most skeletal muscle is derived from segmented groups of pluripotent cells called somites (reviewed in Buckingham, 1992). As somites begin to differentiate, compartmentalize and become somite myotome, a subset of cells begins to express muscle-specific transcription factors myf-5 and myogenin (8 and 8.5 dpc respectively in the mouse). These skeletal muscle precursor cells, called myoblasts, migrate to destinations in the thorax and limbs, where they proliferate and differentiate into primary muscle fibers.

At an intracellular level, myogenesis is one of the best-characterized developmental events in mammalian embryogenesis. Muscle-specific transcription factors, e.g., myogenin, myf5 and MyoD, have been shown to be crucial for muscle formation in mice (Hasty et al., 1993; Rudnicki et al., 1993). However, the extracellular molecules that (1) control determination of the muscle lineage (myotome) in the somites, (2) trigger myoblast migration and (3) induce terminal differentiation, are largely undetermined. One source of inducer molecules is the axial structures (notochord and neural tube), since it has been shown that these structures can influence myogenic specification within the somite (Rong et al., 1992; Buffinger and Stockdale, 1994).

Studies of muscle cells in culture have begun to shed light on the extracellular regulation of myoblast differentiation.

First, it is known that maintenance of myoblasts in media containing 20% fetal bovine serum (maintenance medium) suppresses differentiation, whereas medium with lower concentrations (2-10%) of horse serum (differentiation medium) promotes myotube formation. These results indicate that extracellular molecules can modulate myogenesis. Second, it has been shown that specific serum components, e.g., peptide growth factors bFGF and TGF β , can modulate myogenesis (Olson et al., 1986; Salminen et al., 1991, Cusella-De Angelis et al., 1994). However, the role of growth factors and their receptors in skeletal muscle formation is complex. For example, the effect of TGF β is dependent on the extracellular environment, since this growth factor can have both positive and negative effects on myoblast differentiation depending on the culture conditions (Filvaroff et al., 1994). Furthermore, embryonic and fetal myoblasts respond differently to TGF β (Cusella-De Angelis et al., 1994). Therefore, it seems likely that there are a number of molecules in the extracellular milieu that influence myogenic determination and differentiation.

In this paper, we show that aprotinin, a Kunitz-type inhibitor of serine proteases (reviewed in Dingle and Gordon, 1988), stimulates differentiation of mouse myoblasts in culture, and results in increased levels of myogenin and muscle-specific proteins. The possible involvement of a Kunitz-domain-containing molecule in myogenesis is intriguing for several reasons. The Kunitz domain is a found in a growing family of proteins identified in organisms ranging from snail to human.

This family of molecules includes a class of serine protease inhibitors, some of which have been isolated by virtue of their effect on cell maintenance and differentiation (McKeehan et al., 1986). Kunitz-type protease inhibitors could affect cell growth and differentiation by inhibiting proteolytic activation of latent growth factors. For example, TGF β -like molecules are assembled as precursors that must be proteolytically processed to have activity (Massague, 1987; Lyons et al., 1990; for review see Lyons et al., 1991), and active TGF β inhibits differentiation of myoblasts in a dose-dependent fashion (Olson et al., 1986). Our results suggests that aprotinin inhibits activation of latent TGF β , which leads to reduced levels of active TGF β , and may be the mechanism by which aprotinin stimulates differentiation. These findings form a striking parallel with recent findings on neural formation in *Xenopus*, which can be induced by either the Kunitz-domain protein noggin (Lamb et al., 1993), or by inhibiting the action of the TGF β -like molecule activin (Hemmati-Brivanlou and Melton, 1992, 1994; Hemmati-Brivanlou et al., 1994). The results presented here further suggest that the Kunitz family of molecules is involved in inductive interactions, and can play diverse physiological roles during development.

MATERIALS AND METHODS

Cell culture

The G8-1 (ATCC Rockville, MD), and C₂C₁₂ mouse skeletal muscle cell lines (obtained from Dr Joav Prives, SUNY Stony Brook) were maintained in DME + 20% Hyclone FBS (GibcoBRL, Gaithersburg, MD), 5 U/ml penicillin and streptomycin. Cells were induced to differentiate by replacing the media with DME + 10% horse serum (HS) (G8-1) or 2% HS (C₂C₁₂). Aprotinin (Sigma A3428, St Louis, MO), affinity-purified aprotinin (Sigma A4529), recombinant aprotinin (Miles inc., Kankakee, IL), soybean trypsin inhibitor (Sigma, T-9003), pepstatin, E64 (Boehringer Mannheim, Indianapolis, IN) and/or active TGF β (CalBiochem, San Diego, CA) were added to cells 12 hours prior to addition of differentiation promoting media, and media was replenished 3 days after induction for G8-1 cells and every other day for C₂C₁₂ cells. The mink lung epithelium cell line used as a bioassay for active TGF β (from Drs Lynn Wilson and Daniel Rifkin, NYU Medical Center) had been stably transfected with the TGF β responsive promoter of PAI 1 driving expression of the luciferase reporter gene. The cell line was cloned and maintained in DME + 10% FBS, 5 U/ml pen/strep, and 200 μ g/ml geneticin (GibcoBRL).

Assays for differentiation of skeletal muscle

Myotube formation was determined by fixing cells in methanol, staining the nuclei with 0.1% Giemsa stain in 5% glycerol, 5% methanol in water and quantifying the per cent of nuclei contained within multinucleated myotubes. In separate experiments, four random fields of view were chosen per time point per condition, each containing an average of 300-700 nuclei per field. Creatine kinase assays were performed as described (Munson et al., 1982). Briefly, cells were washed once with PBS, lysed in 1% NP40, 100 mM Hepes pH 6.75, and assayed for creatine kinase activity in a two-step reaction. The first stage contains (all from Sigma); 1 mM ADP, 10 mM MgCl₂, 0.2% BSA, 0.1 mM P¹,P⁵ diadenosine, 5' pentaphosphate, 2.5 mM DTT, 3 mM D-glucose, 25 mM phosphocreatine and 20 U/ml hexokinase, and was stopped after 30 minutes at 37°C with 0.25 M NaOH. 10 μ l of the reaction mixture was added to 190 μ l of a second reaction mixture containing 50 mM imidazole pH 7, 0.82 mM NADP, and 1 U/ml glucose 6-P dehydrogenase, and formation

of NADPH was assayed by spectrophotometry (A₃₄₂). These assays were performed on duplicate samples, and controls were performed in the absence of phosphocreatine to adjust for background activity in the extracts. AChR levels were assayed by specific binding of ¹²⁵I α -bungarotoxin to duplicate monolayers of cells at each time point (Olson et al., 1986).

Isolation of total cellular RNA

Total cellular RNA was isolated as described (Sambrook et al., 1990). Briefly, cell monolayers were lysed in 4 M guanidinium thiocyanate + 0.1 M β -mercaptoethanol. The extract was passed through an 18-gauge needle to shear the DNA and centrifuged through 5.7 M cesium chloride containing 0.1 M EDTA. RNA pellets were resuspended in RNase-free water + 0.3 M sodium acetate and ethanol precipitated. Ethanol precipitates were dried and resuspended in 1 \times northern running buffer containing 50% deionized formamide, 6% formaldehyde.

Labeling cDNA probes

The cDNA for myogenin was from Dr Eric Olson (MD Anderson, Houston, TX), and contains the entire coding sequence of myogenin cloned into the unique *Eco*RI site of pKS (+) (Stratagene, La Jolla, CA). This clone was linearized with *Acc*I for transcription of labeled antisense RNA. The cDNA for mouse protease nexin-1 was the kind gift of Dr Dominique Belin (University Medical Center, Geneva, Switzerland) and contains most of the coding sequence of PN-1 cloned into the unique *Bam*HI site of pGEM3Z (Promega, Madison, WI). The PN-1 clone was linearized with *Xba*I for transcription of labeled antisense RNA. pT7 18S RNA clone from Ambion (Austin, TX) was used to generate labeled antisense 18S RNA. Clones were transcribed with T7 RNA polymerase (Boehringer Mannheim, Indianapolis, IN) in the presence of [³²P]UTP (NEN, Boston, MA).

Northern blot hybridization

Formaldehyde-agarose gel electrophoresis and northern blot hybridization were performed as described (Carroll et al., 1993). Briefly, 7.5 μ g of total RNA was electrophoresed on an 0.8% agarose gel containing 6% formaldehyde. Gels were blotted onto a Duralon nylon membrane (Dupont, Wilmington, DE), and UV crosslinked using a Stratagene crosslinker. Blots were prehybridized and hybridized in 50% formamide, 0.8 M NaCl, 2 mM EDTA, 0.1% SDS, 2.5 \times Denhardt's solution, 0.1 mg/ml sonicated salmon sperm DNA, and 50 mM Pipes (pH 6.7) for at least 18 hours at 58°C. Filters were washed in successively more stringent conditions, the last wash being 0.2 \times SSC, 0.1% SDS, 0.1% sodium pyrophosphate at 72°C. Filters were exposed to Kodak XAR-5 film (Rochester, NY) at -80°C between intensifying screens.

Luciferase assay for active TGF β

Mink lung epithelium cells (MLECs) containing the stably integrated luciferase reporter gene under the transcriptional control of the plasminogen activator inhibitor-1 (TGF β -responsive) promoter were plated onto 96-well plates (5 \times 10³ cells/well) and allowed to attach overnight. MLECs were then incubated with conditioned media from G8-1 cells overnight. MLECs were washed twice with PBS, lysed in 1 \times lysis buffer (Promega) containing 1% Triton X-100, and assayed for luciferase activity in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). The luciferase assay reagent from Promega contained 20 mM Tricine, 1.07 mM (MgCO₃)₄Mg(OH)₂·5H₂O, 2.67 mM MgSO₄, 0.1 mM EDTA, 270 μ M coenzyme-A, 530 μ M ATP, 33.3 mM DTT and 470 mM luciferin. Latent TGF β was activated by heating aliquots of the G8-1 conditioned medium to 80°C for 5-10 minutes and assaying as described. Dilutions of purified, active TGF β were used to obtain a standard curve with which to quantify TGF β levels in the G8-1 cell conditioned medium. Values represent the mean and standard deviation of triplicate samples.

RESULTS

Several independently derived myoblast cell lines are available that remain undifferentiated in medium containing high concentrations of fetal bovine serum (maintenance medium), which is growth factor rich. However, differentiation can be induced when these myoblasts are placed in low concentration of horse serum (differentiation medium), with the formation of multinucleated muscle myotubes that have physiological (e.g. contractile) and biochemical properties of skeletal muscle *in vivo* (for example, see Fig. 4A control). To identify molecules controlling myogenesis, specific components of the medium can be manipulated and the effect on differentiation observed. We have used this system to investigate extracellular molecules that might be involved in the differentiation pathway.

Aprotinin, a Kunitz-domain containing inhibitor of serine proteases, stimulates skeletal muscle differentiation

We initially investigated if perturbing the proteolytic balance in myoblast cultures would affect differentiation. Mouse G8-1 myoblasts were plated DMEM + 20% FBS (maintenance medium), in which they remain undifferentiated. When cells reached approximately 40-50% confluence, different protease inhibitors were added to the culture media and cells were incubated overnight. Cells were then switched to differentiation-promoting media (DMEM + 10% horse serum \pm protease inhibitor) and incubated for 7 days. Cells grown in

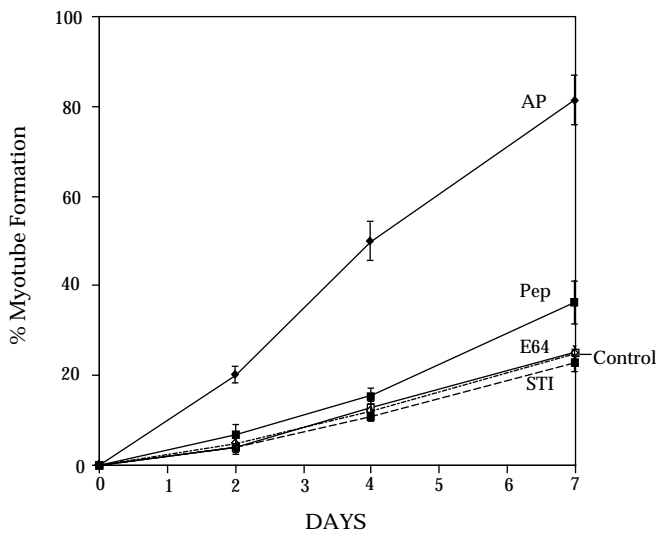


Fig. 1. Effect of different protease inhibitors on G8-1 myoblast differentiation. G8-1 cells were cultured in DME + 20% fetal bovine serum, protease inhibitors were added and the cells were induced to differentiate 12 hours later (day 0) by addition of differentiation media (DME + 10% horse serum) alone, or containing the indicated protease inhibitor. Protease inhibitors were: AP, aprotinin (2 trypsin inhibitory units/ml (TIU)); STI, soybean trypsin inhibitor (2 TIU/ml); E64 (20 μ M); and Pep, pepstatin (20 μ M). At each time point the extent of fusion (% myotube formation) was determined by counting the number of nuclei found in myotubes compared to the total number of nuclei. The values represent the mean and standard deviation of four random fields.

the absence of protease inhibitor (control, 10% HS) formed 25% myotubes (\pm 5%) after 7 days in culture (Fig. 1), and continued to differentiate for up to 14 days until greater than 70% of the culture formed myotubes. The serine protease inhibitor aprotinin (AP) effected a 3- to 4-fold increase in myotube formation by day 7, reaching a level comparable to that seen in day 12-14 control cultures. Neither soybean trypsin inhibitor (STI, another inhibitor of serine proteases), E64 (a cysteine protease inhibitor), or pepstatin (an aspartic acid protease inhibitor) significantly affected myotube formation at concentrations known to inhibit proteolysis (Beyon and Bond, 1990). These data indicate that the effect of aprotinin is specific, and not simply a general inhibition of proteolytic activity.

The stimulation of differentiation by aprotinin is a general property of skeletal myoblasts, since aprotinin also accelerates differentiation of C₂C₁₂ cells (Fig. 2B). This stimulation is not due to impurities in the protein preparation, since affinity-

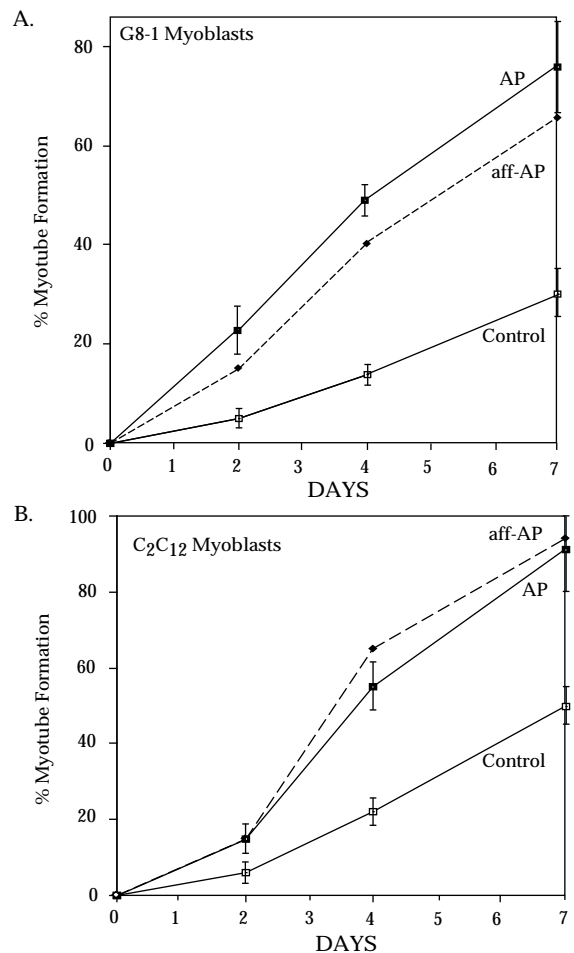


Fig. 2. Aprotinin and affinity-purified aprotinin stimulate differentiation of two independently derived skeletal muscle cell lines. (A) G8-1 or (B) C₂C₁₂ cells were induced to differentiate in the presence of aprotinin (2 TIU/ml) or affinity purified aprotinin (2 TIU/ml). Cells were cultured, fixed and stained and % myotube formation was calculated as described in Fig. 1 and Materials and Methods. Values represent the mean and standard deviation of four random fields from duplicate experiments.

purified aprotinin (Fig. 2), and aprotinin expressed in the yeast *Pichia pastoris* (data not shown) evoke a comparable response.

Aprotinin elicits early accumulation of myogenin transcripts and increased expression of muscle-specific proteins

The increase in myotube formation by aprotinin could be caused by a stimulation of membrane fusion or by an induction of the myogenic program. To address these possibilities, the effect of aprotinin on expression of myogenin and muscle-specific proteins was analyzed. G8-1 myoblasts were plated in maintenance medium (DME + 20% FBS), and allowed to reach approximately 50% confluence. Aprotinin was then added to the experimental cultures, cells were incubated for 12 hours and total RNA was extracted from 0 day cultures (day 0, Fig. 3A). Remaining cultures were induced to differentiate by differentiation-promoting media (DMEM + 10% horse serum, \pm aprotinin). RNA was extracted at 1 day intervals and all samples assayed for myogenin mRNA by northern blot analysis. In each case, hybridization to 18S RNA was used to determine the integrity and quantity of the RNA loaded. Myoblasts cultured in the presence of aprotinin showed a striking increase in myogenin transcripts over the control

cultures (Fig. 3A), especially prior to the addition of the differentiation promoting media (compare day 0 of control column to day 0 + aprotinin column). In contrast, it has been demonstrated that TGF β can inhibit myogenin transcription and differentiation of C₂C₁₂ cells (Olson et al., 1986; Salminen et al., 1991), which was also observed with G8-1 myoblasts (Fig. 3A). Myogenin transcripts are undetectable in cultures treated with 1 ng/ml active TGF β indicating that either maintenance (growth factor rich) medium (20% FBS) or addition of growth factors can inhibit accumulation of myogenin transcripts.

Stimulation of differentiation by aprotinin and inhibition of differentiation by active TGF β were also reflected in the expression of muscle-specific proteins. Aprotinin-treated cultures showed a 2- to 4-fold increase in both muscle creatine kinase activity and acetylcholine receptor (AChR) number (Fig. 3B) between 2 and 6 days after the cells were induced to differentiate. The increase in muscle proteins and myotube formation could be explained by the early presence of myogenin, since myogenin stimulates differentiation, and transcription from the muscle creatine kinase (MCK) promoter (Brennan and Olson, 1990). In contrast, including TGF β in these cultures inhibited expression of MCK and AChR, which could also be mediated by repressing the expression of muscle regulatory molecules like myogenin.

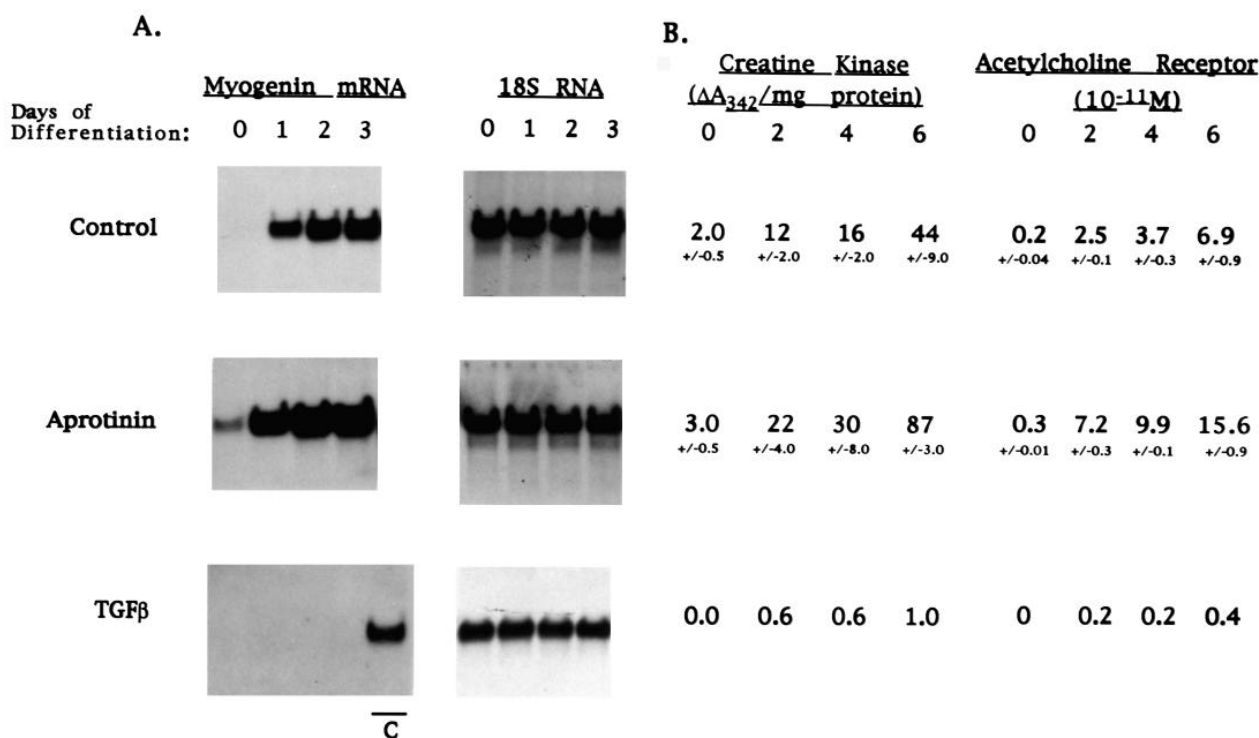


Fig. 3. The effect of aprotinin and TGF β on the accumulation of myogenin mRNA and muscle-specific proteins. G8-1 myoblasts were cultured in DME + 20% FBS, and aprotinin (2 TIU/ml) or TGF β (1 ng/ml) was added 12 hours before the cells were induced to differentiate. At the indicated times (days of differentiation), total RNA was isolated and 7.5 μg subjected to (A) Northern blot analysis for myogenin and 18S RNA. RNA isolated from control cultures (10% HS) or cells grown in the presence of aprotinin were electrophoresed on the same gel and hybridized simultaneously. RNA from TGF β -treated cultures were electrophoresed on a separate gel and hybridized separately. **C** indicates control RNA from G8-1 cells differentiated in 10% HS to insure that the myogenin probe was able to hybridize. All blots were stripped and reprobbed with radiolabeled 18S antisense RNA to determine relative amounts of total RNA loaded. (B) Cells were assayed for muscle creatine kinase activity and AChR number as described in Materials and Methods. Values for MCK and AChR represent the mean and standard deviation from duplicate samples of two separate experiments.

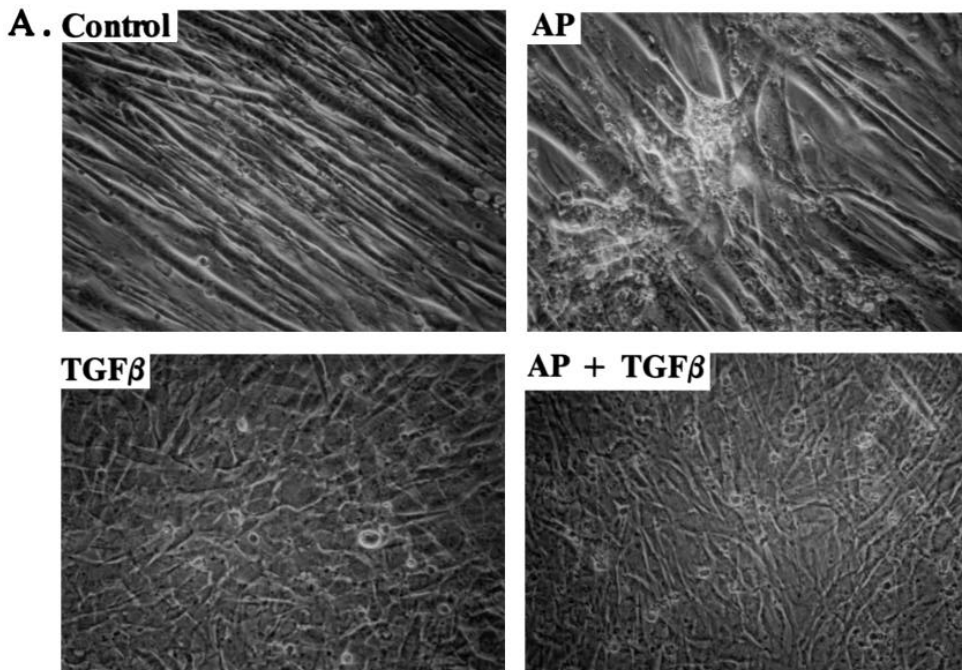
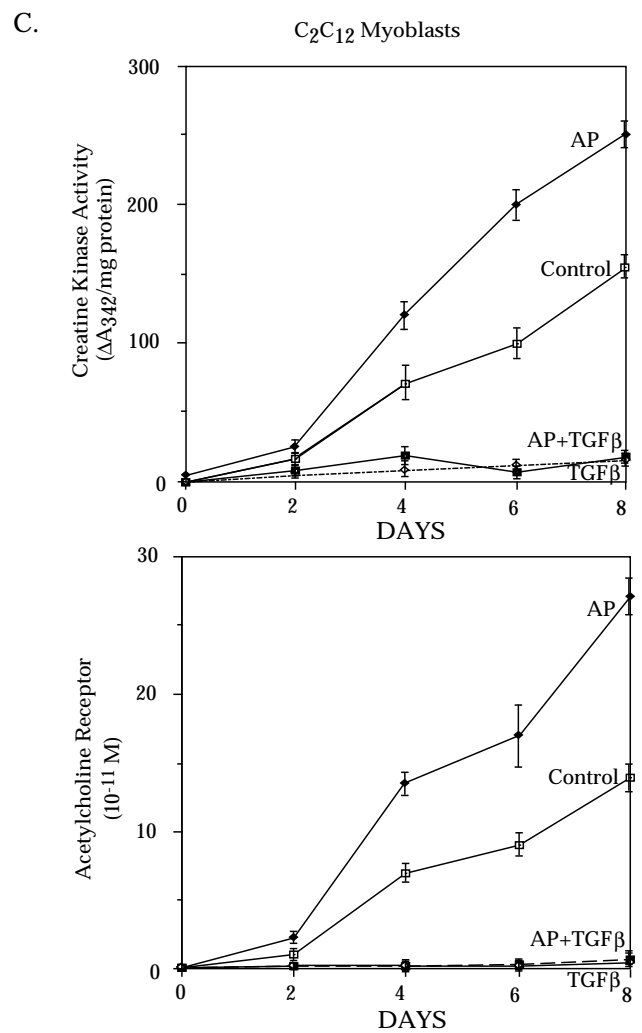
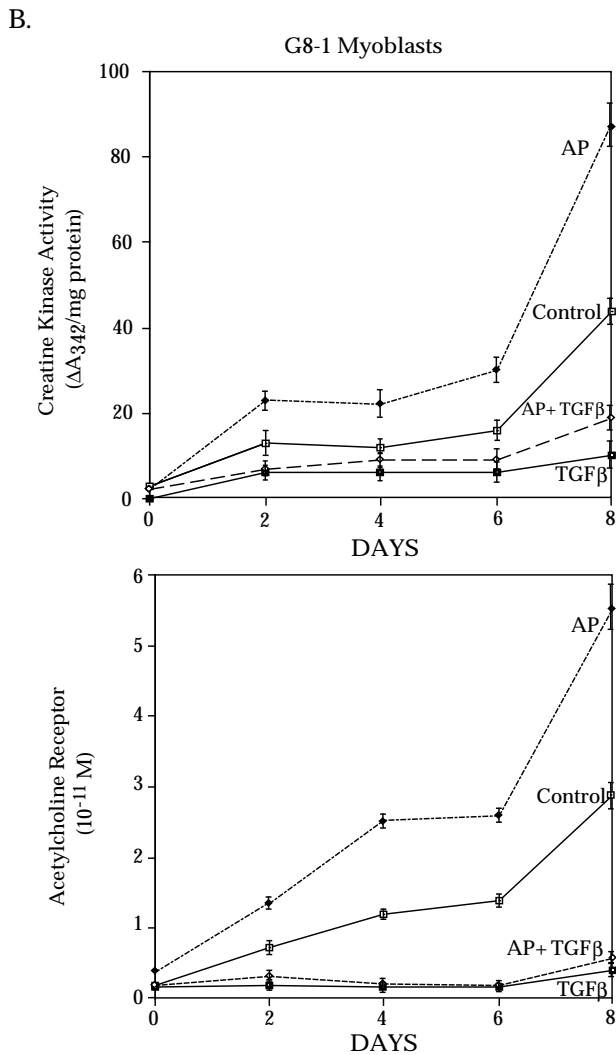


Fig. 4. Active TGFβ reverses the stimulation of differentiation seen with aprotinin morphologically and molecularly in two independently derived cell lines. (A) G8-1 cells were induced to differentiate by replacing DME + 20% FBS with DME + 10% HS alone or media containing aprotinin (2 TIU/ml), TGFβ (2 ng/ml), or aprotinin + TGFβ. (B) G8-1 cells were differentiated as described in A and assayed for MCK activity and AchR levels to quantify differentiation. (C) C₂C₁₂ cells were differentiated by replacing DME + 20% FBS with DME + 2% HS alone or media containing aprotinin (2 TIU/ml), TGFβ (2 ng/ml), or aprotinin + TGFβ and assayed for MCK activity and AChR levels to quantify differentiation. Values represent the mean and standard deviation of duplicate samples.



Active TGF β blocks the stimulation of myogenesis mediated by aprotinin

Since aprotinin is a protease inhibitor that stimulates differentiation and TGF β is a growth factor that requires proteolytic activation and inhibits differentiation, our data raised the possibility that aprotinin acts by inhibiting activation of latent TGF β . To address this possibility, G8-1 myoblasts were induced to differentiate in the presence of either aprotinin, 1 ng/ml TGF β , or both aprotinin and TGF β , and assayed for differentiation morphologically (Fig. 4A) and biochemically (Fig. 4B). The results demonstrate that the increase in myoblast differentiation promoted by aprotinin is blocked by active TGF β . This result was reproduced with C₂C₁₂ cells (Fig. 4C), suggesting that the inhibition of differentiation by TGF β and the reversal of the aprotinin stimulatory effect is a general property of differentiating skeletal muscle cells. These data indicate that active TGF β acts downstream of aprotinin.

Levels of active and latent TGF β change in response to treatment with aprotinin

If aprotinin is eliciting its effect by inhibiting proteolytic activation of latent TGF β , the endogenous levels of active and latent TGF β in G8-1 cultures should be perturbed by the inclusion of aprotinin. Therefore, we used a bioassay to measure the levels of active TGF β in the culture media of G8-1 myoblasts treated with aprotinin. The bioassay for active TGF β used a mink lung epithelium cell line that had been stably transfected with a TGF β -responsive promoter driving expression of the luciferase reporter gene (E. L. Wilson and D. B. Rifkin, unpublished results, see Materials and Methods). 12 hours after the addition of aprotinin, G8-1 cultures showed reduced levels of active TGF β (60 pg/ml active TGF β in control cultures, and 30 pg/ml active TGF β in aprotinin-treated cultures, Fig. 5A). The difference in levels of active TGF β could result from increased clearance of TGF β from the media, an inhibition of TGF β activation, or a combination of the two.

To test these possibilities, latent TGF β remaining in the culture medium was activated by heating the above samples to 80°C for 5 minutes, a treatment that converts the latent form to the biologically active form and the activated TGF β was quantified (Fig. 5B) using this bioassay. This analysis showed that (1) unconditioned media (20% FBS) contains an abundant store of latent TGF β (up to 2 ng/ml); (2) latent TGF β was depleted from the medium of control cultures, so that by the time of serum depletion (24 hours after plating), roughly 50% of the latent TGF β was depleted (0d, C) and (3) latent TGF β remained at stable levels in aprotinin-treated cultures and

was not depleted (0d, AP). A reasonable interpretation of these results is that proteolytic activation of TGF β is being blocked by aprotinin so that TGF β remains in the latent form, and this leads to a decrease in steady-state levels of active TGF β . Since G8-1 myoblasts respond to active TGF β in a relatively linear manner within the concentration ranges measured in media of cultured cells (data not shown), a decrease in generation of active TGF β may be the mechanism by which aprotinin stimulates differentiation (Fig. 7).

Production and regulation of an endogenous serine protease inhibitor by G8-1 cells: protease nexin-1 (PN-1)

The ability of aprotinin to stimulate differentiation, possibly by inhibiting proteolytic activation of latent TGF β , prompted us to analyze G8-1 myoblasts for the production of inhibitors of serine proteases that might mediate growth factor activation. It has been shown (Festoff et al., 1991) that G8-1 cells produce the serine protease inhibitor protease nexin-1 (PN-1). PN-1 and aprotinin have many similar inhibitory properties, so we determined whether PN-1 mRNA levels change in a manner consistent with a role in regulating myogenesis. PN-1 mRNA expression is low in undifferentiated myoblasts grown in high serum and levels increase when cells are shifted to differentiation-promoting medium (Fig. 6). When G8-1 myoblasts are differentiated in the presence of aprotinin,

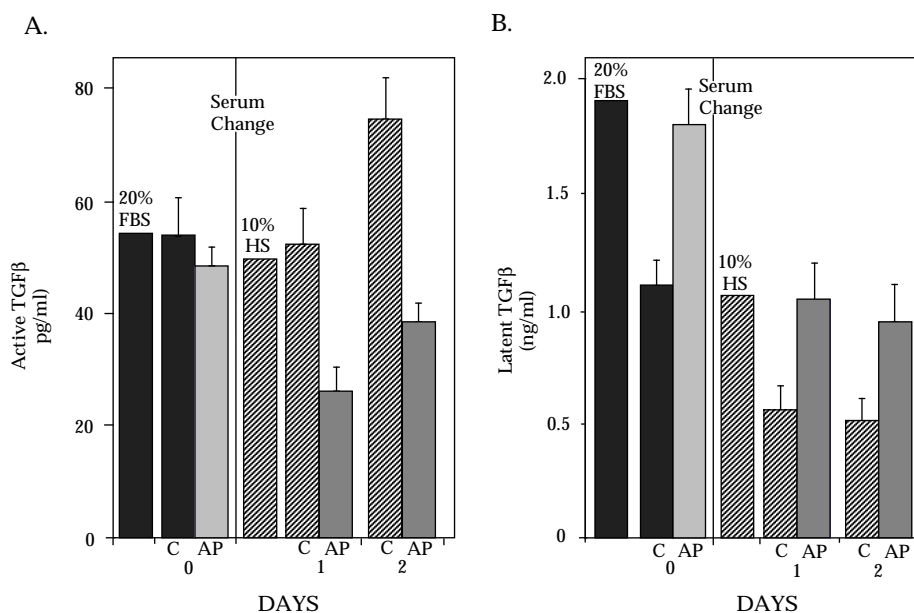


Fig. 5. Effect of aprotinin on levels of active and latent TGF β in cultures of differentiating G8-1 myoblasts. Medium from G8-1 myoblasts cultured in the presence or absence of aprotinin (2 TIU/ml) was assayed for active TGF β using a mink lung epithelium cell line (MLEC) containing a stably integrated luciferase reporter gene under control of a TGF β inducible promoter as described in Materials and Methods. (A) Active TGF β levels were measured by luciferase enzyme activity and quantified by comparing the luciferase activity to MLECs grown in DME + 1% BSA containing increasing concentrations of active TGF β . AP, aprotinin-treated cultures; C, control cultures; FBS, fetal bovine serum; HS, horse serum. (B) Latent TGF β was activated by heat treating aliquots of the G8-1 media to 80°C for 5 minutes and the activated TGF β was quantified as in A. The drop in levels of latent TGF β between 0 and 1 day is due to the change of serum supplement from 20% fetal bovine serum to 10% horse serum, which induces differentiation. Values represent means and standard deviations from triplicate samples.

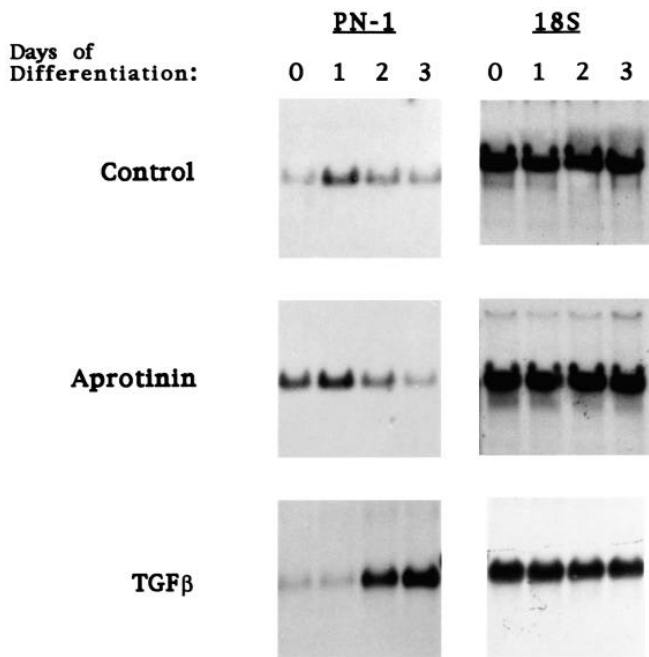


Fig. 6. Effects of aprotinin or TGFβ on protease nexin-1 mRNA levels in differentiating G8-1 myoblasts. Northern blot analysis was performed on total RNA harvested from G8-1 myoblasts differentiated in the presence of aprotinin (2 TIU), or TGFβ (1 ng/ml). 7.5 μg total RNA was electrophoresed for all samples. RNA isolated from control cultures or cells grown in the presence of aprotinin were electrophoresed on the same gel and hybridized simultaneously. RNA from TGFβ-treated cultures were electrophoresed on a separate gel and hybridized separately. All blots were stripped and reprobed with radiolabeled 18S antisense RNA to determine relative amounts of total RNA loaded.

levels of PN-1 mRNA increase prior to induction of differentiation and attain higher levels than control cultures.

When G8-1 myoblasts are grown in the presence of 1 ng/ml TGFβ, cells remain as an undifferentiated monolayer, and PN-1 mRNA is at a low level. After cells are induced to differentiate and the exogenously added TGFβ is exhausted (TGFβ was not replenished), PN-1 mRNA levels increase 5- to 10-fold. This result indicates that PN-1 transcription is repressed by high concentrations of active TGFβ, but increases as active TGFβ gets depleted. An increase in PN-1 at this time could further block proteolytic activation of latent TGFβ and result in an amplified signal to differentiate (Fig. 7).

Since PN-1 protein is not readily available, we have not yet determined if this protease inhibitor can function like aprotinin to stimulate skeletal muscle differentiation. However, the temporal and spatial

expression of PN-1 in culture and in vivo (Mansuy et al., 1993) suggest a role in myogenesis. Recent findings show four MyoD binding sites in the PN-1 promoter (Erno and Monard, 1993), further suggesting that PN-1 is being regulated during myogenesis.

TGFβ activation in cultured myoblasts is not predominantly mediated by plasmin, or other known mechanisms

Since the activation of latent TGFβ1 has been well studied in other systems, the mechanism of activation in myoblasts was investigated. The trypsin-like protease plasmin, which can proteolytically activate TGFβ in co-cultures of bovine aortic endothelial and smooth muscle cells (Sato et al., 1993) is not involved, since including plasminogen, plasminogen activators (Barlovatz-Meimon et al., 1994), α₂-antiplasmin or plasminogen activator inhibitor (PAI-1) had no effect on the differentiation of muscle cells (data not shown). Similarly, addition of mannose-6-P (Dennis and Rifkin, 1991), known to inhibit cell surface activation of latent TGFβ, had no effect.

DISCUSSION

The extracellular control of skeletal muscle myogenesis, which starts with pluripotent mesoderm cells and results in differentiated muscle fibers, remains a conundrum. Our results show that aprotinin stimulates myoblasts to differentiate, as measured morphologically and by the elevated expression of myogenin mRNA and muscle-specific proteins. Since active TGFβ inhibits differentiation of skeletal muscle myoblasts and can be generated via limited proteolysis of latent TGFβ, aprotinin could stimulate differentiation by inhibition of this proteolytic activation (Fig. 7). This line of reasoning may be relevant to the effect of various serum supplements on myoblast differentiation. For example, a shift in proteolytic balance brought about by a change in serum supplements could lead to altered growth factor activation.

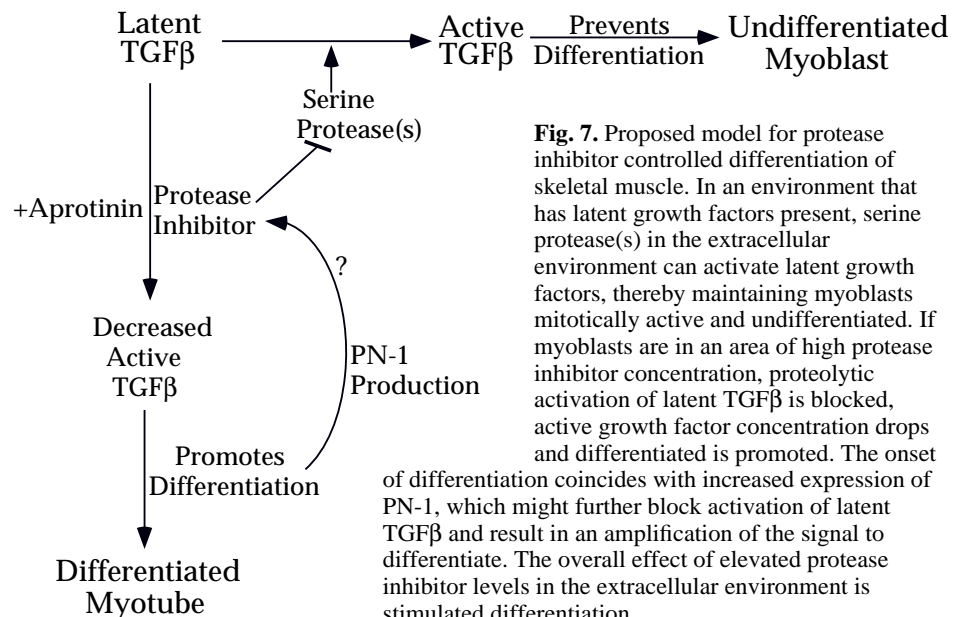


Fig. 7. Proposed model for protease inhibitor controlled differentiation of skeletal muscle. In an environment that has latent growth factors present, serine protease(s) in the extracellular environment can activate latent growth factors, thereby maintaining myoblasts mitotically active and undifferentiated. If myoblasts are in an area of high protease inhibitor concentration, proteolytic activation of latent TGFβ is blocked, active growth factor concentration drops and differentiation is promoted. The onset of differentiation coincides with increased expression of PN-1, which might further block activation of latent TGFβ and result in an amplification of the signal to differentiate. The overall effect of elevated protease inhibitor levels in the extracellular environment is stimulated differentiation.

Further evidence supports a role of TGF β activation in myoblast differentiation. Myoblasts grown in medium with 20% fetal bovine serum (maintenance medium) remain undifferentiated for several days. In these cultures, latent TGF β levels are high (2 ng/ml latent TGF β , Fig. 5B). However, addition of aprotinin to myoblasts maintained in medium with 20% FBS induces differentiation (data not shown). This result demonstrates that aprotinin can elicit an effect in the presence of 2 ng/ml latent TGF β . However, our experiments show (Fig. 4) that aprotinin has no stimulatory effect in the presence of 2 ng/ml active TGF β . These observations indicate that activation of latent TGF β is necessary for the maintenance of undifferentiated myoblast cultures. If the activation process is inhibited by aprotinin, differentiation is stimulated (Fig. 7).

Aprotinin is found both intracellularly and extracellularly, and has a K_d for trypsin of 10^{-14} M. The high affinity of aprotinin for serine proteases indicates that it could efficiently interact with extracellular proteases in the culture medium or at the cell surface. If aprotinin is functioning to stimulate myogenesis via protease inhibition, its effects could be explained in the following model: myoblasts proliferate, migrate and remain undifferentiated in an environment in which latent TGF β is activated, either by cell derived proteases, or those in the extracellular milieu. If myoblasts encounter or produce the appropriate protease inhibitors, activation of latent TGF β would be inhibited, thereby inducing differentiation and formation of primary muscle fibers (Fig. 7). The ability of aprotinin to stimulate differentiation is not a general property of the molecule because it has no detectable effect on the pluripotent teratocarcinoma cell line F9 (data not shown).

Aprotinin, also known as bovine pancreatic trypsin inhibitor, is the charter member of the Kunitz-domain superfamily. Kunitz-domain proteins have been found in many organisms including human, pig, rat, snake, fish, frogs, silkworm and snail (reviewed in Dingle and Gordon, 1988). Many of the proteins that contain a Kunitz domain are protease inhibitors, and some have been shown to affect cellular growth. For example, human endothelial cell growth factor-2b (ECGF-2b), isolated by virtue of its effects on cell maintenance and differentiation, is identical to HI-30, a Kunitz-domain containing protease inhibitor. Also, the *Xenopus* protein noggin, which acts during late mesoderm formation to induce neural tissue, contains a Kunitz-like domain (Lamb et al., 1993; McDonald et al., 1993; Creighton and Kemmink, 1993).

Although the mechanism by which Kunitz family members such as ECGF-2b and noggin control cellular differentiation is not known, it is possible that they act to regulate the processing of growth factors. TGF β -like molecules are assembled as precursors that must be proteolytically processed to have activity (Massague, 1987; Lyons et al., 1990; for review see Lyons et al., 1991), and establishment of germ layers in *Xenopus* is in part under the control of these molecules (Asashima et al., 1990). It has been demonstrated that blocking activin signalling results in neural induction (Hemmati-Brivanlou and Melton, 1992, 1994; Hemmati-Brivanlou et al., 1994). Noggin also induces neural tissues. Therefore, noggin may act as a neural inducer by inhibiting proteolytic activation of a TGF β -like molecule such as activin. Noggin, like several of the protease inhibitor members of the Kunitz family, contains a lysine in the active site, indicating that it could act as a protease inhibitor. Furthermore, unlike other signalling

molecules, high concentrations of purified noggin (up to 1 mg/ml) are necessary to induce neural-specific gene expression (Lamb et al., 1993), which is reminiscent of a protease inhibitor that acts stoichiometrically.

The importance of regulated TGF β activation is suggested in other systems. For example, in *Drosophila* the activity of the *decapentaplegic* gene (*dpp*), which encodes a member of the TGF β family and directly influences dorsa-ventral pattern formation (Irish and Gelbart, 1987; Padgett et al., 1987), can be modified by the *tolloid* (*tld*) gene, which has homology to metalloproteases (Schimell et al., 1991). In *Xenopus*, the gene *Vg1* encodes a TGF β -like molecule that remains biologically inactive unless it is proteolytically cleaved, whereby it acts to signal mesoderm induction and axis formation (Thomsen and Melton, 1993), suggesting that proteolysis can regulate *Vg1* action.

The description of the extracellular molecules that regulate myogenesis still lags far behind our information about intracellular events. The fact that the Kunitz family member aprotinin can induce differentiation of cultured myoblasts suggests the existence of an endogenous molecule that could be a novel regulator of myogenesis. In conjunction with the results seen with noggin, this report implicates a broad role for Kunitz-domain containing molecules in the inductive interactions in the developing embryo.

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