INTRODUCTION

Growing skeletal muscle requires a pool of dividing mononucleated muscle precursors since differentiated muscle cells are mitotically quiescent (Olson, 1992). The size of the proliferating cell population depends on the rate of proliferation, the rate of differentiation, and the rate at which cells are eliminated through cell death. Intercellular signalling provides essential information about when and where to proliferate, to differentiate, or to be eliminated through cell death, which ensures development of correctly sized and positioned muscle (Amthor et al., 1998).

Muscle development of the limb is an excellent model in which to study how signalling molecules balance proliferation, differentiation, and apoptosis. A small number of dividing muscle precursors migrate from the lateral edges of the somites and invade the limb mesenchyme (Chevallier et al., 1977; Christ et al., 1974). The embryo uses this limited population of cells to form all the musculature of the limb. We have previously shown that dividing muscle precursors of the developing limb can be marked by the expression of Pax-3 (Amthor et al., 1998), a gene which encodes a transcription factor of the Paired-box family (Strachan and Read, 1994; Tassabehji et al., 1994). Myogenic cells differentiate only after having down-regulated Pax-3 expression, become postmitotic, and express MyoD (Amthor et al., 1998), a gene which encodes a transcription factor of the myogenic regulatory family (Emerson, 1993; Sassoon et al., 1989). We have shown that, in the absence of proliferation-enhancing signals, myogenic cells automatically and precociously differentiate, which initially enlarges muscle masses. However, further muscle growth is arrested because the pool of Pax-3-expressing muscle precursors is exhausted (Amthor et al., 1998, 1999). Conversely, stimulation of Pax-3 expression after exposure of...
developing limb muscle to Sonic Hedgehog is linked to a transient delay of muscle differentiation, which results in excessive muscle growth (Amthor et al., 1998; Duprez et al., 1998).

Growth factors of the bone morphogenetic protein (BMP) family have been shown to be key signals that regulate embryonic muscle growth based on their sites of expression, which are situated adjacent to dividing muscle cells, and their ability to up-regulate Pax-3 expression (Amthor et al., 1998; Pourquie et al., 1996). In addition to stimulating muscle growth, BMPs can restrict muscle growth by inducing apoptosis. These opposing activities are concentration-dependent because BMP protein applied at low concentrations stimulates Pax-3 expression, whereas BMP applied at higher concentrations induces apoptosis (Amthor et al., 1998).

However, the characterisation of secreted factors, including Follistatin, Noggin, and Chordin, which modulate the activity of BMPs in a variety of developmental systems, challenge the rather simple model of a morphogen gradient being established simply by diffusion (Fainsod et al., 1997; Piccolo et al., 1996; Sasai et al., 1995; Wolpert, 1978; Zimmerman et al., 1996). Previous work has demonstrated that concentration gradients of BMP and counter gradients of Noggin specify paraxial, intermediate, and lateral mesoderm in vertebrates (Neave et al., 1997). Furthermore, even within the paraxial compartment, Pourquie et al. (1996) have demonstrated that BMP maintains myogenic cells in an undifferentiated Pax-3-expressing state in the lateral somite halves, whereas Noggin induces MyoD expression and initiates differentiation in cells of the medial part of the somites (Capdevila and Johnson, 1998; Hirsinger et al., 1997). A similar organisation is found during limb development. Ectodermal, subectodermal, and marginal BMP expression is countered by Noggin expression in the mesenchymal core. Pax-3-expressing cells are located adjacent to the BMP source, and MyoD-expressing cells are located closer to the Noggin source (Amthor et al., 1998).

Follistatin is expressed in muscle precursors of somites, in myotomal, and in limb muscle (Amthor et al., 1996; Connolly et al., 1995). Genetic deletion of Follistatin in mice results in generalised growth retardation, decreased muscle mass, especially of respiratory muscle, and postnatal asphyxia (Matzuk et al., 1995). However, the molecular mechanism which enables Follistatin to support muscle growth is not known. Follistatin is a secreted glycoprotein of approximately 32–39 kDa and was originally characterised by its ability to antagonise activin-stimulated secretion of follicle-stimulating hormone (Michel et al., 1993; Patel, 1998). During early Xenopus development, Follistatin, like Noggin and Chordin, antagonises BMP signalling, which enables specification of dorsal mesoderm and neuroectoderm (Fainsod et al., 1997). Follistatin interacts directly with BMP but does not prevent the ligand binding to its receptor (Iemura et al., 1998). However, it has been suggested that conformational changes of BMP caused by Follistatin binding prevent receptor activation (Iemura et al., 1998).

In this study, we aimed to determine the role of Follistatin during embryonic limb muscle development. We applied beads soaked in Follistatin protein to developing chick wing buds and found that Follistatin promotes expression of the proliferation marker Pax-3 and inhibits the expression of the differentiation marker MyoD. We compared effects of Follistatin with BMP and used beads soaked in BMP-7 protein as a representative of this family. Low concentration BMP-7 stimulates Pax-3 expression, but high concentration BMP-7 induces apoptosis and loss of muscle. When Follistatin was applied together with a high concentration of BMP-7, it enhanced the effect of BMP-7 to stimulate Pax-3 expression, prevented BMP-7-induced apoptosis, and resulted in excessive muscle growth. We compared the ability of Follistatin to induce muscle with another BMP-binding protein, Noggin. In contrast to the muscle-promoting ability of Follistatin, Noggin inhibits Pax-3 expression, which results in muscle growth retardation. This shows that Noggin blocks the muscle growth-stimulation effect of BMP on muscle. In order to fully understand the nature of the interaction of Follistatin with BMPs, we studied the binding kinetics between these proteins using surface plasmon resonance. We present data which show that Follistatin interacts with BMP-2 and -7 at low affinities. We present a model to explain the experimental findings and suggest that Follistatin acts to present BMPs to muscle at concentrations which permit stimulation of embryonic muscle growth.

MATERIALS AND METHODS

Preparation of Chick Embryos

Fertilised chicken eggs were incubated at 38°C, and the embryos were staged according to Hamburger and Hamilton (1992). Experiments were performed on embryos at stage 17-24, reincubated for a total of 6 days, sacrificed, and processed for whole-mount hybridisation or antibody staining.

BMP-7 and Follistatin Bead Preparation

BMP-7 was provided by Creative BioSciences as part of a material transfer agreement between KP and Creative BioSciences. BMP-2 was supplied by Genetics Institute. Follistatin was provided by National Hormone and Pituitary Program. Proteins were applied to 80- to 120-μm heparin acrylic beads (Sigma, UK). BMP-7 was used at concentrations stated in the text. Follistatin was used at a concentration of 1 mg/ml. Proteins were loaded onto beads as described by Cohn et al. (1995).

Bead Application and Microsurgical Procedures

For bead implantation, the dorsal ectoderm and mesenchyme of the right wing or somites were punctured with a electrolytically sharpened tungsten needle, and beads were inserted into the punctured mesenchyme by using a blunt glass needle.
Apoptosis Assay

Acridine Orange is a cationic fluorescent dye that accumulates in condensing chromatin of apoptotic nuclei, which can be visualised as fluorescent spots in the FITC spectrum (Erenpreisa et al., 1997). Wing buds were dissected free of all unwanted tissue and then incubated in Acridine Orange (100 ng/ml in PBS) at 37°C for 30 min. Specimens were then washed two times for 5 min in PBS and then flattened on a microscope slide under a coverslip (Weil et al., 1997). Samples were analysed and photographed immediately by using fluorescence illumination and confocal microscopy and then were fixed in 4% paraformaldehyde for in situ hybridisation.

Whole-Mount in Situ Hybridisation

All chick embryos were washed in PBS and then fixed overnight in 4% paraformaldehyde at 4°C. Anti-sense RNA probes were labelled with digoxigenin, and whole-mount in situ hybridisation was performed as described by Nieto et al. (1996). The following probes were used in this study: Bmp-2, PCR cloned fragment (nucleotides 1-1797); Bmp-4, PCR cloned fragment (nucleotides 1-953); Bmp-7, full-length fragment, 1.1 kb (gift from Dr. Anthony Graham); Follistatin, full-length, 1.1-kb fragment; MyoD, clone CMD9 full-length, 1.5-kb fragment (gift from Bruce Patterson); Noggin, full-length clone, approximately 700 bp; and Pax-3, 645-bp fragment corresponding to nucleotides 468-1113 (gift from Dr. Martin Goulding). Whole-mount embryos were partially cryosectioned for further histological examination.

Immunohistochemistry on Whole Mounts

Embryos were fixed overnight in 4% paraformaldehyde (PFA), dehydrated into 100% methanol/H2O2, washed in PBT (PBS containing 0.5% Triton), incubated for 1 h in 10% horse serum (in PBT), incubated overnight with an anti-Desmin monoclonal antibody (DAKO; 1:100, in horse serum/PBT), washed in PBT, incubated overnight in secondary antibody (AP-conjugated goat anti-mouse Ig antibody; Sigma; 1:300, in horse serum/PBT), washed in PBT, transferred in AP-buffer (Nieto et al., 1996), incubated for 5-10 min in colour reagent (4.5 μl NBT and 3.5 μl BCIP in 1 ml AP-buffer; Boehringer Mannheim), washed in AP-buffer and cleared in dimethylformamide, and stored in 4% PFA.

Virus Preparation and Injections

Noggin-RCAS constructs were kindly provided by Dr. Randy Johnson. Noggin virus was produced according to Capdevila and Johnson (1998), and viral titres of 10^8 pfu/ml were obtained. For injections, 1 μl of Fast Green (0.01% stock) was mixed with 9 μl of virus to aid visualisation and was injected at various pulse times by using a PM1000 cell microinjector (Micro Data Instruments).

Cell Grafting

Noggin expression chick "O" Line fibroblasts were trypsinised and pelleted in growth medium at 1000g for 5 min. The pellet was subsequently resuspended in 1 ml of culture medium in a 2-ml Eppendorf tube. The cells were pelleted at 1000g for 10 min. The pellet was removed by running a wire loop between the pellet and the bottom of the tube. The pellet was placed into a sterile plastic plate and bathed in culture medium and then cut into fragments for grafting.

BMP-Follistatin Interaction Analysis by Surface Plasmon Resonance

Binding experiments were performed by using the BIACORE 2000 (Biacore AB). Purified recombinant human Follistatin was immobilised onto the surface of a CM5 sensor chip (587.3 resonance units) by using amine-coupling chemistry. Purified recombinant BMP-2 and BMP-7 were injected over the sensor chip surfaces at a flow rate of 5 μl/min at 25°C. For controls, the BMPs were run over derivatised sensor chip surfaces which lacked Follistatin. Hepes-buffered saline (HBS; 10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20, pH 7.4) was used for sample dilution and as a running buffer. All curves were corrected by subtraction of the blank run. Biacore evaluation software was used for the mathematical adaptation of experimental curves.

RESULTS

Expression of Follistatin, BMPs, and Muscle Markers during Wing Bud Development

Distribution of Follistatin mRNA was analysed in muscle of HH stage 28–29 chick wings by in situ hybridisation and was compared with the Pax-3-expressing population of proliferating muscle precursors, with the MyoD-expressing population of differentiating myogenic cells, and with the Desmin-expressing population of terminally differentiated muscle cells (Fig. 1).

At HH stage 28, differentiated limb muscle identified by Desmin expression extended from the shoulder girdle into the autopod, and proximal muscle had begun to split into individual blocks (Fig. 1A). Transverse sections of the zeugopod showed uniform levels of Desmin expression in the dorsal and ventral limb muscle at HH stage 28 (Fig. 1B). Follistatin was also expressed in the wing muscle but did not extend as far distally as Desmin expression (Figs. 1A and 1G). Transverse sections additionally revealed Follistatin expression localised in subectodermal mesenchyme at HH stage 28 (data not shown) and more predominantly in muscle at HH stage 29 (Fig. 1H). Distribution of MyoD and Pax-3 transcripts was almost identical to Desmin expression (Figs. 1A, 1C, and 1E), and transverse sections of the zeugopod at HH stage 29 showed expression of both genes in muscle (Figs. 1D and 1F). Pax-3 transcripts, however, were not as uniformly expressed as Desmin and MyoD. High levels of expression rimmed the MyoD and Desmin domains at the distal tip of autopod muscle. Pax-3 expression in proximal muscle of stylopod and zeugopod were localised to prospective individual muscle, which corresponded approximately to the sites of Follistatin expression (Figs. 1A, 1C, 1E, and 1G).

In previous work, we reported that the subdivision of premyoscle masses in Pax-3-expressing cells were situated subectodermally, whereas MyoD-expressing cells were situated more centrally during early limb bud stages (Amthor et al., 1998). However, concomitant to the up-regulation of Follistatin in muscle at later limb bud stages (from HH stage 26 onwards), MyoD-, Pax-3-, and Follistatin-
FIG. 1. Normal expression profile of Desmin, MyoD, Pax-3, Follistatin, and BMP-2, -4, and -7 in chick wing buds. View on dorsal side of wing buds at HH stage 28 and on transverse sections at zeugopod level at HH stages 28 and 29. (A) Desmin protein expression shows splitting of muscle proximally whilst a compact muscle block is maintained distally (arrow). (B) Transverse section shows Desmin expression of dorsal (arrow) and ventral muscle (arrowhead) at HH stage 28. (C) Distribution of MyoD transcripts correlates to position of muscle (arrow, compare to A). (D) Transverse section shows MyoD expression at zeugopod level after splitting of muscle at HH stage 29 (arrows). (E) Distribution of Pax-3 transcripts correlates to position of wing muscle (compare to A) with regionalised high expression at the zeugopod level (arrow) and at the distal front of the expression domain (arrowheads). (F) Transverse section shows Pax-3 expression at zeugopod level after splitting of muscle at HH stage 29 (arrows). (G) Distribution of Follistatin transcripts in autopod and zeugopod (arrow) correlates to position of muscle as shown in (A). (H) Transverse section shows predominant Follistatin expression in muscle of HH stage 29 zeugopod (arrows). (I) Expression of BMP-2, -4, and -7 after simultaneous hybridisation by using one chromophor (arrows). BMPs are expressed at the periphery of muscle masses when compared to (A). (J) Transverse section shows BMP-2, -4, and -7 expression in some but not all muscles (arrows), subectodermally and perichondrally at HH stage 29.
FIG. 2. Effect of beads soaked in Follistatin on muscle development. (A) Position of Follistatin beads after implantation in wing buds at HH stage 23. (B) Up-regulation of Pax-3 expression 1 day after Follistatin bead implantation (arrow) in the right wing compared to left. (C) Down-regulation of MyoD expression 1 day after Follistatin bead implantation (arrow) in the right wing compared to left. (D) Up-regulation and broadening of the MyoD expression domain (arrow) 2 days after Follistatin bead implantation in the right wing compared to left. (E) Transverse section at zeugopod level shows down-regulation of Desmin expression predominantly in the dorsal muscle (arrow) 2 days after Follistatin bead implantation in the right wing compared to left. Bead (green arrowhead). (F) Transverse section at zeugopod level shows Desmin expression 4 days after Follistatin bead implantation. No significant alteration of muscle size between operated right (arrow) and unoperated left side.

FIG. 3. Effect of beads soaked in 500 \( \mu \)g/ml BMP-7 on muscle development. (A) Position of BMP-7 beads after implantation in wing buds at HH stage 23. (B) Lack of Pax-3 expression at site of BMP-7 bead implantation 1 day after operation (arrow) in the right wing compared to left. Some proximal and distal expression remained (arrowheads). (C) Lack of MyoD expression at the site of BMP-7 bead implantation 1 day after operation (arrow) in the right wing compared to left. Some proximal expression remained (arrowhead). (D) MyoD expression 2 days after BMP-7 bead implantation shows smaller wing diameter, lack of dorsal zeugopod muscle, and bending of ventral muscle to the dorsal side (arrow). (E) Implantation of BMP-7 beads resulted in a malformed wing, lack of most of the zeugopod (arrow), and some remaining muscle at the stylopod (arrowhead) 4 days after operation as shown by Desmin expression. (F) Transverse section at level of bead implant of the wing shown in (E) demonstrates a single bone rudiment (asterisks) and some remaining muscle (arrow). Bead (green arrowhead).

FIG. 4. Effects of simultaneous application of beads soaked in Follistatin and beads soaked in 500 \( \mu \)g/ml BMP-7. (A) Position of Follistatin and BMP-7 beads after implantation in HH stage 23 wing buds. (B) Up-regulation and broadening of the Pax-3 expression domain (arrow) after Follistatin and BMP-7 bead implantation 1 day after operation in the right wing compared to left. Pax-3-positive cells scatter off distally of the expression domain (arrowhead). (C) Lack of MyoD expression 1 day after Follistatin and BMP-7 bead implantation (arrow) in the right wing compared to left. (D) Transverse section at zeugopod level shows up-regulation and broadening of the dorsal (arrow) and ventral MyoD expression domain (arrowhead) 2 days after Follistatin and BMP-7 bead implantation. Bead (green arrowhead). (E) Transverse section at zeugopod level shows lack of Desmin expression 2 days after Follistatin and BMP-7 bead implantation compared to contralateral (arrows). Bead (green arrowhead). (F) Transverse section at zeugopod level shows gain in intensity and size of Desmin expression (arrows) 4 days after Follistatin and BMP-7 bead implantation in the right wing compared to left. Long bone rudiments are enlarged in diameter (asterisks) compared to left.
BMPs, whose activity can be modulated by Follistatin, have been analysed at later limb bud stages by in situ hybridisation against BMP-2, -4, and -7 transcripts simultaneously and visualised by using a single chromophore. At HH stage 28, BMPs were expressed in close proximity to limb muscle (Fig. 1I, compare to Fig. 1A), but not in limb muscle when sections of the zeugopod were analysed (data not shown). Interestingly, at HH stage 29, BMP expression was found in some but not all muscle of the wing zeugopod after transverse sectioning (Fig. 1J). For expression of individual BMPs, refer to Amthor et al. (1998), Francis et al. (1994), and Merino et al. (1999).

Effect of Follistatin on Pax-3 and MyoD Expression

In the next set of experiments, we explored the mechanism of how Follistatin influences muscle growth. We tested the effect of Follistatin protein on the proliferating and differentiating myogenic cell population by analysing the expression of Pax-3, MyoD, and Desmin. We implanted beads soaked in Follistatin protein into wing buds at stages prior to the onset of Follistatin expression and thereby avoided the effects of the endogenous protein. One to six Follistatin beads were applied to the dorsal wing mesenchyme of HH stages 17–23, and distribution of Pax-3 and MyoD transcripts and Desmin protein were examined at different times after the operation (Fig. 2A). We found an up-regulation of Pax-3 expression in dorsal and ventral premuscle masses 12–34 h after operation in whole-mount wings and on transverse sections of the prospective zeugopod region compared to the unoperated left wing (38 of 44 cases; Fig. 2B). Two days after Follistatin bead implantation, no difference was found in the intensity of Pax-3 expression between operated and contralateral unoperated wings (5/5; data not shown). Examination of MyoD transcripts after Follistatin bead implantation showed a smaller domain of expression after 14–28 h (20/25; Fig. 2C). The MyoD-suppressing activity of Follistatin was only transient, since 2 days after bead implantation the expression domain was broader especially in the region of stylopod muscle of the operated whole-mount wing and after transverse sectioning compared to contralateral (4/4; Fig. 2D). However, 4 days after Follistatin bead implantation, no significant difference in MyoD expression could be detected between operated and unoperated wings (4/4; data not shown). Even though the domain of MyoD expression expanded 2 days after Follistatin bead implantation, Desmin expression was down-regulated in dorsal premuscle masses after such an operation (4/4; Fig. 2E). Again, the reduction of Desmin expression was only transient, since 4 days after Follistatin bead implantation no clear differences were found either in size of muscle or in intensity of Desmin expression between manipulated and contralateral nonmanipulated wings both on whole mounts and on transverse sections of the zeugopod (4/4; Fig. 2F).

In summary, Follistatin stimulated Pax-3 expression and transiently prevented the activation of MyoD and Desmin expression. After being maintained for a prolonged period in a Pax-3-expressing state, the myogenic cells eventually differentiated. Thus, Follistatin acted as a proliferative signal; however, this stimulation of muscle growth was not strong enough to effect muscle size.

The Interaction of Follistatin and BMP-7 during Limb Muscle Development

Next, we asked whether Follistatin could modulate BMP activity during muscle development. First, we implanted five beads soaked in different concentrations of BMP-7 (10–500 μg/ml) into the dorsal wing mesenchyme of HH stage 22/23 embryos. The BMP-7 beads were implanted around a control bead soaked in PBS in the centre of the dorsal wing mesenchyme (Fig. 3A). In a second experiment, we replaced the PBS bead by a Follistatin bead, and in a third experiment, we additionally positioned five Follistatin beads between the BMP-7 beads (Fig. 4A). Sixteen hours to 4 days after bead implantation, wings were examined for the expression of Pax-3, MyoD, and Desmin and the presence of BMP-induced apoptosis.

Beads soaked in 500 μg/ml BMP-7 induced massive levels of apoptosis around the beads, judged by the incorporation of Acridine Orange 16–24 h after implantation (8/8; Figs. 5A–5C). Apoptotic activity was confined predominantly to the prospective zeugopod region. The diameter of the limb was reduced, indicating loss of cellular material due to cell death. However, the beads still had approximately the same position and distance to each other compared to their location following implantation. In situ hybridisation revealed a loss in Pax-3 expression over the area where apoptosis was observed 17–24 h after bead implantation (10/10; Fig. 3B). Some Pax-3 expression was conserved proximally and distally to implanted beads (Fig. 3B). Pax-3 expression of the ventral premuscle mass was not affected, which was confirmed after transverse sectioning of the operated right wing compared to left (data not shown). We also found a lack of MyoD expression around the operated region 17–24 h after bead implantation compared to contralateral (7/7; Fig. 3C). Again, some of the proximal and distal most ventral MyoD expression was conserved at this stage (Fig. 3C). Two days after BMP-7 bead implantation, loss of tissue, especially in the zeugopod region, was more obvious (Fig. 3D). The diameter of the zeugopod was greatly reduced and originally, even spaced beads now were situated next to each other. Examination of MyoD and Desmin expression showed that dorsal zeugopod and autopod muscle were completely missing (8/8; Fig. 3D). Examination of sections from these limbs revealed that the ventral musculature was present and bent towards the dorsal side (Fig. 3D). Four days after bead implantation, the wing was completely malformed and twisted (5/5; Fig. 3E).
Transverse sectioning of such a wing failed to reveal a recognisable zeugopod (Fig. 3F). Instead, a single long bone rudiment was found, which was linked directly to the wrist and a relatively normal autopod. It was difficult to determine whether the bone rudiment was part of the humerus or the remnant of the radius or ulna. Examination of MyoD and Desmin expression demonstrated the presence of some muscle surrounding the single long bone rudiment. Autopod muscle was found only on the ventral side (Figs. 3E and 3F).

Therefore, exposure of limb mesenchyme to high concentration of BMP-7 protein resulted in induction of high levels of apoptosis. The application of BMP to the prospective zeugopod mesenchyme resulted in a highly dysmorphic limb which lacked most of the zeugopod.

In the next set of experiments, we analyzed whether BMP-7 alone could induce the development of muscle at a low concentration. We titrated BMP-7 to find the highest concentration of BMP-7 that does not induce apoptosis. We found that BMP-7 at a concentration of 25 µg/ml did not induce apoptosis (3/3). In a manner identical to our previous work on BMP-4, we found that BMP-7 beads at this concentration enhanced Pax-3 expression (3/3) during the first 24 h after protein application (see Amthor et al., 1998). We extended this study and determined the effects of low concentration of BMP-7 on the long-term development of muscle. Application of 25 µg/ml BMP-7 led to the down-regulation of MyoD expression (3/3) for up to 48 h after protein application (Fig. 6B). In the same time period, Desmin expression was completely inhibited in the developing limb bud (Fig. 6D). After a total of 72 h after BMP-7 application, the expression of MyoD was higher than the control limb bud (Fig. 6F). During this time period, not only was the expression of Desmin reinstated to its normal pattern, but transverse sections of the zeugopod region demonstrated that BMP-7 had induced the formation of extra muscle (Fig. 6H).

Thus, at subapoptotic concentration, BMP-7 acts as a proliferation factor on myogenic cells. Implantation of one Follistatin bead encircled by five beads soaked in 500 µg/ml BMP-7 prevented induction of apoptosis around the Follistatin bead, but apoptotic nuclei could still be detected at the periphery of the BMP-7 beads 16 h after bead implantation (6/6; data not shown). Implantation of five BMP-7 beads with an additional six Follistatin beads completely prevented induction of apoptosis when analysed 16–23 h after operation (6/6; Figs. 5D–5F). A few fluorescent nuclei were detected at the anterior margin of the operated and contralateral unoperated wing, indicating naturally occurring apoptosis in the anterior necrosis zone. Examination of Pax-3 17–23 h after operation showed an up-regulation and a broadening of the expression domain both of the dorsal and ventral premuscle masses and a thickening of the Pax-3-expressing cell layer on transverse sections compared to contralateral (8/8; Fig. 4B). At the distal tip of the premuscle masses, Pax-3-positive cells dispersed away from the main block of Pax-3-expressing tissue and migrated into regions of limb mesenchyme where they would not normally be localised. Examination of MyoD expression revealed a complete lack of transcripts in the operated wing in both the dorsal and ventral sides 16–25 h after manipulation (7/7; Fig. 4C). Two days after operation, MyoD expression was up-regulated and the expression domains were enlarged both dorsally and ventrally, and transverse sections revealed that the MyoD-expressing cell layer had thickened (4/4; Fig. 4D). However, Desmin expression was almost completely down-regulated at this stage of development compared to the contralateral unoperated wing (4/4; Fig. 4E). Four days after operation, limbs had a normal macroscopic appearance. MyoD and Desmin expression were up-regulated in all limb muscles, and transverse sections of the zeugopod revealed a massive gain in muscle size and a stronger expression of Desmin in all muscle compared to contralateral wing (5/5; Fig. 4F). Interestingly, the correct muscle pattern was well preserved. Most surprisingly, not only did the muscle increase in size but so did the long bone rudiments (Fig. 4F).

These results show two effects of Follistatin. First, it can completely antagonise apoptotic activity of BMP-7. Second, the effect of Follistatin to induce Pax-3 expression is drastically enhanced in combination with BMP-7. Both molecules together exert strongest proliferative activity during muscle growth.

In the next experiment, we analysed whether Follistatin antagonises the Pax-3-inducing effect of low concentration BMP-7. We simultaneously implanted Follistatin beads and beads soaked in 25 µg/ml BMP-7 to HH stage 21 wing buds. We found an up-regulation of Pax-3 expression 1 day after operation compared to the contralateral unoperated wing in whole mounts as well as after transverse sectioning (3/3; data not shown).

Thus, Follistatin specifically antagonises the apoptosis-inducing activity but not the Pax-3-inducing activity of BMP-7.

Control Experiments

In previous work, we demonstrated that implantation of heparin acrylic beads into wing bud mesenchyme soaked in PBS did not alter expression of Pax-3 or MyoD nor did they interfere with the ultrastructure of the tissue (Amthor et al., 1998). Here, we performed control experiments and implanted six beads soaked in PBS into HH stage 22/23 wing bud mesenchyme to mimic the multiple bead implants used in our experiments. Apoptosis was not induced 17–24 h after the implantation of six beads soaked in PBS (6/6; data not shown). Fluorescent nuclei were observed only in anterior necrosis zone in both operated and unoperated wings. In situ hybridisation failed to detect any alterations in the expression of Pax-3 (4/4) or MyoD (4/4) 24 h after PBS bead implantation (data not shown). We previously analysed the effect of BMPs applied at high concentration on the ultrastructure of limb mesenchyme and found no signs of unspecific necrosis, such as an
FIG. 5. (A–C) Implantation of beads soaked in 500 μg/ml BMP-7 (C) resulted in massive induction of apoptosis as shown by fluorescent spots after Acridine Orange staining (B, arrows) 1 day after operation compared to the unoperated contralateral side (A) at which apoptotic nuclei are found only at the anterior necrosis zone (arrowhead). (D–F) Implantation of beads soaked in Follistatin and beads soaked in 500 μg/ml BMP-7 (F) prevented apoptosis at the site of bead implantation (E) Apoptotic nuclei are only found in the anterior necrosis zone (arrow) similar to the contralateral unoperated wing (D, arrowhead).

FIG. 6. Effect of the application of low doses of BMP-7 (25 μg/ml) to limb muscle development. Five beads soaked in BMP-7 were applied to stage 20/21 limb buds and examined for myogenic markers following reincubation for 48 (stage 26) or 96 h (stage 30). (A) Stage 26 contralateral control showing strong uniform expression of MyoD. (B) Operated side of (A) shows that MyoD was slightly down-regulated in proximal regions (arrowheads) corresponding to the position of the BMP-7 beads. (C) Stage 26 contralateral control showing strong expression of Desmin in the limb (arrowhead). (D) Operated side of (C) shows that, following BMP-7 implantation, Desmin levels were significantly reduced (arrowhead). (E) Stage 30 contralateral control shows MyoD expression in individual muscles (arrowheads). (F) Stage 30 operates, side of (E) shows similar expression profile to unoperated side with higher levels of MyoD in proximal muscle (arrowheads). (G) Transverse section of contralateral control stage 30 limb at the zeugopod level showing Desmin expression located in discrete muscle blocks (arrowheads). (H) Transverse section of operated side of (G) at zeugopod level showing increased levels of Desmin expression following implantation of BMP-7 (arrowheads).
FIG. 7. (A) Expression of Noggin (arrow) 36 h after Noggin-RCAS virus injection of the right wing compared to left. (B) Transverse section of the Noggin transfected wing which is shown in (A) demonstrates scattered expression of Noggin throughout the wing mesenchyme. (C) Down-regulation of Pax-3 expression 36 h after Noggin transfection of the right wing compared to left. (D) Down-regulation of Pax-3 expression 62 h after Noggin transfection of the right wing compared to left. (E) Smaller expression domain of MyoD 36 h after Noggin transfection of the right wing compared to left. (F) Smaller expression domain of MyoD shows retarded muscle development 62 h after Noggin transfection of the right wing compared to left.

FIG. 8. Kinetic of Follistatin–BMP-7 protein interaction by using surface plasmon resonance (Biacore). Association of Follistatin–BMP-7 complexes is shown by increasing resonance units (RU) as the Follistatin flow cell was exposed to different concentrations of BMP-7 for approximately 200 s. Dissociation of Follistatin–BMP-7 complexes is shown by decreasing RU for the different BMP-7 concentrations after exposure of the Follistatin flow cell to buffer for approximately 800 s.
amorphous mass of cell debris, tissue coagulation, and swelling of cells (Amthor et al., 1998). We also examined whether the deposition of a high concentration of protein into limb mesenchyme could nonspecifically result in apoptosis as a result of an alteration in the colloidosmotic pressure. We inserted six beads soaked in Follistatin into the dorsal wing mesenchyme of HH stage 17 embryos and tested for induction of apoptosis 17 h after operation. Follistatin did not induce apoptosis, and the only sign of cell death was found in the anterior necrosis zone (2/2; data not shown).

**The Effect of Noggin on Limb Muscle Development**

Our results suggested that Follistatin only partially antagonises BMP signalling on limb muscle. This raised the question of whether this feature was specific to Follistatin or whether it was a general feature common to other BMP-binding proteins.

We questioned the role of Noggin during limb muscle development and infected HH stage 17 wing buds with replication-competent retrovirus encoding the Noggin gene. Approximately 36–48 h after infection, the limb buds had developed to HH stage 24/25 and Noggin was expressed throughout the wing bud mesenchyme (3/3; Figs. 7A and 7B). Infected wing buds appeared slightly shorter in proximodistal axis than the noninfected contralateral wing. Examination of Pax-3 expression in Noggin-infected wing buds showed a drastic down-regulation of the gene 36 h after operation (2/3; Fig. 7C). Analysis for MyoD expression showed a proximodistal truncation of the expression domain concomitant to truncation of the wing (3/3; Fig. 7E). Analysis of infected wings 62 h after Noggin infection showed severe shortening of the wing bud together with an anterior extension of the apical ectodermal ridge in most cases. Pax-3 expression was either totally abolished (1/3) or severely reduced (2/3; Fig. 7D). MyoD expression was slightly down-regulated and the musculature was reduced in size as the whole limb was shortened (3/3; Fig. 7F).

An alkaline-phosphatase (AP)-RCAS construct was used as a control. Ten embryos injected with the AP virus at an identical stage to the Noggin virus resulted in normal expression of Pax-3 and MyoD (data not shown).

Next, we determined the consequences on muscle development of locally expressed Noggin. We grafted RCAS cells carrying the Noggin gene into the centre of HH stage 20–22 wing bud mesenchyme. Twenty-four hours after grafting, Noggin expression was found in a centrally located band along the proximodistal axis (5/5; data not shown). Wing buds appeared shorter after ectopic Noggin expression compared to contralateral nontreated wings. Molecular analysis showed down-regulation of the Pax-3 expression 17–24 h after grafting (10/11; data not shown). MyoD expression was found to be normal 15 h after grafting but the expression domain was split due to grafted cells (3/4; data not shown). However, longer reincubation resulted in shortened wings and concomitantly in a shortened domain of MyoD expression (5/5; data not shown).

These results demonstrate that Noggin inhibits Pax-3 expression, which leads to a reduced size of premuscle masses as marked by MyoD expression. Thus, Noggin impairs continuous growth of embryonic muscle and this is in contrast to the growth-stimulation effect of Follistatin.

**The Kinetic of the Follistatin-BMP Interaction**

We investigated the nature of interaction between Follistatin and BMP-7 in order to understand the ability of Follistatin to potentiate the Pax-3-inducing capacity of BMP-7. To elucidate this phenomenon, we analysed the kinetics of Follistatin–BMP protein interaction using a surface plasmon resonance biosensor (Biacore). The same Follistatin and BMP-7 proteins were used as for the bead experiments. In a first step, Follistatin was irreversible bound with 657.3 response units onto a dextran-coated float cell. In a second step, the same Follistatin-coated cell was exposed to different concentrations of BMP-7 and BMP-2 proteins. The Follistatin–BMP interaction was measured in response units from which association and dissociation constants were calculated by using Biacore evaluation software.

Follistatin–BMP-7 interaction was studied at 1.563, 3.125, 6.25, and 12.5 nM (Fig. 8). Experimental curves of the kinetics were mathematically adapted by Langmuir curves, which resulted in an average association rate of $K_a = 2.06 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ and average dissociation rate of $K_d = 1.66 \times 10^{-3}$ s$^{-1}$. From the association and dissociation rate constants, the affinity ($K_0$) of Follistatin for BMP-7 was calculated to be 80 nM.

Follistatin–BMP-2 interaction was studied at 1.563, 3.125, 6.25, 12.5, and 25 nM (data not shown). Experimental curves of the kinetics were mathematically adapted by Langmuir curves which resulted in an average association rate of $K_a = 1.29 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ and average dissociation rate of $K_d = 6.82 \times 10^{-3}$ s$^{-1}$. From the association and dissociation rate constants, the affinity ($K_0$) of Follistatin for BMP-2 was calculated to be 5.29 nM.

These results show that Follistatin and BMP-7, which were used throughout the study, could interact directly. The calculated affinities for Follistatin to bind BMP-7 and BMP-2 are very similar to the affinity calculated by Iemura et al. (1998) for Follistatin to binding to BMP-4.

**DISCUSSION**

The biological activity of polypeptide growth factors is specifically regulated by various binding proteins (Christian, 2000). Here, we present data which suggest that the ability of BMPs to stimulate myogenic proliferation and embryonic muscle growth is promoted by Follistatin but inhibited by Noggin. Thus, two BMP-binding proteins modulate the effect of BMP on muscle in opposite ways.
In our previous work, we demonstrated that BMPs support growth and positioning of muscle in a dose-dependent manner (Amthor et al., 1998). We showed that, at high concentrations, BMPs induce apoptosis in muscle cells, and we noted that, during normal chick limb development, migrating muscle precursors avoid regions of high BMP expression that could enable exact positioning of premuscle masses. However, at low concentrations, BMPs maintain a pool of Pax-3-expressing proliferating muscle precursors. Muscle growth occurs as cells are recruited from the precursors. Once they escape the influence of the proliferation supporting signal, they enter a differentiated, postmitotic muscle state signified by the onset of MyoD expression. We showed that the ectoderm and subectodermal mesenchyme express low levels of BMPs and are adjacent to populations of cells that express Pax-3. Differentiated muscle was found towards the centre of the limb mesenchyme. Signals from the ectodermal regions maintain this organisation. After ectoderm removal, all Pax-3-expressing precursors differentiate immediately, which enlarges muscle in short term but arrests muscle growth in long term because the pool of proliferating Pax-3-expressing cells is depleted. Thus, proliferation signals from the ectoderm suffice to sustain muscle growth during early limb bud stages when the precursors are present as a continuous layer immediately underneath the ectoderm. However, it would be difficult to implement such an organisation later during development when muscle is bigger and forms individual units at various positions.

Biological assays based on Xenopus dorsal mesoderm formation and neuroectoderm induction have been used to identify molecules which negatively regulate the inhibitory actions of the BMPs in these processes. This list includes Noggin, Follistatin, Chordin, Gremlin, Cerberus, and DAN (Fainsod et al., 1997; Piccolo et al., 1996, 1999; Stanley et al., 1998; Zimmerman et al., 1996). In the context of muscle development, one molecule, Noggin, fits all the criteria of a classical BMP antagonist. During somite development, ectopic expression of Noggin results in a down-regulation of Pax-3 expression and an up-regulation of MyoD expression. During limb development, Noggin prevents BMP-induced apoptosis (Capdevila and Johnson, 1998; Hirisinger et al., 1997). In this study, we have shown that overexpression of Noggin represses Pax-3 expression, which leads to impairment of muscle growth in the chick limb.

It is therefore remarkable that myogenic cells exposed to Follistatin actually increase Pax-3 expression and transiently delay differentiation. Thus, Follistatin, originally described as a BMP antagonist during Xenopus development, acts as a proliferation-promoting reagent during muscle development, and therefore Follistatin acts as an agonist of BMP signalling.

Furthermore, we showed that application of Follistatin together with BMP-7 at a concentration which would normally induce apoptosis completely prevents the apoptotic effect. Instead of loss of premuscle masses, Follistatin together with BMP-7 stimulate Pax-3 expression and delayed the onset of differentiation for at least 24 h. Such an enormous burst of precursor proliferation results in excessive-sized muscle after myogenic cells eventually differentiated. Thus, Follistatin acts as an agonist of BMP-7 in its activity to promote Pax-3 expression but antagonises BMP-7 in its activity to induce apoptosis and muscle loss.

The effect of Follistatin to partially function as an agonist and antagonist of BMP-7 activity is reflected by the low affinity of Follistatin to bind BMP-7 of 80 nM. This affinity is similar for Follistatin binding to BMP-2 as well as BMP-4 (Iemura et al., 1998) but is approximately 240-fold weaker than the affinity of Noggin to bind BMP (Zimmerman et al., 1996). Furthermore, by studying the association and dissociation kinetics of BMPs and Follistatin, we found that Follistatin binds BMPs quite rapidly but was also able to dissociate.

These results suggest that Follistatin binds BMPs, and in the bound form, the activity of the BMP is neutralised. However, the binding seems to be reversible and released BMP regains biological activity. We propose that Follistatin stores and presents BMPs in a subapoptotic concentration which promotes continuous muscle growth.

Although we have demonstrated the ability of Follistatin to bind and to lower the biological concentration of BMP-7 in an experimental scenario, we are aware that in vivo there are members of the TGF-β family other than the BMPs which influence muscle development. Activin and Myostatin are expressed in developing skeletal muscle (Link and Nishi, 1997; McPherron et al., 1997). Follistatin can bind activin with high affinity and prevents activin signalling in various biological systems (Hemmati-Brivanlou et al., 1994; Michel et al., 1993; Schneyer et al., 1994). Activin can repress muscle differentiation in culture, but the exact role of activin and possible interaction with Follistatin during muscle development remains to be determined (Link and Nishi, 1997). Myostatin prevents hyperplasia and hypertrophy muscle, and in vitro, it inhibits muscle differentiation (McPherron et al., 1997; Taylor et al., 2001; Thomas et al., 2000; Zhu et al., 2000). However, Myostatin is unlikely to be a target molecule of Follistatin because preliminary data of protein interaction using surface plasmon resonance failed to show binding between these molecules (unpublished observations).

Our previous study and data presented in this study show that BMPs are able to promote muscle growth (Amthor et al., 1998). Therefore, if Follistatin would act as a BMP antagonist, then genetic deletion of the Follistatin gene should result in increased muscle mass. However, the opposite is actually the case because Follistatin−/− mice present muscle defects which result in postnatal asphyxia (Matzuk et al., 1995). Thus, the Follistatin−/− phenotype supports the mode of action suggested by this study.

We have shown that muscle in late-stage limb buds expresses Follistatin as well as BMPs and that Pax-3-, MyoD-, and Desmin-expressing cells appear intermingled. The simple linear organisation of muscle seen during early limb bud development (growth factor adjacent to precursor...
cells adjacent to differentiated cells) has clearly broken down. We suggest that during later muscle development Follistatin acts as a store of BMPs within the muscles themselves and thereby maintains a pool of proliferative active myoblasts necessary for continuous muscle growth and secondary fibre formation. By distributing muscle growth-regulating factors locally, individual muscles could develop at different rates.

We have also shown that Follistatin in combination with BMP-7 induces enlargement of long bone rudiments. These results suggest that Follistatin may also regulate the development of tissues other than muscle. Indeed, Follistatin deficient mice are generally reduced in size and show abnormalities in the skeletal system (Matzuk et al., 1995).

The results from this study suggest a new role for Follistatin in which it enhances the action of BMPs by acting as a reservoir. There are clear differences between the biochemical properties of Follistatin and the other BMP-interacting proteins. First, whereas Noggin and Chordin inhibit BMPs interacting with the BMP receptors (Piccolo et al., 1996; Zimmerman et al., 1996), Iemura et al. (1998) have recently shown that BMPs bound to Follistatin can still complex to its receptor. They suggest that, even though the Follistatin—BMP complexes can bind the BMP receptor, the conformation of receptor attachment is altered and the BMP cannot induce a signalling cascade. Secondly, the affinity for Follistatin to bind BMPs is much weaker compared to Chordin and Noggin (Iemura et al., 1998; Piccolo et al., 1996; Zimmerman et al., 1996). And lastly, recent work by Iemura et al. (1999) has demonstrated the presence of molecules which specifically regulate the binding of Follistatin to BMPs. They have shown that Ep45, when bound to BMP-4, prevents Follistatin interacting with BMPs, and under these conditions, the BMP is refractory to the action of Follistatin. Ep45, however, cannot prevent the inhibitory action of Chordin or Noggin on BMPs. The deployment of molecules such as Ep45 could be used to further regulate the activity of BMPs by inhibiting the inhibitors. It will be interesting to determine whether molecules such as Ep45 are expressed during chick limb development at stages when Follistatin becomes localised to muscle.

In summary, we have presented data indicating that Follistatin does not act as a classical BMP antagonist. The low affinity of Follistatin for BMPs indicates that the binding of these proteins is irreversible. We suggest that Follistatin expressed in muscle may locally store and present BMPs at a defined concentration which enables continuous muscle growth.

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