Inhibin $\alpha$- and $\beta_A$-subunit immunoreactivity in the chicken embryo during morphogenesis

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Summary

Antibodies against synthetic peptides selected from the amino acid sequences of human inhibin $\alpha$- and $\beta_A$-subunits were used to examine the distribution of inhibin subunit immunoreactivity in chicken embryos during the first week of development. Inhibin $\alpha$-subunit immunoreactivity was localized in skeletal and smooth muscle myoblasts as well as developing cardiac muscle cells. In somites, immunostaining was seen exclusively in myotomes. The appearance of $\alpha$-subunit immunoreactivity was correlated with myogenic differentiation; immunoreactivity was not seen in non-differentiated mesenchymal cells or in terminally differentiated adult muscle cells. In cardiac muscle, some immunopositive myocytes were seen also in the adult. In the adult heart, the Purkinje fibers were strongly immunoreactive, suggesting a possible role of the immunoreactive protein in the impulse-conducting function of these specialized cells. Inhibin $\alpha$-subunit immunoreactivity was also seen in the visceral and parietal cells of the Bowman’s capsule in both mesonephric and metanephric kidneys. In addition to mesodermal derivatives, $\alpha$-subunit immunoreactivity was localized in neuroepithelial cells and axons in the developing central nervous system. Immunoblotting with anti-$\alpha$(1-32) revealed two protein bands with $M_r$ values of 50 000 and 32 000 in cytosol samples of whole embryos under nonreducing conditions. In reduced samples an approximately 14 000 $M_r$ protein species was detected.

Inhibin $\beta_A$-subunit immunoreactivity was detected only in chondrocytes, suggesting that the immunoreactive protein might represent a chicken homologue of the various cartilage and bone morphogenetic proteins expressed in mammals.

Key words: inhibin, activin, differentiation, chicken embryo.

Introduction

Several members of the transforming growth factor-$\beta$ (TGF-$\beta$) family of growth and differentiation factors have been shown to be involved in the regulation of embryonic development. The amino acid sequences of these factors are well conserved during evolution and closely homologous members of the TGF-$\beta$ family have been found in chordates as well as in arthropods. These include the Müllerian inhibiting substance (MIS), which leads to the regression of the Müllerian ducts during male development in mammals (Cate et al., 1986), and the decapentaplegic (dpp) protein in Drosophila, which participates in dorsal-ventral specification during embryogenesis and later in correct morphogenesis of the imaginal disks in the larva (Padgett et al., 1987). Also a group of differentiation factors involved in cartilage formation and bone morphogenesis have close homology to TGF-$\beta$ (Seyedin et al., 1986, 1987; Wozney et al., 1988).

Members of the TGF-$\beta$ family, as well as those of another group of regulatory proteins termed the fibroblast growth factor (FGF) family (Slack et al., 1987), have been extensively studied for their capacity to induce mesoderm in vitro (Whitman and Melton, 1989). TGF-$\beta$s themselves have been shown to induce ectodermal explants of the Xenopus embryo to form various mesodermal tissues either independently (Rosa et al., 1988) or in synergy with FGF (Kimmelman and Kirschner, 1987). Activins and inhibins, members of the TGF-$\beta$ family, which in the adult have a hormonal role in regulating the expression and release of follicle-stimulating hormone, also appear to have regulatory functions during embryogenesis. Like other active molecules of the TGF-$\beta$ family, activins and inhibins are dimers of polypeptide subunits linked together by disulphide bridges. Activins occur as homodimers of two closely similar $\beta$-subunits $\beta_A$ or $\beta_B$ (activin A and activin B, respectively) or as $\beta_A\beta_B$ heterodimers (activin AB). Inhibins consist of an inhibin-specific $\alpha$-chain paired with either $\beta_A$ in inhibin $\alpha_A$ or $\beta_B$ in inhibin B. Activin A has recently been shown to be capable of inducing mesoderm in explants of
the Xenopus ectoderm (Asashima et al., 1990; van den Eijnden-van Raaij et al., 1990; Smith et al., 1990; Thomsen et al., 1990). Activin B, found to be expressed in the hypoblast of the chick, is suggested to be the endogenous inducer of the body axis during avian embryogenesis (Mitrani et al., 1990). Asashima et al. (1990) have reported that also inhibin A has a low mesoderm-inducing activity and may enhance the capacity of activin A to induce mesodermal structures. In the experiments of Thomsen et al. (1990), however, these effects were not observed.

Recently, the expression and localization of various growth factors in embryonic tissues have been described. Using in situ hybridization Millan et al. (1991) and Schmidt et al. (1991) have studied the expression of TGF-β1, TGF-β2 and TGF-β3 during mouse embryogenesis and Roberts et al. (1991) the localization of inhibin and activin subunit mRNAs in rat embryos. Pelton et al. (1991) and Flanders et al. (1991) have used an immunohistochemical approach to study the distribution of TGF-βs in developing mouse embryos.

In this study, we examined the presence and distribution of inhibin α- and βA-subunit immunoreactivity in chicken tissues during the first week of embryonic development, at the time when the major morphogenetic events take place. As tools, we used antibodies against synthetic human inhibin α- and βA-subunit peptides.

Materials and methods

Peptide synthesis

The peptide containing the first 32 N-terminal amino acids of the human inhibin α-subunit was obtained from Peninsula Laboratories, Belmont, MA. A peptide corresponding to amino acids 88-102 of the inhibin βA-subunit was synthesized using a model 430 A peptide synthesizer from Applied Biosystems (USA). The peptide was purified with a preparative HPLC-column (Waters Bondapak C18) using a linear TFA-acetonitrile gradient. The α(1-32) peptide was conjugated to carrier proteins, thyroglobulin (for immunization) and ovalbumin (for direct EIA) with M-maleimido benzoyl-N-hydroxysuccinimide ester (Liu et al., 1979; Green et al., 1982) and the peptide βA(88-102) with glutaraldehyde (Geysen et al., 1985). The peptide-to-carrier molar ratios in the reaction mixture were 40 for thyroglobulin and 2 for ovalbumin.

Antibody preparation

Polyclonal antibodies to the thyroglobulin-conjugated peptides α(1-32) and βA(88-102) were raised in California rabbits. 30-100 µg of peptides as conjugates emulsified with Freund’s complete adjuvant were given at intervals of about one month. The sera were tested by direct EIA for antibody titer against the corresponding ovalbumin-conjugated peptides.

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Microtiter plates were coated with the ovalbumin-conjugated peptides diluted to approximately 0.5 µg of conjugate/ml with 0.06 M carbonate-bicarbonate buffer (pH 9.6) by incubation overnight at 4°C. After washing the coated plates three times with washing buffer (PBS, pH 7.4, containing 0.5% Tween 20), unreacted protein-binding sites were blocked with a solution containing 1% BSA and 0.05% Tween 20 in PBS, pH 7.4. Rabbit serum diluted with the same solution was incubated for 1 hour at 37°C. After three washes, goat anti-rabbit IgG conjugated to horseradish peroxidase (Cappel, West Chester, PA) (1:5000) was added and allowed to react for 1 hour at 37°C. After washing the wells three times, the substrate solution (0.4 mg O-phenylenediamine and 1.5 µl H2O2 in 1 ml citric acid/Na2HPO4-H2O2 buffer) was added. The colorimetric reaction was stopped with 2 M H2SO4. Absorbance values were read at 492 nm. The sera were precipitated with 40% ammonium sulfate. The pellet obtained after centrifuging at 10 000 g for 5 minutes was redissolved in distilled water and incubated overnight against PBS, pH 7.4.

Characterization of the antibodies

Specificity of the purified gammaglobulin fractions to inhibin subunits was assessed by immunoblotting of recombinant human activin A (rh-Act A) and inhibin A (rh-Inh A) (Genentech, Inc., South San Francisco, CA) using the method described below.

Embryos

Fertilized White Leghorn chicken eggs (strain b-15, Piikkiö, Finland) were placed into a humidified forced draft incubator one day after laying and incubated at 38 ± 0.5°C. Embryos were removed from the eggs at 24 hour intervals during a period of seven days. Removed embryos were fixed in phosphate-buffered 4% formaldehyde, pH 7.3, for 2 to 12 hours depending on the size of the embryo, and embedded in paraffin. To examine the distribution of inhibin-like immunoreactivity in certain fully differentiated chicken tissues, a 4-week-old chicken was killed by decapitation and the tissue samples were fixed as described above.

Immunohistochemistry

Deparaffinated 8 µm-thick sections were treated with 0.5% H2O2 in methanol for 10 minutes to block endogenous peroxidase activity and thereafter incubated with 10% normal goat serum for 15 minutes at room temperature. The sections were then incubated overnight at 4°C with anti-α(1-32) (2 µg/ml) or anti-βA(88-102) (5 µg/ml) Further incubations were performed with biotinylated goat anti-rabbit IgG (1:1000; Vector, Burlingame, CA) and then with avidin-biotin-peroxidase complex (Vectastain Elite ABC kit, Vector, Burlingame, CA), both for 30 minutes at room temperature. Staining for peroxidase activity was accomplished using 0.02% 3,3′-diaminobenzidine (Sigma, St.Louis, MO), 10 mM imidazole and 0.01% H2O2 in 0.5 M Tris buffer (pH 7.6). Control sections were incubated with anti-peptide antibodies preabsorbed with a 50-fold molar excess of the corresponding synthetic peptides, or with normal rabbit IgG or PBS instead of the specific primary antibodies.

Polyacrylamide gel electrophoresis and immunoblotting

7-day-old embryos were homogenized 1:4 (w/v) in PBS, pH 7.4, containing 1 mM phenylmethylsulphonyl fluoride (PMSF) at 0°C and the homogenate was centrifuged for 1 hour at 4°C and 100 000 g. The supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmlli, 1970). Samples were prepared by boiling for 10 minutes in 3 vol of sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.05% bromophenol blue with (reducing conditions) or without (nonreducing conditions) 10% 2-mercaptoethanol. Proteins were separated on SDS-PAGE and thereafter electrophoretically transferred to nitrocellulose membranes. The membranes were blocked by incubation in 50 mM Tris, 0.9% NaCl (TBS, pH 8.0), containing 5% bovine serum albumin (BSA) for 1 hour at 37°C. The membranes were then incubated overnight at 4°C with the primary antibodies diluted to 5 µg/ml in TBS containing 1% BSA. After three 10 minute washes in TBS, the membranes were incu-
Inhibin subunits in the chicken embryo

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Results

Antibody specificity

The specificity of anti-α(1-32) and anti-β(88-102) was assessed by immunoblotting of rh-Inh A and rh-Act A (Fig. 1). Anti-α(1-32) was shown to react with both the 32 000 M_τ inhibin dimer and the 18 000 M_τ α-subunit in nonreduced and reduced samples of rh-Inh A, respectively (lanes A and B). The 32 000 M_τ inhibin dimer was detected also with anti-β_A(88-102) (lane A). Anti-β_A(88-102) reacted with nonreduced activin A (approximately 25 000 M_τ) (lane C) and the 14 000 M_τ β_A-subunit in reduced samples of rh-Inh A and rh-Act A (lanes B and D).

Inhibin α-subunit immunoreactivity in chicken tissues

Mesodermal derivatives

At the very earliest stages of development (0-24 hours), no staining was observed with anti-α(1-32). After two days of incubation, an intense staining appeared in myotomes and remained equally strong during the one week’s period examined (Fig. 2A). At the same time, staining appeared also in the walls of the aorta and in the myocardium (Fig. 3F,G). Two days later, immunoreactivity was seen also in the walls of smaller blood vessels. In limb bud myoblasts, staining appeared after 5 days of incubation (Fig. 3A,B,C).

For comparison, samples of striated skeletal muscle and cardiac muscle from a 4-week-old chicken were examined for inhibin α-subunit immunoreactivity. Staining was not seen in skeletal muscle and, in cardiac muscle (Fig. 3H), only a few muscle cells were immunopositive. Cells of the impulse-conducting system (Purkinje fibers) were intensely stained. In gut mesoderm (Fig. 3D,E), the smooth muscle myoblasts were stained after 3 days of incubation and in Wolffian ducts after 5 days. In mesonephros, cells of the parietal and visceral layers of the Bowman’s capsule were immunopositive (Fig. 4A). The same cells were immunopositive also in the kidney of a 4-week-old chicken (Fig. 4B).

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Fig. 3. Localization of inhibin α-subunit immunoreactivity in developing muscle tissue. (A, B, C) Sections through limb buds of embryos of different ages showing the appearance of immunostaining in skeletal muscle myoblasts after 5 days of incubation concomitantly with skeletal differentiation. Developing limb bud cartilage is marked with an asterisk. (D, E) Immunoreactive smooth muscle myoblasts surrounding the gut. (F, G, H) Localization of inhibin α-subunit immunoreactivity in cardiac muscle. (F,G) Immunoreactivity is localized in embryonic myocardium. (H) In the adult heart, the Purkinje fibers are intensely stained (arrow). Weaker staining is seen in some cardiac muscle cells (arrowheads). The number in the top right hand corner indicates the time of incubation in days; a, adult. Bar, 50 µm.
rounded cells lying in the neuroepithelium next to the lumen and in differentiating axonal processes in the mantle of the neural tube (Fig. 2A). In the sensory retina, immunostaining appeared after 4 days of incubation. The staining was localized predominantly at the inner surface of the optic cup (data not shown).

**Inhibin βA-subunit immunoreactivity**

With anti-βA(88-102), immunostaining was observed exclusively in cartilage cells from day 6 onwards (Fig. 5B). Before the appearance of histologically identifiable cartilage cells, no staining was seen in sclerotomal mesenchyme (Fig. 5A).

No immunostaining was seen in control sections (Figs 2B, 5C).

**Immunoblotting**

Immunoblotting with anti-α(1-32) revealed two protein bands with Mr values of 50 000 and 32 000 in cytosol samples of whole embryos under nonreducing conditions (Fig.6, lane A). In reduced samples, an approximately 14 000 Mr protein species was detected (lane B).

No protein bands were detected with anti-βA(88-102) (data not shown).
Fig. 6. Immunoblot analysis of immunoreactive protein species in 7-day-old embryos. Embryos were homogenized in PBS containing 1 mM PMSF and centrifuged for 1 hour at 4°C and 100,000 g. Nonreduced (A) and reduced (B) cytosol samples were electrophoresed on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose. Blots were reacted with anti-α(1-32) followed by peroxidase-conjugated anti-rabbit IgG.

Discussion

Inhibins and activins were originally characterized by their ability to regulate FSH secretion: inhibins suppress the production of FSH while activins stimulate its release from the pituitary (reviewed by Woodruff and Mayo, 1990). Later, inhibin subunit mRNAs and proteins have been found to be produced also in various extragonadal tissues, including placenta, bone marrow and nervous tissue, and the proteins have been suggested to act not only as hormones but also as local regulators of cell growth and/or differentiation (Meunier et al., 1988; Petraglia et al., 1987; Bronxymeyer et al., 1988; Sawchenko et al., 1988; Schubert et al., 1990).

More recently, the role of inhibins and activins as mesoderm inducers in Xenopus has been extensively studied (Aashima et al., 1990; van den Eijnden-van Raaij et al., 1990; Smith et al., 1990; Thomsen et al., 1990; Arizumi et al., 1991). In the present study, we have demonstrated that proteins identical or closely related to inhibin subunits are produced in embryonic chicken tissues and that their appearance correlates with certain differentiation processes.

During the first week of development, inhibin α-subunit immunoreactivity appeared in a variety of tissues and organs of mesodermal and ectodermal origin in the chicken embryo. Most conspicuously, inhibin α-subunit immunoreactivity was observed to be associated with the differentiation of muscular tissue. α-subunit immunoreactivity appeared in the smooth muscle myoblasts in gut mesoderm and in the walls of Wolffian ducts at the time when smooth muscle fibers differentiate to form the muscular compartments of these organs. In developing somites, only the myotomes were intensely stained. The correlation of the appearance of α-subunit immunoreactivity with muscular differentiation was, however, seen most clearly in the limb bud where the differentiation of limb muscle coincides with cartilage differentiation. Immunostaining did not appear in developing limb bud myoblasts until after 5 days of incubation, at the time when the conversion of skeletal mesenchyme into limb cartilage had become histologically evident. Inhibin α-subunit immunoreactivity was no longer seen in fully differentiated skeletal muscle fibers.

Inhibin α-subunit immunoreactivity was observed in the myocardium from the very earliest stages of cardiac differentiation. As development proceeded, the staining gradually disappeared from the majority of cardiac muscle fibers, so that after hatching immunoreactivity was seen only in scattered fibers. An interesting finding was that strong inhibin α-subunit immunoreactivity persisted in the specialized muscle cells (Purkinje fibers) forming the impulse-conducting system of the heart. Whether the immunoreactive protein might have a regulatory role in the impulse-conducting function of these cells in the avian heart is not known. Nevertheless, an intriguing finding was that α-subunit immunoreactivity was found in two different cell types able to conduct electrical impulses: in Purkinje cells of the heart and in neurons.

The disappearance of inhibin α-subunit immunoreactivity from myocytes during terminal differentiation is comparable to the downregulation of acidic and basic FGF mRNAs in skeletal murine and rat myoblasts in culture (Moore et al., 1991). FGFs are known to be potent mitogens for skeletal myoblasts (Gospodarowicz et al., 1976) and they have been shown by several authors to inhibit myoblast differentiation (Lathrop et al., 1985; Spizz et al., 1986; Clegg et al., 1987). Moore et al. (1991) suggest that a decrease in the endogenous production of FGF at the transcriptional level as well as a coordinate decrease in myoblast FGF receptors may be factors participating in the process of myogenic differentiation. The association of an inhibin α-related protein with myocyte differentiation detected in the present study indicates also that proteins of the TGF-β family may play a role in myocyte development.

The exact structural and functional nature of this protein species remains to be determined.

In another mesodermal derivative, the kidney, inhibin α-subunit immunoreactivity was observed exclusively in the cells of the Bowman’s capsule. Since the staining appeared in the same cells also in adult renal corpuscles, it is probable that, rather than being a differentiation factor in the developing urinary system, the inhibin α-related-peptide might be in some way connected to the functioning of the Bowman’s capsule.

Previously, all three inhibin subunits have been shown to be expressed in the adult rat nervous tissue, with α-subunit mRNA being the most abundant inhibin mRNA species both in brain and in spinal cord (Meunier et al., 1988). During rat embryogenesis βα- and ββ-subunit mRNA expression has been detected in the developing brain (Roberts et al., 1991). In the present immunohistochemical work, only inhibin α-subunit immunoreactivity was
detected in developing chicken nervous tissue. The rounded form and the location of the immunoreactive cell soma next to the lumen of the neural tube suggest that they probably are proliferating neuroepithelial cells undergoing mitosis. Another predominant site of inhibin α-subunit immunoreactivity was close to the outer surface of the neural tube where the differentiation of neuroepithelial cells into neurons takes place. Here the staining was seen in the developing axonal processes. As development of the chicken nervous system proceeds, α-subunit immunoreactivity appears first in the ventral root axons and later also in dorsal roots (data not shown). It might be possible that the inhibin α-subunit immunoreactivity described in the present paper has two separate functions in nervous tissue. During the earliest phases of development, it might correspond to a protein involved in nerve cell proliferation and differentiation. Later, the protein might be participating in intraneuronal and/or interneuronal functions as previously suggested for an inhibin β-subunit-like peptide found in certain areas of the rat brain (Sawchenko et al., 1988).

Immunoblot analysis of whole-embryo cytosols with anti-α(1-32) revealed two immunoreactive protein bands in samples electrophoresed under nonreducing conditions. The apparent 32 000 Mr of the more intense band is in agreement with the molecular weight of the mammalian inhibin dimer. The approximately 14 000 Mr band detected in reduced samples is, however, smaller than the 18 000 Mr α-subunit monomer. The 50 000 Mr protein band also found in nonreduced samples might represent a larger precursor form of an avian inhibin-like protein. As precursors of the inhibin α-subunit are known to have independent biological functions in the adult, dissimilar to those of inhibin (Robertson et al., 1989; Schneyer et al., 1991), it might be possible that they would have independent regulatory activities also during embryogenesis. Further studies are needed to determine the degree of homology that the detected avian protein species have with the mature mammalian inhibin α-subunit and its precursors.

With anti-βA(88-102), immunoreactivity was localized exclusively in cartilage cells. When differentiation of the sclerotomal mesenchyme into cartilaginous vertebrae was examined, it was observed that, before the appearance of histologically identifiable chondrocytes, which occurs after 5-6 days of incubation, no staining was seen in non-differentiated mesenchymal cells. This does not rule out the possibility that the immunoreactive protein might be present also in mesenchymal cells, but at levels below the detection limit of the immunohistochemical method used. A number of differentiation factors closely related or identical to TGF-β have been isolated from bovine bone with the ability to regulate cartilage and bone formation. Two cartilage-inducing factors, CIF-A and CIF-B, have been found to induce cultures of fetal rat mesenchymal cells to express a chondrocyte phenotype (Seyedin et al., 1986, 1987). Of these two factors, CIF-A has been immunohistochemically localized in fetal bovine chondrocytes and osteocytes (Ellingsworth et al., 1986). In addition, three distinct bone morphogenetic proteins have been characterized which are able to induce cartilage formation in rats in vivo (Wozney et al., 1988). The inhibin βA-subunit immunoreactivity detected in the present study in embryonic chicken chondrocytes might correspond to a chicken homologue of these morphogenetic proteins. The immunoreactivity could be due to inhibin βA-subunits whose mRNA expression has recently been observed in developing rat skeletal structures (Roberts et al., 1991) or to related proteins having enough structural similarity to allow cross-reaction with the anti-βA-subunit antibody.

In summary, this study shows the expression of inhibin α- and βA-subunit-related proteins in the chicken embryo during morphogenesis. Inhibin α-subunit immunoreactivity was most prominently associated with the development of mesodermal derivatives and the central nervous system whereas βA-immunoreactivity was confined to differentiating skeletal structures. The data suggest that proteins related to inhibin subunits may be morphogenetic regulators in the avian embryo.

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