Activin B: Precursor Sequences, Genomic Structure and in Vitro Activities

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We report here the complete amino acid sequence of the human inhibin βB-subunit as deduced from the sequence of cDNA and genomic clones. The primary translation product of the βB mRNA predicts a protein of 407 amino acids, containing a prepro region of 292 amino acids separated by basic amino acids from the mature C-terminal 115 amino acids. Mammalian tissue culture cells transfected with a βB-subunit expression plasmid secreted an activin B homodimer of approximately 22K mol wt. Coexpression of the βA- and βB-subunit mRNAs resulted in the secretion of the three forms of activin, A, AB, and B. Purified activin B was shown to elicit FSH release in an in vitro pituitary assay and trigger the accumulation of hemoglobin in K562 cells. The potency of activin B in both of these assays (ED₅₀ ~2 ng/ml) was indistinguishable from that observed for activin A. (Molecular Endocrinology 3: 1352-1358, 1989)

INTRODUCTION

Activins are proteins of gonadal origin which stimulate FSH release in vitro (1-3). Two forms of activin have been isolated from porcine follicular fluid and shown to be either a disulfide-linked homodimer of the inhibin βA-subunit (activin-A) or a heterodimer composed of a βA- and a βB-subunit protein (activin AB) (1-3). Activin A has also been shown to promote differentiation of a variety of erythroleukemic cells in vitro and synergize with erythropoietin in stimulating erythroid colony formation in primary bone marrow cultures (4-6). Activin A has been reported previously that inhibit A (αβₐ) and inhibit B (αβₐ) exist in porcine follicular fluid. Inhibin A and inhibin B have similar biological potencies in the rat primary pituitary assay (7), and inhibin A has been shown to antagonize the actions of activin A on K562 cells and bone marrow cultures (5, 6). Activin B (βBβB) has never been isolated from follicular fluid, suggesting that it is either not synthesized or is present at much lower levels than activin A and activin AB. In the present study we sought to synthesize activin B firstly to ascertain whether such a molecule could be formed and secondly to determine if it possesses similar biological activities and potencies as those of the two previously described activins.

RESULTS

Complete Sequence of βB Precursor

In our earlier work (8) we reported a partial cDNA sequence of the human inhibin βB-subunit. Subsequent attempts at obtaining full-length cDNA clones from specifically primed cDNA libraries were frustrated by the presence of a GC-rich sequence at the 5' end of the βB mRNA. Translation of the longer βB cDNA clones started with an unusual proline-rich sequence. To confirm this sequence information, a Charon 4A human genomic library was screened with an oligonucleotide whose sequence was derived from the 5'-end of a βB cDNA clone. One X clone was obtained and restriction enzyme mapping revealed a 12-kilobase (kb) EcoRI fragment which hybridized to both 5' and 3' end βB DNA probes. The 12-kb EcoRI fragment was subcloned into pBR322, and a detailed restriction map was deduced as shown in Fig. 1A. A 600-basepair (bp) PstI fragment and an 800-bp BamHI fragment which hybridized to the 5' end of a βB cDNA clone. One X clone was obtained and restriction enzyme mapping revealed a 12-kilobase (kb) EcoRI fragment which hybridized to both 5' and 3' end βB DNA probes. The 12-kb EcoRI fragment was subcloned into pBR322, and a detailed restriction map was deduced as shown in Fig. 1A. A 600-basepair (bp) PstI fragment and an 800-bp BamHI fragment which hybridized to the 5'-oligonucleotide were subcloned into M13 vectors and completely sequenced. The complete sequence of the 5' end of the βB gene, commencing at a BamHI site, is shown in Fig. 1B. Translation of the DNA sequence revealed an open reading frame colinear with the previously deduced protein sequence. An initiator methionine was followed by a characteristically hydrophobic signal sequence and the proline-rich sequence previously observed in βB cDNA clones. This sequence predicts a prepro inhibin βB-subunit of 407 amino acids. The proline-rich region, which contains 12 proline residues in the space of 16 residues, is located between the signal sequence and the first two cysteine residues.
of the \( \beta_n \) precursor. The corresponding region of the human \( \beta_n \) precursor contains no such related sequence (9).

The \( \beta_n \) coding sequences are contained within two exons, with a 2.5-kb intron splitting the sequence at amino acid position 169 (Fig. 1B). No TATAAA-like sequence is present in the 5' flanking region of the \( \beta_n \) gene, which is extremely GC rich. The 5' flanking sequence contains a total of eight potential Sp1-binding sites, with the first one located 85 bp 5' of the initiator ATG (9, 10). Mammalian and viral promoters which use Sp1 generally have the cap site located 40-70 bp 3' of the first Sp1-binding sites (10). Although the 5' end of the \( \beta_n \) mRNA has not been mapped, it is tempting to postulate that the cap site or sites are located within the 85 bp 3' of the first Sp1-binding site. The sequence 5' of the Sp1-rich sequence contains three potential cAMP regulatory sequences (11). This is not unexpected, since Sertoli cell production of inhibin has been shown to be up-regulated by cAMP via the actions of FSH (12). This is likely to involve the \( \beta_n \) gene, since it is the major \( \beta \)-chain expressed in adult male rats (13). We cannot discount that further 5' exons may exist within the 5' untranslated sequence of the \( \beta_n \) gene.

Expression of Activin B

To construct a full-length coding sequence for the \( \beta_n \)-subunit, a 5' end genomic fragment of the \( \beta_n \) gene was linked in frame to a partial \( \beta_n \) cDNA clone, as outlined in Fig. 2. This DNA fragment containing the complete coding region was inserted into a cytomegalovirus promoter expression vector, creating the plasmid pRK\( \beta_n \) (14, 15). A mammalian kidney cell line transfected with pRK\( \beta_n \) was shown by metabolic labeling to secrete a band of about 22K mol wt (Fig. 3, lane 5). The 22K protein was reduced with \( \beta \)-mercaptoethanol to a band of about 20K (Fig. 3, lane 5). The amounts of both homodimers formed are significantly greater than that of the heterodimer, suggesting that each chain preferentially forms homodimers. As previously shown for activin A biosynthesis, the pro region of \( \beta_n \) precursor is secreted as an approximately 40K band with the activin dimer. Unpublished observations suggest that the pro region of the activins may function as a binding protein for the activin dimer, and that the mature and pro regions are secreted as a noncovalently linked complex.

To construct a stable cell line secreting activin B, a mammalian kidney cell line was cotransfected with pRK\( \beta_n \) and a plasmid containing a neomycin resistance gene. A total of 24 G418 resistant clones were screened for activin B secretion by metabolic labeling. Supernatants from each of the clones that secreted the 22K \( \beta_n \) dimer were shown to contain biologically competent activin B, as judged by the rat pituitary bioassay (data not shown). Unpublished recombinant activin A as a standard, clone 1106 was found to secrete between 1-2 \( \mu \)g/ml activin-like biological activity. Forty liters of clone 1106 were grown up in roller bottles, and the supernatants were used as starting material to purify activin B. Standard protein chemistry methods were used to purify activin B to homogeneity (Schmelzer, C., in preparation).

Biological Activities of Activin B

Purified activin B stimulated FSH secretion by cultured pituitary cells. A representative dose-response curve is shown in Fig. 4A. The ED\(_{50}\) was 2.1 ± 0.6 ng/ml (mean ± SEM; \( n = 5 \)), which is comparable to that for activin A (2.1 ± 1.1 ng/ml (mean ± SD; \( n = 2 \)). Purified activin B also stimulated hemoglobin accumulation in the K562 human erythroleukemia cell line (Fig. 4B). The ED\(_{50}\) in this assay was 2.2 ± 0.4 ng/ml (mean ± SE; \( n = 5 \)), which again agrees well with that obtained for activin A (14).

DISCUSSION

In this study we have determined the complete amino acid sequence of the prepro form of human inhibin \( \beta_n \)-subunit. The human \( \beta_n \) gene was shown to consist of

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**Fig. 1. Genomic Structure and Sequence of Human Inhibin \( \beta_n \) Subunit**

A, Restriction map of a 12-kb EcoRI fragment containing the human \( \beta_n \) gene. Location of restriction sites (E, EcoRI; H, HindIII; B, BamHI; P, PstI; N, NcoI; S, Sall; SI, SalI; Pv, PvuII) are indicated by vertical lines. The coding regions of exons 1 and 2 are shown as boxes. The open box region indicates the signal sequences; the dotted box indicates the precursor sequences, and the stippled box indicates the mature sequences. The 5' and 3' ends of the primary transcription unit are not known and, thus, are indicated by an open-ended thick black line. B, DNA sequence of the human \( \beta_n \) inhibin gene. The possible Sp1-binding sites and cAMP response elements are underlined in the sequence. The coding region has been translated, and the numbering commences at the initiator methionine. The intron sequence is shown in smaller case printing.
Fig. 2. Construction of Human βB-Subunit Expression Vector

As detailed in Materials and Methods, the full-length βB coding sequence was constructed from a genomic fragment, synthetic DNA, and a human ovarian βB DNA clone. The plasmid pRKβB was used for transfections.

Fig. 3. Metabolic Labelings of Transfected Cells

A. Autoradiogram of a SDS-polyacrylamide gel of 35S-labeled proteins. Supernatants from untransfected cells (2), βA activin stable cell line (3), cells transfected with pRKβA and pRKβB (4), and cells transfected with pRKβB (5). Lane 1 shows mol wt markers (kilodaltons). B. Autoradiogram of a SDS-polyacrylamide gel of 35S-labeled proteins. Lane 1, Mol wt markers; lane 2, cell supernatant of pRKβB-transfected cells; lane 3, immunoprecipitation of the same supernatant with an activin A polyclonal antibody; lane 4, cell supernatant of cells transfected with pRKβA and pRKβB; lane 5, immunoprecipitation of the pRKβA/pRKβB supernatant with an activin A polyclonal antibody.

two exons which contain all of the coding sequences; this is analogous to the genes for the human α and βA inhibin subunits (Mason, A., and L. Berkemeier, in preparation). The conservation of genomic structure suggests a common origin for these three related proteins. Interestingly, the genomic structure of the related transforming growth factor-β and Mullerian inhibiting substance genes has diverged considerably since these members of the transforming growth factor-β superfamily are encoded on seven and five exons, respectively (16, 17). The sequence of the βB 5’ flanking region contains a total of eight potential Sp1-binding sites spread over approximately 180 bp. No TATAAA-like sequences are present in the 766 bp of 5’ flanking, suggesting that the Sp1 sites may act as the major transcriptional activator sequences for this gene. The
Fig. 4. In Vitro Activities of Recombinant Human Activin B 
A. Stimulation of FSH secretion by human activin A (□) and activin B (●). Pituitary cells were cultured for 2 days in the presence of the indicated concentrations of activin A and activin B. Medium samples were then assayed for FSH. The curve was derived with a four-parameter curve-fitting program. 
B. Induction of hemoglobin accumulation by recombinant human activin B. K562 cells were cultured for 4 days in the presence of the indicated concentrations of activin B. Cells were then assayed for hemoglobin content (see Materials and Methods). The curve was derived with a four-parameter curve-fitting program.

Comparison of the βA and βB prepro amino acid sequences revealed an overall homology of approximately 45%, with the majority of the homology located in the mature C terminal sequences (~70%) (8, 18). The individual precursor regions display several regions of homology with each other, particularly in the NH₃-terminal half of the molecule. The βB precursor contains an unusually proline-rich sequence which is not present in the βA precursor. A similarly unusual sequence of lysine and glutamic acid repeats is contained near the COOH terminus of the βA precursor. Such unusual sequences have been seen in other proteins (19-22). The function of these repeat motifs is not known.

In summary, recombinant activin B has been produced using recombinant DNA technology. The data presented here show that activin B, which has never been purified from natural sources, has the potential to modulate FSH secretion and act as an erythroid differentiation factor. The use of recombinant activin B to generate monoclonal antibodies specific for activin B should enable us to determine when and where it is produced in vivo. Such results may provide clues as to possible individual roles for activin A and activin B.

MATERIALS AND METHODS

Isolation of βB Genomic Clones
An end-labeled synthetic oligonucleotide of sequence 5’-GGATCCCCGGGTGGCTCGCAGGACACCTGTACGTCGTG-3’ was synthesized and used as a probe in in situ hybridization experiments to detect the presence of βB mRNA in the primate ovary.
CGGCCGCGTCCGGCGCGCCAGAGGAGCT-3' was used to sequence screen a total of 1 × 10^6 clones from a Charon 4A genomic library. Clones were screened, and positives were purified as described by Maniatis et al. (25). A 12-kb EcoRI and a 4.2-kb BamHI fragment derived from the λ clone λO5' were subcloned into the plasmid pBR322. Purified plasmid was used to perform multiple restriction digests. Digests were analyzed by agarose gel electrophoresis and Southern blotting (25). A mixture of synthetic and cDNA-derived probes were used to map the exon-intron arrangement of the β2 gene. Relevant fragments were subcloned into appropriate M13 vectors and sequences by the dideoxy method (26). These included M13β5', which contains the 5' coding sequence contained on a 575-bp PstI fragment (Fig. 2).

Assembly of β2 Expression Vector and Transfections

The sequence AAGGCA, located 7 bp 3' of the β2 stop codon, was changed by in vitro mutagenesis to an HindIII restriction site. This facilitated the removal of β2 3' untranslated region. Single stranded M13β5'3'Hd DNA was made double stranded in a Klenow reaction using the universal 17-mer lac primer. The ds DNA was digested with HindIII and PstI, and the 1099-bp fragment was purified by polyacrylamide gel electrophoresis (PAGE). This fragment was ligated to a similarly prepared 261-bp NcoI-PstI from M13β5' along with a 25-bp EcoRI-NcoI adaptor (sequence Fig. 2). The resultant 1385-bp EcoRI-HindIII fragment was cloned into the EcoRI and HindIII sites of the cytomegalovirus vector pRK5. Plasmid pRK5β2 was purified by CsCl gradients (Fig. 2). The pRK5 expression vector contains the CMV enhancer, promoter, and splice donor sequence, and an immunoglobulin variable splice acceptor followed by the poly(A) addition site and transcription terminator of the early region of SV40 (15).

Transient transfections were performed by the calcium phosphate precipitation method using 5 μg CsCl gradient-purified plasmid per 60-mm dish (27). Stable clones were selected from transfections which included 0.1 μg pRSV neo in 800 μg/ml G418. Cells were cultured in either Dulbecco's Modified Eagle's Medium-Ham's F-12 (1:1) containing 10% fetal bovine serum or, for serum-free culture, Dulbecco's Modified Eagle's Medium-Ham's F-12 (1:1) plus 5 μg/ml insulin.

Metabolic Labeling and Immune Precipitations

Transiently transfected or stable clones were grown to confluency in a 60-mm dish and labeled for 1 h in 1 ml serum-free medium supplemented with 100–250 μCi/ml [35S]methionine and [35S]cysteine (Amersham Corp, Arlington Heights, IL). One hour later, cells were rinsed with PBS and chased for 5 h in 1 ml serum-free medium. Labeled supernatants were then either analyzed by sodium dodecyl sulfate-PAGE (SDS-PAGE) or immunoprecipitated before SDS-PAGE (28). For the immunoprecipitations, a goat polyclonal antibody prepared against recombinant activin A was used. Fifty microtiter of supernatant were incubated with 2 μg activin A-affinity-purified immunoglobulin G at room temperature for 1 h. Immunocomplexes were precipitated using protein A. Samples were boiled in SDS loading buffer for 3 min before analysis on 12% polyacrylamide SDS-gels. Polyacrylamide gels were fixed in acetic acid, treated with Enhance (New England Nuclear, Boston, MA), and dried before autoradiography.

Pituitary Cell Bioassay and K562 Bioassay

The ability of activin B to stimulate FSH secretion was asessed in cultured pituitary cells, as described previously (14). Induction of hemoglobin was measured in the K562 human erythroleukemia cell line, as described previously (14). Hemoglobin was measured by the method of Clarke et al. (29). ED_50 values were derived using a four-parameter curve-fitting program based on the algorithm described by Marquardt (30).

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