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MOLECULAR CLONING AND BINDING PROPERTIES OF THE HUMAN TYPE II ACTIVIN RECEPTOR

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SUMMARY: A full-length cDNA for the type II human activin receptor was cloned by hybridization from a human testis cDNA library. The sequence encodes a 513 amino acid protein that is 99% identical, at the amino acid level, with the mouse type II activin receptor. The type II human activin receptor consists of an extracellular domain that specifically binds activin A with a Kd of 360 pM, a single-membrane spanning domain, and an intracellular kinase domain with predicted serine/threonine specificity.

Activins are members of a functionally diverse group of growth and differentiation factors including the inhibins, the transforming growth factors-β, Mullerian duct inhibiting substance, the Drosophilia *decapentaplegic* gene product, several bone morphogenetic proteins, and the Vg-related genes (1). Activins have a broad anatomic distribution and are involved in the regulation of multiple biological events, including control of the secretion and expression of the anterior pituitary hormones, FSH, GH, and ACTH (2), proliferation of many cell lines (3-5), erythropoiesis (6, 7), neuron survival (8, 9), hypothalamic oxytocin secretion (10), and early embryonic development (11-13). There are three activins (A, B, and AB) which are hetero/homodimers of two closely related β subunits ($\beta_A\beta_A$, $\beta_B\beta_B$, and $\beta_A\beta_B$, respectively) (2, 14). Activin binding sites have been identified on a number of activin-responsive cells (5, 15-18), and chemical cross-linking suggests that multiple binding species exist on the cell surface (18-20). The predominant cross-linked bands at approximately 65kDa and 85kDa represent the type I and type II activin receptors, respectively, based on the nomenclature defined for the TGF-β receptors (21).

DNAs coding for a type II activin receptor (ActRII) were cloned from AtT20 mouse corticotrophic cells using an expression strategy (22). The mouse (m) ActRII is predicted to be a 513 amino acid protein comprised of an extracellular domain that binds activin A with high affinity, a single membrane-spanning domain, and an intracellular kinase domain with predicted serine/threonine specificity. We report here the sequence of the human (h) ActRII, which is 99% identical to mActRII. The type II activin receptor thus appears to be an unusually highly conserved protein among mammals.

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MATERIALS AND METHODS

Recombinant human (rh) activin A was generously supplied by Anthony Mason, Laura Bald, Ralph Schwall, and Jennie Mather of Genetech Incorporated.

Library screening: Three different libraries were screened using either the full-length mouse type II activin receptor or a fragment representing the kinase domain of mActRII. All probes were labeled with [α^{32}P]dCTP using a Multiprime DNA labeling kit from Amersham. Approximately 10^6 λphage plaques were screened in each of the following libraries: human placenta cDNA library in λgt10 (23) plated on *E. coli* strain C600, human adult liver cDNA library in λzap (24) plated on *E. coli* strain XL1-blue, and human testis cDNA library in λgt11, purchased from Clontech (Palo Alto, CA), plated on *E. coli* strain Y1090. The plaques were lifted onto Nylon membranes (Schleicher & Schuell), denatured with 1.5 M NaCl, 0.5 M NaOH, neutralized with 1.5 M NaCl, 0.5 M Tris, pH 8.0, and rinsed with 3XSSC. The membranes were prehybridized 1-3 hrs at 42°C in a hybridization buffer (4XSSC, 4X Denhardt's, 40 mM Na phosphate, pH 6.5, 170 μg/ml salmon sperm DNA, 0.1% SDS, 20% formamide) and then hybridized 16-20 hrs at 42°C in the same buffer solution with ~10^6 cpm per ml of labeled probe. After hybridization the filters were washed 4X with 2XSSC, 0.1% SDS at 42°C.

Positive clones were isolated and purified through three successive rounds of screening-hybridization. Helper phage R408 (Biorad) was used for *in vivo* excision of λzap clones from the liver library. Clones from the placental and testis libraries were subcloned into pBluescript II SK (Stratagene). Double stranded DNA was sequenced by the dideoxy chain termination method using the Sequenase kit (United States Biochemical Corporation).

Cell culture and transfection: A BamHI-XhoI fragment containing the full-length hActRII clone was subcloned into pcDNA1 (Invitrogen). 2x10^5 COS cells, in Dulbecco's Modified Eagle's medium (DME) with 10% fetal calf serum (FCS), were plated in six-well plates and allowed to attach overnight. Cells were subjected to DEAE-Dextran mediated transfection as follows. Cells were washed once and 1.5 ml of serum-free DME containing 100 μM chloroquine was added to each well. A mixture of DME, 100 μM chloroquine containing 500 μg/ml DEAE-Dextran and 1 μg of hActRII was added to each well in a volume of 200 μl. The cells were incubated at 37°C for 4 hrs, then the media was removed and the cells were treated with 10% DMSO in HEPES buffered saline (HDB) for 2 min. Fresh DME, 10% FCS was added and the cells were incubated for 3 days at 37°C and then assayed.

Iodination of activin A: 5 μg activin A was iodinated with Na[^{125}I] by chloramine T oxidation to a specific activity of 50-90 μCi/μg. ^{125}I-labeled activin A was purified on a 0.7x20 cm G-25 column in 50 mM phosphate pH 7.4, 1% BSA.

Radioreceptor assay: Transfected COS cells were washed 2X with HDB, 0.1% BSA. 0.5 ml of HDB, 0.1% BSA containing 100,000 cpm (approximately 1 ng, 75 pM) ^{125}I activin A was added to each well, with varying doses of unlabeled competitor hormone, and the cells were incubated at 22°C for 90 min. Following binding, the cells were washed 3X with cold HDB, solubilized in 0.5 ml 0.5 N NaOH, removed from the dish, and radioactivity was measured in a gamma counter. Data are expressed as the percent of bound counts over total counts of ^{125}I-labeled activin A added to each well. Binding parameters were determined using the program LIGAND (25).

Chemical cross-linking: Transfected COS cells or K562, human erythroleukemia, cells were washed 2X with HDB, scraped off the dish, incubated for 90 min at 22°C under constant rotation in 0.5 ml HDB containing 10^6 cpm (approximately 750 pM) ^{125}I activin A with or without 500 ng (36 nM) unlabeled activin A. Cells were diluted with 1 ml HDB, pelleted by centrifugation and resuspended in 0.5 ml HDB. Disuccinimidyl suberate (freshly dissolved in DMSO) was added to 500 μM and the cells incubated at 0°C for 30 min. The cross-linking was terminated by addition of 1 ml 50 mM Tris-HCl pH 7.5, 100 mM NaCl. The cells were then pelleted by centrifugation, resuspended in 100 μl 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 100 KIU/ml trasylol, and incubated at 0°C for 60 min. The samples were centrifuged 5 min at 13,000xg and the Triton-soluble supernatants analyzed by SDS-PAGE, in the presence of 2 mM DTT, using 8.5% or 10% gels. The gels were dried and subjected to autoradiography for 3-4 days.

RESULTS AND DISCUSSION

cDNA isolation, sequencing, and analysis: Three different human cDNA libraries were screened by hybridization in order to obtain a full-length clone for hActRII. Clones of 1.6 kb from a

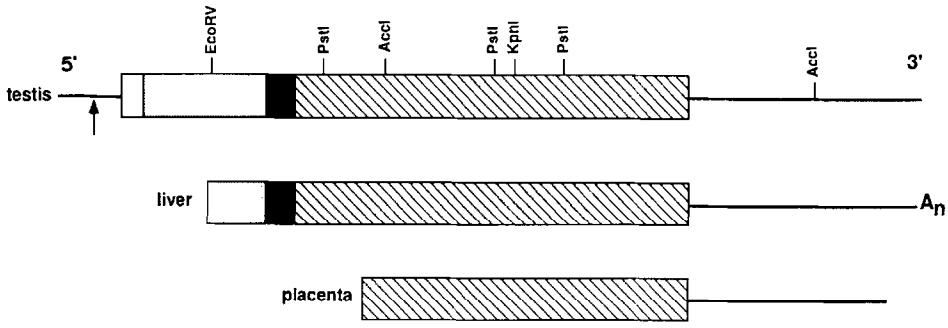

Figure 1. Structure and restriction map of hActRII. Boxes represent coding sequences: white is the signal peptide, grey is the extracellular ligand binding domain, black is the transmembrane domain, and hatched is the intracellular kinase domain. Polyadenylation of the liver clone is indicated. The site of divergence with mActRII is marked by an arrow.

placenta library and 2.0 kb from a liver library were incomplete at the 5' end; a full-length clone (2.5 kb) was obtained from a testis library (Fig. 1). The insert was subcloned into Bluescript and completely sequenced. The placenta and liver clones were partially sequenced and found to be identical to the testis clone. Further experiments were conducted using the full-length testis hActRII.

The human clone, with a 173 bp 5' untranslated region and 672 base pairs of untranslated 3' DNA, codes for a 513 amino acid protein (Fig. 2). As in mActRII, the 19 amino acid stretch at the amino terminus is assumed to be a signal peptide and a single putative 26 residue membrane-spanning region occurs between amino acids 117-142 (22). Therefore, the mature activin receptor is predicted to be a 494 amino acid membrane protein of Mr 56,000 with a 116 amino acid N-terminal extracellular ligand binding domain, and a 352 amino acid intracellular signalling domain (Fig. 1). The position of all cysteines, as well as sites of N-linked glycosylation are conserved. The nucleotide sequences of hActRII and mActRII were 91% identical overall and 95% identical in the coding region; the amino acid sequences were over 99% identical, with only two amino acid differences (mouse arginine vs. human lysine at position 39 and mouse isoleucine vs. human valine at position 92). Although the degree of conservation is unusually high, the possibility of contamination by a second allele of the mouse gene is unlikely because of the identity between the clones from three independent human libraries. Similar to ActRII, the respective activin β_A and β_B subunits are strongly conserved (26-28), suggesting that the structures of these molecules are likely to be critical for biological function.

The 3' untranslated regions of the human and mouse clones are 91% identical; however, the 5' untranslated regions are only 60% identical. That conservation is due to 96% identity in the

Figure 2. Nucleotide and translated amino acid sequence of hActRII. The signal peptide, transmembrane domain, and polyadenylation site are indicated by a single underline. Potential sites of N-glycosylation are indicated by a double underline. The ends of the kinase domain are indicated by arrows. The site of divergence with mActRII is indicated with an asterisk.

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   1 GGGGCCGCCCCTTCCCCGCGCCGCAGCCGCCTCGCCGCCACCGCCGCGAGCTCGGCCGCCAGTGGTCCTCGGACTTTAGGTGTCTGGGTT   90

  91 GAAGGAGGTTTGTCTCCGAGGAAGACCCAGGGAACTGGATATCTAGCGAGAACTTCCTCCGGATTCCCCGGCGCCTCGGGAAAATGGGAG  180
 -46   *                                                                                 M G A  -17

 181 CTGCTGCAAAGTTGGCGTTTGCCGTCTTTCTTATCTCCTGTTCTTCAGGTGCTATACTTGGTAGATCAGAAACTCAGGAGTGTCTTTTCT  270
 -16  A A K L A F A V F L I S C S S G A I L G R S E T Q E C L F F  14

 271 TTAATGCTAATTGGGAAAAAGACAGAACCAATCAAACTGGTGTTGAACCGTGTTATGGTGACAAAGATAAACGGCGGCATTGTTTTGCTA  360
  15   N A N W E K D R T N Q T G V E P C Y G D K D K R R H C F A T  44

 361 CCTGGAAGAATATTTCTGGTTCCATTGAAATAGTGAAACAAGGTTGTTGGCTGGATGATATCAACTGCTATGACAGGACTGATTGTGTAG  450
  45   W K N I S G S I E I V K Q G C W L D D I N C Y D R T D C V E  74

 451 AAAAAAAAGACAGCCCTGAAGTATATTTTTGTTGCTGTGAGGGCAATATGTGTAATGAAAAGTTTTCTTATTTTCCGGAGATGGAAGTCA  540
  75   K K D S P E V Y F C C C E G N M C N E K F S Y F P E M E V T  104

 541 CACAGCCCACTTCAAATCCAGTTACACCTAAGCCACCCTATTACAACATCCTGCTCTATTCCTTGGTGCCACTTATGTTAATTGCGGGGA  630
 105   Q P T S N P V T P K P P Y Y N I L L Y S L V P L M L I A G I  134

 631 TTGTCATTTGTGCATTTTGGGTGTACAGGCATCACAAGATGGCCTACCCTCCTGTACTTGTTCCAACTCAAGACCCAGGACCACCCCCAC  720
 135  V I C A F W V Y R H H K M A Y P P V L V P T Q D P G P P P P  164

 721 CTTCTCCATTACTAGGTTTGAAACCACTGCAGTTATTAGAAGTGAAAGCAAGGGGAAGATTTGGTTGTGTCTGGAAAGCCCAGTTGCTTA  810
 165   S P L L G L K P L Q L L E V K A R G R F G C V W K A Q L L N  194

 811 ACGAATATGTGGCTGTCAAAATATTTCCAATACAGGACAAACAGTCATGGCAAAATGAATACGAAGTCTACAGTTTGCCTGGAATGAAGC  900
 195   E Y V A V K I F P I Q D K Q S W Q N E Y E V Y S L P G M K H  224

 901 ATGAGAACATATTACAGTTCATTGGTGCAGAAAAACGAGGCACCAGTGTTGATGTGGATCTTTGGCTGATCACAGCATTTCATGAAAAGG  990
 225   E N I L Q F I G A E K R G T S V D V D L W L I T A F H E K G  254

 991 GTTCACTATCAGACTTTCTTAAGGCTAATGTGGTCTCTTGGAATGAACTGTGTCATATTGCAGAAACCATGGCTAGAGGATTGGCATATT 1080
 255   S L S D F L K A N V V S W N E L C H I A E T M A R G L A Y L  284

1081 TACATGAGGATATACCTGGCCTAAAAGATGGCCACAAACCTGCCATATCTCACAGGGACATCAAAAGTAAAAATGTGCTGTTGAAAAACA 1170
 285   H E D I P G L K D G H K P A I S H R D I K S K N V L L K N N  314

1171 ACCTGACAGCTTGCATTGCTGACTTTGGGTTGGCCTTAAAATTTGAGGCTGGCAAGTCTGCAGGCGATACCCATGGACAGGTTGGTACCC 1260
 315   L T A C I A D F G L A L K F E A G K S A G D T H G Q V G T R  344

1261 GGAGGTACATGGCTCCAGAGGTATTAGAGGGTGCTATAAACTTCCAAAGGGATGCATTTTTGAGGATAGATATGTATGCCATGGGATTAG 1350
 345   R Y M A P E V L E G A I N F Q R D A F L R I D M Y A M G L V  374

1351 TCCTATGGGAACTGGCTTCTCGCTGTACTGCTGCAGATGGACCTGTAGATGAATACATGTTGCCATTTGAGGAGGAAATTGGCCAGCATC 1440
 375   L W E L A S R C T A A D G P V D E Y M L P F E E E I G Q H P  404

1441 CATCTCTTGAAGACATGCAGGAAGTTGTTGTGCATAAAAAAAAGAGGCCTGTTTTAAGAGATTATTGGCAGAAACATGCTGGAATGGCAA 1530
 405   S L E D M Q E V V V H K K K R P V L R D Y W Q K H A G M A M  434

1531 TGCTCTGTGAAACCATTGAAGAATGTTGGGATCACGACGCAGAAGCCAGGTTATCAGCTGGATGTGTAGGTGAAAGAATTACCCAGATGC 1620
 435   L C E T I E E C W D H D A E A R L S A G C V G E R I T Q M Q  464

1621 AGAGACTAACAAATATTATTACCACAGAGGACATTGTAACAGTGGTCACAATGGTGACAAATGTTGACTTTCCTCCCAAAGAATCTAGTC 1710
 465   R L T N I I T T E D I V T V V T M V T N V D F P P K E S S L  494

1711 TATGATGGTTGCGCCATCTGTGCACACTAAGAAATGGGACTCTGAACTGGAGCTGCTAAGCTAAAGAAACTGCTTACAGTTTATTTTCTG 1800

1801 TGTAAAATGAGTAGGATGTCTCTTGGAAATGTTAAGAAAGAAGACCCTTTGTTGAAAAATGTTGCTCTGGGAGACTTACTGCATTGCCGA 1890

1891 CAGCACAGATGTGAAGGACATGAGACTAAGAGAAACCTTGCAAACTCTATAAAGAAACTTTTGAAAAAGTGTACATGAAGAATGTAGCCC 1980

1981 TCTCCAAATCAAGGATCTTTTGGACCTGGCTAATGGAGTGTTTGAAAACTGACATCAGATTTCTTAATGTCTGTCAGAAGACACTAATTC 2070

2071 CTTAAATGAACTACTGCTATTTTTTTTAAATCAAAAACTTTTCATTTCAGATTTTAAAAAGGGTAACTTGTTTTTATTGCATTTGCTGTT 2160

2161 GTTTCTATAAATGACTATTGTAATGCCAATATGACACAGCTTGTGAATGTTTAGTGTGCTGCTGTTCTGTGTACATAAAGTCATCAAAGT 2250

2251 GGGGTACAGTAAAGAGGCTTCCAAGCATTACTTTAACCTCCCTCAACAAGGTATACCTCAGTTCCACGGTTGCTAAATTATAAAATTGAA 2340

2341 AACACTAACAAAATTTGAATAATAAATCGATCCATGTTTCCC                                                2382
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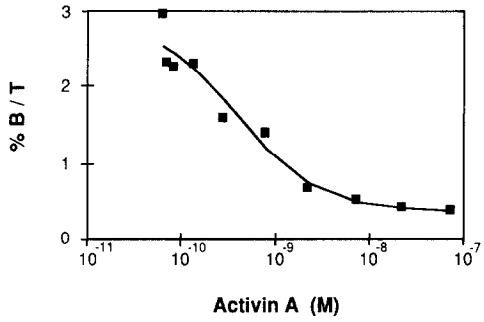

Figure 3. ^{125}I-labeled activin A binding to COS cells transfected with hActRII. Binding was performed on cell monolayers as described in Materials and Methods and was competed with varying doses of unlabeled activin A. Data are expressed as percent total binding.

80 bp preceding the open reading frame; the sequences of the two clones 5' to that point diverge, suggesting alternate processing of the RNAs. All three human clones contain a consensus polyadenylation signal in a site analagous to one observed, but not found to be utilized, in mActRII. In the liver clone a poly A tail followed the site, implying that it is used *in vivo*.

Binding properties of hActRII: To study the binding properties of hActRII, the full-length cDNA was subcloned into the expression vector pcDNA1, transiently transfected into COS cells, and assayed for binding of activin A (Fig 3). Competition binding experiments indicated that the Kd

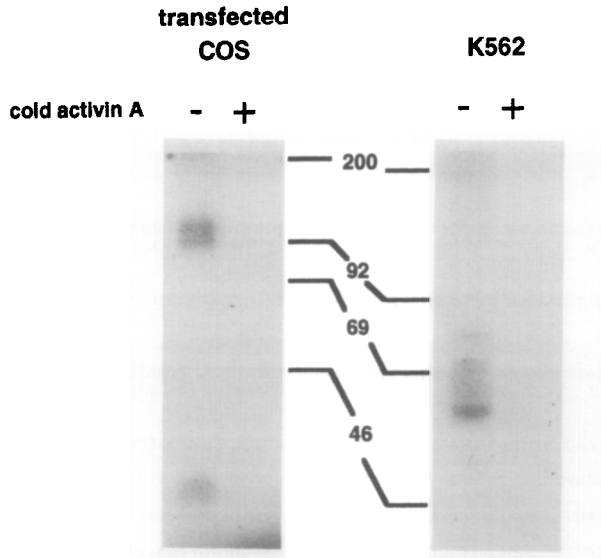

Figure 4. Cross-linking of ^{125}I activin A to transfected COS cells and K562 cells. Iodinated activin A was bound to COS cells transfected with hActRII or K562 cells, cross-linked with DSS, and run on SDS-PAGE in the presence of 2mM DTT as described in Methods. Binding was performed in the absence or presence of unlabeled activin A. The sizes of molecular weight standards are indicated in kDa.

for hActRII is 360 pM. This Kd is within the range of values reported for other cloned activin receptors (22, 29) or for activin binding to various cell lines (15, 16, 18, 19, 30). The sizes of the activin receptors in transfected COS cells, as well as human K562 erythroleukemia cells, were analyzed by affinity cross-linking of ^{125}I activin A, using the bifunctional chemical cross-linker disuccinimidyl suberate (Fig. 4). Two major bands are seen for transfected COS cells expressing hActRII. The 85 kDa band represents the receptor affinity labeled with one β-subunit of activin. This would yield a 70 kDa protein after subtracting a 15 kDa monomer of labeled activin A. The difference between the predicted molecular weight of 56 kDa, based on the hActRII amino acid sequence, and the 70 kDa cross-linked band is likely due to N-glycosylation of the receptor protein. The 100 kDa band may be receptor cross-linked to both β-subunits, or may contain other post-translational modifications. Cross-linking ^{125}I-activin A to K562 cells yields bands at 84, 78, and 65 kDa, as previously observed in K562 and erythroid progenitor cells (31). The 84 and 78 kDa bands represent the type II activin receptor or a closely related family member. Recent data report the type IIB activin receptor, which has four alternatively spliced isoforms, and specifically binds activin A. Cross-linked bands in the range of 78-88 kDa have been reported for cells transfected with ActRII and ActRIIB (29, 32). The 65 kDa band represents the type I activin receptor.

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