Expression and regulation of human activins and their receptors

by

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Academic dissertation

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List of original publications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I Hildén K, Tuuri T, Erämaa M, Ritvos O: Expression of type II activin receptor genes during differentiation of human K562 cells and cDNA cloning of the human type IIB activin receptor. Blood 83:2163-2170, 1994

II Tuuri T, Erämaa M, Hildén K, Ritvos O: The tissue distribution of activin β_A- and β_B-subunit and follistatin messenger ribonucleic acids suggests multiple sites of action for the activin-follistatin system during human development. J Clin Endocrinol Metab 78:1521-1524, 1994

III Erämaa M, Hildén K, Tuuri T, Ritvos O: Regulation of inhibin/activin subunit messenger ribonucleic acids (mRNAs) by activin A and expression of activin receptor mRNAs in cultured human granulosa-luteal cells. Endocrinology 136:4382-4389, 1995

IV Hildén K, Tuuri T, Erämaa M, Ritvos O: Co-ordinate expression of activin A and its type I receptor mRNAs during phorbol ester-induced differentiation of human K562 erythroleukemia cells. Mol Cell Endocrinol 153:137-145, 1999

Abbreviations

8-Br-cAMP	8-bromo cyclic adenosine monophosphate
aa	amino acid
ActR	activin receptor
Acvr	activin receptor gene
ALK	activin receptor-like kinase
AMH(R)	anti-Müllerian hormone (receptor)
BMP(R)	bone morphogenetic protein (receptor)
bp	base pair
BSA	bovine serum albumin
C-terminal	carboxyterminal
cDNA	complementary DNA
co-Smad	common mediator Smad
CRE	cAMP responsive element
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
dpp	decapentaplegic
ECL	enhanced chemiluminescense
EDF	erythroid differentiation factor
FAST	forkhead activin signal transducer
FCS	fetal calf serum
FISH	fluorescence in situ hybridisation
FS	follistatin
FSH	follicle stimulating hormone
FSRP	follistatin-related protein
GDF	growth/differentiation factor
GL	granulosa luteal
hCG	human chorionic gonadotropin
I-Smad	inhibitory Smad
kb	kilobase
kDa	kilodalton
mRNA	messenger RNA
N-terminal	amino-terminal
NCS	newborn calf serum
PAGE	polyacrylamide gel electrophoresis
R-Smad	receptor-activated Smad
RT-PCR	reverse-transcription polymerase chain reaction
SARA	Smad anchor for receptor activation
SBS	Smad-specific binding site
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
TβR	transforming growth factor–β receptor
TBS	Tris buffered saline
TGF-β	transforming growth factor-β
TPA	12-0-tetradecanoyl phorbol 13-acetate
TRE	TPA responsive element
Xnrs	*Xenopus* nodal related proteins

Introduction

Activins are dimeric polypeptide growth factors which belong to the transforming growth factor-β (TGF−β) superfamily. This extended family comprises a large number of structurally related polypeptide growth factors that regulate many fundamental cellular processes, such as cell proliferation, lineage determination, differentiation and apoptosis (1, 2). In the TGF−β superfamily, activins are unique and multifunctional factors that can stimulate hormone production in ovarian and placental cells, support neuronal cell survival, influence cell-cycle progress positively or negatively depending on cell type, and induce mesodermal differentiation at least in amphibian embryos (3-6). Moreover, erythroid differentiation factor, isolated from the conditioned medium of stimulated human monocytic cell line, has been found to be identical to activin A (7). In several tissues, activin signalling is antagonized by its related heterodimer, inhibin. The nomenclature of inhibins and activins reflects their discovery as functional antagonists in the release of follicle-stimulating hormone (FSH) from the pituitary: inhibin prevents whereas activin promotes FSH secretion and synthesis (8). In addition, other proteins may regulate activin bioactivity as well. The activin binding-protein, follistatin (FS), is well characterized and the effects of activin can be counteracted by follistatin in all systems tested (4).

Learning the molecular basis of activin receptors provides a better understanding of the multifunctional nature of activins. To date extensive evidence obtained suggests a general signal transduction mechanism for TGF−β and related ligands. Two distinct transmembrane serine/threonine kinases known as type I and type II receptors are needed for signal transduction. Ligand induced association of type I and II receptors results in the phosphorylation and consequent activation of the type I receptor, which is required for downstream signal cascades. Smad proteins activated by type I receptors carry signal to the nucleus, and together with other proteins direct transcriptional responses (9-13).

At the beginning of the 1990s, when this study began, relatively little was known about the expression and regulation of activin β-subunits in human tissues. There was some experimental evidence that activins are involved in regulation of embryogenesis (14, 15) but there was no information concerning the expression of activin subunits or its receptors in developing human embryo. At that time only the mouse type II activin receptor had been discovered in vertebrates although high affinity binding sites for activin were detected in several different cell culture models, such as human erythroleukemia K562 cells (16, 17) and ovarian granulosa cells (18). The aim of this thesis was to clone human type II activin receptor cDNAs. Additionally, we wanted to study how activin β-subunits and activin receptor transcripts are regulated in activin responsive cell culture models.

Review of the literature

1. Structure and function of activin and activin binding proteins

1.1 Isolation of activins

Inhibin was isolated in 1985 from porcine ovarian follicular fluid, by four independent research groups, as a protein, which displayed potent activity to suppress follicle-stimulating hormone (FSH) secretion in cultured rat anterior pituitary cells (19-22). It was found to be a glycoprotein hormone of 32 kD consisting of two subunits, a common 18 kD α-subunit and either a 14.7 kD β_A-or a 14 kD β_B-subunit, yielding inhibin A or inhibin B, respectively. In side fractions during purification of inhibin 25 kD proteins that could promote FSH secretion *in vitro* were also isolated (23, 24). Surprisingly, these proteins turned out to be homo- or heterodimers of the β_A- or β_B-subunits linked together by disulphide bonds. The proteins were subsequently named as activin A ($\beta_A\beta_A$), activin B ($\beta_B\beta_B$) and activin AB ($\beta_A\beta_B$) (Fig. 1). Molecular cloning of β_A- and β_B-subunits from an human ovarian cDNA library revealed that the β-subunits were 70% homologous to each other, and the position of seven cysteine residues in the carboxy-terminus were perfectly conserved indicating structural similarity to other members of TGF-β superfamily (25, 26).

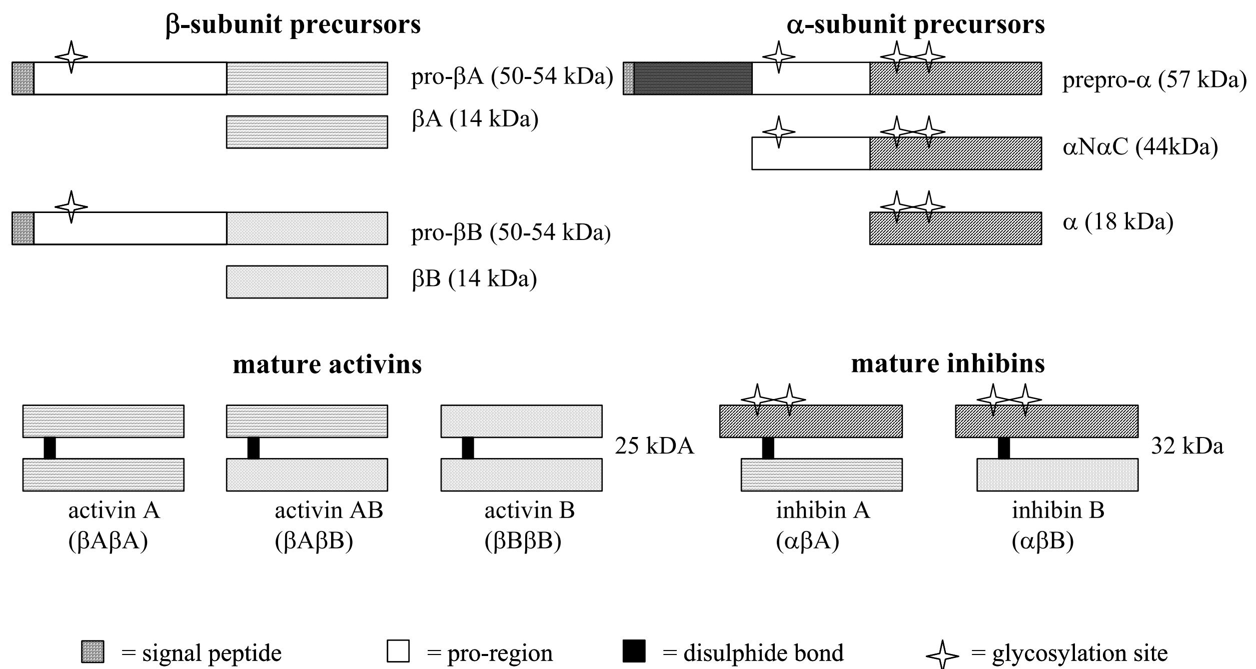


Figure 1. Structure of activin and inhibin polypeptide chains. The α- and β-subunits are produced as larger precursor proteins, prepro-α, pro-β_A and pro-β_B, that include a signal peptide and pro-region, both of which are cleaved to form the mature α- or β-subunit. Activins are dimers consisting of two β-subunits joined by disulphide bridges. Inhibins are heterodimers of α- and either β_A- or β_B-subunits. Size in kilodaltons (kDa) is indicated to the right of each subunit.

1.2 Activin subunits and their regulation

1.2.1. Human β_A-subunit

The human β_A-subunit mRNA encodes a 426 aa pre-proprotein containing an N-terminal 28 aa signal peptide followed by a pro-region (25). The C-terminal mature 116 aa polypeptide is proteolytically cleaved from the pre-pro-region at an arginine rich processing site (RXXR) to form the dimeric, bioactive protein (25, 27, 28). The proregion of β_A-subunit is involved in the correct folding, dimerization and secretion of the activin protein (29). In addition, all of the nine conserved cysteine residues in the mature part of the activin β_A-subunit are essential for either the biosynthesis or biological activity of activin A (30). Human (25), porcine (31), bovine (32), rat (33, 34) and mouse (35, 36) β_A-subunits are highly conserved and the mature parts of the proteins are identical between these species.

The human activin β_A-subunit gene is assigned to chromosome 7 locus 7p15-p14 (37). It consists of three exons, the first of which contains only 5' noncoding sequences (26, 38, 39). The human β_A-gene is transcribed to several distinct mRNAs in the size range of 1.7- to 6.4-kb (7, 40-43), probably reflecting the use of alternative polyadenylation signals (44) or different transcription start sites (45). The 5' noncoding region has been characterized in detail, leading to the identification of conserved promoter sequences with several potential enhancer sites. Of those sites, at least the 12-0-tetradecanoyl phorbol 13-acetate (TPA) responsive element (TRE) and cyclic AMP responsive element (CRE) binding sites are functionally active and are involved in regulation of β_A-subunit gene expression in human fibrosarcoma HT1080 cells (39). Recently, the structure of rat activin β_A-subunit gene has been analysed, showing strong conservation of the potential transcription factor binding sites (46). In human bone marrow cells two essential regions for the promoter activity have been found, one between 0.2- and 0.25-kb and the other 2.5- and 3.6-kb upstream of the start codon (45). The distal promoter region contains TATA-box whereas the other region does not contain TATA or CAAT boxes, but instead has several potential Sp1- binding sites.

1.2.2. Human β_B-subunit

The β_B-subunit is synthesized as a 407 aa precursor protein containing a hydrophobic signal sequence and a proregion of 292 aa separated from the mature 115 aa C-terminal by basic amino acids (aa) (47). Like the mature part of the β_A-subunit, the activin β_B-subunit also lacks putative glycosylation sites. The β-subunits share similar a cysteine distribution and their mature regions show about 70% aa sequence homology with each other (31).

The human β_B-subunit gene is located on chromosome 2qcen-q13 (37) and consists of two exons separated by a 2.5 kb intron (47). According to the DNA sequence analysis, no TATA or CAAT-like elements have been identified. However, the promoter region is extremely GC-rich with multiple potential Sp1- binding sites and three CRE sequences (47). Two β_B-subunit mRNAs of 3.8- and 4.8-kb in length have been observed in human tissues (48). In rat granulosa cells β_B-subunit transcripts originate from two independent transcriptional start sites of the gene, and they are also differentially regulated (49).

1.2.3. Other β-subunits

The properties of the more recently described activin subunits β_C (50), β_D (51) and β_E (52), are largely unknown. Human activin β_C- and β_E- subunits have been cloned from a human liver cDNA library. They consist of 352 aa and 350 aa from which a mature C-terminal fragment is 116 aa and 114 aa, respectively. Both subunits show about 50% aa identity to mature β_A- and β_B-subunits (50, 52). Activin β_C- and β_E-subunit genes are organized similarly, consisting of two exons (53). It has been suggested that the β_C- and β_E-subunit genes have been generated by tandem duplication of an ancestral gene because they are closely linked to each other. They also share a similar liver specific gene expression pattern in adult mouse. Activin β_D-subunit has been cloned from *Xenopus laevis* and it shows highest homology to human β_C-subunit (60%) although the putative mature part of the protein is slightly shorter (114 aa) (51). The mRNA microinjection studies have shown that β_D-subunit is able to stimulate mesoderm induction in early development of *Xenopus laevis* in a similar manner to activin A (51). Although the functions of β_C- and β_E- subunits are not yet known, recent studies demonstrate that all possible homo- and heterodimers can be formed by recombinant co-expression of the β_A-, β_C- and β_E-subunit cDNAs (54-56).

1.2.4. Transgenic mice models of activin β-subunits

Gene targeting by homologous recombination has been used to address the physiological roles of the activin subunits (Table 1). Activin β_A-subunit deficient mutant mice, Act$\beta_A^{-/-}$, develop normally throughout fetal life but die within 24 h of birth from craniofacial abnormalities that prevent suckling. These defects include cleft palate, absence of whiskers and lack of lower incisors (57). These findings suggest that the activin β_A-subunit is essential only in the development of a few tissues.

Mice homozygous for a deletion of the β_B-subunit gene, Act$\beta_B^{-/-}$, are viable and fertile but suffer eyelid defects. Moreover, female mice fail to nurse their newborns properly (58, 59). This phenotype suggests that the proteins activin B, activin AB and inhibin B are not essential for murine embryonic development and that the presence of the β_A-subunit may possibly replace the missing β_B-subunit. Mice deficient in both activin β_A- and β_B-subunits, Act$\beta_A^{-/-}$ x Act$\beta_B^{-/-}$ display the defects of both activin β_A and β_B mutant mice but no additional defects are seen (57).

Recently, it has been shown that the sequence encoding the mature part of the β_B-subunit can function as a hypomorphic allele of the β_A-subunit, rescuing in part the phenotype of the activin β_A-subunit deficient mice in early development (60). However, the biological effects of activin β_A in testis and ovary development cannot be fully substituted by activin β_B. Mutant mice deficient in both liver-specific activins, Act$\beta_C^{-/-}$ x Act$\beta_E^{-/-}$, are viable and fertile suggesting that activin β_C- and β_E –subunits are not essential for liver growth, differentiation or regeneration (61). Normal mesoderm formation in activin β-subunit deficient mice shows that activins are not required for mesoderm induction in mammals although activins have been identified as a potent mesoderm inducing factor in *Xenopus*. The lack of such defects in null mice may be due to functional compensation of other activin subunits or TGF-β superfamily members.

Table 1. Targeted inactivation of activin subunits

Transgenic mouse model	Phenotype	References
Activin $\beta_A^{-/-}$	postnatal lethality craniofacial anomalies; cleft palate, lack of whiskers and incisors and lower molars	(57, 62)
Activin $\beta_B^{-/-}$	viable nursing defects reproductive abnormalities in females defects in eyelid closure at birth	(58, 59)
Activin $\beta_C^{-/-}$	viable; no obvious abnormalities	(61)
Activin $\beta_E^{-/-}$	viable; no obvious abnormalities	(61)
Activin $\beta_A^{-/-}$ x $\beta_B^{-/-}$	postnatal lethality eyelid closure defects at birth craniofacial anomalies	(57)
Activin $\beta_C^{-/-}$ x $\beta_E^{-/-}$	viable; no obvious abnormalities	(61)

1.3 TGF-β superfamily

The TGF−β superfamily is a large family of structurally related growth and differentiation factors named according to the first family member identified [for review see, Massagué *et al.*, Kingsley *et al.* and Hu *et al.* (63-65)]. TGF−β was originally identified based on its ability to induce anchorage-independent growth of normal rat kidney fibroblasts (66). Subsequently it was found to be involved in a wide variety of cellular functions, which can be either growth promoting or growth inhibiting depending on cell type. For example, it controls the production and remodelling of the extracellular matrix, modulates immune and inflammatory responses and regulates angiogenesis, embryogenesis and gonadal differentiation. There are three highly related mammalian TGF−β isoforms (TGF-β_1, -β_2 and -β_3), chicken TGF-β_4 and frog TGF-β_5. Like activins, various members of the TGF−β superfamily are initially synthesized as larger precursor molecules, which are cleaved to release mature the C-terminal protein. Pro-domains are usually poorly conserved between different family members and the mature region contains most of the sequence similarities by which new family members are usually recognized.

The TGF−β superfamily includes over 40 members divided into several subgroups according to their structural and biological similarities (10) (Fig. 2). In addition to TGF−β and activin subfamilies, the bone morphogenic proteins (BMPs) form a large and heterogenous subgroup of TGF−β family members. Some family members, such as inhibin α, growth differentiation factor 9 and anti-Müllerian hormone, are only distantly related to other members of the TGF−β superfamily and do not belong to any particular subgroup.

Although the mature regions of activin A and TGF−β share nine conserved cysteines, most of the members of the TGF−β superfamily contain only seven of these nine cysteine residues. Crystallography studies on TGF-β_2 and OP-1/BMP-7 have shown that in biologically active proteins six of these cysteines are closely grouped to form a rigid structure called a cystine knot (67-69). The knot is formed by intrachain disulphide bonds and probably accounts for the strong resistance of many TGF−β family members to heat, denaturants and extremes of pH. The remaining cysteine residue in each monomer forms an additional disulphide bond that links two monomers into a dimer. It has been predicted that due to the conservation of cysteines, other TGF−β related molecules contain a similar general folding pattern.

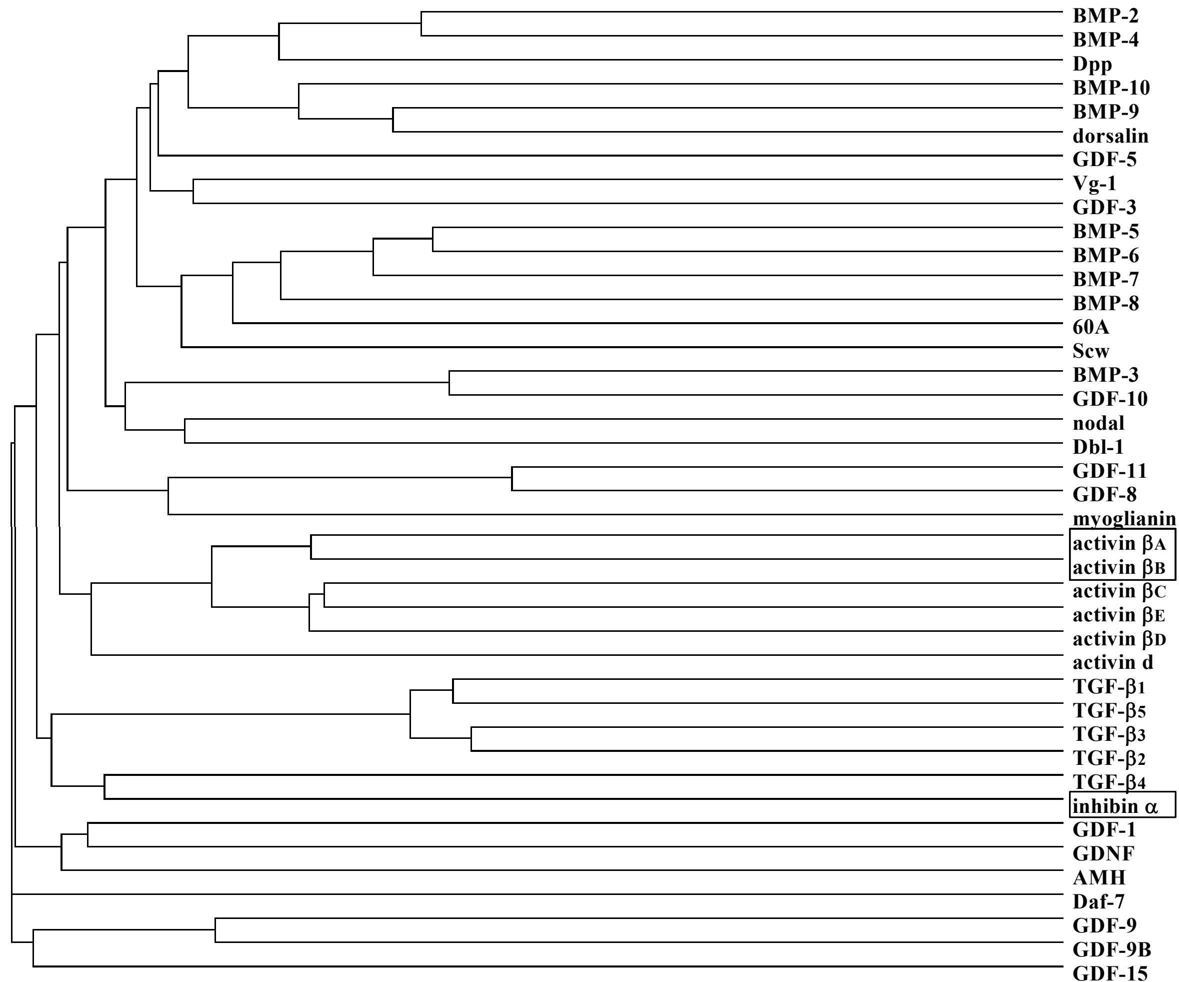


Figure 2. Dendrogram of the TGF−β superfamily ligands. The alignment tree indicates the relative level of amino acid sequence identity between TGF-β superfamily members. The amino acid sequences used are mature parts of TGF-β superfamily ligands from human, unless otherwise indicated. The sequences have been compared using the ClustalW program. The Genbank accession numbers for ligands are as follows: inhibin α (P05111), activin β_A (P08476), activin β_B (A40150), activin β_C (JC2347), *X. laevis* activin β_D (D49543), activin β_E (P58166), *D. melanogaster* Activin β (AF054822), TGF-β_1 (224622), TGF-β_2 (557563), TGF-β_3 (AAA61161), TGF-β_4 (O00292), TGF-β_5 (AAB64441), BMP-2 (P12643), BMP-3 (P12645), BMP-4 (BMHU4), BMP-5 (P22003), BMP-6 (P22004), BMP-7 (P18075), BMP-8 (P34820), BMP-9 (AAD56960), BMP-10 (AAC77462), GDF-1 (NP001483), *M. musculus* GDF-3 (NP032134), GDF-5 (JC2347), Myostatin/GDF-8 (AAB86694), GDF-9 (NP005251), GDF-9B (O95972), GDF-10 (NP004953), GDF-11 (NP005802), GDF-15 (NP004855), AMH (P03971), GDNF (P39905), *G. gallus* dorsalin (AAA48752), *D. melanogaster* 60A (A43918), *D. melanogaster* Scw (AAA56872), *D. melanogaster* myoglianin (AF132814), *D. melanogaster* Dpp (P07713), *X.laevis* Vg-1 (P09534), *M. musculus* nodal (S29718), *C. elegans* DBL-1 (AF004395) and *C. elegans* Daf-7 (U72883).

1.4. Follistatin and other activin binding proteins

Activins interact with various binding-proteins, of which follistatin has been most extensively studied. Follistatin was originally purified from follicular fluid as a monomeric glycoprotein that could inhibit FSH secretion from cultured rat pituitary cells (70, 71). Follistatin exists as three different isoforms produced from a single gene either by alternative mRNA splicing (72) or by proteolytic modification of the core protein (73). Structurally it is composed of a signal peptide, followed by the N-terminal domain and three follistatin domains (72). Follistatin neutralizes the biological effects of activin in a number of biological systems by preventing activin to bind its type II receptors (74, 75). It has been shown that a single follistatin has one activin binding site, which can bind either activin β_A- or β_B-subunits (76-78) whereas activins have two binding sites for follistatin (76). Follistatin binds to activin A with high affinity and under physiological conditions the interaction can be considered to be virtually irreversible (79, 80). Follistatin-activin complexes bound to plasma membrane proteoglycans are internalised and then degraded through the lysosomal pathway (81). Follistatin does not affect the bioactivity of inhibins, despite its capability to bind to the β-subunit present in inhibin (76).

Follistatin-related protein (FSRP) is a recently described new activin binding protein (82, 83). FSRP and follistatin share many structural features like a primary sequence homology, a common exon/intron arrangement and domain structure. However, the affinity of FSRP for activin A is lower than that of FS and they differentially neutralize exogenous *vs*. endogenous activin, suggesting nonoverlapping cellular functions (84). A serum protein, α_2-macroglobulin, has been implicated as a low-affinity binding protein of activin A (85). It as been suggested that α_2-macroglobulin may play a role as a carrier for activin in the circulation (86). The extracellular protein Cerberus has been shown to block signalling by activin in a *Xenopus* animal cap model (87).

Another group of activin binding proteins are accessory proteins and pseudoreceptors. Endoglin is a cell surface molecule, which can interact with several TGF−β family members. As an accessory protein it binds to multiple receptor complexes, such as those containing ActR-II or ActR-IIB, modulating the activity of the receptor kinase complexes (88). The pseudoreceptor, named BMP and activin membrane- bound inhibitor (BAMBI), has a type I receptor-like structure but lacks a full intracellular domain (89). It competes with ActR-Is for association with ActR-II, and hence inhibits signal transduction by activin (Fig. 5).

2. Activin-mediated signalling

TGF−β superfamily members exert their diverse biological effects through a receptor complex formed by two distantly related types of transmembrane serine/threonine (ser/thr) kinase proteins that fall into two classes, type I receptors and type II receptors (13, 90). Vertebrate type I receptors can be divided into two different groups according to their sequence homology at the kinase domain and their signalling activities (Table 2). One group includes type I TGF-β receptor (TβR-I), type IB activin receptor (ActR-IB) and activin-like receptor-7 (ALK-7), and the other one includes type I and IB BMP receptors (BMPR-I and BMPR-IB), type I activin receptor (ActR-I) and activin-like receptor 1 (ALK-1).

Table 2. Mammalian type I and II receptor families.

Type I receptors	References	Type II receptors	References
ALK-1	(91, 92) (93)	ActR-IIA	(94)
ActR-I (ALK-2)	(91, 92, 95-98)	ActR-IIB	(99)
BMPR-I (ALK-3)	(92)	TβR-II	(100)
ActR-IB (ALK-4)	(13, 91, 92, 101)	BMPR-II	(102-105)
TβR-I (ALK-5)	(91, 106)	AMHR-II	(107)
BMPR-IB (ALK-6)	(108)		
ALK-7	(109, 110)		

In activin and TGF−β signaling, ligand induced activation of the type I and type II receptors allows the type II receptor to phosphorylate serine and threonine residues in the GS-domain (= gly and ser-rich domain) of the type I receptor, thus, inducing its kinase activity (Fig. 3) (11, 13, 111, 112). Type I receptors for activin and TGF−β can only recognize ligand that is bound to the type II receptor (95, 96, 106, 113). Type II receptors can bind ligand independently of the type I receptor, but they are unable to signal without the type I receptor (90, 95, 114, 115). This phenomenon was originally revealed by analyzing the receptor phenotype in TGF−β resistant cell mutants (116). Ligand binding does not increase the overall phosphorylation of type II activin receptors or their kinase activity *in vitro* (90), suggesting that type II receptors are constitutively active kinases that require the ligand to interact with type I receptor that is considered as a substrate. Once phosphorylated, the type I receptor activates the downstream target. Thus it appears that the specificity in signalling is mainly determined by the type I receptor. Co-operative binding mode is typical for BMP subfamily receptors. BMPs bind weakly to the type I receptor in the absence of type II receptor, but the presence of type II receptors accelerates the ligand binding to type I receptors (Fig. 3) (13, 117, 118).

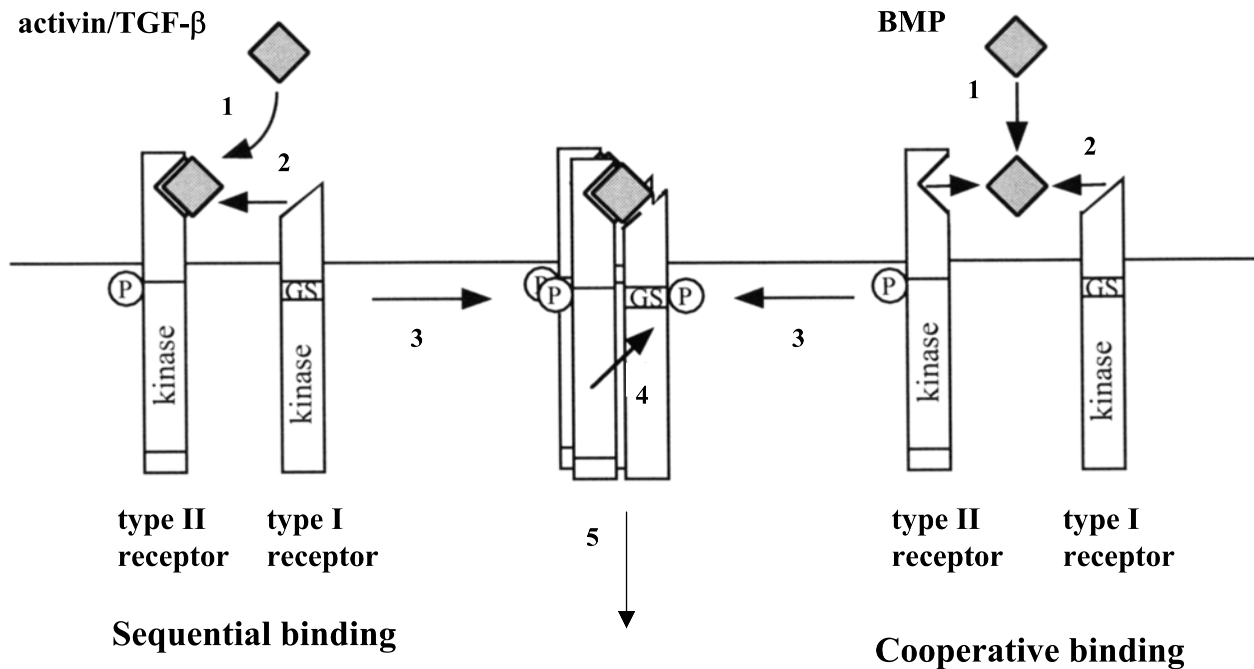


Figure 3. Two modes of ligand binding in TGF−β receptor family members. In sequential binding, ligand binding to the type II receptor kinase (1) is followed by formation of a complex with the type I receptor (2), whereas the co-operative mode requires both type I and type II receptors for high affinity ligand binding. In the heterotetrameric receptor complex (3), the constitutively active, dimeric type II receptor kinase complex phosphorylates the GS-domain of the dimeric type I receptor complex (4) inducing activation of type I receptor kinases and transduction of various intracellular signals (5).

2.1. Type II serine/threonine kinase receptors

2.1.1. Type II activin receptor (ActR-II)

The mouse type II activin receptor cDNA encodes a 513 aa protein (94). The extracellular ligand binding domain with two N-linked glycosylation sites is relatively short compared to receptor tyrosine kinases. It contains eight conserved cysteines determining the general fold of this region. A cysteine-rich motif found near the transmembrane domain is characteristic to TGF−β receptor family members (119). Recently the crystal structure of ActR-II extracellular domain has been resolved, and surprisingly, it has seven anti-parallel β-strands that form a three-finger toxin fold similar to the folds of several cobra cardiotoxins (120). Mutagenesis studies have shown that three hydrophobic amino acid residues predicted to be surface-exposed on the concave face of the ActR-II are each required for activin A binding (121).

A common feature for serine/threonine (ser/thr) kinase receptors is a single transmembrane domain and a cytoplasmic domain with a juxtamembrane region and a kinase domain (Fig. 4). Neural tissue specific variant of ActR-II, ActRIIA-N, has a 24 bp insert between the transmembrane region and kinase domain suggesting that it may be an alternatively spliced product of the type II receptor gene (122). The ActR-II kinase domain has 11 conserved subdomains characteristic of the serine kinases (94). The mouse ActR-II gene is encoded by 11 exons and spans over 66 kb (123). It is mapped to

chromosomal locus 2q22.3-q23.2 (124). In the 5' region of the gene, no TATA or CCAAT boxes are found in the vicinity of the putative transcription start site. However, there are several putative transcription factor binding sites that may play important roles in complex transcriptional regulation of this gene.

Phylogenetic analysis of the ActR-II compared with other protein kinase sequences revealed that ActR-II and a previously identified receptor, Daf-1, from *Caenorhabditis elegans* (125) constitute a separate subfamily of receptor ser/thr kinases. Analysis of type II receptor sequences from various species has revealed high evolutionary conservation, with only two aa differences between mouse and human sequences, and greater than 90% identity between species as divergent as chicken and man (94, 126-128).

Mice deficient in the ActR-II gene (*Acvr2*) (62) were expected to be a phenocopy of the activin β_A, β_B or $\beta_A\beta_B$ double mutant mice (57, 59). However, they demonstrate only minimal overlap in phenotype compared to activin-deficient mutant mice (Table 3). The majority of ActR-II deficient mice developed to adults. Suppressed FSH levels and defects in their reproductive performance confirm an important role of ActR-II in reproduction. A smaller population of ActR-II-deficient mice die at birth or have skeletal and facial abnormalities. The lack of phenotype similarity between the ActR-II-deficient mice and the activin deficient mutant mice suggests that other ligands in addition to activins may signal through ActR-II.

2.1.2. Type IIB activin receptor (ActR-IIB)

Subsequently, another ser/thr kinase receptor homologous to the ActR-II was characterized in mouse based on the sequence similarity between ActR-II and daf-1 cDNAs (99). The new receptor, called ActR-IIB, showed 50-60% identity with ActR-II in the ligand-binding domain and 60-70% identity in the kinase domain. Alternative splicing of mouse ActR-IIB mRNA gives rise to four different isoforms (Fig. 4). Two of the ActR-IIB isoforms differ from each other by inclusion of a 24 aa alternatively spliced segment in the cytoplasmic juxtamembrane domain. A second alternative splicing event generates two additional isoforms that lack an eight aa proline-rich cluster in the external juxtamembrane region. The receptor isoform that includes both segments is designated ActR-IIB_1 and it codes for a protein of 536 aa. The isoforms that include only the first segment or the second segment are designated ActR-IIB_2 and ActR-IIB_3, respectively, and the isoform that lacks both segments is designated ActR-IIB_4. The ActR-IIB_2 isoform is predominant in the mouse. The ActR-IIB isoforms differ in their ligand-binding affinity. Those isoforms that have the full extracellular domain sequence have a slightly higher affinity to activin A. Soon after the mouse ActR-IIB was published we characterized the human ActR-IIB cDNA, which was found to correspond to the mouse ActR-IIB_2 isoform (Study I). Subsequently, ActR-IIB_1 and ActR-IIB_2 isoforms have been characterized in human teratocarcinoma cells (129). Recently the genomic organization of this gene has been resolved. Human ActR-IIB consists of 11 exons and spans about 30 kb in chromosome 3p22 (124, 130). A unique type of alternative splicing has been observed in bovine ActR-IIB. Activin receptor type IIB_5 lacks a sequence coding a 14 aa proline-rich region located between the transmembrane domain and kinase domain (131) (Fig. 4).

Disruption of the type IIB receptor expression leads to cardiac malformations, defects in axial patterning and disturbance of left-right asymmetry in mice (132). Although ActR-IIB deficient mice develop to term they die shortly after birth because of complicated heart defects. Among human left-right axis malformation cases mutations in ActR-IIB gene are present only rarely (129). Gastrulation of mice deficient of both type II receptors, ActRII *(Acvr2)*$^{-/-}$ x ActR-IIB (*Acvr2b*)$^{-/-}$, is

severely impaired (133). Hence, type II receptors are suggested to be required for egg cylinder growth, mesoderm formation and primitive streak formation (Table 3).

2.1.3. Other type II serine/threonine kinase receptors

In vertebrates, the type II receptor subfamily includes TGF−β type II receptor (TβR-II) (100), anti-Müllerian hormone type II receptor (AMHR-II) (107) and BMP type II receptor (BMPR-II) (102-105). These receptors have been cloned from various species and they share high homology to each other. They also exist in alternatively spliced forms. A recently characterized alternatively spliced variant of TβR-II, TβR-IIB, with an insertion of 26 amino acids at the N-terminus, is a TGF-β_2 binding receptor (134-136). Alternative splicing of the AMHR results in a 61 aa insertion at the same location as TβR-IIB (107). BMPR-II also exists in two different isoforms, one of which has a long C-terminal extension that might arise from an alternative splicing (102, 103). Although the functional significance of these variants is still unknown, it is interesting that insertions at the N-terminus of the receptor are only seen in the ligand-binding subunit.

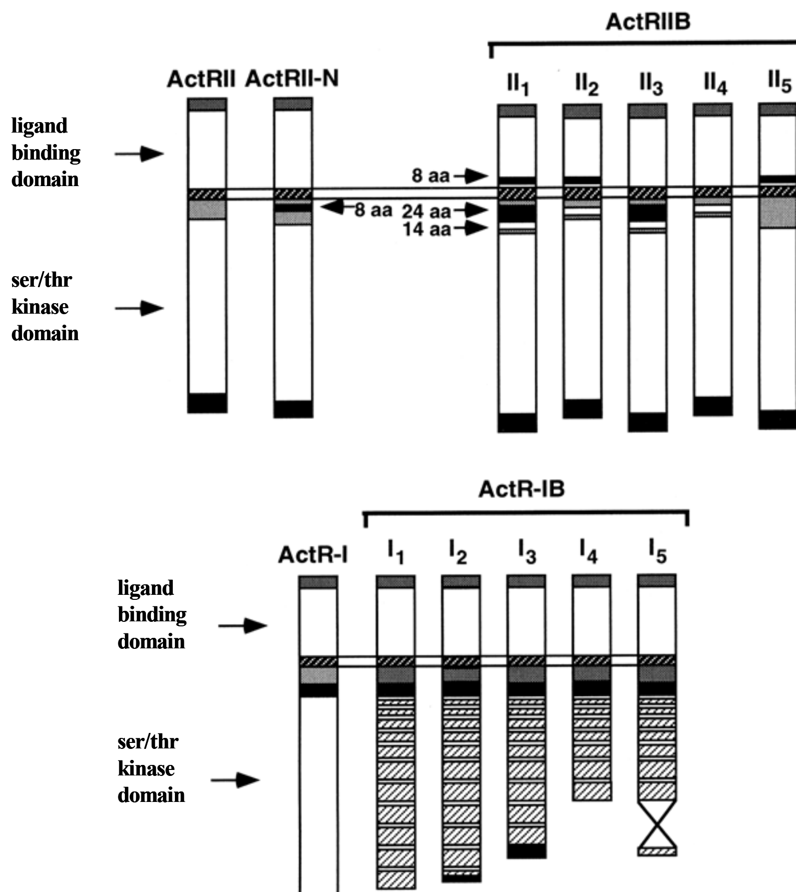


Figure 4. Schematic representation of activin type II and type I receptors and their alternatively spliced variants. The receptors are transmembrane proteins composed of a ligand-binding extracellular domain with a signal sequence (stippled), a transmembrane domain (dark stripes), and a cytoplasmic domain with ser/thr kinase activity. Black boxes indicate juxtamembrane alternatively spliced regions or ser/thr-rich C-terminal regions in type II and IIB receptors. The white box in the cytoplasmic juxtamembrane region is 14 aa proline-rich sequence deleted from ActR-IIB_5. In type I receptors, a black box indicates GS-domain or additional C-terminal tail in ActR-IB_2 and ActR-IB_3. ActR-IB is alternatively spliced at the kinase subdomains VII-XI (striped boxes) generating truncated forms of the receptor. In ActR-IB_5 the kinase subdomain VII is spliced to subdomain X.

2.2 Type I serine/threonine kinase receptors

2.2.1 Type I activin receptors

A number of additional ser/thr kinase receptors were cloned by PCR using oligonucleotide primers based on type II ser/thr kinase receptor sequences. These receptors were initially considered orphan receptors because they did not bind activins, inhibins, TGF−βs or BMPs (97). As a result of being simultaneously cloned by different groups, most type I receptors have received different names before their identity and ligands were established. One practice has been to use the neutral nomenclature ALK (activin receptor-like kinase). The first receptor characterized was ALK-2 (also known as SKR-1, Tsk7L, R1 and ActX1R) (91, 92, 95-98). It is commonly referred to as type I activin receptor (ActR-I) although it is able to mediate only certain activin responses, suggesting that there may be other ligands than activins (95, 137-140). Recently it has been demonstrated that at least AMH may use ActR-I as its type I receptor (141, 142). The type I activin receptors are structurally related to the type II receptor family displaying ser/thr kinase activity. Type I activin receptors are slightly smaller than type II receptors displaying a shorter extracellular domain and lacking the ser/thr rich C-terminal tail after the kinase domain (Fig. 4) (13). The extracellular ligand-binding domains of type I receptors contain seven cysteine residues at nearly invariant positions in addition to the cysteine box. A unique feature of type I receptors is a highly conserved 30 aa region preceding the kinase domain. Because of the characteristic TTSGSGSG sequence this region is called the GS domain (115). Ligand-induced phosphorylation of the ser and thr in the GS domain of the ActR-IB by type II receptor is required for signal activation (90, 111). Immediately following the GS domain, type I receptors have a leu-pro motif that serves as a binding site for the cytoplasmic immunophilin FKBP-12 protein (143). Binding of FKBP-12 protects TGF−β type I receptor against phosphorylation, thus preventing the spontaneous, ligand-independent activation of type I receptor by type II receptor *in vitro* (11, 144).

The predominant type I activin receptor in mammalian cells is ActR-IB (ALK-4) (13, 91, 92, 101). A study of the ActR-IB gene predicted several possible mRNA species, generated by alternative splicing (145). The full length ActR-IB (ALK-4) has eleven kinase subdomains (I-XI) and is called ALK4-1. ALK4-2 lacks almost completely the kinase subdomain XI and ALK4-3 lacks subdomains X and XI, but both have a carboxy-terminus with novel sequences not found in ALK4-1. ALK4-4, the most truncated splicing variant, lacks kinase subdomains IX-XI and part of the subdomain VIII. ALK4-5 is identical to ALK4-4, except for the addition of a ser and pro at its carboxy-terminus (146) (Fig. 4). Activin type I and IB receptors are in different subgroups within the ALK family (Table 2). The extracellular domains share little sequence identity between ActR-I and ActR-IB and the distribution of mRNA expression of these two receptors in various tissues is distinct (91, 92). However, the kinase domain of the type I and type IB activin receptors are over 60% homologous to each other. Human ActR-I (*Acvr*1) and ActR-IB (*Acvr1b*) genes have been localized to chromosomes 2q23-q24 and 12q13, respectively (147).

The genomic structure of the mouse ActR-I has been resolved (148). The mouse ActR-I gene consists of 10 exons of which the first one is not translated. According to primer extension studies, alternative transcription start sites might be used for expression of the mouse ActR-I gene resulting in variable size of the 5' untranslated region. The roles of ActR-I and IB in activin signalling have been studied by gene targeting in mice (Table 3) (149-151). ActR-I is required for gastrulation and ActR-IB functions in organizing the egg cylinder before gastrulation and primitive streak formation indicating that activin type I receptors mediate signals required for early embryonic development.

Table 3. Targeted inactivation of activin receptors

Transgenic mouse model	Phenotype	References
ActR-II$^{-/-}$ (*Acvr2*$^{-/-}$)	viable infertility in females delayed fertility in males small gonads mandibular defects	(62)
ActR-IIB$^{-/-}$ (*Acvr2b*$^{-/-}$)	postnatal lethality cardiovascular, splenic and renal defects disturbance of left-right asymmetry defects in axial patterning	(132)
ActR-II$^{-/-}$ x ActR-IIB$^{-/-}$	embryonic lethality	(133)
ActR-I$^{-/-}$ (*Acvr1*$^{-/-}$)	embryonic lethality	(149, 150)
ActR-IB$^{-/-}$ (*Acvr1b*$^{-/-}$)	embryonic lethality	(151)

2.2.2. Other type I serine/threonine receptors

Presently, seven different type I receptors are known. They can be classified into two different groups according to which downstream signalling proteins, Smads, they are connected with (see chapter 2.4). ActR-IB, TGF−β type I receptor (ALK-5, XTrR-I) (106, 152) and ALK-7 (109, 110) acting as the type I receptor for mouse Nodal and *Xenopus* nodal related protein-1 (Xnr-1) (153) form the first group. ActR-I, BMP type I and IB receptors (ALK-3 and ALK-6) (91, 92, 108, 118) and ALK-1 (also known as TSR-I), which participates in TGF−β signalling in endothelial cells (9, 154), form the other group.

2.3. Ligand-receptor interactions and mechanism of receptor activation

In the case of activin A both type I receptors (ActR-I and ActR-IB) are able to form complexes with type II activin receptors (ActR-II and ActR-IIB) (95, 98, 101). Further, it has been suggested that ActR-IB, but not ActR-I, mediates growth-inhibitory and extracellular matrix responses in concert with activin type II receptors (101). Thus, activin A may induce distinct cellular responses depending on which particular receptors are expressed in the cell. The diversity of cellular responses to different TGF−β ligands may be accounted for by the type I receptors (Table 3). Interestingly, it has been observed that in the presence of ActR-II both BMP-7/OP-1 and GDF-5 are able to use BMPR-IB and ActR-I, respectively, for signal transduction (118, 138, 155). On the other hand, BMP-7/OP1 and BMP-2 can induce a transcriptional response via a BMPR-II-ActR-I receptor complex (103). BMP-2 is also able to signal either by a BMPR-II-BMPRI/BMPR-IB or an ActR-IIB-BMPR-I/BMPR-IB complex (156). In *Xenopus* embryo explant experiments constitutively active ActR-I mimics the mesoderm ventralizing activity of BMP-4, which suggests that ActR-I may function as a BMP receptor *in vivo* (137). As activin does not have any BMP-like effects, it is conceivable that ActR-I is not a real activin receptor but rather a receptor mediating

BMP signals. Inhibin also binds to ActR-II (94) sharing the same binding site with activin A on ActR-II (121). According to current knowledge, after binding to cell surface betaglycan co-receptor, inhibin binds to ActR-II, preventing activin from binding to ActR-II and thereby antagonizing the activin signal (Fig. 5) (157).

Table 3. Mammalian TGF−β family member ligands and their receptors

Ligand	Receptor II	Receptor I
Activin	ActR-II ActR-IIB	(ActR-I) (ALK-2) ActR-IB (ALK-4)
TGF-β	TβR-II	TβR-I (ALK-5) ALK-1 (ALK-2) (ALK-7)
BMP-2 BMP-4 BMP-7/OP-1 GDF-5	BMPR-II ActR-II ActR-IIB	BMPR-I (ALK-3) BMPR-IB (ALK-6) ActR-I
Nodal	ActR-II ActR-IIB	ALK-7 ActR-IB
AMH	AMHR-II	ActR-I BMPR-IB
GDF-9	BMPR-II	?

2.4. Intracellular signalling of activin

The proteins of the SMAD family are so far the only identified substrates for type I receptor ser/thr kinases (Fig. 5). Three functionally distinct subfamilies of Smads have been defined: receptor-regulated Smads (R-Smads: Smad1, Smad2, Smad3, Smad5 and Smad8), the common-mediator Smads (co-Smads: Smad4) and the antagonistic or inhibitory (I-Smads: Smad6 and Smad7) [for review see Attisano *et al.*, Itoh *et al.*, Massagué and Chen, ten Dijke *et al.* and Roberts and Derynck (10, 12, 139, 158, 159)]. Activin receptor complexes activate the Smad pathway by interacting and phosphorylating specific R-Smads, Smad2 and Smad3. Phosphorylation of R-Smads dissociates them from the activin receptors and leads to formation of complexes with co-Smad. Thereafter, the Smad complex is translocated to the nucleus, where it binds to DNA and associates with transcription factors such as the forkhead activin signal transducer (Fast) (160) and Jun (161). Antagonistic Smad7 inhibits the binding of R-Smads to the receptor subunits by competing for the receptor interaction and marking the receptors for degradation. Like TGF−β superfamily ligands and receptors the Smad family is highly conserved and the homologues of these classes have been identified in *X.laevis*, *D.melanogaster* and *C.elegans*.

Identification of several Smad-associated proteins has provided new insights into how Smad activity is controlled. In the activin signalling cascade Smad/receptor accessory protein, Smad anchor for receptor activation (SARA), facilitates binding of Smad2 to the heteromeric activin receptor complex. The stable interaction of SARA with non-phosphorylated Smad2 also prevents nuclear import of Smad2 (162). Newly described activin receptor-interacting protein-1 associates with ActR-II and with Smad3, enhancing Smad3-mediated signalling in response to activin (163, 164). Activin receptor-interacting protein-2 interacts with both ActR-II and ActR-IIB and enhances

endocytosis of ActR-IIs, suppressing activin-induced transcription (165). The nature of these interactions appears to determine whether Smads positively or negatively regulate activation of target genes (158, 164).

Although Smads are central elements in the activin signal transduction pathway, activin is also able to activate the p38 mitogen-activated protein kinase (MAPK) pathway (166). Phosphorylation of p38 MAPK leads to phosphorylation of ATF2 transcription factor and growth inhibition of breast cancer cell lines *in vitro*.

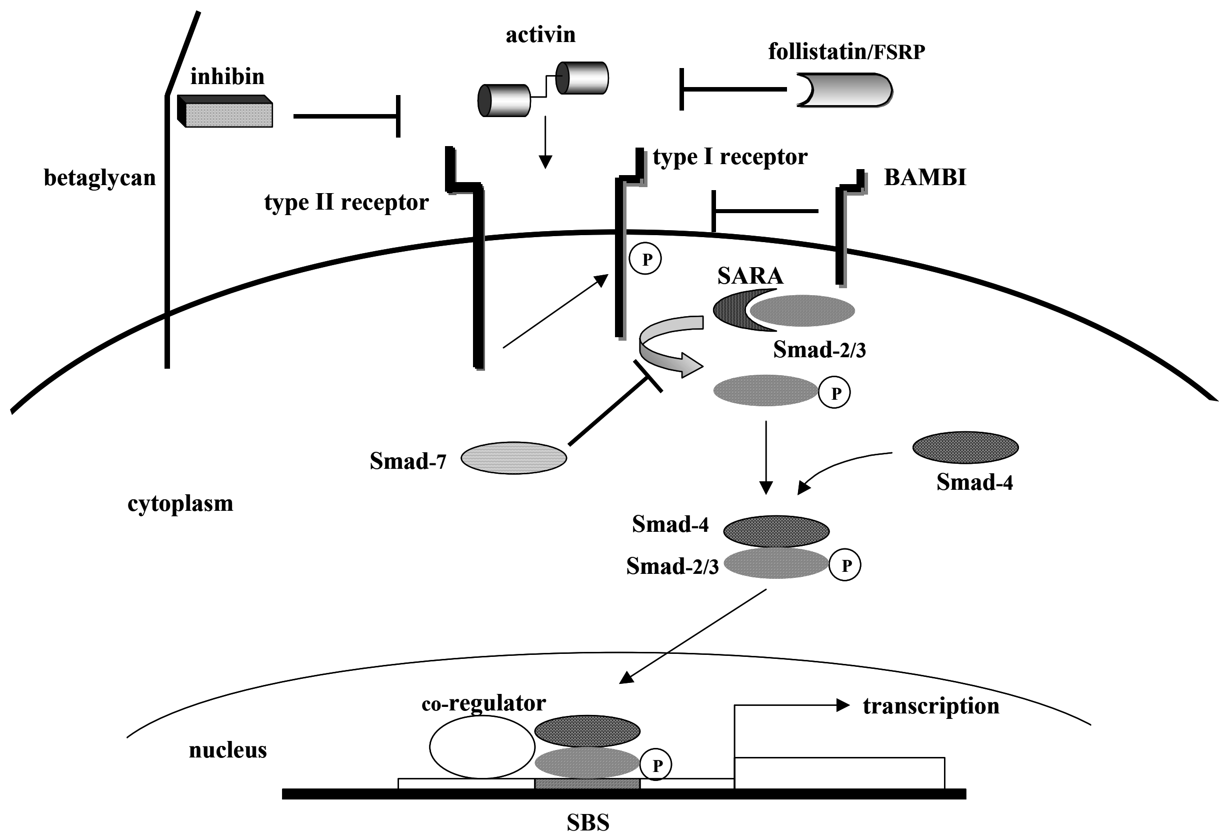


Figure 5. A simplified model for transducing activin signals into the cells. The binding of activin to type II receptors induces the phosphorylation of the type I receptors. Activated receptor complex leads to phosphorylation of Smad2 and Smad3 that permits the interaction with Smad4 and translocation of this complex to the nucleus. In the nucleus Smad-complex regulates specific gene promoters through the interaction with Smad-specific binding site (SBS) and specific coregulators. SARA interacts with Smad2 and the receptor complex and assists in the phosphorylation of Smad2 by the type I receptor. Negative regulation is provided by different growth factor-sequestering proteins. Follistatin or FSRP binds activin by inhibiting its interaction with activin receptors. In the presence of betaglycan inhibin competes with activin for the binding to type II receptors. Smad7, in turn, inhibits the activation of Smad2 and Smad3, thereby blocking the activin signalling pathway. BAMBI, which is structurally related to type I receptors, lacks the intracellular kinase domain. Acting as a pseudoreceptor, it prevents homodimerization of type I receptors. (adapted from Findlay *et al.* (167).

3. Expression and regulation of activin/inhibin subunits, follistatin and activin receptors

3.1. The tissue distribution of activin/inhibin subunits, follistatin and activin type I and II receptor mRNAs

For many years the primary information available for activins and its receptors has mainly been the expression pattern in different cells and tissues. Although activins were initially isolated as gonadal proteins, the expression of their mRNAs has been detected in a number of extragonadal tissues in all germ layers of fetal and adult tissues. Table 2 presents the distribution pattern of activin and inhibin subunits, activin receptors and FS mRNA expression in adult murine tissues from references (4, 91, 94, 97, 98, 122, 145, 168-174) and http://www.ncbi.nlm.nih.gov/UniGene/.

Table 2. Tissue distribution of inhibin and activin subunits, FS and activin receptor transcripts in adult murine tissues.

Tissue	α	β_A	β_B	FS	ActRII	ActR-IIB	ActR-I	ActR-IB
Ovary	+	+	+	+	+	+	+	+
Testis	+	+	+	+	+	+	+	?
Pituitary	+	-	+	+	+	+	+	?
Adrenal	+	+	+	+	?	?	?	?
Brain	+	+	+	+	+	+	+	+
Bone marrow	-	+	-	+	?	?	?	?
Spleen	+	+	-	-	?	?	+	+
Placenta	+	+	+	-	+	+	?	?
Heart	?	?	?	+	+	+	+	+
Lung	?	?	?	+	+	?	+	+
Thymus	+	?	+	+	+	+	+	+
Skeletal muscle	?	?	+	+	?	?	?	+
Uterus/decidua	-	+	-	+	+	+	?	?
Kidney	+	-	-	+	?	?	+	+
Pancreas	+	+	+	+	+	+	+	?
Liver/hepatocytes	+	+	-	?	+	?	+	+

3.2. Expression and regulation of activin subunit and activin receptor mRNAs in the ovary

Inhibin and activin have important roles in folliculogenesis, oocyte maturation and in corpus luteum function in rodents and primates. In the rat ovary inhibin and activin subunit mRNAs are expressed in granulosa cells of growing follicles (8). The expression patterns and regulation of the mRNAs by a variety of hormones and growth factors have been well documented (175, 176). In the human ovary, β_A-subunit mRNA expression is found in the granulosa cells and the corpus luteum whereas the β_B-subunit messenger is detected in the granulosa cells of small antral follicles but not in dominant follicles or the corpus luteum (177). Activin A protein is present exclusively in the granulosa cells of mature follicles and in the corpus luteum (178).

Many studies have demonstrated that activin A has a direct effect on the synthesis of many ovarian hormones. Activin-induced proliferation has been observed in cultured rat granulosa cells from both small and large follicles (179, 180) and in human granulosa luteal (GL) cells (181), suggesting a role for activin in early follicular development. Activin A also increases inhibin α-subunit transcription and protein biosynthesis in cultured rat granulosa cells (182). Sites of activin production are widespread and often overlap with their sites of action, suggesting paracrine or autocrine role in reproduction.

The expression of activin receptor genes has been detected in ovarian follicles. In ligand binding studies activin A binds to the granulosa cells during all phases of the rat ovarian cycles (183). Expression of the activin receptor type II and IIB mRNAs has also been detected in ovarian granulosa cells, corpus luteum and oocytes of mouse, rat and human (172, 184). The presence of four activin receptor subtypes has been demonstrated in human granulosa cells and oocytes as well as in postnatal rat ovary (173, 184). Recently, the expression of ActR-II and IIB proteins in human follicles has been confirmed by immunohistochemistry (185).

3.3. The effects of activin A in normal erythropoiesis and in erythroleukemia cells

Several lines of evidence indicate that activin A functions as a physiological regulator of erythroid lineage cells. Erythroid differentiation factor (EDF) was first found in the conditioned medium of 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-stimulated human monocytic leukemia cells, based on its ability to induce erythroid differentiation of murine Friend erythroleukemia cells (186). Later, EDF was found to be identical to human activin A (7). Early studies indicate that in the presence of erythropoietin, activin A promotes the colony formation of early (187, 188) and late stages (189) of erythrocyte differentiation in the bone marrow cell cultures. It drives immature erythroid precursors to form hemoglobinized cells, thereby rendering the colonies recognizable as erythroid colonies (189-191). Purified erythroid progenitor cells also possess receptors for activin on their surface (191). Recently, it has been observed that activin A commits the erythroid progenitor cells to apoptosis without erythropoietin costimulation required for further maturation (192). Activin enhances proliferation of normal erythroid precursor cells not only in culture systems but also when administered to rodents *in vivo* (193, 194). Endogenous activin A production by bone marrow cells supports the importance of activin A in regulating erythropoiesis (195-197).

Human K562 cells, derived from a patient with chronic myelogenous leukemia with the BCR/ABL oncogene of the Philadelphia chromosome translocation (198), provide a model system for studying erythroid differentiation. The addition of activin A to K562 cells causes them to become hemoglobin positive and inhibits their proliferation (189). Further, the accumulation of globin transcripts is induced by activin A (199). It has been indicated that the K562 cells possess specific binding sites for activin on their surface (16). Both type II activin receptors and activin receptor type IB are required for activin-induced transcriptional activity (200). Moreover, by using an inducible promoter the levels of ActR-IB and ActR-II can be increased, which enhances the effects of activin on erythroid differentiation (201). The treatment of K562 cells with TPA leads to loss of erythroid properties. Although the K562 cell line is not an ideal model for megakaryoblastic differentiation, several megakaryoblastic characteristics, such as TGF−β expression, can be detected by TPA induction (202, 203).

Aims of the present study

The aim of the present study was to investigate where human activins and their signalling components are expressed, and how they are regulated in different activin responsive cell models as well as in human developing tissues.

The specific aims of the study were to determine:

- the primary structures of human type II activin receptors

- the distribution of the activin β-subunits, follistatin and type II activin receptor transcripts in human developing tissues

- the expression and regulation of activin and inhibin subunits and activin receptor transcripts in human granulosa-luteal cells

- the regulation of activin β_A-subunit and activin receptor transcripts in human erythroleukemia K562 cells

Materials and methods

1. Cell cultures

Human K562 [CCL-243; American Type Culture Collection (ATCC), Rockville, MD, USA] and HL-60 cells (CCL-240; ATCC) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; GIBCO Laboratories, Grand Island, NY, USA). Mouse NIH-3T3 fibroblasts (CRL-1658; ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% newborn calf serum (NCS), 2 mmol/l L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin (GIBCO) at 37˚C in a humified 95% air-5% CO_2 atmosphere. The experiments were performed in RPMI 1640 medium or DMEM supplemented with 1% FCS or NCS. Human GL cells were aspirated from women undergoing hormone treatment for *in vitro* fertilization. For each experiment, the cells from two to four patients were pooled and enzymatically dispersed and separated from red blood cells by centrifugation through Ficoll-Paque (Pharmacia, Uppsala, Sweden), as previously described (204). They were plated at a density of 2-5 x 10^5 cells/well in 35-mm six-well dishes (Costar, Cambridge, MA) and cultured in DMEM (GIBCO) supplemented with 10% FCS, 2 mM L-glutamine, and antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin-B; GIBCO). Cell culture media were changed every other day, and hormone treatments were performed on days 2 to 7 of culture. Prior to hormone treatments the human GL cells were transferred to DMEM medium supplemented with 2.5% FCS and antibiotics.

2. Treatments of the cell cultures

For megakaryocytic differentiation, K562 cells were induced with increasing concentrations of TPA [0.003 to 100 ng/ml; Sigma Chemical Co, St Louis, MO, USA; dissolved in dimethyl sulfoxide (DMSO)] (203, 205). For erythroid differentiation, K562 cells were treated with recombinant human activin A (189) (0.1 to 30 ng/ml; a gift from Dr. Y. Eto, Ajinomoto Co, Kawasaki, Japan) or TGF−β (1 ng/ml; R&D Systems, Minneapolis, MN, USA). To study the effect of cyclic AMP (cAMP) on β_A-subunit mRNA levels the K562 cells were treated with the cell permeable cAMP analog, 8-bromo-cAMP (8-Br-cAMP; 1 mM; Sigma). The K562 cells were also treated with protein kinase inhibitor H7 (10 µM; Seigaku America, Inc., St. Petersburg, FA, USA) (76) with or without TPA (10 ng/ml). HL-60 cells were induced to either monocyte/macrophage or granulocyte-like cells by 10 ng/ml TPA or 1.2% DMSO, respectively (206).

To study the regulation of inhibin subunits, the GL cells were treated with different concentrations of activin A (0.3 to 100 ng/ml) for indicated time periods. To test the effect of recombinant human follistatin (288-aa follistatin, generously provided by Dr. Raiti, University of Maryland School of Medicine, Baltimore, MD) on basal and activin A-stimulated β_B-subunit mRNA expression, increasing concentrations of follistatin (4-700 ng/ml) were first incubated with or without 50 ng/ml activin at 37˚C for 1 h in 50 ml phosphate buffered saline (PBS) containing 0.1 % bovine serum albumin (BSA) before given to the cells in 2 ml DMEM-2.5% FCS for 24 h. To study the effect of human chorionic gonadotropin (hCG) alone (CR-127 preparation was donated by the National hormone and pituitary programme, NIDDK, NIH) or in combination with activin A, the cells were stimulated with 100 ng/ml hCG and/or 60 ng/ml activin A for 24 or 48 h. Purified bovine inhibin A was from Peninsula Labs Europe (Merseyside, UK).

3. Human fetal tissues

Human fetuses were obtained from the Helsinki Maternity Clinic by approval of the hospital's ethics committee and by informed consent of the mother undergoing legal abortion. The gestational ages varied from 15-17 weeks as estimated from fetal foot lengths (207). Tissues were dissected 1-4 h post-mortem and rapidly frozen in liquid nitrogen for storage at –70˚C prior to RNA extraction.

4. RNA extraction and blotting

Total RNA was isolated from K562 and HL-60 cells and human fetal tissues by the guanidinium isothiocyanate/CsCl method (208). Polyadenylated RNA was isolated from total K562 and NIH-3T3 cell RNA as well as human fetal brain tissue by a PolyATtract System IV kit (Promega, Madison, WI, USA). The modified Nonidet P-40 lysis method (209) was used for extracting cytoplasmic RNA from human GL cells, K562 cells and NIH-3T3 fibroblasts. The RNAs were quantitated by absorbance at 260 nm. For Northern blots, 3.9 micrograms of glyoxylated polyadenylated RNA or 10 µg of total RNA samples were size-fractioned in 1.5% agarose gels, and transferred to Hybond-N nylon membranes (Amersham International, Aylesbury, Buckinhamshire, UK). For dot blots, 1-2 µg of cytoplasmic RNA was denatured and spotted onto nylon membranes using a 96-well Minifold device (Schleicher and Schuell, Keene, NH, USA). The RNA blots were baked for 1-2 h at 80˚C and thereafter UV cross-linked for 6 min with a UV illuminator (Reprostar II, Camag, Switzerland).

5. RT-PCR, cDNA cloning and sequencing

Reverse transcription-polymerase chain reactions (RT-PCR) were performed according to previously described protocols (210). One microgram of total RNA from the adequate tissue source was used for cDNA synthesis. The 10 µl reaction volume contained 100 U Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories [BRL], Gaithersburg, MD), 0.2 µg oligo $d(T)_{15}$ primer (Boehringer Mannheim, Mannheim, Germany), 2 µl of 5 X RT buffer (BRL), the four dNTPs (at 0.5 mmol /l final concentration of each; Perkin-Elmer Cetus Corp, Norwalk, CT), and 5 U human placental RNAse inhibitor (Amersham) at 37˚C for 60 min.

One microliter of the RT mixture was subjected to PCR, which was performed according to the instructions provided with the GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer, Cetus). The primers for amplification of full length ActR-II and ActR-IIB were designed according to published rodent sequences (94, 99, 211), and are listed in Table 1 (I). Amplification of K562 cell cDNA with primers no. 7 and 8 yielded a 556-bp DNA product homologous to mouse ActR-II sequence. Primers no. 1 and 2 were used for amplification of a 959-bp human ActR-IIB fragment from the same cDNA. These fragments were used as hybridization probes to screen Northern blots of human fetal tissue RNA samples. For obtaining the full open reading frame of human ActR-II and ActR-IIB, we used human fetal kidney and brain cDNA, respectively, to amplify the missing 5' and 3' sequences. The amplification conditions consisted of initial denaturation at 94˚C for 2 minutes followed by 45 cycles of amplification with denaturation at 94˚C for 30 seconds, annealing at 55˚C for 30 seconds and extension at 72˚C for 1 minute and 30 seconds.

All probes used in hybridization analysis were produced by the RT-PCR. Amplification of activin β_A-subunit and β-actin fragments was performed with previously described specific oligonucleotide primers using untreated granulosa-luteal cell RNA as a template (41). Human ActR-I, ActR-IB, TGF−β and α-globin amplification, the cDNA cloning procedure and primers used for PCR are

detailed in Studies I and III. The primers used for inhibin α- and β_B-subunit and the cDNA clones obtained have been described elsewhere (36, 42). A genomic 417-bp DNA fragment for the activin β_C-subunit was derived by PCR performed on human K562 cell DNA (212).

The amplified cDNAs were ligated to commercial T-vectors pCR1000 (InVitrogen, San Diego, CA, USA) or pGEM-T (Promega) and characterized by sequencing the double stranded plasmid templates using cloned T7 DNA polymerase (Sequenase 2.0, U.S. Biochemical Corp., Cleveland, OH, USA).

6. Southern blotting

For Southern blotting of amplified cDNA fragments, the samples were electrophorezed through 3.5 or 4% agarose gels, stained with ethidium bromide and illuminated under UV light. The samples were then transferred on Hybond N nylon membranes (Amersham) according to standard protocols (213) or by the downward alkaline capillary transfer method (214).

7. Probe labeling, hybridizations and quantification of the data

Double stranded cDNA inserts were labeled with [α-^{32}P]deoxy (d)CTP (3000Ci/mmol; DuPont, Boston, MA, USA or Amersham) by the random priming method (Random priming (30) labelling kit; Boeringer Mannheim or Prime-a-gene kit; Promega). If the signal was weak single stranded cDNA probes were prepared by linear PCR amplification. Both single- and double-stranded cDNA probes were purified with Nick columns (Pharmacia Biotech, Uppsala, Sweden) or NucTrap columns (Stratagene, La Jolla, CA) and used at 1-3 x 10^6 cpm/ml in hybridization solution containing 50% formamide, 6 X SSC (1 X SSC = 0.15 M NaCl and 0.015 M Na-citrate, pH 7.0), 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA, 100 μg/ml salmon sperm DNA, 100 μg/ml yeast RNA, and 0.5% sodium dodecyl sulfate (SDS). Northern and Southern blots were hybridized for 16 h at 42˚C and washed three times for 20 minutes with 1 X SSC-0.1% SDS at 55˚C. Filters were exposed to X-ray film with Trimax 16T intensifying screens (3M, Ferrania, Italy) at –70˚C. The relative densities of dot blot hybridization signals were detected using transmission densitometer (model 331, X-rite Co., Grand Rapids, MI). Alternatively, hybridized filters were analyzed by Fujifilm IP-reader Bio-Imaging Analyzer BAS 1500 (Fuji Photo co. Ltd., Tokio, Japan) with the MacBas software supplied by the manufacturer.

8. Analysis of RNA data

For single comparisons the data were analysed by the Student's t-test. For multiple comparisons the data were first analysed by one-way analysis of variance, and statistical significance was determined by Scheffe's multiple comparison test using the Exstatics program (Select Micro Systems, Yorktown Heights, NY) on a Macintosh personal computer.

9. Western blotting

For Western analysis the culture media were concentrated with Ultrafree MC 10.000 NMWL filter unit concentration tubes (Millipore Products Division, Bedford, MA, USA). Duplicate 30 ml aliquots equivalent to 800 μl of medium were run under reducing and non-reducing SDS-polyacrylamide gel electrophoresis (PAGE) conditions and transferred to Hybond-C extra nitrocellulose filters (Amersham) using a Bio-Rad Mini Trans-Blot apparatus (Bio-Rad Laboratories, Richmond, CA, USA). Before immunoblot analysis the membranes were incubated in blocking solution [Tris buffered saline (TBS), 3% BSA] at room temperature for 1-2 hours.

Subsequently, the blots were exposed to a 1:500 dilution of rabbit polyclonal β_A-antiserum (#560) (41) or to a 1:2000 dilution of mouse monoclonal E4 anti-β_A antibody (Serotec, Oxford, UK) and incubated at +4 C overnight. As negative controls, the polyclonal β_A-antiserum was pre-incubated with synthetic β_A (93-105)-NH_2 peptide (50 µg/ml) and the mouse monoclonal E4 antibody was pre-incubated with activin A (250 ng/ml) overnight at +4 C prior to incubation with membranes. The blots were then rinsed with the TBS/Tween buffer and incubated with peroxidase conjugated goat-anti-rabbit IgG or goat-anti-mouse IgG (1:3000) (Bio-Rad). The immunoreaction was detected using Enhanced Chemiluminescence (ECL) system according to the manufacturer's instructions (Amersham).

10. Radioiodination of follistatin and ligand blotting

Activin binding protein, follistatin 288, obtained from the National Institute of Child Health and Human Development, NIH, was iodinated using the Iodo-Gen method according to manufacturer's instructions (Pierce, Rockford, IL, USA) and the labelled protein was purified by PD10 columns (Pharmacia). The proteins in the culture media of unstimulated and TPA-induced cells were separated on SDS-PAGE and transferred onto nitrocellulose filters. The filters were incubated with ^{125}I follistatin (200 000 cpm/ml) overnight at +4˚C (76), washed with the TBS/Tween buffer and exposed to X-ray film for 1 to 3 days at –70˚C. As a specificity control, ^{125}I-labeled follistatin was pre-incubated with activin A (250 ng/ml) overnight at +4˚C prior to incubation with membranes.

Results

1. The primary structure of human activin receptor type II and IIB (I)

Human ActR-II and ActR-IIB cDNAs were amplified by RT-PCR using primers designed according to published rodent ActR-IIs sequences. The 556-bp and 959-bp fragments of ActR-II and ActR-IIB, respectively, were amplified from human K562 cell cDNA covering part of the extracellular domains, transmembrane regions and part of the intracellular kinase domains. Since type II activin receptors were expressed at relative low levels in K562 cells we delineated the tissue distribution pattern of ActR-II and ActR-IIB mRNAs in other sources to facilitate their cloning. A strong expression level of ActR-II was detected in human fetal kidney. The expression of ActR-IIB was highest in the developing brain. Therefore, human fetal kidney and brain cDNAs were used to amplify RT-PCR clones representing the missing 5' and 3' sequences to obtain the full reading frame of hActR-II and hActR-IIB, respectively.

To evaluate the possible alternative splicing of hActR-IIB mRNA, RT-PCR was performed on human K562 cells and human fetal brain polyadenylated mRNAs and mouse NIH-3T3 cell RNA using primers flanking the region of the cDNA encompassing the alternative spliced regions. Southern hybridization with the 544-bp hActR-IIB_2 probe defined only a fragment corresponding to the ActR-IIB_2 isoform. This fragment was amplified from K562 cell and human fetal brain cDNA. However, under similar experimental conditions, the four mouse splicing variants were detected in Balb/c 3T3 fibroblast cDNA (99). In NIH-3T3 cells the ActR-IIB_2 isoform was clearly more abundantly expressed than the other three variants.

2. The tissue distribution of inhibin α, activin β_A- and β_B-subunit, follistatin and ActR-II and -IIB messenger ribonucleic acids in human fetal tissues (I and II)

During human development only the adrenal glands expressed the 1.6-kb inhibin α-subunit transcripts. The strongest expression of the β_A-subunit was detected in neural and muscular tissues. These 1.7-, 4.0- and 6.0-kb β_A-subunit transcripts were also detected in several exocrine tissues as well as in bone marrow. The 3.8- and 4.8-kb transcripts of the β_B-subunit were also detected in most of these tissues but the expression levels differed compared to the β_A-subunit. The β_B-subunit transcripts were most abundant in neural tissues, adrenal glands and salivary glands. The major 2.5-kb and the minor 1.5-kb transcripts of follistatin were seen in several fetal tissues with the highest expression levels in the kidneys, the liver and skeletal muscle.

Similar to activin β-subunit mRNAs, the highest expression levels of the 3.0- and 6.0-kb ActR-II and 2.5- and 10-kb ActR-IIB transcripts were detected in developing neural tissues such as cerebrum and spinal cord. Moderate expression levels of both ActR-II and ActR-IIB were detected in muscular tissues such as skeletal muscle, heart and stomach. Low but detectable expression of ActR-IIB mRNA was also observed in several glandular tissues (kidney, salivary, and pancreas) and hematopoietic tissues (spleen, liver, thymus, and bone marrow). The adrenal glands, which consists of both glandular (cortex) and neural (medulla) tissue, also expressed ActR-IIB mRNA. Although ActR-II and ActR-IIB mRNAs were coexpressed in several human fetal tissues ActR-IIB showed a wider tissue distribution than ActR-II.

3. Expression and regulation of β_A-subunit transcript and polypeptide, and type I and II receptor mRNAs in K562 cells (I and IV)

In Northern analysis of untreated K562 cell RNA no hybridization signal of β_A-subunit mRNA was detected. However, β_A-subunit transcripts of 1.8-, 2.8-, 3.0-, 4.3-, and 6.4-kb were seen in TPA-stimulated K562 cells. The expression of β_A-subunit mRNA was also confirmed by RT-PCR analysis. A 786-bp fragment was amplified from TPA-induced K562 cells whereas no amplification product was seen in untreated cells. As a positive control for β_A-subunit primers granulosa-luteal cell RNA was used as a template. No transcripts for the inhibin α-, activin β_B- or β_C-subunits were detected by RT-PCR followed by Southern blotting of the PCR products of untreated or TPA-treated K562 cells (data not shown).

TPA regulates the expression of β_A-subunit transcript in both a time- and concentration dependent manner in K562 cells. The induction of the β_A-subunit transcripts with 10 ng/ml TPA was seen after eight hours incubation, and the maximal responses were obtained with 1.0-100 ng/ml of TPA. In contrast neither activin nor TGF-β, which differentiate K562 cells toward the erythroid lineage, stimulated β_A-subunit mRNAs (data not shown). The induction of activin β_A-subunit mRNA expression by TPA could be blocked with a potent protein kinase C inhibitor H7 (10 μM).

In Western blot analysis we used the reduced samples of culture medium of K562 cells treated for 72 h with 10 ng/ml of TPA. Polyclonal β_A-antibody (#560) detected a 14 kD protein in these samples. The immunoreaction was prevented by pre-incubation of the polyclonal anti-β_A antiserum with the synthetic β_A peptide against which the antibody was raised confirming the specificity of the β_A-antibody. The monoclonal E4 anti-β_A antibody was used to recognize dimeric β_A-subunits. The predicted 25 kD protein was recognized in the culture medium of TPA-treated K562 cells whereas no signal was detected when the E4 β_A-antibody was pre-incubated with activin A. The follistatin binding capacity of the 25 kD protein was determined by ligand blotting experiments with ^{125}I-labelled activin-binding protein follistatin. A 25 kD protein was consequently detected in conditioned medium of TPA-treated K562 cells. Pre-incubation of ^{125}I-follistatin with activin A prior to ligand blotting prevented the recognition of the 25 kD protein confirming the specificity of the signal. To determine the time- and concentration dependence of the effect of TPA on activin A protein secretion K562 cells were also used in Western and ligand blotting analyses. Our results indicate that β_A-subunit monomers and dimers become detectable in the culture medium after a 24 h stimulation with 10 ng/ml of TPA and that maximal levels are reached by 48 h. Concentration dependence experiments indicated that TPA concentrations of 1.0 ng/ml, and above, strongly induced the secretion of activin A in these cells.

The expression of activin type II and type I receptor transcripts were studied by Northern blot analysis of polyadenylated K562 cell RNAs. The specific 10- and 2.5-kb transcripts and a very weak 2.1-kb hybridization signal were observed with the ActR-IIB probe. For ActR-II, 6.0- and 3.0-kb transcripts were detected. The expression levels of both type II receptors were relatively low in K562 cells when compared with eg. human fetal brain. Although no exact quantitative determination of mRNA levels was performed, ActR-IIB appeared to be more abundantly expressed than ActR-II in K562 cells based on the comparison of hybridization signals obtained after similar exposure times of Northern blots.

The regulation of ActR-II and ActR-IIB mRNA levels during the differentiation of K562 cells was studied by Northern blot and dot blot hybridization. The K562 cells were treated with increasing concentrations of activin A (0.1 to 30 ng/ml) for 72 hours for erythroid differentiation. Activin A

increased α-globin mRNA expression in K562 cells in a concentration- and time- (data not shown) dependent manner. However, neither ActR-II nor ActR-IIB mRNA levels were affected by activin A treatment. On the other hand, during TPA-induced (0.01 to 10 ng/ml) megakaryocytic differentiation the expression of TGF-β_1 mRNA was stimulated in a concentration-dependent manner in K562 cells although it had no effect on ActR-II and ActR-IIB mRNA levels. The relative expression levels of both ActR-II and ActR-IIB mRNAs were not altered by induced differentiation.

For ActR-I and ActR-IB the specific 4.0-kb and 5.2-kb transcripts were detected. Both type I activin receptors were relatively weakly expressed in untreated K562 cells, but induction with TPA (10 ng/ml) increased their expression in a time-dependent manner. However, neither ActR-I nor ActR-IB mRNA levels were affected by activin A (20 ng/ml) or TGF-β_1 (1 ng/ml) treatments.

Since 8-Br-cAMP has been shown to increase β_A-subunit mRNA levels in several different human cell culture models we determined whether it affects β_A-subunit and type I activin receptor mRNA expression in K562 cells. Neither β_A-subunit nor type I activin receptor transcript levels were induced by 8-Br-cAMP (1 μM). Interestingly, the effect of TPA on β_A-subunit mRNA expression was significantly increased when the K562 cells were treated with TPA (10 ng/ml) and 8-Br-cAMP (1 μM) together. However, TPA-induced type I activin receptor mRNA levels were not affected by a co-treatment with 8-Br-cAMP.

4. Regulation of α-, β_A- and β_B-subunit and activin receptor mRNAs in human GL-cells (III)

Northern analysis of activin A (30 ng/ml) treated human granulosa-luteal cells showed that the expression of a specific 4.8-kb β_B-subunit mRNA was induced within 24 h whereas the expression of the 1.6-kb α-subunit or β-actin transcripts (used as a loading control) were not affected. No effect on the β_A-subunit mRNAs was detected (data not shown). Dot blot hybridization experiments indicated that the α- and β_A-subunit transcripts were not induced by activin A at any time point between 2-48 h. By contrast, after 8 h stimulation with activin A the expression levels of β_B-subunit mRNAs were significantly increased and the maximal effect was detected at 48 h. On the other hand, the mRNA levels of cytochrome P450 (P450scc), which is a rate-limiting enzyme in progesterone synthesis, slightly decreased at 24 and 48 h by activin. The maximal effects of activin A were detected in concentration range of 25-100 ng/ml. hCG (30 ng/ml) treated granulosa cells were used as a positive control for the α-subunit and P450scc mRNA expression.

To study the effect of FS on the basal and activin A-stimulated β_B-subunit mRNA levels, increasing amounts of follistatin (up to 350 ng/ml) were pre-incubated with or without activin (25 ng/ml). Follistatin inhibited the effect of activin A on β_B-subunit mRNA levels in concentration-dependent manner, but has no effect by itself. Inhibin A had no effect on β_B-subunit mRNA levels at any concentration or time point tested in the presence or absence of activin A.

Inhibin α-subunit and p450scc mRNA levels were significantly induced when the granulosa cells were stimulated with hCG for 24-48 h. The β_B-subunit transcript levels were not affected. However, co-stimulation of granulosa cells with hCG and activin A prevented the stimulatory effect of activin A on β_B-subunit mRNA levels. Interestingly, activin A slightly decreased hCG-stimulated inhibin α-subunit transcript levels. Activin A did not affect hCG-induced β_A-subunit mRNA levels (data not shown).

The expression of activin receptor mRNAs was studied by Northern blot analysis of RNA extracted from freshly isolated human preovulatory granulosa cells as well as human GL cells cultured for 5 or 6 days. The Northern blots were hybridized with single or double stranded DNA probes for human ActR-I, ActR-IB, ActR-II and ActR-IIB. Specific transcripts of the expected sizes were detected for all four receptor subtypes in both the freshly isolated preovulatory granulosa cells and cultured GL cells. The expression of the specific transcripts of inhibin and activin subunit mRNAs in preovulatory granulosa cells was also shown.

Discussion

1. Cloning and characterization of human type II activin receptors (I)

The open reading frame of hActR-II and hActR-IIB cDNA sequences was constructed from several partial overlapping cDNA clones amplified from K562 cells, and human fetal kidney and brain RNA, respectively. Human ActR-II cDNA encodes a protein of 513 aa and was 99% identical at the amino acid level with the mouse ActR-II (94). In hActR-II we observed one nucleotide difference in sequence comparisons to hActR-II cDNAs published by others (126, 127). However, the difference did not alter the deduced amino acid sequence. Like the type II activin receptor (94, 126, 127, 215, 216) the amino acid sequence of hActR-IIB shows more than 98% homology with its rodent counterparts (99, 211). The human ActR-IIB cDNA encodes a 512 aa transmembrane protein with an extracellular ligand binding domain and an intracellular signalling domain with ser/thr specificity. The human sequence differs only by five amino acids from the corresponding mouse sequence (99). Three substitutions in the signal peptide, one in the ligand binding domain, and one in the C-terminal region was found.

Because four different type IIB activin receptor transcript isoforms are generated in mouse by alternative splicing events (99), we examined whether this feature is also characteristic of the human ActR-IIB gene. However, based on our RT-PCR studies we did not find any other receptor transcript isoforms than the ActR-IIB_2 variant in K562 cell and human fetal brain samples. Under similar experimental conditions we detected all four splicing variants in mouse NIH-3T3 fibroblast RNA, corresponding to earlier results reported by Attisano *et al.* (99). Peng *et al.* have confirmed our observation by showing that ActR-IIB_2 is the only variant of the ActR-IIB in human brain, placenta and granulosa-luteal cells (217). However, in human teratocarcinoma cells two isoforms, ActR-IIB_1 and ActR-IIB_2, have been characterized later (129). The ActR-IIB_2 variant appears to be the most prevalent isoform in the mouse and ActR-IIB sequences reported in other species resemble ActR-IIB_2 in structure (211, 218-220). The biological relevance of different isoforms is still unclear, but the ActR-IIB_1 and ActR-IIB_2 isoforms appear to bind activin with higher affinity than the ActR-IIB_3 and ActR-IIB_4 variants, which have binding abilities comparable to that of ActR-II receptor (99). This suggests that cells bearing ActR-IIB_1 and ActR-IIB_2 isoforms may bo more sensitive to activin than those harbouring type IIB_3, IIB_4, or II receptors. Different ActR-IIB isoforms may also have different binding properties on the other TGF-β family ligands that use ActR-IIB in their signal transduction.

Our study suggests that human ActR-IIB RNA is processed to encode the high affinity ActR-IIB_2 receptor. Although we did not characterize the binding properties of ActR-IIB_2 Zhou *et al.* have used our cDNA clone in studies determining the signalling properties of ActR-IB isoforms (221). Activin A-induced phosphorylation of ActR-IIB complexed with ActR-IB was significantly increased compared with that seen with basal phosphorylation. In another study, soluble ActR-IIB_2 protein produced by us has been used to block molar tooth development in mandibular explants in mouse (222).

2. The tissue distribution of inhibin α, activin β_A- and β_B-subunit, activin type I and II receptor and follistatin mRNAs in human fetal tissues (I, II)

Our studies were originally the first reports to describe the expression of activin α- and β-subunits, activin receptor and follistatin transcripts during human development. The highest expression levels of the activin signalling components were detected in developing neural and muscular tissues. Activin subunit transcripts are differentially expressed and the mRNA levels varied in various brain regions. The strongest hybridization signals of β_A- and β_B subunits were observed in developing neural tissues in the cerebrum and spinal cord, whereas no expression of β-subunits was detected in the cerebellum. Interestingly, of the neural tissues studied, follistatin was expressed only in the cerebellum. Prior to this study the β-subunit transcripts had been localised in developing rat (223) and *Xenopus* (224) central nervous systems, and later a more detailed expression pattern in developing rat brain was reported by Andreasson *et al.* (225). In several models of acute brain injury up-regulation of β_A-subunit transcript as well as activin A protein has been observed, suggesting an important role for activin in neuroprotection (226).

Further confirming the role of activin during the development of the nervous system, we found that ActR-IIB and ActR-II mRNAs were abundantly expressed in developing human neural tissues such as brain and spinal cord. Subsequently, it has been shown that all four types of activin receptor mRNAs are expressed in embryonic rat brain (227), and throughout the adult rat brain the expression of ActR-II mRNA is stronger than that of ActR-IIB (172). Studies on non-mammalian vertebrates have shown the expression of ActR-II in chicken embryo neural tissues (128) and ActR-IIB in *Xenopus* tissue undergoing neurulation (218). Activin promotes neural cell survival *in vitro* (228), stimulates proliferation and inhibits terminal differentiation of several neural cells in culture conditions (229). However, homozygous mice carrying null mutations of activin A and/or activin B or ActR-II genes do not show any obvious defects in neural development (57-59, 62). This discrepancy in the effects of activin in neural tissues may be explained by other TGF-β ligands signalling through ActR-IIB, and some compensation by maternal activins in the embryo.

In the developing human muscle, including heart, skeletal muscle and smooth muscle cells of the stomach, activin β_A-subunit and follistatin transcripts as well as both type II activin receptor transcripts were relatively abundantly expressed. The strongest hybridization signal of the follistatin was detected in the skeletal muscle. The expression of follistatin is also detected in developing muscle of chicken limb (230). Recent data confirms the role of follistatin and type II activin receptors in regulating muscle growth. Myostatin, which negatively regulates skeletal muscle mass, is suggested to use ActR-IIs in signalling, and its activity can be blocked by follistatin (231). Further, the expression of follistatin or the dominant-negative form of ActR-IIB under the myosin light-chain promoter/enhancer increases muscle mass similar to that seen in myostatin deficient mice (232).

The β_A-subunit mRNA expression was strongest in heart. In line with our findings the expression of the β_A-subunit has been detected in the developing rat (223) and mouse heart (233). However, no β_B-subunit transcripts were detected in the developing murine heart at the same stage (234). The expression of ActR-II has been shown in the developing mouse (235) and chicken heart, and in myotomes of developing chicken embryos (128). Recently, it was reported that disruption of the ActR-IIB gene results in lateral asymmetry of heart and lungs in developing mice (132) suggesting that this gene may be mutated in left-right axis abnormalities in humans as well. However, ActR-IIB mutations were detected only in rare cases of left-right axis malformations (129).

The detection of β_A-subunit expression in human fetal hematopoietic tissues (bone marrow, spleen, liver) is in line with earlier evidence for activin as a hematopoietic regulator (3, 189). In human bone marrow cultures and peripherical blood activin is able to increase the proportion of DNA-synthesizing erythroid progenitors (190). In contrast to human liver, no expression of the β_A-subunit transcript was detected in developing mouse embryos (233). Based on ample evidence of the role of activin as a hematopoietic regulator (186, 187, 189, 192-197) it is not surprising that several human hematopoietic tissues express activin receptors. In conclusion, the similar distribution pattern of activin β-subunits as well as activin type II receptors in the midgestational fetus suggests an important role of activin during human development.

The expression of the β-subunits and follistatin was detected in some developing exocrine glandular organs. Relatively low but detectable expression levels of ActR-IIB were also found in kidney, salivary, pancreas, and adrenal tissues, as well as in fetal hematopoietic tissues including spleen, thymus, liver and bone marrow. Mutations of ActR-IIB have been shown to disrupt the development of the stomach, pancreas and spleen in mouse (236). In developing rat salivary gland the β_B-subunit mRNA has been shown to be highly expressed (223), correlating well with our findings. Moreover, by *in vitro* organ cultures of the developing mouse kidney, salivary gland and pancreas rudiments we have observed that the epithelial branching morphogenesis of these tissues is severely disturbed by the addition of exogenous activin (36). In transgenic mice expressing the truncated ActR-II under the β-actin promoter the attenuated signalling of activin A or related ligands is suggested to increase the number of nephrons by enhancing the branching of ureteric buds in the developing kidney (237). A role for activin in the development of the kidney is also supported by the study showing ActR-II and ActR-IIB transcripts in chicken (128) and mouse (233) developing kidney, respectively. The effects of activin A in the branching morphogenesis have been recently reviewed in Ball and Risbridger (238). Although the wide tissue distribution of activin β-subunits suggests the importance of activin in developing human tissues, gene targeting studies have shown that in mice deficient in both activin β_A- and β_B-subunits the structure and function of the organs studied were not affected (57). Thus, it seems that other activin subunits or growth factors using the same signalling components are able to substitute for the effects of activin.

3. The expression and regulation of activin β_A-subunit during induced differentiation of human erythroleukemia K562 cells (IV)

In human erythroleukemia K562 cells, activin regulates both mitogenesis (189, 239) and differentiation-related phenomena such as hemoglobin production (189, 199, 239). In addition to activin, TGF-β is also able to induce erythroid differentiation of K562 cells (189, 240). However, neither of these growth factors affects activin β_A-subunit mRNA or protein expression in these cells. Instead the expression of the activin β_A-subunit mRNA and protein is induced by the phorbol ester TPA which activates protein kinase C (241) and megakaryocytic properties in K562 cells (205). The 5' untranslated region of the human activin β_A-subunit gene includes promoter sequences and various potential enhancer sites of which TPA and cAMP-responsive elements are functionally necessary for transcriptional regulation of β_A-subunit gene in human fibrosarcoma HT1080 cells (39). TPA-induced expression of β_A-subunit mRNAs was clearly observed in K562 cells as well. By Northern blotting five different β_A-subunit mRNA species (6.4-, 4.3-, 3.0-, 2.8-, and 1.8-kb) were detected, with main sizes of 3.0- and 2.8-kb. Consistent with our findings, the expression of multiple β_A-subunit mRNAs in the size range of 1.7- to 6.0-kb has also been reported in other human cell culture models (7, 31, 40, 42, 44, 48, 242). Different transcription start sites

(45) or alternative polyadenylation signals (44) may be responsible for the existence of the differently sized β_A-subunit mRNAs.

TPA induces the expression of activin β_A-subunit mRNA in K562 cells both in a time- and concentration-dependent manner. The maximal response was seen at 24 h whereafter the mRNA levels decreased. However, in other cultured cells TPA-induced β_A-subunit mRNA expression shows differential kinetics. In human fibrosarcoma HT1080 cells (243) and in murine bone marrow stromal cell lines, MC3T3-G2/PA6 and ST2 (197), TPA increases β_A-subunit mRNA expression rapidly and transiently with maximal levels at 6h and 4h, respectively. By contrast, in human granulosa-luteal cells (212) and in human fetal adrenal cells (42) β_A-subunit mRNAs are rapidly induced within 2-4 h and the levels remain increased up to 48 h. The TPA-induced expression of β_A-subunit mRNA in K562 cells resembles that seen during TPA treatment of human THP-1 (7) and HL-60 cells (242), where β_A-subunit transcripts are slowly induced and remain elevated up to 48 h. Thus, the regulation of β_A-subunit mRNAs by TPA seems to differ in distinct cell types suggesting tissue specific regulation of the β_A-subunit gene.

Using Western and ligand blotting analyses we evaluated whether the TPA-induced changes of β_A-subunit mRNA levels are reflected of the protein level in K562 cells. We observed that K562 cells secrete fully processed immunoreactive 25 kD activin A protein in response to TPA stimulation and in reducing conditions the protein is seen as the expected 14 kD monomer. Labelled follistatin was used in ligand blotting analyses to further confirm that the 25 kD protein exhibits follistatin binding capacity. Thus, K562 cells secrete considerable amounts of processed activin A protein. In line with our results, it has been reported that TPA-stimulated K562 cells produce erythroid differentiation factor bioactivity (244). Thus, the production of immunologically and biologically active activin A protein is clearly characteristic for K562 cells induced toward the megakaryocytic differentiation lineage by TPA.

We also determined the role of the cAMP-activated signalling pathways in the regulation of activin β_A-subunit mRNA levels in K562 cells. Prior to this study cyclic AMP had been shown to induce activin β_A-subunit mRNAs at least in human placental cells (245), granulosa-luteal cells (212), adrenal cells (42) and in HT1080 cells (39, 243). However, in K562 cells no induction of β_A-subunit transcript by cAMP-analogue, 8-Br-cAMP, was observed. Thus it seems that the regulatory mechanisms controlling the mRNA levels of the β_A-subunit differ between cell lineages. Interestingly, co-treatment of K562 cells with both 8-Br-cAMP and TPA significantly increased the expression of activin β_A-subunit mRNA levels compared with mRNA levels induced by TPA alone. The synergistic induction of the activin gene expression by cAMP and TPA has been reported also in human fibrosarcoma HT1080 cells (243).

4. The expression and regulation of activin receptors in human K562 erythroleukemia cells (I, IV)

We have demonstrated that all four known activin receptor mRNAs are expressed in human K562 erythroleukemia cells, which fits well with the finding that kinase activities of both type II and type I receptors are required for activin signalling (200). Kinase-deficient ActR-II abrogates activin-induced erythroid differentiation (246) and the dominant-negative mutant of ActR-IB inhibits transcriptional activation and growth suppression induced by activin in K562 cells (221). Furthermore, increasing the levels of ActR-IB and ActR-II using an inducible promoter enhanced

the effects of activin on erythroid differentiation in K562 cells, demonstrating that both ActR-IB and ActR-II are physiologically important receptors for activin (201). Consistent with this observation, ActR-II in concert with activated mutants of either ActR-I or ActR-IB induces hemoglobin expression in another erythroleukemia cell line, mouse F5-5 cells (247). On the other hand, only ActR-IB and ActR-II were detected by RT-PCR in mouse F5-5.fl cell line, in which erythrodifferentiation is induced by activin (248).

By Northern analysis we detected the specific 4.0- and 5.2-kb transcripts for ActR-I and ActR-IB, respectively. Results from primer extension studies suggest that tissue specific alternative transcription start sites might be used for expression of the mouse ActR-I gene, resulting in variable sizes of the 5' untranslated region (148). Two major transcripts, 3.0- and 6.0-kb and 2.5- and 10-kb, were observed for both ActR-II and ActR-IIB, respectively. A very weak signal of 2.1-kb was also detected with the ActR-IIB probe. If ActR-II transcripts are structural counterparts of the respective mouse mRNAs they are likely to arise from the use of different polyadenylation signals during the transcription of the ActR-II gene. In the mouse ActR-II gene a transcription start site together with a polyadenylation signal gives rise to the 3.0-kb transcript (123). When mActR-II was originally cloned, a cDNA clone expanding beyond the 3.0-kb transcript polyadenylation signal was reported, suggesting that a longer transcript also arises during mActR-II transcription, and supporting our finding of the existence of a 6.0-kb mRNA for the human receptor (94). The relative expression level of these two transcripts is regulated in a tissue- and developmental-stage-manner in mammalian tissues (169, 249, 250). Although the genomic organization of hActR-IIB has been reported (130), there are still not sufficient data available on the 3' and 5' untranslated regions of the ActR-IIB gene from any species to predict the nature of these different transcripts. However, different transcript sizes have been detected by Northern analysis at least in P19 mouse embryonic carcinoma cells (2.4-, 3.8-, 11- and 14-kb) (233) and bovine tissues (2.0-, 2.3- and 10-kb) (131), the latter correlating well with the length of human ActR-IIB mRNA species.

The expression levels of type I and IB transcripts are relatively low in untreated K562 cells but they are up-regulated during megakaryocytic differentiation of these cells by TPA in a time-dependent manner. By contrast, TPA did not affect the steady-state levels of hActR-II and IIB mRNAs. Similarly, TPA in human HL-60 promyelolytic leukemia cells does not regulate the expression of ActR-IIB either. Although we did not observe regulation of ActR-II or ActR-IIB mRNAs in K562 or HL-60 cells, these mRNAs are regulated in other systems in a tissue- or cell-specific manner (251-253). In K562 cells, the ActR-IIB mRNAs are somewhat more abundantly expressed than ActR-II transcripts and induction of K562 cells by TPA does not affect their relative expression levels. Although the expression of type I receptor mRNAs were stimulated by TPA treatment, neither type I receptor nor type II transcripts were affected during activin-induced erythroid differentiation. The co-ordinated up-regulation of activin β_A-subunit and its type I receptors by TPA suggests that activin might play a role in megakaryopoiesis. According to present knowledge the overall role of activin in blood formation is considered to be secondary, not primary. Thus, activin fine-tunes rather that drives the differentiation of hematopoietic progenitor cells.

5. The regulation of activin β_B-subunit mRNA in cultured human granulosa-luteal cells (III)

The data regarding the expression and regulation of activin and inhibin subunits and follistatin suggest that these proteins are likely to have important roles in folliculogenesis, oocyte maturation and the function of corpus luteum [reviewed in Knight and Glister (176)]. In our studies, we have used human granulosa-luteal (GL) cells as an activin responsive cell model. These cells are

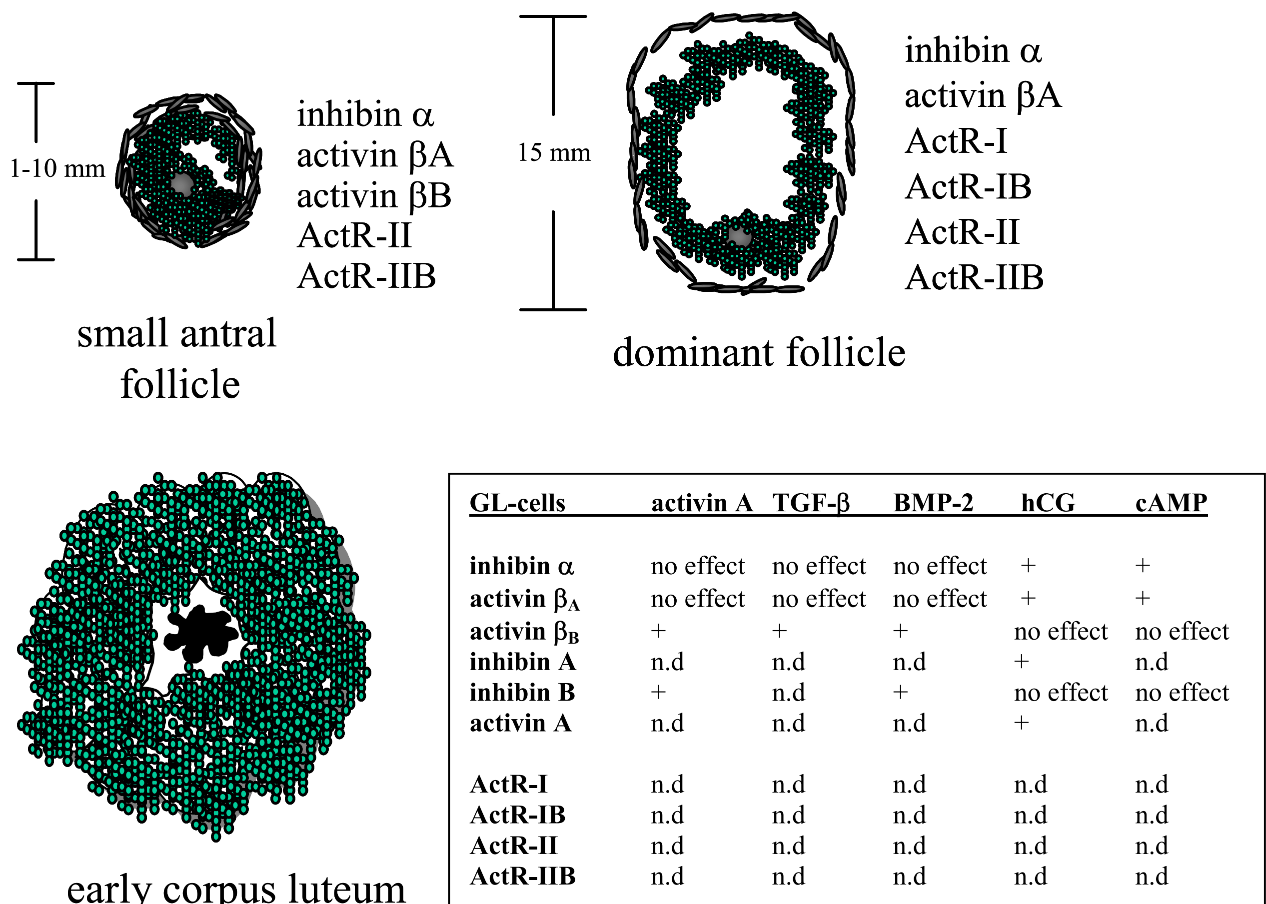
obtained from women undergoing infertility treatment for *in vitro* fertilization program. GL cells can be maintained in primary cultures and they provide a model for studying hormonal regulation of late follicular to early luteal granulosa cells. We have observed that human granulosa-luteal cells express inhibin α-subunit and activin β_A- and β_B-subunit genes (204). Confirming our findings, activin A and inhibin A proteins are produced by human GL-cells (181, 254). To further analyse their biological role in ovarian physiology, we studied whether activin or inhibin are able to regulate their subunit or receptor mRNA levels. The clear induction of activin β_B-subunit mRNA levels was observed by activin A in concentration- and time-dependent manner without affecting basal α- and β_A-subunit mRNAs in human GL cells. Recently this finding has been confirmed by Liu *et al.* (255). In addition, activin A increases inhibin B secretion in human GL-cells (256). We have observed in this study that follistatin prevents the stimulatory effect of activin A on β_B-subunit mRNA levels further confirming the specificity of the stimulatory effect of activin A. In addition to activin A, TGF-β and BMP-2 are also able to induce the expression of activin β_B-subunit expression in human GL cells (257, 258). We have detected that the ser/thr kinase receptors and Smad signalling proteins needed for activin A, TGF-β and BMP-2 signalling are expressed in human GL cells. Recent studies confirm that BMP-2 and activin A activate the Smad1 and Smad2 pathways, respectively, as well as inhibin B production in human GL cells (259). The expression of inhibin, activin and activin receptors in developing human follicles and GL-cells is presented in figure 6.

Our results on the regulatory effects of activin A on inhibin and activin subunit mRNA levels differ markedly from those reported in rat granulosa cells. Activin-induced expression of both the α- and β_A-subunit mRNAs has been shown previously in rats, but no data on β_B-subunit mRNA levels have been presented (182). However, activin A-induced production of both inhibin A and B has been recently reported in cultured rat granulosa cells (260). Several reports have indicated that the distribution of inhibin/activin subunits in the primate ovary is different from that in ovaries of non-primate mammals (261). In human and other higher primate ovaries α- and β_B-subunits are the major mRNA species expressed in small antral follicles, whereas α- and β_A-subunits are expressed by dominant follicles (177, 261, 262). By contrast, the expression of α-subunit mRNA gradually increases from the early primordial follicle to the preovulatory follicle in rat, and the β_A- and β_B-subunit mRNA levels are induced in antral follicles concomitant with the LH and FSH surge (182, 263). However, in the rat corpus luteum levels of the three subunits have been low, or undetectable (182). In human corpus luteum, strong inhibin α and moderate activin β_A-subunit mRNA signal has been detected, but no β_B-subunit mRNA was observed (177).

Although hCG induces α- and β_A-subunit mRNA levels in human GL-cells (264) we were unable to detect any induction of basal β_B-subunit mRNA levels by hCG (Fig. 6). In agreement with our studies, it has been shown at the protein level that hCG and recombinant human LH, which uses the same receptor as hCG, induce inhibin A and activin A secretion but not inhibin B production (254, 265). Interestingly, recombinant human LH even down-regulates the expression of the β_B-subunit mRNA (255).

Our studies on the effect of gonadotropins on inhibin/activin subunit levels indicate that the three subunits are regulated through distinct mechanisms in human GL cells (Fig. 6). The α-subunit is induced by gonadotropins by relatively slow kinetics (264), while activin A decreases hCG-induced α-subunit mRNA levels. The β_A-subunit mRNA is rapidly and transiently induced by gonadotropins (264), but activin A does not influence its levels. The expression of β_B-subunit mRNA is not induced by hCG, but hCG prevents the stimulatory effect of activin A. Subsequently, it has been shown that the second messenger of hCG, cAMP, has no stimulatory effect on secreted

inhibin B levels in human GL-cells (256). This data fits well with our data on β_B-subunit mRNA expression. The inability of hCG or cAMP to induce β_B-subunit mRNA levels in human GL cells was somewhat surprising, as the human β_B-subunit gene promoter region is known to contain several cAMP-responsive elements (47). Further, β_B-subunit mRNA levels are induced by cAMP in human fetal testicular cell cultures (48) and placental cells (245). Although no cAMP-responsive elements have been identified in the rat β_B-subunit gene promoter, the expression of both β_B-subunit transcripts is increased by cAMP (49, 266). Hence the putative AP-2 binding sites, able to mediate transcriptional induction by the protein kinase-A pathway (267), may mediate the effects of cAMP on β_B-subunit gene transcription. The activity and regulation of the β_B-subunit promoter has been examined by CAT assay, revealing that in mouse Leydig tumour cells neither of the two β_B-subunit promoter regions is responsive to cAMP (268) although cAMP induced β_B-subunit promoter activity has been observed in the mouse Sertoli cell line (269). This indicates that cAMP may regulate the activity of the β_B-subunit promoter in a cell-specific manner in the mouse. The regulation of β_B-subunit promoter activity has not been studied in humans.



GL-cells	activin A	TGF-β	BMP-2	hCG	cAMP
inhibin α	no effect	no effect	no effect	+	+
activin β_A	no effect	no effect	no effect	+	+
activin β_B	+	+	+	no effect	no effect
inhibin A	n.d	n.d	n.d	+	n.d
inhibin B	+	n.d	+	no effect	no effect
activin A	n.d	n.d	n.d	+	n.d
ActR-I	n.d	n.d	n.d	n.d	n.d
ActR-IB	n.d	n.d	n.d	n.d	n.d
ActR-II	n.d	n.d	n.d	n.d	n.d
ActR-IIB	n.d	n.d	n.d	n.d	n.d

Figure 6. Expression of inhibin, activin and activin receptors in developing human follicles and GL-cells. In granulosa cells of small antral follicles inhibin α-, β_A- and β_B-subunit mRNAs and polypeptides as well as ActR-II and ActR-IIB proteins are expressed. In dominant follicles the expression of the α- and β_A- subunit, and type I and type II activin receptor mRNAs can be detected. Human GL-cells, which probably correspond to granulosa cells of the early corpus luteum, express inhibin α-, activin β_A- and β_B-subunits as well as inhibin A, B and activin A proteins. Transcripts of type I and type II receptors are also detected. The effects of induction by different factors are shown. (+ = induction of expression, n.d = not determined)

6. The expression of activin receptor mRNAs in human granulosa-luteal cells (III)

We have shown that freshly isolated human granulosa-luteal cells as well as cultured GL cells express the specific transcripts of all four activin receptors, results confirmed later by others (Fig. 6) (217, 270). There is a strong correlation between the transcriptional activity of ActR-I and ActR-II, and ActR-IB and ActR-IIB in human granulosa cells, suggesting that these receptors might be functionally linked in activin signal transduction (271). The transcript sizes of activin type I receptors in human GL cells correspond to those observed in human erythroleukemia K562 cells and other human tissues (92, 97). The major transcripts for ActR-II and ActR-IIB were 3.0- and 2.5-kb, respectively, whereas the 6.0- and 10-kb transcripts were hardly detectable. Interestingly, in bovine reproductive tissues (ovary, corpus luteum, uterus) (250) and in rat ovaries (169) the major band for the type II activin receptor was 6-kb. The expression of mRNAs for all four activin receptors has subsequently been detected in human, mouse and rat oocytes (184, 272). In mice deficient of ActR-II follicles arrest at an early antral stage, consistent with the key role for activin in GC proliferation and differentiation (273). Recently, Pangas *et al.* (185) have reported the co-localization of the proteins involved in the activin signal transduction cascade in human follicles. The expression of ActR-IIB is mainly restricted to granulosa cells of the small antral follicles, whereas ActR-II is intensively expressed also in atretic follicles. Co-localization of activin signalling components is limited to a few developmental stages including granulosa cells of early atretic follicles. However, dominant follicles and corpus luteum, which best corresponds our GL-cell model, were not included in this study.

7. ActR-IIB interacts with multiple TGF-β superfamily ligands

Considerable effort has been devoted to identify the receptors for individual TGF-β superfamily ligands. Although over 40 ligands have been identified only five type II ser/thr kinase receptor genes and seven type I receptor genes are known in vertebrates so far. The diversity of ligands suggests that some receptors probably bind multiple ligands and form several type II/type I receptor complex combinations in signalling. Among the known type II receptors AMHR-II and TβR-II (100) have been shown to be ligand specific with little or no cross-reactivity to other factors. In contrast, activin type II receptors have shown to interact with multiple ligands.

ActR-IIB binds not only activin A and B, but also OP-1/BMP-7, BMP-2 and GDF-5 in the presence of BMP type I receptors (Fig. 7) (138, 155, 156). Confirming the cross-talk between the activin signalling system and BMP receptors, membrane glycoprotein endoglin interaction with BMP-2 and BMP-7 is detected in the presence of ActR-IIB but not when BMPR-II forms the signalling complex (88). Mesoderm formation induced by activin, Vg1, BMP-4 or *Xenopus* nodal-related proteins (Xnrs) can be blocked by truncated dominant-negative ActR-IIB in developing *Xenopus* embryos (5, 274). BMP-4 induces distinct cellular responses in *Xenopus* embryogenesis depending on the receptor combination that it uses for signalling (275). Recently, it has been detected that ActR-IIB can act as type II receptor for Nodal and Xnr1 together with ActR-IB and ALK-7 (Fig. 7) (153). Mice lacking ActR-IIB have defects in left-right axis formation, which is characteristic of Nodal activity as well (132, 133). In addition, the ActR-IB mediated pathway activates Smad-2, which has been shown to affect left-right patterning in mesoderm-formation (276, 277). Myostatin/GDF-8 binding to ActR-IIB has been detected both *in vitro* and *in vivo* using transgenic mice with dominant-negative ActR-IIB (231). Truncated ActR-IIB increases muscle mass in these mice resembling the phenotype of myostatin deficient mice. Myostatin binding can be inhibited by follistatin, and it has been proposed that myostatin presumably activates the same signalling pathway as activin although the type I receptor involved is yet to be defined. Gene targeting studies

have shown that none of the phenotypes observed in ActR-IIB deficient mice were related to the defects seen in mice deficient in activin β_A- and β_B-subunits (57, 132) confirming the hypothesis that ActR-IIB probably transduces the signals of multiple TGF-β family ligands during normal development.

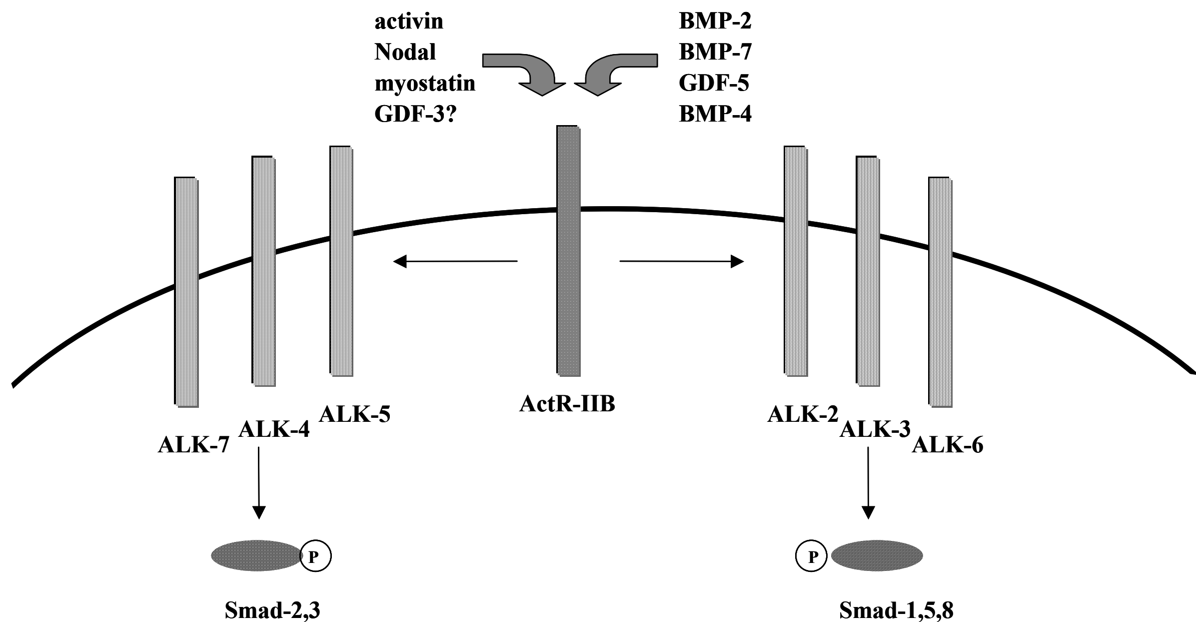


Figure 7. Schematic illustration of the signalling mechanism of TGF-β family members via ActR-IIB. In addition to activin, several other TGF-β family members use ActR-IIB in their signal transduction. Ligand-induced complex formation between specific type I receptor (ALK2-7) and ActR-IIB determines which intracellular downstream components, Smads, are activated by phosphorylation. In the nucleus activated Smad complexes together with other transcription factors regulate target gene responses.

Summary and concluding remarks

During the last decade the expression of activin β-subunits has been extensively studied in order to understand the roles of activins as autocrine/paracrine regulators of cell functions. To elucidate the possible physiological functions of activins it is crucial to detect where activin signalling components are expressed and how they are regulated. We investigated the expression and regulation of activin subunits and its receptors in developing human tissues as well as in different activin responsive cell cultures. Human erythroleukemia K562 cell line contains a functional activin signalling pathway and is commonly used as a model system for erythroid differentiation and activin signalling. In addition to facilitating studies for erythroid differentiation, the K562 cells offer a tool to approach early steps of megakaryoblast commitment and differentiation. In the ovary activins are local growth factors which regulate folliculogenesis. Human granulosa luteal cells used in this study were obtained from women undergoing hormone treatment for *in vitro* fertilization. These cells are well on the pathway to luteinization and thus represent cells forming corpus luteum rather than granulosa cells from growing follicles. However, this model has turned out to be a suitable model for studying the regulation of activin and inhibin subunits *in vitro*.

In the first study we characterized the primary structure of human ActR-II and ActR-IIB. According to cDNA sequences, human ActR-II and ActR-IIB appear to be transmembrane ser/thr kinase receptors corresponding to mouse sequences. Human ActR-IIB represents an ActR-IIB_2 alternatively spliced variant. Like TGF-β superfamily ligands, type II activin receptors are highly conserved between mammalian species.

We demonstrated the tissue distribution pattern of activin and inhibin subunits, follistatin and type II activin receptors in developing human tissues. Strong co-expression of type II activin receptor and activin β-subunit mRNAs were detected in developing human neural and muscular tissues. In glandular tissues both activin β-subunit and ActR-IIB mRNA expression levels were low but detectable. In bone marrow only activin β_A-subunit and ActR-IIB were observed. Follistatin was not detected in any of the hematopoietic tissues. According to our findings inhibin appears to be restricted to the adrenals and testes. Taken together, all activin receptors studied here show wide distribution pattern, and they seem to be controlled in tissue- and gene-specific manner during human development.

We investigated the regulation of activin A and its type I and II receptors in K562 cells. During megakaryocytic differentiation the expression of both of the type I activin receptors were co-ordinately upregulated with the expression of the activin in K562 cells by TPA. 8-Br-cAMP enhanced the inducing effect of TPA on expression of activin β_A-subunit mRNA. In contrast, neither activin nor TGF-β_1 induced β_A-subunit, ActR-I or ActR-IB expression during erythroid differentiation. The expression levels of activin type II receptor transcripts were not regulated during K562 cell differentiation. Our data suggest that activin may play a role in megakaryopoiesis, and that type I and II activin receptors are differentially regulated in K562 cells.

Cultured human GL-cells express all three activin/inhibin subunit mRNAs. We studied the effect of activin A on the expression of activin β_A- and β_B-subunit as well as inhibin α-subunit mRNA levels in human GL-cells. Activin A induced the low basal levels of activin β_B-subunit transcripts without affecting β_A- or α-subunit mRNA levels. Although hCG has been shown to induce activin β_A-subunit and inhibin α-subunit transcript levels it did not affect the β_B-subunit transcripts. By contrast, hCG suppressed the effects of activin A on β_B-subunit mRNA expression. Thus regulation

of the β_B-subunit clearly differs from that of β_A- and α-subunits in human GL-cells. The expression of all currently known activin receptor mRNAs was detected in cultured GL-cells as well as preovulatory granulosa cells, confirming that activin A may act as an inducer of β_B-subunit mRNA levels in these cells.

Taken together, our data suggests that the expression of activins and its receptors are differentially regulated in distinct human tissues and cell models. The multifunctional nature of activin requires tight control. The expression profiles of activin receptors and Smads in the target cell suggest which particular cellular responses are induced by activins or other ligands using activin receptors. Studies on transgenic mice models suggest that activins are perhaps developmentally less important than previously anticipated. In contrast, mice lacking activin signalling pathway genes have severe defects that cannot be rescued by other factors. The Smad pathway seems to be the core of the activin signalling mechanism, but the transcriptional response to activins also depends on what other signals are being received by the cell. In addition, transcription factors present in the cell determine the response to Smad interaction. Thus, the final response depends as much on the activity of receptors, Smads and co-modulators that control and determine targets as it does on the activin signal itself.

Recent studies support the hypothesis that the inappropriate activation or inactivation of the activin receptor mediated signalling pathways could contribute to human diseases. Mutations in the ActR-IIB gene have been associated with left-right axis malformation in humans (129). Additionally, mutated ActR-IB has been detected in pancreatic cancer (278). Smads may inhibit cell division, and, consequently, mutations in Smad proteins have been reported to be involved in several cancers (279). New technologies hold a promise for better understanding of the contribution of activin signalling components to various disease conditions. One important task for the future is to identify those genes that respond to TGF-β superfamily signals. If activin receptors, Smads or co-regulators could be organ-specifically targeted in humans, that could provide new therapeutic possibilities within biomedical science.

As the human genome has been sequenced, it is important to obtain a general view on how the over 40 TGF-β receptor family ligands signal through the currently known 12 receptors and what relevant co-receptors are needed. Several growth factors are known to bind to the same type II ser/thr kinase receptor, which enables signalling by multiple growth factors via a limited set of available receptor types. How many more genes of TGF-β family ligands or receptors will still be found and what is the impact of heterodimerization of ligand monomers remains to be resolved. The newly developed functional genomics and the ability to monitor gene expression at the RNA and protein levels in detail provide an important approach for the future.

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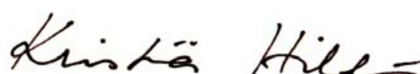

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