Expression and dimerization of the rat activin subunits β_C and β_E : evidence for the formation of novel activin dimers

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Abstract

Activins are cytokines of the transforming growth factor β family, which plays a central role in the determination of cell fate and the regulation of tissue balance. Family members are composed of two subunits and this dimerization is critical for liganding their cognate receptors and execution of proper functions. In the current study we focused on the localization of activin β_A , β_B , β_C and β_E subunits in the adult rat and analyzed the composition of putative activin β dimers. By dissecting tissue distribution of various activins, we found that the liver, in particular the hepatocytes, is the major source for activin β_C and β_E transcripts, since other tissues almost failed to express these isoforms. In sharp contrast, the emergence of activin β_A and β_B appeared ubiquitous. Using a highly selective proteome approach, we were able to identify homo- as well as heterodimers of individual activin subunits, indicating a high redundancy of ligand composition. Certainly, this broad potential to homo- and heterodimerize has to be considered in future studies on activin function.

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Introduction

Activins are members of the transforming growth factor- β (TGF- β) superfamily of growth factors (Kingsley 1994). This extended family encompasses disulfide-linked dimeric proteins characterized by a conserved cysteine-knot motif. Most family members appear to be involved in differentiation and control of proliferation. The first described activins were originally purified from porcine follicular fluid as stimulators of follicle-stimulating hormone secretion, and were found to be homo- or heterodimers of the previously characterized inhibin β subunits A and B (Ling *et al.* 1986, Vale *et al.* 1986). Today they are most commonly referred to as activin A ($\beta_A\beta_A$ subunit structure) and activin AB ($\beta_A\beta_B$). Activin B ($\beta_B\beta_B$) was demonstrated a few years later (Mason *et al.* 1989). Activin subunits β_A and β_B have been shown to be expressed in various tissues and the mature factors

are thought to be involved in biological processes as diverse as reproduction, development, hematopoiesis, tumor development and the immune system (for review see Woodruff 1998, DePaolo 1997).

Based on similarity to known activins, two further mammalian subunits, termed activin β_C and β_E , have been cloned more recently (Hötten *et al.* 1995, Schmitt *et al.* 1996, Fang *et al.* 1996, O'Bryan *et al.* 2000). So far, however, no biological role for activin β_C and β_E has been elucidated. We have now isolated the cDNAs encoding activin β_C and β_E from the Norway rat and performed a comprehensive analysis of activin gene expression in male and female rat tissues. As the three subunits β_A , β_C and β_E were found to be coexpressed in hepatocytes, we furthermore analyzed their potential to homo- or heterodimerize. Since dimerization is critical for activin function, the variety of homo- and heterodimers formed by the subunits β_A , β_C

Table 1 Primers used in the cloning of rat activin subunits β_C and β_E

Primer pair	Primer sequences	Fragment length	Covered nucleotides*
5actbC4 3actbC2	AGG CTA TCC TCC AGC AAT CCT CGA CCA CCA TGT CAG GTA	1055 bp	1–1039
5actbC5 3actbC5	CCC AAC ACC ACC CAG ACC A GCC TGT ATC ACC CAT AAG CTA ACT	654 bp	421–1056
5actbE3 3actbE1	GAG CCA TCT ACC TGG AGC AT GCC ACA GGC CTC TAC TAC CAT	1062 bp	1–1044
5actbE1 3actbE5	GCT CTA GAC CCC CTT ATG TTG AGG CCC TGT TGC TAG CTG	342 bp	728–1053

*Nucleotide positions refer to the A of the ATG initiation codon of rat activin subunit β_C or β_E , respectively, as +1.

and β_E could be a way of modulating activin bioactivity and availability, thereby generating funtional diversity.

Materials and methods

Cloning

The complete coding sequences of the rat activin subunits β_C and β_E were each cloned as two overlapping fragments. Rat liver cDNA was prepared from poly(A) selected RNA of a female Wistar rat using avian myeloblastosis virus (AMV) reverse transcriptase. PCR primers were derived from the mouse cDNA sequences and are given in Table 1. For PCR the high-fidelity polymerase *PfuTurbo* (Stratagene) was used. Fragments were cloned and three independent plasmid clones of each fragment were sequenced by a commercial sequencing service (MWG-Biotech). Nucleotide sequences and the derived peptides were analyzed with MacVector (Oxford Molecular) bioinformatics software.

Preparation of tissues

Anesthetized rats were perfused with ice-cold PBS, the tissues were subsequently dissected on ice, snap-frozen in liquid nitrogen-cooled isopentane, and stored at $-80\text{ }^{\circ}\text{C}$ until further use.

Preparation of liver cell subpopulations

After the preparation of rat liver cells by collagenase perfusion as previously described

(Parzefall *et al.* 1989), parenchymal cells were purified by three low-speed sedimentations (50 *g*) and subsequent centrifugation through Percoll to separate hepatocytes from remaining non-parenchymal cells (Kreamer *et al.* 1986). The supernatant of the first sedimentation step was used for the preparation of the non-parenchymal cell fraction. These cells were purified by centrifugation through a Percoll gradient based on the method of Smedsrod & Pertoft (1985). The cells from the interphase were collected. Two preparations were pooled and used for RNA isolation (see below).

RNA analysis

Preparation of total RNA

Total RNA from various tissues of male and female rats was isolated using the TRIzol Reagent (Life Technologies) according to the instructions of the manufacturer. RNA was dissolved in 3 mM EDTA and the concentration determined photometrically.

RNA probes

Probes complementary to the following regions of the respective cDNAs were used in RNase protection experiments (nucleotide positions generally refer to the A of the ATG initiation codon as +1): rat activin β_A (268 bp; corresponding to nucleotides (-)33–235) (Woodruff *et al.* 1987); rat activin β_B (334 bp; 40–373 of the part encoding the mature peptide; the complete coding sequence of rat activin β_B is currently not available); rat activin β_C (196 bp; 861–1056); rat activin β_E (180 bp; 722–901); rat transforming growth factor (TGF) β_1

(202 bp; 343–544) (Qian *et al.* 1990); rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (101 bp; 866–966) (Tso *et al.* 1985); and rat hepatocyte nuclear factor 4 (HNF4) (124 bp; 916–1039) (Sladek *et al.* 1990).

Plasmid DNA templates were linearized with appropriate restriction enzymes and *in vitro* transcriptions were carried out as previously described (Rossmannith *et al.* 1997) with the following minor modifications: probes were labeled with 4 $\mu\text{Ci}/\mu\text{l}$ α -[^{32}P]UTP (800 Ci/mmol); UTP at 100 μM was included in the synthesis of the GAPDH probe to generate a low specific activity riboprobe; after gel purification the riboprobes were eluted and employed in RNase protection assays on the same day.

RNase protection assay

RNase protection assays were performed as previously described (Rossmannith *et al.* 1997) with the following minor modifications: hybridizations were carried out at 52 °C; RNase A and RNase T1 were used at 15 and 1 $\mu\text{g}/\text{ml}$, respectively. Dried gels were analyzed with a PhosphorImager and ImageQuant software (Molecular Dynamics).

In situ hybridization

Cryostat sections (10 μm) were postfixed in 4% paraformaldehyde (in PBS) for 20 min, treated twice with 0.1% diethylpyrocarbonate in PBS for 15 min, followed by equilibration in $5 \times \text{SSC}$ ($1 \times \text{SSC}$ is 150 mM NaCl, 15 mM Na-citrate, pH 7) for 15 min at room temperature and incubation in hybridization buffer (50% formamide, $5 \times \text{SSC}$, 40 $\mu\text{g}/\text{ml}$ salmon sperm DNA) for 2 h at 58 °C. The specimens were subsequently incubated overnight at 58 °C with 400 ng/ml digoxigenin-labeled antisense or sense transcripts of the complete coding sequence of rat activin β_E or β_A , respectively, in hybridization buffer prepared according to the instructions of the manufacturer (Roche). After hybridization the slides were washed for 30 min in $2 \times \text{SSC}$ at room temperature, and subsequently at 65 °C in $2 \times \text{SSC}$ and in $0.1 \times \text{SSC}$ for 1 h each. Hybridized RNAs were detected with anti-digoxigenin-alkaline phosphatase Fab fragments (Roche) diluted 1:500 in 100 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.5% blocking reagent (Roche). After washing and equilibration to pH 9.5,

alkaline phosphatase was stained by the Nitro Blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate reaction overnight and the sections were subsequently counterstained with Methyl Green.

Production of conditioned media

Plasmids

The complete coding sequences of the rat activin subunits β_A (1275 bp) (Woodruff *et al.* 1987) and β_E (1053 bp), and β_C (1056 bp) were cloned in either pTracer-CMV (β_A and β_E) or pcDNA3 (β_C) (Invitrogen). The sequences preceding the initiation codon were changed to a Kozak consensus sequence (Kozak 1987) by PCR mutagenesis in each case. Plasmid DNA was purified using a plasmid maxi kit (QIAGEN).

Transfections

Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with sodium pyruvate (1 mM) and fetal calf serum (10%). The day before transfection cells were seeded at a density of 2×10^4 cells/cm² in T75 flasks. DNA transfections were performed using the calcium phosphate coprecipitation technique (Graham & van der Eb 1973). Plasmid DNA (27 μg) was sterilized by ethanol precipitation and subsequently dissolved in 1.5 ml 250 mM CaCl₂. One and a half milliliters of $2 \times \text{HBS}$ (280 mM NaCl, 1.5 mM Na₂HPO₄, 50 mM Hepes-Na, pH 7.08) were added dropwise under constant mixing. The mixture was left at room temperature for about 20 min and then added to the medium. Fifteen hours later cells were washed and provided with fresh medium, after a further 8 h the cells were shifted to serum-free medium. The conditioned medium was harvested after 3 days, spun at 1000 g to pellet cells and stored at 4 °C.

Antibodies

Activin β_A antiserum

An affinity-purified rabbit activin β_A subunit antiserum, raised in rabbits immunized with conjugated cyclic Ac-inhibin β_A (81–113)-NH₂, was used in this study (Vaughan *et al.* 1989).

Activin β_C monoclonal antibody

The preparation, cloning, isotyping and purification of monoclonal activin β_C subunit antibodies was recently described (Mellor *et al.* 2000). However, instead of clone 1 as used by Mellor *et al.* (2000), we used clone 68 of the described preparation in this study.

Activin β_E monoclonal antibody

A synthetic peptide of sequence ARRPLSLLYLD HNGNVVKTDPDMVVEAC, corresponding to amino acids 319–347 of the mouse activin β_E subunit (Fang *et al.* 1996), was synthesized by fluorenylmethoxycarbonyl chemistry (Atherton & Sheppard 1989). Peptide authenticity was evaluated by laser desorption mass spectrometry and reverse phase HPLC. Female Balb/C mice were immunized monthly over a period of 4 months. Tail bleeds were screened using a standard ELISA procedure to determine reactivity with the peptide. The mice with high responses were boosted and killed. Their spleens were removed and splenocytes fused to SP2/0 myeloma cells using a standard polyethylene glycol fusion protocol (Harlow & Lane 1988). Six wells gave a positive ELISA signal when screened on 96-well plates coated with recombinant β_E monomer kindly provided by Biopharm GmbH. Four of the positive clones were expanded and checked for cross-reactivity with activin β_C by ELISA screening against recombinant material kindly provided by Biopharm GmbH. The clone (2R) that gave minimal cross-reactivity was chosen.

Structure analysis

Two-dimensional polyacrylamide gel electrophoresis (2d-PAGE)

Proteins were precipitated from conditioned media with one volume of acetone. Protein samples were then dissolved in loading buffer without reducing agents (10 M urea, 4% CHAPS, 0.5% SDS). Samples were supplemented with 2% pH 7–9 carrier ampholytes and 0.03% Bromophenol Blue before loading. 2d-PAGE was performed according to Gerner *et al.* (2000). Briefly, isoelectrofocusing (IEF) was carried out in tube gels (70 mm $\times$ 1 mm) containing a gel composed of 8.6 M urea, 3.9% acrylamide, 0.1% piperazine diacrylamide, 0.08% CHAPS, 0.03% Nonidet P-40, 1.3% pH 3.5–10,

1.3% pH 4–8, and 2.6% pH 5–7 carrier ampholytes (Merck) 10 min at 500 V and 3.5 h at 750 V using the Mini-Protean II system (Bio-Rad). In the second dimension proteins were separated by denaturing SDS-PAGE (1 mm thick, 12% polyacrylamide gels) according to Laemmli (1970).

Western blotting

Gels were equilibrated in 25 mM Tris, 192 mM glycine, 20% methanol, and 1% β -mercaptoethanol to post-reduce activin dimers and proteins were subsequently electroblotted on Hybond-P membrane (Amersham Pharmacia Biotech). Non-fat dried milk at 5% in TBST (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.1% Tween 20) was used to block non-specific binding. The activin β_A antibody was used at 0.4 μ g/ml, the activin β_C antibody at 5 μ g/ml and the activin β_E antibody at 28 μ g/ml in TBST containing 1% non-fat dried milk. The ECL+Plus Western blotting detection system (Amersham Pharmacia Biotech) was used according to the manufacturer's instructions.

Results

Molecular cloning and structure of rat activin β_C and β_E cDNAs

The complete coding sequences of the rat activin subunits β_C and β_E were cloned by high-fidelity PCR from rat liver cDNA using primers derived from the homologous mouse genes (Table 1). The sequences were deposited in GenBank under the accession numbers AF140031 and AF140032 for activin β_C and β_E , respectively. The properties of both cDNAs and the encoded polypeptides are summarized in Table 2. The sequence of rat activin subunit β_E differs from a recently published sequence (O'Bryan *et al.* 2000) by four nucleotide positions, two of which affect the amino acid sequence of the propeptide (amino acid 160 and 161).

Expression of rat activin subunits

A wide variety of male and female rat tissues were analyzed for the expression of the four activin subunits β_A , β_B , β_C and β_E by multiprobe RNase protection analysis (Fig. 1). Activin β_A and β_B showed a broad tissue distribution. In fact activin

Table 2 Properties of rat activin subunits β_C and β_E

	Activin β_C	Activin β_E
Number of amino acids of the putative pre-pro-protein	351	350
Position of the predicted cleavage site	235–236	236–237
Number of amino acids of the mature peptide subunit	116	114
Position of potential <i>N</i> -linked glycosylation sites	110, 142, 160	198
Predicted molecular weight of the mature peptide subunit (kDa)	12.81	12.47
Identity to the mouse*/human† coding nucleotide sequence	92%/81%	94%/81%
Similarity of the predicted pre-pro regions to the corresponding mouse*/human† peptide sequences	92%/83%	94%/79%
Similarity of the mature peptides to the mouse*/human† peptide sequences	97%/95%	99%/97%
Similarity of the mature peptides to each other	82%	
Similarity of the mature peptides to rat activin β_A ‡	69%	65%

*Derived from Genbank accession numbers X90819 (activin β_C ; Schmitt *et al.* 1996) and U96386 (activin β_E ; Fang *et al.* 1996). †Derived from Genbank accession number X82540 (activin β_C ; Hötten *et al.* 1995), and from Celera gene ID hCG39662 (activin β_E ; Venter *et al.* 2001). ‡Derived from Genbank accession number M37482 (Woodruff *et al.* 1987).

β_B mRNA was detected in all tissues but the liver. Particularly strong expression was displayed in fat, lung, uterus and the gonads. Activin β_A mRNA was most prominent in adipose tissue, liver, epididymis, ovary and uterus. In sharp contrast, strong expression of activin subunits β_C and β_E was only seen in the liver. In addition, very low levels of activin β_C and β_E were found in skeletal muscle and heart of the male rat, and in the female kidney. Traces of activin β_E were furthermore detected in the lung. These results were confirmed by RNase protection experiments using activin β_C and β_E probes only (data not shown). Regarding expression in the liver, levels of all three activin mRNAs were higher in the male than in the female rat.

In situ hybridization with antisense RNA indicated an uneven distribution of the β_E mRNA in the rat liver; staining appeared stronger in hepatocytes surrounding the portal triad (Fig. 2a). In contrast, activin β_A expression appeared more homogeneously distributed throughout the liver (Fig. 2b). In both cases staining was most prominent in the perinuclear region of hepatocytes; non-parenchymal cells generally displayed no significant β_E or β_A mRNA staining (Fig. 2c and f). The specificity of the hybridizations was assessed by use of a probe transcript from the opposite strand (sense RNA): no staining was observed (Fig. 2d and e).

We did not succeed with *in situ* hybridization for activin β_C using either full-length or partial activin β_C antisense transcripts as probes (data not shown).

Thus to confirm and extend the *in situ* hybridization result on the distribution among different liver cell populations, we isolated liver cells by collagenase perfusion of a rat liver, and separated parenchymal and non-parenchymal cells by differential and percoll step centrifugations. The mRNAs of the three liver activins were predominantly found in the parenchymal (hepatocyte) fraction, while TGF- β_1 was exclusively expressed in non-parenchymal cells (Fig. 3). The non-parenchymal fraction also contained low amounts of activin mRNAs. Since, however, low amounts of HNF4 were also found in this fraction, it cannot be ruled out that this finding is due to hepatocyte contamination.

Dimer structures of rat activins

Mature activins are dimeric molecules composed of two β subunits. As activin β_A , β_C and β_E mRNAs are coexpressed in hepatocytes one may envisage that, in analogy to the heterodimerization of activin β_A and β_B in the ovary (Ling *et al.* 1986), activins could possibly form all different kinds of heterodimers in addition to the homodimers. Since the different dimers are very similar in size (Table 3), they cannot be definitely discerned by simple SDS-PAGE. However, they are well discriminated by their isoelectric points (pI) (Table 3). Therefore we used 2d-PAGE to first separate the proteins according to their pI, followed by SDS-PAGE. Non-reducing conditions were used throughout the

Figure 1 Expression of the activin β subunits in tissues of an adult male (m) and female (f) rat. Thirty micrograms of total RNA isolated from the indicated tissues were analyzed by RNase protection assay using riboprobes hybridizing to activin β subunits and GAPDH (labeled to low specific activity), as indicated to the right.

separation process, to preserve the dimer structure. The subsequent reduction of the resolved dimers, before blotting on membranes, was found to significantly improve the immunological detection by the activin β_A , β_C or β_E peptide antibodies used. As a straightforward detection of liver activins from crude preparations by immunoblotting was not possible, we used recombinant DNA technology to produce amounts of activin proteins sufficient for detection. 293T cells were transfected with plasmids encoding activin β_A , β_C and β_E cDNAs either alone or in pairs to allow heterodimerization. Cells were cultured for 3 days and samples of conditioned media were used for 2d-PAGE analysis of the dimer structure. The activin A homodimer was detected as a single spot with a molecular weight of approximately 26 kDa

in the basic region of the IEF dimension as predicted by data in Table 3 (Fig. 4a). In contrast, activin C was reproducibly resolved into two spots of the same molecular weight, yet of different pI, indicating formation of two isoforms (Fig. 4b). Analysis of the activin β_A - β_C cotransfections with activin β_C antibody did not reveal the two activin C isoforms (Fig. 4e). Instead the two spots had shifted to a more basic pI, consistent with a complete heterodimerization of the two activin β_C isoforms with activin β_A . In addition, the activin A spot was detected with the same antibody, indicating cross-reactivity of the activin β_C antibody. As predicted, activin E had the lowest molecular weight and the most acidic pI within the family of rat liver activins and migrated as a single spot (Fig. 4c). Furthermore, we discovered two novel,

Figure 2 Expression of activin β_E and β_A in the rat liver as analyzed by *in situ* hybridization. Cryostat sections of the liver of a male rat were hybridized with digoxigenin-labeled activin β_E (a and c) or activin β_A (b and f) antisense riboprobes. The specificity of the *in situ* hybridization was assessed through parallel hybridizations using sense activin β_E (d) or activin β_A RNAs (e). The purple color (NBT/BCIP staining) indicates *in situ* hybridization of riboprobes. Nuclei are stained with Methyl Green. Granules of black precipitate, visible at higher magnification (c and f), are a staining artefact. Representative central veins (C) and portal triads (P) are indicated.

hitherto undescribed heterodimers, activin CE and AE. They were identified using the same non-reducing 2d-PAGE approach (Fig. 4f and g). However, detection of the activin AE and CE heterodimers was hampered by the apparently much lower levels of recombinant expression of activin β_E and the lower reactivity of the activin β_E antibody. Long exposure times led to a strong, spread-out activin A spot (Fig. 4g). For comparison, a similarly treated activin A Western blot is shown

in Fig. 4 (d). Blots of the CE and AE coconditioned media were probed with the more reactive activin β_C and β_A antibodies (Fig. 4f and g), respectively. In both cases a single heterodimer spot was detected at the predicted position in addition to the much stronger activin A and C homodimer spots.

In addition to the described mature activin forms a number of other spots were observed in most Western blots (Fig. 4). The low molecular weight spots appear to be the monomeric activin subunits,

Figure 3 Distribution of the expression of the activin β subunits among hepatocellular subpopulations. Parenchymal (P) (hepatocytes) and non-parenchymal cells (NP) from livers of male rats were separated and total cellular RNA isolated. Ten micrograms of total RNA were analyzed by RNase protection assay using riboprobes hybridizing to activin β subunits, TGF- β , HNF4, and GAPDH (labeled to low specific activity), as indicated.

which are about 12·5 to 13 kDa in size. The high molecular weight proteins (about 66 kDa), also observed in a number of blots, are likely to be proforms with different posttranscriptional modifications, probably within the proregion.

Discussion

The basic structure of the activin β_C and β_E peptides as derived from the respective cDNAs is highly similar to that of activin subunits β_A and β_B as well as of other TGF- β family members. They are composed of a pre-prodomain, made up by the signal peptide and the prodomain with its potential glycosylation site(s), followed by a C-terminal

mature peptide domain. In both peptides a stretch of basic amino acids precedes the predicted proteolytic cleavage site and both contain the nine cysteines required to form the cysteine knot structure characteristic of TGF- β family peptides. The high homology to each other in size, nucleic acid as well as peptide sequence justifies their classification as a subgroup within the inhibin β (activin) subunit family as proposed on the basis of the mouse sequences (Fang *et al.* 1997). Their proximity in the human and mouse genome, 5·5 kb apart in both cases, furthermore suggests that the closely related genes probably arose by a tandem duplication of an ancestral gene (based on an analysis of the corresponding sequences on human chromosome 12 (Venter *et al.* 2001), and on the analysis of Fang *et al.* (1997).

Activin β_C and β_E not only display significant structural similarity, but they also share a highly unique expression pattern, distinct from the other activin β subunits. While activin β_A and β_B mRNAs were easily detectable in almost all tissues analyzed, strong activin β_C and β_E expression was only seen in the liver. The liver furthermore expressed activin β_A , but was the only tissue where activin β_B was not detectable. Expression of activin β_E displayed a slight zonation: mRNA levels appeared higher in hepatocytes surrounding portal triads. While a zoned gene expression pattern is not unusual in the liver, notably if genes are involved in hepatic metabolism, speculations about the biological relevance of the activin β_E expression pattern will have to await the elucidation of its function.

In contrast to previous reports on the expression of human activin β_C (Loveland *et al.* 1996, Thomas *et al.* 1998, Mellor *et al.* 2000) we did not see expression of activin β_C or β_E in rat reproductive tissues. Nevertheless, traces of activin β_C and β_E mRNA were detected in some other tissues. With the exception of the lung, they were coexpressed in all these cases. Yet, it has to be pointed out that the mRNA levels of activin β_C and β_E in the liver exceeded those found in any of the other tissues by several orders of magnitude. Although such restricted expression pattern is rare among members of the TGF- β family, most of which show a rather broad tissue distribution, a further member with liver-specific gene expression, termed growth-differentiation factor 15, has recently been described (Hsiao *et al.* 2000).

Table 3 Calculated isoelectric points (pI) and molecular weight (MW) of activin homo- and heterodimers

	pI	MW (kDa)
Activin A	7·1	25·9
Activin AC	6·7	25·8
Activin C	6·3	25·6
Activin AE	6·1	25·4
Activin CE	5·7	25·3
Activin E	5·4	24·9

Interestingly, activin expression in the liver is distinguished from that of TGF- β by its cellular origin. Subcellular fractionation as well as *in situ* hybridization indicated that hepatocytes are the main source of all three activin subunits synthesized in the liver. Expression of TGF- β on the other hand was restricted to the non-parenchymal cell fraction as previously reported (Jakowlew *et al.* 1991). Thus, while TGF- β appears to act primarily in a paracrine manner in the liver, activins may exert paracrine as well as autocrine functions.

Activin A and TGF- β nevertheless appear to play a similar role in liver growth regulation (for review

see Rossmanith & Schulte-Hermann 2001). Both have been shown to inhibit DNA synthesis and induce apoptosis in primary hepatocyte cultures as well as in the liver of rats and mice *in vivo* (Oberhammer *et al.* 1991, Oberhammer *et al.* 1992, Schwall *et al.* 1993, Hully *et al.* 1994). However, so far it is unclear whether activin C or E have any similar potential. In liver regeneration activin A, C and E showed a distinct expression pattern (compare Esquela *et al.* 1997, Zhang *et al.* 1997, Lau *et al.* 2000). Activin β_A mRNA like that of TGF- β builds up to reach high levels in later phases of regeneration, possibly associated with the termination of DNA synthesis (for discussion see Rossmanith & Schulte-Hermann 2001). In the case of activin β_C mRNA, different expression patterns in regeneration have been observed by different researchers, although in neither case was there any upregulation later than 24 h after partial hepatectomy (Esquela *et al.* 1997, Zhang *et al.* 1997, Lau *et al.* 2000). Expression of activin β_E was highly upregulated after 6 h, but declined rapidly thereafter (Lau *et al.* 2000). This induction was also observed in rats treated with lipopolysaccharide (O'Bryan *et al.* 2000) reminiscent of an acute phase response (Moshage 1997). Collectively, these results indicate diverse functions of the activins expressed in the liver. Moreover, activin C and E appear to be less redundant than expected from their high similarity in structure and tissue distribution.

Mature activins are dimeric proteins composed of two β subunits. As there are two subgroups of

Figure 4 Homo- and heterodimers formed from rat activin subunits β_A , β_C and β_E . 293T cells were transfected with plasmids encoding cDNAs for activin β_A , β_C or β_E , either alone or in pairs. The recombinantly produced dimers secreted in the media were separated under non-reducing conditions by 2d-PAGE, blotted on membranes and detected by use of activin-specific antibodies. (a and d) Transfection of rat activin subunit β_A cDNA, detected with an activin β_A antibody; (b) transfection of rat activin subunit β_C cDNA, detected with an activin β_C antibody; (c) transfection of rat activin subunit β_E cDNA, detected with an activin β_E antibody; (e) cotransfection of rat activin subunits β_A and β_C cDNAs, detected with an activin β_C antibody; (f) cotransfection of rat activin subunits β_C and β_E cDNAs, detected with an activin β_C antibody; (g) cotransfection of rat activin subunits β_A and β_E cDNAs, detected with an activin β_A antibody. Positions of the molecular mass markers are shown. Homodimers are indicated by arrows, heterodimers by circles.

activin β subunits that differ in structure and expression, it is of utmost biological interest if these groups do form heterodimers other than AB or CE. By heterodimerization, normal activin homodimer function could be modulated, altered, or abolished. *In vivo* colocalization of all activin subunits within one cell type is essential for the formation of different dimers, because dimerization appears to be a strictly intracellular event (Gray & Mason 1990). Since hepatocytes express three different activin β subunits, three different heterodimers may form in addition to the respective homodimers: activin AC, activin AE, and activin CE. We were able to demonstrate the formation of all the possible homo- and heterodimers by recombinant coexpression of their cDNAs in a human cell line. On the one hand this confirms the recently published data on the formation of an activin AC heterodimer by Mellor *et al.* (2000), on the other hand this is the first demonstration of the formation of activins CE and AE, as well as the homodimeric activin E.

So far no biological activity or function has been assigned to activin β_C or β_E . Cell lines responsive to activin A did not show any response after treatment with recombinant activin C (Mellor *et al.* 2000, S Vejda and W Rossmannith, unpublished observations), neither did the deletion of one or both of the mouse activin β_C or β_E genes elucidate any function for these peptides (Lau *et al.* 2000). Therefore, it has recently been suggested that the role of activin β_C is to inactivate activin β_A and β_B by the formation of inactive heterodimers (Phillips 2000, Mellor *et al.* 2000). However, assignment of such a role to heterodimerization will have to await the characterization of purified heterodimers in suitable bioassays. Furthermore, the relative proportions of the different dimers in tissues coexpressing activin β subunits will have to be determined. This would certainly be a major undertaking due to the generally low levels of these growth factors even in 'high' expressing tissues like the liver (W Rossmannith, unpublished observations). The strictly tissue-specific expression of activin β_C and β_E in contrast to that of activin β_A or β_B would be even more surprising in the light of such a potential role. In any case the potential of the different activin β subunits expressed in the liver to form all kinds of homo- and heterodimers should be considered in future studies on activin function.

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