Identification of Two Amino Acids in Activin A That Are Important for Biological Activity and Binding to the Activin Type II Receptors*

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Activins are members of the transforming growth factor-β family of growth and differentiation factors. In this paper, we report the results of a structure-function analysis of activin A. The primary targets for directed mutagenesis were charged, individual amino acids located in accessible domains of the protein, concentrating on those that differ from transforming growth factor-β2, the x-ray crystal structure of which is known. Based on the activities of the recombinant activin mutants in two bioassays, 4 out of 39 mutant proteins (D27K, K102A, K102E, and K102R) produced in a vaccinia virus system were selected for further investigation. After production in insect cells and purification of these four mutants to homogeneity, they were studied in bioassays and in cross-linking experiments involving transfected receptor combinations. Mutant D27K has a 2-fold higher specific bioactivity and binding affinity to type II receptors, whereas mutant K102E had no detectable biological activity and did not bind to any of the activin receptors. Mutant K102R and wild type activin bound to all the activin receptor combinations tested and were equipotent in bioassays. Our results with the Lys-102 mutants indicate that the positive charge of amino acid 102 is important for biological activity and type II receptor binding of activins.

The TGF-β family consists of a large group of structurally related, but functionally diverse polypeptides that control the growth and differentiation of many cell types in vitro and in vivo (1–4). TGF-βs, activins, and bone morphogenetic proteins (BMPs) exert their biological effects through binding to two types of serine/threonine kinase receptors, termed type I (≥ 53 kDa) and type II (≥ 70 kDa) receptors (1, 5, 6). Type I and II receptors can form high affinity receptor complexes at the cell surface and this is necessary for signal transduction (7–11). Overexpressed type II receptors can bind ligand in the absence of type I receptor with moderate affinity, while it is generally accepted that type I receptors require type II receptors to bind ligand in the high affinity receptor complex. The type II receptor phosphorylates the type I receptor after ligand binding, and the latter propagates the signal to downstream effectors, the Smad proteins (see Ref. 12).

TGF-β proteins are biologically active as dimers. Like other members of the TGF-β family, the activins are synthesized as large precursor proteins consisting of a signal peptide, a glycosylated prodomain and a mature domain. The maturation of activin requires intracellular cleavage by protein convertases, such as furin, at the basic cleavage site which separates the mature chain from the prodomain (13, 14). Removal of the prodomains from the precursor dimer is necessary for biological activity of the mature 25-kDa dimer, since unprocessed high molecular weight forms of activin A display no biological activity (13, 15, 16).

Thus far, TGF-β2, TGF-β3, and BMP-7 (also called osteogenic protein-1 (OP-1)) have been crystallized, and the three-dimensional structures of the mature, dimeric molecules have been elucidated (17–19). These proteins share a common three-dimensional polypeptide folding pattern, although their amino acid sequence identity is limited to 36% (BMP-7 compared with TGF-β2). Hence, it is likely that this structure is the prototype for the whole family and might be extrapolated to activins as well. The common fold of the monomer is defined by seven cysteines that are conserved throughout the family. Six of these form intrachain disulfide bonds and make up the cystine knot, while the seventh cysteine forms an interchain disulfide bond that stabilizes the dimer (17–19). By analogy with a left hand, the monomeric structure consists of the N-terminal thumb region, two antiparallel pairs of β-strands that build up four fingers, two loops that connect the fingers (loop 1 connects finger 1 and 2; loop 2 connects finger 3 and 4), and a long α-helix at the heel of the hand (see Fig. 1). This prototype structure defines four solvent-accessible, flexible and divergent regions, which may contain putative receptor-binding sites, i.e. the N terminus, loop 1, loop 2, and the C-terminal end of the long α-helix (17–19).

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† The abbreviations used are: TGF-β, transforming growth factor-β; ActR, activin receptor; ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; FS, follistatin; FSH, follicle-stimulating hormone; GDF, growth and differentiation factor; hCDMP, human cartilage-derived morphogenetic protein; OP, osteogenic protein; RIA, radioimmunoassay; TGF-β receptor; vv, vaccinia virus; PAGE, polyacrylamide gel electrophoresis; BS², bis-sulfosuccinimidyl suberate.
Amino acids important for biological activity have been defined by limited structure-function analysis of TGF-β members and by molecular characterization of naturally occurring mutations that cause drastic phenotypes in different organisms. Mutation analysis revealed that the nine cysteines, including the seven conserved cysteines in the family, of mature activin A are essential for either the biosynthesis or the (full) biological activity. In the seven conserved cysteines in the family, of mature activin A, the seven conserved cysteines in the family,

Mutagenesis—Oligonucleotide-directed mutagenesis was performed using plasmid PTZ18R, which contains a mouse activin A cDNA cloned in the sense orientation with respect to the T7 promoter, and the Muta-Gene phagemid in vitro mutagenesis kit (Bio-Rad). Mutations were introduced using the single-stranded sense oligonucleotides listed in Table I.

| E3A: | 5’-GTAGCCGCTGGGTGGAGTGCAGCATGGCAGGTTG-3’ |
| D5A: | 5’-GGTGCAGGAGGGTCCCCAGCTCCG-3’ |
| K7A: | 5’-GGATGCGGACGGCCGGTCCAAATTTGCTG-3’ |
| K13A | 5’-CAAACATTGGCTGCACAAAGTCTTCTTGCT-3’ |
| K13T: | 5’-CAAACATTGGCTGCACAAAGTCTTCTTGCT-3’ |
| K14A: | 5’-CAAACATTGGCTGCACAAAGTCTTCTTGCT-3’ |
| K14E: | 5’-CAAACATTGGCTGCACAAAGTCTTCTTGCT-3’ |
| R21A: | 5’-CTTGTGCACTTCCGCCGACATTGCC-3’ |
| R21T: | 5’-CTTGTGCACTTCCGCCGACATTGCC-3’ |
| R21E: | 5’-CTTGTGCACTTCCGCCGACATTGCC-3’ |
| D22A: | 5’-CACTGCTTGGTCAAGCAGGACATTCTTCTTGCT-3’ |
| D27A: | 5’-GCTGGTAAATGTGCTATATGCCCATTTCCCC-3’ |
| D27K: | 5’-GCTTCAGGATATCCGGTGAATAAGGATATTGCC-3’ |
| V18I/S19D: | 5’-GAAACAGTCTTCTATCGATTTCAGGACATTGCC-3’ |
| H7A: | 5’-GAGGCCTCAAGGCCGTAGACGAGCTTCTTGGTCCG-3’ |
| H7A: | 5’-GAGGCCTCAAGGCCGTAGACGAGCTTCTTGGTCCG-3’ |
| H7A: | 5’-GAGGCCTCAAGGCCGTAGACGAGCTTCTTGGTCCG-3’ |
| H7A: | 5’-GAGGCCTCAAGGCCGTAGACGAGCTTCTTGGTCCG-3’ |
| H7A: | 5’-GAGGCCTCAAGGCCGTAGACGAGCTTCTTGGTCCG-3’ |
| K55A: | 5’-GCTGTGTCGCAAGCGCTGAGCGCCCACCT-3’ |
| R87A: | 5’-GTCGCCACACAGGGTTGCCCAGACTTCTGCT-3’ |
| D95A: | 5’-GCTGTATACGCTGAGGCCAAAATAC-3’ |
| D96A: | 5’-GCTGTATACGCTGAGGCCAAAATAC-3’ |
| K102A: | 5’-GCTCAAATAGTCCAAAGGATATCCAAAACATG-3’ |
| K102E: | 5’-GCTCAAATAGTCCAAAGGATATCCAAAACATG-3’ |
| K102R: | 5’-GCTCAAATAGTCCAAAGGATATCCAAAACATG-3’ |
| K103A: | 5’-CACTACACAGGATATCAGACAAAACAAAG-3’ |
| Q116A: | 5’-CACTACAAAGAGATACCCAAACATGATG-3’ |
| E111A: | 5’-CACTACAGGGAGGTCCGAGTGGCTCAGGC-3’ |
| E112A: | 5’-CACTACAGGGAGGTCCGAGTGGCTCAGGC-3’ |
| G114A: | 5’-CACTACAGGGAGGTCCGAGTGGCTCAGGC-3’ |
| G114K: | 5’-CACTACAGGGAGGTCCGAGTGGCTCAGGC-3’ |
added to three wells, and the RIA for FSH was performed in duplicate using the FSH-RIA kit (NIDDKD, National Institutes of Health, Rockville, MD) according to Denef et al. (26).

Mesoderm Induction Assays in Xenopus—Xenopus embryos were obtained in vitro fertilization (27). They were maintained in 10% New Amphibian Medium (28) and aged according to Nieuwkoop and Faber (29). Animal pole regions were dissected from mid-blastula (stage 8) embryos (30) and cultured in 75% New Amphibian Medium containing 0.1% (w/v) bovine serum albumin and wild type or mutant activin (2.5 ng/mL). A preliminary assessment of mesoderm induction was based on the elongation of the animal caps. Animal pole regions were then frozen on dry ice, and expression of the mesoderm-specific gene Brachyury (Xbra) (31) was assayed by RPA. An analysis as described by Jones et al. (32).

Radioiodination of Activins and Follistatin—Wild type and mutant activins, and follistatin, were iodinated using a modified chloramine-T method (33). Two μg of protein (in 10 μL of 30% acetonitrile, 0.1% trifluoroacetic acid) were diluted with 10 μL of 600 mM sodium phosphate (pH 7.5) and 5 μL of NaCl(1250 μCi; Amersham Pharmacia Biotech) and 5 μL of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na2HPO4, and 1.5 mM KH2PO4). To initiate the radioiodination, 10 μL of chloramine-T (100 μg/mL in 50 mM sodium phosphate (pH 7.5); Sigma) was added. After 2 min, the iodination was stopped by addition of 20 μL of 50 mM N-acetyl-t-tyrosine (Sigma), 200 μL of 60 mM sodium iodide and 16 μL of 0.1% (w/v) bovine serum albumin. Subsequently, the reaction mixture was passed over a Sephadex G-25 column (Amersham Pharmacia Biotech), which was equilibrated and eluted with phosphate-buffered saline containing 0.1% (w/v) hemoglobin (Sigma). Peak fractions, with specific activities of 30–100 μCi/μg of protein, were routinely obtained, pooled, and stored at −80 °C.

Activin/Follistatin Cross-link—Radioiodinated follistatin288 (FS288) was cross-linked to cold wild type and mutant activins using bis-sulfosuccinimidyl suberate (BS3; Pierce) (33). Approximately 2 ng of iodinated FS288 (5 μL) was incubated with 500 μL of activin-containing conditioned medium prepared as described above. After 2 h of incubation at 4 °C on a rotatory shaker, 125 μL of 5 mM BS3 in HEPES-buffered saline (150 mM NaCl and 20 mM HEPES; Life Technologies, Inc.) was added and the reaction was incubated for 1 h at 4 °C. Activin/follistatin complexes were purified using wheat germ agglutinin-agarose (Sigma) beads (33). They were separated by SDS-PAGE under reducing conditions and visualized by autoradiography.

Receptor Binding Studies—PK15 cells (28-cm2 dishes) were transfected with different combinations of activin receptors using the vaccinia virus-T7 system as described above. On the second day, the cells were infected with different combinations of activin receptors using the baculoviral polyhedrin promotor. Recombinant baculovirus was generated using a baculovirus expression system. The mutant cDNA was inserted in the baculoviral polyhedrin promotor. Recombinant baculovirus was generated by homologous recombination in Sphingostiera frugipera cells. SDS-cotransfected recombinant transfer construct and BaculoGold™ virus AcNPV DNA (PharMingen). Recombinant virus was plaque-purified and amplified to high titer stock for production. Activins were purified from conditioned medium of recombinant baculovirus infected SF9 cells, harvested 72 h after infection.

Purification of wild type and mutant activins was performed by use of an optimized four-step purification protocol in which the conditioned medium is dialyzed and concentrated in the presence of six strand urea and loaded onto an anion exchange column (Fracotag-(EMD)-TMMAE, Merck). The flow-through is then loaded on a Protein Pack Sulfonyl (Millipore) cation exchange column. The 150 mM NaCl fraction is then adjusted to 10% acetonitrile, 0.1% trifluoroacetic acid (v/v) and separated by RPC-4 (Phenyl-glycine) reversed phase chromatography. Mutant activins are recovered in the 30–34% acetonitrile fraction (34) and further purified on a RPC-8 (Brownlee octyl) column run as a polishing step. Quantification of these pure activins was obtained through amino acid composition analysis.

RESULTS

Mutagenesis—Some mutations in structurally important regions of TGF-β family members have been reported to lead to improper biosynthesis of these ligands (15, 16, 20, 35, 36). In our study, this was also the case when severe changes in activin A were introduced. For example, the substitution of loop 1 of activin A by the equivalent region of TGF-β2 led to undetectable protein expression levels in the vaccinia virus T7 system, both in the secreted and in the intracellular fraction (data not shown). It is likely that intracellular degradation occurred, as has been suggested for most cysteine mutants of activin A and TGF-β1 (20, 36). To avoid synthesis and intracellular trafficking problems due to structural changes, we anticipated that the majority of mutant activins used in this study should be generated by single amino acid substitution.

Four solvent-accessible regions can be deduced from the three-dimensional structure of TGF-β2 and BMP-7 (17–19): the N terminus, loop 1, loop 2, and the C-terminal segment of the long α-helix (18; see also Fig. 1). These regions are the most flexible structures in the dimer, and their sequences are divergent throughout the family, which marks them as good candidates for receptor interaction. Most of the mutations introduced are single alanine substitutions at charged residues in these domains (Fig. 1A). A large panel of 39 activin A mutants was constructed by oligonucleotide-directed mutagenesis (Fig. 1B).

Synthesis and Secretion of the Mutant Polypeptides—Synthesis of this large panel of activin polypeptides was first analyzed in HeLa cells using the T7 vaccinia virus-based expression system. As suggested previously, these cells have sufficient levels of endogenous furin to support correct and efficient processing of the activin A precursor (13). Synthesized proteins were visualized by metabolic labeling followed by SDS-PAGE. We assessed both the maturation of activin A mutants to a 25-kDa dimer as well as their capacity to heterodimerize with zebravtail activin B; a secreted activin AB heterodimer can be resolved in SDS-PAGE because activin A homodimers have a slower migration than activin B homodimers. Nearly all activin A mutant polypeptides were processed like the wild type precursor and they heterodimerized efficiently (and predominantly) with activin B, as observed previously (Ref. 37 and data not shown; only activin dimers of D27K, K102A, K102E and K102R, respectively, are shown in Fig. 2). This indicates that the overall structure of the precursor polypeptides and their intracellular folding and dimerization in the rough endoplasmic reticulum are not altered. As well as analyzing their ability to dimerize, the ability of the mutants to bind follistatin (FS), an antagonist of binding protein of activin, was tested by cross-linking. All mutant activins tested (including K102A and K102E), formed complexes with FS288 like wild type activin (shown for K102A and K102E in Fig. 3). This again suggests that their overall structure is not dramatically altered, if at all.

A modified version of the vaccinia virus-T7 expression system was used to produce all mutant activins (see “Experimental Procedures”). De novo synthesized proteins were pulse-labeled early in infection and then chased with excess cold amino acids. Assuming that their production rates do not dif-
Activin Mutants

**FIG. 1.** A, schematic diagram of the activin A monomer (taken from Ref. 20). The proposed structure is based on the known structures of TGF-β2 and BMP-7 (17–19). Residues that, when mutated individually or in combination (as indicated in panel B), did not significantly alter wild type activity, are highlighted in light gray. Mutant activins with higher or lower activity compared with wild type are highlighted in dark gray (i.e., Asp-27 and Lys-102, respectively). Cysteine residues are boxed and cysteine bonds are presented as solid, gray bars. The cysteine bond at residue 80 represents the intermolecular disulfide bond. B, list of mutations introduced in activin A/TGF-β1, chimera activin/TGF-β protein in which amino acid V18-P32 from activin is replaced by the corresponding residues of TGF-β (loop 1: 22IDFKRDLGWKWI-HEPα, TGF-β numbering). The quadruple activin mutant protein containing D27G was obtained because of misincorporation during oligonucleotide synthesis.

**FIG. 2.** Detection of metabolically labeled activins (wild type and Asp-27 and Lys-102 mutants shown only; data not shown for other mutants) secreted from HeLa cells, using the vaccinia virus-T7 system. ActA-ActB heterodimers (βα−ββ) as well as homodimers (βα−βα and ββ−ββ) can be distinguished (37). [35S]Methionine- and [35S]cysteine-labeled proteins secreted from the cells were separated by SDS-PAGE under non-reducing conditions on 15% gels. Mock, non-infected cells; T7, vaccinia virus-infected but not DNA-transfected cells; +, cotransfection with T7 plasmids containing the respective inserts.

Biological Activities of vv-T7 Produced Mutant Activins—The crude conditioned media described above were used for a preliminary characterization of the bio-activities of the different mutant activins. The first bioassay was based on the stimulatory effect of activin on the production of FSH by pituitary cells (38). Wild type activin A stimulated FSH production in a dose-dependent manner with a maximum stimulation of 2–2.5-fold compared with non-stimulated cells at concentrations of 2.5–5 ng of activin/ml. Purified activin A exhibited a half-maximal stimulation (ED_{50}) of FSH production at a concentration of 0.4 ng/ml in our modified FSH assay (25), which is more sensitive than previously used assays (38). Most of the unpurified mutant activin preparations behaved like wild type activin A in this assay (data not shown). However, mutant K102E was not significantly active, while K102A consistently displayed a lower activity than wild type activin A. Loss of bioactivity of K102E was restored to wild type levels when this lysine (Lys-102) was replaced with another positively charged residue (mutant K102R). Mutant activin A bearing a D27K substitution appeared to be more active in this assay (data not shown, but see below).

Activin causes animal cap explants of early Xenopus embryos to undergo a rapid and dramatic morphogenetic response (39), allowing a provisional assessment of the mesoderm-inducing activities of different activin mutants. Mesoderm-inducing activities of the crude vv-T7 produced mutant activins were first assessed by observing the elongation of Xenopus animal caps cultured in activin-containing media. The animal cap assay is very sensitive, since it has an ED_{50} (50% of the animal caps show elongation) of 0.2 ng of activin/ml. Nearly all activin variants induced weak to strong elongation (like wild type activin) of animal caps, but mutant K102E showed no elongation (data not shown).

Based on these two biological assays, we selected four activin mutants for further analysis. Three mutants bear amino acid substitutions at Lys-102 (Ala, Glu, and Arg), and the positive charge at this position is apparently critical for biological activity (see also below). The D27K mutant was also selected because it displayed higher specific activity.

**Biological Activities of Purified Mutant Activins**—In order to analyze the selected mutant proteins in more detail, they were produced in large amounts using the baculovirus expression system. Four baculovirus recombinants were generated and used to infect insect cells (Spodoptera frugiperda [SF] cells). The secreted 25-kDa dimer was purified from 1.5–3 liters of conditioned medium. The mutant proteins were purified to homogeneity in four steps using a modification of a previously published purification protocol for activin A (34). This yielded pure activins, as judged by SDS-PAGE followed by silver stain-
ing (data not shown). Quantification of these pure activins was obtained through amino acid analysis.

In order to confirm the data obtained with conditioned media, the mesoderm-inducing activities of the purified mutant activins were studied using the animal cap assay. At a concentration of 2.5 ng/ml, all mutant activins and wild type activin A caused clear elongation of the animal caps, except for mutant K102E (data not shown). The mesoderm-inducing activities of the activins were confirmed by studying expression of *Xenopus Brachyury* (*Xbra*), which is induced in an immediate-early fashion in amphibian embryos by activin. Both D27K and K102R induced expression of Xbra to levels similar to those induced by wild type ligand, whereas virtually no Xbra expression was detected in animal caps incubated with K102E activin (Fig. 4). Mutant K102A was less potent than wild type activin A in this assay.

The FSH release assay was also repeated with purified activins. Different dilutions of the mutant proteins were tested in order to generate a dose-response curve for each mutant activin. Mutant D27K stimulated FSH levels to 240% of the unstimulated control (100% level), whereas maximum stimulation was detected by wild type activin A. The ED₅₀ values for D27K and K102E were about 350 pM, and these values were comparable to wild type activin A and mutant D27K, suggesting that D27K has a higher affinity for the receptor complex.

The K102A and K102E mutants were less efficient at FSH stimulation. Their maximal stimulation was 160% of the unstimulated control and this required very high concentrations of ligand, which are known for wild type activin to result in non-specific effects in the assay (including luteinizing hormone stimulation; Ref. 13). Also, the onset of stimulation occurred at a 4-fold lower concentration compared with wild type activin A and this mutant activin also generated a higher level of FSH stimulation than wild type.

**Receptor Binding of Purified Mutant Activins**—In order to test the binding of mutant activins to type I and type II receptors, affinity cross-linking experiments were performed on cells overexpressing different (mouse) activin receptor combinations. These receptors (IIA, IIB, ALK-2, and ALK-4) were expressed in kidney (PK15) cells using the vaccinia virus-T7 expression system (40). Radiolabeled wild type activin, and mutants D27K and K102R, all bound to the activin receptors tested (type II receptors and type II-I receptor combinations, respectively), whereas no binding could be detected with the K102E mutant (Fig. 6). K102A binds to ActRII/ALK-4 and ActRIIB/ALK-4, but interacts only very weakly with type II/ALK-2 combinations. The fact that activins bind better to complexes containing ALK-4 than those containing ALK-2 suggests that ALK-4 is the type I receptor that responds to activin in *vivo*, a conclusion consistent with previous observations on the binding of activin to primary pituitary cells (25). The lack of detectable cross-linked complexes with the K102E mutant was not due to the method of cross-linking itself, BS³ uses lysine residues to cross-link, and Lys-102 in activin A is not essential for cross-linking by BS³, since mutant K102R could still be cross-linked to receptor complexes in a manner similar to wild type.

In order to compare the binding affinities of the different mutant proteins with that of wild type activin, competition cross-linking experiments were performed on PK15 cells transfected with ActRIIA and ALK-4. These transfected cells were affinity-labeled using a constant amount of 125I-activin A (150 pM) in the presence of increasing concentrations (5-, 10-, and 20-fold excess) of cold mutant or wild type activin. Wild type activin A and mutant D27K competed for binding to the ActRIIA/ALK-4 receptor complex efficiently, whereas mutants K102A and K102E did not (Fig. 7). Interestingly, K102R competed efficiently for binding to ALK-4 in the ActRIIA/ALK-4 receptor combination, but very poorly for binding to ActRIIA alone (data not shown).

Quantification (using a PhosphorImager) indicates that D27K (Kᵢₙ 350 pM) has a 2-fold higher affinity than wild type activin.
PK15 cells were transfected (using the vaccinia virus-T7 system) with cDNAs encoding different activin receptor: ActRIIA (IA), ActRIIB (IB), ActRIIA (ALK-2), and/or ActRIIB (ALK-4), as indicated. The cells were affinity-labeled using 125I-wild type activin (WT), 125I-D27K, 125I-K102E, 125I-K102A or 125I-K102R activins, and then incubated with BS3 cross-linking agent. Samples were analyzed by SDS-PAGE (8% gels) followed by autoradiography (data not shown), and analysis using a PhosphorImager. Affinity cross-linking experiments indicate that Lys-102 is crucial for interaction with the type II receptor and, as predicted by the current model of receptor activation, also for binding to a type II (AB)-type I (ALK-2/4) receptor complex while mutant D27K can be cross-linked to the ActRIIA/ALK-4 receptor complex with a 2-fold higher efficiency than wild type activin.

In the present study, we have identified two individual amino acids in activin A that are important for biological activity as assessed by their ability to stimulate FSH release by gonadotropin pituitary cells and to induce mesoderm in Xenopus animal cap assays: residue K102, located in loop 2 of each subunit of the dimer, and D27, in loop 1. Substitution of the positively-charged amino acid (K102) with a neutral (A) or negatively charged (E) residue greatly reduces activin function, whereas mutant K102R has no effect on activin bio-activity, suggesting that a positive charge at position 102 is crucial for activity in these assays. Substitution of D27 with K results in a mutant protein with a 2-fold higher specific activity than wild type activin. This study adds important new results to previously obtained data concerning the structure and function of activins, which have demonstrated that phenylalanine 21 of zebrfish activin B and 2 cysteine residues (Cys-4 and Cys-12 in the mature protein) of human activin A are important for biological activity (16, 20). However, the precise level at which the phenylalanine 21 mutant affects the biological activity of activin has not been determined.

Studying the influence of the substitution at position 102, we find that substitution of D27 with K results in a mutant protein with a 2-fold higher specific activity than wild type activin. The 2-fold stronger binding of D27K for the ActRIIA/ALK-4 receptor complex is consistent with the results from the FSH assay, where mutant D27K had a 2-fold lower ED50 than wild type activin. The relative binding affinities of mutants K102A, K102E, and K102R for the ActRIIA-ALK-4 receptor complex are also in agreement with their activities in the FSH assay.

**Discussion**

In the present study, we have identified two individual amino acids in activin A that are important for biological activity as assessed by their ability to stimulate FSH release by gonadotropin pituitary cells and to induce mesoderm in Xenopus animal cap assays: residue K102, located in loop 2 of each subunit of the dimer, and D27, in loop 1. Substitution of the positively-charged amino acid (K102) with a neutral (A) or negatively charged (E) residue greatly reduces activin function, whereas mutant K102R has no effect on activin bio-activity, suggesting that a positive charge at position 102 is crucial for activity in these assays. Substitution of D27 with K results in a mutant protein with a 2-fold higher specific activity than wild type activin. This study adds important new results to previously obtained data concerning the structure and function of activins, which have demonstrated that phenylalanine 21 of zebrfish activin B and 2 cysteine residues (Cys-4 and Cys-12 in the mature protein) of human activin A are important for biological activity (16, 20). However, the precise level at which the phenylalanine 21 mutant affects the biological activity of activin has not been determined.

Affinity cross-linking experiments indicate that Lys-102 is crucial for interaction with the type II receptor and, as predicted by the current model of receptor activation, also for binding to a type II (AB)-type I (ALK-2/4) receptor complex while mutant D27K can be cross-linked to the ActRIIA/ALK-4 receptor complex with a 2-fold higher efficiency than wild type activin. Since D27K displays a higher biological activity, we do not believe that this more efficient cross-linking occurs because of the introduction of an additional lysine, but that it rather reflects a higher binding affinity of D27K for the receptor combination tested here. The latter could be the result of a higher rate of association and/or a lower rate of dissociation.

Lys-102 is positioned in a region (loop 2) of the ligand previously shown to be important for high affinity binding of TGF-β1 to TβRII (21). Other approaches, using antagonistic peptides that block binding of TGF-β to its receptors, have defined the W/XXXD motif of the N-terminal segment of the long α-helix of TGF-β as a primary determinant for receptor binding (41). However, the W/XXXD motif does not seem to be involved specifically in type II receptor binding, as a peptide containing this motif also blocks binding of TGF-β to the high molecular weight type III and type V receptors (42, 43). Moreover, such peptide studies usually need high concentrations of peptides to interfere with the function of the wild type molecule, which can lead to nonspecific effects, as reported previously (44).

All mutant activins, including K102A and K102E, can be cross-linked to the activin-binding and inhibitory protein follistatin (FS288). Many conclusions can be drawn from this observation. First of all, together with the fact that all activin A variants can form mature homodimers and heterodimers with activin B, this indicates that their overall three-dimensional structure is not dramatically altered. However, at this stage it is not clear whether Lys-102 or Asp-27 introduces local structural changes in the receptor binding pocket of the ligand or whether these mutations are directly involved in interaction with the receptor. This is difficult to assess, since conformation-specific monoclonal antibodies for ligands of the TGF-β family are not available. In addition, it is significant that Lys-102 and Asp-27 are located in the most flexible and solvent-accessible loop regions of the ligand, which favor the hypothesis that they interact directly with the receptors. Strikingly, Lys-102 and Asp-27 are conserved in BMP-7 and GDF-5, which have been
shown to bind to and signal through ActRIIA or ActRIIB containing receptor complexes (25, 45). In contrast, TGF-βs have, respectively, a lysine (Lys) and a glutamic acid (Glu) residue at these positions, and TGF-βs do not bind ActRIIA or IIB. Both these observations with BMP-7 and GDF-5 (25, 45) supported the notion that these amino acids (Asp-27 and Lys-102) are important for (type II) receptor recognition.

Second, since K102E does not bind to the activin type IIa and IIB receptors, this suggests that the receptor binding determinant of activin is (at least in part) distinct from the follistatin binding determinant. Consistent with this idea, we note that BMPs can also bind follistatin and that follistatin can form a trimERIC complex with BMP and its receptor (46). A peptide approach has defined two contact sites in activin that are necessary for interaction with follistatin (47). These sites encompass amino acids 15–29 and 99–116 in activin A. Although Lys-102 is localized in one of these regions, it is not necessary for binding to follistatin, suggesting that follistatin may act by masking this amino acid and thus prevents activin from binding to the activin type II receptors, as reported previously (33).

An ideal antagonistic ligand would be able to interfere with wild type ligand function by binding to its receptor(s) without activating the signal transduction cascade. In the TGF-β family, such an antagonist would bind with a normal or even higher affinity to a type II receptor, but not at all to a type I receptor. Although we performed an extensive mutagenesis study, such an antagonistic activin variant was not found. Possibly, a more drastic change is needed to interfere with binding to type I receptors or no strict separate binding determinants exist for binding to type II and type I receptors. A dominant-negative ligand, distinct from an antagonist, might interfere with wild type function in two ways: either by altering the affinity of the mutant/wild type heterodimer for its receptor(s) or by interfering with the processing of the wild type ligand. Co-translation of the wild type and dominant-negative ligands would thereby deplete the endogenous pool of activin. Such dominant-negative variants of activin B, BMP-7 and BMP-4, have been described, and in these the consensus cleavage site for the protein convertase is modified into a noncleavable sequence (16, 35). In addition, certain cysteine mutants of different TGF-β members have been identified as dominant negative; however, such mutations may result in nonspecific inhibition of ligand secretion (20, 23). The K102E mutant is likely to act as a dominant negative construct of the first kind, because homodimers and heterodimers with activin B are still secreted but, at least in the case of the K102E homodimer, cannot interact with the type II receptor. Future studies, for example using RNA injection experiments in Xenopus embryos, can investigate this question in more detail. Future work should also try to extrapolate our data to other ligands of the TGF-β family. In this way, our work will contribute to the design of agonistic, dominant negative, and antagonistic variants of TGF-β members. These variants might help in the development of new therapeutic agents, e.g. for use in bone repair, wound healing, fibrosis, immune modulation, and acute kidney insufficiency.

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