Residues in the C-terminal region of activin A determine specificity for follistatin and type II receptor binding

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Abstract

Activin is a secreted growth factor that signals by binding two related classes of single transmembrane receptors at the cell surface. The interaction of activin with its receptors is highly regulated by other cell surface receptors, antagonistic ligands, and high affinity extracellular binding proteins such as follistatin. Two activin A mutants, the deletion mutant des[85–109]-activin A and the point mutant K102E-activin A (K102E), were investigated with respect to their ability to bind cell surface receptors and the binding protein follistatin. The deletion mutant exhibits low affinity for both receptors

and follistatin whereas the point mutant fails to bind cell surface receptors but binds follistatin-288 with high affinity. K102E is able to compete with wild type activin to bind to follistatin and can thus increase the concentration of activin available for receptor binding and signaling. These findings underline the importance of the C-terminal region of activin for binding interactions and show that different residues in this region are involved in cell surface receptor and follistatin interactions.

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Introduction

Activin A is a member of the transforming growth factor beta (TGF- β) superfamily of growth and differentiation factors. Besides its effects on growth and differentiation in many tissue types, activin A also plays a role in reproduction by regulating follicle-stimulating hormone (FSH) release from the pituitary. Activin A is a homodimer consisting of two disulfide-linked polypeptide chains, termed β_A , of 116 amino acids in length (Vale *et al.* 1986). It signals through two classes of single transmembrane cell surface receptors which possess a cytosolic serine/threonine kinase domain. Activin A binds initially to the type II receptor (ActRII) (Mathews & Vale 1991). The type I receptor is then recruited into this complex and a cross-phosphorylation event takes place in which the type I receptor is phosphorylated in its intracellular domain by the type II receptor (ten Dijke *et al.* 1994, Wrana *et al.* 1994). The type I receptor, in turn, phosphorylates downstream signaling molecules known as SMADs (Wrana & Attisano 2000).

The three-dimensional structures of several activin-like members of the TGF- β superfamily in complex with the extracellular domains (ectodomains, ecd) of their receptors have recently been solved. These include bone morphogenic protein-2 (BMP2) in complex with its type I receptor ectodomain (Kirsch *et al.* 2000), BMP7 in

complex with the activin receptor type II ectodomain (Greenwald *et al.*, personal communication), and TGF- β 3 in complex with its type II receptor ectodomain (Hart *et al.* 2002). Evidence from mutation data on the ActRIIecd suggests that the interaction of activin with its type II receptor is similar to that observed in the BMP7–ActRIIecd complex. This interaction involves a hydrophobic pocket on the concave surface of the ActRIIecd and residues on the ligand's 'fingers' – two pairs of antiparallel β -strands extending from the disulfide-linked core of the structure. Each of the monomeric subunits of the ligand interacts with one type II receptor ectodomain.

Activin's access to its receptors is regulated by a number of high and low affinity extracellular binding proteins (Phillips 2000, Phillips & de Kretser 1998). The most important one of these is follistatin (Ueno *et al.* 1987), which binds activin with sub-nanomolar affinity and renders it unable to interact with receptors (Nakamura *et al.* 1990). Follistatin is a glycoprotein and exists in two splice variants, FS288 and FS315. Different functions have been proposed for these two variants. FS315 is the predominant circulatory form (Schneyer *et al.* 1996) whereas FS288 associates with the cell surface and is involved in activin internalization and degradation (Hashimoto *et al.* 1997). Both mechanisms lead to a diminished bio-availability of activin. The activin–follistatin complex is thought to consist of one activin

dimer and two follistatin molecules (Shimonaka *et al.* 1991).

Although a clearer picture of the interaction of activin with its cell surface receptors is emerging, it is not understood which parts of the ligand interact with follistatin. For this study we generated both deletion and point mutants of activin to determine whether similar regions are involved in the interaction with signaling cell surface receptors and the extracellular regulatory protein follistatin. Mutants that exhibit specificity for one class of binding protein would be useful tools for the modulation of activin bio-availability.

Selection of the mutant activins investigated in this study was based on our survey of activin mutants in which predicted surface regions were deleted. Of these, a mutant with a deletion in the C-terminal portion, predicted to form an extended beta structure, exhibited the lowest biological activity. A point mutation in this region described by Wuytens *et al.* showed low biological affinity but was able to bind to follistatin as shown in cross-linking experiments (Wuytens *et al.* 1999). Here we investigate the actions of the deletion and point mutants with respect to their binding affinities and their behavior in biological assay systems.

Materials and Methods

Mutagenesis and protein expression

To generate the [85–109] deletion mutant, a PCR mutagenesis procedure using the ExSite (Stratagene, La Jolla, CA, USA) kit was employed. A FLAG epitope sequence was added to the N-terminus of the mature activin β_A sequence by replicating the entire plasmid including its insert and adding the FLAG sequence between residues G1 and L2 of the mature activin sequence. This plasmid (pGem3zf(-), Promega, Madison, WI, USA) was used as a substrate for successive PCRs to introduce deletion or point mutations. The deletion mutant was generated with an antisense primer starting at the site corresponding with T84 and a sense primer starting at V110. In order to maintain high fidelity of the PCR, approximately 0.1 unit of cloned Pfu DNA Polymerase (Stratagene) in addition to 5 units of Taq DNA Polymerase (New England Biolabs) was used. Twelve cycles of PCR were performed under high stringency conditions. The PCR mixture was then treated with endonuclease Dpn 1 to remove parental plasmids. The mutated cDNA was subcloned into the expression vector (pVL1392, Invitrogen) and the expression was carried out by standard baculovirus protocols.

Mutagenesis of K102E mutant activin was carried out using an oligonucleotide primer designed to contain a point mutation, where the lysine codon (AAA) was altered to a glutamic acid codon (GAA). The sequences of this and all other plasmids were confirmed by dideoxy sequencing. The cDNA containing K102E mutation was subcloned

into a baculovirus transfer vector (pVL1392, Invitrogen) and transfected into Sf9 insect cells (Invitrogen) along with linearized BaculoGold DNA (Pharmingen, La Jolla, CA, USA). Resulting recombinant virus was used to express the mutant protein in High 5 insect cells.

Since the mutant protein is a secreted recombinant protein, conditioned media were harvested, filtered and purified using batch purification with anti-FLAG M2-agarose affinity gel (Sigma). This was followed by reversed phase HPLC purification. The amount of protein was quantitated by comparing the peak area to that of wild type activin A (which had previously been quantified by amino acid analysis) in a separate HPLC analysis. Purities were estimated, from both HPLC and SDS-PAGE analysis, to be in excess of 95%. The intact molecule mass was determined by MALDI-MS (matrix-assisted laser desorption ionization mass spectrometry) analysis on an ABI-Perceptive DE-STR mass spectrometer in the linear mode employing sinapinic acid as the matrix.

Biological activity (FSH release) assay

Biological activity of the mutant activins was assessed by their ability to promote the release of FSH from rat anterior pituitary cells in primary culture. The details of this assay are described elsewhere (Vale *et al.* 1983). Briefly, anterior pituitary cells from male Sprague–Dawley rats were dispersed with collagenase and plated in 48-well plates (0.15×10^6 cells per well). After a 72 h recovery in complete medium (β PJ) supplemented with 2% fetal bovine serum (FBS) and appropriate growth factors (Vale *et al.* 1983), the cells were washed three times with the same medium and treated for 72 h, as indicated. The secretion of FSH was quantified by radioimmunoassay using kits generously provided by the National Pituitary and Hormone Distribution Program of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK).

ActRII binding assay

Receptor binding was determined by employing the human erythroleukemia cell line K562 stably transfected with ActRII and activin receptor-like kinase (ALK4) (KAR6 cells) (Lebrun & Vale 1997). Receptor expression in these cell lines is controlled by a lac repressor/promoter system and therefore is isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible. Cells were grown in suspension culture in RPMI 1640 medium supplemented with 10% FBS and 2 mM glutamine. Induction was achieved by addition of 1 mM IPTG 16 hr prior to the assay. Cells were isolated by centrifugation and washed twice in binding buffer (HDB [137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 , 25 mM HEPES, 100 $\mu\text{g}/\text{mL}$ gentamycin, pH 7.4] supplemented with 5 mM MgSO_4 , 1.6 mM CaCl_2 , 1 mg/mL BSA). Binding was

carried out in microcentrifuge tubes with $1\text{--}2 \times 10^6$ cells per tube in the presence of a constant amount of ^{125}I -labeled activin A (50 fmol = $\sim 100\,000$ c.p.m.) and varying concentrations of activin A mutant or wild type proteins in a total volume of 100 μl . After incubation at room temperature for 90 min, the tubes were placed on ice and 1 ml of ice-cold binding buffer was added. This was followed by centrifugation to separate the cell pellet. The supernatant was aspirated, the bottom of the tube containing the pellet was cut off and c.p.m. from bound ligand were determined.

Follistatin binding assay

The follistatin binding assay employed a polyclonal antiserum against follistatin that binds the protein with high affinity but does not interfere with its binding to activin (Bilezikjian & Vaughan, unpublished observations). The antiserum against follistatin (PBL #5542, 8/21/95 bleed) was raised in our laboratory in rabbit using recombinant human FS288 (rhFS288) produced by Chinese hamster ovary cells (Inouye *et al.* 1991), purified and provided by Dr Nicholas Ling (Neurocrine, La Jolla, CA, USA) under contract with National Institute of Child Health and Human Development (NICHD). The rhFS288 antigen was a heterogenous preparation of non-glycosylated, mono-glycosylated and di-glycosylated forms of 31, 35 and 40 kDa rhFS288.

rhFS288 (1–2 ng) was incubated with a constant amount of ^{125}I -labeled activin A (20 fmol = 40 000 c.p.m.) and varying concentrations of mutant or wild type activin A in the presence of the anti-follistatin antibody (1:500 final dilution). Binding was carried out in a volume of 100 μl at room temperature for 90 min. After this time, the tubes were chilled on ice and normal rabbit serum (1:300 final dilution) and sheep anti-rabbit IgG (1:200 final dilution) were added. After an additional 30 min, PEG was added (7% final concentration) and the precipitate was collected by centrifugation after 30 min of incubation on ice. The supernatant was aspirated and the bottoms of the tubes were cut off and bound counts were quantified.

p15-Luciferase assay in HepG2 cells

Tissue culture and generation of HepG2 cell line stably transfected with p15 HepG2 cells (obtained from American Type Culture Collection, Manassas, VA, USA, ATCC# HB-8065) were cultured in 90% (v:v) α -MEM (Mediatech, Herndon, VA, USA)/10% FBS (Gemini Bioproducts, Calabasas, CA, USA) at 37 °C in 5% CO_2 . Every 4 days cells were dispersed with trypsin and equal numbers of cells were transferred to new dishes. Viable cells were counted in a hemocytometer using trypan blue exclusion staining. Stable cell lines were generated by transfecting HepG2 cells with linearized DNA at a ratio of 9 μg pcDNA3.1-Hygromycin (Invitro-

gen) to 1 μg of p15^(INK4B) promoter-pGL2-Basic (provided by Dr X-F Wang, Duke University, Raleigh, NC, USA) (Li *et al.* 1995) by electroporation. Transfected cells were selected by culture in 250 $\mu\text{g}/\text{ml}$ Hygromycin-B. After 3 weeks individual colonies were selected. The stable clone HepG2-p15.1 was routinely cultured without Hygromycin-B with no loss of luciferase activity or Hygromycin-B resistance, even after more than 4 months in culture.

Luciferase assays in the HepG2-p15.1 stable cell line

For luciferase assays, HepG2-p15.1 cells were plated at 0.5×10^4 per well in 48-well plates in 200 μl 90% α -MEM/10% FBS. Treatments were added in triplicate for 18 h in a final volume of 100 μl with α -MEM/10% FBS as vehicle. Luciferase harvest and assay were carried out as follows: Cells were rinsed with 250 μl Hepes dissociation buffer (HDB) and lysed on the plate by adding 100 μl of luciferase assay lysis buffer (25 mM HEPES pH 7.8, 10 mM MgCl_2 , 5 mM EGTA, 1% Triton-X-100) per well and incubating on a tilting platform shaker for 20 min at 4 °C. For luciferase assays 20 μl of lysate was injected with 100 μl of luciferin dilution buffer (25 mM HEPES pH 7.8, 10 mM MgCl_2 , 5 mM EGTA, 1 mM ATP, 0.25 mM luciferin) in a luminometer and light emission was measured for ten seconds after a two second delay. Relative luminometer units were recorded and statistical analysis was performed employing Prism (version 3.0) using non-linear regression and sigmoidal dose-response parameters.

Statistical analyses

For all assays described above, the data points were determined in triplicate. Statistical analyses were performed employing the GraphPad Prism program (San Diego, CA, USA), version 3.0. Binding assays were analysed using non-linear regression parameters for one-site competition. For activity assays, non-linear regression parameters for a sigmoidal dose-response were used. The error bars in the graphs represent S.E.M. Each assay was carried out at least three times.

Results

Deletion mutant des[85–109]-activin A exhibits low affinity for follistatin and cell surface receptors

To examine regions of activin A that are involved in interaction with cell surface receptors and other binding proteins, a series of activin mutants was generated in which predicted surface regions were deleted. The predictions were made based on homology to TGF- β (Daopin *et al.* 1992, Schlunegger & Grütter 1992) and BMP (Griffith *et al.* 1996, Scheufler *et al.* 1999) molecules for

Figure 1 (A) Receptor binding assay. Displacement of ^{125}I -activin A from KAR6 cells by activin A (■) and by des[85–109]-activin A (□). Whole cells were precipitated by centrifugation to separate bound from free ligand. (B) FSH release from rat anterior pituitary cells by the [85–109] deletion mutant. Rat anterior pituitary cells in primary culture were treated for 72 h with activin A (■) or des[85–109]-activin A (□). FSH release was quantified by radioimmunoassay. Data points were determined in triplicate and error bars represent S.E.M.

which three-dimensional structures were available. The deletions were introduced in regions of the molecule that would minimize overall structural disturbance, i.e. outside the disulfide linked core of the dimer. Three of these deletion mutants, des[1–9]-C12S-activin A, des[21–32]-activin A and des[68–75]-activin A, exhibited FSH releasing bioactivity with less than a tenfold reduction in potency compared with activin A. A fourth deletion mutant, des[85–109]-activin A, exhibited the lowest biological activity as assessed by its ability to release FSH from rat anterior pituitary cells (Fig. 1B). The receptor binding affinity of the mutant, as measured by its ability to compete with radiolabeled activin A for binding to cells stably transfected with ActRII and ALK4 (Lebrun & Vale 1997), was found to be very low ($\text{EC}_{50} > 100 \text{ nM}$) (Fig. 1A). Competition binding to follistatin (FS288) was also found to be low ($\text{EC}_{50} > 100 \text{ nM}$) (Fig. 2). This region of

the ligand is therefore likely to contain major binding determinants for both the cell surface receptors as well as for follistatin.

Point mutant K102E exhibits high affinity for follistatin but low affinity for type II receptor

An activin point mutant that exhibited low biological activity but was able to bind to follistatin in cross-linking experiments was identified by Wuytens *et al.* (1999). The point mutation, which replaces a positively charged lysine in position 102 with a negatively charged glutamic acid, is in the region absent in the deletion mutant described above. We generated microgram quantities of this mutant. The binding affinity of the K102E mutant to cell surface receptors was greatly reduced (Fig. 3A) as was its ability to release FSH from rat anterior pituitary cells (Fig. 3B). This demonstrates the importance of the long C-terminal 'finger' in receptor interactions. In contrast, the affinity of the point mutant for follistatin was reduced by less than one order of magnitude compared with wild type activin A, and was determined to be in the nanomolar range (Fig. 4).

K102E competes with wild type activin A for follistatin binding

Activin bound to follistatin is not available for receptor binding and does not contribute to signaling events (Inouye *et al.* 1991). It was therefore investigated if K102E could increase the concentration of wild type activin A in a system where competition between signaling receptors and soluble follistatin occurs. In most activin-responsive systems, follistatin is present in either its membrane-associated or soluble form. A mutant such as K102E that binds follistatin with nanomolar affinity yet exhibits low receptor affinity could therefore be used to compete for

Figure 2 Follistatin binding assay of the [85–109] deletion mutant. Displacement of ^{125}I -activin A from FS288 by activin A (■) and by des[85–109]-activin A (□). Follistatin-activin complexes were immunoprecipitated to separate bound from free ligand. Data points were determined in triplicate and error bars represent S.E.M.

Figure 3 (A) Displacement of ¹²⁵I-activin A from KAR6 cells by activin A (■) and by K102E (□). Whole cells were precipitated by centrifugation to separate bound from free ligand. (B) FSH release from rat anterior pituitary cells in primary culture treated for 72 h with activin A (■) or K102E (□). FSH release was quantified by radioimmunoassay. Data points were determined in triplicate and error bars represent S.E.M.

follistatin binding sites and liberate wild type activin sequestered in such a complex. Follistatin and activin form a 2:1 (molar ratio) complex (Shimonaka *et al.* 1991). The ability of the K102E mutant to release FSH from rat anterior pituitary cells was therefore tested in the presence of a constant ratio of FS288 and wild type activin A. As controls, constant concentrations of either follistatin or submaximal doses of activin A were added. The mutant by itself exhibited low, but significant, FSH releasing bio-activity ($P < 0.02$ at 4 nM). As expected, activin A (0.2 nM) and follistatin (0.5 nM) stimulated and inhibited FSH release from rat anterior pituitary cells, respectively (Fig. 5). Basal FSH release was also suppressed in the presence of a constant ratio of activin:follistatin (0.2:0.5 nM). The K102E mutant did not significantly modify the effect of a constant concentration of activin A alone. However its presence did stimulate FSH release in a concentration-dependent manner when added to rat

anterior pituitary cells along with either follistatin alone or a constant amount of activin:follistatin complex at doses > 2 nM ($P < 0.02$) (Fig. 5). This indicates that K102E can liberate wild type activin that is complexed with follistatin and thus increase the effective activin concentration.

Primary cultures of rat anterior pituitary cells are known to secrete both activin and follistatin into the medium (Bilezikjian *et al.* 1993a,b). The possibility therefore arose that the increase in FSH release observed in the presence of the K102E mutant was caused by the displacement of activin from the endogenous activin–follistatin complex in this system. To investigate this further, we employed an artificial activin–responsive system consisting of the human liver cell line, HepG2, stably transfected with the activin responsive p15 promoter (Li *et al.* 1995) fused to the luciferase gene. These cells respond to activin by producing luciferase which can be conveniently assayed. Wild

Figure 4 Follistatin binding assay of K102E. Displacement of ¹²⁵I-activin A from FS288 by activin A (■) and by K102E (□). Follistatin–activin complexes were immunoprecipitated to separate bound from free ligands. Data points were determined in triplicate and error bars represent S.E.M.

Figure 5 FSH release from rat anterior pituitary cells in primary culture treated for 72 h with the K102E mutant in the presence and absence of activin A and follistatin. K102E mutant alone (□). K102E in the presence of a constant concentration of follistatin (0.5 nM) (○). K102E in the presence of a constant concentration of activin A (0.2 nM) (◇). K102E in the presence of a constant concentration of activin A (0.2 nM) and follistatin (0.5 nM) (△). Data points were determined in triplicate and error bars represent S.E.M.

Figure 6 Luciferase activity assay. HepG2 cells stably transfected with a p15-Luc reporter construct were treated for 18 h with the K102E mutant in the presence and absence of activin A and follistatin. K102E mutant alone ($\square$). K102E in the presence of a constant concentration of follistatin (1.2 nM) ($\circ$). K102E in the presence of a constant concentration of activin A (0.5 nM) ($\diamond$). K102E in the presence of a constant concentration of activin A (0.5 nM) and follistatin (1.2 nM) ($\triangle$). The inset (top right) shows the effect of activin A by itself. RLU, relative luminometer units. Data points were determined in triplicate and error bars represent S.E.M.

type activin A stimulated luciferase activity with an EC_{50} of 394 ± 106 pM ($n=4$, Fig. 6 inset). In this system, K102E was devoid of any significant activin-like activity (Fig. 6). A constant concentration of 1.2 nM follistatin alone or together with a constant amount of activin A (0.5 nM) did not have a great effect on baseline luciferase activity suggesting that endogenous activin does not significantly contribute to the basal luciferase activity, unlike the situation in rat anterior pituitary cells. The K102E-activin A mutant stimulated luciferase reporter activity when added to cells incubated with a constant ratio of activin: follistatin (0.5/1.2 nM) with an EC_{50} of 2.4 ± 1.3 nM ($n=4$) but not with follistatin alone (Fig. 6). Similar effects were observed in the assay regardless of whether or not the activin A-FS288 complex was pre-incubated for 8 h or not. Similar to rat pituitary cells, the K102E mutant did not have an effect on luciferase reporter activation in the presence of a constant submaximal concentration of activin A (Fig. 6).

These findings indicate strongly that K102E-activin A is able to compete with wild type activin for follistatin binding. Activin that has been engaged in a complex with follistatin can be liberated and become available for signaling interactions. The low, but significant, release of FSH observed in response to the point mutant in the pituitary cell assay is most likely a result of the mutant's ability to release endogenous activin from activin-follistatin complexes present in the rat anterior pituitary cell culture system. The low baseline of reporter gene expression in the HepG2 cell system indicates that such endogenous complexes do not play a significant role in this assay.

Discussion

Two activin A mutants that exhibit greatly impaired receptor binding affinity and low biological activity were investigated in this study. The deletion mutant, des[85–109]-activin A, was able to bind to cells overexpressing activin receptors with an approximately 300-fold reduced affinity (100 nM compared with 0.3 nM for wild type activin A). Its ability to bind follistatin was similarly diminished. The point mutant, K102E (Wuytens *et al.* 1999), showed no measurable affinity for cell surface receptors but bound to FS288 with nanomolar affinity. It was therefore investigated whether the K102E point mutant could be employed to modulate the availability of wild type activin in biological systems. The bio-availability of activin is extensively regulated by a variety of factors (Phillips 2000). The propagation of a biological response requires ligand interaction with type II and type I cell surface receptors. Access to these receptors is regulated by the high affinity binding protein follistatin, of which one form (FS288) is thought to be mainly membrane-associated (Hashimoto *et al.* 1997) whereas the other (FS315) is thought to be the major circulatory form (Schneyer *et al.* 1996). Other regulatory events include the low affinity interaction with α_2 -macroglobulin (Vaughan & Vale 1993) and the presence of a pseudo-type I receptor, BAMBI (Onichtchouk *et al.* 1999).

Both forms of follistatin bind activin with high affinity and diminish its biological activity (Inouye *et al.* 1991). This is likely to be due to the formation of a high affinity complex that is unable to interact with cell surface receptors. We employed two activin responsive systems to investigate the ability of the K102E mutant to compete for follistatin binding and thus increase the bio-active concentration of wild type activin. The well established rat anterior pituitary system responds to activin treatment by releasing FSH and has been employed extensively for investigating activin bio-activity. The K102E mutant by itself exhibited a low, but significant, activity in this assay. The fact that the bioactivity of the mutant was evident in the presence of FS288 or FS288-activin A suggests that the mutant displaced activin from endogenous or exogenous complexes. This primary cell culture assay system is likely to be a good representation of events in the anterior pituitary *in vivo*. Cultured rat anterior pituitary cells have been reported to secrete both activin and follistatin during the course of the 72 h assay (Bilezikjian *et al.* 1993a,b). The presence of endogenous amounts of activin and follistatin might explain the low but significant activity caused by the treatment with K102E by itself. The intrinsic activity observed would be unlikely given the extremely low receptor affinity of the mutant. To further investigate this question, an artificial activin-responsive cell culture system was used. The liver cell line HepG2 was stably transfected with the promoter of the activin-responsive p15 gene linked to a luciferase reporter construct. Activin A was

able to stimulate luciferase activity approximately three-fold in these cells after an 18 h treatment. In this system, the K102E mutant did not exhibit any activity. Addition of exogenous activin A and FS288 rendered the cells responsive to K102E in a dose-dependent manner. It is not known how much, if any, activin or follistatin is secreted by the HepG2 cells. The lack of any luciferase activity through addition of K102E or FS288 alone suggests that the endogenous concentrations of activin and follistatin are low. In addition, the much shorter treatment time makes interference from endogenous quantities of these proteins less likely. In both cell culture systems, the K102E mutant can effectively compete for follistatin binding thus freeing up wild type activin in the endogenous or exogenous complexes.

The three-dimensional structure of activin is not known but is likely to be similar to those of other members of the TGF- β superfamily. The BMPs and TGF- β s are likely to share a cysteine bonding pattern with activin, that is known as the cystine knot motif. Each monomeric subunit of the ligands can be described as a hand from which two finger-like beta-sheets extend. The mutations described in this study are located in the more C-terminal long finger. The corresponding region in TGF- β has been implicated in receptor binding (Qian *et al.* 1996). A number of contacts between ligand and receptor were also identified in this region in the recently solved structure of the BMP7-ActRIIcd complex (Greenwald *et al.*, personal communication). The residue equivalent to the activin K102 is L125 in BMP7. In the complex, this residue is in close contact with a region of the ActRIIcd that has been identified as being important in activin binding (Gray *et al.* 2000). The introduction of a negative charge in the ligand at this location can explain the inability to bind, as a glutamic acid residue (E29) on the receptor side could cause electrostatic repulsion. The fact that the neutral substitution (leucine) in BMP7 enables the ligand to bind, whereas the neutral substitution in activin A (K102A, Wuytens *et al.* 1999) abolishes binding, indicates that other factors may play a role in the interaction. One possible explanation could involve steric requirements on both the receptor and ligand sides.

The interaction of activin with follistatin is not as well characterized as that with its type II receptors. There is evidence that one activin dimer binds two follistatin molecules (Shimonaka *et al.* 1991). We had previously found that a monomeric activin mutant, [Ser80]-activin A, had greatly diminished affinity for follistatin but high affinity for type II receptors (Fischer *et al.* 1995, Hüsken-Hindi *et al.* 1994). Evidence has been presented that two distinct domains of follistatin are involved in activin binding (Sidis *et al.* 2001, Wang *et al.* 2000). Our finding that the des[85–109]-activin A deletion mutant has greatly diminished follistatin binding affinity indicates that this region is either directly involved in the interaction or lies close to the interaction site. The high affinity of the

K102E mutant for FS288 indicates that this particular residue is not essential for the interaction. Further mutagenesis studies are required to elucidate the activin–follistatin interaction in more detail.

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