Structural and Biophysical Coupling of Heparin and Activin Binding to Follistatin Isoform Functions*

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Follistatin (FS) regulates transforming growth factor-β superfamily ligands and is necessary for normal embryonic and ovarian follicle development. Follistatin is expressed as two splice variants (FS288 and FS315). Previous studies indicated differences in heparin binding between FS288 and FS315, potentially influencing the physiological functions and locations of these isoforms. We have determined the structure of the FS315-activin A complex and quantitatively compared heparin binding by the two isoforms. The FS315 complex structure shows that both isoforms inhibit activin similarly, but FS315 exhibits movements within follistatin domain 3 (FSD3) apparently linked to binding of the C-terminal extension. Surprisingly, the binding affinities of FS288 and FS315 for heparin are similar at lower ionic strengths with FS315 binding decreasing more sharply as a function of salt concentration. When bound to activin, FS315 binds heparin similarly to the FS288 isoform, consistent with the structure of the complex, in which the acidic residues of the C-terminal extension cannot interact with the heparin-binding site. Activin-induced binding of heparin is unique to the FS315 isoform and may stimulate clearance of FS315 complexes.

Ligands of the transforming growth factor-β (TGFβ) superfamily regulate a diverse set of cellular and physiological functions, including early embryonic development, cellular growth and proliferation, and reproduction (1–3). Activin A, a member of the TGFβ superfamily, is necessary to the regulation of both the male and the female reproductive axes by stimulating the release of follicle-stimulating hormone from the pituitary (4, 5). Activin A also has other important physiological roles, because activin A-deficient mice die shortly after birth and exhibit multiple defects in craniofacial development (6, 7). Comparative studies of the similarities and differences between TGFβ ligands have lead to a better understanding of the many physiological effects of this multipotent ligand superfamily.

TGFβ superfamily members typically exist as covalently linked dimers and fall into three main sub-categories: TGFβs, activins/inhibins/nodals, and bone morphogenetic proteins (BMPs) (8). Structurally, these proteins all share a cysteine-knot fold and signal by engaging type II and type I receptors (9). Activin and BMPs bind to their type II receptors through a structural feature known as the “knuckle” region (10–12). They have similar type I receptor binding sites on the concave surfaces of the molecule, which span both monomers of the ligands (13–16). Although specific ligand:receptor pairs have been characterized, multiple ligands share several receptors providing complexity and precise control to the system. The members of this superfamily are produced as prepro-proteins, and the pro-domain often regulates biological functions. In the absence of the pro-domain, TGFβ-1 and -2, as well as BMP-2, -4, and -7, can bind directly to the cell surface through heparin binding sites (17, 18). This provides a mechanism for internalization and degradation of the bioactive ligand not associated with a receptor. Many TGFβ superfamily members, such as activin, do not directly associate with heparin, yet are cleared from the extracellular space through the adaptor protein follistatin. Thus the local concentration of these ligands is precisely controlled by heparin binding interactions.

Several extracellular inhibitors of ligands of the TGFβ superfamily have been identified and characterized. Two such ligand antagonists, noggin and follistatin, have been characterized structurally and both are observed to disrupt type I and II receptor binding (19–21). Noggin specifically inhibits BMP signaling, whereas follistatin modulates signaling by activin, myostatin, and a subset of BMPs to varying extents, displaying a wide range of affinities for these ligands (22–26). The specific details of the noggin and follistatin inhibitory mechanisms are distinct, and in particular distinguished by differing stoichiometries of complex formation. Noggin binds ligands with a 1:1 stoichiometry, whereas two follistatin molecules bind a single activin dimer (27). Follistatin is composed of an N-terminal domain (ND), and three follistatin domains (FSD1–3; see Fig. 1A), each of which is further divided into epidermal growth factor-like and a kazal protease inhibitor-like sub-domains (28). FSD1, FSD2, and FSD3 adopt different internal arrangements of the two subdomains, which appear to act as separately folded units with structural but not functional linkage to epidermal growth

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¶ The abbreviations used are: TGFβ, transforming growth factor-β; BMP, bone morphogenetic protein; ND, N-terminal domain; FS, follistatin; FSD, follistatin domain; HBS, heparin binding site; MES, 4-morpholineethanesulfonic acid; r.m.s.d., root mean square deviation.

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factor ligands or protease inhibitors. The ND comprises a modified TB fold, found in latent TGFβ-binding proteins and fibrillins (20). A helical segment within the ND presents a phenylalanine, which is spatially conserved in type I TGFβ receptors. This striking molecular mimic interferes with type I receptor binding (20). Type II receptor interactions are abrogated by FSD1 and -2, which bury a surface area of 810 Å² on the type II receptor interface (20, 21). The mechanism of follistatin binding, together with varying ligand affinities, also permits the formation of a 1:1:1 complex of follistatin-BMP-type I receptor (29), potentially providing additional degrees of signal modulation, in addition to complete inhibition.

Follistatin exists physiologically in three forms: FS288, -303, and -315, ending at residues 288, 303, and 315, respectively. FS288 and -315 arise from alternative splicing of follistatin mRNA, whereas the FS303 form results from proteolysis of the C terminus of FS315 (30, 31). Most follistatin mRNA corresponds to the FS315 form, with <5% coding for the FS288 form (32). FS288 has the highest affinity for activins, with an affinity for activin A of ~45 pM and a virtually non-existent dissociation rate. A 10-fold lower affinity between activin and FS315 is attributed to a 10-fold slower association rate of complex formation (33). Activin found in follicular fluid associates with FS288 or FS303, whereas FS315 appears to be the major form found in serum (34). These data imply that isoform compartmentalization is important for the physiological functions of follistatin.

Previous biochemical studies have suggested that the different follistatin isoforms have different binding affinities for heparin, with FS288 having a high affinity, followed by FS303 with intermediate affinity, and finally FS315, which appeared in these studies to have a very low affinity (35, 36). These data, together with the apparent physiological compartmentalization of the isoforms, lead to the proposal that FS288 represents a predominantly cell-bound bioneutralizing antagonist, whereas FS315 is a soluble, serum scavenger for activin (34). The heparin binding site (HBS) of follistatin has been mapped to a lysine- and arginine-rich sequence within residues 75–86 in FSD1 (Fig. 1A) (37). Mutations in this region have identified lysines 75, 76, 81, and 82 as critical for cell surface association (38). Asparagines 80 and arginine 86 have also been shown to be important in binding to small heparin analogs (39). The affinity differences in cell surface association between FS315, -303, and -288 have been attributed to the highly acidic nature of the C-terminal amino acids present in the longer isoforms of follistatin, where 11 of 13 contiguous residues are either glutamic acid or aspartic acid in FS315. Together, these observations suggest that, in the free state, FS315 could adopt an autoinhibited conformation, where the acidic C-terminal tail folds back and associates with the HBS in FSD1 (Fig. 1B), competing for heparin binding. When binding to activin or other members of the TGFβ superfamily, FS315 would have to first disengage this interaction to extend to a conformation capable of binding ligand, potentially explaining its slower on-rates and reduced affinity as compared with FS288. FS288 would lack this autoinhibited interaction and should be more capable of ligand binding. This model is consistent with differences in both on-rates and cell surface association through heparin and heparan sulfates; however, the mechanistic differences between follistatin isoforms and their implications for associated physiological functions remain to be fully understood.

To examine the structural and mechanistic differences between follistatin-288 and -315 isoforms, we have determined the x-ray structures of FS-activin A complexes and used quantitative heparin binding assays. The structure of FS315 in complex with activin reveals structural changes in FSD3 that are associated with an apparent binding of the C-terminal extension within an extended peptide-binding groove. The heparin binding characteristics of FS288 and FS315, both alone and in complexes with activin A, are also quantitatively compared, revealing activin-dependent changes in FS315 affinity. These structural and biophysical studies of the two follistatin isoforms suggest a novel mechanistic role of this vital regulator of TGFβ signaling.

**EXPERIMENTAL PROCEDURES**

**Production and Purification of Activin and Follistatin—**Activin A and follistatin 288 were produced and purified as previously described (11, 20), using Chinese hamster ovary cell lines obtained from Genentech and S. Shimasaki, respectively. Initial crystal screening was performed with recombinant human FS288 purified previously (40). Subsequently, FS315 was purified as described for FS288. Briefly, supernatants from Chinese hamster ovary cells overexpressing follistatin were obtained from the National Cell Culture Centre, concentrated, dialyzed into 20 mM Tris (pH 8.0), and applied to a HiPrep 16/10 heparin FF column (GE Healthcare). A gradient to 1.5 M NaCl was used to elute bound protein, and fractions containing FS were pooled. These fractions were dialyzed into 20 mM MES, 150 mM NaCl (pH 5.0) and applied to a Bio-Scale S2 cation-exchange column (Bio-Rad). A gradient from 150 mM to 1.25 M NaCl was used to elute the protein, and FS-containing fractions were applied to a HiLoad S200 Superdex gel-filtration column (GE Healthcare) for final purification. FS288 or -315 were mixed with activin in a 2.5:1 ratio, incubated for 30 min, and reapplied to the HiLoad S200 gel-filtration column to purify the complexes. Proteins were analyzed on Coomassie-stained 4–20% polyacrylamide gels (Bio-Rad) and by Western blot, using antibody specific for FS315 (provided by DSL Laboratories).

**Structural Analysis—**Activin A-FS315 was concentrated to 10 mg/ml and subjected to crystallization trials. Diffraction quality crystals were grown in 20–23% polyethylene glycol 1000, 200 mM MgCl₂ (pH 6.5), with 3% EtOH and 20 mM trimethylamine-HCl added. Crystals belonging to space group P222, diffracted x-rays to 3.4 Å resolution using a synchrotron x-ray source (DND-CAT, Advance Photon Source). Data processing was performed using the CCP4 Program Suite (41). Data were indexed, integrated, and scaled in Mosflm and SCALA packages. The structure of the complex was solved by molecular replacement using one activin monomer and ND, FSD1, and FSD2 of one follistatin molecule of the activin-FS288 complex (PDB ID 2B0U) as a search model using the program PHASER. Data refinement was carried out in REFMAC5 (41) and CNS (42). Electron density for the C-terminal extension of FS315 was improved by NCS averaging using the program DM (41).
Structure of the Activin-Follistatin 315 Complex

NCS restraints were applied during refinement but provided only minor improvement of the final statistics. Model building was performed using O (43), and figures were generated using PyMol.4

Fluorescence Polarization—Fluorescence polarization assays were performed at Northwestern University’s Keck Biophysics Facility on a Beacon 2000 (Invitrogen). Assays were performed at 1 nm fluorescein-labeled heparin (Invitrogen) and were repeated at least three times in triplicate, carried out in 20 mM HEPES buffer (pH 7.0) with 0.1% Triton X-100 and 150 mM or 250 mM NaCl. Competition assays using unlabeled porcine intestinal heparin (Sigma) were done at 25 nm follistatin. NaCl titration experiments showed that differences in heparin affinity were magnified at 250 mM NaCl, thus competitions were done at this concentration. For FS-activin complexes, proteins were mixed in a 1:1 ratio, ensuring that all follistatin present would exist in an activin-bound state. Samples were incubated for 1 h prior to measurement. Direct binding curves were generated using equations generated by Roehrl et al. (45), and heparin competition curves were fit using the Michaelis-Menten equation for a competitive inhibition. Data were plotted using KaleidaGraph.

Coordinate Deposition—Coordinates and structure factors have been deposited in the RCSB protein data bank (accession code 2P6A).

RESULTS

FS288 Binding to Heparin Is Not Competed by FS289–315 Peptide—Given the highly acidic nature of the C-terminal FS315 extension, it seemed possible that this peptide region could potentially exert its effects on follistatin function by directly mimicking heparin binding. In this scenario, a synthetic peptide corresponding to the FS315 C-terminal region might be expected to act independently as an inhibitor of heparin binding. To study heparin binding to FS288 and FS315, and the potential inhibitory capability of a synthetic C-terminal peptide, we established a fluorescence polarization assay using fluorescein-labeled heparin. We readily observed FS288 and activin-FS288 associating with fluorescein-labeled heparin, by monitoring increases in the fluorescence polarization of the labeled heparin, as a function of follistatin or follistatin complex concentration, with a maximum value of ~100 mP (milli-polarization units) (Fig. 1C). These interactions are specific, because the addition of unlabeled heparin competes with the polarization signal efficiently at nanomolar concentrations (Fig. 1C). We then tested whether a synthetic peptide corresponding to residues 289–315 of FS315 could act as a competitor. When added, the synthetic peptide was unable to compete for heparin binding to FS288 alone or in complex with activin (Fig. 1C), at concentrations comparable to those used for heparin. These data indicate that any interaction between the peptide and the FS HBS must be substantially weaker than the heparin affinity itself. Because the acidic tail is covalently linked in FS315, it exists locally at a much higher concentration. We increased the peptide concentration to 10,000-fold excess over the labeled heparin but still observed no competitive effect (Fig. 1C, inset). These results demonstrate that the C-terminal extension of FS315 does not, on its own, efficiently block activin-FS288 or FS288 interactions with heparin.

Structure of the Activin-Follistatin 315 Complex—To further understand the role of the C-terminal tail of FS315 in heparin binding, we determined the crystal structure of FS315 in com-
TABLE 1
Processing and refinement statistics for the activin-FS315 complex

| Statistic                  | Value               |
|----------------------------|---------------------|
| Space group                | P222                |
| Unit cell dimensions       | a = 104.64 Å, b = 106.58 Å, c = 87.57 Å; α = β = γ = 90° |
| Resolution (Å)             | 3.4                 |
| Wavelength (Å)             | 1.00                |
| No. unique observations    | 13,978 (1994)a      |
| Completeness (%)           | 99.7 (100)c         |
| Redundancy                 | 7.2 (7.3)           |
| Rmerge (%)                 | 16 (70.8)b          |
| Rwork (%)                  | 4.0 (1.0)           |

Refinement statistics

| Statistic                  | Value               |
|----------------------------|---------------------|
| No. unique reflections     | 13,672              |
| No. Resides                | 816                 |
| No. atoms                  | 5,952               |
| Rmerge/Reffects (%)        | 22.3/32.4           |
| Figure of merit            | 74.3                |
| r.m.s.d.                   | 0.014               |
| Angles (°)                 | 1.945               |
| Mean B-factor (Å²)         | 57.307              |
| Activin A                  | 46.005              |
| FS315                      | 55.297              |
| FS315                      | 68.921              |
| Ramachandran statisticsb  |                     |
| Most favored (%)           | 64.9                |
| Additionally allowed (%)   | 27.0                |
| Generously allowed (%)     | 5.6                 |
| Disallowed (%)             | 2.6                 |

a Numbers in parentheses refer to data in the highest resolution shell.
b Distribution is within the allowed range for this resolution.

Structure of the Activin-Follistatin 315 Complex

FSD3 Clamps onto the FS315 C-terminal Extension—Comparisons of the FS315 complex to the previously determined FS288 complex reveal that the most significant structural changes occur in FSD3. Compared with the activin-FS288 structure, the FS315 kazal-like domain of FSD3 shifts toward FSD2, clamping down onto the C-terminal extension (Fig. 3A). This shift demonstrates previously unseen flexibility within the sub-domain architecture of follistatin and appears to be linked to the binding of the C-terminal residues of FS315.

The C terminus of FS315 initially extends away from the complex before looping back through an extended groove formed by FSD2 and FSD3, toward activin (Fig. 3B). Interestingly, most of the C-terminal residues bound in this region are likely to be acidic in nature, suggesting that FSD2 and FSD3 play a role in ordering the highly charged tail. Several basic residues reside within this groove (Arg-140, Arg-200, and Arg-237, most notably), potentially forming hydrogen-bonding and charge-charge interactions with the C-terminal extension. Glu-297 forms a hydrogen bond with Arg-200. Residues Glu-280 and Glu-299 are in close proximity to the side chain of Arg-237, providing some charge neutralization and likely H-bond interactions. We have assigned residues in one of the follistatin molecules, based on modeling...
Continuous electron density from the C terminus of FSD3. Coloring these residues by charge, the acidic C-terminal extension is clear and would likely enable ionic interactions with the highly positive region near the basic HBS (Fig. 3C). These observations raise the possibility that the final two follistatin domains could bind and present a structured acidic tail to the HBS, in the absence of activin, forming a composite surface of interaction that could extend beyond the C-terminal charged residues.

**Structural Implications for Heparin Binding**—An important structural feature of activin-bound FS315 is highlighted by the direction of residues 289–299. In a simple model for the function of the acidic C-terminal amino acids, these residues could be imagined to extend toward the HBS, neutralizing the charges in both these regions. However, of the 27 residues unique to FS315, the final 11 (from 305–315) contain only one negatively charged residue (Glu-314; Fig. 4A). Most of the negatively charged residues are clustered between residues 292 and 304, a region that does not appear to be capable of reaching the HBS on either follistatin molecule, despite the movement observed in FSD3. In fact, measurement within the structure places the HBS 43–58 Å from residues 295–299 (Fig. 4B), whereas an extended peptide of the acidic residues 295–304 would only span ~38 Å. The HBS of the adjacent follistatin molecule lies somewhat closer at a distance of 25–53 Å, suggesting that, in the activin-bound state, FS315 molecules might inhibit heparin binding across the complex (Fig. 4C). This also appears unlikely, however, as a majority of the acidic residues would be incapable of reaching the heparin binding site. In addition, if these C-terminal residues remain bound to the FSD2/3 groove as suggested by the structure, their ability to compete for heparin binding in the complex would be expected to diminish significantly. These structural data suggest that free FS315 could interact with heparin differently than activin-bound FS315.

**Sensitivity of Heparin Binding to Ionic Strength Changes**—To quantitatively evaluate the effects of TGFβ ligand binding on heparin affinity, fluorescence polarization was used to compare heparin interactions for the free and activin-bound follistatins. Surprisingly, differences in heparin affinity between FS288 and FS315 were much more subtle than expected, based on previously reported studies. In fact, initial experiments at physiological pH and in the presence of 150 mM NaCl showed little difference in
heparin affinity for free and activin-bound follistatin isoforms (Table 2). Based on this observation, we tested whether ionic strength differentially affects the dissociation of heparin from each form of follistatin (Fig. 5A). This analysis shows that heparin binding by FS288 and FS315 indeed exhibit different sensitivities to ionic strength. The trend in Fig. 5A shows very little difference in heparin affinity between isoforms at low salt but much larger differences at higher concentrations with FS315 exhibiting a greater sensitivity to the higher salt concentrations.

Interestingly, when activin is bound to either FS315 or FS288, the two isoforms behave quite similarly and exhibit relatively minor differences in this assay. At salt concentrations >250 mM, a significant difference in heparin binding was observed for the free follistatin isoforms (Fig. 5A), whereas follistatin isoforms in complex with activin A consistently retained higher affinity for heparin and behaved more similarly to free FS288. This suggests that, after binding activin and likely other members of the TGFβ superfamily, the FS315 HBS may be structurally more accessible for heparin binding and develop a binding affinity for heparin comparable to FS288.

**FS315 Changes Its Affinity for Heparin upon Activin Binding**—Because the salt titration appeared to selectively decrease the heparin affinity for FS315 more readily than it did for activin-FS315, we anticipated that activin-FS315 would associate with heparin differently in its free and activin-bound states.

**TABLE 2**

| Protein      | 150 mM $K_D$ (nM) | 250 mM $K_D$ (nM) | EC$_{50}$ (nM) |
|--------------|-------------------|-------------------|----------------|
| Activin-FS288| 2.63 ± 0.50       | 2.32 ± 0.58       | 0.53 ± 0.23    |
| Activin-FS315| 1.78 ± 0.36       | 3.16 ± 0.36       | 0.96 ± 0.38    |
| FS288        | 2.03 ± 0.27       | 3.21 ± 0.31       | 0.86 ± 0.27    |
| FS315        | 1.92 ± 0.29       | 16.04 ± 1.75      | 8.65 ± 1.03    |

**FIGURE 3. A novel role for FSD3 clamping onto the acidic tail.** A, alignment of FS288 and FS315 FSD2 (light blue) reveals a shift of the kazal region of FSD3 between FS288 (blue) and FS315 (purple). The shift demonstrates a clamping effect of the kazal-like region of FSD3 onto the C-terminal extension, unique to FS315. B, surface representation of the direction of the C terminus of FS315. The tail (residues 289–299 shown in red) extends out away from the ligand before looping back between FSD2 and FSD3 toward activin (yellow). C, surface representation where all residues built were colored based on charge (blue, basic; red, acidic). The acidic tail forms a negative patch between FSD2 and FSD3, while a positive region within FSD1 contains the basic HBS. Lying between FSD2 and FSD3, the tail is likely more ordered for specific presentation to the HBS, when FS315 is not bound to a ligand.

**FIGURE 4. Structural implications for heparin binding.** A, the sequence of the acidic C terminus, unique to FS315. The distance from follistatin residue 299 (the last residue observed in this structure) to the end of the acidic stretch is ~19 Å if in an extended conformation. Measurement from residues 295–299 (red), the beginning of the acidic tail, to the HBS (blue) shows that this acidic portion of the C terminus is incapable of directly competing for heparin binding (B). This is also true for competition across the complex (C) where the C terminus is somewhat closer to the HBS of the adjacent molecule (shown in green), but would still be unable to place the negatively charged C-terminal residues near the positively charged HBS. This structural observation suggests that FS315 may associate with heparin differently in its free and activin-bound states.
Indeed a significant increase in heparin affinity is induced for FS315 upon activin binding. In direct binding assays at 250 mM NaCl, free FS315 bound heparin with lower affinity than free FS288 or activin-bound FS288 (Act:FS288) or FS315 (Act:FS315) (Fig. 5B). Free FS315 bound with a $K_D$ value of $\sim 16$ nM, while free FS288, activin-FS288 and, surprisingly, activin-FS315 bound with an $\sim 5$- to 8-fold tighter $K_D$ of $\sim 2$–3 nM (Table 2).

Competition experiments using unlabeled heparin were also carried out and yielded similar quantitative differences in affinity (Fig. 5C), with FS315 having a EC$_{50}$ of $\sim 8.65$ nM and FS288, activin-FS288 and activin-FS315 having EC$_{50}$ values of $\sim 0.5$–1 nM. The affinities observed here for FS288 binding to heparin are very similar to those observed in previous studies (46). Also, consistent with our observations, it has previously been reported that activin binding to follistatin can increase the amount of radioactive heparin retained in a filter binding assay (36).

**DISCUSSION**

Two isoforms of follistatin, FS288 and FS315, arise from alternative mRNA splicing and are present in the pericellular environment or in the serum, respectively. The localization of both isoforms has been studied and correlated to the differing abilities of the isoforms to bind heparin (34). It has been proposed that the ability of FS288 to bind heparin with greater affinity could restrict this inhibitor to specific tissues, such as the ovary. In contrast, weaker interactions of FS315 with heparin would enable this isoform to avoid cell surface localization, and indeed the FS315 isoform is the predominant isoform found in serum. These studies suggest more generally that FS288 could act locally within the pericellular region, providing localized control of TGFβ signaling and interacting with heparin within the extracellular matrix to form a bioneutralizing barrier for ligands or to inhibit endogenously produced activin. FS315 found predominantly in serum could alternatively serve as a soluble surveillance molecule, blocking exogenous sources of activin or other TGFβ ligands. The two follistatin isoforms are not only compartmentalized to different locations, but also differ in their affinities for TGFβ superfamily members. The mechanism by which the C-terminal 27 residues of FS could impact both the heparin- and activin-binding activities of the two FS isoforms is incompletely understood, limiting a complete understanding of FS physiology. Our biophysical and structural characterization of FS isoforms, both alone and in complex with its ligands activin and heparin, has uncovered a number of key insights.

**FIGURE 5.** Fluorescence polarization assays of heparin binding to various follistatin species. A, the differences in affinity observed between follistatin species are highly sensitive to [NaCl]. When measured at physiological [NaCl], little to no differences in affinity were observed for all forms. At 250 mM NaCl, however, a large difference between free FS315 and other species becomes apparent, demonstrating the sensitivity of this simplified system to environment. To demonstrate the differences between free and activin-bound FS315, assays shown in B and C were carried out at 250 mM NaCl. B, titration of free and activin-bound FS288 and FS315 shows that FS288, activin-FS288, and surprisingly activin-FS315 all bind heparin with comparable affinities. Only free FS315 shows a deviation from this trend and binds with weaker affinity. C, competition assays with unlabeled heparin confirm that the binding is specific and yields similar results as those seen in B. For both FS288 and FS315, activin-bound forms have an increase in affinity for heparin over the free forms.
Structure of the Activin-Follistatin 315 Complex

First, the structures of activin-FS315 and activin-FS288 (20) show that FS288 and FS315 form similar ligand complexes, and both inhibit signaling by competing for type I and type II receptor binding sites. The FS315 splice variant contains an acidic extension at the C terminus of 27 residues and has been shown to have a lower affinity or inhibitory potency for several of its TGFβ superfamily ligands compared with FS288 (25, 33, 35, 36, 40, 47, 48). In the case of activin, this difference in affinity is ~10-fold and is a result of a slower on-rate (33), consistent with the structures of the FS288 and FS315 complexes. Once a ligand is bound by either of the two different follistatin isoforms, we observe no substantial structural differences in their interactions caused by the presence or absence of the C-terminal extension. These data support a model in which follistatin isoform-specific differences in TGFβ superfamily ligand binding are due to internal structural differences within follistatin. For example, FS288 may exist in a more open conformation that is able to bind ligands more rapidly, whereas FS315 may exist in a more closed or rearranged conformation caused by interactions of the acidic C-terminal extension and the basic HBS within FSD1 (48), interfering with its ability to bind both heparin and TGFβ superfamily members (Fig. 6, A and B).

A second unexpected observation in the FS315 complex with activin is that the FS315 extension appears to interact with a groove formed between FSD2 and FSD3, suggesting that, in the free state, this acidic tail could be ordered in this crevice for presentation to the HBS in a specific orientation. In this scenario, FSD2 and FSD3 could also participate in direct interactions with the HBS, generating a “composite” surface for autoinhibition of FS315.

Finally, a quantitative heparin binding assay was used to measure the binding affinities of FS288 and FS315, revealing differences between the two isoforms and the unique coupling of activin and heparin for the FS315 isoform. Binding interactions between heparin and FS315 were surprisingly similar to those measured for FS288, with larger differences becoming apparent with increases in solvent ionic strength. We have also clearly demonstrated that activin binding induces a significant increase in the heparin binding affinity of FS315 and that the binding of these two FS315 ligands are linked. This behavior was in part predicted from the FS315 complex structure, which shows that the acidic stretch of the FS315 C terminus is incapable of reaching the HBS when activin is bound. Thus FS288, activin-FS288, and activin-FS315 all have similarly accessible binding sites with comparable heparin affinity (Fig. 6, C and D).

The enhanced binding of FS315 for heparin in the presence of activin also implies that the binding of heparin to FS315, but not FS288, would lead to increases in activin affinity. These observations raise the interesting possibility that FS315 that is bound to heparin prior to activin binding could act similarly to FS288, providing local control of TGFβ signaling. In addition, the coupled binding of these two follistatin ligands suggests that circulating FS315 that engages a ligand of the TGFβ superfamily would have increased affinity for heparin, potentially leading to more effective clearance or sequestering of these complexes.

Follistatin isoform compartmentalization has been correlated with its physiological functions, with FS315 being the main form found in serum and FS288 being found in abundance in follicular fluid (34). Upon administration of heparin during cardiovascular treatments, a rapid release of activin and follistatin from the vascular endothelium is observed (49). Our observations that the FS315 isoform in complex with activin has a higher affinity for heparin are consistent with the possibility that this heparin-released protein is composed of the FS315 form. Despite the fact that FS315 is produced at different locations from FS288, there may be local environments of heparin density or specific modifications that allow FS315 to associate with the cell surface and act similarly to FS288.

In previous studies, FS288 and FS315 have been shown to associate differently with the surfaces of cultured cells (25, 35, 46, 47). In contrast to these results, however, a comparison of
Structure of the Activin-Follistatin 315 Complex

the Xenopus follistatin isoforms showed that both short and long forms of follistatin diffuse at comparable rates and remain largely restricted to the vicinity of their sites of production (48), suggesting similar cell surface-binding properties. These conflicting observations are in part resolved by our quantitative studies of purified follistatin proteins. Our assays revealed that differences in isoform affinities for heparin are very sensitive to ionic strength and that, depending on the salt concentration, and likely the specific sulfation of heparin, the differences in these affinities can vary by 5- to 10-fold. Previous cell-based studies of follistatin interactions with heparin utilized Dulbecco's modified Eagle's medium, which has been shown to have a higher ionic strength than other media. Slight variations in the ionic strength of growth media have been shown to have a surprising effect on differential cell surface association of other proteins, which differ by only one residue (50). In vitro, FS288 has also been characterized as having a high affinity for heparin and FS315 as having very low affinity (35, 36); however, these experimental systems also utilized buffers with high salt concentrations, potentially accounting for the observed differences in affinity.

Differences in extracellular matrix composition between cell types likely play a role in follistatin interactions, because intravenous 125I-follistatin administration in rats results in specific localization to both the liver and kidneys (44). Cell surface environments that potentiate differences in heparin affinity between FS288 and FS315 could provide an advantage to a higher affinity for activin-bound FS315. FS315 that is secreted away from the site of production could theoretically bind activin and then be internalized for degradation with higher affinity for activin-bound FS315. FS315 that is secreted away from the site of production could theoretically bind activin and then be internalized for degradation with higher efficiency than free FS315. Internalization and degradation has already been characterized quite exquisitely for the activin-FS315 complex (47) and would therefore represent a likely characteristic of the activin-FS315 complex. Ongoing research into the structural and physiological roles of follistatin isoforms will certainly provide a better understanding of the unique characteristics of these proteins.

Follistatins act as general regulators by eliciting a broad range of binding affinities for different classes of TGFβ superfamily ligands. Associations with heparin and heparan sulfate, even broader effectors of various cellular processes, guide follistatin proteins in their physiological roles. Through subtle differences in heparin affinity, which can be modulated by the local environment, follistatin isoforms have evolved a mechanism of selectivity for cell surface binding and compartmentalization. Finally, the ability of FS315 to greatly enhance its affinity for heparin upon ligand binding represents a possible endocytic clearance mechanism of bioneutralized ligands into the cell. These observations reveal significant mechanistic insights into the biological functions of follistatin.

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REFERENCES

1. Whitman, M., and Mercola, M. (2001) Sci. STKE 2001, RE1
2. Siegel, P. M., and Massague, J. (2003) Nat. Rev. Cancer 3, 807–821
3. Knight, P. G., and Glist, C. (2006) Reproduction 132, 191–206
4. Lerch, T. F., Xu, M., Jardetzky, T. S., Mayo, K. E., Radhakrishnan, I., Kazer, R., Shea, I. D., and Woodruff, T. K. (2006) Mol. Cell. Endocrinol. 267, 1–5
5. Woodruff, T. K. (1998) Biochem. Pharmacol. 55, 953–963
6. Matzuk, M. M., Kumar, T. R., and Bradley, A. (1995) Nature 374, 356–360
7. Matzuk, M. M., Kumar, T. R., Vassalli, A., Bickenbach, J. R., Roop, D. R., Jaenisch, R., and Bradley, A. (1995) Nature 374, 354–356
8. Lin, S. J., Lerch, T. F., Cook, R. W., and Jardetzky, T. S., and Woodruff, T. K. (2006) Reproduction 132, 179–190
9. Shi, Y., and Massague, J. (2003) Cell 113, 685–700
10. Greenwald, J., Groppe, J., Gray, P., Wiater, E., Kwiatkowski, W., Vale, W., and Choe, S. (2003) Mol. Cell 11, 605–617
11. Thompson, T. B., Woodruff, T. K., and Jardetzky, T. S. (2003) EMBO J. 22, 1555–1566
12. Greenwald, J., Vega, M. E., Allendorph, G. P., Fischer, W. H., Vale, W., and Choe, S. (2004) Mol. Cell 15, 485–489
13. Harrison, C. A., Gray, P. C., Fischer, W. H., Donaldson, C., Choe, S., and Vale, W. (2004) J. Biol. Chem. 279, 28036–28044
14. Cook, R. W., Thompson, T. B., Kurup, S. P., Jardetzky, T. S., and Woodruff, T. K. (2005) J. Biol. Chem. 280, 40177–40186
15. Keller, S., Nickel, J., Zhang, J. L., Sebald, W., and Mueller, T. D. (2004) Nat. Struct. Mol. Biol. 11, 481–488
16. Kirsch, T., Sebald, W., and Dreyer, M. K. (2000) Nat. Struct. Biol. 7, 492–496
17. Rider, C. C. (2006) Biochem. Soc. Trans. 34, 458–460
18. Lyon, M., Rushton, G., and Gallagher, J. T. (1997) J. Biol. Chem. 272, 18000–18006
19. Groppe, J., Greenwald, J., Wiater, E., Rodriguez-Leon, J., Economides, A. N., Kwiatkowski, W., Affolter, M., Vale, W. W., Belmonte, J. C., and Choe, S. (2002) Nature 420, 636–642
20. Thompson, T. B., Lerch, T. F., Cook, R. W., Woodruff, T. K., and Jardetzky, T. S. (2005) Dev. Cell 9, 535–543
21. Harrington, A. E., Morris-Trigg, S. A., Ruotolo, B. T., Robinson, C. V., Ohnsuma, S., and Hyvonen, M. (2006) EMBO J. 25, 1035–1045
22. Abe, Y., Minegishi, T., and Leung, P. C. (2004) Growth Factors 22, 105–110
23. Gumienny, T. L., and Padgett, R. W. (2002) Trends Endocrinol. Metab. 13, 295–299
24. Glist, C., Kemp, C. F., and Knight, P. G. (2004) Reproduction 127, 239–254
25. Sidis, Y., Mukherjee, A., Keutmann, H., Delbaere, A., Sadatsuki, M., and Schneyer, A. (2006) Endocrinology 147, 3586–3597
26. Otuka, F., Moore, R. K., Iemura, S., Ueno, N., and Shimazaki, S. (2001) Biochim. Biophys. Res. Commun. 289, 961–966
27. Shimonaka, M., Inouye, S., Shimazaki, S., and Ling, N. (1991) Endocrinology 128, 3313–3315
28. Phillips, D. J., and de Kretser, D. M. (1998) Front. Neuroendocrinol. 19, 287–322
29. Iemura, S., Yamamoto, T. S., Takagi, C., Uchiyama, H., Natsume, T., Shimazaki, S., Sugino, H., and Ueno, N. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9337–9342
30. Shimazaki, S., Koga, M., Esch, F., Cooksey, K., Mercado, M., Koba, A., Ueno, N., Ying, S. Y., Ling, N., and Guillemin, R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4218–4222
31. Tashiro, K., Yamada, R., Asano, M., Hashimoto, M., Muramatsu, M., and Shiokawa, K. (1991) Biochem. Biophys. Res. Commun. 174, 1022–1027
32. Michel, U., Albiton, A., and Findlay, J. K. (1990) Biochem. Biophys. Res. Commun. 173, 401–407
33. Hashimoto, O., Kawasaki, N., Tsuchida, K., Shimazaki, S., Hayakawa, T., and Sugino, H. (2000) Cell. Signal. 12, 565–571
34. Schneyer, A. L., Wang, Q., Sidis, Y., and Sluss, P. M. (2004) J. Clin. Endocrinol. Metab. 89, 5067–5075
35. Sugino, K., Kurosawa, N., Nakamura, T., Takio, K., Shimazaki, S., Ling, N., Titani, K., and Sugino, H. (1993) J. Biol. Chem. 268, 15579–15587
36. Yamane, Y., Tohno-oka, R., Yamada, S., Furuya, S., Shiokawa, K., Hirabayashi, Y., Sugino, H., and Sugahara, K. (1998) J. Biol. Chem. 273, 7375–7381
37. Inouye, S., Ling, N., and Shimasaki, S. (1992) Mol. Cell. Endocrinol. 90, 1–6
38. Sidis, Y., Schneyer, A. L., and Keutmann, H. T. (2005) Endocrinology 146, 130–136
39. Innis, C. A., and Hyvonen, M. (2003) J. Biol. Chem. 278, 39969–39977
40. Inouye, S., Guo, Y., DePaolo, L., Shimonaka, M., Ling, N., and Shimasaki, S. (1991) Endocrinology 129, 815–822
41. CCP4 (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763
42. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. D. Biol. Crystallogr. 54, 905–921
43. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
44. Kogure, K., Zhang, Y. Q., Kanzaki, M., Omata, W., Mine, T., and Kojima, I. (1996) Hepatology 24, 361–366
45. Roehrl, M. H., Wang, J. Y., and Wagner, G. (2004) Biochemistry 43, 16056–16066
46. Nakamura, T., Sugino, K., Titani, K., and Sugino, H. (1991) J. Biol. Chem. 266, 19432–19437
47. Hashimoto, O., Nakamura, T., Shoji, H., Shimasaki, S., Hayashi, Y., and Sugino, H. (1997) J. Biol. Chem. 272, 13835–13842
48. Yamamoto, T. S., Iemura, S., Takagi, C., Shimasaki, S., and Ueno, N. (2000) Int. J. Dev. Biol. 44, 341–348
49. Phillips, D. I., Jones, K. L., McGaw, D. J., Groome, N. P., Smolich, J. J., Parsson, H., and de Kretser, D. M. (2000) J. Clin. Endocrinol. Metab. 85, 2411–2415
50. Lee, P., Knight, R., Smit, J. M., Wilschut, J., and Griffin, D. E. (2002) J. Virol. 76, 6302–6310