Vol. 208, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 8, 1995 Pages 1-9

# THE HEPARIN BINDING SITE OF FOLLISTATIN IS INVOLVED IN ITS INTERACTION WITH ACTIVIN

Shoji Sumitomo[1], Satoshi Inouye[2], Xin-Jun Liu[3], Nicholas Ling[3] and Shunichi Shimasaki[4]

*Department of Molecular Endocrinology, The Whittier Institute for Diabetes and Endocrinology, La Jolla, California 92037*

Received January 23, 1995

Whether the heparin-binding site of follistatin would interact with activin has been examined. When a mixture of recombinant human follistatin-288 (rhFS-288) and -315 (rhFS-315) was applied to an activin-coupled affinity column, followed by stepwise elution of the column using 4M urea, 8M urea, 1M guanidine-HCl and 2M guanidine-HCl, rhFS-315 was eluted with 4M urea, while rhFS-288 was eluted with 2M guanidine-HCl. This finding implies that the carboxyl-terminal 27 amino acid extension of rhFS-315, which is not present in rhFS-288, affects the binding of follistatin with activin. Addition of heparin (50 μg/ml) to the elution solvent caused rhFS-288 to elute with 4M urea, whereas rhFS-315 was not affected. These data suggest for the first time that these two structurally related follistatin molecules interact with activin by different modes of binding and, in the presence of heparin, the interaction of rhFS-288 with activin is indistinguishable from that of rhFS-315. Two analogs of rhFS-288 mutated at the heparin binding site were eluted with 8M urea or 1M guanidine-HCl, distinct from the elution profile of the intact rhFS-288. These results indicated that mutation at the heparin binding site alters the activin binding affinity. In addition, bioassay of the two mutants showed that they were less potent than the rhFS-288. These findings suggest that the heparin binding site of follistatin also contributes to its binding for activin, and heparin may play an important role in the bioactivity of follistatin.



Follistatin (FS) is a monomeric protein originally isolated from ovarian follicular fluid based on its ability to specifically inhibit pituitary follicle-stimulating hormone (FSH) secretion (1,2). Subsequently, it was also identified as a binding protein for activin (3), a member of the TGF-β protein superfamily. Aside from its original identification as a stimulator of FSH secretion (4~6), activin was later found to have a wide spectrum of activities in various cell types and tissues (7).

[1]Present affiliation: Kawasaki Medical School, Kurashiki, Japan.

[2]Present affiliation: Chisso Co., Yokohama, Japan.

[3]Present affiliation: Neurocrine Biosciences, Inc., La Jolla, CA.

[4]To whom all correspondence should be addressed: Shunichi Shimasaki, Ph.D. Present address: Department of Cell Biology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037. FAX:(619) 457-5534.

The abbreviations used are: recombinant human follistatin, rhFS; follicle-stimulating hormone, FSH; Chinese hamster ovary, CHO; acidic residue rich, AR; polymerase chain reaction, PCR; sodium dodecylsulfate-polyacrylamide gel electrophoresis, SDS-PAGE; Gu, guanidine.

0006-291X/95 $5.00

Molecular cloning analysis of the FS cDNA and gene revealed that there are two types of FS mRNAs encoding precursor proteins of 317 and 344 amino acid residues with a common 29-amino acid signal peptide sequence (8~11). The two types of FS precursors originated from alternatively spliced mRNAs and mature FS with 315 amino acids (FS-315) differs from mature FS with 288 amino acids (FS-288) by having an extra 27-amino acid sequence at the carboxyl terminus (8~11). Using recombinant human (rh) FS-288 and -315 expressed in Chinese hamster ovary (CHO) cells, we found that rhFS-288 was 8~10 times more potent than rhFS-315 in suppressing FSH release from pituitary cells and the potency of rhFS-288 is similar to that of inhibin-A (12). Moreover, when the FSH-suppressing activity of rhFS-288 was compared to that of inhibin-A in ovariectomized adult rats, rhFS-288 was found to be more potent and longer acting than inhibin-A (12). Similar potencies were recently reported for native porcine FS-288 and -315 (13).

The biological action of FS has been postulated to occur through its binding to activin. Indeed, it has been shown that two molecules of FS could bind to one molecule of activin (14). In addition to binding activin, FS also associate with heparin/heparan sulfate (1,13,15,16), with FS-288 exhibiting a higher affinity than FS-315 (13,16). The heparin binding site of FS was recently located at amino acid sequence 72-86 which is a basic amino acid-rich region (16). Since the structural difference between FS-288 and -315 is the absence of a carboxyl-terminal 27 amino acid sequence in FS-288, in which 44 % of the amino acids are acidic residues, hereinafter designated as the AR (Acidic residue Rich) domain, the structure of these two molecules might be pictured by imagining as follows: The heparin binding domain (basic residue-rich region) present in FS-315 is structurally hindered by the AR domain, whereas the haparin binding site of FS-288 is bare because of the lack of the AR domain (9,10). As such, the difference in bioactivity of these two molecules might be explained by the difference in their ability to bind heparin/heparan sulfate.

To test this hypothesis we have now examined the binding of rhFS-288 and -315 for activin using an immobilized activin affinity column. We found for the first time that their binding to activin is distinct, implying a role for the AR domain in activin binding. Addition of heparin to the elution solvent abolished the difference in their binding affinity to activin. Moreover, mutants of rhFS-288 at the heparin binding site showed different binding affinity to activin than the intact rhFS-288 and they are less active than FS-288 in the pituitary culture assay. Taken together, the results of the present study suggest that the heparin binding site of the FS molecule is also involved in activin binding, and the AR domain interferes with FS binding to activin.

## MATERIALS AND METHODS

*Construction of hFS cDNA mutants by site-directed mutagenesis*

Site-directed mutagenesis was carried out by methods involving polymerase chain reaction (PCR) using primers containing a mutation (17). Four primers were synthesized by a Cyclone Plus DNA synthesizer (Milligen/Biosearch, Novato, CA). They are: primer A, 5'-CGTCAAGCGAAGAACGGCCG-3' (nucleotide #130-149 of the human FS cDNA clone described in figure 2 of reference 9); primer B, 5'-GATACACTTTCCCTCATAGG-3' (nucleotide #747-728, complementary); primer C, 5'-GTGGACCTGGGTCAAAATGC-3' (nucleotide #325-344) containing the replacement of AA (wild) by TC (mutant) at #337-338 to substitute Lys by Ser at the amino acid 75; and primer D, 5'-AGCGGGGTTTGTTCGTCTTG-3' (nucleotide #376-355, complementary) containing the replacement of T (wild) by G (mutant) at #359 to substitute Lys by Thr at the amino acid 82.

Two PCR experiments using primers A and D or B and C were first performed separately using pSV2HF-288 as a template (12), resulting in the production of the DNA segments of nucleotide #130-376 and #325-747 containing an amino acid mutation at residues 82 and 72, respectively. These PCR products were purified from agarose gel and the purified products were mixed together and a small aliquot of the mixture was used for another PCR using primers A and B, which generated DNA segments #130-747 containing a mutation at either amino acid 75 or 82. These PCR products were digested with NarI and StyI located at nucleotides #277-282 and 507-512, respectively, and the DNA segments containing NarI and StyI sites at the termini were gel-purified. They were used to replace the corresponding regions of the plasmid encoding hFS-288 and, after DNA sequencing of the resulting plasmid subclones, rhFS-288 mutants at amino acid 75 (Lys to Ser) or 82 (Lys to Thr) were obtained. The coding region of these two plasmids were then subcloned into the cloning sites (BamHI and XhoI) of pcDNAIneo (Invitrogen Co., San Diego, CA). The plasmids containing a mutation at either amino acid 75 or 82 were designated as rhFS-288K75S or rhFS-288K82T.

*Expression of the hFS mutants in CHO cell*

Expression of the rhFS-288K75S and rhFS-288K82T was performed as described previously (12) with a slight modification. Briefly, dhfr-deficient CHO cells (CHO-DG44) were seeded at a density of 2 x $10^6$ cells/10-cm dish in 10 ml Minimum Essential Medium alpha (αMEM; Gibco, Grand Island, NY) with 10 % heat-inactivated fetal calf serum (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco). Cells were cultured at 37°C in a humidified 5 % $CO_2$ and 95 % air incubator for 16 hr and the culture medium was replaced with fresh medium. Cells were cultured further for 3 hr and then transfected by the calcium phosphate precipitation method (18) with 20 μg of the expression plasmid. After 2 days the cells were trypsinized, reseeded at the same condition as above, and cultured for 16 hr. The transfected cells were then selected by the addition of 500 μg/ml of Geneticin (Gibco) to the medium. Approximately two weeks later, transformants which tolerated Geneticin and formed colonies were picked up by toothpicks and transfered to a 24-well culture dish. The transformed cells were cultured for 2~3 days and expression of the recombinant proteins in the conditioned media was monitored by Western immunoblotting analysis (12).

Both FS mutants were purified by using activin-coupled affinity column as described previously (12) and their amino-terminal amino acid sequence was ascertained by microsequencing. The yield of the purified proteins was 50-100 μg/l in the conditioned medium.

*Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blotting analysis*

SDS-PAGE was performed essentially as described by Laemmli (19) under non-reducing conditions using a 0.1 x 8 x 6-cm, 12 % polyacrylamide fractionating gel at 25 mA for 2 hr. The gels were stained with Coomassie brilliant blue R-250 (Serva, Westbury, NY) or subjected to Western immunoblotting analysis as described previously (12).

*Activin-coupled affinity chromatography*

An activin-coupled affinity column described previously (12) was used. After loading the sample, the column was washed with 50 ml 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl. The adsorbed rhFS was eluted with the elution solvent described in each figure legend. The flow rates were 0.6 ml/min for loading and 0.3 ml/min for elution, and the eluate fractions (2.1 ml/fraction) were monitored by UV absorbance at 280 nm. The eluted protein fractions were pooled, dialyzed against 20 mM phosphate buffer and lyophilized.

*Bioassay of the expressed rhFSs*

The inhibitory activity of the rhFSs on FSH secretion was measured using a primary culture of rat anterior pituitary cells according to a previously reported procedure (21). Handling and experimental manipulation of the animals was done in strict accord with the NIH Guide for the Care and Use of Laboratory Animals. FSH concentrations were determined in duplicate using a rat FSH RIA kit supplied by the National Hormone and Pituitary Program supported by NIDDK.

## RESULTS AND DISCUSSION

In order to explore whether the two species of FS, FS-288 and -315, have different binding affinities for activin, we first examined their elution characteristics from an activin-coupled affinity

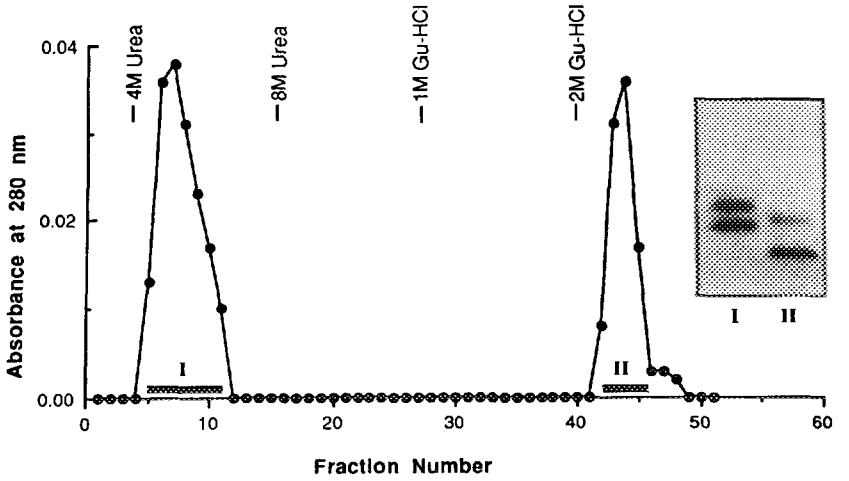


**Fig. 1. Activin-coupled affinity column chromatography of rhFS-288 and -315.** A mixture of 300 mg of rhFS-288 and -315 was applied to the column and then eluted with a stepwise gradient of developers as indicated. Two peaks denoted by solid bars I and II were analyzed by SDS-PAGE followed by Coomassie staining of the gel (photo insert).

column. Preliminary experiments using a gradient of 0 to 8 M urea demonstrated that rhFS-315 was eluted from the activin column at 4 M urea. However, rhFS-288 was not eluted from the column even with 8 M urea and it would only be eluted with 2 M guanidine-HCl (data not shown). Therefore, we attempted a stepwise elution using in increasing order: 4 M urea, 8 M urea, 1 M guanidine-HCl and 2 M guanidine-HCl. To carry out these experiments, a mixture of 300 μg each of rhFS-288 and -315 was applied to an activin-coupled column. After washing the column with phosphate buffer containing 0.5 M NaCl, the proteins were eluted with the stepwise gradient of developers. As shown in Fig. 1 two distinct elution peaks were observed. One was eluted with 4 M urea and the other with 2 M guanidine-HCl. SDS-PAGE analysis of these two pooled fractions demonstrated that two protein bands at 35 and 38 kDa were eluted with 4 M urea (pool I), which corresponded to the non- and N-glycosylated forms of rhFS-315, respectively, while another protein bands at the 31 and 35 kDa were eluted with 2 M guanidine-HCl (pool II), which corresponded to the non- and N-glycosylated forms of rhFS-288, respectively, as described in our previous observation (12). Since urea and guanidine-HCl are reagents which interefere with hydrogen bonding and ionic interaction, respectively, this data suggest that the association between activin and rhFS-315 or rhFS-288 is mainly through hydrogen bonding or ionic interaction, respectively. Moreover, since the only difference in structure between rhFS-288 and -315 is the absence or presence of the AR domain, respectively, the AR domain seems to interfere with the ionic interaction between FS and activin.

Since FS is known to bind heparin (1,13,15,16) and previous data suggested that the heparin-binding affinity of FS-288 is higher than that of FS-315 (13,16), we next tested the effect of heparin on their activin binding. For this purpose we performed similar experiments to those described in Fig. 1, with the exception that protein elution was carried out in the presence of 50

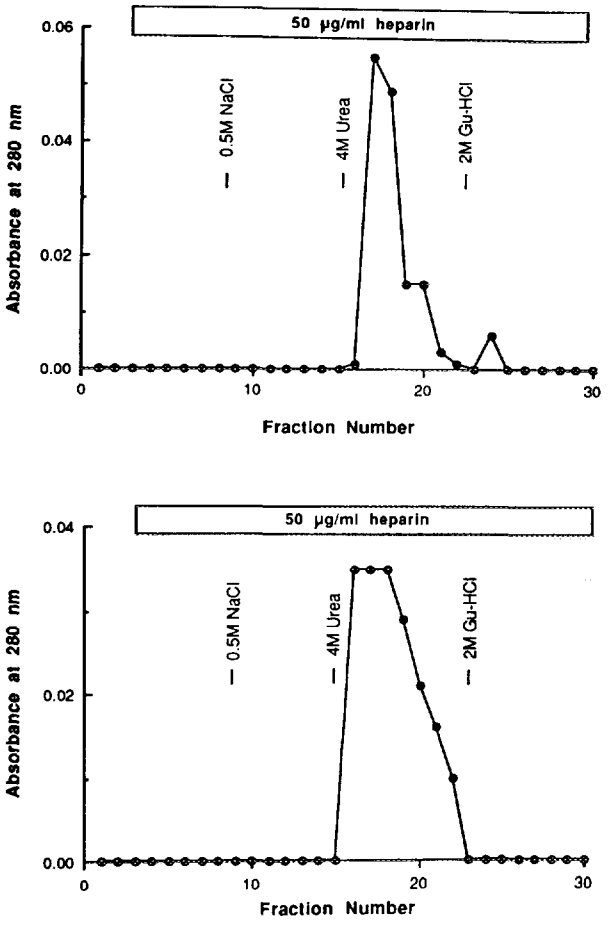


**Fig. 2. Activin-coupled affinity column chromatography of rhFS-288 and -315 in the presence of heparin.** Four hundred mg of rhFS-288 (upper panel) or -315 (lower panel) was applied to the column and then eluted with a stepwise gradient of developers containing 50 µ g/ml heparin as indicated.

µg/ml heparin. As shown in Fig. 2, rhFS-288 was eluted with 4 M urea in the presence of 50 µg/ml heparin, while rhFS-315 was eluted with 4 M urea, regardless of the presence or absence of heparin (see also Fig. 1). Consequently, the ionic interaction of rhFS-288 to activin seems to be broken by the addition of 50 µg/ml heparin into the elution solvent and, thus, both rhFS-288 and -315 were eluted with 4 M urea. These findings led us to hypothesize that the heparin binding site (basic residue-rich region) of FS-315 is structurally masked by the AR domain and, thus, FS-315 interacts with activin mainly by hydrogen bonding, whereas FS-288 interacts by both hydrogen bonding and ionic interaction. Therefore, when the ionic interaction is masked by heparin, rhFS-288 behaves similar to rhFS-315 in binding activin. Importantly, the fact that the activin binding affinity of rhFS-288 is affected by the presence of heparin implies that the heparin-binding site contributes to its binding to activin.

To further explore the role of the heparin-binding site of the FS molecule for its activin binding as well as its biological activity, we prepared two analogs of rhFS-288 mutated at the heparin-

binding site. Fig. 3 shows the elution profile of the two mutants rhFS-288K75S and rhFS-288K82T from the activin-coupled affinity column. Greater than 80% of the rhFS-288K75S was eluted as a single peak with 8 M urea, while rhFS-288K82T eluted as two peaks, one with 8 M urea and the other with 1 M guanidine-HCl. Although we could not explain why the rhFS-288K82T was eluted as two peaks, the elution of both mutants was very distinct from that of intact rhFS-288 (see also Fig. 1). These observations support the notion that the heparin-binding site contributes to the binding of FS to activin.

The biological activity of the FS mutants was examined by measuring their inhibitory activity on FSH release from pituitary cells (Fig. 4). As was observed previously (12), the potency of rhFS-315 ($ED_{50}$=2.2 x $10^{-10}$M) was about 10 times lower than that of rhFS-288 ($ED_{50}$=2.0 x $10^{-11}$M), while the potency of the two rhFS-288 mutants ($ED_{50}$=3.8 and 4.0 x $10^{-11}$M, respectively) was about 2 times lower than that of rhFS-288. Based on the notion that the role of FS in the suppression of FSH secretion from the pituitary cells is to block the autocrine/paracrine action of activin through its binding with the endogenous activin (22), these data are in agreement with the results that the ionic interaction of the rhFS-288 mutants to activin was attenuated

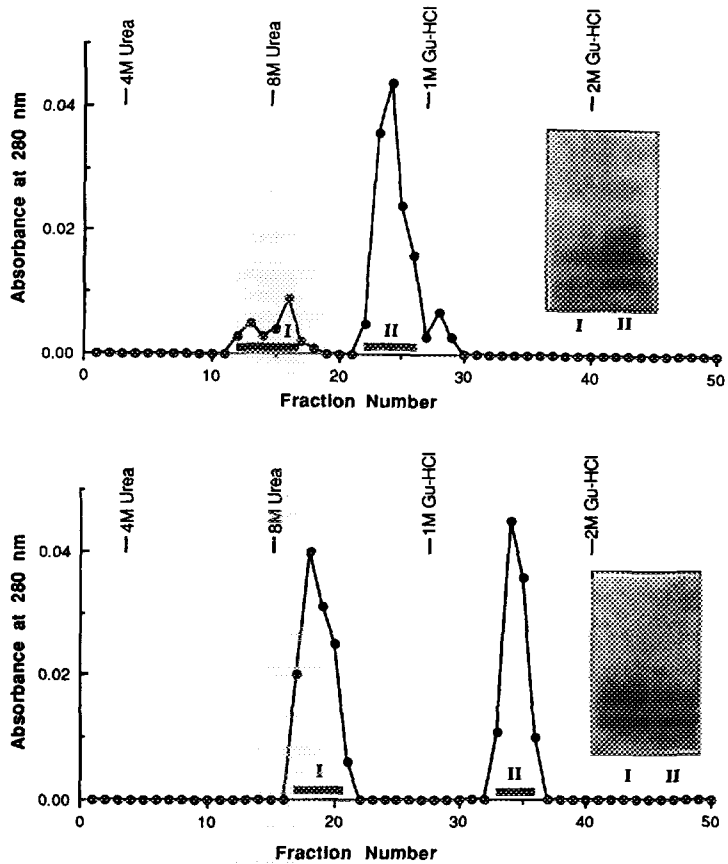


**Fig. 3. Activin-coupled affinity column chromatography of rhFS-288 mutants.** Four hundred mg of rhFS-288K75S (upper panel) or rhFS-288K82T (lower panel) was applied to the column and then eluted with a stepwise gradient of developers as indicated. Two peaks denoted by solid bars I and II were analyzed by SDS-PAGE followed by Coomassie staining of the gel (photo insert).

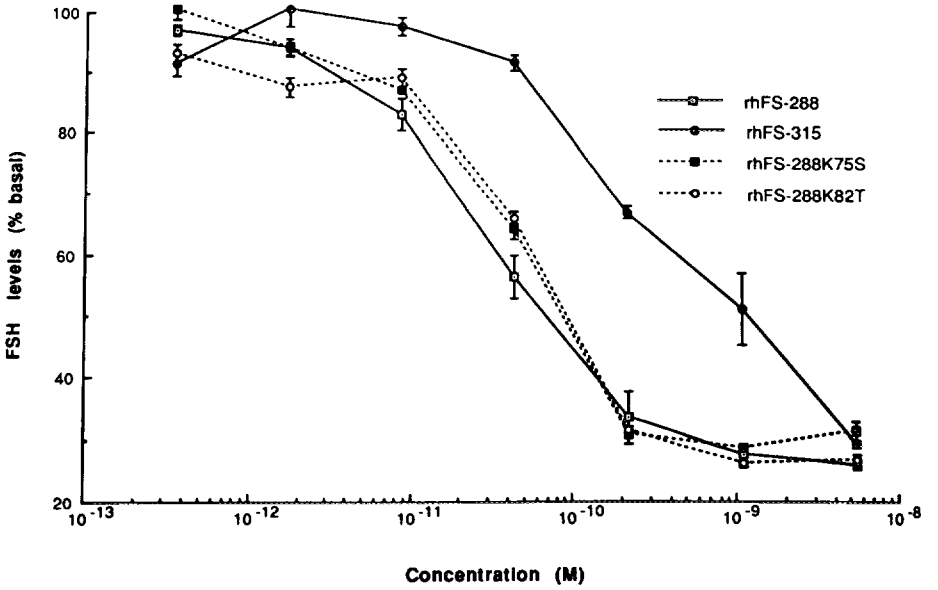


**Fig. 4. Dose-response curve of rhFS-288, rhFS-315, rhFS-288K75S and rhFS-288K82T in the suppression of FSH secretion from cultured rat anterior pituitary cells.** Protein concentrations were determined by amino acid analysis. The data are presented as the percent change in FSH concentrations in the medium compared with the concentration of FSH in the medium from untreated cells, which was normalized to 100%. The vertical bars in the graph represent the SEM (n=3). The data presented in this figure represent one of two similar experiments.

compared to that of the intact rhFS-288. In a recent collaboration with Sugino's laboratory, we measured the inhibitory activity of FSH release for purified porcine FS-288 and -315 and determined that native porcine FS-288 is 10 times more potent than FS-315, as is the case with rhFS-288 and -315 (13). The activin-binding affinity of the two native molecules, however, was found to be similar (Kd=540~680 pM) by Sugino's laboratory when they determioned the dissociation constants for the complex of activin and native porcine FS-288 or -315 by the polyethylene glycol precipitation method using $^{125}$I-activin-A (13). Since we were not successful in obtaining the reproducible Kd values for the rhFS-activin complex by the same method, we employed the immobilized activin affinity column to examine the affinity of the rhFSs for activin. Although this method can't provide the Kd value, it is straightforward and highly reproducible. The present study clearly demonstrated that there are two distinct modes of binding for FS to activin. It remains to be investigated whether the different binding modes reflect their Kd values in these molecules.

In a prior publication we reported that, using a heparin column with a gradient of NaCl (0 to 3 M), a mixture of rhFS-288 and -315 was co-eluted from the column at a NaCl concentration between 0.8 and 1.0 M. However, the subsequent SDS-PAGE analysis of the column fractions showed that rhFS-288 had a slightly higher affinity than rhFS-315 (16). Recently, Sugino's group also showed that native porcine FS-288 binds to the rat granulosa cell surface (Kd=2 x $10^{-9}$ M) as well as to a heparan sulfate column, whereas FS-315 had no affinity for either of them (13).

Therefore, the difference in biological activity between FS-315 and -288 might be due to their different cell surface association ability through heparan sulfate proteoglycans on the rat pituitary cells (13,16), although this speculation is based on the assumption that the activin-binding affinity for these two FS molecules is the same. Our present study, however, suggests another mechanism to explain why FS-288 is more potent than FS-315 on the suppression of FSH secretion. FS-288 interacts with activin by both hydrogen bonding and ionic interaction, and the site responsible for the ionic interaction is the heparin binding site. On the other hand, FS-315 interacts with activin mainly by hydrogen bonding because the site for ionic interaction in the FS-315 molecule is masked by the AR domain, making FS-315 less potent than FS-288. The present study further suggests that the heparin-binding site of the FS molecule is also part of the activin-binding site, because of the fact that the interaction of FS-288 with activin was altered by heparin, probably due to the dissociation of the ionic interaction between rhFS-288 and activin by heparin. In contrast, the binding of FS-315 to activin was unaffected by heparin, because the heparin binding site in FS-315 is already masked by the AR domain. These findings point to an important role of heparin in the bioactivity of FS.

## ACKNOWLEDGMENTS

The authors are grateful for excellent technical assistance from G. Mickey, R. Schroeder, and M. Regno-Lagman. We also thank Drs. Yuzuru Eto and Hiroshiro Shibai at Ajinomoto Co., Japan, for the generous gift of recombinant activin-A. This work was supported by a grant from the Andrew W. Mellon Foundation and Program Project Grant HD-09690 from the National Institutes of Health.

## REFERENCES

1. Ueno, N., Ling, N., Ying, S.-Y., Esch, F., Shimasaki, S., and Guillemin, R. (1986) Proc. Natl. Acad. Sci. USA. 84, 8282-8286.
2. Robertson, D.M., Klein, R., de Vos, F.L., McLachlan, R.I., Wettenhall, R.E.H., Hearn, M.T.W., Burger, H.G., and de Kretser, D.M. (1987) Biochem. Biophys. Res. Commun. 149, 744-749.
3. Nakamura, T., Takio, K.,Eto, Y., Shibai, H., Titani, K., and Sugino,H. (1990) Science 247, 836-838.
4. Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D., and Spiess, J. (1986) Nature 321, 776-779.
5. Ling, N., Ying, S.-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M., and Guillemin, R. (1986) Nature 321, 779-782.
6. Ling, N., Ying, S.-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M., and Guillemin, R. (1986) Biochem. Biophys. Res. Commun. 138, 1129-1137.
7. DePaolo, L.V., Bicsak, T.A., Erickson, G.E., Shimasaki, S., and Ling, N. (1991) Proc. Soc. Exp. Biol. Med. 197, 500-512.
8. Esch, F.S., Shimasaki, S., Mercado, M., Cooksey, K., Ling, N., Ying, S., Ueno, N., and Guillemin, R. (1987) Mol. Endocrinol. 1, 849-855.
9. Shimasaki, 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. USA. 85, 4218-4222.
10. Shimasaki, S., Koga, M., Esch, F., Mercado, M., Cooksey, K., Koba, A., and Ling, N. (1988) Biochem. Biophys. Res. Commun. 152, 717-723.
11. Shimasaki, S., Koga, M., Buscaglia, M.L., Simmons, D.M., Bicsak, T.A., and Ling, N. (1989) Mol. Endocrinol. 3, 651-659.
12. Inouye, S., Guo, Y., DePaolo, L., Shimonaka, M., Ling, N., and Shimasaki, S. (1991) Endocrinology 129, 815-822.
13. Sugino, K., Kurosawa, N., Nakamura, T., Takio, K., Shimasaki, S., Ling, N., Titani, K., and Sugino, H. (1993) J. Biol. Chem. 268, 15579-15587.
14. Shimonaka, M., Inouye, S., Shimasaki, S., and Ling, N. (1991) Endocrinology 128, 3313-3314.

15. Nakamura, T., Sugino, K.,Titani, K., and Sugino, H. (1991) J. Biol. Chem. 266, 19432-19437.
16. Inouye, S., Ling, N., and Shimasaki, S. (1992) Mol. Cell. Endocrinol. 90, 1-6.
17. Huguchi, R. (1989) In: Erlich, H.A. (ed) PCR technology, Stockton press, New York, pp61-70.
18. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745-2752.
19. Laemmli, U.K. (1970) Nature 277, 680-685.
20. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA. 76, 4350-4354.
21. Ying, S.-Y., Becker, A., Swanson, G., Tan, P., Ling, N., Esch, F., Ueno,.N., Shimasaki, S., and Guillemin, R. Biochem. Biophys. Res. Commun. 149, 133-139, 1987.
22. Corrigan, A.Z., Bilezikjian, L.M., Carroll, R.S., Bald, L.N., Schmelzer, C.H., Fendly, B.M., Mason, A.J., Chin, W.W., Schwall, R.H., and Vale, W. (1991) Endocrinology 128, 1682-1684.