Vol. 179, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1991 Pages 352-358

Site-specific mutagenesis of human follistatin

Satoshi Inouye, Yili Guo, Nicholas Ling and Shunichi Shimasaki*

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

Received June 27, 1991

Follistatin is a monomeric protein originally discovered in ovarian follicular fluid as a suppressor of pituitary follicle-stimulating hormone (FSH) secretion, and later identified as a binding protein for activin. To explore the role of the Asn-linked carbohydrate chains on the follistatin molecule in regard to the inhibition of FSH secretion and activin binding ability, site-specific mutations were introduced at either or both of the two potential Asn-linked glycosylation sites of human follistatin with 315 amino acids (hFS-315). The three types of follistatin mutants were expressed individually in Chinese hamster ovary cells. When tested for their ability to inhibit FSH secretion and to bind activin, each mutant was found to have a similar property as the non-mutated recombinant hFS-315, suggesting that glycosylation of the follistatin molecule has no effect in these functions. However, a two amino acid insertion in between the second and the third amino acid residues in hFS-315 caused the resulting compound to lose completely its inhibitory activity on FSH secretion from the pituitary as well as its binding ability to activin. This finding suggests that the amino-terminal region of the follistatin molecule is critical for both of these functions.

Follistatin (FS) is a monomeric protein originally isolated from ovarian follicular fluid based on its ability to suppress follicle-stimulating hormone (FSH) secretion from the pituitary (1,2). Molecular cloning analyses of its cDNA and genomic DNA revealed that there are two types of alternatively spliced FS mRNAs encoding 317 and 344 amino acid residues, which include a common 29-amino acid signal peptide sequence (3-6). Mature FS with 315 amino acids (FS-315) differed from mature FS with 288 amino acids (FS-288) by having an extra 27-amino acid sequence at the carboxy-terminal. The predicted primary structures of FS in human (3), pig (4,5), and rat (6) are highly conserved (>98%), including the two potential Asn-linked glycosylation sites. Bioassays using rat anterior pituitary cells revealed that the potency of FS on the suppression of FSH secretion was only 10-33% of that of inhibin on a molar basis. As a result, the physiological significance of FS was considered trivial. However, a recent discovery rekindled our interest in follistatin, because Nakamura *et al.* (7) found that FS is a binding protein for activin. This prompted us to produce FS by gene expression to further study its functions.

Our efforts have led to the establishment of an expression system in Chinese hamster ovary (CHO) cells to produce recombinant human FS-288 (rhFS-288) and -315 (rhFS-315) with a yield

*To whom correspondence should be addressed.

Abbreviations: FSH, follicle-stimulating hormone; hFS, human follistatin; CHO, Chinese hamster ovary; kDa, kilodalton; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RIA, radioimmunoassay; ED_{50}, half effective dose.

0006-291X/91 $1.50

of 3-5 mg/liter conditioned medium after a single step of affinity chromatography purification on an activin-coupled Affi-Gel column (8). Using the rhFSs as molecular mass markers, Western blotting analysis with specific antibodies demonstrated that native FS in porcine follicular fluid was composed of at least six different molecular forms (major bands of 32, 35, and 39 kDa and minor bands of 31, 36, and 37 kDa) under non-reducing conditions, and the core protein sequence of a majority of native FS was neither FS-315 nor FS-288, but was composed of 300 amino acids in various forms of glycosylation (8). A minor component (<1%) with a core protein sequence corresponding to FS-288 was also detected.

To explore the function of the carbohydrate chains in FS in regard to FSH suppression and the binding of activin, we undertook a structure-function relationships study by substituting some of its amino acid residues. The substitutions were carried out by site-directed mutagenesis of the FS-315 cDNA followed by expression of the mutated cDNA in CHO cells. The expressed mutants were assayed for their ability to bind activin and to suppress FSH secretion, and the results are presented in this paper.

MATERIALS AND METHODS

Materials

All restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase and Klenow fragment of DNA polymerase were purchased from Stratagene (La Jolla, CA). Purified native porcine FS was prepared as described previously (1). Recombinant activin (9) was kindly provided by Dr. R. Schwall (Genentech, Inc., South San Francisco, CA). [γ-^{32}P]-A' P (5000 Ci/mmol) for labeling of the synthetic primers was obtained from Amersham (Arlington, Heights, IL).

Construction of hFS cDNA mutants by site-specific mutagenesis

The oligonucleotides used for the site-specific mutagenesis are shown in Table 1. They were synthesized by the phosphoramidate method using a Cyclone Plus DNA synthesizer (Milligen/Biosearch, Novato, CA) and purified with the cartridge column (Applied Biosystems Inc., Foster City, CA). Construction of the mutated hFS cDNAs was carried out by the method of oligonucleotide-directed site-specific mutagenesis (10) using the gapped duplex DNA of the pSV2HF-315 plasmid (8). The circular form of the mutated plasmid DNA was then used to transform *E. coli* JM 83 (11) and the bacterial clones harboring the mutated plasmid were identified by the colony hybridization method (12), using the corresponding ^{32}P-labeled synthetic oligonucleotide used to construct the mutated plasmid as a probe. Because the plasmid DNA prepared from a positive bacterial colony may contain both mutant and wild type forms, we repeated the transformation and colony hybridization steps using the isolated plasmid. The positive

Table 1. Synthetic oligonucleotides for site-specific mutagenesis

Name of Mutant	Oligonucleotide	Mutation or Insertion
	* *	
T97A	5'-C AAC ATC GCT TGG AAG GG-3'	$Thr^{97}\rightarrow Ala^{97}$
	N I **A** W K G	
	95 97	
	* *	
T261A	5'-C AAT GCC GCA TAT GCC AG-3'	$Thr^{261}\rightarrow Ala^{261}$
	N A **A** Y A S	
	259 261	
	*** ***	
449	5'-CAG GCT GGG AAC AAG CTT TGC TGG CTC CGT-3'	Insertion between residues 2 and 3
	Q A G N **K** **L** C W L R	
	-2 -1 1 2 3 4 5 6	

*Asterisks indicate the positions of nucleotide substitution or insertion. The numbers in the FS sequence correspond to the positions of the residues.

bacteria clones identified at the second hybridization, which contained only the mutated plasmid, were isolated and the nucleotide sequence of the plasmid DNA isolated from the bacterial culture was confirmed by DNA sequencing (13). One of the mutated plasmid DNAs, named TWA, which has double mutations at the two potential Asn-linked glycosylation sites, was constructed by assembling parts of the two mutated plasmids, T97A and T261A, with Ser^{97} mutated to Ala^{97} and Thr^{261} mutated to Ala^{261}, respectively. Specifically, the *BglII-XbaI* (5.45 kb) fragment from the T97A plasmid was ligated to the *BglII-XbaI* (0.33 kb) fragment from the T261A plasmid. Each constructed plasmid contained the simian virus-40 (SV40) promoter, intron and poly(A) signal sequence as described previously (8).

Expression of the hFS mutants in CHO cells

The method used to express the hFS mutants in CHO cells was the same as that reported previously (8). A dhfr-deficient CHO cell line (CHO-DG44) (14) was co-transfected by the calcium phosphate method (15) with the mutated plasmid plus the pSV2dhfr plasmid as a selectable marker (16). The stable transformants that secreted the FS mutant into the conditioned medium were isolated for further biological and immunological analyses. The samples for the FSH suppression bioassay and the activin binding assay were prepared as follows: After culturing the transformant cells in a nucleotide free MEM-alpha medium (GIBCO, Grand Island, NY) containing 10% dialyzed fetal calf serum (Hyclone, Logan, UT) in 75 cm^2 flasks (Corning Lab, Corning, NY) for 2 days, the culture medium was changed to a serum-free MCDB 302 medium (Sigma, St. Louis, MO) containing 5 μg/ml leupeptin (Sigma), and the cells were further cultured for 2 days. The serum-free medium was then harvested and centrifuged to remove cell debris. The supernatant obtained was dialyzed against phosphate buffered saline (PBS) (Sigma), concentrated in a Speed-Vac concentrator and then subjected to analysis.

Western-blotting analysis

SDS-PAGE was performed essentially as described by Laemmli (17) under non-reducing conditions using a polyacrylamide fractionating gel (1 mm thickness, Novex, Encinitas, CA) at 25 mA for 1.5 hr. The proteins were transferred electrophoretically onto nitrocellulose membranes (pore size, 0.45 μm; Bio-Rad, Richmond, CA) with the Mini-Cell apparatus (Novex) at 150 mA for 2 hr (18). The membranes were blocked with casein/TBST [1% casein (Sigma), 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20 (Bio-Rad)] for 1 hr at room temperature, and then incubated with the polyclonal antibody Rb-32 (1:500 diluted in casein/TBST), raised against native porcine FS in a rabbit, for 16 hr at 4° C. After washing with TBST, the membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Calbiochem, San Diego, CA) for 2 hr at room temperature. The specifically bound antibodies were visualized by treatment with 300 μg/ml nitroblue tetrazolium chloride (Sigma) and 150 μg/ml 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (Sigma) dissolved in 100 mM Tris-HCl, pH 9.5, containing 100 mM NaCl, 5 mM $MgCl_2$.

Activin-binding analysis

Binding of labeled activin to the expressed rhFSs was determined by the ligand-blotting method of Hossenlopp *et al.* (19). Recombinant activin-A (2 μg) was iodinated using 500 μCi $Na^{125}I$ (Amersham) by the chloramine-T method (20). The reaction mixture was loaded onto a 0.7 x 30 cm column of Sephadex G-25 (Pharmacia LKB, Piscataway, NJ) precoated with PBS/5% BSA (Fraction V, Sigma) and eluted with PBS/0.1% BSA. The labeled activin fractions were pooled and stored at -20° C until used. For ligand blotting, the electro-blotted filter from the SDS-PAGE gel fractionation was soaked in 3% Nonidet P-40 (Sigma) dissolved in TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 10 min at 4° C and then blocked with casein/TBST for 2 hr followed by incubation with ^{125}I-labeled activin A (approximately 150,000 cpm/ml) in casein/TBST for 16 hr at 4° C. The filters were washed 3 times at 10 min each with TBST and then exposed to Kodak XAR film (Rochester, NY) for 72 hr in the presence of an intensifying screen at -80° C. After activin-binding analysis, the same filters were also used for Western-blotting analysis as described above.

Radioimmunoassay (RIA) and bioassay of expressed rhFSs

The amount of the expressed rhFSs in the conditioned medium was determined by RIA (21) using purified porcine FS as a standard. The potency of the expressed proteins on the inhibition of FSH secretion was determined in rat anterior pituitary primary culture according to a previously reported procedure (22).

RESULTS AND DISCUSSION

Figure 1 shows the schematic representation of the expression plasmids used in the present study. The region encoding the FS cDNA with the five distinct domains of the FS-315 molecule (3-6) is blown-up to show the potential Asn-linked glycosylation sites at Asn^{95} and Asn^{259}. We have recently reported that FS isolated from porcine follicular fluid was composed of at least six different molecular forms of 31, 32, 35, 36, 37, and 39 kDa under non-reducing conditions (8). But, when this mixture of native FSs was treated with N-glycosidase-F, only a major band at 32 kDa and a minor band at 31 kDa were detected. These findings together with other evidences (8) suggest that native FSs are a mixture of non-, mono- and di-glycosylated forms with 288 and 300 amino acids.

To determine the significance of the carbohydrate moieties in the follistatin molecule on the inhibition of FSH secretion and activin binding, we produced a series of rhFSs mutated at either or both of the potential Asn-linked glycosylation sites. Because substitution by Ala at the third residue of the consensus Asn-linked glycosylation site (Asn-X-Ser or Asn-X-Thr) (23) is a more homologous substitution than replacement of Asn by any other amino acids, we mutated the Thr at position 97 and 261 to Ala to remove the corresponding potential glycosylation sites. Thus, hFS-315 mutants, T97A and T261A, which lost one of the two potential glycosylation sites as well as TWA, which lost both sites, were produced. In the course of constructing the mutated plasmids, we have also prepared another plasmid, named 499, which has two extra amino acids (Lys-Leu) inserted in between the second and the third amino acids of hFS-315. The purpose for the construction of this plasmid was to introduce a *Hin*dIII restriction site at the amino-terminal region so as to use this plasmid for the expression of any domain of the FS molecule to determine its function.

The mutated proteins expressed by the CHO cells and secreted into the conditioned medium were detected by Western-blotting analysis using the Rb-32 antibody (21). Figure 2 shows a typical blot pattern of rhFS-315, which exhibited two major bands at 35 and 38 kDa and a minor band at 42 kDa, while the TWA mutant which has no glycosylation sites showed a single band at 35 kDa. The molecular size of the TWA mutant is in agreement with the results obtained from N-glycosidase F digestion of the purified rhFS-315 reported previously (8). By contrast, the T97A mutant exhibited two distinct bands at 35 and 38 kDa, and since the 35 kDa form corresponded to the non-glycosylated hFS-315, the 38 kDa form would correspond to the mono-glycosylated hFS-315 with the carbohydrate chain at Asn^{259}. The T261A mutant also showed two bands at 35 and

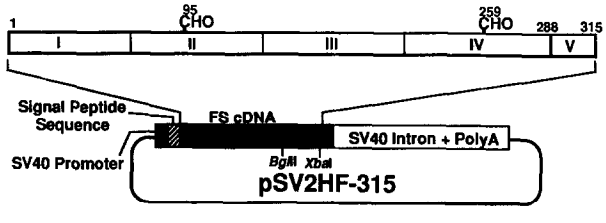

Fig. 1. Schematic representation of the structure of hFS-315 (blow-up portion) and its expression vector pSV2HF-315.
The Roman numerals I, II, III, IV, V represent the five domains of hFS-315 and CHO represents the potential carbohydrate chains attached at Asn^{95} and Asn^{259}.

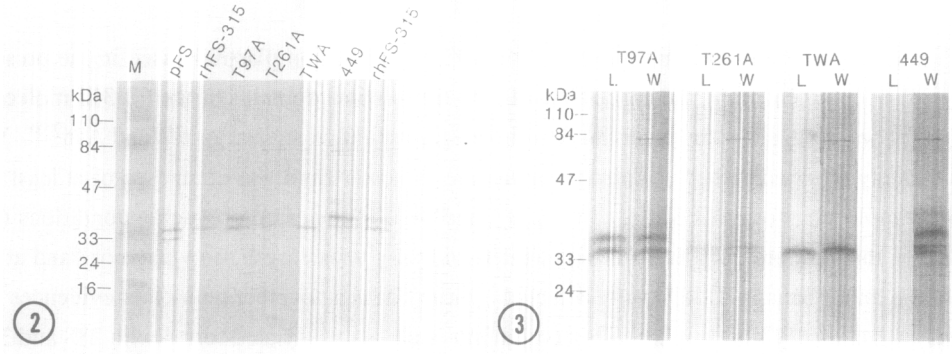

Fig. 2. Western-blotting analyses of porcine (p) FS, rhFS-315 and its mutants expressed in the conditioned medium.

SDS-PAGE was carried out using a 8-16% gradient fractionating gel under non-reducing conditions. Thirty microliters of the serum-free conditioned medium from the mutants were used for the analysis as described in Materials and Methods. The numbers on the left margin represent the molecular size of the pre-stained protein molecular mass standard (Bio-Rad, Richmond, CA).

Fig. 3. Activin ligand-blotting (L) and Western-blotting (W) analyses of the hFS-315 mutants.

Five hundred microliters of the serum-free medium from the mutants were concentrated and subjected to the SDS-PAGE using a 12% fractionating gel under non-reducing conditions as described in Materials and Methods.

42 kDa and, by analogy, the 42 kDa band would correspond to the mono-glycosylated hFS-315 with the carbohydrate chain at Asn^{95}. However, because the relative intensity of the 42 kDa band to the 35 kDa band in the T261A mutant is much lower than that of the 38 kDa band to the 35 kDa band in the T97A mutant, the Asn^{95} seems to be much less susceptible for incorporation of the carbohydrate chain than the Asn^{259}. The 449 mutant showed the same banding pattern as rhFS-315 because a two amino acid insertion in the amino-terminal of the molecule could not be differentiated by this method of molecular weight analysis.

To determine whether the hFS mutants possess activin-binding ability, ligand-blotting analyses were performed with each mutated protein using ^{125}I labeled activin-A. As shown in Figure 3, mutants T97A, T261A and TWA were able to bind labeled activin-A under non-reducing conditions. Under reducing conditions, however, they completely lost their binding ability (date not shown). These results suggest that the binding ability of FS to activin strictly depends on the properly folded conformation of the protein but is independent of the carbohydrate chains. Interestingly, the 449 mutant, which has two extra amino acids inserted at the amino-terminal region, did not bind the labeled activin. This finding suggests that the amino-terminal region of FS could be responsible for its binding to activin because the insertion of two amino acids between the second and third residues is unlikely to destroy the conformation in the rest of the molecule.

The biological activities of these mutants were determined by an *in vitro* bioassay using rat anterior pituitary cells. The protein concentration in each mutant was determined by RIA (21). The control medium from CHO cells did not inhibit FSH secretion. Dose-response curves for the suppression of FSH secretion exhibited by the mutants, T97A, T261A, TWA and rhFS-315 are shown in Figure 4. The ED_{50} values calculated for each of these four proteins were similar (2.5 ± 0.5 ng/ml). These results suggest that the carbohydrate chains of the FS molecule have no effect on the suppression of FSH secretion. By contrast, the 449 mutant did not show any inhibition of

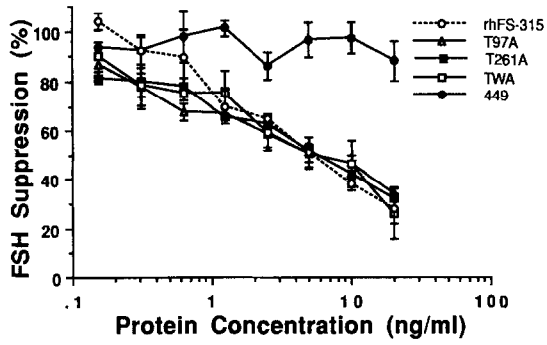

Fig.4. Dose-response curves of rhFS-315 and its mutants on the suppression of FSH secretion in cultured rat anterior pituitary cells.
The protein concentrations were determined by RIA as described in Materials and Methods. The data was presented as percent inhibition of FSH secreted into the culture medium in comparison with the FSH concentration in the medium from untreated cells, which was normalized to 100%. The vertical bars in the graph represent the SEM (n = 3).

FSH secretion up to a dosage of 20 ng/ml. Moreover, our preliminary experiment with another mutant, named C3S, in which the third amino acid of cysteine is mutated to serine also completely lost both the activin-binding ability and the inhibition of FSH secretion. These findings strongly suggest that the amino-terminal region of FS is critical for these functions.

In conclusion, we have determined in this study that the carbohydrate chains in FS are not required for its binding to activin and the suppression of FSH release from the pituitary. However, in this same study, we have also uncovered that an intact amino-terminal region is absolutely required for FS to exhibit both of these activities. Since insertion of two amino acids at the second and third positions of FS is not likely to affect the conformation of the rest of the molecule, our results strongly point to the amino-terminal domain as the most critical portion of molecule for activin binding and FSH suppression.

ACKNOWLEDGMENTS

We thank R.L. Schroeder, and M. Regno-Lagman for their technical assistance and E. Exum for preparing the manuscript. We also thank Genentech Inc. for the recombinant activin-A and the National Hormone and Pituitary Program of NIDDK for providing the rat FSH RIA kit. This work was supported by a grant from the Andrew W. Mellon Foundation, NICHD contract N01-HD-0-2902, 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. (1987) Proc. Natl. Acad. Sci. USA 84, 8282-8286.
2. Ling, N., DePaolo, L.V., Bicsak, T.A., and Shimasaki, S. (1990) *In:* Clinical Obstetrics and Gynecology (E.Y. Adashi, Ed.), pp. 690-702. J.B. Lippincott Co., Hagerstown, MD.
3. 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.
4. Esch, F.S., Shimasaki, S., Mercado, M., Cooksey, K., Ling, N., Ying, S., Ueno, N., and Guillemin, R. (1987) Mol. Endocrinol. 1, 849-855.

5. Shimasaki, S., Koga, M., Esch, F., Mercado, M., Cooksey, K., Koba, A., and Ling, N. (1988) Biochem. Biophys. Res. Commun. 152, 717-723.
6. Shimasaki, S., Koga, M., Buscaglia, M.L., Simmons, D.M., Bicsak, T.A., and Ling, N. (1989) Mol. Endocrinol. 3, 651-659.
7. Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K., and Sugino, H. (1990) Science 247, 836-838.
8. Inouye, S., Guo, Y., DePaolo, L., Shimonaka, M., Ling, N., and Shimasaki, S. (1991) Endocrinology (in press)
9. Schwall, R.H., Nikolics, K., Szonyi, E., Gorman, C., and Mason, A.J. (1988) Mol. Endocrinol. 2, 1237-1242.
10. Inouye, S. and Inouye, M. (1987) *In:* Synthesis of DNA and RNA (S.A. Narang, Ed.), pp. 181-206. Academic Press, New York.
11. Vieira, J. and Messing, J. (1982) Gene 19, 259-268.
12. Wallace, R.B., Johnson, M.J., Hirose, T., Miyake, T., Kawashima, E.H., and Itakura, K. (1981) Nucleic Acids Res. 9, 879-894.
13. Hattori, M. and Sakaki, Y. (1986) Anal. Biochem. 152, 232-238.
14. Urlaub, G. and Chasin, L.A. (1980) Proc. Natl. Acad. Sci. USA 77, 4216-4220.
15. Chen, C. and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745-2752.
16. Kaufman, R.J. and Sharp, P.A. (1982) J. Mol. Biol. 159, 601-621.
17. Laemmli, U.K. (1970) Nature 277, 680-685.
18. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.
19. Hossenlopp, P., Seurin, D., Segovia-Quinson, B., Hardouin, S., and Binoux, M. (1986) Anal. Biochem. 154, 138-143.
20. Greenwood, R.C., Hunter, W.M., and Glover, J.S. (1963) Biochem. J. 89, 114-124.
21. Sugawara, M., DePaolo, L., Nakatani, A., DiMarzo, S.J., and Ling, N. (1990) J. Clin. Endocrinol. Metab. 71, 1672-1674.
22. Ling, N., Ying, S-Y., Ueno, N., Esch, F., Denoroy, L., and Guillemin, R. (1985) Proc. Natl. Acad. Sci. USA 82, 7217-7221.
23. Gavel, Y. and von Heijne, G. (1990) Protein Engineering 3, 433-442.