Follistatin and its role as an activin-binding protein

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Abstract : Follistatin (FS), a specific binding protein for activin, neutralizes the diverse actions of activin by forming an inactive complex with activin. FS is a monomer derived from two polypeptide core sequences of 288 (FS-288) and 315 (FS-315) amino acids originated from alternatively spliced mRNA. We purified six molecular forms of FS from porcine ovaries. Their structural differences were caused by truncation of the COOH-terminal region and/or the presence of carbohydrate chains, resulting in the formation of FS-288, FS-315 and FS composed of 303 amino acids (FS-303) in various forms of glycosylation on the two potential Asn-linked glycosylation sites. All six molecular species have almost the same activin binding activity (Kd=540-680 pM). By contrast, the COOH-terminal truncated form, FS-288, showed much higher affinity for heparan sulfate proteoglycans of the cell surface than FS-303,whereas the intact form of FS, FS-315, had no affinity. Furthermore, FS-288 more effectively blocked the suppression of follicle-stimulating hormone (FSH) secretion from rat pituitary cells by activin. This implies that activin binds to the cell surface through FS-288 which adheres to the cell surface. To clarify the physiological role of cell-associated FS, we then investigated the binding of activin to cell-associated FS and the fate of cell surface-bound activin and FS using primary cultured rat pituitary and ovarian granulosa cells. When the cells were incubated with ^{125}I-activin A in the presence of FS-288 or 315, the binding of activin A to the cell surface was promoted much more markedly by FS-288 than by FS-315. The amounts of radioactivity recovered in trichloroacetic acid-soluble fractions (degraded activin) from the incubation medium were greatly increased by the addition of FS-288. This increase was abolished by heparan sulfate, monensin (an endocytosis inhibitor), chloroquine (a lysosome function inhibitor) and several lysosomal enzyme inhibitors. These results suggest that cell-associated FS-288 accelerates the internalization of activin into the cells, leading to its degradation by lysosomal enzymes, and that cell surface-associated FS therefore plays a role in the clearance system of activin. J. Med. Invest. 44 : 1-14, 1997

Key Words : *follistatin, activin, heparan sulfate*

INTRODUCTION

The interplay of various hormones originating from the hypothalamic-pituitary-gonadal axis regulates the development and maintenance of mammalian reproduction. The central regulators in this system are the pituitary gonadotropins, i.e., leutenizing hormone (LH) and follicle-stimulating hormone (FSH). The synthesis and secretion of both of these hormones are controlled by a combination of hypothalamic gonadotropin-releasing hormone (GnRH) and the gonadal steroids. The gonadal steroids exert feedback actions on the brain to attenuate the release of GnRH and on the anterior pituitary gland to inhibit the secretion of LH and FSH. In recent years, several hypophysiotropic protein factors that can regulate the release of FSH by pituitary cells have been identified in mammalian gonads. These include activin (1, 2), which stimulates FSH release, and inhibin (3-6) and follistatin (FS) (7), which inhibit FSH release. These protein factors have added a new dimension to the complexity of this system.

Although functionally antagonistic, activin and inhibin are structurally related ; inhibin is a heterodimer composed of an α-subunit linked by disulfide bonds to one of the related β-subunits, whereas activin is a dimer composed of the inhibin β-subunits (8-13). Five β-subunits have been reported : βA, βB, βC (14), βD (15) and βE (16), whereas only a single α-subunit has been identified. There is an extensive array of possible dimers ; inhibin A (αbA), inhibin B (αbB) (5, 6), activin A (βAβA) (1), activin

Received for publication June 16, 1997 ; accepted July 31, 1997.

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AB (βAβB) (2) and activin B (βBβB) (17) have been isolated as dimeric proteins. DNA sequence analyses showed that all of the subunits are initially synthesized as large precursor proteins with the mature subunits residing at the COOH-terminal regions of the several clusters of multiple basic residues which can serve as potential proteolytic processing sites (Fig.1). The sizes of the mature, most abundant forms of each subunit are 18 kDa for the α-subunit and 14 kDa for the βA-and βB-subunits. Thus, depending on the combination of the gene products, protein molecules having quite different biological activities could be derived from a limited number of genes.

The finding of a homodimeric form of a β-subunit is noteworthy in view of the fact that the members of the transforming growth factor-β (TGF-β) superfamily are all homodimers. Structural studies have shown that these proteins constitute the TGF-β superfamily with diverse regulatory effects on the growth and differentiation of numerous cell types. These findings prompted us to elucidate the full spectrum of actions of activin and inhibin apart from their effects on FSH secretion. There is increasing evidence from various areas of study that activin has multiple functions in a wide variety of biological systems, e.g., the regulation of the secretion of anterior pituitary hormones such as growth hormone, prolactin and corticotropin (1,18-22), the induction of erythropoiesis (23-26), the modulation of ovarian and testicular cell functions (27-31), the promotion of survival of nerve cells (32,33), the initiation of early embryonic development of *Xenopus* (34-40), the stimulation of insulin secretion (41), and the enhancement of bone formation (42-44). In view of the wide spectrum of bioactivities exerted by activin, it is not surprising that the mRNA encoding β-subunits is found in a multitude of diverse tissues, including the gonads, pituitary, placenta, brain, spleen, heart, bone marrow and others (45). Activin is a growth and differentiation factor that is produced by and exert effects on a broad range of cells and tissues (Fig.2).

In contrast, inhibin is a functional antagonist to activin in some systems. This antagonism was first observed with regard to FSH secretion by pituitary cells, but has also been documented for a number of other responses. There are, however, many activin responses that do not appear to be regulated by inhibin ; inhibin-independent effects have been described.

Despite the various effects of members of the TGF-β superfamily on cell phenotype and physiology, until recently very little was known about the structures of their receptors or the signaling mechanisms. An activin receptor was recently cloned from the mouse pituitary tumor-derived cell line AtT 20 (46). The receptor sequence predicted a transmembrane serine/threonine-specific protein kinase. This result was unexpected, because at that time all known cytokine receptor kinases were tyrosine-specific. With the use of the activin receptor cDNA as a low-stringency hybridization probe or to direct the construction of degenerate oligonucleotides for polymerase chain reaction (PCR), a number of additional receptor serine kinases have been cloned. These are divided into two classes, called type I and type II receptors, both of which contain a serine/threonine kinase domain in their intracellular domains. Activin and other members of the TGF-β superfamily interact with both types of receptors. Activin and TGF-β can bind their individual type II receptor but fail to bind the type I receptor in the absence of the type II receptor. The ligand-bound type II receptor can associate with the type I receptor. This ligand-induced heteromerization between the type I and type II receptors results in the ligand signal transduction (Fig.3). In the current model, the function of the type II receptor is confined to ligand binding, type I receptor recruitment and transphosphorylation, whereas the type I receptor is the signaling unit in the complex (reviewed in Ref.47). Furthermore, intracellular mediators such as the Smad's have been identified and used for the clarification of signaling mechanisms for activin and other

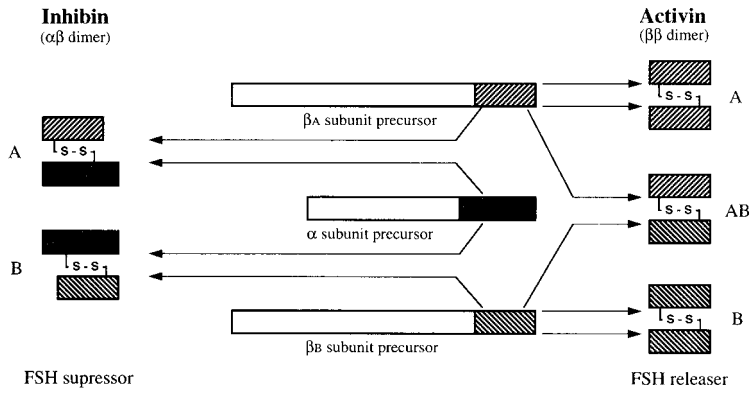

Fig. 1. Structures of inhibin and activin. The multiple arrangement of gene products from the α, βA and βB subunit precursors through disulfide bridging yields FSH releasers (activin) and suppressors (inhibin).

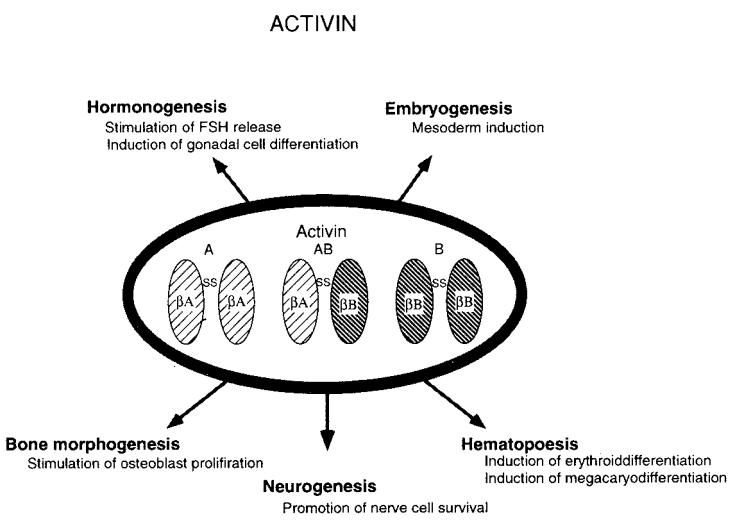

Fig. 2. Multiple functions of activin.

members of the TGF-β superfamily.

Additionally, in 1987, a new class of gonadal protein factor, named FSH-suppressing protein (FSP) or FS, was identified in a side fraction derived from the purification of bovine and porcine ovarian inhibins and activins (7, 48). FS was characterized initially by its ability to suppress pituitary cellular FSH secretion *in vitro*. The action of FS appears to be similar to that of inhibin, but it is structurally quite different. FS is a single-chain protein that occurs in 31-to 39-kDa molecular mass forms, all of which have similar amino acid compositions and identical amino-terminal amino acid sequences. In a recent study, we purified an activin-binding protein which was specific to and had a high affinity for activin, from rat ovary (49) and bovine pituitary (50), and we demonstrated that it was identical to FS. Interest in the biological significance of FS had diminished, because of its weak inhibitory activity compared with that of inhibin. However, our finding that FS is an activin-binding protein sheds new light on the understanding of its physiological role ; FS may participate in the regulation of the multiple actions of activin. Indeed, FS mRNA transcripts have been detected in a wide variety of tissues, which may indicate that FS, like activin, has tissue-specific effects or that FS has a universal action on different cell types. The ability of FS to bind the preiotropic growth and differentiation factor activin and thereby neutralize activin action makes this glycoprotein a potentially important regulatory factor, capable of modulating autocrine and paracrine functions and the processes of differentiation and development. In this review, we describe the biochemical properties of FS and the biological implications of FS-activin interaction in a local regulatory system.

MOLECULAR HETEROGENEITY OF FOLLISTATIN

FS was originally detected as a mixture containing several bioactive polypeptides in bovine and porcine follicular fluids. Thereafter, the FS gene product processing was investigated and it was revealed that alternative splicing generated two types of FS precursor (51, 52). One is a pre-FS and the other its COOH-terminal 27-amino acid truncated homologue. The corresponding mature FS isoforms comprise 315 (FS-315) and 288 (FS-288) amino acids, respectively.

We have established a conventional procedure for purifying FS proteins from mammalian and amphibian sources. To date, we have purified FS from rat ovary (49), porcine (17) and human (53) follicular fluids, bovine pituitary (50) and *Xenopus* XTC cell-culture medium (54). Every preparation demonstrated multiple bands at a 32-38 kDa molecular mass range when subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. After reduction, all these bands migrated characteristically at a higher molecular mass range (40-45 kDa). In order to determine the exact chemical and biological properties of each FS isoform, we isolated each one from porcine follicular fluid and found that there are at least six (and probably more) FS molecular forms, which resulted from truncation of the COOH-terminal region and/or the presence of N-linked carbohydrate chains (Fig.4) (55). The six FS isoforms can be divided into three groups, according to the extent of truncation ; i.e., full-length core proteins with 315 amino acids (FS-315), those with 303 amino acids (FS-303), and a COOH-terminal truncated protein with 288 amino acids (FS-288). Characteristically, both FS-315 and FS-303, but not FS-288, contain a highly acidic carboxyl-terminal extension. The FS-303 molecular form is a major component of FS from mammalian sources. Interestingly, unlike mammalian FSs, the core protein of *Xenopus* FS may be of a single type (54), and its heterogeneity may be attributable to the number of carbohydrate chain moieties, since the N-glycanase treatment of the *Xenopus* preparation resulted in convergence of the multiple bands on SDS-PAGE. It has yet to be established as to which isoform group the *Xenopus* FS produced by XTC cells belongs. All six of the molecular species of FS we have isolated demonstrated almost identical activin-binding activity (Kd=540-680 pM). The presence of various forms of FS may

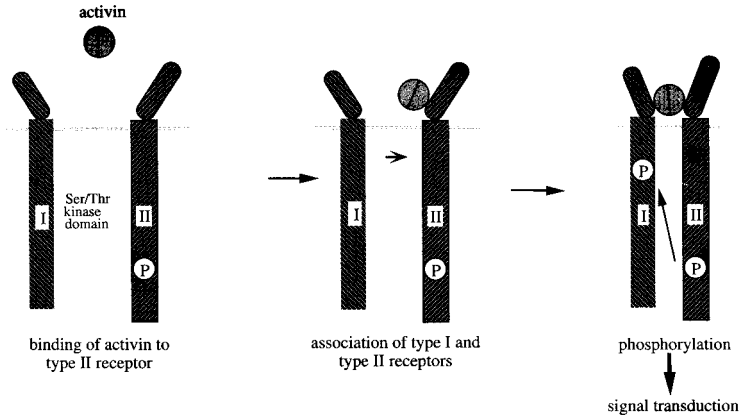

Fig. 3. A general model for the initiation of signaling by the activin receptor. Receptor II is the primary activin receptor and is a constitutively active serine/threonine kinase that recruits receptor I by means of bound activin. The subsequent phosphorylation of receptor I by receptor II allows the receptor I kinase to propagate the signal to downstream substrates.

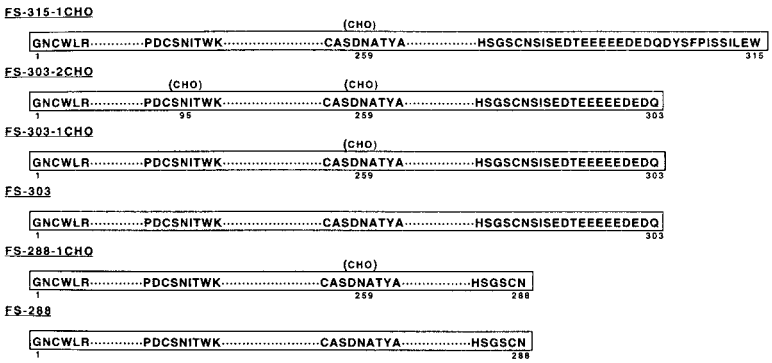

Fig. 4. Structures of six isoforms of follistatin (FS) from porcine follicular fluid. CHO : N-linked carbohydrate chain.

be physiologically significant in the regulation of the many actions of activin.

TISSUE LOCALIZATION OF FOLLISTAIN

We studied the distribution of FS in rat tissues using immunohistochemistry (56) with a polyclonal antibody directed against an FS peptide (residues 123-134) as a specific immunological probe. In addition to the ovary and testis, the antiserum bound to almost all of the tissues tested, i.e., the pituitary, oviduct, uterus, kidney, adrenal gland, liver, spleen and brain cortex. In the testis, a unique immunostaining pattern was obtained with intense and specific FS immunoreactivity evident in spermatogenic cells. Interestingly, the predominant staining was in the spermatocytes and spermatid nuclei, but no immune reaction was observed in spermatogonia or spermatozoa. To our knowledge, no information has been obtained regarding how FS gets into the cell nucleus. The pituitary gland also demonstrated a characteristic staining profile. The stained spots were scattered sporadically over the anterior lobe, indicating that a specific group of anterior pituitary cells, but not all, synthesize FS. The results of our recent double immunostaining studies suggested that the cells stained with FS antibodies may be somatotrophs (57). This staining profile coincided with the distribution profile of FS mRNA determined using Northern and S 1-nuclease analyses (58). Our observations clearly indicate that FS protein is ubiquitous in tissues. A similar broad tissue localization has also been demonstrated for inhibin β-subunit mRNA expression. Taken together, these findings lead us to suggest that FS, in view of its activin-binding activity, can regulate various activin actions in many tissues.

MODULATION OF ACTIVIN ACTIONS BY FOLLISTAIN

The widespread tissue distributions of activin and FS imply, but do not demonstrate conclusively, that both factors are synthesized locally and that FS regulates the physiological actions of activin in a paracrine or autocrine manner. Several lines of indirect evidence suggest that the biological activities of activin are neutralized by its binding to FS. In rat granulosa cell cultures, FS (50 ng/ml) negated the stimulatory effects of activin (30 ng/ml), including the expression of FSH and LH receptors and inhibin and progesterone production (Fig.5) (59). A neutralizing effect of FS on activin activity was demonstrated in the mouse Friend cell (erythroleukemia cell) bioassay. When cellular differentiation was induced by 80 ng/ml activin, an inhibitory neutralizing effect of FS on erythropoiesis *in vivo* was observed. The continuous intraperitoneal administration of FS to normal mice resulted in a decrease of the erythroid progenitors BFU-E and CFU-E in the bone marrow and spleen (60). This supports the hypothesis that endogenous activin participates in murine erythropoiesis, a process in which FS exerts a regulatory function. In amphibian systems, activin appears to be responsible for mesodermal induction, which was also found to be inhibited by FS in a dose-dependent manner in the *Xenopus* animal cap assay.

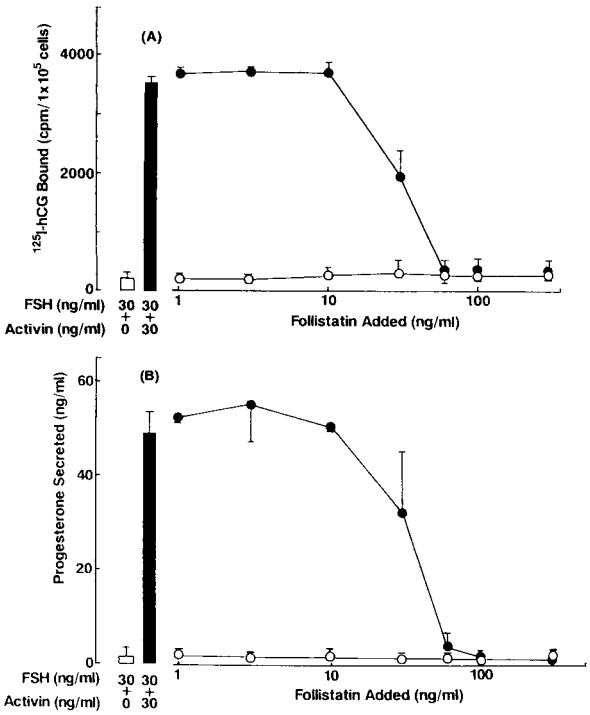

Fig. 5. Effect of FS on activin-stimulated human chorionic gonadotropin (hCG)/LH receptor expression (A) and progesterone production (B) by rat granulosa cells. Granulosa cells (1 x 10^5 cells/well) obtained from diethylstilbestrol-treated rats were cultured for 72 h in the presence of FSH (30 ng/ml) without (○) or with (●) activin (30 ng/ml).

The coincubation of a constant amount of activin with increasing concentrations of FS prevented mesodermal tissue formation : a roughly 3-fold excess of FS (on a weight basis) was needed to abolish the effect of activin (61). This also implies that there is a physiological interaction between activin and FS.

COMPLEX FORMATION BETWEEN ACTIVIN AND FOLLISTAIN

The ability of FS to bioneutralize the activities of activin in various assay systems suggests that there is a stoichiometric relationship between FS and activin. We therefore investigated whether activin binds to FS to form a biologically inactive complex *in vivo* (17). Porcine follicular fluid was collected on ice by aspiration from ovaries of various sizes and subjected directly to gel filtration. The immunoblotting analysis of the resulting fractions indicated that more than enough FS exists in follicular fluid to generate an activin-FS complex. The absence of free activin was confirmed by the finding that no FSH-release stimulating activity was detected in any fraction. Next, the activin-FS complex was purified by successive steps of affinity chromatography on dextran sulfate-Sepharose and fast protein liquid chromatography (FPLC) gel permeation on a Superose 12 HR column. This procedure exploits the high affinity of FS and its complex for sulfated polysaccharides. The complex thus obtained was subjected to reverse-phase high-performance liquid chromatography (HPLC), which caused it to dissociate and yield its components, activin and FS. Interestingly, three isoforms of activins, A, AB and B, were found to be

present as complexes with FS in the follicular fluid. It is also noteworthy that the biological activity of activin B was significantly lower than those of activins A and AB in various assay systems, such as the stimulation of FSH secretion, the induction of erythrodifferentiation and the potentiation of gonadotropin receptor expression on ovarian cells, whereas activin B was a potent inducer of *Xenopus* mesoderm. Activin B may therefore play a specific role in early embryonic development. These results indicate that mammalian ovarian cells, probably granulosa cells, synthesize and secrete three activin isoforms and FS, which easily generate an inert complex in follicular fluid. Accordingly, to establish the precise physiological significance of the interplay between FS and activin in the ovary, it is important to identify the changes that the components of the complex undergo in relation to the development of the ovary or during the reproductive cycle.

ACTIVIN BINDING CAPACITY OF FOLLISTAIN

Although the relationship between activin and FS has not been characterized fully, there appears to be a stoichiometric interaction between them. We examined their interaction using gel permeation FPLC with two Superose 12 HR columns connected in tandem. The amounts of the proteins produced were calculated from the amino acid composition analysis results. When activin and FS were premixed in a 1 : 2 molar ratio and subjected to FPLC, a single activin-FS complex peak was observed. Neither free activin nor free FS peaks were detected. Even when increasing amounts of FS were added, the complex peak remained unchanged and a free FS peak emerged (Fig.6), which indicates that one mole of activin bound to two moles of FS to form an inactive large molecular weight complex, and that activin therefore has two binding sites for FS. A 1 : 1 molar mixture of activin and FS showed significant FSH-release stimulatory activity (about 40% of the initial activin activity remained), whereas no such activity was detected in a 1 : 2 molar mixture (containing a 2-fold molar excess of FS). This stoichiometric relationship between FS and activin closely reflects the antagonistic effect of FS on the activin actions described above. In studies of the inhibitory effect of FS on activin action, at least two moles of FS were needed to neutralize the bioactivity of one mole of activin. This raised the question of whether inhibin can also bind to FS, because the inhibin molecule contains a β-subunit derived from activin. Therefore, we used the gel permeation FPLC procedure to determine the capacity of inhibin binding to FS. When a 1 : 1 molar mixture of inhibin and FS was gel-filtered, the elution peak shifted forward and the formation of a large molecular complex was observed. A 2-fold molar excess of FS to inhibin caused no change in the height of the complex peak, which indicates that inhibin bound to FS to form a 1 : 1 molar complex. These results demonstrate that activin has two binding sites (whereas inhibin has only one) for FS, and imply that FS binds to both through the common β-subunit.

This explanation of the activin and inhibin FS-binding capacities is consistent with the results obtained by Shimonaka *et al.* (62). However, these studies give no information about the affinity of inhibition for FS compared with that for activin. Therefore, we tested the abilities of inhibin and activin to displace ^{125}I-activin bound to FS ; activin competed for binding with an ED_{50} of 20 ng/ml, whereas inhibin was at least 1,000-fold less potent in this respect (ED_{50} of 24 μg/ml). This association of inhibin with FS may not lead to the neutralization of the activity of either inhibin or FS. Further studies are needed to elucidate the physiological implications of the interaction between inhibin and FS.

Another binding protein for inhibin and activin, α2-macroglobulin (α2M), found in human serum and follicular fluids was recently identified (63, 64). To test its ability to bind activin in human serum, SDS-PAGE under non-reducing conditions of human serum was performed using 3.6% gels, and the proteins were transferred to Immunobilon membranes, which were incubated with ^{125}I-activin and subjected to autoradiography. The major α2 (α2M) M band was observed at 370 kDa, which indicated that α2M may be the primary activin-binding protein in serum. However, the affinity of activin for α2M is much lower than that for FS, since typical stoichiometric binding

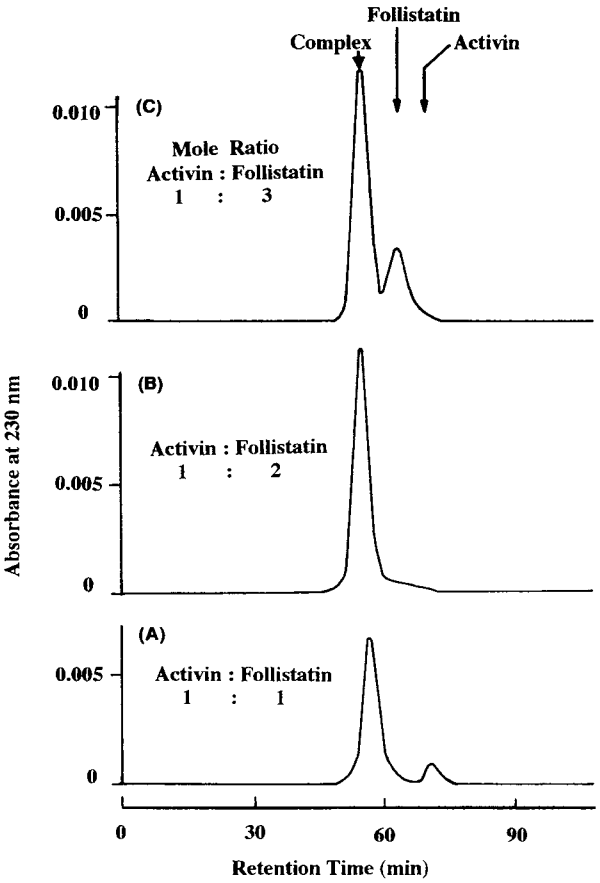

Fig. 6. Complex formation between activin and FS. Activin, 5 μg, plus FS,6.6 μg (molar ratio : 1 : 1) (A), activin, 5 μg, plus FS 13.2 μg (molar ratio : 1 : 2) (B) and activin, 5 μg, plus FS, 19.8 μg (molar ratio : 1 : 3) (C) were separately incubated in 20 mM Tris/HCl (pH 7.3) containing 0.1% CHAPS and 1 M NaCl, and each solution was applied to tandemly connected FPLC Superose 12 HR columns.

was not observed in the gel filtration experiments. In addition, several other bands, including some at around 500 kDa and 180 kDa, were also detected on the blots. Although these serum proteins that are capable of binding activin and probably inhibin have not been identified, they may serve as binding proteins for activin, inhibin and possibly other factors as well as α2M to protect these molecules from proteolytic degradation and/or affect their clearance from the bloodstream.

AFFINITY OF FOLLISTAIN FOR HEPARAN SULFATE OF PROTEOGLYCANS

In the course of an FS purification study, we noticed that it possessed a unique and strong affinity for sulfated polysaccharides, such as dextran sulfate, heparin and sulfated cellulose. It was recently proposed that the binding of basic fibroblast growth factor (FGF) to its receptor requires prior binding to the heparan sulfate side chains of proteoglycans or heparin(65). We therefore attempted to ascertain the physiological significance of FS-sulfated polysaccharide interactions in regulating activin signal transduction. Rat granulosa cells cultured in serum-free medium were incubated with various concentrations of FS, and the amounts of cell-bound FS were determined by adding ^{125}I-activin and counting the specifically bound radioactivity ; this yielded a typical ligand saturation curve with an apparent Kd of 5 nM. Heparin and heparan sulfate, but not chondroitin, keratan or dermatan sulfate competed strongly for this binding. When granulosa cells were treated with various glycosaminoglycan-degrading enzymes before or after adding FS to the cultures, heparinase and heparitinase treatment resulted in significant suppression of the binding. These results show that FS has a high affinity for the heparan sulfate side chains of granulosa cell surface proteoglycans (66).

As discussed above, our FS preparation contains at least six isoforms, which result from truncation at the COOH-terminal region and/or the presence of carbohydrate chains. Interestingly, the affinity for the heparan sulfate chains was found to differ quite markedly depending on the sequence of core proteins(55). The COOH-terminal truncated form (FS-288) showed a very high affinity for the granulosa cells, with a Kd of 2 nM. In contrast, the full-length form (FS-315) showed no affinity, whereas the midsized isoforms (FS-303) showed moderate affinity (Fig.7). The carbohydrate chains did not appear to affect their affinity for the cell surface. The association of the short and midsized FS isoforms with the cell was abolished by adding heparan sulfate or heparin. High concentrations of the sulfated polysaccharides (>10 μg/ml) had no effect on the interaction between activin and FS. To obtain direct evidence of FS binding to heparan sulfate, we carried out heparan sulfate-Sepharose affinity chromatography. Almost all of the short isoform (FS-288) applied bound to a heparan sulfate-Sepharose column, whereas the full-length isoform (FS-315) was recovered quantitatively in the breakthrough fraction, indicating that it had no affinity for this column. These results clearly indicate that the amino acid sequence of the COOH-terminal is important for FS binding to the heparan sulfate chains of cell surface proteoglycans.

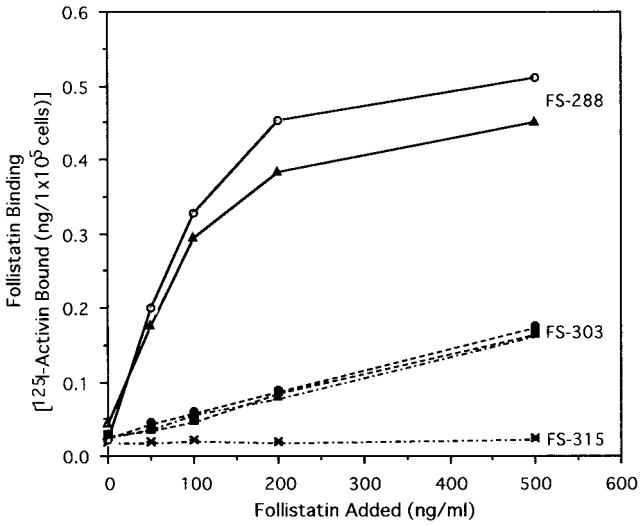

Fig. 7. The cell-adhesiveness of FS isoforms to granulosa cells. ○ : FS-288, △ : FS-288-1 CHO, ● : FS-303, ▲ : FS-303-1 CHO, ■ : FS-303-2 CHO, x : FS-315-1 CHO

The sequence dependence of FS isoforms on its cellular association was also confirmed by determining the transient expression of FS cDNA in COS cells (55). Cells transfected with the FS-288 cDNA secreted FS-288, which bound to the COS cells themselves, whereas cells transfected with the FS-315 construct produced the FS-315 protein, which did not bind to the cells. This result shows that the short form of FS (FS-288) expressed and secreted by COS cells can bind to the cell surface with a much higher affinity than that of the long form (FS-315).

The biological response first associated with FS was inhibition of activin-induced FSH secretion from cultured rat pituitary cells. To determine whether this inhibitory activity is affected differentially by the various FS isoforms, the potencies of the short, midsized and long FS forms were compared in this system *in vitro*. The COOH-terminal truncated FS (FS-288) was more potent than FS-303 and FS-315. There was a 5-fold difference between the inhibitory effects of FS-288 (ED_{50}=2 ng/ml) and FS-303 (ED_{50}=10 ng/ml), and a 2-fold difference between those of FS-303 and FS-315 (ED_{50}=20 ng/ml), results which are consistent with the differences in the binding affinities of these forms to cell surfaces (55). Moreover, a recent study demonstrated that the *in vivo* FSH suppressant activity of recombinant FS-288 in ovariectomized rats was greater and longer-lasting than that of inhibin A, which suggests that the COOH-terminal region of FS is important for inhibiting the FSH-releasing activity of activin and probably for exerting other bioneutralizing effects on the various actions of activin (67). Proteoglycans similar to those on ovarian granulosa cells may also be present in the pituitary, and various FS isoforms found locally therein may be bound by them.

INTERFERENCE BY FOLLISTATIN WITH ACTIVIN BINDING TO ACTIVIN RECEPTORS

Activin receptor mRNAs have been reported to be

distributed in the rat pituitary (68), but the receptor protein in the pituitary has yet to be identified. We attempted to analyze the pituitary activin receptor by affinity cross-linking ^{125}I-activin A to rat pituitary cells using the bifunctional chemical cross-linker DSS (69). Very faint, but definite, cross-linked bands of 80 and 100 kDa were observed (Fig.8), which corresponded well with those of types I and II activin receptors coexpressed transiently in COS-7 cells (Fig.8). The binding of labeled activin to both types I and II activin receptors was abolished in the presence of excess FS-288 or FS-315 (Fig.8). These findings indicate that the activin-FS complex cannot bind to activin receptors and would account for the inhibitory effect of FS on the activin-induced stimulation of FSH secretion by pituitary cells. De Winter *et al*. recently demonstrated that the preincubation of radioiodinated activin A with FS abolished the activin binding to type II activin receptors and consequently, its binding to type I activin receptors, and they proposed that FS can neutralize activin bioactivity by interfering with activin binding to type II activin receptors (70). Our affinity cross-linking experiments also showed inhibition by FS of activin binding to its receptors on rat pituitary cells. This result can be explained by the hypothesis that FS masks the as yet unidentified receptor binding domain of the activin molecule, resulting in the failure of activin to transduce its signal in responsive cells.

ASSOCIATION OF FOLLISTAIN WITH RAT PITUITARY CELL SURFACES

Although the formation of activin-receptor complexes was prevented by adding FS, a broad band with a molecular mass ranging from 45 to 65 kDa was visible after treatment with either FS-288 or FS-315 (Fig.8). This band with a low molecular mass was not related to activin receptors, because it was also yielded by COS-7 cells that were not transfected with activin receptor DNAs (Fig.8) and the labeling of this band was completely inhibited by incubation with heparan sulfate (Fig.8) and with excess unlabeled activin, indicating that labeled activin stays on the cell surfaces via FS bound to the heparan sulfate side chains of proteoglycans. It should be noted that FS-288 yielded a much more intense band than that yielded by FS-315 (Fig.8), which was consistent with our above-mentioned finding that FS-288 showed much higher affinity than FS-315 for heparan sulfate proteoglycans on rat ovarian granulosa cells (55). To test this finding applied to pituitary cells, rat pituitary cells were incubated with various concentrations of radioiodinated FS. As expected, FS-288 showed a quite high affinity for the pituitary cells, whereas the affinity of FS-315 was low. The association of FS with the cells was completely suppressed by excess heparan sulfate or heparin, indicating that FS-288 binds to heparan sulfate on pituitary cell surfaces.

It is well documented that the interaction between FGF and heparin-like molecules in the extracellular matrix is important for various biological functions, such as the protection of this factor against proteolytic degradation and its concentration on cell surfaces (65). The role of heparin-like molecules in signal transduction of FGF is noteworthy : the binding of basic FGF (bFGF) to its receptor requires prior binding either to the heparan sulfate side chains of cell-surface glycosaminoglycan or to free heparin to present the ligand to the receptor. De Winter *et al*. attempted to determine whether cell surface-bound FS-288 presents activin A to the activin receptors on human erythroleukemic K 562 cells, and they found that FS-288 and the activin A-type IIA activin receptor complex were not coprecipitated by an anti-type IIA activin receptor antibody (70), suggesting that, unlike bFGF, cell surface-associated FS cannot present ligands to signaling receptors. Judging from these results, FS appears to be nothing more than a negative regulator for activin, and its function is to form an inactive complex with activin to neutralize the activity of activin.

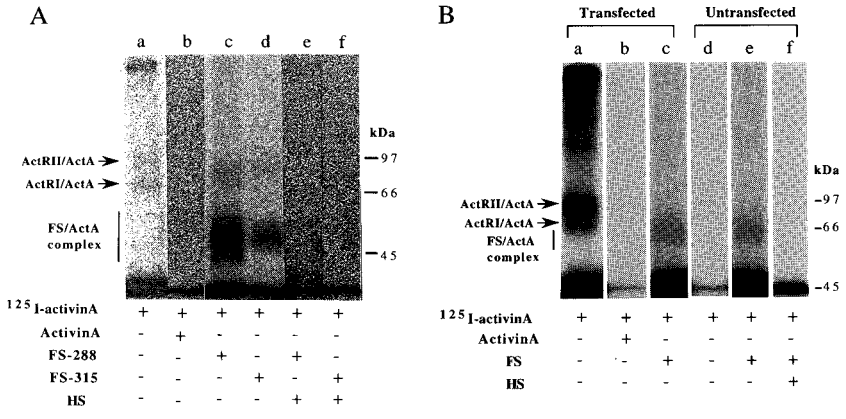

Fig. 8. Inhibitory effects of FS on ^{125}I-activin A binding to activin receptors on pituitary cells and COS-7 cells transfected with activin receptor cDNAs. A, rat pituitary cells (5×10^5cells) cultured in 24-well plates were incubated with ^{125}I-activin A (40 ng/ml) in the absence (a) or presence (b) of unlabeled activin A (400 ng/ml), and in the presence of FS-288 (200 ng/ml) (c) FS-315 (200 ng/ml) (d), FS-288 (200 ng/ml) and heparan sulfate (10 μg/ml) (e), or FS-315 (200 ng/ml) and heparan sulfate (10 μg/ml) (f), cross-linked with DSS, and analyzed by SDS-PAGE (8% gels). B, COS-7 cells cultured in 6-well plates (2×10^5 cells) and transfected with type IA and type IIA activin receptor cDNAs were incubated with ^{125}I-activin A (40 ng/ml) A in the absence (a) or presence (b) of activin A (400 ng/ml), or porcine FS (200 ng/ml) (c), cross-linked with DSS, and analyzed by SDS-PAGE (7.5% gels). Untransfected COS-7 cells were also analyzed under the same conditions in the absence (d) or presence (e) of porcine FS (200 ng/ml) or porcine FS (200 ng/ml) and heparan sulfate (10 μg/ml) (f).

FATE OF ACTIVIN ASSOCIATED WITH FOLLISTATIN ON CELL SURFACES

To investigate the behavior of cell surface-associated activin via FS, we examined the binding of activin to FS associated with pituitary cell surfaces (69). In the presence of various concentrations of FS-288 or FS-315, rat pituitary cells were incubated with increasing amounts of ^{125}I-activin A (0-100 ng/ml), and the cell-bound radioactivities (activin-FS complex) were then determined (Fig.9). The binding activity of activin A alone was difficult to detect, probably due

to the very small number of activin receptors on pituitary cells. However, as expected, FS-288 markedly increased the affinity of activin A for cell surfaces, in a dose-dependent manner, whereas FS-315 did not enhance the activin A binding to cell surfaces. These results suggest that activin A can adhere strongly to cells by forming a complex with FS-288 on the cell surfaces. Next, we followed the behavior of the cell-associated activin-FS complex and found that it was degraded endocytotically. Rat pituitary cells were incubated with radioiodinated activin A (40 ng/ml) in the presence of increasing concentrations of FS-288 or FS-315 for various incubation periods, after which the radioactivities recovered from the trichloroacetic acid (TCA)-soluble fractions (degraded activin) of the incubation media were determined using a γ-spectrometer. As shown in Fig.10, FS-288 stimulated the activin A degradation significantly time- and dose-dependently to a greater extent than did FS-315. This stimulatory effect of FS-288 was abolished by adding heparin or heparan sulfate to the culture medium. This result reflects the cell-surface adhesiveness of the complex : the more strongly the activin A-FS-288 complex binds to cell-surface heparan sulfate, the more easily it is degraded. Moreover, this degradation was dependent on the number of pituitary cells : increasing their number stimulated the degradation of activin A bound to the cell surfaces via FS. These degradation data were obtained by monitoring the degradation of ^{125}I-activin. SDS-PAGE and gel filtration of the complex samples demonstrated that the FS component of the activin-FS complex was also degraded.

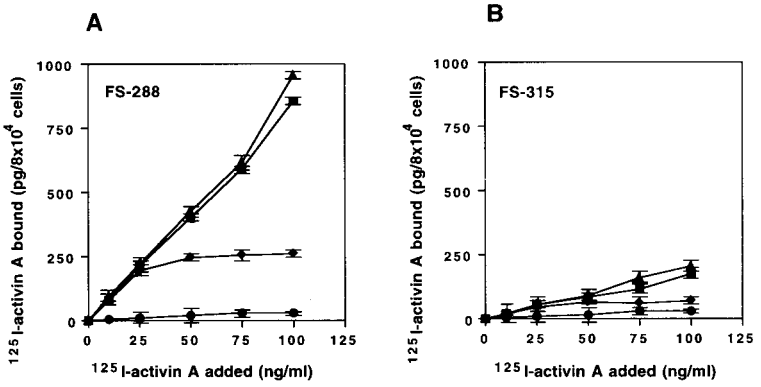

Fig. 9. Effect of FS on the binding of ^{125}I-activin A to pituitary cells. Rat pituitary cells cultured in 96-well plates (8 x 10^4 cells/well) were incubated with increasing concentrations of ^{125}I-activin A (0-100 ng/ml) in the presence of various concentrations of FS-288 (A) or FS-315 (B) at 37℃. After a 2 h incubation, specific binding was determined. The concentrations of FS added were 0(●), 25(◆), 100(■), and 400 (▲) ng/ml. The results are expressed as means ±S.D. from triplicate experiments.

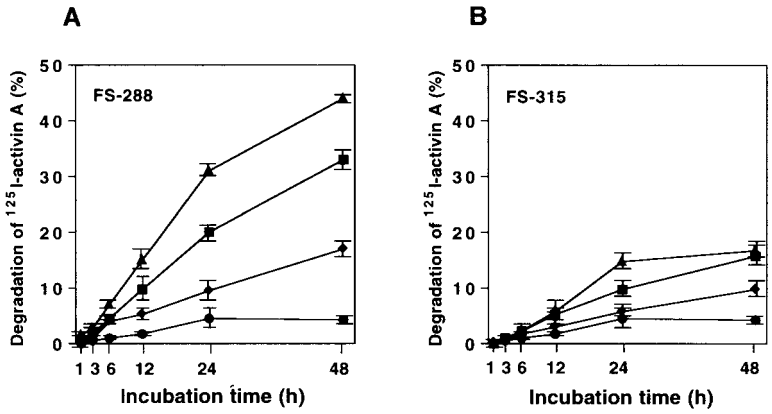

Fig. 10. Effect of FS on endocytotic degradation of ^{125}I-activin A by pituitary cells. Rat pituitary cells cultured in 96-well plates (8 x 10^4 cells/well) were incubated with 40 ng/ml ^{125}I-activin A in the presence of various concentrations of FS-288(A) or FS-315 (B) at 37℃. The medium was collected at the indicated time points and subjected to trichloroacetic acid treatment. The radioactivity of the trichloroacetic acid-soluble fraction (degraded ^{125}I-activin A) was determined in a γ-counter. The concentrations of FS added were 0 (●), 40 (◆), 120 (■), and 400 (▲) ng/ml. The results are expressed as means±S.D. from triplicate experiments.

ENDOCYTOTIC DEGRADATION OF ACTIVIN A IN LYSOSOMES

The degradation of cell-bound activin and/or FS by pituitary cells led us to hypothesize that endocytotic internalization occurs in the cells and the resulting endocytotic vesicles ultimately fuse with lysosomes, after which most of the vesicle contents are rapidly broken down. To explore this idea, we examined the effects of various types of lysosomal enzyme inhibitor on activin A degradation in rat pituitary cells (69). The results are summarized in Table 1. The lysosomal enzyme inhibitors reduced the degradation significantly, but the serine protease inhibitor aprotinin had no effect. The addition of chloroquine, which increases the pH inside lysosomes, to intact cultured cells inhibited the activin A breakdown markedly. Both heparin and heparan sulfate significantly suppressed the activin degradation, strongly suggesting that the degradation does not occur until FS binds to the pituitary cells. The almost complete inhibition of the degradation by monensin indicated that the endocytotic process is essential for the degradative process.

Autoradiographic experiments using radioiodinated activin A were also performed (Fig.11). Pituitary cells were incubated with ^{125}I-activin A at 37℃ in the presence or absence of FS, heparan sulfate and chloroquine for 12 h, and the cells were washed with acid/salt buffer to strip ^{125}I-activin A from their surfaces and then autoradiographed. FS-288 accelerated the uptake of activin A by pituitary cells markedly, and to a greater extent than did FS-315. Heparan sulfate significantly suppressed the uptake, a result which agreed well with the degradation data described above. The coincubation with chloroquine inhibited the degradation of activin A taken up by the cells, probably in the lysosomes, resulting in activin A accumulation within the cells. Microscopic

observations supported our hypothesis that activin A bound to pituitary cell surfaces via FS-288 becomes internalized and packaged into endocytic vesicles, which fuse with lysosomes, followed by proteolytic degradation of their contents.

Table 1. Effects of various lysosomal enzyme inhibitors on FS-288 -induced endocytotic degradation of activin A by rat pituitary cells

Inhibitors (concentration)	Degradation
	%
None	100±7.5
Lysosomal protease inhibitors	
E-64 (1 mM)	64±6.8
Leupeptin (1 mM)	58±4.7
1,10-Phenanthroline (0,05 mM)	68±5.1
Pepstatin A (1 mM)	89±5.8
Serine protease inhibitor	
Aprotinin (200 μg/ml)	103±7.2
Lysosome function inhibitor	
Chloroquine (0.1 mM)	14±4.8
FS binding inhibitor	
Heparin (10 μg/ml)	26±3.8
Heparan sulfate (10 μg/ml)	40±4.3

Rat pituitary cells (8×10^4 cells/well) cultured in 96-well plates were incubated with ^{125}I-activin A (40 ng/ml) and FS (200 ng/ml) in the presence of various inhibitors for 24 h at 37℃, after which the media were collected and ^{125}I-activin A degradation was measured. The activin degradation in the absence of inhibitors was designated as 100%. The results are expressed as means ±S.D. from triplicate experiments.

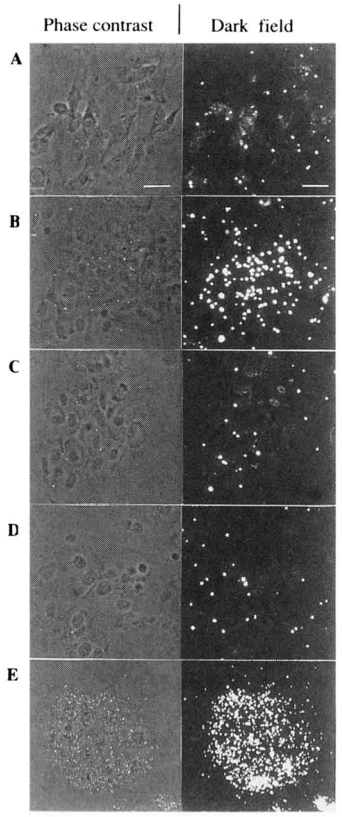

Fig. 11. Uptake of ^{125}I-activin A by rat pituitary cells. Rat pituitary cells cultured in chamber slides were incubated with ^{125}I-activin A (40 ng/ml) in the absence (A) or presence (B) of FS-288 (200 ng/ml), FS-315 (200 ng/ml) (C), FS-288 (200 ng/ml) and heparan sulfate (10 μg/ml) (D) or FS-288 (200 ng/ml) and chloroquine (0.1 mM) (E) for 12 h at 37℃. After extensive washing, the cells were fixed and subjected to autoradiography. Scale=50 μm.

The number of growth factors and cytokines found to bind to heparin and heparan sulfate is steadily increasing ; these include FGF, granulocyte-macrophage colony stimulating factor, interleukin-3, pleiotrophin, hepatocyte growth factor, vascular endothelial growth factor and midkine, among others. On the basis of our results described above, we speculated that, like the activin A-FS-288 complex, these growth factors bind to cell-surface heparan sulfate proteoglycans, then become internalized and eventually are degraded in lysosomes. Indeed, we found that ^{125}I-bFGF bound to rat cultured pituitary cells in a dose-dependent manner and that its intracellular degradation was time-dependent. It is therefore conceivable that such endocytotic degradation of growth factors is a common mechanism for eliminating signaling molecules from cell surfaces.

SUPPRESSION OF FSH SECRETION-INHIBITING ACTIVITY OF FOLLISTAIN BY HEPARAN SULFATE

FS was originally identified as an inhibitor of FSH secretion by cultured pituitary cells, but its potency was only 10-30% of that of inhibin (7,48). The mechanism by which FS acts is unclear, but it has been suggested that it binds to endogenous activin and neutralizes activin-stimulated FSH secretion. In an attempt to elucidate how the interaction between FS and proteoglycans participates in the FSH-suppressing action of FS, we examined the effect of heparan sulfate on the inhibitory activity of FS on rat pituitary cells(69). Dose-response curves for the inhibition of basal FSH secretion into the culture medium by FS-288 and FS-315 in the presence and absence of heparan sulfate (10 μg/ml) were plotted, and revealed that FS-288(ED_{50}=5 ng/ml) was about 7 times more potent than FS-315(ED_{50}=35 ng/ml), and that heparan sulfate reduced the inhibitory activity of FS-288 by about 50%, whereas it had no effect on that of FS-315. These results suggest that cell-associated FS-288 plays a more positive role than FS-315 in controlling the action(s) of activin at cell surfaces, and show the importance of the cell-surface adhesiveness of FS to its role in controlling activin activity.

DEGRADATION OF ACTIVIN IN RAT CULTURED GRANULOSA CELLS

We reported that activin stimulated the FSH-induced differentiation of rat granulosa cells and enhanced the induction by FSH of LH receptor expression in and progesterone production by rat cultured granulosa cells (27). As previously observed with cultured pituitary cells, the presence of FS-288 markedly increased the affinity of activin for heparan sulfate on granulosa cell surfaces, whereas FS-315 did not enhance the activin binding to the surfaces of these cells.

We demonstrated that rat cultured granulosa cells produced FS protein and secreted it into the medium and that FSH, but not LH, stimulated these events (71). Rat granulosa cells were incubated with ^{125}I-activin A (40 ng/ml) in the absence or presence of FSH (100 ng/ml) or/ and heparin (10 mg/ml), and FSH stimulated activin A

degradation by about 2-fold compared with that in the absence of FSH. This stimulatory effect was abolished by the addition of heparin. Indeed, ligand blotting of the culture medium demonstrated that both FS-315 and FS-288 were produced and secreted into the medium in the presence of FSH. Moreover, both FS-315 and FS-288 mRNA expressions were confirmed by reverse transcription (RT)-PCR. These results indicate that, in response to FSH, granulosa cells produce and secrete FS-288, which itself adheres to the cells, and that the cell-associated FS-288 can capture activin, leading to its endocytotic degradation.

ROLE OF FOLLISTATIN IN NEURAL INDUCTION IN XENOPUS EMBRYOS

Hemmati-Brivanlou *et al.* recently constructed a truncated type II activin receptor and demonstrated that the inhibition of the signal transduced by this receptor led to neuralization in developing *Xenopus* embryos (72). They proposed that FS RNA neuralized *Xenopus* ectodermal explants directly in the absence of detectable mesoderm (73). It has been argued that heparan sulfate proteoglycans are involved in mesoderm development and neural induction in *Xenopus* embryos. Furuya *et al*. found that heparan sulfate proteoglycans were expressed exclusively in the animal hemisphere at the early gastrula stage of *Xenopus* embryos and appeared predominantly on the sheath of the neural tube, the notochord and epithelium (74). In light of these recent findings, we speculated that FS bound to heparan sulfate proteoglycans removes activin signals from cell surfaces, as observed with rat pituitary and ovarian granulosa cells, and we therefore examined the affinity of FS for *Xenopus* heparan sulfate as follows. *Xenopus* heparan sulfate was purified from *Xenopus* embryos at the tadpole stage, labeled with tritium acetate, and incubated with the required FS. The resulting incubation mixture was filtered through nitrocellulose membranes, which trapped heparan sulfate bound to FS. FS-288 was found to bind to *Xenopus* heparan sulfate in a dose-dependent manner, whereas FS-315 showed only slight affinity for it. When a PCR was carried with a pair of suitable primers to amplify the *Xenopus* short form of FS, the short-form FS band did not appear until the gastrula stage. Since there is no information about the entire cDNA sequence of *Xenopus* long-form FS, its expression could not be detected. Taken together, our preliminary results strongly suggest that the short form of *Xenopus* FS binds to embryonic cell surfaces, and eliminates the activin signal from the activin target cells, thereby making them ready for neural induction.

Two organizer factors, chordin and noggin, were recently found to be bone morphogenetic factor (BMP)-binding proteins (75, 76). These two factors bind to BMP with high affinity and abolish its activity by blocking binding to all cognate surface receptors. Interestingly, both chordin and noggin were observed to have affinity for heparin and heparan sulfate, like FS. Therefore, BMP may bind to its target cells via cell surface-bound chordin or noggin, followed by endocytotic degradation, whereby BMP signaling may be removed from its target cell surfaces.

CONCLUSION

FS binds stoichiometrically to activin to form an inactive complex, which results in the blockade of various activin bioactivities. However, the physiological significance of this complex formation is not fully understood. In addition, little is known about the importance of the cell-surface adhesiveness of FS regarding its role in controlling activin bioavailability.

Heparan sulfate reduced the FSH-suppressing activity of FS-288 more effectively than it did that of FS-315, lending support to the hypothesis that FS isoforms have different affinities for cell surfaces as well as differing roles in the local modulation of activin function. Significant binding of radioiodinated activin A to pituitary cell surfaces was observed only in the presence of FS. As expected, FS-288 promoted this binding markedly and to a greater extent than FS-315. When incubated with pituitary cells in the presence of FS-288, activin A in the medium appeared to be trapped by cell-associated FS-288. Therefore, after being captured on the cell surface, activin A may, together with FS-288 and proteoglycans, be ingested by endocytotic vesicles, which ultimately fuse with primary lysosomes and are degraded. Most of the vesicle contents were found to be hydrolyzed into small breakdown products and secreted to the exterior. There is little doubt that activin A is broken down by such an endocytotic degradation process, because various types of inhibitor of each stage of this process significantly blocked the activin A degradation : the inhibitors tested included monensin, a proton ionophore and an endosome-lysosome fusion inhibitor ; chloroquine, a lysosome

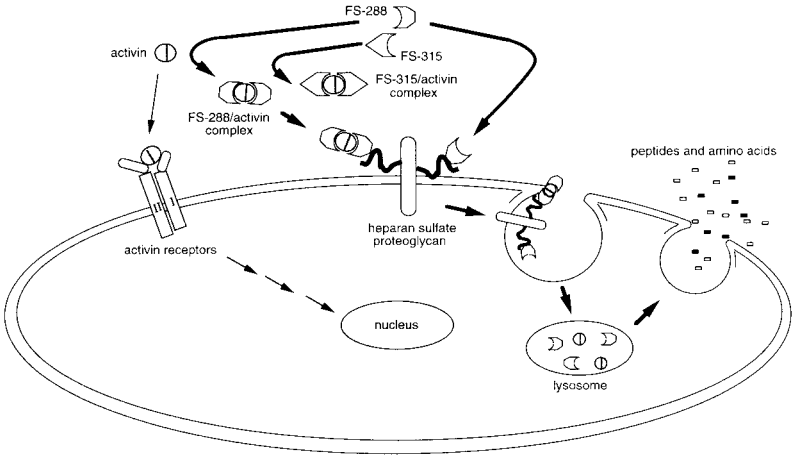

Fig.12. Regulatory mechanism of activin signal transduction by FS. Activin binds and activates the transmembrane serine/threonine kinase of the type I/II receptor complex. FS can neutralize activin bioactivity by interference with the binding of activin to activin type II receptor. Cell surface-bound FS accelerates the endocytotic internalization of activin to its degradation.

function inhibitor, and several lysosomal protease inhibitors. As was the case with radioiodinated activin A, the proteolytic degradation of ^{125}I-labeled FS-288 in pituitary cells was observed. Taking these findings together, we hypothesized that the endocytotic degradation of growth factors via cell-surface heparan sulfate is necessary to erase the growth factor signals from the surrounding cell surfaces when they become excessive and thus useless. It has been established that the binding of a signaling ligand to its receptor stimulates a biological response and triggers a sequence of events leading to cellular desensitization to the ligand in order to regulate the responsiveness of the target cell to the ligand. We propose that, in addition to such receptor-mediated endocytosis, there must be a scavenger mechanism for clearing signaling molecules away from their target cell surfaces (Fig.12). Our results demonstrated that cell-associated FS-288 (carboxy-terminal truncated FS) accelerates the endocytotic internalization of activin into rat pituitary cells, rat granulosa cells and probably *Xenopus* animal hemisphere cells, leading to its degradation by lysosomal enzymes. Cell-associated FS therefore plays a role in the system responsible for clearing the activin signal from cell surfaces.

ACKNOWLEDGEMENTS

We thank Dr. Y. Eto for providing recombinant human activin A, Dr. S. Shimasaki for the gift of recombinant human follistatins, and Dr. Y. Hasegawa for donating bovine inhibin A. We are grateful to the NIDDK Pituitary Hormone Program for supplying rat FSH RIA kit. This work was supported in part by grants from the Ministry of Education, Science, and Culture of Japan, from the Naito Foundation, the Uehara Memorial Foundation and the Sankyo Foundation of Life Science.

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