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# PRODUCTION OF ACTIVIN-BINDING PROTEIN BY RAT GRANULOSA CELLS *in vitro*

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**SUMMARY** We have developed an assay method for activin-binding protein, which exploits its high affinity for sulfated polysaccharides. We used this method to investigate the production of activin-binding protein by rat ovarian granulosa cells, *in vitro*. The production of activin-binding protein by granulosa cells was dependent on the cell density; the maximum was observed at 6 x $10^5$ cells/ml. Follicle-stimulating hormone (FSH), but not luteinizing hormone (LH), enhanced production significantly. Sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) and ligand blotting analyses of the activin-binding protein secreted by rat granulosa cells demonstrated it was the same protein molecule as that purified from rat ovaries. It is inferred from these results that the granulosa cell is a source of ovarian activin-binding protein and that its secretion is regulated by FSH. 

Activin was initially obtained from mammalian gonads as a protein with a capability of differential enhancement of follicle-stimulating hormone (FSH) by pituitary cells (1,2). The structural and biological studies demonstrated that it is a member of the transforming growth factor β (TGF-β) gene family (3) and that it has numerous functions; regulation of erythropoiesis (4,5), modulation of follicular granulosa cell differentiation (6,7), stimulation of insulin secretion (8), induction of mesoderm in *Xenopus laevis* (9,10) and inhibition of neural differentiation (11).

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Recently, we purified activin-binding protein from rat ovary and bovine pituitary and proved that it is identical to follistatin (12,13), which is a protein factor that was isolated from ovarian extracts (14,15). The biological activity that was first attributed to follistatin was the inhibition of secretion of FSH from cultured pituitary cells. However, the wide tissue distribution of follistatin mRNA suggests follistatin also has other physiological roles (16,17,18). This was endorsed, at least in part, by our recent findings that many of activin's effects were suppressed by activin-binding protein/follistatin. This activin-binding protein bound activin and formed an inactive equimolar complex with neither stimulatory nor inhibitory effects on FSH secretion by cultured pituitary cells (13).

The rat cultured granulosa cell system appears to be an appropriate *in vitro* model with which to study the physiological significance of activin-binding protein and activin for several reasons: follistatin/activin-binding protein mRNA has been observed in granulosa cells (16), granulosa cells possess specific receptors for activin (19) and granulosa cell functions are modulated by activin (6,7). However, no convenient method to assay activin-binding protein is available yet and, furthermore, it is still not known whether granulosa cells actually secrete activin-binding protein.

In this communication, we describe the development of a convenient assay method for the measurement of activin-binding protein in culture medium and also report that granulosa cells produce and secrete activin-binding protein in response to FSH.

## MATERIALS AND METHODS

**Hormones and Reagents** Recombinant human activin A (erythroid differentiation factor) was a kind gift from Drs. H. Shibai and Y. Eto (Ajinomoto Co., Inc., Central Research Laboratories, Kawasaki, Japan) (20). Standard activin-binding protein was purified from porcine ovaries as described previously (13) and $^{125}$I-activin (≈20000 cpm/ng) was prepared by the chloramin-T method (6). Equine FSH was provided kindly by Dr. D. N. Ward (University of Texas, M. D. Anderson Cancer Center, Houston, Texas), rat luteinizing hormone (LH) was obtained from the National Institute of Arthritis Diabetes, Digestive and Kidney Disease (NIHDDK) Pituitary Hormone Program and Sulfate-Cellulofine (sulfated cellulose gel) was

obtained from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). All other chemicals were of analytical reagent grade or the highest quality commercially available.

**Cell Culture** Rat granulosa cells were collected from immature female Wistar rats (21-23 days old), which had been pretreated with 1 mg of diethylstilbestrol (DES) in 0.1 ml sesame oil, subcutaneously, daily for 3 days. The cells were cultured in 1 ml Ham's F-12/Dulbecco's modified Eagle medium (DMEM) (1:1, v/v) medium supplemented with 14.3 mM sodium bicarbonate, gentamicin sulfate (40 μg/ml) and fungizone (1 μg/ml) at a cell density of 5 x $10^5$ per well in 24-well plates. The plates were incubated in the absence or presence of FSH and/or LH at 37 °C in a 5% $CO_2$- 95% air atmosphere.

**Affinity Gel Assay of Activin-Binding Protein** The assay buffer was 20 mM Tris-HCl, pH 7.3, containing 0.3 M NaCl, 0.5% BSA, 0.2% Triton X-100 and 2 mM EDTA. Sulfate-Cellulofine gels were washed with 20 mM Tris-HCl, pH 7.3, containing 0.3 M NaCl, and suspended to give a final concentration of 1 g/ml in the washing buffer. Samples or standard solutions (200 μl) and 200 μl of the assay buffer were added to a disposable glass culture tube (10 x 75 mm), which contained 50 μl of the gel suspension, and shaken at room temperature overnight. The suspension was centrifuged at 3000 rpm for 5 min, the supernatant was discarded and the gels were washed with 500 μl of the assay buffer. Fifty microlitres each of the assay buffer and $^{125}$I-activin solution (100 ng $^{125}$I-activin/ml) were added to the washed gels and shaken at room temperature for 2 h. In order to determine the non-specific binding, a 100-fold excess of unlabeled activin was added. After incubation, 1.5 ml of the assay buffer was added, the tubes were centrifuged at 3000 rpm for 5 min, the gels were washed with 1.5 ml of the assay buffer and the radioactivity of each tube was counted in a γ-counter.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Ligand Blotting** Analysis by SDS-PAGE on 10 % gels was performed as described by Laemmli (21) and ligand blotting was carried out as described previously (12).

## RESULTS

During the process of activin-binding protein purification, we found that it has a high affinity for sulfated polysaccharides, such as heparin and dextran sulfate. Therefore, we exploited this property and our previous finding that activin-binding protein binds activin stoichiometrically to form a 1:1 molar complex (13) and developed a conventional assay to determine the amount of activin-binding protein in the culture medium of rat granulosa cells. Sulfate-Cellulofine gels (sulfated cellulose gels) and samples, which contained activin-binding protein, were

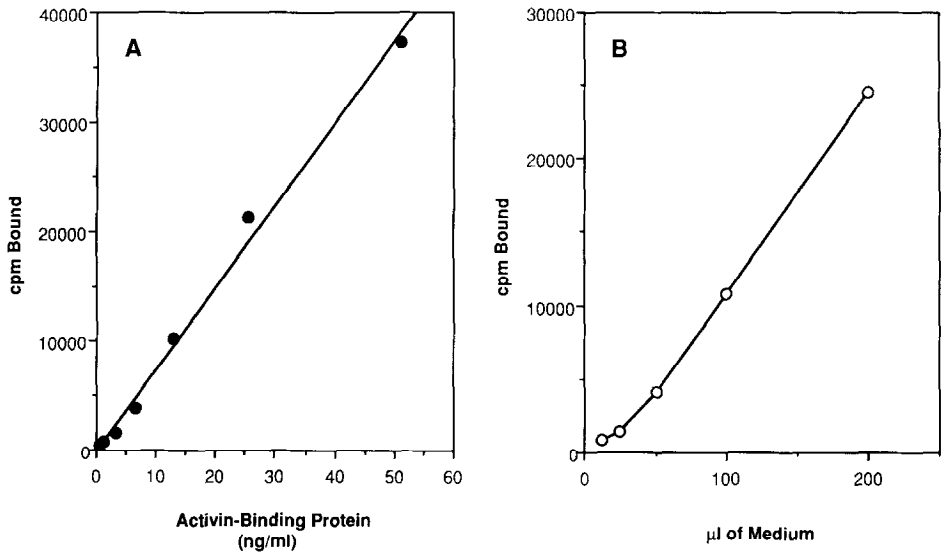



**Fig. 1.** Affinity gel assay for activin-binding protein. (A) Standard curve for purified activin-binding protein from rat ovaries, and (B) concentration-response curve for cultured medium of rat granulosa cells.

incubated with constant shaking and then the required amount of $^{125}$I-activin (usually 5 ng) was added to the washed gels in order to determine the amounts of the binding-protein bound to the gels. After shaking, the radioactivity of the collected gels was measured and the amounts of binding protein in the culture medium were calculated from the standard curve. A linear concentration-response curve was obtained over the concentration range of 1 to 50 ng/ml of standard activin-binding protein (Fig. 1-A). Serial dilutions of serum-free conditioned medium from rat granulosa cell cultures also showed a linear response (Fig. 1-B) and the conditioned medium had no effect on the response of standard binding protein (data not shown). Other structurally related factors, inhibin and TGF-β, and gonadotropins, LH and FSH, did not cross-react in the assay.

An increase in the density of the granulosa cells caused a logarithmic acceleration of activin-binding protein secretion (Table I). At the initial density of 1 x $10^5$ cells/ml no binding protein was detected in the medium, even after 72 h-culture, whereas at 6 x $10^5$ cells/ml confluent culture was observed and binding protein production per cell reached its uppermost limit. Therefore, the initial cell density of 5 x $10^5$ cells/ml was used in the following experiments.

Table I. Effect of Cell Density on Production of Activin-Binding Protein by Rat Granulosa Cells

| Cell Density (cells/ml) | Activin-Binding Protein Secreted (ng/ml/72h) |
|---|---|
| $1 \times 10^5$ | <1.0 |
| $3 \times 10^5$ | 1.0±0.6 |
| $6 \times 10^5$ | 3.8±0.3 |

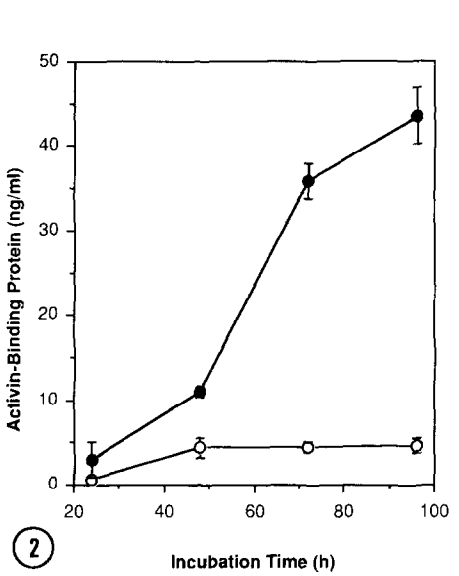
Granulosa cells obtained from DES-treated rats were cultured for 72h, and activin-binding protein secreted into the medium was measured by affinity gel assay, as described in MATERIALS AND METHODS. The results are means±SE for three wells.

FSH stimulated the secretion of activin-binding protein in a concentration-dependent manner: at an FSH concentration of 30 ng/ml secretion reached a plateau level of 35 to 40 ng/ml in the 72 h-culture (Table II). The time-dependent effect of FSH is shown in Fig. 2; FSH-induced secretion of activin-binding protein increased over the period of 24 to 96 h. However, LH had no effect on either secretion or FSH-induced augmentation of secretion (Table II).

Table II. Effect of FSH and/or LH on Activin-Binding Protein Production in Granulosa Cells

| | Hormone Added (ng/ml) | Activin-Binding Protein (ng/ml/72h) |
|---|---|---|
| None | | 3.3±0.1 |
| FSH | 10 | 11.0±4.3 |
| | 30 | 36.3±4.8 |
| | 100 | 43.0±4.5 |
| LH | 10 | 3.9±3.9 |
| | 30 | 3.8±0.3 |
| | 100 | 4.3±0.7 |
| FSH+LH | 30+30 | 42.0±5.2 |

Granulosa cells ($5 \times 10^5$ cells/ml) obtained from DES-treated rats were cultured for 72h with FSH and/or LH, and activin-binding protein secreted into the medium was measured by affinity gel assay, as described in MATERIALS AND METHODS. The results are means±SE for three wells.



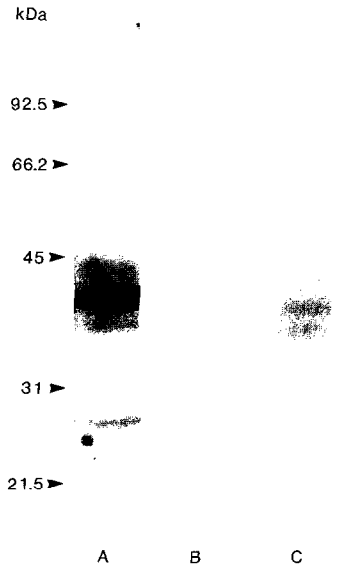




**Fig. 2.** Time course of production of activin-binding protein by rat granulosa cells. Granulosa cells (5 x $10^5$ cells/well) obtained from DES-treated rats were cultured for 72h with (-●-) or without (-○-) FSH (30 ng/ml). Activin-binding protein secreted into the medium was measured by affinity gel assay, as described in MATERIALS AND METHODS. The results are means±SE for three wells.

**Fig. 3.** Western blotting pattern of partially purified activin-binding protein from cultured rat granulosa cells. The culture medium (70 ml) was incubated with Sulfate-Cellulose gels (1 ml) at 4 °C overnight, and activin-binding protein bound to the gels was eluted with 20 mM Tris-HCl, pH 7.3, containing 2 M NaCl. The eluate was dialyzed and lyophilized. Following SDS-PAGE of the samples, the proteins were electroblotted onto an Immobilon membrane (Millipore) and incubated with $^{125}$I-activin in the absence of unlabeled activin (A) and in the presence of a 100-fold excess of unlabeled activin (B). The pattern of purified activin-binding protein from rat ovaries is shown in (C).

In order to confirm that the activin-binding protein determined using Sulfate-Cellulofine gels was indeed the same protein molecule as the one we had purified previously from rat ovaries (12), Western blotting analyses were carried out with samples of the partially purified preparations, which were obtained from the culture medium of rat granulosa cells, by affinity chromatography on Sulfate-Cellulofine gels. Incubation of blots with $^{125}$I-labeled activin followed by autoradiography resulted in labeling of multiple bands in the Mr 32 kDa-38 kDa range, with a pattern quite similar to that of rat ovary preparations (Fig. 3). Labeling of the bands on ligand blots could be prevented completely by incubation with excess unlabeled

activin (Fig. 3-B). This was confirmed by staining the blots with antiserum directed against activin-binding protein peptide 123-134 (22). The immunoblotting analysis gave a similar pattern to that shown by ligand blotting analysis (data not shown). These results indicate clearly that rat granulosa cells do produce activin-binding protein and secrete it into the medium.

## DISCUSSION

These experiments have demonstrated that rat granulosa cells secreted activin-binding protein, *in vitro*, which was stimulated by FSH, and also that the amount of the secreted protein in the culture medium could be determined by the affinity gel method using Sulfate-Cellulofine gels.

Recent studies on activin and activin-binding protein/follistatin suggest that activin-binding protein possesses an important physiological role in the regulation of diverse activin actions (12,13,16). In order to clarify the mechanisms of the actions of activin-binding protein appropriate assay methods for activin-binding proteins need to be developed. Therefore, we attempted to develop a novel method for measuring activin-binding protein in culture medium using sulfated polysaccharide gels. In principle, the high affinity of activin-binding protein for Sulfate-Cellulofine gels should make it easy to concentrate the binding protein in the medium on to the gels. In order to estimate the amount of the binding protein bound on the gels $^{125}$I-activin is added to the gel suspension and the bound radioactivity is determined with a $\gamma$-counter. This method is very convenient as it does not need an antibody, the operation is simple and the culture medium is assayed directly without any pretreatment. Furthermore, a wide range of concentrations (1 to over 50 ng/ml) of activin-binding protein can be measured. There is, however, a problem to be solved, that is the low sensitivity of the assay; the lowest limit of detection is about 1 ng/ml, which differs considerably from that achieved with ordinary radioimmunoassay systems. This may be due to a lower affinity of activin-binding protein for the sulfated polysaccharides (association constant, $\approx 10^8$ M), than that of the interaction between antigens and antibodies (association constant, $>10^9$ M). Nevertheless, this demerit is compensated for by

several merits, at least in our experiments, which enable the production mechanism for activin-binding protein in cultured granulosa cell systems to be studied.

Shimasaki *et al* (16), Michel *et al* (17) and Kaiser *et al* (18) demonstrated, by Northern analyses in rat tissues, that the follistatin gene is expressed not only in the ovary but also in other tissues, including the kidney and brain. Their *in situ* hybridization studies also revealed that significant levels of follistatin mRNA are present in the granulosa cells of the growing secondary and tertiary follicles (16). However, granulosa cells have not been shown to produce and secrete activin-binding protein molecule, *in vitro*, nor has activin-binding activity been detected in the culture medium of granulosa cells. Therefore, our present finding provides the first evidence that granulosa cells have the capability of synthesizing and releasing the protein, *in vitro*. Addition of FSH to the cultured granulosa cells augmented the production of activin-binding protein significantly in a concentration-dependent manner. This correlates well with our preliminary observation that administration of equine chorionic gonadotropin (eCG) to immature female rats caused a remarkable increase in the ovarian activin-binding protein content (H. Sugino, unpublished data). Shimasaki *et al* (16) and Michel *et al* (17) also reported that the follistatin/activin-binding protein mRNA level in the immature rat ovary was increased by eCG injection. We found recently that activin stimulated FSH-induced differentiation of rat granulosa cells and enhanced FSH-induction of LH receptor expression and progesterone production (6). Hasegawa *et al* (23) found that activin induced expression of FSH receptors in rat cultured granulosa cells and suggested that activin may play a role in the initiation of the cytodifferentiation of ovarian granulosa cells. These findings and our results indicate that granulosa cells can produce activin-binding protein in response to FSH and one can conjecture that activin-binding protein/follistatin plays an important role in the activin-modulated differentiation of granulosa cells and probably various other types of cells. Accordingly, more observations on the regulation for activin-binding protein production in granulosa cells may lead us to a clearer understanding of cytodifferentiation of granulosa and others cells. Therefore, this study may have

provided a good *in vitro* model system and a convenient assay method to pursue studies of dynamic changes of activin-binding protein in cell culture.

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## REFERENCES

1. Vale, W., River, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D., and Spiess, J. (1986) Nature 321, 776-779.
2. Ling, N., Ying, S.-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M., and Guillemin, R. (1986) Nature 321, 779-782.
3. Ying, S.-Y. (1988) Endocr. Rev. 9, 267-293.
4. Eto, Y., Tsuji, T., Takezawa, M., Takano, S., Yokogawa, Y., and Shibai, H. (1987) Biochem. Biophys. Res. Commun. 142, 1095-1103.
5. Yu, J., Shao, L. E., Lemas, V., Yu, A. L., Vaughan, J., Rivier, J., and Vale, W. (1987) Nature 330, 765-767.
6. Sugino, H., Nakamura, T., Hasegawa, Y., Miyamoto, K., Abe, Y., Igarashi, M., Eto, Y., Shibai, H., and Titani, K. (1988) Biochem. Biophys. Res. Commun. 153, 281-288.
7. Hutchinson, L. A., Findlay J. K., de Vos, F. L., and Robertson, D. M. (1987) Biochem. Biophys. Res. Commun. 146, 1405-1412.
8. Totsuka, Y., Tabuchi, M., Kojima, I., Shibai, H., and Ogata, E. (1988) Biochem. Biophys. Res. Commun. 156, 335-339.
9. Smith, J. C., Price, B. M. J., van Nimmen, K., and Huylebroeck, D. (1990) Nature 345, 729-731.
10. van den Eijnden-Van Raaij, A. J. M., van Zoelent, E. J. J., van Nimmen, K., Koster, C. H., Snoek, G. T., Durston, A. J., and Huylebroeck, D. (1990) Nature 345, 732-734.
11. Hashimoto, M., Kondo, S., Sakurai, T., Etoh, Y., Shibai, H., Muramatsu, M. (1990) Biochem. Biophys. Res. Commun. 173, 193-200.
12. Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K., and Sugino, H. (1990) Science 247, 836-838.
13. Kogawa, K., Nakamura, T., Sugino, K., Takio, K.,Titani, K., and Sugino, H. (1991) Endocrinology 128, in press.
14. Ueno, N., Ling, N., Ying, S.-Y., Esch, F., Shimasaki, S., and Guillemin, R. (1987) Proc. Natl. Acad. Sci. USA 84, 8282-8286.

15. 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 Krester, D. M. (1987) Biochem. Biophys. Res. Commun. 149, 744-749.
16. Shimasaki, S., Koga, M. Buscaglia, L. M., Simmons, D. M., Bicsak, T. A., and Ling, N. (1989) Mol. Endocrinol. 3, 651-659.
17. Michel, U., Albiston, A., and Findlay, J. K. (1990) Biochem. Biophys. Res. Commun. 173, 401-407.
18. Kaiser, M., Gibori, G., and Mayo, K. E. (1990) Endocrinology 126, 2768-2770.
19. Sugino, H., Nakamura, T., Hasegawa, Y., Miyamoto, K., Igarashi, M., Eto, Y., Shibai, H., and Titani, K. (1988) J. Biol. Chem. 263, 15249-15252.
20. Murata, M., Onomichi, K., Eto, Y., Shibai, H., and Muramatsu, M. (1988) Biochem. Biophys. Res. Commun. 151, 230-235.
21. Laemmli, U. K. (1970) Nature (London) 227, 680-685.
22. Saito, S., Sugino, K., Yamanouchi, K., Nakamura, T., Titani, K., Shiota, K., Takahashi, M., and Sugino, H. (1991) Endocrinol. Japon. Submitted.
23. Hasegawa, Y., Miyamoto, K., Abe, Y., Nakamura, T., Sugino, H., Eto, Y., Shibai, H., and Igarashi, M. (1988) Biochem. Biophys. Res. Commun. 156, 668-674.