E_2 and E_4. Originally thought to be unstable, E_2 and E_4 appeared unexpectedly as stable points escorted by two unstable equilibria, one on either side of the originals, as seen in Fig. 2. Once again, the ratio H/L passes through a pitchfork bifurcation making E_2 and E_4 stable while spawning pairs of unstable equilibria. After the fact, we found the mathematical explanation. Eliminating G from Eq. 19 produces a quadratic equation in $\sin g$. Previous analysis restricted g to be a multiple of $\pi/2$ to ease the algebraic complexities. Not until the ratio H/L becomes very small does the discriminant of the equation in $\sin g$ become positive, providing for real roots and new equilibria.

We have now come full circle. Analytical study of a dynamical system prompted graphical representations to support our results. Improvements in the visualization techniques revealed new phenomena, which brought us to refine our mathematical analysis.

REFERENCES AND NOTES

1. See, for instance, the cover of *Science*, 30 October 1987, courtesy of C. Grebogi, E. Ott, and J. A. Yorke.
2. A. A. Orlov, *Soobshch. Gos. Astron. Inst. Mosk. Gos. Univ.* **88–89**, 3 (1953); *Tr. Gos. Astron. Inst. Mosk. Gos. Univ.* **24**, 139 (1954).
3. A. Deprit, *J. Guidance Control* **4**, 201 (1981); S. L. Coffey and A. Deprit, *J. Guidance Control Dyn.* **5**, 366 (1982).
4. S. L. Coffey, A. Deprit, E. Deprit, L. Healy, B. R. Miller, in *Proceedings of the Conference on Computer-Aided Proofs in Analysis* (Institute for Mathematics and Its Applications) (Springer-Verlag, New York, in press).
5. C. Delaunay, *C. R. Acad. Sci.* **22**, 32 (1846).
6. S. Coffey, A. Deprit, L. Healy, in preparation; P. Exertier, thèsis, Observatoire de Paris (1988).
7. S. L. Coffey, A. Deprit, B. R. Miller, *Celestial Mech.* **39**, 365 (1986).
8. S. Hughes, *ibid.* **25**, 235 (1981).

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Activin-Binding Protein from Rat Ovary Is Follistatin

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Activin, a member of the transforming growth factor β protein family, was originally isolated from gonadal fluids and stimulates the release of pituitary follicle-stimulating hormone (FSH). Activin has numerous functions in both normal and neoplastic cells. Various cells synthesize activin and have a specific binding site for this peptide. However, the molecular basis for its actions is unknown. A binding protein for activin was purified from rat ovary and was identical to follistatin, a specific inhibitor of FSH release. It is likely that the binding protein participates in the diverse regulatory actions of activin.

SEVERAL HYPOPHYSIOTROPIC PROteins that can suppress or enhance follicle-stimulating hormone (FSH) secretion by pituitary cells have been identified from mammalian gonads. These include inhibin (*1*) and follistatin (*2*), which inhibit FSH release, and activin (*3*), which stimulates FSH release. They have potential roles in reproduction and their structures have been elucidated by protein chemistry and cDNA cloning techniques (*4, 5*).

Activin is a member of the transforming growth factor β (TGF-β) gene family and may have a variety of similar functions. Activin acts outside the reproductive system as erythroid differentiation factor (EDF). Although EDF was initially isolated from a human leukemia cell line as a protein factor that causes differentiation of erythroid progenitor cells (*6*), protein chemistry and cDNA cloning analyses of the factor proved that EDF is the same molecular species as activin (*7*). Activin (EDF) has diverse biological roles: modulation of follicular granulosa cell differentiation (*8*), regulation of erythropoiesis (*9*), stimulation of insulin secretion by rat pancreatic islets (*10*), and modulation of several types of anterior pituitary cells (*11*). Specific binding sites for activin on rat granulosa cell (*12*) and human and murine erythroleukemia cells (*13*) have been identified; activin binding may be responsible for its biological activities. Nevertheless, regulation of the multiple functions of activin remains obscure.

During our purification of activin receptors we noticed binding proteins for activin in rat ovary homogenates. We therefore

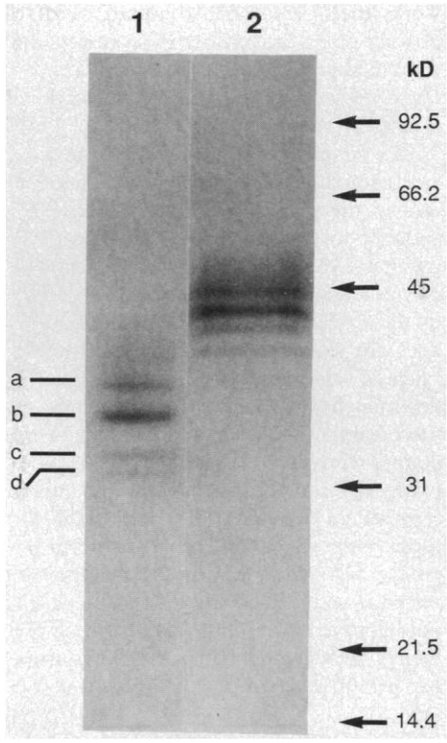

Fig. 1. SDS-PAGE of purified activin-binding protein under nonreducing (lane 1) and reducing (lane 2) conditions. The ovaries (188 ovaries: wet weight, 21 g) were homogenized in 200 ml of Buffer A [20 m*M* tris-HCl (*p*H 7.2) with 150 m*M* NaCl, 5 m*M* benzamidine-HCl, 1 m*M* phenylmethylsulfonyl fluoride, 1 m*M* *N*-ethylmaleimide, 2 m*M* diisopropylfluorophosphate, 2 m*M* EDTA, and 20% glycerol] with a Polytron homogenizer. The homogenate was centrifuged; the resulting supernatant was added to an equal volume of Buffer A (with 25% polyethylene glycol 6000 instead of 20% glycerol), stirred for 30 min at 4°C, centrifuged, and the supernatant decanted. The pellet was suspended in Buffer A with Triton X-100 at a final concentration of 2%, stirred, and centrifuged. The supernatant was mixed with the activin–Affi-Gel 10 [5 mg of recombinant activin (EDF) (*21*) was coupled to 5 ml Affi-Gel 10 (Bio-Rad)] and incubated overnight with a gentle stirring at 4°C. The gel was first washed with 0.1% Triton X-100 in Buffer A and then with 0.1% Triton X-100 in 50 m*M* sodium acetate buffer (*p*H 4.0), resuspended in 0.1% Triton X-100 in Buffer A, and poured into a column. Activin-binding protein was eluted with Buffer B (Buffer A with 2*M* guanidine-HCl and 0.1% Triton X-100). The active fractions desalted by a Bio-Gel P-10 column were applied to an activin–Affi-Gel 10 column, and the column was washed with 0.1% Triton X-100 in Buffer A. The activin-binding protein was eluted with Buffer B. Active fractions were desalted using a Bio-Gel P-10 column equilibrated with 0.001% SDS. SDS-PAGE of the preparation was done in 12.5% gels and proteins were stained with Coomassie brilliant blue (*22*). Bands a to d identify activin-binding protein.

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attempted to purify and characterize it from the ovaries of immature female Wistar rats that were treated with diethylstilbestrol (DES) and equine chorionic gonadotropin (eCG) (*14*). The binding protein was extracted by incubating the ovary homogenate in a buffer containing 2% Triton X-100. Two cycles of affinity chromatography of the extract on an activin-immobilized column resulted in a 22,000-fold purification, yielding 24 μg from 21-g (wet weight) ovaries, with 44% recovery of activity.

SDS–polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions of the purified binding protein gave two major bands, a and b, of 38 and 36 kD, and two minor ones, c and d, of 33 and 32 kD (Fig. 1, lane 1). After reduction, all these bands migrated in a similar pattern, but at a higher molecular mass range from 40 to 45 kD (Fig. 1, lane 2).

To establish that the isolated protein does indeed have activin-binding activity, we did ligand blotting with samples of purified binding protein (Fig. 2). Incubation of blots with ^{125}I-labeled activin resulted in labeling of bands in the 32- to 38-kD range with a pattern similar to Coomassie-stained bands after SDS-PAGE under nonreducing conditions (Fig. 2A). Labeling of the bands on ligand blots could be completely prevented by incubation in the presence of excess unlabeled activin (Fig. 2B). These results indicate that all the molecular forms of the protein observed on the gels bind activin. This was further confirmed by detection of the binding activity in gel-slice extracts from the 32- to 38-kD region (*15*).

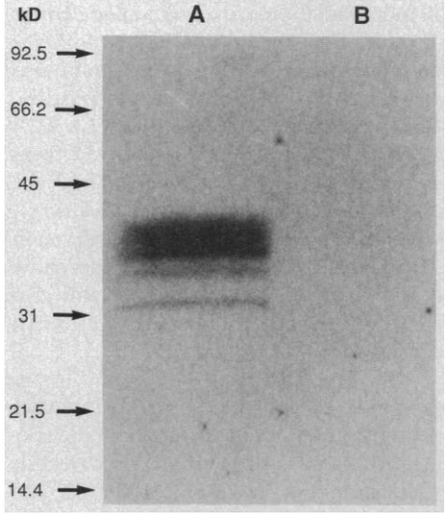

Fig. 2. Activin-binding activity of purified activin-binding protein as demonstrated by ligand blotting. Purified protein (0.5 μg) was dissolved in SDS-PAGE sample buffer in the absence of 2-mercaptoethanol without heating. Electrophoresis was done in 12.5% gels and the protein transferred to Immobilon membranes (Millipore). The membrane was blocked with 10% skim milk, washed, and incubated with ^{125}I-activin (final concentration of 10 ng/ml) in the absence (**A**) or presence (**B**) of unlabeled activin (500 ng/ml). Blots were then washed three times, air-dried, and exposed to Kodak XAR film for 4 days at −80°C.

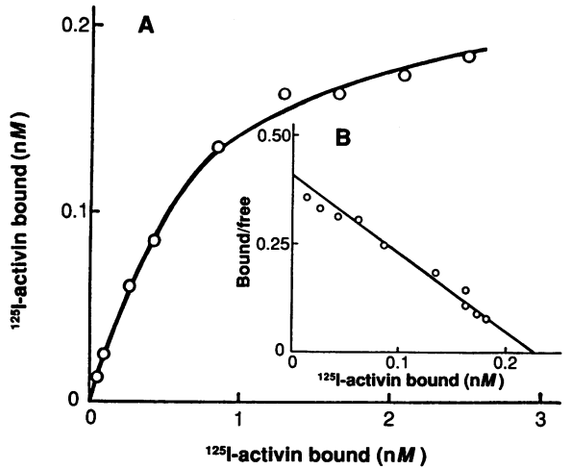

Fig. 3. (**A**) Saturation studies of activin-binding protein. The activin-binding activity was determined by a polyethylene glycol precipitation method. The binding protein [3.2 ng dissolved in 150 μl of 25 m*M* tris-buffered saline (*p*H 7.2) (TBS) with 0.1% BSA (TBS-BSA)] was incubated with various amounts of ^{125}I-labeled activin dissolved in 100 μl of TBS-BSA for 90 min at room temperature. To separate bound and free ^{125}I-activin, 50 μl of TBS-BSA, 100 μl of 5% bovine γ-globulin, and 400 μl of 25% polyethylene glycol 6000 were added to the incubation mixture and mixed vigorously. The mixture was centrifuged at 1900*g* for 20 min. The radioactivity of the pellet was counted in a γ-spectrometer. Nonspecific binding was determined by measuring the amount of ^{125}I-activin that could be precipitated in the presence of 100-fold excess unlabeled activin. Activin was iodinated by the chloramine-T method as described previously (*12*). (**B**) Scatchard analysis of the binding data presented in (A).

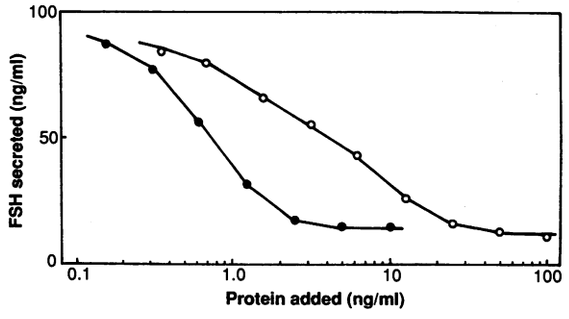

Fig. 4. Concentration dependence of activin-binding protein and bovine inhibin on the suppression of FSH release in cultured rat pituitary cells. FSH–release inhibiting activity was assayed with in vitro bioassay by the use of immature female rat pituitary monolayer cultures (*1*). Activin-binding protein, ○; bovine inhibin, ●.

Binding properties of the activin-binding protein were characterized by Scatchard analysis. The dissociation constant (K_d) and maximum binding capacity (B_{max}) values were estimated to be 590 ± 230 p*M* and 18,300 ± 3,500 pmol/mg, respectively (Fig. 3). Ligand specificity was examined in competition experiments with ^{125}I-activin and unlabeled protein. Little or no competition was demonstrable with the structurally related peptides, inhibin and TGF-β, nor with gonadotropins, FSH, and luteinizing hormone (LH) (*16*).

We determined the NH_2-terminal sequence of each protein band observed on SDS-PAGE by electroblotting each of the protein bands (Fig. 1, bands a to d) onto an Immobilon membrane and directly sequencing each with an Applied Biosystems 477A Protein Sequencer (*17*). Although the third and thirteenth amino acids could not be decisively identified, all the bands were shown to possess a single NH_2-terminal sequence: Gly-Asn-X-Trp-Leu-Arg-Gln-Ala-Lys-Asn-Gly-Arg-X-Gln-Val-Leu-Tyr-Lys-Thr-Glu- (X denotes unidentified amino acids). This sequence was found by a search of the Protein Identification Resource protein sequence database to be identical to the NH_2-terminal sequence of rat follistatin (*5, 18*).

Two forms of follistatin (32 and 35 kD) were initially isolated from porcine follicular fluid and found to be factors having the ability to specifically inhibit the secretion of FSH from the pituitary (*2*). Since then, the primary structures of porcine, human, and rat follistatins have been elucidated by molecular cloning of their cDNAs (*5*). These results have demonstrated that follistatin is a glycosylated single-chain protein distinct from inhibin in spite of its similar effect on FSH secretion. The difference between the two forms of follistatin is due to glycosylation or COOH-terminal truncation.

The activin-binding protein purified in the present study was also found to be glycosylated by means of retardation on a wheat germ agglutinin column. Comparing the various characteristics so far obtained for rat activin-binding protein with those of follistatin, the two proteins are likely to be

identical to each other and the two major forms of the activin-binding protein correspond to the 32- and 35-kD forms of follistatin.

To confirm this conclusion, we examined the effect of activin-binding protein on FSH release from cultured pituitary cells for follistatin-like activity (Fig. 4). The binding protein significantly suppressed FSH release in a concentration-dependent manner in the range of 0.4 to 100 ng/ml [median effective dose (ED_{50}), 5.2 ng/ml]. When compared with the concentration dependence of bovine inhibin, similar slope and maximal effect were obtained. However, the potency of the binding protein is 10 to 20% that of inhibin. This FSH suppression and activity ratio compared to inhibin is much like that reported for follistatin (*2, 19*). This supports the identity of the activin-binding protein and follistatin.

We obtained preliminary evidence that cultured rat ovarian granulosa cells produce activin-binding protein and secrete it into medium. We also purified an activin-binding protein from bovine pituitary that had the same chemical and biological characteristics as those of the binding protein from rat ovary (*20*). Together with various modulatory actions of activin on granulosa cells and pituitary cells, our findings support the idea that the binding protein may have an autocrine or paracrine function in the ovary and pituitary. Studies of the activin-binding protein from various cells may lead to a clearer understanding of the regulation of activin.

REFERENCES AND NOTES

1. D. M. Robertson *et al.*, *Biochem. Biophys. Res. Commun.* **126**, 220 (1985); K. Miyamoto *et al.*, *ibid.* **129**, 396 (1985); N. Ling *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7217 (1985); J. Rivier, J. Spiess, R. McClintock, J. Vaughan, W. Vale, *Biochem. Biophys. Res. Commun.* **133**, 120 (1985); M. Fukuda *et al.*, *Mol. Cell. Endocrinol.* **44**, 55 (1986); D. M. Robertson *et al.*, *ibid.*, p. 271; L. J. Leversha *et al.*, *J. Endocrinol.* **113**, 213 (1987).
2. N. Ueno *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8282 (1987); D. M. Robertson *et al.*, *Biochem. Biophys. Res. Commun.* **149**, 744 (1987).
3. W. Vale *et al.*, *Nature* **321**, 776 (1986); N. Ling *et al.*, *ibid.*, p. 779.
4. A. J. Mason *et al.*, *ibid.* **318**, 659 (1985); R. G. Forage *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3091 (1986); A. J. Mason, H. D. Niall, P. H. Seeburg, *Biochem. Biophys. Res. Commun.* **135**, 957 (1986); F. S. Esch *et al.*, *Mol. Endocrinol.* **1**, 388 (1987); K. E. Mayo *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5849 (1986); A. G. Stewart, H. M. Milborrow, J. M. Ring, C. E. Crowther, R. G. Forage, *FEBS Lett.* **206**, 329 (1986).
5. F. S. Esch *et al.*, *Mol. Endocrinol.* **1**, 849 (1987); S. Shimasaki *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4218 (1988); S. Shimasaki *et al.*, *Mol. Endocrinol.* **3**, 651 (1989).
6. Y. Eto *et al.*, *Biochem. Biophys. Res. Commun.* **142**, 1095 (1987).
7. M. Murata, Y. Eto, H. Shibai, M. Sakai, M. Muramatsu, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2434 (1988).
8. H. Sugino *et al.*, *Biochem. Biophys. Res. Commun.* **153**, 281 (1988); Y. Hasegawa *et al.*, *ibid.* **156**, 668 (1988); L. A. Hutchinson, J. K. Findlay, F. L. de Vos, D. M. Robertson, *ibid.* **146**, 1405 (1987).
9. J. Yu *et al.*, *Nature* **330**, 765 (1987).
10. Y. Totsuka, M. Tabuchi, I. Kojima, H. Shibai, E. Ogata, *Biochem. Biophys. Res. Commun.* **156**, 335 (1988).
11. M. Kitaoka, I. Kojima, E. Ogata, *ibid.* **157**, 48 (1988).
12. H. Sugino *et al.*, *J. Biol. Chem.* **263**, 15249 (1988).
13. C. A. Campen and W. Vale, *Biochem. Biophys. Res. Commun.* **157**, 844 (1988); S. Kondo *et al.*, *ibid.* **161**, 1267 (1989); M. Hino *et al.*, *J. Biol. Chem.* **264**, 10309 (1989).
14. Immature female Wistar rats (21 to 23 days old) were injected subcutaneously with DES (2 mg in 0.1 ml of sesame oil) daily for 3 days. On day 4, eCG (50 IU) was injected subcutaneously. Two days after the injection of eCG, ovaries were obtained from rats killed by CO_2 inhalation. The organs were trimmed free of fat, frozen on dry ice, and stored at −85°C until use. This treatment augments the activin-binding activity in rat ovary by six to eight times greater than in nontreated rats.
15. After SDS-PAGE under nonreducing conditions, the gel was cut into 0.2-cm slices and the gel slices were placed overnight at 4°C in 1% Triton X-100 in 20 m*M* tris-HCl (*p*H 7.2) containing 150 m*M* NaCl and 0.1% bovine serum albumin (BSA). The activin-binding activity of the extracts was determined by the binding assay as described in the legend to Fig. 3.
16. Incubation of the activin-binding protein with 1.5 ng of ^{125}I-activin and increasing amounts of unlabeled activin resulted in progressive inhibition of the tracer binding; at 0.1 μg/ml the competition was 100%. In contrast, in the presence of inhibin, TGF-β, FSH, or LH at 10 μg/ml, the inhibition was less than 20%.
17. P. Matsudaira, *J. Biol. Chem.* **262**, 10035 (1987).
18. The third and thirteenth residues are deduced to be Cys by cDNA cloning analysis.
19. S.-Y. Ying *et al.*, *Biochem. Biophys. Res. Commun.* **149**, 133 (1987).
20. T. Nakamura and H. Sugino, unpublished observations.
21. M. Murata, K. Onomichi, Y. Eto, H. Shibai, M. Muramatsu, *Biochem. Biophys. Res. Commun.* **151**, 230 (1988).
22. U. K. Laemmli, *Nature* **227**, 680 (1970).

23. We thank D. N. Ward and G. R. Bousfield for critical reading of the manuscript and providing eCG; M. Igarashi, Y. Hasegawa, and K. Miyamoto for the gift of bovine inhibin and helpful suggestions; and the National Institute of Arthritis, Diabetes, and Digestive and Kidney Disease (NIADDK) Pituitary Hormone Program for supply of rat FSH assay kit and rat FSH. This work was supported in part by grants from the Ministry of Education, Science and Culture, Japan.

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Endocranial Features of *Australopithecus africanus* Revealed by 2- and 3-D Computed Tomography

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The earliest hominid from South Africa, *Australopithecus africanus*, is known from only six specimens in which accurate assessment of endocranial capacity and cranial venous outflow pattern can be obtained. This places a severe limit on a number of hypotheses concerning early hominid evolution, particularly those involving brain–body size relationships and adaptations of the circulatory system to evolving upright posture. Advances in high-resolution two- and three-dimensional computed tomography (CT) now allow the inclusion of another important specimen to this list, MLD 37/38 from Makapansgat. A new computer imaging technique is described that "reconstructs" the missing portions of the endocranial cavity in order to determine endocranial capacity. In addition, CT evaluation allows assessment of cranial venous outflow pattern even in cases where the endocranial cavity is completely filled with stone matrix. Results show that endocranial capacity in this specimen is less than originally proposed and also support the view that gracile and robust australopithecines evolved different cranial venous outflow patterns in response to upright postures.

BRAIN SIZE IS AN IMPORTANT BIOlogical parameter and its measurement should be as accurate as possible, particularly in problems involving allometric growth and brain-body relationships (*1*). Even though it has been more than 60 years since *Australopithecus africanus* was first described (*2*), endocranial capacity estimates for this earliest South African hominid species are still based on only six individuals (Taung, Sts 60, Sts 5, Sts 19, Sts 71, and MLD 37/38) (*3–8*). Endocranial capacity estimates for one of these specimens, MLD 37/38 from Makapansgat, have varied from 480 cm^3 (*3*) to 435 cm^3 (*4, 9*), a difference of approximately 10%. Obtaining a reliable estimate of endocranial capacity in MLD 37/38 is difficult for two reasons: the endocranial cavity is filled with stone matrix, and part of the cranium anterior to the coronal suture is missing (Fig. 1). It would be impossible to extract the natural endocast intact from the calvaria; even to remove it

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