acquire anchorage-independent growth properties by mechanisms not involving the activation of *ras* oncogenes. None of the MNNG-treated transformed cell lines derived from 118 cells were tumorigenic in vivo (*18*). These results indicate that oncogene activation may be required to drive guinea pig cells to a fully neoplastic phenotype. Yet DNA's isolated from two other tumorigenic clones, 123-C1 and HM9-C1 (*17*), have so far failed to induce transformation of NIH/3T3 cells. However, it is possible that tumorigenicity in vivo could also be acquired by mechanisms not involving activation of the *ras* oncogene.

Guinea pig cells require several months of subculturing before they acquire anchorage-independent growth (*17*). This property is usually associated with the ability of these transformed cells to induce tumors in newborn guinea pigs or nude mice (*17, 18*). To examine when oncogenes became activated during the progression of carcinogen-treated guinea pig cells toward malignancy, we analyzed parental cultures, which had been stored in liquid nitrogen, before the development of tumorigenicity. They included passages 17, 40, and 61 of the MNNG-treated 107 cell line; passages 33, 67, and 86 of the DEN-treated HM2 series; and passages 31, 58, and 77 of the MNNG-treated HM4 line. In each case, the last passage used was the mass cell culture used to derive clones 107-C3, HM2-C1, and HM4-C1, respectively (*17, 25*). Cells were thawed, expanded, and harvested for DNA extraction. DNA's were tested for transformation of NIH/3T3 cells in transfection assays. In each of the series, early nontumorigenic passages did not show detectable anchorage-independent growth, and their DNA's did not induce morphologic transformation of NIH/3T3 cells (Table 1). In contrast, each of the DNA's isolated from the cell lines at later passages, when they had developed tumorigenic properties, showed transforming activity (Table 1). These results indicate that activation of a specific *ras* oncogene in carcinogen-treated fetal guinea pig cells is associated with the acquisition of tumorigenicity.

Cellular transforming genes isolated by molecular cloning techniques can efficiently induce malignant transformation of immortalized cells (*6, 26–31*). However, they have failed to transform primary cultures or senescent cells (*32–34*). In our studies, we observed the appearance of the oncogene in fully neoplastic guinea pig cells after several passages in culture. Thus, if the oncogene became activated immediately after exposure to the carcinogen, some biological event, such as acquisition of immortality (*32–34*), ought to occur before the transforming gene can be phenotypically expressed. Alternatively, oncogene activation may represent a well-defined step within the carcinogenic process, which is triggered but not directly caused by the initiating carcinogenic event. Isolation and characterization of the *ras* oncogene specifically activated in carcinogen-treated, tumorigenic guinea pig cells may provide the necessary tool to investigate, at the molecular level, the mechanisms involved in oncogene activation and its role in the multistep process of carcinogenesis.

SARASWATI SUKUMAR
SIMONETTA PULCIANI
Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Maryland 20205
JAY DONIGER
JOSEPH A. DIPAOLO
CHARLES H. EVANS
Laboratory of Biology, National Cancer Institute
BERTON ZBAR
Laboratory of Immunobiology, National Cancer Institute
MARIANO BARBACID
Laboratory of Cellular and Molecular Biology

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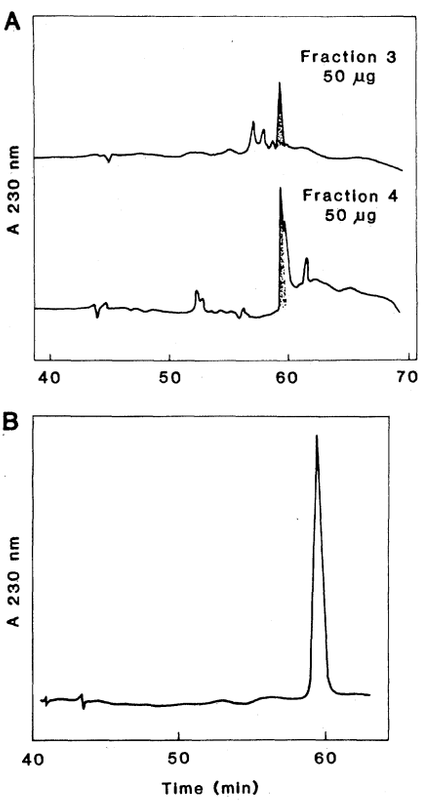
Isolation, Structure, and Synthesis of a Human Seminal Plasma Peptide with Inhibin-Like Activity

Abstract. *A basic peptide isolated from pooled human seminal plasma exhibited inhibin-like activity by suppressing pituitary follicle-stimulating hormone secretion in vitro and in vivo. The peptide has been characterized and sequenced, and a 31-amino-acid synthetic replicate showed full biological activity in vitro.*

A substance called "inhibin," believed to be present in aqueous extracts of testis, was imputed (*1*) to have an important if not exclusive role in the secretion of pituitary follicle-stimulating hormone (FSH) in the male. An ovarian nonsteroidal component also exerts a similar effect in the female (*2*) and participates in the regulation of cyclical events in the hypothalamo-pituitary-gonadal axis (*3, 4*). Since the name was proposed half a century ago (*1*), activity resembling that of inhibin has been detected in various gonadal and related fluids from at least six species (*5*). Peptide or proteinaceous extracts of testis, seminal plasma, spermatozoa, rete-testis fluid, testicular lymph, ovary, ovarian follicular fluid, and culture media of granulosa and Sertoli cells have been reported to contain a factor (or factors) (*3–8*) that inhibit pituitary FSH secretion and that

can be assessed by direct and indirect methods. However, the active substance from any source has not yet been identified. We now present evidence that a basic peptide isolated from human seminal plasma exerts inhibin-like activity; this peptide has been characterized and subjected to structural analyses and has been synthesized chemically.

Inhibin-like activity in human seminal plasma is associated with fractions of differing molecular weights (> 100,000, 45,000, and 15,000) (*9, 10*), including smaller peptides (~ 5000 daltons) (*11, 12*). The smaller peptide was isolated from ethanolic powders of sperm-free extracts of human ejaculates by a combination of procedures (*12*) including ion-exchange chromatography (Sulphopropyl-Sephadex C50 and DEAE-Sephadex A25), gel filtration (Sephadex G-50), and successive high-performance liquid chromatography (HPLC) (Waters C18) micro-Bondapak column). Figure 1A shows the HPLC separation of the material extracted from the gel filtration column into several discrete fractions, one of which showed inhibin-like activity when assessed by luteinizing hormone–releasing hormone (LHRH) challenge



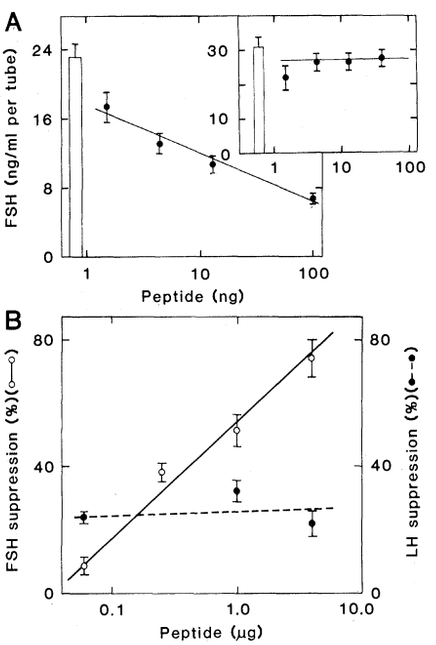


Fig. 1 (left). (A) High-performance liquid chromatography of human seminal plasma fractions 3 and 4 eluted from the gel filtration column (*12*). Since we were interested in investigating the smaller peptide, attention was focused on fraction 4. A 50-μg equivalent of the fraction was injected into a HPLC column equilibrated with 0.05 percent trifluoroacetic acid. Acetonitrile gradient was established in a stepwise fashion from 5 to 17 percent. At 17 percent acetonitrile, the flow rate was reduced to 0.8 ml/min. Absorbance was recorded at 230 nm (0.2 absorbance units full-scale setting). The shaded area showed inhibin-like activity in the short-term incubation assay on whole mouse pituitary in vitro (*13*). (B) The rerun of the peptide from fraction 4 collected from the above runs. Fig. 2 (right). Action of human seminal plasma inhibin-like peptide derived from fraction 4 on gonadotropin release by mouse pituitary (*13*) (open bars represent control values). Whole pituitaries from 21-day-old mice were first incubated with the peptide for 60 minutes in 0.5 ml of Dulbecco-modified Eagle's medium, and 3 ng of synthetic LHRH in 0.5 ml of the medium was then added. The incubation at 37°C was continued for three more hours in an O_2/CO_2 (19:1 by volume) atmosphere. The FSH and LH released into the medium was measured by specific radioreceptor assays (*13*). Highly purified ovine FSH and LH prepared in our laboratory were used as standards in the respective radioreceptor assays (N = 5 per group). (A) Cumulative data [mean ± standard error of the mean (S.E.M.) from ten experiments showing suppression of FSH release (slope = −0.177, Y-intercept = 3.14, r = −0.986; Student's t test). (Inset) Effect on LH release in the same incubations (slope = 0.209, Y-intercept = −4.48, r = 0.870). LH release was not significantly suppressed ($P > 0.05$) at any dose. The seminal plasma peptide alone had no effect on the specific FSH and LH radioreceptor assays or on the integrity of the LHRH. Suppression of FSH release cannot be accounted for by destruction of either FSH or LHRH (*13*). (B) Effect of human seminal plasma inhibin-like peptide on FSH and LH release in castrated male rats (FSH: slope = 193.9, Y-intercept = −1.541, r = 0.992; LH: slope = 345.3, Y-intercept = −0.57, r = 0.24). The rats were castrated on day 34 (N = 5 per group), and 0.2-ml subcutaneous injections were administered soon after the animals recovered. Two additional injections were administered after 10 and 24 hours, and blood was drawn after 30 hours. Animals in the control group received an equivalent volume of 0.5 percent gelatin in 0.9 percent saline. The total dose of the peptide administered is shown in micrograms. The amounts of FSH and LH in the serum was estimated by radioimmunoassay (*13*). The FSH and LH values in the intact control group were 2.4 ± 0.3 and 7.5 ± 0.5 ng/ml (mean ± S.E.M.), respectively; those in the castrated control group were 6.5 ± 1.6 and 64.3 ± 7.2 ng/ml, respectively. Percent suppression was calculated by comparing the hormone values in the treated animals with these values; the marginal LH suppression, which was independent of dose, was not significant ($P > 0.1$).

	1					5					10					
Inhibin-like peptide	HIS	- ASN	- LYS	- GLN	-	GLU	- GLY	-	ARG	- ASP	- HIS	-	ASP	- LYS	- SER	- LYS	- GLY	-
Baker's yeast enolase	HIS	- GLU	- ALA	- LEU	-	GLU	- MET	-	ARG	- ASP	- GLY	-	ASP	- LYS	- SER	- LYS	- TRP	-
Yeast enolase	HIS	- GLU	- ALA	- LEU	-	GLU	- MET	-	ARG	- ASP	- GLU	-	ASP	- LYS	- SER	- LYS	- TRP	-

	15					20					25					
Inhibin-like peptide	HIS	- PHE	- HIS	- ARG	-	VAL	- VAL	- ILE	- HIS	- HIS	-	LYS	- GLY	- GLY	- LYS	- ALA	-
Baker's yeast enolase	MET	- GLY	- LYS	- GLY	-	VAL	- LEU	- HIS	- ALA	- VAL	-	LYS	- ASN	- VAL	- ASN	- ASP	-
Yeast enolase	MET	- GLY	- LYS	- GLY	-	VAL	- MET	- ASN	- ALA	- VAL	- ASN	- ASN	- VAL	- ASN	- ASN	-	

	30		31
Inhibin-like peptide	HIS	- ARG	- GLY
Baker's yeast enolase	VAL	- ILE	- ALA
Yeast enolase	VAL	- ILE	- ALA

Fig. 3. Amino acid sequence of inhibin-like peptide derived from human seminal plasma. The sequence of the first 31 amino acids obtained in three runs is shown. Phenylthiohydantoins obtained at each cycle were identified by HPLC (*14*). The carboxyl terminal sequence could not be identified because yields dropped after the 31st residue; digestion with carboxypeptidase Y also yielded equivocal results. The amino acid composition (molar ratio with Ala taken as one residue) of the peptide is Lys_5 His_7 Arg_3 Asp_4 Ser_{1-2} Glu_{2-3} Gly_6 Ala_1 Val_2 Ile_1 Phe_1. The structural homology between the peptide and two other proteins is also shown (blocked regions). The homology is based on a computer data bank search (*17*) which showed that, out of 2,145 sequences and about 365,573 30-residue segments of proteins, only Baker's yeast (*18*) and yeast enolases (*19*) exhibited any significant homology. The sequence proposed for the human seminal plasma inhibin-like peptide is therefore a new structure.

test in 21-day-old whole mouse pituitaries incubated in vitro (*13*). The purity of the fraction was verified by rechromatography on HPLC (Fig. 1B) as well as by polyacrylamide gel electrophoresis at *p*H 4.5. The basic nature of the material is suggested by its behavior during ion-exchange chromatography and rapid migration on gel electrophoresis at *p*H 4.5.

Incubation of whole mouse pituitaries (*13*) with small amounts of the purified peptide inhibited the subsequent action of LHRH. Its action was dose-dependent and preferential (Fig. 2A) in that the secretion of FSH but not luteinizing hormone (LH) was significantly inhibited. The purified peptide has no effect on the basal release of FSH in these 4-hour incubations.

When injected into 34-day-old castrated male rats, the peptide inhibited the rise in circulating FSH levels that normally occurs within 24 hours. Serum LH was not suppressed at any dose of the peptide (Fig. 2B), an indication of the specific nature of the inhibition observed in the in vitro experiments (Fig. 2A).

Gel filtration on a calibrated column of Sephadex G-50 (superfine) implied an apparent molecular weight of 4000 to 5000 for the peptide with inhibin-like activity. Hydrolysis with 6*N* HCl and analysis for amino acids indicated that approximately 33 to 35 residues were present in the peptide (Fig. 3). The ultraviolet absorption spectrum of the peptide correlated with the amino acid composition; aromatic residues, which absorb at 280 nm, were not present. The observed high proportion of basic amino acids was also consistent with the mobility on electrophoresis at *p*H 4.5. Amino acid composition (see legend to Fig. 3) also indicated that carbohydrates (hexosamines) and half-cystine were not present, suggesting that the active substance was a simple linear polypeptide.

Automatic microsequencing was performed (*14*) on a Beckman 890C sequenator equipped with a sequemat P-6 autoconverter. The sequence of the first 31 amino acids (Fig. 3) could be identified unequivocally. We were unable to identify the residue at the carboxyl terminus or the sequence of amino acids at this end; however, by comparing the residues sequenced thus far with the available data (see Fig. 3), we believe that there may be only a few more amino acids near the carboxyl terminus. In all our attempts, we could not identify the amino acid residue beyond position 31 with certainty because of the sudden drop in the yield of phenylthiohydantoin amino acid. Further, digestion of the peptide with carboxypeptidase Y did not result in the identification of the carboxyl terminus, an experiment that suggests the presence of a resistant sequence.

The proposed 31-amino-acid partial structure of the peptide with inhibin-like activity does not bear resemblance to the structures of either of the human pituitary gonadotropins LH and FSH, or to urinary human chorionic gonadotropin (*15*) or LHRH (*16*). This accounts for the failure of the peptide to affect the specific receptors or radioimmunoassays of these highly purified hormones. However, a computer search with the protein sequence data bank (*17*) revealed that the sequence of the human seminal plasma peptide had some degree of structural homology (about 30 percent) with yeast enolase, which has been analyzed (*18, 19*) (Fig. 3). Similar homologies have been found in other instances, such as mammalian LHRH compared to yeast α-mating factor; mammalian corticotropin-releasing factor compared to the frog skin peptide sauvagine and fish urotensin; and human platelet–derived growth factor compared to sarcoma virus gene products (*20*).

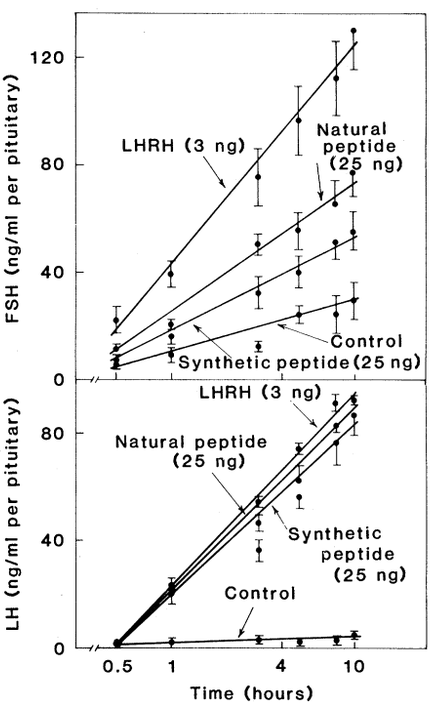


Fig. 4. Effect of human seminal plasma inhibin-like peptide (HPLC fraction, see Fig. 1B) and a synthetic replicate of the proposed structure (Fig. 3) on the cumulative release of FSH and LH by mouse pituitary in vitro (values are mean ± S.E.M.). The individual pituitaries ($N = 5$ per group) were exposed simultaneously to test materials (25 ng per pituitary) and LHRH (3 ng per pituitary). At the indicated time intervals, the spent medium was removed and replaced with fresh medium containing the test substance and LHRH. All the samples were stored at −20°C immediately after the incubation until the gonadotropin radioreceptor assays were performed. FSH release in the groups exposed to the peptide was significantly lower than LHRH control ($P < 0.01$ or 0.005) at each time interval. The LH values in these same solutions were not significantly different.

In order to explore the possibility that the sequence we determined contains portions of the molecule responsible for biological activity, synthetic investigations were undertaken (*21*). A synthetic replicate of the 31-amino-acid sequence prepared by the solid-phase method showed full biological activity in vitro. An example of one of these tests is shown in Fig. 4. The synthetic peptide inhibited the release of FSH induced by LHRH but had no such effects on the release of LH in the same incubations. From these data we concluded that the carboxyl terminal of the peptide beyond residue 31 may not be crucial for biological activity in vitro.

The consistent specific inhibition of FSH release (Figs. 2 and 4) leads to the question of whether or not this inhibin-like peptide behaves as an FSH-releasing hormone antagonist; however, the existence of a separate FSH-releasing hormone is still debated. The molecule has not yet been isolated as a distinct chemical entity from the hypothalamus or other tissues.

K. RAMASHARMA
M. R. SAIRAM*
N. G. SEIDAH
M. CHRÉTIEN
P. MANJUNATH
P. W. SCHILLER
Reproduction Research Laboratory, Protein-Pituitary Hormone Laboratory, and *Laboratory of Chemical Biology, Clinical Research Institute of Montreal, Quebec H2W 1R7, Canada*
D. YAMASHIRO
CHOH HAO LI
Laboratory of Molecular Endocrinology, University of California, San Francisco 94122

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* To whom correspondence should be directed.

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6 January 1984

Health Effects of Dioxin

The evidence of deleterious health consequences from the environmental use of substances containing 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has appeared in a multitude of studies. Philip H. Abelson's editorial (*1*) on the dioxin issue is based on a number of misleading inaccuracies about this evidence.

First, the results of the accident at Seveso, Italy, are not limited to mild cases of chloracne, despite Abelson's statement that "No significant change was observed in the incidence of spontaneous abortions, congenital malformations, or postnatal development." The Seveso data (*2*) show a sharp increase in spontaneous abortions during the first trimester of 1977, followed by a slow decrease to 1976 levels and significant increases in risk of malformations. For example, there was a 100 percent increase in the rate of spina bifida, a 71 percent increase in the rate of neural tube defects, an elevenfold increase in hypospadias, and a 110 percent increase in polydactyly. A number of these malformations are frequently observed in animals exposed to TCDD. The Seveso data are still being analyzed for postnatal effects.

Second, the National Institute of Occupational Safety and Health (*3*) and recently the Environmental Protection Agency (*4*) followed workers exposed to TCDD in industrial accidents and found, in sharp disagreement with scientists who analyzed that data for industry, a multiple increase in soft tissue carcinomas and lymphomas.

Third, the question is not whether TCDD has to be ingested before it is toxic (obviously it has to make effective contact) but whether there is an effect from the presence of elevated environmental levels of TCDD, especially as a result of herbicide spraying (*5*). There is ample evidence that the latter is the case. Multiple studies by Swedish investigators, notably, Axelson and Sundell (*6*) and Hardell and Erikson and their colleagues (*7*) show an increase in soft tissue carcinomas in railway and forestry workers exposed to environmental TCDD. Observations from Vietnam (*8*) have reaffirmed increased liver cancer among populations exposed to Agent Orange during the Vietnam War (*8*). Spontaneous abortions, stillbirths, and malformations are still reported in these areas (*9*).

Fourth, it is misleading to stress the great variability of the median lethal dose (LD_{50}) when commenting on the value of animal experiments. It would be more accurate to point to the uniformly low effective doses for producing carcinogenic and teratogenic effects (*10*).

THEODOR D. STERLING
Faculty of Interdisciplinary Studies, Department of Computer Science, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6

References and Notes

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2. P. Bruzzi *et al.*, "Birth defects in TCDD polluted areas" (Provisional Report, Seveso Birth Defects Registry, Seveso, Italy, 1981); *Reporto Conclusino Sui Defetti Congeniti Ed Altri Esiti Sfavorevoli Di Gravidanza Relaveti Nella Popolazione Del L'Area Di Seveso Interasata Dall' Inquinamento Da TCDD IL 10.7.1976* (Registro Delle Malformazioni, Seveso, 28 June 1983).

Pro and con evidence and differing points of view on questions concerning the toxicity of dioxin are cited in a large number of articles and books. Three publications that contain a total of hundreds of references are

1) *Further Review of the Safety for Use in the U.K. of the Herbicide 2,4,5-T* from the Advisory Committee on Pesticides, London, December 1980;

2) *Agent Orange Dioxin—The Health Effects of "Agent Orange" and Polychlorinated Dioxin Contaminants*, a technical report prepared by the Council on Scientific Affairs of the Advisory Panel on Toxic Substances of the American Medical Association, Chicago, Illinois, October 1981; and

3) *Human and Environmental Risks of Chlorinated Dioxins and Related Compounds*, edited by Richard E. Tucker, Alvin L. Young, and Allan P. Gray (Plenum Press, New York, 1983).

As Sterling points out, a number of investigators have taken the position that TCDD has been a causative agent of soft tissue sarcomas. However, to other experts, the evidence is not compelling.

The Agent Orange report includes the following statement (p. 28): "While 2,4,5,-T and 2,4-D pesticides (phenoxy herbicides in Agent Orange) have been used in agriculture, forest management, and residential landscaping for over 30 years, there is still no conclusive evidence that they and/or TCDD (a contaminant of Agent Orange) are mutagenic, carcinogenic, or teratogenic in man, nor that they have caused reproductive difficulties in the human."

—PHILIP H. ABELSON

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