Additional derived cryptodiran characters are ventral process of the prefrontal contacting vomer; supramarginal scutes absent; carapacial caudal notch absent; and 12th marginal scutes in mutual contact.

7. In addition to the presence of a cryptodiran processus trochlearis oticum, *Kayentachelys* also has two other hypothesized cryptodiran synapomorphies. The posteriorly directed processus pterygoideus externus with a vertical plate laterally is absent in *Proganochelys* and occurs only in *Kayentachelys* and other cryptodires. The vomer-prefrontal contact, present in *Kayentachelys* and other cryptodires is apparently absent in *Proganochelys*, although the area is poorly preserved and not definitely determinable.
8. G. H. Schumacher, in *Biology of the Reptilia*, C. Gans and T. Parsons, Eds. (Academic Press, New York, 1973), vol. 4, pp. 101–199.
9. The presence of frogs and other amphibians in the fauna together with the occurrence of limestone lenses and ripple marks at various levels within the formation is corroborative evidence of local aquatic conditions during Kayenta time.
10. The Selmacryptodira (Fig. 2) is a new term for the monophyletic group defined by the pterygoid process flooring the middle ear (*selma*, Greek for floor) and consisting at present of all cryptodires except *Kayentachelys*. For those using the classification of E. S. Gaffney (*5*) this requires the addition of a new category, the Capaxorder (*capax*, Greek for large) to be inserted between megaorder and hyperorder. The resultant higher classification of cryptodires would then be

Megaorder Cryptodira
  Capaxorder Kayentachelydia
    Family Kayentachelyidae
  Capaxorder Selmacryptodira
    Hyperorder Pleurosternoidea
    Hyperorder Daiocryptodira

11. We thank C. R. Schaff, W. W. Amaral, K. K. Smith, K. Padian, T. B. Rowe, J. M. Clarke and other members of the Harvard and Berkeley field teams for collection of specimens; W. W. Amaral, O. Simonis, K. Kishi, and A. Burke for preparation; F. Ippolito for illustration of the shell; and P. Meylan and E. E. Williams for thoughtful criticism and advice. We thank the Navajo Tribal Council and the Coal Mine Mesa Chapter (T. T. Nez, president) for permission to conduct paleontological exploration on Navajo land, and the National Geographic Society for support of the fieldwork. Also supported in part by NSF grants DEB 8002885 and BSR 8314816 to E.S.G. and DEB 78-01327 to F.A.J.

24 February 1987; accepted 11 May 1987

Reversible Inhibition of Mammary Gland Growth by Transforming Growth Factor–β

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Transforming growth factor–β (TGF-β) can stimulate or inhibit growth of cells in vitro, as well as induce the transformed phenotype. Although widely distributed in animal tissue, the effects of TGF-β in vivo are largely unknown, and a physiological role for the peptide hormone has not been demonstrated. The effect of TGF-β on developing epithelial tissue in situ was studied by using slow-release plastic pellets containing TGF-β to treat developing mouse mammary gland. Powerful inhibition of mammary growth and morphogenesis was observed. This growth-inhibited mammary tissue was histologically normal, and the inhibitory effect was fully reversible. Under the conditions of these experiments, TGF-β displayed many of the characteristics expected of a physiologically active growth-regulatory molecule.

THE PEPTIDE GROWTH FACTOR called transforming growth factor–β (TGF-β) is found in cultured normal cells from connective tissues, various epithelia, and the immune system (*1–4*) and has been shown to inhibit proliferation of primary or secondary cultures of epithelial cells. The cell types affected include endothelium (*5*), bronchial epithelial cells (*6, 7*), mammary cells (*6, 8*), and hepatocytes (*9*). In some cases, inhibition is associated with terminal differentiation (*7*), while in others the inhibition is fully reversible (*10*).

Although the effects of TGF-β on cells and tissues in vivo are poorly understood, the wide distribution of both the peptide and its high-affinity receptors suggests that this peptide has a physiologic role (*3, 11*). The only known effects of TGF-β on tissues are rapid induction of fibrosis and angiogenesis when the factor is administered subcutaneously in wound chambers (*12*) or by injection (*13*). There have been no reports of TGF-β effects in vivo on any epithelial tissue, nor are there any examples of negative control by TGF-β in animals.

The effects of TGF-β on ductal growth in virgin mice were investigated with ethylene vinyl acetate copolymer (EVAc) implants that allow the slow release of bioactive molecules to small zones of the mammary gland (*14*). Implants containing TGF-β were placed in front of the mammary end buds (Fig. 1A, solid arrows), which are the focus for regulatory influences on ductal growth in the glands of the subadult virgin (*15*). End buds are bulbous, highly mitotic, multilayered epithelial structures found only during active growth; the numbers of end buds reliably indicate the rate of enlargement of the ductal tree (*16*). As an end bud approaches the edge of the gland's fatty stroma or is confined by ductal epithelium, forward growth ceases and the bulbous tip shrinks to the size of the duct (Figs. 1 and 2).

Human platelet–derived TGF-β, implanted for 4 days (Fig. 1B), caused the complete inhibition of end buds; a control implant containing bovine serum albumin (BSA) had no effect on the contralateral gland (Fig. 1A). Both treatment and control implants were placed in approximately the same location with respect to the growth zone, and it is clear that ducts in the treated gland did not elongate. TGF-β inhibition was limited to the implanted gland, with no observable perturbation of either the contralateral or ipsilateral glands, indicating that the factor was acting directly on the gland and not through a systemic intermediary. Thus TGF-β, acting locally, can inhibit the growth and morphogenesis of normal mammary epithelial tissue.

The implant contained 543 ng of TGF-β mixed with BSA as a carrier; the latter, as the major protein component of the implant, determined the release kinetics for TGF-β (*17*). From previous experience with the release of peptides from implants of this type (*18*), we estimate the TGF-β concentration in a 1-ml hypothetical volume around the implant to range from 8 ng/hour during the first 24 hours down to 1.3 ng/hour during the next 2 days. This range is comparable to TGF-β concentrations shown to affect cells in vitro (*2, 7, 10*).

Thymidine autoradiography was used to investigate the effects of TGF-β on glandular DNA synthesis. Growing end buds had numerous labeled cells (Fig. 2A), and previous studies have shown similar structures to have labeling indices of 15 to 20% (*19*). TGF-β treatment (Fig. 2C) reduced epithelial DNA synthesis to levels virtually identical with those seen in untreated, growth-quiescent ducts (Fig. 2B and Table 1). Stromal DNA synthesis normally accompanies ductal growth and is reduced in growth-quiescent glands (*20*). Levels of stromal DNA synthesis in TGF-β–treated glands were similar to those seen around terminal ducts from control tissue (Fig. 2) and in both cases were somewhat lower than in stroma around growing end buds (Table 1).

To investigate the reversibility of TGF-β–induced inhibition, we treated glands for 4 days to induce inhibition, after which the implant was surgically removed. Eleven days after TGF-β removal, end bud growth in the treated and contralateral control glands was determined. At 4 days, TGF-β had reduced end bud number by about 75% (Table 2). After the 11-day recovery period, end bud numbers in the treatment and control glands were the same, demonstrating complete recovery from inhibition. The distance that TGF-β–treated ducts grew beyond the implant is also indicative of growth inhibition and its subsequent recision. In 11 days after

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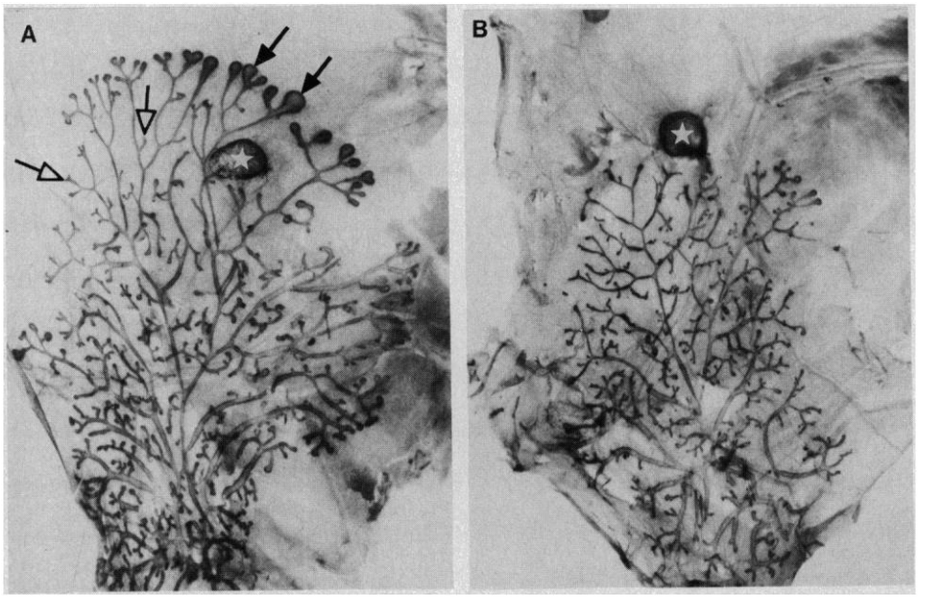

Fig. 1. Photomicrograph illustrating the effect of TGF-β on ductal growth. Five-week-old, C57 female mice were treated for 4 days with an EVAc implant (*26*) containing 543 ng of TGF-β. (**A**) Control. Arrows point to end buds at the ductal growth front which have grown around and past a BSA-containing implant (☆). Blunt-tipped, growth-quiescent, terminal ducts (open arrows) can be seen on either side of the end bud array and are also prominent in the middle of the gland where end bud growth is normally restricted (×5). (**B**) Treatment (gland is contralateral to the pictured control). EVAc implant containing TGF-β (☆) caused regression of end buds and inhibition of ductal growth (×5).

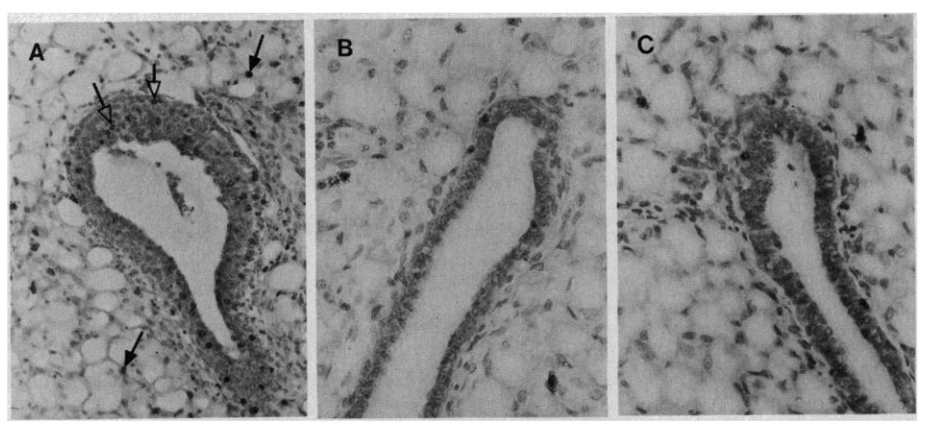

Fig. 2. [^{3}H]Thymidine autoradiographs illustrating the effects of TGF-β on glandular DNA synthesis. (**A**) End bud from control gland shows considerable labeling in both epithelium (open arrows) and stroma (solid arrows) (×190). (**B**) Terminal duct from control gland (×230). (**C**) Terminal duct from gland treated with 300 ng of TGF-β for 4 days (×230). The technique for preparing thymidine autoradiographs with mouse mammary tissue has been described in detail elsewhere (*18*).

Table 1. Effect of TGF-β on mammary gland DNA synthesis. For epithelium, the data are expressed as percentage of cells labeled ± SD. The labeling index for end buds was determined with tissue from four animals (*19*). Labeling indices for terminal ducts were determined by counting cells in alternate 5-μm sections from three representative ducts. A total of six sections and 416 and 610 cells in tissue from a single animal were counted for control and treatment ducts, respectively. For the determination of both epithelial and stromal labeling, glands were treated for 4 days with 300 ng of TGF-β. For stroma, the data are expressed as the average number of labeled cells ± SD. The stroma's cellular heterogeneity makes the determination of a labeling index difficult and of questionable meaning. For this reason the range of stromal DNA synthetic activity was estimated by counting labeled stromal cells in a standard field of 0.39 mm^2 (×20 magnification, alternate 5-μm sections). The percentage of the field occupied by epithelium was estimated, and the reported values were normalized to 100% stroma. Stromal labeling in tissue from two untreated animals was estimated around two end buds of typical morphology and labeling pattern; 13 alternate sections were scored and 776 labeled cells were counted. Labeling around terminal ducts was likewise estimated by scoring 14 sections in two glands from a single animal for treated tissue and seven sections in a single gland from a single animal for the control tissue.

Tissue	Labeled cells
Epithelium	
End buds	17.7 ± 5.4
Terminal ducts	
Treated	0.3 ± 1.1
Control	0.7 ± 0.5
Stroma, in vicinity of	
End buds	59.7 ± 15.5
Terminal ducts	
Treated	32.1 ± 9.6
Control	41.4 ± 7.8

implant removal, treated glands grew one-third the distance of the controls.

Ductal growth inhibition is a normal feature of glandular development and, by several criteria, TGF-β–inhibited ducts closely resembled growth-quiescent ducts in untreated glands (Figs. 1 and 2). Both TGF-β–treated and normal terminal ducts were blunt-tipped, thin-walled structures surrounded by a fibrous connective tissue tunic. In addition, the patterns and level of epithelial and stromal DNA synthesis were similar in treated and control tissue (Fig. 2 and Table 1). The apparent normalcy of this histological picture was underscored by the lack of obvious TGF-β–induced tissue disruption or cytotoxicity (the latter judged by the absence of pynotic nuclei in the epithelium). Inhibition was reversible and was accompanied not only by the structural reorganization of terminal ducts into end buds but also the resumption of ductal elongation (Table 2). This evaluation suggests that implanted TGF-β is either mimicking a natural, negative growth regulator or is itself such a regulator.

Although in culture TGF-β can be mitogenic either by itself or in combination with epidermal growth factor (EGF) or TGF-α, effects on the mammary gland were consistently inhibitory. Single implants, in place for 26 days in the glands of hormonally intact animals, did not stimulate either hyperplastic or dysplastic outgrowths, nor was secretory differentiation stimulated. Because only a single implant was used in each mouse, we cannot now comment on the effects of continued exposure to higher levels. TGF-β was also implanted to the regressed glands of ovariectomized animals and did not stimulate either end bud or secretory differentiation, nor did it synergize with implanted EGF. These results suggest that the effects of TGF-β on the mammary gland are limited to growth inhibition.

The mechanism of the inhibitory action of TGF-β on mammary ductal growth can only be a subject for speculation, but two ideas are suggested by our current understanding of mouse mammary growth regulation and by the known effects of TGF-β in other systems. TGF-β antagonizes the mitogenic effects of other peptide growth factors (*3*), some of which are thought to be mammogenic (*21*). Therefore it is not unreasonable

Table 2. Reversibility of TGF-β inhibition of mammary ductal growth. Ten 4-week-old C57 female mice were implanted with 200 ng of TGF-β in the number 3 and 4 glands on the right-hand side of the animal and with BSA-containing implants in the contralateral glands. At 4 days, five animals were killed, and glands were scored for end bud number and growth. TGF-β implants were surgically removed from the other five animals and replaced with a marker implant containing India ink particles to facilitate later estimation of growth. Values are shown with 95% confidence intervals.

Treatment	Number of end buds per gland	Distance grown (mm)
4 Days		
TGF-β	2.64 ± 2.3	0
Control	9.90 ± 3.0	0.98 ± 0.73
15 Days		
TGF-β (implant removed at day 4)	14.9 ± 2.6	2.2 ± 1.1
Control	16.1 ± 3.1	5.8 ± 1.5

to suggest that the effects of implanted TGF-β reflect interference with unidentified mammogenic peptides. In addition, TGF-β can stimulate a fibrotic response in vivo (*13*) and collagen (*22*) and fibronectin (*23*) synthesis in vitro. Normal inhibition of ductal growth is accompanied by the progressive envelopment of the end bud by a collagen-rich (*24*) and fibronectin-rich (*25*) tunic, a process that may in itself inhibit growth and that may be accelerated by implanted TGF-β.

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26. To prepare slow-release implants, we mixed human platelet TGF-β, purified by high-performance liquid chromatography (HPLC), with 15 mg of BSA dissolved in 0.25 ml of distilled water and lyophilized the mixture. The lyophilized material was mixed with 0.125 ml of a 20% solution of EVAc in dichloromethane; the mixture was quick-frozen in acetone–dry ice; and the resulting pellet was dried. Pellets typically weighed about 35 mg and implants weighed between 0.5 and 1 mg. Implants prepared with the acetonitrile–trifluoroacetic and HPLC carrier plus BSA had no effect on ductal growth.

27. We thank A. Roberts and M. Sporn for their gift of TGF-β, DuPont for their gift of EVAc (Elvax 40P), and P. Strickland for helpful discussions concerning this work. Supported by PHS grant 01050 from the National Institute on Aging and by NSF grant PCM83-0814.

18 February 1987; accepted 11 May 1987

Functional Analysis of a Complementary DNA for the 50-Kilodalton Subunit of Calmodulin Kinase II

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The calcium-calmodulin–dependent protein kinase II is a major component of brain synaptic junctions and has been proposed to play a variety of important roles in brain function. A complementary DNA representing a portion of the smaller 50-kilodalton subunit of the rat brain enzyme has been cloned and sequenced. The calmodulin-binding region has been identified and a synthetic analog prepared that binds calmodulin with high affinity in the presence of calcium. Like the 50-kilodalton kinase polypeptide, the concentration of the messenger RNA varies both neuroanatomically and during postnatal development of the brain. The broad tissue and species cross-reactivity of the complementary DNA suggests that the 50-kilodalton subunit found in rat brain is evolutionarily conserved and is the product of a single gene.

CALCIUM-CALMODULIN–DEPENDENT protein kinase II (CaM-KII) belongs to a family of enzymes known as Ca^{2+}- and calmodulin-dependent multifunctional protein kinases because of their broad substrate specificity (*1, 2*). First detected in mammalian brain (*3*), enzymes closely related to CaM-KII are present in many vertebrate tissues and have also been found in a variety of invertebrate organisms (*1, 2, 4–6*). The CaM-KII enzyme has been proposed to play a role in synaptic vesicle translocation and neurotransmitter release in neural tissue (*5*), neuronal modulation in *Aplysia* (*4*), and visual adaptation in *Drosophila* (*6*). In rat forebrain, the enzyme is concentrated at asymmetric synaptic junctions where it comprises a large part of the submembranous structure called the postsynaptic density (*7*). Whereas the native molecular weight of CaM-KII is 650,000 to 750,000, it is an oligomer of two subunits with molecular weights of 50,000 and 60,000, respectively. The stoichiometry of subunits varies between brain regions (*8*). The two subunits are related, since they share antigenic determinants and common tryptic and chymotryptic peptides (*7*). The Ca^{2+}-bound form of calmodulin (Ca^{2+}-calmodulin) interacts with and promotes autophosphorylation of each subunit and is required to activate the enzyme (*1, 2, 9, 10*). Once autophosphorylated, CaM-KII no longer requires Ca^{2+} or calmodulin for activity (*11*). These observations imply a functional interaction between autophosphorylation and calmodulin-binding domains. However, in molecular terms, the precise relations between the subunits and their functional domains are poorly understood.

We have used antibodies to obtain and identify complementary DNA (cDNA) clones encoding a large part of the 50-kD subunit of rat brain CaM-KII. Partial re-

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