Cell Lines of the Pituitary Gonadotrope Lineage Derived by Targeted Oncogenesis in Transgenic Mice

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Study of the molecular and cellular biology of the gonadotropin hormones would be greatly facilitated by the availability of immortalized anterior pituitary gonadotrope cell lines. We directed expression of the simian virus-40 (SV40) T-antigen (Tag) oncogene to specific cells in the anterior pituitary of transgenic mice using the promoter/enhancer region from the human glycoprotein hormone α-subunit gene. Transgenic mice carrying this fusion gene developed anterior pituitary tumors. Clonal cell lines established from these tumors express the endogenous mouse α-subunit gene and synthesize and secrete α-subunit protein. However, they do not express β-subunit genes. Alpha-subunit mRNA is induced by GnRH in a dose- and time-dependent manner, but is not regulated by TRH. Thus, we have targeted tumorigenesis in transgenic mice to anterior pituitary cells of the gonadotrope lineage to immortalize this specific endocrine cell while maintaining several highly differentiated functions unique to gonadotropes. (Molecular Endocrinology 4: 597–603, 1990)

INTRODUCTION

Reproduction in mammals is regulated by two anterior pituitary hormones, the gonadotropins LH and FSH, which are produced in a subset of anterior pituitary cells, the gonadotropes. These hormones are members of a larger family, the glycoprotein hormones, which also includes TSH, produced in pituitary thyrotropes, and CG, produced in the placenta of primates, but not rodents. Each of these hormones is a heterodimer, sharing a common α-subunit, but having a unique β-subunit (1).

Studies of the regulation of expression of these hormones has been hampered by the lack of established gonadotrope cell culture systems. Thus, molecular analysis of LH and FSH regulation has depended largely on primary pituitary cultures, which have limited viability and show progressive loss of function (2). Such studies are further complicated by the cellular heterogeneity of the anterior pituitary, which, in addition to gonadotropes and thyrotropes, consists of endocrine cells producing GH, PRL, and FOMC as well as nonendocrine endothelial cells, folliculo-stellate cells, and stromal cells (3).

The ability to target expression of oncogenes to specific cell types in transgenic mice provides a method for immortalizing rare cell types (4). We have used this approach to direct expression of the simian virus-40 T-antigen (SV40 Tag) oncogene to specific pituitary cells by driving its expression with the promoter and associated regulatory domains of the human glycoprotein hormone α-subunit gene (5). Transgenic mice bearing this fusion gene heritably develop pituitary tumors. These tumors have been cultured to develop clonal cell lines that maintain differentiated functions of gonadotropes, including glycoprotein hormone (α-subunit) synthesis and secretion, and responsiveness to GnRH.

RESULTS

Pituitary Tumorigenesis in Transgenic Mice

A fusion gene containing approximately 1.8 kilobases (kb) of 5’ flanking sequences of the human glycoprotein hormone α-subunit gene linked to the protein-coding sequences of the SV40 T-antigen oncogene (Fig. 1) was used to generate 17 founder transgenic mice. The α-subunit gene is expressed only in anterior pituitary gonadotrope and thyrotrope cells in rodents (6, 7), and thus, Tag-driven tumorigenesis was expected to be anterior pituitary specific (8). More than half of the founder mice developed pituitary tumors, and in only a
Fig. 1. Map of the α-Tag Plasmid

The α-Tag transgene contains the promoter region of the human glycoprotein hormone α-subunit gene from the EcoRI site at approximately −1.8 kb to the BamHI site at +40 (S) linked to the SV40 early region from the BglII site to the BamHI site (23). This SV40 fragment contains the protein-coding region for large T- and small t-antigens, including the translation initiation and transcription termination sites, but lacks the SV40 early promoter/enhancer. The transgene was excised from the plasmid vector by EcoRI/SalI digestion for microinjection, and thus contained 275 basepairs of pBR322 vector sequences at its 3’ terminus.

new instances were other tumors observed. Figure 2 demonstrates the tissue-specific expression of Tag in a representative founder animal, αT3, a male who was killed at 49 days of age and found to have a large encapsulated pituitary tumor. This tumor was also found to be expressing α-subunit mRNA, consistent with gonadotropic or thyrotropic origin.

Establishment of Pituitary Tumor Cell Lines

Cells from the tumor of αT3 as well as tumors from several other founder mice were dispersed and placed in culture. The initial cultures were very heterogeneous, including endocrine cells, fibroblasts, and blood cells. To enrich for the endocrine cells, we took advantage of the fact that fibroblasts attached readily to plastic culture dishes, while the endocrine cells attached more slowly and could be dislodged easily. Eventually, stable cultures were established from three independent tumors (from the αT2, αT3, and αT4 founder mice), and several clonal cell lines were derived from each by serial dilution (e.g. αT3–1 and αT3–2).

A direct correlation was observed between the rate of tumor development and the ability to establish cell lines. Cells from the tumor obtained from αT3 at 49 days of age were the most readily established, while cells of the tumors from αT2 and αT4, killed at 92 and 93 days of age, respectively, grew significantly more slowly and were more difficult to clone. We were unsuccessful at maintaining cells from the tumor of αT6, a mouse killed at 143 days of age. The αT3–1 and αT4–1 cell lines have been cultured continuously for approximately 2 yr without apparent change in phenotype, and can be frozen in liquid nitrogen and thawed successfully. They remain stably diploid. The cells double in culture every 30 h (αT3–1) or 20 h (αT4–1).

Analysis of α-Subunit and Tag Expression in Tumors and Cells

The abundance of Tag and α-subunit mRNAs in the uncloned cell populations and in clonal cell lines was compared to that seen in the original tumors (Fig. 3). In all cases the cells expressed higher levels of Tag mRNA than did the tumor from which they were derived. This is not surprising, since the tumors are heterogeneous in cellular composition, and the cells expressing the highest levels of Tag should have a growth advantage in culture and, thus, should be selected. Among the various cell lines there is a direct relationship between the levels of Tag and α-subunit mRNA, consistent with the fact that expression of both is driven from an α-subunit gene promoter. In addition, α-subunit protein is
synthesized and secreted by these cell lines. Secretion of \( \alpha \)-subunit from the \( \alpha T4-1 \) cell line was 140 ± 15.5 ng/plate·24 h, and the cells contain 15 ± 1.73 ng/plate (where one plate contains ~2 × 10⁶ cells), assayed by RIA.

All of the tumors examined expressed \( \alpha \)-subunit mRNA, with the exception of \( \alpha T-2 \). The cell lines derived from the \( \alpha T-2 \) tumor were also negative for \( \alpha \)-subunit mRNA. It is surprising that the exogenous \( \alpha \)-subunit promoter driving Tag expression can be active in a cell in which the endogenous \( \alpha \)-subunit gene is silent, suggesting that the oncogene has escaped the normal control acting on the endogenous gene.

**Characterization of Pituitary Hormone Gene Expression in \( \alpha T3-1 \) Cells**

Poly(A)⁺ mRNA was prepared from \( \alpha T3-1 \) cells and hybridized to cDNA probes for each of the pituitary hormones, including \( \alpha \)-subunit, the \( \beta \)-subunits of LH, FSH, and TSH, as well as GH, PRL, and POMC. Since the \( \alpha \)-subunit is expressed in both gonadotropes (as part of LH and FSH) and thyrotropes (as part of TSH), we expected to obtain cells representing one cell type or the other, or possibly a developmental precursor of the two. The patterns of \( \beta \)-subunit expression should distinguish between these possibilities. However, Fig. 4 demonstrates that \( \alpha T3-1 \) cells do not express any of the glycoprotein hormone \( \beta \)-subunit genes, consistent with these cells being precursors of gonadotropes and/or thyrotropes that express the \( \alpha \)-subunit but none of the \( \beta \)-subunit genes. The cells are also negative for expression of GH, PRL, and POMC, as would be expected since the \( \alpha \)-subunit gene is silent in the cell types that produce these hormones.

**Hormone Responsiveness of Various \( \alpha \)-Tag Cell Lines**

Both mRNA synthesis and secretion of LH and FSH are stimulated by GnRH, while TSH is regulated by TRH (9). A number of different cell lines derived from the \( \alpha T3 \) and \( \alpha T4 \) tumors were, therefore, tested for response of \( \alpha \)-subunit mRNA levels to either Nafarelin (a GnRH analog) or TRH (Fig. 5). The cell lines from both tumors fall into two classes: those that respond to GnRH by elevating \( \alpha \)-subunit mRNA levels, but do not respond to TRH, and those that do not respond to either hormone. Two of four \( \alpha T3 \) cell lines and two of three \( \alpha T4 \) cell lines responded to GnRH. In cell lines that responded, the endogenous mouse \( \alpha \)-subunit RNA and the \( \alpha \)-Tag transgene RNA (data not shown) were induced by 3- to 8-fold. No cell lines that responded to TRH were obtained. Since none of the cell lines derived from the \( \alpha T2 \) tumor express \( \alpha \)-subunit mRNA, cells were tested for GnRH responsiveness of the \( \alpha \)-Tag transgene mRNA. However, no response was observed (data not shown). Thus, at least some of the cell lines from the \( \alpha T3 \) and \( \alpha T4 \) tumors appear to be of the gonadotrope lineage, since they respond to GnRH but not TRH. Other cell lines derived from each of the three tumors cannot be classified on the basis of hormone responsiveness.

To further characterize the stimulation of \( \alpha \)-subunit mRNA levels by GnRH, a concentration-time curve and time course were performed using one of these cell lines, \( \alpha T3-1 \) (Fig. 6). A strong response was observed by 3 h and continued to increase through 48 h. The induction could be observed with as little as \( 10^{-9} \) M Nafarelin and increased with increasing concentrations of the analog. The increase at 24 h with \( 10^{-7} \) M Nafarelin was 8.6-fold. In addition, the response to Nafarelin was completely blocked by simultaneous addition of an equal concentration of a GnRH antagonist (0.1 mm; \([\text{Ac-d2Nle}-\text{d4CpHe}-\text{d3Pac}, \text{Arg}6,4-(\text{p-methoxybenzoyl)lo-2}-\text{aminobutyric acid}, \text{dAla}9] \text{GnRH} \); data not shown) (10). The fact that the GnRH response is dose dependent and is blocked by a specific antagonist is consistent with GnRH acting through the GnRH receptor. Thus, a number of these cell lines are of the gonadotrope lineage, since the presence of GnRH receptors represents a specialized function of pituitary gonadotropes.

**DISCUSSION**

Cultured cell lines that maintain specific differentiated phenotypes have been invaluable in the study of cell
Fig. 4. Analysis of Pituitary Hormone mRNA Expression in αT3-1 Cells

Five micrograms of poly(A)^+ RNA from αT3-1 cells and 10 μg total RNA from control female pituitaries were run in seven sets on a Northern gel and hybridized to probes derived from cDNA clones of each of the indicated pituitary hormones. The cDNA probes used were: mouse α-subunit (25), rat LHβ (6), rat FSHβ (26), mouse TSHβ (27), rat GH (28), rat PRL (29), and rat POMC (a genomic fragment containing the third exon) (30).

Fig. 5. GnRH and TRH Treatment of Various αT3 and αT4 Cell Lines

Cells were incubated for 16 h without hormone or with 0.1 mM Nafarelin (a GnRH analog; see Materials and Methods) or 0.1 mM TRH. Total RNA was prepared, and the following amounts were run on a Northern gel: αT3-6, 5 μg; αT3-1, 10 μg; αT3-3, 10 μg; αT4-2, 5 μg; and αT4-3, 10 μg. The RNAs were then hybridized to the α-subunit cDNA probe.

Fig. 6. Time Course and Concentration Curve for Response of αT3-1 Cells to GnRH

αT4-1 cells were incubated with the indicated concentration of Nafarelin or TRH, second lane, for the indicated length of time. Total RNA was prepared, and 20 μg of each sample were run on a Northern gel. The RNAs were first hybridized to the α-subunit cDNA probe, then the filter was washed, and they were rehybridized to a histone probe (31) to control for the amount of RNA loaded. Results correlate approximately with those found by Huber et al. (32) for rat primary pituitary cell cultures.

The endocrinology and molecular biology of the anterior pituitary hormones GH, PRL, and POMC have been substantially advanced due to the development of the somatotropic/lactotrope ACTH-20 cell line (11, 12) and the corticotrope ACTH-20 cell line (13, 14). In contrast, study of the regulation and synthesis of the pituitary glycoprotein hormones has been impeded by the lack of analogous cell lines representing gonadotropes or thyrotropes. We have used targeted oncogenesis in transgenic mice to transform this subset of pituitary cells and have established clonal cell lines from the resulting tumors. Tag expression was driven by the 5' flanking region of the human glycoprotein hormone α-subunit gene, which is normally expressed in both gonadotropes and thyrotropes. Thus, we anticipated that the resulting cell lines would represent gonadotropes, thyrotropes, or a precursor cell in that lineage. Most of the cell lines expressed the glycoprotein hormone α-subunit, but none of the lines expressed any of the β-subunits.

One explanation for the absence of β-subunit expression is that precursor cells that have not fully differentiated into gonadotropes or thyrotropes were transformed and arrested in an early developmental stage.
This is consistent with observations that α-subunit expression appears in ontogeny before LHβ (15). In support of this idea, pituitary tumors from transgenic mice carrying the human or rat LHβ promoter linked to T-antigen (Windle, J. J., D. B. Whyte, and P. L. Mellon, manuscript in preparation) produce both the α-subunit mRNA and those for the LH and FSH β-subunits. These tumors are apparently derived from fully differentiated gonadotropes and, thus, may have been arrested in a later developmental stage by coupling T-antigen expression to the LHβ promoter.

An alternative explanation for the observed pattern of expression is that β-subunit expression correlates with the fully differentiated state of gonadotropes and thyrotropes, and transformation by Tag results in partial dedifferentiation and loss of this function. It is unlikely that dedifferentiation has occurred as a result of culturing, since the pattern of α, Tag, and β expression in the cultured cells matches that in the original tumors. We are currently investigating the possibility that dedifferentiation occurs in vitro after initiation of Tag expression using lines of α-Tag transgenic mice that develop pituitary tumors at a fairly consistent age.

It is interesting that these cell lines resemble a class of human anterior pituitary tumors termed nonfunctioning adenomas (16), which often express the glycoprotein hormone α-subunit in the absence of the β-subunits. Since these tumors presumably arise long after full differentiation of the anterior pituitary, they are consistent with a model for transformation-mediated dedifferentiation.

An additional differentiated characteristic of gonadotropes and thyrotropes is their responsiveness to specific hypothalamic releasing hormones. Several of the cell lines were responsive to GnRH but not TRH, indicating that they represent cells of the gonadotrope lineage. GnRH-binding sites appear several days earlier in development than either α- or β-subunits (17), consistent with the pattern of expression of these cell lines. Other cell lines were nonresponsive to both GnRH and TRH and, thus, may have been arrested in an earlier stage of development. It is also possible that they represent dedifferentiated cells. Alternatively, they could be precursor cells in the thyrotrope lineage but lacking TRH responsiveness if TRH receptor expression occurs later in development than α-subunit and GnRH receptor and, thus, might be absent at the stage when they were transformed.

Another possible explanation for the lack of TRH-sensitive cell lines may stem from the specificity of the 1.8-kb fragment of the 5′ flanking α-subunit gene used to direct expression. Fox and Solter (8) found expression of a 17-kb fragment containing the whole α-subunit gene (with 5.7 kb of 5′ flanking) in an identical population of pituitary cells as the endogenous mouse α-subunit. Perhaps the 1.8-kb fragment used in our studies contains only specificity for gonadotropes, and thyrotrope specificity is determined by sequences either up-stream or within the coding sequence of the gene itself.

Study of the molecular and cellular biology of the gonadotrope cell will be greatly facilitated by the availability of stably transformed gonadotrope cell lines. These cells are proving useful for studies of the mechanisms involved in α-subunit gene expression, synthesis, and secretion, as well as the second messengers and cis-acting sequences in the α-subunit gene that are involved in the response to GnRH. In addition, since these cells display a strong response to GnRH, they will provide the first cell model system for the study of the GnRH receptor.

We have successfully used genetic targeting of tumorigenesis to transform and immortalize anterior pituitary cells of the gonadotrope lineage and to derive cell lines that maintain both synthesis and secretion of the α-subunit protein and responsiveness to GnRH. A similar strategy was employed by Efrat et al. (18) in a recent report on the establishment of pancreatic β-cell lines from tumors of transgenic mice, in which Tag expression is driven by the insulin promoter. In addition, we have derived clonal cell lines of the hypothalamic neurosecretory neurons that produce GnRH using this same approach (Mellon, P. L., J. J. Windle, P. C. Goldsmith, C. A. Padula, J. L. Roberts, and R. L. Weiner, manuscript submitted). Thus, this strategy is effective for deriving cell lines from rare cell types for which cell-specific promoters have been cloned.

MATERIALS AND METHODS

Microinjection and DNA Analysis

The α-Tag plasmid is described in Fig. 1. The transgene was excised from the plasmid by digestion with EcoRI and Sall and purified by agarose gel electrophoresis and binding to glass beads (Bio101, Inc., La Jolla, CA; GeneClean). Approximately 1–2 pl of a solution of DNA at a concentration of 2 μg/ml were microinjected into the pronuclei of fertilized one-cell mouse embryos (19). The F2 embryos were derived from matings of C57Bl/6J(C57Bl/6J x BALB/cJ) males and females, obtained from the Jackson Laboratory (Bar Harbor, ME). The Injected embryos were reimplanted into CD-1 pseudopregnant mice (19). The presence of the transgene in the resulting mice was determined by preparing genomic DNA from a small piece of tail and assaying by Southern blot analysis (20).

Cell Culture

Tumors were minced into small fragments and incubated at 37°C in Hank's Buffered Saline with 10 mg/ml collagenase (Cooper Biomedical, Malvern, PA) and 10 μg/ml DNAse (Sigma, St. Louis, MO) for 30 min, followed by trituration and plating in Dulbecco's Modified Eagle's Medium (DME) with L-glutamine (Sigma), 15% fetal calf serum (FCS; Irvine Scientific, Santa Ana, CA), nonessential amino acids (Sigma), 4.5 mg/ml glucose, and 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma). The unattached and weakly attached cells were frequently transferred to new plates, until contamination by fibroblasts was eliminated. After growth in culture for 2–6 months, during which time the cells became more adherent and began to grow more rapidly, they were cloned by limiting dilution. Clonal cell lines were maintained in DME with 5% FCS, 5% equine serum (HyClone Laboratories, Logan, UT), 4.5 mg/ml glucose, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.
Hormone Treatment of Cells

The cells were plated in DME containing 12.5% FCS (Fig. 4) or 5% FCS, and 5% equine serum (Fig. 5). When cells reached approximately 50% confluency, hormone was added at the indicated concentration and for the given length of time. TRH was obtained from Sigma, and a 10⁻⁶ M stock was prepared as described by the supplier. Naltrexol was obtained from Syntex (Palo Alto, CA), and a 10⁻⁷ M stock was prepared.

RNA Analysis

Total RNA from both tissues and cells was prepared by the method of Chirgwin et al. (21). For Fig. 4, poly(A)⁺ RNA was selected by oligo(dT)-cellulose chromatography (New England Biolabs, Beverly, MA). Northern blotting was performed as described by Maniatis et al. (22). The probes were generated from plasmids encoding the cDNAs for the indicated hormone genes (as described in the figure legends) or SP6 or T7 polymerase by nick translation (20). Where indicated, the reticulocyte lysates were washed twice (5 min/wash) in 0.1 x SSPE-0.1% sodium dodecyl sulfate (0.1 x SSPE = 18 mM NaCl, 1.0 mM NaH₂PO₄, and 0.1 mM EDTA) at 100 C, for rehybridization to a second probe.

α-Subunit RIA

α-Subunit secretion was measured from cells incubated in DME with 0.1% FCS. A double antibody RIA using specific reagents for rat LH were obtained from the NIDDK. Extracts of mouse anterior pituitary showed close parallelism for displacement of labeled α-subunit compared to purified rat LH.

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