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ISOLATION AND CHARACTERIZATION OF A PROLACTIN-REGULATING FACTOR (PRF) FROM A MOUSE PITUITARY INTERMEDIATE LOBE CELL LINE

A dissertation submitted to the Division of Research and Advanced Studies of the University of Cincinnati in partial fulfillment of the requirement for the degree of

DOCTORATE OF PHILOSOPHY (Ph.D.)

Neuroscience in the Department of Cell Biology, Neurobiology and Anatomy of the College of Medicine

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by

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ABSTRACT

Prolactin (PRL) gene expression and release is regulated by inhibitory and stimulatory factors from the hypothalamus and pituitary. Dopamine is the primary PRL-inhibitory factor, but acute elevations in PRL require stimulation by a PRL-releasing factor (PRF). A PRF peptide that differed from known PRL secretagogues was initially discovered in the rat posterior pituitary (PP). This PRF localized to a subpopulation of pituitary intermediate lobe (IL) cells and it rapidly stimulated PRL release. Partial purification of PRF by sequential chromatography from bovine PP and IL tumors revealed the presence of multiple species that differed in size and chromatographic properties. However, the primary structure of PRF has not been resolved.

Our working hypothesis was: a subpopulation of IL cells secretes a novel peptide that functions as a PRF. Transgenic POMC-Tag mice that develop IL tumors were used to investigate the following objectives: 1) determine if POMC-Tag mice with IL tumors develop hyperprolactinemia, 2) establish and characterize an IL cell line that produces PRF, 3) determine the biochemical properties of PRF, 4) purify PRF to homogeneity and determine its amino acid sequence.

No hyperprolactinemia was observed in either male or female POMC-Tag mice from day 20 to 120 of tumor progression. Cells from two distinct IL tumors were cloned and expanded into mIL cell lines that differed in morphology and cellular characteristics. As assessed by RT-PCR the mIL39, but not mIL5, cells expressed POMC and D2R gene products, characteristic of IL melanotrophs. The cell lineage of the mIL5 cells remains undefined and they may represent a novel IL cell subtype. Only the mIL5 cells stimulated PRL gene expression and release from coculture with GH3/luc cells.

The PRF from mIL5 cells was a secreted product that bound to heparin. Two heparin-binding proteins, FGF2 and HB-EGF, were identified by RT-PCR in mIL5 cells. Both were potent PRL inducers, but they did not significantly contribute to the PRF activity from mIL5 cells as judged by Western blotting, heparin-affinity and immunoneutralization.

Purification of PRF from mIL5 cells by heparin-affinity chromatography indicated multiple species that differed in chromatographic properties. Sequential chromatography was used to isolate PRF from mIL5 cell extract and sequencing identified a peptide identical to an internal sequence of murine heparin/heparan-interacting protein (HIP/L29). The expression of HIP protein by RT-PCR was ubiquitous and two anti-HIP antibodies were ineffective in the attenuation of PRF activity from mIL5 cells.

Isolated PRF from mIL5-conditioned media was subjected to mass spectrometry that revealed an estimated mass of 14,968 Da. N-terminal sequence analysis failed to resolve the primary structure of PRF.
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ABBREVIATIONS

Å Angstrom
ACE angiotensin-converting enzyme
AcN acetonitrile
ACTH adrenocorticotrophic hormone
á-MSH alpha-melanocyte stimulating hormone
amu atomic mass units
AII angiotensin II
AP anterior pituitary
ARN arcuate nucleus
â-END beta-endorphin
cAMP cyclic-adenosine monophosphate
CCK cholecystokinin
CDK cyclin-dependent kinase
CM conditioned media
CNS central nervous system
CRH corticotrophin releasing hormone
CT calcitonin
D2R dopamine type-2 receptor
DA dopamine
Da Dalton
DAG diacylglycerol
DAT dopamine transporter
DOPAC 3,4-dihydroxyphenylacetic acid
E2 17-â-estradiol
EGF epidermal growth factor
ER estrogen receptor
ERE estrogen response element
ESMS electrospray ionization mass spectrometry
ET endothelin
FBS fetal bovine serum
FGF1 acidic-fibroblast growth factor
FGF2 basic-fibroblast growth factor
FGF4 fibroblast growth factor-4 (hst/protein)
FPLC fast protein liquid chromatography
FS folliculo-stellate cells
FSH follicle stimulating hormone
GABA gamma-aminobutyric acid
GAL galanin
GFAP glial fibrillary acidic protein
GH growth hormone
GnRH gonadotropin releasing hormone
HA histamine
HB-EGF heparin-binding epidermal-like growth factor
HIP heparin/heparan sulfate interacting protein
<table>
<thead>
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<th>Abbreviation</th>
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<td>HD</td>
<td>high-density</td>
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<tr>
<td>HPLC</td>
<td>high-pressure liquid chromatography</td>
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<td>HS</td>
<td>horse serum</td>
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<td>IL</td>
<td>intermediate lobe</td>
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<tr>
<td>IP3</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
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<tr>
<td>LD</td>
<td>low-density</td>
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<tr>
<td>LH</td>
<td>luteinizing hormone</td>
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<td>LOBEX</td>
<td>posterior pituitary lobectomy</td>
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<tr>
<td>MALDI-MS</td>
<td>matrix assisted laser desorption ionization-mass spectrometry</td>
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<tr>
<td>ME</td>
<td>median eminence</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
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<tr>
<td>MW</td>
<td>molecular weight</td>
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<tr>
<td>NGF</td>
<td>nerve growth factor</td>
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<td>NIL</td>
<td>neural-intermediate lobes</td>
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<td>neural lobe</td>
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<tr>
<td>NP</td>
<td>neurophysin</td>
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<td>NPY</td>
<td>neuropeptide Y</td>
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<td>OT</td>
<td>oxytocin</td>
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<tr>
<td>P4</td>
<td>progesterone</td>
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<tr>
<td>PACAP</td>
<td>pituitary adenylate cyclase activating polypeptide</td>
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<td>PB</td>
<td>phosphate buffer</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PeVN</td>
<td>periventricular nucleus</td>
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<tr>
<td>PHDA</td>
<td>periventricular hypophyseal dopaminergic neurons</td>
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<tr>
<td>PIF</td>
<td>prolactin inhibitory factor</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>PL</td>
<td>placental lactogen</td>
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<td>pro-opiomelanocortin</td>
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<td>posterior pituitary</td>
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<td>PPP</td>
<td>primary portal plexus</td>
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<td>PRF</td>
<td>prolactin releasing/regulating factor</td>
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<td>PRL</td>
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<td>PRL-R</td>
<td>prolactin receptor</td>
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<td>PrRP</td>
<td>prolactin-releasing peptide</td>
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<td>PVN</td>
<td>paraventricular nucleus</td>
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<td>RIA</td>
<td>radioimmunoassay</td>
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<td>Rb</td>
<td>retinoblastoma</td>
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<tr>
<td>RP</td>
<td>reversed-phase</td>
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<td>RPL19</td>
<td>ribosomal protein L19</td>
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<tr>
<td>RT</td>
<td>reverse transcription</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SFM</td>
<td>serum-free media</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>SON</td>
<td>supraoptic nucleus</td>
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<td>SP</td>
<td>substance P</td>
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<td>simian virus 40</td>
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<tr>
<td>Tag</td>
<td>large-T antigen</td>
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<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
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<tr>
<td>TGFα</td>
<td>transforming growth factor-alpha</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor-beta</td>
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<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
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<tr>
<td>THDA</td>
<td>tuberohypophyseal dopaminergic neurons</td>
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<tr>
<td>TIDA</td>
<td>tuberoinfundibular dopaminergic neurons</td>
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<tr>
<td>TRH</td>
<td>thyrotropin releasing hormone</td>
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<tr>
<td>TSH</td>
<td>thyroid-stimulating hormone</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>VIP</td>
<td>vasoactive intestinal polypeptide</td>
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<tr>
<td>VP</td>
<td>vasopressin</td>
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<tr>
<td>5HT</td>
<td>serotonin</td>
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Section 1. Hypothalamo-pituitary axis

Endocrine function in vertebrates is directed primarily through the hypothalamo-pituitary axis. The hypothalamus produces releasing and inhibitory factors that regulate pituitary hormone secretion. In turn, circulating levels of pituitary hormones control the secretion of various endocrine glands. Hormones released by these glands modulate various physiological processes including their own secretion through positive and negative feedback.

A. Anatomical structure and embryonic origin

The rat pituitary gland (Fig.1) is composed of three distinct lobes: anterior, intermediate and neural. The intermediate and neural lobes are collectively called the posterior pituitary (PP) or neuro-intermediate lobe (NIL). The entire pituitary is connected to the hypothalamus by an infundibulum or stalk, which contains specialized vascular and neuronal elements (368). The anterior pituitary (AP) is embryologically derived from an outgrowth of the ectoderm lining the roof of the stomodeum called Rathke’s pouch. In contrast, the neural lobe (NL) is derived from the neuroectoderm of the diencephalic floor. Rathke’s pouch is eventually pinched off by constriction caused by growth of the surrounding mesoderm and envelopes the infundibular process. Cells from Rathke’s pouch that are adjacent to the neural tube differentiate into the epithelial cells of the intermediate lobe (IL) (260,460).

B. Pituitary innervation

The NL is primarily composed of unmyelinated axons from the magnocellular neurons containing vasopressin (VP) and oxytocin (OT), located in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus (202). The AP receives no direct neuronal input from the hypothalamus. In contrast, the IL, also of ectoderm origin, is innervated by dopaminergic neurons located in the periventricular nucleus (PeVN; A₁₄ neurons) of the hypothalamus (213). In addition, the PP contains axonal terminals from serotonergic (158,214), noradrenergic (416), and neuropeptidergic neurons (50). The products from these neuronal afferents play an important modulatory role in pituitary function including hormone synthesis and secretion.

C. Portal vasculature and the median eminence

The internal carotid arteries bifurcate into the superior and inferior hypophyseal arteries, which provide the pituitary blood supply. The superior hypophyseal arteries divide into anterior and posterior hypophyseal arteries that feed the pituitary stalk, optic chiasm and portions of the hypothalamus including the median eminence (ME) (352,460). The ME is a specialized neuroendocrine structure that serves as a functional bridge between the hypothalamus and the pituitary. It is divided anatomically into an internal and external layer. The internal layer consists of descending axons projecting to the PP. The external layer contains axon terminals; projections from specialized ependymal cells called tanyocytes, and capillary loops. This specialized primary portal plexus (PPP) consists of endothelia with high permeability in close
Fig 1. A schematic diagram of the rodent pituitary gland depicting the three pituitary lobes, hormone composition, innervation, and vascular connections.
proximity to the hypothalamic neurosecretory axon terminals (270). These capillaries drain into the long portal vessels that transverse the infundibular stalk and deliver the neurosecretory products to the secondary capillary plexus in the ventral and rostral AP. The posterior hypophyseal arteries supply blood to the NL and gives rise to the short portal vessels that reach the dorsal and caudal capillary plexus of the AP (460). The secondary capillary plexus of the portal vasculature containing secreted pituitary hormones drains into the venous cavernous sinus toward general circulation. Blood flow in the portal system is primarily toward the AP in the anterograde direction. However, limited retrograde blood flow has been reported that may provide a rapid mechanism by which AP hormones feedback to the hypothalamus and PP to regulate their own secretion (353). In contrast to the rich capillary and sinusoidal network in the AP and NL, the IL is unusual for an endocrine tissue in that it is poorly vascularized. A small number of blood vessels are limited to the boundary between NL and along the residual space of Rathke’s pocket that separates the IL from the AP (9,193).

**D. Innervation of the median eminence (neurosecretion)**

Neurosecretion is the release of neuronal secretory products from nerve terminals into the bloodstream. This is accomplished by diffusion of exocytosed neurosecretory products through the extracellular space to adjacent blood vessels. Specialized junction complexes control movement of secreted substances across basement membranes into the perivascular space and through fenestrated capillary endothelia before reaching the lumen of blood vessels (9,353). Dopaminergic perikarya located in the PeVN (A14 neurons) and arcuate (ARN; A12 neurons) nuclei of the hypothalamus supply dopamine (DA) to the pituitary (358). Functionally, these neurons are grouped according to the course of their projections and final destination. The ME contains three major dopaminergic systems: the tuberoinfundibular (TIDA), tuberohypophyseal (THDA), and periventricular hypophyseal (PHDA). Neurons of the A12 TIDA system terminate in the external zone of the ME (289,520). They release DA into the PPP where it is carried down the infundibular stalk via the long portal veins to the AP (34). The PHDA (A14) and THDA (rostral A12) neurons pass through the internal zone of the ME and infundibular stalk and innervate the IL and NL, respectively (188,297). DA released into the NL from the THDA neurons can reach the AP via the short portal vessels (346,384).

Innervation of the ME is not limited to dopaminergic neurons. Noradrenergic neurons originating from the locus coeruleus (375) and superior cervical ganglia (162) as well as serotonergic neurons from the dorsal raphe nucleus (489) terminate in the ME and modulate neurosecretory activity by axo-axonal influences (138). Many parvicellular neurons distributed throughout the hypothalamus contain peptide releasing and inhibiting factors that also terminate in the ME. These peptides include: thyrotropin releasing hormone (TRH), gonadotropin releasing hormone (GnRH), growth hormone releasing hormone (GHRH), somatostatin and corticotropin releasing hormone (CRH). Theses peptides serve as potent regulators of AP hormone synthesis and secretion and their release into the PPP of the ME is tightly regulated (244,382). Furthermore, a variety of other peptidergic neurons impinge on the axonal terminals in the ME and modulate secretion of the pituitary regulatory factors (275,360).
Section 2. The pituitary gland

A. Genetics of pituitary development

Successive expression of homeobox genes in primordial tissues throughout embryonic development coordinates pattern formation that culminates in the final pituitary phenotype (49). A better understanding of the ontogeny of pituitary development has emerged from targeted disruption of these homeobox genes in mice (380,499). Disruption of the thyroid transcription factor-1 gene (Titf1) results in pituitary aplasia (266). This homeodomain protein functions in the early genetic pathways necessary for the induction of Rathke’s pouch. Two members of the LIM-homeobox gene family, Lhx3 and Lhx4, are important in the formation and expansion of Rathke’s pouch (212). The expression of Lhx3 is an early marker for AP and IL cells. Mouse embryos deficient in Lhx3 show arrested pituitary development i.e., Rathke’s pouch begins to form, but fails to expand or differentiate (438). Transgenic mice lacking Lhx4 display a mild hypopituitarism with reduced hormone levels resulting from decreased cell number (290). An overlapping pattern of Lhx3 and Lhx4 is supported by the phenotype of the double Lhx3-4 mutant mouse. These mice have a more severe arrest in early pituitary development than either mutant alone (437).

B. The neural lobe (NL)

1. Cellular organization

Peptidergic magnocellular neurons originating in the hypothalamus and terminating in the NL are the primary neuronal element in this tissue. Large dilated axonal terminals containing dense core vesicles terminate without forming true synapses in perivascular spaces near fenestrated capillaries (202). Neurons carrying both classical neurotransmitters and neuropeptides also innervate the NL and their axonal terminals are dispersed among those of the magnocellular neuron fields (50).

The NL also contains a population of specialized astroglia called pituicytes. These cells interdigitate among the axonal processes of the magnocellular neurons and can modulate hormone release from neurosecretory terminals under various physiological states (167,201). One possible mechanism by which pituicytes regulate neurosecretion is engulfment. They can extend and retract cellular processes between the axon terminal and the basement membrane when hormone demand is low or high, respectively, during dehydration, parturition, or lactation (203,503). Both magnocellular axons and pituicytes express a variety of neurotransmitter and peptide receptors. The interaction of specific ligands with these receptors can directly modulate neurosecretion from magnocellular neurons or indirectly through changes in pituicytes function (46,390).

2. Neurochemical composition

The two major neuropeptides, oxytocin (OT) and vasopressin (VP), are synthesized by distinct magnocellular neurons and released from the NL following depolarization. Initially, each of these peptides is part of larger precursor proteins that are cleaved by proteases in secretory
vesicles during axoplasmic transport to yield mature nonapeptides and respective neurophysin (NP) carrier proteins (3). The best-characterized function of VP is stimulation of water reabsorption from the kidney to maintain blood osmolarity (339). In females, OT stimulates milk letdown during suckling and uterine contraction at parturition. In males, the physiological role of pituitary OT is unclear (247). Many other peptides have been identified in the NL and several of these coexist within neurons containing VP or OT. Galanin (449), endothelin-1 (ET-1), ET-3 (410) and dynorphin (415) have been co-localized with VP in the NL. Cholecystokinin (CCK) (325), ET-1, ET-3 (410), dynorphin and enkephalin (415) have been co-localized with OT in the NL. Additional nerve terminals in the NL contain gamma-aminobutyric acid (GABA) (376), DA, serotonin (5HT) (463), nitric oxide (NO) (5), CRH, substance P (SP), vasoactive intestinal peptide (VIP), neuropeptide Y (NPY) and somatostatin (329). This large and complex milieu of neuro-modulatory factors within the NL represents only one of many regulatory systems governing neurosecretory activity.

C. The Intermediate lobe (IL)

1. Cellular organization

In most vertebrates, the IL appears as a small band interposed between the AP and NL that is composed of multiple cell layers (58). The human fetus has a distinct IL but the integrity of this structure is lost postnataally as it becomes indistinguishable from the AP (17,324). Melanotrophs are the only recognized endocrine cells in the IL and comprise 90-95% of the total cells. They are polyhedral cells with smooth, ovoid nuclei, extensive Golgi and endoplasmic reticulum, abundant mitochondria and coated cytoplasmic secretory vesicles (85,86). Melanotrophs form lobular structures with agranular glial-like interstitial cells that extend cytoplasmic processes between them (72). The IL is separated from the AP by a colloid-filled space (Rathke’s cleft) that is lined by a single layer of ciliated agranular marginal epithelial cells (143,240). These cells may represent a germinal layer that support continued IL cell proliferation during postnatal development (73).

2. Susceptibility to tumor formation

Unlike most AP cells, no factor has been identified that defines the cell lineage of the melanotrophs (31). The postnatal onset of dopaminergic innervation and subsequent increase in DA content in the IL suppresses the proliferation of melanotrophs (86,169). This antiproliferative activity of DA in the IL is mediated by high-density dopamine type-2 receptors (D2R) on melanotrophs that are negatively coupled to adenyl cyclase (305,327). A dysfunction in dopaminergic regulation may contribute to the susceptibility of the IL to tumorigenesis (330,364).

The regulation of cell division involves activation of cyclin-dependent kinases (CDK) and the inactivation of retinoblastoma (Rb) protein, the product of the Rb tumor suppressor gene. The Rb protein is a target of CDKs and phosphorylation of Rb results in its inactivation. Many studies support the notion that CDK inhibitors and Rb are part of a pathway regulating a cell's commitment to divide.
Heterozygous transgenic mice with a germline mutation in the retinoblastoma gene (Rb) develop spontaneous multi-focal IL tumors with near complete penetrance (199,226). The reintroduction of the Rb gene by adenoviral gene transfer to IL tumors decreased cell proliferation, reestablished dopaminergic innervation and inhibited tumor expansion (406). However, the expression of N-terminally deleted Rb transgenes (RbdeltaN) only delayed IL tumor onset and progression, but did not prevent formation (405).

Inhibitors of CDKs are involved in the regulation of cell cycle, thereby functioning as tumor suppressor genes. A member of the CDK family, the p27/kip1 gene, encodes an inhibitor of several CDK complexes that control the progression of the cell cycle from G1 to S-phase. Lack of p27/kip1 function produces a complex phenotype associated with the development of specific IL tumors (108). The development of IL tumors in Rb+/- mice correlated with a reduction in p27 expression. In Rb+/-p27/- mice, IL tumorigenesis has an earlier onset that may result from the cooperative action of these two proteins on different integrative regulatory signals (378). Mice deficient in the CDK p18 (INK4c) develop gigantism and widespread organomegaly. Loss of p18, like that of p27, but not other CDK inhibitor genes, leads to IL tumorigenesis. Hence, both p18 and p27 mediate two pathways that suppress IL tumorigenesis, likely by controlling the function of Rb (155).

3. Pro-opiomelanocortin (POMC) peptides

POMC is a prohormone that is differentially processed by enzymatic cleavage depending on its cellular localization. In melanotrophs, POMC is cleaved by two prohormone convertases (PC1 and PC2) that are co-localized in secretory vesicles (112,306). Cleavage of POMC by these enzymes yields two major peptide products. The first is alpha-melanocyte-stimulating hormone (α-MSH), a 13-amino acid peptide that regulates skin color during background adaptation in amphibians (412) and skin homeostasis in mammals (450,471). The second is beta-endorphin (â-end), a 31-amino acid peptide that is N-terminal acetylated to yield N-acetyl β-endorphin (1-31) with additional C-terminal processing to yield 27- and 26-amino acid forms characteristic of melanotrophs (137). These acetylated forms of β-end do not exhibit high-affinity binding for brain opiate receptors in vitro and possess no significant analgesic properties (121). Their physiological function might be to antagonize opioid mediated analgesia (172). The expression of these peptides is not restricted to melanotrophs, thereby confounding their physiological roles.

4. Melanotroph heterogeneity and hormone regulation

Several lines of evidence support morphological and functional heterogeneity among IL melanotrophs (191). These cells can be separated into high-density (HD) and low-density (LD) populations (184). The HD cells contain more α-MSH than LD cells, have a slower rate of secretion, and are less responsive to acute α-MSH release by TRH (182,183). Dopamine inhibits α-MSH release from LD melanotrophs (119), but reduces KCL-stimulated cytosolic free calcium concentrations in both LD and HD cells (115). In addition, DA decreases the accumulation of POMC mRNA and the rate of acetylation in LD, but not in HD cells. In rats, individual melanotrophs respond differently to DA. In some cells, POMC mRNA was reversibly downregulated by DA, while other cells remained unaffected (29). The spatial organization of
DA innervation could contribute to the induction or maintenance of melanotroph heterogeneity (87). In general, DA exerts a tonic inhibition on POMC biosynthesis and hormone release from IL melanotrophs. Peptide secretion can be facilitated indirectly by decreasing DA or directly through activation of beta-adrenergic receptors (100,475). In addition to DA, the direct innervation of the IL by adrenergic, 5HT, and GABA neurons provide regulatory signals that participate in the modulation melanotroph function (158,463).

5. Neurotrophic activity

In adult rats, the non-hormone producing cells of the IL are immunoreactive for the acidic calcium binding protein S-100. All these cells express the intermediate filament protein vimentin, but only a subpopulation express glial fibrillary acidic protein (GFAP) (171). The number and distribution of these GFAP positive cells differs with the animal gender and its pattern of expression varies during lactation, adrenalectomy and salt loading without affecting that of S-100 (170,399). However, during prenatal and early postnatal development, melanotrophs express S-100 protein that does not co-localize with vimentin (422). The transient expression of S-100 in melanotrophs coincides with DA innervation of the IL. Similar to its function in the developing CNS, S-100 may function as a neurotrophic or differentiation factor in the IL (292,309). The spatial and temporal expression of multiple neurotrophic and differentiation factors by IL cells may have important functional and organizational consequences. The neurotrophic glycoproteins L1 and laminin are both developmentally expressed by interstitial cells and melanotrophs, respectively (37). Before innervation of the IL, polysialic acid-neural cell adhesion molecules (PSA-NCAMs) are expressed in interstitial cells, but not melanotrophs (38,283). The transient postnatal expression of insulin-like growth factor binding protein-2 (IGFBP-2) in melanotrophs and interstitial cells may also serve as a neurotrophic agent during IL innervation (281,519). Finally, fetal GABAergic and dopaminergic neurons co-cultured with melanotrophs can form morpho-functional synapses, further supporting the expression of neurotrophic and differentiation factors by melanotrophs (402).

D. The anterior pituitary (AP)

The AP is a complex and specialized endocrine tissue that contains a functionally heterogeneous population of hormone secreting cells. Hypothalamic peptides regulate the synthesis and secretion of six major hormones from five distinct cell types. 1) The lactotroph produces and secretes the lactogenic hormone prolactin (PRL) whose primary target is the mammary gland. 2) The somatotroph produces and secretes growth hormone (GH) which targets most tissues. 3) The thyrotroph produces and secretes thyroid-stimulating hormone (TSH) whose primary target is the thyroid gland. 4) The gonadotroph produces and secretes both leutinizing hormone (LH) and follicle stimulating hormone (FSH) whose primary target is the gonads. 5) The corticotroph produces POMC and primarily secretes adrenocorticotropic hormone (ACTH) whose primary target is the adrenal gland. Positive and negative feedback from hypothalamic factors or circulating peripheral hormones coordinates AP hormone secretion. Hence, a balance between positive and negative signals maintains the appropriate endocrine status for any given physiological state.
1. Development and differentiation

The endocrine cells of the AP differentiate from progenitor cells in response to autocrine and paracrine signals during ontogeny. Pathophysiological disorders in mice and humans have provided evidence for the nature of these differentiation factors affecting multiple cell lineages. Two dwarf mouse lines (Ames; df) and (Snell/Jackson; dw or dwJ) are a result of spontaneous mutations in homeobox genes (493). The Snell and Jackson dwarf mice have mutations in the Pit-1 gene that encodes the POU-homeodomain transcription factor necessary for the cellular differentiation of somatotrophs, lactotrophs and thyrotrophs. The loss of these three cell types as a consequence of Pit-1 mutation suggests their origin from a common progenitor (8,404). The phenotype of the Ames dwarf mouse is a result of a mutation in the paired-like homeobox gene Prop-1 that mimics the effect of Pit1 mutation on cellular differentiation (455). Expression of Prop-1 occurs only in the developing pituitary and is required for Pit-1 activation (161). These two transcription factors likely function sequentially on proliferation and differentiation of somatotrophs, lactotrophs and thyrotrophs (54). Although rare, humans were identified who manifest a combined pituitary hormone deficiency and exhibit similar problems to those of the dwarf mice. This human phenotype is a result of multiple, yet distinct, mutations in the PROP-1 and POU1F1 (human homologue of Pit1) genes (385). In families with PROP-1 mutations there is also a reduction in LH and FSH, suggesting an additional regulatory role for PROP-1 on gonadotroph development (505). Multiple POU1F1 mutations have been observed in several families, all of which show severe reductions of GH and PRL, but variable effects on TSH (94,95). Recent genetic analysis has implicated a variety of other homeobox genes in pituitary development (269,499). These include; Rpx (208), Pitx1 (282), Pitx2 (433) and Ftzf1 (237), which are expressed in restricted spatial and temporal locations during development (248). Further characterization of these genes is required to identify their roles in AP cell proliferation and differentiation. A more detailed understanding of the genetic hierarchy involved in pituitary development, cell lineage commitment and differentiation is emerging (6,499). However, future efforts will be needed to elucidate the cascade of regulatory events specific for each of these homeobox genes and the consequence of their combined expression on pituitary development and final determination of cell fate.

2. Cellular organization

The pituitary cell types were first distinguished by morphological and cytological differences (197). The isolation of the major hormone products and their genes helped in characterization of these cell types based on their chemical composition. The ontogeny of endocrine AP cells occurs in the following sequence: corticotrophs, thyrotrophs, gonadotrophs, somatotrophs and lactotrophs (68,79). The percentage of each cell population in the adult AP is shown in Table 1, but this proportion varies between sexes and during some endocrine conditions such as pregnancy and lactation.

In addition to endocrine cells, the AP has a large population of folliculo-stellate (FS) cells with a characteristic star-like morphology. The ontogeny of these cells has not been resolved. They express the cell markers S-100 and GFAP, suggestive of a glial origin from neuroectoderm (93). However, they also express cytokeratins that are characteristic of epithelial cells derived from ectoderm (479). Although a supportive role is attributed to these cells, their function in the AP
<table>
<thead>
<tr>
<th>CELL TYPE</th>
<th>Corticotroph</th>
<th>Thyrotroph</th>
<th>Gonadotroph</th>
<th>Somatotroph</th>
<th>Lactotroph</th>
<th>Folliculo-stellate</th>
</tr>
</thead>
<tbody>
<tr>
<td>% OF AP</td>
<td>5%</td>
<td>5%</td>
<td>5%</td>
<td>20-25%</td>
<td>25-30%</td>
<td>25-30%</td>
</tr>
<tr>
<td>HORMONE PRODUCT</td>
<td>ACTH</td>
<td>TSH</td>
<td>LH/FSH</td>
<td>GH</td>
<td>PRL</td>
<td>Cytokines and Growth factors (?)</td>
</tr>
<tr>
<td>TARGET TISSUE</td>
<td>Adrenal</td>
<td>Thyroid</td>
<td>Gonads</td>
<td>Most tissues</td>
<td>Mammary</td>
<td>AP cells</td>
</tr>
<tr>
<td>POSITIVE REGULATOR</td>
<td>CRH</td>
<td>TRH</td>
<td>GnRH</td>
<td>GHRH</td>
<td>PRF</td>
<td>?</td>
</tr>
<tr>
<td>NEGATIVE REGULATOR</td>
<td>Corticosteroids</td>
<td>Thyroid hormones</td>
<td>Gonadal steroids</td>
<td>Somatostatin, IGF-1</td>
<td>Dopamine</td>
<td>?</td>
</tr>
</tbody>
</table>

Table 1. Cellular and hormonal composition of anterior pituitary gland.
remains obscure. They may serve as a source of growth factors or even as undifferentiated stem cells (238). FS cells produce two angiogenic factors, vascular endothelia growth factor (VEGF) and basic-fibroblast growth factor (FGF2), which are involved in modulating pituitary angiogenesis and vascular permeability under both physiological and pathophysiological conditions (178).

3. Hormone regulation

With few exceptions, hypothalamic releasing/inhibiting factors are small peptides that function either as positive or negative regulators of AP hormone secretion. These releasing factors are secreted in nanogram quantities into the ME and the closed portal vasculature is necessary to maintain an effective concentration (285). In general these factors are extremely potent and interact with specific high affinity membrane receptors to rapidly stimulate hormone release. Pituitary hormones circulate to distant endocrine glands where they act on specific receptors to induce hormone release from that target tissue. These hormones in turn feedback on both the CNS and pituitary to regulate their secretory activity. Thus, a fine tuned regulatory loop is established that can rapidly adapt to the changing environment. This classic paradigm of hormone regulation serves as a foundation for our understanding of the hypothalamo-pituitary-endocrine axis (303,401). However, many other less characterized factors are emerging as stimuli that contribute directly to the control of hormone release (167,275).

There are two types of hormone releasing systems in the hypothalamus. The first produces a single releasing factor that positively controls the secretion of a specific AP hormone. These include thyrotropin-releasing hormone (TRH), gonadotropin-releasing hormone (GnRH) and corticotropin-releasing hormone (CRH). All stimulate hormone release from AP cells that express their specific membrane receptors coupled to an intracellular signal transduction pathway (303). Exocytosis of hormone-containing vesicles usually follows an elevation in intracellular calcium (478). Depending on the nature of the receptor-second messenger system in a given cell, ligand-receptor interaction can lead to stimulation of gene expression, hormone secretion, or both (82,254,383).

A second hormone releasing system in the hypothalamus produces a counteracting pair of hormones, one acting positively and the other negatively to modulate pituitary hormone secretion. This arrangement is particularly important in the regulation of GH and PRL, hormones that do not have specific target organ products for feedback inhibition. Growth hormone releasing-hormone and somatostatin stimulate and inhibit GH secretion from somatotrophs, respectively. GHRH is produced by hypothalamic neurons in the ARN and somatostatin by the PVN (271). GHRH and somatostatin each interacts with specific receptors on somatotrophs. GHRH is positively coupled to the cAMP-adenylate cyclase second messenger system, whereas somatostatin inhibits this pathway (45). The control of prolactin (PRL) involves both positive and negative regulators that will be discussed in detail in subsequent sections.
Section 3. The lactotroph

A. Ontogeny

Transcription factors that bind to DNA regulatory elements play critical roles during development by modulating cellular gene expression, growth and differentiation. The pituitary specific transcription factor Pit-1 was identified by its ability to transactivate PRL, GH and the β-TSH genes (381). Although other endocrine cell types in the AP express Pit-1 transcripts, only lactotrophs, somatotrophs and thyrotrophs express the Pit-1 protein (445). This 33-kDa protein belongs to the helix-turn-helix group of transcription factors. They share a bipartite DNA binding motif consisting of two conserved regions that are tethered by a variable linker region. The site-specific DNA binding of these proteins requires both a N-terminal POU-specific domain and a C-terminal POU-homeodomain (429). Initial expression of Pit-1 during embryogenesis coincides spatially and temporally with PRL and GH gene expression and is likely responsible for the initial transcriptional activation of both genes (445). The importance of Pit-1 as a regulator of AP development has been demonstrated in spontaneous Pit-1 mutations in mice and humans as discussed in the previous section (94). The expression of Pit-1 is essential for lactotroph differentiation and is first detected in the rat AP on embryonic day 15 (ED15). This expression precedes PRL transcription that occurs on ED19. Lactotrophs are the last AP endocrine cell to differentiate with detectable PRL protein observed only postnatally (391).

Pit-1 POU domains bind the 5' cis-regulatory elements in the PRL promoter as a homodimer (429). Interactions with either a distal enhancer (-2.5 to -1.2 kb) or a proximal region (-422 to +33 bp) of the PRL promoter can induce the PRL gene (190). However, a synergistic interaction between these two regions is necessary for high levels of PRL expression (236). Pit-1 is essential for the initiation of PRL gene transcription, but other factors are necessary to maintain high PRL expression in the mature lactotroph (236,436). In females, the estrogen receptor (ER) mediates progressive induction of the PRL gene characteristic of the maturing lactotroph. Estrogen exerts its effects via binding of ER to the estrogen response element (ERE) that is located in the distal enhancer of the PRL gene, adjacent to a Pit-1 site (445).

In the rat, the lactotrophs increase significantly in number between postnatal days 6-20 (288). The spatial distribution and temporal expression of several homeodomain transcription factors may contribute to the proliferation and differentiation of lactotrophs by interacting with Pit-1 (379,476). Notably, Prophet of Pit-1 (Prop-1) is required to activate Pit-1 expression. Prop-1 deficiency, as in the Ames dwarf mouse, results in the failure of the Pit-1 cell lineage to develop and hypopituitarism, including the absence of lactotrophs (385,455).

Other signaling molecules can affect the proliferation and differentiation of developing lactotrophs. Cortisol and corticosterone suppressed lactotroph differentiation in AP cultures from 13.5-day-old fetal rats (425). Transgenic mice lacking gonadotrophs have reduced numbers of lactotrophs at birth. This appears to be a result of direct stimulatory effects from gonadotrophs on developing lactotrophs during fetal or early postnatal life (434). Vasoactive intestinal polypeptide (VIP) can stimulate lactotroph differentiation from the chick AP in vitro (504). Transgenic mice lacking functional galanin (GAL) have reduced AP and plasma PRL. These animals have lactotrophs that fail to proliferate in response to estrogen and lactation is
abolished (509). Factors such as DA from the hypothalamic may also play an important regulatory role in the maturing lactotroph (196).

B. Cellular characteristics

Lactotrophs and somatotrophs exhibit dynamic structural and functional heterogeneity. Together they account for over 60% of all cells in the AP. Although they each represent a distinct population of hormone producing cells, an intermediary cell type, referred to as the somatomammotroph, has been identified that secretes both PRL and GH. Cellular heterogeneity and bi-hormonal expression in the AP is not limited to these two cell types, but also exists in gonadotrophs.

1. Bihormonal secretion

The reverse hemolytic plaque assay used sequentially provided the first evidence that an individual rat AP cell can secrete both GH and PRL (156). The presence of these cells has been confirmed in many species including the human fetus (344). In cultures of male rat AP cells, one third of all PRL and GH producing cells were dual secretors (156). In male bovine, approximately 9% of all pituitary cells were dual secretors, whereas 21% released solely GH and 45% PRL. This population changed in castrated males, with somatomammotrophs increasing to 22% of pituitary cells, sommatotrophs decreasing to 8%, and lactotrophs remaining at 46% (267). The changing cell populations suggest that these cells may be interconvertible (393).

The distribution of dual hormone secreting cells within the intact rat pituitary is not uniform. When AP tissue is divided into an inner and outer zone, the outer zone had a larger proportion of dual hormone secretors (51). Estradiol increases the number of lactotrophs especially in the outer zone, whereas progesterone decreases the number in both zones (225). Furthermore, the rate of PRL release in response to TRH and DA differed between cells from these zonal regions. Outer zone cells increased PRL release in response to TRH compared to cells in the inner zone. Dopamine was an effective inhibitor of PRL release from inner zone cells, but not in cells from the outer zone. Changes in GH release were not observed under similar conditions (51).

2. Heterogeneity

Morphologically lactotrophs vary in size. Differences exist in intracellular PRL content, secretory rate and sensitivity to PRL secretagogues between large granulated and small agranular cells (234,483). Density gradient separation of AP cells established two lactotroph subpopulations that differed in resting membrane potential, basal and stimulated PRL secretion (294). Combined in situ hybridization and reverse hemolytic plaque assay demonstrated lactotroph heterogeneity in both PRL gene expression and secretion. In ovariectomized rats the nonsecretory lactotrophs displayed higher PRL gene expression than those that were actively secreting PRL (427). Individual lactotrophs can release PRL in either a constant or episodic manner and gonadal steroids may contribute to the relative abundance of each cellular phenotype (76). Indeed, repeated measures of PRL gene expression from individual lactotrophs, using a modified luciferase assay, revealed a subpopulation of cells that exhibited dramatic fluctuations in PRL gene expression under basal conditions (77). Furthermore, PRL gene expression and
release differed between distinct subpopulations of lactotrophs under basal and modified culture conditions (77,302). Not only does a subset of lactotrophs differentially respond to a given stimulus, but also subpopulations differ in their ability to respond to a stimulus at all (118,488).

3. Other secretory products

In addition to PRL, lactotrophs produce and secrete a host of biologically active peptides that can function as autocrine or paracrine regulatory molecules within the pituitary. Dual immunocytochemical analysis has localized proteins of the renin-angiotensin system within lactotrophs (418). These proteins were detected in secretory granules with renin being the most abundant. Localization of these proteins in AP endocrine cells is not restricted to the lactotroph, although they showed the highest level of expression in this cell type (485). A large lactotroph population produces and secretes ET-1, which may function in the autocrine regulation of PRL release (256). Nerve growth factor (NGF) expression in the AP appears restricted to lactotrophs and NGF is released with PRL from secretory granules (335).

Galanin is an important PRL regulatory molecule and immunoreactive GAL has been detected in lactotrophs (231). Co-expression of GAL and PRL within secretory granules is dependent on estrogen (229), which induces GAL mRNA up to 3000-fold (224,231). Notably, secretion of GAL is from a minority of lactotrophs and estrogen increases the number of cells secreting this peptide (507). Transgenic mice deficient in GAL as well as those over-expressing GAL support a paracrine role for this peptide on PRL expression and lactotroph proliferation (71,508,509).

Transforming growth factor-beta-1 (TGFβ-1) mRNA and immunoreactivity have been detected in lactotrophs and estrogen decreases the number of TGFβ-1 positive cells (67). TGFβ-1 may suppress lactotrophs function through estrogen-sensitive TGFα type-2 cell surface receptors (120). Furthermore, TGF beta type-1 and type-2 receptors may mediate different functions for TGFβ-1 on lactotrophs, with type-1 receptors controlling PRL transcription whereas type-2 receptors modulating lactotroph proliferation (424).

Co-localization of other PRL regulatory peptides within lactotrophs remains less certain. Identification of the peptide VIP in lactotrophs remains controversial, with some groups reporting detection in a small subpopulation of PRL positive lactotrophs (278) and other reporting no detectable co-localization (75). A similar discrepancy has been reported for co-localization of SP with PRL (13,343).

C. Cell lines

Primary AP cell cultures provided a valuable model for the study of lactotroph cell function and PRL regulation. However, the small size of the AP combined with its heterogeneity has hindered experimentation. Several lactogenic cell lines have been cloned from rodent pituitary tumors and have provided useful in vitro models. Although their usage is often confounded by aberrant cellular characteristics, they have been widely used to investigate lactotroph properties. Only a few human pituitary cell lines have been developed that produce PRL or GH but none remained stable over time (84,221,263).
1. GH cell lines

The best-characterized lactogenic cell line, GH, was cloned from a radiation induced transplantable pituitary tumor (MtTW) from a female rat (468). Two original lines, GH1 and GH3, are somatomammotrophs that produces GH and PRL in different ratios. Subcloning of these cells has produced additional cell lines that express more (GH4C) or less (GC) PRL (268,467). The GH3 line differs from most pituitary endocrine cells by having a limited storage capacity that results in continuous hormone release (25). Additionally, these cells do not respond to some PRL and GH secretagogues such as DA and GHRH (101,517).

The GH3 cells express functional receptors for a variety of hormone secretagogues. TRH increases the production and secretion of PRL and somatostatin inhibits the synthesis of both PRL and GH (511). Estrogen (E2) increases PRL production and the sensitivity of GH3 cell to TRH by increasing the number of TRH receptors (174). Insulin, E2, and epidermal growth factor (EGF) all increase the storage capacity of GH3 cells as reflected by elevated intracellular PRL content (426). Several secretagogues can increase PRL gene expression and release from GH3 cells. However, DA, which is the primary in vivo inhibitor of PRL release, is ineffective due to the lack of functionally coupled D2R (80,101). The introduction of D2R constructs into GH3 cells shows that their insensitivity to DA may be the result of a transcriptional block in D2R expression and not because of a downstream signaling malfunction (153). This is consistent with the ability of growth factors such as EGF and NGF to up-regulate functional expression of D2R in GH3 cells (168,336).

It is often assumed that GH or PRL producing pituitary cell lines represent homogenous cell populations. However, most of these cell lines are composed of a mixture of hormone cells that include individual, bi-hormonal or nonsecretory cells. Furthermore, the relative proportion of functional cell types is dynamic and can be altered by exposure to various secretagogues (258,338). The GH3, GH4C1 and GH1 cell lines are comprised of mixed populations of GH, PRL and dual secretors (268). In GH3 cell cultures, exposure to estradiol shifts the cell types to predominantly somatomammotrophs, whereas TRH results in a mainly lactotroph cell population (52). Hence, factors that alter PRL secretion may also change the type of secretory cell present and this indicates a multipotential cell may serve as an intermediary between GH and PRL secretors. The mechanism responsible for this phenotypic switch is unclear, but may involve the reciprocal suppression and induction of each gene by a common transcription factor.

2. Other PRL-producing cell lines

A predominantly lactotroph cell line, MMQ, was established from the transplantable rat 7315a pituitary tumor (279). These cells, unlike the GH3 somatomammotrophs, secrete primarily PRL that is inhibited by DA via a D2R mediated mechanism (252,253). The SUP1 lactotroph cell line, also cloned from a transplantable 7315a tumor is similar to MMQ cells in its responsiveness to DA (241). Another cloned cell line, 235-1, secretes PRL but is unresponsive to DA agonists or antagonists even though these cells possess D2R (102,103). This lack of responsiveness may result from the absence or inactivation of a guanine nucleotide binding protein in the D2R complex due to a structural anomaly (57).
D. Pathophysiology

Hyperprolactinemia is an inappropriate and sustained elevation in serum PRL that can lead to galactorrhea and/or infertility. Excess production and secretion of PRL from the AP often results from disruption of the negative hypothalamic input by various medications, including neuroleptics, antidepressants, opiates and cocaine. Pathological states such as cirrhosis, hypothyroidism, and renal insufficiency can also lead to excess PRL production (340,388). Neurogenic stimulation and lesions of the pituitary stalk or hypothalamus also disrupt PRL regulation and result in its over-secretion. Furthermore, ectopic neoplasms or most commonly a pituitary prolactinoma is a major cause of hyperprolactinemia.

1. Prolactinomas

Prolactinomas are prolactin secreting pituitary tumors that are the most common among pituitary tumors. They occur more frequently in females and are categorized according to size, invasiveness and secretory activity (340). Visual disturbances are reported with macroadenomas that compress the optic nerve. Malignant prolactinomas are extremely rare but have been reported (39). Serum PRL levels in excess of 50 ng/ml is a diagnostic indicator for the presence of a prolactinoma. Treatment generally begins with the ergot alkaloid bromocriptine that acts as a DA agonist to inhibit PRL synthesis and release (41). Continued treatment usually results in normalization of serum PRL levels, alleviation of clinical symptoms and tumor regression (89). Patients who are non-responsive to chemical therapy can be treated with transsphenoidal surgical tumor removal. However, this approach achieves only a 50% long-term success rate for microadenomas with significantly less for macroadenomas (96). Although defects in the dopaminergic transduction cascade may be the cause of the insensitivity of some prolactinomas to DA, no mutation in the D2R gene has been detected (157). However, cells from these tumors respond to NGF by modifying their phenotype to a DA-sensitive cell type and this may prove a useful short-term treatment for patients where conventional pharmacology fails (334). No clear molecular alterations in oncogenes or tumor suppressor genes have been detected to explain formation of prolactinomas (26).

2. Lactotroph hyperplasia

In the adult pituitary, hyperplasia is unique to lactotrophs and many factors can contribute to this process. Most notably is the lactotroph hyperplasia that occurs during pregnancy. This is a result of both cell division and recruitment of somatotrophs into somatotrophs or lactotroph phenotypes (456). Estrogen plays both a direct and indirect role in lactotroph hyperplasia during pregnancy. Some of the action of estrogen may be mediated through its stimulation of GAL, which participates in estrogen-induced lactotroph proliferation (224,507). In the Fisher 344 inbred rat strain, estrogen induces both lactotroph hyperplasia and hypertrophy (20). Lactotroph subpopulations from estrogen treated F344 rat pituitaries exhibit differential responsiveness to bromocriptine. A small subpopulation of lactotrophs responds to bromocriptine by suppression of proliferation. Most lactotrophs also respond to DA agonists by reversing cell hypertrophy with concomitant inhibition of PRL production and release (386). Dopamine may be necessary to keep normal lactotrophs from spontaneous hyperplasia. Indeed, transgenic mice lacking D2R exhibit hyperprolactinemia with progressive lactotroph hyperplasia.
that eventually leads to tumor formation. Females deficient in D2R have an earlier onset of
tumor formation and higher PRL levels than males (16,261,417).

Section 4. Prolactin

Prolactin (PRL) is a lactogenic hormone originally identified as a product of the anterior pituitary
gland. However, extra pituitary sites of PRL production have since been identified that include
the brain, decidua and immune cells (33). Furthermore, a family of prolactin-related proteins has
emerged including placental lactogens, growth hormone variants, proliferin, and decidual
lutotropin (110,494). The widespread distribution of PRL-R combined with the spatial and
temporal pattern of PRL and PRL-related protein expression underscores the diverse functions of
these proteins. The nature of their regulation and the extent that they contribute to various
physiological processes such as lactation is not completely understood.

A. Gene structure and regulation of expression

PRL is a member of a gene family that includes GH and placental lactogen (PL) (98). It appears
that PRL and GH have evolved from a common ancestral gene by duplication and evolutionary
divergence (28). A large 16-kilobase fish gene coding somatolactin also belongs to the PRL/GH
gene family. Somatolactin shares 24% identity with PRL and GH and the structural organization
of the gene suggest that it may represent an ancestral precursor to both PRL and GH (465). In
humans, PRL is a single gene located on chromosome 6 and spans approximately 10 kilobases
(374). It is composed of five exons of variable length that are separated by four larger introns
that code a kilobase mRNA transcript (Fig. 2b) (477).

Upstream of the rat PRL gene are two distinct promoter domains that are essential for regulation
and tissue-specific expression. The proximal 5’-flanking region (-38 /-200 bp) and a more distal
enhancer (-1530/-1718 bp) are located upstream from the transcription initiation site. Highly
conserved DNA sequences located in the PRL promoter direct tissue-specific expression of the
PRL gene through binding to the pituitary transcription factor Pit-1 (Fig. 2a) (141). Additional
forms of the Pit-1 protein have been identified in the pituitary. A 54-amino acid truncated
variant that lacks part of the POU-homeodomain may be involved in repression of PRL
transcription (113). Alternatively, the Pit-1 beta variant contains a 26-amino acid insert and has
distinct transcriptional properties. A bipartite DNA element in the rat PRL promoter that maps
to –96/-87 and –76/-67 interacts with Ets-transcription factors and may mediate EGF, insulin and
TRH induced PRL gene transcription (243). Interactions of Pit-1 and Pit-1 beta with Ets-1, a
member of the Ets-transcription factor family, differs and may account for their unique
properties on the PRL gene (59). The 3P-DNA element in the proximal rat PRL promoter also
contains a consensus Ets-binding site and mutations within this domain decrease EGF, but not
basal, PRL gene transcription (222). These data support a functional synergism between Pit-1
and Ets-transcription factors in mediating hormone induced PRL gene transcription.

The regulation of PRL transcription requires interaction of proteins bound to DNA elements in
the distal and proximal promoter. There is evidence for structural changes in the chromatin that
juxtaposes these two regions allowing for physical interactions between bound proteins
(105,514). For example, the ER complex, through interaction with the ERE in the distal
enhancer region results in a chromatin “loop” that facilitates PRL gene transcription. This is likely mediated by stabilization of this loop structure resulting in protein-protein interactions between the juxtaposed distal and proximal enhancer (187). This structure may be the distinguishing feature that delineates cell specific expression of PRL.

A DNA element (-101/-92) in the proximal PRL promoter binds the CCAAT enhancer-binding protein alpha (C/EBPα) and is involved in basal and hormone induced PRL transcription (242). Calcium mediated induction of PRL transcription requires the first 395 bp of the 5’-flanking region of the proximal promoter (111). Methylation of the PRL promoter may modulate the action of transcription factors that leads to changes in PRL transcription. Hypomethylation of CpG dinucleotides between -277/-97 are correlated with high PRL gene transcription (362). Site-specific methylation may contribute to the tissue-specific expression of PRL.

The DA-mediated inhibition of PRL transcription involves a cAMP-responsive pathway, but the actual nuclear events that control downregulation of the PRL gene by DA remain unclear. The decrease of intracellular calcium in lactotrophs following DA exposure partially mediates the action of DA on the PRL gene (123). It appears that a Pit-1 site is necessary and sufficient to mediate DA repression of the PRL gene. This may be accomplished by inhibition of Pit-1 transcription conferred by a DA-responsive DNA element between −92/ +8 of the Pit-1 promoter (140). This is in contrast to the suppression of PRL gene transcription by TFGβ that does not modify Pit-1 gene expression (124). Glucocorticoids can inhibit PRL gene transcription directly through a regulatory element between −200/+75 of the PRL gene, perhaps through a similar mechanism as estrogen (454).

B. Structural heterogeneity of prolactin

PRL is a protein hormone of approximately 23-kDa molecular weight and 199 amino acids. PRL is present in all vertebrates and there is considerable heterogeneity in amino acid sequence among species (363). Most PRLs are composed of 197-199 amino acids and contain six conserved cysteine residues that result in three disulfide bridges that form two short loops at the N- and C-terminals and a large intramolecular loop (446). The three-dimensional structure of PRL has not been resolved, but modeling predicts that human PRL is folded in a four-helix bundle in the up-up-down-down configuration similar to GH (181). In addition to the predominant 23-kDa form of PRL, several variants of different sizes have been identified as a result of post-translational modifications that include proteolytic cleavage, glycosylation, and phosphorylation (90,371,448). There is considerable variation in both the temporal and spatial distribution of these PRL variants during development and reproduction (48,307). The physiological significance of these modified forms of PRL remains uncertain. Cleaved forms of PRL include a 16-kDa protein that inhibits mitogen induced endothelial cell proliferation independent of the PRL-R (150). The extent of glycosylation varies among species with rat and human possessing 8-15% of total PRL in glycosylated forms as compared to porcine that has 40-50% glycosylated (447). Modifications of PRL can result in altered immunoreactivity, receptor binding and biological action (69,308,431,451).
**Fig 2.** (A). Schematic representation of 5’- flanking region of the rat prolactin gene. (B). Processing of the human prolactin gene. Exons are numerical (1-5) and introns are letters (A-D). Abbreviations: ERE is estrogen response element and S-S indicates disulfide bridges.
C. Physiological actions

PRL has been implicated in a wide variety of physiological processes in vertebrates. Its best-characterized function is on the mammary gland. In most species, PRL along with ovarian steroids, insulin and cortisol are the principle hormones required for the development and differentiation of the mammary gland during pregnancy and lactation (154,218). However, PRL is the primary hormone responsible for lactogenesis by induction of milk protein genes in mammary epithelial cells (371). PRL also suppresses resumption of hypothalamic-pituitary-ovarian cycle and thus functions as a contraceptive agent during lactation (501). In women, hyperprolactinemia is associated with abnormal menses and infertility (96). The generation of PRL-deficient mice has confirmed the essential role of PRL in lactation and the development of the mammary gland (219,491).

The effect of PRL on reproduction can be both facilitatory and inhibitory, depending on the reproductive status of the animal (323,470). In rats, abnormal elevation of PRL is associated with a reduction in gonadotropin secretion that may be mediated by the suppression of hypothalamic GnRH synthesis and release (452). PRL can increase LH receptors in the ovary during corpus luteum formation and in the testis during puberty (44,131). PRL stimulates steroidogenesis in the corpus luteum while it is inhibitory in granulosa cells (44,106). The stimulation and growth of ovarian follicles is also affected by PRL (414,480). PRL-deficient transgenic female mice, but not males, are infertile due to absence of corpus luteum development (457,491). The function of PRL as a luteotropic hormone is unique to rodents.

The immune system is also a target of PRL. The rat Nb2 lymphoma cells are dependent on lactogenic hormones for growth, but this cell line may not represent the typical response of lymphocytes to PRL (215). There is some evidence that PRL plays a modulatory role in lymphocytes via autocrine or paracrine actions (314,315,326). However, the effect of PRL on immune cells does not appear essential since both PRL and PRL-R knockout mice show no recognizable alterations in the immune system (180,219). PRL also functions in the regulation of water and salt balance (320), growth and morphogenesis (469), and metabolism (217) in a variety of vertebrates. The brain is also a target of PRL that appears to modulate maternal (62,179), sexual (132) and ingestive (280) behaviors in many species.

D. Prolactin receptors

The PRL-R belongs to the class-1 cytokine receptor superfamily that shares conserved sequences in their extracellular domains (262). All PRL receptors have an extracellular ligand-binding domain, a single transmembrane region and the variable length cytoplasmic tail that mediates intracellular signaling (262). There are several PRL-R isoforms that differ in the length of their cytoplasmic domain as a result of alternate transcript splicing (369). PRL binding to its receptor leads to dimerization and activation of an intracellular cascade mediated by the JAK/STAT signal transduction pathway (47). The Janus kinase JAK2 is constitutively associated with the PRL-R and is activated upon PRL binding. Following activation of JAK2, tyrosine residues on the PRL-R and the transcription factor Stat5 are phosphorylated (198). Activated Stat proteins translocate into the nucleus where they bind DNA consensus motifs to mediate activation of target genes (235).
The PRL-R is widely distributed in vertebrate tissues and its expression levels can vary with reproductive status (47,373). The widespread distribution of the PRL-R is consistent with the diverse biological actions of PRL. However, the physiological significance of PRL-R in many of these tissues is not well understood. The regulation and function of the PRL-R is tissue dependent. For example, in the mammary gland the density of the PRL-R is increased during early lactation (129), while PRL-R in the choroid plexus mediates transport of PRL from the blood into the CSF (495). The PRL-R has been localized within the CNS and in cultured fetal dopaminergic neurons (11,23). The level of PRL-R expression in the hypothalamus is differentially regulated by ovarian steroids and during lactation (289,387). These data are consistent with PRL regulation of its own secretion from the pituitary via direct interaction with the THDA and TIDA neurons in the hypothalamus.

In male and female transgenic mice with heterozygous mutation of the PRL-R, reproduction was normal. However, homozygous males had a delayed fertility, while females were sterile with multiple abnormalities including failure of embryo implantation that could be corrected by administration of progesterone. Mammary gland development was impaired in heterozygous female mice with normal lactation occurring after few pregnancies. The homozygous females are infertile and do not lactate (47,370). No impairment has been detected in the immune system and the only behavioral deficit observed is in maternal care (56,301).

Section 5. Regulation of pituitary prolactin

A. Negative hypothalamic regulators

The neuroendocrine control of PRL secretion is a multifactorial process that involves both stimulatory and inhibitory molecules (Fig. 3). Pituitary lactotrophs are active secretory cells that maintain a high rate of basal secretion in the absence of an inhibitory signal. Disruption of the hypothalamic-pituitary connection by transection of the pituitary stalk, hypothalamic lesion or pituitary transplantation augments PRL release (32). Similar effects on PRL release are observed in vitro from isolated pituitary fragments or dissociated AP cells. This is unique to PRL since the release of all other major pituitary hormones is attenuated.

1. Hypothalamic dopamine

Dopamine is the principle PRL inhibitory factor (PIF). Physiologically a low basal rate of PRL secretion is maintained by DA release from hypothalamic TIDA neurons into the ME. The concentration of DA in the portal blood is sufficient to mediate the tonic inhibition of PRL release (194). The rate of DA biosynthesis and its release from TIDA neurons into hypophyseal portal blood has been effectively evaluated in the rat. Changes in the activity of tyrosine hydroxylase (TH), the rate-limiting enzyme in DA biosynthesis, turnover rate of DA, concentration of 3,4-dihydroxyphenylacetic acid (DOPAC), a DA metabolite, have been used to measure dopaminergic neuronal activity. Alterations in the activity of TIDA neurons reflect changes in PRL release and an inverse relationship between the concentration of DA in portal blood and plasma PRL has been documented (34).
Fig 3. Model depicting the complex regulation of PRL secretion. The release of PRL from lactotrophs is controlled by stimulatory and inhibitory endocrine, paracrine and autocrine factors. Stimulatory factors are shown in black and inhibitory factors in red.
PRL regulates its own secretion through a short loop feedback mechanism. Elevated PRL levels increase TIDA neuronal activity and DA release, thereby increasing the inhibitory signal that reduces pituitary PRL secretion (403). Hyperprolactinemia and hypoprolactinemia increase and decrease, respectively, TH mRNA levels in TIDA neurons and DOPAC accumulation in the ME (10). In lactating rats, suckling stimulates a rapid rise in plasma PRL and the level of DA in the ME is reduced within five minutes (125). Pup separation decreases plasma PRL and increases the concentration of DA in the ME and AP. The concentration of DA continues to rises in these tissues as the time interval of pup separation and non-suckling increases (83). In female rats, long-term estrogen treatment decreases TIDA responsiveness to PRL. Central PRL administration can increase the rate of DOPA accumulation in the ME of control animals but not in rats treated chronically with E2 (126). Systemically administered PRL antibodies are effective in neutralizing basal plasma PRL and decrease DOPAC in the ME of female, but not male rats. A similar reduction in basal TIDA neuronal activity was observed following central infusion of PRL antibodies (207). In males, hyperprolactinemia, induced by the DA receptor antagonist haloperidol, increased DOPAC in the ME. A sustained hyperprolactinemia (>3h) was required for DOPAC elevation and was blocked by central PRL antibodies (207). The activation of TIDA neurons by PRL is biphasic, involving a rapid response to acute changes in PRL concentrations and a delayed response that is activated by long-term alteration in plasma PRL.

2. Posterior pituitary dopamine

Dopamine released into the PP from THDA and PHDA neurons reach the AP via the short portal vessels and contribute to PRL inhibition (35). This was first demonstrated by PP lobectomy (LOBEX). In anesthetized male rats, LOBEX results in a rapid elevation in plasma PRL that is reversible by intracarotid injection of DA (384). Cycling female rats exhibit a faster elevation in PRL that is of shorter duration than males and is dependent on ovarian steroids (35). Chronic LOBEX in female rats results in elevated PRL that persists for 3 days before declining to near normal levels as a result of short loop feedback on the TIDA neurons (347). Further evidence supporting a role for DA derived from THDA and PHDA neurons of the PP in the suppression of PRL comes from lactating rats and sheep. During lactation and suckling, DA content and turnover rate in the ME and PP decreases (104,213). The selective suppression of THDA neurons caused by dehydration in the rat also reduces the PRL response to suckling (357). Pituitary stalk transection induces a gradual rise in plasma PRL over time that plateaus after 2 week. Despite the separation of the AP from the hypothalamus and loss of TIDA input, the PP maintains PRL inhibition while DA is gradually depleted (32). In sheep, the PP may contribute most of the DA, via the short portal vessels, that is responsible for the negative regulation of PRL (472). Alterations in either hypothalamic or PP DA pools may result in differential regulation of PRL release.

3. Dopamine type-2 receptors (D2R)

The D2 receptors are the product of a single gene that is alternately spliced into transcripts that code a long (444 amino acid) and short (451 amino acid) isoform. These membrane receptors contain seven-transmembrane domains and are coupled to G-proteins. Activation of D2R inhibits adenylyl cyclase activity and decreases intracellular calcium (65). The AP contains both the long and short forms of the D2R, but the long form predominates (109). DA binding to the
D2R mediates the tonic inhibition of PRL release from lactotrophs. PRL secretion is elevated following brief removal of DA and this is likely the result of the rapid dissociation of DA from the D2R. Intracellular cAMP increases after DA withdrawal through activation of protein kinase-A, protein kinase-C and phospholipase-C. The removal of DA is sufficient to release PRL and can potentiate the action of other PRL secretagogues through activation of second messenger pathways and increases in intracellular calcium (88,311). Disruption of the D2R in transgenic mice supported the involvement of these receptors in the negative regulation of PRL release and lactotroph proliferation. Both male and females animals exhibit chronic hyperprolactinemia with a progressive increase in lactotroph number and pituitary size during aging (261). Plasma PRL levels in D2R-deficient females is 6-fold higher than in males and tumor formation has an earlier onset. The PRL-R shows a concomitant elevation with tumor expansion (417). Sexual differences in plasma PRL and lactotroph hyperplasia in D2R-null mice may be the result of a hypersensitive subpopulation of lactotrophs to negative regulation by DA and emphasizes the role of estrogens on lactotroph function. Paradoxically, DA at very low concentrations can stimulate PRL release under certain experimental conditions. The mechanism responsible for this effect is poorly understood, but limited evidence suggests that both the D1R and D5R may mediate this PRL induction via activation of adenylyl cyclase (392).

4. Dopamine transporter (DAT)

The DA transporter (DAT), a member of the family of (Na+/Cl-) dependent transporters, mediates reuptake of DA from the synaptic cleft by the presynaptic membrane (211). The mouse DAT is a 619 amino acid protein that has been detected in the hypothalamic-pituitary axis (506). Pharmacological blockade of DAT by cocaine (non-specific) and mazidol (specific) decrease PRL mRNA in the AP and increase TH mRNA in the hypothalamus. Estradiol induced PRL release is abolished by the blockade of DAT. The formation of DOPAC decreases in the ME, IL and NL following DAT blockade. Because DOPAC formation requires reuptake, its decrease indicates a functional loss of a DA reuptake mechanism by axon terminals at these sites. The presence of immunoreactive DAT in the ME, IL and NL support a functional DAT system located in TIDA, THDA and PHDA neuron populations (127). Dysfunction of the DAT system should result in increased concentration of DA reaching the AP via the long and short portal vessels. Indeed, transgenic mice with deletion of DAT exhibit AP hypoplasia, dwarfism and an inability to lactate. Although animals have normal plasma PRL there is a reduced number of lactotrophs and somatotrophs and an altered spatial distribution of these cell types in the AP (55). The elevated DA levels in DAT-null mice downregulate the D2R and commensurate alteration in lactotroph sensitivity to DA may explain unchanged plasma PRL.

5. Peptide factors

Although hypothalamic DA is the predominant inhibitory molecule regulating PRL secretion, factors produced within the pituitary gland can also decrease PRL production and release. TGFβ1, a member of the TGFβ family, is important in the regulation of extracellular matrix, cell proliferation and differentiation. Lactotrophs in the AP produce and secrete TGFβ1 and intrapituitary administration of TGFα1 inhibits plasma PRL and pituitary weight (332). In primary AP cultures, TGFβ1 selectively decreases basal PRL production and release in a dose and time-dependent manner (466). Furthermore, TGFβ1 suppressed estradiol-induced PRL
secretion and lactotroph proliferation (423). In female rats, 60% of TFGβ1 immunoreactivity in
the AP was located in lactotrophs and estrogen treatment reduces this number (67). The effects
of TGFβ1 are mediated through interaction with the TGFβ1 type II receptors (TGFβ-R2) that
have been localized to the surface of lactotrophs. Estradiol treatment decreases the specific
binding of 125I-TGFβ1 and TGFβ1-R2 mRNA in lactotrophs (120). In GH3 cells, TGFβ1
specifically reduces basal and calcium-stimulated PRL mRNA levels. This inhibition is
mediated by a Pit-1 independent transcriptional mechanism (124). The TGFβ1 inhibitory
response maps to position -116/-54 in the rat PRL promoter, but no single cis-acting element
within this region of DNA appears sufficient to mediate this action (148). Immunodepletion of
TGFβ1 by specific antibodies increases PRL transcription in a subpopulation of lactotrophs in
vitro (2). These data support a negative regulatory role for TGFβ1 in a subpopulation of
lactotrophs expressing the TGFβ1-R2 through autocrine or paracrine interaction. Alterations in
the sensitivity of lactotrophs to TGFβ1 by estrogen-dependent changes in TGFβ1-R2 suggest a
physiological role for this factor during the reproductive cycle.

Other inhibitors include calcitonin (CT), Endothelin (ET) and gamma amino butyric acid
(GABA). CT inhibits basal and TRH-induced PRL mRNA and release from rat AP cells through
inhibition of a calcium-inositol tri-phosphate signaling pathway. CT-like peptides are released
from cultured AP cells and CT-antiserum stimulates PRL release from these cultures (435). ET-
1 and ET-3 have been identified in the pituitary and both inhibit PRL release from AP cell
cultures. ET-1 is more potent than ET-3 in AP cells grown in serum containing media. Cell in
serum-free media exhibit a biphasic response with low concentrations inhibiting PRL release and
high concentrations stimulating a transient rise in PRL (135). The signals responsible for the
inhibitory and stimulatory responsiveness of PRL to ET remain undefined. GABA inhibits PRL
secretion directly by interaction with high affinity receptors in the AP (318). Lactating rats have
elevated GABA content in the AP and these levels decline in parallel with plasma PRL following
pup removal (396). However, GABA levels in portal blood is not significantly higher than those
in general circulation (345), suggesting that little secretion of GABA occurs in the ME.
GABAergic neurons innervating the PP may provide the AP with GABA via the short portal
vessels (64).

B. Positive hypothalamic regulators

Many physiological events can elevate plasma PRL levels. In mammals serum PRL is elevated
during the reproductive cycle, early post-partum, lactation, and after suckling. PRL release is
sensitive to numerous stressors including anesthesia and surgery. In addition, there is a nocturnal
rise in serum PRL from episodic secretory bursts that occurs during sleep. Like other AP
hormones PRL is released in a pulsatile manner throughout the day and night. This episodic
pattern of PRL release is mediated by a neural mechanism and its physiological significance is
not understood. In rodents, PRL has an important additional supporting role in initiating and
maintaining the corpus luteum of the ovary (30,91).

Unlike the other AP hormones, a singular hypothalamic PRL releasing factor (PRF) has not been
identified. The removal of hypothalamic dopaminergic tone rapidly elevates PRL release and
stimulates biosynthesis. The physiological requirement for a fast-acting positive regulator of
PRL release remains dubious. Yet, a diverse group of molecules have been implicated in the
stimulation of both PRL release and gene expression. The complexity of their contributions to PRL regulation is exemplified by the diversity of their actions throughout the entire neuroendocrine axis. This is further complicated by both a synergistic and antagonistic action on PRL release that involves a heterogeneous lactotroph population. Furthermore, the time scale of their action on PRL release varies and is often dependent on the reproductive status. Although evidence from in vitro studies has demonstrated the potency of many of these factors on PRL release, only a few have been established in the physiological regulation of PRL.

1. Compounds that affect PRL by disinhibition of dopamine

Pharmacological evidence in vivo has implicated several hypothalamic neurotransmitter pathways in the stimulation of PRL release by inhibiting DA. Neurons originating from the dorsal raphe nucleus that produce 5HT stimulate PRL release (27). Destruction of these abolishes PRL-induction by 5HT and stress (333). Selective activation of 5HT1 and 5HT2 receptors increases plasma PRL levels (409). More recently, the 5HT3 receptor has been implicated in mediating stress-induced PRL release (366). An intact PP is essential for PRL-induction by the 5HT precursor 5-hydroxytryptophan (5-HTP), suggesting an indirect mechanism of action (249). No 5HT receptors have been localized to lactotrophs suggesting that 5HT indirectly activates an intermediary molecule in the PP that stimulates PRL.

GABA also functions as both a positive regulator of PRL release by reducing TIDA neuronal activity (321,396). This action is likely mediated by high affinity GABA-B receptors identified in the hypothalamus, but absent in the pituitary (264). Some evidence supports hypothalamic GABAergic activation as a mechanism by which 5HT stimulates PRL (4).

Histamine (HA) stimulates PRL release via H2-receptors following central administration and H1-receptors following systemic infusion. The induction of PRL by HA occurs at different sites within the hypothalamus through alteration of dopaminergic and serotonergic neuronal activity (272). The portal concentration of DA decreases following central or systemic HA infusion and blockade of DA or 5HT receptors prevents HA stimulated PRL release (273). No evidence for direct action of HA on pituitary lactotrophs has been reported.

2. Thyrotropin releasing hormone (TRH)

Posttranslational processing of a larger precursor protein, proTRH, yields several copies of the TRH peptide. TRH is a tripeptide composed of pyroglutamyl, histidyl, and proline amide. An intact amide group and a cyclized glutamic acid terminus are essential for TRH activity (365). In addition to the rapid stimulation of TSH from thyrotrophs, TRH is as effective in stimulating PRL release from lactotrophs. TRH neurons from the PVN innervate both the ME and PP (286). TRH levels in portal blood show some correlation to elevated PRL during the afternoon of proestrus and suckling (116,152). Furthermore, TRH antiserum blocks both proestrus and suckling-induced elevations in plasma PRL (216,439). Hence, decreased hypothalamic DA may elevate basal plasma PRL during lactation and TRH might stimulate the acute elevation in PRL following suckling.
Intravenous administration of TRH results in a rapid elevation in serum PRL in many species (122,481). The action of TRH on PRL release is through binding to the TRH-receptor (TRH-R) (291,515) a seven-transmembrane domain G-protein coupled receptor. Ligand binding activates PKC (461) and this leads to increased inositol tri-phosphate (IP3) that causes calcium discharge from the endoplasmic reticulum. Elevated intracellular calcium rapidly releases PRL from storage granules (175,462). A smaller but more prolonged rise of PRL results from influx of extracellular calcium that is mediated in part by TRH-R activated diacylglycerol (DAG) (276).

The GH3 cell line has proven useful in elucidating the role of TRH and the TRH-R in PRL regulation and lactotroph function. These cells express TRH-R and responds to TRH with the rapid release of PRL and increased gene expression (117,354). Activation of MAPK by a PKC-dependent pathway results in phosphorylation of Ets-transcription factors that bind to the PRL promoter and increases PRL gene transcription (114,496).

The regulation of PRL release by TRH is confounded by the difference in the temporal release of PRL and TSH from the AP when serum PRL is elevated (274,408). Thus, the physiological status of an animal may differentially alter the sensitivity of lactotrophs or thyrotrophs to TRH and consequently hormone release. Indeed, estrogens can increase the number of TRH-R in primary AP and lactotroph cell lines without changing their affinity (174). Upregulation occurs by increased transcription of the TRH-R and stabilization of its mRNA (265). TRH itself can downregulate TRH-R mRNA via a PKC-dependent mechanism (159). Within a given population of GH3 cells, individual cells display a unique pattern of PRL gene expression in response to TRH (487).

3. Vasoactive intestinal polypeptide (VIP)

VIP and pituitary adenylate cyclase activating polypeptide (PACAP) are two structurally related peptides that belong to the VIP/secretin/glucagon protein family (36,142). There are two structurally distinct receptors that recognize these peptides with similar affinity, PACAP/VIP R-1 and PACAP/VIP R-2. An additional receptor, PACAP-Type I, is alternately spliced into multiple isoforms and has a high affinity for PACAP, but not VIP (12,500). These receptors are G protein-coupled with seven-transmembrane domains and many have been localized to the hypothalamus and AP (209,389).

Originally isolated from the gut, VIP is a 28 amino acid peptide that stimulates PRL release in vivo and in vitro (259,413). It has been identified in portal blood at concentrations sufficient to stimulate PRL (440). VIP is less potent than TRH and the temporal stimulation of PRL is delayed and more gradual (43,310). VIP neurons have been identified in the PVN and their activation by 5HT neurons from the dorsal raphe are thought to mediate stress-induced PRL release (400,484). Indeed, activation of hypothalamic VIP neurons during chronic, but not acute, stress increases VIP levels in portal blood and serum PRL levels (173). Antiserum to VIP reduces basal PRL release from cultured AP cells in vitro (195) and suckling-induced PRL release in vivo (1). Like TRH, VIP may contribute to the elevation of PRL during suckling, but it does not appear to participate in the preovulatory PRL surge (498).
In addition to the hypothalamus, VIP mRNA and immunoreactivity has been detected in the pituitary (15). Female rats have a higher VIP content in both the hypothalamus and pituitary compared to males. Moreover, hypothalamic and pituitary VIP levels are regulated in opposing directions (407). Lactotrophs can express VIP and this may be dependent on the status of thyroid and ovarian hormones (81,377). Pituitary VIP may function in an autocrine or paracrine manner to stimulate PRL release (24,356) and it may be the reason for a high rate of basal PRL release from cultured lactotrophs. The action of TRH and VIP on PRL release is additive, indicative of independent signaling pathways for these peptides in the lactotroph (142,189). Although the PACAP/VIP receptors are coupled to several different signal transduction systems, activation of PKA and elevations in cAMP likely mediate the stimulation of PRL release from lactotrophs by VIP (97,189). The role of PACAP in PRL regulation is unclear since its action is broad, stimulating the release of several pituitary hormones.

4. Angiotensin II (AII)

Renin is a 347-amino acid enzyme secreted by the kidney that converts the pro-hormone angiotensinogen into inactive angiotensin I that is further processed by angiotensin-converting enzyme (ACE) into the bioactive octapeptide angiotensin II (AII) (136). In addition to the stimulation of aldosterone from the adrenal gland and vasoconstriction, AII regulates the secretion of pituitary hormones (165). The action of AII is mediated by three distinct receptors, AT1A, AT1B and AT2 (92). The AT1A and AT1B represent a receptor subfamily, but have unique gene promoters that result in differential regulation and tissue distribution (92). The AT1 receptors have been implicated in the mediation of most neuroendocrine effects of AII. Central administration of AII inhibits PRL release by activating TIDA neurons in the hypothalamus (165,355). This action of AII is mediated by AT1A whose induction may be dependent on ovarian steroids (251). Conversely, in the AP AII stimulates PRL release via the AT1B receptor. Ovarian hormones decrease the expression of AT1B mRNA and protein levels in the AP (74,251). The pituitary produces renin-angiotensin proteins and several endocrine cell types express AT1B receptors (165,342). The production of angiotensinogen and its conversion to AII may be accomplished by more than one pituitary cell type. Gonadotrophs do not contain significant angiotensinogen, but ACE and AII co-localize with the β-subunit of LH (164,166). Interestingly, lactotroph-enriched AP cell cultures incubated with gonadotrophs enhance PRL release by AII (128). The AT1 receptors are coupled to multiple signal transduction pathways including adenyl cyclase, phospholipase-C (PLC) and the PKC pathways (18,342). Although AII is a potent PRL secretagogue in vitro, it may play only a limited physiological role in PRL release. Indeed, intravenously administered antiserum to AII has no effect on the suckling-induced plasma PRL rise (359).

5. Oxytocin (OT)

The PP peptide OT is necessary for uterine contraction during delivery and the suckling-induced milk ejection during lactation. OT exerts both stimulatory and inhibitory control over PRL release depending on its route of administration. The action of OT is mediated by the OT receptor (OT-R) that belongs to the seven-transmembrane-spanning G-protein-coupled receptor family. Activation of the OT-R stimulates IP3 that mediates calcium release from stored intracellular pools and influx through membrane channels (144). The central infusion of OT into
the third ventricle inhibits PRL via activation of TIDA neurons (516). Conversely, OT stimulates PRL release from pituitary AP cell cultures (419). The expression of the OT-R is restricted to lactotrophs in the AP, suggesting a direct effect of OT on PRL release (61). The physiological role for OT regulation of PRL is controversial. Serum OT levels rise just before the suckling-induced PRL surge and infusion of OT antiserum into lactating rats delays and reduces PRL release after suckling (419). However, OT-R antagonism blocks suckling-induced milk ejection but not the acute PRL rise (250). A direct action of OT on PRL release is supported by in vitro stimulation, but the rather weak potency of OT relative to other secretagogues makes its contribution to the physiological regulation of PRL questionable (232).

6. Opioid peptides

The opioid peptides are derived from three preprohormones: POMC, proenkephalin, and prodynorphin. Multiple receptor classes have been identified and generally enkephalins activate delta-receptors, dynorphins the kappa-receptors and β-endorphin target both delta- and mu-receptors (331). POMC is a 241 amino acid protein produced by, but not limited to, hypothalamic arcuate neurons, AP corticotrophs and IL melanotrophs. The mechanism regulating POMC biosynthesis, processing and secretion is tissue specific (78). In the PP, POMC peptides are localized to the IL whereas proenkephalin and prodynorphin neurons innervate the NL (42). There may also be local production of these two opioids within the NL by glial cells (428). Proteolytic processing of POMC yields bioactive peptide products that include the endogenous opioid β-endorphin.

In many species, morphine, β-end, enkephalins and dynorphins all stimulate PRL release following central or systemic administration (177,482). The failure of β-end to stimulate PRL release following pituitary stalk transection (497) and lack of effect on isolated AP cells (232,296) supports a hypothalamic site of action. Opioid peptides inhibit DA production and release via the mu- and kappa-opioid receptors (453) located on dopaminergic neurons of the ME and PP (192,200,298). Antiserum against β-end reduces both basal and stress induced PRL release (397). The opioid receptor antagonist naloxone can reverse opioid stimulated PRL release (130). Naloxone alone reduces basal PRL in male rats, but not females, and this effect is dependent on androgens (453). Naloxone also reduces stress, suckling- and estrogen-induced PRL release, suggesting a role for endogenous opioids during these physiological states (32). Although alterations in pituitary DA may be the primary mechanism of opioid induced PRL release, there is evidence for the participation of other hypothalamic factors (14). Opioid receptors have not been identified in pituitary lactotrophs, consistent with an indirect role for opioid peptides on PRL release.

7. Prolactin-releasing peptide (PrRP)

Recently, a novel peptide (PrRP) was isolated from the hypothalamus by reverse pharmacology using an orphan seven-transmembrane domain containing pituitary receptor (hGR3) as ligand bait. This 31 amino acid peptide stimulates a modest, yet specific, release of PRL in vitro within one hour (210). These investigators have since reported that PrRP caused a dose-dependent increase in PRL release both in vitro and in vivo (316,317). They proposed that PrRP was the hypothalamic PRF and its action at the hGR3 receptor mediates PRL release from AP
lactotrophs. This proposal was quickly refuted when several groups demonstrated that PrRP is absent in the ME and pituitary (312,341,512). Although the AP contains a high density of hGR3 receptors, the mechanism of PrRP transport from the hypothalamus to the pituitary is unclear. In the absence of de novo PrRP synthesis within the pituitary the role of PrRP in PRL release is questionable. Indeed, PrRP has a weak stimulatory effect on PRL release from AP cell cultures (421). Similarly, PrRP has a weak potency in vivo, requiring supraphysiological doses and dependency on estrogen to release PRL (473).

The detection of PrRP in discrete hypothalamic and brain stem nuclei suggests a broader role for this peptide in brain function (411). Intracerebroventricular infusion of PrRP results in a significant increase in both OT and VP release from female rats, but only OT from males (313). Central infusion of PrRP also increases blood pressure (421). These functional and anatomical data indicate that the primary action of the PrRP peptide is not on PRL release.

C. Other positive regulators

1. Estrogens

The estrogens are 18-carbon steroids that interact throughout the neuroendocrine axis to regulate pituitary hormone release. In females, the ovaries are the primary source of estrogens, but during pregnancy, the fetal-placental unit is responsible for the massive increase in circulating progesterone and estrogen. Males produce limited amounts of estrogens de novo by aromatizing testosterone (367,442). Estrogen binds two soluble cytoplasmic receptors, ERα and ERβ. Following activation, the ER complex translocates to the nucleus where it interacts directly with unique DNA sequences (ERE) to regulate gene transcription (322). The existence of a membrane ER that may mediate non-transcriptional effects of estrogen has been reported (398). The distribution of ERα and ERβ does not completely overlap and they may serve unique functions within the same cell (443).

The lactotroph is an established target of estrogen. Although both ERα and ERβ mRNA have been detected in the lactotroph, the low level of ERβ expression suggests that it is not the primary mediator of estrogen action in lactotrophs (337,502). In transgenic mice lacking functional ERα, PRL mRNA and lactotroph cell number are reduced (432). These transgenic mice demonstrate the importance of ERα in PRL gene transcription and proliferation, but not on lactotroph differentiation. In certain rat strains (F344), chronic estrogen treatment induces hyperprolactinemia that is accompanied by lactotroph hyperplasia and eventual prolactinoma formation (160). The mechanism by which estrogen stimulates lactotroph proliferation is unclear but a direct mitogenic effect is supported by in vitro studies (295,444).

Systemic administration of 17-β-estradiol (E2), but not progesterone (P4), stimulates pituitary PRL gene expression and release. Females are more sensitive to E2 than males and show higher pituitary PRL content and gene induction (474). In both primary AP cultures and lactotroph cell lines, estrogens stimulate PRL gene expression and release. Estrogen treatment also stimulates non-secretory lactotrophs to release PRL and increases the amount of PRL released by individual lactotrophs (293). Estrogens also act at CNS sites to suppress DA and consequently stimulate PRL release (245,287).
2. Galanin (GAL)

Galanin (GAL) is a 29 amino acid peptide implicated in the neuroendocrine regulation of PRL. Three receptor subtypes (GAL1-3), belonging to the G-protein coupled seven-transmembrane domain superfamily, modulate GAL action (60). The GAL2 receptor has been identified in the pituitary gland and likely mediates the direct action of GAL on lactotrophs (510). Neurons containing immunoreactive GAL synapse in the ME and GAL is detectable in portal blood (299,372). In the arcuate nucleus GAL immunoreactivity co-localizes with TH and ERα in TIDA neurons (220,223). In the rodent pituitary gland, GAL is expressed predominantly in lactotrophs where it is co-localized in secretory granules with PRL (229). Expression of this peptide is extremely sensitive to estrogen, which dramatically increases GAL mRNA and release (257) as well as the number of lactotrophs secreting GAL (70). Estrogen-induced PRL rise and lactotroph proliferation can be reduced with GAL antiserum (205,507), suggesting that the estrogenic effects are mediated in part by autocrine or paracrine actions of GAL from the AP. The secretion of GAL from the AP is inhibited by DA and somatostatin and stimulated by TRH (230,231). Intracerebroventricular, but not intravenous, injection of GAL elevates plasma PRL and this is suppressed by VIP antiserum (277). Superfused rat hypothalamic fragments release VIP in response to GAL suggesting central GAL can modulate PRL by stimulating VIP release (239).

Transgenic mice deficient in GAL exhibit endocrine abnormalities that relate to PRL expression. These mice have reduced PRL mRNA, serum PRL levels, and females fail to lactate. Their lactotrophs do not proliferate in response to high doses of estrogen, nor do they up-regulate PRL gene expression or release (508). Male and female transgenic mice that carry the GAL gene linked to the PRL promoter have elevated GAL mRNA and peptide levels in the AP. Female mice overexpressing GAL show a gradual elevation in pituitary PRL content and mRNA between 2-4 months of age, but it takes 6 months to increase serum PRL and 11 months for an increase in lactotroph cell number. Overexpression of GAL in male mice did not alter PRL expression or lactotroph cell number. However, estrogen treatment of these male mice increased pituitary weight and PRL secretion (71). These data support the hypothesis that GAL acts as an autocrine or paracrine regulator of PRL expression and lactotroph growth and mediates estrogen actions on the lactotroph.

3. Epidermal growth factor family

Epidermal growth factor (EGF) and transforming growth factor-alpha (TGFα) belong to the same protein family that binds to EGF receptors. The EGF receptors are tyrosine kinases and ligand-binding results in receptor dimerization followed by the activation of multiple signaling pathways. The mRNA for both EGF and TGFα are expressed by somatotrophs and gonadotrophs, but lactotrophs express only TGFα. All pituitary cell types express EGF receptors (146). EGF stimulates PRL genes expression and release in vitro without modifying Pit-1 mRNA (518). EGF activates the same cis-elements of the proximal PRL promoter that is responsible for TRH induction of the PRL gene, but does so via different intracellular pathways (40). EGF treatment increases the proportion of cells secreting PRL from primary AP and GH3 cell cultures (149). This increase does not involve mitosis, but rather a transdifferentiation (255).
Hence, EGF may facilitate PRL expression directly by activating gene transcription and release, but also by increasing the proportion of cells secreting only PRL.

Estrogen induces TGF\(\alpha\) mRNA in the AP and this effect can be reversed by bromocriptine. Neither treatment alters EGF receptor mRNA, suggesting that this receptor is not co-regulated with TGF\(\alpha\) (53). In transgenic mice, overexpression of TGF\(\alpha\) in lactotrophs induces hyperplasia in females at 6 months of age. By 12 months, all homozygous females develop prolactinomas. Male transgenic mice develop neither pituitary hyperplasia nor adenomas (319). The induction of TGF\(\alpha\) by estrogen in the AP precedes lactotroph hyperplasia and the specificity of this action may contribute to the formation of prolactinomas.

4. Heparin-binding growth factors

Among the heparin-binding growth factors reported to stimulate PRL, basic-fibroblast growth factor (FGF-2) is the most potent. This 146 amino acid protein is concentrated in the pituitary gland from which it was initially isolated (185,186). FGF-2 is a member of a large family of heparin-binding proteins that interact with a few common tyrosine-kinase receptors (246,464). In general, they all participate in cell growth and differentiation, but several, including FGF-2, have been implicated in the progression of pituitary tumors (145,204). FGF-2 can stimulate PRL gene expression and release \textit{in vitro} from both AP cells and pituitary tumor cell lines (304). The induction of PRL by FGF-2 requires prolonged exposure and does not involve mitosis (22). This may reflect a primary action of FGF-2 on PRL transcription via induction of the PRL promoter and a secondary effect on PRL release. In GH4 cells, FGF-2 activate MAP kinase-dependent Ets factors that interact with two Ets binding sites in the proximal rat PRL promoter to activate transcription (430).

The non-hormone producing folliculo-stellate cell in the AP and IL is the primary source of FGF-2 in the pituitary gland (151). The FGF-2 precursor protein lacks a signal peptide and consequently FGF-2 is not packaged into secretory granules (328). Although the mechanism of FGF-2 secretion is poorly understood, it is clearly released from cells. The recent identification of a FGF binding-protein may provide a mechanism for FGF-2 transport (107). The high affinity of FGF-2 for heparin sulfate proteoglycans contributes to its mobilization and possibly its activity (464,490). The pituitary is a rich source of heparin sulfate proteoglycans; in particular the colloid material produced by IL cells and concentrated in Rathke’s cleft (176,486,492). The liberation of these growth factors may be regulated by activation of binding proteins or heparinases (139,147). Recently, the estrogen inducible protein TGF\(\beta\)3 has been shown to stimulate FGF-2 release from FS cells \textit{in vitro} (206). Hence, the regulated release of FGF-2 from FS cells indicates that it may act as a paracrine factor to regulate lactotroph function.

Another member of the FGF family, acidic-FGF or FGF-1, was also isolated from the pituitary gland (163). This 140 amino acid protein is the product of a distinct gene and differs from FGF-2 in its heparin-binding properties (21). FGF-1 also stimulates PRL gene expression and release \textit{in vitro}, but is less potent than FGF-2 (304). Although both FGF-1 and FGF-2 bind to the same receptor, FGF-2 has a much greater affinity and this is likely responsible for the differences in potency (361). Lactotroph differentiation from neonatal rat AP cell cultures is stimulated by
FGF-1 albeit with less potency than FGF-2 (394). The cell type that produces the low levels of FGF-1 found in the pituitary has not been identified.

Human prolactinomas express heparin-binding secretory transforming gene (hst) that encodes FGF-4. FGF-4 expression is restricted to cells during early development and is not expressed in normal adult tissues (63). Unlike FGF-1 and FGF-2, FGF-4 contains a signal peptide and is secreted by the typical secretory pathway (395). The expression of this gene in adult animals is associated with oncogenic transformation. Primary rat AP cells and GH3 cells treated with FGF-4 exhibit a dose-dependent increase in PRL gene expression and release (441). The significance of FGF-4 on PRL induction may relate to its function following lactotroph transformation.

These heparin-binding proteins may be involved in the growth and differentiation of pituitary cells during physiological and pathophysiological states. Although their contribution to PRL regulation in vivo has not been established their potency in vitro suggests they may be important modulators of PRL gene expression and release.

**Section 6. Prolactin-releasing factor (PRF) from the posterior pituitary**

1. Historical perspective

PRL is essential for lactation and its release in response to suckling facilitates newborn survival by ensuring adequate maternal milk. In lactating rats the onset of suckling rapidly increases plasma PRL up to 100-fold compared to non-suckled animals (66,348). The termination of the suckling stimulus gradually decreases plasma PRL to pre-surge levels. A decline in pituitary DA during lactation elevates basal PRL, but this alone is insufficient to account for the suckling-induced PRL surge. Additional factors are necessary to achieve this rapid and pronounced elevation in PRL. Although several hypothalamic peptides have been implicated in the stimulation of PRL during suckling, no clear physiological PRL-releasing factor (PRF) has been identified.

The typical hypothalamic releasing factor is a peptide, characterized by a high concentration in the ME and a potent action through high-affinity receptors on specific hormone secreting cells in the AP. Most hypothalamic peptides that act directly on the lactotroph to stimulate PRL release lack specificity. The multiplicity of their action suggests that PRL induction is secondary to their primary function in other cell systems. In addition, most of these peptides are weak stimulators of PRL release in vivo and their action in vitro is often slow, requiring hours to significantly elevate PRL. Furthermore, blockade of their action by specific antagonists or antisera fails to abolish PRL release under many conditions. Evidence has accumulated over many decades to support the existence of a PRF and it remains the last unidentified hypophysiotropic hormone.

2. Detection and physiological significance

The first evidence for the existence of a potent PRF in the PP was shown in LOBEX rats that failed to respond to suckling or estrogen administration with a PRL surge (348,350). Although basal PRL was elevated in these animals due to the loss of PP dopamine, treatment with a 5HT precursor, TH blockade, or exposure to ether increased PRL to the same levels of control animals.
This demonstrates that LOBEX animals have functional DA and 5HT hypothalamic systems and their disruption is not responsible for the loss of function. LOBEX results in diabetes insipidus and failed milk ejection during lactation due to losses of VP and OT, respectively. The administration of exogenous VP and OT analogs to lactating-LOBEX rats restores both water balance and milk ejection without increasing PRL (348). This PP PRF is also involved in the PRL surge at the peak phase, but not the plateau, of proestrus (351). LOBEX attenuates the nocturnal PRL rise during early pregnancy but has no effect on the preovulatory LH surge or ovulation (19). These data demonstrate that the PP is the source of a PRF and several independent laboratories have confirmed this localization (420,513).

3. Characterization

The initial in vitro characterization of PRF showed that acid extracts of the rat PP elicit a specific, rapid and concentration-dependent stimulation of PRL release from perfused AP cells (232). Hypothalamic extracts result in a weak stimulation of PRL release that is blocked by TRH receptor antagonists. The action of TRH, OT, and AII peptides on PRL release were inhibited by specific receptor antagonists and were shown not to significantly contribute to PRL induction by PP extracts. Systemic injection of rat PP extracts results in a concentration-dependent elevation of plasma PRL, but not GH or LH (228). Size exclusion chromatography estimates the molecular weight of PRF from PP extract to be less than 5-kDa. This PRF peptide is insensitive to enzymatic digestion by trypsin, but is inactivated by chymotrypsin or proline-specific endopeptidase. In addition, PRF bioactivity is resistant to permanganic acid oxidation, a treatment that inactivates OT and VP. Furthermore, PRF-induced PRL release was unaffected by pretreatment with the opioid antagonist naloxone (227). These biochemical features of PRF differentiate it from other PRL secretagogues like TRH, AII and OT.

4. Cellular origin

The transection of the pituitary stalk diminishes PRF activity from the PP (233). This suggests that PRF may be produced by the hypothalamus and transported to the PP where it accumulates and is released. Several lines of evidence have ruled out the hypothalamus as a site of origin for PRF. First, in cultured PP cells the protein synthesis inhibitor cycloheximide suppresses PRF activity, suggesting de novo synthesis. Second, the concentration of DA, OT and α-MSH gradually decrease in PP cultures, but PRF activity remains stable (284). Third, the separation of PP cells by density gradients shows that PRF activity is confined to a subpopulation of IL cells that are not melanotrophs (459). Finally, PRF activity has been demonstrated in pituitary IL’s of many species including; rats, mice, bovine, ovine, fish and primates (32). Although adult humans do not have a distinct pituitary IL, it is thought that cells from the fetal structure migrate and disperse into surrounding AP and PP tissue. Indeed, the adult human pituitary contains PRF activity in both the AP and PP (32). The loss of PRF activity following pituitary stalk transection may be due to the loss of trophic hypothalamic factors that support PRF production. Alternatively, the suppression of PRF activity from cultured PP cells following the addition of PRL suggests a role of PRL negative feedback in PRF regulation (284).
5. Long-term action

The long-term action of PRF on lactotroph function was evaluated by co-culture of PP cells with primary AP or GH3 cells. In AP cells, intra-cellular PRL content and its accumulation in culture media increase proportionally to the number of co-cultured PP cells (133,232). This increase is specific for PRL and PRF has no effect on LH and GH cell content or release. Co-culture also potentates the action of: TRH, AII, potassium, PKC activation and the calcium ionophore A23187 on acute PRL release (134). Conditioned media from PP cells mimic the effect of co-culture on PRL release. Co-culture of GH3 cells with PP cells increases PRL, but not GH mRNA (99). This elevation in PRL gene expression parallels an increase in PRL cell content and PRL secretion (458). There is no change in the proliferation rate of GH3 cells during co-culture with PP cells. The sensitivity of GH3 cells to PRF action on PRL gene expression and release indicates their usefulness as a cell model for the characterization and identification of PRF from the PP.

6. PRF in IL tumors from transgenic mice

Transgenic mice generated using a truncated POMC promoter ligated to the large T-antigen (POMC-Tag) of the transforming simian 40 virus develop large IL tumors (300). Tumor formation was restricted to the IL and was the result of melanotroph expansion. Adult mice with primary IL tumors were reported to have elevated serum PRL. Extracts from primary IL tumors and secondary tumors, generated from subcutaneous injection of primary IL tumors cells into athymic nude mice, showed PRF activity \textit{in vitro} (7). The utilization of these POMC-Tag mice for the generation of IL tumors as a source for the isolation of PRF represents the foundation of my thesis work.

7. Previous attempts to purify PRF

Several groups have tried and failed to identify PRF from the PP. Partially purified bovine and ovine PP extracts suggests that there may be several peptides with similar PRF bioactivity. It is uncertain if these multiple PRFs represent distinct peptides or altered forms of a single molecule. PRF from the PP can stimulate PRL release \textit{in vivo} in the presence of physiological concentrations of DA and is critical for the PRL surges of lactation, ovulatory cycle and in response to estrogen. In addition to stimulating PRL release, PRF participates in lactotroph recruitment and PRL gene expression (232). This PRF activity has been localized to a subpopulation of cells in the IL that are not melanotrophs. Several factors have hindered the isolation of PRF and these include; the small size of the IL, low concentration, lactotroph heterogeneity and the sensitivity of PRL release to multiple factors. Transgenic mice that develop IL tumors provide a renewable source for the isolation and identification of a distinct PRF.
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CHAPTER II

SPECIFIC AIMS OF THESIS RESEARCH
Specific aim 1: To determine if POMC-Tag transgenic mice with pituitary intermediate lobe tumors develop hyperprolactinemia.

Rationale: The pituitary intermediate lobe (IL) is an established source of an unidentified prolactin releasing factor (PRF). This PRF is produced \textit{de novo} in the IL and is necessary to achieve the physiological elevations in plasma PRL following suckling. Transgenic mice carrying a transgene consisted of a truncated POMC promoter ligated to the large-T antigen of the simian 40-virus develop massive IL tumors. These IL tumors result from multifocal expansion of melanotrophs driven by the expressed T-antigen. Acid extracts from both primary IL tumors and secondary tumors generated in athymic mice stimulate rapid release of PRL from AP cells \textit{in vitro}. Female mice with primary IL tumors, but not nude mice with secondary tumors, have elevated plasma PRL levels. These data suggest that IL tumors produce a PRF that stimulates PRL release \textit{in vivo}. The following questions were addressed: 1) do POMC-Tag mice with primary IL tumors have elevated plasma PRL? 2) When do these transgenic mice develop hyperprolactinemia and how long does it last?

Objectives: 1) to establish and maintain a breeding colony of POMC-Tag transgenic mice, 2) to determine if male and female POMC-Tag transgenic mice develop hyperprolactinemia, and 3) to examine time related changes in plasma PRL levels associated with the development and progression of IL tumors in these mice.

Methods: Transgenic mice were confirmed by PCR and blood was repeatedly collected by tail clip from male and female mice between 20-120 days of life. Mouse plasma PRL was measured by radioimmunoassay (RIA). The presence of an IL tumor was confirmed in each transgenic mouse at time of sacrifice.

Specific aim 2: To establish and characterize a PRF-producing cell line from pituitary IL tumors of POMC-Tag mice.

Rationale: The minute size of the IL has been a major obstacle impeding the successful isolation of PRF. This is further exasperated by evidence that only a small subpopulation of non-melanotroph IL cells produce PRF. The difficulty in isolating a single protein from a complex and heterogeneous tissue extract is compounded by the sensitivity of PRL release to many compounds. A homogenous cell population that produces and secretes PRF would provide a renewable source of material for purification. Since no structural information is known about PRF, a bioassay has to be used to follow PRF activity during purification. Previous bioassays used primary rat AP cells and RIA to measure PRL release following treatment with PRF-containing fractions. Although this assay is useful for measuring PRF activity, it is slow and labor intensive. To facilitate the characterization and identification of PRF the lactotroph cell line GH3 was stably transfected with a plasmid containing the PRL promoter ligated to the luciferase reporter gene. This GH3/luc cell model offers a dual measure of PRF activity by quantification of PRL gene induction using luminometry and PRL release using RIA. Thus, GH3/luc cells provide a rapid (15h) high thorough-put (96-well) bioassay to assess PRF activity in vitro.
Objectives: 1) to establish a clonal cell line (mIL) from a mouse primary or secondary IL tumor, 2) to determine if mIL cells express characteristic pituitary gene products, 3) to determine if mIL cells produce or secrete a PRF, and 4) to establish GH3/luc cells as a model bioassay for characterization of PRF from mIL cells.

Methods: Mammalian cell culture techniques were used to establish and clone mIL cell lines from primary IL tumors. The MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) proliferation assay was used to determine cell growth rates. PRF activity was assessed by co-culture of mIL cells with primary AP cell cultures and RIA was used to measure both PRL and GH to assess the hormonal specificity of PRF action. PRL gene induction and release were quantified using GH3/luc cells incubated with mIL cells or conditioned media (CM). RT-PCR was used to examine pituitary gene products expressed by mIL cells and determine their cell lineage.

Specific aim 3: To characterize PRF from the mIL5 cell line.

Rationale: The mIL5 cell line was established from a primary IL tumor of a female POMC-Tag mouse. These cells, derived from a single clone, do not express cellular markers characteristic of pituitary melanotrophs. The nature of their cellular transformation and lineage remain unknown and they may represent a unique pituitary IL cell type. The mIL5 cells produce and secrete a potent PRF and therefore, serve as a renewable source for PRF purification. In addition, they provide a homogenous cellular model for future studies on the regulation of PRF in vitro. Although production of PRF by mIL5 cells offer a distinct advantage over crude starting material, the diversity of substances that can stimulate PRL in vitro confounds any purification strategy for PRF. We address the following questions: 1) what are the biochemical characteristics of mIL5 PRF? 2) Does this PRF represent a previously identified protein? 3) Do mIL5 cells produce more than one PRF?

Objectives: 1) to compare the biochemical properties of PRF from mIL5 cell extract and conditioned media (CM), and 2) to determine the contribution of FGF-2 and HB-EGF to the PRF activity produced by mIL5 cells.

Methods: PRL gene induction and release from GH3/luc cells was used to characterize PRF activity from mIL5 cells. Affinity and ion exchange chromatography were used to evaluate the biochemical features of PRF. RT-PCR was used to screen mIL cells for the expression of proteins with similar biochemical characteristics as PRF. Western blotting was used to confirm protein expression of relevant gene products. Neutralizing antibodies against FGF-2 and HB-EGF were used to evaluate their contributions to the PRF activity produced by mIL5 cells.
**Specific aim 4:** To isolate PRF from mIL5 cell extract and conditioned media (CM) and determine its amino acid sequence.

**Objectives:** 1) to define a purification strategy using mIL5 cell extract as a source of PRF, 2) to determine if PRF from mIL5 cell extract is identical to that secreted by the cells, and 3) isolate PRF from serum-free mIL5 CM and obtain a partial amino acid sequence.

**Methods:** Sequential chromatography was used to isolate PRF from both mIL5 cell extract and CM. Heparin-affinity and strong cation exchange FPLC were followed by analytical HPLC. Isolated PRF is defined by a single protein peak, measured at a wavelength of 280nm that corresponds to a single fraction with bioactivity. This protein was subjected to analysis by mass spectrometry and Edman N-terminal sequencing to obtain a partial amino acid sequence.
CHAPTER III

POMC-Tag TRANSGENIC MICE WITH PITUITARY INTERMEDIATE LOBE TUMORS DO NOT DEVELOP HYPERPROLACTINEMIA
INTRODUCTION

The pituitary IL is the source of a potent PRF that has not been identified. This PRF is produced de novo by a minor subpopulation of IL cells (10,16). PRF was initially characterized in vivo by loss of function following the surgical removal of the PP (7). In these LOBEX rats, removal of the PP resulted in a failed suckling-induced PRL surge (12). The importance of this PRF in the generation of PRL surges on the day of proestrus and following estradiol treatment was also demonstrated using LOBEX rats (13,14). Systemic injections of PP extract into rats specifically increased serum PRL levels (8). Acid extracts of PP from several animal species rapidly stimulates PRL release from primary AP cell cultures in a dose-dependent and hormone-specific manner (7,9). In addition, the co-culture of PP with GH3 cells stimulates both PRL gene expression and release (2,4,15). The production of several lines of transgenic mice that develop IL tumors may provide novel models to study the over expression of PRF (3,5,6,11).

The POMC-Tag transgenic mouse was generated by targeted tumorigenesis using a construct containing the POMC gene promoter ligated to the simian 40 virus large-T antigen (SV40-Tag). These mice develop pituitary IL tumors from multifocal expansion of pituitary melanotrophs. Primary IL tumors synthesize and process POMC-related peptides and the progressive enlargement of these tumors constricts the brain and leads to death. Subcutaneous transplantation of IL tumor cells into athymic nude develops into solid secondary tumors that have attenuated POMC expression and altered peptide processing (11). Acid extracts of both primary and secondary IL tumors result in a rapid and dose-dependent increase in PRL release from GH3 cell cultures. Female POMC-Tag mice with IL tumors at 90 days of age have elevated serum PRL, but not nude mice bearing secondary IL tumors (1). Although plasma PRL was elevated in the above study, the size of the sample was small and was limited to females of variable age.

Our specific aim was to determine if POMC-Tag transgenic mice with pituitary IL tumors develop hyperprolactinemia. We pursued the following objectives: 1) to establish and maintain a breeding colony of POMC-Tag transgenic mice, 2) to determine if male and female POMC-Tag transgenic mice develop hyperprolactinemia, and 3) to examine age related changes in plasma PRL levels associated with the developmental progression of IL tumors in these mice.

MATERIAL AND METHODS

Mice

POMC-Tag transgenic mice were produced at the Vollüm Institute (Portland, OR) in the laboratory of Malcolm Low by pronuclear injection of B6D2F2 hybrid oocyte with a fusion gene encoding SV40-Tag under the transcriptional control of the rat POMC gene regulatory sequence (-706 to +64) (11). Heterozygote male mice from the F_{22} generation were backcrossed to female Swiss-Webster outbred stock (Harlan Industries, Indianapolis, IN), establishing a breeding colony in Cincinnati. Mice used in this study were from the F_{24-30} generations. At 20 days of age, DNA was extracted from tail clips and genotyped by PCR using primers for SV40-Tag. Animals were housed in same sex pairs and maintained under constant temperature with food and water ad libitum on a 12h light/dark cycle.
**PCR genotyping**

Tail clips were dissolved in digestion buffer containing proteinase-K (0.5 mg/ml) and DNA was extracted using the phenol:chloroform:isoamyl method. PCR was performed on 1 µg of genomic DNA using primers for SV40 large T antigen (sense 5'-GCAATCGAAGCAGTAGCAATC-3' and antisense 5'-CAGCTAATGGACCTTCTAGG-3') with an expected product size of 395 bp. Plasmid containing the POMC-Tag transgene was used as a control. A PCR master-mix containing Taq DNA polymerase (GIBCO-BRL, Gaithersburg, MD) was used to amplify DNA for 30 cycles using the following conditions: 94 C for 30 sec, 58 C for 30 sec and 72 C for 30 sec. Products were separated on a 1.5% agarose gel containing ethidium bromide and photographed.

**Primary IL tumors**

Primary IL tumors began to develop between 6-12 weeks of age in both male and female POMC-Tag transgenic mice. All POMC-Tag mice developed soft primary IL tumors, albeit of variable size, as confirmed by necropsy at the time of sacrifice on day 120.

**Blood collection**

Mice were repeatedly bled by tail clip without anesthesia on days 20, 30, 40, 50, 60, 90, and 120 between 1500-1700 hours. Briefly, a small end of the tail was removed using a razor blade and 100-200 µl of blood was extruded by gentle milking of the tail between fingers into a heparinized capillary tube. This procedure took between 1-3 minutes with minimal stress and tail wounds were cauterized before returning mice to cages. Blood was centrifuged and plasma was stored at –70 C until assayed for PRL.

**PRL determination**

The concentration of PRL in mouse serum was determined using a mouse PRL NIDDK RIA kit (mouse PRL standard AFP-6476-C) with an assay sensitivity of 50 pg/well. Briefly, plasma was diluted 1:5 in 10mM PBS with 0.1% BSA and duplicate samples of 30 µl were added to the same buffer in white 96-well plates to a final volume of 100 µl. After adding 50 µl of anti-mouse PRL (1:40,000) and iodinated mouse PRL (15,000 cpm/well), samples were incubated for 3 days at 4C. Protein A (50 µl of 1:5) was added to each sample at room temperature for 10 minutes and the plates were centrifuged at 4000 X g for 10 minutes. The supernatant was aspirated and the pellet dissolved in 20 µl of 0.1N NaOH followed by the addition of 200 µl of scintillation fluid (Microscint 20, Packard Instrument Co, Downers Grove, IL). Plates were covered with TopSeal (Packard), vortexed and radioactivity was counted using a Packard TopCount.

**RESULTS**

*Transgenic mice with primary IL tumors do not develop hyperprolactinemia*

As shown in Fig. 1, there are no significant differences in plasma PRL between female POMC-Tag and wild-type mice at any time point tested. Similar results were obtained with males.
(Fig. 2) as serum PRL levels of POMC-Tag male mice did not differ from those of wild-type controls. By day 120 all transgenic mice had developed IL tumors of variable size. At this age, POMC-Tag mice exhibit no other obvious pathophysiological changes.

CONCLUSIONS

The failure of female POMC-Tag mice to develop hyperprolactinemia differs from previously published results (1). This discrepancy may be explained by the high variability of serum PRL in POMC-Tag mice and the small sample size used in the previous report. There is no difference in plasma PRL levels from either male or female POMC-Tag transgenic mice when compared to their wild-type controls. These results are unlikely due to dysregulation of PRL release from AP lactotrophs, since pregnant or lactating female POMC-Tag mice between 40-120 days are hyperprolactinemic (data not shown).

The cell specific activation of the POMC promoter appears to limit expression of the transforming large-T antigen to melanotrophs in the POMC-Tag mouse. Indeed, primary IL tumors from these mice are composed predominantly of melanotrophs that process and secrete POMC peptides. A great deal of effort has shown that the major peptide products of the IL and PP are not PRF (1,7-9). This is supported by evidence from AP co-culture experiments with IL cells demonstrating that only a minor subpopulation of cells distinct from melanotrophs produce PRF (10,16). Therefore, it is hypothesized that POMC-Tag mice with melanotroph tumors would not produce PRF in sufficient amounts and consequently their expansion would have no effect on serum PRL.

How do these data reconcile the stimulation of PRL release by primary IL tumor extracts? First, a 10 mg equivalent of IL tumor was necessary to achieve a 3-fold increase in PRL from GH3 cells (1). This suggested that PRF in these IL tumors is a negligible component and not likely derived from melanotrophs. Second, a minor cell population within IL tumors may produce PRF. These cells may represent a subpopulation of melanotrophs that do not express POMC or an intermediary cell that produces a unique complement of factors. Other possible sources of PRF include glial cells that may expand in IL tumors or an invading hematopoietic cell type that takes up residence in tumors.

The stimulation of PRL release by IL tumor extract in vitro may be a cumulative effect of several regulatory factors. Indeed, partial purification of IL tumors separated PRF activity into two components that differed in size and another fraction that inhibited PRL release from GH3 cells (1). Perhaps, a balance between these factors in primary IL tumors of POMC-Tag mice antagonizes a PRF induced-rise in serum PRL. Interestingly, partially purified extract of secondary IL tumors from nude mice contain only one of two major PRL stimulatory fractions. Similar reasons may explain the inability of this PRF to stimulate PRL release in vivo. In addition, dilution effects and degradation of PRF before reaching the AP may also explain the normal plasma PRL levels in nude mice with secondary IL tumors.

In conclusion: 1) the POMC-Tag mouse is not a useful in vivo model to study PRF, 2) primary and secondary IL tumors may provide an enriched source for the purification and structural elucidation of multiple PRF proteins, 3) PRF-producing cell lines from IL tumors may serve as a
good source of PRF and a cell model to examine the regulation of PRF gene expression and release.

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Fig. 1. Female POMC-Tag transgenic mice with primary IL tumors do not develop hyperprolactinemia. There is no significant difference in plasma PRL levels between transgenic and control mice at any time point. Blood was repeatedly collected by tail bleed and each PRL value represents a mean ±SEM. Sample size for POMC-Tag and (wild-type) mice: day 20, n=9(8); day 30, n=8(6); day 40, n=8(7); day 50, n=9(8); day 60, n=9(8); day 90, n=9(9); day 120, n=9(8).
Fig. 2. Male POMC-Tag transgenic mice with primary IL tumors do not develop hyperprolactinemia. There is no significant difference in plasma PRL levels between transgenic and control mice at any time point. Blood was repeatedly collected by tail bleed and each PRL value represents a mean ± SEM. Sample size for POMC-Tag and (wild-type) mice: day 20, n=7(11); day 30, n=11(10); day 40, n=11(12); day 50, n=14(16); day 60, n=12(16); day 90, n=13(15); day 120, n =10(14).
CHAPTER IV

PITUITARY LACTOTROPH HYPERPLASIA AND CHRONIC HYPERPROLACTINEMIA IN DOPAMINE D2 RECEPTOR-DEFICIENT MICE

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INTRODUCTION

Hypothalamic dopamine (DA) tonically inhibits PRL release from AP lactotrophs by activation of the dopamine type-2 receptor (D2R) (1,3). The D2R belongs to a group of seven transmembrane domain receptors that utilize dopamine as a common ligand. Activation of D2R generally inhibits adenylate cyclase and lowers intracellular calcium and consequently suppresses PRL secretion from lactotrophs (2,6). Elevated plasma PRL results from decreased hypothalamic DA or pharmacological blockade of D2R. Dopamine agonists like bromocriptine are used in the medical treatment of prolactinomas to suppress plasma PRL and cause tumor regression (8,9). The generation of a transgenic mouse that lacks functional D2 receptors provides a unique animal model for evaluating the contribution of the D2R to PRL release in vivo. Dr. Malcolm Low at the Vollum Institute in Oregon generated this transgenic mouse line.


MATERIAL AND METHODS

Mice

Briefly, a neo cassette was used to replace exon 7 and a portion of exon 8 from a clone of the mouse D2R gene. The subcloned vector was targeted by homologous recombination to the endogenous mouse D2R locus. This deletion results in a truncated transcript that fails to encode the putative third intracellular loop through the carboxy terminus of the D2R. This mutation abrogates specific binding of radiolabeled D2R antagonists to adult striatal membranes from homozygote D2R mice. In heterozygote mice, the density (B_max) of D2 receptors is half that of wild type, but the affinity (K_d) remains unchanged. Mice used in these studies were either F2 on a mixed genetic background of 129/Sv x B6 or congenic N5 generated by backcrossing to the B6 strain for 5 generations (4).

Serum PRL measurements

Plasma for basal PRL measurement was obtained from trunk blood collected following decapitation from stress-free mice. PRL secretory response to Haloperidol challenge was assessed within subject and between genotypes. Pretreatment blood samples were obtained by tail bleed one hour after intraperitoneal (i.p.) injection of saline. This was followed by i.p. injection of 5 mg/kg of haloperidol. The post-treatment blood samples were collected one hour after the second injection. The concentration of PRL in mouse serum was determined in duplicate using a mouse PRL NIDDK RIA kit (mouse PRL standard AFP-6476-C) with an assay sensitivity of 50 pg/tube as described in the previous section for POMC-Tag mice.

RESULTS

By six weeks of age, homozygote D2R deficient mice (−/−) have basal serum levels 3-fold higher than wild type siblings of the same sex (Fig.1). The constitutively elevated PRL levels in
homozygote D2R mice were not further increased when treated with haloperidol (Fig. 2 and Fig. 3). This differs from male and female wild type (+/+) and heterozygote (+/-) siblings that showed a significant increase in plasma PRL in response to the same dose of haloperidol. PRL values in females were significantly higher than in males regardless of genotype. This is likely due to effects of estrogen on PRL gene expression, storage and release. In aged (9 months) homozygote female mice (n=4) PRL values increased dramatically to $1670 \pm 427$ ng/ml while those in males remained stable over time (Fig. 4). The 15-fold increase in serum PRL in aged females compared to males is a result of lactotroph hyperplasia that is restricted to the female -/- genotype. Plasma PRL is elevated in D2R deficient mice on both the F$_2$ 129 x B6 (129/SvEv) and the congenic B6 (C57BL/6J) backgrounds compared to same sex siblings. However, plasma PRL levels in female D2R (-/-) C57BL/6J mice were 6-fold higher compared to age-matched 129/SvEv females (Fig. 5).

DISCUSSION

The absence of dopaminergic tone, normally provided by the inhibitory action of D2 receptors, elevates circulating PRL levels in both male and female D2R -/- mice. Despite the functional loss of D2 receptors, sexual dimorphism in PRL secretion persists in these mice. This indicates the important independent role of sex steroids on PRL synthesis and accumulation in secretory granules. In female mice, estrogen promotes PRL gene transcription and storage. This assures that adequate PRL reserves are available to respond to the physiological demands of the reproductive cycle, pregnancy or lactation (5). The dramatic PRL response to haloperidol in wild-type females compared to basal PRL in D2R deficient mice is explained by the high secretory rate of lactotrophs in the -/- mice. Haloperidol at a high dose was without effect on serum PRL levels in D2R -/- mice indicating that no other known DA receptors are important for dopaminergic regulation of PRL release from lactotrophs. Although D2R +/- mice have only half the number of D2 receptors as that of wild-type mice, they maintain a normal secretory response to Haloperidol. The reason for this responsiveness is unclear, but it may be due to functional excess in D2 receptors within the pituitary (7).

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Fig. 1. Basal serum PRL levels are elevated in homozygote (-/-) male and female D2R deficient mice (*p<0.0001). No difference in plasma PRL was observed between heterozygote (+/-) and wild-type (+/+) mice. In all groups, females have significantly higher PRL levels compared to males (p<0.05). PRL values represent the mean ±SEM. Female mice (-/-) n=7; (+/-) n=13; (+/+) n=15. Male mice (-/-) n=7; (+/-) n=18; (+/+) n=14.
Fig. 2. Plasma PRL levels remain unchanged in female D2R deficient mice (-/-) following haloperidol (5 mg/kg BW) treatment. Heterozygote (+/-) and wild-type (+/+) mice respond to haloperidol with an acute increase in serum PRL levels (*p<0.0001). PRL values represent the mean ±SEM. Female mice (-/-) n=6; (+/-) n=16; and (+/+ n=6.
Fig. 3. Plasma PRL levels remain unchanged in male D2R deficient mice (-/-) following haloperidol treatment. Heterozygote (+/-) and wild-type (+/+), mice respond to haloperidol with an acute increase in serum PRL (*p<0.0001). In these mice the haloperidol-induced rise in serum PRL does not surpass basal PRL levels of -/- mice. PRL values represent the mean ±SEM. Male mice (-/-) n=7; (+/-) n=14; (+/+) n=7.
**Fig. 4.** Female D2R-deficient mice (-/-) at 9 months of age have highly elevated circulating PRL levels. Plasma PRL levels in male -/- mice remain stable over time. PRL levels represent mean ±SEM. All female -/- mice (n=4) had pituitary hyperplasia at the time of sacrifice. No pituitary hyperplasia was observed in age-matched male -/- mice (n=2).
Fig. 5. Female D2R-deficient mice (-/-) on the C57BL/6J genetic background have higher serum PRL levels than age-matched animals on the 129/SvEv genetic background. PRL values represent the mean ±SEM (129/SvEv, n=6 and C57BL/6J, n=5).
CHAPTER V

TWO DISTINCT PITUITARY CELL LINES FROM MOUSE INTERMEDIATE LOBE TUMORS: A CELL THAT PRODUCES PROLACTIN REGULATING FACTOR (PRF) AND A MELANOTROPH

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Two distinct pituitary cell lines from mouse intermediate lobe tumors: A cell that produces prolactin regulating factor (PRF) and a melanotroph

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ABSTRACT

The intermediate lobe (IL) of the pituitary produces a prolactin (PRL) regulating factor (PRF). Targeted tumorigenesis, using the proopiomelanocortin (POMC) promoter ligated to SV40 large T antigen (Tag), generated transgenic mice that develop IL tumors with PRF activity. Our goal was to establish and characterize a PRF-producing cell line. Two cell lines, which differ markedly in size and morphology, were independently developed from IL tumors and designated mIL5 and mIL39. These cells are transformed, as judged by rapid proliferation, low serum requirements and generation of secondary tumors in nude mice. RT-PCR revealed that mIL39, but not mIL5 cells, express POMC and dopamine D2 receptors, typical of a melanotroph phenotype. Although mIL5 cells originated from an IL tumor, they do not express mRNA for SV40 Tag.

The bioassay for PRF utilized GH3 cells stably transfected with the PRL promoter ligated to a luciferase reporter gene (GH3/luc). Co-culture of mIL5 with GH3/luc cells induced cell-density dependent increases in PRL gene expression and release, whereas mIL39 cells showed negligible PRF activity. Incubation of GH3/luc cells with conditioned media from mIL5, but not mIL39 cells, stimulated PRL gene expression and release up to 10 fold. Co-culture of mIL5 cells with primary rat anterior pituitary cells stimulated PRL, but not GH, release. Fractionation of mIL5 cell extracts by reverse phase HPLC resolved PRF activity into one major and one minor peak.

In conclusion, we have developed two novel and distinct cell lines from mouse intermediate lobe tumors. The first reported melanotroph cell line, mIL39, could provide a valuable model for studying dopaminergic regulation of POMC gene expression and release. In contrast, the mIL5 cells do not express POMC, D2 receptors or SV40 Tag and appear to have been immortalized by a spontaneous mutation(s). These cells produce and secrete a potent PRF and could be used for the purification and biochemical characterization of PRF.
INTRODUCTION

The mouse pituitary gland is composed of three distinct lobes. The predominant anterior pituitary (AP) contains a heterogenous population of hormone secreting cells of ectodermal origin and is devoid of nerve endings. The neural lobe (NL) is derived from the neuroectoderm and consists of hypothalamic neurosecretory terminals and pituicytes (astroglial cells). The much smaller intermediate lobe (IL), juxtaposed between the two lobes, is primarily composed of pro-opiomelanocortin (POMC)-producing melanotrophs. Like the AP, the IL develops from the oral cavity (Rathke’s pouch), but differs from the AP by having rich innervation (1,2) and poor vascularization (3). Given the proximity of the IL to the NL, the combined tissue is often referred to as the neurointermediate lobe or posterior pituitary.

Recent evidence indicates that the IL participates in the regulation of prolactin (PRL) secretion. PRL release is modulated by both inhibitory and stimulatory factors that originate from the hypothalamus and pituitary (4,5). Whereas dopamine is well established as the primary inhibitor of PRL secretion (6), the identity of the physiological stimulator of PRL release remains to be determined. Our laboratory has reported that an intact posterior pituitary is necessary for the suckling- and estradiol-induced rises in PRL (7,8), suggesting the presence of a PRL regulating factor (PRF) in this tissue. This was supported by in vitro experiments demonstrating that PRF is a potent stimulator of both PRL gene expression (9,10) and release (11,12) and is distinct from other PRL secretagogues (12-14). Cell separation on density gradients indicated that PRF is produced by a subpopulation of IL cells (15).

The small size of the mammalian IL has hampered efforts to isolate and purify PRF. This impediment could be overcome by the availability of PRF-producing cells. Targeted tumorigenesis (16) and gene knockout strategies (17-19) have generated several lines of transgenic mice that develop IL tumors. In particular, the use of a transgene composed of a truncated POMC promoter ligated to the transforming simian virus 40 large T antigen (POMC-Tag) resulted in tumor formation exclusively in the IL (16). We previously reported that primary tumors from heterozygous POMC-Tag mice, as well as secondary tumors developed in athymic nude mice, exhibited PRF activity which was distinct from known PRL secretagogues or POMC-derived products (14). Given the time and expenses required for generating tumors of relatively small size, an enriched and renewable source of PRF was clearly needed. The objective of the present investigation was to establish a PRF-producing cell line from such tumors and determine its cellular and biochemical characteristics.

MATERIAL AND METHODS

Animals

Mice. Transgenic mice were produced as previously described (16) at the Vollum Institute, Portland, OR. Heterozygote male mice from the F22 generation were back crossed to female Swiss-Webster outbred stock (Harlan Industries, Indianapolis, IN), establishing a breeding colony at Cincinnati. At 22-28 days of age, DNA was extracted from tail clips and subjected to genotyping by PCR using primers for SV40 large T antigen (20). Adult female athymic nu/nu mice (Harlan) were maintained individually in isolation cages and were used as hosts to generate secondary tumors.
Rats. Ovariectomized adult female Fischer 344 rats (Harlan) were used as donors of AP cells for
the PRF bioassay. All animals were maintained under constant temperature with food and water
ad libitum. Animal experimentation was performed under an institutionally approved protocol
according to the USPHS Guide for the Care and Use of Laboratory Animals.

Primary and secondary tumors
Primary IL tumors begin to develop between 6-12 weeks of age in both male and female
heterozygous mice. Mice bearing large tumors were easily recognized by lack of grooming and
change in the shape of the skull as the IL tumor expanded and displaced the brain. Mice were
sacrificed by cervical dislocation and the tumors aseptically removed. Portions of the tumors
were either processed for histological examination, dispersed by trypsinization and the cells
placed in culture or injected subcutaneously into nude mice to generate secondary tumors.
Approximately 1x10^7 primary tumor cells were used for induction of secondary tumors, which
developed as early as 20 days after inoculation.

Cell culture
mIL cell lines. Of more than 15 primary IL tumors placed in culture, cells from only two females,
designated mIL5 and mIL39, remained viable. Repeated serial dilutions generated clonal cell
lines that have been carried for over 70 passages. Cultures are maintained in RPMI 1640
medium supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (Pen-
Strep; GIBCO-BRL, Gaithersburg, MD) at 37C under 5% CO_2. Both mIL5 and mIL39 cells
were used for the following: a) generation of secondary tumors in nude mice, b) determination of
selected gene expression by reverse transcriptase-PCR (RT-PCR), c) assessment of PRF activity
by coculturing with either GH_3/luc cells or primary AP cells, d) production of conditioned media
(CM), and e) morphological characterization following growth on glass chamber slides
(LabTech, Naperville, IL).

Primary rat AP cells. Anterior pituitary glands were removed from ovariectomized Fischer 344
female rats and dispersed as previously described (21). Cells were plated at a density of 10,000
cells per well (10K) in 96 well plates (NUNC, Copenhagen, Denmark) and cultured for three
days in serum free medium (SFM) composed of DMEM/F10 medium (50/50;Gibco BRL)
supplemented with 1% ITS^ Premix (Collaborative Research, Bedford, MA), 1% nonessential
amino acids and Pen-Strep. The mIL cells were trypsinized, added to the AP cells, and co-
cultured for three additional days. Media were removed and analyzed for PRL and GH by RIA.

GH_3/luc cells. GH_3 cells, obtained from the American Type Culture Collection (ATCC,
Rockville, MD), were stably transfected with 2.5 Kb rat PRL promoter ligated upstream of a
firefly luciferase reporter gene as previously described (10,22). The GH_3/luc cells were
maintained in Ham’s F-10 medium supplemented with 15% gelding serum and 50 ìg/ml
geneticin (G418; Sigma Chemical Co, St. Louis, MO); basal luciferase activity remained stable
for over 18 months. The GH_3/luc cells were used to evaluate PRF activity as judged by both
PRL gene expression and release as described below.

Cell proliferation
The growth rate of mIL5 and mIL39 cells was determined by the MTT (3-[4,5-Dimethylthiazole-
2-yl]-2,5-diphenyltetrazolium bromide) assay as previously described (23). Briefly, cells were
plated at 5K cells per well in 96 well plates (NUNC) precoated with protamine (2.5 mg/ml; Sigma) and Nu-Serum (Collaborative Research) and cultured in RPMI 1640 medium containing 10% FBS. At the designated times, MTT (1 mg/ml; Sigma) was added and incubated for three hrs. After removal of the medium, 100 ul of a developing solution (0.04M HCl/isopropyl alcohol) were added and optical density at 540 nm was determined using a Dynatech (Chantilly, VA) MR700 Microplate Reader.

**RT-PCR**

Total RNA from cells and tissues was extracted by Tri Reagent (Molecular Research Center, Cincinnati, OH) and 5ig were reverse transcribed using Superscript II (GibcoBRL) and random hexamers as described (22). PCR was performed on 10% of the RT product using the following primers: 1) *POMC*, sense primer 5'-TGCCGAGATTCTGCTACAGTCG'3' and antisense 5'-GGAAGTGACCCATGACGTACTT-3', with an expected product size of 246bp; 2) *Dopamine receptor (D₂ long and short)*, sense primer 5'-CGÇAGCAGIQCGAGCTTTCCAGA-3' and antisense 5'-GCTCATCGTCTTAAAGGAGGT-3' with expected product sizes of 402bp (long form) and 315bp (short form); 3) *Estrogen receptor a (ERα)*, sense primer 5'-GGTCÇAATTCTGACAATCGACG-3' and antisense 5'-CAGCTATCCCCTTCATC-3' with an expected product size of 309bp; 4) *SV40 large T antigen (SV40 Tag)*, sense primer 5'-GCAATCGAAGCAGTAGCAATC-3' and antisense 5'-CAGCTATGGAACTTCTAGG-3' with an expected product size of 424bp. All PCR reactions also contained primers for the housekeeping gene ribosomal protein L19 (RPL19), sense primer 5'-AGTAGTTAGCTACAGAAG-3' and antisense 5'-TTCCCTGTTGCTTAGACCTGCG-3' with an expected product size of 500bp. All primer sets, except for SV40 Tag, were designed to span introns. Underlined nucleotides represent mismatches between mouse and rat sequences. For SV40 Tag, RNA was treated with DNase to remove contaminating DNA. Cycle conditions were: 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec for 32 cycles. Products were separated on a 1.5% agarose gel containing ethidium bromide and photographed.

**Bioassay for PRF**

PRL gene expression. GH₃/luc cells (20K) were plated on protamine/Nu-Serum-coated 96 well plates and incubated in SFM for three days. The cells were then washed and subjected to the following treatments: a) co-culture with mIL cells, b) incubation with CM from mIL cells, or c) incubation with reconstituted HPLC fractions. After 18h, media were removed for PRL and GH analysis and the cells lysed by adding 50il of lysis buffer (Promega, Madison WI). After incubation for 15 minutes at 37°C, 20il aliquots in duplicate were transferred to black 96 well plates (Packard Instrument Co, Downers Grove, IL) and 80il of luciferin (Promega) were added. Luciferase activity, as a measure of PRL gene expression, was quantitated by luminometry using a Packard TopCount.

**PRL and GH release.** The concentrations of PRL and GH in media from both GH₃/luc and primary AP cells were determined by a modified RIA. NIDDK rat PRL and rat GH RIA kits with rPRL RP-3 and GH RP-2 as reference preparations, respectively, were used. Briefly, media aliquots were diluted in PBS containing 0.1% BSA in opaque white 96 well plates (Packard) to a final volume of 100il. After adding 50il each of primary antibody and iodinated hormone, the plates were incubated for 2 days at 4°C. Protein A (50il) was then added and the plates centrifuged at 4000xg for 10 min. The supernatant was aspirated and the pellet dissolved in 20il
of 0.1N NaOH followed by 200ìl of scintillation fluid (Microscint 20, Packard). The plates were sealed with TopSeal (Packard) and after vigorous mixing; radioactivity was counted using a Packard TopCount.

**HPLC fractionation**
Approximately 18x10^6 mIL5 cells were pelleted, washed with saline and extracted by sonication in 1N acetic acid. After freeze-thaw, extracts were centrifuged at 10,000xg for 20 min at 4C. The supernatant was loaded on an analytical C-18 reversed phase column (4.6 mm x 25 cm; Rainin, Woburn, MA) and fractionated with 0.1% trifluoroacetic acid at an increasing gradient of acetonitrile (AcN) from 0-60% over 60 min at 1 ml/min as previously described (14). One ml fractions were collected and aliquots were pooled from every 4 fractions. After lyophilization, fractions were reconstituted in SFM and incubated with GH_3/luc cells as described above.

**Data analysis.**
All experiments were performed at least three times. PRF values are expressed as a percentage of control values, i.e., GH_3/luc or primary AP cells incubated alone. Data were analyzed by analysis of variance followed by Student’s t-test.

**RESULTS**

*Morphology and histology of primary and secondary tumors*

Heterozygous mice expressing the POMC-Tag transgene developed tumors of the IL as previously described (16). Fig 1, top left panel compares a brain with attached pituitary from a wild type mouse with two brains from transgenic animals with massive IL tumors. These tumors grow at an unpredictable rate and can become 100 fold larger than a whole pituitary. In spite of their massive size, the tumors do not appear to infiltrate the brain proper. At an advanced tumor stage, animals begin to lose weight and eventually die from complications caused by brain compression. The two females whose IL tumors are shown in Fig 1 were one year old at the time of sacrifice. A representative section of a primary IL tumor stained with hematoxylin and eosin (H&E) is shown in Fig 1, lower left panel. The cells are small and ovoid with prominent nuclei and variable cytoplasmic staining. The tumor is highly vascularized, as evident by the presence of blood vessels filled with red blood cells. A nude female bearing a secondary tumor that was derived from the mIL5 cell line (passage 65), is shown in Fig 1, upper right panel. Within 45 days of inoculation, the tumor reached a 1 cm diameter and weighed 0.7 g. An H&E stained section of this tumor (lower right panel) depicts large, spindle-shaped cells with uniform cytoplasmic staining. In contrast to the slow growth rate of this tumor, secondary tumors generated from either primary tumor cells or from mIL39 cells grew rapidly. In fact, mIL39-derived tumors have reached sizes as large as 4 cm in diameter and weighed as much as 3.5g (data not shown).
**Cellular characteristics of the mIL cell lines**

Although both mIL5 and mIL39 cells originated from primary IL tumors, they differ dramatically in size and morphology. The mIL5 cells, seen by phase contrast microscopy in Fig 2, panel A, are gigantic cells with numerous branched cytoplasmic extensions of variable length that make focal contacts with neighboring cells. As shown in detail in panel C, these cells have big polymorphic nuclei containing numerous nucleoli and a large cytoplasmic volume. Extremely large multinucleated cells are commonly observed in less confluent cultures. The mIL39 cells, depicted in panel B and D, are much smaller and bipolar. These cells have a low cytoplasmic to nuclear ratio and project long and unbranched processes. Clearly, these photographs underscore the dramatic difference in cell size and nuclear diameter between mIL5 and mIL39 cells. The presence of several mitotic figures in both cell types was indicative of their fast replication rate.

**Differences in growth rate between mIL5 and mIL39**

The growth rate of mIL5 and mIL39 cell lines was determined by the MTT colorimetric assay (Fig 3). Under the standard culture conditions (RPMI containing 10% FBS), the mIL5 cells have a fast doubling time of 15h, whereas mIL39 cells double every 21h. This growth rate remained relatively stable over a period of 6-8 months. The cells also proliferate, albeit slower, under reduced serum conditions, e.g. 1% FBS.

**Characterization of cell phenotype by RT-PCR**

RT-PCR was used to determine whether both mIL cell types can be classified as melanotrophs, whose defining gene is POMC (24,25). As expected, POMC is expressed by the normal mouse pituitary (mAP) and by both primary IL tumors and secondary tumors derived from primary tumor cells (Fig 4, upper panel). However, of the two cell lines, only mIL39 expressed POMC. Further analysis for expression of SV40 Tag revealed that only mIL39, but not mIL5 cells, were positive for SV40 Tag (Fig 4, lower panel). Although mIL5 cells do not express SV40 Tag mRNA, they carry the POMC-Tag transgene, as judged by PCR of genomic DNA (data not shown).

Dopamine D2 receptor expression differentiates IL melanotrophs from POMC-producing AP corticotrophs (26,27). As shown in Fig 5, upper panel, dopamine receptors are expressed by all samples examined except for mIL5. The long form of the D2 receptor predominates, but the short form (315 bp) is also weakly expressed by the same tissues. Since we previously reported that estrogen increases PRF activity in the IL (8,22), we examined the expression of estrogen receptors in these cells. As shown in Fig 5, lower panel, ERα is expressed by all tested samples, except for rPP8, a newly developed rat posterior pituitary endothelial cell line (unpublished observation) that served as a negative control.

**Comparison of PRF activity in mIL cell lines using GH3/luc cells**

The advantage of the stably transfected GH3/luc cells as a bioassay for PRF is the concurrent measurement of both PRL gene expression and release. Coculturing mIL5 cells for 18h with GH3/luc cells induced a significant cell density-dependent rise in luciferase gene activity (Fig 6, left panel). As few as 2.5x10^3 mIL5 cells stimulated PRL gene expression 2.5-fold. In contrast, as many as 40x10^3 mIL39 cells were needed to increase PRL gene expression 2-fold, with little evidence for density-dependence. To determine whether PRF is a secreted product, 4x10^5 mIL5
or mIL39 cells were grown in SFM and the CM collected after three days. As shown in Fig 6, right panel, 12.5, 25 and 50% CM from mIL5 cells induced 4, 5 and 10 fold increases in PRL gene expression, respectively. In contrast, at all concentrations tested, CM from mIL39 cells resulted in only 2-3 fold increases. The pattern of PRL release from the same GH3/luc cells paralleled the luciferase activity, supporting the presence of substantial PRF activity in mIL5, but not mIL39 cells (Fig 7). The release of GH was unchanged by coculture with mIL cells or by incubation with their CM (data not shown). Coculture of mIL cells with non-transfected GH3 cells induced a similar rise in PRL release to that obtained using GH3/luc cells (data not shown).

**PRF activity determined by coculturing mIL cells with primary rat AP cells**
Coculture of mIL5 cells with primary rat AP cells for three days resulted in a cell density-dependent increase in PRL release (Fig 8, upper panel). PRF activity of mIL39 cells, compared to mIL5, was significantly lower (p<0.01 at 20K). As evident, GH release from these cocultures was not significantly changed (Fig 8, lower panel), further supporting the hormonal specificity of PRF activity.

**Resolution of PRF activity from mIL5 on reverse phase HPLC**
Acetic acid extract of mIL5 cells was fractionated on an analytical C-18 reverse phase HPLC column and aliquots from pooled fractions were analyzed for PRF activity by incubation with GH3/luc cells. As shown in Fig 9, PRF activity resolved into a major peak at 45-48% AcN and a minor peak at 38-40%. PRL release, both in elution pattern and magnitude, closely paralleled luciferase gene activation.

**DISCUSSION**
We have successfully developed two cell lines from IL tumors of POMC-Tag transgenic mice. These cells are transformed, as judged by their fast doubling time, low serum requirement and ability to generate secondary tumors in athymic nude mice. The two cell lines differ markedly in size, morphology and biochemical characteristics. The mIL5 cell line produces and secretes PRF but does not possess classical melanotroph attributes. This supports and extends our previous report (14) that PRF is not a POMC-derived product. Surprisingly, these cells do not express Tag and appear to have been transformed by a spontaneous mutation(s). In contrast, mIL39 cells express both POMC and dopamine D2 receptors, consistent with a melanotroph phenotype, but have weak PRF activity.

The mouse IL is a minute tissue that contains only 2x10^5 cells (28). Unlike most of its neighboring AP cells, IL cells maintain a robust proliferative capability, as evident by increased cell division in response to pituitary stalk section (3) or treatment with haloperidol (29) or interleukin-1α (30). The IL expresses a very high density of D2 receptors (26,27) and dopamine exerts an inhibitory control over both POMC gene expression (29,31) and melanotroph proliferation (32,33). Indeed, the onset of dopaminergic innervation of the IL in early postnatal life coincides with cessation of cellular proliferation (32). Whether maintenance of a relatively constant number of IL cells during adulthood reflects a balance between proliferation and apoptosis remains to be determined.

A common strategy for targeted tumorigenesis is via transgenes encoding a transforming viral
protein (e.g., SV40 Tag) under the transcriptional control of tissue-specific gene promoters. The rat 5'-flanking sequences (-706 to +64) of the POMC gene used to generate the POMC-Tag transgenic mice appear insufficient for transcriptional activity in extrapituitary sites (34). However, the reason for tumor formation in IL melanotrophs, but not AP corticotrophs, is not clear. Presumably, SV40 Tag induces neoplastic transformation by binding to the protein products of the tumor suppressors p53 and retinoblastoma (Rb) genes (35), which are involved in cell cycle regulation, differentiation and survival. It is of interest that large IL tumors also develop in 95% of Rb+/− heterozygous mice (19) subsequent to a loss of the remaining wild-type RB allele (28). Further, disruption of the p27kip1 gene, whose protein product inactivates cyclin/cyclin-dependent kinase complexes, also caused selective neoplastic growth in the IL (17,18). These, together with the high incidence of spontaneous IL tumors in several mammalian species (36), suggest that IL cells, for yet unknown reasons, are especially sensitive to loss of negative regulators of cell cycle progression.

In spite of the general notion that the IL is composed of homogeneous cells, two distinct subpopulations of melanotrophs have been identified which differ in secretory activity, staining properties and receptor expression (37-39). The IL also contains several non-POMC expressing cells, including marginal, folliculo stellate and interstitial cells (40-42), whose function is poorly understood. Microscopic and biochemical observations revealed that IL tumors in either POMC-Tag (16) or Rb+/− (19) heterozygous mice begin as multifocal nodules that progress into large tumors by clonal expansion. Given the heterogeneity of the IL and the polyclonal origin of IL tumors, the emergence of two cell lines with different properties was not unexpected. Yet, although both mIL cell lines were derived from transgenic mice with IL tumors, only mIL39 cells expressed SV40 Tag. Presumably, only the POMC-expressing mIL39 cells should drive the production of Tag, resulting in cell transformation. Therefore, it appears that mIL5 cells have originated from a cell other than melanotroph that became immortalized by a spontaneous mutation(s). Alternatively, mIL5 cells represent a stem cell or a dedifferentiated melanotroph that no longer expresses typical cellular markers. Future studies will explore cellular markers that might reveal the origin and identity of mIL5 cells.

Pituitary hormone research has benefitted from the availability of immortalized cell lines such as the rat somatomammotroph GH3 cells (43) and the ACTH-producing mouse AtT20 cells (44). Cell lines provide a renewable homogeneous population of cells that can be manipulated under controlled conditions. Although both melanotrophs and corticotrophs produce POMC, they differ in their expression of POMC processing enzymes (24), main secretory products (45), and expression of receptors for glucocorticoids (46), CRH (47) and dopamine D2 receptors (26,27). Work is underway to determine whether mIL39 cells process POMC in a melanotroph-specific pattern and whether they secrete POMC peptides such as α-MSH and α-endorphin in a regulatable manner.

Regardless of its cellular origin or stage of differentiation, the mIL5 cell line has remained stable for many generations. In validation of PRF production by these cells, several criteria were fulfilled, including stimulation of PRL gene expression and release and lack of effect on GH. PRL release in response to co-culture with mIL5 cells increased in both the somatomammotroph GH3 cell line and primary rat AP cells. The robust stimulation of PRL gene expression and release by serum-free CM from mIL5 cells confirms that PRF is a secreted product, as would be
expected from our in vivo demonstration that the IL participates in the control of PRL release (7,8). Furthermore, in support of our previous report (14), the HPLC elution profile of PRF extracted from mIL5 cells resembles that extracted from primary IL tumors and differs from POMC products and known PRL secretagogues. Finally, the small size of the IL and the time-consuming task of producing sufficient IL tumors have made previous purification attempts an arduous task. Given the development of the PRF-producing cell line, the challenge of PRF isolation and structural determination should be finally met.

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Fig. 1. Photomicrographs of primary and secondary IL tumors. The upper left panel compares the brain and pituitary of a wild type mouse with two POMC-Tag females with IL tumors. The upper right panel shows an athymic nude mouse with a secondary tumor, generated from subcutaneous injection of $1 \times 10^7$ mIL5 cells. The lower left panel is a H&E stained section of a representative primary IL tumor and the lower right panel is a H&E stained section of the secondary tumor from mIL5 cells at the same magnification. Calibration bars = 100 μm.
Fig. 2. Photomicrographs of mIL cell lines. Panels A and B are phase contrast images of mIL5 and mIL39 cells at the same magnification. *Calibration bars* for the *top panels* = 40 μm. Panels C and D show H&E stained mIL5 and mIL39 cells grown on glass chamber slides. *Calibration bars* for the *bottom panels* = 20 μm.
Fig. 3. Growth rates of the two mIL cell lines. Optical density (540 nm), as determined by the MTT assay, is proportional to cell number. Each value is the mean ± SEM of five determinations from a representative experiment.
Fig. 4. POMC and SV40 Tag gene expression by IL tumors and cells as determined by RT-PCR. 
Top panel: POMC expression in primary and secondary IL tumors, mL39 and mL5 cells and 
wild type mouse anterior pituitary tissue (mAP); expected product size is 246bp. Bottom panel: 
expression of SV40-Tag with an expected product size of 424bp. The POMC-Tag plasmid is 
included as a positive control and a mAP from a wild type animal as a negative control. All 
samples included primers for RPL19, with an expected product size of 500bp, as an internal 
control. Ladder = 100bp.
Fig. 5. Dopamine (D_{2}R) and estrogen (ERα) receptor expression by IL tumors and cells as determined by RT-PCR. Top panel: dopamine D_{2} receptor expression with expected product size of 402bp (long isoform) and 315bp (short isoform). Bottom panel: estrogen receptor expression with an expected product size of 309bp. A rat posterior pituitary endothelial cell line (rPP8) is included as a negative control. All samples included primers for RPL19, with an expected product size of 500bp, as an internal control. Ladder = 100bp.
Fig. 6. Induction of PRL gene expression in GH3/luc cells by mIL cells. Left panel: Cell density-dependent stimulation of luciferase activity following coculture with mIL5 and mIL39 cells for 18h. Right panel: Concentration-dependent stimulation of luciferase activity by CM from mIL cells. Each value is a mean ± SEM of four determinations from a representative experiment.
Fig. 7. Stimulation of PRL release from GH<sub>3</sub>/fluc cells by mIL cells. PRL concentrations in media were determined after 18h of coculture (left panel) or incubation with CM (right panel). See Fig 6 for other details.
Fig. 8. Stimulation of PRL release (upper panel) but not GH (lower panel) upon coculturing mL cells with primary rat AP cells for 3 days. Each value is a mean ± SEM of four determinations.
Fig. 9. Elution profile of PRF activity from mIL5 cell extracts on analytical reverse phase C-18 HPLC column. Fractionation was performed using 0.1% trifluoroacetic acid and an increasing gradient of AcN from 0-60% over 60 min at 1ml/min. Pooled aliquots from every four fractions were analyzed for PRF activity using GH3/luc cells. Top panel: optical density (OD) at 220 nm. PRF activity eluted as one major peak (45-48% AcN) and a minor peak (38-40% AcN), as shown by increased luciferase activity (middle panel) and PRL release (lower panel). Each value is a mean ± SEM of four replicates.
CHAPTER VI

PROLACTIN REGULATION BY HEPARIN-BINDING GROWTH FACTORS EXPRESSED IN MOUSE PITUITARY CELL LINES

Submitted to Endocrine
Prolactin Regulation by Heparin-Binding Growth Factors Expressed in Mouse Pituitary Cell Lines

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ABSTRACT

Prolactin (PRL) secretion is regulated by complex interactions between inhibitory and stimulatory factors. Dopamine is the primary inhibitor, but multiple peptides and growth factors can stimulate PRL gene expression and release. These stimulators can be divided into two categories, those that rapidly stimulate PRL release from secretory granules and those that induce the PRL gene followed by increased PRL release. Collectively, they can be termed prolactin-regulating factors (PRFs). The pituitary intermediate lobe (IL) has been reported to contain a PRF that rapidly stimulates PRL release. From a mouse IL tumor, we recently established a non-melanotroph cell line, mIL5, which secrete a factor that stimulated PRL gene expression and release \textit{in vitro}. Unexpectedly, we found that this PRF activity did not rapidly stimulate PRL release and bound to heparin. The objective was to examine the regulation of PRL by heparin-binding proteins and characterize the PRF produced by the mIL5 cells.

PRL gene expression and release was determined using GH3 cells stably transfected with a PRL promoter/luciferase reporter (GH3/luc). After screening mIL5 cells for various genes by RT-PCR, we found that they expressed two heparin-binding growth factors, basic fibroblast growth factor (FGF-2) and heparin-binding EGF-like growth factor (HB-EGF), which were considered strong candidates for PRF activity in these cells. Both growth factors were potent stimulators of PRL gene expression and release. To determine whether PRF activity produced by mIL5 cells is attributed to FGF-2 or HB-EGF, three complimentary approaches were used: heparin-affinity chromatography, Western blotting and immunoneutralization. PRF in conditioned media (CM) eluted from heparin with 1M NaCl whereas both FGF-2 and HB-EGF eluted with >1M NaCl. Neither growth factor was detectable in mIL5 cells by Western blotting. Antibodies directed against FGF-2 or HB-EGF, alone or together, did not abolish the PRF activity from mIL5 cells.

In conclusion, both FGF-2 and HB-EGF are potent regulators of the PRL gene and should be included within the growing list of PRFs. However, these growth factors do not account for most of endogenous PRF activity produced by mIL5 cells, suggesting the presence of a distinct heparin-binding PRF that remains to be identified.
INTRODUCTION

Prolactin (PRL) is a protein hormone produced by anterior pituitary lactotrophs. The regulation of PRL secretion is complex, involving both stimulatory and inhibitory factors that can act either directly on the lactotrophs or indirectly at the hypothalamus [7,20]. Dopamine, acting via dopamine type-2 receptors (D_2R) expressed by the lactotroph, is the primary inhibitor of PRL release [6]. However, numerous peptides and growth factors are capable of stimulating PRL release and gene expression \textit{in vitro}. These prolactin-regulating factors (PRFs) can be broadly divided into two categories: those that rapidly stimulate PRL release from storage granules and those that induce the PRL gene with a subsequent increase in PRL secretion.

Peptide releasing factors such as TRH [16], oxytocin [34], angiotensin II [11], and vasoactive intestinal peptide [1] rapidly stimulate PRL release. Their action is mediated by activation of specific membrane receptors, ultimately resulting in a calcium-mediated release of PRL from secretory granules. A delayed induction of the PRL gene by these peptides often follows hormone release. Growth factors such as FGF-1 and FGF-2 also stimulate PRL release, but their action is less rapid, paralleling the increase in PRL gene expression [5]. The physiological role played by these growth factors in the overall regulation of PRL remains undefined.

Several lines of evidence suggests that the posterior pituitary (neurointermediate lobe) participates in the control of PRL secretion under several physiological conditions [12,19,25,35]. In addition to dopamine, the intermediate lobe (IL) contains a fast acting PRF that differs from known peptide releasing factors [18,39], but its structure remains unresolved. Using IL tumors from POMC-Tag transgenic mice [22], we previously confirmed the presence of PRF in these tumors [2]. Recently, we developed two distinct cell lines from primary IL tumors, named mIL5 and mIL39, which differ in cellular characteristics as well as endogenous PRF activity [17].

The mIL39 cells express the POMC and D_2R genes, which define them as melanotrophs. Co-culture experiments with GH3 cells, a somatolactotroph cell line, showed that mIL39 cells contain little, if any, PRL regulating activity. However, the mIL5 cells, whose cellular phenotype is unknown, secrete a potent regulator of PRL [17]. Preliminary characterization of this PRF activity indicates that it is a heparin-binding protein (>5000 MW) that does not rapidly stimulate PRL release \textit{in vitro}. The present paper compares genes expressed by the two mIL cell lines, investigates the regulation of PRL by heparin-binding growth factors, and further characterizes the PRF produced by the mIL5 cells. The specific objectives were to: 1) examine the expression of select heparin-binding growth factors by these cell lines and evaluate their ability to induce the PRL gene, 2) compare the heparin-binding property of these growth factors to that of PRF activity in mIL5 cell extract and conditioned media (CM), and 3) determine if these growth factors account for the endogenous PRF activity in mIL5 cells.
MATERIAL AND METHODS

Reagents

Chemicals. Beetle luciferin and reporter lysis buffer (Promega, Madison, WI). Tri Reagent (Molecular research Center Inc., Cincinnati, OH). Protease inhibitors: leupeptin, aprotinin, pepstatin A, PMSF and NaF (Sigma Chemical Co., St. Louis, MO). Superscript II, RNAsin, DNAse I, and Taq DNA polymerase (GIBCO BRL, Gaithersburg, MD).

Culture media. RPMI 1640, Ham’s F10, Dubucco’s modified eagles medium (DMEM), Trypsin-EDTA, fetal bovine serum (FBS), horse serum (HS), penicillin-streptomycin (Pen-Strep; GIBCO-BRL, Gaithersburg, MD). ITS and ITS+ premix supplement (Collaborative Biomedical Products, Bedford, MA). Poly-D-lysine and geneticin (G418; Sigma). Gelding serum (Colorado Serum Co).


Animals

Mice. POMC-Tag transgenic mice were generated by Dr. Malcolm Low (Vollum Institute, Portland, OR) as previously described [22] and used to establish a breeding colony at Cincinnati. Genotyping on DNA extracted from tail clips was done by PCR with primers for SV40 large T-antigen [17]. Primary IL tumors were removed from adult heterozygote females. Tumors were homogenized in 50mM phosphate buffer (PB) with 50mM NaCl, pH 7.4, in the presence of protease inhibitors to generate the cellular extract used for heparin-affinity chromatography and Western blots. Total RNA was obtained with Tri-Reagent for RT-PCR analysis. To generate secondary tumors, primary tumors were dissociated with 0.1% trypsin and 5x10^6 cells/0.1ml saline was injected s.c. into female athymic nu/nu mice (Harlan, Indianapolis, IN ). Mice were sacrificed after large secondary tumors had developed (2-4 weeks) and RNA was extracted as above.

Cell culture

mIL cell lines. The mIL5 and mIL39 cell lines were established from primary IL tumors of adult female POMC-Tag transgenic mice as previously described [17]. Cultures were maintained in RPMI 1640 medium supplemented with 10% FBS and 1% Pen-Strep at 37C under 5% CO2. CM from mIL5 cells grown to 75% confluence in Nunclon 185 cm^2 flasks (NUNC, Copenhagen, Denmark) were collected after a 3 day incubation with 30ml of serum-free medium (SFM) composed of DMEM:F10 (1:1) supplemented with 1% ITS. Protein and RNA extracts were
obtained as described above.

_C6 glioma cells_. Rat C6 glioma cells obtained from the American Type Culture Collection (ATCC, Rockville, MD), were grown in F10 medium supplemented with 12.5% HS, 2.5% FBS, and 1% Pen-Strep at 37°C under 5% CO2. Cells were grown to 75% confluence, harvested by scraping and protein extracts were obtained as above.

_GH3/luc cells_. GH3 cells, obtained from the ATCC, were stably transfected with 2.5-kb rat PRL promoter ligated upstream of a firefly luciferase reporter gene as described [37,38]. The GH3/luc cells were maintained in F-10 medium supplemented with 15% heat-inactivated gelding serum and 50µg/ml G418. Under these conditions the cells were maintained as floating colonies with a low and stable basal luciferase activity. The GH3/luc cells were used to evaluate PRL regulation, as judged by both gene expression and release, described below.

**RT-PCR**

Total RNA (5µg) was reverse transcribed using Superscript II and random hexamers as described [17]. PCR was performed on 0.5-1 µg of the RT products using Taq DNA polymerase. The composition of the primer pairs, all designed to span introns, is described in Table 1. Each PCR reaction also included a primer pair for ribosomal protein L19 (RPL19), serving as an internal control. Mouse tissues served as positive controls and omission of the RT step was used as a negative control. Annealing temperatures varied with the different primer sets but the melting and extension temperatures were 94C and 72C, respectively. Each temperature step in the PCR cycle was 45 seconds for a total of 30 cycles. The PCR products were separated on a 1.5% agarose gel containing eithidium bromide and photographed.

**PRL induction**

_PRL gene expression_. GH3/luc cells (10,000 cells/well) were plated on poly-D-lysine coated 96 well plates and incubated in SFM with 1% ITS+ for 24h. Cells were washed twice with SFM and incubated (200 µl/well) with the test materials for 18h. Media were analyzed for PRL release by RIA (see below) and the cells were lysed in 60 µl lysis buffer, and duplicate 20 µl samples were transferred to black 96 well plates (Packard Instrument Co, Downers Grove, IL). After adding 80 µl of luciferin substrate, luciferase activity, as a measure of PRL gene expression was quantified by luminometry using a Packard TopCount.

_PRL release_. The concentration of PRL in medium collected from GH3/luc cells was determined by a modified RIA [17] using a rat PRL RIA kit from the NIDDK with RP-3 as a reference preparation. Briefly, 20 µl aliquots were diluted in 10mM PBS containing 0.1% BSA and added to white 96 well plates (Packard) in a final volume of 100 µl. After adding 50 µl each of primary antibody (S9; 1:45,000) and iodinated rat PRL (18,000 cpm), the plates were incubated for 2 days at 4C. Protein-A (1:5; 50 µl) was then added and the plates centrifuged at 4,000 x g for 10 min. The supernatant was aspirated and the pellets were dissolved in 20 µl of 0.1M NaOH, followed by the addition of 200 µl of scintillation fluid (Microscint 20, Packard). The plates were sealed with TopSeal (Packard) and after vigorous shaking; radioactivity was counted using a Packard TopCount. The limit of sensitivity for the RIA was 125 pg/well.
**Heparin-affinity chromatography**

One or five ml heparin-sepharose columns were equilibrated in 50mM PB with 50mM NaCl at pH 7.4. Cell extract, CM or recombinant growth factors were fractionated step wise with NaCl (0.05-2.5M) in 50mM PB at pH 7.4. Fractions were desalted and concentrated by centrifugation using spin filters (Ultrafree-15 or –4; Millipore, Bedford, MA) with a 5000 MW cutoff. Aliquots were taken either for the GH$_3$/luc bioassay, Western blotting, or immunoneutralization as described below.

**Western Blotting**

Extracts or CM from mIL5 cells or rat C$_6$ glioma were fractionated on a heparin affinity column as described above. Each fraction was dialyzed (3500 MW cutoff) against water overnight at 4C, concentrated, denatured under reducing conditions and 50 µg proteins from each fraction were separated by SDS-PAGE on a 13.5% gel. Proteins were transferred to nitrocellulose, incubated with FGF-2 antibodies (3 µg/ml) overnight at 4C and were detected using an ECL chemiluminescence kit and x-ray film (Amersham).

**Immunoneutralization**

Recombinant human FGF-2 and HB-EGF were diluted in SFM to a concentration of 2 ng/ml. Aliquots (0.5 ml) of FGF-2, HB-EGF as well as mIL5 extract, heparin fractions or CM were incubated with either anti FGF-2 or anti HB-EGF (at a final concentration of 20 µg/ml) for 18h at 4C. After 3h incubation with 50 µl of protein A-coupled agarose beads, samples were centrifuged at 10,000 x g to pellet the antibody-antigen complexes. The supernatants were diluted with equal volumes of SFM containing 1% ITS+, and 200 µl/well were incubated for 18h with GH$_3$/luc cells and analyzed for PRL induction as described above.

**Data analysis**

All experiments were performed at least twice. Data from the GH$_3$/luc bioassay represent 4 samples, each analyzed in duplicate. PRL values are expressed as a percentage of control, i.e. GH$_3$/luc cells incubated alone. Data were analyzed by ANOVA followed by Student’s t test and significance was accepted with a $P < 0.05$.

**RESULTS**

*Selected genes expressed by mIL cell lines, IL tumors and mouse pituitary*

During the initial characterization of PRF activity from mIL5 cells, we found that it binds heparin. Consequently, we examined whether mIL cells express FGF-1, FGF-2, FGF-BP or HB-EGF, all of which are heparin-binding proteins that may function as PRL inducers. Utilizing RT-PCR, we compared the expression of these heparin-binding proteins in mIL cells, mouse pituitary as well as primary and secondary POMC-Tag tumors. As evident in Fig 1, the mIL cell lines differ in their pattern of gene expression. The mIL5 cells express FGF-2, but not FGF-1,
whereas mIL39 cells express FGF-1, but not FGF-2. Of the two cell lines, only mIL39 express FGF-BP, but both express HB-EGF. The normal mouse pituitary gland expresses FGF-1, FGF-2 and FGF-BP, with a weak and inconsistent expression of HB-EGF. Primary IL tumors express only FGF-BP, whereas secondary tumors express both FGF-1 and HB-EGF, but not FGF-BP.

In an attempt to identify the cell lineage of mIL5 cells, we used RT-PCR to screen for select gene products known to be expressed in the pituitary (Tables 1 and 2). Among the hormones examined, only follistatin (a heparin-binding protein) was expressed by both cell lines, while POMC was expressed exclusively by mIL39 cells. The only receptor expressed by both cell lines was estrogen receptor \( \alpha \) (ER\( \alpha \)), while mIL39 expressed receptors for dopamine, corticotropin releasing factor (CRF) and leptin. Notably, ER\( \beta \) and the PRL receptor were undetectable in either cell line. Table 2 also summarizes the expression of heparin-binding growth factors by the two cell lines. This gene screening failed to resolve the lineage of mIL5 cells.

**Comparison of PRL induction by heparin-binding growth factors.**

The stimulation of PRL gene expression in GH\(_3\)/luc by FGF-1 and FGF-2 is shown in Fig 2. FGF-1 stimulates the PRL gene in a linear manner from 10-200 ng/ml, reaching a 5-fold induction at the highest dose tested, with an \( ED_{50} \) of 30 ng/ml; FGF-1 below 5 ng/ml is ineffective. FGF-2 is 100-fold more potent than FGF-1, with an \( ED_{50} \) of 0.2 ng/ml or 10 pM. The induction of the PRL gene by FGF-2 results in a Gaussian-shaped curve which plateaus at 1 ng/ml and declines at concentrations above 20 ng/ml. No change in cell number was observed with either FGF-1 or FGF-2 at any dose tested during the 18h incubation period (data not shown). The next experiment demonstrates that HB-EGF can stimulate both PRL gene expression and release from GH\(_3\)/luc cells (Fig 3). As evident, at 0.5 ng/ml, HB-EGF and FGF-2 increase PRL release 5- and 9-fold, respectively \((P<0.05)\). Neither of the growth factors examined stimulates PRL release in less than 4h (data not shown).

**Heparin-binding properties of FGF-2, HB-EGF, mIL5 cell extract and CM.**

Since FGF-2 and HB-EGF are expressed by mIL5 cells (Fig 1) and stimulate PRL (Fig 3), it was important to examine whether these growth factors account for the PRF activity in mIL5 cells. In the first experiment, we compared the heparin-binding profiles of FGF-2, HB-EGF and PRF activity in mIL5 cell extract and CM. Fig 4 shows that both FGF-2 and HB-EGF elute from heparin with \( >1M \) NaCl. All the PRF activity in either CM or cell extract was heparin bound as no activity was detected in the column flow-through. The PRF activity in CM elutes from heparin between 0.05-1M NaCl while that in the cell extract elutes between 0.05-1.5M NaCl, showing partial overlap with either FGF-2 or HB-EGF (Fig 4).

**FGF-2 and HB-EGF are undetectable by Western blotting in mIL5 cells.**

The next experiment examined the presence of FGF-2 and HB-EGF proteins in mIL5 cells by Western blotting. Fig 5 shows that FGF-2 is undetectable in any heparin fraction of mIL5 cell extract, at a detection limit of <5 ng. Rat C\(_6\) glioma cells, which express FGF-2 [30], were used as a positive control. FGF-2 is detected only in the 1.5M NaCl heparin fraction of C\(_6\) cell extract.
(Fig 5, lower panel). FGF-2 was undetectable in heparin fractions of CM from either mIL5 or C6 cells (data not shown). In addition, HB-EGF was undetectable by Western blotting in heparin fractions from either mIL5 cell extract or CM (data not shown).

**Immunoneutralization with anti FGF-2 and anti HB-EGF.**

Because FGF-2 and HB-EGF stimulate PRL at concentrations undetectable by Western blotting, we used neutralizing antibodies to determine if either growth factor accounted for the PRF activity in mIL5 cells. As shown in Fig 6, the antibodies decreased the PRL gene induction by recombinant FGF-2 (200 pg/ml) by 90% ($P<0.05$), but decreased the PRF activity in nonfractionated mIL5 extract (input) by only 18% ($P<0.05$). The FGF-2 antibodies did not significantly reduce PRF activity in any heparin fraction of mIL5 extract (Fig 6). Comparable results were obtained with mIL5 CM (data not shown). Fig 7 demonstrates that HB-EGF antibodies abolished the PRL gene induction by recombinant HB-EGF (1 ng/ml), but had no effect on PRF activity in mIL5 CM. A combination of both antibodies was also ineffective in neutralizing the majority of PRF activity in mIL5 CM (data not shown).

**DISCUSSION**

We have shown that two heparin-binding proteins, FGF-2 and HB-EGF, are potent stimulators of PRL gene expression and release from GH3 cells *in vitro*. The mouse pituitary mIL5 cells express the mRNA for both growth factors and secrete a protein that binds heparin and stimulates PRL. However, the heparin-binding properties of FGF-2 and HB-EGF differ from the endogenous PRF activity in mIL5 cells and Western analyses did not detect either FGF-2 or HB-EGF proteins in mIL5 cells. Furthermore, antibodies against both FGF-2 and HB-EGF effectively blocked the induction of PRL by these growth factors but were ineffective in reducing the majority of PRF activity in mIL5 cell extract or CM. These data suggest that FGF-2 and HB-EGF are not responsible for most of the PRL induction by mIL5 cells, and raise the possibility of a distinct heparin-binding protein with PRL regulating properties.

Several heparin-binding growth factors, known to regulate cell growth, differentiation and survival, are expressed in the pituitary [32]. Heparan sulfate proteoglycans are abundant in the pituitary extracellular matrix and the residual cleft that separates the anterior from the intermediate lobe [15]. They play a role in sequestering certain proteins via sequence-specific interactions. Changes in the extracellular environment, such as ion concentration or activation of proteolytic enzymes, can liberate proteins bound to heparin and make them readily available to target cells [9]. Both FGF-1 and FGF-2 were isolated from the pituitary, but their precise cellular origin remains unclear [4]. They differ in their heparin-binding properties, with FGF-1 eluting from heparin with 0.5M NaCl and FGF-2 eluting with 1.5M NaCl [4]. Both growth factors can stimulate proliferation of fibroblasts, smooth muscle, and endothelial cells, but neither affected GH3 cell growth under our culture conditions. Since both lack putative signal sequences, the mechanism by which they are released from the cells is unclear [24]. Trafficking of these proteins to the extracellular environment and their release from proteoglycans might involve apoptosis, proteolysis and/or the FGF-BP.
FGF-1 and FGF-2 have been reported as PRL stimulators [21,23,29], and the FGF-BP has been implicated as a general mediator of their action [10]. Our results demonstrate that FGF-2 is one of the most potent inducers of the PRL gene, with a half-maximal stimulation at 10pM (Fig 2). In contrast, TRH, a well-characterized regulator of PRL, requires 10nM to equally stimulate PRL. FGF-2 is extremely potent at low doses, but its dose response curve is Gaussian, with higher doses producing less induction of the PRL gene. FGF-1 requires much higher doses than FGF-2 to stimulate the PRL gene and produces a linear dose response. One might speculate that a combination of these growth factors could have a significant effect on PRL production by lactotrophs.

The mIL5 cells, established from a primary POMC-Tag IL tumor but of an unknown cell lineage, produce and secrete a potent PRF. The PRF activity required a minimum of 4h incubation with GH3/luc cells to induce either PRL gene expression or release. Preliminary data obtained with primary anterior pituitary cells also show a slow effect on PRL release. Therefore, this PRL stimulatory activity does not conform to the properties of classical peptide releasing factors that affect hormone release within minutes. Instead, it belongs to a class of heparin-binding proteins, e.g., FGF-2, that exerts an indirect effect on PRL release via induction of the PRL gene. Our data suggest that mIL5 produces a PRF that is distinct from FGF-2. First, it has a different heparin-binding property than FGF-2. Second, although FGF-2 expression was detected by RT-PCR, the protein was undetectable by Western blots of cell extract or CM, while it was detected in C6 glioma cells. Third, to rule out the possibility that the FGF-2 protein was translated at very low levels, we used neutralizing antibodies that suppressed PRL activity by recombinant FGF-2, but caused minor reduction of PRF activity of mIL5 cells. Collectively, these data suggest that the bulk of PRF activity in mIL5 cells is not due to FGF-2.

Other heparin-binding proteins expressed by mIL5 cells, including HB-EGF, VEGF and follistatin, were examined as possible candidates for PRF activity. HB-EGF, a member of the EGF family of growth factors, is a membrane bound protein that is processed to yield a 74 amino acid secreted product [26]. HB-EGF stimulates growth of fibroblasts and smooth muscle cells but not endothelial cells [31], while its action on PRL has not been reported. This study is the first demonstration of FGF gene induction and release by HB-EGF (Fig 3). The action of HB-EGF is likely mediated by the EGF receptor [3], but it also binds to the ErbB4 receptor, which is expressed in the pituitary [28]. The RT-PCR data (Fig 1) show that HB-EGF is not consistently expressed in the normal mouse pituitary or in primary POMC-Tag tumors, but is detectable in both mIL cell lines and secondary tumors. Although HB-EGF binds heparin, it differs in affinity from PRF activity in mIL5 cells. Furthermore, the lack of detection of HB-EGF by Western blot in mIL5 cells and ineffective immunoneutralization of endogenous PRF activity by HB-EGF antibodies (Fig 7) suggest that HG-EGF is not produced in large enough quantities to account for PRL activation by mIL5 cells.

Follistatin is a 288 amino acid heparin-binding protein that interacts with the β-subunit of both activin and inhibin and modulates FSH secretion [27]. Follistatin did not stimulate PRL gene expression or release from GH3/luc cells at any dose tested. Another heparin-binding protein, VEGF, is a potent mitogen and permeability factor for the endothelia that exists in several isoforms [14]. Like follistatin, VEGF did not affect PRL. Notably, FGF-4/hst protein also binds
heparin and can stimulate the PRL gene [36]. However, since the mRNA for this protein was not detected by RT-PCR in any mIL cells, FGF-4 was not further explored.

Another objective was to further characterize the two-mIL cell lines, which differ in morphology, growth rates and gene expression [17]. Unlike mIL5 cells that do not express POMC or D2R, mIL39 cells are classified as melanotrophs. Using RT-PCR we have demonstrated the expression of FGF-1 and HB-EGF, possibly accounting for the weak stimulation of PRL by CM from mIL39 [17]. These endogenous growth factors may play a role in the robust proliferation of these cells in vitro or in IL tumor formation in vivo. The proliferative capacity of melanotrophs is suppressed by dopamine, acting through D2R [8]. The maintenance of cell number in the adult IL is due to a balance between proliferation and apoptosis, and a disruption of this balance might account for the sensitivity of the IL to tumorigenesis [13,33]. The expression of both CRF-R and leptin-R by mIL39 cells provide a valuable cellular model for studying the involvement of these receptors and their ligands in POMC regulation in the melanotrophs.

In summary, this investigation addressed several important issues. First, compared to classical releasing/inhibiting factors, the role of heparin-binding growth factors in pituitary physiology in general and PRL regulation in particular, has been under-appreciated. Second, the availability of two distinct cell lines from the intermediate lobe provides new opportunities for elucidating the function of this inaccessible tissue. Finally, the identification of a novel PRF from the IL has been hampered by the sensitivity of PRL to multiple factors and will require a creative approach as well as luck.

ACKNOWLEDGEMENTS

We wish to thank The National Hormone and Pituitary Program NIDDK for the gift of PRL RIA reagents Laura Hudson and Jana Peterson are recognized for their technical assistance.

REFERENCES


Fig. 1. Expression of FGF-1, FGF-2, FGF-BP and HB-EGF in mL5, mL39, mouse pituitary and IL tumors (1° and 2°), as determined by RT-PCR. *Top left panel:* FGF-1, expected product size 440 bp. *Top right panel:* FGF-2, expected product size 450 bp. *Bottom left panel:* FGF-BP, expected product size 291 bp. *Bottom right panel:* HB-EGF, expected product size 403 bp. All samples included primers for RPL19, an internal control, expected product sizes of 500 or 333 bp. Ladder, 100 bp.
Fig. 2. Induction of PRL gene expression in GH<sub>3</sub>/<i>luc</i> cells by FGF-1 and FGF-2. Cells were incubated with the growth factors for 18h, followed by quantitation of luciferase activity in cell lysates by luminometry. Each value is a mean ±SEM of four determinations from a representative experiment.
Fig. 3. Stimulation of PRL gene expression (upper panel) and release (lower panel) from GH/luc cells by FGF-2 and HB-EGF. The concentration of PRL in culture media was determined by RIA after 18h incubation with the growth factors. See Fig 2 for other details.
Fig. 4. Comparison of the elution profiles of FGF-2, HB-EGF and PRF activity from mIL5 cell extract or CM from heparin-affinity columns. Fractionation was performed with a discontinuous NaCl gradient at pH 7.4. Aliquots were analyzed for PRL gene induction using GH/luc cells. See Fig 2 for other details.
Fig. 5. Western blot analysis for FGF-2 in mIL5 and C₆ cell extracts fractionated panel, FGF-2 was detected in the 1.5M NaCl heparin-fraction of C₆ cell extract. Recombinant human FGF-2 was used as a standard, with a detection limit of <5ng. This is a representative experiment repeated 2-3 times. M, molecular on heparin. Upper panel, FGF-2 (17 kDa) was undetectable in any heparin fractions of mIL5 cell extract. Lower weight markers.
Fig. 6. Comparison of the ability of FGF-2 antibodies to neutralize the PRL gene induction by recombinant FGF-2 and mIL5 extract. The antibodies reduced the PRL gene induction of FGF-2 (200 pg/ml) to 10% of control (FGF-2 alone) but to 82% of the PRF activity from non-fractionated cell extract (input). The FGF-2 antibodies were ineffective in reducing PRF activity in any heparin fraction of the cell extract. See Materials and Methods and Fig 2 for other details.
Fig. 7. Comparison of the ability of HB-EGF antibodies to neutralize the PRL gene induction by recombinant HB-EGF and mIL5 CM. The antibodies abolished the PRL gene induction of HB-EGF (1 ng/ml), but have no effect on PRF activity of the CM (100 µl/well). See Materials and Methods and Fig 2 for other details.
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Abbreviations: CRF-R, corticotropin releasing factor receptor; D<sub>2</sub>R, dopamine type-2 receptor; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; FGF-1, acidic-fibroblast growth factor; FGF-2, basic-fibroblast growth factor; FGF-4, hst/K-fibroblast growth factor; FGF-BP, FGF-binding protein; GH, growth hormone; HB-EGF, heparin-binding epidermal-like growth factor; POMC, pro-opiomelanocortin; PRL, prolactin; PRL-R, prolactin receptor; RPL19, ribosomal protein L19; VEGF, vascular endothelial growth factor. Underlined bases designate mismatches against mouse gene sequences (derived from rat gene sequences).
Table 2. Genes expressed in mouse pituitary cell lines, as determined by RT-PCR (see Table 1 for abbreviations).

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<td>GH</td>
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CHAPTER VII

PURIFICATION AND CHARACTERIZATION OF PROLACTIN REGULATING FACTOR (PRF) FROM A MOUSE PITUITARY CELL LINE
INTRODUCTION

The establishment of the mIL5 cell line from an IL tumor has provided a unique opportunity to identify the structure of PRF. These cells constitute a renewable source of secreted PRF that has been shown to stimulate both PRL gene expression and release from GH3/luc cells in vitro (5). Our discovery that this PRF binds to heparin, suggested that it might be a member of the FGF family of growth factors that had been shown to stimulate PRL release in vitro (2,11,14). RT-PCR screening of gene products expressed by mIL5 cells revealed two heparin-binding proteins, FGF2 and HB-EGF, as potential PRFs. Although both of these growth factors are potent inducers of the PRL gene in vitro, they do not significantly contribute to the PRF activity from mIL5 cells (4).

The biochemical characterization of PRF indicated that it was not a peptide, but a protein of an estimated molecular weight between 10-20 kDa. However, PRF activity from mIL5 cells separated on heparin-affinity chromatography into multiple species, all with MW >5,000 Da. This was similar to the PRF activity from bovine PP extract, which also fractionated into several molecular species, albeit with molecular weights of <5,000 Da (7). In addition, the PRF activity from extracts of POMC-Tag IL tumors fractionated into three major species, a protein >70 KDa and two smaller proteins <15 KDa (1). Heparin-affinity chromatography was not part of the paradigm used to separate proteins from bovine PP or IL tumor extract, so it is unknown if these PRFs share heparin-binding properties with that from mIL5 cells. The separation of PRF into multiple species may indicate multiple gene products whose combined activity functions in an additive manner to stimulate PRL gene expression and release. Alternatively, multiple species of PRF may represent an unprocessed protein together with its derivatives that differ in chromatographic properties but maintain bioactivity. The later hypothesis is supported by the fact that all species of PRF from mIL5 cells bind to heparin.

It is uncertain if any of the PRF proteins from PP, IL tumors or mIL5 cells are the same or related. Furthermore, the PRF(s) from the PP responsible for the suckling-induced rise in serum PRL may or may not be represented in IL tumors or mIL5 cells. All of the identified hypothalamic releasing/inhibiting hormones are small peptides that rapidly stimulate or inhibit hormone release from AP cells. Therefore, it is unlikely that a protein of 15,000 Da, acting primarily by stimulating the PRL gene, fulfills the criteria of a typical hormone-releasing factor. Indeed, two classes of PRF have become apparent: 1) those that rapidly (<1h) stimulate PRL release and may subsequently stimulate PRL gene expression, or 2) those that stimulate PRL gene expression followed by a delayed (>4h) PRL release. The first class of PRFs represent acute PRL-releasing factors that act primarily on lactotrophs to stimulate PRL release, while the second class are modulatory PRL-regulating factors that act primarily through the induction of the PRL gene. The latter proteins include several growth factors, typified by FGF2, that interact with heparan-sulfate proteoglycans. The final classification of the protein from mIL5 cells responsible for PRF activity requires a pure compound, thereby underlying the final objective: to isolate and identify the PRF from mIL5 cells.
MATERIAL AND METHODS

Cell extract

The mIL5 cells used for extraction were grown to 90% confluency in 72 cm$^2$ flasks (NUNC, Copenhagen, Denmark) containing RPMI 1640 supplemented with 10% FBS and penicillin-streptomycin (GIBO-BRL, Gaithersburg, MD) at 37 C under 5% CO$_2$. Cells were harvested by brief trypsinization (0.1% Trypsin-EDTA; GIBCO-BRL) and centrifuged at 800 X g. Pellets were re-suspended in saline, counted and centrifuged. Pellets containing approximately 45 x 10$^6$ cells were frozen at –70 C prior to extraction. After thawing, 15 ml of extraction buffer (50mM PB + 50mM NaCl + protease inhibitor mix; pH 7.3) was added and sonication on ice for 3-5 min. Extracts were centrifuged at 14,000 X g for 20 min and the supernatants were frozen-thawed and centrifuged again at 14,000 X g for 30 min. A 150 ml pool of cleared cell extract from 450 x 10$^6$ cells was stored at –70 C until chromatography.

Cell conditioned media (CM)

The mIL5 cells were grown to 70% confluency as described above. Growth medium was removed and the attached cells were washed twice in RPMI 1640 alone for 2 min at 37 C. Cell were then incubated in serum-free media that contains DMEM:F10 (1:1) supplemented with 1% ITS Premix (Collaborative Biomedical Products, Bedford, MA) for 3 days at 37 C under 5% CO$_2$. This CM was filtered (0.2 µm) into sterile glass bottles and stored at 4 C. A 20X concentration of 6500 ml was achieved by repeatedly pumping CM through a 3,000 MW cutoff hollow-fiber cartridge (Millipore, Bedford, MA) under 10-15 psi using an Amicon CH2 concentrator (Millipore/Amicon).

Bioassay for PRF

PRL gene expression. GH3/luc cells (10K) were plated on poly-D-lysine (20 µg/ml; Sigma Chemical Co, St. Louis, MO) coated 96-well plates (NUNC) and incubated in serum-free media (SFM) that contains DMEM:F10 (1:1) supplemented with 1% ITS+ Premix (Collaborative Biomedical Products) for 2 days. The attached cells were then washed with fresh media and subjected to the following treatments (200 µl/well) in quadruplicate: 1) SFM as control, 2) CM:SFM (1:1) as 50% CM internal control, 3) 25 µl aliquots from 2.5ml FPLC fractions in 1 ml SFM, 4) 100 µl aliquots from 0.6 ml HPLC fractions dried and reconstituted in 1 ml of SFM. After 18h of incubation, the media were removed and the cells disrupted by adding 50 µl of lysis buffer (Promega, Madison WI). After 10 min of lysis at 37 C, plates were rocked for an additional 5 min then two 20 µl aliquots from each well were transferred to black 96-well plates (Packard Instrument Co, Downers Grove, IL) and 80 µl of luciferin (Promega) was added. Luciferase activity, as an indirect measure of PRL gene expression, was quantified by luminometry using a Packard TopCount.
Fractionation on FPLC

Heparin-affinity chromatography

Cell extract. Extract from approximately 150 x 10^6 mIL5 cells in 50 ml of 50mM phosphate buffer with 50mM NaCl at pH 7.3 (start buffer) was loaded on a 5 ml HiTrap heparin-affinity column (Amersham-Pharmacia Biotech, Piscataway, NJ) at 1ml/min using a 50 ml superloop on a dual FPLC/HPLC ACTA Explorer instrument (Pharmacia). Protein absorbance was monitored at 215 and 280 nm wavelengths. Unbound proteins were washed from the column at 1 ml/min for 30 min with the start buffer, after which the UV absorbance had returned to baseline levels. Heparin-bound proteins were eluted at 2.5 ml/min for 30 min with an increasing linear NaCl gradient (0.05 – 2.00M) in 50mM phosphate buffer at pH 7.3. Fractions of 2.5 ml were collected; vortexed, and 25 µl aliquots from every two fractions were pooled and added directly to SFM. PRF activity was determined using the GH3/luc bioassay. This process was repeated with the two remaining 50 ml aliquots of mIL5 cell extract. Fractions containing PRF activity that eluted from heparin at specific NaCl concentrations were divided into separate pools with bioactivity.

CM. Approximately 100 ml of the 20X concentrated mIL5 CM (2200 ml original volume) was loaded on a 5 ml HiTrap heparin-affinity column (Pharmacia) at 1 ml/min from a 50 ml superloop. Proteins were monitored, eluted and assayed for PRF activity as described for cell extract.

Strong-cation exchange chromatography

Cell extract and CM. The heparin-bound fractions with PRF activity were desalted and concentrated (10X) using centrifugal concentrators (Ultrafree-15, 5,000 MW cut off; Millipore). Sample were diluted in equal volume of 50mM PB (pH 7.3) and loaded on a 5 ml HiTrap SP column (Pharmacia) at 0.5 ml/min. Proteins were monitored as described for heparin chromatography and 20 ml of the start buffer was used to elute unbound proteins. Cation-bound proteins were eluted at 2.5 ml/min with an increasing linear NaCl gradient (0.05-1.65M) in 50mM PB (pH 7.3). Eluted fractions were assayed for PRF activity and pooled as described for heparin.

HPLC fractionation

Cell extract. Three sequential HPLC purification steps were conducted using two analytical reversed-phase columns: 1) C4 silica column (4.6 x 100mm; Pharmacia) containing 5 µm spherical particles with 300Å pores, and 2) PRP-3 poly(styrene-divinylbenzene) column (4.1 x 150mm; Hamilton Co, Reno, NV) containing 10 µm spherical particles with 300Å pores. Prior to the first analytical HPLC step, PRF containing fractions from the SP column were desalted and concentrated as described for FPLC. Samples were injected on the C4 column and proteins were eluted at 0.5 ml/min using a 0-70% acetonitrile (AcN) gradient over 30 min containing 0.1% trifluoroacetic acid (TFA; Sigma) as an ion-pairing agent. Proteins were monitored by absorbance at 215 and 280 nm wavelengths. Fractions of 0.5 ml were collected into tubes containing 0.1 ml of 0.2 M ammonium acetate to adjust the pH of fractions to 4.6. All buffers...
were volatile and aliquots for PRF determination were dried under vacuum using a SpeedVac. The fractions containing PRF activity were pooled, concentrated under vacuum and injected on the PRP-3 column, eluted with a 20-60% linear AcN gradient over 20 min. The final analytical HPLC step was re-fractionation of PRF on the PRP-3 column under a 20-45% linear AcN gradient over 16 min.

**CM.** A single analytical HPLC purification step was used with mIL5 CM following FPLC fractionation. Sample containing PRF activity from SP was desalted, concentrated and injected on an analytical reversed-phase C3 silica column (Zorbax 300SB-C3, 4.6 x 250mm; Hewlett-Packard, Palo Alto, CA) containing 5 µm spherical particles with 300Å pores. Proteins were monitored as above while eluted at a flow rate of 0.5 ml/min using a 15-65% linear AcN gradient over 30 min. Fractions of 0.5 ml were collected into 0.1 ml of 0.2 M ammonium acetate. Aliquots from each fraction were dried under vacuum and reconstituted in SFM for PRF determination using the GH3/luc bioassay.

**Electrospray ionization mass spectrometry (ESMS)**

The molecular weight of PRF was estimated by ESMS using a Micromass Q-Tof Electrospray/Quadrupole Time-of-Flight mass spectrometer (Micromass, Manchester, UK) in positive ion mode. A 10% aliquot was removed from the fractions with PRF activity from mIL5 CM following isolation on the C3 RP-HPLC column and diluted in 150 µl of 0.1% TFA. This sample was desalted using a ZipTip (Millipore) containing C18 silica. Peptides were eluted with 3 µl of 100% AcN and injected into the ESMS. The ESMS raw data spectrum was converted to a molecular mass scale using the MaxEnt transformation. The average mass error was ±0.02%. This work was conducted at Yale Cancer Center Mass Spectrometry Resource/W.M. Keck Foundation Biotechnology Resource Laboratory.

**Enzymatic fragmentation**

Prior to digestion, the PRF sample was reduced and the cysteines alkylated to carboxamidomethylated cysteine. The protein fraction was dried under vacuum, dissolved in 60 µl of dH2O and dried again. This sample was dissolved in 30 µl of 10mM sodium bicarbonate at pH 8.0 and incubated in 2.5 µl of 45mM dithiothreitol (DTT) at 37 C for 20 min. This was followed by the addition of 2.5 µl of 100mM iodoacetamide (IAN) and incubation for 20 min at room temperature. Enzymatic digestion was done with 0.5 µg of trypsin in 5 µl at 37 C for 21 hours. A blank tube without protein was run in parallel as control. Yale did this work.

**Microbore HPLC**

Tryptic PRF peptides were fractionated on a Hewlett Packard 1090 HPLC instrument using a C18 reversed-phase microbore HPLC column (1mm X 25cm; Vydac, Hesperia, CA) containing 5 µm spherical silica with 300Å pores. Protein absorbance was monitored at 210 nm wavelength and elution was at 30 µl/min under the following gradient conditions: buffer-A is 0.06% TFA and buffer-B is 80% AcN with 0.052% TFA; gradient 1) 2-37% buffer-B from 0-60 min, 2) 37-75% buffer-B from 60-90 min, and 3) 75-98% buffer-B from 90-105 min. Fractions were collected by protein peak. Yale did this work.
Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS)

Following PRF fragmentation by trypsin, a 5% aliquot was passed through a ZipTip (Millipore) as described for ESMS. The peptides were eluted with 3 µl matrix solution (alpha-cyano-4-hydroxy-cinnamic acid; CHCA) and 2 µl spotted on target. To obtain peptide mass accuracy, 50 fmol bradykinin, which has a protonated, monoisotopic mass of 1060.57 and 125 fmol ACTH Clip, which has a protonated, monoisotopic mass of 2465.2 were used as internal calibrates. Mass analysis was done using a Micromass ToFSpec MALDI SE instrument operated in positive linear ion mode at an accelerating voltage of 25 kV and equipped with a nitrogen laser (337 nm). Yale did this work.

Edman chemistry

N-terminal sequence analysis of PRF was done using Edman chemistry on an Applied Biosystems 494 cLC sequencer (Applied Biosystems, Foster City, CA) with online HPLC identification of phenythiohydantion (PTH) amino acid derivatives. Yale did this work.

RT-PCR for HIP

Total RNA was obtained from mIL5 cells using Tri Reagent (Molecular Research Center, Cincinnati OH) and 5 µg was subjected to DNase treatment to remove contaminating genomic DNA. This was followed by reverse transcription of 1 µg RNA using Superscript II (GIBCO-BRL) and random hexamers as described (5). PCR was performed on 10% of the RT product using the following primers: 1) Murine heparin/heparan sulfate (HP/HS) interacting protein (HIP/L29); sense primer 5’-AACCACGGTGCAAAAGATACG-3’ and antisense primer 5’-TGGCAGGAGTCATTCTATGGG-3’ with an expected product size of 431 bp; 2) RPL19, sense primer 5’-AGTAGTCTTAGGCTACAGAAG-3’ and antisense primer 5’-TTCCCTGTGCTTACAGCTCG-3’ with an expected product size of 500 bp, as an internal control. The mouse HIP transcript (GenBank accession # L08651) is a single open reading frame, so all PCR reactions included controls without RT that ensured genomic DNA was not detectable. Cycle conditions for the PCR reaction were: 96 C for 30 sec, 55 C for 30 sec, and 72 C for 45 sec for 30 cycles. Products were separated on a 1.5% agarose gel containing ethidium bromide and photographed.

Immunodepletion

Rabbit polyclonal antibodies generated against two synthetic peptides derived from the HIP protein sequence were gifts of Dr. Daniel D. Carson from the University of Delaware. These two peptides and their corresponding antibodies were as follows: 1) MRFAKKHNKGLKKM (amino acids 43-57; FAKK antibody) and 2) CQPKPKVQTKAGAKA (amino acids 117-131; GAKA antibody). Immunohistochemistry and Western blotting by the Carson laboratory have validated the utility of these antibodies (6). Aliquots of mIL5 extract or mIL5 CM (0.5 ml) were incubated with anti-FAKK, anti-GAKA or both (at a final concentration of 20 µg/ml) for 18h at 4C. After 3h incubation with 50 µl of protein A-coupled agarose beads, samples were centrifuged at 10,000 X g to pellet the antibody-antigen complexes. The supernatants were
diluted with equal volume of SFM, incubated for 18 hours with GH3/luc cells (200 µl/well), and cell lysate analyzed for PRL gene expression.

**Data analysis**

All experiments were performed at least twice. Data from the GH3/luc bioassay represent 4 wells, each analyzed in duplicate. PRL values are expressed as a percentage of control, i.e. GH3/luc cells incubated alone. Data were analyzed by ANOVA followed by Student’s t test and significance was accepted with a P<0.05.

**RESULTS**

**Isolation of PRF from mIL5 cell extract**

The schematic in Fig 1 depicts the purification strategy used for the isolation of PRF from mIL5 cells. This paradigm utilized sequential chromatography, mass spectrometry, and Edman sequencing to identify partial amino acid sequence from PRF. As shown in Fig 2, PRF from mIL5 cell extract bound to a heparin-affinity column. No PRF activity was found in the flow through fraction that contained the majority of proteins (data not shown). Two major peaks of PRF bioactivity eluted from the heparin-affinity column; one that bound heparin with a strong affinity, >1.5M NaCl (D), and was associated with very few proteins and a second that was associated with the majority of heparin-bound proteins and eluted from heparin between 0.5-1.25M NaCl. All PRF activity was separated into four arbitrarily defined pools designated A-D. The PRF activity from pools A, C and D were used in subsequent chromatographic steps, but these were never resolved into isolated proteins suitable for amino acid sequencing. However, the PRF activity that eluted from heparin between 0.72-0.98M NaCl and designated pool B (yellow bar) was loaded onto a strong-cation exchange (SP) column.

As shown in Fig 3, the PRF activity from pool B eluted between 0.5-0.9M NaCl from the SP column. No PRF activity was found in the flow through fraction that contained the majority of proteins. In addition, PRF did not interact with a strong-anion exchanger (data not shown). The SP fraction with PRF activity that eluted between 0.8-0.9M NaCl (yellow bar) had the least amount of proteins and was therefore further purified on an analytical C4 column. The majority of PRF activity eluted from the C4 column between 37-47% AcN (Fig. 4). The fraction with greatest PRF bioactivity (37-42% AcN; yellow bar) was associated with two major protein peaks at 215nm. The PRF activity eluting at 32% AcN (yellow bar) was repurified on the same column under different conditions. Fig 6 shows isolated PRF protein as an asymmetrical peak that eluted between 28-31% AcN (yellow bar) and this was subjected to Edman N-terminal sequence analysis.

**Identification of truncated HIP protein from mIL5 cell extract**

Protein sequencing, done at the University of Michigan protein facility, revealed a 30-amino acid peptide that was identical to an internal sequence of murine heparin/heparan sulfate (Hp/Hs)-interacting protein (HIP/L29). Fig 7 depicts the partial amino acid sequence identified from the
purified fraction of cell extract with PRF bioactivity (red highlight). These amino acids correspond to amino acids 43-73 of the mouse HIP protein. As shown in Fig 8, the expression of HIP mRNA was detected by RT-PCR in mIL5 cells. The HIP transcript was also detected in normal mouse pituitary gland and the mIL39 cell line. Since a pure source of the HIP protein is unavailable, its PRF bioactivity could not be assessed. However, two antibodies (FAKK and GAKA) generated against synthetic peptides derived from the mouse HIP protein were used to indirectly evaluate HIP as a PRF. As shown in Fig 9, the PRF activity from mIL5 CM was not significantly attenuated by immunodepletion with FAKK, GAKA or their combination.

**Isolation of PRF from mIL5 CM**

Serum-free mIL5 CM (6.5 liters) was concentrated 20-fold using a hollow-fiber cartridge with a 3,000 MW cutoff. No PRF activity was found in the flow through and the concentration effectively increased the potency of PRF in the CM (data not shown). As shown in Fig 10, PRF activity eluted from a heparin-affinity column in two peaks. The first PRF peak eluted between 0.7-0.89M NaCl and the major peak eluted between 1.03-1.36M NaCl. The fractions with PRF activity eluting between 1.20-1.36M NaCl (yellow bar) were loaded on a strong-cation exchanger. Fig 11 shows a major peak of PRF bioactivity that eluted between 0.68-0.77M NaCl from a SP column. These fractions (yellow bar) were loaded onto a C3 RP-HPLC column and eluted with a 10-65% linear AcN gradient. As shown in Fig 12, PRF bioactivity eluted between 38-42% AcN and corresponded to a single sharp protein absorbance at 280nm. This PRF activity was restricted to fractions 15 and 16 and both tubes were subjected to ESMS and Edman sequencing at the University of Michigan. The samples were lost and no data was obtained.

The PRF activity from mIL5 CM that eluted from the heparin-affinity column between 1.03-1.19M NaCl (blue bar; Fig 10) was subjected to the same sequential chromatographic steps as described for the PRF activity that eluted between 1.20-1.36M NaCl (yellow bar; Fig 10). Fig 13 compares the protein profiles (280 nm) of these two fractions following final separation on the C3 column. The PRF activity from both fractions eluted from C3 between 38-42% AcN and were associated with single corresponding protein peaks (Fig 13; red and blue lines). This second run of mIL5 CM provided two fractions (15 and 16) with PRF bioactivity that was similar to that from the first run.

**Identification of PRF from mIL5 CM**

To determine sample homogeneity and establish the molecular weight of PRF, electrospray ionization mass spectrometry (ESMS) was performed on fraction-15 (40% AcN) from the second purification run of mIL5 CM. As shown in Fig 14, raw ESMS spectra data was dominated by low mass ions (top panel). However, the most intense peaks were two high mass ions observed at 14782 and 14968 atomic mass units (lower panels). The remainder of fraction-15 was cleaved into small peptides by trypsin and loaded on a microbore C18 RP-HPLC column. Fig 15 shows the peptide elution profile (210nm) of fraction-15 and the major peptides that eluted between 50-70 min. All major peptide peaks were collected, and the selected fractions (numbered peaks in Fig 15) were subjected to MALDI-MS. Mass analysis screen of these HPLC fractions indicated that they were all complex mixtures (Table 1) and not ideally suited for amino acid sequencing. However, the MALDI-MS spectrum of peak-1, shown in Fig 16 (upper panel), was the least
heterogeneous, with an estimated purity of 80%. The major mass specie was 4675.9 (lower panels) dominating as a 7:1 major-to-minor mass ratio (m/z). No structural information was obtained following Edman sequence analysis of this peptide.

DISCUSSION

This report describes the isolation and characterization of PRF protein from mIL5 cells. PRF resolved into at least two major species that differed in heparin-binding properties. Sequential chromatography of cell extract isolated a peptide determined to be identical to an internal sequence of murine HIP/L29. The biochemical properties of HIP/L29 and failure of two anti-HIP antibodies to neutralize PRF bioactivity from mIL5 cells suggests that it is not PRF. Coincidental isolation of HIP/L29 during purification of PRF could be the result of similar heparin-binding properties. The purification of PRF from CM isolated a protein with an estimated MW of 15,000 Da. Fractions containing PRF bioactivity were lost during subsequent mass and sequence analysis. The primary structure of PRF from mIL5 cells remains unresolved.

Sequential chromatography was used to purify PRF from mIL5 cells. The heparin-affinity of PRF was exploited as the initial purification step and effectively removed the majority of proteins. Although all the PRF bioactivity bound to heparin, it eluted at several different salt concentrations. This variable affinity of PRF for heparin suggests the presence of several PRF species that may represent distinct proteins. However, these PRF species are neither FGF2 nor HB-EGF, both heparin-binding proteins that stimulate PRL gene expression and release (4). Alternatively, the processing of a single protein precursor into derivatives may alter their heparin-affinities. This may represent enzymatic cleavage, modification of amino acid side chains or interaction with binding proteins. The specificity of PRF for heparan sulfates compared to other proteoglycan side chains such as chondroitin, dermatan and keratan sulfates has not been determined. The interaction of PRF with heparin might be via a specific sequence of amino acids or through the strong negative charge of the sulfate ion. PRF also binds to the negatively charged strong cation exchanger, but did not bind to an anionic exchanger. The positive charge of PRF undoubtedly interacts with the strong negative charge of the sulfate ions in the heparin side chains and this may contribute to the broad elution of PRF from heparin.

The PRF activity from cell extract that eluted from heparin with >1.5M NaCl (Fig. 2; pool D) was well separated from other proteins. This PRF was not consistently observed in CM and may represent an unprocessed precursor protein that is not secreted. Subsequent chromatographic purification of this fraction resulted in complete loss of PRF bioactivity. The non-specific adsorption and instability of this PRF at acidic pH may have contributed to its loss. Indeed, adsorption of PRF proteins became worse as purification progressed and immediate neutralization of pH following RP-HPLC was necessary to recover bioactivity.

A fraction containing PRF bioactivity from cell extract that eluted from heparin between 0.75-1.00M NaCl (Fig. 2; pool B) and further purified on HPLC was sequenced and shown to be identical to HIP/L29. The 160 amino acid HIP protein is the product of a single gene. This 18-kDa protein was initially characterized as an accessory ribosomal protein L29 (13). However, since its discovery, HIP protein has been implicated in a variety of functions including blood coagulation (10), cell adhesion (9), embryo implantation (3), and as a component of the
extracellular matrix (12). HIP binds specifically to heparan sulfate proteoglycans and elutes from heparin-affinity columns with 0.75M NaCl (6,8). Murine HIP is expressed in a cell-specific manner in adult tissues (6). Although HIP may function as an accessory ribosomal protein, its restricted distribution suggests an additional extraribosomal role. The amino acid sequence of HIP does not contain a canonical signal peptide sequence or a potential transmembrane spanning domain. Although HIP protein is not a secreted protein it can be found associated with the cell membrane. The peptide sequenced from cell extract begins at amino acid 44 of the HIP protein, adjacent to an internal methionine residue at position 43. This suggests an alternate translation start site at the first methionine residue in the HIP sequence. The functional consequence of a truncated HIP protein is unknown, but the first 42 amino acids do not affect the heparin binding properties of HIP (8). It is interesting to note that the first 50 amino acids of the HIP protein share the most sequence identity (>58%) with five other non-mammalian proteins identified in the Swissprot data bank.

Although the HIP protein may have different roles, it is not likely our PRF. The expression of HIP mRNA was not restricted to the mIL5 cell line. Indeed, the mIL39 melanotroph cell line also expressed HIP mRNA while these cells produced no PRF activity. Two polyclonal antibodies generated against synthetic HIP peptides, one (FAKK) that was identical to the first 15 amino acids of our peptide sequence, were ineffective in attenuating PRF activity from mIL5 cells. Although the neutralizing capability of these anti-HIP antibodies has not been validated, they have been used to detect HIP protein by Western blotting and immunohistochemistry (6). Unfortunately, recombinant HIP protein was not available so we did not directly test HIP for PRF bioactivity. It is probable that the HIP protein co-purified with PRF and was sequenced because it dominated the fraction with PRF bioactivity. Indeed, the asymmetrical shape of the corresponding protein absorbance peak suggested more than one protein component. It is possible that HIP represents a binding protein for PRF. It may mediate interactions with heparin or play a functional role in PRF bioactivity.

If PRF from mIL5 cells is a physiological regulator of PRL then it must be a secreted protein. The use of CM provided a source of secreted PRF and eliminated many proteins present in cell extracts. Only three chromatographic steps were necessary to isolate PRF from CM. The primary CM fraction with PRF bioactivity was associated with a symmetrical protein peak. Although this fraction appeared suitable for protein identification, no sequence or mass information was obtained. The failure of direct Edman sequencing was not surprising, since many eukaryotic proteins have blocked N-termini that render the chemistry ineffective. However, the failure to obtain mass information from either ESMS or MALDI-MS was troubling. These instruments can easily detect fmol quantities of peptide and the failure to detect any mass lead us to conclude the problem was operator error. Unfortunately, the mishandling of this sample resulted in its complete loss.

The same chromatography steps were used on a secondary CM fraction with PRF activity and this was resolved to a single protein peak that matched the profile obtained from the previous CM fraction. Although similar protein peaks were resolved from these two adjacent heparin fractions, this secondary material contained many more proteins. Indeed, the UV profile indicated less separation between the PRF protein peak and minor contaminating proteins. These components would confound subsequent structural analysis. The PRF bioactivity split between
two fractions (15 and 16) and ESMS of fraction 15 revealed two major mass components, 14,782 and 14,968 that differed by the incremental mass of tryptophan (186 amu); the series of smaller peaks, that differ in mass of 38 amu, that accompanied these major peaks is characteristic of potassium adducts. A MW of 15,000 Da is consistent with our estimation based on its passage or retention through dialysis membranes. The predominance of low mass species in this fraction was confirmed by MALDI-MS analysis, which estimated 10-20 pmol of the major protein components. Tryptic peptides from fraction 15 were analyzed by MALDI-MS and the spectra map did not produce a significant match from the search of several databases. This trypsinized sample was separated by microbore HPLC and several peptide peaks were analyzed by MALDI-MS. All peptide peaks selected for analysis contained complex mass spectra (Table 1). This may represent true heterogeneity of the orginal sample or derivatives of the major protein resulting from side chain modification of amino acids or degradation. The suitability for N-terminal sequencing requires a 10:1 major-to-minor mass ratio to achieve peptide identification at >85%. The best sample we obtained for sequence analysis had a 7:1 major-to-minor mass ratio with an unusually large major peptide of 4,676 Da. This could have resulted from incomplete fragmentation or lack of trypsin cleavage sites in the parent protein. The sequencing of half this sample using Edman chemistry failed to yield a useful amino acid sequence. The remaining half was lost following refractionation by RP-HPLC on a C8 microbore column.

ACKNOWLEDGEMENTS

The two anti-HIP antibodies (FAKK and GAKA) were the generous gift of Dr. Daniel D. Carson at the University of Delaware. Dr. Philip Andrews at the University of Michigan for mass analysis and protein sequencing of HIP and the primary fraction of PRF from mIL5 CM. The Yale Cancer Center Mass Spectrometry Resource/W.M. Keck Foundation Biotechnology Resource Laboratory for analysis of the secondary PRF fraction from mIL5 CM.

REFERENCES


Fig. 1. Schematics of the purification strategy used to isolate a prolactin-regulating factor (PRF) from cell-conditioned media (CM) of a mouse pituitary intermediate lobe (mIL5) cell line.
Fig. 2. Elution profile of PRF activity from mIL5 cell extract on a heparin-affinity column. The column was eluted with an increasing linear NaCl gradient. Aliquots of 10 µl were incubated with GH3/luc cells and luciferase activity was measured by luminometry. PRL gene induction is expressed as a % of control and each value represents the mean ±SEM of four determinations. Fractions 11-14 (yellow bar), designated peak B, were pooled and used in the next chromatographic step.
Fig. 3. Elution profile of heparin-bound PRF (Peak B from Fig. 2) on a strong-cation exchanger (SP). The column was eluted with an increasing linear NaCl gradient. See Fig. 2 for other details. Fractions 15-16 (yellow bar) were pooled and used in the next chromatographic step.
Fig. 4. Elution profile of SP-bound PRF (from Fig. 3) on an analytical C4 reversed-phase HPLC column. The column was eluted with an increasing linear acetonitrile (AcN) gradient. Aliquots of 20 µl were dried, reconstituted in culture media and incubated with GH3/luc cells. See Fig. 2 for additional details. Fractions 17-18 (yellow bar) were pooled and used in the next chromatographic step.
Fig. 5. Elution profile of PRF from C4 pool (fractions 17-18 from Fig. 4) on an analytical polymeric PRP3 RP-HPLC column. The column was eluted with an increasing linear acetonitrile (AcN) gradient. See Fig. 4 for other details. Fraction 6 (yellow bar) was used in the final chromatographic step.
Fig. 6. Elution profile of PRF (from Fig. 5) repurified on an analytical polymeric PRP3 column. The column was eluted and aliquots were assayed as described in previous figures. Fractions 6 and 7 (yellow bar) were subjected to N-terminal sequence analysis using Edman chemistry.
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**Fig. 7.** Amino acid sequence of murine heparin/heparan sulfate (Hp/HS)-interacting protein (HIP/L29). The amino acids shown in red were obtained by Edman sequence analysis of the isolated protein peak from mIL5 cell extract containing PRF activity (Fig. 6). Two antibodies generated against the underlined HIP amino acids 43-57 (FAKK) and 117-131 (GAKA) were gifts of Dr. Daniel D. Carson.
Fig. 8. Detection of mouse HIP gene in mIL5 cells by RT-PCR. The HIP gene was also expressed in normal mouse pituitary and mIL39. All samples were treated with DNase and controls (no RT) were included to detect contaminating genomic DNA. RPL19 was included in all reactions as an internal control. The expected product size of HIP is 431 bp and RPL19 is 500 bp. Ladder is 100 bp.
Fig. 9. Inability of two antibodies (FAKK and GAKA) generated against mouse HIP peptides to abolish PRF activity from mIL5 CM. No recombinant HIP protein is currently available to assay for PRF activity or validate the neutralizing properties of these antibodies. Immunodepleted mIL5 CM was incubated with GH3/luc cells and luciferase activity determined by luminometry. Data is expressed as a % of control and represents the mean ±SEM of four determinations.
Fig. 10. Elution profile of PRF activity from mIL5 cell conditioned media (CM) on a heparin-affinity column. The column was eluted with an increasing linear NaCl gradient. Aliquots of 10 µl were incubated with GH3/luc cells and luciferase activity was measured by luminometry. PRL gene induction is expressed as a % of control and each value represents the mean ±SEM of four determinations. Fractions 13-16 (yellow bar) were pooled and used in the next chromatographic step.
Fig. 11. Elution profile of heparin-bound PRF from mIL5 CM (from Fig. 10) on a strong-cation exchanger (SP). The column was eluted with an increasing linear NaCl gradient. See Fig. 10 for other details. Fractions 15-16 (yellow bar) were pooled and used in the final chromatographic step.
Fig. 12. Elution profile of cation-bound PRF from mIL5 CM (from Fig. 11) on an analytical C3 reversed-phase HPLC column. The column was eluted with an increasing linear acetonitrile (AcN) gradient. Aliquots of 20 µl/well were dried, reconstituted in culture media and incubated with GH3/luc cells. PRL gene induction was assayed as previously described and expressed as a % of control. See Fig. 10 for additional details. Fractions 15 and 16 (yellow bar) were subjected to mass analysis by electrospray ionization mass spectrometry (ESMS) and N-terminal sequencing by Edman chemistry.
Fig. 13. Comparison of protein elution profiles between an alternate mIL5 CM fraction (green bar in Fig. 10 shown in blue UV profile) with that shown in Fig. 12 (yellow bar in Fig. 10 shown in red UV profile) on an analytical C3 reversed-phase HPLC column. The column was eluted with an increasing linear acetonitrile (AcN) gradient. See Fig. 12 for other details. Fractions 15 and 16 (yellow bar) from the blue UV profile were subjected to mass analysis by ESMS and N-terminal sequencing by Edman chemistry.
Fig. 14. Electrospray mass spectrometry (ESMS) spectrum from bioactive PRF fraction of mIL5 CM (fraction 15 from Fig. 13 blue UV profile). MaxEnt transformation of raw spectrum onto a molecular mass scale. Raw data was dominated by low mass ions, but two high mass ions were detected. The most intense peaks from the two components were observed at 14782.6 and 14968.6 with a mass difference of 186 amu, which is the incremental mass of tryptophan. These peaks are accompanied by a series of peaks which differ in mass by a nominal 38 amu which is characteristic of potassium adducts. The average mass error is 0.02%.
Fig. 15. Elution profile of trypsin digested PRF fraction (see Fig 14) on microbore HPLC. Numbered peaks were selected for MALDI-MS screening prior to possible Edman sequencing.
Fig. 16. MALDI-MS spectrum from HPLC peptide peak 1 (from Fig. 15). The major mass species is 4675.91 with a doubly charged species at 2339.61. The ratio of major to minor masses is 7:1, indicating sample purity of 80%.
<table>
<thead>
<tr>
<th>PEAK #</th>
<th>Purity</th>
<th>Mass-to-charge ratio (m/z)</th>
<th>Ratio(major:minor)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80%</td>
<td>1533.03, 1804.02, 2339.61</td>
<td>7:1</td>
<td>4675.91 is the major mass with a double charged specie at 2339.61</td>
</tr>
<tr>
<td>10</td>
<td>60%</td>
<td>2150.39, 2217.77, 2465.66</td>
<td>5:1</td>
<td>4298.3 is the major mass with a double charged specie at 2150.39</td>
</tr>
<tr>
<td>14</td>
<td>50%</td>
<td>1420.31, 1436.50, 1953.71</td>
<td>2:1</td>
<td>1420.31 is the major mass with a met/trp oxidation at 1436.5</td>
</tr>
<tr>
<td>20</td>
<td>50%</td>
<td>824.03, 1593.80, 1650.71</td>
<td>5:1</td>
<td>1593.8 is the major mass</td>
</tr>
<tr>
<td>21</td>
<td>50%</td>
<td>696.06, 712.06, 824.20</td>
<td>2:1</td>
<td>1465.79 is the highest peak, followed by 696.06</td>
</tr>
<tr>
<td>23</td>
<td>50%</td>
<td>1422.29, 1449.46, 1497.33</td>
<td>7:5</td>
<td>1497.33 is the major mass followed by 1449.46</td>
</tr>
</tbody>
</table>

**Table 1.** Summary of MALDI-MS data obtained from selected peaks following microbore HPLC (numbered peaks from Fig. 15). Estimated fraction purity determined from the observed linear masses (M/Z) and the ratio of major to minor mass species.
CHAPTER VIII

PERSPECTIVE AND CONCLUSIONS
DISCUSSION

Tonic inhibition of PRL release by DA is unique among pituitary hormones. Circulating PRL reflects changes in the level of DA reaching the pituitary. DA is low at times of elevated PRL secretion such as during lactation. However, acute increases in serum PRL following the suckling stimulus suggested the existence of a PRF. Although several hypothalamic peptides stimulate PRL release, their physiological role remains uncertain. A novel PRF represents the last of the hypophysiotrophic releasing hormones to be identified. The initial search for PRF focused on the hypothalamus, the established origin of all releasing/inhibiting hormones, but it was discovered in the PP. In rats, an intact PP was necessary to achieve acute elevations in serum PRL following suckling or estrogen treatment. This PRF was localized to the IL, but it was not a POMC peptide produced by melanotrophs. Instead, the cellular source of PRF was from a subpopulation of IL cells. The biochemical characterization of PP-PRF showed that it stimulated the rapid release of PRL, was a small peptide, and was effective in the presence of DA. The partial purification of PRF from bovine PP demonstrated multiple bioactive species. The identity of these PRF peptides was not resolved and they may represent derivatives of a precursor protein or products of distinct genes.

The inaccessibility of the pituitary IL has hindered experimental manipulation and the lack of established IL cell lines has limited the scope of in vitro investigations to primary cell cultures. Although processing of POMC peptides by melanotrophs has been well established, their cellular lineage remains undefined and even less is known about minor cell populations distributed among the melanotrophs. The small size of the IL has been a major obstacle in the purification of the PRF peptide.

The generation of transgenic POMC-Tag mice that develop IL tumors provided a novel model to investigate the IL and PRF. IL tumors produced PRF and partial purification revealed two major PRF species that differed in molecular weights. Our data clearly show that neither male nor female POMC-Tag mice develop hyperprolactinemia with the progression of IL tumors. These results disagree with a previously published observation that female POMC-Tag mice with primary IL tumors have elevated PRL. Theoretically, hyperprolactinemia could be caused by the disruption of DA delivery to the AP due to expansion of the tumors. However, increasing tumor size did not affect serum PRL levels. In addition, female POMC-Tag mice with large IL tumors have increased serum PRL during pregnancy and lactation, indicating an intact regulatory system. Furthermore, we conclude that since the melanotroph is not the likely source of PRF, IL tumors resulting from their transformation and expansion would not necessarily increase PRF production or consequently affect PRL release. The PRF activity observed in IL tumor extracts represents a minor component detectable by a sensitive bioassay and produced by a subpopulation of secondary tumor cells.

The PRF in mice may not be the same as that characterized in rats. Indeed, differences may exist in its biochemical properties or mechanism of action. The function of mouse PRF during suckling or in the presence of DA has not been examined and it is assumed that mouse PRF acts in a similar manner to that established for the rat. Furthermore, the use of the rat GH3/luc bioassay may not reflect the true potency of mouse PRF on PRL gene induction or release.
IL tumors from POMC-Tag mice were used to establish two novel cell lines, mIL5 and mIL39, which differed in morphological and biochemical characteristics. The mIL39 cells were identified as melanotrophs based on the expression of typical cellular markers such as POMC and D2R. The mIL5 cells had none of the cellular characteristics of melanotrophs and their cell lineage remains enigmatic. The cells also differed in their ability to stimulate PRL release from primary rat AP and GH3/luc cells. The mIL39 cells did not stimulate PRL gene expression or release, consistent with the notion that melanotrophs were not the source of IL PRF. The mIL5 cells did produce and secrete a potent PRF that selectively stimulates both PRL gene expression and release. However, this PRF required >4h to stimulate PRL release and this paralleled an increase in PRL gene expression. This is inconsistent with a typical releasing hormone that acts within minutes to affect hormone release. Tissue extracts from the rat PP increased PRL release in <1h and this difference in the time course of PRF action suggests distinct proteins. However, the sensitivity of the GH3/luc bioassay to mouse PRF might not be the same as that of rat or bovine PP extract. Furthermore, GH3/luc cells had been adapted to growth conditions that did not facilitate PRL storage. These cells require priming with EGF and E2, both stimulators of the PRL gene and release, to store sufficient PRL for rapid release determination. In fact, growing GH3/luc cells in a specially formulated heat-inactivated gelding serum provided a low and stable basal luciferase background that created a responsive PRL gene to activation by PRF from mIL5 cells. Cell culture conditions dramatically affect the sensitivity of hormone secreting cells to regulatory molecules by either enhancing or inhibiting their responsiveness. Although no evidence supports that PRF from mIL5 cells is a fast-acting releasing hormone, a final assessment of this attribute would be better suited with a pure compound.

The dependence of PRF purification on a bioassay necessitates the establishment of the GH3/luc cell model. These cells were stably transfected with a plasmid containing a construct with the rat PRL promoter ligated to the luciferase reporter. This provided a convenient assessment of PRL gene expression by measuring luciferase activity. PRL release could be measured by RIA from the same cells and paralleled changes in gene expression. This adaptation to the more labor-intensive release assay was exploited during the purification of PRF from mIL5 cells.

Previous evidence from partial purification of PRF activities from bovine PP and IL tumors suggests the existence of several PRF molecules. A major difficulty in the identification of PRF has been the hypersensitivity of PRL release to a plethora of stimulatory molecules that include peptides and cytokine growth factors. The following biochemical characteristics of mIL5 PRF suggested that it is a growth factor: 1) heparin-binding properties, 2) time course of action, and 3) molecular weight. RT-PCR was used to evaluate gene expression in mIL5 cells and two candidate growth factors with similar properties to PRF were identified. FGF-2 and HB-EGF are both potent stimulators of PRL gene expression and release \textit{in vitro}. Although no physiological role for these growth factors in PRL regulation has been established they were detected within the normal mouse pituitary. In fact, FGF2 was initially isolated from the bovine pituitary gland. Their contribution to the PRF activity in mIL5 cells was extensively evaluated using heparin-elution profiles, Western blotting, and immunodepletion. We conclude that PRF, from mIL5 cells, is neither FGF2 nor HB-EGF.

The purification of PRF was deemed a realistic goal by the following considerations; 1) the mIL5 cells provided a stable and renewable source of PRF material, 2) the cells could be
maintained in serum-free medium and still secrete PRF, 3) a rapid and sensitive bioassay for measuring PRF activity was available, and 4) PRF binds to heparin enabling the efficient use of affinity chromatography. Initially, mIL5 cell extract was used to define the purification strategy. The identification of multiple fractions with PRF activity that eluted from heparin-affinity columns added considerable complexity toward the resolution of PRF protein. However, sequential chromatography of mIL5 cell extract resulted in an isolated fraction with PRF activity. The protein sequence obtained from this fraction was identical to 30-amino acids of an internal sequence of the heparin-binding protein HIP/L29. The biochemical characteristics of mouse HIP, together with the failure of two anti-HIP antibodies to attenuate PRF activity from mIL5 CM, suggested this protein is not a PRF. The isolation of the truncated HIP protein from mIL5 cell extract appeared to be due to co-purification with PRF.

The physiological relevance of a PRF requires it to be a secreted protein. The use of serum-free CM provided a cleaner source of PRF than cell extract. The successful isolation of a single protein peak coinciding with a single bioactive PRF fraction was followed by the disappointment of protein identification. It is unclear why our Ann Arbour collaborator was unsuccessful in obtaining structural information from the isolated PRF protein, but one possibility includes N-terminal blockade. Non-specific adsorption is also a common problem with purified proteins and this may be exasperated with mIL5 PRF due to its strong hydrophobicity.

A final attempt was made to isolate PRF from mIL5 CM on a second sample that was run in parallel with the first. Although this fraction contained more extraneous proteins, a protein peak with PRF activity was successfully isolated on RP-HPLC. The elution of PRF activity in this sample at 42% AcN was identical to the first sample and the protein absorbance peaks from both HPLC runs overlapped. Therefore, we assumed that the same protein represented the PRF activity in these two fractions. Under contract with the protein core at the Yale Cancer Center we attempted to identify this PRF sample. Using ESMS they obtained a mass estimate of 14968 ±0.02% for the major protein species, which agreed with our size estimate for PRF. A database screen of mass spectra obtained following enzymatic cleavage and MALDI-MS failed to match the peptides to a parent protein. Microbore HPLC separation of the tryptic peptides and subsequent MALDI-MS screen indicated that all fractions were complex mixtures. The least heterogeneous sample with a 7:1 major-to-minor mass spectrum was N-terminally sequenced by Edman chemistry. The success rate of identifying a sample of this purity was 50%. Unfortunately, this failed to provide an adequate sequence of amino acids and the structure of PRF from mIL5 remains unresolved. Any future efforts to identify PRF will require repurification or an alternate approach.

In conclusion, we have: 1) demonstrated that POMC-Tag mice with IL tumors do not develop hyperprolactinemia, 2) established and characterized two novel pituitary IL cell lines, a melanotroph and a cell that produces and secretes a potent PRF, 3) biochemically characterized PRF, and 4) isolated PRF by sequential chromatography but failed to obtain primary structure.