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Prolactin in Human Breast Cancer

by

David R. Gould

Department of Medicine, Division of Experimental Medicine, McGill University, June,1992

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Abstract

The function of prolactin in human breast cancer was studied using four different approaches. First, purification and characterization of the prolactin receptor from breast cancer cells indicated that the receptor has a molecular mass of 88 000 Da, 67 000 Da being protein, and the other 21 000 Da presumably carbohydrate. Secondly, prolactin was tested for mitogenic activity in breast cancer *in vitro*. No consistent mitogenic response to prolactin could be demonstrated in these experiments. Thirdly studies upon the regulation of the prolactin receptor in breast cancer cells indicated that the prolactin receptor is stimulated by lactogen, estrogen and progesterone at the protein level. Estrogen, progesterone, thyroid hormone, and forskolin (but not lactogen) increase prolactin receptor steady state RNA levels, and the phorbol ester PMA and retinoic acid inhibited receptor RNA levels. However, effects at the RNA level were of a much lesser magnitude than effects at the protein level. Mechanisms other than transcriptional regulation alone are likely involved in prolactin receptor regulation. Fourthly, prolactin receptor and prolactin inducible protein / gross cystic disease fluid protein (PIP/GCDFP-15) RNA levels were examined in breast cancer tumors. Highly significant correlations were observed between the prolactin receptor and the progesterone receptor; the prolactin receptor and PIP/GCDFP-15; and PIP/GCDFP-15 and progesterone receptor.

Résumé

Le rôle de la prolactine dans le cancer du sein chez l'humain a été étudié selon quatre approches. Premièrement, la purification du récepteur de la prolactine à partir des cellules cancéreuses du sein, de même que l'étude de ses caractéristiques portent à croire que le récepteur a une masse moléculaire de 88 000 Da, dont 67 000 Da serait protéique et les 21 000 Da restant, se composeraient probablement d'hydrate de carbone. Deuxièmement, nous avons testé la prolactine pour connaître son activité mitogénique sur les cellules cancéreuses du sein et ce, *in vitro*. Cependant, aucun effet significatif de la prolactine n'a pu être démontré par ces expériences. Troisièmement, nous avons étudié la régulation du récepteur de la prolactine chez les cellules cancéreuses du sein. Cette étude nous indique que le lactogène, l'estrogène, et la progestérone stimulent le récepteur de la prolactine au niveau protéique. La progestérone, l'estrogène, l'hormone thyroïdienne, et forskolin (mais non le lactogène) stimule le récepteur de la prolactine au niveau de l'état d'équilibre de l'ARN, et l'acide rétinoïque, et l'ester de phobol inhibent le récepteur de la prolactine. Cependant, les effets au niveau des ARN étaient de moindre magnitude que ceux observé au niveau des protéines. Des mécanismes autres que la seule régulation de la transcription sont également impliqués dans la régulation du récepteur de la prolactine. Quatrièmement, le niveau des ARN du récepteur de la prolactine et du *prolactin inducible protein / gross cystic disease fluid protein* (PIP/GCDFP-15) a été examiné dans les tumeurs cancéreuses du sein. Des corrélations hautement significatives furent observées entre le récepteur de la prolactine et le récepteur de la progestérone, entre le récepteur de la prolactine et le PIP/GCDFP-15 et enfin, entre le PIP/GCDFP-15 et le récepteur de la progestérone.

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Abbreviations

^{125}I-hGH ..... iodinated human growth hormone
ABTS ..... 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid
ANOVA ..... analysis of variance
ATP ..... adenosine triphosphate
bp ..... base pair
CNTF-R ..... ciliary neurotrophic factor receptor
cpm ..... counts per minute
DABA ..... diaminobenzoic acid
DMEM ..... delbecco's modified eagles medium
dNTP ..... deoxynucleotide
DTT ..... dithiothreitol
E ..... indicates exponential to the power of 10
EDTA ..... Ethylenediaminetetra-acetic acid
EGF ..... epidermal growth factor
EMEM ..... Earl's modified eagles medium
Epo-R ..... erythropoietin receptor
ER ..... estrogen receptor
FBS ..... fetal bovine serum
G-CSF-R .... granulocyte-colony stimulating factor
GM-CSF-R ... granulocyte macrophage-colony stimulating factor receptor
GM-CSF-Rβ .. GM-CSF-R associated protein
GTC ..... guanidinium thiocyanate
HBS ..... hepes buffered saline
HEPES ..... (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
hGH ..... human growth hormone
HRP ..... horse radish peroxidase
IFG-1 ..... insulin like growth factor
Ig ..... immuno-globulin
IL-2-R ..... interleukin-2 receptor
IL-3-R ..... interleukin-3 receptor
IL-4-R ..... interleukin-4 receptor

IL-5-R ..... interleukin-5 receptor

IL-6-R ..... interleukin-6 receptor

IL-6-Rβ ..... IL-6-R associated protein gp130

IL-7-R ..... interleukin-7 receptor

LIF-R ..... leukemia inhibitory factor receptor

LSNBS ..... lactogen stripped newborn bovine serum

MCF-7M ..... MCF-7 cells obtained form the Michigan Cancer Foundation.

MCF-7T ..... MCF-7 cells obtained from the American Type Culture

..... Collection

MCF-7V ..... MCF-7 cells obtained from Dr. Vonderhaar, NIH

NBS ..... newborn bovine serum

NIADDK .... National Institute of Arthritis, Diabetes, Digestive Diseases and

..... Kidney

oPRL ..... ovine prolactin

PR ..... progesterone receptor

PAGE ..... polyacrylamide gel electrophoresis

PBS ..... phosphate buffered saline

PEG ..... polyethylene glycol

PIP/GCDFP-15 prolactin inducible protein/ gross cystic disease fluid protein-15

PMA ..... phorbol 12-myristate 13-acetate

PMSF ..... phenylmethylsulfonylfluoride

SDS ..... sodium dodecylsulfate

sulfoEGS .... ethylene glycol*bis*(sulfosuccinimidylsuccinate)

TBE ..... Tris-HCl, Borate, EDTA buffer.

TGF-β ..... transforming growth factor beta

Preface

Contributors to the Thesis Work

In Chapter 2., Prolactin Receptor: Protein Characterization and Sequence Analysis, the candidate was responsible for all of the experimental work with the exception of the production of the rat prolactin receptor short form mutant which lacks the third glycosylation site Rat (d3) which was prepared by Mrs. Maria Rozakis-Adcock.

In Chapter 3., Prolactin Receptor Regulation, all work was performed by the candidate.

In Chapter 4., Breast Cancer Responses to Prolactin, all work was performed by the candidate.

In Chapter 5., Prolactin Receptor Expression in Breast Tumors, all work was performed by the candidate with the exception of the Semi-Quantitative PCR. The candidate prepared all the RNA samples, but the PCR analysis was performed by friend and fellow student, Mr. Makoto Nagano in Paris.

Contributions to Original Knowledge

The following novel findings and observations have been demonstrated in this thesis.

1. A method for purification of the human prolactin receptor from breast cancer tissue was developed. It was discovered that the human prolactin receptor was unique in that it was much more labile than the prolactin receptor from the rabbit or rat.
2. The human prolactin receptor was shown to have a molecular mass of 88 000 Da by chemical crosslinking of the receptor to ^{125}I-hGH.
3. Immunoprecipitation of human prolactin receptor showed that the major species of prolactin receptor after purification had a molecular mass of 38 000 kDa
4. Sequence analysis indicated that the human receptor lacks the normal third potential glycosylation site. It was shown that the absence of this site in the receptor from the rat could not reproduce the uniquely labile nature of the human receptor.
5. Lactogens were shown to stimulate prolactin receptor binding in breast cancer cells.
6. Progesterone, estrogen, thyroid hormone, and forskolin have significant stimulatory activity on prolactin receptor steady state RNA levels. Retinoic acid, and PMA have significant inhibitory activity on prolactin receptor steady state RNA levels. Lactogen, insulin, IGF-1, EGF, TGFβ, had no significant regulatory activity at the RNA level. With the exception of progesterone, the magnitude of regulation at the RNA level was less than 2-fold. This contrasts sharply with the effects of estrogen, and especially lactogen, at the prolactin receptor protein level, where the magnitude of induction at the protein level is much greater than the degree of induction at the steady state RNA level. These observations point to the existence of regulatory mechanisms beyond the regulation of prolactin receptor mRNA.
7. Prolactin receptor RNA was measured in breast cancer tumors. A highly significant correlation between levels of prolactin receptor and PIP/GCDFP-15 RNA was observed. In addition a highly significant correlation between levels of prolactin receptor and progesterone receptor was observed.

Acknowledgements

I wish to express my gratitude to the many people who have assisted me in during the course of this work.

- First I thank my parents Kay and Ross Gould, and also the numerous teachers I have been fortunate to have known, for their guidance, encouragement, and inspiration.
- I thank the Cancer Research Society of Montreal Inc. for their generous support during six years of research work. I dedicate this work to the hope of those who donate money to cancer research. I also hope that this work may in some unguessed way, improve the lives of those who fall ill to cancer.
- I thank Joseph Zachweija, Sally Raguet, Guylaine Benoit, and Cathy Russo for their expert technical assistance.
- I recall the numerous friendships I have made at the Laboratory of Molecular Endocrinology: their stimulating conversation, and warmth proved to be the antidote to the occasional frustrations and disappointments which occur in research. Special thanks go to Makoto Nagano, Suhad Ali, Isabelle Pellegrini, and Joseph Zachweija for the many hours spent in their good company.
- To my special friend Claire Vézina who has been my companion and greatest supporter. Thank you for your care and understanding.
- I wish to especially thank my supervisor, Dr. Paul Kelly, for his very generous support, for his great patience, and for his good humour. I wish also to thank Dr. Hans Zingg for his vey kind help and useful suggestions.

Chapter 1. Introduction

Prolactin was first characterised for its lactational activity in rabbits in 1928,[1] and was first purified from the pituitary in 1932.[2] Since its discovery, a great diversity of actions for prolactin have been identified. In the last 15 years, knowledge of the receptor for prolactin has accumulated, starting with the development of binding assays, then purification schemes, and recently the DNA sequence isolation.

This Introduction discusses the biological actions of prolactin, reviews what is known about the structure and function of the prolactin receptor, what is known about the regulation of the prolactin receptor, and examines the possible role for prolactin in breast cancer. In this way, the research reviewed here provides the context in which the experiments described in the following sections were performed.

Biological Actions

There are at least 85 different functions for prolactin in the vertebrate subphylum.[3] These actions can be classified into seven categories:

1. Osmoregulation
2. Ectodermal and Integument Effects
3. Reproduction and Lactation
4. Metabolism
5. Morphogenesis
6. Growth
7. Behavior
8. Immunoregulation

The earliest function for prolactin was that of osmoregulation in fish, and many of prolactin's functions can be regarded as specializations of this action, the most familiar example being the mammary gland, which is derived from the sweat glands.

Osmoregulation

Osmoregulation controlled by prolactin is most clearly observed in fish.[4] Prolactin acts to prevent sodium loss from the plasma into the water environment, an action which is especially important in fresh-water fish.[5] Prolactin also reduces the water permeability of the gills, intestinal tract and urinary bladder of fish.[6] In mammals, there is evidence that prolactin similarly regulates the flow of fluid: milk in the mammary gland,[7] urine in the kidney,[8,9] perspiration by the sweat gland,[10] and cerebral spinal fluid through the choriod plexus of the brain.[11] The presence of prolactin receptors in the chorion laeve of the placenta has led to the speculation of an osmoregulatory function there as well.[12,13]

Ectodermal and Integument Effects

Prolactin's functions in skin are diverse. In addition to the osmoregulation that occurs across the skin, prolactin plays a role in skin pigmentation, mucus, nasal, sebaceous, and preputial gland secretions, and feather and hair development.[14]

Reproduction and Lactation

Prolactin stimulates secretions of the male[15] and female reproductive tissues, and participates in gametogenesis, steroidogenesis, and gestation. In the male prolactin stimulates the growth of the lateral lobe of the prostate by up-regulating androgen receptors in this tissue,[16] although prolactin action may be independent of androgens.[17] Prolactin also stimulates growth of the seminal vesicles.[18] In the testes prolactin up-regulates LH receptors in rats,[19,20] though, in hyperprolactinemic men there is a reduced testicular response to hCG.[21] In women, hyperprolactinemia can cause amenorrhea and infertility.[22,23] Treatment of patients with bromocryptine, which suppresses prolactin secretion, is an effective treatment in 80-90% of cases.[24] In the ovary prolactin is both luteotrophic and luteolytic. That prolactin is luteolytic has been demonstrated in rats treated with bromocryptine: in these animals the ovaries are enlarged due to an accumulation of corpora lutea. In animals treated this way, if a single prolactin injection is given, the ovaries do not appear enlarged and contain a normal number of corpora lutea.[25] Prolactin is necessary for the normal development of the follicle.[26,27] It directly regulates α_2-macroglobulin expression in granulosa cells during

corpus luteum formation,[28] and has been observed to up-regulate LH receptors, and to support progesterone synthesis.[29,30] Others, however, have found prolactin to have the opposite effect on both LH receptor regulation and progesterone synthesis.[31,32] The confusion over the precise action of prolactin in the ovary is most likely due to the fact that other hormones and the timing of these various signals have important effects on prolactin's function.

Lactation can be said to occur in two classes of vertebrates- in mammals and in birds, although in birds it is not a feature common to the whole class, and the production of crop milk is not traditionally thought of as lactation. The pigeon is the classical model for the production of crop milk in birds,[33] and recently 4 cDNA clones have been isolated that encode separate milk proteins. mRNA levels of three of the clones are induced 2-3 fold by prolactin, but for the forth there is an induction of 70 fold.[34]

Breast development involves several steps, from the stimulation of growth and partial differentiation during sexual maturation, to the final differentiation of the gland during gestation, and the maintenance of lactation during the period of suckling.[35] Ablation and replacement experiments in the 1950`s showed that mammary gland development and lactation could be produced only in the presence of a combination of hormones: estrogen, progesterone, cortisol, thyroid hormone, placental lactogens, insulin and prolactin.[36] *In vitro* and *in vivo* studies indicate that attachment factors and cell-cell interactions are also important.[37,38] Depending upon the species, prolactin plays a role in the growth and development of the mammary gland as well as in the synthesis of the various milk proteins, of lactose and of milk lipids. For example, casein mRNA levels are increased by prolactin both by transcriptional activation and increased mRNA stability.[39,40] Prolactin usually requires insulin and cortisol in cultures in order to have its action, although depending upon the specific milk protein and upon the species tested, prolactin alone can be sufficient.[41] It is interesting to note that lactation (with normal nutritional content) can occur outside of the normal progression of events in women who experience hyperprolactinemia, or hyperstimulation of the nipple.[42] Nipple stimulation results in the release of oxytocin and β-endorphin which in turn stimulate PRL release.[43,44,45]

Metabolism

During lactation, prolactin may induce metabolic changes, especially on fat metabolism.[46,47,48]

Morphogenesis

A morphogenic action is seen in the amphibians. Prolactin retards metamorphosis from the larval to the adult form by antagonizing thyroid hormone induction.[49] In newts, the second metamorphosis, a return to larval characteristics which are needed for breeding (which takes place in the water) is induced by prolactin.[50] Prolactin also elicits the reproductive "water drive" behaviour in these species.[51]

Growth

Prolactin's mitogenic action is seen in the pigeon crop sac,[52] mammary gland,[53] intestine,[54] seminal vesicles,[55,56] and follicles,[57] in the β cells of the pancreas,[58] cells of the immune system[59] and liver,[60] though the effect in liver is controversial.[61] One way in which prolactin stimulates cell growth is through the stimulation of ornithine decarboxylase, the rate limiting enzyme in polyamine synthesis. Prolactin has been shown to regulate ornithine decarboxylase activity in mammary gland explants, liver, kidney, brain, adrenals, spleen, thymus, and heart.[62] Prolactin may also contribute to growth through the induction of insulin-like-growth factor-1 (IGF-1).[63]

Behavior

As cited above, prolactin induces the newt species to return to the water habitat for breeding. Most of prolactin's behavioural effects are associated with reproduction, and often require the presence of estrogen and progesterone to occur. Such actions include migration in birds,[64] and parental behaviour in fish,[65] birds, and mammals[66,67]

Immunoregulation

The earliest clue that prolactin was important in immune function came from the observation of involution of the thymus and reduced immune responsiveness in hypophysectomized rats.[68,69] If, in these animals, prolactin was replaced by a pituitary allograft, or by injection, immune function returned to a normal state.[70] More

specifically, prolactin has been shown to stimulate the thymus to release thymulin in mice.[71] Prolactin also seems to play a role in the development the of immune secretions of the mammary gland.[72] The best *in vitro* model of prolactin function in the immune system is the rat lymphoma cell line Nb2,[73] which is dependent on prolactin for growth. Recently, the immediate-early response gene interferon-regulatory factor-1 was shown to be transcriptionally regulated by prolactin in these cells.[74] Co-workers at the Laboratory of Molecular Endocrinology have determined that the prolactin receptor in the Nb2 cells is a truncated mutant, which never the less maintains its biological activity.[75] It may be this truncation that gives the Nb2 receptor its 4-fold greater affinity for prolactin. Other actions of prolactin in the immune system have recently been reviewed.[76]

Prolactin Receptor and its Regulation

Binding of Prolactin to its Receptor

Prolactin binding sites have been found in many tissues, (refer to Table 1) and for several of these tissues the physiological role of prolactin is unknown. The first binding assay was developed in 1973 using a rabbit mammary gland cell membrane preparation.[77] These experiments showed that human growth hormone (hGH) could bind to the lactogen receptor, thus confirming earlier studies that primate growth hormones could have lactogenic activity.[78] It has recently been discovered that hGH binds lactogenic receptors only in the presence of zinc ions: zinc supplies a positive charge in the receptor binding domain that is necessary for high affinity binding.[79] Non-membrane bound soluble prolactin receptors have been found in cytosol fractions,[80] and in milk.[81]

The kinetics of prolactin-receptor dissociation are slow: the energy required for dissociation, at 25 degrees C, is about 2.5-fold higher than that needed for association.[82] Therefore, binding sites must be desaturated with a chaotropic agent (concentrated $MgCl_2$) to determine the total number of receptor sites in a tissue.[83] Studies of the factors which perturb prolactin/receptor binding and of the thermodynamics of prolactin binding suggest that hydrophobic interactions are the principle force involved in binding.[82,84]

Table 1. Tissue Distribution of Prolactin Receptors

Tissue	Species	Reference
Adipose	Sheep	85,86
	Rabbit	87
Adrenal	Rat	88,89,90,91,92
	Pig	93,94
	Cow	82
	Sheep	82
	Mouse	95
	Rabbit	96,97
	Hamster	98
Brain	Human	99
... Choriod Plexus	Rabbit	11
	Xenopus	100
	Toad	101
	Rat	102
... Ependymal lining 3rd ventricle	Rat	103
... Hypothalamus	Rabbit, Rat	104
... Hypothalamus, Substantia Nigra	Rabbit	105
... Gill	Tilapia	106
Immune System		
... Mast Cells	Rat	107
... Erythrocytes, Lymphocytes	Human	108
... Lymphocytes	Human	109
... Lymphoma	Rat	110
... Thymocytes	Rat	63
... Monocytes	Rat	111
... Macrophages	Rat	112
Intestine	Python	113
	Fish (Tilapia)	114
Kidney	Rat	84,115,116,117
	Rabbit	118,119,120
	Cow	121
	Mink	122
	Python	104
	Frog	123,124,125
	Toad	93,126
	Xenopus	92,127
	Tilapia	128
	Carp	129
Liver	Mouse	130,131
	Cow	132,133
	Sheep	78,134
	Hamster	135
	Rabbit	136
	Tilapia	97
	Eel	118
	Carp	118
	Xenopus	137,117

....	....	.....	Frog	.....	115,138
....	....	.....	Tilapia	.....	117,139
....	....	.....	Guinea Pig	.....	140
....	....	.....	Rat	.....	84,141,142
Lung	....	.....	Rabbit	.....	143
....	....	.....	Rat	.....	144,145
Mammary Gland	Tumour	.....	Rat	.....	146,147,148149,
....	Tumour	.....	Dog	.....	150
....	Tumour	.....	Human	.....	151,152,153154
....	....	.....	Mouse	.....	155,156
....	....	.....	Cow	.....	132,133,157
....	....	.....	Rat	.....	92,115,158
....	....	.....	Pig	.....	94,159,160
....	....	.....	Rabbit	.....	97,161,164,162
....	....	.....	Sheep	.....	86,163,164165
....	....	.....	Tammar Wallaby		166
Ovary	....	.....	Rat	.....	88,167,168,169
....	....	.....	Rabbit	.....	97,170
....	....	.....	Human	.....	171,172,173
....	....	.....	Guinea Pig	.....	174
....	....	.....	Hamster	.....	175
....	....	.....	Mouse	.....	176
....	....	.....	Mink	.....	177
....	....	.....	Tilapia	.....	114
Pancreas	.. β-Cells	.....	Rat	.....	178,179,180,181
Placenta	....	.....	Human	.....	182,183,184,185
Prostate	....	.....	Human Tumors		186,187
....	....	.....	Human BPH		188
....	....	.....	Rat	.....	91,189,190,191
Salivary Gland ...	....	.....	Human	.....	192
Skin	....	.....	Xenopus	.....	127,137
....	....	.....	Fish (Triturus)		193
Testis	....	.....	Rat	.....	194,195,196,197
Leydig Tumor	....	.....	Rat	.....	198
....	....	.....	Hamster	.....	199,200
....	....	.....	Human	.....	201
....	....	.....	Tilapia	.....	202
Uterus	....	.....	Rabbit	.....	87,203,204
....	....	.....	Pig	.....	205,206

After prolactin binds to the plasma membrane, it is rapidly internalized into internal membrane vesicles (endosomes). iI then gradually moves into the lysosomal compartment where the hormone receptor complex is degraded.[207,208,209] Lysosomotropic agents (which interfere with lysosomal functioning) delay receptor down regulation, which thus appears to be due to receptor internalization and degradation.[210]

Prolactin Receptor Purification

Many laboratories have purified the prolactin receptor, and depending upon the techniques used, different results have been obtained (refer to Table 2). The first purification protocols used ligand affinity purification. The relative molecular weight of the receptor have been estimated by gel chromatography, hydrodynamic properties during centrifugation, and by SDS-PAGE. One puzzling feature of these experiments was the large differences in the molecular weights reported for the receptor. These differences may have derived in part from the methods used during purification. For example, gel chromatography gave the largest molecular weights for the receptor. This was thought to be due to a quality of the detergents used to solubilize the receptor which can form micelles of different sizes in association with the receptor. It was also suggested that the receptor might exist in a complex with other proteins. SDS-PAGE under reducing conditions, which completely denatures protein yielded much smaller molecular weights. However, even with this technique, differences in receptor size were observed. The recent discovery of the unusual dimer conformation of the hGH binding protein with hGH could shed some light on the discrepancy of receptor sizes reported in the past,[211,212] since receptors could exist as monomers, dimers or even oligomers. Once the receptor was partially purified, monoclonal antibodies were made,[141] which permitted the use of more effective immuno-affinity purifications. Material purified in this way was eluted as a 42 000 kDa band from SDS-PAGE gels and microsequenced,[213] providing the information needed for the first isolation of the cDNA sequence for the receptor.[214]

Table 2. Molecular Weight Estimates of Purified Prolactin Receptor

Tissue	Detergent	Size	Method	Reference
Rabbit Mammary	Triton X-100	220 000	Chromatography	[215]
Frog Liver	Triton X-100	170 000	Chromatography	[216]
Rabbit Mammary	Zwittergent	320 000	Chromatography	[217]
Rat Liver	Triton X-100	99 800	Chromatography	[218]
Mouse Liver	CHAPS	37 000	SDS-PAGE	[219]
Mouse Liver	CHAPS	37 000	Chromatography	[220]
Rabbit Mammary	Triton X-100	35 000	SDS-PAGE	[221]
Frog Tail Fin	Triton X-100	47 100	Hydrodynamic	[222]
Rat Liver	CHAPS	340 000	Chromatography	[223]
Rat Liver	Triton X-100	73 000	Hydrodynamic	[224]
Rabbit Mammary	CHAPS	42 000	SDS-PAGE	[225]
Rabbit Mammary	CHAPS	32 000	SDS-PAGE	[226]
Mouse Liver	CHAPS	37 000	SDS-PAGE	[227]
Human Placenta	CHAPS	37 000	SDS-PAGE	
Rabbit Mammary	CHAPS (5mM)	74 000	Chromatography	[228]
	(7.5mM)	36 800	and Hydrodynamic	
Rat Ovary	Triton X-100	88 000	SDS-PAGE	[229]
		41 000	SDS-PAGE	
		180 000	Chromatography	
		300 000	Chromatography	
Rat Liver	Triton X-100	92 000	Hydrodynamic	[230]
		87 000	SDS-PAGE	
		40 000	SDS-PAGE	
		35 000	SDS-PAGE	
Pig Mammary	CHAPS	45 000	SDS-PAGE	[231]
Rat Ovary	Triton X-100	80 000	SDS-PAGE	[232]
		40 000		
		34 000		

Cloning of Prolactin Receptors

From cDNA cloning experiments it is now known that there are several different sizes of prolactin receptor, which vary according to the species and the tissue examined. The prolactin receptor has now been cloned from the rat liver,[206] ovary,[233,234] and the Nb2 lymphoma,[235] rabbit,[236] mouse,[237] human,[238] and from the cow (Krivi G ;in press). These studies show that there are two main forms of prolactin receptor: long and short form (refer to Figure 1). The short form (291 amino acids) first cloned from the rat liver, has an extracellular region of 210 amino acids, a transmembrane sequence of 24 amino acids and a short intracellular domain of 57 amino acids. The long form in the rat, cloned from the ovary (591 amino acids) has the same extracellular domain with a longer intracellular sequence of 357 amino acids.

When the expression of the prolactin receptor gene is examined, a number of mRNAs of varying sizes are found. The number of transcript sizes varies with the species and tissue studied. In the rat, a short form specific probe hybridizes almost entirely to a transcript of 1.8 kb, whereas the long form specific probe hybridizes to three transcripts of 2.5 ,3.0, and 5.5 kb.[233] In the liver the majority of the mRNA is the 1.8 kb size, and in the ovary, which contains mostly long form, the three longer bands are prevalent. Transcripts were observed in female liver, prostate, kidney, ovary, adrenal and mammary gland. One other shorter size band of 0.6 kb appears in the testis, albeit the significance of this particular band is puzzling, since this size is too short to code for the full length protein.[214] These different sized mRNAs result from alternative splicing of one primary transcript. Analysis of the structure of the receptor gene revealed the existence of one gene composed of at least 11 exons which spans more that 70 kb of DNA.[239] The difference between the long and short forms is in the last two exons: exon 11 encodes the short form, and exon 10 encodes the long form. In the rabbit four transcripts have been observed, three major transcripts of 2.7, 3.4, and 10.5, and a minor form of 6.2 kb. These bands were present and expressed in the same ratios in all tissues tested. No short form of the receptor was found in the rabbit. All four bands encoded the long form of the receptor with differences existing only in the 3' and 5' non-coding regions.[240] Transcripts were identified in adrenal, mammary gland, ovary, jejunum,

Figure 1. Prolactin Receptor Forms

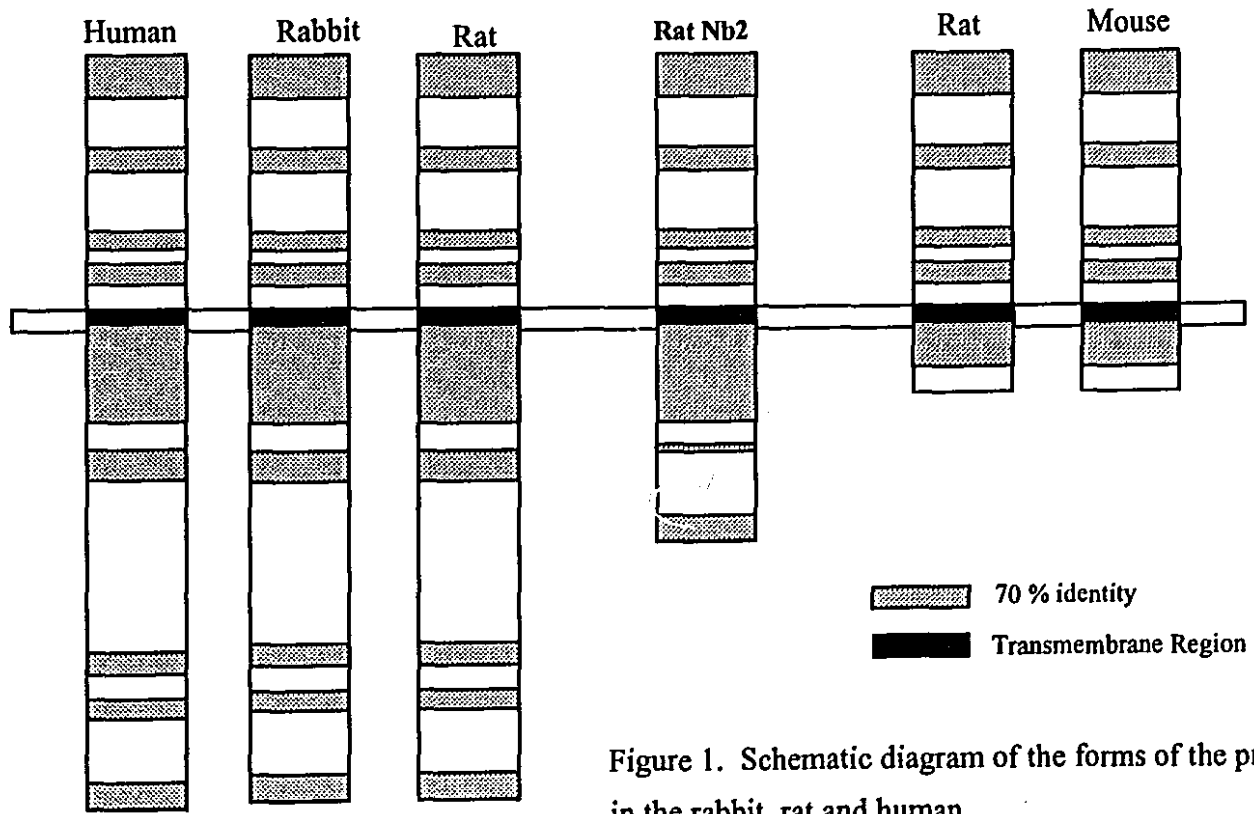


Figure 1. Schematic diagram of the forms of the prolactin receptor in the rabbit, rat and human

uterus, liver, kidney, pancreas, testis and seminal vesicle. In the human, transcripts sizes of 2.5, 3.0, and 7.2 have been reported in the T-47D breast cancer cell line and in chorion laeve.[214] In the mouse, only the short form of the receptor has been cloned, though recently larger transcript sizes which may correspond to a longer form have been reported to exist in mammary gland (M. Edery, P.A Kelly: personal communication). Transcripts were also identified in liver, ovary, mammary gland, and intestine.[241]

In the species examined to date, the short form has been found only in the rat and mouse, where it is the most abundant transcript.[242] It is the long form that has been cloned in the rabbit, cow and the human. It is possible to speculate that there is a connection between the existence of the short form in rats and mice and the large number of liver receptors in these two species since in other species which do not have the short form of receptor, the abundance of prolactin receptors in the liver is lower.

The prolactin receptor in the rat ovary was reported to have a consensus sequence for an ATP/GTP binding site.[234] This could imply endogenous kinase activity for the prolactin receptor. However, careful analysis of this sequence by Southern blotting, PCR, and S1 nuclease analysis failed to detect the sequences required for such a site,[243] confirming the sequence published in the first report for the rat long form sequence.[233]

When PCR analysis was performed on reverse transcribed RNA obtained from the prolactin dependent Nb2 rat lymphoma cell line, a single band was seen that was neither the short nor the long form. Sequencing of clones obtained from an Nb2 cDNA library revealed a protein of 393 amino acids, which appeared to be due to a deletion of 594 bp in the intracellular domain of the long form. Therefore, the Nb2 cell line possesses a unique version of the prolactin receptor. Could this mutation in the intracellular domain explain the higher affinity of the receptor seen in this line? The observation that the Nb2 prolactin receptor is actually a mutant receptor could question the meaningfulness of its use as a model system for the function of prolactin in the immune system.

The prolactin/growth hormone receptor family. The comparison of the sequence of the prolactin receptor with other sequences known at that time suggested that the prolactin receptor was closely related to the growth hormone receptor.[214] Both receptors have two regions of similarity in the extracellular domain, each with a pair of cysteines. These two regions probably play a crucial role in the conformation of the binding site of the receptor. When these cysteines are replaced by serine the resulting protein looses its

ability to bind the ligand.[244] Ellman analysis of proteolytic fragments of the growth hormone receptor determined that the cysteines within these two regions formed disulfide bridges.[245] The other regions of similarity, both extracellular and intracellular, shared in the prolactin receptor of different species, are present in the growth hormone receptor as well (see Fig. 1.). A great deal of work has gone into the identification of the amino acids involved in hormone receptor interactions that is beyond the scope of this review.[211,212,246,247,248,249] Despite the similarity of the growth hormone receptor to the prolactin receptor, no membrane bound growth hormone receptor short form has been identified. However, a growth hormone binding protein that is produced from an alternately spliced exon, that replaces the transmembrane and intracellular domains has been identified.[250] Thus short and long forms of both prolactin and growth hormone receptors exist, one being a cellular receptor, the other, a circulating binding protein.

The presence of the two forms of prolactin receptor explains to some degree the variation in molecular weights observed during the purification of the receptor protein. Glycosylation is another factor which contributes to receptor apparent molecular mass. Mutational analysis of the short form of the rat prolactin receptor indicates that each of the three potential glycosylation sites Asn^{35}, Asn^{80}, and Asn^{108} are glycosylated, the three together contributing 8 000 of the 42 000 Da molecular mass of the receptor.[244] The extent to which glycosylation contributes to receptor size seems to vary with the from of receptor, or the tissue in which the receptor is expressed. For example purification of the rat ovary prolactin receptor gave a binding subunit with a molecular weight of 80 000 in the presence of reducing agents[232] (refer to Table 2). This protein is probably the translation product of the long form of the receptor, which codes for a 68 000 Da molecular mass protein. Thus, the portion of this receptor that is carbohydrate is $\cong$ 12 000 Da.

Mechanism of Action

Contrary to hopes at the time, the sequence information obtained for the prolactin receptor provided little new information about how the receptor transduces its signal to the intracellular region of the cell. The receptor was clearly not a member of the tyrosine kinase family of receptors, nor of the seven transmembrane domain family of G-protein linked receptors. The mechanism of prolactin receptor action is still unknown.

Like many other receptors, it appears that one of the first events to occur after prolactin binding is receptor dimerization. It may be that the sole role of prolactin in signal transduction is to cause the formation of receptor dimers. Antibodies to the receptor, which, because of their two antigen binding sites, cause dimerization, are able to stimulate milk gene expression in the mammary gland,[251] and mitogenesis in Nb2 cells.[252]

X-ray crystallographic data of human growth hormone in complex with the human growth hormone receptor extracellular domain show a receptor dimer surrounding one ligand molecule of hGH.[212] In this configuration, one of the receptors was in a slightly higher position compared to the other, and, if the extracellular structure can be applied to the full length receptor containing a transmembrane and intracellular domain, it would appear that dimerization causes one of the receptors to shift upward from the plane of the plasma membrane.[211] This change in position of one receptor in relation to the plasma membrane might be enough to transduce the binding signal.

As previously noted, once prolactin is bound, the ligand is internalized and degraded in lysosomes. This process seems to be unimportant for signal transduction in the mammary gland, since lysosomotropic agents do not interfere with milk protein induction by prolactin.[253] It is unlikely, therefore, that this degradation might release a factor which would act as a second messenger to convey an intracellular signal.

However, mitogenesis in the immune system might involve another pathway. In the T-helper lymphocyte clone L2 and in splenocytes, prolactin and prolactin receptor have been observed to translocate from the extracellular milieu into the nucleus. This was demonstrated with indirect immunofluorescence using antiserum to both prolactin and the prolactin receptor,[254] and with colloidal gold electron microscopy.[255] This is contrary to autoradiographic measurement of ^{125}I-PRL in T-47D cells which indicate very little prolactin near the nucleus.[256] The presence of prolactin in the nucleus seems to be involved in prolactin action in Nb2 cells since transfection of these cells with a prolactin construct containing a nuclear translocation sequence from the SV40 large T antigen (which directs the protein to the nucleus) stimulates cell growth.[257] Another report has suggested that prolactin interacts with a putative nuclear receptor in the rat liver to activate protein kinase C.[258]

In Nb2 cells, prolactin has been shown to involve activation of protein kinase C,[259,260,261,262] cellular protein phosphorylation[263,264] mobilization of calcium 254,[265] a $^{+}/H^{+}$ antiporter activity[266,267] phospholipid metabolism[268,269,270] and G proteins, where chemical crosslinking studies suggest an association between prolactin receptor and G proteins.[271,272,273] In addition ,prolactin has been shown to activate growth related genes such as c-myc[265],[274] c-fos,[275] hsp70 homologue,[276] β -actin ,[266] and IRF-1.[277]

Prolactin has also recently been shown to increase cytosolic free calcium in the liver.[278] In the pigeon crop sac, one of the proteins induced by prolactin is a member of the calpactin/lipocortin family.[279]

In the mammary gland, production of milk proteins is only partially regulated by cyclic nucleotides,[280,281] and prostaglandins.[282] There seems to be no evidence for a role of protein kinase C in milk protein regulation, as has been demonstrated in the Nb2 cells. From studies of growth hormone signalling,[283] protein kinase C can stimulate ornithine decarboxylase activity in mammary gland explants but not milk protein expression,[284] and phorbol esters or inhibitors of protein kinase C have no effect on casein synthesis.[285]

Considering the diversity of prolactin's actions, it is possible that there is more than one pathway by which prolactin binding is signalled to the cell interior. Another aspect of prolactin physiology which complicates the search for second messengers is prolactin's usual requirement for the coordinated signaling of several hormones at once. The effects of cyclic nucleotides or prostaglandins upon milk proteins, for instance, may be important, but their effects probably are due to the influence of other hormones.

Functional Assays

In order to identify pathways which might be involved in prolactin signal transduction, a good *in vitro* system is necessary. Efforts to establish functional assays from mammary tissue have been largely unsuccessful. This may be due to the requirement of extracellular matrix and a complex hormonal environment for a normal response in these cells to be observed. Mammary tumour cells, although less fastidious, rarely produce milk proteins in measurable quantities. (see in vitro studies of breast cancer). Therefore, some engineering has been necessary to obtain a functional system: prolactin receptor cDNA and a fusion gene containing the regulatory sequences of a

responsive gene linked to a reporter gene were co-transfected into host cells. This has been successful in transfections of the β-lactoglobulin promoter coupled to chloramphenicol acetyltransferase (CAT) in Chinese hamster ovary cells. In serum free media prolactin can sinduce a 12-20 fold induction of CAT activity.[286] In the presence of prolactin, the long form of the receptor can give an induction of 17 fold, yet the short form of the receptor has no activity in this assay. Therefore, it is probably the long-form of receptor which transduces the lactogenic signal in the mammary gland. In another functional system in CHO cells, using cotransfections of the rat β-casein promoter linked to CAT, the natural deletion mutant found in Nb2 cells was able to induce CAT activity.[287] It appears that the 198 amino acids that are missing from the Nb2 receptor are not absolutely required for receptor signalling, at least in the case of mitogenesis and milk protein regulation. The function of the short form of prolactin receptor remains unknown. It could form heterodimers with the long form of receptor in some cells and promote activity, or alternatively, act to block activity by competing with the long form for prolactin binding.

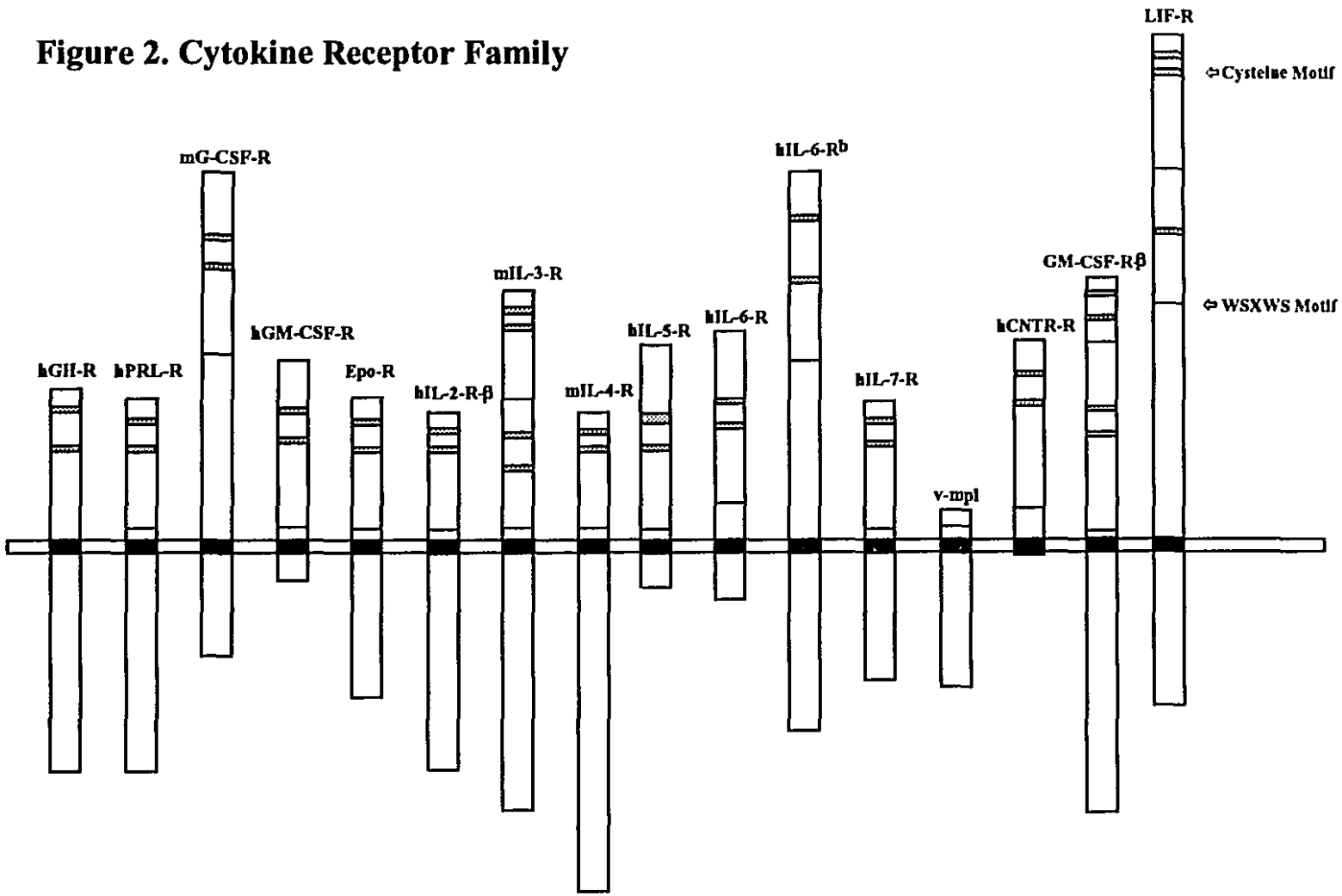
Mutational analysis of the receptor has been carried out using the β-lactoglobulin system. When the intracellular domain, including the transmembrane anchor, were transfected there was no binding of prolactin to these cells and no induction of CAT activity. Transfections with the sequence for the extracellular domain alone, produced a soluble binding protein with a 10-fold higher affinity, but also failed to induce CAT activity. However when the two constructs were transfected together, CAT was induced to a similar extent as the wild-type long form.[288] These experiments indicate that both the extracellular and the intracellular domains of the receptor are necessary for signal transduction. Yet these experiment pose a question: how is it that normal signal transduction can occur despite the uncoupling of the extracellular and intracellular domains?

The Prolactin/Growth Hormone Cytokine Receptor Family

Similar experiments, where the truncated soluble binding domain was able to transmit ligand signal to an intracellular end response, have been performed with the interleukin-6 receptor (IL-6-R).[289] Activity of this receptor was shown to occur by association with another membrane protein via an interaction in the extracellular

domain. Cloning of this associated protein, and subsequent transfection experiments have established that the associated protein, known as gp130, converts the interleukin-6 receptor from a low affinity to a high affinity state, and that it is gp130 itself which transduces IL-6 binding into an intracellular signal.[290] Interestingly, gp130 has regions which are similar to the prolactin and growth hormone receptors. Indeed, many of the cytokine receptors have been shown to contain these common regions.[291] These include the receptors for granulocyte colony-stimulating factor (G-CSF-R),[292] erythropoietin (Epo-R),[293] granulocyte macrophage-colony stimulating factor (GM-CSF-R),[294] the p75 or β-chain of interleukin-2, (IL-2)[295] IL-3-R,[296] IL-4-R,[297] IL-5,[298] IL-6,[299] IL-7,[300]the IL-6-R associated protein gp130 (IL-6-Rb),[279] the GM-CSF-R associated protein (GM-CSF-Rb),[301] ciliary neurotrophic factor receptor (CNTF-R),[302] and leukemia inhibitory factor receptor (LIF-R).[303] (see Figure 2 for a schematic diagram of the cytokine/prolactin/growth hormone receptor family). The arrows in Figure 2 indicate two common motifs found in the family: the two cysteine repeats and the WSXWS sequence. One other molecule which is not a receptor contains the cysteine regions and the WSXWS motif: the p40 subunit of natural killer cell stimulatory factor (NKSF).[304] This factor could be a natural example of the experimental signaling of the extracellular soluble domain of the IL-6-R through the gp130 subunit.[290] All of the receptors in the family share the two cysteine regions near the N terminal of the extracellular domain; these cysteines form disulfide bridges in the growth hormone receptor molecule, so this is probably the case for all the family members. The WSXWS motif, usually found near the transmembrane region, is common to all family members except the growth hormone receptor (YGEFS). The function of the WSXWS motif is still unknown, although it is tempting to speculate that it might be important in binding of the α and β forms of receptors. Point substitutions by alanine or replacement of the prolactinreceptor WSXWS motif with the growth hormone homologue results in a reduction in the binding affinity of this mutant receptor.[305] Similar studies on the IL-2 receptor indicate that high affinity binding and signal transduction requires the presence of this motif in the β-subunit.[306] Perhaps the prolactin receptor associates with another subunit to produce a high affinity state, in a way similar to the IL-2-R,[295] IL-3-R,[296] IL-5-R,[298] IL-6-R,[288] and GM-CSF-R.[294] This possibility is consistent with the crystal structure of

Figure 2. Cytokine Receptor Family



the human growth hormone receptor-ligand complex which indicates that the WSXWS motif lies on the outside surface, away from the ligand binding and receptor dimerization surfaces.[211] Despite the number of receptors which belong to this family, only the LIF-R and gp130 share common sequences in the cytoplasmic domain, the rest all differ. Could it be that each receptor has a unique mechanism for transduction, or is it more likely that signalling passes through a gp130-like associated protein?

Receptor Regulation

Just as prolactin action usually depends upon the co-ordinated action of several hormones, the regulation of the receptor is a complex interplay of many hormonal signals. The review of the regulation of prolactin binding by Shiu[307] illustrates this situation. In general, receptor regulation depends upon the species, the sex, the tissue, and the physiological stage. For instance, estrogen induces and testosterone inhibits binding in rat liver, but in the testis, kidney and adrenal, the opposite applies (refer to Table 3). As was true in the past, most of these studies have been performed *in vivo* so that whether hormonal manipulation leads to direct or indirect effects is unclear.

One example of this, is the effect of gonadotropins on PRL-R regulation. In cycling hamsters, the proestrous gonadotropin surge was accompanied by a loss of prolactin binding in both the interstitium, the follicular thecae, and granulosa cells.[308] If the gonadotropin surge was blocked by phenobarbitol, prolactin binding did not drop. In rat mammary gland, the contrary was true: prolactin binding was highest during estrous,,,[309] yet, in the rat liver, receptor number remained constant.[310] In the rat testis, FSH and LH can increase prolactin binding, but a gonadotropin releasing hormone agonist reduces prolactin binding.[311] Photoperiod regulates receptor number in the hamster testis, with short day long nights causing a marked reduction in prolactin receptors.[312] In the Syrian hamster, gonadotropins have been shown to be involved in this decline, but in the Djungarian hamster, no such effect could be demonstrated.[313]The use of in vitro models may not alleviate the situation, for factors such as the state of differentiation of the cell model, the influence of extracellular matrix, and the possibility that responses to test hormones might differ in the presence of other hormones make in vitro studies cumbersome.

Table 3. Prolactin Receptor Regulation

Treatment	Species	Tissue	Effect	Method	Reference
Estrogen	Rat	Liver	↑	RNA Blotting	314
	Rat		↑	Binding	
	Rat	Liver	↑	Binding	315,316,317
	Rat	Liver	↑		318
(Tamoxifen)	Rat	Liver	↓		
		Mammary	≅	Binding	319
	Mouse	Prostate	↓	Binding	320
		Testis	↓		
	Human (*in vitro* breast Ca)	EFM-19	↑	Binding	321
	Pig	Granulosa	↑	Binding	322,323
Progesterone	Rat	Liver	↓	Binding	324,325
	Rat	Mammary	↓	Binding	326
	Pig	Granulosa	↑	Binding	323
	Human (*in vitro* breast Ca)	EFM-19	↓	Binding	321
	Human (*in vitro* breast Ca)	T-47D	↑	Binding	327,328,329
Testosterone	Rat	Liver	↓	Binding	330,315,316,317
	Pig	Granulosa	↑	Binding	323
	Human (*in vitro* breast Ca)	EFM-19	↑	Binding	
Cortisol	Rat	Liver	↓	Binding	331
	Rat	Kidney	↓	Binding	332
	Rat	*Adrenals*	↓	Binding	
Diet: Glucose	Rat	Liver	↓	Binding	333
Fat, Protein			≅		
Glucagon			↑		
		Mammary	≅		
Thyroid . (hypo)	Rat	Liver	↓	Binding	334
. (hypo)	Rat	Liver	↓	Binding	335
(hyper)			↑		
(hypo)	Rat	Prostate	↑	Binding	117
		Adrenal	↑		
		Kidney	↓		
		Testis	↓		
(hypo)	Rat	Mammary	↓	Binding	336
(hyper)			↑		
Growth Hormone	Rat	Liver	↑	Binding	337,338,339
	Rat	Liver	↑	RNA Blotting	340
			↑	Binding	
	Rat *In Vitro*	Hepatocytes	↑	Binding	341
	Rat	Liver	↑	RNA Blotting	342
Prolactin	Rat	Liver	↑	Binding	315
	Rat	Liver	≅	Binding	337,338,339
	Rat	Liver	↑	Binding	343,344
	Rat *In Vitro*	Hepatocytes	↑	Binding	341
bromocryptine	Rat	Liver	↓	Binding	319

... bromocryptine	...	Hamster	..	Testis	↓	Binding		98
....	....	....	....	Adrenal	≅			
....	....	....	....	Liver	≅			
... bromocryptine+PRL	..			Testis	↑			
....	....	....	....	Adrenal	≅			
....	....	....	....	Liver	↑			
....	....	....	...	Hamster	↑			312
....	....	....	...	Tilapia	↑	Binding		106
....	....	....	...	Rabbit	↑	Binding		326
Somatostatin	.....	...	Rat	Liver	↓	Binding		345
....	....	....	...	Rat Tumor	↓	Binding		346
LH or FSH	...	Hamster	..	Testis	↑	Binding		312
hCG	..	....	...	Rat	↑	Binding		347
LHRH agonist	....	...	Rat	Testis	↓	Binding		311
FSH	..	....	...	Rat	↑	Binding		301
LHRH	....	...	Rat Tumor	Prostate	↓	Binding		187
PMA	.....	...	Rabbit	Mammary	≅	Binding		348
....	....	....	...	Mouse	↓	Binding		349
....	....	....	...	Rat	↓	Binding		350

In general, prolactin receptor binding and RNA levels in the liver are increased by estrogen. This effect is sex specific, for even if estrogen is given to male rats the response to estrogen is less than in females.[317] This effect is established neonatally, for if newborn male rats are castrated, estrogen injection in the adult resembles the female response, whereas if testosterone is administered during the first three days to castrated newborns, the male pattern is maintained. Just as prolactin function differs with the tissue, the regulation of the receptor appears to depend on the tissue: tamoxifen treatment in rats reduced liver receptors numbers but had no effect on the number of mammary gland receptors.[315]

Prolonged treatment of rats with high doses of medroxyprogesterone acetate (MPA) reduced prolactin binding in rat liver. However, this was probably due to the lowered levels of estrogen and prolactin in these animals.[324] If estrogen replacement was given to these animals, prolactin receptor levels were not decreased, but increased due to the estrogen.[325] In the mammary gland, progesterone was shown to antagonize the induction of prolactin receptors by prolactin itself. However, no direct effect of progesterone on prolactin binding was shown.[326] In the T-47D breast cancer cell line, progesterone has been shown to increase prolactin receptor binding, however in the EFM-19 cell line progesterone decreased binding. (refer to Table 3)

Diet also seems to regulate liver prolactin receptors: glucose feeding for three days caused a 57% reduction in hepatic prolactin binding, but no change in bGH binding. When a dose of glucagon which did not alter serum glycemia was administered to these animals, prolactin binding was higher than in animals with glucose alone. It is possible that binding is lower in glucose fed rats, since glucose lowers serum glucagon levels.[333]

Regulation at the RNA Level. Analysis of mRNA levels for the prolactin receptor have been performed in the rat. In rat liver the developmental expression of prolactin receptor was studied. Prolactin receptor mRNA was undetectable in fetuses and neonates. On day 21 mRNA levels start to rise until day 40, when they plateau. The expression of the receptor parallelled mRNA levels, except that receptor binding continued to rise to over twice the day 40 levels, even when RNA levels remained constant. This same difference between RNA and receptor binding was observed in

animals stimulated by estrogen. In adult female rats, mRNA levels rose 3-fold on day 3 and then were stable, however receptor binding continued to rise up to at least day 7.[314]

In immune cells, prolactin receptor mRNA steady state levels are increased 2 fold in cells stimulated with IL-2 or concanavalin-A.[254] This contrasts with the 7 fold induction of prolactin receptor protein measured by flow cytofluorometry. The cytofluorometric data suggests that the increase in prolactin receptor levels is associated with cell activation and growth.

In rat mammary gland, both the long and the short form of the receptor are found. mRNA levels are quite low in virgin females and remain so until the end of pregnancy, when levels increase.[242] Surprisingly, there is a much greater induction of the short form than of the long form, with the short form comprising 70% of the mRNA on day 5 of lactation. From day five onward short form mRNA declines, but the long form continues to increase until day 15, when it too starts to decline. In the liver, the pattern is different. In this tissue, the short form accounts for 85% of receptor mRNA and mRNA levels start to increase half-way through pregnancy, reaching a maximum near birth. During lactation PRL-R mRNA falls to levels lower than that found in virgin animals. Receptor binding in the mammary gland displays the same pattern as in the liver. Even though mRNA levels peaked at day 15 and were declining by day 20, the binding levels continued to increase. There are several questions which arise from these studies. How is it that steady state levels of short and long forms have different pattern of regulation? Why do receptor protein levels increase even when steady state mRNA levels are constant or declining? The responses are probably related to differing transcriptional regulation, to independently regulated mRNA stability, and to some form of translational regulation.

Prolactin in Breast Cancer

Breast cancer is unusual in that the development of breast cancers and their cure may rely on hormonal signals. The current view is that oncogenesis develops from mutations in the genes involved in the regulation of growth. Carcinogenic mutations can occur in genes involved in the mechanics of mitogenesis, genes involved in mitogenic signal

transduction, and genes which act as inhibitors of growth. Estrogen is the primary hormonal cause of breast cancer developement and growth. The preferred method of treatment for breast cancer after surgery involves antiestrogens. Antiestrogen therapy is successful because approximately 60% of breast cancers retain their dependence upon environmental growth signals (estrogen) and respond to such therapy.[351] Antiestrogen therapy is also widely used because it has a low toxicity. The success of the therapy has stimulated a search for other hormonal factors which might play a role in breast cancer growth. Since prolactin is required for normal mammary gland function, its role in tumor development has received attention.

Animal Models

Experimental breast cancer models in rats clearly indicate that prolactin is the major factor implicated in the induction and maintenance of tumors. Rats that receive pituitary grafts (which secrete large quantities of prolactin) have a higher incidence of mammary tumor formation than control animals.[352] The growth of spontaneous tumors in rat is inhibited by treatment with bromocryptine.[353] Mammary gland tumors induced by the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) are also dependent upon prolactin. Pituitary grafts[354] and drugs which increase prolactin levels increase tumor growth.[355,356] Hypophysectomy and ergot drugs (like bromocryptine) reduce the number and size of these tumors.[357,358,359] The growth of such tumors can be reinstated in hypophysectomized animals by reinjection with prolactin. It has also been observed that there is a direct correlation between serum prolactin levels and the incidence of carcinogen induced tumors in various strains of rats.[360] It may be that in carcinogen induced tumors the prolactin receptor plays a role: the level of prolactin receptor in DMBA induced tumors is threefold higher than in normal lactating mammary glands.[361] This may be due to differences in the processing of the hormone receptor complex after internalization.[362]

Clinical Studies

Hyperprolactinemia: The observations made in rat mammary gland cancer stimulated the study of the role of prolactin in human breast cancer. If prolactin was a factor in the progression of normal breast tissue toward a cancer state, it is likely that higher serum

prolactin levels would be an indicator of risk. However, a clear relationship between circulating prolactin levels and breast cancer incidence has not been observed.[363,364,365]

High serum prolactin is more likely to cause galactorrhea. One study reported the presence of breast lumps in five patients with hyperprolactinemia. The breast tissue was benign dysplasia showing secretory vesicles in the epithelial layer. In two cases the foci resembled lactating breast tissue.[366]

However, it has been observed that patients with high serum prolactin levels tend not to respond as well to hormonal therapy and have a reduced survival time.[367,368,369] The same observation has been made in patients treated with chemotherapy.[370] In metastatic breast cancer, there is a significant elevation in serum prolactin levels compared with non-metastasized disease. Hyperprolactinemia was associated with 88% of cases of progressive disease. In patients who experienced several cycles of remission and relapse, prolactin levels increased with each relapse. Prolactin levels returned to normal after remission in chemotherapy treated patients.[371] There is a significant correlation between prolactin blood levels and estrogen and progesterone receptors in these patients. Patients with estrogen and progesterone receptor negative tumors had higher circulating levels of prolactin.[372] Another group found this to be true only between serum prolactin and estrogen receptors.[373] Clearly, prolactin blood levels can serve as a prognostic indicator or response and survival in previously identified breast cancer patients.[374,375]

The significance of prolactin's role, however, is unclear. Are elevated prolactin levels a causal factor in disease progression, or are high prolactin levels a response to other factors? For example, mastecomy causes an increase in serum prolactin that lasts several months.[376,377] The degree of prolactin elevation has been positively correlated to tumor size.[378] There are means by which breast physiology may influence prolactin secretion, for example though nipple stimulation (see Biological Activities: Reproduction and Lactation). It can be speculated that metastasizing breast cancer perturbs breast physiology in a way which stimulates prolactin release. However, whatever these findings mean to the physiology of prolactin and breast cancer, the relationship is clear: that the least favourable prognosis is associated with the highest levels of circulating prolactin.[379]

Prolactin Receptors in Breast Cancer: Prolactin receptors have been measured in breast cancer tumors.[380,381,382,383,384,385,386] When the amount of prolactin binding was compared with estrogen receptor and progesterone receptor, more tumors contained prolactin receptors than steroid receptors,[387,388] though this was not the case in all studies.[394] Studies on the levels of prolactin receptor in tumors showed that tumors with the highest prolactin binding tended to have the lowest steroid receptor binding.[389] This contrasts with results obtained by others who found that, although prolactin binding did not correlate with serum levels of prolactin, estrogen or progesterone, there was a positive correlation between prolactin and steroid receptor levels.[390,391,392,388] However ,a ten year study with 308 patients found no correlation between prolactin receptors and estrogen or progesterone receptors.[393]

This same study found that prolactin receptors had no independent prognostic significance when tested by Cox analysis. Also, prolactin receptors were not a prognostic indicator of response to tamoxifen treatment. Desaturation of endogenous ligand from tumor prolactin receptors showed that, while only 43% of tumors had positive free receptors, 72% of tumors were receptor positive after receptor desaturation.[394] In this study, neither free nor total prolactin receptors correlated with overall survival. However, prolactin receptors (total) correlated with improved relapse free survival in patients with positive lymph nodes, although, when tested by Cox analysis neither free receptors nor total receptors had independent prognostic significance.

It has been reported that prolactin receptors are more frequently detected in stage three breast cancer compared to stage one, or two[395]. In this study, recurrence free survival time was not related to prolactin receptor, but prolactin receptor positive patients had a poorer overall survival.

Hormone Therapy: Many clinical trials have been performed to examine whether serum prolactin suppressing drugs could improve prognosis in breast cancer. There has been little success. For a review of this work refer to Nagasawa.[363] More recent work has shown that bromocryptine treatment has no effect on tumor prolactin binding, but significantly increases estrogen receptor levels in the premenopausal subgroup.[396] A study which tested for differences in response between tamoxifen treatment and

tamoxifen plus bromocryptine treatment, found no differences in response rate or overall survival.[388] If bromocryptine was given five days prior to and three to ten days after surgery, there was a significant reduction in prolactin levels and in the S-phase fraction of tumour cells. It was thought that this might be important in controlling the proliferation of micrometastatic cells shed during the time of surgery.[397] A case report of a patient with advanced breast cancer, chemotherapy resistance, and hyperprolactinemia who went into remission following bromocryptine treatment stimulated a follow-up study with 18 patients with similar disease. Patients were given bromocryptine as well as continued chemotherapy. One patient had a partial remission which was thought to be due to a delayed response to chemotherapy and not to suppressed prolactin levels. It was concluded that prolactin did not serve to restore tumor sensitivity to chemotherapy.[398] Another study that examined the effect of bromocryptine in hyperprolactinemic patients with metastatic disease also showed no response to bromocryptine.[399]

One critique of past work is that these studies failed to take into account that human growth hormone is also lactogenic and therefore growth hormone levels must also be controlled. In a trial of ten patients with advanced disease, when treated with both bromocryptine and somatostatin analogue to reduce both prolactin and growth hormone levels, one patient experienced disease stabilization consisting of less than 50% regression of skin nodules and pleural effusion, a decline in CEA titer, and an improved performance status lasting 7 months.[400] Another possible confounding factor is the observation that the hormones estradiol, progesterone, and prolactin in breast fluid may be concentrated up to a hundred fold greater than serum levels.[401,402]

Normal Breast Development

The development of the breast can be divided into 5 stages.[403] To begin with, surface epithelial cells descend into the mesenchyme, differentiate to form the nipple, and a rudimentary set of duct forms, which are composed of two cell types: an epithelial layer, and a stromal layer. These structures are formed during fetal development and remain static until puberty, when a second round of growth and differentiation occurs. A final round of growth and differentiation takes place during pregnancy. Breast growth and development, both in puberty and pregnancy, is under hormonal control. Breast growth

in puberty is closely linked to the rise in serum estradiol which is triggered by changes in the pattern of GnRH pulsatile secretion of the pituitary.[404,405]

Breast growth during puberty occurs mostly in the stromal connective and adipose tissues. In the post pubertal breast, epithelial tissue comprises at most only 20% of breast volume[406] At the end of puberty, the development of stromal elements is complete, but there is extensive growth and differentiation of glandular epithelial tissue starting in the second trimester of pregnancy. This is due, again, to the action of estrogen (mitogenesis) and the effect of progesterone (differentiation).[407,408] Histological studies of nulliparous and involuted parous breast tissue reveal a much higher proportion of two-layered epithelium in nulliparous breast, and it has been proposed that the underlaying second layer may consist of stem cells which would grow in response to the hormonal conditions surrounding pregnancy.[406]It is thought that these cells, due to their latent mitogenic potential may be the cell type from which breast cancers are likely to arise.

The role of prolactin in breast growth is unclear. Increases in serum prolactin occurs only late in puberty, after the start of breast growth.[409] In cases of macromastia in boys, prolactin levels were normal.[410] Pituitary hormones have a direct role in breast development, since, in girls with gonadal dysgenesis, estrogen treatment can produce normal breast development when hypothalamo-hypophyseal function is normal, but, in cases of gonadotropin deficiency estrogen therapy is insufficient.[411] This pituitary factor is probably not prolactin. In primates, bromocryptine suppression of prolactin to undetectable levels in the serum had no effect on mammary gland development in *Macaca nemestrina* or *Macaca mulatta*.[412] Some pituitary factor is important, however, since hypophysectomy interferes with gland development, even in estrogen replaced animals. However, lactogen replacement in these animals with hGH made no difference in breast development in estrogen treated animals. When estrogen was given with an extract of human pituitary in hypophysectomized animals, normal development was seen.[413]

In Vitro Studies

Prolactin Receptors On Cultured Breast Cancer Cells: Prolactin receptors were first documented in the T-47D, MCF-7, BT-474, MDA-MB-231, Hs578T and HBL-100 cell

lines. Receptor levels were three times higher in the T-47D line than the next highest prolactin receptor expressing cell line, MCF-7.[414] Histochemical analysis of prolactin binding sites on T-47D cells determined that prolactin receptor number was heterogeneous: there was a range of staining intensity with some cells showing no staining at all. Similar results were seen by autoradiography. After a one hour incubation with prolactin, most grains were observed near the cytoplasm and very few in the nucleus.[256] Studies of the specificity of binding in T47-D and MCF-7 cells showed that hGH bound slightly better than hPRL, with placental lactogen and rat prolactin affinities being an order of magnitude lower.[415]

When binding studies were performed on a number of breast cancer cell lines, prolactin receptors were observed in the MCF-7, R27, R98, T-47D, ZR-75-1, BT-474, MDA-MB-361, and MDA-MB-134 cell lines but not in the MDA-MB-157, MDA-MB-330, MDA-MB-231, BT-20, Hs578T, HBL-100 cell lines.[390] This study found a significant positive correlation between estrogen receptors and prolactin receptor in these cell lines. In T-47D cells, prolactin receptor levels were increased by progesterone,[416,417] and this effect could be blocked by the antiprogestin RU486.[418] Human growth hormone causes a down regulation of prolactin binding, but only at concentrations of 500 ng/mL or more . This is in disagreement with previous work.[415].

More recently the EFM-19,[419] and VHB-1[420] cells lines have been developed and have been shown to contain prolactin receptors.

Cell Growth and Other Responses

Several studies have examined the regulation of growth in normal human mammary epithelium *in vitro*. Estrogen is mitogenic in normal human mammary epithelial cells, and this can be blocked by anti-estrogens.[421] Estrogens had effects on cell morphology in normal cells in a way similar to tumor cells, however estrogen did not stimulate the secretion of p52 (a factor thought to be involved in metastasis) in normal cells.[422] Linoleic acid is mitogenic when co-cultured with EGF and insulin. This effect was probably mediated via prostaglandin synthesis, since indomethacin was able to abolish this effect. Linoleate had no effect in co-cultures of EGF, insulin and cortisol. Normal mammary cells were found to produce a growth inhibiting factor that was capable of inhibiting breast cancer cell growth. Organ cultures of normal breast

examined for α-lactalbumin production responded to prolactin. One biopsy from pregnant breast produced 200 times more than normal breast. Of ten breast tumors, six produced α-lactalbumin but none responded to prolactin.[424]

Numerous studies support the concept that breast cancer cells loose their responsiveness to prolactin for milk protein production.[424] When α–lactalbumin levels are measured in normal or tumor tissue from the breast, 80% of tumors contained less α-lactalbumin than normal tissue from the same subject.[425] In the breast cancer cell lines BT-20, BT-474, SW527, SW613, MCF-7, EVSA-T, and ZR-75-1, prolactin did not stimulate α-lactalbumin production.[417],[426] However, some tumors retain responsiveness. In 22 organ cultures of normal breast, α-lactalbumin production could not be induced, whereas in organ cultures of tumors, 3 out of 33 samples responded to prolactin. α-lactalbumin is more likely to be found in differentiated tumors such as lobular carcinoma.[427] No correlation could be found between serum α-lactalbumin levels and prolactin receptors in tumor samples,[428] or between α-lactalbumin staining in tumors measured by immunohistochemistry and serum prolactin levels.[429] As for lipid synthesis, in the cell line T-47D progesterone stimulated triglyceride synthesis and prolactin did not alter this action of progesterone.[430] In another study, prolactin did have a stimulatory effect on lipid drop accumulation in T-47D cells.[431]

Prolactin was found to stimulate the secretion of several proteins in T-47D cells. The major protein, known as prolactin inducible protein (PIP),[432] is identical to gross cystic disease fluid-15 (GCDFP-15)[433] and secretory actin binding protein (SABP),[434] and has been identified in breast fluid, saliva, in extracts of the submandibular gland, and seminal vesicles, in tumors of the breast, prostate, salivary gland, and sweat glands, and in amniotic fluid.[435,436] Studies have found that either thirty[437] or seventy-four[435] or ninety percent of tumors express this protein and that its expression is significantly positively correlated with estrogen receptors.[437,438,429] Another study found PIP/GCDFP-15 expression was restricted to estrogen receptor positive tumors and was correlated with the presence of progesterone receptors.[439] However, other reports find no correlation with estrogen receptors, but confirmed a positive correlation with progesterone and androgen receptor.[440] Others have reported a high number of positive

samples in breast cancers except in tumors without apocrine features in which only 23% of tumors were positive.[441] These authors found no correlation with estrogen receptors, but noted a positive correlation between PIP/GCDFP-15 and lymph node involvement. A study which compared tumor grade with immunohistochemical staining for PIP/GCDFP-15 showed a significant decrease in PIP/GCDFP-15 from grade 1 tumors to grade 3 tumors.[442] It was noted that tumors that responded to therapy tended to have high estrogen receptor and low PIP/GCDFP-15, thus suggesting that PIP/GCDFP-15 could serve as a marker for estrogen receptor positive tumors that don't respond to treatment. In a pilot study, a patient given four recurrent injections of baboon anti-PIP/GCDFP-15 antibody experienced partial regression of subcutaneous metastatic growths without toxicity.[443]

After it was discovered that PIP/GCDFP-15 was regulated by prolactin, it was also determined that hydrocortisone, and dihydrotestosterone,[444] stimulated PIP/GCDFP-15 expression in T-47D cells.[445] In the ZR-75-1 cell line, estrogen reduced the expression of PIP/GCDFP-15 and the progesterone agonist R5020 counteracted this effect.[446] PIP/GCDFP-15 levels have a negative relation to estrone glucuronide in breast cyst fluid.[447] PIP/GCDFP-15 also correlates positively with epidermal growth factor in breast cyst fluid.[448]

That elevated serum prolactin levels can serve as an indicator of poor prognosis suggests the possibility that prolactin might be involved in metastasis. In a study that examined whether prolactin effected basement membrane digestion by breast cancer lines, a serum factor was observed to inhibit digestion in the most active cell lines. No hormonal treatment, including hGH altered basement membrane digestion by T-47D cells.[449]

Just as a pituitary factor is necessary for normal or primate mammary gland development, similar results have come from experiments with human breast cancer cells transplanted into athymic nude mice. Tumors growth could be stimulated by estrogen, but co-transplantation with a prolactin and growth hormone secreting pituitary tumor enhanced the estrogen effect.[450,451] Yet purified prolactin or human growth hormone was not able to duplicate this effect in T-47D tumors.[452] Furthermore, conditioned media from this pituitary tumor stimulated the growth of several breast cancer cell lines in serum free cultures.[453] However, when the MCF-7 cell line was used

to make tumors in these animals, prolactin seemed to have a very slight effect, though significance was not reported.[454]

***Growth of Normal Mammary Epithelium In Vitro*:** Several studies have examined prolactin-induced growth in normal breast epithelial cultures. In primary monolayer cultures form normal breast and tumor tissue, prolactin reduced the cell population doubling time in both normal and tumor tissue, though more often in normal than in tumor tissue.[455] Another study of primary cultures of mammary epithelial cells in serum free medium found that, in the presence of hydrocortisone, insulin, epidermal growth factor, and cholera toxin (IHEC), a combination of progesterone and prolactin had no effect on cell growth, but, if either insulin or EGF was removed from the IHEC medium, the progesterone/prolactin combination did have an effect.[456] In explant cultures, insulin supported ^{3}H-thymidine incorporation and human prolactin was able to stimulate ^{3}H-thymidine incorporation on top of the insulin effect.[457] Another study of explant cultures that examined the effects of prolactin, hydroxycoritsone, progesterone, hCH, and insulin found that the combination of insulin and hydroxycortisone with the addition of prolactin was mitogenic.[458] One study used slices of normal tissue transplanted into athymic nude mice and examined growth by an autoradiographic analysis of labelling with ^{3}H-thymidine. Human placental lactogen (hPL) alone had no stimulatory effect, but treatments of hPL together with estrogen significantly augmented the growth response seen with estrogen alone.[459] It should be noted that in these experiments were done in tissue explants or primary cultures, and that most often prolactin was able to stimulate cell growth only in the presence of other factors.

Growth of Tumors In Vitro In six of thirty-eight (16%) tumor slice cultures, prolactin stimulated thymidine incorporation. Only a fraction of prolactin receptor positive tumors responded by DNA synthesis. No relation was found between this response and the presence of estrogen or progesterone receptors or the stage of disease of the patient.[460] These results confirm earlier work which showed that 15% of breast tumors incorporated ^{3}H-thymidine, when prolactin was added with insulin in tumor explant cultures.[461] An early study demonstrated that 32% of organ cultures had increased pentose-shunt activity after oPRL incubation, when compared to uncultured control tissue.[462] In organ cultures of benign tumors, hPL alone (5ug/mL) had mitogenic activity, whereas oPRL at a similar concentration had no effect.[463] Specificity for human

lactogen was also reported in a primary culture of a single tumor which responded to human prolactin or growth hormone, but not to ovine prolactin.[464] However, in the clonogenic assay, oPRL significantly stimulated colony formation in 12 out of 17 tumors.[465] Taken together, these studies suggest that prolactin can have a mitogenic action in a subset of breast tumors, especially in the presence of other hormones.

Established breast cancer cell lines have also been tested for growth stimulation by prolactin. Prolactin was found to stimulate the growth of the cell lines BT-20 and 734B.[455] In the CAMA-1 cell line, prolactin alone had no growth stimulating activity, but it had a significant potentiating effect on estrogen stimulated growth. Prolactin was found to stimulate cell growth in the EFM-19 cell line in the presence of thyroxine and insulin.[466] Seven out of ten cell lines developed in that lab also responded to prolactin.[467]

In serum free experiments with the MCF-7 cell line grown on extracellular matrix support, dexamethazone, insulin, thyroid hormone and prolactin together significantly inhibited growth.[468] Prolactin alone or in combination with other hormones was not tested. Another study of serum free conditions identified a cocktail of physiological concentrations of insulin, transferrin, EGF, prostaglandin $F_2\alpha$, and cold-insoluble globulin, that permitted MCF-7 cells to grow in a way identical to 10% fetal bovine serum.[469] The addition of prolactin to this culture had no effect. Another study also reported prolactin had no mitogenic effect on the MCF-7 cells.[470]On the other hand, when fetal bovine serum was extensively stripped of endogenous prolactin with dextran coated charcoal, human prolactin was able to elicit a response in the MCF-7 cells.[471]

Overall, these of the data suggest that prolactin is able to stimulate the growth of normal mammary tissue and of some breast cancer tumors under certain conditions. Prolactin is more likely to be mitogenic in experiments that use tissue explants, primary cultures, or at the least, early passages of established cultures. There is a tendency for established cell lines to evolve with passaging and sensitivity to prolactin may be a feature of breast cancer cell cultures which is lost. Specific conditions may be required to elicit prolactin response *in vitro,* since prolactin is often observed to function only in the presence of other hormones including estrogen, progesterone, thyroid hormone, insulin, and hydrocortisone. Unlike the case of Nb2 cells which have an absolute dependence on PRL for growth, no such cell line from normal or neoplastic breast tissue has been isolated. The literature provides a mixed review of the importance of prolactin

in breast cancer cell growth. There are reports of growth stimulation by prolactin in breast cancer, but this far from true in all cases. One wonders how many negative studies have gone unpublished.

Chapter 2. Prolactin Receptor: Protein Characterization, and Sequence Analysis

Materials and Methods

Studies were performed on the established cell line T-47D.[472] Earls modified eagles medium, fetal and newborn bovine serum, NUNC culture plastics, and restriction enzymes were obtained from Gibco/BRL Canada (Burlington ON). Standard laboratory chemicals were obtained from Fisher Scientific Montreal, PQ, or from BDH (Ville St. Laurent, P.Q.). Falcon culture ware was obtained from Fisher. Keyhole limpet hemocyanin, HEPES, EDTA, BSA, PMSF, Tris-HCl sodium metaperiodate, chloramine-T, sodium cyanoborohydride, benzamidine, leupeptin, control pore glass, Triton X-100, Triton X-114, Lubrol PX, digitonin, ABTS and estradiol were obtained from Sigma (St. Louis, Mo). Sephadex G-100, protein-A affigel-10, and benzamidine-sepharose 6B were obtained from Pharmacea (Baie D'Urfe, Quebec). DeoxyBIGCHAP, CHAPS, CHAPSO, Brij-35, and sulfoEGS, were obtained from Pierce (Prof. Diagnostics, Edmonton, AB). Pansorbin, and the Zwittergent series of detergents were obtained from Calbiochem, SanDiego CA. 96-well filter plates were obtained from Millipore (Mississauga, ON). Sodium ^{125}Iodide was obtained from ICN Chemicals, Mississauga, ON. Taq polymerase was purchased from Perkin Elmer (Canada), Rexdale ON, and reverse transcriptase was obtained from Life Sciences Inc., St Petersburg Fl. Ovine prolactin was obtained from the NIADDK and human growth hormone was a generous gift from Dr. H. Friesen (University of Manitoba).

Cell Culture

A subclone of the T-47D cell line containing the highest amount of prolactin binding sites was grown in gram quantities in Cell Factories (NUNC) for purification experiments. Cells were cultured in 2L EMEM with 5% fetal bovine serum, 5% newborn bovine serum, 10 mM HEPES, 100 pM estrogen, and 10 ng/mL ovine prolactin, and harvested with 5 mM EDTA/PBS. The cell pellets were weighed and frozen at -80°C.

Prolactin Binding

Hormone iodinations were perform using a modification of the Hunter- Greenwood[473] method. A low quantity of chloramine-T (500ng in 10 μL) was added to a solution containing hormone (5 μg in 10 μL ammonium bicarbonate 0.05M), sodium phosphate buffer (25 μL of a 3.0 M solution), and Na ^{125}I (750 μCi in 15μL H_2O). The reaction was allowed to proceed for 4 min. after which incorporated iodine was separated from free on a sephadex G-75 (0.9 x 60 cm) column. Specific activitiy of iodinated hormone ranged from 45 to 100 μCi/μg.

Binding on whole cells was performed in the following way. Cells were harvested with a rubber policeman, pooled and 2 million cells per tube were incubated in a binding buffer containing 500 μL of EMEM which had been de-gassed and pH re-equilibrated to pH 7.5 with 50 mM HEPES and 0.1% BSA; 100 000 cpm of ^{125}I-hGH, and for nonspecific binding tubes, 1μg of cold oPRL.

To make microsomes, frozen cell pellets were thawed in microsome buffer containing Tris-HCl (25mM) pH 7.4, magnesium chloride (25mM), PMSF (1mM), benzamidine (10 mM), and Leupeptin (7ug/mL), and homogenized with a Brinkman polytron at the setting "50" for 3 bursts of 30 seconds while on ice. The homogenate was centrifuged at 100 000 x g in a Sorval ultracentrifuge for 1 hr at 4°C. Microsomes from cultured cells were produced by another simpler method. The cell pellet was resuspended in microsome buffer an then treated to two cycles of freezing at -80°C and thawing. Cell membranes were collected from this preparation by ultracentrifugation, or for small samples, by centrifugation in a microcentrifuge at 18 000 rpm for 30 min. at 4°C.

Binding was performed with buffer containing TRIS (25mM) pH7.4, magnesium chloride (25mM), and .1% BSA, with 100 000 cpm of ^{125}I-hGH, plus 1 μg/mL of cold oPRL for nonspecific binding tubes, with a final volume of 500 μL. ^{125}I-hGH was used as the labelled ligand instead of ^{125}I-hPRL, since hGH was more easily iodinated and proved to be more stable. The binding reaction was allowed to incubate between 15 to 18 h. For solubilized receptors, binding was carried out in the above buffer containing detergent: 0.2% for Triton X-100, and 4 mM for deoxyBIGCHAP. Four mL of cold buffer was added to the binding reaction followed by centrifugation at 4000 x g for 15 minutes, and decanting to remove the bound ^{125}I-hGH from the unbound. For solubilized receptor, bound was separated from free by the addition of 500 μL of 0.1% γ-globulin in 0.1M phosphate buffer (pH 7.4) and 1 mL of 24 % polyethylene glycol, followed by vortexing, and centrifugation at 4000 x g for 15 min. Samples were counted in a LKB gamma counter. A microsomal protein concentration of 400 μg/tube was used in all binding experiments (refer to Figure 3). Protein concentration was determined by the method of Lowry. [474]

Purification

The cell pellets, frozen at -80°C, were thawed in microsome buffer, and homogenized with a polytron. The resulting pellet was resuspended in microsome buffer and used immediately or and frozen at -80°C. Solubilizations were performed on the microsome pellet resuspended in microsome buffer plus 10 mM deoxyBIGCHAP detergent, at a concentration of 10 mg/mL for 30 min at 20°C with gentle agitation. The solubilized supernatant was then collected by centrifugation at 100 000 x g for 1 hr. at 4°C. Solubilized receptors were loaded onto a benzamidine-sepharose 6B protease affinity column (Pharmacea), and then onto a hGH affinity column. Figure 4 shows a schematic diagram of the purification protocol.

Coupling of hGH to control pore glass followed the protocol of Spencer.[475] Control pore glass was activated with 10 mL of 0.1% sodium metaperiodate at 20°C for 30 min, then washed with 20 mL of cold water. 10 mg of hGH and 5 mg of sodium cyanoborohydride in 5 mL of PBS was added to the gel and incubated overnight at 4°C with gentle agitation. After washing the gel again with 20 mL of cold water, uncoupled

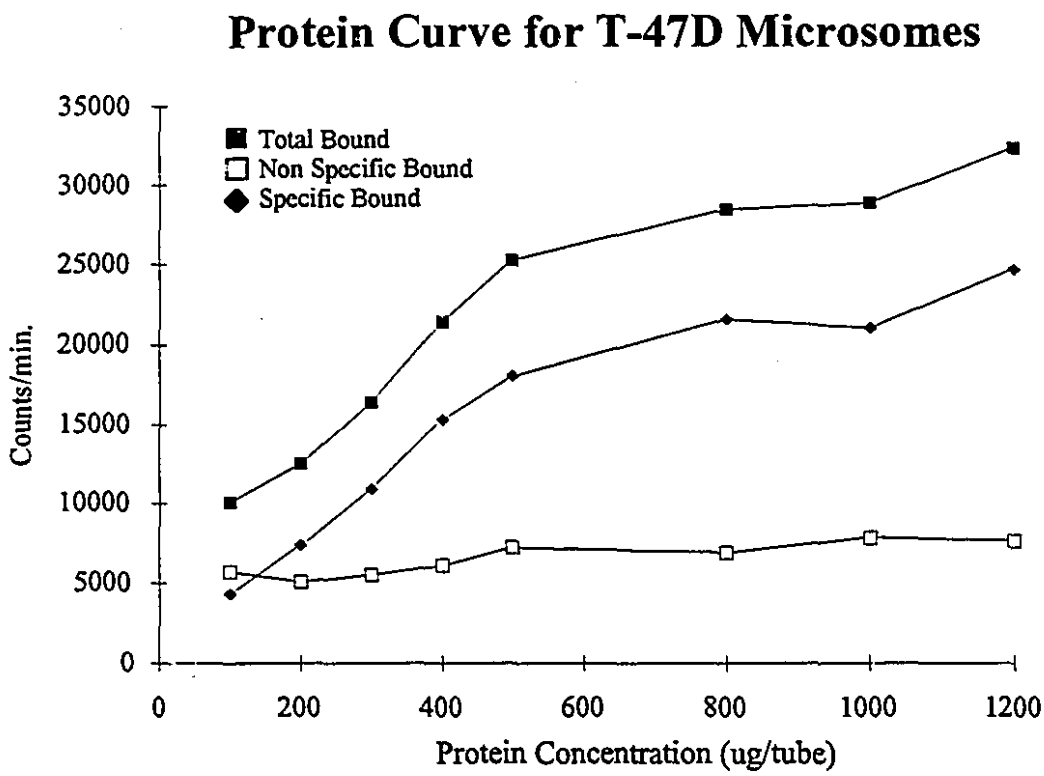


Figure 3. Binding on T-47D microsomes was done at different concentrations of protien/tube to determine the optimal concentration of protein to use in binding experiments. A concentration of 400 ug/tube was chosen.

Receptor Purification Schema

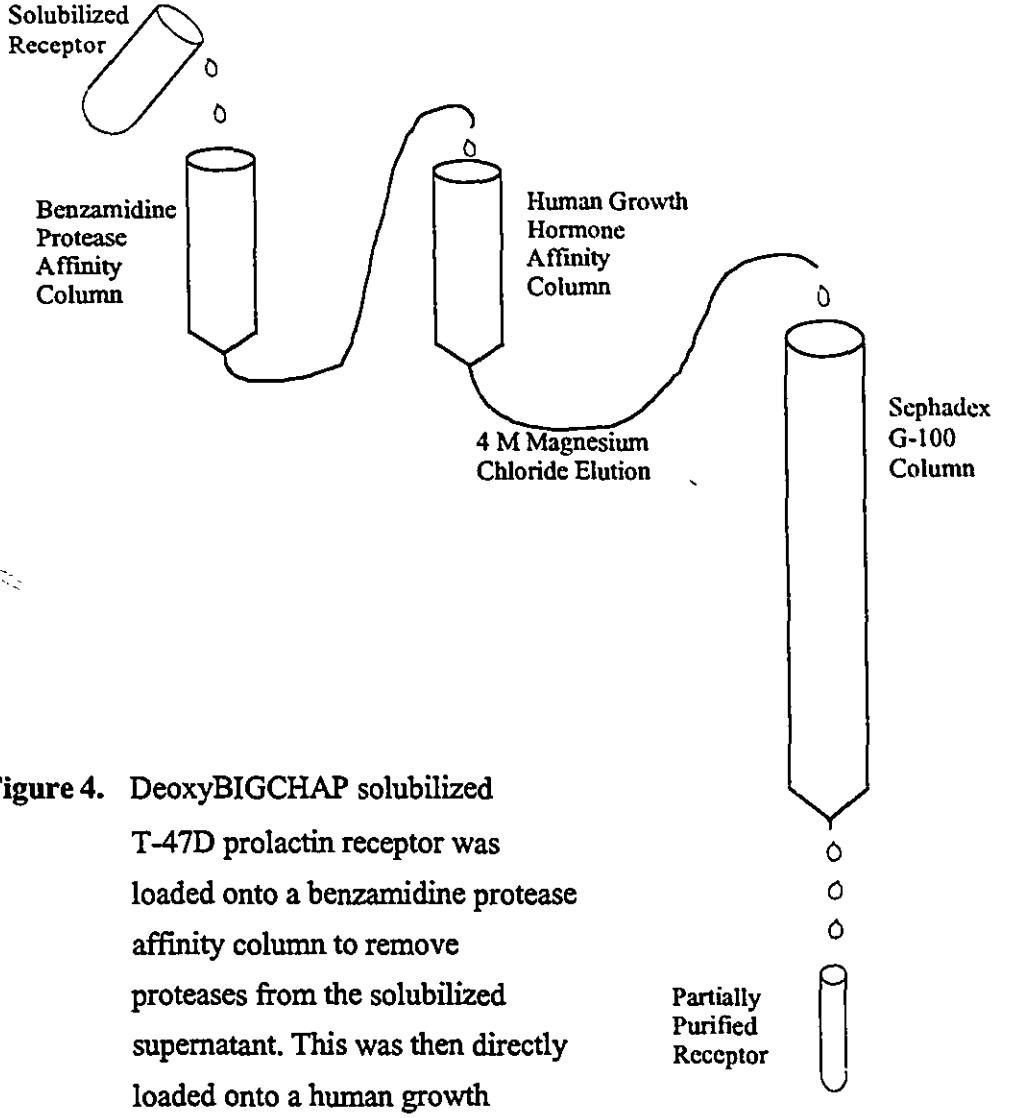


Figure 4. DeoxyBIGCHAP solubilized T-47D prolactin receptor was loaded onto a benzamidine protease affinity column to remove proteases from the solubilized supernatant. This was then directly loaded onto a human growth hormone affinity column. After washing with buffer, the hGH column was eluted with 4 M $MgCl_2$. The eluate was then loaded onto a sephadex G-100 molecular sieve column to remove the $MgCl_2$ and to further purify the receptor.

sites were blocked with 10 mL of 1 M ethanolamine, containing 5 mg sodium cyanoborohydride, and incubated for 48 h at 4°C with gentle agitation. Finally the gel was washed three with three cycles of 5 mL sodium acetate 0.5 M pH 4.0, and 5 mL Tris-HCl 25 mM pH 8.5, NaCl 0.5M. The gel was then washed with urea 6M, and magnesium chloride 4M, and finally equilibrated with several washes of Tris-HCl 25 mM deoxyBIGCHAP.

Solubilized receptor (50 mL) was loaded on the hGH affinity column at 20 mL/h at 20°C, then washed with 30 mL of dBC buffer (microsome buffer with 10 mM deoxyBIGCHAP), 20 mL of dBC buffer containing NaCl 0.5M, and another 30 mL of dBC buffer. The receptor was eluted from the column with 5 mL of $MgCl_2$ 4M containing deoxyBIGCHAP 15 mM. Refractive index readings were taken on 1 mL fractions to determine the $MgCl_2$ front, and fractions containing $MgCl_2$ plus the fraction immediately before the $MgCl_2$ peak were pooled and loaded onto a large sephadex G-100 column. G-100 fractions (1.5 mL) were measured for binding and positive samples were pooled and used for Scatchard analysis. The binding assays for the magnesium eluted fractions was incubated for only 6h, and for the G-100 fractions for only 3h at 30°C in order to minimize the time between the elution and the Scatchard analysis binding. Scatchard analyses were performed under the standard optimal conditions of 16 hr at 20°C.

Monoclonal Antibody Production

Methods used in immunizations and hybridoma production have been previously described.[141] Immunizations were performed with several forms of human prolactin receptor antigen. Briefly, immunizations were performed with 10 μg of protein, four times, at intervals of 2 weeks. Fusions were made with the SP2/0 cell lines by the PEG method. Before the human prolactin receptor was purified, immunizations were performed using T-47D microsome preparations. Immunizations were also performed with preparations of partially purified prolactin receptor, and with a synthetic peptide (ELAVEVKQPEDRKPY) corresponding to amino acids 108 to 122 of the mature prolactin receptor sequence. A peptide hapten was produced by conjugation to keyhole limpet hemocyanin by the glutaraldehyde method.[476]

Hybridoma supernatants were screened by binding inhibition, immunoprecipitation, and whole cell ELISA. Binding assays were carried out with T-47D microsomes in a way similar to a standard binding except that in place of the cold prolactin used for nonspecific measurement, hybridoma supernatant was used.

Immunoprecipitation assays were performed once a successful solubilization protocol was determined. Solubilized T-47D membranes and ^{125}I-hGH were incubated overnight at 20 °C to produce a hormone-receptor complex. Aliqouts of complex containing 100 000 cpm of ^{125}I-hGH and 250 µg of solubilized membrane protein were combined with 100 µL of hybridoma supernatant and incubated overnight at 4°C. Then pansorbin (20µL) was added and incubated with the hormone-receptor-antibody complex for 30 min at 20 °C whereupon 4 mL binding buffer, containing 4 mM deoxyBIGCHAP, was added. The samples were then centrifuged at 4000 x g, decanted, and counted.

In the whole cell ELISA, T-47D cells were harvested in EDTA/PBS, washed in PBS, and added to 96-well filter plates at 200 000 cells per well. These cells were incubated in 250 µL of hybridoma supernatant for 60 min at 4°C. After washing the cells 3 times in 4°C PBS, 150 µL of 1:500 dilution of goat antimouse-Ig-HRP conjugated antibody in PBS was added and incubated for 60 min at 4°C. After 5 washings in cold PBS, ABTS in a buffer containing 0.1M sodium citrate pH 4.2, 0.2 M sodium chloride, and 1 µL/mL 30% hydrogen peroxide, was added and incubated for 20 min. The colored reaction product was measured by spectrophotometry at 415 nm in a 96 well plate reader (Flow, Mississauga ON).

Immunoprecipitation

Immunoprecipitations were performed with iodinated partially purified human prolactin receptor. Protein-A affigel-10 (25µL) was washed in buffer (HEPES 25 mM pH 7.4, NaCl 150 mM, Triton X-100 1%, $MgCl_2$ 25 mM, leupeptin 7 µg/mL) and then incubated with 20µL of anti rat prolactin receptor rabbit serum or non-immune serum rabbit serum for 2 h at 20°C. The gel was washed 4 times with buffer, then 5 000 000 cpm of iodinated partially pure human prolactin was added to the nonimmune serum coated gel sample, and incubated with receptor for 3 h at 20°C. The

supernatant from this step was incubated with the anti-receptor serum coated gel overnight at 4°C with gentle agitation. Both gels were washed once each with HEPES 50 mM pH 8.0, Triton X-100 0.2%, SDS 0.1%, NaCl 500 mM; then HEPES 50 mM pH 8.0, Triton X-100 0.2%, SDS 0.1%, NaCl 150 mM; and finally HEPES 50 mM pH 8.0, Triton X-100 0.2%, SDS 0.1%. The samples were then loaded onto reducing SDS-PAGE, and visualized by autoradiography.

Crosslinking

Chemical crosslinking of the human prolactin receptor to ^{125}I-hGH was carried out on T-47D cells on 100 mm^2 plates. Cells were cultured in 10% LSNBS for 2 days before crosslinking, then were rinsed 2 times with binding buffer (EMEM containing 25 mM $MgCl_2$, 10 μM $ZnCl_2$, and 50 mM HEPES, and diluted with water to keep the correct osmolarity). Binding was performed for 3h at 20°C with 5 000 000 cpm/mL ^{125}I-hGH in 3.5 mL of binding buffer. To a second plate, 1μg/mL of unlabelled hGH was added for the determination of nonspecific binding. The cells were washed 2 times with ice cold PBS, and incubated for 30 min on ice with the crosslinker sulfoEGS. Then the cells were washed, 3 times in cold binding buffer and scraped off the plates with 5 mL buffer containing HEPES 10 mM, EDTA 1 mM, PMSF 1 mM, benzamidine 10 mM, leupeptin 7 μg/mL, and glycine 1 M. The cells were spun, resuspended in the same buffer without the glycine, and an aliqout was loaded onto reducing SDS/PAGE.

Sequence Analysis

Amino acid comparisons of growth hormone receptors and prolactin receptors were performed with the DOS based PC-GENE collection of analysis algorithms (IntelliGenetics Inc., Mountain View, CA). These algorhithms are designed to compute probable secondary structure of a protein given the primary amino acid sequence. Sequence alignment was performed using the algorithm by Higgins,[477] with a k-tuple value of 1, a gap penalty of 5, a window size of 10, an open gap cost of 10, and a unit gap cost of 10. Secondary structure analysis was performed with algorithms by Garnier (using a helix coefficient of -75 and an extended coefficient of -88)[478] Gascuel (using a helix coefficient of 1.2311, and extended coefficient of 1.5451)[479] Novotny,[480] and

Karplus .[481] Hydrophobicity analysis was performed with the algorithms of Rao,[482] and Kyte.[483] In addition a homology scan upon the rat, human, and rat prolactin receptors was performed manually with a window size of 20 amino acid, counting only identical amino acids between the three sequences.

Transfections

Transfections were performed using the calcium phosphate precipitate method. COS-7 cells were grown in P-150 flasks in DMEM supplimented with 10% fetal bovine serum. At 50% confluency the media was replaced with 18 mL DMEM containing 2.5% serum, plus 2 mL of a solution containing 125 mM calcium phosphate in HBS, plus 25 μg of vector DNA. After 4 h the cells were exposed for 3 min to 15% glycerol in DMEM, then washed with 2 times 10 mL of DMEM whereupon the cells were cultured in media containing 10% fetal bovine serum. Cells were harvested two days after transfection. Transfections were made using the pECE expression vector.[484]

Polymerase Chain Reaction Mutagenesis

Mutagenesis upon the human prolactin receptor cDNA was performed. Oligomer primers were synthesized with a Biosearch DNA synthesizer, and purified by PAGE followed by C_{18} reverse phase batch chromatography. Primers were chosen to remove the 3' and 5' untranslated regions of the human prolactin receptor cDNA, and to produce a perfect Kozak[485] consensus sequence. H 103 was the 5' primer which contained 5 substitutions, to produce the Kozak sequence and a Sal 1 restriction enzyme site. H 106 was the 3' primer and contained an Eco RI restriction enzyme site.

H 103 GAG AAG GTC GAC ACC ATG GAG GAA AAT GTG GCA TCT GCA ACC G
H 106 CAT TCG AAT TCG TCA AGC TAT CAG TC

PCR was performed on 100 ng of human prolactin receptor cDNA and 100 pmol each of purified primers for twenty cycles with an annealing temperature of 45°C,in a buffer containing Tris-HCl (10mM), KCl (50mM) and $MgCl_2$ (5mM). The PCR product was digested with the restriction enzymes Sal I and Eco RI and then ligated into the pECE vector which also had been digested with Sal I and Eco RI. The selected

recombinant clones were sequenced by the dideoxy-chain termination method[486] to confirm the correct form of the new insert.

PCR was performed on reverse transcribed T-47D RNA. Five μg of T-47D RNA in a buffer with Tris-HCl (50 mM) pH 8.3, KCl (75 mM), $MgCl_2$ (3 mM), and DTT (10 mM) was reverse transcribed.. The equivalent of 1 μg of RNA was used in the PCR reaction, using conditions similar to those described above except the reaction was performed for 30 cycles.

Results

Cell Culture

The use of cell factories was an effective way to produce large quantities of cells. Typically, T-47D cells were subcultured with 20 T-175 flasks into 2 cell factories once a week, with an average cell harvest of 4.5g. Each gram of cells contained an average of $2.56x10^8$ cells and produced an average of 58.9 mg of membrane protein. Cell cultures grown for use in binding or purification were harvested with 5mM EDTA/PBS since trypsin digestion destroyed prolactin binding.

Prolactin Binding

Previous reports indicated that the T-47D cell line contained the highest amount of prolactin binding.[414] This was confirmed in our binding experiments. It was determined that binding experiments using cell membranes, produced either by homogenization or by freeze-thawing, were more effective than binding experiments using whole live cells. This is due presumably to receptors present in intracellular compartments which become accessible in membrane preparations. The T-47D cell line was cloned to isolate cells with the highest binding: binding among clones ranged from 0.5 to 1.7 % in the whole cell binding assay. The clone with the highest binding was used for all further purification work. Figure 3 shows a typical binding assay with T-47D microsomes.

From this experiment, it was determined that 400 μg/tube of T-47D membrane protein would be used for further binding studies.

Purification

Attempts were made to optimize solubilization of the human prolactin receptor. Lubrol PX, Brij-25, the Zwittergent series, Triton X-114, Triton X-100, CHAPS, CHAPSO, deoxyBIGCHAP, and digitonin were tested for there effectiveness in solubilizing receptor. Some detergents such as Triton X-100, and the larger Zwittergent detergents were more effective in the solubilization of protein from T47-D membranes, but left the solubilized fraction with negligible specific binding of ligand. Figure 5 shows that this was due to high nonspecific binding values which were observed in bindings with solubilized receptor. The human prolactin receptor seems to be much more labile than the rat or rabbit receptor and easily looses its biological activity. The detergent CHAPSO produced good specific binding, but later Scatchard analysis revealed that this detergent produced a large increase in affinity and actual receptor number was lower than with receptor solubilized with CHAPS. The detergent which was best able to preserve receptor binding was deoxyBIGCHAP (see Table 4). The yield of receptor was low, however, the amount of receptor extracted being less than half of that present in microsomes. It was determined later that digitonin also gave a stable extraction of prolactin receptor. Receptor solubilizations for purification were performed with deoxyBIGCHAP.

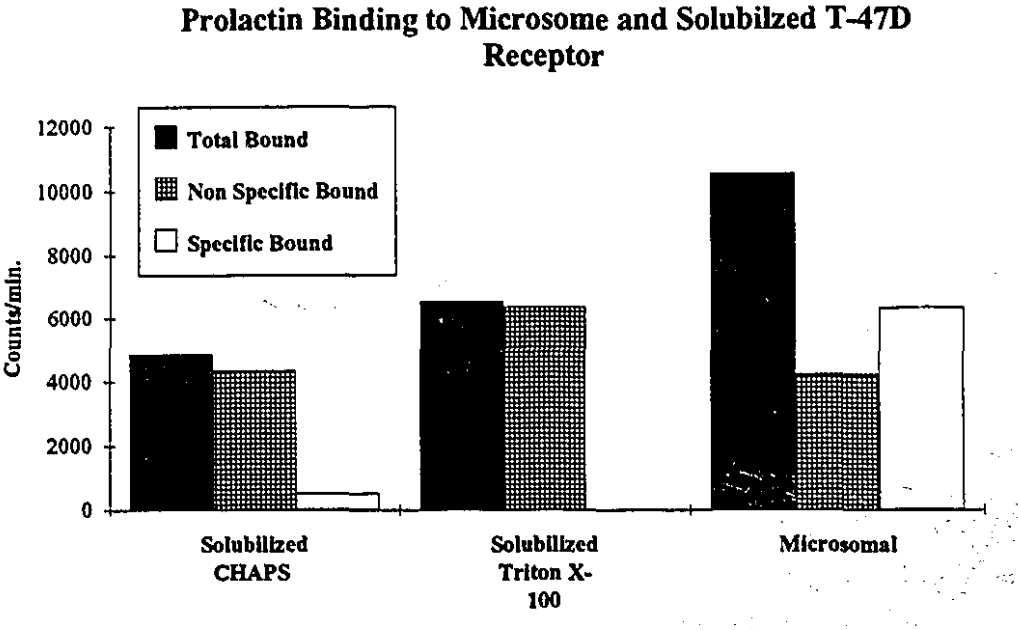


Figure 5. T-47D microsomes were solubilized with 1% triton X-100 and CHAPS 20mM with 10 mg/mL microsomal protein. Binding was done with 101000 cpm ^{125}I-hGH. Protein concentrations in the binding assays were 1/10 of the amount recovered in the solubilized supernatant: 101 ug/tube for triton X-100 assay, and 165ug/tube for CHAPS. For reference, binding upon the original microsomal fraction at 400ug/tube protein was performed.

Table 4. Comparison of Chaps and DeoxyBIGCHAP Solubilizations

	No. Sites / mg	Total No. Sites	Affinity K_d (nM)
deoxyBIGCHAP	74.7	744,012	0.22
CHAPS	96.6	669,438	0.52

Once the receptor was solubilized, it was loaded onto a benzamidine-sepharose 6B column, connected in series to a hGH affinity column. The column was washed and eluted with 4 M $MgCl_2$. Figure 6 shows receptor elution from the affinity column. Fractions 3 to 6 were pooled and loaded onto a G-100 column for desalting and further purification. Figure 7 shows the protein (measured by spectrophotometry at 280 nm) and the specific binding of fractions collected from the G-100 column. Fractions 19 to 32 were pooled and used for Scatchard analysis .

The affinity of the prolactin receptor increases following purification, with a K_d of 1.6 nM for homogenate and a K_d of 0.5 nM for purified, as can be seen from Figure 8. Table 5 presents the data collected from Scatchard analysis for homogenate, microsome, solubilized and purified fractions from one prolactin receptor purification. The procedure started with 13.4 g of packed T-47D cells and ended with 1.97 mg of partially purified receptor with a purity of 0.12%. The final recovery of receptors from the homogenate was 25.8% with a purification factor of 130.

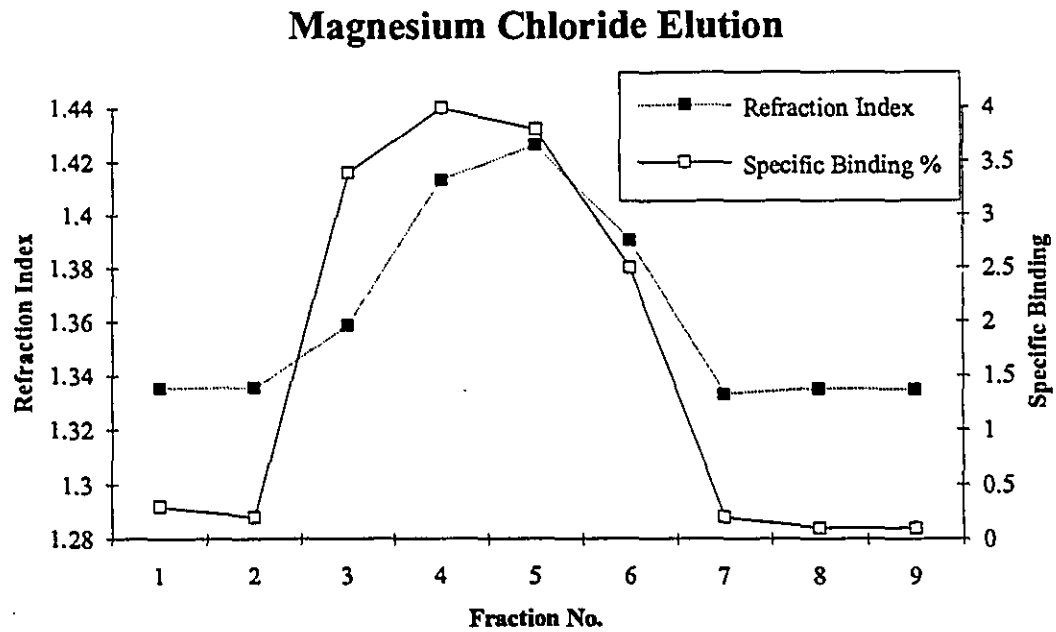


Figure 6. Eluted of the hGH affinity column with 4M $MgCl_2$. The refraction index indicates the concentration of $MgCl_2$.

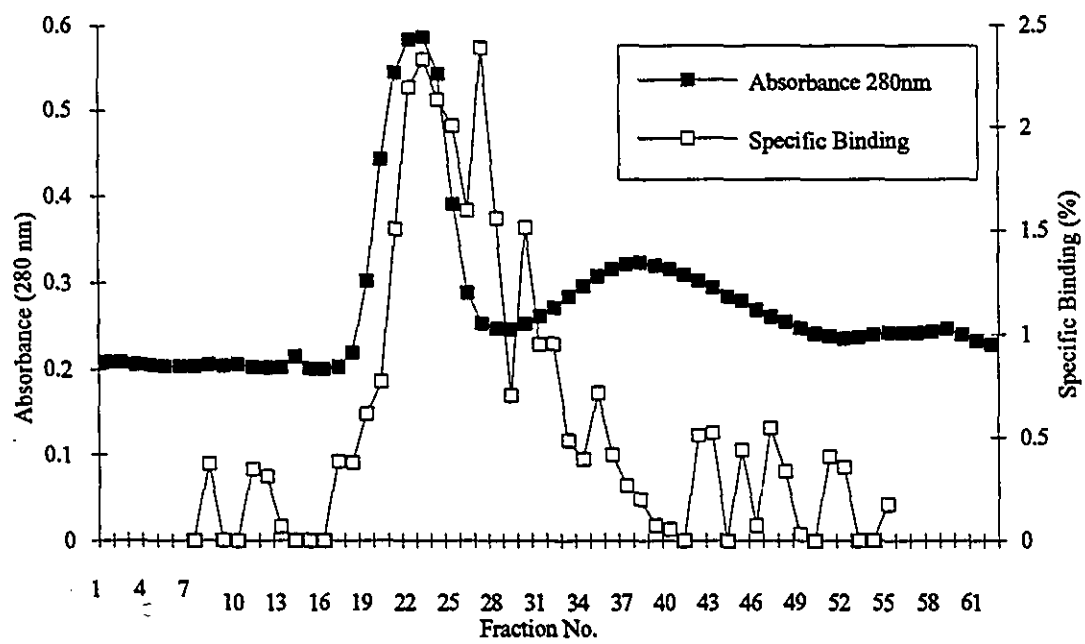


Figure 7. Fractions from the $MgCl_2$ elution were pooled and loaded onto a G-100 column for desalting. The binding assay was performed at 30°C for 3 h. Scatchard analysis was performed on pooled fractions 19 to 32.

Human Prolactin Receptor Purification Scatchard Analysis

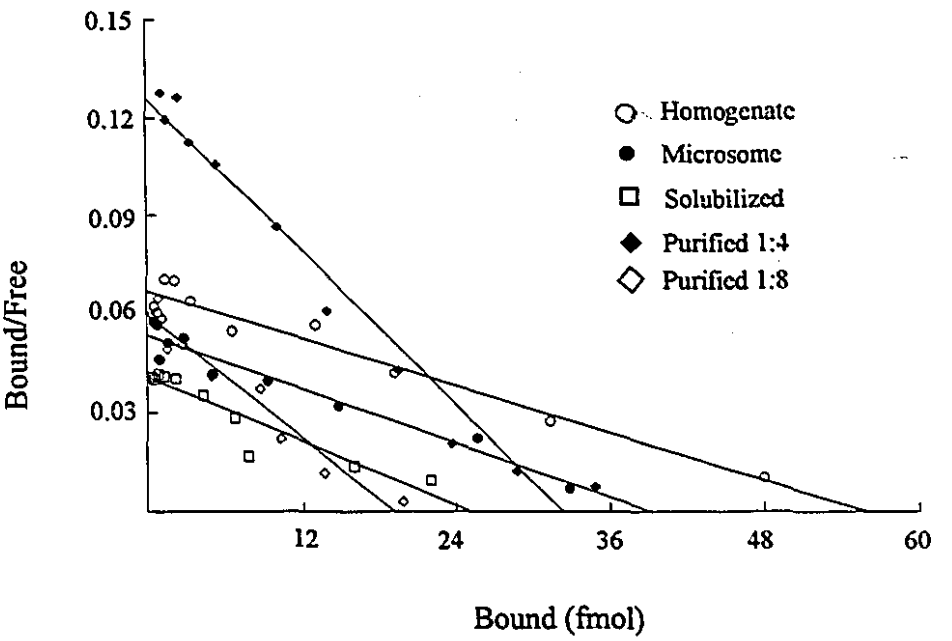


Figure 8. Scatchard analysis of human prolactin receptor from different steps of the purifiaction procedure. The homogenate series contained 500µg protein/tube, microsome series 300µg/tube, solubilized 90µg/tube, purified 1:4 4.8µg/tube, and purified 1:8 2.4µg/tube. Scatchards were done with ^{125}I-hGH and cold hGH.

Table 5. Purification Data

Fraction	Protein (mg)	No. Sites (fmol/mg)	No. Sites (fmol)	Affinity K_d (nM)	Purification	Recovery
Homogenate	990	107	105 903	1.6	1:1	100.0%
Microsome	770	125	96 250	1.4	1:1.2	90.9%
Solubilized	381	200	76 160	1.1	1:1.9	71.9%
Purified	1.97	13 878	27 340	0.5	1:130	25.8%

$$\text{Purity} = \frac{S \times M}{P}$$ Where S is Number of Sites

M is Molecular Weight

$$\text{Purity} = \frac{27340 \times 10^{-15}\text{mol} \times 88000\text{g/mol}}{.00197\text{g}} = .0012$$

$$= 0.12\%$$

Monoclonal Antibody Production

Monoclonal antibody production using T-47D microsomes as antigen was attempted on two different occasions. Screening with the whole cells ELISA produced many positive clones, though none showed activity to the prolactin receptor when determined by binding inhibition. The same was true for monoclonals raised to a partially purified receptor preparation. When monoclonals were made to the synthetic peptide (ELAVEVKQPEDRKPY) several clones possessed high affinity for the peptide when tested in ELISAs with the peptide antigen, yet no interaction with the prolactin receptor could be observed in binding inhibition, immunoprecipitation, or immuno-blotting experiments.

Immunoprecipitation and Crosslinking

Crosslinking of the prolactin receptor to live T-47D cells with ^{125}I-hGH produced several bands on reducing SDS/PAGE. It can be seen in Figure 9 that these bands correspond to specific binding of ^{125}I-hGH to receptor since in the presence of excess cold hGH these bands are absent. Based on a measurement of an aliquot of the binding, specific binding was 3.4% of the ^{125}I-hGH added, and nonspecific binding comprised 9% of the total amount of bound radioactivity.

Determination of the relative molecular weight of the receptor, requires that the relative molecular weight of hGH must be subtracted from the weight of the hormone receptor complex observed on the gel. Katoh,[226] determined that under reducing conditions the relative molecular weight of oPRL was 27 200, compared to 23 600 with non-reducing conditions, an increase in apparent molecular weight of 3 600. If hGH behaves in a similar manner its relative molecular weight under reducing conditions would be 25 000, though since growth hormone has only 2 disulfide bonds compared to 3 for prolactin this can only be an estimated mobility. If it is assumed that the hormone receptor complex contains only one molecule of ligand the lowest possible relative molecular weight of the receptor itself would be 88 000 (refer to Table 6 for the relative molecular weights calculated from the other specific bands).

Table 6. Relative Molecular Weights of Crosslinked Bands

Rf: Hormone Receptor Complex	290 000	235 000	205 000	137 000	125 000	113 000
Rf: With Hormone Subtracted	265 000	210 000	180 000	112 000	100 000	88 000

The molecular weight of the protein component of the human prolactin receptor without the signal sequence is 66 888. This would suggest that the portion of the receptor composed of carbohydrate would have a relative molecular weight of 21 000.

Immunoprecipitation of iodinated partially purified human prolactin receptor with the anti-rat prolactin receptor polyclonal antiserum "212", revealed a band of molecular

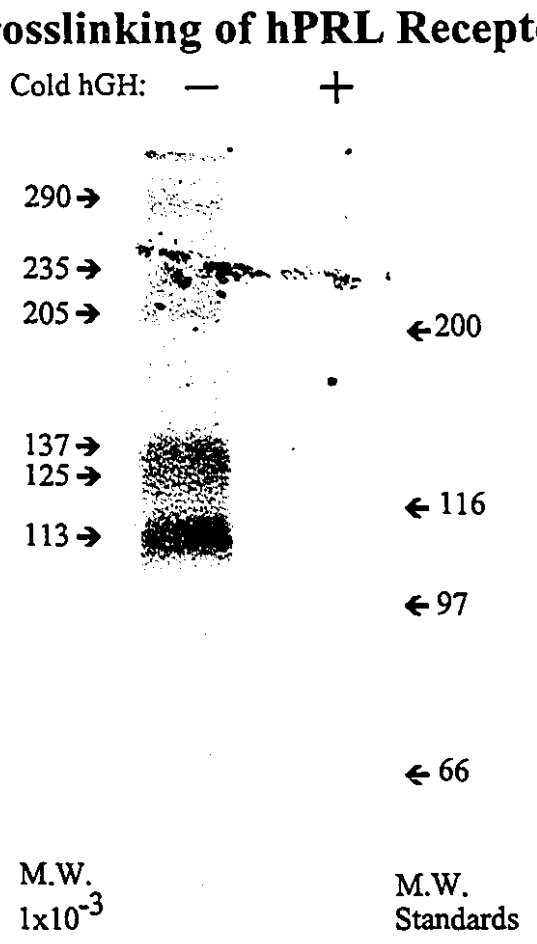


Figure 9. Human prolactin receptor from T-47D cells was crosslinked to iodinated ^{125}I-hGH with EGS, and then run on SDS-PAGE, and exposed for 2 weeks with intensifying screens. After subtraction of the mass of the hormone from the hormone receptor complex the strongest band of molecular mass 113 000 would have a size of 88 000.

weight 38 000 (refer to Figure 10). This band is specifically adsorbed, since no such band was observed when normal rabbit serum was used. When iodinated partially purified human prolactin receptor was run on SDS/PAGE a number of bands were seen, confirming the low degree of purity calculated for this purification protocol.

Sequence Analysis

The sequence of the human, rabbit and rat prolactin receptors were compared and analyzed using several different methods. Since the human prolactin receptor is extremely labile (it has a much greater tendency to loose its biological activity following membrane solubilization or treatment with concentrated $MgCl_2$) the following questions were asked: could an interspecies analysis of prolactin receptor sequences point to some difference which could explain the unique features of the human prolactin receptor? Algorhithms were used which compute probable secondary structure of a proteins given the primary amino acid sequence. These analyses indicated that there were several regions of the human prolactin receptor with a propensity for secondary structure which differed from those of both the rabbit and the rat (refer to Table 7). Only differences which existed between the human, and both the rabbit and the rat together are indicated in Table 7. Specifically, the region centred around amino acid 50 is more hydrophobic than the rat and rabbit receptors as determined by the methods of Rao *et al*,[482] and Kyte *et al*,[483] and has a greater propensity for forming an α-helix as determined by the methods of Rao (refer to Figure 11), and of Gascuel *et al*.[497] The second region, surrounding amino acid 95 is less likely to form a β-sheet or β-turn as determined by Novotny *et al*,[480] or an extended configuration as calculated by Gascuel, and more likely to take an α-helical configuration as determined by Novotny, Gascuel, and Garnier *et al*[478] (refer to Figure 12). It is unlikely that these computed propensities indicate that the human receptor contains α-helicies where the other receptors do not, especially since x-ray crystallographic data indicates that for the human growth hormone receptor there are no α-helices in the extracellular domain.[212] However these computations do indicate how the primary sequence of the human prolactin receptor could result in the increased instability of this protein compared to the receptor in other species. A third region

Immuno-adsorbtion of Purified Human PRL-R

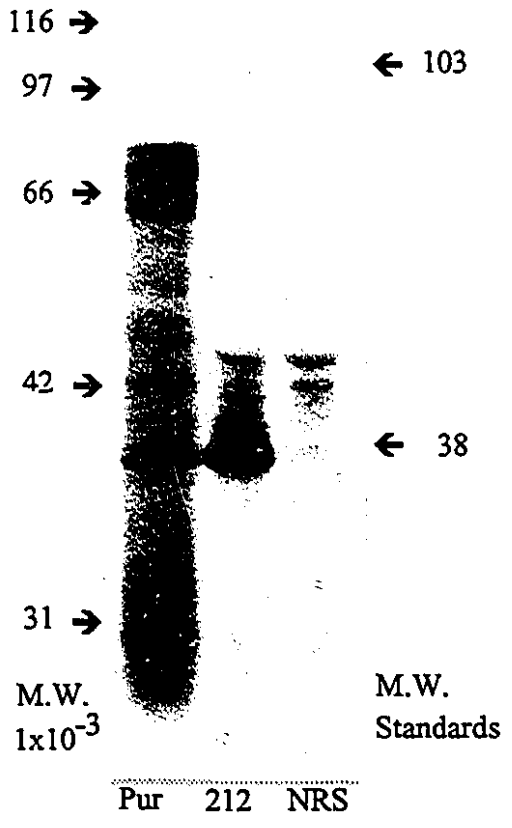


Figure 10. Purified human prolactin receptor was iodinated and adsorbed to protein A- coated affigel coated with either immune or nonimmune rabbit serum. The first lane contains unadsorbed iodinated partially purified human prolactin receptor, lane two contains the material in lane one adsorbed to the anti-rat PRL-R polyclonal antibody 212, and lane three is similar to lane 2 using normal rabbit serum.

Buried Helix Analysis: Rat, Rabbit, and Human Sequences

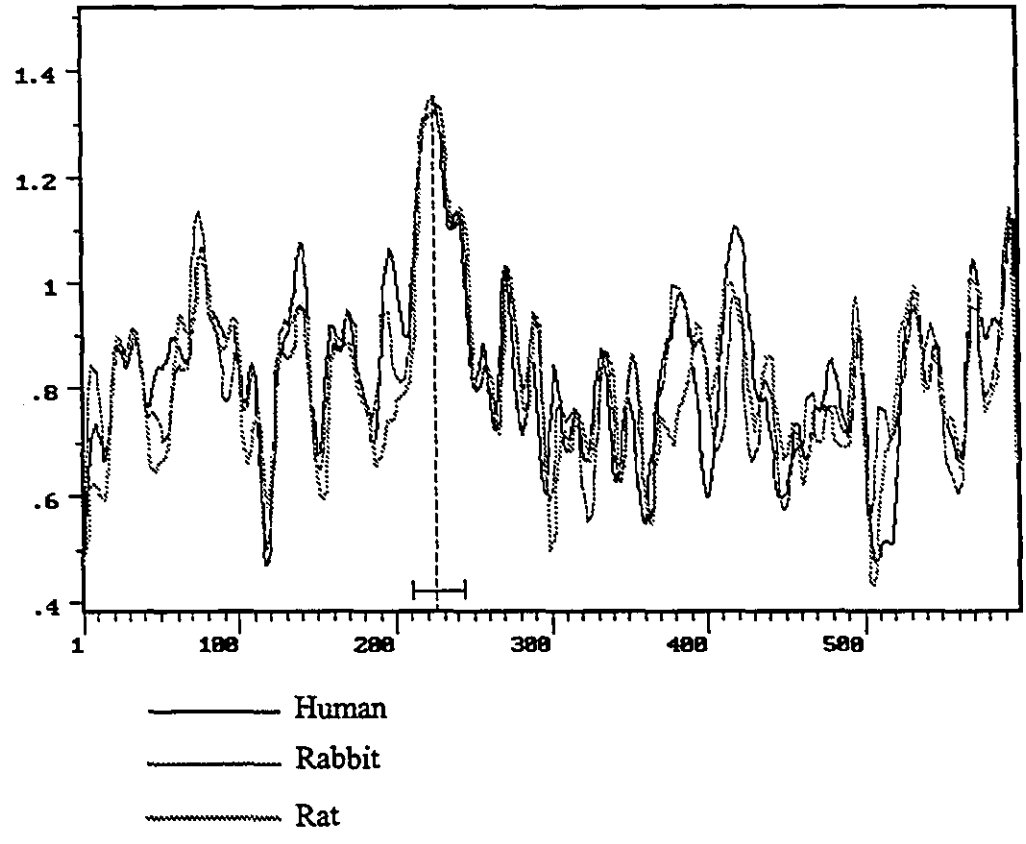


Figure 11. Rat, rabbit and human sequences were analyzed by the algorythm of Rao to predict burried helix formation. Differences between rat, rabbit and human sequences are apparent near amino acid 50, and 200.

Garnier Helix Analysis on Rat Rabbit and Human PRL-R

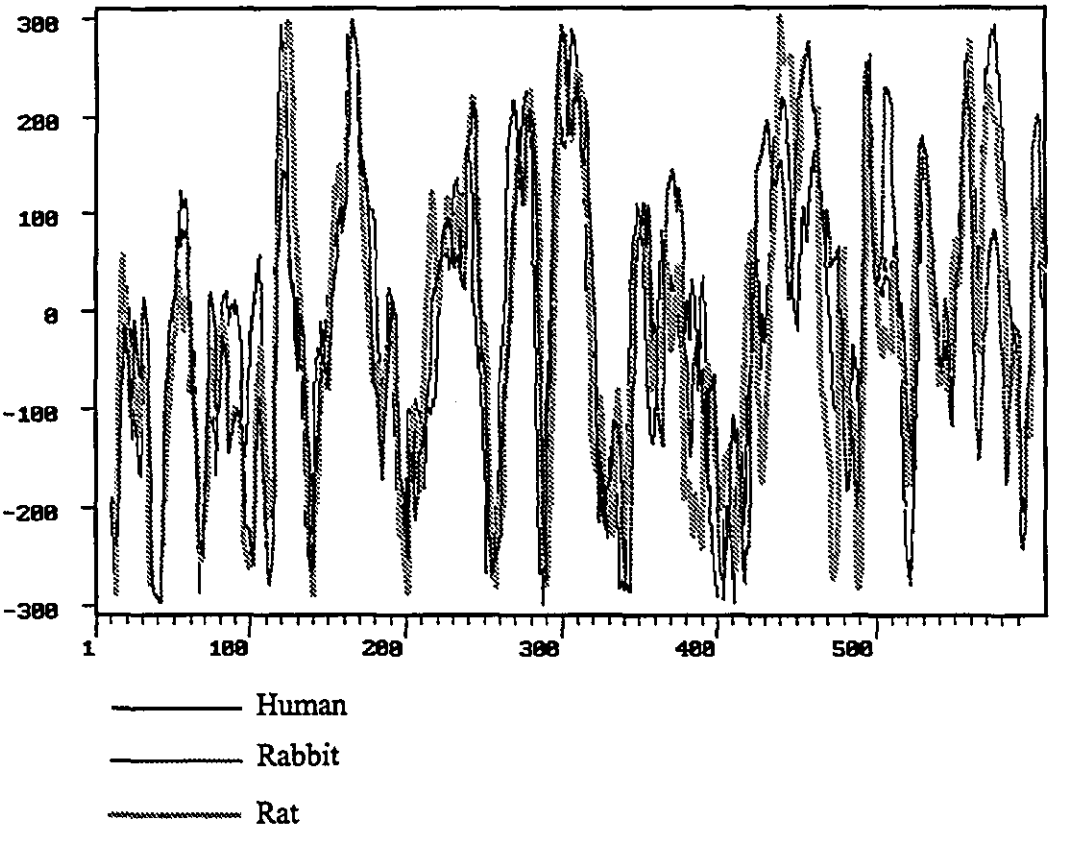


Figure 12. Sequences for the rat, rabbit and human prolactin receptor were examined for probable α-helix configurations with the algorhithm of Garnier. The human receptor differs from the rat and rabbit in the region from amino acid 70 to amino acid 100.

spanning from amino acid 195 to 205 is more hydrophobic in the human than in the rabbit or rat (determined by the methods of Novotny, Rao, and Kyte). A region, centred around amino acid 210 has much less chain flexibility compared to the rat and rabbit sequences as when analysed by the algorithm of Karplus and Schulz. Figure 13 shows the degree of conservation in the amino acid sequence for the prolactin receptor between the human rabbit and rat. Figure 14 is an alignment of PRL and GR receptor sequences using the method of Higgins *et al.*[477]

Table 7. Protein Structure Analysis

Amino Acid Position	Characteristic Analyzed	Type of Analysis	Tendency in Human
60-70	Hydrophobicity	Novotny	Less
90-100	Hydrophobicity	Novotny	Less
195-205	Hydrophobicity	Novotny	Greater (opposite)
210-215	Hydrophobicity	Novotny	Less
70-95	α-Helix	Novotny	Greater
100-110	α-Helix	Novotny	Greater
80-100	β-Turn	Novotny	Less
70-80	β-Sheet	Novotny	Less
60-85	Extended	Gascuel+Golmard	Less
75-100	α-Helix	Gascuel+Golmard	Greater
45-55	α-Helix	Gascuel+Golmard	Greater
190-195	Flexibility	Karpus+Shulz	Less (opposite)
80-100	Turn	Garnier	Less
95-110	Turn	Garnier	Less
165-175	Turn	Garnier	Less (opposite)
85-95	Random Coil	Garnier	Less (opposite)
80-105	α-Helix	Garnier	Greater (opposite)
40-70	Buried Helix	Rao+Argos	Greater (opposite)
195-210	Burried Helix	Rao+Argos	Greater

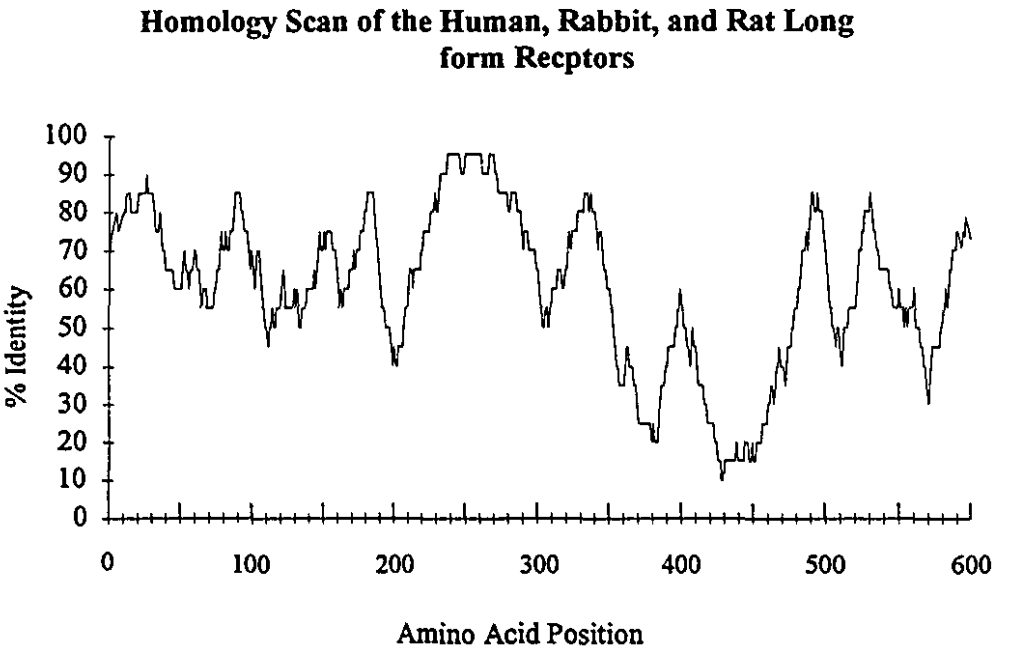
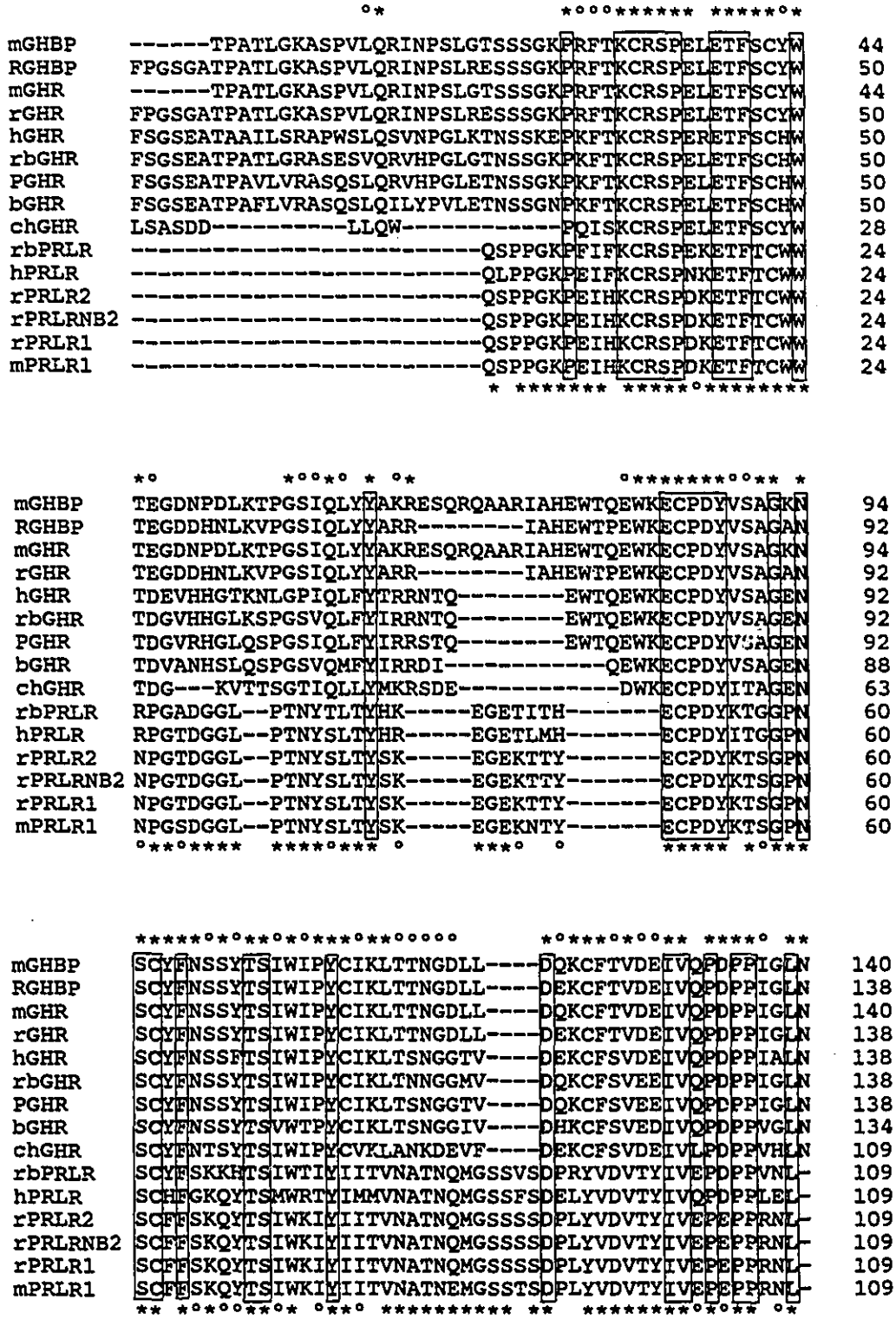
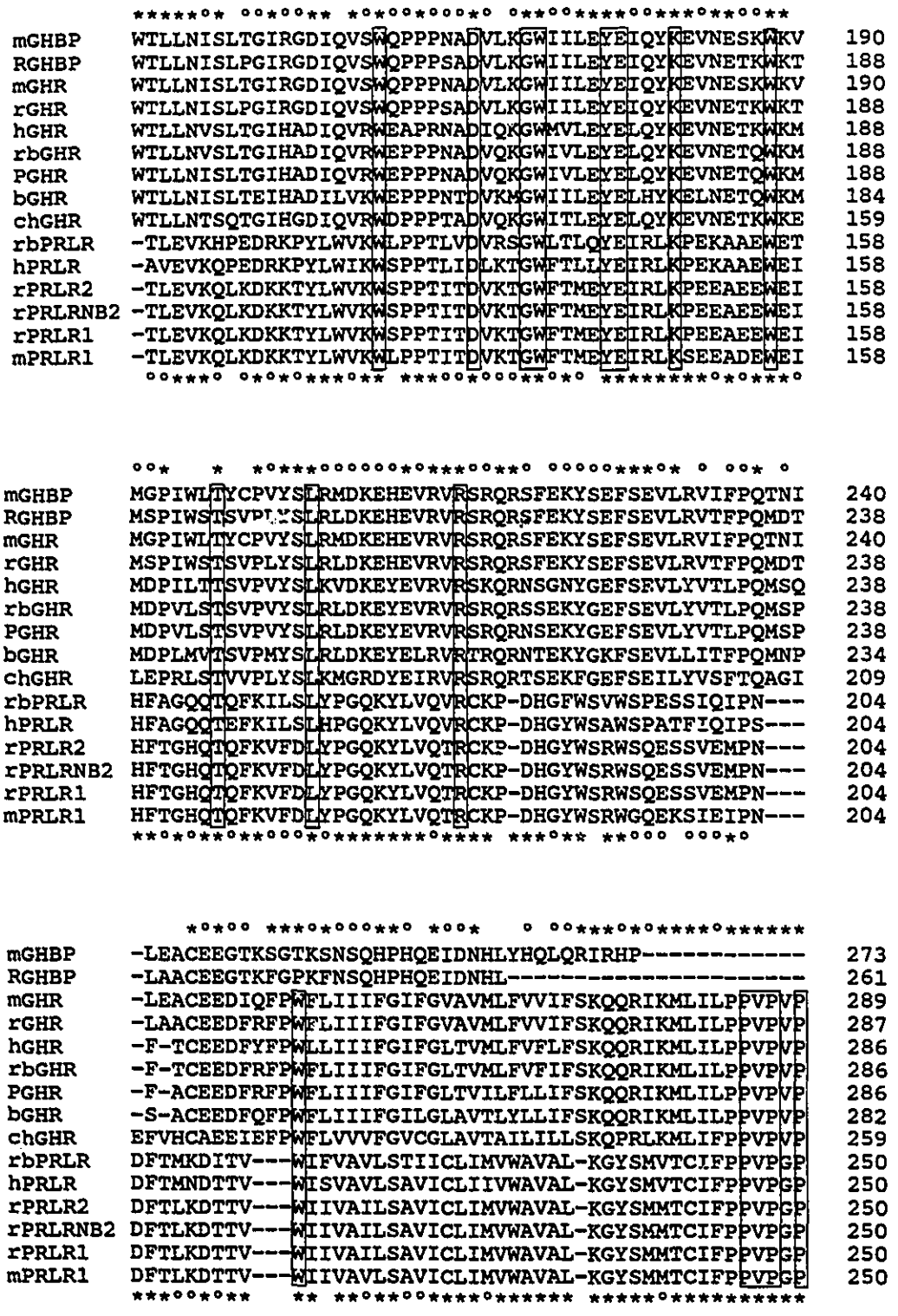


Figure 13. Scan of the identity between the human rabbit and rat prolactin receptors. Comparisons were done upon the amino acid sequence of the mature protein with a window size of 20 amino acids.

Protein Sequence Alignment for PRL and GH Receptors

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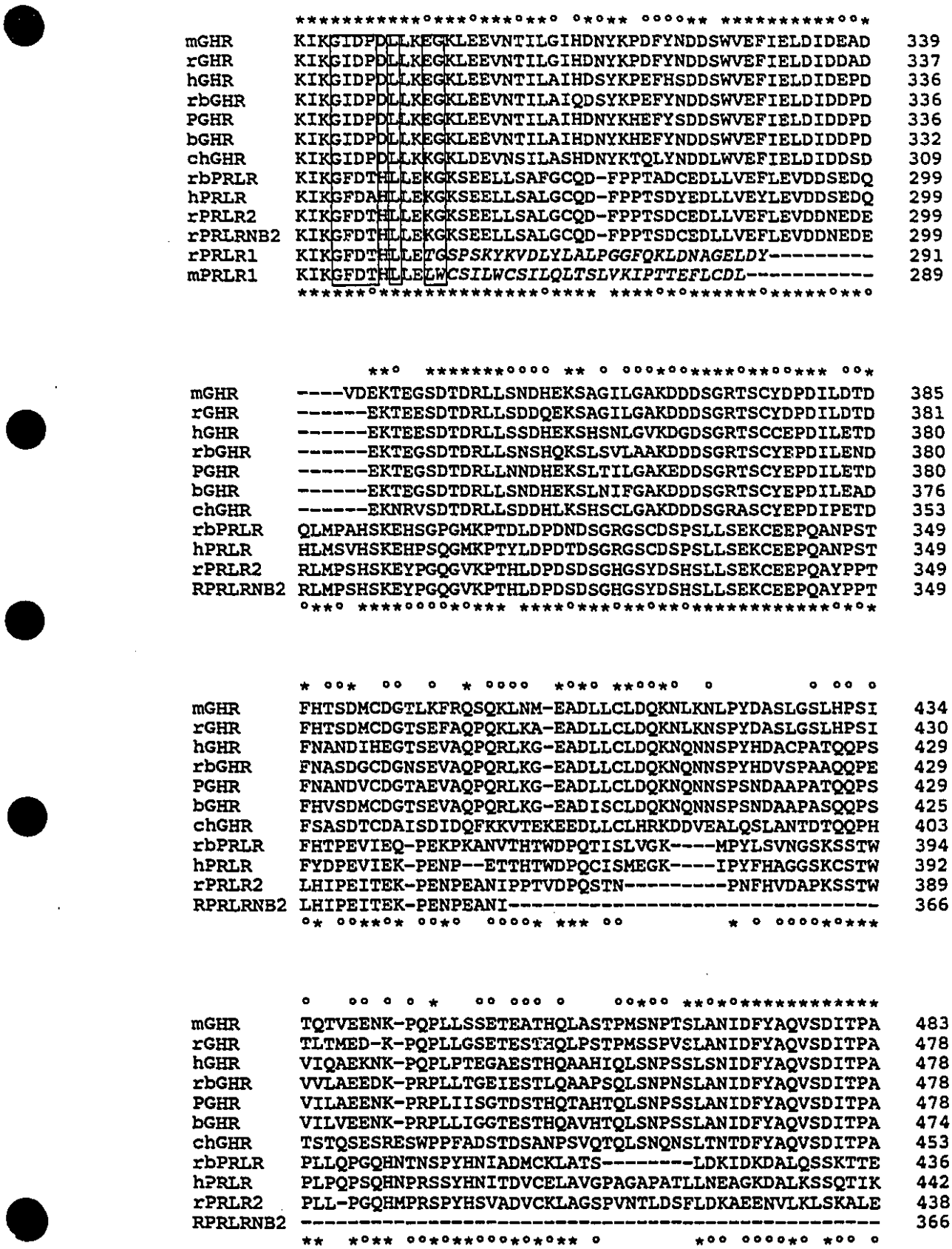
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hGHR      GSVVLSPGQKNKAGMSQCDMHPE---MVSLCQENFLMDNA--YFCEADAK   523
rbGHR     GSVVLSPGQKNKAGNSQCDAHPE---VLSLCQTNFIMDNA--YFCEADAK   523
PGHR      GSVVLSPGQKNKAGISQCDMHLE---VVSPCPANFIMDNA--YFCEADAK   523
bGHR      GNVVLSPGQKNKTGNPQCDTHPE---VVTPCQANFIVDTA--YFCEVDAK   519
chGHR     GSVVLSPGQKSKVGRAQCESCTE---------QNFTMDNA--YFCEADVK   492
rbPRLR    AAGEEKATKQREVESSHSKAEQDTGWLLPKEKPPFISPKPLDYVEIHKVN   486
hPRLR     SREEGKATQQREVESFHSETDQDTPWLLPQEKTPFGSAKPLDYVEIHKVN   492
rPRLR2    T-GEEEVAKQKGAKSFPSDK-QNTPWPLLQEKSPTVYVKPPDYVEIHKVN   486
RPRLRNB2  ----------------------------------------------------   366
          ° °*°°°°°*°°°°* °*°  *°* * * °**°*    ** *********

          *°*°  °° ° °° °     °***°*°********°°    °° ° °*° *
mGHR      KCIAVARRMEATSCIKPSFNQEDIYITTESLTTTA-QMSETADIAPDAEM   577
rGHR      KCIAAAPHMEATTCVKPSFNQEDIYITTESLTTTA-RMSETADTAPDAE-   571
hGHR      KCIPVAPHIKVESHIQPSLNQEDIYITTESLTTAA-GRPGTGEHVPGSEM   572
rbGHR     KCIAVAPHVDVESRVEPSFNQEDIYITTESLTTTA-ERSGTAEDAPGSEM   572
PGHR      KCIAMAPHVEVESRLAPSFNQEDIYITTESLTTTA-GRSATAECAPSSEM   572
bGHR      KYIALAPHVEAESHVEPGFNQEDIYITTESLTTTA-GRSGTAEHVPSSEI   568
chGHR     KCIAVISQEEDEPRVQEQSCNEDTYFTTESLTTTGINLGASMAETPSMEM   542
rbPRLR    KDGALSLLLKQKENGDQTGKAGTPETSKEYAKVSRVMDNNILVLVQDPGA   536
hPRLR     KDGALSLLPKQRENSGKPKKPGTPENNKEYAKVSGVMDNNILVLVPDPHA   542
rPRLR2    KDGVLSLFPKQRENN-QTEKPGVPETSKEYAKVSGIMDNNILVLVPDSRA   535
RPRLRNB2  ----------------------------------LVLVPDSRA   375
          ***°***° **°***° °° *°*°***°°******** °**********°*° *

          °******°°*°*°**°**°***°°*°°°
mGHR      SVPDYTTVHTVQSPRGLILNATALPLPDKKNFPSSC-------GYVSTDQ   620
rGHR      PVPDYTTVHTVKSPRGLILNATALPLPDKKKFLSSC-------GYVSTDQ   614
hGHR      PVPDYTSIHIVQSPQGLILNATALPLPDK-EFLSSC-------GYVSTDQ   614
rbGHR     PVPDYTSIHIVQSPQGLVLNAASLALA----------------------   599
PGHR      PVPDYTSIHIVQSPQGLVLNATALPLPDK-EFLSSC-------GYVSTDQ   614
bGHR      PVPDYTSIHIVQSPQGLVLNATALPLPDK-EFLSSC-------GYVSTDQ   610
chGHR     PVPDYTSIHIVHSPQGLVLNATALPVPEK-EFNMSC-------GYVSTDQ   584
rbPRLR    QNVALFEESTKEAPPSPSQNQAEKDLSSFSTAPSDCRLQQGGLDYLDPAC   586
hPRLR     KNVACFEESAKEAPPSLEQNQAEKALANFTATSSKCRLQLGGLDYLDPAC   592
rPRLR2    QNTALLEESAKKAPPSFEADQSEKDLASFTATSSNRRLQLGRLDYLDPTC   585
RPRLRNB2  QNTALLEESAKKAPPSFEADQSEKDLASFTATSSNRRLQLGRLDYLDPTC   425
          °*°* °****°*°**** °°°*°***°*°°*°°°°*° *** * *******°*

          ****°
mGHR      LNKIMQ   626
rGHR      LNKIMQ   620
hGHR      LNKIMP   620
rbGHR     LNKILP   620
PGHR      LNKIMP   620
bGHR      LNKIMP   616
chGHR     LNKIMP   590
rbPRLR    FMHSLH   592
hPRLR     FTHSFH   598
rPRLR2    FMHSFH   591
RPRLRNB2  FMHSFH   393
          * **°*
```

Figure 14. The sequence alignment was performed upon the mature protein sequences of published growth hormone and prolactin receptors. Boxes indicate amino acid that are the same in both PRL and GH receptors; * indicates an amino acid identity, and ° indicates a conservative divergence within each of the PRL and GH receptor groups.

Comparison of the PRL-R's among different species revealed that the number of potential N-linked glycosylation sites is normally three, and that these three sites are in identical positions among most species. However, the human receptor is different, as the third site has a glycine in place of asparagine, and therefore lacks the normal third glycosylation site (see Table 8). However, there is a potential site at Asn^{209}, although it is unlikely that this site is glycosylated, since it lies only one amino acid away from the putative transmembrane domain, and the threonine which completes the "Asn-Xaa-Ser/Thr" glycosylation signal, lies buried in the membrane. Therefore, it is likely that the human receptor is glycosylated at only two positions. As mentioned in the Introduction, studies indicate that all three potential glycosylation sites of the rat prolactin receptor are glycosylated, and that the degree of glycosylation may differ between either the short and long form of the receptor, or the tissue in which the receptor is expressed.

In order to see if the third glycosylation site might play a role in the susceptibility of the receptor to lose activity during solubilization, three different prolactin receptor species were tested for their stability in the detergents Triton X-100 (1%), and deoxyBIGCHAP (10mM). These were the rat short form receptor wild type (F3) in pECE; the rat mutant receptor (d3) containing an asparagine to aspartic acid substitution to remove the third glycosylation site; and the human prolactin receptor from T-47D cells. Figure 15 indicates that the absence of the third glycosylation site in the rat receptor has no effect of the biological activity of this receptor in the presence of detergents. Thus, it is unlikely that the instability of the human receptor can be explained by the absence of glycosylation at this site.

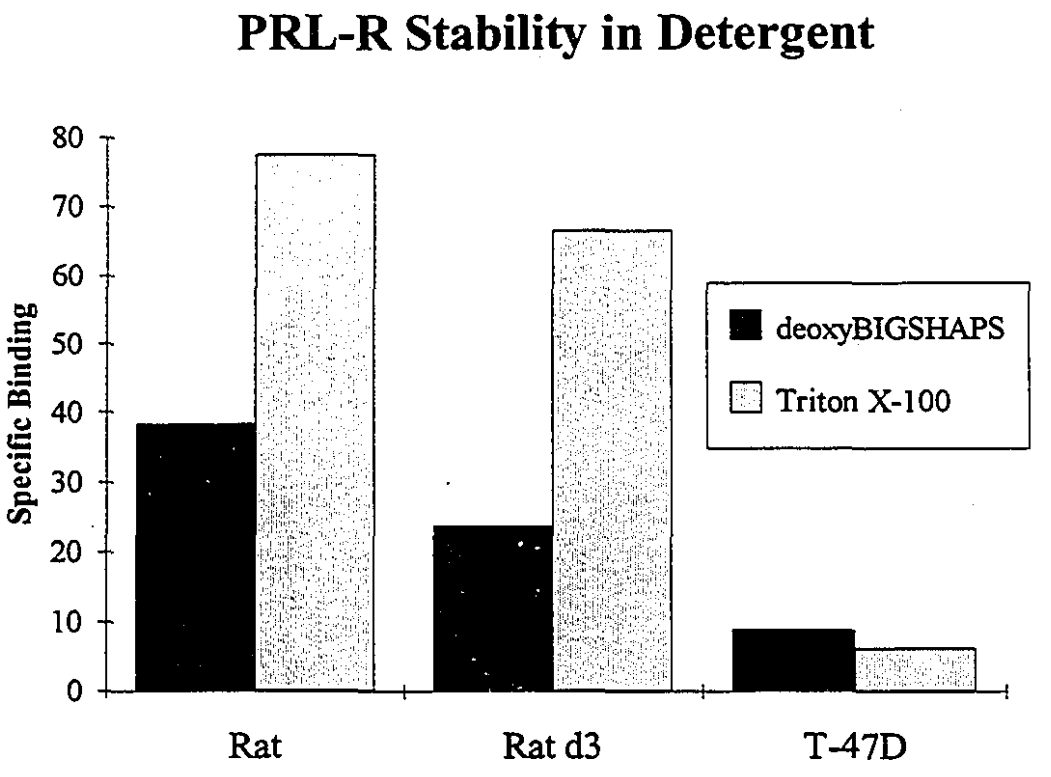

Figure 15. Three different prolactin receptor species were tested for their stability in the detergents Triton X-100 (1%), and deoxyBIGCHAPS (10mM) : Rat (the rat short form receptor wild type in pECE); Rat d3 (rat short form with an asparagine to alanine substitution that removes the third glycosylation site); T-47D (untransfected wild type human prolactin receptor from the T47-D cell line).

Table 8. Potential Glycosylation Sites in Prolactin Receptors

Mouse	G	G	L	P	T	N	Y	S	L	T	Y
Rat	G	G	L	P	T	N	Y	S	L	T	Y
Rabbit	G	G	L	P	T	N	Y	T	L	T	Y
Human	G	G	L	P	T	N	Y	S	L	T	Y
Mouse	Y	I	I	Y	V	N	A	T	N	E	M
Rat	Y	I	I	T	V	N	A	T	N	Q	M
Rabbit	Y	I	I	T	V	N	A	T	N	Q	M
Human	Y	I	M	M	V	N	A	T	N	Q	M
Mouse	P	E	P	P	R	N	L	T	L	E	V
Rat	P	E	P	P	R	N	L	T	L	E	V
Rabbit	P	D	P	P	V	N	L	T	L	E	V
Human	P	A	P	P	L	G	L	A	V	E	V
Alternate Site 3 in Human	S	D	F	T	M	N	D	T	T	V	W
								Membrane →			

Polymerase Chain Reaction

The polymerase chain reaction was used to modify the sequence of the human prolactin receptor cDNA (see "Transfections" below). PCR was also performed on reverse transcribed T-47D RNA with primers from the 3' and 5' untranslated regions, to

determine if there was any variation in the size of the receptor mRNA in these cells. Multiple sizes of prolactin receptor RNA are seen in these cells, yet Figure 16 shows that all of these forms appear to result in a transcript containing a coding region of uniform size. Therefore the differences in size of the several RNA species probably results from differences in either the 5' or 3' untranslated regions.

Transfections

Transfections with the human prolactin receptor using the pECE vector in COS-7 cells gave very low binding near to that of COS-7 untransfected COS-7 controls. This contrasted with experiments using the rat PRL-R with the same vector and cell line, where specific binding of 30% of total radioactivity was routinely obtained. In an attempt to improve transfection of the human receptor, mutations were introduced into the cDNA using PCR, to produce a complete Kozak[485] sequence, and second to remove the 5' and 3' untranslated regions from the pECE/hPRL-R construct. Unfortunately, no improvement in specific binding was observed in transfections with this construct. Therefore it is unlikely that the untranslated regions, or the incomplete Kozak sequence have an inhibitory effect on the expression of the receptor.

PCR Analysis of the Coding Region of T-47D RNA

Figure 16. PCR analysis of T-47D RNA. Primers from position 213 and 2318 of the cDNA sequence were used to produce a PCR product spanning the full length of the coding region. The control lane contained a reverse transcription reaction containing no RNA.

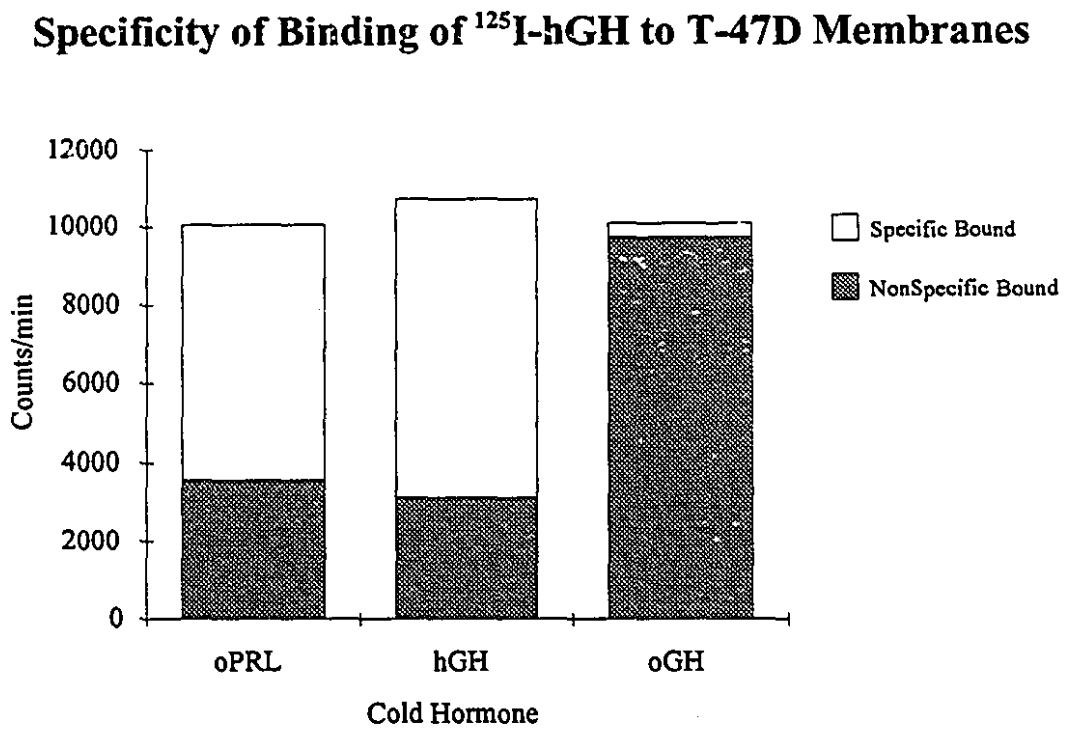

Figure 17. Binding specificity of ^{125}I-hGH to the T-47D membranes. The standard binding proceedure with the addition 1 μg of cold hormone for the calculation of specific binding.

Discussion

Purification

One of the most difficult aspects of purifying the human prolactin receptor was determining the optimal conditions for receptor solubilization. The binding observed with solubilized receptor had a much higher nonspecific binding than for membrane bound receptor. The high nonspecific binding was not due to an effect of the detergent on the ligand, since binding experiments using the same ligands and detergents with rat liver prolactin receptor had excellent specific binding despite high nonspecific binding (refer to Figure 15). Higher nonspecific binding with solubilized receptor could be due in part to the way in which bound labelled ligand was separated from free: precipitation of the ligand receptor complex with polyethylene glycol. Solubilizations were routinely performed with deoxyBIGCHAP, since it was the detergent that best preserved receptor activity.

During purification, a benzamidine-sepharose 6B protease affinity precolumn was included, since this column increased the yield of the rat prolactin receptor.[213] In pilot experiments, the final purified fraction contained no prolactin binding activity. Purification was therefore attempted without any lengthy manipulation such as dialysis, or delay such as overnight storage on ice or freezing. Binding assays of the $MgCl_2$ elution and G-100 elution fractions were incubated for 6 hr and 3 hr, respectively, at 30°C in order to reduce the time between the column elutions and the Scatchard analysis binding. These short incubation times produced a low amount of specific binding compared to values which could have been obtained at optimum conditions of 15 to 18 hr incubation at 20°C. Using this rapid approach, however, it was possible to obtain biological activity in the purified fractions. The yield of this purification was only 130 fold. In comparison, the yield observed for rat receptor purification using a similar ligand affinity purification technique was between 300 to 500 fold.[141] The lower degree of purification and the low recovery (25%) for the human receptor is probably due to its tendency to loose binding activity during purification.

Monoclonal Antibody Production

Approximately 4000 hybridoma supernatants were tested for reactivity to the human prolactin receptor. The lack of successs of these procedures can be explained in two ways. First, the fusions utilized T-47D membranes, which simply did not contain enough antigen to produce a useful immune response: only 0.0000001 % of membrane protein was prolactin receptor. Secondly, hybridoma supernatants were screened by binding inhibition using T-47D membranes, which often gave a specific binding no higher than 5.0%. It is thus probable that some weak antibodies were missed. Specific binding to T-47D microsomes improved after it was discovered that hGH had better binding to the human prolactin receptor in the presence of zinc ions.[79] Thirdly, screening by binding inhibition detects only antibodies with epitopes near the hormone binding domain, thus antibodies to other regions of the receptor were not detectable.

As for the fusion performed with the peptide ELAVEVKQPEDRKPY, several antibodies had high affinity for the peptide itself (by ELISA), but none had binding activity to the native receptor from T-47D cells. Comparison of the human prolactin receptor with the human growth hormone receptor (refer to Figure 14) reveals that this region in the growth hormone receptor comprises amino acids 136 to 152. In the human growth hormone receptor, the amino acids in this region are involved in a β-sheet on the surface of the molecule which forms the interface between the two receptor molecules in the hormone receptor complex.[212] This surface is composed of two noncontiguous segments of sequence: the region around the turn between β-strand C and C', and the beginning of the β-strand F.[212] Thus, if the human prolactin receptor has a conformation similar to the human growth hormone, it is possible that the peptide ELAVEVKQPEDRKPY is part of an epitope containing two noncontiguous sequences, which may explain why antibodies which bound very well to the peptide, failed to bind to the native receptor.

Immunoprecipitation

Immunoprecipitation of iodinated partially purified human prolactin receptor with the anti rat prolactin receptor polyclonal serum "212" produced a specific band of 38 000, which probably represents a degradation product of the receptor. Bands of similar size

have been observed in immunoprecipitation experiments with the pig and rabbit prolactin receptor.[487] This fragment is approximately the right size to be the extracellular domain of the prolactin receptor which, containing 210 amino acids, has an approximate molecular mass of 24 000 Da, plus the speculated size contribution of glycosylation of 19 000.

On the other hand, some evidence suggests that the fragment may not come from the extracellular domain. If it is supposed that the band seen in the human corresponds to those seen in the rabbit, it is probable that the break-point among the sequences would occur in the same place. It is also simplest to suppose that this fragment (which is relatively large) is produced from a single cleavage. If the fragment were to start at the N-terminal, it would contain the glycosylated segment of the sequence. Since there are differences in the potential glycosylation sites (the rabbit having three, and the human most likely having two) the differences in glycosylation would probably produce fragments of different sizes. Therefore, it is also possible to argue that this fragment could be derived from the intracellular region.

Crosslinking

Crosslinking of ^{125}I-hGH to the T-47D prolactin receptor produced several bands on SDS-PAGE. (refer to Table 6). It is unlikely that any of these bands represents the growth hormone receptor, since binding studies have indicated that ^{125}I-hGH cannot be displaced from the binding sites on T-47D membranes by non-lactogenic growth hormones (refer to Figure 17). It is proposed that the smallest band of 113 000 is a complex containing one molecule of hormone (25 000) and one molecule of receptor (88 000). The other bands may be combinations of the ligand and receptor molecules. Refer to Table 9 for the possible combinations of receptor and ligand which might constitute these bands. In particular, the combination of two molecules of receptor (88 000) and one molecule of ligand (25 000) for the band of 205 000 is especially interesting, since this represents the crystal structure observed in the human growth hormone binding protein - hGH binding complex. [212] The combinations presented in Table 9 are only speculative, and other possibilities exist. Other proteins may be involved in ligand binding and signal transduction of the prolactin receptor in a manner

similar to that observed for other cytokine receptors, and therefore may contribute to some of the bands observed in this experiment.

Table 9. Possible Subunit Structure of Crosslinked Bands

Size of Band	Subtract Ligand (25)	Possible Combinations
290	265	88+88+88+25=289
235	210	100+100+25=225
205	180	88+88+25=201
137	112	88+25+25=138
125	100	
113	88	

Associated Proteins

Recent experiments with the IL-6, IL-2, and GM-CSF receptors, have demonstrated that these receptors depend upon additional associated proteins for full biological activity. It is therefore possible that the same situation exists for the prolactin receptor. What evidence is there to support this possibility? Solubilization of the receptor often leads to an increase in receptor affinity. This could be explained by the influence of phospholipids and detergents upon receptor conformation or energy, or by the influence of associated proteins, whose coupling to the receptor may be influenced by detergent. As was pointed out in the Introduction, the affinity of several cytokine receptors is influenced by associated proteins, usually leading to an increase in receptor affinity for the ligand.

Crosslinking data, showing multiple high molecular weight specific bands, strongly points to the possibility of associated proteins existing for the prolactin receptor. For instance, the second most intense band of 235 kDa could be composed of one receptor molecule, one hormone molecule and one gp130 molecule (combined size of 243 kDa), and if the associated protein was a bit smaller than 130, the largest band of 290 kDa could be composed of one hormone molecule, two receptor molecule and one associated protein.

Observations made studying the growth hormone receptor have shown that there is a tyrosine kinase activity which is tightly associated with the receptor.[488] Testing this activity with various inhibitors indicated that the kinase is not protein kinase C or A, casein kinase, or a calcium/calmodulin-dependent protein kinase, and is probably a MAP kinase.[489] Other studies have demonstrated that there is a protein with kinase activity associated with the growth hormone receptor of molecular mass 120 kDa.[490] For the prolactin receptor, recent work demonstrates the existence of an associated tyrosine kinase activity which co-precipitates with the prolactin receptor in Nb2 cells.[491] This associated protein also has a molecular weight of 120 000.

Another recent observation suggests the possibility that G proteins may be associated with the prolactin receptor. [^{32}P]-ADP-ribosylated G-proteins crosslinked to neighbouring proteins with EGS, a chemical crosslinker, possessed immunoreactivity to an anti-prolactin receptor antiserum.[272]

Protein Instability

Clearly, there are differences in stability between the prolactin receptor in humans and that found in other species. Both solubilization and $MgCl_2$ desaturation greatly reduce the biological activity of the human receptor. Sequence analysis revealed several differences in the human receptor. There are three regions of divergence, centred around amino acids 50, 95, and 200. Essentially, the human receptor differs from other species in that it is more hydrophobic in these regions and has a greater tendency to form α-helices in two of these regions. The first and third region of divergence correspond to regions of low amino acid conservation as seen in Figure 13.

These analyses indicate divergences in the propensity for specific conformations in the human sequence. The tests used to determine these differences compute the probabilities of protein conformation based on the physico-chemical properties of the amino acids constituting a sequence, and on the form such sequences take in known protein structures. As such, these tests are not concrete, and definite images of the differences in conformation between the human and other prolactin receptors may require x-ray crystallography. Even so, these tests provide a basis for further analysis of receptor structure by such methods as mutagenesis.

The human receptor most likely contains only two N-linked glycosylation sites while the receptors from other species contain three. In spite of this, the contribution of carbohydrate to the relative molecular weight in the human is 21 000, while in the rat it is 8 to 12 000.[234,244] The absence of glycosylation in the third position is not enough to explain the human receptor's lability, since, unlike the human receptor, the rat mutant receptor missing the third site was not adversely affected during Triton X-100 solubilization (refer to Figure 15). Whether or not the higher proportion of carbohydrate in the human is important here could be tested by deglycosylation studies, either through enzymatic removal, or by point mutation removal of the glycosylation sites. In order for mutational analysis to work however, the difficulty in expressing human receptor in transfections would have to be overcome.

Human prolactin receptor instability, and poor binding activity in transfections, could be explained by an increased dependence upon an associated protein. It is possible that solubilization, and $MgCl_2$ desaturation during purification, could remove this associated protein. Likewise, the COS-7 host cells in the transfections performed here might lack such an associated protein, and thus the ability to produce high affinity binding.

Chapter 3. Prolactin Receptor Regulation

Materials and Methods

Studies on the regulation of prolactin receptor were performed on T-47D cells,[472] and, to a lesser extent, on MCF-7 cells.[515] Cell culture reagents, and standard laboratory chemicals were obtained as indicated in Chapter 2. Phenol red free EMEM, estrogen, progesterone, testosterone, cortisol, thyroid hormone, PMA, and insulin were obtained from Sigma Chemical Co., St. Louis, Mo.. EGF, TGF-β, and IGF-1, and T4 polynucleotide kinase were obtained from Gibco/BRL Canada, Burlington, ON. Forskolin and retinoic acid were obtained from ICN Chemicals, Mississauga ON. C_{18} Sep-paks and oligomer synthesis reagents were obtained from Millipore, Missisauga, ON. Nitrocellulose and nylon membranes were obtained from Amersham (Amersham Canada Ltd., Oakville, ON). Oligo-dT cellulose, and S1 nuclease was obtained from Pharmacia.

Cell Culture

T-47D cells were routinely cultured in EMEM with 5% NBS and 5% FBS. Prior to the addition of hormones, cells were cultured in phenol red free medium containing 10% lactogen stripped serum (LSNBS) for two days, with a media change each day. Phenol red free medium was used to avoid the estrogenic activity of phenol red in cell culture.[492] For the studies of RNA regulation, cells were rinsed with EMEM and cultured in phenol red free serum free medium, containing EMEM; 0.1% lipid free BSA; transferrin, 1 μg/mL; glutamine, 4 mM; pyruvate, 1 mM; inositol, 18.2 μg/mL; vitamin B_{12}, 100 ng/mL; ascorbic acid, 25 μg/mL; $CuSO_4 \cdot 5H_2O$; 25 ng/mL; SeH_2O_3, 1

ng/mL; and $ZnSO_4 \cdot 7H_2O$, 100ng/mL. Serum was stripped of lactogens using the protocol of Biswas [471]. Steroids were dissolved in 100% ethanol, then serially diluted in 50% ethanol in sterile water. Steroids were added to cultures in 50% ethanol with a concentration and volume such that the final ethanol concentration in the medium was 0.01%. Cells were cultured either in T-175 tissue culture flasks or P-150 tissue culture plates.

Prolactin Receptor Protein Measurement

Whole cell binding and Scatchard analysis were performed in a way similar to that reported in Chapter 2.

RNA Extraction

RNA was extracted from cultured cells by the acid/phenol method of Chomczynski *et al.*[493] Five mL of denaturing solution (containing guanidinium thiocyanate (GTC) 4 M, sodium citrate, pH 7, 25 mM, sarcosyl, 0.5%, and 2-mercaptoethatnol 0.1 M) was added to each P-150 culture dish. Once the cells were dissolved, this solution was transfered to a 15 mL conical centrifuge tube and homogenized for 10 s to cleave the DNA. Then 250 µL of sodium acetate 4 M was added, followed by 5.0 mL of water saturated phenol, and 1.0 mL of 49:1 chloroform isoamylalcohol, with vortexing after the addition of each solution. The mixture was cooled on ice for 15 minutes and then centrifuged at 3500 rpm at 4°C for 15 min. The aqueous supernatant was removed and mixed with one volume of isopropanol and stored overnight at -20°C then centrifuged to precipitate the RNA. The RNA pellet was resuspended in 3 mL of denaturing solution and precipitated with 2.5 volumes of ethanol at -20°C overnight followed by centrifugation. The RNA pellet was washed two times in 70% ethanol in water to remove any traces of GTC. RNA was measured by spectrophotometry at 260 nm. The ratio of 260/280 was rarely lower than 1.7.

Northern Blotting

T-47D RNA was extracted as above and purified on oligo-dT cellulose.[494] Poly A RNA was loaded onto a 1.2% agarose gel, and transferred to nitrocellulose or nylon membranes by capillary transfer in 1 M sodium chloride. After baking the membranes at

80°C for two hours, membranes were prehybridized in hybridization buffer (50% deionized formamide; sodium phosphate buffer, pH 6.5, 50 mM; sodium chloride, 0.8 M; SDS, 0.5%; EDTA, 1mM) containing 500 μg/mL yeast RNA, and 100 μg/mL denatured salmon sperm DNA, for 2 hours. Hybridization was performed in hybridization buffer overnight at 42°C with 500 000 cpm/mL of probe. Membranes were washed 3 times in washing solution containing sodium chloride, 12.5 mM; and SDS, 0.125%.

Quantitative S1 Nuclease Protection Analysis

S1 nuclease protection experiments were done with synthetic oligonucleotides based upon published methods.[495] Sequences were chosen which contained no more than three contiguous A's or T's, since it was found that S1 nuclease had background cleavage activity on double stranded sequences of more that three A's or T's in a row. To ensure adequate hybridization, probes were no shorter that 40 bp. In order to determine the efficiency of digestion, a noncomplementary sequence was placed on the 3' end so that digested and nondigested probe could be resolved after separation on an 8% polyacrylamide sequencing gel. Hybridization of the probes was verified by Northern blotting with T-47D RNA.

Table 10. Quantitative S1 Nuclease Protection Assay Probes

HPR3S1 5'**CCCAGCTGGAGCCTGCACTTGCTTGATGTTGCAGTGAAGTTGGCCAGGG***AGGGAGACTA*3'

S141S1 5'**CATACCCGAGCGGGCAAGGGCTGTGAGGGCCGACTGGGC***AAACTTGAAGTTTTAAA*3'

Table 10 indicates the probes used in the quantitative S1 nuclease protection assay. The sequence in bold indicates the complementary region of the probe and the region in italics indicates the non-complementary region used to determine the efficiency of enzyme digestion.

Oligomer sequences were synthesized with a Biosearch Cyclone DNA Synthesizer (New Brunswick Scientific), and released from the column matrix with incubation for 2

h in 30% ammonium acetate. The matrix was removed by centrifugation, and the supernatant incubated overnight at 55°C. Oligomers were then lyophilized. Purification of oligomers was performed by PAGE, followed by diffusion elution from the section of the gel containing the oligo. C_{18} reverse phase batch chromatography was performed upon the eluate. A C_{18} sep-pak was washed with 10 mL acetonitrile, 10 mL methanol, and 10 mL water whereupon the sample was applied to the column by gravity. The column was washed with 10 mL water and then the sample was eluted with 60% methanol in water.

Purified oligomers were labelled with T4 polynucleotide kinase under conditions which transferred ^{32}P to 92% of oligomer molecules. 10 μL of oligomer (30 pmol), 15 μL of [^{32}P] ATP (7000 Ci/mmol), 3.1 μL of 10X kinase buffer (containing Tris-HCl pH 7.6, 500 mM; $MgCl_2$, 100 mM; DTT, 50 mM; spermidine hydrochloride, 1 mM; and EDTA, 1 mM) and 3μL of T4 polynucleotide kinase were mixed, and incubated for 2 h at 37°C. The labelled oligomer was separated from the free [^{32}P]ATP by electrophoresis on a 8% nondenaturing polyacrylamide gel , followed by diffusion elution from the excised band with two volumes of 500 μL water.

Probes were hybridized to 50 μg of sample total RNA with excess probe (100 000 cpm, or 0.3 ng), in a total volume of 30 μL, with 9 μL of buffer containing sodium chloride, 3 M; HEPES, pH 7.5, 0.5 M; and EDTA, pH 8.0, 1 mM. Samples were heated for 10 min. at 75°C, then incubated for 3 h at 70°C.

The samples were then digested. S1 nuclease, 550 U, 2 μL, was added along with 3 μL denatured calf thymus DNA, 115 μL water, and 150 μL of buffer containing sodium chloride, 0.56 M; sodium acetate, pH 4.5, 0.1 M; and zinc sulphate, 9 mM. Digestion was allowed to proceed for 1 hr. at 37°C, whereupon the reaction was terminated with the addition of 3 μL EDTA, pH 8.0, 0.5 M; 1 μL transfer RNA, 10 mg/mL; and 700 μL ethanol. Samples were stored overnight at -20°C. The samples were centrifuged, and the RNA pellet was resuspended in 10 μL sodium hydroxide, to which 10 μL formamide loading buffer was then added.

Samples were loaded onto an 8% sequencing gel, and exposed to Kodak XAR film for 2 to 7 days. Quantification of samples was performed by scanning densitometry, and values for prolactin receptor mRNA were normalized according to S14 gene expression.

Experiments were carried out using the T-47D cell line, and the abundance of human prolactin receptor was measured, using the expression of the S14 ribosomal protein gene[496] as a control. Each experiment was performed in triplicate.

Results

Prolactin Receptor Protein Regulation

Figure 18 shows a Northern blot using the HPR1S1 probe, used in the S1 nuclease protection assay to detect the prolactin receptor mRNA in T-47D cells. Major bands of 2.8 kb and 3.6 kb were detected. Figure 19 illustrates the quantitative nature of the S1 nuclease protection assay using T-47D mRNA. The darker bands at the top of the figure are a remnant of undigested probe. Although it appears intense here it represents a small fraction of probe added to the reaction mix. Since the protected fragment was run on a sequencing gel, it is possible to observe slight variations in the size of the protected region of duplex DNA. It appears that S1 nuclease is able to digest the ends of a DNA duplex during random "breathing" of the duplex. Both the remnant of undigested probe, and the "fuzzy" nature of S1 nuclease digestion are normal observations for this technique and cannot be avoided.[497]

Regulation of prolactin receptor by prolactin was observed in T-47D cells in whole cell binding assays. Figure 20 shows the effect of oPRL on specific binding of ^{125}I-hGH to whole T-47D cells. The percentage of receptor occupancy was calculated based on the affinity of the prolactin receptor measured by Scatchard analysis, and the concentration of hormone in the culture media. With high concentrations of hormone in the culture media, most receptors would have been already occupied prior to the binding assay. Therefore, reduced specific binding could be

Northern Blot Analysis of T-47D RNA with S1 Nuclease Probe for Human Prolactin Receptor

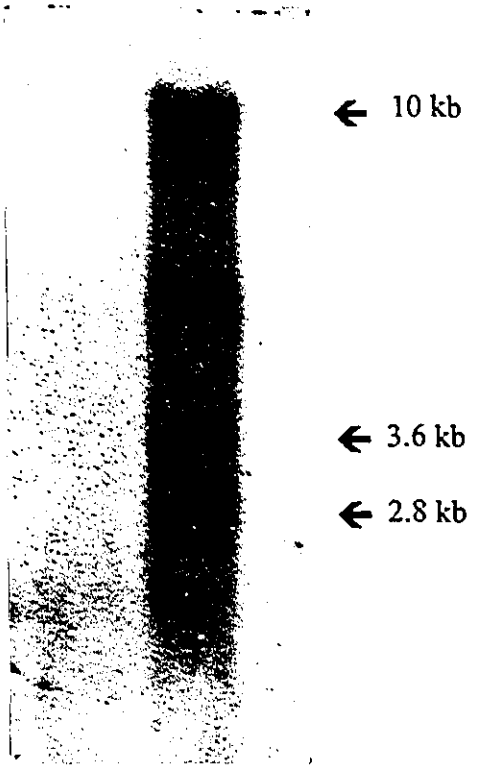

Figure 18 Northern Analysis was performed with 17 μg of T-47D mRNA, and probed with the S1 nuclease protection assay probe HPR1S1. Major bands had the relative size of 10, 3.6, and 2.8 kb, and several minor band of relative size 9.2, 8.4, 5.9, 5.2, and 1.6 kb appeared.

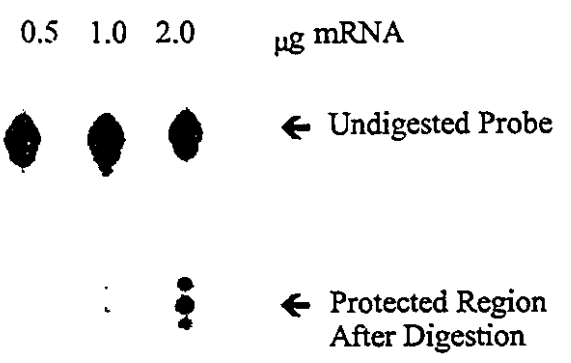

Figure 19. Three quantities of T-47D mRNA were hybridized to the HPR1S1 probe, and digested with S1 nuclease as described in Materials and Methods.

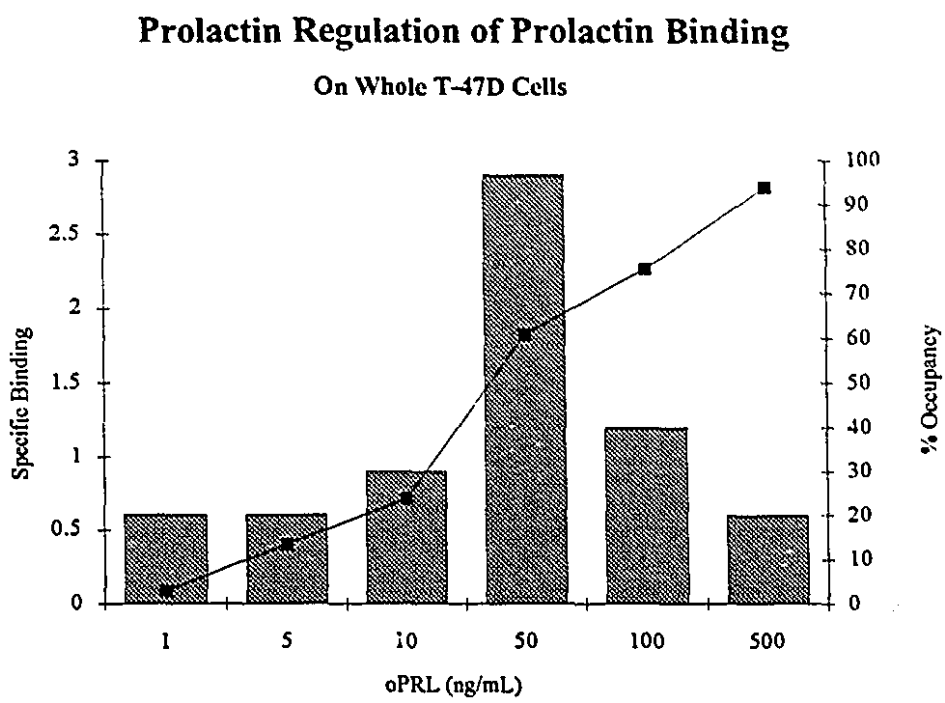

Figure 20. T-47D cells were cultured were cultured in EMEM with insulin (5 ug/mL), estradiol (1e-10 M), 5%NBS and 5%FBS for 3 days with various concentrations of oPRL. Cells were washed 1 hr. in EMEM, then equal numbers of cells (determined by hemocytometer) were assayed for prolactin receptors in a 4 hr. whole cell binding assay with ^{125}I-hGH as label in a buffer containing EMEM, .1% BSA, and 50 mM HEPES (pH 7.4) at 20°C. Receptor occupancy was calculated from the affinity constant for prolactin binding in microsomes of 1.4 nM.

attributed to two things, either receptor occupancy, or a reduction in receptor number (or down regulation). When the calculated percentage occupancy is added to the observed specific binding, the "theoretical" number of prolactin receptors appears to increase in a dose dependent manner even up to 500 ng/mL.

In Scatchard analysis, a significant induction of receptor numbers is seen with estrogen and progesterone. Figure 21 shows that estrogen has a maximum induction of prolactin receptor at 100 pM, and progesterone has maximal response at 10 nM. When estrogen and progesterone are combined there is an additive effect on receptor induction with the overall highest induction of receptor being observed at concentrations of estrogen at 1 nM and progesterone at 1 nM.

Prolactin Receptor RNA Regulation

The effects of estrogen, progesterone, cortisol, human growth hormone, insulin, thyroid hormone, testosterone, insulin like growth factor (IGF-1), epidermal growth factor (EGF), transforming growth factor beta (TGF-β), fetal calf serum, phorbol 12-myristate 13-acetate (PMA), forskolin, and retinoic acid were tested for their effect on the regulation of human prolactin receptor. Figure 22 shows the effect of hGH and insulin on receptor expression after 2 days incubation. There were no significant differences on receptor expression, as determined by single factor ANOVA. Figure 23 shows the effect of progesterone and estrogen on receptor expression after three days. There appears to be some change in expression, especially in response to progesterone, although this failed to achieve statistical significance at the 0.05 when tested by single factor ANOVA. However when single treatments were compared against the control, by the two-sample F-test, all treatments except estrogen at $1x10^{-10}$ M were significantly different at the 0.05 level and progesterone at $2x10^{-9}$ M was significantly different at the 0.01 level. When IGF-1, EGF, TGF-β, and FBS where tested, single factor ANOVA showed no significant differences among these treatments (refer to Figure 24.)

Figure 25 shows the effects of cortisol, forskolin, retinoic acid and thyroid hormone on receptor expression. Single factor ANOVA indicated a highly significant difference ($p< 0.005$) among treatments. Analysis by the two sample for variance F-test showed that thyroid hormone and forskolin stimulation of prolactin receptor RNA was

Prolactin Receptor Regulation in T-47D Cells

As Determined By Scatchard Analysis

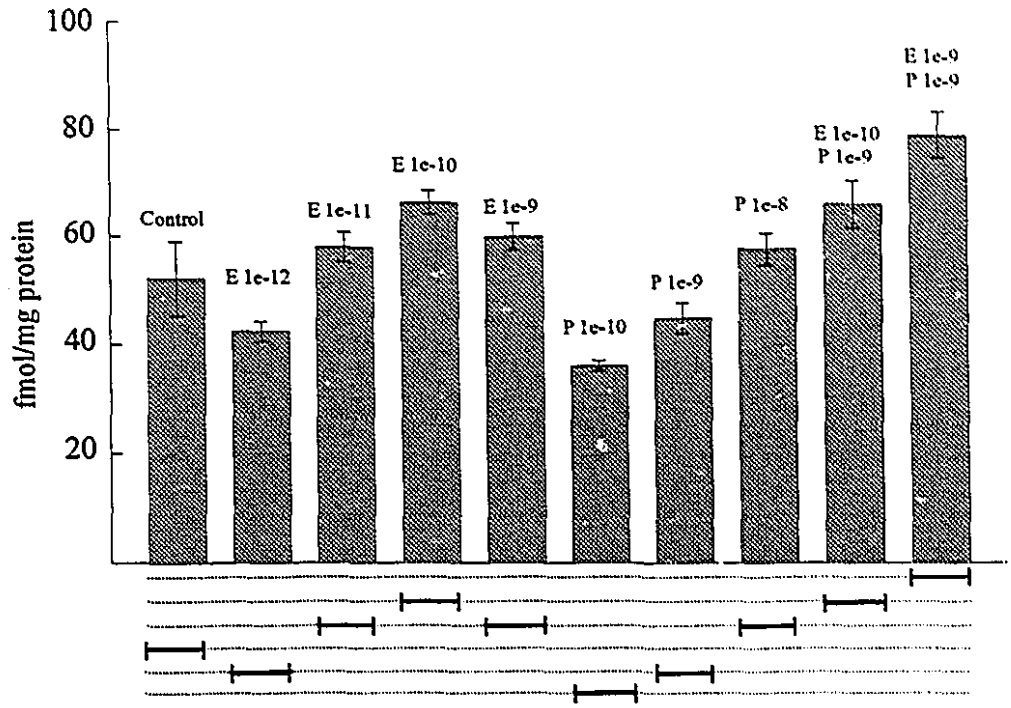

Figure 21. T-47D cells were cultured for 3 days in phenol red free EMEM with 10% lactogen stripped serum with various concentrations of estradiol and progesterone. Scatchard analysis was determined by competition between ^{125}I-hGH and cold hGH with 400ug/tube of cell membrane preparation. Error bars indicate the standard deviation around the X intercept of the Scatchard plot. Significant differences among the treatments tested as determined by the Newman-Keuls multiple comparisons test at the 0.05 significance level are indicated by bars at the bottom of the graph. Bars on different lines indicate significant differences.

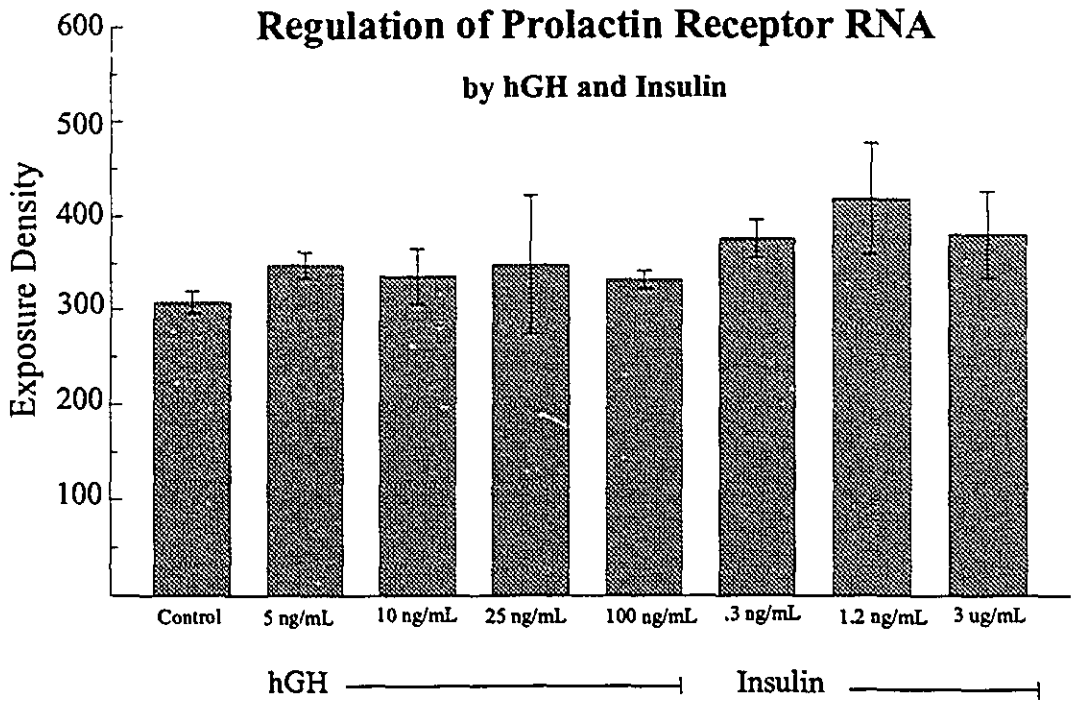

Figure 22. The effect of human growth hormone (hGH) and insulin on human prolactin receptor mRNA steady state levels was tested in the T-47D cell line. Cultures were incubated in hormone for 2 days. Each treatment was performed in triplicate. There were no significant differences among the treatments tested as determined by single factor ANOVA.

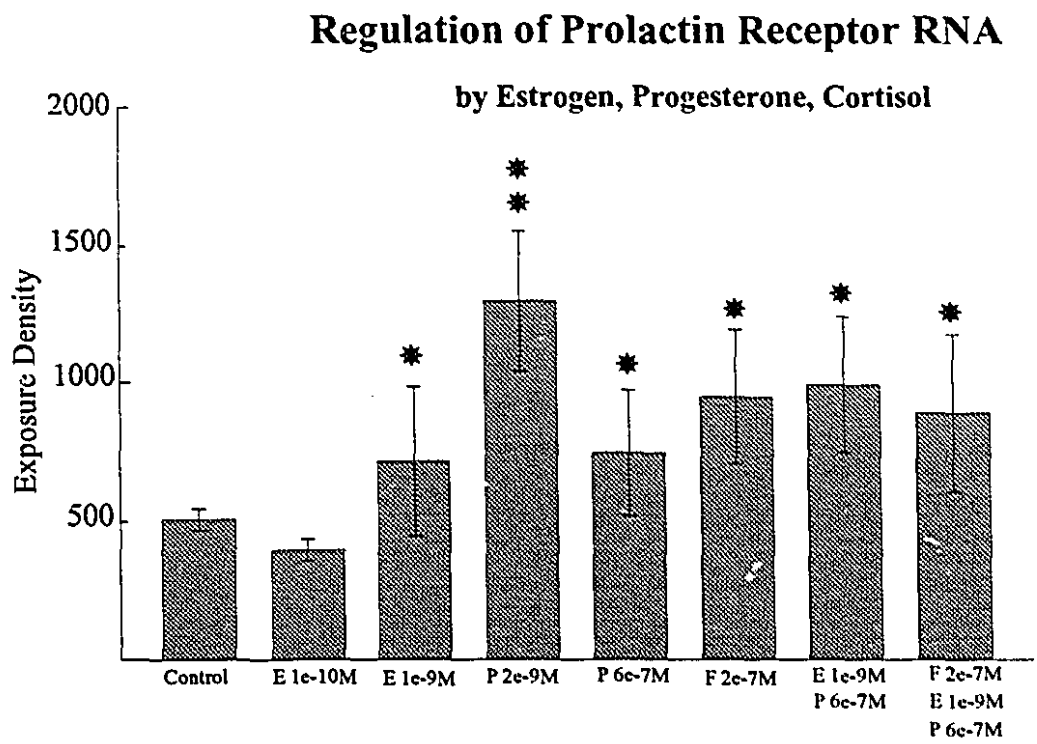

Figure 23. Estrogen (E), progesterone (P) and Coritsol (F) were tested for their effect on the steady state levels of human prolactin receptor mRNA in T-47D cells. The unit represented is mol/L (M). Cells were cultured in hormone for three days. Each treatment was performed in triplicate. There was no significant difference among treatments tested as determined by single factor ANOVA, although a highly ($p<0.01$) significant difference was found between progesterone at 2×10^{-9} M and control using the 2 factor for variance F-test. Significant differences ($p<0.05$) were indicated for all treatments except for estrogen at $1x\ 10^{-10}$ M.

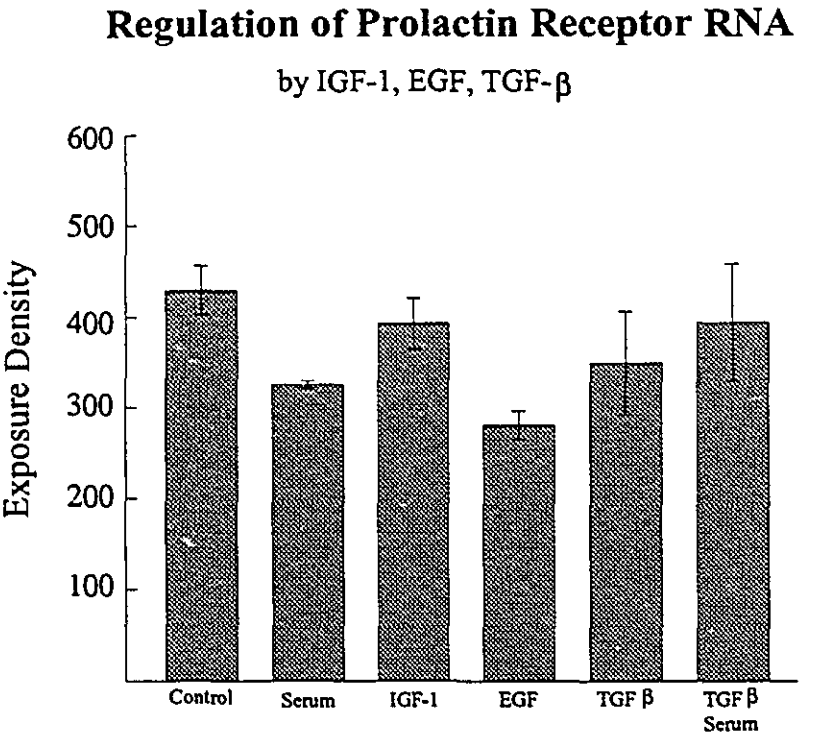

Figure 24. T-47D cells were incubated for 2 days in either 5% FBS, insulin-like growth factor-1 (IGF-1, 10 ng/mL), epidermal growth factor (EGF, 5 ng/mL), transforming growth factor-β (TGF-β, 10 ng/mL). Each treatment was performed in triplicate. There were no significant difference among treatments as determined by single factor ANOVA.

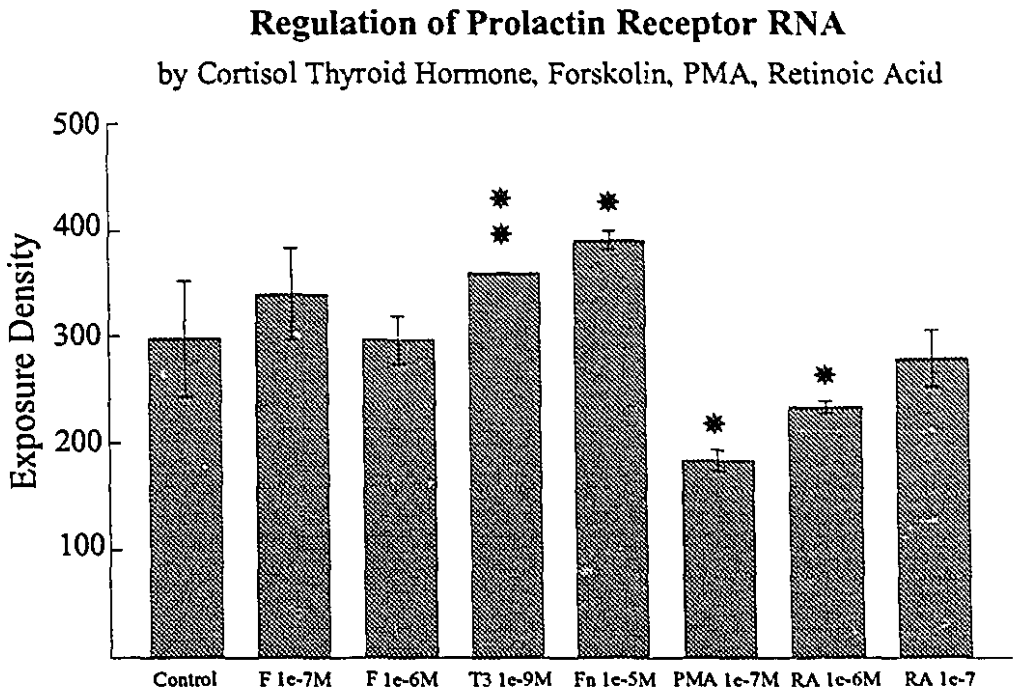

Figure 25. For 3 days T-47D cells were cultured in cortisol (F), thyroid hormone (T3), forskolin (Fn), phorbol-myristol-acetate (PMA), and retinoic acid (RA). Each treatment was performed in triplicate. Each treatment was performed in triplicate. A highly significant difference ($p<0.005$) among treatments was indicated by single factor ANOVA. The two factor for variance F-test indicated significant differences between control and thyroid hormone, forskolin, Phorbol-myristol- acetate, and retinoic acid.

significant, and that the inhibition of receptor RNA by PMA, and the high dose of retinoic acid was significant.

Discussion

Prolactin secretion is under complex negative control from the CNS. Prolactin secretion serves as a signal, a method of communication between the brain and the distant target organs. Prolactin receptors convey the information contained in the concentration of serum prolactin to the interior of the target cell, where the final response occurs. Prolactin receptor number may therefore be an essential factor in the modulation of the intensity of prolactin action. Thus it is useful to study the regulation of prolactin receptor expression in breast cancer cells to further the understanding of the role of prolactin in breast cancer. Human tissues in general contain low concentrations of prolactin receptor, breast cancer cells are one of the few models which permit the study of the prolactin receptor in humans.

The present study shows that the prolactin receptor protein in breast cancer cells is regulated by lactogenic hormones. Treatment of T-47D cultures with oPRL causes a 5 fold increase in specific binding. This response is probably much greater, since some receptor would not have bound ^{125}I-hGH, because of prior occupancy due the oPRL treatment. The effect of lactogenic hormones on the regulation of prolactin receptor has been well studied in the rat liver, where there has been some controversy over whether or not lactogens do induce prolactin receptors. A number of studies have shown that prolactin causes both an increase in receptor binding, at low to moderate concentrations, and a down regulation of receptor binding at high concentrations of the treatment hormone (Refer to Table 3). These observations were made in both liver tissue from treated animals and in *in vitro* experiments on rat hepatocytes. In *in vivo* experiments, treatment with bromocryptine reversed this effect. It must be noted, however, that several studies found an increase in receptor binding only when somatogenic hormones were tested and found no change in binding when lactogenic hormones were tested.[337,338,339] Lactogenic hormones have also been observed to increase receptor

binding in rat testis, hamster testis, and *Tilapia* gill and kidney (Refer to Table 3). Prolactin receptor binding in the mammary gland has been shown to be stimulated by prolactin in pseudopregnant rabbits. The experiment shown in Figure 20 is the first study to demonstrate that lactogens increase receptor binding in human mammary tissue.

Figure 22 shows the effect of hGH and insulin on prolactin receptor regulation at the RNA level. In contrast to the effect seen on receptor protein, lactogens seem to have no effect on receptor mRNA levels in T-47D cells. It has been suggested that prolactin can regulate RNA by both transcriptional control, and RNA stability.[39] The results presented here demonstrate that prolactin can regulate the number of receptor protein molecules without producing any marked effects at the RNA level, suggesting that prolactin may produce an effect upon the regulation of translation, or through changes in receptor protein turnover. Similar observations have been made in other species. For example, when the normal expression of prolactin receptor was studied in female rats, receptor mRNA levels start to rise on day 21, and continue until day 40, when they plateau. Receptor number rose along with RNA levels. However, by day 80, receptor number continued to rise to over twice the day 40 levels, despite a constant level of receptor RNA.[314] In the same study, in adult female rats treated with estrogen, mRNA levels rose 3-fold by day 3 then levelled off, yet receptor binding continued to increase past day 3 up to at least day 7.[312] In pregnant rabbits, even though mRNA levels peaked at day 15 of pregnancy, and were declining by day 20, the binding levels continued to increase.[242]

The experiment in shown in Figure 21 shows that estrogen and progesterone have a significant stimulatory effect on prolactin receptors in T-47D cells. Previous work has shown that estrogen increases receptor number in rat liver (Refer to Table 3). It is thought that estrogen induces prolactin receptor in the rat liver via an indirect mechanism, through stimulation of prolactin release at the pituitary. Since estrogen was not able to induce prolactin receptors in hypophysectomized rats.[92] Evidence presented here concurs with observations made in EFM-19 cells that estrogen has a direct effect on prolactin receptor number in mammary tissue.

The effect of progesterone is also seen at the RNA level (refer to Figure 23). Progesterone produced the greatest induction of prolactin receptor RNA of any hormone tested. There is some controversy about the action of progesterone on prolactin receptor regulation. It has been demonstrated that progesterone inhibits the ability of prolactin

injection to increase prolactin binding in pseudopregnant rabbit mammary gland.[326] Blockage of progesterone action by the progesterone antagonist RU 486 produced an accumulation of prolactin receptor RNA prior to the normal start of prolactin receptor expression in pregnant rabbit mammary gland.[242] Progesterone has an inhibitory action on milk protein synthesis, and it has been suggested that the inhibitory action of progesterone includes an inhibitory effect on prolactin receptor expression. However in the experiments cited above, progesterone was only shown to counter the induction of prolactin receptors by prolactin, and so it is unclear from these studies whether the inhibitory effect of progesterone on prolactin action includes a direct inhibition of prolactin receptor expression. However, other evidence promotes the notion that progesterone inhibits the expression of prolactin receptor. Progesterone at concentrations of $1x10^{-7}$ and $1x10^{-6}$ M significantly reduced specific binding in comparison to control cultures in the EMF-19 cell line.

Nevertheless, other groups have also observed an increase in prolactin receptor binding in response to progesterone. All these observations share one common feature: all have been made in the T-47D cell line. Is the T-47D cell line unique? Very few breast cancer cell lines have been tested for regulation of prolactin receptor by progesterone. Most studies of prolactin receptor in human breast cancer cells have focused on the T-47D cell line, since this cell line has a relatively high number of prolactin receptors. It is thus difficult to asses the normal situation: an increase of prolactin receptor in T-47D cells, or a decrease in prolactin receptor number as seen in EFM-19 cells. However, there is one unique feature of T-47D cell line. These cells have a constituitively high number of progesterone receptors. T-47D cells have four copies of the chromosome containing the progesterone receptor on chromosome 11.[498] The progesterone receptor is under the regulation of estrogen. However, in T-47D cells, progesterone receptor is expressed in the absence of estrogen, indicating that there may be some abnormality in progesterone receptor expression in at least one of these four copies. What form this abnormality takes, and whether this could effect regulation of the prolactin receptor in these cells is still obscure.

When growth factors and serum were tested for effects on prolactin receptor RNA, as seen in Figure 24, no significant differences were seen in treatments with EGF, IGF-1,

TGFβ, or 5% FBS. However in another experiment, PMA had a significant inhibitory effect on T-47D RNA levels, as seen in Figure 25.

PMA had no effect on prolactin binding in rabbit mammary explant cultures, but inhibited prolactin induction of casein RNA.[348] In rat lymphoma cell Nb2, short term incubation of 4 h caused a 20% increase in cell surface receptor number, and a 20% decrease in intracellular receptor number,[499] whereas longer tern incubations inhibited the level of prolactin binding.[350] In mouse mammary epithelial cell cultures, PMA caused a 50% reduction in prolactin binding,[349] as well as inhibiting milk protein expression. In mammary epithelial cells, PMA acts as a mitogen,[500] and so cells treated with PMA loose their differentiated characteristics as they move to the proliferative state. Even in species, such as the mouse, where prolactin is a mitogen in the mammary gland prolactin receptor numbers are inhibited by PMA. EGF and PMA appear to share a similar signalling pathway[349], and although EGF failed to achieve a statistical significance in the experiment in Figure 24, EGF tended to reduced prolactin receptor RNA expression. It appears that EGF and prolactin have opposing effects in the mammary gland when it comes to the regulation of milk proteins and the prolactin receptor.

Forskolin, an adenyl cyclase activating drug, induces prolactin receptor RNA (refer to Figure 25). It is unknown whether this action is due to the effect of cAMP on protein kinase A, or to the action of cAMP on one of the cAMP binding protein transcription factors.

Retinoic acid significantly reduces prolactin receptor expression at a dose of $1x10^{-6}$ M. This is consistent with the known actions of retinoic acid in the mammary gland. Retinoic acid inhibits prolactin's morphogenic and mitogenic action in mouse mammary gland organ cultures.[501] Retinoic acid has little direct effect on mammary physiology, but rather acts as a permissive factor, modulating the effects of other hormonal signals. For example, in mouse mammary explant cultures, retinoic acid enhances EGF inhibition of milk protein expression, and mitogenicity, although it has no effect on these parameters itself.[502] However retinoic acid does upregulate EGF receptor expression in this system.

Thyroid hormone induces prolactin receptor expression in the mouse mammary gland, and does so in T-47D cells as well (shown in Figure 25). It would be interesting

to investigate the effect of combinations of retinoic acid and thyroid hormone on prolactin receptor expression, since although they have cooperative effects in the induction of MMTV expression,[503] each one alone has opposite effects on the expression of prolactin receptor. The interactions of retinoic acid and thyroid hormone are complex, as their receptors both interact with a similar *cis* element in DNA sequences.

Based on these studies it can be concluded that the prolactin receptor RNA in mammary epithelia is regulated by external signals in only a slight way since changes in expression did not exceed a 2-fold difference from control. These results indicate that future research should focus on non-transciptional methods expression regulation such as the regulation of translation, or changes in receptor protein turnover.

The prolactin receptor sequence contains features which could be involved in the control of translation. Efficient translation depends upon 5 factors: i.) the m7G cap, ii.) the sequence adjacent to the AUG (Kozak sequence), iii.) the presence of upstream AUG's, iv.) secondary structure, and v.) the leader sequence. The prolactin receptor transcript possess 6 upstream AUG sequences (refer to Table 11). Two of these have the -3 A which in itself is enough to cause most ribosomes to start translation.[504] The presence of upstream AUG's in good context would completely preclude translation of the transcript from the normal start site at 285, if it were not that these AUG's begin small open reading frames which terminate before position 285. Under these conditions, reinitiation of translation can occur,[504] though the net effect is a reduction in efficiency of translation from the downstream AUG at position 285.[494] The 5' untranslated region of the human prolactin cDNA is GC rich, which indicates a tendency to form secondary structures. This would have the effect of reducing the efficiency of translation since recognition of the start site by the initiation complex could be blocked by secondary structure, and scanning of the complex along the leader sequence could be slowed by secondary structure.

Another intriguing feature of the prolactin receptor is the existence of multiple exons 5' to the start of translation. Each of these exons may become the leader sequence in the mature mRNA through differential splicing, depending upon the tissue (Dr. D. Banville, personal communication). An example where this phenomenon leads to regulation of expression is the rat preproenkephalin mRNA: differences in expression between testes and brain derive from the use of different promoters which produce leader sequences.[505]

Similarly, switching to a leader sequence which permits a more efficient translation can be stimulated by an external cue such as serum, or retinoic acid.[506]

A recent analysis of vertebrate mRNA's has revealed that many regulatory genes, such as proto-oncogenes, growth factors, cytokines and their receptors, kinases and transcription factors, are burdened with GC rich leader sequences and upstream start sites. This has lead to the speculation that inefficient translation might be necessary to control the expression of these important molecules. It is possible that translation of these transcripts be regulated by derepression, through phosphorylation of the translational machinery, or the activation of helicases.[506]

Table 11. Start Sites for Translation in the hPRL-R transcript.

Position				-3			+1 Start			4
111-149	C	A	C	**A**	C	A	A	U	G	U
121-171	A	G	C	U	U	C	A	U	G	U
152-229	C	G	A	C	U	G	A	U	G	U
199-249	C	U	A	U	U	C	A	U	G	**G**
218-229	C	U	G	**A**	G	G	A	U	G	C
229-249	U	U	C	C	A	C	A	U	G	A
285-2150	G	C	C	**A**	A	C	A	U	G	A
Kozak Sequence	G	C	C	**A** G	C	C	A	U	G	**G**

Chapter 4. Breast Cancer Responses to Prolactin

Materials and Methods

DABA was purchased from Sigma Chemical. Standard laboratory chemicals were obtained from Fisher Scientific Montreal, PQ, or from BDH Ville St. Laurent, P.Q.. Studies were performed on the established cell lines EFM-19,[507] obtained from the authors; MCF-10[508] obtained from the authors; ZR-75-1,[509] HBL-100,[510] BT-474,[511] MDA-MB-134-VI,[512] T-47D,[472] Hs578T,[513] MDA-MB-231,[512] MDA-MB-361,[514] MCF-7,[515] were all obtained from the American Type Culture Collection. Additional MCF-7, lines were obtained from Dr. B. Vonderhaar, Laboratory of Tumor Immunology and Biology, Bethesda, Maryland; and the Michigan Cancer Foundation. MCF-7 cells from the American Type Culture Collection are designated as MCF-7T, from Dr. Vonderhaar as MCF-7V, and from the Michigan Cancer Foundation as MCF-7M. Earls modified eagles medium, and fetal and newborn bovine serum, NUNC culture plastics, and restriction enzymes were obtained from Gibco/BRL Canada (Burlington ON).

Cells were routinely grown in EMEM supplemented with 10 mM HEPES, 10 pM estrogen, and 10 ng/mL oPRL with 5% NBS and 5% FBS. For growth studies, cells were plated at a density of 10 000 cells/cm^2 in either serum free medium (see page 78) or in phenol red free EMEM with 10 mM HEPES, and 5 or 10% LSNBS. Steroids were dissolved in 100% ethanol, then serially diluted in 50% ethanol. A thousand fold concentration of steroid was added to culture such that the final ethanol concentration in the medium was 0.01%.

DNA was measured by a modification of the method of Kissane and Robbins.[516] Cells were harvested in 5 mM EDTA/PBS by scraping with a rubber policeman, and pelleted in a desk-top centrifuge. To the cell pellets, 100 μL of water was added, and the

cell suspension was exposed to two cycles of freezing and thawing to rupture cell membranes. DABA (100 μL) was then added to the cell suspension, and to DNA standards of 100 μL/tube, at a concentration of 0.4 g/mL. The samples were gently mixed, then incubated at 60°C for one hour. Then 2 mL of hydrochloric acid, 1 M, was added to each tube and fluorescence was measured at an excitation wavelength of 420 nm, and an emission wavelength of 510 nm.

Results

Human breast cancer cell lines were tested for mitogenic responsiveness to prolactin. Figure 26 shows the effect of prolactin and estrogen on the growth of the MCF-7V, EFM-19, ZR-75-1, and MCF-7T cell lines. In this experiment, none of the cell lines tested responded to prolactin, whether alone or in the presence of $1x10^{-10}$ M estrogen. In the presence of estrogen, the ZR-75-1 cells produced a slight growth response.

Figure 27 shows the responses of the cell lines T-47D MCF-7T, MCF7V, ZR-75-1, Hs578T, HBL-100 MDA MB 361, and MDA MB 231. A slight but significant response to prolactin was observed in the MCF-7V and ZR-75-1 cells. No response was seen in another MCF-7 subclone, MCF-7T. Estrogen produced significant growth responses in T-47D, MCF-7V, HBL-100, and MDA MB 361 cells. The largest response was a six fold increase in DNA seen with estrogen stimulation in T-47D cells.

The experiment in Figure 28 was performed to determine if a larger response to prolactin could be observed in cultures with a lower concentration of estrogen than that used in Figure 27. No responses to prolactin were observed. However, the MCF-7V, and T-47D cells lines produced responses to estrogen, even at $1x10^{-11}$ M. To determine if the amount of lactogen stripped serum could influence responsiveness to prolactin, the experiment shown in Figure 29 was performed. In this experiment, there was no significant growth response to prolactin, and it appears that a range of concentrations of 0 to 5% lactogen stripped serum had no effect on the response of MCF-7V cells

Effects of Prolactin on Breast Cancer Cell Growth

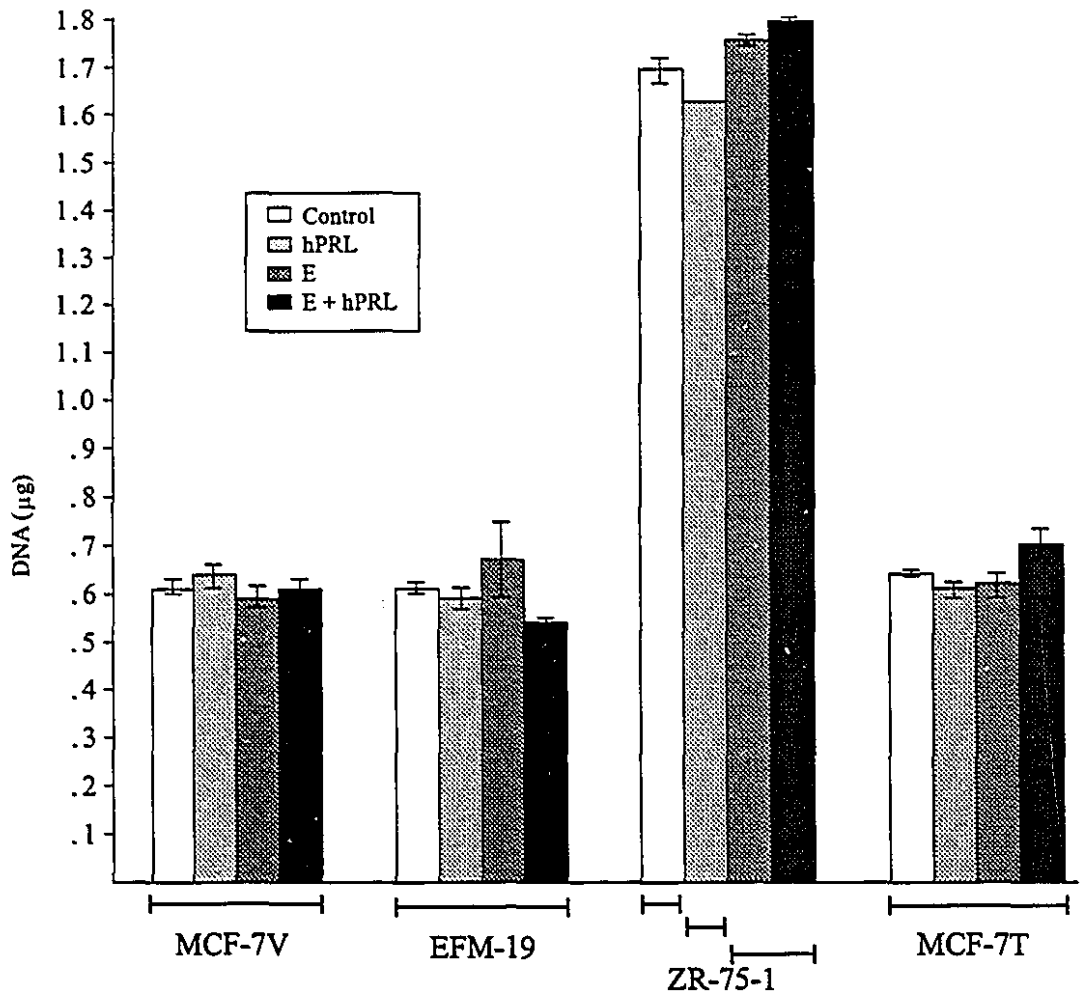

Figure 26. Prolactin growth stimulation in the ZR-75-1, EFM-19, MCF-7V (from Vonderhaar) and the MCF-7T (from the American Tissue Type Collection). Hormones were added at a concentration of 1e-10 M for estradiol, and 100ug/mL for human prolactin, in phenol red free EMEM with 10% lactogen stripped serum. cells were cultured for 6 days. Each treatment was performed in triplicate. Bars at the bottom of the graph indicate significant differences as determined by the Newman-Keuls multiple comparisons test.

Estrogen and Prolactin Effects on Breast Cancer Cell Growth

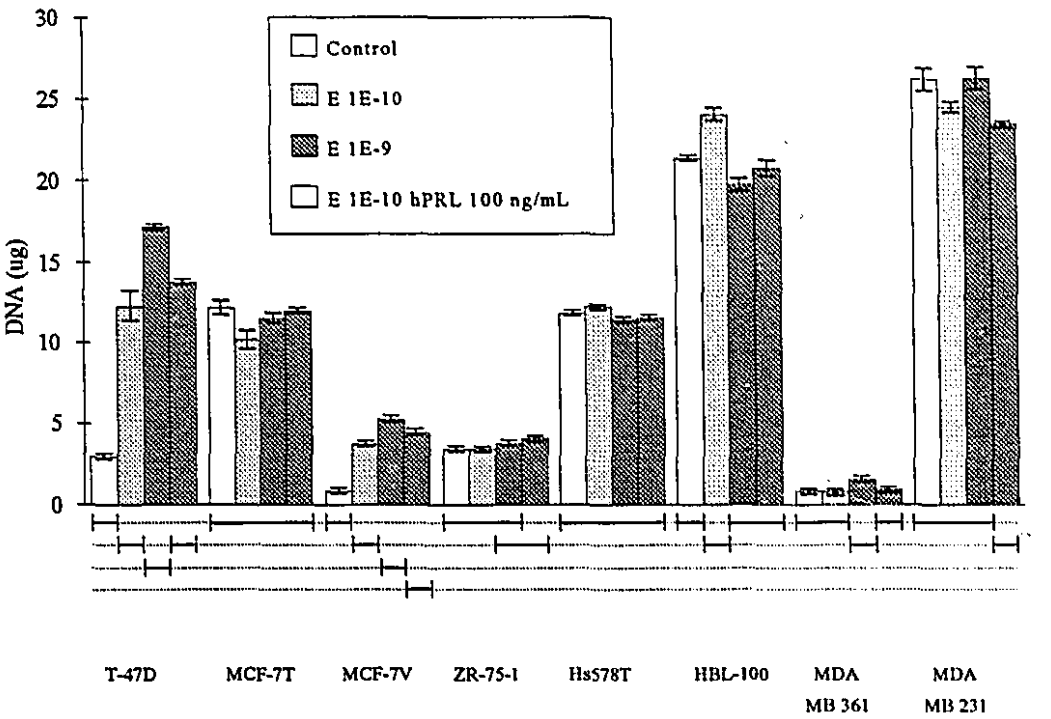

Figure 27. Breast cancer cell lines were cultured in 5% lactogen stripped serum for 11 days. Prolactin was added at 100µg/mL and Estradiol at 1e-9 M and 1e-10 M. MCF-7T were obtained from the American Tissue Type Collection; MCF-7V from Dr. Vonderhaar. Each treatment was performed in triplicate. Bars on different lines indicate significant differences as determined by the Newman-Keuls multiple comparisons test at the 0.05 significance level.

Growth of Breast Cancer Cell Lines

Effects of Varying Concentrations of Estrogen and Prolactin

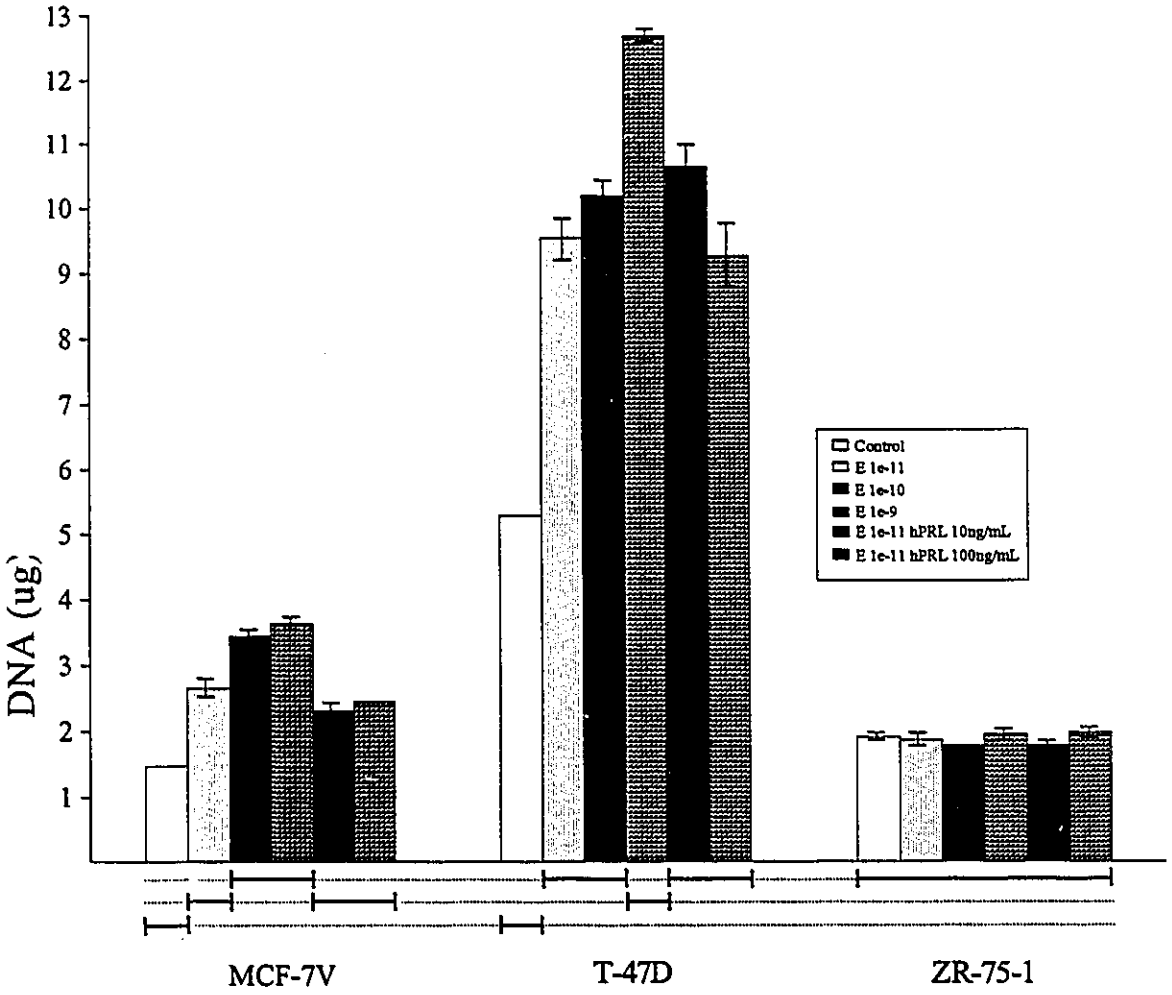

Figure 28. Effects of Estrogen and Prolactin (Estrogen from $1x10^{-11}$ to $1x10^{-9}$ and human prolactin at 10-100 µg/mL. Cells were grown for 6 days in phenol red free EMEM with 5% lactogen stripped serum. Each treatment was performed in triplicate. Significant differences among the treatments tested as determined by the Newman-Keuls multiple comparisons test at the 0.05 significance level are indicated by bars at the bottom of the graph. Bars on different lines indicate significant differences.

Prolactin and Estrogen Stimulation of Growth

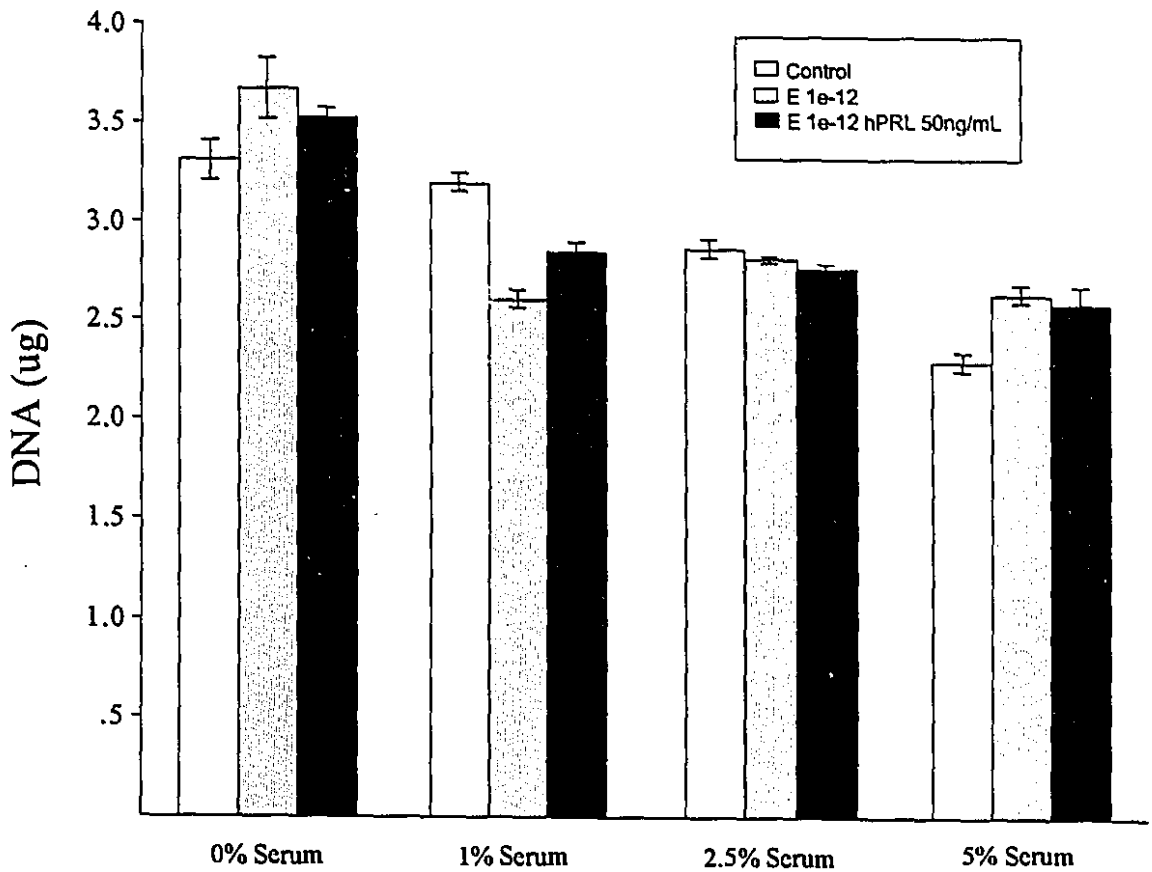

Figure 29. The effect of serum concentration on responsiveness of the MCF-7V cell line to prolactin and estrogen. Serum was stripped of lactogens as described in Materials and Methods. Cells were cultured in phenol red free EMEM. Each treatment was performed in triplicate. There were no significant differences among the treatments tested within each serum group as determined by the Newman-Keuls multiple comparisons test at the 0.05 significance level.

to prolactin. An experiment was performed to assess the role of thyroid hormone and insulin in the responsiveness of breast cancer cells to lactogen. Figure 30 shows no growth response of breast cancer cells to hGH, either alone or in combination with thyroid hormone and insulin.

In another experiment presented in Figure 31, different breast cancer cell lines were cultured together in order to determine if a complimentarity effect could reveal a mitogenic response to prolactin. First the cell lines T-47D, MCF-7V, HBL-100, ZR-75-1, and MDA MB 231 were tested alone, with prolactin, or prolactin with estrogen and progesterone. Then the cell lines were combined, two at a time, and growth responses to prolactin, or prolactin with estrogen and progesterone were measured. There was no significant growth response to prolactin in the cell lines T-47D, MCF-7V, HBL-100, or ZR-75-1, either alone or in mixed cultures. Strangely the MDA MB 231 cell line produces a mitogenic response to prolactin in this experiment. This response could not be repeated in further experiments.

Discussion

Observations made by Vonderhaar's group suggested that stripping the serum used in cell cultures of lactogens, with large quantities of dextran coated charcoal, was necessary to demonstrate an effect of prolactin on MCF-7 cell growth. However, using this protocol , we were unable to see any effect of prolactin. Dr. Vonderhaar confirmed that not all MCF-7 subclones responded to prolactin (personal communication), and she kindly provided the subclone that was used in their studies (MCF-7V). However this subclone produced only minimal and inconstant responses to prolactin. Further communication with Dr. Vonderhaar indicated that their subclone was changing in its sensitivity to prolactin in their hands as well.

In Figure 26, two subclones of MCF-7 (MCF-7V, and MCF-7T), as well as EFM-19 showed no responsiveness to either prolactin or estrogen. The ZR-75-1 cell line did show a slight response to estrogen. This contrasts with other experiments, such as that in

Effect of Lactogen, Thyroid Hormone and Estrogen on Breast Cancer Cell Growth

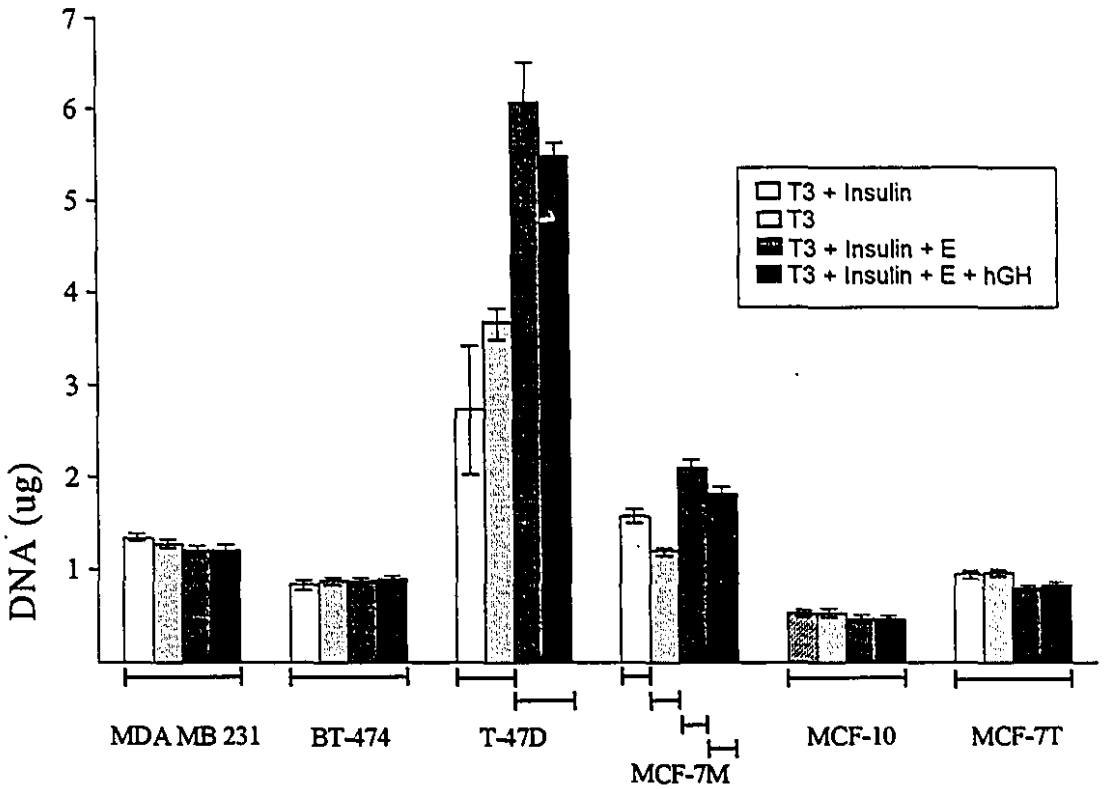

Figure 30. Several breast cancer cell lines were grown in phenol red free EMEM with 10% lactogen stripped serum for 10 days in the presence of various hormones. Thyroid hormone was added at a concentration of 1e-9 M, insulin at 1e-10 M, estradiol at 1e-12 M and human growth hormone at 200 ng/mL. The two MCF-7 cell lines come from different sources: MCF-7T form the American Tissue Type Collection, and MCF-7M from the Michigan Cancer Foundation. Each treatment was performed in triplicate. Different bars at the bottom of the graph indicate significant differences in growth as determined by the Newman-Kuels multiple comparison test.

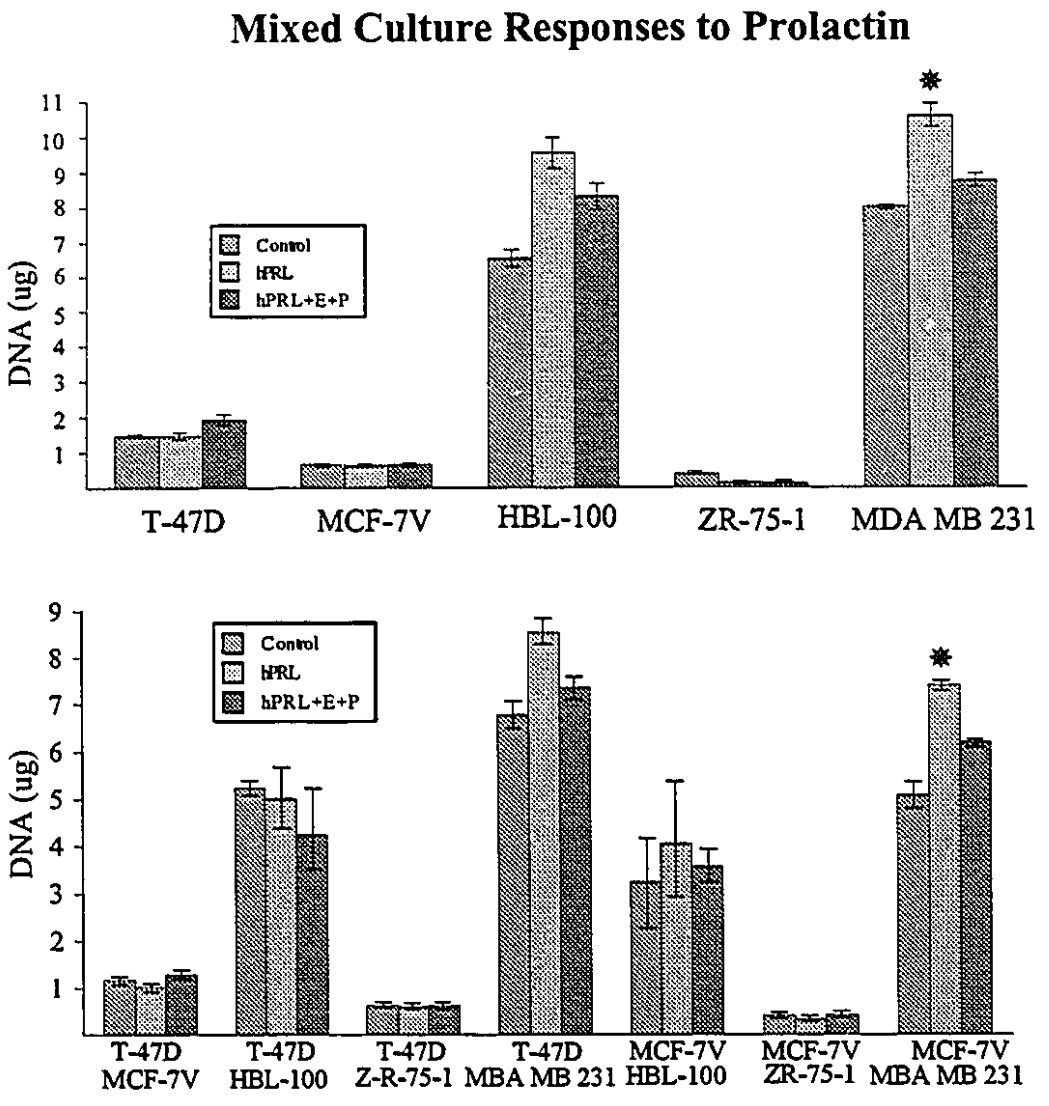

Figure 31. Co-cultures of breast cancer cell lines were performed to determine if various cell lines could compliment each other in a mitogenic response to prolactin. **A:** Separate cell cultures to indicate individual growth responses. Cells were grown in phenol red free EMEM with 10% lactogen stripped serum for 5 days. Human prolactin was added at a concentration of 100 ng/mL, estradiol at 1e-10 M and progesterone at 1e-9 M. **B:** Co-cultures were seeded with one half each the cell density compared to panel A. Each treatment was performed in triplicate. The symbol ✱ indicates a significant difference determined by the Newman-Kuels multiple comparison test at the .05 level.

Figure 27, where the MCF-7 cell line responded to estrogen, even though the MCF-7T, and ZR-75-1 cell lines did not. Differences in the response to estrogen can be explained in two ways: the nature of cancer cell lines may change with time, as is seen with the evolution of differing characteristics within the MCF-7,[517] and Nb2 cell lines. The experiments shown here were performed over the course of several years. Another variable is the serum used in cell culture. Some serum batches have been observed to inhibit estrogen responses in MCF-7 cells.[518,519] Although there was some variability in cell responsiveness to estrogen, the conditions used in these experiments resulted in an up to 6 fold increase in cellular DNA in estrogen treated cultures.

A significant response to *prolactin* was observed in MCF-7V cells in one experiment (refer to Figure 27), although the magnitude of this response was very slight compared to the stimulation of growth seen with estrogen. In Figure 26, some cell lines displayed a slight although non-significant increase in DNA, in cultures of prolactin and estrogen together, compared to prolactin, or estrogen alone. Since prolactin normally exerts its action in coordination with other hormones, low doses of estrogen were included in experiments thereafter, in order to mimic *in vivo* conditions. Since the prolactin response in the presence of $1x10^{-10}$ M estrogen was slight, the experiment in Figure 28, with a concentration of estrogen of $1x10^{-11}$ M was performed in order to determine if a greater response to prolactin could be observed. In this experiment, no response to prolactin was seen. Similarly, no response to prolactin was seen at concentrations of $1x10^{-12}$ M (as shown in Figure 29). Figure 29 also shows that the percentage of lactogen stripped serum made no difference in prolactin responsiveness. Since thyroid hormone increases prolactin receptor expression, and lactogen stripping of serum also likely removes many hormones from serum, the experiment in Figure 30 was done. Thyroid hormone, as well as insulin, was added back into the culture medium. In the presence of thyroid hormone and insulin, estrogen was strongly mitogenic in T-47D, and MCF-7M cells. When lactogen was included in this hormone cocktail, no additional growth was observed. On the contrary, there was an inhibition of cell growth with the addition of hGH to these cultures. This raised the intriguing possibility, that lactogen might slow breast cancer cell growth if it was presented in a context which elicited the differentiating action of prolactin. The analysis of this question would require culturing together a range of hormones, including, thyroid hormone, insulin, estrogen,

progesterone, cortisol, and perhaps other factors such as retinoic acid, attachment factors, and tissue matrix, each of which could have individual effects on cell proliferation and differentiation. This interesting question therefore, remained outside the bounds of this study.

Another approach to examining whether cofactors are necessary for prolactin to elicit a mitogenic action in breast cancer cells is to culture breast cancer cells with other cells. The products of other cells may have a complementary action, possibly revealing a prolactin-induced mitogenesis. Breast stromal cells would have been the ideal cell type for such co-cultures, however these cells were unavailable. Therefore, different breast epithelial cancer cell lines were cultured together (refer to Figure 31). In particular, the highly tumorigenic, steroid receptor negative cell line MDA MB 231, and the non-cancerous HBL-100 cells were cultured with the MCF-7V and T-47D cell lines. As was expected, no mitogenic response to prolactin was seen when each cell line was cultured by itself, with the exception of the MDA MB 231 cell line, which produced a significant increase in DNA in the presence of prolactin. This observation was not confirmed in repeated experiments, and would have been unusual if confirmed, since this cell line has neither detectable receptor binding nor RNA hybridization to the prolactin receptor cDNA. When cell lines were cultured together, no mitogenic response to prolactin was seen. This means that neither the diffusible factors released by these cells, nor the cell-cell contacts established between these cells had an effect on prolactin action. Several studies have shown that the behavior of both normal and tumorigenic mammary epithelial cells differs in the presence of stromal, or non-tumorigenic epithelial cells in the mouse.[520,521,522] However, the developmental state of the animal from which normal epithelial and stromal cells were taken was important. Therefore, in order to determine if such an effect exists for human breast cancer cells, the stromal cells would need to be taken form pubertal or pregnant breast tissue.

Chapter 5. Prolactin Receptor Expression in Breast Tumors

Materials and Methods

Materials were obtained as described in previous chapters. Tumors were obtained by the kindness of Dr. Tremblay at the Royal Victoria Hospital, Montreal; Dr. Pollack at the Jewish General Hospital, Montreal; and Dr. Duguid, at the Montreal General Hospital, Montreal. Values for tumor size, lymph node involvement, estrogen receptor, and progesterone receptor were obtained from hospital records.

RNA Extraction

RNA was extracted from cultured cells by the acid/phenol method of Chomczynski *et al*,[493] Depending upon the mass of the tumor, from 10 to 25 mL of denaturing solution (containing guanidinium thiocyanate (GTC) 4 M, sodium citrate, pH 7, 25 mM, sarcosyl, 0.5%, and 2-mercaptoethatnol 0.1 M) was added to each tumor. The tumors were homogenized for 30 seconds. Then a 1/20th volume of sodium acetate 4 M was added, followed by one volume of water saturated phenol, and 1/5th volume of 49:1 chloroform isoamylalcohol, with vortexing after the addition of each solution. The mixture was cooled on ice for 15 min. then centrifuged at 3500 rpm at 4°C 15 min. The aqueous supernatant was removed and mixed with one volume of isopropanol and stored overnight at -20°C to precipitate the RNA. The RNA pellet was resuspended in 10 mL of denaturing solution and precipitated with 2.5 volumes of ethanol at -20°C overnight. The RNA pellet was washed two times in 70% ethanol in water to remove any traces of GTC. RNA was measured by spectrophotometry at 260 nM. The ratio of 260/280 was rarely lower than 1.7.

Measurement of PIP/GCDFP-15 RNA

The S1 nuclease protection assay was performed as described in Chapter 3, Materials and Methods, with the exception that samples were processed in quintuplicate, with 10 μg of RNA per tube. After hybridization and S1 nuclease digestion, samples were loaded onto an 8% sequencing gel, and exposed to Kodak XAR film for 2 to 10 days. Quantification of samples was performed using a BioImage scanning densitometer (Millipore, Mississauga, ON). Four samples were processed at once, and the values for PIP/GCDFP-15, and cyclophilin were normalized against an internal control consisting of a triplicate sample of T-47D RNA. The PIP/GCDFP-15 expression was then normalized according to the expression of the cyclophilin house keeping gene.[523]

Table 12. Quantitative S1 Nuclease Protection Assay Probes

PIP3S1 5'**GGCCCCCAACTGCAGGCAGAGAACCAGGAGCAGGGTGGCAGGGCTGGC***TTCAGGTG*3'

CYC1S1 5'**GGAGACGCGGCCCAAGGGCTCGCCGTCGACGGCAATGTCG***TTATTTTTAAAAAAG*3'

Table 12 indicates the probes used in the quantitative S1 nuclease protection assay for tumor samples. PIP/GCDFP-15 expression was measured with the PIP3S1 probe, and CYC1S1, the probe taken from the cyclophilin house keeping gene. The sequence in bold indicates the complementary region of the probe and the region in italics indicates the non-complementary region used to determine the efficiency of enzyme digestion.

Measurement of Prolactin Receptor RNA

Prolactin receptor in tumor samples was originally measured by S1 nuclease protection assay, however this method proved to be not sensitive enough to accurately detect the low levels of prolactin receptor mRNA in these samples. Therefore, the tumor RNA samples were remeasured using a semi-quantitative polymerase chain reaction technique.[524,525]

Complimentary DNA was prepared from 3.2 μg of total RNA, 200 units of MMLV reverse transcriptase (BRL), and 1 μg of oligo-dT primer in 20 μL, containing Tris-HCl,

pH8.3, 20 mM; KCl, 5 mM; $MgCl_2$ 5 mM; DTT, 10 mM; 20 units of RNAsin (Promega) and 1 mM of each deoxynucleotide. The reaction proceeded at 37°C for 1 hr. Negative control reactions differed only in that the RNA was replaced by DEPC treated water. After incubation, the reaction mix was boiled for 5 min. and then chilled on ice to heat inactivate the enzymes and denature the DNA-RNA duplex.

A three-fold dilution series of six points of the reverse transcription product, starting at 1 μg, was mixed with 1x PCR buffer (Tris-HCl, pH 8.3, 20 mM; KCl, 50 mM; $MgCl_2$, 2 mM; each dNTP, 200 μM; and 1 unit of Taq polymerase; plus 50 mmol of each of the upstream and downstream primers, in a reaction volume of 50 μL. In addition 1 x 10^6 cpm of the primer H101 was added to the reaction in order to measure the reaction product. The amplification for the prolactin receptor alone was performed in a Perkin-Elmer-Cetus thermocycler with denaturation for 30 s. at 94°C, annealing for 75 s. at 60°C, and elongation for 90 s. at 72°C, for 9 cycles. At this point 50 pmol of each of the cyclophilin primers was added, plus 1 x 10^6 cpm of the labelled Cy2 primer. The PCR reaction was continued for another 24 cycles with the same reaction conditions.

Primers were ^{32}P-labeled using 50-100 pmol of primer plus 2μL of 10x buffer (described in Chapter 3, Materials and Methods), 2 μL of T4 PNK (BRL), and 10 μL of [^{32}P]ATP (7000 Ci/mmol). This mix was incubated 1 hr. at 37°C, whereupon another 1 μL of enzyme was added and the incubation continued for another 1 hr. at 37°C.

Ten μL of PCR reaction product was electorphoresed using a 5% non-denaturing polyacrylamide gel in TBE buffer. After staining with ethidium bromide, the appropriate bands were excised and the radioactivity was determined by liquid scintillation in a β-counter.

Table 13. Primer Sequences Used in Semi-Quantitative PCR.

Prolactin Receptor Primers

Upstream Primer: ..... H1 ..... $^{5'}$TGAGGATGCTTTCCACATGAACCC$^{3'}$

Downstream Primer: ..... H2 ..... $^{5'}$GTGGCCCCAACTCCTGC$^{3'}$

Length of amplified sequence: .... 324 bp

Cyclophilin Primers

Upstream Primer: ..... C1 ..... $^{5'}$CCGCGTCTCCTTTGAGCTGTTTGCAG$^{3'}$

Downstream Primer: ..... C2 ..... $^{5'}$GCTCTCGCTGCAGTTCCCTTTGGGT$^{3'}$

Length of amplified sequence: .... 568 bp

Results

Measurement of PIP/GCDFP-15 by S1 Nuclease Protection Assay

Figure 32 illustrates the quantitation of PIP/GCDFP-15 and cyclophilin transcript from increasing amounts of T-47D total RNA. For experiments performed on tumor RNA the probes for cyclophilin and PIP/GCDFP-15 were added together to control for variations in digestion efficiency. PIP/GCDFP-15 was detectable in all samples, with the range being from 720 to 142 924 in arbitrary units, with the mean value being 7773.

Semiquantitative PCR

The expression of the transcript for cyclophilin was much higher in tumor RNA than that for the prolactin receptor. In order to balance the competition for reagents within the reaction tube, and to bring the intensities of the two products into the same range, the polymerase chain reaction was performed with only the primers for prolactin

Quantitative S1 Nuclase Protection Assay

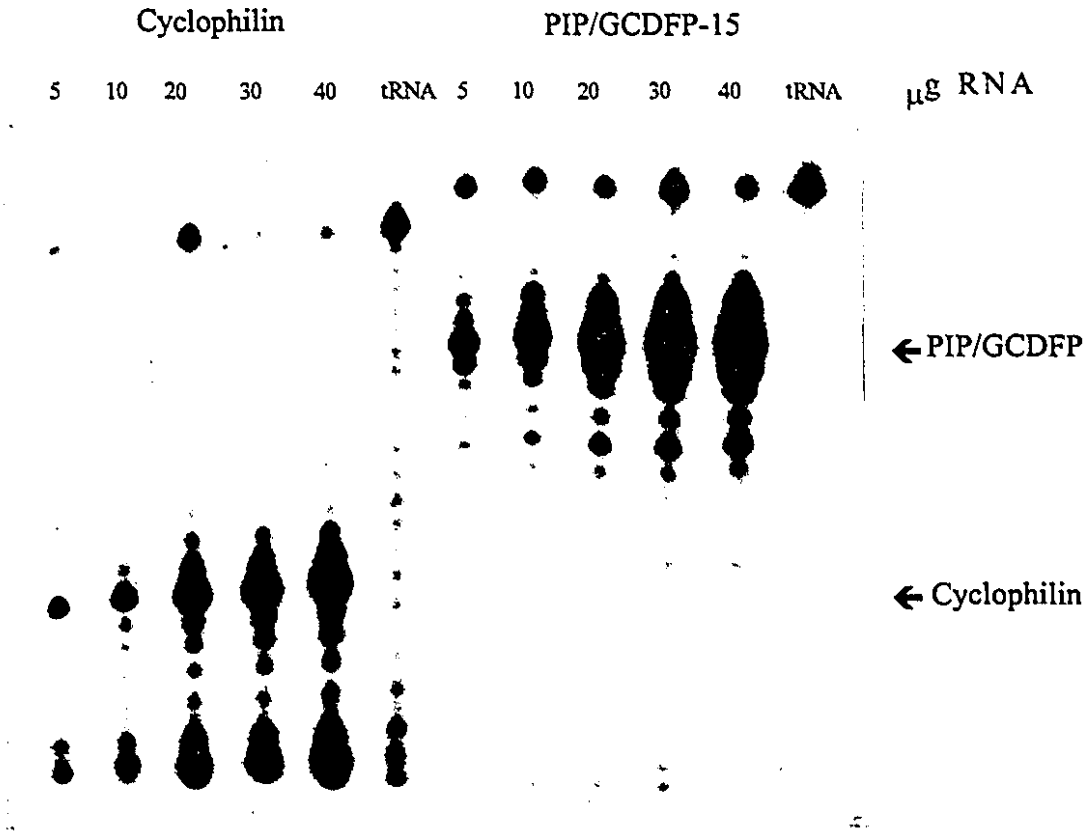

Figure 32. T47D total RNA was hybridized to probes for cyclophilin, and PIP/GCDFP-15, following the protocol used for quanitification of human prolactin receptor and S14 RNA indicated.

receptor for 9 cycles, prior to the addition of the cyclophilin primers. Figure 33 shows a typical PCR reaction. The amplified product for cyclophilin produced a band of 568 bp, and for the prolactin receptor, of 329 bp.

To calculate the expression of prolactin receptor and cyclophilin, a linear regression was performed upon the $\log_{10}$ values of the serial dilution of the reverse transcribed tumor RNA. The average r^2 value for prolactin receptor and cyclophilin regressions was 0.976, with the lowest r^2 value being 0.886. Figure 34 shows a typical regression plot for tumor number 6. First, using the regression coefficients for the prolactin receptor, the signal for 300 ng of total RNA was calculated. Then using the regression coefficients for cyclophilin, the amount of total RNA needed to produce this same signal by cyclophilin was calculated. Prolactin receptor expression was expressed as a ratio of 300 ng over the amount of RNA needed to give an equivalent cyclophilin signal. The converse calculation was also made, and the two ratios were averaged to correct for the deviation of the slopes from a non-parallel state.

A correction was made for the 9 cycles in which the prolactin receptor mRNA was amplified alone (theoretically, the prolactin signal would be 2^9 or 512 times greater than that for cyclophilin in the original tumor RNA).

Relations Between PIP/GCDFP-15 and PRL-R RNA and Other Clinical Markers

Figure 35 shows the relation between six measurements taken from the tumor samples collected. Tumor size (largest dimension), number of involved lymph nodes, estrogen receptor (fmol/mg), progesterone receptor (fmol/mg), PIP/GCDFP-15 RNA, and prolactin receptor RNA. In order to make comparisons of each parameter easier, adjustments in scale were made.

Linear Regression analysis was used to examine the relationships between each of the parameters measured against PIP/GCDFP-15, and prolactin receptor. As can be seen from Figure 35, the data for each tumor is incomplete. Not every sample was tested for estrogen or progesterone receptor, examination of lymph node status was not always performed. In this regard, each statistical analysis for correlation was performed upon a subset of tumors which contained data for each parameter, including tumors in which the parameter measured was undetectable. Significant relationships were found between

Semi-Quantitative PCR Analysis of Prolactin Receptor and Cyclophilin RNA

Figure 33. Six serial dilututions of reverse transcribed breast tumor RNA were amplified by PCR using primers for cyclophilin and prolactin receptor, including 1 x 10^6 cpm of labelled PRL-R and cyclophilin primers to permit quantitation by β emission. Samples were run on a 5% non-denaturing polyacrylamide gel. The gel was stained with ethidium bromide, and the bands were excised and counted. Lanes were loaded in the order shown above to aid in the acurate excision of the lower intensity bands.

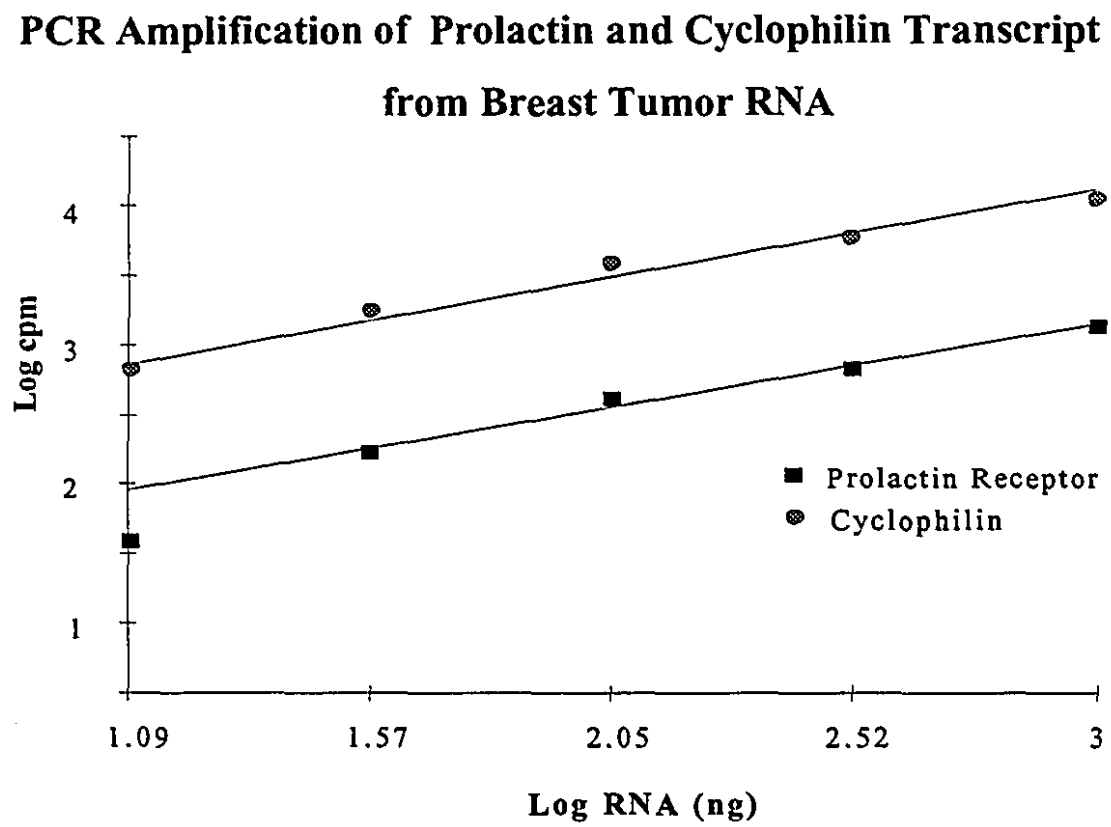

Figure 34. Serial dilutions of reverse transcribed breast tumor RNA were amplified by PCR. ^{32}P-labelled primer permited the measurement of amplification, and these values were treated to linear regression analysis, as a step in the calculation of prolactin receptor signal in relation the control cyclophilin housekeeping gene. The example shown here is from tumor number seven.

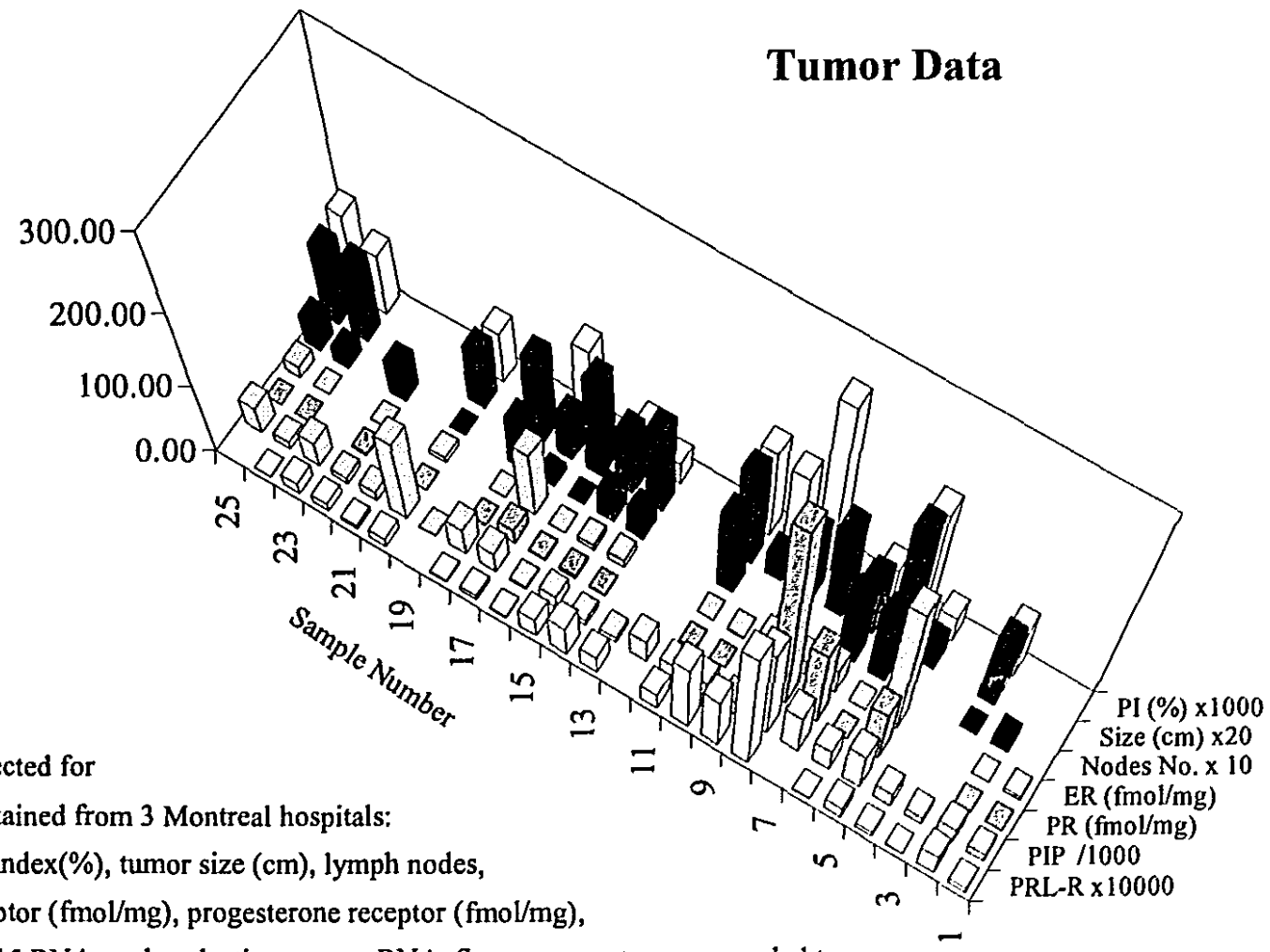

Figure 35. The data collected for 25 tumors obtained from 3 Montreal hospitals: proliferation index(%), tumor size (cm), lymph nodes, estrogen receptor (fmol/mg), progesterone receptor (fmol/mg), PIP/GCDFP-15 RNA, and prolactin receptor RNA. Some parameters were scaled to permit easy comparison, as indicated in the z axis.

PIP/GCDFP-15 and progesterone receptor, and PIP/GCDFP-15 and prolactin receptor. A significant relation was also seen between prolactin receptor and progesterone receptor. There was no correlation between PIP/GCDFP-15 or prolactin receptor and estrogen receptor, size, mitotic index, or the number of involved lymph nodes. Table 14 summarizes the results of these statistical tests.

Table 14. Correlations Between PIP/GCDFP-15 or PRL-R and other Clinical Measurements of Breast Tumors

Measures Tested	r^2	p value
PIP/GCDFP-15 vs. Progesterone Receptor	**0.563**	**0.00029**
PIP/GCDFP-15 vs. Estrogen Receptor	0.046	0.39
PIP/GCDFP-15 vs. Size	0.0038	0.94
PIP/GCDFP-15 vs. Nodes	0.0039	0.82
PIP/GCDFP-15 vs. Mitotic Index	0.0138	0.71
Prolactin Receptor vs. Progesterone Receptor	**0.58**	**0.00052**
Prolactin Receptor vs. Estrogen Receptor	0.0021	0.87
Prolactin Receptor vs. Size	0.00013	0.97
Prolactin Receptor vs. Nodes	0.019	0.65
Prolactin Receptor vs. Mitotic Index	0.144	0.18
Prolactin Receptor vs. PIP/GCDFP-15	**0.307**	**0.0089**

Discussion

Technical Considerations

Quintuplicate samples of 10 μg each, for the measurement of PIP/GCDFP-15, and prolactin receptor were prepared. However, unlike the T-47D cell line, breast cancer tumor RNA contained too little prolactin receptor mRNA to be measured by the S1 nuclease protection assay protocol. A more sensitive nuclease protection assay could involve the use of different probes. Chain extension using DNA polymerase, would produce a probe with the incorporation of multiple ^{32}P-nucleotides, compared to the single ^{32}P present introduced by T4 PNK to the oligomer probe used in the present experiments. The RNase protection assay has the advantage of the use of probes containing multiple ^{32}P-nucleotides, as well as the stronger hybridization of RNA-RNA duplexes, and therefore would be the most sensitive of the enzyme protection assays. The semi-quantitative PCR protocol was used to measure prolactin receptor in this instance because of its sensitivity, and also because it required the use of only small amounts of RNA.

For both prolactin receptor and PIP/GCDFP-15, values were expressed in terms of cyclophilin expression. Cyclophilin was originally characterized for its binding activity to cyclosporin A, but since then it has been identified as a highly conserved protein present in all eukaryotic cells tested and to be identical to peptidyl-prolyl cis-trans isomerase, an enzyme which assists in protein folding.[526] Although cyclophilin is a house-keeping gene, there is no definite evidence that the cyclophilin the gene is expressed to the same degree in all tumors. However, the use of this control gene was necessary to correct for variations in digestion efficiency in the S1 nuclease protection assay, and to account for possible differences in cell density and necrosis among tumors.

Correlations between PRL-R, PIP/GCDFP-15 and Progesterone Receptor

A highly significant correlation exists between the prolactin receptor and PIP/GCDFP-15. This is consistent with the stimulatory effect that prolactin has on PIP/GCDFP-15 gene expression.[445] Prolactin receptor and PIP/GCDFP-15 expression were also found to correlate with progesterone receptor expression.

Previous studies have found that PIP/GCDFP-15 is correlated with estrogen receptor,[437,438] when measured at the RNA level[437], or by release of PIP/GCDFP-15 in explant cultures.[438] Other groups failed to observe any significant correlation with estrogen receptor,[441] when measured in explant cultures,[440] or by immunohistochemistry.441 The present study does not agree with that which measured PIP/GCDFP-15 at the RNA level. Clearly, there does not appear to be any clear relationship between estrogen receptor levels and PIP/GCDFP-15 levels. There is one report which shows that estrogen reduces PIP/GCDFP-15 expression,[446] suggesting that there would be no positive correlation between these parameters.

Among the tumors tested in the present study, prolactin receptor mRNA expression correlates with progesterone receptor levels, but not with estrogen receptor levels. Earlier studies have reported that the prolactin receptor is correlated with the estrogen recetor,[380,390,391,392] with one exception.[393] The obvious difference in the present study is that here prolactin receptor was measured at the RNA level, while, in previous studies, prolactin receptor was measured by receptor binding assay. Studies on the regulation of prolactin receptor described in this thesis have shown only minor changes in prolactin receptor RNA levels in spite of larger changes in receptor protein levels. Therefore, the small changes in prolactin receptor RNA may obscure a possible correlation between the level of prolactin and estrogen receptors.

While prolactin receptor, PIP/GCDFP-15 and progesterone receptor correlate well, do they have similar a prognostic significance for breast cancer treatment? In a study of 638 patients, 73% of patients who responded to treatment had positive progesterone receptor, and only 23% of non-responsive patients were progesterone receptor positive.[527] For PIP/GCDFP-15, a significant reduction in histochemical staining was observed in tumors with advanced (grade III) tumors compared to grade I tumors.[442] However, tumors which responded to treatment tended to have low PIP/GCDFP-15. When total prolactin receptors were measured after desaturation with $MgCl_2$, they were found to correlate with improved relapse free survival[494], although Cox analysis failed to show that prolactin receptor status was an independent indicator of prognosis[493,494]. In another study, prolactin receptors were correlated with poorer survival.[395] On the whole, the presence of progesterone receptor seems to bode well for response to treatment.

However, the is no clear prognostic significance for either PIP/GCDFP-15, or prolactin receptor. Therefore, although prolactin receptor, progesterone receptor, and PIP/GCDFP-15 expression correlate in this study, there does not appear to be a similar relation in the prognostic significance of these factors.

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