Peptide Hormone Receptors

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Editors
M.Y. Kalimi · J. R. Hubbard

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Foreword

It is becoming increasingly clear that receptor proteins play a vital role in the molecular mechanism, physiology and pathology of hormone action. For this reason, a great deal of research has recently been directed towards understanding these receptor molecules. This exciting work has greatly advanced our knowledge in this area, but it is scattered throughout various basic science and clinical journals in such fields as endocrinology, biochemistry, molecular biology, cell biology, and physiology.

In order to consolidate and clarify the considerable body of information concerning hormone receptors "Principles of Receptorology",edited by M.K. Agarwal, was published, which focussed on steroid hormone receptors. The present volume deals with the second major category of hormone receptors, namely peptide hormone receptors. These important receptors differ from their steroid counterparts not only in their hormone specificity, but also in their intracellular location and molecular mechanism(s) of action.

It is our sincere hope that this text, together with the previous one on steroid receptors, will be of value to both new investigators in the field as well as to those researchers who have already contributed by their researches to the scientific information contained in these volumes. We also wish to express our deep appreciation to the many notable scientists who have participated in the writing of the present volume.

Richmond

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GROWTH HORMONE RECEPTORS

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I. INTRODUCTION

Growth hormone (somatotropin or somatotropic hormone) is a protein of about 21,500 daltons, which has many physiological and metabolic functions (1-8). Growth hormone (GH) promotes the growth of nearly all body tissues *in vivo*. This occurs by increased cell size, cell number and extracellular matrix synthesis. Metabolically, GH (directly or indirectly) exerts the following actions:

a. increases synthesis of protein, RNA and DNA

b. decreases use of carbohydrates for energy and promotes glycogen production

c. increases use of fats for energy

d. increases retention of calcium and phosphate

Peptide Hormone Receptors

Many biological effects of GH *in vivo* have been difficult to mimic *in vitro*. For example, the anabolic action of GH on cartilage and muscle are not clearly evident in *in vitro* systems (2,6). This discrepancy has led to the "somatomedin hypothesis", which states that many effects of GH *in vivo* (particularly on skeletal growth) occur indirectly via GH-dependent generation of somatomedin (or insulin-like growth factor) which acts directly on cartilage and other tissues (1-13). Somatomedin appears to be produced primarily in the liver, however, the mechanism of somatomedin generation is not completely understood. Although the somatomedin hypothesis is commonly accepted, the physiological role(s) and action(s) of somatomedin *in vivo* have not been clearly demonstrated. In addition, Isaksson et al. (14) reported that human GH injected directly into the proximal growth plate accelerated longitudinal bone growth.

Because of the many important biological effects of growth hormone, elucidation of its mechanism(s) of action is of significant scientific and clinical interest. Like other peptide hormones, GH binds to high affinity receptors on target tissues. This binding has been shown to be specific, saturable and reversible. Hormone-receptor interaction is believed to trigger a series of biochemical and molecular events which eventually elicit a response at the cell, tissue and organism levels (1-3).

Since the interaction of GH with its receptor appears to be a vital step in the molecular mechanism of GH action, many studies have been performed to measure, regulate, purify and characterize this protein. However, numerous problems have retarded efforts to characterize these receptors. The major difficulties include:

a. lack of definitive *in vitro* responses to GH in many systems
b. little direct evidence that binding sites mediate GH responses
c. cross-receptor binding of GH and prolactin to plasma membrane receptors
d. heterogeneity of GH receptor molecules
e. prolactin contamination in some GH preparations
f. dearth of specific GH antagonists and agonists

Despite these obstacles, considerable progress has been made in GH receptor research. Because the liver contains a high concentration of GH receptors and is a well defined target tissue (being the site of GH-dependent production of somatomedin, ATPase, tyrosine amino transferase, ornithine decarboxylase and tryptophan pyrolase production) the hepatic receptors are perhaps the most vigorously characterized.

In this chapter, a comprehensive review of the advances and methodology in GH receptor investigation is provided, with emphasis on the most recent concepts and contributions in the field.

II. MEASUREMENT OF GROWTH HORMONE RECEPTORS

Quantification, purification and characterization of GH receptors requires detection methodologies which are specific, reproducible and sensitive. Procedures have been developed for a number of experimental systems such as whole cells, cell membranes, and solubilized receptors.

Preparation of Labeled GH -

To conduct radioreceptor assays for the GH receptor, highly purified radiolabeled and unlabeled GH is required. In a study by Maes et al. (15), bovine GH was purified by the method of Dellacha and Sonenberg (16). In most instances, the GH was obtained from the National Institutes of Health and the National Pituitary Agency. GH is generally labeled by iodination with ^{125}I using lactoperoxidase (17-19) and chloramine-T (20-24) methodologies. Both procedures commonly produce specific activities in the range of 50-100 μCi/μg GH (15,25-27). In a report by Gordin and Goodman (28), a modified chloramine-T procedure (20,23) yielded a specific activity of 220-230 uCi/ug, which averaged about 3 iodine atoms per GH molecule. In most procedures the labeled hormone was separated from unreacted iodine and hormone aggregates by gel chromatography (such as Sephadex G-50, G-75 or G-110). The iodinated and polyiodinated GH molecules have been shown to retain biological activity (29). Just prior to a binding assay, the ^{125}I-labeled GH is frequently repurified by gel filtration to remove free ^{125}I-label and damaged hormone.

Because of cross-receptor binding, it can be difficult to determine if a GH binding site is somatogenic or lactogenic in nature. In addition to consideration of the lactogenic or nonlactogenic function of particular target tissues, the source of GH used can help distinguish these receptor forms as previously described (30).

A. Liver Membrane Receptor Assays-

Liver cell membrane GH receptors have been extensively investigated. An assay for rat hepatic microsomal membrane receptors has been described by Baxter et al. (31), using a slightly modified method of Kelly et al. (32). $MgCl_2$ was used to strip bound endogenous hormone from the receptors so that total receptor binding could be determined using radiolabeled GH. Briefly, microsomal membranes (1-2 mg protein) were placed in 3 ml of Tris-HCl buffer, pH 7.4. To some samples 3.8 M $MgCl_2$ was added until a final concentration of 3.0 M was achieved. Samples were incubated 5 min at 21°C and then diluted with 9 ml of cold Tris-HCl buffer containing 0.1% BSA and 10 mM $CaCl_2$. The membranes were centrifuged and the pellets washed with 9 ml Tris-HCl and then sedimented. Tris buffer (0.6 ml) containing 10 mM $CaCl_2$ and 0.1% BSA was added, and membranes were suspended by glass-glass homogenization. Aliquots of 0.1 ml were used for radioreceptor measurements.

In the study for Baxter and Turtle (33), ^{125}I-human GH was incubated with rat hepatic membranes (200 ug membrane protein) in

300 ul of 25 mM Tris-HCl, pH 7.4, containing 10 mM $CaCl_2$ and 0.5% BSA. Samples were incubated at 22°C for 16 h. Maes et al. (15) performed binding studies on liver homogenates, in which 0.2 - 0.3 ng ^{125}I-GH was incubated with 2.0 mg (protein) liver sample and 0-500 ng unlabeled GH in 300 ul total volume of 25 mM Tris-HCl, pH 7.4, 0.1% BSA and 10 mM $CaCl_2$. Samples were incubated for 2 h at 22°C. Cold Tris-HCl buffer was then added, and homogenates were then pelleted by centrifugation at 1,500 X g for 30 min at 4°C. The washed pellets were then measured for radioactivity. Approximately 80% of receptor binding was membrane bound, while about 20% was solubilized. Membrane binding assays have also been performed at low temperature for 1.5 h with overnight incubation and at physiological (37°C) temperature for 1.5 h (34).

B. Detection of Solubilized Receptors-

In some studies GH receptor assays have been carried out on solubilized receptors. In a report by McIntosh et al. (35), liver microsomal membrane proteins were solubilized by treatment with Triton X-100 (1 mg/ml protein) in Tris-HCl, pH 7.5. Samples were mixed for 30 min and then centrifuged at 200,000 X g for 1 hr. The supernatants were assayed by incubation with ^{125}I-GH (300-500 pg) in Tris-HCl, pH 7.5 with 10 mM $MgCl_2$, γ-globulin (0.025%), and 0.1% BSA for 18-24 h at room temperature. Polyethylene glycol (12.5% final concentration) was added and the samples were centrifuged. The pellets were then counted for radioactivity.

Similarly, Herington and Veith (25) incubated liver membranes (5-10 mg protein/ml) with 1% (vol/vol) Triton X-100 in 25 mM Tris-HCl, pH 7.4, containing 10 mM $CaCl_2$ and 0.1% (wt/vol) BSA. Samples were incubated at room temperature for 30 min and then centrifuged for 2 h at 200,000 X g. Supernatants were then used for radioreceptor assays using 150-200 ug protein to 30-50,000 cpm ^{125}I-GH with and without excess unlabeled hormone(s). The incubation buffer consisted of 0.5 ml of 25 mM Tris-HCl, pH 7.4, with 10 mM $CaCl_2$ and 0.1% wt/vol BSA with 0.06-0.13% Triton X-100. The reaction was stopped with 0.5 ml cold 0.1 M NaH_2PO_4, pH 7.5, and then 1 ml of cold 25% (wt/vol) polyethylene glycol. Samples were mixed, stood for 30 min at 4^{o}C, and then centrifuged at 1500 X g for 45 min to separate free (supernatant) hormone from bound (pellet).

Spontaneous release of GH receptors from human lymphocytes (IM-9) into a soluble fraction was reported by McGuffin et al. (26). Cultured lymphocyte cells were used when they reached a stationary growth phase. Lymphocyte pellets were washed with cold phosphate-buffered saline solution (PBS), pH 7.0, and then suspended (2-3 x 10^8 cells/ml) in PBS containing the proteinase inhibitor iodoacetamide (0.02 M). Cells were then gently mixed for 90 min at 30^{o}C, after which they were pelleted at 600 X g. The supernatant was removed and centrifuged at 20,000 X g for 1 h at 4^{o}C and then for 2 h at 100,000 xg to remove particulate matter. This supernatant was then used in receptor assays. About 40% of original receptor binding was recovered using this procedure. The specific binding was determined by incubating 10^{-9}-10^{-10}M ^{125}I-human GH

with or without 10^{-6}M unlabeled GH for 90 min at 30°C in phosphate buffered saline (PBS). Bound and free hormone were separated at Sephadex G-75 (1 x 55 cm columns) chromatography at 4°C.

C. Binding Assays Using Cell Suspensions and Cell Monolayers-

GH receptors can also be measured using cell suspensions and cell culture monolayers. A cell suspension assay for rat adipocyte GH receptors was recently used by Gorin and Goodman (28). Rat epididymal fat was cut into small pieces and incubated at 37°C for 20 min in Krebs-Ringer phosphate buffer (KRP), pH 7.4, containing 1 mg/ml crude collagenase, 5.5 mM glucose and 4% BSA. Cells were filtered through silk and washed several times with KRP buffer containing 5.5 mM glucose and 1% BSA. Cells were incubated at 37°C in KRP buffer containing ^{125}I-human GH with 1% BSA and 5.5 mM glucose in the presence or absence of 0.25 mM unlabeled ligand. After incubation, 100 µl aliquots were placed into polyethylene microcentrifuge tubes. The bound ^{125}I-GH was separated from free ^{125}I-labeled hormone by centrifuging the samples through dinonylphthalate. The containers were cut, and the cell containing upper layer was counted in a gamma camera. Specific binding was calculated as total binding (no unlabeled GH) minus binding in the presence of 0.25 mM unlabeled GH. Specific binding was commonly about 70% of total binding.

Because of the relative ease of obtaining blood samples from patients, GH receptor measurements using blood cells could be of

great use in clinical investigations. In 1981, Solis-Wallckermann et al. (36) reported the development of a GH receptor assay using human red blood cells. Erythrocytes were obtained from heparinized blood and incubated for 150 min at pH 7.5 in HEPES-NaCl containing radiolabeled GH with and without different concentrations of unlabeled ligand. Erythrocytes were then separated by centrifugation and the radioactivity was determined.

Recently, Kiess and Butenandt (37) characterized receptor binding in human peripheral mononuclear cells (PMC). PMC were isolated using Ficoll-Isopaque centrifugation. Tris-HCl buffer (25 mM at pH 7.4 containing 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1 mM Titriplex III, 15 mM Na-acetate, 10 mM dextrose, and 1 mg/ml BSA) was added and the cells were suspended in an overnight preincubation without alteration of cell viability. In the binding assay approximately 10^6 PMC were incubated in the Tris buffer (1 ml) containing 10-30 x 10^3 CPM ^{125}I-human GH with unlabeled hormones ranging in concentration from 0-800 ng/ml. The assay incubation was conducted for 120 min at 37^{o}C with constant shaking. Ice-cold Tris buffer (0.5 ml) was added at the end of the incubation period and the cells were centrifuged at 280 X g for 10 min. The cell pellets were then counted for radioactivity. Binding was shown to be hormone-specific, reversible and time-dependent (37).

Billestrup and Martin (38) used cell culture monolayers in a study on GH receptors of rat insulinoma RIN-5AH cells. Briefly, confluent 60 mm cell culture dishes seeded with RIN-5AH cells

were washed with 0.01 M HEPES buffer, pH 7.4, containing 2.5 mM NaH_2PO_4, 0.13 mM NaCl, 4.7 mM KCl, 1.24 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 1% human serum albumin (HEPES buffer). The monolayer was incubated with about 0.14 ng ^{125}I-human GH (80-90 uCi/ug) generally for 1 h at 37°C in a shaking water bath. Some samples also received 100 ug GH/ml to determine nonspecific binding. The cultures were then washed 6 times with cold HEPES buffer. Cells were removed from the plate by incubating with 0.05% trypsin in Ca^{2+}- and Mg^{2+}-free Hanks' Balanced Salt Solution with 0.3 mM EDTA for 5 min at 37°C. Samples were then counted for radioactivity. Again specific binding was estimated by subtracting nonspecific binding from total binding.

D. In Vivo Receptor Measurements-

In 1984, Roguin et al. (39) compared the biological activities of chemically modified GH to their *in vitro* and *in vivo* binding activities. The *in vivo* binding assay was a modification of the procedure described by Turyn and Dellacha (40). Anaesthetized Long-Evans female rats were injected with ^{125}I-GH (10^6c.p.m.) with or without unlabeled GH or chemically modified GH into the jugular vein. Liver samples were taken 20 min after injection, washed with 0.15 M NaCl, blotted dry and weighed. Radioactivity was then determined. While both active and inactive forms of GH were equally effective in their isolated cell and cell liver membrane binding assays, only the derivatives which increased body weight appeared to bind to receptors *in vivo*. Since serum clearance of all GH forms was

similar, differences in biological *in vivo* binding activities were not due to alteration of hormone levels. Their results, therefore, suggest that *in vivo* receptor binding assays correlate closer with biological activity than *in vitro* methods (40).

III. RELATIONSHIPS BETWEEN RECEPTOR BINDING AND BIOLOGICAL ACTIONS OF GROWTH HORMONE

Demonstration that receptor binding correlates with biological actions of a hormone serves to support the concept that detectable receptors are involved in the mechanism of hormone action. While this has been shown for GH receptors in some systems, this is an area that needs considerably more investigation.

One of the earliest GH receptor studies was in 1973, when Tsushima and Friesen (41) compared the biological potencies of five different GH samples to their ability to displace ^{125}I-GH from heptic plasma membrane preparations from rabbits. The hormone samples were found to displace ^{125}I-GH in proportion to their biological activities, supporting the relationship between receptor binding and biological actions of GH.

The low level of GH receptor sites in epiphysial cartilage is consistent with the lack of direct metabolic effects of GH on cartilage *in vitro* (42).

The relationship between receptor binding and biological action of human GH was also examined using cloned rat insulinoma RIN-5AH cells (38). GH was shown to increase insulin concentration 80% in these cells, as well as DNA, protein and cell number. Half-maximal effect was found at 10 ng/ml GH, which was approximately the amount needed for half-maximal displacement of ^{125}I-GH in cell receptor binding studies. Maximal biological response occurred with 100 ng/ml GH, which was the concentration needed for maximal high affinity receptor occupancy. Their results, therefore, suggest a stoichiometric relationship between GH receptor binding and induction of insulin concentration in RIN-5AH cells. Their studies also indicated that this biological action of GH occurred independent of somatomedin production.

Insulin-deficient diabetes mellitus is characterized by growth retardation and low sensitivity to GH in many tissues. In a study by Baxter et al. (43) streptozotocin-induced diabetic animals were shown to have a reduced number by hepatic GH receptors which was reversed by insulin therapy. Similarly, a decreased level of GH receptors was produced by fasting-induced hypoinsulinemia in rats. While indirect, these experiments suggest a general correlation between low GH receptor levels and diminished for GH response in diabetic animals.

As previously mentioned in "measurement of growth hormone receptors", in a study by Roguin et al. (39) the biological potency of GH was altered by chemically introducing different numbers of modified residues in GH molecules. While in vivo binding assays correlated with the ability of the GH preparation

to increase body weight in rats, other *in vitro* methods using isolated hepatocytes or hepatic membrane preparation appeared to be far less discriminating.

In other studies, a correlation between receptor binding and biological action of GH was not apparent. Cultured preadipose 3T3 cells differentiate into adipose cells in response to GH. In a study by Nixon and Green (44) the GH receptors of 3T3 cells of differing susceptibility to GH-mediated transformation was examined. Both the readily converted and insusceptible cells bound approximately 10^4 GH molecules per cell and had a Kd of about 10^{-9}M. GH internalization and degradation rates were also similar. Thus differences in the biological response of preadipose 3T3 cells were not clearly apparent, though discriminating mechanisms may have occurred in post-receptor binding events.

The relationship between receptor binding and GH action has also been studied in rat adipocytes (45). Isolated adipocytes showed insulin-like responses to human GH 3 h after excision, but were refractory by the 4'th hour. About 20,000 specific GH receptors were measured per cell in both GH responsive and non-responsive time points.

While studies on the relationship receptor binding and GH action are somewhat conflicting, the "receptor hypothesis" of GH action is widely accepted. Development of new GH-sensitive *in vitro* systems may greatly aid research in this area. In addition, studies where GH receptor levels and biological action(s) did not

correlate may be due to differences or alterations in many other non-receptor properties of the cells.

IV. REGULATION AND CLINICAL ASPECTS OF GROWTH HORMONE RECEPTORS

It is increasingly evident that GH receptors exist in a dynamic state of flux and regulation. Because these receptors appear to be essential to the molecular mechanism of GH action, modulation of these proteins could significantly alter biological responses to GH. Investigation of the regulatory influences on this protein is, therefore, of significant experimental and possibly clinical interest. In this section, information concerning the regulation (particularly endocrine, age, sex and chemical modulators) of the GH receptor is discussed. In addition, the relationship between GH receptor levels and clinical disorders is considered.

A. Regulation by Growth Hormone-

Many hormones have been shown to regulate their own receptors. For example, insulin down-regulates its receptor in many systems (46-48). Investigation of the possible control of GH receptors by the hormone ligand is therefore of considerable interest. As with insulin, the GH receptor appears to be down-regulated in some systems (49-54). For example, Lesniak and Roth (52) found that human GH depressed IM-9 lymphocyte receptor levels. The degree of down-regulation was dose-dependent with 50% reduction

at 2 X 10^{-10}M (5.0 ng/ml, the *in vivo* basal concentration of GH) and 80% loss at 20 ng/ml at 30°C steady state condition. This reduction in receptor binding appeared to be due to decreased receptor concentration rather than alteration of receptor affinity or cell number. Removal of GH from the media restored receptor levels with 50% recovery in 6-8 h and full replenishment by 24 h. Restoration of the receptors seemed to require protein synthesis as recovery was significantly inhibited by 10^{-4}M cycloheximide (52). Similar results with IM-9 lymphocytes were reported by Rosenfeld and Hintz (54), who used this phenomenon to develop a radioassay for human GH. In their study, 10% receptor loss occurred with 1.25 ng/ml GH, and a 50% decrease was found with 6-8 ng/ml GH (54). It is of interest to note that this down-regulation also occurred in response to ovine and human placental lactogen and ovine prolactin (55).

Down regulation of fibroblast GH receptors was also observed in human fibroblasts (53). A 24 h preincubation of cultured fibroblasts with human GH reduced receptor binding approximately 20% at 5 ng/ml and 55% at 500 ng/ml GH (53).

In many other biological systems, GH appears to actually induce its own receptor levels. For example, a study by Herington et al. (56) showed that high GH levels produced by transplantable GH producing tumors correlated with increased rat liver GH receptor binding. However, injection of 100 or 500 μg of bovine GH/day for 5-10 days caused no apparent change in GH binding sites. In 1978, Furuhashi and Fang (57) studied the relationship between hepatic GH receptors and serum GH levels in normal and

GH_3 tumor secreting rats. GH receptors were found to be elevated in tumor-bearing rats (with high GH levels), suggesting that GH induces its own receptor. The increased binding was due to an enhanced number of binding sites, with little or no change in the affinity constant (Ka). In normal animals, GH receptor binding correlated with rat serum GH levels (58).

Further evidence that GH enhances hepatic receptors was reported by Vezinhet et al. (58) and Posner et al. (59) using rabbit and sheep. In their studies, hypophysectomy greatly reduced GH receptor levels in both experimental animals. When hypophysectomized sheep were injected with 1 mg/kg ovine or bovine GH every other day for 19-21 days, the GH receptor levels were significantly enhanced. Receptor binding reflected an alteration of GH receptor concentration, not changes in affinity for the ligand.

However, Herington (30) found no clear relationship between rat hepatic receptor binding and known age-dependent patterns of GH levels, suggesting that endogenous GH may not exert significant control over its own receptor in normal *in vivo* conditions.

In an investigation by Baxter et al. (60), GH levels were raised about 200-fold in MtT/Wl5 pituitary tumor-bearing Wistar-Furth rats. Initial liver GH receptor measurements were unchanged in male rats and decreased by over 75% in female animals in the tumor bearing animals. However, desaturating the GH receptors by incubation with 3.2 M $MgCl_2$ for 5 min, resulted in GH

receptor level measurements 2-3 fold higher in tumor-bearing animals compared to controls. While this study supports the contention that GH induces its own receptor, alteration of the receptor levels may have been due to prolactin which is also secreted by MtT/W15 tumors.

Further investigation of GH regulation of its receptor was conducted by Baxter et al. (31) using implanted osmotic minipumps to release rat GH or ovine GH into female rats. Again, $MgCl_2$ treatment was used to release endogenous hormone from receptor sites so that total receptor levels could be measured. At hormone release rates of 150-400 μg rat GH/day the GH receptor levels were enhanced 2-3 fold. Unlike rat GH, ovine GH infused 75-400 ug/day did not consistently effect GH receptors. Baxter and Zaltsman (61) showed that enhancement of GH binding sites by infusion of 200 μg rat GH/day for 7 days occurred in both normal and hypophysectomized rats.

In a recent study by Gorin and Goodman (63), GH receptors were resolved into 3 molecular weight bands by SDS gel electrophoresis (as described in section VI - growth hormone receptor characterization). After hypophysectomy, GH receptor binding was reduced approximately 50%, however, no change in the relative proportion of the 3 species was observed.

Recently, Gause and Eden (64) showed that the mode of GH replacement greatly influenced GH receptor modulation in rat adipocytes. As in many other tissues, hypophysectomy greatly reduced receptor binding. The hypophysectomized rats were given

T_4 and cortisone therapy which only partially restored binding levels. GH was administered as 2 injections/day, 4 injections/day or by an osmotic minipumping system (64). Rats injected with GH twice a day showed little alteration in receptor levels. On the other hand, GH injected 4 times a day or by osmotic minipumps significantly enhanced GH receptor levels if assayed up to 6 h (but not 12 h) post-GH-treatment.

Endocrine regulation of non-hepatic GH receptors has also been investigated. As previously mentioned, hypophysectomy depressed GH receptor binding in rat adipocytes (64). This decrease was not restored by cortisone acetate treatment (50 µg/100 g-day), but was enhanced to about 50% recovery by cortisone acetate plus T_4 (1 µg/100 g-day) treatment (64).

In 1983, Stewart et al. (62) showed that GH could induce its own receptor in man. In their study, human GH treatment was administered to GH-deficient children. 2 1/2 h After injection lymphocyte GH receptors were found to be elevated over 4-fold, and at 5 h they were increased almost 3-fold over low initial values. Children injected with chorionic gonadotropin showed no alteration of receptor binding.

B. Effect of other (non-GH) hormones-

In addition to GH, several other hormones influence GH receptors. (Many of these studies have utilized the rat hepatic system.) In 1976, Herington et al. (56) reported that rat hepatic receptors

were enhanced by a 10-12 day treatment with β-estradiol (25 ug/day). This finding would seem to support other studies which reported enhanced binding in females (65,66). However, Furuhashi et al. (57) found little or no relationship between serum estrogen, corticosterone or prolactin levels and rat liver GH receptor binding. Interestingly, they also reported an inverse correlation between GH receptors and rat serum testosterone levels (57).

Baxter and Turtle (33) investigated the effect of diabetes on rat liver GH receptor levels. In their studies, GH receptors were reduced about 50-80% in streptozotocin-induced diabetic rats. Insulin treatment significantly restored GH receptor levels. Immunoreactive GH levels were unaltered by streptozotocin, indicating that changes in GH levels did not cause the alteration in receptor binding in the diabetic animals. Insulin status had little or no effect on the affinity constant, which was reported at $5.6 \times 10^{9} M^{-1}$ (33). It is also interesting to note that 3-day fasting in rats caused a 67% decrease in immunoreactive insulin, which was correlated with a fall in hepatic GH receptor binding. While many interpretations are possible, it was speculated that hypoinsulinemia produced the fall in GH receptors.

Many of the biological actions of GH have been attributed to the generation of somatomedin (1-14). Studies have, therefore, been conducted to determine if GH-mediated regulation of GH receptors might be due to somatomedin effects. In recent studies (31,61)

using GH infusion into normal rats, serum GH concentrations and hepatic GH receptors were increased, while somatomedin-C was unaltered or lowered in these normal animals. These results suggest that somatomedin is probably not responsible for changes in hepatic GH receptor levels.

C. Influence of Sex, Age and Pregnancy-

A number of studies have shown an age, sex and pregnancy dependence of GH receptor levels. Overall, puberty and pregnancy are characterized by significant increases in GH receptor binding. While binding does not increase in males after puberty, females usually show an increase in adulthood. Examples of specific findings in these areas of regulation are discussed below.

In 1974, Kelly et al. (65) reported that GH binding sites on liver membranes were higher in female rats (compared to male controls) of all age groups ranging from 10 days to adult (over 40 days). While binding in female rats increased with age, the GH receptor binding in male animals remained almost constant. In a more recent investigation, DeHertogh et al. (66) found that rat liver GH binding was greater in females between 50-120 days, but binding was about equal to males at ages of 40 days or less. Binding in female samples steadily increased (from about 6-8 fmol/mg protein at 8 days of age to over 60 fmol/mg protein at 120 days) with age. In the male animals binding rose up to 30-40 days of age (about 6 fmole/mg protein at 8 days to almost 40

fmol/mg protein at 35 days), appeared to decrease between 35 and 50 days (down to about 15 fmoles/mg protein), and finally increased again reaching about 30 fmole/mg protein at 120 days. A study by Baxter and Zaltsman (61) showed that induction of GH receptors by infusion of GH occured in both sexes.

In a recent study by Husman et al. (67), no difference in hepatic receptors was found in $MgCl_2$-treated samples from male and female rats. The apparent dissociation constants (Kd) were also essentially identical with 0.24×10^{-9}M in female and 0.27×10^{-9}M in male rat preparations (67).

Herrington (30) reported that receptor binding (before and after $MgCl_2$-treatment) to bovine GH was not significantly different in male and female rats until attaining adulthood; at which time receptor binding was significantly higher in female animals.

The early work of Kelly et al. (65) also showed that rat liver membrane GH binding sites were slightly greater than controls during early pregnancy, and was over 200% of nonpregnant female control values from 20-day pregnant rats. Similarly, Herington et al. (56) reported that the hepatic membranes from pregnant rats had about twice the number of GH binding sites than from female controls. In a recent study (67), 2-4 enhanced binding in pregnant rat hepatic preparations was found before and after $MgCl_2$-treatment (to remove endogenous ligand). The Kd of pregnant rat preparation was $0.44 \times 10^{9}M^{-1}$, while that of nonpregnant rat samples was $0.27 \times 10^{9}M^{-1}$.

D. Starvation-

In 1982, Postel-Vinay et al. (68) reported that hepatic GH receptors were low in starved rats. Thus only 45% of control binding was observed in microsomal membranes and 52% of control in plasma membrane preparations. This result was independent of GH levels since immunoactive GH concentration was the same in fasted and normal rats. Similar results were reported by another group (69).

Recently, Gorin and Goodman (73) showed that the relative proportion of 3 different molecular weight forms of the receptor (discussed in section VI - Growth Hormone Receptor Characterization) was not altered by fasting.

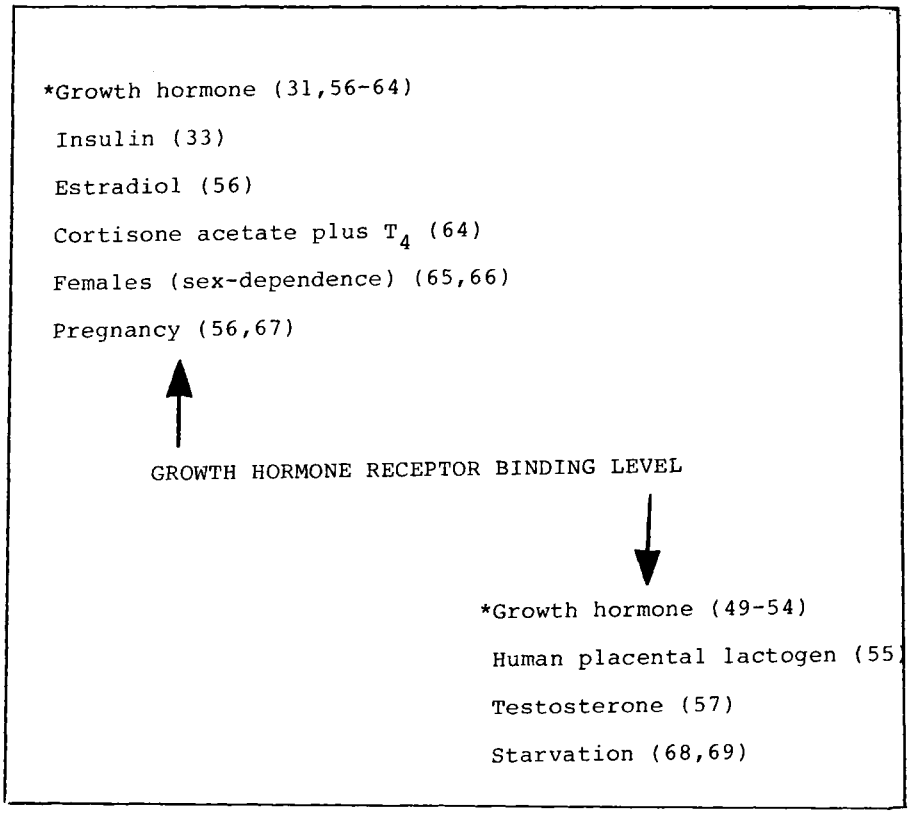

Fig. 1 Physiological Regulators of Growth Hormone Receptor Binding Levels . The physiological factors which increase (↑) and decrease (↓) GH receptor binding are shown above. Reference numbers are in parentheses. Growth hormone has been shown to increase and decrease receptor binding depending on the tissue examined (*).

E. Chemical modulators-

Several chemical reagents have been shown to alter GH (70) receptor binding. Such studies can lend insight into:

a) how receptor binding could be altered by physiological regulators
b) how receptor binding might be chemically/pharmacologically regulated *in vivo* and *in vitro*
c) the chemical nature of the GH receptor and hormone-receptor interaction

In 1976, Van Obberghen et al. (70) investigated the influence of microfilament and microtubule modifying agents on human lymphocyte (IM-9) GH receptors. When lymphocytes were incubated with 10 µg/ml cytochalasin A, B, and D, receptor binding was reduced 60%. This loss of binding was due to a reduction in the number of binding sites and was not reversible by removal of cytochalasins. On the other hand, the anti-microtubule reagents vincristine, colchicine and vinblastine had no apparent effect on the GH receptor binding properties. These results therefore suggested that microfilaments, but not microtubules, were involved in expression of GH receptors on the surface of human lymphocytes (70).

In another study, the effect of plant lectin concanavalin A (Con A) on rabbit and rat hepatic GH receptor binding was studied

(71). Con A depressed binding (about 30%) in particulate and soluble microsomal membrane preparations in a concentration-dependent manner. The Con A competitor, α-methyl-mannoside prevented the action of Con A on receptor binding properties. Con A appeared to have little or no effect on receptor binding affinity, but depressed the number of binding sites possibly by binding directly to the receptor protein (71).

In a report by Tsim and Cheng (72), the thiol-reactive agent p-chloromercibenzene (1 mM) had no effect on rat hepatic GH receptor binding properties. On the other hand, this reagent completely inhibited prolactin binding to hepatic receptors.

Martal et al. (73) chemically modified highly purified GH preparations to study structural components on the GH molecule that are required for receptor binding. Methylation, ethylation, quanidination and acetimidination all significantly disrupted binding to liver homogenates. The lysine or arginine groups at positions 41, 64, 70 and 115 were implicated as residues that may be important in hormone-receptor interaction.

In another study, methoxylglycyl residues were introduced into the GH molecule to determine their influence on receptor binding (39). Carboxylate groups were chemically reacted with glycine methyl ester and water-soluble carbodi-imide. While modified GH molecules containing up to 7-8 methoxylcyl residues appeared to have similar potency as native GH in *in vitro* membrane and hepatocyte binding assays. Only forms (3 methoxyglycl residues)

which retained biological activity in a growth assay displaced labeled GH in an *in vivo* binding method.

In a study by Blossey (74), GH binding to rabbit liver membranes was slightly reduced by dithiothreitol, β-mercaptoethanol and N-ethylmelaimide, while enhanced by 20 mM L-cysteine. Membranes treated with phospholipase A_2, C and D bound hormone similar to controls, while DNase and RNase slightly enhanced binding. Neuraminidase appeared to have no effect on binding, whereas α-and β-galactosidase greatly reduce binding. A schematic diagram showing the influence of various chemicals and enzymes on GH receptor binding is shown in Fig. 2.

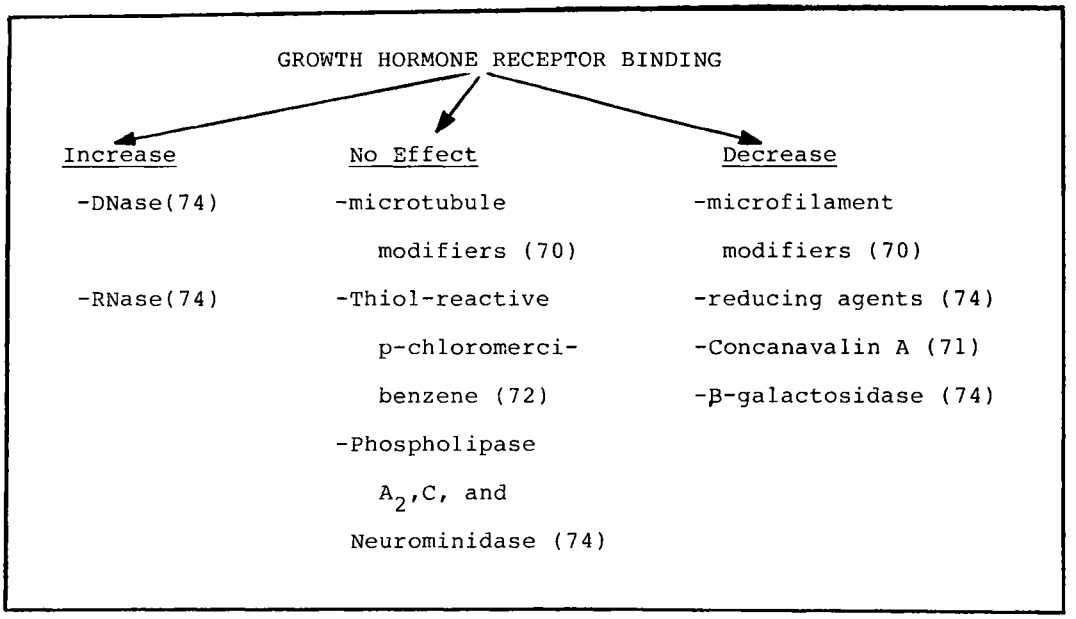

Fig. 2. Chemical and Enzymatic Modulators of Growth Hormone Receptor Binding. The effect of chemical and enzymatic modulators of GH receptor binding (increase, decrease or have no effect) are shown above. Reference numbers are in parentheses.

F. Clinical Aspects of Growth Hormone Receptors-

The clinical significance of GH receptor levels and modulation is largely unexplored. One possible correlation that has been investigated involves Laron-type dwarfism which is characterized by short stature but high blood levels of GH (1,2,75,111). The circulating GH in these dwarfs appears to be active in other systems, and exogenous GH does not enhance somatomedin levels or increase growth rates. A tissue-level defect was therefore

postulated for the cause of this dwarfism. In a rather preliminary but provocative clinical study, essentially no GH receptor binding was detected in liver biopsies from two patients with Laron-type dwarfism (75). On the other hand, liver samples from all 6 healthy subjects demonstrated a significant number of specific binding sites. The liver from Laron dwarf patients did possess binding sites for ^{125}I-insulin. These results support the contention that Laron-type dwarfs may have defective hepatic GH receptors which could account for depressed production of somatomedin (75). Since GH depresses GH receptor levels in some (not all) tissues, exogenous GH may not provide help to short children due to receptor reduction. On the other hand, short children with low levels of endogenous GH may be more responsive to GH-treatment due to availability of functional receptors (112).

V. GROWTH HORMONE RECEPTOR PURIFICATION

Purification of GH receptors is important for:

a. chemical characterization of the protein (including amino acid composition(s) and sequence(s), subunit analysis, chemical modifications such as phosphorylated residues, and determination of microheterogeneity)
b. understanding the physicochemical nature of GH-receptor interaction
c. generation of specific antibodies to the GH

receptor for further analysis such as subcellular location (see Section VII - preparation and use of antibodies to growth hormone receptors).

Purification of this protein has been difficult, in part due to the problem of separating GH receptors from prolactin receptors. At high concentrations prolactin is able to displace nearly all GH from its liver receptor, and likewise GH is capable of displacing some of the prolactin from prolactin-specific membrane receptors (16). Radioimmunoassays have indicated that cross-contamination of the hormones was not the cause of this phenomenon, but rather there was a degree of receptor site sharing. In mammary tissue the prolactin receptor appears to be much more specific (76,77).

After detergent solubilization of the receptor, affinity chromatography using covalently linked GH (usually human) has proven to be one of the most effective procedures in purification of the GH receptor. Affinity chromatography using Concanvalin A is also effective since the carbohydrate portion of the receptor binds to this lectin. For example, Tsushima et al. (110) showed that GH receptors would bind Concanavalin A, and then could be selectively eluted using methyl-glucoside.

GH receptor purification attempts utilizing affinity chromatography were initiated in the 1970's. Gottsmann et al. (78) solubilized rabbit liver microsomes with 1% Triton X-100 (at $37^{o}C$ for 30 min). Receptor binding was detected using ^{125}I-labeled human GH ligand. After removal of the Triton by

Sephadex G-200 chromatography, receptor purification was performed using affinity chromatography in which 4 mg of human GH was chemically attached to 1 g of CNBr-activated Sepharose 4B. Elution of the receptor was carried out with 4 M ammonium thiocyanate.

McIntosh et al. (35) solubilized microsomes from livers of pregnant rabbits using Tris-HCl, pH 7.5 containing Triton X-100 (1 mg/mg protein). Approximately 70-80% of the receptor binding was solubilized (35). In order to prepare an affinity column, human GH (in 0.1 M $NaHCO_3$, pH 8.6) was chemically coupled to the N-hydroxy-succinimide ester of 3,3´-diaminodipropyl-amino-succinyl agarose (Bio-rad, Affi-Gel 10). After mixing for 4 h at 4^oC glycine (100 ml, 1M) was added. The affinity gel was then washed with 6 M guanidine hydrochloride (200 ml), 8 M urea (500 ml), and 0.1 M $NaHCO_3$ (2000 ml, pH 0.5). Columns were equilibrated with 50 mM Tris-HCl, pH 7.0-9.5, containing 0.1% Triton X-100 and 10 mM $MgCl_2$. Maximal binding was observed at pH 7.5. The receptor was eluted using 5 M $MgCl_2$ with a recovery of about 67%.

Unfortunately, these early purification attempts (35,78) did not clearly separate GH receptors from prolactin receptors. In 1979, Waters and Friesen (76) reported a technique to correct this problem. The pregnant rabbit liver system was chosen because of the advantage of having a particularly high level of GH receptors (76,79). The 3-stage purification procedure first utilized human GH affinity columns. Separation of prolactin-specific receptors from GH-specific receptors was achieved by use of Triton, which

increased the K_A of the prolactin-specific receptor 5-fold (5 x $10^{9}M^{-1}$ to 2 x $10^{10}M^{-1}$), while slightly decreasing the K_A of the GH receptor from 3 x $10^{-9}M^{-1}$ to 2 x $10^{9}M^{-1}$. Under these conditions the affinity of prolactin and GH receptors for GH differed 10-fold, so that the GH receptor could be eluted from the affinity column with 4 M urea, while the prolactin-specific receptor required 5 M $MgCl_2$. Purification was then continued using preparative isoelectric focusing and Sepharose 6B gel chromatography (76).

The GH affinity gels were prepared coupling human GH (hGH) or bovine GH (bGH) to Affi-Gel 10 at pH 8.5 using the procedures provided by Bio-Rad (76). The reaction was quantitated by use of ^{125}I-labeled hGH or bGH in the coupling mixture (50 mg samples). After coupling overnight at 4°C, 5 ml of ethanolamine-HCl (1 M, pH 8.5) was added per 10 g of gel to stop the coupling reaction. This mixture was incubated for 2 h at 22°C. The gel (30 ml samples) was then washed with $NaHCO_3$ (2000 ml, 0.1 M), 8 M urea (500 ml in 0.1 M $NaHCO_3$), $NaHCO_3$ (1000 ml, 0.1 M), 5 M guanidine-HCl (300 ml in 0.1 M $NaHCO_3$), $NaHCO_3$ (1000 ml, 0.1 M), NaCl (1000 ml, 2 M), sodium acetate/acetic acid, pH 4.0 (1000 ml, 0.05 M), $NaHCO_3$ (1000 ml, 0.1 M), NaH_2PO_4 (1000 ml, 0.2 M), Tris-HCl (2000 ml, 0.025 M with 0.1% Triton X-100, pH 7.4) (Tris/Triton), $MgCl_2$ (500 ml, 5 M in the Tris/Triton X-100 solution) and Tris/Triton X-100 (1000 ml). To remove "labile" bound hormone, the gel was incubated for 2 h at 22°C with 2 vol/vol of a rabbit liver soluble membrane preparation in the presence of the proteinase inhibitors Trasylol (5000 Kallikrein

inactivity units) and phenylmethanesulfonyl fluoride (0.3 mM). The gel was then washed with 6 M urea, 5 M $MgCl_2$ in Tris/Triton X-100 and the Tris/Triton X-100 (2000 ml). The gel was washed again with Tris-Triton X-100 just before addition of Triton solubilized samples. Liver membranes prepared by the procedure described by Tsushima and Friesen (41). Membranes were suspended in 25 mM Tris-HCl buffer at pH 7.4 with a protein concentration of 5-10 mg/ml. Receptors were solubilized by addition of Triton X-100 or Triton X-305 (1% v/v final) and mixed for 10 min at 25°C. In bulk scale purification (starting with 250 to 500 g of pregnant rabbit liver) the membranes were initially treated twice with Triton X-305 since it solubilizes fewer membrane proteins than Triton X-100. Five volumes of Triton-extract were added per 1 volume of affinity gel and mixed in 10 mM $MgCl_2$, Trasylol (5000 Kallikrein inactivity units/100 ml extract), and 0.3 mM $PhCH_2SO_2F$. After incubation for 2 h at 22°C the gel was put into a column and washed with over 150 volumes of cold Tris/Triton X-100 solution. The affinity gel was slowly eluted with 3 volumes of 4 M urea in Tris/Triton X-100 solution and samples were collected. The gel was then treated with 5 volumes of 6 M urea in Tris/Triton X-100, Tris/Triton X-100 and finally 3 volumes of Tris/Triton X-100 containing 5 M $MgCl_2$. Urea eluted samples (as well as the others in preliminary studies) were then dialyzed 2 times at 4°C against Tris/Triton X-100 solution for binding studies and further purification. The affinity step yielded approximately 70-fold purification and SDS gels revealed about 15 protein bands.

Further purification by preparative isoelectric focusing showed most GH receptor binding in the pH 4-5 range (76). Focusing was carried out in 0.1% Triton X-100 for 48 h at 4°C. Binding assays were performed after adjusting the pH to 7.5 with 2 M NaOH or 2 M HCl (76).

The final major purification step utilized preparative Sepharose 6B gel chromatography. Samples (20 ml aliquots) were filtered on 118 x 2.55 cm columns using a Tris/Triton X-100 buffer. The chromatrography was run on a 60 cm head with a flow rate of 17 ml/h at 4°C. An 8,000-fold purification of the GH receptor was achieved by Waters and Friesen (76).

Receptor techniques yielding still greater purification in simpler more efficient systems are still being sought. One technique of potentially great usefulness is monoclonal antibody (to GH receptors) affinity columns. Such a procedure was initiated by Simpson et al. (80) as described in Section VII (preparation and use of antibodies to growth hormone receptors).

While these studies have been very important in GH receptor research, large scale purification of receptors from various animal and tissue sources is greatly needed in future investigations.

Table I

Growth Hormone Receptor Purification Studies

Investigators	Year	Receptor Source	Reference Number
Gottsman et al.	1976	rabbit liver	78
McIntosh et al.	1976	pregnant rabbit liver	35
Waters & Friesen	1979	pregnant rabbit liver	76
Simpson et al.	1983	rabbit liver	80

VI. GROWTH HORMONE RECEPTOR CHARACTERIZATION

The growth hormone receptor(s) from various animals and tissues has been characterized by a variety of biochemical techniques. In this section the methodologies used to characterize purified and partially purified GH receptors will be discussed. While still uncertain, it should be emphasized that multiple receptor forms may exist. Thus the specific animal, tissue and ligand may effect data concerning GH receptor properties. Evidence for GH receptor heterogeneity comes from antibody studies (see section VII), characterization of receptor forms from different sources, and differential binding of different GH forms. For example, receptor binding characteristics of rat GH, 22k human GH and 20k human GH was compared (81). While each of these forms of GH were equally effective in inhibiting ^{125}I-rat GH binding to rabbit hepatic receptors, the 20k human GH and rat GH were much less

potent than the 22k human GH in blocking 22k ^{125}I-labeled human GH to the same membranes. This difference was not due to lactogenic receptors, but rather the 20k human GH and rat GH were believed to bind to a smaller subset of GH receptors.

In a recent review by Hughes et al. (104) a model was presented in which 3 classes of rabbit liver GH binding sites were identified according to their abilities to bind different GH molecules. GH receptor-1 was a low capacity binding protein which bound human GH, rat GH, rabbit GH and a 20k varient human GH with similar affinities. GH receptor-2 was characterized by high affinity for human GH and low affinity for the other GH molecules. This second receptor accounted for 85-90% of GH receptor binding capacity in the rabbit liver membranes. The third binding site was identified as a prolactin receptor, which binds human GH and the 20k varient, with low affinity for rat and rabbit GH.

A. General GH Receptor Binding Characteristics-

The basic characteristics of GH to its receptor(s) has been investigated in many systems. For example, Gavin III et al. (82) showed that GH receptor binding in isolated rat adipocytes was reversible and time, temperature and pH dependent. Optimal specific binding occurred in about 40 min at 37^{o}C, pH 7.4 (82). Linear Scatchard plots showed a Ka of approximately $10^{9}M^{-1}$ with about 15,000 binding sites per cell. The adipocyte receptor did not significantly discriminate between

rat, monkey, porcine and bovine GH. Specificity was shown by the lack of binding to human placental lactogen and prolactin.

In a recent study by Gorin and Goodman (28) the rate of turnover of rat adipocyte GH receptors was investigated. In this report, preincubation of fat cells with either 20 μg/ml cycloheximide (a protein synthesis inhibitor) or 200 μg/ml puromycin (an inhibitor of translation) caused a steady loss of specific GH receptor binding following first order kinetics. Loss of GH receptor binding had a half-life of approximately 45 min, which was unaltered by the presence or absence of GH. When fat cells were treated wth 0.1 mg/ml trypsin for 10 min, receptor binding was destroyed. However, binding sites returned to near normal levels 2 h after trypsin removal. This recovery could be prevented by addition of cycloheximide after trypsin was removed.

Binding of ^{125}I-GH to human peripheral mononuclear cells (PMC) was shown to be maximal at 2 h incubation at 37^{o}C (37). Saturation was found with 25 mg ^{125}I-GH per 10^{6} PMC. Half-maximal receptor binding inhibition was observed at 12-25 ng unlabeled GH in incubations containing about 10^{6} PMC in 1 ml Tris-HCl buffer. The binding was not very sensitive to potassium concentration or pH, while sodium, calcium, and magnesium ion concentration significantly altered GH binding.

Maximal binding of ^{125}I-human GH to human fibroblasts occurred in 2 h at 30^{o}C (53). 30 ng/ml GH produced half-maximal binding in these cells. Scatchard analysis indicated a single class of receptor sites with an affinity

constant of 1.07 x $10^{9}M^{-1}$. 50% dissociation occured in about 1.5 h at 30°C and 3 h at 15°C. In addition they found no apparent change in specific binding with alteration of pH from 7.4-8.9 (53).

The time and temperature dependent binding of ^{125}I-labeled human GH to the insulin-secreting cell line RIN-5AH has also been studied (38). A steady state binding was achieved in 60 min at 37° and 120 min at 24°C. Approximately 80% dissociation occured in GH-free media after about 120 min. Half-maximal receptor binding occured with 3 x 10^{10}M GH. The receptor also bound to rat GH and human placental lactogen, but with less affinity than the human GH. Scatchard analysis suggested about 2,700 high affinity receptor sites per RIN-5AH cell.

GH receptors of rat liver microsomes and golgi fractions were recently characterized by Husman et al. (67). Binding was shown to be protein, time and temperature-dependent, with maximal binding at 15-20 h in microsomal membranes and 15-16 h in golgi fractions at 22°C. Receptors appeared to be somatogenic, as 50% binding was inhibited by 5-130 ng bovine, rat or human GH, while a much greater amount (500 ng) of rat prolactin was required for 50% displacement. Treatment of membranes with 3 M $MgCl_2$, to remove endogenous ligand, enhanced binding 2- to 3-fold. Subcellular fractionation experiments showed about 20- to 25-fold higher concentration of receptors in golgi/endosomal preparations compared to total membrane fraction. Only low receptor binding was located in lysosomal fractions and

non-golgi/endosomal microsomes contained 2-fold enhanced receptor binding.

An investigation by Burstein et al. (83) using mixed recombinants of human GH and chorionic somatomamanotropin indicated that the initial 134 residues of GH are involved in hormone-receptor interactions. Similarly, an N-terminal fragment (Mr of 15,000 daltons) of human GH was shown to bind to receptors on IM-9 human lymphoblastic cells (84). Table II summarizes some of the various species and tissues where GH receptors have been identified.

Table II

Growth Hormone Receptors Identified

in Various Animals and Tissues

Tissue Source	Animal Source	Reference No.
Liver	rat	33,67
	rabbit	78
	pregnant rabbit	35,76
	mouse	106
	sheep	105,107
	human	108
Adipose	rat	28,82,86
	sheep	107
Lymphocytes	human	62,84
Fibroblasts	human	53
Thymocytes	bovine	109
	mouse	109
Insulinoma	rat	38
Peripheral mononuclear cells	human	37

B. Gel filtration-

Gel filtration has been used by many groups to purify and characterize GH receptors from various sources. McGuffin et al. (26) estimated a molecular weight of 200,000 or more for human lymphocyte GH receptors using Sephadex G-200 chromatography of soluble receptors prepared without the use of detergent. In a study by Gottsmann and Werder (78), pregnant rabbit liver ^{125}I-human GH binders were calculated at Mr of about 250,000 and 500,000 on 3 x 80 cm Sephadex G-200.

Using a 90 x 1.5 cm Sepharose 6B column, and an elution buffer consisting of 50 mM Tris-HCl, 0.1% Triton and 10 mM MgCl at pH 7.6, McIntosh et al. (35) calculated Triton solubilized rabbit liver GH receptor Mr to be about 200,000 daltons. However, due to association of Triton with the receptor, the Mr of Triton-solubilized receptors may be overestimated (85). In a more recent study by Waters and Friesen (76), the rabbit liver GH receptor was separated from possible contamination and cross-receptor binding from prolactin receptors. Molecular size was estimated using a 118 x 2.55 cm Sepharose 6B column eluted with 0.025 M Tris-HCl containing 0.1% Triton X-100, pH 7.4. The column was run with 60 cm head and a flow rate of 17 ml/h at 4^{o}C (76). The 8,000 fold purified receptor was calculated to have a Mr of 300,000 daltons and Stokes radius of 62 A. A smaller peak of less than 40,000 daltons was also noted, which could represent dissociated subunits or a receptor cleavage fragment.

C. Electrophoresis-

Electrophoretic analysis has indicated that the GH receptor may contain subunits or is attached to non-receptor proteins. In a recent study by Gorin and Goodman (63) rat adipocyte GH receptors were studied by SDS gel electrophoresis. ^{125}I-labeled human GH was cross-linked to intact adipocytes using the bifunctional coupler disuccimimidyl suberate (1 mM). Samples were then solubilized in 1% SDS in the presence or absence of DTT (usually 100 mM) before applying to 7.5% or 5% polyacrylamide gels. Electrophoresis separation of proteins was conducted for 4-5 h at 30 mA constant current. Gel proteins were then visualized by staining with 0.05% coomassie blue R in 25% propanol-7% acetic acid. Gels were destained with 5% isopropanol-7% acetic acid. ^{125}I-Hormone-receptor complexes were detected on destained gels by autoradiography. In the absence of DTT, 3 radioactive bands were observed with Mr of 56,000, 130,000 and 250,000 on 7.5% gels. The presence of excess unlabeled GH during binding of ^{125}I-GH to adipocytes resulted in the absence of all three binding species. Taking into account the molecular weight of GH (about 22,000 daltons) the receptor proteins averaged Mr of approximately 32,000, 108,000 and over 230,000 (63). The presence of a reducing agent diminished the high molecular weight band and enhanced the 130,000 species suggesting that 130,000 molecular weight subunits could be generated from the high molecular weight receptor. About 42% of the radioactivity was found in the high molecular weight species, 39% in the 130,000 dalton binder and 19% in the low molecular weight form in the absence of DTT. When DTT was present 25% of

the high molecular weight activity shifted to the 130,000 dalton band.

When the samples were analyzed on 5% gels the high molecular weight band was resolved into 2 bands of 240,000 and 310,000 daltons. The inclusion of protein inhibitors, N-ethyl maleimide or sulfhydryl alkylating reagents in the preparation of the receptor did not alter their results, supporting the contention that multiple molecular weight binding species were not artefactually generated (63). In a later study, Gordin and Goodman (28) showed that the 56,000, 130,000 and 250-300,000 Mr species were reduced with a similar half-life after treatment of adipocytes with cycloheximide.

Carter-Su et al. (86) also studied the biochemical characteristics of rat adipocyte GH receptors using SDS gel electrophoresis. Receptors were covalently labeled with ^{125}I-GH by incubating cells with 0.4 mM disuccinimidyl suberate for 15 min at 15^{o}C. The cross-linking reaction was stopped by addition of excess buffer (10 mM Tris, 0.25 M sucrose, 1mM EDTA, pH 7.8). Autoradiographs of SDS gels revealed a major band at Mr=134,000 when samples were reduced. This peak could be eliminated when samples were incubated with a large excess of unlabeled GH (but not insulin or prolactin). At low concentrations of reductant molecular weights of 135,000 and 270,000 daltons were observed, suggesting that the receptor may contain intrachain disulfide bonds. As the reducing agent was increased the Mr=270,000 form was reduced with a corresponding increase in the Mr=134,000 species. After accounting for the

weight of GH, the receptor protein (in reduced form) was calculated to be about Mr=112,000. These results therefore agree quite well with studies by Gorin and Goodman (63).

Disuccinimidyl suberate was used to covalently couple ^{125}I human GH (5 nM) to rat hepatocytes for biochemical analysis (87). 7.5% Polyacrylamide gel electrophoresis indicated complexes of Mr=220,000 and 300,000. Reduction with 100 mM dithiothreitol led to the generation of a Mr=130,000 form with concommitant reduction in the higher molecular weight species. Subtracting the molecular weight of GH from this complex, the major reduced binding protein had a molecular weight of about 100,000.

In a later study on rat hepatic GH receptors, Yamada and Donner (88) observed multiple binding protein complexes with molecular weights of 300,000, 220,000, 130,000, 65,000 and 50,000 daltons. Unlike the larger complexes, prolactin inhibited binding to the 65,000 and 50,000 dalton species. After accounting for the hormone itself, the receptor proteins were calculated to have Mr of 280,000, 200,000 and 100,000.

In a study (76) using highly purified rabbit liver GH receptors, 3 bands were observed on mercaptoethanol reduced SDS slab gels with the major protein at about 80,000 Mr. The proteins were visualized by staining with 0.2% Coomassie blue in 50% trichloroacetic acid. Destaining was conducted using methanol/acid acid/water. A second investigation of rabbit liver receptors showed major SDS bands at 56,000, 68,000, and 76,000 Mr (74).

A summary of electrophoretic estimations of GH receptor(s) molecular weights is shown in Table III.

Table III

Electrophoretic Estimations of Growth Hormone Receptor Molecular Weights

Reference Number	Receptor Source	Mr of Growth Hormone Receptor	
		Not Reduced	Reduced
63	rat adipocyte	32,000	32,000
		108,000*	108,000*
		240,000*	230,000
		310,000*	
86	rat adipocyte	112,000	112,000
		250,000	
87	rat liver	200,000	100,000
		280,000	
88	rat liver	100,000	100,000
		200,000	
		280,000	
76	rabbit liver	80,000	
74	rabbit liver	56,000	
		68,000	
		76,000	

*designates the major receptor form

D. Isolectric Focusing-

Waters and Friesen (76) characterized highly purified rabbit liver GH receptors by analytical slab gel isoelectric focusing. Gels consisted of riboflavin-polymerized 3.5% acrylamide, 0.1% methylene bisacrylamide and 0.1% Triton X-100. A pH gradient of 3.5 to 9.0 was used. Samples were applied to the area corresponding to pH 7.0. Focusing was conducted in $4^{o}C$ for 4 h at 8 watts. 0.5 cm samples were taken from the gel and eluted at $4^{o}C$ in 0.1 M Tris/Triton buffer, pH 7.5. The pI of the Triton X-100 solubilized receptors was approximately 4.6 with considerable charge heterogeneity. Interestingly, treatment of the receptor preparation with neuraminidase for 30 min at $37^{o}C$ at pH 5.85 caused a shift in the pI to about 6.2 with slightly less charge heterogeneity, suggesting that the receptor is a sialoglycoprotein and that differential sialic acid content contributed to the observed heterogeneity seen with isoelectric focusing.

Further evidence that the GH receptor is a glycoprotein was indicated by the decreased number of IM-9 lymphocyte receptor sites resulting from treatment with tunicamycin, an antibotic which blocks N -glycosylation (89).

E. Two-dimensional gel electrophoresis-

Two-dimensional gel electrophoresis is a very powerful tool in characterizing proteins. In a study by Carter-Su et al. (86), two-dimensional gel electrophoresis was used to study the

adipocyte GH-receptor complexes. Rat adipocytes were incubated with 60 ng/ml ^{125}I-human GH for 2 h at 37^{o}C. Cells were incubated with the cross-linking agent, ethylene glycol bis (succinimidyl succinate) prior to plasma membrane preparation. Samples were reduced with 10 mM dithiothreitol and solubilized with 1% sodium dodecyl sulfate. Analysis revealed that the mononeric 22,000 Mr human GH molecule bound to a membrane receptor protein of approximately 112,000 Mr in reduced form assuming a stoichiometry of 1:1 for hormone and receptor. While this molecular weight is larger than that reported using pregnant rat and rabbit liver (90), rabbit liver (90,91) and rabbit mammary glands glands (90), these smaller molecular weight proteins may represent lactogenic GH receptor molecules which bind prolactin with even higher affinity than GH. Prolactin rat liver receptors of about 60,000 and 37,000 Mr have previously been reported (92,93).

VII. PREPARATION AND USE OF ANTIBODIES TO GROWTH HORMONE RECEPTORS

Preparation of specific antibodies to the GH receptor(s) is of great importance for investigating the structure, function, heterogeneity, location and molecular mechanism of the GH receptor. Initial experiments generated polyclonal antibodies, and later monoclonal techniques yielded antibodies of much greater specificity to allow more definitive interpretation of results.

A. Polyclonal Antibodies to GH Receptors-

In 1978, Tsushima (94) reported generation of polyclonal antibodies against rabbit liver GH receptors. However, the purity and specificity of this receptor preparation (purified by chromatography on concanavalin-A Sepharose, DEAE-cellulose, and Sepharose 6B) was unclear, particularly in light of hormone cross-binding of prolactin and GH receptors (94,95).

Later, Waters and Friesen (95) produced antibodies in guinea pigs to rabbit liver GH receptors. The receptors were purified using a receptor-specific affinity chromatograph technique (76,95). In this procedure (95), female guinea pigs were innoculated dorsally at 10-20 intradermal sites with 10-50 µg samples of purified rabbit liver GH receptor preparations in 2 volumes of Freund's adjuvant (total volume of 1.5 ml). Injections took place at 14-day intervals. Following 3 or more injections the animals were bled every 14 days. Crude γ-globulin samples were prepared by ammonium sulfate precipitation using 1 volume of saturated ammonium sulfate in 0.05 M sodium phosphate buffer, pH 7.4. The immunoactivity precipitated in the 20-40% ammonium sulfate fraction. Precipitates were washed 2 times and dialyzed against 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl. Th antisera generated blocked binding of ^{125}I ovine GH and ^{125}I-ovine prolactin to liver membrane receptors, but did not inhibit binding of ^{125}I-ovine prolactin to rabbit specfic mammary gland GH receptors, ^{125}I-insulin to human placental membrane and rabbit liver membranes, or ^{125}I-bovine follitropin to porcine testicular cell membranes. Similarities

of immunological determinants of hepatic GH receptors from rabbits, mouse, human, and sheep species were indicated in inhibition studies using this antisera.

B. Monoclonal Antibodies to GH Receptors-

In 1983, Simpson et al. (80) utilized hybridoma technology to generate specific monoclonal antibodies to rabbit liver GH receptors. GH receptors were purified using human GH affinity columns and Sepharose 6B chromatography by the method of Waters and Friesen (76). Female $CB5F_1/j$ mice were immunized with 26 ug GH receptors injected s.c. in Freund's Complete Adjuvant every 7 days up to the 28th day. Animals were then injected i.p. with 260 ug GH receptor 4 times every 14 days. Mice were killed 3 days after the final injection and the spleens were aseptically excised.

In polyethylene glycol fusion procedures, 25 x 10^6 P3x20 myeloma cells were added to about 10^8 isolated spleen cells and pelleted by centrifugation (80). RPMI/1640 (2 ml) containing 7.5% (vol/vol) dimethyl sulfoxide and 50% (wt/vol) PEG were added to the pellet. The cells were then carefully resuspended for30 sec and allowed to stand for another 30 sec. While gently agitating the cells, 10 ml of fresh media were added an ml at a time over a 90 sec period. After allowing the cells to stand for 2-3 min, the cells were centrifuged and then resuspended in 5 ml of medium. In cloning procedures, 50 ul of cells were placed in four 24-well plates with 1 ml HAT medium containing approximately 10^6 spleen cells obtained from mice that had not been

immunized (as feeders). RPMI/1640 (0.5 ml) containing aminopterin, hypoxanthine, and thymidine was added on days 5-7 when needed. After 10-11 days hybridomas were confluent, and 0.5 ml of media were removed for analysis of antibody production. Positive wells were cloned twice by limiting dilution. About 10^7 monoclonal cells in 0.5 ml medium were injected i.p. into $CB6F_1/j$ mice (previously injected i.p. with 0.5 ml 2,6,10,14-tetra- methylpentadecane) to prepare ascite fluid. The antibody activity was detected by inhibition of ^{125}I-human GH binding to rabbit liver membranes. At a dilution of 1:10,000 ascitic fluid approximately 50% inhibition was obtained, and 95% inhibition was observed at higher concentrations (80). Experiments using protein A-Sepharose and ELISA assays for mouse Ig subclasses indicated that the antibody was an IgE type protein.

Simpson et al. (80) used the monoclonal antibodies to prepare an immunoaffinity column for GH receptor purification studies. For preparation of this affinity gel, 82 mg of antibody protein was precipitated with saturated $(NH_4)_2SO_4$ and then chemically coupled to 10 ml of activated Sepharose-4B using procedures provided by Pharmacia. The antibody affinity gel (1 ml) was put into a disposable pipette tip with a Tris 0.1% Triton X-100 buffer. 250 ml of unpurified solubilized receptor preparation were added to the gel and incubated for 48 h at 4^oC. The antibody activity could be dissociated with 0.1 M glycine, producing about 10-fold purification.

The production of monoclonal antibodies to affinity purified rabbit liver GH receptors (76) was also reported by Barnard et al. (96). In their procedure male BALB/C mice were given three 20 μg (emulsified in complete Freund's adjuvant) injections of purified rabbit GH receptor protein every 2 weeks. Two weeks later, an iv boost injection of 10 μg in saline was given. Four days later, the serum of these immunized mice, at 1:800 dilution, was able to inhibit 50% of ^{125}I-human GH binding to membrane receptors.

The splenic lymphocytes from 3 mice were mixed in culture medium (RPMI-1640 containing 15% fetal calf serum, 2 x 10^{-3}M glutamine, 100 IU/ml penicillin and 100 ug/ml streptomycin). About 10^{8} splenic white blood cells (SWBC) were fused with 25 x 10^{6} NS-1 myeloma in a 42% (wt/vol) of PEG/RPMI-1640 with 15% (vol/vol) dimethyl sulfoxide. The cells were washed and pelleted. 0.05 ml of cell suspension were added to 24-well plates containing 10^{6} feeder cells (BALB/C SWBC) in 1.0 ml of culture medium containing 10^{-4}M hypoxanthine, 1.6 x 10^{-5}M thymidine, 4 x 10^{-7}M aminopterin, and 4 x 10^{-5}M 2-mercaptoethanol (HAT medium). Cells were incubated at 37^{o}C and refed on day 5 and then when needed. To screen for antibody production Barnard et al. (96) used the immunoprecipitation method described by Waters and Friesen (95). Clones producing antibody were subcloned by limiting dilution. The best antibody producers were injected into pristene-primed BALB/C mice in order to generate ascitic fluid. The antibodies were extracted from the ascitic fluid by ammonium sulfate precipitation or DEAE chromatography. ^{125}I-antibody purity was determined by

quantitation of the maximum degree of precipitability of ^{125}I-antibody using specific antimouse immunoglobulin G.

In a recent study, four monoclonal antibodies raised against rabbit liver GH receptors and one to rat liver receptors were used as probes for investigating structural heterogeneity of GH receptor molecules (97). Using these antibodies to immunoprecipitate solubilized receptors and inhibit binding of ^{125}I-ovine GH to membrane binding sites and solubilized receptors , Barnard et al. (97) proposed three types of GH receptors in rabbit hepatic plasma membranes. Type 1 receptors were postulated to be involved in the anabolic action of GH and reacts with all the monoclonal antibodies tested. Type 2 binding sites did not possess the epitope for the anti-(rat GH receptor) antibody in the GH-binding region of the molecule and was believed to be the cytosolic GH receptor, although it was found in the plasma membrane. A third binding site (type 3) was lost during purification procedures and did not contain the epitope for an anti-(rabbit GH receptor) monoclonal antibody. The rabbit plasma membrane appears to contain approximately 30% type 1, 50% type 2, and 20% type 3 receptors (97). As can be seen in Table IV below, antibody work on GH receptors is still rather limited. Generation of antibodies to GH receptors from sources other than rabbit liver (particularly human sources) should be initiated.

Table IV

Generation of Antibodies to Growth Hormone Receptors

Reference Number	Type of Antibody	Source of Receptor
94	polyclonal	rabbit liver
95	polyclonal	rabbit liver
80	monoclonal	rabbit liver
96	monoclonal	rabbit liver
97	monoclonal	rabbit liver

C. Monoclonal Antibodies to GH-

Retegui et al. (98) used monoclonal antibodies to human GH to study hormone-receptor interaction. They found that the Fab fragments of three monoclonal antibodies blocked hormone binding to IM-9 human lymphoid and pregnant rabbit liver receptors. Similar inhibitory potencies were obtained in both the liver and lymphocyte systems indicating that both binding sites may react with the same area of the GH molecule. Their inhibitory antibodies also reacted to synthetic peptides corresponding to residues 19-128, 73-128 and 98-128 suggesting that the amino portion of the GH molecule participates in receptor binding (98). Monoclonal antibodies against human GH were also used by Cadman et al. (99) to study hormone-receptor interactions. A concern with these studies was that the antibodies would not identify

sites on the GH molecule that were specific for GH and prolactin receptors, possibly due to overlapping binding regions on the GH molecule for these two receptors (85).

VIII. FUTURE TRENDS IN GROWTH HORMONE RECEPTOR RESEARCH

Although considerable progress has been made in the study of GH receptors, many basic science and clinical questions are yet to be resolved and pose an exciting challenge to the research community. For example, few studies have demonstrated a clear relationship between GH-receptor interaction and biological responses to GH. The role and importance of the receptor protein is therefore mostly speculative. In addition, little is known about the precise chemical nature (such as amino acid sequence and 3-dimensional conformation) of GH receptors and whether these differ significantly between species and tissues. The sequence of molecular events ranging from control factors influencing expression of the GH receptor gene, to transcription of the GH receptor RNA and post-translational modifications are all yet to be elucidated.

The chemical interaction between GH and its receptor is also a subject of interest. The key functional groups on each molecule and the nature of their orientation with one another is only beginning to be explored. Since the GH receptor is located in the cell membrane, investigation of how the membrane environment influences GH receptor properties could prove valuable. The fate of GH and receptor molecules after interaction has occurred must

also be studied and could lend insight into molecular mechanism on turnover of these proteins.

While many groups have begun to explore regulators of GH receptors, the actual physiological and pathophysiological role these factors play *in vivo* is largely unknown. It is quite likely that many other endocrine and paracrine regulators of GH receptor expression are yet to be discovered. In addition, the synergistic and antagonistic interplay between these modulators has not been investigated. The mechanism by which these regulators alter binding levels is essentially unexplored. Control may occur at synthesis, degradation, chemical modification or internalization. For each of these possible mechanisms, modulating enzymes can be envisioned which are as yet unidentified and characterized. Another aspect of regulation which is to be rigorously investigated is how GH receptor modulation may be involved in various disease states.

Elucidation of how the GH receptor fits into the biochemical mechanism of GH action is of great interest. That is, how does the binding of GH to its receptor lead to molecular, biochemical, cellular, physiological and anatomical changes of the target tissues? A great gap in understanding still exists concerning the events that occur after GH-receptor interaction. Unlike some other peptide hormones which cause the production of specific effector second messengers such as cAMP, a definitive second messenger generated after GH-receptor interaction has not been found. Our understanding of the role of the receptor in GH mechanism must therefore continue to evolve. For example, a

recent report by Fletcher and Greenan (100) discussed the role of human chorionic gonadotropin (hCG) receptor occupancy. In their study, they observed that cells did not have to bind hCG to have a hormone-like response if they contacted a cell which had hormone-receptor interaction. Whether similar processes exist for GH-receptor interaction are not known. Studies of receptor-mediated second messengers must continue to be explored, since hormone-receptor interaction is surely only the first step in a cascade of molecular processes which regulate cell and tissue activity. In light of he tyrosine-specific protein kinase activities associated with certain recptors, growth factors and viral oncogenes, studies on GH receptor-depedent covalent modifications may produce important information about receptor mehanism (85,101-103).

Two recent achievements may be of particular importance in resolving many questions about the GH receptor. First the purification of GH receptors is of significant importance for chemical characterization studies and generation of specific antibodies to the receptor protein. Secondly, the greatly expanded preparation and use of monoclonal antibodies to the GH receptor is likely to be of great importance in future research. The monoclonal system can provide essentially unlimited amounts of highly specific antibodies for GH receptor study. It is possible for example, that these antibodies could be used in the development of radioimmunoassays for easier measurement of GH receptors, as well as used in GH receptor localization, purification and characterization.

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REFERENCES

1. Williams R.H. In: Textbook of Endocrinology, W.B. Saunders Co., Philadelphia.

2. Hubbard, J.R., M.Y. Kalimi, R.J. Witorsch. In: Review of Endocrinology and Reproduction, The Renaissance Press, Inc., Richmond (in press).

3. Guyton, A.C. 1976. In: Textbook of Medical Physiology, W.B. Saunder Co., Philadelphia.

4. Hubbard, J.R., J.P. Liberti. 1982. Endocrinology 110 , 1483-1488.

5. Hubbard, J.R., J.P. Liberti. 1981. Biochim. Biophys. Acta 673 , 332-338.

6. Isaksson, O.G.P., S. Eden, J.O. Jansson. 1985. Ann. Rev. Physiol. 47 , 483-499.

7. Alzuler, N. 1974. In: Handbook of Physiology (Knobil, E., Sawyer, W.H., eds.) Vol. 4, pp 233-252, Am. Physiol. Soc., Washington, D.C.

8. Daughaday, W.H. 1981. In: Endocrine Control of Growth (Daughaday, W.H., ed.) pp 1-24, Elsevier, New York.

9. Salmon, W.D., Jr., W.H. Daughaday. 1957. J. Lab. Clin. Med. 49 , 825-836.

10. Hubbard, J.R., J.P. Liberti. 1980. Biochim. Biophys. Acta 627 , 207-214.

11. VanWyk, J.J., L.E. Underwood. 1978. In: Biochemical Actions of Hormones (Litwack, G., ed.) Vol 5, Academic Press, New York.

12. Philips, L.S., R. Vassilopaulou-Sellin. 1980. N. Engl. J. Med. 307 , 371-380.

13. Liberti, J.P., M. Miller. 1980. J. Biol. Chem. 255 , 1023-1029.

14. Isaksson, O.G.P., J.O. Jannson, I.A.M. Gause. 1982. Science 216 , 1237-1239.

15. Maes, M., R. DeHertogh, P. Watrin-Grangen, J.M. Ketelslegers. 1983. Endocrinology 113 , 1325-1332.

16. Dellacha, J.M.,M. Sonenberg. 1964. J. Biol. Chem. 239 , 1515-1520.

17. Ketelslegers, J.M., K.J. Catt. 1974. J. Clin. Endocrinol. Metab. 39 , 1159-1162.

18. Thorell, J.I., B.G. Johansson. 1972. Biochim. Biophys. Acta 251 , 363-369.

19. Shiu, R.P.C., P.A. Kelly, H.B. Friesen. 1973. Science 180 , 968-971.

20. Lesniak, M.A., J. Roth, P. Gorden, J.R. Gavin III. 1973. Nature (New Biol) 241 , 20-21.

21. Hunter, W.M., F.C. Greenwood. 1962. Nature 194 , 495-496.

22. Greenwood, F.C., W.M. Hunter, J.S. Glover. 1983. J. Biochem. 89 , 114.

23. Roth, J. 1975. In: Methods in Enzymology, part B. Vol 37, pp 223, Academic Press, New York.

24. Apinyocharti, P., R.C. Baxter. 1979. Clin. Chem. 25 , 161-163.

25. Herington, A.C., N.M. Vieth. 1977. Endocrinology 101 , 984-987.

26. McGuffin, W.L., J.R. Gavin III, M.A. Lesniak, P. Gorden, J. Roth. 1976. Endocrinology 98 , 1401-1407.

27. Baxter, R.C., J.M. Bryson, J.R. Turtle. 1980. Endocrinology 107 , 1176-1181.

28. Gorin, E., H.M. Goodman. 1985. Endocrinology 116 , 1796-1805.

29. Goodman, H.M.,L.K. Levy. 1983. Endocrinology 113 , 2017-2023.

30. Herington, A.C. 1982. Horm. Metabol. Res. 14 , 422-424.

31. Baxter, R.C., Z. Zaltsman, J.R. Turtle. 1984. Endocrinology 114 1893-1901.

32. Kelly, P.A., G. Leblanc, J. Djiane. 1979. Endocrinology 104 , 1631-1638.

33. Baxter, R.C., J.R. Turtle. 1978. Biochem. Biophys. Res. Commun. 84 , 350-357.

34. Gerasino, P., J. Djiane, P.A. Kelly. 1979. Mol. Cell. Endo. 13 , 11-23.

35. McIntosh, C., J. Warnecke, M. Nieger, A. Barnes, J. Kobberling. 1976. FEBS Lett. 66 , 149-154.

36. Solis-Wallckermann, J., O. Robinson, M. Caballero, R. Barbosa, E.S. Canaley, C.Y.A.Zarate. 1981. Rev. Invest. Clin. 33 , 37-43.

37. Kiess, W., O. Butenandt. 1985. J. Clin. Endo. Met. 60 , 740-746.

38. Billestrup, N., J.M. Martin. 1985. Endocrinology 116 , 1175-1181.

39. Roguin, L.P., J.M. Delfino, N. Vita, A.C. Paladini. 1984. Biochem. J. 224 , 535-540.

40. Turyn, D., J.M. Dellacha. 1978. Endocrinology 103 , 1190-1195.

41. Tsushima, T., H.G. Friesen. 1973. J. Clin. Endocrinol. Metab. 37 , 334-337.

42. Kan, K.W., R.L.Cruess, B.I. Posner, H.J. Guyda, S. Solomon. 1983. J. Endocr. 103 , 125-131.

43. Baxter, R.C.,J.M. Bryron, J.R.Turtle. 1980. Endocrinology 107 , 1176-1181.

44. Nixon, T., H.Green. 1983. J. Cell Physiol. 115 , 291-296.

45. Grichting, G., L.K. Levy, H.M. Goodman. 1983. Endocrinology 113 , 111-1120.

46. Gavin, J.R.,J. Roth, D.M. Neville, P. DeMeyts, N. Buell. 1974. Proc. Natl. Acad. Sci. USA 71 , 84-88.

47. Blackard, W.G., P.S. Guzelian, M.E. Small. 1978. Endocrinology 103 , 548-553.

48. Vigneri, R., N.B. Pliam, D.C. Cohen, V. Pezzino, K.Y. Wong, I.D. Goldfine. 1978. J. Biol. Chem. 253 , 8192-8197.

49. Picard, F., M.C. Postel-Vinay. 1984. Endocrinology 114 , 1328-1333

50. Messina, J.L., J.L. Kostyo, S. Eden. 1982. 64'th Annual Meeting of the Endocrine Society, San Francisco, CA, pp 99 (Abstract).

51. Smal, J., J. Closset, G. Hennen, P. Demeyts. 1985. Biochem. J. 225 , 283-289.

52. Lesniak, M.A., J. Roth. 1976. J. Biol. Chem. 251 , 3730-3729.

53. Murphy, L.J., E. Vrhovseh, L. Lazarus. 1983. Clin. Endocrinol. Metab. 57 , 117-1124.

54. Rosenfeld, R.G., R.L. Hintz. 1980. J. Clin. Endocrinol. Metab. 50 , 62-69.

55. Lesniak, M.A., P. Gorden, J. Roth. 1977. J. Clin. Endocrinol. Metab. 44 , 838-849.

56. Herington, A.C., L.S. Phillips, W.H. Daughaday. 1976. Metabolism 25, 341-353.

57. Furuhashi, W., V.S. Fang. 1978. Endocrinology 103 , 2053-2060.

58. Vezinhet, A., B. Posner, B. Patel, J. Charrier. 1980. Reprod. Nutr. Develop. 20 , 289-294.

59. Posner, B.I., B. Patel, A. Vezenhet, J. Charrier. 1980. Endocrinology 107 , 1954-1958.

60. Baxter, R.C., Z. Zaltsman, J.R. Turtle. 1982. Endocrinology 111 , 1020-1022.

61. Baxter, R.C., Z. Zaltsman. 1984. Endocrinology 115 , 2009-2014.

62. Stewart, C., S. Clejan, F. Fugler, T. Cheruvanky, P.J. Collipp, 1983. Arch. Biochem. Biophysics 220 , 309-313.

63. Gorin, E., H.M. Goodman. 1984. Endocrinology 114 , 1279-1286.

64. Gause, I., S. Eden. 1986. Endocrinology 118 , 119-124.

65. Kelly, P.A., B.I. Posner, T. Tsushima, H.G. Friesen. 1974. Endocrinology 95 , 532-539.

66. DeHertogh, M., P. Watrin-Granger, J.M. Ketelslegers. 1983. Endocrinology 113 , 1325-1332.

67. Husman, B., G. Andersson, G. Norstedt, J.A. Gustafsson. 1985. Endocrinology 116 , 2605-2611.

68. Postel-Vinay, M.C., E.C. Cohen-Tanugi, J. Charrier. 1982. Mol. Cell. Endo. 28 , 657-669.

69. Grichting, G., V. Coiro, H.M. Goodman. 1983. 65th Annual Meeting of the Endocrine Society, San Antonio, TX pp 216, Abst. #542.

70. Van Obberghen, E., P. DeMeyts, J. Roth. 1976. J. Biol. Chem. 251 , 6844-6851.

71. Herington, A.C., D. Elson. 1979. Mol. Cell. Endo. 16 , 91-97.

72. Tsim, K.W.K.,C.H.K. Chong. 1984. Mol. Cell. Endo. 38 , 61-66.

73. Martal, J., N. Chene, P. delaLlosa. 1985. FEBS Letters 180 , 295-297.

74. Blossey, H.L. 1979. Horm. Metab. Res. 11 , 616-621.

75. Eshet, R., Z. Laron, A. Pertzelan, R. Arnon, M. Diatzman. 1984. Isr. J. Med. Sci. 20 , 8-11.

76. Waters, M.J., H.G. Friesen. 1979. J. Biol. Chem. 254 , 6815-6825.

77. Shiu, R.P.C., H.G. Friesen. 1974. Biochem. J. 140 , 301-311.

78. Gottsmann, M., K.V. Werder. 1976. Acta Endocrinol. 82 , Suppl. 202, 40-41.

79. Posner, B.I., P.A. Kelly, R.P.C. Shiru, H.G. Friesen. 1974. Endocrinology 96 , 521-531.

80. Simpson, J.S.A., J.P. Hughes, H.G. Friesen. 1983. Endocrinology 111 , 2137-2141.

81. Hughes, J.P., E. Tokuhiro, J.S.A. Simpson, H.G. Friesen. 1983. Endocrinology 113 , 1904-1906.

82. Gavin III, J.R., R.J. Saltman, S.E. Tollefsen. 1982. Endocrinology 110 , 637-643.

83. Burstein, S., M.M. Grumbach, S.L. Kaplan, C.H. Li. 1978. Proc. Natl. Acad. Sci. 75 , 5391-5394.

84. Aston, R., J. Ivanyi. 1983. EMBO J. 2 , 493-497.

85. Hughes, J.P., H.G. Friesen. 1985. Ann. Rev. Physiol. 47 , 469-482.

86. Carter-Su, C., J. Schwartz, G. Kikuchi. 1984. J. Biol. Chem. 259 , 1099-1104.

87. Donner, D.B. 1983. J. Biol. Chem. 258 , 2736-2743.

88. Yamada, K., D.B. Donner. 1984. Biochem. J. 220 , 361-369.

89. Keefer, L.M., P. DeMeyts. 1981. Biochem. Biophys. Res. Commun. 101 , 22-29.

90. Hughes, J.P., J.S.A. Simpson, H.G. Friesen. 1983. Endocrinology 112 , 1980-1985.

91. Tsushima, T., H. Murakami, K. Wakai, O. Isozaki, Y. Sato, K. Shizume. 1982. FEBS Lett. 147 , 49-53.

92. Borst, D.W., N.L. Sayare. 1982. Biochim. Biophys. Res. Commun. 105 , 194-201.

93. Liscia, D.S., B.K. Vonderhaar. 1979. Proc. Natl. Acad. Sci. U.S.A. 79 , 5930-5934.

94. Tsushima, T. 1978. In: Proceedings of the Sixth Asian and Ocean Congress of Endocrinology, Singapore Vol. I, pp 365-373, Singapore.

95. Waters, M.J., H.G. Friesen. 1979. J. Biol. Chem. 254 , 6826-6832.

96. Barnard, R., P.G. Bundesen, D.B. Rylatt, M.J. Waters. 1984. Endocrinology 115 , 1805-1813.

97. Barnard, R., P.G. Bundesen, D.B. Rylatt, M.J. Waters. 1985. Biochem. J. 231 , 459-468.

98. Retegui, L.A., P. DeMeyts, C. Pena, P.L. Masson. 1982. Endocrinology 111 , 668-676.

99. Cadman, H.F., M. Wallis, J. Ivanyi. 1982. FEBS Lett. 137 , 149-152.

100. Fletcher, W.H., J.R.T. Greenan. 1985. Endocrinology 116 , 1660-1662.

101. Carpenter, G. 1983. Mol. Cell. Endocrinol. 31 , 1-19.

102. Jacobs, S., F.C. Kull Jr., H.S. Earp, M.E. Svoboda, J.J. Van Wyk. 1983. J. Biol. Chem. 258 , 9581-9584.

103. Marx, J.L. 1984. Science 223 , 806.

104. Hughes, J.P., H.P. Elsholtz, H.G. Friesen. 1985. In: Polypeptide Hormone Receptors (Posner, B.I., ed.) pp 157-199, Marcel Dekker, Inc., New York.

105. Posner, B.I., P.A. Kelly, R.P.C. Shiu, H.G. Friesen. 1984. Endocrinology 95 , 521-532.

106. Posner, B.I. 1976. Endocrinology 98 , 645-654.

107. Chan, J.S.D., H.A. Robertson. 1978. Endocrinology 102 , 632-660.

108. Carr, D., H. G. Friesen. 1976. J. Clin. Endocrinol. Metab. 42 , 484-493.

109. Arrenbrecht, S. 1974. Nature 252 , 255-256.

110. Tsushima, T., N. Sasaki, Y. Imai, F. Matsuzaki, H.G. Friesen. 1980. Biochem. J. 187 , 499-492.

111. Laron, Z., A. Pertzelan, S. Mannheimer. 1966. Isr. J. Med. Sci. 2 , 152-155.

112. Davies, T.F. 1978. J. Roy. Cell. Phycns. 12 , 379-397.

PROLACTIN RECEPTORS: THE STATUS OF KNOWLEDGE AND CURRENT CONCEPTS CONCERNING THE MECHANISM OF ACTION OF PROLACTIN

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Introduction

Perhaps more speculation exists about the mechanism of action or signal transduction of the anterior pituitary hormone prolactin than that of any other protein/peptide hormone. Part of this uncertainty is due to the fact that this hormone is rather ubiquitous and varied in its biologic actions. Moreover, in some of the principal tissues used to study prolactin receptors and mechanisms, such as liver and prostate, little is known about the fundamental role of the hormone. Furthermore in some tissues,

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such as mammary gland and prostate, prolactin responsiveness is dependent upon the presence of other hormones. Another factor that accounts for the uncertainty about prolactin signal transduction is that it apparently does not appear to act via the conventional adenylate cyclase mediated second messenger pathway.

The purpose of this chapter is to provide an overview and critical analysis of the past, current, and possibly future concepts regarding the mechanism of action of prolactin. In this review we attempt to cover this vast field in three parts. In the first part we review the physiological action of prolactin and its relationship to other hormones. The second part deals with the prolactin receptor. Here we discuss prolactin radioreceptor methodology, the distribution and regulation of prolactin receptivity in mammalian target tissues, and some recent studies pertaining to the role of membrane phenomena in receptor regulation. The third part covers the concepts of prolactin signal transduction. In this section we discuss the available information and prevailing views regarding intracellular mediators for prolactin as well as the entry of prolactin into its target cells. How prolactin internalization relates to signal transduction is also be discussed. Finally, some recent work concerning the target organ proteolytic processing is reviewed as well as its possible relationship to internalization and signal transduction.

Physiological Actions of Prolactin

Prolactin as a lactogenic hormone

The anterior pituitary hormone prolactin (PRL) has a wide variety of effects in many different vertebrate species. Although Bern and Nicoll (1,2) and deVlaming (3) have catalogued more than one hundred reported actions of PRL, the function for which the hormone received its most widely used name derives from its effect on the mammary gland. The maturation of the mammary gland and the production of milk require a complex interaction of many hormones other than PRL.

As reviewed by Lyons et al. (4) and Topper (5), and more recently by Vonderhaar and Bhattacharjee(6), all of the anterior pituitary hormones participate either directly or indirectly in the development of the mammary gland and lactogenesis. Of course growth hormone (GH) in certain circumstances can be a lactogenic hormone (7); it might therefore serve as a "fail-safe" lactogen. Nonetheless, given the normal hormonal milieu of pregnancy and parturition, the appropriately primed mammary gland appears to produce milk in response to PRL secretion. One method of assessing this primary function has been to measure stimulation of the production of the milk protein, casein, by PRL (8). As recently reviewed by Rosen (9), measurement of casein mRNA in response to PRL has begun to provide clues to the mechanism of PRL's action in the mammary gland. Using midpregnant rat mammary glands in chemically defined culture medium containing insulin and cortisol, Rosen and colleagues demonstrated that PRL caused a moderate increase in casein mRNA synthesis and a dramatic increase in the half-life of casein mRNA (10). One of the enzyme subunits of lactose synthetase, alpha-lactalbumin, is also stimulated by PRL (11). As demonstrated by Vonderhaar (12), triiodothyronine is important to demonstrate PRL's effect on this specific milk protein. The mRNA for alpha-lactalbumin also appears to be stimulated by PRL (13).

The effect of PRL on the other components of milk have not been as comprehensively studied. It has been postulated that PRL controls lipoprotein lipase in the mammary gland and peripheral adipose tissue, switching activity depending on the lactational state of the animal (14). Falconer and Rowe demonstrated that PRL decreases sodium uptake into mammary glands (15), a possible mechanism to control milk sodium concentrations. Since milk calcium is mainly protein bound, PRL may indirectly increase this cation by stimulating protein synthesis. PRL's osmoregulatory effects (vide infra) suggest that the hormone may regulate the water content of milk. Recent investigations of PRL's effects on immune function (vide infra) were preceded by evidence (16) that PRL augments the migration of plasma cells that synthesize IgA to the mouse mammary gland. As with other effects of PRL, the mice

were primed with other hormones, in this case estrogen and progesterone, in order to demonstrate this effect. This may be the mechanism of transfer of immunoglobulins into milk.

Recent work from Kleinberg's laboratory (17) and Shiu's laboratory (18) support the hypothesis that the anterior pituitary secretes mammary stimulating hormones other than PRL and GH. Kleinberg et al. (17) suggest that while in the rodent PRL is a crucial requirement for full mammary development, in the primate other pituitary factors are more important. Shiu's laboratory demonstrated that the rat pituitary tumor cell lines (GH 3 and GH$_c$1) and normal transplanted pituitaries stimulated a human breast cancer cell line, T-47D, via a non-PRL mitogenic substance. It is clear that the anterior pituitary contains substances in addition to the classic hormones. For example a fibroblast growth factor (19) and a chondrocyte stimulating factor (20) have been found in bovine and human pituitaries, respectively. In growth hormone cells of rat pituitary gland immunoreactivity specific for human placental lactogen has been demonstrated (21). How these novel or unsuspected substances relate to PRL structurally and/or functionally is a subject of great interest and experimental activity.

Two important points summarize the role of PRL in the mammary gland. One is that PRL does not exist or function by itself. As reported above, most, if not all, PRL effects in the mammary gland require other hormones. These other hormones are often under pituitary control, and their effects are consistent with the concept that for lactation, at least, the anterior pituitary is indeed a "master gland." The existence of newer mammary-stimulating adenohypophysial hormones may make the concept more complex but no less true. The second point is that PRL plays a multitude of roles even in the mammary gland. It appears to affect protein, fat, and carbohydrate metabolism, ion transport, and immune function. It is not surprising, therefore, that PRL would have similar effects in other organs in mammals and in animals not having mammary glands.