CHARACTERIZATION AND REGULATION OF GROWTH HORMONE RECEPTORS IN GOLDFISH (*CARASSIUS AURATUS* L.)

A Thesis

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by

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ABSTRACT

The goal of this research was to characterize growth hormone (GH) receptor (GHR) and binding protein (GHBP) and to investigate the physiological regulation of GHR and GHBP in the goldfish (*Carassius auratus*). Recombinant carp (*Cyprinus carpio*) growth hormone (rcGH) was used to develop a goldfish hepatic GHR binding assay. A single class of high affinity and low-capacity binding sites, with an association constant (K_a) of 1.9×10^{10} M^{-1} and a maximum binding capacity (B_{max}) of 9 fmol mg^{-1} protein was identified in goldfish hepatic membranes. A similar K_a was also found in goldfish serum GHBP. The level of serum GHBP was highly correlated with the number of hepatic GHR. GH displayed an antimitogenic effect in cultured goldfish hepatocytes. *In vivo* and *in vitro* experiments suggested that GH regulated its own receptors, and that nutrition also had an important modulatory effect on GHR and GHBP in the goldfish. Together, results of this thesis suggest that goldfish is a good model to study the roles of GH, GHR and GHBP in endocrine regulation of somatic growth in teleosts.

Perhaps, the most novel finding in this thesis was the identification of a single class of high-affinity and low-capacity binding sites for rcGH and recombinant rainbow trout GH (rtGH) in rabbit and rat liver membranes. Also, rcGH and rtGH were found to have biological activity equivalent to bGH in the mammalian 3T3-F442A cell line. Covalent cross-linking of rcGH or bovine GH (bGH) to goldfish, rabbit or rat liver membrane proteins resulted in the same specifically labeled bands, suggesting that the GHR was similar in all three species. This was the first demonstration that teleost GH may highly cross-react with mammalian GHR, and

challenged the long-held dogma that fish GH is inactive in mammals. The cross-reactivity of teleost GH with mammalian GHR was hypothesized to be related to structural similarities between teleost and mammalian GH. The new hypothesis proposed in this thesis may better explain the species-specificity of GH-GHR interactions in vertebrates.

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A very special thanks to my family for their love and unconditional support during the many years of my education. I would like to dedicate my thesis to my parents, Gui Qin Yang and Feng Lu Zhang. Mom and Dad, this thesis shows the wonderful results of your unlimited love and care. I love you with all my heart and soul. I hope the completion of this thesis brings you long-lasting joy. The unselfish contributions to the completion of my thesis from my wife, Yu-Li Shi, my son, Ray, and my daughter Marina can never be acknowledged enough.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
APROT	aprotinin
B/Bo	specific binding of iodinated hormone in presence of unlabeled hormone over maximum specific binding of the hormone
BAC	bacitracin
B/F	specifically bound ligand over free ligand
bGH	bovine growth hormone
B_{max}	maximum binding capacity
bPRL	bovine prolactin
BSA	bovine serum albumin
cPRL	common carp prolactin
C terminal	carboxyl terminal
DMEM	Dulbecco's modified Eagle's medium
DTT	dithiothreitol
ED_{50}	half-maximal effective dose
EDTA	ethylenediaminetetraacetic acid disodium salt
EGTA	[ethylene-bis(oxy-ethylenenitrilo)]tetraacetic acid disodium salt
gfGH	goldfish growth hormone
GH	growth hormone
GHBP	growth hormone binding protein
GH-I	type I of growth hormone
GH-II	type II of growth hormone
GHR	growth hormone receptor
GHRH	growth hormone releasing hormone
HEPES	N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid
hGH	human growth hormone
hGHBP	human growth hormone binding protein
hGHR	human growth hormone receptor
Hx	hypophysectomized
IGF-I	insulin-like growth factor-I
JAK	Janus kinase
K_a	association affinity constant
KD	kilodaltons
LSI	liver-somatic index
MAPK	mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
M_r	apparent molecular weight
MS222	tricaine methane sulfonate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NSB	non-specific binding
N-terminal	amine terminal
OD	optical density

oGH	ovine growth hormone
PBS	phosphate-buffered saline
PMSF	phenylmethane sulfonyl fluoride
PRL	prolactin
rcGH	recombinant common carp growth hormone
rGH	rat growth hormone
rhGH	recombinant human growth hormone
rhIGF-I	recombinant human insulin-like growth factor-I
RIA	radioimmunoassay
rsbGH	recombinant seabream growth hormone
rPRL	rat prolactin
rtGH	recombinant rainbow trout growth hormone
rsbIGF-I	recombinant sea bream insulin-like growth factor-I
SB	specific binding
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of mean
SFM	serum-free medium
SL	somatolactin
SRIF	somatostatin
STAT	signal transducer and activator of transcription
T_3	triiodothyronine
T_4	thyroxine
TB	total binding
TPCK	N-tosyl-L-phenylalanine chloromethylketone
Tris	Tris (hydroxymethyl) methylamine

CHAPTER 1 LITERATURE REVIEW

Somatic growth in teleosts and other vertebrates is controlled by the brain-growth hormone (GH)-insulin-like growth factor-I (IGF-I) axis (for reviews see: Peter & Marchant 1995, Goffin & Kelly 1996, Peng & Peter 1997, Goffin *et al.* 1998). In this endocrine pathway, GH and IGF-I play key roles in regulating whole body growth and metabolism. GH and IGF-I actions are initiated by interaction with specific receptors for each hormone distributed on the surface of cellular membranes (for reviews see: Hughes & Friesen 1985, Kelly *et al.* 1991, Postel-Vinay & Finidori 1995, Goffin & Kelly 1996, Goffin *et al.* 1998). Circulating GH (Barnard & Waters 1997) and IGF-I (Clark 1997, Duan 1997, 1998) are mainly present in serum combined with specific binding proteins. In the following sections, a brief review about the structure and function of GH, its receptor (GHR) and binding proteins (GHBP) is given with respect mainly to mammals and teleosts, followed by an outline of the objectives of the research presented in subsequent chapters.

1.1 Growth Hormone

1.1.1 Structure of GH

In vertebrates, GH is a 21-22 kilodalton (KD) single chain polypeptide consisting of approximately 191 amino acids in mammals (Kawauchi & Yasuda 1989, Wallis 1996) and 188 amino acids in teleosts (Chen *et al.* 1994, Venkatesh & Brenner 1997). The most recent information from the GenBank database

(http://www.ncbi.nlm.nih.gov/PubMed/) indicates that the primary structure of GH from 43 teleosts has been determined. Sequence analysis of GH genes from these teleosts reveals that the intron pattern of GH genes might serve as a natural marker in understanding the evolutionary relationship of various teleost and mammalian species (Yowe & Epping 1995, Venkatesh & Brenner 1997, May *et al.* 1999). The GH genes from goldfish (*Carassius auratus*) (Law *et al.* 1996, Mahmoud *et al.* 1996), various catfish species (Tang *et al.* 1993, Lemaire *et al.* 1994) and several carps (Chao *et al.* 1989, Koren *et al.* 1989, Chang *et al.* 1992, Chiou *et al.* 1990, Zhu *et al.* 1992, Hong & Schartl 1993, Rand-Weaver *et al.* 1993) consist of four introns and five exons, similar to avian and mammalian GH genes (Kawauchi & Yasuda 1989, Wallis 1996). In contrast, the GH genes from the majority of other teleosts have an additional intron within the last exon (Chen *et al.* 1994, Yowe & Epping 1995, Venkatesh & Brenner 1997, May *et al.* 1999). These findings suggest that at least some teleost GH may be divergent from the main line of tetrapod evolution in terms of their distinct GH gene structure.

There is also evidence indicating that GH, prolactin (PRL) and somatolactin (SL) evolved from a common ancestral gene by duplication and divergence, and the ancestral gene, in turn, may have arisen by repeated duplication of a smaller gene or coding domain and insertion of additional domains (Chen *et al.* 1994). Two forms of GH, derived from separate genes, termed GH-I and GH-II, were reported for salmonids (Du *et al.* 1993, Forbes *et al.* 1994, Kavsan *et al.* 1994, Rubin & Dores 1995), Nile tilapia (*Tilapia nilotica*) (Ber & Daniel 1993), and goldfish (Law *et al.* 1996, Mahmoud *et al.* 1996). The presence of two forms of GH genes has been suggested to be the result of a

tetraploidization event that occurred in a common ancestor to salmonids (Du *et al.* 1993, Forbes *et al.* 1994, Kavsan *et al.* 1994, Rubin & Dores 1995) and goldfish (Law *et al.* 1996, Mahmoud *et al.* 1996). In Nile tilapia, the GH gene sequence seems to have undergone duplication (Ber & Daniel 1993). Thus, it is necessary to take into account the molecular differences of both inter-species and the two intra-species variants, GH-I and GH-II in GH actions.

The amino acid sequence alignment of GH has shown that there is only approximately 25-27% amino acid sequence identity between teleost and mammalian GH (May *et al.* 1999). Approximately 15% of the remaining residues in the GH molecule are highly conserved. Although there are significant differences in amino acid sequences between teleost and mammalian species, the structure of GH is considered to be fairly well conserved throughout vertebrate evolution (Nicoll *et al.* 1987, Chen *et al.* 1994). Overall, the vertebrate GH molecule can be divided into four highly conserved domains, A, B, C and D (Chen *et al.* 1994, May *et al.* 1999). These domains are thought to be important in receptor binding or in maintaining the correct conformation of the hormone (Chen *et al.* 1994, May *et al.* 1999).

1.1.2 Release and distribution of GH

In vertebrates, GH is largely secreted by anterior pituitary somatotrophs and released in a pulsatile manner (for reviews see: Peter & Marchant 1995, Peng & Peter 1997, Clark 1997, Ray & Melmed 1997, Sower 1998). In mammals, secretion of GH is primarily controlled by the interplay of two hypothalamic hormones, somatostatin (SRIF) and GH releasing hormone (GHRH) (Harvey 1993, Clark 1997, Ray & Melmed

1997, Sower 1998). Evidence of the inhibitory effect of SRIF on GH secretion has also been found in teleosts (Peter & Marchant 1995, Peng & Peter 1997). However, the inhibitory effects of SRIF in teleosts are balanced by two major stimulatory factors, GHRH and gonadotropin-releasing hormone, and by a number of other hypothalamic factors that stimulate GH secretion directly at the level of the somatotrophs (Peter & Marchant 1995, Peng & Peter 1997). Such hypothalamic factors include dopamine, neuropeptide Y, thyrotropin-releasing hormone, cholecystokinin, bombesin and activin (Peter & Marchant 1995, Peng & Peter 1997).

In addition to the primary control mechanism of GH secretion by hypothalamic substances, there is strong evidence for peripheral feedback mechanisms in mammals (Scanlon *et al.* 1996, Ray & Melmed 1997, Sower 1998) and teleosts (Peter & Marchant 1995, Peng & Peter 1997). A negative feedback action of GH on its own release at the pituitary level has been reported in rat (Cella *et al.* 1990) and human (Ross *et al.* 1987). IGF-I was found to block GH secretion from rat pituitary cells via a pituitary IGF-I receptor-mediated process (Scanlon *et al.* 1996, Ray & Melmed 1997). In mammals, the secretion of GH may also be regulated by other peripheral products including glucose, glucocorticoids, estradiol, testosterone, triiodothyronine (T_3), and free fatty acids (Scanlon *et al.* 1996). A similar IGF-I action has also been found in teleosts (Björnsson 1997, Duan 1998), suggesting that IGF-I may serve as an important regulator of GH secretion in vertebrates through negative feedback mechanism.

Several extrapituitary sites also have the ability to synthesize and release GH in mammals (Scanlon *et al.* 1996, Ray & Melmed 1997). Such sites include discrete

neuronal populations within the central nervous system (Hojvat *et al.* 1982, Gossard *et al.* 1987), epithelial cells of the mammary gland (Mol *et al.* 1995) and thymus (Maggiano *et al.* 1994), endothelial cells of blood vessels (Wu *et al.* 1996), fibroblasts (Palmetshofer *et al.* 1995), and cells of the immune system (Weigent *et al.* 1991). In humans, placenta tissue was found to synthesize variant forms of GH (Scippo *et al.* 1993). The significance of GH production and release by this non-endocrine system remains obscure.

1.2 Growth Hormone Receptor

1.2.1 Structure of GHR

In avian and mammalian species, GHR is identified as a single chain glycosylated protein composed of typical class 1 cytokine receptor extracellular, transmembrane and cytoplasmic domains (Kelly *et al.* 1991, Goffin & Kelly 1996, Bole-Feysot *et al.* 1998, Goffin *et al.* 1998). The GHR in mammals consists of an extracellular hormone-binding domain of approximately 246 amino acids, a short hydrophobic transmembrane domain and a cytoplasmic domain of approximately 350 amino acids (Goffin *et al.* 1998).

The structure of the GHR is well conserved in mammals, with greater than 70% sequence similarity among the various species studied thus far (Goffin *et al.* 1998). For example, an amino acid sequence identity of 84% was found between rabbit and human GHR (Leung *et al.* 1987, Kelly *et al.* 1991). Two highly conserved features are also found in the class 1 cytokine receptor extracellular domains. The first is the presence of two pairs of disulfide-linked cysteines in the amino (N)-terminal subdomain. These cysteines may be involved in forming ligand-binding pockets characteristic for each

specific ligand (Wells & de Vos 1996, Clackson *et al.* 1998). The second is a pentapeptide termed the WSxWS motif (Tryptophan-Serine-any amino acid-Tryptophan-Serine) found near the carboxyl (C)-terminal of the subdomain. There are conservative substitutions in the GHR WSxWS motif (Goffin *et al.* 1998), and it is probably required for correct folding and cellular trafficking of GHR rather than for ligand binding (Goffin *et al.* 1998). The cytoplasmic domain of cytokine receptors displays more restricted sequence similarity than the extracellular domain. Two regions, called Box 1 and Box 2 are relatively conserved (Goffin *et al.* 1998). Box 1 is a membrane-proximal region composed of eight amino acids highly enriched in prolines and hydrophobic residues. A conserved PxP motif (Proline-any amino acid-Proline) within Box 1 is assumed to adopt the consensus folding specifically recognized by transducing molecules. The second consensus region, Box 2, is much less conserved than Box 1 and consists of a succession of hydrophobic, negatively charged residues (Goffin *et al.* 1998). The transmembrane domain of GHR is approximately 24 amino acids (Goffin *et al.* 1998). However, the involvement of any crucial amino acids in GH-GHR interactions within this domain is unknown.

The three-dimensional structure of human GHR (hGHR) extracellular domain has been determined by crystallographic analysis (de Vos *et al.* 1992). Each of the two subdomains contains seven β-strands that fold in a sandwich formed by two antiparallel β-sheets, one composed of three strands and the other composed of the four remaining strands (de Vos *et al.* 1992, Wells & de Vos 1996, Goffin *et al.* 1998). In humans, the GH-GHR complexes were found to be composed of a single molecule of hormone bound to two molecules of binding protein, indicating the occurrence of

ligand-induced receptor dimerization (de Vos *et al.* 1992, Wells & de Vos 1996, Clackson *et al.* 1998). To date, there is no three-dimensional structural information available for the cytoplasmic domain of the mammalian GHR.

Multiple types of GHR were found in mammals (Barnard *et al.* 1985, Smith & Talamantes 1987, Breier *et al.* 1988) and teleosts (Ng *et al.* 1991, Gray & Tsai 1994) by electrophoresis. The apparent molecular weights for these multiple forms of GHR vary from 20 KD to 400 KD. It has been hypothesized that the multiple forms of GHR in mammals are attributed to post-translational modifications of the GHR gene product (Mathews 1991). However, further research is needed to determine whether these multiple forms of GHR are the subunits of one receptor or different receptors with same affinity constants.

1.2.2 Tissue distribution of GHR

The liver has long been known to be the primary target organ for GH and the major source of GHR in mammals (Goffin *et al.* 1998). However, GHR has also been demonstrated in a variety of other tissues including heart, adipose tissue, kidney, lung, brain, pancreas, intestine, gonadal tissues, cartilage, and skeletal muscle (Roupas & Herington 1989, Kelly *et al.* 1991).

As in mammals, teleost liver is also an abundant source of GHR. Although specific GH binding was detected in other tissues of teleosts (Yao *et al.* 1991, Le Gac *et al.* 1991, Péréz-Sánchez *et al.* 1991, Sakamoto & Hirano 1991), GH-specific receptors are mainly present in liver membranes as reported in the tilapia (*Oreochromis mossambicus*) (Fryer, 1979, Ng *et al.* 1992), coho salmon (*Oncorhynchus kisutch*)

(Fryer & Bern, 1979, Gray *et al.* 1990), Japanese eel (*Anguilla japonica*) (Mori *et al.* 1992), rainbow trout (*O. mykiss*) (Ikuta *et al.* 1989, Yao *et al.* 1991), and snakehead fish (*Ophiocephalus argus*) (Sun *et al.* 1997). In these studies, analysis of the binding characteristics reveals a single class of high affinity and low-capacity binding sites which are highly specific for teleost GH.

1.3 Growth Hormone Binding Protein

1.3.1 Structure of GHBP

Circulating GHBP has sequence identity with the extracellular domain of GHR which is approximately 246 amino acids in length (Barnard & Waters 1997). The GH binding domain of the high affinity GHBP is identical to that of GHR, indicating a strict relationship between GHBP and GHR (Barnard & Waters 1997). GHBP is derived either by translation of an alternatively spliced GHR mRNA lacking the appropriate transmembrane and intracellular domains of the GHR (Smith *et al.* 1989, Baumbach *et al.* 1989, Edens *et al.* 1994) or by direct cleavage from GHR (Leung *et al.* 1987, Amit *et al.* 1996, Mullis *et al.* 1997).

As a result of alternative splicing between exons 7 and 8, the GHBP in the rat and mouse possesses a hydrophilic 17 or 27 amino acid C-terminal sequence, respectively (Baumbach *et al.* 1989, Smith *et al.* 1989, Sadeghi *et al.* 1990). This hydrophilic amino acid C-terminal sequence is generally referred to as the 'tail' sequence. In rodents, the GHBP transcript encodes a short hydrophilic 'tail' sequence instead of the transmembrane and cytoplasmic domains characteristic of the full length GHR (Smith *et al.* 1989, Baumbach *et al.* 1989, Edens *et al.* 1994). In humans and rabbits, GHBP is

largely generated from proteolytic cleavage of the membrane-anchored receptor (Barnard & Waters 1997). The cleavage site was found to be adjacent to a cysteine residue at position 241 close to the transmembrane boundary (Leung *et al.* 1987). Similar findings were also reported for human IM-9 lymphocytes (Trivedi & Daughaday 1988, Saito *et al.* 1995), and transfected human HepG2 (Harrison *et al.* 1995) and COS-7 cells (Sotiropoulos *et al.* 1993). These studies indicate that the loss of a reactive thiol group, probably cysteine at position 241, destabilizes the GHR and renders it susceptible to endopeptidase cleavage (Alele *et al.* 1998).

In all species studied, the circulating GHBP have been found to be present in multiple forms that are structurally distinct. Major circulating GHBP of 50 KD and 100 KD have been found in humans (Baumann *et al.* 1986, Hocquette *et al.* 1990, Baumann & Shaw 1990, Amit *et al.* 1996) and rabbits (Ymer & Herington 1985, Leung *et al.* 1987). The 100 KD GHBP was also identified in the rat (Baumbach *et al.* 1989, Massa *et al.* 1990, Amit *et al.* 1992), mouse (Peeters & Friesen 1977, Smith *et al.* 1989) and sheep (Amit *et al.* 1992). A recent study has demonstrated four structurally distinct GHBP of 55, 74, 158 and 363 KD in guinea pig serum (Ymer *et al.* 1997). As in mammals, the structural heterogeneity of GHBP also exists in avian (Vasilatos-Younken *et al.* 1991, Davis *et al.* 1992) and reptilian (Sotelo *et al.* 1997) species. The significance and origin of the multiple forms of GHBP remain unclear.

1.3.2 Release and distribution of GHBP

The major source of circulating GHBP is liver tissue as found for GHR (Herington *et al.* 1986, Tiong & Herington 1991, Lobie *et al.* 1992). Partial

hepatectomy decreases circulating GHBP in the rat (Baruch *et al.* 1993). GHBP was found to be expressed and released in human and rodent hepatoma cell lines (Mullis *et al.* 1995). GHBP have also been found to be present in human (Barnard *et al.* 1989), rabbit (Postel-Vinay *et al.* 1991) and bovine (Devolder *et al.* 1993) milk. Other sources of GHBP include human follicular fluid (Amit *et al.* 1993), urine (Hattori *et al.* 1990) and lymph (Maheshwari *et al.* 1995). GHBP was found to be expressed in rat adipocytes (Frick *et al.* 1994) and mouse placental cells (Barnard *et al.* 1994). However, these cell types do not appear to be capable of releasing GHBP (Barnard *et al.* 1994).

1.4 Roles of GH, GHR and GHBP in Somatic Growth and Metabolism

1.4.1 Molecular basis for interactions between GH and GHR/GHBP

The initial step in GH action is the binding of GH to specific membrane-associated GHR (for reviews see: Hughes & Friesen 1985, Kelly *et al.* 1991, Postel-Vinay & Finidori 1995, Goffin & Kelly 1996, Goffin *et al.* 1998). Recent discoveries of the binding stochiometry (Cunningham *et al.* 1991) and the crystallization (de Vos *et al.* 1992) of the human GH (hGH)-hGHR complex have advanced our knowledge in understanding the molecular basis for interactions between hGH and hGHR/human GHBP (hGHBP). The crystallographic structure of hGH-hGHR complex shows that one molecule of hGH binds to two molecules of identical hGHR (de Vos *et al.* 1992). The hGHR contains two β-sandwich-like binding domains, with one binding domain interacting with hGH and the second contacting with the other hGHR (de Vos *et al.* 1992). hGH binds to hGHR in a 1:2 ratio through two regions called binding sites 1 and

2 (de Vos *et al.* 1992). Binding site 1 in hGH is formed by amino acids located in helix IV and the loop between helix II and III; binding site 2 in hGH is formed by amino acids in helix I and III on the opposite side of the hormone (de Vos *et al.* 1992). A recent study has found that the key amino acids of hGHR in the formation of hGH-hGHR complex are two tryptophan residues, Trp-104 and Trp-169, which participate in aliphatic-aromatic stacking interactions with hGH (Clackson *et al.* 1998).The remaining hGHR residues interacting with hGH are part of a more hydrophilic periphery surrounding the hydrophobic tryptophan patch (Clackson *et al.* 1998). Thus, the binding affinity of hGH-hGHR is determined by the central hydrophobic patches which are formed by Trp-104 and Trp-169 of hGHR and the side-chains participating in alkyl-aromatic stacking interactions (Clackson *et al.* 1998).

The hGH-hGHR interaction model has also made it possible for identification of the amino acids in the hormone and receptor that might be responsible for species-specificity. hGH is known to be able to bind GHR from a variety of species including teleosts (Goodman *et al.* 1996). However, only primate GH can bind with hGHR (Souza *et al.* 1995, Goodman *et al.* 1996, Behncken *et al.* 1997). The low cross-reactivity of non-primate GH with hGHR appears to result from the incompatibility of a single arginine at position of 43 in the hGHR with a histidine at position of 171 in non-primate GH (Souza *et al.* 1995, Goodman *et al.* 1996, Behncken *et al.* 1997). In hGHR, other amino acid residues that may also have some importance in determining species-specificity include glutamine at positions 44 and 127 and aspartic acid at position 164 (Clackson *et al.* 1998). Since the hGH-hGHR model is the only three-dimensional model available so far, further studies in other vertebrates are needed if the

molecular basis of the species-specificity of interactions between GH and GHR is to be fully understood.

1.4.2 Involvement of GHR in signal transduction pathways

Dimerization of the extracellular portion of the hGHR induced by one hGH appears to bring the intracellular domains of the hGHR together so that they interact as illustrated in Figure 1.1. A hGH antagonist that binds only a single hGHR was found to be unable to exert hormonal effect on humans (Fuh *et al.* 1992). Thus, dimerization of GHR is an essential step for GHR signal transduction pathways (Wells & de Vos 1996). GHR dimerization induced by GH activates the signal transduction pathway of the Janus kinase (JAK) and signal transducers and activators of transcription (STAT) cascade (Postel-Vinay & Finidori 1995, Goffin & Kelly 1996, Goffin *et al.* 1998). Several other signal transduction pathways, including the mitogen-activated protein kinase (MAPK) cascade, appear to also be activated following GHR dimerization (Goffin *et al.* 1998). However, more evidence is needed to determine the precise interactions that occur between the JAK-STAT and MAPK cascades, and their relationship to the other intracellular cascades thought to be involved in the GHR signal transduction pathways (Goffin *et al.* 1998).

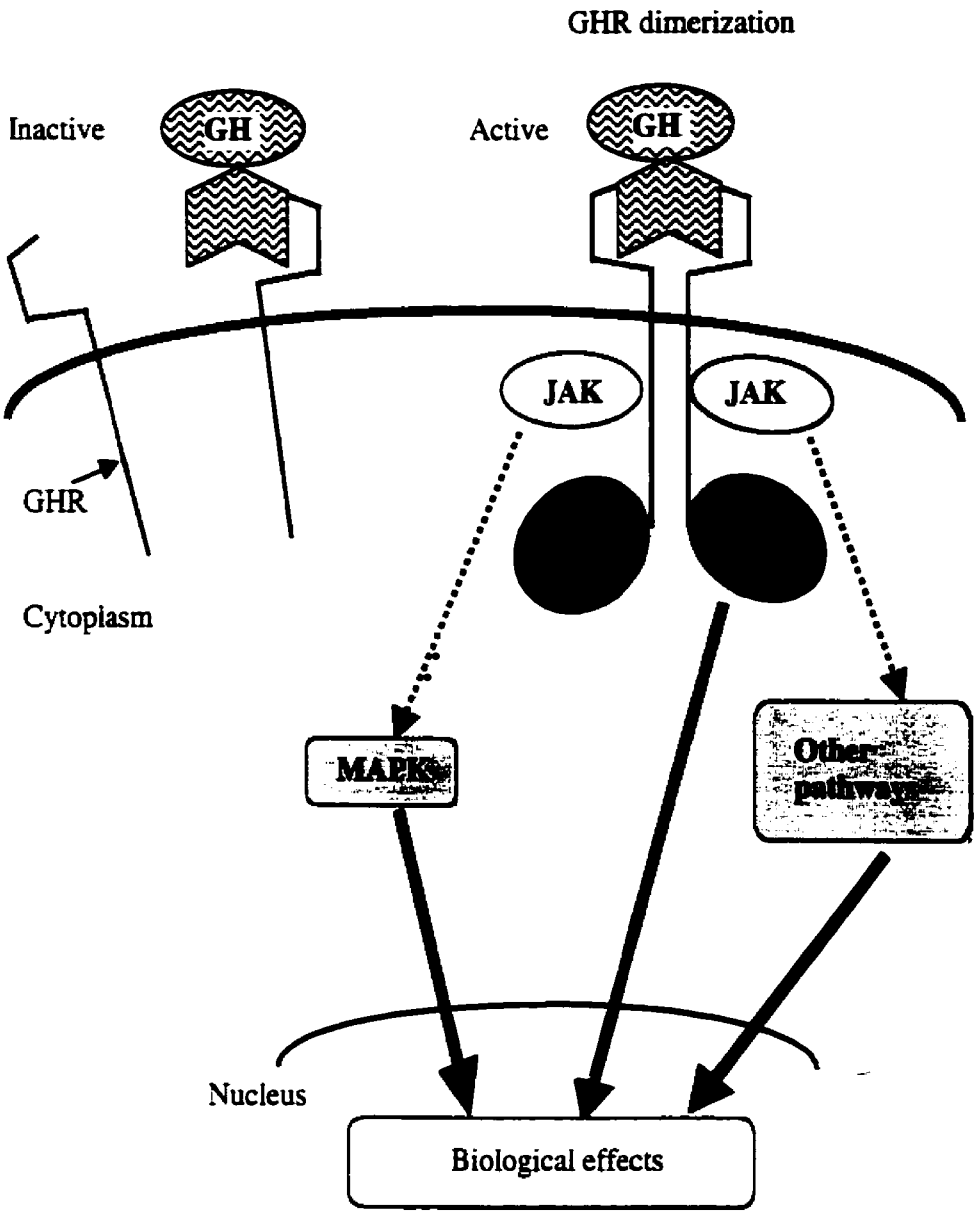

Figure 1.1. Schematic representation of the GHR signaling pathways (redrawn from Goffin *et al.* 1998). GH first interacts with GHR through its binding site 1, forming an inactive GH-GHR complex. Then GH binds to a second receptor through its binding site 2, which leads to receptor dimerization. GHR dimerization activates the JAK-STAT, MAPK, and possible other intracellular signaling pathways.

1.4.3 *In vivo* and *in vitro* regulation of GHR

In mammals, GH has been found to play a role in regulating its own receptor. A relatively long-term GH treatment was reported to induce an up regulation of GHR in rat (Baxter *et al.* 1984), pig (Chung & Etherton 1986), and rabbit and lamb (Posner *et al.* 1980). Recently, it has been shown that elevated levels of ovine GH expressed as a transgene in mice are capable of inducing hepatic GHR (Orian *et al.* 1991). In contrast, the absence of circulating GH is associated with low hepatic GHR numbers in the hypophysectomized rat (Gause *et al.* 1985). This reduced GH binding can be partially restored by treatment with other hormones such as estrogen (Maes *et al.* 1983), insulin (Liang *et al.* 1999), thyroxine (T_4) (Gause & Eden 1985), and cortisone (Gause & Eden 1986).

Although GH may have a role in long-term maintenance of GHR, it also appears to cause a short-term down-regulation of GHR. Such effects of GH have been reported in cultured human IM-9 lymphocytes (Rosenfeld & Hinz 1980), mouse fibroblasts (Murphy & Lazarus 1984), and rat adipocytes (Roupas & Herington 1986). Interpretation of acute down-regulation must be made with some caution since GH binding may involve a rapid and complex pathways for the physiological turnover (dimerization, internalization, degradation, recycling, synthesis) of GHR.

In teleosts, hepatic GH binding was reported to be decreased in the Japanese eel (Mori *et al.* 1992) and long-jawed mudsucker (*Gillichthys mirabilis*) (Gray & Kelley 1991) following hypophysectomy. In these species, hepatic GH binding was further decreased after GH treatment (Mori *et al.* 1992, Gray & Kelley 1991). In intact Japanese eel, however, hepatic GH binding was increased 5 days after GH injection

(Mori *et al.* 1992). Together, these results suggest that endogenous GH may also regulate hepatic GHR in teleosts, although further studies are needed to clarify the short-term and long-term effects of GH on hepatic GHR.

Nutritional status also appears to alter hepatic GHR in mammals and teleosts. Significant reduction in the number of hepatic GHR was reported for fasted rat (Maes *et al.* 1983, Mulumba *et al.* 1991), salmonids (Duan *et al.* 1994), and gilthead seabream (*Sparus aurata*) (Pérez-Sánchez *et al.* 1994). A similar relationship between hepatic GHR levels and nutritional status was also reported for the steer (Breier *et al.* 1988), Japanese eel (Duan & Hirano, 1992), and coho salmon (Gray *et al.* 1992). Although a recent study on rabbit indicated that fasting induced a significantly higher level of GHR messenger ribonucleic acid (mRNA) in growth plate and muscle tissue than those of fed animals (Heinrichs *et al.* 1997), poor nutritional status appears to be generally correlated with hepatic GHR down-regulation in both mammals and teleosts.

1.4.4 Biological actions of growth hormone

GH is a primary regulator of somatic growth, and appears to promote body growth through a "dual-effector" mechanism in mammals (for reviews see: Isaksson *et al.* 1991, Jones & Clemmons 1995). According to this model, IGF-I is the major cellular growth stimulator, with GH stimulating *in vivo* growth via the promotion of IGF-I production in the liver or at the tissue level. GH also acts directly on prechondrocytes to promote their differentiation into chondrocytes which are sensitive to IGF-I and produce IGF-I locally (Isaksson *et al.* 1991, Jones & Clemmons 1995). Thus, IGF-I actions involve endocrine, paracrine and autocrine pathways.

In mammals, linear skeletal growth occurs through interstitial growth and expansion of chondrocytes (Isaksson *et al.* 1991). IGF-I induced by GH was found to be directly involved in fetal and postnatal growth in a number of mammalian species (Jones & Clemmons 1995). Evidence from *in vitro* studies also supports the "dual effector" hypothesis. IGF-I was found to stimulate the differentiation of myoblasts (Florini *et al.* 1986), osteoblasts (Schmid *et al.* 1984), adipocytes (Smith *et al.* 1988), and chondrocytes (Isaksson *et al.* 1987). These studies indicated that GH stimulated local production of IGF-I which in turn promoted the clonal expansion of cells that had already started to differentiate.

Although the autocrine and paracrine roles of IGF-I in teleosts remain unclear, the endocrine action of teleost IGF-I clearly influences body growth and development (Duan 1997, 1998). In teleosts, administration of GH has been shown to stimulate IGF-I mRNA expression in the liver of coho salmon (Cao *et al.* 1989, Duan *et al.* 1993), Japanese eel (Duan & Inui 1990, Duan & Hirano 1992), rainbow trout (Duan *et al.* 1994), and gilthead seabream (Duguay *et al* 1996). *In vitro* studies have demonstrated that mammalian IGF-I, but not GH, directly stimulates cartilage proteoglycan synthesis in teleosts, including the Japanese eel (Duan & Hirano 1990), coho salmon (McCormick *et al.* 1992, Tsai *et al.* 1995), long-jawed mudsucker (Gray & Kelley 1991), goldfish (Marchant & Moroz 1993), and common carp (*Cyprinus carpio*) (Cheng & Chen 1995). These results provide strong evidence that IGF-I mediates at least some of the growth-promoting effects of GH in teleosts as in mammals.

A variety of other biological effects of GH have also been described. In teleosts, GH has been demonstrated to stimulate protein synthesis (Foster *et al.* 1991), lipid mobilization (O'Connor *et al.* 1993), glycogen breakdown (Björnsson 1997), oxygen consumption (Seddiki *et al.* 1995), hypoosmoregulatory ability (Gray *et al.* 1990, Sakamoto & Hirano 1991), appetite (Johnson & Björnsson 1994), and efficiency of food conversion (Fine *et al.* 1993b, Garber *et al.* 1995). These results indicate the multifunctional nature of GH action on somatic growth and metabolism.

1.5 Research Objectives

The overall goal of this research is to identify and characterize GHR and GHBP and to investigate the physiological regulation of GHR and GHBP in the goldfish. Although our knowledge about the role GHR (Goffin & Kelly 1996, Goffin *et al.* 1998) and GHBP (Barnard & Waters 1997) play during somatic growth in mammals has been greatly advanced, our understanding on GHR in teleosts is still limited, particularly in cyprinid species. A serum GHBP has been reported to be present in rainbow trout (Sohm *et al.* 1998), but the role of serum GHBP during somatic growth in rainbow trout and other teleosts is not clear. Thus, it is important to determine how various aspects of teleost GHR and GHBP resemble or differ from other vertebrate GHR and GHBP, including the biochemical nature of teleost GHR and GHBP, cross-reactivity of GH, GHR, and GHBP among various species, and the roles of teleost GH, GHR, and GHBP in somatic growth.

In this research, I addressed several specific objectives with a variety of experimental approaches. A goldfish GH binding assay was established and developed

with recombinant common carp GH (rcGH) as the ligand, making it possible to directly study GHR in the liver membranes of the goldfish (Chapter 2). The biochemical nature of the GHR in the goldfish was investigated using a covalent hormone-receptor cross-linking technique (Chapter 3) and the biological activities of rcGH, bovine GH (bGH), recombinant rainbow trout GH (rtGH) and recombinant sea bream GH (rsbGH) were compared in the 3T3-F442A bioassay (Chapter 4). The cross-reactivity of rcGH to rabbit and rat liver GHR was also studied using GH binding assays (Chapter 4). A circulating GHBP in the serum of goldfish and from cultured goldfish hepatocytes was identified using a ligand blotting technique (Chapter 5). The goldfish GHBP was also compared with GHBP from rabbit and rat sera using ligand blotting and GH binding assays (Chapter 5). The effects of GH injection, hypophysectomy, and fasting on goldfish growth physiology *in vivo* were examined (Chapter 6). Parameters measured in these experiments included growth rate, serum GH, GHBP, thyroid hormones and glucose levels, and hepatic GHR (Chapter 6). The last objective was to examine the *in vitro* regulation of GHR in cultured goldfish hepatocytes (Chapter 7). Factors that may influence the *in vitro* regulation of GHR were investigated, including IGF-I, insulin, thyroid hormones, GH, and PRL (Chapter 7). Together, these results provide considerable new knowledge about the biochemical characteristics and physiological roles of GHR and GHBP in the goldfish, and greatly increase overall understanding of the endocrine regulation of somatic growth in teleosts.

CHAPTER 2 DEVELOPMENT OF A GOLDFISH GROWTH HORMONE RECEPTOR BINDING ASSAY*

2.1 Introduction

Since the first report of GHR in the rabbit liver (Tsushima & Friesen 1973), our knowledge about the role GH receptors play during somatic growth in vertebrates has been greatly advanced (for reviews see: Kelly *et al.* 1991, Peter & Marchant 1995, Goffin & Kelly 1996, Peng & Peter 1997, Goffin *et al.* 1998). In teleosts, receptor binding assays have been employed to detect GH binding sites in tilapia (Fryer 1979, Ng *et al.* 1992), coho salmon (Fryer & Bern 1979, Gray *et al.* 1990), rainbow trout (Sakamoto & Hirano 1991, Yao *et al.* 1991), Japanese eel (Hirano 1991; Mori *et al.* 1992), gilthead seabream (Pérez-Sánchez *et al.* 1994), striped bass (*Morone saxatilis*) (Gray & Tsai 1994), and snakehead fish (Sun *et al.* 1997). These studies have indicated that GH receptors are highly concentrated in the liver of teleosts, although GH specific receptors have also been reported to be present in the brain (Pérez-Sánchez *et al.* 1991), gonads (Sakamoto & Hirano 1991, Le Gac *et al.* 1991), gill, intestine and kidney (Sakamoto & Hirano 1991, Yao *et al.* 1991). Scatchard (Scatchard 1949) analyses indicate that a single class of low-capacity, high affinity GH binding sites, highly specific for teleost GH, are present in these tissues.

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* Portions of the results presented in Chapter 2 have been published elsewhere (Zhang & Marchant 1996).

Although GH receptor binding sites have been reported in a fairly wide range of teleosts, there is a general lack of information on GH receptors in cyprinid teleosts. One reason for this is that a sufficient quantity of purified cyprinid GH has not been available for receptor binding assays. Recently, however, a common carp GH cDNA was cloned (Koren *et al.* 1989) and expressed in *Escherichia coli* (Fine *et al.* 1993a, 1993b) to produce a rcGH. This rcGH was found to be biologically active *in vivo* (Fine *et al.* 1993a) and to bind specifically to liver membranes from common carp (Fine *et al.* 1993b). These findings suggest that rcGH may be useful in the characterization of GH receptors in common carp and related cyprinids. The goal of the present study was to establish a GH receptor binding assay based on rcGH in the goldfish.

2.2 Materials and Methods

2.2.1 Experimental animals

Goldfish (common and comet varieties) were purchased from Grassyforks Fisheries Co. (Martinsville, IN). Animals were maintained at 21-25°C in flow-through aquaria under a simulated natural (Saskatoon) photoperiod for a minimum of two weeks prior to use in the experiments. All studies were conducted during the summer months (May to July) with reproductively immature fish (10-20 g) of mixed sex. The fish were fed to satiation twice daily with Nutrafin Goldfish food purchased from R.C. Hagen Co. (Edmonton, Canada).

2.2.2 Hormones and reagents

rcGH containing five cysteine residues (Fine *et al.* 1993a, b) was kindly provided by Dr. A. Gertler (The Hebrew University of Jerusalem, Israel). This rcGH corresponds to type I of the two carp GH variants (Law *et al.* 1996). Iodination of rcGH was performed according to the methods of Cook *et al.* (1983). The specific activity of ^{125}I-rcGH prepared in this way was routinely more than 60 μCi μg^{-1} when determined using self-displacement (Calvo *et al.* 1983) in the receptor binding assay or rcGH radioimmunoassay (Fine *et al.* 1993a). Purified pituitary goldfish GH (gfGH) and common carp prolactin (cPRL) were gifts from Dr. R. E. Peter (University of Alberta, Canada). bGH, bovine prolactin (bPRL), ovine GH (oGH), rat GH (rGH) and rat prolactin (rPRL) were gifts from the National Hormone and Pituitary Program (NIDDK, Baltimore, MD). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or BDH Chemicals (Edmonton, AB).

2.2.3 Membrane preparation

Goldfish liver membranes were prepared using methods modified from Haro and Talamantes (1985). Fish were anesthetized in 0.05% tricaine methanesulfonate (MS222) and killed by spinal transection. The liver was removed quickly, weighed, washed in 0.7% saline and homogenized with a Polytron homogenizer in 4 volumes (wt/vol) of ice-cold homogenization buffer. The homogenate was then centrifuged at 9,600 g for 30 min at 4°C. The supernatant from 9,600 g was then centrifuged at 100,000-g for 1 h at 4°C. The 100,000-g pellet was collected and suspended in ice-cold suspension buffer to a protein concentration of 20-30 mg ml^{-1}. The membranes were then frozen on dry ice and stored at -20°C. Protein concentration in the membrane preparation was determined using the method of Lowry *et al.* (1951). Membranes from the other tissues were also prepared according to the above procedure. Where applicable, endogenous GH was removed by incubating membranes with 4 M $MgCl_2$ (Kelly *et al.* 1979, Maiter *et al.* 1988). In the present study, the number of total and free binding sites refers to specific ^{125}I-rcGH binding to membranes treated with or without $MgCl_2$, respectively.

2.2.4 Buffers and enzyme inhibitors

To ensure that GH binding in the present study was performed under optimal ionic and buffer conditions (Haro & Talamantes 1985), the influence of a variety of buffer combinations on hepatic rcGH binding was studied. Two types of

homogenization buffers were examined; a glycine buffer (200 mM glycine, 150 mM NaCl, 50 mM EGTA, 50 mM EDTA, 300 mM sucrose, at pH 9.0) and a Tris buffer (100 mM Tris, 150 mM NaCl, 50 mM EGTA, 50 mM EDTA, 300 mM sucrose, at pH 9.0). The suspension buffers examined were a Tris buffer (25 mM Tris, 10 mM $MgCl_2$, at pH 7.6) and a phosphate buffer (10 mM phosphate, 10 mM $MgCl_2$, at pH 7.6). The assay buffers included a Tris buffer (100 mM Tris, 500 mM sodium acetate, 10 mM $MgCl_2$, 0.1% BSA at pH 7.6) and a phosphate buffer (10 mM phosphate, 150 mM NaCl, 10 mM EDTA, 0.1% BSA, 0.1% NaN_3 at pH 7.6). The effectiveness of various enzyme inhibitors during tissue homogenization was also examined. These included 1 mM phenylmethane sulfonyl fluoride (PMSF), 100 KIU ml^{-1} aprotinin (APROT), 0.1 mM *N*-tosyl-L-phenylalanine chloromethylketone (TPCK), 0.02 μg ml^{-1} bacitracin (BAC), and 1 mM PMSF plus 100 KIU ml^{-1} APROT.

2.2.5 Receptor binding

Goldfish membranes were diluted in 300 μl of assay buffer to a final protein concentration of 800 μg. The membranes were then incubated in a final volume of 0.5 ml of assay buffer with approximately 25,000 cpm ^{125}I-rcGH in the presence or absence of unlabeled rcGH (500 ng). Separation of bound ligand from free ligand was performed by addition of 1.0 ml ice-cold assay buffer followed by centrifugation at 4000 g for 30 minutes. Radioactivity corresponding to ^{125}I-rcGH in the pellet was then measured in a gamma counter.

2.2.6 Statistics

In each assay, total binding (TB) of ^{125}I-rcGH to the membrane preparation was determined as the total radioactivity remaining in the pellet following incubation of the membranes in the absence of unlabeled rcGH. Non-specific binding (NSB) of ^{125}I-rcGH was determined as the radioactivity remaining in the pellet following incubation of the membranes with excess unlabeled rcGH (500 ng per tube). Specific binding (SB) of ^{125}I-rcGH was then calculated as the difference between TB and NSB. Thus, SB represents displaceable binding whereas NSB represents non-displaceable binding of ^{125}I-rcGH in this study. All binding parameters were expressed as a percentage of the total radioactivity present during incubation. The association affinity constant (K_a) and maximum binding capacity (B_{max}) of rcGH were calculated using the LIGAND computerized program (Munson & Rodbard 1980). Other data were analyzed with Student's t-test or analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple comparison test.

2.3 Results

The SB of ^{125}I-rcGH to the 100,000-g liver membrane fraction was 1.7 times higher than 9,600 g fraction and 6.6 times higher than 600 g fraction (Fig. 2.1). Membranes prepared with TPCK displayed higher SB (10.4%) when compared to the SB (6.4%) observed in membranes prepared without enzyme inhibitor (Fig. 2.2). Membranes prepared with the other enzyme inhibitors (PMSF, APROT, BAC, and a combination of PMSF and APROT) displayed a SB between 7.0% and 8.0% (Fig. 2.2). The combination of Tris homogenization and suspension buffers and a phosphate assay buffer resulted in the highest SB of all the buffer combinations tested (Table 2.1). Consequently, all subsequent binding assays were performed using these buffers and the 100,000-g membrane fraction prepared with TPCK, as this protocol consistently resulted in the highest SB and lowest NSB.

Binding of ^{125}I-rcGH to goldfish liver membranes was dependent on incubation pH, time and temperature (Fig. 2.3 & 2.4). Significant binding occurred over a wide pH range between 4.4 and 11.6, although NSB increased as pH of the assay buffer decreased (Fig. 2.3). The optimal pH for the rcGH receptor binding assay was chosen to be 6.5 because a high SB with a NSB of approximately 5% or less was achieved at this pH (Fig. 2.3). Following 24 h of incubation, SB reached a steady state at 24°C and 30°C (Fig. 2.4). In contrast, equilibrium was not reached until after 36 h at 4°C and 15°C (Fig. 2.4). Following 24 h incubation, NSB was approximately 8% at 30°C compared to 5% at 24°C. Thus, all assays were subsequently performed using a 24 h incubation at 24°C.

The effect of increasing amounts of liver membrane protein on the binding of ^{125}I-rcGH to goldfish liver membranes is shown in Figure 2.5. Specific binding increased as the amount of liver membrane protein was increased. A plot of the reciprocal of SB versus the reciprocal of membrane protein revealed a linear relationship (Fig. 2.5, inset). From the ordinate intercept of this plot (Calvo *et al.* 1983), the fraction of ^{125}I-rcGH that would bind to an infinite receptor concentration was calculated to be 53% of the total radioactivity added.

Prior to $MgCl_2$ treatment, only liver, spleen and gut tissues were observed to have specific binding of ^{125}I-rcGH (Table 2.2). In contrast, $MgCl_2$-treated membranes from all tissues displayed specific binding of ^{125}I-rcGH (Table 2.2). Liver tissue had the highest SB of the tissues examined; displacement of endogenous GH by $MgCl_2$ resulted in approximately 40% increase in SB in the liver membranes.

The K_a of ^{125}I-rcGH to goldfish liver membranes was indicated by the negative slope of the Scatchard plot (Scatchard 1949) and B_{max} was indicated by the x-intercept of this plot. LIGAND analysis of binding of ^{125}I-rcGH to goldfish liver membranes revealed a single class of binding sites with a K_a of 1.9 x 10^{10} M^{-1} and B_{max} of 9 fmol mg^{-1} protein (Fig. 2.6).

Displacement of ^{125}I-rcGH from goldfish liver membranes by various unlabeled hormones is shown in Figure 2.7. The K_a for each hormone was calculated using the LIGAND program in order to determine the relative cross-reactivities of the various hormones with rcGH as the reference; the K_a of unlabeled rcGH was determined as 3.9

($\pm$0.8) x 10^9 M^{-1} (mean $\pm$ SEM) from three different experiments. The relative cross-reactivities of gfGH, cPRL, bPRL and rPRL were found to be 92.5%, 1.8%, 0.5% and 0.4%, respectively. All mammalian GH displayed a similar relative cross-reactivity of 11.4%.

2.4 Discussion

An important goal of this study was to achieve maximum specific binding of ^{125}I-rcGH to goldfish tissues through the optimization of binding assay conditions. The results indicate that buffer, enzyme inhibitor, temperature and pH are important factors in the goldfish GH receptor binding assay. The optimum buffer combination is Tris as a homogenization and suspension buffer and phosphate as an assay buffer. Similar buffer conditions were found to be optimal in the bovine GH receptor (Haro *et al.* 1984) and the mouse PRL receptor (Haro & Talamantes 1985) binding assays. During hepatic membrane preparation, PMSF is widely used as an enzyme inhibitor to prevent receptor degradation in teleosts (Fryer 1979, Gray *et al.* 1990, Hirano 1991, Pérez-Sánchez *et al.* 1991, Mori *et al.* 1992, Ng *et al.* 1992, Sun *et al.* 1997) and mammals (Haro & Talamantes 1985). The present study demonstrates, however, that TPCK is the most effective enzyme inhibitor in the goldfish GH binding assay. The lack of an effect of PMSF during membrane preparation in the goldfish is not surprising as PMSF was originally found to be completely ineffective in blocking the activity of acetycholinesterase in brain tissue from electric eel (*Electrophorus electricus*) (Turini *et al.* 1968) and goldfish (Moss & Fahrney 1978). Recently, TPCK was also found to be more effective in coho salmon and striped bass (Gray & Tsai 1994).

The binding of ^{125}I-rcGH to goldfish liver membranes was a pH-dependent process, with an optimum pH at 6.5 which allows the highest SB with a low NSB of approximately 5%. When the goldfish GH binding assay was performed at a pH of 7.2-7.5, a pH used for most other teleost GH receptor binding assays (Fryer 1979,

Hirano 1991, Yao *et al.* 1991, Pérez-Sánchez *et al.* 1991, 1994, Mori *et al.* 1992, Ng *et al.* 1992, Sun *et al.* 1997), specific binding decreased by more than 30%. A lower optimum pH (7.0) was also reported for coho salmon (Gray *et al.* 1990). In mammals, the optimum binding of recombinant bovine GH to bovine hepatic membranes occurs at pH 7.8 (Haro *et al.* 1984), whereas the optimum pH for binding of mouse PRL to mouse hepatic membranes is 8.3 (Haro & Talamantes 1985). Other mammalian GH receptors are often found to bind GH with high affinity at neutral pH (Mellman *et al.* 1986). The reason why the optimum pH for the goldfish GH receptor binding assay is lower than in other teleost or mammalian species is not clear. It is possible that pH may cause slight structural changes to rcGH and/or the goldfish hepatic binding sites, which, in turn, would alter the overall binding parameters.

In the goldfish GH receptor binding assay, the optimum temperature was determined to be 24°C at which binding equilibrium was reached by 24 h with a low NSB. Higher incubation temperature resulted in a shorter time for the reaction to reach equilibrium, but also resulted in a higher level of NSB. In teleosts, the optimum incubation temperature for GH receptor binding assays are reported to vary according to species: 20°C for tilapia (Ng *et al.* 1992), 15°C for coho salmon (Gray *et al.* 1990), rainbow trout (Sakamoto & Hirano 1991, Yao *et al.* 1991) and gilthead seabream (Pérez-Sánchez *et al.* 1994). These differences in optimum temperatures for teleost GH receptor binding assays possibly reflect the different *in vivo* temperature requirements of the various species. The stability of labeled hormones *in vitro* may also account for the temperature differences (Yao *et al.* 1991).

LIGAND analysis of ^{125}I-rcGH binding to goldfish liver membranes indicated a single class of high affinity and low-capacity binding sites with a K_a of 1.9 x 10^{10} M^{-1} and B_{max} of 9 fmol mg^{-1} protein. Fryer (1979) reported a similar K_a (1.5 x 10^{10} M^{-1}) in a tilapia GH binding assay. However, in other teleost GH binding assays, the K_a is usually reported to be approximately 10-fold lower (Gray *et al.* 1990, Hirano 1991, Sakamoto & Hirano 1991, Yao *et al.* 1991, Ng *et al.* 1992, Pérez-Sánchez *et al.* 1994). In these teleost GH binding assays, B_{max} generally varies between 26 fmol mg^{-1} protein and 360 fmol mg^{-1} protein. Thus, the goldfish hepatic GH binding sites are of a higher affinity and lower capacity than those reported for most other teleosts.

Analysis of displacement of ^{125}I-rcGH from goldfish liver membranes by various unlabeled hormones indicated that ^{125}I-rcGH binding to goldfish liver membranes was highly specific for teleost GH. Goldfish GH was slightly less potent than rcGH, but was more potent than mammalian GH. In turn, bGH, oGH and rGH had higher cross-reactivities than cPRL, oPRL and rPRL. These results indicate that displacement of ^{125}I-rcGH from goldfish liver membranes by various unlabeled hormones reflects the general pattern reported for other teleosts (Fryer 1979, Gray *et al.* 1990, Hirano 1991, Sakamoto & Hirano 1991, Yao *et al.* 1991, Ng *et al.* 1992, Pérez-Sánchez *et al.* 1994, Sun *et al.* 1997).

A significantly higher SB was found in goldfish liver membranes than in other tissues, indicating that the liver is a major source of GH binding sites. This result is consistent with findings reported for other vertebrates (for reviews see: Kelly *et al.* 1991, Peter & Marchant 1995, Peng & Peter 1997, Goffin *et al.* 1998). In the goldfish,

specific GH binding was also found in kidney, gill, gut, brain, heart, spleen, skeletal muscle, and blood cells after removal of endogenously bound GH. A wide tissue distribution of GH binding sites was also reported for tilapia (Fryer 1979, Ng *et al.* 1992) and various salmonid species (Gray *et al.* 1990, Sakamoto & Hirano 1991, Yao *et al.* 1991). These results suggest that a variety of tissues may be targets for GH action in the goldfish and other teleosts.

The present study is the first time that a fully characterized GH receptor binding assay in a cyprinid species has been reported. The goldfish hepatic GH binding sites meet two of the general criteria of a GH receptor by displaying a high affinity and low-capacity binding for ^{125}I-rcGH and a specificity for teleost GH. Study of the binding of ^{125}I-rcGH to goldfish tissue membranes revealed similarities to the general pattern of GH receptor binding observed in other teleosts. However, hepatic GH binding in the goldfish is different from that reported for other teleosts in several aspects, including optimal conditions for microsomal preparation, pH, buffer composition, and incubation temperature. These findings emphasize the importance of optimizing binding assay conditions when GH binding sites are to be studied in a new species.

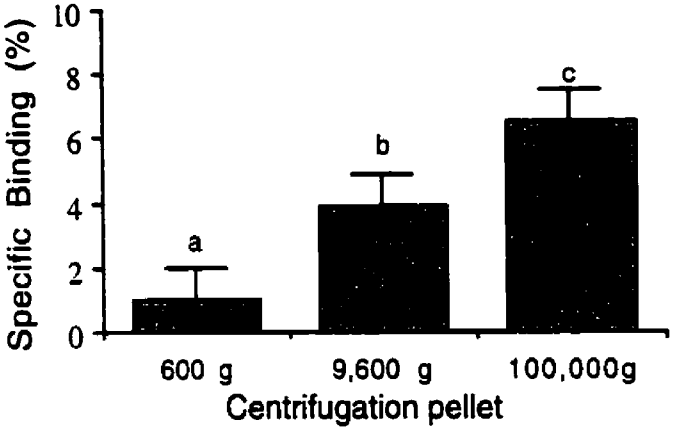

Figure 2.1 Specific binding of ^{125}I-rcGH to various goldfish liver membrane fractions. Data, expressed as mean±SEM, were obtained by pooling results from three experiments, each carried out in triplicate and with liver membranes from different animals. Specific binding of rcGH at various fractions was analyzed using the Student-Newman-Keuls multiple comparison test ($p<0.05$); groups with different superscript letter displayed a different value for SB.

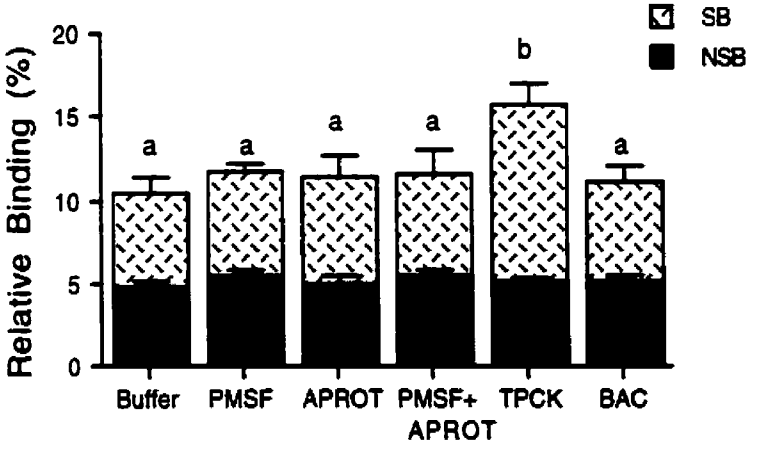

Figure 2.2 Effect of various enzyme inhibitors during homogenization on the binding of ^{125}I-rcGH to goldfish liver membranes. The concentrations of each enzyme inhibitors used in this experiment are described in the Materials and Methods. Data, expressed as mean±SEM, were obtained by pooling results from three experiments, each carried out in triplicate and with liver membranes from different animals. Specific binding of rcGH corresponding to each enzyme inhibitor was analyzed using the Student-Newman-Keuls multiple comparison test ($p<0.05$); groups with same superscript letter displayed a similar value for SB.

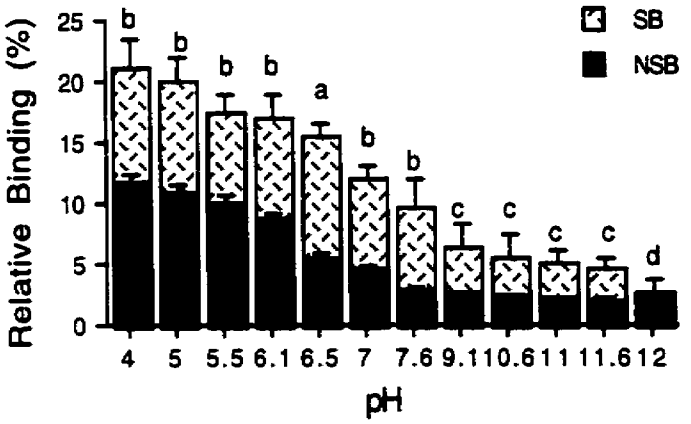

Figure 2.3 Effect of assay buffer pH on the binding of ^{125}I-rcGH to goldfish liver membranes. Data, expressed as mean±SEM, were obtained by pooling results from three experiments, each carried out in triplicate and with liver membranes from different animals. Specific binding of rcGH at different pH was analyzed using the Student-Newman-Keuls multiple comparison test ($p<0.05$); groups with same superscript letter displayed a similar value for SB.

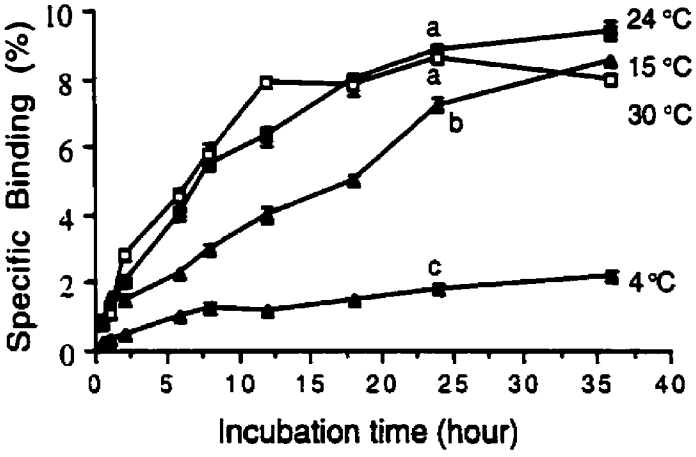

Figure 2.4 Specific binding of ^{125}I-rcGH to goldfish liver membranes over time at various incubation temperatures. Each point represents the mean±SEM of triplicate determinations in a single assay. Similar results were obtained in two additional experiments. Specific binding of rcGH at the various temperatures at 24 h was compared using the Student-Newman-Keuls multiple comparison test ($p<0.05$); groups with same superscript letter displayed a similar value for SB.

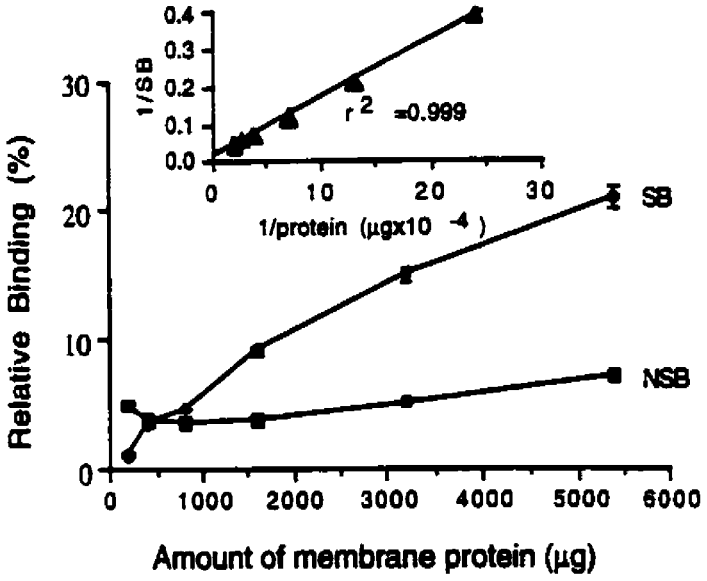

Figure 2.5 Effect of increasing amounts of membrane protein on the binding of ^{125}I-rcGH to goldfish liver membranes. Data are presented as mean±SEM of triplicate determinations from three different experiments, each carried out with liver membranes from different animals. The inset represents a reciprocal plot of SB versus the reciprocal of protein concentration.

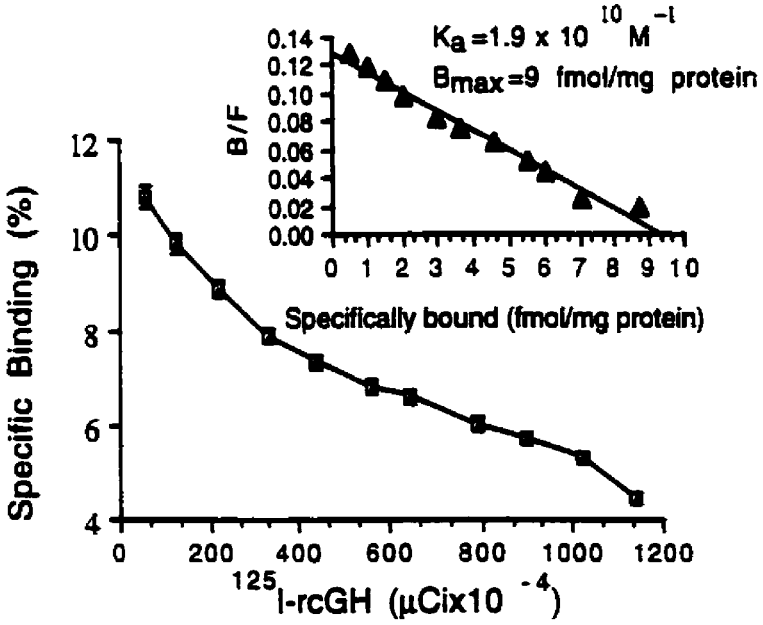

Figure 2.6 Displacement curve produced by incubating increasing amounts of ^{125}I-rcGH with 800 µg of goldfish liver membranes. Data are presented as mean±SEM of triplicate determinations in a single assay. The inset graph represents the derived Scatchard plot. Similar results were obtained in two additional experiments.

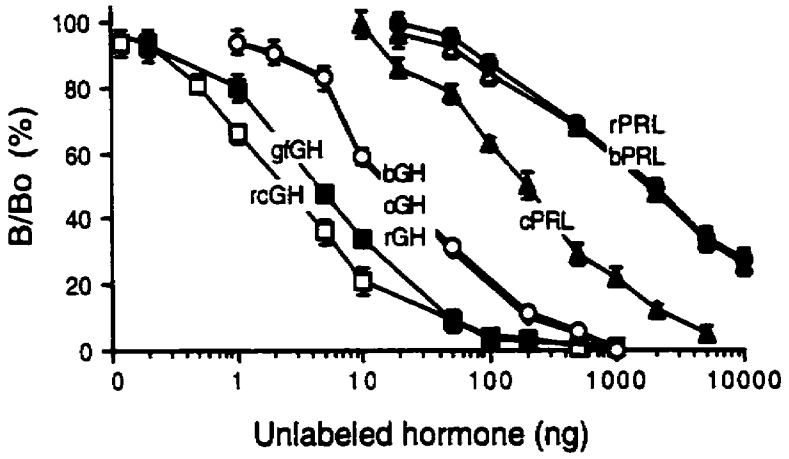

Figure 2.7 Displacement of ^{125}I-rcGH from goldfish liver membrane proteins by increasing amounts of various unlabeled hormones. Data, expressed as mean±SEM, were obtained by pooling results from three experiments, each carried out in triplicate and with liver membranes from different animals.

Table 2.1 Relative binding of ^{125}I-rcGH to goldfish liver membranes under various buffer combinations. Data are presented as mean ± SEM of triplicate determinations in a single assay.

Buffer			Relative binding (% of total radioactivity)		
Homogenization	Suspension	Assay	TB	NSB	SB
Glycerine	Tris	Tris	9.1±6.9	6.9±0.1	2.1±0.1
		Phosphate	9.4±0.3	5.8±0.1	3.6±0.1
	Phosphate	Tris	11.3±0.4	7.1±0.2	4.2±0.1
		Phosphate	9.8±0.2	5.1±0.1	4.7±0.1
Tris	Tris	Tris	13.2±0.3	6.1±0.1	7.1±0.1
		Phosphate	15.2±0.2	5.6±0.1	9.6±0.2
	Phosphate	Tris	10.9±0.3	5.8±0.1	5.1±0.1
		Phosphate	10.5±0.3	5.5±0.1	5.0±0.1

Table 2.2 Specific binding of ^{125}I-rcGH to membranes from various goldfish tissues. Data are presented as mean ± SEM from a triplicate determinations in a single assay.

Tissue	%SB Before $MgCl_2$ treatment	%SB After $MgCl_2$ treatment
Liver	5.49 ± 0.12	10.70 ± 0.24
Kidney	0	1.85 ± 0.02
Spleen	0.21 ± 0.01	2.15 ± 0.03
Gut	0.35 ± 0.12	2.43 ± 0.04
Brain	0	1.28 ± 0.01
Heart	0	1.48 ± 0.02
Gill	0	1.49 ± 0.05
Blood cells	0	1.36 ± 0.06
Muscle	0	1.83 ± 0.11

CHAPTER 3 COMPARISON OF THE BIOCHEMICAL NATURE OF GOLDFISH AND MAMMALIAN GROWTH HORMONE RECEPTORS

3.1 Introduction

Covalent hormone-receptor cross-linking studies have been employed to study the GHR in rat hepatocytes (Yamada & Donner 1984), rat adipocytes (Carter-Su *et al.* 1984, Gorin & Goodman 1984), and hepatic membranes from rat (Hughes *et al.* 1983, Husman *et al.* 1988), rabbit (Hughes *et al.* 1983, Ymer & Herington 1987), mouse (Smith & Talamantes 1987, Orian *et al.* 1991) and sheep (Breier *et al.* 1994). All these studies indicate the presence of multiple forms of the GHR. The mammalian GHR generally occurs as a glycoprotein with an 22 KD extracellular binding subunit, a short 36 KD transmembrane domain, and a 51 KD cytoplasmic domain (Baumann 1991). Intact GHR can be rapidly cleaved to these lower molecular weight forms (Goffin *et al.* 1998).

The biochemical nature of the GHR in teleosts remains largely undefined. There is evidence from tilapia (Ng *et al.* 1991) and striped bass and coho salmon (Gray & Tsai 1994) indicating the presence of multiple forms of GHR as found in mammals. The tilapia GHR was found to be a glycoprotein of M_r approximately 400 KD (Ng *et al.* 1991) whereas in striped bass and coho salmon the GHR were N-linked glycoproteins of M_r approximately 80 KD or 112 KD (Gray & Tsai 1994). In the present study, the biochemical nature of goldfish hepatic GHR was studied using the covalent hormone-

receptor cross-linking technique. Cross-linking studies on rabbit and rat hepatic GHR were also conducted and compared to goldfish GHR using the same SDS-PAGE gel. Such direct comparisons provide new information on the biochemistry of goldfish hepatic GHR and the similarities or differences between goldfish and the mammalian GHR.

3.2 Materials and Methods

3.2.1 Experimental animals

Goldfish of the common or comet varieties were maintained as previously described (Chapter 2). Livers from male New Zealand White rabbits and female Wistar rats were kindly provided by Mr. R. Risling and Dr. A. Richardson (Departments of Physiology and Anatomy and Cell Biology, University of Saskatchewan). All animals were used in accordance with guidelines established by the Canadian Council on Animal Care.

3.2.2 Hormones and reagents

rcGH and bGH were obtained as described previously (Chapter 2). Bis (sulfosuccinimidyl)-suberate was purchased from Pierce Chemical Co. (Brockville, ON). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or BDH Chemicals (Edmonton, AB).

3.2.3 Iodination of the GH and preparation of liver membranes

Iodination of the GH was performed using the lactoperoxidase method as described previously (Chapter 2). Specific activities of ^{125}I-rcGH and ^{125}I-bGH prepared in this way were routinely more than 100 μCi μg^{-1} when determined using self-displacement (Calvo *et al.* 1983) in the GH binding assay. Goldfish liver membranes were prepared as described previously (Chapter 2). Rabbit and rat liver membranes were prepared using the published method of Haro *et al.* (1984).

3.2.4 Covalent hormone-receptor cross-linking

Covalent cross-linking of GH to receptor proteins from goldfish, rabbit and rat was performed using the methods of Gray and Tsai (1994). Briefly, 2 mg of liver protein was incubated with 500,000 cpm of labeled GH in presence or absence of 1 μg unlabeled GH for 20 h at room temperature, followed by centrifugation at 10,000 g for 15 min. The pellet was resuspended in 100 μl of HEPES buffer (10 mM N-2-Hydroxyethylpiperazine-N'2-ethanesulphonic acid, pH 8.0) containing 1 mM of the cross-linking agent, Bis (sulfosuccinimidyl)-suberate, and cross-linking was allowed to proceed for 25 min at room temperature. Samples were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in absence or presence of reducing agents such as dithiothreitol (DTT) or ß-mercaptoethanol (Laemmli 1970). Gels were dried and exposed to X-ray film (Kodak X-Omat AR5) for 7-14 days at -70°C; M_r of the various bands on the autoradiograms were calculated using the BIOMED computer program (MGA Software Inc.). In the present study, cross-linking of ^{125}I-rcGH to rabbit and rat liver membranes was used as a "negative" control because teleost GH including rcGH were not expected to cross-react with the mammalian GHR (Goodman *et al.* 1996).

3.3 Results

Covalent hormone-receptor cross-linking was used to compare ^{125}I-bGH and ^{125}I-rcGH binding sites in rabbit, rat and goldfish liver membranes. The GH binding site in all species consisted of three bands with M_r of 88KD, 142KD, and > 200KD (Fig. 3.1). The appearance of these bands was completely inhibited by the addition of 1 μg of the corresponding unlabeled hormone to the incubation mixture. The three specifically labeled bands were observed in the absence (Fig. 3.1) or presence (Fig. 3.2) of the reducing agent, ß-mercaptoethanol. Incubation of goldfish liver membranes with the reducing agent, DTT also did not alter the position of the three specifically labeled bands (Fig. 3.3). Covalent cross-linking of ^{125}I-rcGH to rabbit and rat liver membrane proteins resulted in the same specifically labeled bands, with M_r of 88KD, 142KD, and > 200KD, as when ^{125}I-bGH was used (Fig. 3.4).

3.4 Discussion

These covalent hormone-receptor cross-linking studies revealed the presence of multiple forms of GH binding sites in the goldfish. The presence of reducing agent such as ß-mercaptoethanol or DTT did not alter the M_r of the multiple GH binding sites in the goldfish. Similar findings were reported for other teleosts (Ng *et al.* 1991, Gray & Tsai 1994) and mammals (Hughes *et al.* 1983, Smith & Talamantes 1987, Ymer & Herington 1987, Husman *et al.* 1988, Orian *et al.* 1991). These results suggest that the various bands observed following SDS-PAGE are not simply GH receptor subunits linked through disulfide bonds.

Recent studies from mammals indicated that the mammalian GHR has extracellular binding subunit, a short transmembrane domain, and cytoplasmic domain (Baumann 1991). These GHR can be rapidly cleaved to lower molecular weight forms (Goffin *et al.* 1998). There is also evidence from hGH-hGHR binding indicating GHR dimerization (de Vos *et al.* 1992). It appears that the combination of one molecule of GH (M_r 22 KD) with two molecules of the extracellular portion of GHR would result in a complex with an M_r of 88 KD. The combination of one molecule of GH with one intact molecule of GHR would have an M_r of approximately 130 KD. One molecule of GH binding to two intact molecules of GHR would make a complex with an M_r > 240 KD. Thus, the 88, 142, and > 200 KD bands observed in my study in goldfish may reflect various GH-GHR complexes formed following GH binding and subsequent GHR dimerization.

Previous cross-linking studies in mammals have shown that the M_r of the GH-GHR complexes varied according to species. For example, the GH-GHR complexes were found to have an M_r of 52 KD, 78 KD and 142 KD in rabbits (Ymer & Herington 1987), 43, 55, 64, and 95 KD in rat (Husman *et al.* 1988), and 56, 62, and 125 KD in mouse (Smith & Talamantes 1987). In the present study, the M_r of the GH-GHR complexes observed in rabbit and rat was slightly different to those reported by other authors (rabbit: Hughes *et al.* 1983, Ymer & Herington 1987, Leung *et al.* 1987, Spencer *et al.* 1988; rat: Husman *et al.* 1988, Husman & Andersson 1993). The M_r of the GH-receptor complexes in goldfish were also different from those reported in other teleosts (Ng *et al.* 1991, Gray & Tsai 1994). These discrepancies could be explained by species differences, variations in experimental protocols, enzymatic degradation of the GHR during incubation, or the calculation error of M_r in SDS-PAGE which may be up to 10% (Weber & Osborn 1975). However, comparison of the three species in my study was done on the same gel under identical conditions which minimizes the error in cross-species comparisons.

The SDS-PAGE banding pattern observed after cross-linking bGH or rcGH to liver receptor proteins was identical in the rabbit, rat and goldfish, indicating that rcGH was able to bind to the same complexes in mammals as bGH. Cross-linking of ^{125}I-rcGH to rabbit and rat liver membranes was originally conducted as a negative control as I expected that rcGH would have negligible cross-reactivity with the mammalian GHR. This expectation was based on the long-held dogma that fish GH are inactive in mammals (Goodman *et al.* 1996). Thus, the finding that rcGH bound to the same GHR complexes as bGH in rabbit and rat was quite surprising. However, this cross-linking

study does not provide information on the binding affinity between rcGH and the mammalian GHR or on the biological activity of rcGH. Therefore, a GH binding assay and a bioassay were employed in the following chapter to determine these parameters.

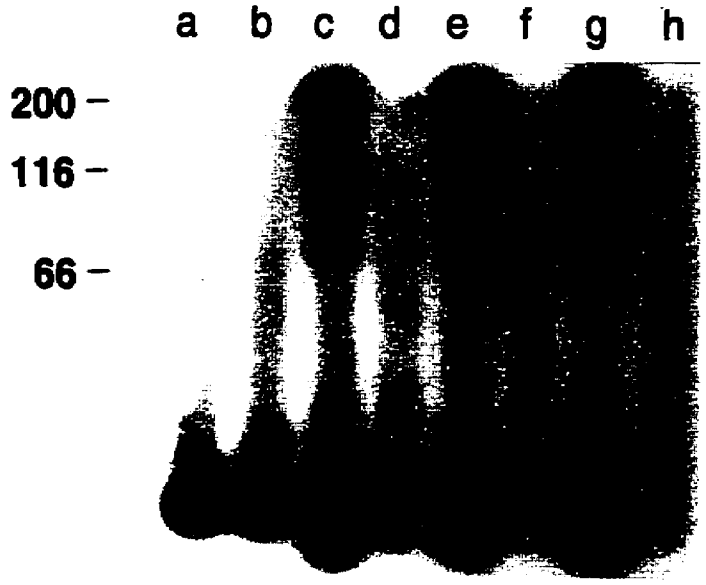

Figure 3.1 Autoradiogram of SDS-PAGE gel (7.5%) showing cross-linking of ^{125}I-rcGH to goldfish liver membranes (lanes c, d) and ^{125}I-bGH to rabbit (lanes e, f) and rat (lanes g, h) liver membranes under non-reducing conditions. Molecular weights (KD) of standards are shown on the left. All bands disappeared when the membrane was incubated with labeled GH in presence of 1 μg of the unlabeled GH (lanes d, f, h). The positions of labeled rcGH (lane a) and bGH (lane b) are also shown in this autoradiogram.

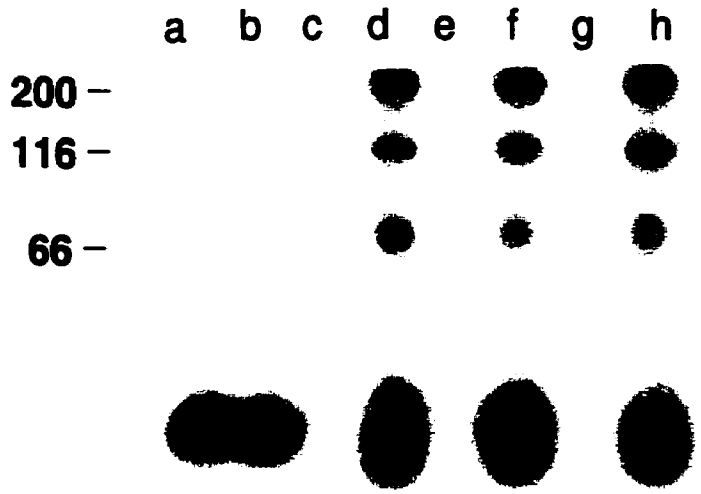

Figure 3.2 Autoradiogram of 7.5% SDS-PAGE gel showing cross-linking of ^{125}I-rcGH to goldfish liver membranes (lanes c, d) and ^{125}I-bGH to rabbit (lanes e, f) and rat (lanes g, h) liver membranes under reducing conditions. Molecular weights (KD) of standards are shown on the left. All bands disappeared when the membrane was incubated with labeled GH in presence of 1 μg of the unlabeled GH (lanes c, e, g). The positions of labeled rcGH (lane a) and bGH (lane b) are also shown in this autoradiogram.

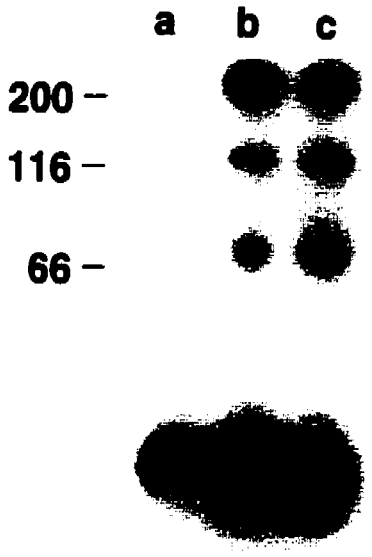

Figure 3.3 Autoradiogram of SDS-PAGE gel (7.5%) showing cross-linking of ^{125}I-rcGH to goldfish liver membranes under non-reducing conditions (lane b) and reducing conditions (lane c). Molecular weights (KD) of standards are shown on the left. The position of labeled rcGH (lane a) is also shown in this autoradiogram.

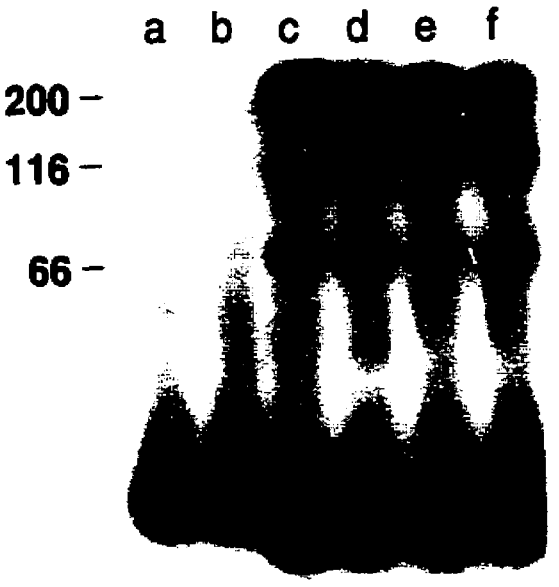

Figure 3.4 Autoradiogram of SDS-PAGE gel (7.5%) showing cross-linking of ^{125}I-rcGH to rabbit (lane d) and rat (lane f) liver membranes and ^{125}I-bGH to rabbit (lane c) and rat (lane e) liver membranes. Molecular weights (KD) of standards are shown on the left. The positions of labeled rcGH (lane a) and bGH (lane b) are also shown in this autoradiogram.

CHAPTER 4 COMPARISON OF THE SPECIES-SPECIFICITY OF THE GROWTH HORMONE-RECEPTOR INTERACTION IN TELEOSTS AND MAMMALS

4.1 Introduction

The concept that 'fish GH is inactive in mammals' is commonly accepted (Goodman *et al.* 1996). Evidence supporting this concept originated in the 1950s when bovine GH was found to stimulate body growth in teleosts (Pickford 1957). However, none of the teleost GH preparations tested at that time appeared to be active in mammals (Pickford 1957). Since then, a number of studies on the species-specificity of GH have been conducted using various techniques, including radioimmunoassay (Hayashida 1975, Hayashida & Lewis 1978, Farmer *et al.* 1981), bioassay (Hayashida 1975, Hayashida & Lewis 1978) and radioreceptor binding assay (Tarpey & Nicoll 1985, LeBail *et al.* 1989). These studies also found that teleost GH have little or no cross-reactivity in mammalian species, whereas mammalian GH have strong cross-reactivity in a wide range of vertebrates, including most teleosts. Thus, teleost GH are usually considered to be divergent from the main line of tetrapod evolution in terms of their structure-activity features (Nicoll *et al.* 1986, Hayashida 1975, Nicoll *et al.* 1987).

According to these early studies, binding of labeled teleost GH to mammalian GH receptors would be expected to be negligible or of low affinity. However, during a study (Chapter 3) of the biochemical nature of GH receptors in the goldfish, I serendipitously found that a recombinant GH from common carp was able to cross-

react with rabbit and rat hepatic GHR. This surprising finding led me to question the validity of the general concept that teleost GH are inactive in mammals. In the present study, the cross-reactivity between mammalian GH receptors and recombinant GH from three teleosts was investigated more fully using GH binding assays and the *in vitro* 3T3-F442A preadipocyte bioassay (Corin *et al.* 1990). These results indicate that at least two teleost GH molecules retain full bioactivity in mammals.

4.2 Materials and Methods

4.2.1 Experimental animals

Goldfish of the common or comet varieties were maintained as previously described (Chapter 2). Male rainbow trout weighing 1.5-2 kg was purchased locally (McNabb Trout Hatchery). Livers from male New Zealand White rabbits and female Wistar rats were kindly provided by Mr. R. Risling and Dr. A. Richardson (Departments of Physiology and Anatomy and Cell Biology, University of Saskatchewan). All animals were used in accordance with guidelines established by the Canadian Council on Animal Care.

4.2.2 Hormones and reagents

Recombinant rainbow trout GH (rtGH) was purchased from Gro-Pep Co. (Adelaide, Australia). This rtGH corresponds to type I of the two trout GH variants (Agellon *et al.* 1988). Recombinant sea bream GH (rsbGH) and rcGH were kindly provided by Dr. A. Gertler (The Hebrew University of Jerusalem, Israel). bGH and rPRL were obtained as described previously (Chapter 2). Carp prolactin (cPRL) was a gift from Dr. R. E. Peter (University of Alberta, Canada). Recombinant human GH (rhGH) was purchased from Bachem Inc. (Torrance, CA). Cell culture media and sera were purchased from Canadian Life Technologies (Burlington, ON). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or BDH Chemicals (Edmonton, AB).

4.2.3 GH binding assays

GH binding assays were used to examine the interaction between the various GH and receptors from rabbit, rat, goldfish and rainbow trout liver membranes. Iodination of the GH was performed using the lactoperoxidase method as described previously (Chapter 2). Specific activities of the iodinated hormones prepared in this way were routinely more than 100 $\mu Ci\ \mu g^{-1}$ when determined using self-displacement (Calvo *et al.* 1983) in the receptor binding assay. Goldfish and rainbow trout liver membrane preparation and GH binding assay were conducted as described previously (Chapter 2). Rabbit and rat liver membrane preparation and receptor binding assay were performed using published methods (Haro *et al.* 1984) with a slight modification to assay buffer pH as described below. The 100,000-g liver membrane fraction was used in the binding assays.

4.2.4 3T3-F442A bioassay

3T3-F442A cells were generously provided by Dr. H. Green (Harvard University, Boston, MA). The antimitogenic activity of various GH in this cell line was determined according to the method of Corin *et al.* (1990) with minor modifications. Briefly, 3T3-F442A cells were plated in 24-well dishes (Falcon) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum at a density of 2×10^5 cells/cm^2. After 4 h of incubation at 37°C in a humidified atmosphere (5% CO_2/95% air), the DMEM was replaced with a serum-free medium (SFM) (Corin *et al.* 1990) containing increasing amounts of GH. The cells were cultured for an

additional 5 days, after which the medium was changed to SFM with 4% fetal calf serum. The cells were then cultured for 2 additional days.

At the end of this incubation period, cell number was determined using a colorimetric assay based on the mitochondrial conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Plumb *et al.* 1989). The cells were washed twice with phosphate-buffered saline (PBS; 0.15 M NaCl, 8 mM Na_2HPO_4 and 5 mM EDTA, pH 7.4) and 100 µl of MTT solution (1 mg ml^{-1} in PBS) was added to each well. The plate was wrapped in aluminium foil and allowed to incubate for 5 h at 37°C in a humidified atmosphere (5% CO_2/95% air). After the incubation, the MTT solution was removed by aspiration, and each well was rinsed once with PBS. The PBS was discarded and 120 µl of dimethyl sulfoxide was added to each well and mixed throughly by trituration to ensure all the formazan crystals were dissolved. The contents of each well were transferred into spectrophotometric cuvettes and diluted with distilled water to a final volume of 400 µl. The formazan concentration in each cuvette was determined with a Beckman DU-7 spectrophotometer at a test wavelength of 570 nm and a reference wavelength of 690 nm. In this assay, cell number is directly proportional to the formazan concentration (Plumb *et al.* 1989) and results are expressed as the optical density (OD) corresponding to the formazan concentration. The ALLFIT (De Lean *et al.* 1978) computer program was used to analyze the *in vitro* dose-response curves and determine the half-maximal effective dose (ED_{50}) for each hormone.

4.2.5 Statistics

Data from GH binding studies were subjected to LIGAND (Munson & Rodbard 1980) analysis to determine the K_a and B_{max} values. When appropriate, displacement curves were also analyzed using the ALLFIT (De Lean *et al.* 1978) computer program. Other data were analysed with ANOVA followed by the Student-Newman-Keuls multiple comparison test.

4.3 Results

4.3.1 Receptor binding studies

The optimal binding of ^{125}I-rcGH to rabbit liver membranes, defined as the highest SB with low NSB was found to occur at pH 6.5 (Fig. 4.1). This is similar to the previous finding for the binding of ^{125}I-rcGH to goldfish liver membranes (Chapter 2). Maximal specific binding of ^{125}I-bGH to rabbit (Fig. 4.1) liver membranes was found to occur at pH 7.4, but was only slightly reduced (<1%) at pH 6.5. The binding of ^{125}I-rsbGH and ^{125}I-rtGH was similar at either pH (Fig. 4.1). Thus, an assay buffer pH of 6.5 was selected for all binding assays in order to allow direct comparisons to be made among the various hormones.

GH binding assays were conducted in two ways: liver membranes were incubated with increasing amounts of either labeled GH or the unlabeled GH in the presence of a constant amount of labeled hormone. LIGAND analysis of ^{125}I-bGH binding to rabbit and rat liver membranes indicated two classes of binding sites: high affinity, low-capacity sites, and low affinity, high capacity sites (Fig. 4.2). In contrast, a single class of high-affinity and low-capacity binding sites for ^{125}I-rcGH and ^{125}I-rtGH was identified in rabbit and rat liver membranes (Fig. 4.2). A single class of binding sites for ^{125}I-rsbGH was also identified in rabbit liver membranes, but with a much lower affinity (Fig. 4.2). The K_a and B_{max} for iodinated bGH, rcGH, rtGH, rsbGH and rhGH in rabbit, rat, goldfish and rainbow trout liver membranes are summarized in Table 4.1.

Displacement of ^{125}I-bGH or ^{125}I-rcGH from rabbit liver membranes by various unlabeled hormones is shown in Figure 4.3. When ^{125}I-bGH was used, ALLFIT analysis revealed that the bGH and rcGH displacement curves displayed similar values for the minimally effective concentration of hormone, slope and ED_{50}. However, the theoretical concentration of rcGH resulting in maximal displacement was significantly higher than that of bGH. When ^{125}I-rcGH was used, all parameters of bGH and rcGH displacement curves were identical (Fig. 4.3). Prolactins displayed relatively little displacement of either labeled GH (Fig. 4.3). When ^{125}I-bGH was used, the relative cross-reactivities of bGH, rcGH, rsbGH, rPRL, and cPRL as determined by LIGAND analysis were found to be 100%, 100.9%, 2.1%, 0.16% and 0.03%, respectively. In the binding assay of ^{125}I-rcGH to rabbit liver membranes, the relative cross-reactivities of rcGH, bGH, rsbGH, cPRL, and rPRL were found to be 100%, 102.4%, 3.2%, 0.2%, and 0.16%, respectively.

4.3.2 Antimitogenic activity of GH

All GH tested displayed antimitogenic activities in 3T3-F442A preadipocyte cell line (Fig. 4.4). ALLFIT analysis of these dose-response curves revealed that rhGH had the highest antimitogenic activity for 3T3-F442A preadipocytes with an ED_{50} of 0.046 $\pm$ 0.005 nM (mean $\pm$ SEM, n=3). In contrast, rsbGH had the lowest antimitogenic activity with an ED_{50} greater than 0.05 M. The antimitogenic activity of bGH was similar to that of rcGH and rtGH, with ED_{50} of 0.093 $\pm$ 0.016 nM, 0.096 $\pm$ 0.012 nM and 0.098 $\pm$ 0.017 nM (mean $\pm$ SEM, n=3), respectively.

4.4 Discussion

This study provides strong evidence that recombinant GH from some teleosts highly cross-reacts with mammalian GHR. Such evidence includes high affinity binding of rcGH and rtGH to rabbit and rat liver membranes, and a biological activity of rcGH and rtGH equivalent to bGH in the 3T3-F442A cell line. These results support my previous finding of an identical banding pattern for rcGH and bGH in SDS-PAGE following covalent receptor cross-linking to rabbit and rat liver proteins (Chapter 3).

LIGAND analysis of ^{125}I-bGH binding to rabbit and rat liver membranes identified two classes of binding sites for bGH. In contrast, only a single class of binding sites for rcGH, rtGH and rsbGH was found in rabbit and rat liver membranes. The single class of rcGH binding sites appears to be very similar to the high affinity bGH binding sites in both rabbit and rat liver membranes. Cross-reactivity of rcGH with only high affinity binding sites was also evident in the displacement of labeled bGH and rcGH from rabbit liver membranes by various unlabeled hormones. In these experiments, displacement curves from unlabeled rcGH and bGH were generally similar. However, the concentration of unlabeled rcGH needed to cause maximal displacement of ^{125}I-bGH was significantly higher than that of unlabeled bGH. This difference can be explained by a lack of cross-reactivity between rcGH and the low affinity bGH binding sites in rabbit liver membranes. In both the ^{125}I-bGH and ^{125}I-rcGH displacement tests, prolactins were considerably less potent than GH suggesting that the rabbit binding sites were specific for GH.

The high affinity site has been reported to be associated with biological responses in mammals (Mellman *et al.* 1986, Sauerwein *et al.* 1991). My finding that rcGH, rtGH

and bGH were equally biologically active in the 3T3-F442A cell line suggests that the high affinity rcGH and rtGH binding sites identified in rabbit and rat liver membranes also leads to a biological response. Human GH displayed the highest antimitogenic activity in 3T3-F442A preadipocytes as has been found in other studies (Vashdi *et al.* 1992, Fine *et al.* 1993b). The higher biological activity of rhGH in this cell line has been attributed to an increased ability to induce post-binding effects such as receptor dimerization (Vashdi *et al.* 1992).

rsbGH displayed a much lower affinity than bGH in the rabbit GH receptor binding assay and little cross-reactivity with rat liver membranes. The considerably reduced affinity of rsbGH in the radioreceptor binding assays also corresponded to a much lower biological activity in the 3T3-F442A preadipocytes. The low cross-reactivity of rsbGH with mammalian GH receptors reflects the general pattern reported for other teleost GH (Tarpey & Nicoll 1985, Nicoll *et al.* 1987, Le Bail *et al.* 1989).

It is not clear why rcGH and rtGH but not rsbGH shows high cross-reactivity with mammalian GH receptors. Sequence analysis of vertebrate GH indicates that the structure of GH is fairly well conserved throughout vertebrate evolution (Nicoll *et al.* 1987, Chen *et al.* 1994). Interestingly, studies on the structures of GH from common carp (Fine *et al.* 1993a, Chen *et al.* 1994), goldfish (Mahmoud *et al.* 1996) and other cyprinid species (Chen *et al.* 1994) have found that GH from these species possess five cysteines, as opposed to four in other vertebrates. In rcGH, four of the cysteines at positions 48, 161, 178, and 186 are structurally homologous to those found in other vertebrate GH (Fine *et al.* 1993a). However, comparison of rcGH to hGH have

revealed that the cysteine at position 123 in rcGH corresponds to a leucine at position 128 in hGH (Fine *et al.* 1993a). This residue contributes to the formation of the core of the four-helix bundle in hGH rather than being directly involved in binding to the receptor (Fine *et al.* 1993a). A recent study has shown that goldfish possess cDNAs encoding for two different GH; one with a cysteine residue at position 123, and the other with a serine at position 123 (Mahmoud *et al.* 1996). These authors suggested that goldfish and other cyprinid GH may require a polar amino acid at position 123 which is not necessarily a cysteine (Mahmoud *et al.* 1996). The present study also suggests that the presence of an extra cysteine residue in rcGH does not contribute to the high cross-reactivity of rcGH to rabbit and rat GHR. This conclusion was further confirmed by the finding of high cross-reactivity between rabbit and rat GHR and rtGH which has only four cysteine residues.

rtGH was also found to highly cross-react with goldfish GHR whereas rsbGH, bGH and rhGH displayed little binding with goldfish GHR. Binding of rcGH, rsbGH, bGH and rhGH to hepatic GHR from rainbow trout was too low to allow LIGAND analysis. These results indicate that the species-specificity for GH and GHR interactions is complex and will require more structural information on teleost GHR to fully understand the biochemical basis for this finding.

To my knowledge, this is the first report where two teleost GH have been found to highly cross-react with mammalian GHR, and contrasts with earlier findings that teleost GH display little or no activity in mammals (Pickford 1957, Hayashida 1975, Hayashida & Lewis 1978, Farmer *et al.* 1981, Tarpey & Nicoll 1985, Nicoll *et al.* 1987, Le Bail *et al.* 1989, Goodman *et al.* 1996). These earlier findings lead to the general

conclusion (Goodman *et al.* 1996) that teleost GH is divergent from other vertebrate GH in terms of structure-activity. However, my results clearly indicate that GH from at least two teleosts has an ability to highly cross-react with mammalian GHR. Thus, broad conclusions about teleost GH may not be valid, and the structure-activity relationship of GH from each teleost should be examined on an individual basis.

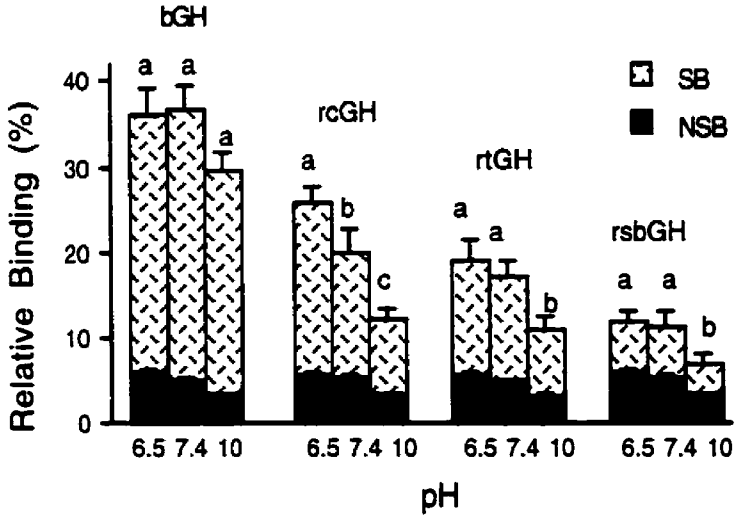

Figure 4.1 Effect of assay buffer pH on the binding of ^{125}I-bGH, ^{125}I-rcGH, ^{125}I-rtGH and ^{125}I-rsbGH to rabbit liver membranes. Data, expressed as mean±SEM, were obtained by pooling results from three experiments, each carried out in triplicate with liver membranes from a different animal. For each GH, specific binding at the different pHs were compared using the Student-Newman-Keuls multiple comparison test ($p<0.05$); groups with same superscript letter displayed a similar level of specific binding.

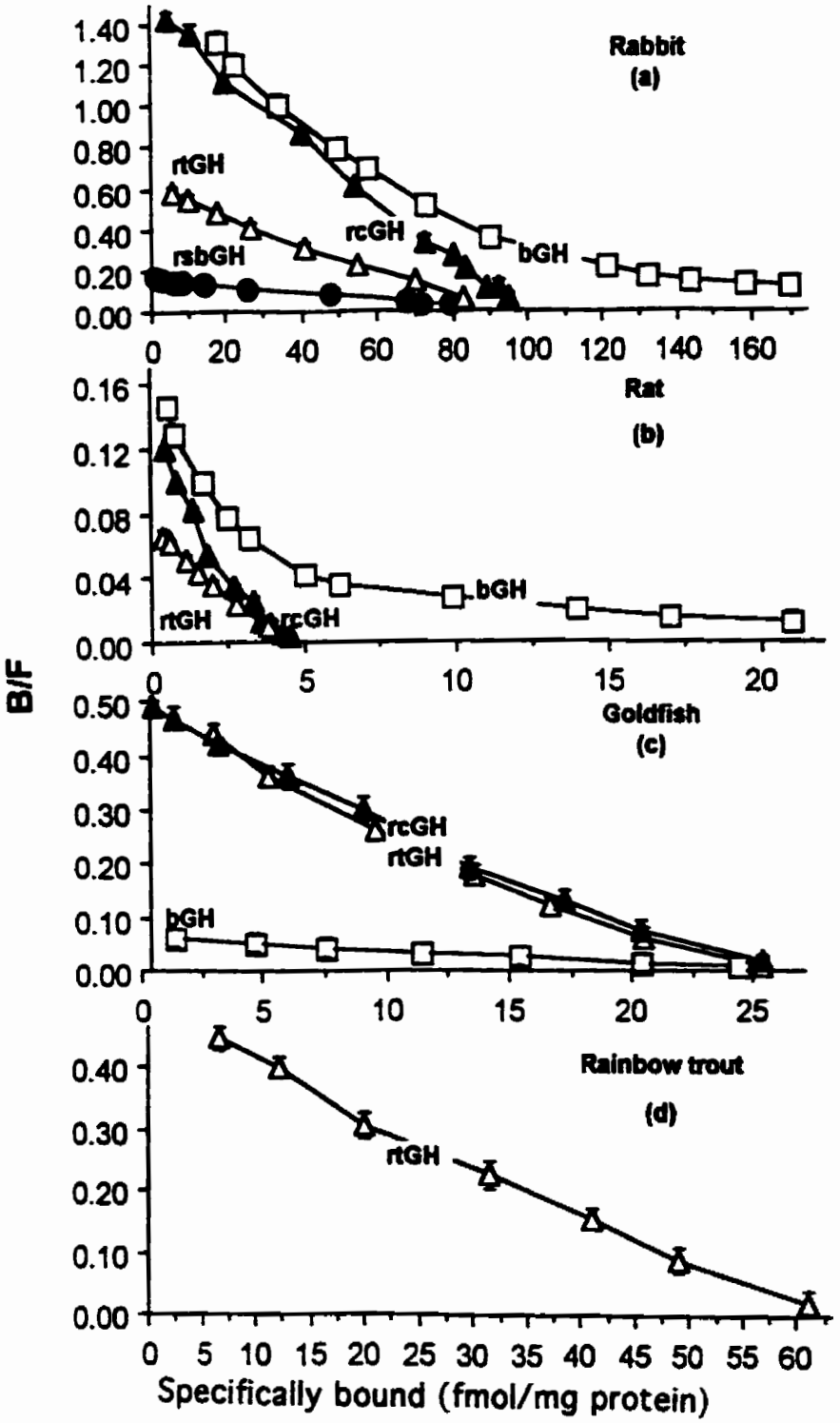

Figure 4.2 Scatchard plots produced by incubating increasing amounts of ^{125}I-bGH, ^{125}I-rcGH, ^{125}I-rtGH and ^{125}I-rsbGH with rabbit (panel a), rat (panel b), goldfish (panel c), and rainbow trout (panel d) liver membranes. Data, expressed as mean±SEM, were obtained by pooling results from four experiments, each carried out in triplicate and with liver membranes from a different animal.

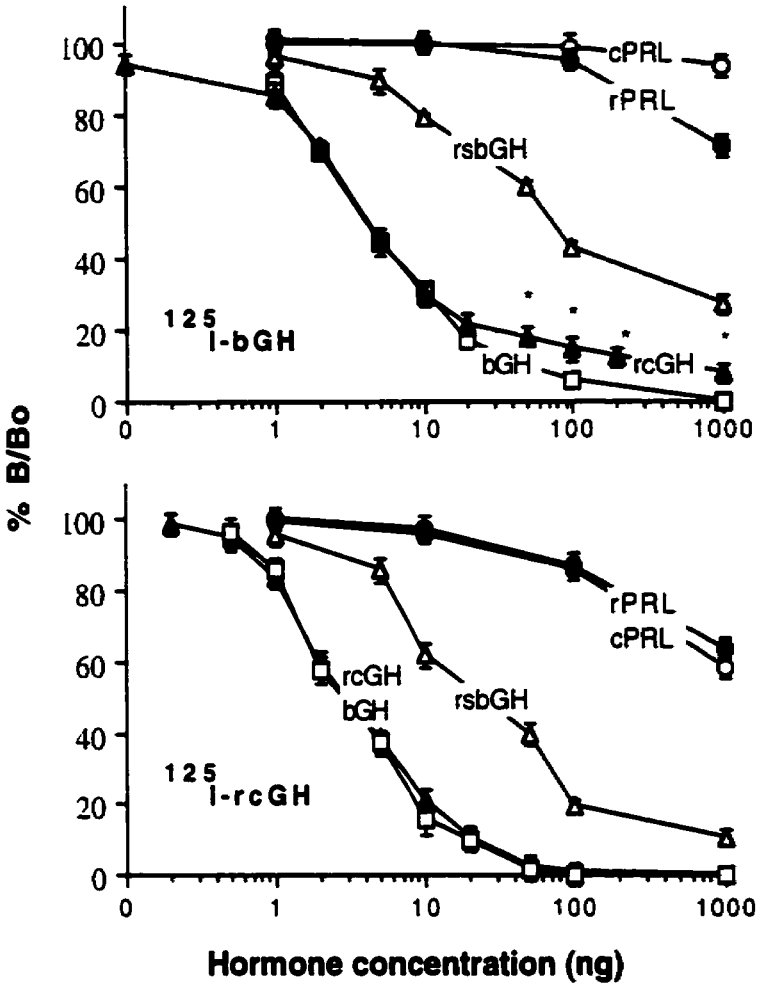

Figure 4.3 Displacement of ^{125}I-bGH (top panel) and ^{125}I-rcGH (bottom panel) from rabbit liver membranes by increasing amounts of various unlabeled hormones. For the ^{125}I-bGH experiment (top panel), ALLFIT analysis indicated that the theoretical concentration of unlabeled hormone causing maximal displacement was significantly different (*$p < 0.05$) between rcGH and bGH. Data, expressed as mean±SEM, were obtained by pooling results from three experiments, each carried out in triplicate and with liver membranes from a different animal.

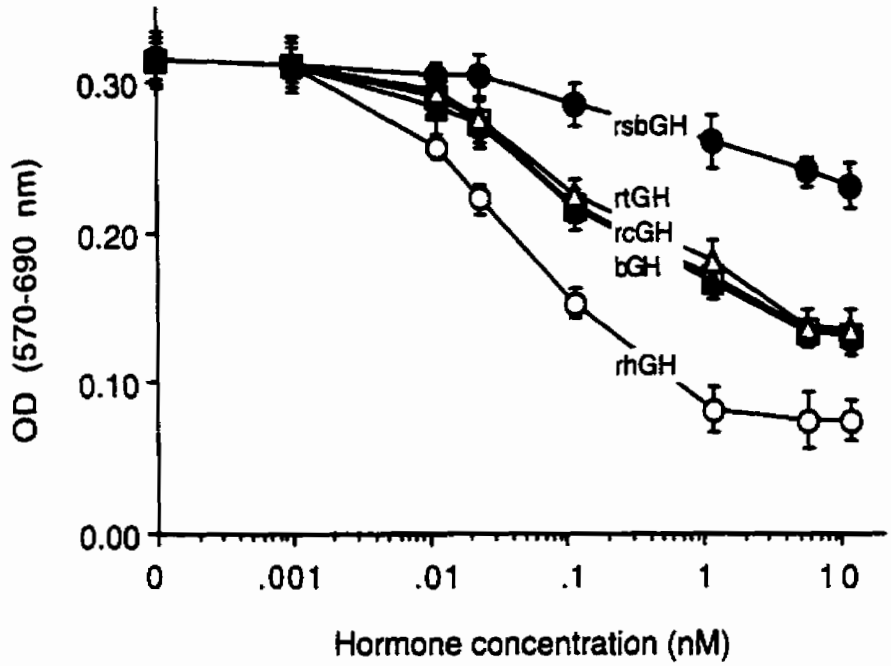

Figure 4.4 Antimitogenic activity of various GH in the 3T3-F442A preadipocyte cell line. Cell number was determined by the MTT assay and data are expressed as the optical density (OD) corresponding to the concentration of formazan. Data, expressed as mean±SEM, were obtained by pooling results from three different experiments, each carried out in triplicate.

Table 4.1 Comparison of bGH, rhGH, rcGH, rsbGH, and rtGH binding sites in liver membranes prepared from rabbit, rat, goldfish, and rainbow trout. The K_a and B_{max} values for liver membranes from rabbit, rat, goldfish, and rainbow trout were obtained by LIGAND analysis of the results presented in Figure 4.2. All data are expressed as mean±SEM (n=3 or 4) and were obtained by pooling results from three or four experiments, each carried out in triplicate and with liver membranes from a different animal.

Source of membranes	Hormone	K_a (10^9 M^{-1})	B_{max} (fmol/mg protein)
Rabbit (n=4)	bGH	10.0 ± 1.0 0.04 ± 0.03	131 ± 7.0 1546 ± 1010
	rhGH	3.1 ± 0.1	413 ± 15
	rcGH	9.9 ± 0.8	104 ± 12
	rtGH	3.5 ± 0.2	98 ± 8
	rsbGH	1.2 ± 0.2	101 ± 10
Rat (n=4)	bGH	27.0 ± 3.0 0.04 ± 0.04	5.2 ± 0.3 157 ± 120
	rhGH	11.0 ± 2.0	28 ± 4
	rcGH	24.0 ± 3.0	5.0 ± 0.4
	rtGH	16.0 ± 1.5	5.3 ± 0.5
	rsbGH	*	*
Goldfish (n=3)	bGH	0.8 ± 0.05	24 ± 2
	rhGH	*	*
	rcGH	19.0 ± 2.0	26 ± 3
	rtGH	18.0 ± 1.6	26 ± 3
	rsbGH	*	*
Rainbow trout (n=3)	bGH	*	*
	rhGH	*	*
	rcGH	*	*
	rtGH	4.3 ± 0.2	65 ± 5
	rsbGH	*	*

* K_a and B_{max} values are not available due to low (<2%) specific binding of the labeled hormone.

CHAPTER 5 IDENTIFICATION OF GROWTH HORMONE BINDING PROTEINS IN GOLDFISH SERUM AND HEPATOCYTE CULTURE MEDIUM*

5.1 Introduction

Circulating GHBP have been identified and characterized in the serum of a number of mammalian species including the mouse (Peeters & Friesen 1977, Smith *et al.* 1989), rabbit (Ymer & Herington 1985), rat (Baumbach *et al.* 1989, Amit *et al.* 1990, Massa *et al.* 1990), dog and pig (Lauteric *et al.* 1988), sheep (Davis *et al.* 1992, Amit *et al.* 1992), goat (Jammes *et al.* 1996), guinea pig (Ymer *et al.* 1997), and human (Baumann *et al.* 1986, Herington *et al.* 1986). In mouse and rat, GHBP is derived by translation of an alternatively spliced GHR mRNA lacking the appropriate transmembrane and intracellular domains of the GHR (Smith *et al.* 1989, Baumbach *et al.* 1989). In humans and rabbits, GHBP is considered to be largely generated from proteolytic cleavage of the membrane-anchored receptor (Barnard & Waters 1997). The mechanistic details of proteolytic shedding of the human and rabbit GHR remain unclear although a recent study in the IM-9 lymphocyte culture (Alele *et al.* 1998) indicated the involvement of a metalloprotease.

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* Portions of the results presented in Chapter 5 have been published elsewhere (Zhang & Marchant 1999).

In other vertebrate groups, studies on GHBP are very limited. There are reports of serum GHBP in domestic poultry (Vasilatos-Younken *et al.* 1991, Davis *et al.* 1992), the turtle, *Chrysemys dorbigni* (Sotelo *et al.* 1997), and the rainbow trout (Sohm *et al.* 1998). The goal of the present study was to investigate the GHBP in the circulation of the goldfish. GH binding assay and ligand blotting techniques were employed to identify GHBP in goldfish serum and from cultured hepatocytes. The binding characteristics and molecular sizes of goldfish serum GHBP were also compared to those in rabbit and rat sera. These results provide clear evidence that a GHBP exists in the goldfish, as in other vertebrates, and indicate that the physiological role of GHBP in teleosts needs to be investigated in future studies.

5.2 Materials and Methods

5.2.1 Experimental animals

Goldfish of the common or comet varieties were maintained as previously described (Chapter 2). All studies were conducted in November with reproductively immature fish (50-70 g) of mixed sex. The goldfish were anaesthetized by immersion in 0.005% (wt/vol) MS222. Blood samples were obtained from each fish by inserting a 25-gauge needle attached to a disposable syringe into the caudal vasculature. The blood was centrifuged at 10,000 g and serum was collected and stored at –20°C. Serum from female New Zealand White rabbits and male Wistar rats was kindly provided by Drs. K. Prasad and A. Richardson (Departments of Physiology and Anatomay and Cell Biology, University of Saskatchewan). All animals were used in accordance with guidelines established by the Canadian Council on Animal Care.

5.2.2 Hormones and reagents

rcGH, rsbGH, bGH, rPRL and cPRL were obtained as described previously (Chapters 2 & 4). Electrophoresis equipment and reagents were purchased from BioRad Co. (Mississauga, ON). Ultrogel AcA54, iodoacetamide, cell culture reagents, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

5.2.3 Iodination and binding studies

Iodination of the GH was performed as previously described, using the lactoperoxidase method (Chapter 2). The specific activity of the labeled hormones was

routinely more than 100 μCi μg^{-1} when determined using self-displacement (Chapter 2) in liver membrane receptor binding assay.

Serum binding studies were carried out using modifications of a published method (Ymer & Herington 1985). Incubation of goldfish, rabbit and rat sera with iodinated hormones was performed at 21-23°C for 4 h using a 10 mM phosphate assay buffer containing 10 mM $MgCl_2$, 0.02% (wt/vol) sodium azide, and 0.1% (wt/vol) BSA, at pH 6.5 in a final volume of 250 μl. Goldfish, rabbit and rat sera were used at a final protein level of 800 μg. Separation of bound ligand from free ligand was performed by gel filtration on AcA54 mini-columns (0.8 x 30 cm) at 21-23°C (Ymer & Herington 1985). Data were subjected to LIGAND (Munson & Rodbard 1980) analysis to determine the K_a and B_{max} values. When appropriate, displacement curves were also analyzed using the ALLFIT (De Lean *et al.* 1978) computer program.

5.2.4 Ligand blotting of serum GHBP

Ligand blotting of GHBP from goldfish, rabbit and rat sera was performed using a slight modification of published methods (Hocquette *et al.* 1990, Vasilatos-Younken *et al.* 1991). Briefly, 20 μg of serum protein was separated by SDS-PAGE on a 7.5% gel under both reducing and non-reducing conditions (Laemmli 1970). Under the reducing conditions, serum proteins were treated with 100 mM DTT or 5% (vol/vol) β-mercaptoethanol prior to separation. Prestained molecular weight standards (BioRad, Richmond, CA) were separated in another lane of the gel. Separated proteins were

transferred to nitrocellulose (0.45 μm pore size) using a BioRad mini transfer unit with Towbin buffer (25 mM Tris, 192 mM glycine, and 20% methanol, at pH 7.4). The nitrocellulose membrane was washed with 3% Nonidet P-40 in Tris saline (10 mM Tris, 150 mM NaCl, pH 7.4) for 30 min and placed in blocking buffer (Tris saline containing 2% skim milk powder, 1% BSA and 0.1% Tween-20, pH 7.4) for 2 h. The nitrocellulose membrane was then incubated with approximately 200,000 cpm of tracer in 50 ml of assay buffer in the presence or absence of excess unlabeled GH (10 $\mu g\ ml^{-1}$) for 24 h at room temperature. Finally, the nitrocellulose membrane was washed, dried, and exposed to X-ray film (Kodak X-Omat AR5 or Biomax MS1) for 4 days at –70°C.

5.2.5 Goldfish hepatocyte culture and iodoacetamide treatment

Preparation of goldfish hepatocytes is described in detail elsewhere (Appendix A). Goldfish hepatocytes cultured for 3 days were used in this study as the physiological function of the hepatocytes was well maintained at this time (Appendix A). In order to release GHBP in the culture medium, hepatocytes were treated with 20 mM iodoacetamide for 1.5 h at 26°C. Similar iodoacetamide treatment has been reported to release a maximum amount of GHBP into the culture medium from IM-9 lymphocytes (Trivedi & Daughaday 1988, Alele *et al.* 1998). At the end of the incubation, the medium was collected and any remaining cells were removed by centrifugation. Twenty micrograms of medium protein was separated by SDS-PAGE on a 12% gel (Laemmli 1970) under both reducing and non-reducing conditions. Separated proteins were then subjected to ligand blotting as described above.

5.3 Results

5.3.1 GH binding studies

All binding assays were carried out at pH 6.5, slightly lower than is routinely used in mammalian binding assays (Ymer & Herington 1985). This change was necessary as the optimal binding of ^{125}I-rcGH to all serum binding proteins, defined as the highest SB with lower NSB, was found to occur at pH 6.5 (Fig. 5.1). This is similar to my previous finding for the binding of ^{125}I-rcGH to goldfish and mammalian liver membranes (Chapters 2 & 4). Specific binding of ^{125}I-bGH to rabbit and rat serum was only slightly reduced (<1%) at pH 6.5 (Fig. 5.1) compared to that at pH 7.4 with no alteration in the shape of the ^{125}I-bGH displacement curve. Thus, an assay buffer pH of 6.5 was selected in order to allow direct comparisons to be made between rcGH and bGH in all species.

Binding of ^{125}I-rcGH to goldfish serum was also dependent on incubation time and temperature (Fig. 5.2). At the optimal pH of 6.5, equilibrium was reached after incubation for 3 h at 24°C (Fig. 5.2). The fraction of ^{125}I-rcGH, ^{125}I-bGH and ^{125}I-rsbGH that would bind to an infinite concentration of serum protein was calculated to be approximately 80% of the total radioactivity added. Therefore, total binding was corrected prior to LIGAND analysis as described previously (Chapters 2 & 4). Typical elution profiles for the total and non-specific binding of ^{125}I-rcGH to goldfish serum are shown in Figure 5.3. Three peaks were observed for the total or non-specific binding profile (Fig. 5.3). Peak I in the total binding profile represented total binding to GHBP which was largely inhibited by the presence of excess unlabeled rcGH in the

incubation mixture prior to gel filtration. Peak I in the non-specific binding profile represented non-specific binding of ^{125}I-rcGH to goldfish serum. Peak II and Peak III represented free ^{125}I-rcGH and Na^{125}I, respectively (Fig. 5.3). Specific binding of ^{125}I–rcGH to serum GHBP was calculated as the difference between the total and non-specific radioactivity corresponding to the Peak I fractions.

LIGAND analysis of ^{125}I-rcGH, ^{125}I-bGH and ^{125}I-rsbGH binding to goldfish serum indicated only a single class of high affinity and low-capacity binding sites (Fig. 5.4). The K_a for ^{125}I-rcGH binding to goldfish serum was approximately 10-fold higher than that of ^{125}I-bGH and 9-fold higher than that of ^{125}I-rsbGH. A single class of high-affinity and low-capacity binding sites for ^{125}I-rcGH and ^{125}I-bGH was also identified in rabbit and rat serum. The K_a and B_{max} for iodinated rcGH, bGH and rsbGH in goldfish, rabbit and rat serum are summarized in Table 5.1.

Displacement of ^{125}I-rcGH from goldfish serum by various unlabeled hormones is shown in Figure 5.5. The K_a for each hormone was estimated using the LIGAND program in order to determine the relative cross-reactivities of the various hormones with rcGH as the reference; the K_a of unlabeled rcGH was estimated as 12 ($\pm$ 4) x 10^9 M^{-1} (mean $\pm$ SEM, n=3). The relative cross-reactivities rsbGH, bGH, cPRL, and rPRL were found to be 13.8, 10.9, 1.7 and 0.4%, respectively.

Displacement of ^{125}I-bGH and ^{125}I-rcGH from rabbit serum by various unlabeled hormones is shown in Figure 5.6. ALLFIT analysis revealed that all

parameters of the bGH and rcGH displacement curves were identical in the ^{125}I-bGH and ^{125}I-rcGH displacement tests (Fig. 5.6). Prolactins displayed relatively little displacement of either labeled GH (Fig. 5.6). When ^{125}I-bGH was used, the relative cross-reactivities of bGH, rcGH, rsbGH, rPRL, and cPRL as determined by LIGAND analysis were found to be 100%, 99.7%, 2.0%, 0.2%, and 0.05%, respectively. In the binding assay of ^{125}I-rcGH to rabbit serum, the relative cross-reactivities of rcGH, bGH, rsbGH, cPRL, and rPRL were found to be 100%, 102.6%, 2.9%, 0.22%, and 0.17%, respectively.

5.3.2 Ligand blotting studies

The GH binding site consisted of multiple bands each with high M_r in goldfish (70, 80, 120, 180, 240, 360 and 400 KD), rabbit (80, 120, 180 and 240 KD), and rat (180 and 240 KD) serum (Fig. 5.7). Serum from all three species contained the 180 KD and 240 KD bands. Two bands with M_r of 80 KD and 120 KD were also found in both goldfish and rabbit serum. The M_r of the labeled bands in all three species was altered under reducing conditions. The M_r of the bands under reducing conditions ranged from 27 to 160 KD in the goldfish and rat serum and 27 to 240 KD in the rabbit serum (Fig. 5.7). A prominent band with an M_r of 66 KD and a minor band with an M_r of 27 KD were observed to occur in serum from all three species under reducing conditions. The appearance of all bands was completely inhibited by the presence of 10 $\mu g\ ml^{-1}$ of the unlabeled rcGH (Fig. 5.7).

Iodoacetamide promoted the shedding of multiple forms of GHBP from the goldfish hepatocyte culture whereas no GHBP was detected in the conditioned medium in the absence of iodoacetamide (Fig. 5.8). The GHBP from goldfish hepatocyte culture consisted of three bands with M_r of 25, 40 and 45 KD (Fig. 5.8). The appearance of these bands was completely inhibited by the presence of 10 $\mu g\ ml^{-1}$ of unlabeled rcGH (Fig. 5.8). The M_r of these bands was not altered under reducing conditions (Fig. 5.8).

5.4 Discussion

The existence of specific GHBP in goldfish serum and cultured hepatocytes was confirmed by rcGH binding and ligand blotting studies. LIGAND analysis of ^{125}I-rcGH binding to goldfish serum indicated a single class of high affinity and low-capacity binding sites with a K_a of 20.1 x 10^9 M^{-1}. In mammals, GHBP can be divided into different types based on GH binding affinity (Amit *et al.* 1992). Type I and Type II GHBP display low affinity GH binding. Type I GHBP include those of the mouse and rat with a binding affinity of 1.2-3.9 x 10^9 M^{-1} (Amit *et al.* 1992). Type II GHBP have even lower GH binding affinity than type I GHBP, and have been found in the sheep (Davis *et al.* 1992, Amit *et al.* 1992), goat (Jammes *et al.* 1996), and cow (Gertler *et al.* 1984, Devolder *et al.* 1993). All type III GHBP have high affinity binding with GH (4.7-9.2 x 10^9 M^{-1}), and are present in the rabbit (Ymer & Herington 1985), dog (Lauteric *et al.* 1988), and horse (Amit *et al.* 1992). In birds, chicken serum GHBP was found to have the highest K_a value for human GH binding (1.55 x 10^9 M^{-1}, Davis *et al.* 1992). In contrast, lower affinity of serum GHBP was reported for turtle (K_a: 3.8 x 10^8 M^{-1}, Sotelo *et al.* 1997) and rainbow trout (K_a: 6.6 x 10^7 M^{-1}, Sohm *et al.* 1998). Among the species studied to date, goldfish serum GHBP has the highest K_a value. This may be attributed to species differences, more optimal conditions for rcGH binding to goldfish serum, or correction of total binding prior to LIGAND analysis.

In goldfish, the affinities of the serum GHBP (K_a: 20.1 x 10^9 M^{-1}) and liver membrane GHR (K_a: 19 x 10^9 M^{-1}, Chapter 2) for rcGH are very similar. This implies

a close relationship between goldfish serum GHBP and the liver membrane GHR. A similar K_a for serum GHBP and liver membrane GHR was also found in the rabbit (Leung *et al.* 1987, Spencer *et al.* 1988), where the GHBP appears to be largely generated from proteolytic cleavage of the membrane GHR (Barnard & Waters 1997). In contrast, the K_a of the rat serum GHBP in the present study was found to be 22-fold lower than that of rat liver GHR (Chapter 4) for ^{125}I-rcGH or ^{125}I-bGH binding. This is similar to previous reports on rat serum GHBP (Massa *et al.* 1990) and liver membrane GHR (Baxter *et al.* 1980). Rat serum GHBP originates from translation of an alternatively spliced GHR mRNA rather than directly from proteolytic cleavage of the membrane GHR (Smith *et al.* 1989, Baumbach *et al.* 1989) which may provide one explanation for the difference between the K_a of the serum GHBP and that of the liver membrane GHR in the rat.

In the goldfish GHBP assay, the K_a value for ^{125}I-rcGH was significantly higher than that for ^{125}I-bGH. Analysis of displacement of ^{125}I-rcGH from goldfish serum by various unlabeled hormones also indicates that ^{125}I-rcGH binding to goldfish serum was highly specific for teleost GH. rsbGH was found to be less potent than rcGH, but was slightly more potent than the mammalian GH. Prolactins were considerably less potent than GH suggesting that the goldfish serum GHBP were specific for somatogenic hormones. This pattern of hormone specificity is similar to that of the goldfish GHR (Chapters 2 & 4), and further indicates that the goldfish GHR/GHBP is very species specific in terms of hormone binding.

The present results also indicate that rcGH, but not rsbGH, highly cross-reacts with serum GHBP from the rabbit and rat. These results are in agreement with previous findings for GHR in rabbit and rat liver membranes (Chapter 4). High cross-reactivity of rcGH with the single class of high affinity serum GH binding sites was also evident in the displacement of labeled bGH and rcGH from rabbit serum by various unlabeled hormones. In these experiments, displacement curves from unlabeled rcGH were similar to those of unlabeled bGH. In both the ^{125}I-bGH and ^{125}I-rcGH displacement tests in the rabbit, prolactins were considerably less potent than GH suggesting that the rabbit binding sites were also specific for GH.

In the present study, the B_{max} value for serum GHBP was found to be considerably higher in the rabbit (~3300 fmol ml^{-1} serum) and rat (~6000 fmol ml^{-1} serum) than in the goldfish (~160 fmol ml^{-1} serum). Amit *et al.* (1992) measured the B_{max} for serum GHBP in a number of mammalian species including rabbit, rat, mouse, sheep, cow, horse, cat, monkey and human, and reported that it varied between 140 fmol/ml serum and 19,200 fmol/ml serum. Among other species studied to date, turtle GHBP was found to have a B_{max} of 1080 fmol ml^{-1} serum (Sotelo *et al.* 1997) whereas the B_{max} values for serum GHBP from rainbow trout (Sohm *et al.* 1998) and chicken (Davis *et al.* 1992) were reported to be remarkably higher, ranging from 5.2 x 10^5 to 1.5 x 10^6 fmol ml^{-1} serum or greater. The physiological significance for such large variations in the B_{max} value among different species remains unknown.

Ligand blotting also identified GHBP in goldfish serum and culture medium of goldfish hepatocytes. Goldfish, rabbit and rat sera contained GHBP of large M_r,

ranging from 120 KD to 360 KD. These large GHBP complexes in rabbit and rat sera have previously been identified by gel filtration (Ymer & Herington 1985, Baumbach *et al.* 1989, Amit *et al.* 1990, Massa *et al.* 1990). In the present study, DTT or ß-mercaptoethanol treatment resulted in the reduction of the M_r of the serum GHBP complexes in all species, suggesting that the large bands observed by ligand blotting contain disulfide bonds. Similar results have been reported for human serum GHBP (Hocquette *et al.* 1990). In the goldfish, the smallest M_r of serum GHBP under reducing conditions was found to be 27 KD. A 27 KD GHBP was also identified in serum of chickens and turkeys (Vasilatos-Younken *et al.* 1991). Previous studies on the primary structure of GHR in rat (Baumbach *et al.* 1989) and mouse (Smith *et al.* 1989) have indicated that the M_r of the extracellular domain of the GHR is approximately 30 KD. Thus, the 27 KD GHBP observed in present study may represent the extracellular portion of the goldfish GHR. The release of small M_r GHBP from cultured goldfish hepatocytes by iodoacetamide suggests that a mechanism of proteolytic cleavage of membrane GHR may also be involved in the generation of goldfish GHBP. However, additional studies will be required to fully determine the nature of the large M_r GHBP in goldfish serum.

The goldfish GHBP resemble GHBP from other species by displaying a specific high affinity and low-capacity binding for GH, presence of multiple forms of GHBP in the circulation, and a close relationship with liver membrane GHR. Although circulating GHBP have been found in all species tested to date, the precise physiological role of GHBP remains unclear (Barnard & Waters 1997). I have previously established and validated a goldfish GH receptor binding assay (Chapter 2). Together, these results

indicate that the goldfish will be a very useful model for studies on the physiological interaction between GH, GHR, and GHBP in teleosts.

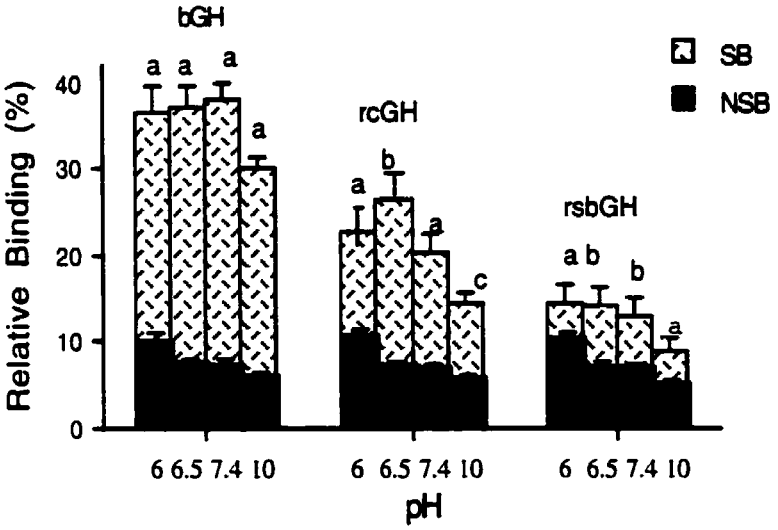

Figure 5.1 Effect of assay buffer pH on the binding of ^{125}I-rcGH, ^{125}I-bGH and ^{125}I-rsbGH to goldfish serum GHBP. Data, expressed as mean±SEM (n=3), were obtained by pooling results from three experiments, each carried out in triplicate with serum from a different animal. For each GH, specific binding at different pH was compared using the Student-Newman-Keuls multiple comparison test ($p<0.05$); groups with same superscript letter displayed a similar level of specific binding.

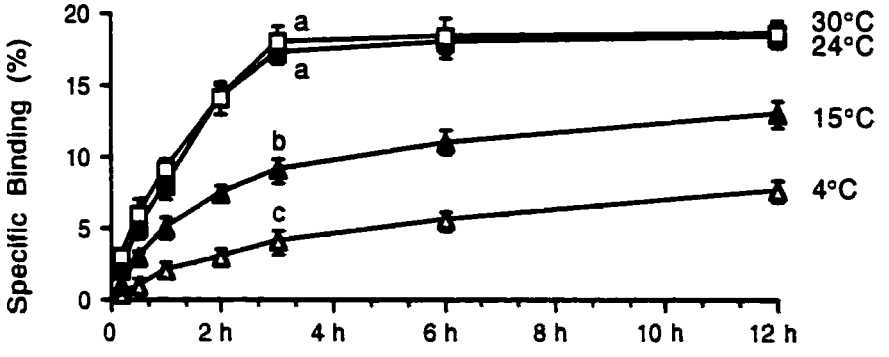

Figure 5.2 Specific binding of ^{125}I-rcGH to goldfish serum over time at various incubation temperatures. Data, expressed as mean±SEM, were obtained from three different experiments, each carried out in triplicate. Specific binding of rcGH at various temperatures at 3 h was compared using the Student-Newman-Keuls multiple comparison test ($p<0.05$); groups with same superscript letter displayed a similar SB.

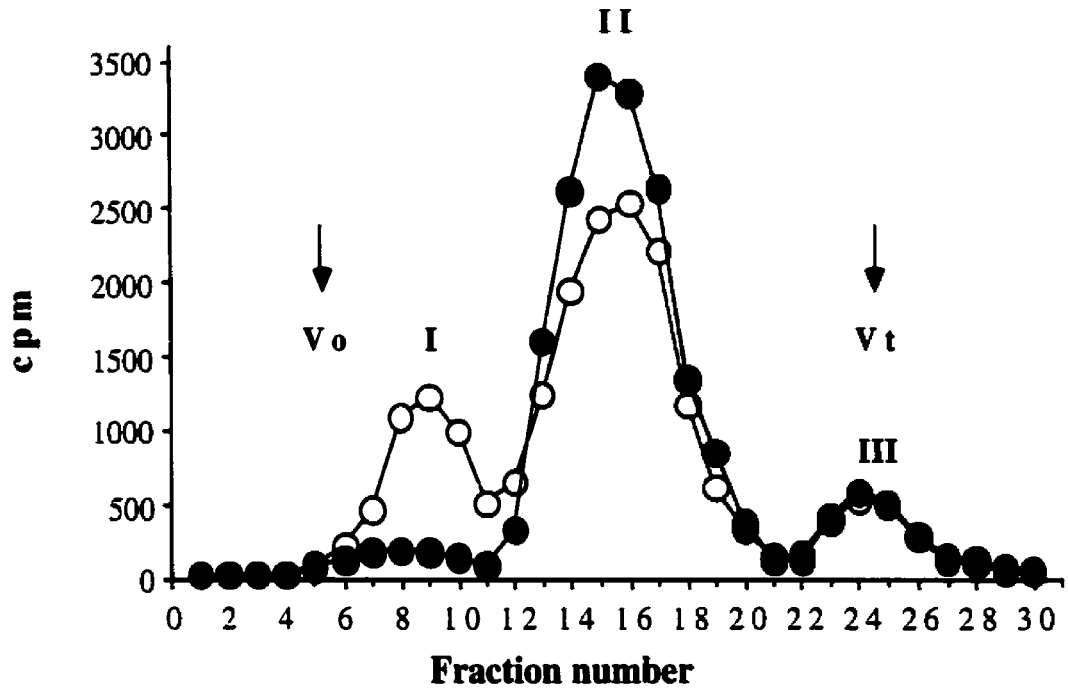

Figure 5.3 Elution profile of goldfish serum incubated with ^{125}I-rcGH following gel filtration on an Ultrogel AcA54 mini-column. An aliquot of 25 µl goldfish serum diluted in 25 µl assay buffer was incubated with approximately 20,000 cpm of ^{125}I-rcGH in the absence (open circles, total binding profile) or presence (closed circles, non-specific binding profile) of excess unlabeled rcGH (1 µg per tube). At the end of incubation, the mixture was eluted with assay buffer at a flow rate of 10 ml h^{-1} and 1.5 min fractions were collected. Fraction pools corresponding to peak I were used to calculate bound ^{125}I-rcGH. Vo, void volume, Vt, total volume.

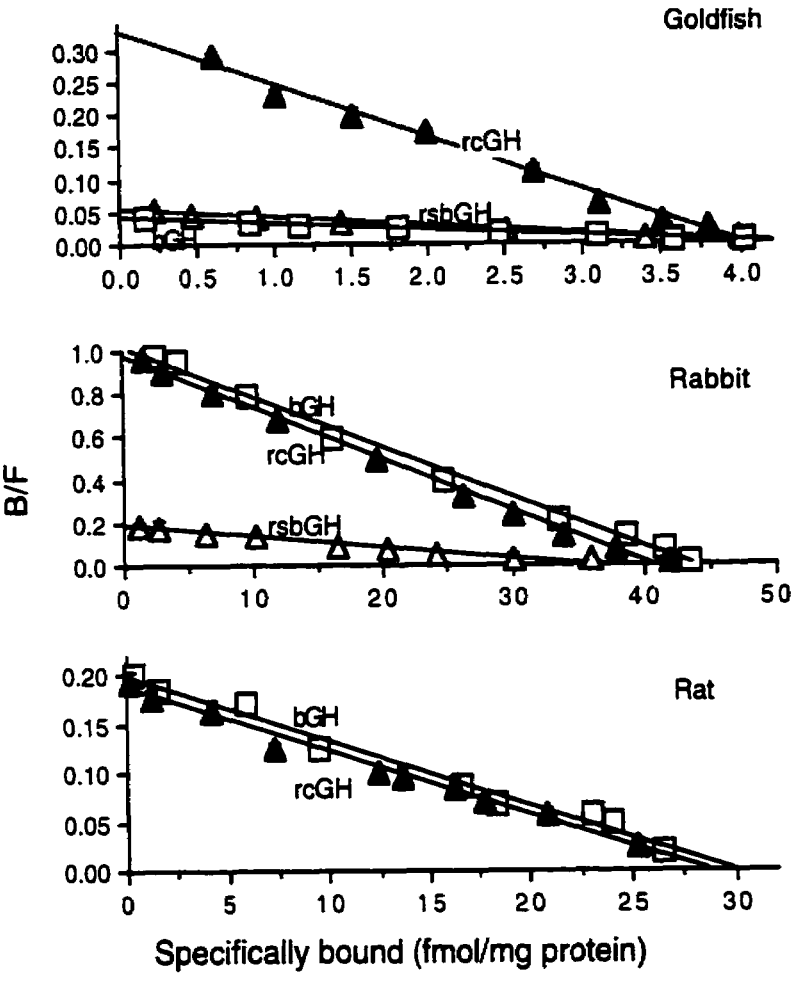

Figure 5.4 Scatchard plots produced by incubating increasing amounts of ^{125}I-bGH, ^{125}I-rcGH or ^{125}I-rsbGH with goldfish (top panel), rabbit (middle panel) or rat (bottom panel) serum. Data, expressed as mean±SEM, were obtained by pooling results from three experiments, each carried out in triplicate and with serum from a different animal.

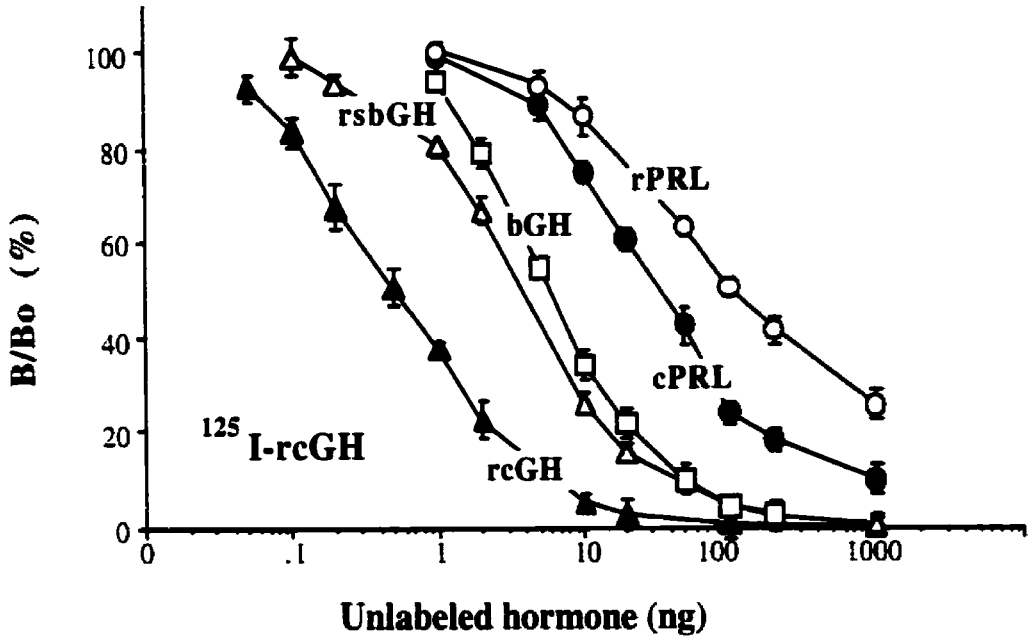

Figure 5.5 Displacement of 20,000 cpm of ^{125}I-rcGH from goldfish serum by increasing amounts of various unlabeled hormones. Data, expressed as mean±SEM, were obtained by pooling results from three different experiments, each carried out in triplicate and with serum from a different animal.

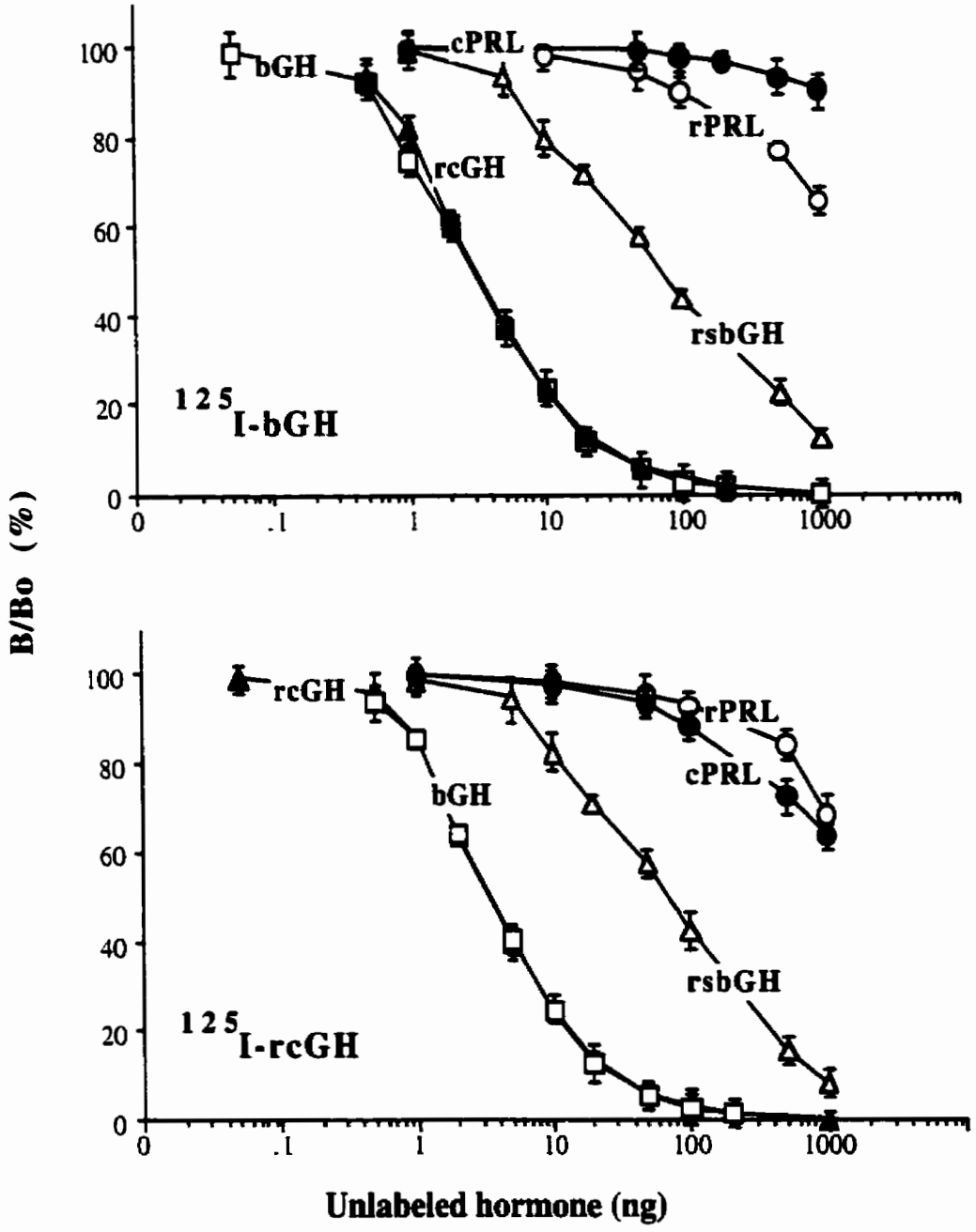

Figure 5.6 Displacement of 20,000 cpm of ^{125}I-bGH (top panel) or ^{125}I-rcGH (bottom panel) from rabbit serum by increasing amounts of various unlabeled hormones. Data, expressed as mean±SEM, were obtained by pooling results from three different experiments, each carried out in triplicate and with serum from a different animal.

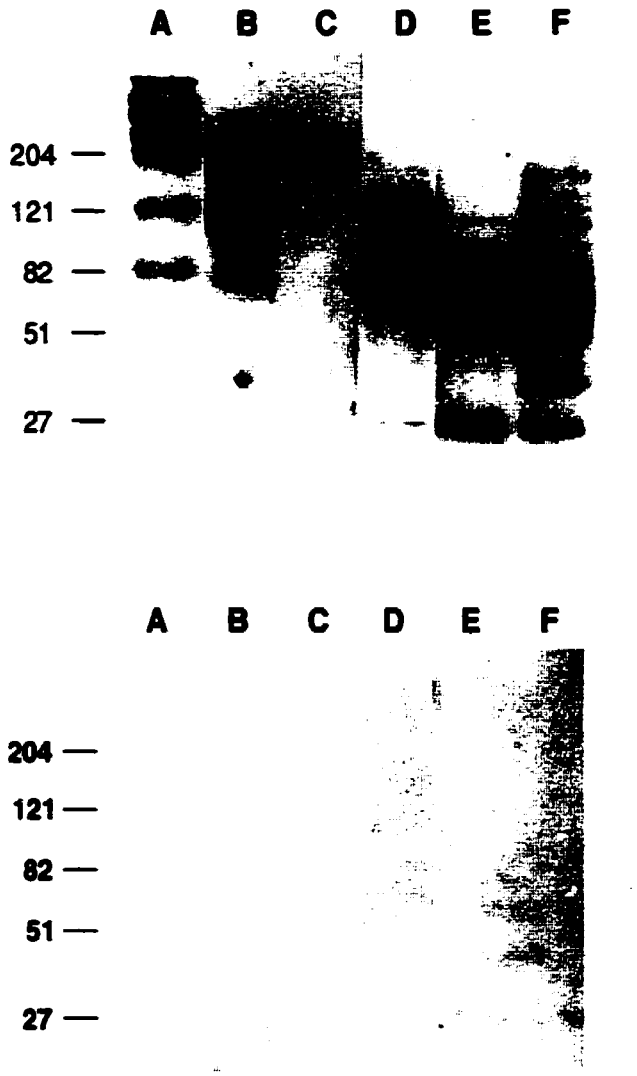

Figure 5.7 Autoradiogram of nitrocellulose membrane following ligand blotting of ^{125}I-rcGH to goldfish (lanes a, d), rabbit (lanes b, e) and rat (lanes c, f) serum (top panel). All samples were separated on 7.5% SDS-PAGE under both non-reducing (lanes a, b, c) and reducing conditions (lanes d, e, f). Molecular weights (KD) of standards are shown on the left. All bands disappeared when the membrane was incubated with labeled GH in presence of 10 μg ml^{-1} of the unlabeled GH (bottom panel).

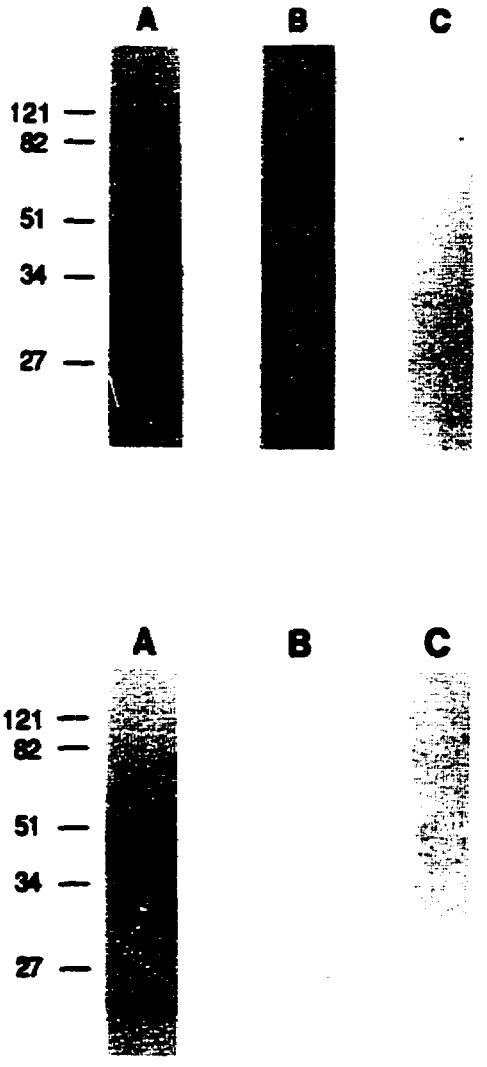

Figure 5.8 Autoradiogram of nitrocellulose membrane following ligand blotting of ^{125}I-rcGH to the culture medium from goldfish hepatocytes treated with (lane a) or without (lane b) iodoacetamide (top panel). Samples were separated on 12% SDS-PAGE under non-reducing conditions. Molecular weights (KD) of standards are shown on the left. All bands disappeared when membranes were incubated with labeled GH in presence of 10 μg ml^{-1} of the unlabeled GH. The M_r of the bands was not altered under reducing conditions (bottom panel). Lane c shows binding of ^{125}I-rcGH to culture medium from hepatocytes treated with iodoacetamide and subsequent incubation of the nitrocellulose membrane with 10 μg ml^{-1} of unlabeled rcGH.

Table 5.1 Comparison of bGH, rcGH and rsbGH binding sites in rabbit, rat and goldfish sera. The K_a and B_{max} values were obtained by LIGAND analysis of the results presented in Figure 4.3. All data are expressed as mean ± SEM.

Source of sera	Hormone	K_a (10^9 M^{-1})	B_{max} (fmol mg^{-1} protein)	B_{max} (fmol mg^{-1} serum)
Goldfish	rcGH	20.1 ± 1.8	4.1 ± 0.2	161 ± 7.8
(n=3)	rsbGH	2.8 ± 0.06	4.1 ± 0.2	158 ± 8.0
	bGH	2.0 ± 0.04	4.0 ± 0.1	162 ± 6.5
Rabbit	bGH	9.1 ± 0.2	49.8 ± 3.2	3540 ± 230
(n=3)	rcGH	9.5 ± 0.3	46.6 ± 3.7	3310 ± 261
	rsbGH	1.1 ± 0.2	41.8 ± 4.5	2970 ± 316
Rat	bGH	1.2 ± 0.1	83.8 ± 8.9	6030 ± 639
(n=3)	rcGH	1.1 ± 0.1	80.2 ± 9.8	5780 ± 706
	rsbGH	*	*	*

* K_a and B_{max} values are not available due to low (<2%) specific binding of the labeled hormone.

CHAPTER 6 *IN VIVO* REGULATION OF HEPATIC GROWTH HORMONE RECEPTORS IN THE GOLDFISH*

6.1 Introduction

In teleosts, GH (Peter & Marchant 1995, Peng & Peter 1997) and nutrition (Duan 1998) have been found to play important roles in regulating hepatic GHR. The effect of exogenous GH on regulation of GHR has been studied in coho salmon (Gray *et al.* 1990, 1992), the long-jawed mudsucker (Gray & Kelley 1991), and the Japanese eel (Mori *et al.* 1992). Hepatic GH binding was reported to be decreased in the hypophysectomized Japanese eel (Mori *et al.* 1992) and long-jawed mudsucker (Gray & Kelley 1991) following GH injection. In intact Japanese eel, however, hepatic GH binding was increased 5 days after GH injection (Mori *et al.* 1992). These results suggest that endogenous GH may regulate hepatic GHR in teleosts.

Nutritional status also appears to alter the hepatic GHR (Duan 1998). Evidence from Japanese eel (Mori *et al.* 1992), coho salmon (Gray *et al.* 1992) and gilthead seabream (Pérez-Sánchez *et al.* 1994) indicates that several weeks of starvation substantially reduced the total number of hepatic GHR. A significant decrease in hepatic GHR in coho salmon (Gray *et al.* 1992) and gilthead seabream (Pérez-Sánchez

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* Portions of the results presented in Chapter 6 have been published elsewhere (Zhang & Marchant 1996).

et al. 1994) was correlated with elevated serum GH concentrations but cessation of animal growth. It appears that the GHR down-regulation induced by food deprivation reflects a general pattern in teleosts.

The goal of the present study was to investigate the roles of GH and nutrition in the regulation of hepatic GHR in goldfish. Comparisons were made between the effect of short-term GH injection in intact goldfish and the effect of relatively long-term GH injection in hypophysectomized goldfish. The effects of other hormones such as prolactin on hepatic GH binding in goldfish were also examined. Parameters measured in the fasting experiments included hepatic GHR, body weight and length, liver-somatic index (LSI), and serum GH, GHBP, T_3, T_4, and glucose levels. Serum GH has been reported to induce glucose production in mammals (Scanlon *et al.* 1996) and teleosts (Björnsson 1997). Thus, the correlation between serum GH and glucose levels was examined. Levels of T_3 and T_4 were measured because the effects of GH on somatic growth of teleosts are potentiated by thyroid hormones (Eales 1988, Leatherland 1994).

6.2 Materials and Methods

6.2.1 Experimental animals

Goldfish of the common or comet varieties were obtained and maintained as described previously (Chapter 2). All studies were conducted in November with reproductively immature fish (50-70 g) of mixed sex.

6.2.2 Hormones and reagents

rcGH and rPRL were obtained as described previously (Chapter 2). Iodination of the rcGH was performed as previously described, using the lactoperoxidase method (Chapter 2). The specific activity of the labeled rcGH was routinely more than 100 $\mu Ci\ \mu g^{-1}$ when determined using self-displacement in liver membrane receptor binding assay (Chapter 2).

6.2.3 Effects of hormone replacement on hypophysectomized goldfish

Hypophysectomy of goldfish (15 to 20 g body weight) was performed using the opercular approach (Yamazaki 1961). Sham operations were performed in a similar manner except that the pituitary was not fully exposed in order to minimize possible disruption of hypothalamic connections to the pituitary. All surgeries were performed in fish deeply anesthetized with 2-phenoxyethanol (0.3% vol/vol). Following surgery, hypophysectomized (Hx) and sham-operated fish were maintained in charcoal-filtered 0.6% (wt/vol) NaCl; the fish were fed to excess twice daily during this recovery period. Mortality during the recovery period was less than 5%. Completeness of hypophysectomy was assessed through change in body color (Chavin 1956, Yamazaki

1961) and by visual inspection for pituitary remnants in the sella region at the time of sacrifice, and was achieved in greater than 95% of the surgeries.

Hormone administration began 10 days after surgery. Groups of fish (n=7 per group) received a total of three intraperitoneal injections of saline, rcGH or rPRL at 1 day intervals. The hormones were dissolved in 0.6% (wt/vol) NaCl. rcGH was injected at a dosage of 0.1, 1.0 or 10 $\mu g\, g^{-1}$ body weight whereas rPRL was injected at a dosage of 5 $\mu g\, g^{-1}$ body weight. Saline-injected animals received an equivalent volume of 0.6% NaCl (10 $\mu l\, g^{-1}$ body weight). 24 h after the last injection, all fish were anesthetized in 0.05% MS222 and killed by spinal transection. The livers from fish in each group were collected, pooled and liver membranes prepared for use in the rcGH receptor binding assay as described previously (Chapter 2). Free and total GH binding sites in liver membranes were measured for each group. Total GH binding sites were measured by treatment of the liver membranes with 4 M $MgCl_2$ to remove endogenous GH from the liver membranes (Chapter 2).

6.2.4 Effects of short-term GH injection on intact goldfish

Groups of fish received a single intraperitoneal injection of saline or rcGH. The hormone was dissolved in 0.6% (wt/vol) NaCl and injected at a dosage of 1.0 $\mu g\, g^{-1}$ body weight. Saline-injected animals received an equivalent volume of 0.6% NaCl (10 $\mu l\, g^{-1}$ body weight). After the injections, groups (n=7) of fish were anesthetized in 0.05% (wt/vol) MS222 and killed by spinal transection at intervals from 1 to 12 h. Livers from fish in each group were collected, pooled and liver membranes prepared for

use in the rcGH receptor binding assay as described previously (Chapter 2). Free and total GH binding sites in liver membranes were measured for each group (Chapter 2).

6.2.5 Effects of short-term fasting on goldfish

Goldfish were allowed to feed themselves through a demand feeder for 4 weeks prior to the start of the experiments. Two separate fasting experiments were conducted. At the beginning of the experiments, fish were weighed, measured for length, divided into different groups, and placed in separate tanks.

The first experiment included two groups of fish, each consisting of 6 fish. Fish in one group were fed continuously through a demand feeder whereas fish in the second group were fasted for 1 week. At the end of the fasting period (Day 7), all fish in the fed and fasted groups were weighed, measured, sacrificed, and blood samples collected for analysis of serum GH, GHBP and glucose. Livers were also collected but were inadvertently destroyed during preparation of the membranes for use in the rcGH receptor binding assay. Thus, data for hepatic GHR are unavailable for this experiment.

In the second experiment, 30 fish were divided into 5 groups. Each group, consisting of 6 fish, was placed in a separate tank. Fish in groups 1, 2 and 4 were fed through a demand feeder whereas fish in groups 3 and 5 were fasted for 3 or 7 days. Groups of fish were weighed, measured, sacrificed at various time intervals (day 0, group 1; day 3, groups 2 and 3; day 7, groups 4 and 5), and liver tissues and blood samples were collected for analysis of total and free hepatic GHR and serum GH, GHBP, T_3, T_4, and glucose. LSI was calculated as the percentage of liver weight over body weight.

6.2.6 Measurement of serum GH, GHBP, T_3, T_4, and glucose levels

Iodination of the GH was performed as previously described (Chapter 2). Serum GH levels were determined using a rcGH radioimmunoassay (RIA) validated for measuring circulating levels of GH in the goldfish (Fine *et al.* 1993b). Serum GHBP levels were measured as described previously (Chapter 5). Iodination of thyroid hormones was performed using the method of Kjeld *et al.* (1975). The specific activity of labeled thyroid hormones prepared in this way was approximately 800 $\mu Ci\ \mu g^{-1}$ for T_3 and 2700 $\mu Ci\ \mu g^{-1}$ for T_4. Serum T_3 or T_4 measurements were performed in unextracted sera by RIA (Chopra 1972) using commercially available T_3 and T_4 antisera (Sigma Chemical Co., St. Louis, MO) in combination with labeled T_3 and T_4, respectively. The minimum detection limit was 6.25 pg l^{-1} for T_3 and 20 pg l^{-1} for T_4. All samples were measured in a single T_3 or T_4 assay. The within assay variability in the T_3 and T_4 RIA was acceptable; the % coefficient of variation for both assays was less than 10%. Serum glucose levels were determined by a glucose oxidase method (Young *et al.* 1975) using a glucose test kit (Catalog No. 315-500) purchased from Sigma Chemical Co. (St. Louis, MO).

6.2.7 Statistics

Specific binding of ^{125}I-rcGH to hepatic GHR and serum GHBP was calculated as described previously (Chapters 2 & 5). Data from hypophysectomy and hormone administration experiments, and from fasting experiments were analyzed with ANOVA

followed by Student-Newman-Keuls multiple comparison test ($p<0.05$). The K_a and B_{max} of ^{125}I-rcGH binding to liver membranes from goldfish in the fasting experiment were determined using the LIGAND computerized program (Munson & Rodbard 1980). Other data were analyzed using Student's t-test ($p<0.05$). The Spearman rank correlation coefficient was used to examine the relationship between between hepatic GHR and serum GHBP in the fasting experiment.

6.3 Results

6.3.1 Effects of hypophysectomy and hormone replacement on goldfish

The effects of hypophysectomy, rcGH and rPRL treatment on binding of ^{125}I-rcGH to pooled liver membranes treated with or without $MgCl_2$ are shown in Figure 6.1. The number of total binding sites in sham-operated goldfish was 1.8 times higher than that of free binding sites. In contrast, all the Hx goldfish had similar numbers of total and free binding sites. Membranes from saline-injected Hx goldfish displayed a significant increase in total binding sites when compared to membranes from saline-injected sham-operated animals (Fig. 6.1). Following injection of rcGH to Hx fish, the number of both total and free binding sites was significantly decreased compared to that of Hx fish injected with saline. The decrease in total and free binding sites was dependent on the dosage of rcGH with dosages of 0.1, 1.0 and 10 $\mu g\ g^{-1}$ resulting in a 27%, 52% and 68% decrease in total binding sites and a 25%, 53% and 65% decrease in free binding sites, respectively (Fig. 6.1). Injection of 5 $\mu g\ g^{-1}$ rPRL into Hx goldfish also resulted in a 32% decrease in total binding sites and a 34% decrease in free binding sites (Fig. 6.1).

6.3.2 Short-term effects of GH injection in intact goldfish

The effects of a single rcGH injection on binding of ^{125}I-rcGH to pooled liver membranes from intact goldfish are shown in Figure 6.2. Administration of rcGH resulted in a rapid down-regulation of hepatic GHR (Fig. 6.2). The lowest levels of both free and total binding sites were reached 2 h after rcGH injection (Fig. 6.2). 12 h

after injection of rcGH, the levels of both free and total binding sites were restored to those observed prior to rcGH treatment and were similar to that observed in saline injected fish (Fig. 6.2). In contrast, membranes from saline-injected goldfish displayed no change in both free and total binding sites over the entire sampling period (Fig. 6.2).

6.3.3 Effects of short-term fasting

Changes in body weight and length of goldfish from both first and second experiments were shown in Figure 6.3. In the first experiment, fasted fish lost approximately 8.3% of their body weight whereas the body weight of fed fish increased by 3.7% at 1 week (Fig. 6.3). The body length of fasted and fed fish in the first experiment decreased by 0.9% and increased by 2.6%, respectively (Fig. 6.3). In the second experiment, in comparison with those of fish at day 0, the body weight and length of fasted fish decreased approximately 3% and 0.2% at day 3 and 9% and 1% at day 7, respectively, whereas those of fed fish increased approximately 1.8% and 0.3% at day 3 and 4.2% and 2.8% at day 7, respectively (Fig. 6.3). Fasting resulted in a significant reduction in LSI of goldfish at both day 3 and day 7 (Fig. 6.4).

In the first experiment, serum glucose (Fig. 6.5a) and GH (Fig. 6.5b) levels in fasted fish increased only slightly whereas serum GHBP (Fig. 6.5c) levels significantly decreased in fasted fish. However, fasted fish from the second experiment had significantly higher serum glucose (Fig. 6.6a) and GH (Fig. 6.6b) levels than the control fish at both day 3 and day 7. A significant reduction in serum GHBP (Fig. 6.6c) and total hepatic GH binding sites (Fig. 6.6d) was also found in fasted fish from the second experiment at both day 3 and day 7. LIGAND analysis of ^{125}I-rcGH binding to liver

membranes from each group of goldfish indicated that K_a was not altered by fasting (Fig. 6.7). A significantly positive correlation was found between hepatic GHR and serum GHBP levels in goldfish from the second experiment (Fig. 6.8). Serum T_3 levels of fasted goldfish from the second experiment were not significant different compared to those of control fish at day 3 and day 7 (Fig. 6.9). In contrast, serum T_4 levels were significantly decreased in fasted fish compared to those of control fish at both day 3 and day 7 (Fig. 6.9).

6.4 Discussion

The number of total ^{125}I-rcGH binding sites in liver membranes from sham-operated goldfish was almost 2-fold higher than the number of free binding sites, indicating that some of the binding sites were occupied by endogenous GH in the sham-operated animals. The existence of endogenous GH in liver membranes has been reported in a variety of teleosts (Gray *et al.* 1992, Mori *et al.* 1992, Pérez-Sánchez *et al.* 1994) and mammals (Kelley *et al.* 1979, Maiter *et al.* 1988). Hepatic membranes from Hx goldfish had similar values for the number of total and free ^{125}I-rcGH binding sites, which reflects the lack of circulating GH in the Hx fish. This result also indicates that the injected rcGH was removed from the circulation system of the Hx fish by the time of sampling. It has been reported that the time required for the total clearance of injected GH is 24 h for Hx Japanese eel maintained at 20°C (Mori *et al.* 1992) and 6 h for Hx rat (Maiter *et al.* 1988).

Hypophysectomy has been reported to reduce the number of total and free GH binding sites in rabbit and sheep (Posner *et al.* 1980), pregnant mouse (Sanchez-Jimenez *et al.* 1990), female rat (Baxter & Zaltsman 1984), long-jawed mudsucker (Gray & Kelley 1991), and Japanese eel (Mori *et al.* 1992). In contrast, hypophysectomy did not alter total and free GH binding sites in the male rat (Baxter & Zaltsman 1984) and coho salmon (Gray *et al.* 1992). These results suggest that the effect of hypophysectomy on hepatic GH binding may vary according to species or sex. Hepatic membranes from Hx goldfish injected with saline had a significantly higher number of total ^{125}I-rcGH binding sites than those of sham-operated fish, indicating an up-regulation of GH binding sites by hypophysectomy in the goldfish. This finding

suggests that endogenous GH may play a role in the regulation of its own receptors in the goldfish.

In vivo administration of rcGH to Hx goldfish reduced the number of ^{125}I-rcGH binding sites in a dose-dependent manner. Rat PRL also reduced ^{125}I-rcGH binding sites, but at a 50-fold higher dose than rcGH. These results suggest that administration of rcGH induced GH receptor down-regulation in the goldfish liver. GH treatment *in vivo* has been reported to cause down-regulation of GHR under some situations in long-jawed mudsucker (Gray & Kelley 1991), coho salmon (Gray *et al.* 1992) and Japanese eel (Mori *et al.* 1992). The finding that rPRL treatment also decreased the number of total ^{125}I-rcGH binding sites in the goldfish provides the possibility that PRL may also have a role in GH receptor regulation. However, whether PRL acts by cross-reacting with GH receptors or indirectly via its own receptor is not clear.

The rapid decrease in both free and total GH binding sites in intact goldfish after a single rcGH injection also suggests that GH may play a role in the short-term down-regulation of its own receptors in the goldfish. The levels of hepatic GHR in intact goldfish were restored to the control levels by 12 h after rcGH injection. In mammals, acute down-regulation of the GHR may involve multiple rapid and complex pathways, including dimerization of GHR, cellular internalization of the GH-GHR complex, and cellular degradation, recycling, or synthesis of GHR (Goffin *et al.* 1998).

Hepatic GHR in fasted goldfish also decreased significantly. A similar reduction in hepatic GHR was reported for salmonids (Gray *et al.* 1992, Duan *et al.* 1994), gilthead seabream (Pérez-Sánchez *et al.* 1994), and Japanese eel (Duan & Hirano 1992). The present study found that lower levels of hepatic GHR were coincident with higher

levels of serum GH. Such higher levels of endogenous GH may induce a down-regulation of hepatic GHR in fasted goldfish as described above.

Serum GHBP levels were also lower in fasted fish than in fed animals. Studies from mammals have found a significant positive correlation between hepatic GHR and serum GHBP in rats (Massa *et al.* 1990) and humans (Baumann *et al.* 1987, Daughaday *et al.* 1987) whereas no relationship between hepatic GHR and serum GHBP was found in rabbit (Heinrichs *et al.* 1997). To date, there is no information available on the physiological regulation of GHBP from teleosts. The present study in goldfish, however, found that hepatic GHR was significantly correlated with serum GHBP in the fasting experiments. Thus, the concentration of serum GHBP appears to reflect hepatic GHR levels in the goldfish, indicating that hepatic GHR may be the primary source of serum GHBP in this species.

There is evidence from mammalian studies that GHBP acts as a GH reservoir by reducing GH clearance rate from the circulation (Barnard & Waters 1997). Based on this effect of GHBP, low levels of GHBP should result in lower levels of serum GH due to increased clearance of GH from the circulation (Barnard & Waters 1997). However, in fasted goldfish, lower levels of serum GHBP were coincident with higher levels of serum GH.

Goldfish GHBP binds GH with high affinity (Chapter 5), and it is possible that the presence of GHBP in serum might also influence the measurement of serum GH levels by RIA. There is a lack of information on how serum GHBP might influence GH RIA in teleosts, although GHBP results in a negligible disturbance in mammalian GH

RIA (Jan *et al.* 1991). However, further research is needed to confirm that teleost GH RIA accurately determine the correct GH levels in serum containing GHBP.

In goldfish fasted for 3 or 7 days, body and liver weights decreased significantly compared to those of the fed fish. The loss of tissue weights in fasted goldfish reflects a general pattern reported for other teleosts (Mommsen & Plisetskaya 1991). The significant reduction in the liver-somatic index during fasting appears to be caused by preferential utilization of liver components such as glycogen and lipid as an energy source in fasted teleosts (Pereira *et al.* 1995). Serum glucose concentrations are usually maintained in fasted fish by reducing the rate of glucose use, increased gluconeogenesis, or increased tissue glycogen breakdown (Pereira *et al.* 1995). There is extensive evidence from carp (Blasco *et al.* 1992a, b), dab (*Limanda limanda*) (Zhang 1993), and Atlantic salmon (*Salmo salar*) (Soengas *et al.* 1996) indicating that tissue glycogen is converted to glucose during the first week of fasting to provide an energy source for fasted animals.

Evidence from mammals (Scanlon *et al.* 1996) and other teleosts (Björnsson 1997) has indicated that GH has an anti-insulin-like effect and may be responsible for the elevated serum glucose levels observed in the present study. However, elevated GH levels induced by food deprivation are not always evident in salmonids (Leatherland 1994) and it has been suggested that the increased circulating GH levels sometimes observed during fasting may be an artifact related to experimental design (Leatherland & Farbridge 1992). Significantly higher serum GH levels in fasted goldfish were also found in only one experiment in the present study, suggesting that the response to fasting is also variable in goldfish. Further research is obviously needed to clarify this

issue and to determine the precise role for the elevated circulating GH during fasting in teleosts.

Fasted goldfish also had lower levels of serum T_4 than fed fish. In teleosts, T_3, but not T_4, is the biologically active hormone produced extrathyroidally by 5'-monodeiodination of T_4 (Eales 1988). There is evidence from salmonids (Eales 1988, Leatherland 1994), goldfish (Spieler 1993), and sea bass (*Dicentrarchus labrax*) and gilthead seabream (Cerdá-Reverter *et al.* 1996), indicating that fasting results in a significant reduction in levels of both circulating T_3 and T_4. Such a decrease in thyroid hormone levels in fasted teleosts indicates an overall reduction in metabolism and the pituitary-thyroidal axis, and would result in a suppression of the growth-promoting effect of GH (Eales 1988, Leatherland 1994). Although goldfish were also fasted for 3 or 7 days similar to those of salmonids (Leatherland 1994) and sea bass and sea bream (Cerdá-Reverter *et al.* 1996), serum T_3 levels remained unchanged in fasted goldfish. However, goldfish fasted for 7 days had lower serum T_4 levels than those fasted for 3 days. These results suggest that fasting did suppress the thyroid axis in goldfish, but a longer period of fasting may be required to alter serum T_3 levels.

The present study provides several lines of new evidence indicating the importance of GH and nutrition in the *in vivo* regulation of GHR in goldfish. In both Hx and intact goldfish, GH administration clearly caused down-regulation of hepatic GHR whereas in the fasted goldfish, elevated circulating GH levels were also coincident with significantly lower levels of hepatic GHR. In response to food deprivation, goldfish displayed a pattern similar to that in other teleosts, including significant reductions in body weight and length, LSI, and serum T_4 levels but not in T_3 levels.

Importantly, the present study provides the first evidence of a direct relationship between hepatic GHR and serum GHBP in a teleost, and suggests that the suppression of somatic growth during fasting may be due, at least in part, to a reduction in hepatic GHR and serum GHBP. Elevation in serum GH and glucose were also found in fasted goldfish, providing for the possibility that changes in the GH-GHR endocrine axis result in the mobilization of short-term energy stores during fasting. Together, these results suggest that the goldfish is a good model to further study the interaction between the GH, GHR and other endocrine pathways during somatic growth and energy partitioning in teleosts.

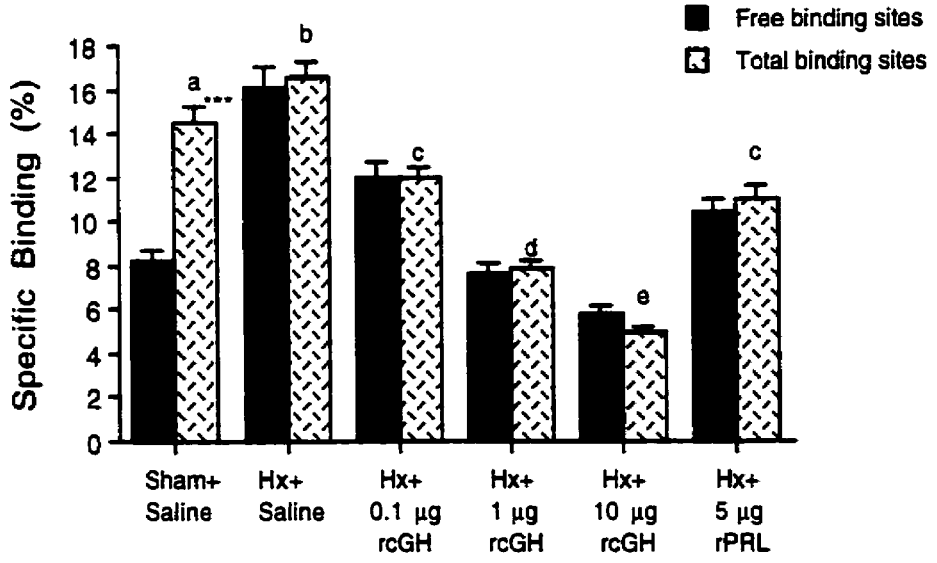

Figure 6.1 Effects of saline, rcGH or rPRL treatment on binding of ^{125}I-rcGH to liver membranes collected and pooled from sham-operated or Hx goldfish. Total binding sites were measured following treatment of liver membranes with $MgCl_2$ to remove endogenous GH; free binding sites were measured in membranes that were not treated with $MgCl_2$. Total binding sites were significantly higher than free binding sites in the sham-operated group (Student's t-test, *** $p<0.001$); total and free binding sites were similar within each of the Hx groups (Student's t-test, $p>0.5$). Total binding sites in the various treatment groups were also compared using the Student-Newman-Keuls multiple comparison test ($p<0.05$); groups with the same superscript letter displayed a similar value for the total binding sites. All data are presented as mean±SEM of triplicate determinations in a single assay.

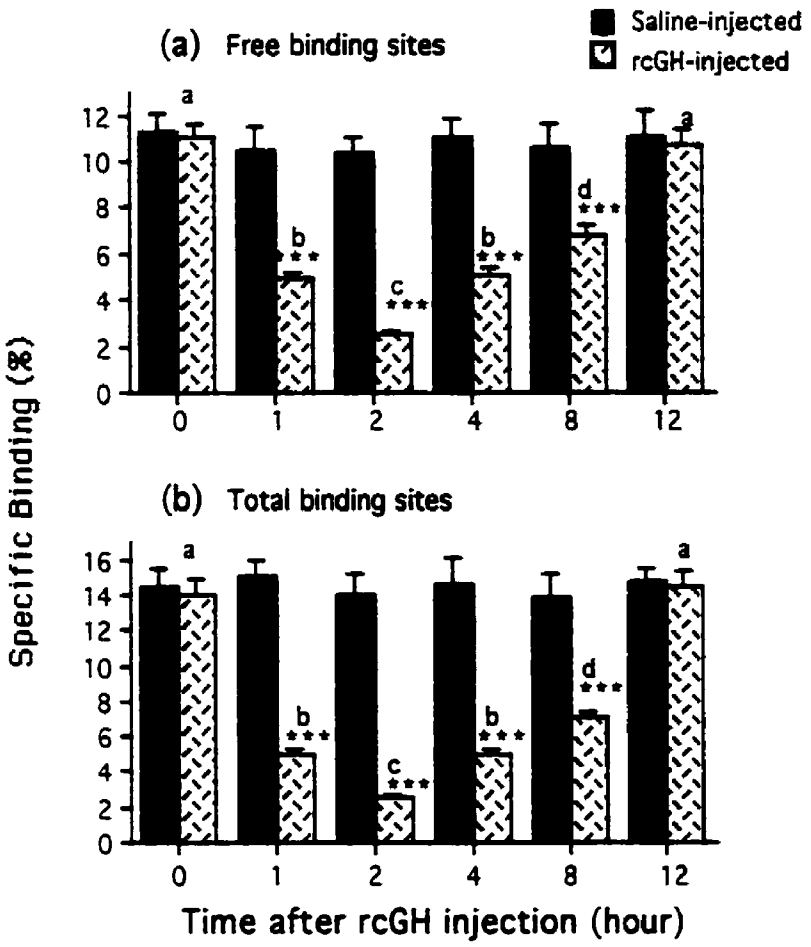

Figure 6.2 Effects of rcGH and saline treatments on binding of ^{125}I-rcGH to liver membranes collected and pooled from intact goldfish. Free (panel a) and total (panel b) binding sites were measured as described previously (Chapter 2). Both free and total binding sites were significantly decreased at 1, 2, 4, and 8 h after rcGH injection compared to those of the saline-injected group (Student's t-test, ***$p<0.001$) and were restored to control levels 12 h after rcGH treatment. Data from the rcGH-injected fish were subjected to the Student-Newman-Keuls multiple comparison test ($p<0.05$); groups with the same superscript letter displayed a similar SB. All data are presented as the mean±SEM of triplicate determinations in a single assay.

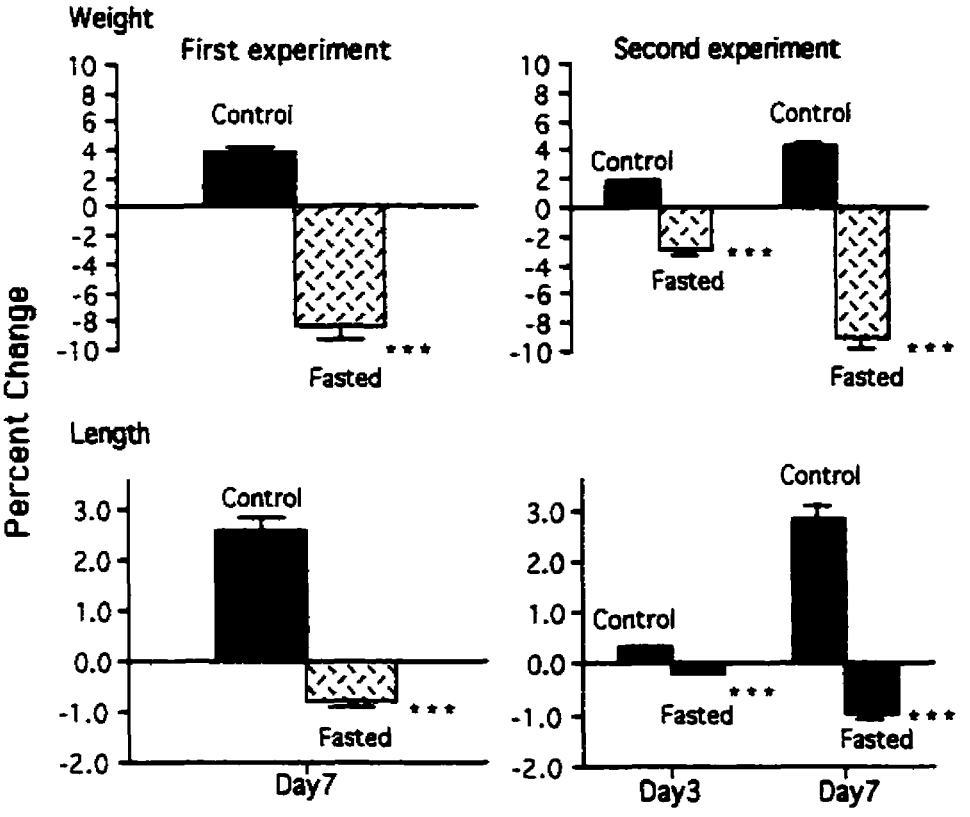

Figure 6.3 Effects of fasting on goldfish growth expressed as percent change in body weight and length of fish from both first (left panel) and second (right panel) experiments. The control fish were fed continuously via demand feeders. Data are expressed as mean±SEM (n=6). Values from the control groups were significantly different from those of the fasted groups at day 3 and day 7 (Student's t-test, ***$p<0.001$).

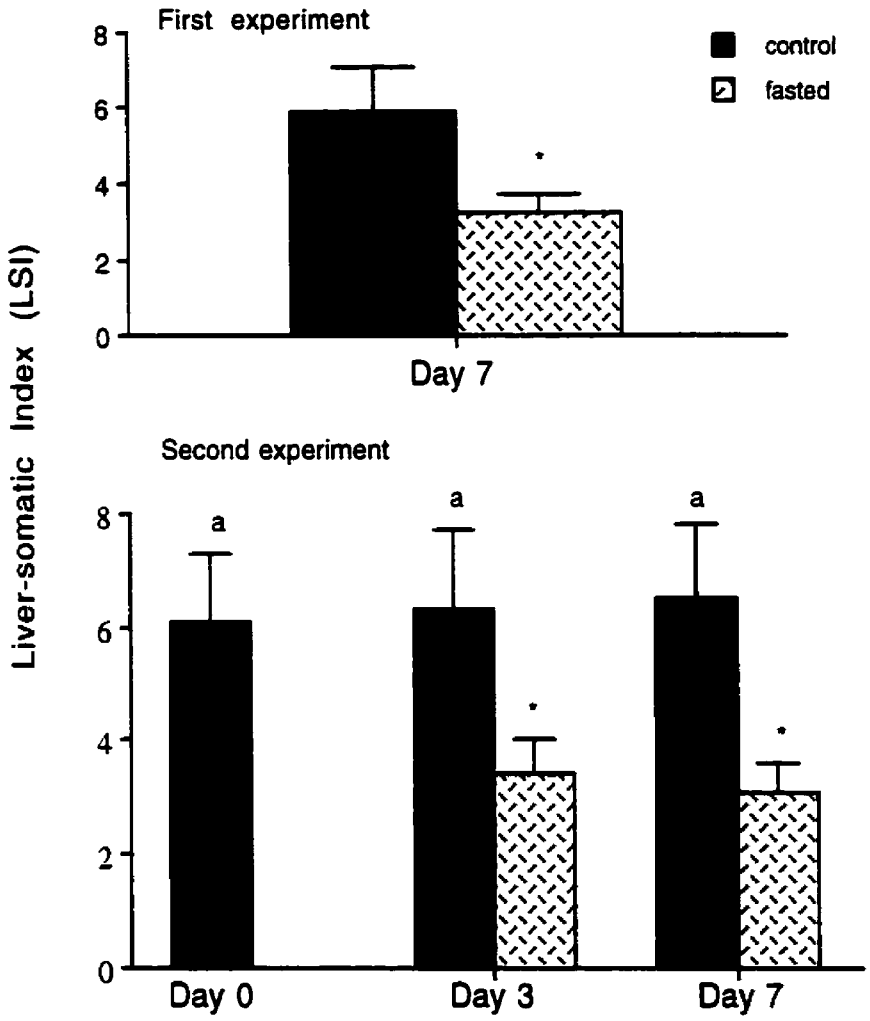

Figure 6.4 Effects of fasting on liver-somatic index of goldfish from both first (top panel) and second (bottom panel) experiments. LSI was significantly decreased in fasted fish at day 3 and day 7 (Student's t-test, *$p<0.05$). Data from the fed (control) groups of the second experiment were subjected to the Student-Newman-Keuls multiple comparison test ($p<0.05$); groups with the same superscript letter displayed a similar LSI. Data are expressed as mean±SEM (n=6).

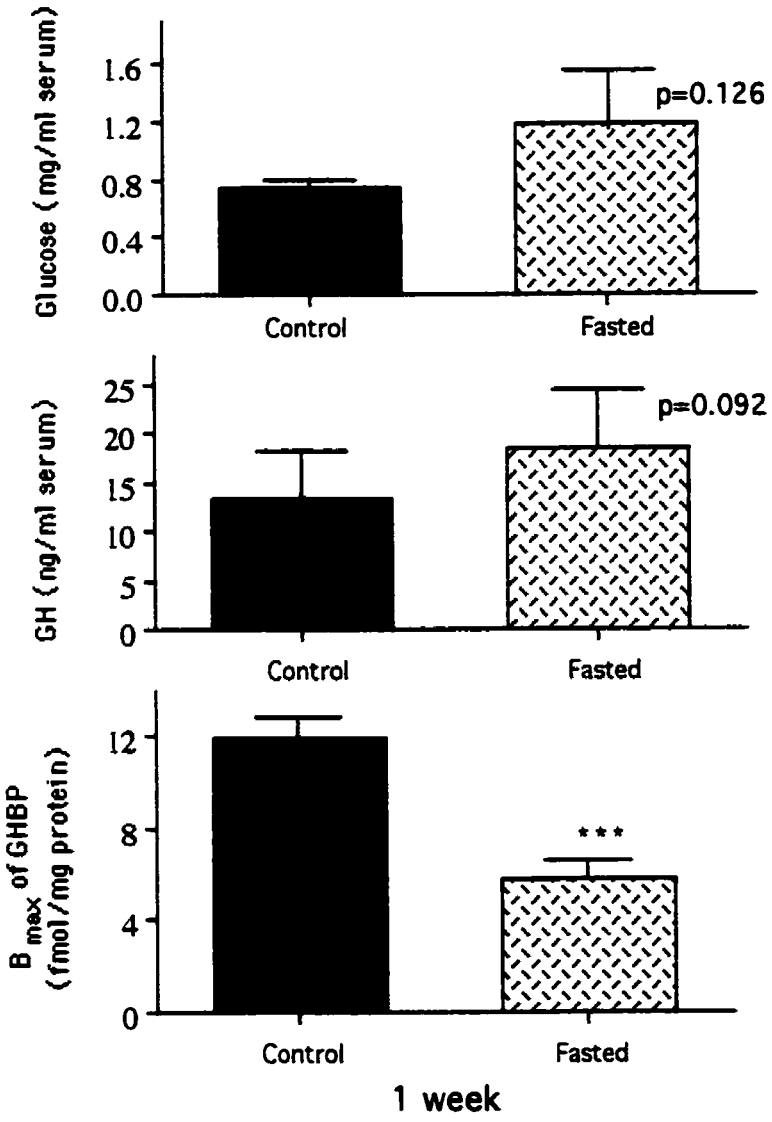

Figure 6.5 Effects of fasting on serum glucose (top panel), GH (middle panel), and GHBP (bottom panel) levels in goldfish from Experiment 1. Serum glucose and GH levels in fasted fish were not significantly different from those of fed (control) fish. The B_{max} of serum GHBP was significantly decreased in fish fasted for 1 week compared to that of control fish (Student's t-test, ***$p<0.001$). Data are expressed as mean±SEM (n=6).

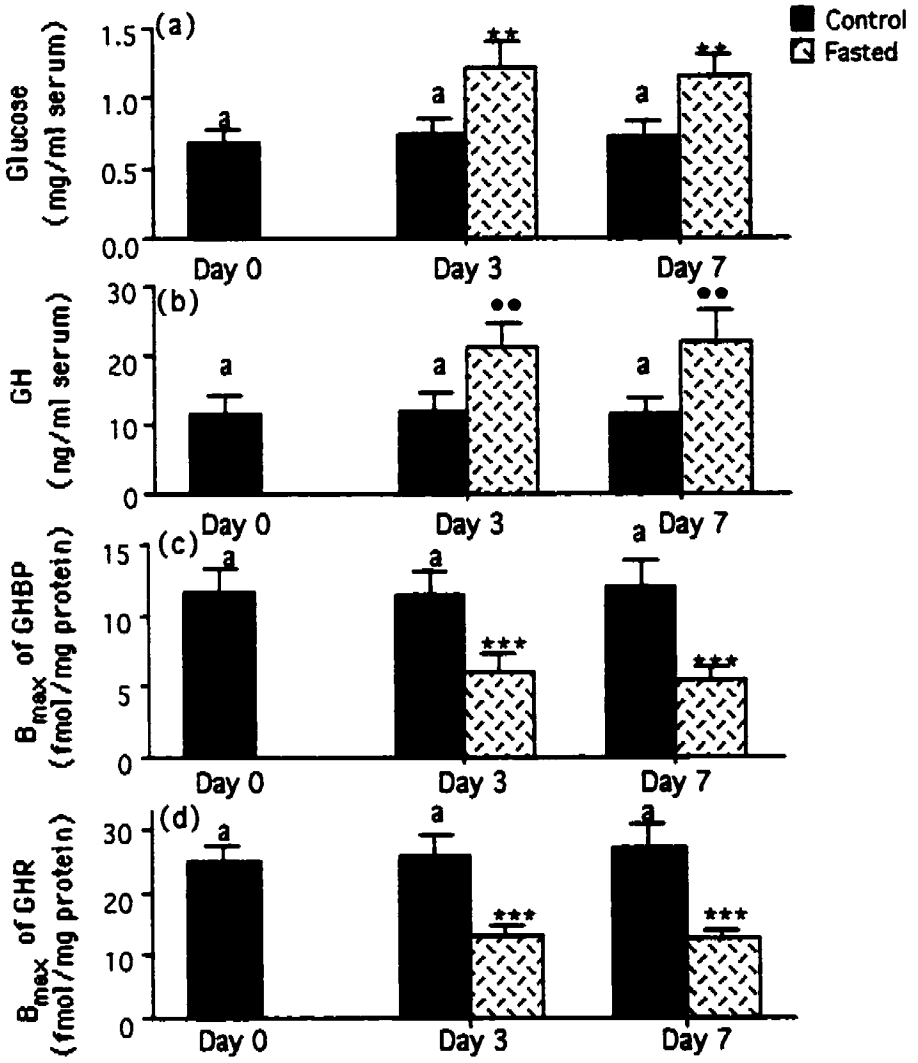

Figure 6.6 Effects of fasting on serum glucose (panel a), GH (panel b), and GHBP (panel c) and hepatic GHR (panel d) levels in goldfish from the second experiment. Serum glucose and GH levels were significantly increased in fasted fish (Student's t-test, **$p<0.01$) compared to those of fed (control) fish. The B_{max} values of serum GHBP and hepatic GHR were significantly decreased in fish fasted for 3 or 7 days (Student's t-test, ***$p<0.001$). Data from the control groups were also subjected to the Student-Newman-Keuls multiple comparison test ($p<0.05$); groups with the same superscript letter displayed a similar value for glucose, GH, or B_{max}. Data are expressed as mean±SEM (n=6).

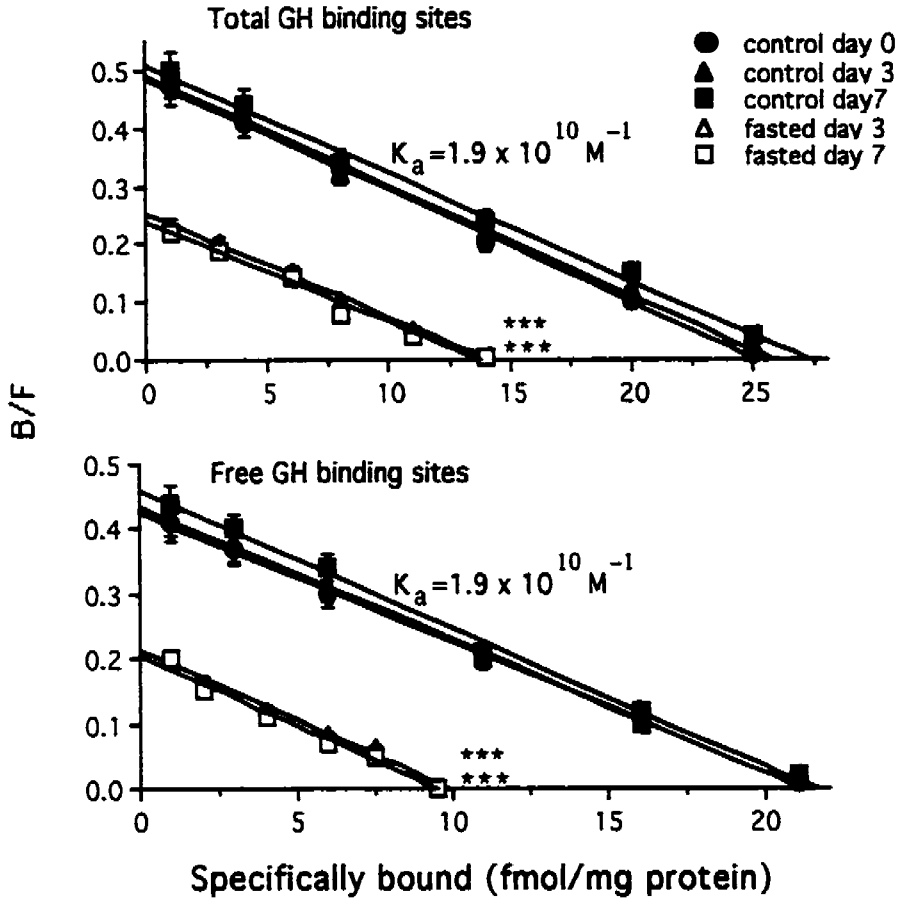

Figure 6.7 Effects of fasting on total (panel a) and free (panel b) GH binding sites, expressed as a Scatchard plot, in hepatic membranes of goldfish. The B_{max} values of both total and free GH binding sites were significantly decreased (Student's t-test, ***$p<0.001$) in fish fasted for 3 or 7 days compared to those of fed (control) groups whereas the K_a was similar in all groups. Data are expressed as mean±SEM (n=6).

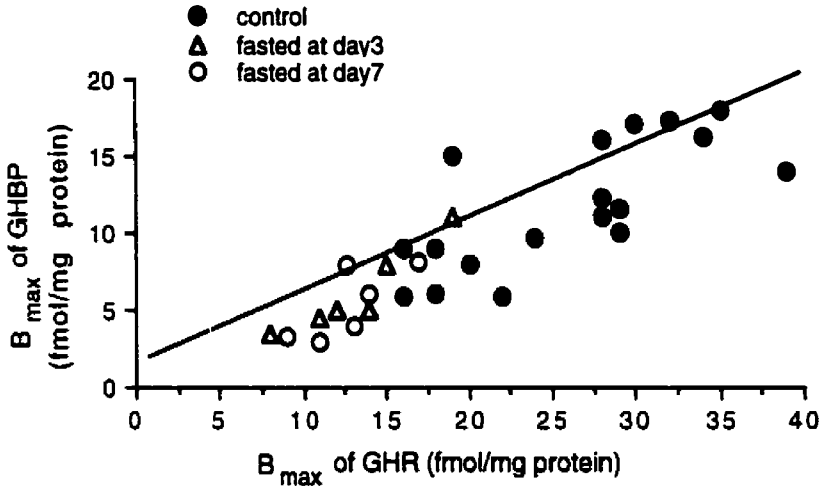

Figure 6.8 Correlation between hepatic GHR and serum GHBP in goldfish. A significantly positive correlation was found between hepatic GHR and serum GHBP ($r=0.86$, $p<0.001$, $n=30$).

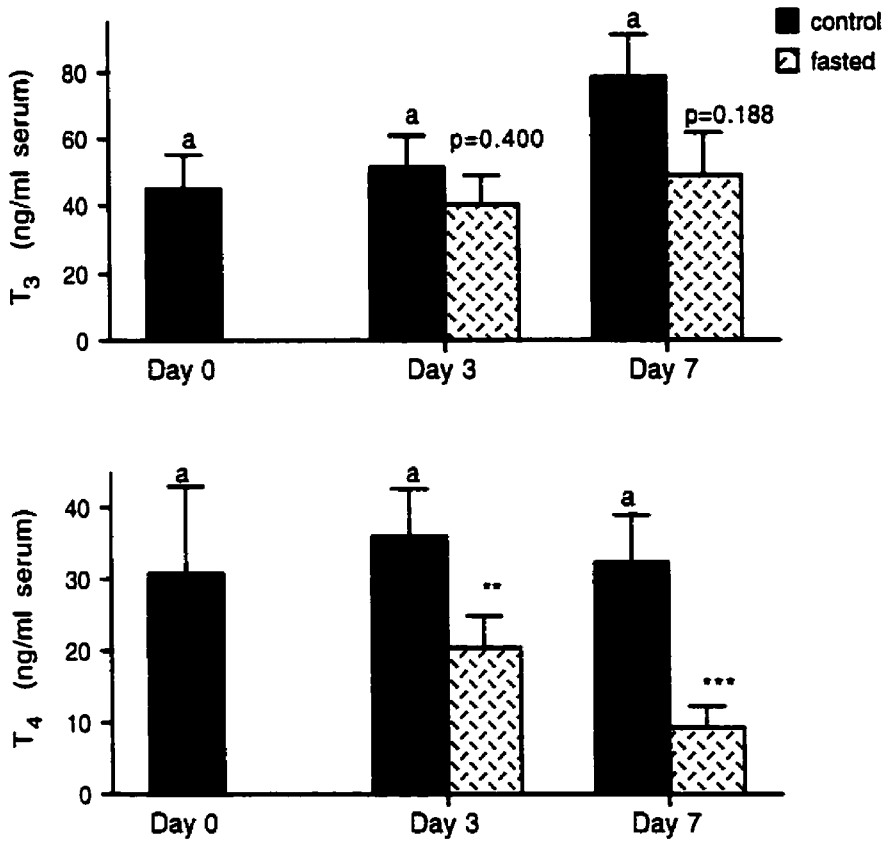

Figure 6.9 Effects of fasting on serum T_3 (top panel) and T_4 (bottom panel) levels in goldfish. Serum T_3 levels in fasted fish were not significantly different from those of fed (control) fish. Serum T_4 levels were significantly decreased in fasted fish at both day 3 and day 7 (Student's t-test, **p<0.01, ***p<0.001). Data are expressed as mean±SEM (n=6).

CHAPTER 7 *IN VITRO* REGULATION OF GROWTH HORMONE RECEPTORS IN THE GOLDFISH

7.1 Introduction

Evidence from *in vivo* experiments (Chapter 6) suggests that GH is involved in the regulation of hepatic GHR in goldfish. *In vitro* studies have revealed that GH induces short-term down-regulation of surface GHR in various types of mammalian cells, including cultured human IM-9 lymphocytes (Rosenfeld & Hinz 1980), mouse fibroblasts (Murphy & Lazarus 1984), rat adipocytes (Roupas & Herington 1986), and Chinese hamster ovary cells (Amit *et al.* 1999). An advantage of using hepatocytes as an *in vitro* model for studies of hepatic physiology and biochemistry is that cultured cells are exposed directly to a reagent at a much lower concentration than with *in vivo* injection where 90% of the reagent is lost by excretion and distribution to tissues other than those under study (Guillouzo & Guguen-Guillouzo 1986).

In rat (Tollet *et al.* 1993) and pig (Brameld *et al.* 1995) hepatocytes, an increase in IGF-I mRNA levels was induced by GH exposure *in vitro*. GH promotion of IGF-I production was also reported for salmon hepatocytes (Duan *et al.* 1993, Shamblott *et al.* 1995). Other hormones such as insulin, PRL and SL were found to have no effect on stimulation of hepatic IGF-I mRNA levels in salmon hepatocytes (Duan *et al.* 1993). These results suggest that hepatic GHR has an important role in mediating GH stimulation of IGF-I production from the liver of teleosts. In the present study, the

effects of GH, PRL, T_3 and IGF-I on the growth of goldfish hepatocytes and the regulation of hepatic GHR were examined *in vitro*.

7.2 Materials and Methods

7.2.1 Experimental animals and preparation of goldfish hepatocytes

Goldfish (common and comet varieties) were maintained as described previously (Chapter 2). All studies were conducted between January and April with reproductively immature fish (50-70 g) of mixed sex. Collection of liver tissue and preparation of viable goldfish hepatocytes are described in Appendix A.

7.2.2 Hormones and reagents

rcGH, bGH, rsbGH, cPRL and rPRL were obtained as described previously (Chapters 2 & 4). Recombinant sea bream IGF-I (rsbIGF-I) was kindly provided by Dr. A. Gertler (The Hebrew University of Jerusalem, Israel). Recombinant human IGF-I (rhIGF-I) was purchased from Bachem Inc (Torrance, CA). T_3, bovine insulin, cell culture reagents, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or BDH Chemicals (Edmonton, AB).

7.2.3 Glucose production by cultured goldfish hepatocytes

7.2.3.1 Influence of epinephrine on freshly isolated cells

Freshly isolated goldfish hepatocytes were placed in sterile 1.5 ml polypropylene vials at a density of 1.16×10^6 cells/vial. The hepatocytes were treated in triplicate with 1 μM epinephrine, 1 μM epinephrine plus 1 μM propranolol, or HEPES buffer alone (control, Appendix A). The hepatocytes were then incubated for 0.5, 1, 2, 4, or 8 h. After incubation, the supernatant was collected by centrifugation. Glucose levels

were determined by the glucose oxidase method (Young *et al.* 1975) using a glucose test kit (Chapter 6).

7.2.3.2 Influence of epinephrine on cultured cells

At 0, 4, 7, 14, or 21 days, goldfish hepatocytes were washed twice with phosphate-buffered saline (PBS; 0.15 M NaCl, 8 mM Na_2HPO_4 and 5 mM EDTA, pH 7.4), and 200 µl of PBS saline with or without 1 µM epinephrine was added to the wells. The hepatocytes were then incubated for 8 h, after which, the supernatant was collected by centrifugation and glucose concentration measured as described above.

7.2.3.3 Influence of rcGH on glucose production

Goldfish hepatocytes cultured for 3 days were treated with various concentrations of rcGH (1, 10, and 100 nM) in triplicate. The treated hepatocytes were then incubated for 1, 4, 8, 16, or 24 h. After incubation, the supernatant was collected by centrifugation and the glucose was measured as described above.

7.2.4 Growth curve of goldfish hepatocytes

Freshly isolated goldfish hepatocytes were seeded in 24-well Primaria plates at a density of 1 x 10^5 cells/well. After cell attachment, the cells were supplemented with medium (DMEM/199 3:1) containing 100 ng rcGH/well or without rcGH. The culture medium was renewed every 2 days. At various time intervals over a 30-day period, the number of viable cells was measured using the MTT assay described previously

(Chapter 4). Population doubling time and level (Freshney 1994) were calculated as follows:

$$\text{Population doubling time} = \frac{\text{Day}_f - \text{Day}_0}{\ln (N_f/N_0)}$$

$$\text{Population doubling level} = \ln (N_f/N_0)$$

where, Day_0 and Day_f represent the start and finish days, respectively, of the exponential cellular growth period. N_0 and N_f represent the number of cells found at the start and finish, respectively, of the exponential growth period.

7.2.5 Influence of various hormones on hepatocyte growth

Freshly isolated goldfish hepatocytes were seeded in 24-well Primaria plates at a density of 1×10^5 cells/well. Cells were supplemented with medium (DMEM/199 3:1) containing various concentrations of rcGH, bGH, rsbGH, cPRL, rPRL, T_3, insulin, rhIGF-I, rsbIGF-I or medium alone (control). The hepatocytes were then incubated for 5 days, with the culture medium renewed every 2 days. At the end of the 5-day incubation period, cell number was determined using the MTT assay described previously (Chapter 4).

7.2.6 *In vitro* GH binding assay

7.2.6.1 Effect of incubation time on ^{125}I-rcGH binding to goldfish hepatocytes

Binding of ^{125}I-rcGH to goldfish hepatocytes cultured for 3 days was measured according to the method of Tollet *et al.* (1993). Binding studies were conducted in triplicate directly in 24-well Primaria plates. Before the addition of radioactive tracer, cells were rinsed once in assay buffer (10 mM phosphate, 150 mM NaCl, 10 mM EDTA, 0.1% BSA at pH 7.4) and preincubated with 400 µl/well assay buffer. After 30 min, 100 µl of assay buffer containing 200,000 cpm ^{125}I-rcGH, with or without 100 ng unlabeled rcGH was added. Cells were further incubated for 0.2, 0.5. 1, 4, or 8 h, after which the assay buffer was removed and cells were washed three times in ice-cold assay buffer. Attached cells were solublized by the addition of 100 µl of 1% (wt/wol) sodium dodecyl sulphate in 0.1 N NaOH, followed by scraping with a rubber stick. The contents of each well were transferred to 12x75 mm glass culture tubes and the radioactivity corresponding to ^{125}I was determined in a gamma counter. TB of ^{125}I-rcGH to the hepatocytes was determined as the total radioactivity in the cells following incubation in the absence of unlabeled rcGH. NSB of ^{125}I-rcGH was determined as the radioactivity present in the cells treated with excess (100 ng/well) unlabeled rcGH. SB of ^{125}I-rcGH was then calculated as the difference between TB and NSB.

7.2.6.2 Scatchard plot

In order to obtain a Scatchard plot (Scatchard 1949), goldfish hepatocytes were incubated with an increasing amount of ^{125}I-rcGH. The amount of specifically bound ^{125}I-rcGH to goldfish hepatocytes and the ratio of specifically bound over free ^{125}I-rcGH (B/F) were then calculated. The fraction of ^{125}I-rcGH that would bind to an infinite receptor concentration was calculated to be 51% of the total radioactivity added. Thus, according to Calvo *et al.* (1983), the calculation of B/F prior to analysis was conducted using only 51% of the total radioactivity added.

7.2.6.3 Internalization of GHR

Bound ^{125}I-rcGH on the cell surface was removed using the method of Haigler *et al.* (1980). Briefly, goldfish hepatocytes cultured for 3 days were rinsed once in assay buffer and preincubated with 400 µl/well assay buffer for 30 min. Incubation of the cells with 100 µl/well of 200,000 cpm ^{125}I-rcGH was performed for 10, 20, 30, 40, or 60 min, after which, the cells were washed three times in ice-cold assay buffer. Surface-bound ^{125}I-rcGH at each incubation time was removed by adding 100 µl of 0.2 M acetic acid containing 0.5 M NaCl (pH 2.5) to each well. The cells were then incubated at room temperature for 10 min. Total binding of ^{125}I-rcGH to the hepatocytes was determined by the radioactivity of cells without acetic acid treatment. The radioactivity of internalized ^{125}I-rcGH was determined using the cells treated with

acetic acid. The radioactivity of surface- bound ^{125}I-rcGH was then calculated as the difference between the radioactivity of total and internalized ^{125}I-rcGH.

7.2.6.4 Effect of rcGH on the in vitro *regulation of GHR*

The effect of unlabeled rcGH on binding of ^{125}I-rcGH to goldfish hepatocytes was determined using cells cultured for 3 days in 35 mm plastic Primaria culture dishes. Cells supplemented with DMEM/199 (3:1) were incubated with (1 μg/dish) or without rcGH for 0.2, 0.5, 2, 4, 8, 12, or 24 h. After incubation, the cells were washed three times in assay buffer, detached by rubber stick and homogenized with a Polytron homogenizer in ice-cold homogenization buffer (100 mM Tris, 150 mM NaCl, 50 mM EGTA, 50 mM EDTA, 300 mM sucrose at pH 9.0). The homogenate was then centrifuged at 100,000-g for 1 h at 4°C. The pellet was collected and suspended in ice-cold suspension buffer (25 mM Tris, 10 mM $MgCl_2$ at pH 7.6), frozen on dry ice, and stored at -20°C. Protein concentration in the microsomal preparation was determined using the method of Lowry *et al.* (1951). The K_a and B_{max} of ^{125}I-rcGH binding to the total binding sites of the hepatocyte microsomes were determined using $MgCl_2$ treated microsomes and the GH receptor binding assay described previously (Chapter 2).

7.2.7 Statistics

All data were expressed as mean ± SEM. The K_a and B_{max} of ^{125}I-rcGH binding to cultured goldfish hepatocytes were determined from the Scatchard plot using the

LIGAND computerized program (Munson & Rodbard 1980). The half-maximal effective dose (ED_{50}) of hormones on hepatocyte growth *in vitro* was estimated using the ALLFIT (De Lean *et al.* 1978) computer program. Other data were analyzed with ANOVA followed by the Student-Newman-Keuls multiple comparison test or Student's t-test ($p<0.05$).

7.3 Results

7.3.1 Glucose production by cultured goldfish hepatocytes

Freshly isolated goldfish hepatocytes released glucose which was further stimulated by the addition of epinephrine (Fig. 7.1). The effect of epinephrine on glucose release was completely inhibited by the ß adrenergic antagonist propranolol (Fig. 7.1). Glucose production declined continuously over time in cultured hepatocytes and the hepatocytes were only responsive to epinephrine for up to 1 week in culture (Fig. 7.1). Administration of rcGH had no effect on glucose production by either freshly isolated cells or cells cultured for various days (Fig. 7.2).

7.3.2 Growth curve of goldfish hepatocytes

Figure 7.3 illustrates a growth curve of goldfish hepatocytes incubated for various times after seeding with or without rcGH. Cultures supplemented with rcGH had a longer lag phase (approximately 6 days) and those cells cultured without rcGH (4 days). The saturation density in cultures without rcGH was reached at 20 days, which was equivalent to 8.06×10^6 cells ml^{-1}. In the cultures without rcGH, the population doubling level and population doubling time were calculated as 4.02 and 53.8 h, respectively. Similar values of saturation density, population doubling level and population doubling time were found in the cultures supplemented with rcGH (Fig. 7.3).

7.3.3 Influence of various hormones on hepatocyte growth

The effects of various hormones on goldfish hepatocyte growth are illustrated in Figure 7.4. All GH displayed an antimitogenic effect on the cultured goldfish hepatocytes. The ED_{50} of the hormone was estimated using the ALLFIT (De Lean *et al.* 1978) computer program. The ED_{50} of rcGH, rsbGH and bGH were 0.11 $\pm$ 0.02 nM, 0.36 $\pm$ 0.04 nM, and 0.38 $\pm$ 0.04 nM, respectively (Fig. 7.4). In contrast, PRL, T_3, insulin, rhIGF-I, and rsbIGF-I had no effect on the growth of cultured goldfish hepatocytes (Fig. 7.4).

7.3.4 *In vitro* GH binding assay

^{125}I-rcGH binding to cultured goldfish hepatocytes was dependent on incubation time (Fig. 7.5). Binding equilibrium was reached 1 h after addition of ^{125}I-rcGH to the hepatocytes (Fig. 7.5). LIGAND analysis revealed a single class of binding site with a K_a of 1.9×10^{10} M^{-1} and B_{max} of 2.0 fmol mg^{-1} protein (Fig. 7.6). Internalization of ^{125}I-rcGH began 10 min after binding to cell surface receptors (Fig. 7.7). Approximately 85% of ^{125}I-rcGH was internalized following 30 min of incubation (Fig. 7.7). Administration of unlabeled rcGH *in vitro* resulted in a rapid down-regulation of total GH binding sites in goldfish hepatocytes (Fig. 7.8). The lowest ^{125}I-rcGH binding to goldfish hepatocytes was reached between 0.5 and 2 h after addition of unlabeled rcGH (Fig. 7.8). After 2 h, binding of ^{125}I-rcGH to goldfish hepatocytes gradually increased and reached control levels by 12 h (Fig. 7.8).

7.4 Discussion

Goldfish hepatocytes cultured *in vitro* displayed proliferation over time, in terms of population doubling time and level, similar to mammalian hepatocytes (Guguen-Guillouzo 1992). Glucose release by hepatocytes in response to epinephrine is used as an indicator of the maintenance hepatocyte function *in vitro* (Birnbaum *et al.* 1976, Guguen-Guillouzo 1992). In the present study, freshly isolated goldfish hepatocytes were very responsive to epinephrine stimulation. The effect of epinephrine was blocked by propranolol, further indicating the maintenance of hepatocyte function *in vitro*. Although goldfish hepatocytes survived for up to 1 month, cells cultured for more than 1 week were not responsive to epinephrine in terms of glucose production. The reason for the loss of responsiveness of the hepatocytes to epinephrine over time remains unclear. However, most primary cultures tend to become dedifferentiated over time *in vitro* (Freshney 1994), and goldfish hepatocytes may also follow this pattern.

Unlike epinephrine, rcGH did not appear to directly stimulate glucose production from cultured goldfish hepatocytes. A similar finding was reported for rat hepatocytes treated with bGH (Blake & Clarke 1989). However, bGH was found to stimulate glucose production by hepatocytes prepared from Hx rats via a possible gluconeogenesis pathway (Blake & Clarke 1989). Previously (Chapter 6), significantly increased serum GH and glucose levels were found to be coincident in fasted goldfish, providing the possibility that GH might be responsible for increased hepatic glucose production during fasting. However, the lack of an effect of GH on glucose production by goldfish hepatocytes *in vitro*, suggests that the GH does not act at the level of the liver to promote glucose release. It is also possible that GH interacts with other

hormones such as insulin at the level of the hepatocyte to influence glucose release, although this was not directly examined in the present study. Further research is needed to clarify the relationship between GH and glucose production in the goldfish, and to determine the exact mechanism by which GH exerts an anti-insulin effect in teleosts (Björnsson 1997). Other possibilities also to be investigated include a reduced rate of glucose utilization *in vivo* (Pereira *et al.* 1995), or increased gluconeogenesis or glycogenolysis by other tissues (Blasco *et al.* 1992a, b, Zhang 1993, Soengas *et al.* 1996).

Although rcGH did not influence glucose release from the goldfish hepatocytes, all GH tested in the present study displayed a clear antimitogenic effect in cultured goldfish hepatocytes. Previous studies have found that GH has an antimitogenic effect on 3T3-F442A preadipocytes (Corin *et al.* 1990, Vashdi *et al.* 1992, Chapter 4). To my knowledge, the antimitogenic effect of GH on mammalian hepatocytes has not been investigated. Thus, the present study provides the first evidence that GH also inhibits the proliferation of hepatocytes and suggests that this effect of GH may be observed in a variety of tissues in vertebrates. T_3, insulin and PRL were found to be unable to alter mitogenesis of goldfish hepatocytes, suggesting that the antimitogenic effect is specific to GH.

A long survival period, up to one month, and proliferation *in vitro* were found in the cultured goldfish hepatocytes. Interestingly, cells treated with rcGH had a longer lag phase prior to undergoing exponential growth than cells treated without rcGH. However, the saturation density and population doubling time and level measured over the period of exponential growth were similar in cultures treated with or without rcGH.

These results also suggest that rcGH has an antimitogenic effect, but only during the early phase of the culture period. The loss of responsiveness of goldfish hepatocytes to rcGH during subsequent stages of cell growth is probably similar to that for epinephrine described above.

Based on the "dual effector" model of GH action, GH treatment stimulates hepatic IGF-I production, which in turn, would promote proliferation of many other cell types (Isaksson *et al.* 1991, Jones & Clemmons 1995, Duan 1998). However, in the present study, adminstration of rhIGF-1 and rsbIGF-I to cultured goldfish hepatocytes did not result in cell proliferation, although rhIGF-I was reported to stimulate proliferation of rat hepatocytes (Kimura & Ogihara 1998). It is not clear why IGF-I is unable to promote proliferation of cultured goldfish hepatocytes. Goldfish cartilage tissue has been reported to be responsive to IGF-I *in vitro* (Moroz 1995). These results suggest that IGF-I action in terms of cell proliferation may be cell or tissue specific.

In addition to rcGH, rsbGH and bGH were found to have an antimitogenic effect on cultured goldfish hepatocytes. However, the ED_{50} values of rsbGH and bGH were much higher than that of rcGH. These results suggest that among the GH examined, rcGH has the highest biological activity. The higher activity of rcGH in goldfish hepatocytes may be attributed to the higher affinity of rcGH for the goldfish hepatic GHR, compared to rsbGH and bGH (Chapters 2 & 4).

LIGAND analysis of ^{125}I-rcGH binding to goldfish hepatocytes indicated a single class of high affinity and low-capacity binding sites with a K_a of 1.9×10^{10} M^{-1} and B_{max} of 2.0 fmol mg^{-1} protein. A similar K_a value was reported for ^{125}I-rcGH binding

to goldfish liver membranes (Chapter 2). The B_{max} (9 fmol mg^{-1} protein) of goldfish liver membranes (Chapter 2) was slightly higher than that of goldfish hepatocytes. The total number of GHR in an individual goldfish hepatocyte was found to be approximately 2900 when calculated using B_{max} of 2.0 fmol mg^{-1} and protein concentration of 2.5 mg/10^6 cells. A similar number of GHR per cell has been reported for rat hepatocytes (Donner *et al.* 1978, Tollet *et al.* 1993).

The number of surface GHR in goldfish hepatocytes decreased shortly after GH binding, indicating that ^{125}I-rcGH initially bound to a surface receptor with the GH-GHR complex subsequently undergoing rapid internalization. Similar findings were reported for human IM-9 lymphocytes (Haigler *et al.* 1980) and Chinese hamster ovary cells (Amit *et al.* 1999). Recent studies on the three-dimensional structure of hGH-hGHR has shown that binding of hGH to hGHR induces receptor dimerization (de Vos *et al.* 1992). Receptor dimerization is an essential step for internalization of GH, and the induction of a biological response (Goffin *et al.* 1998).

In vitro administration of rcGH to goldfish hepatocytes resulted in a significant decrease in the SB of hepatocyte microsomes, indicating a down-regulation of hepatocyte GHR by GH. A similar effect of GH on GHR was also found in goldfish liver membranes during *in vivo* studies (Chapter 6). Down-regulation of GHR may involve dimerization of GHR, increased cellular internalization and degradation of the GH-GHR complex, decreased recycling or *de novo* synthesis of GHR (Goffin *et al.* 1998). Further studies are needed to determine the exact mechanism of GHR down-regulation induced by GH in goldfish.

The present study is the first time that the effects of GH on hepatocyte proliferation and on hepatocyte GHR *in vitro* have been studied in a teleost. The advantages of *in vitro* over *in vivo* studies are many. First, *in vitro* techniques avoid potential influences of animal handling such as hypophysectomy and intraperitoneal injection. Second, an *in vitro* assay to study the biological effects of GH is more rapid and sensitive than an *in vivo* assay. For example, goldfish hepatocytes were found to be responsive to rcGH at concentrations of less than 10 ng/well whereas 1 $\mu g\ g^{-1}$ or more of rcGH needs to be administrated *in vivo* in order to induce a biological response (Chapter 6). Finally, this *in vitro* study provides evidence of GHR internalization following GH binding that cannot be determined by *in vivo* experiments. Together, results of this study indicate that the cultured hepatocytes will be a very useful model in future studies of the GH-GHR interaction in the goldfish.

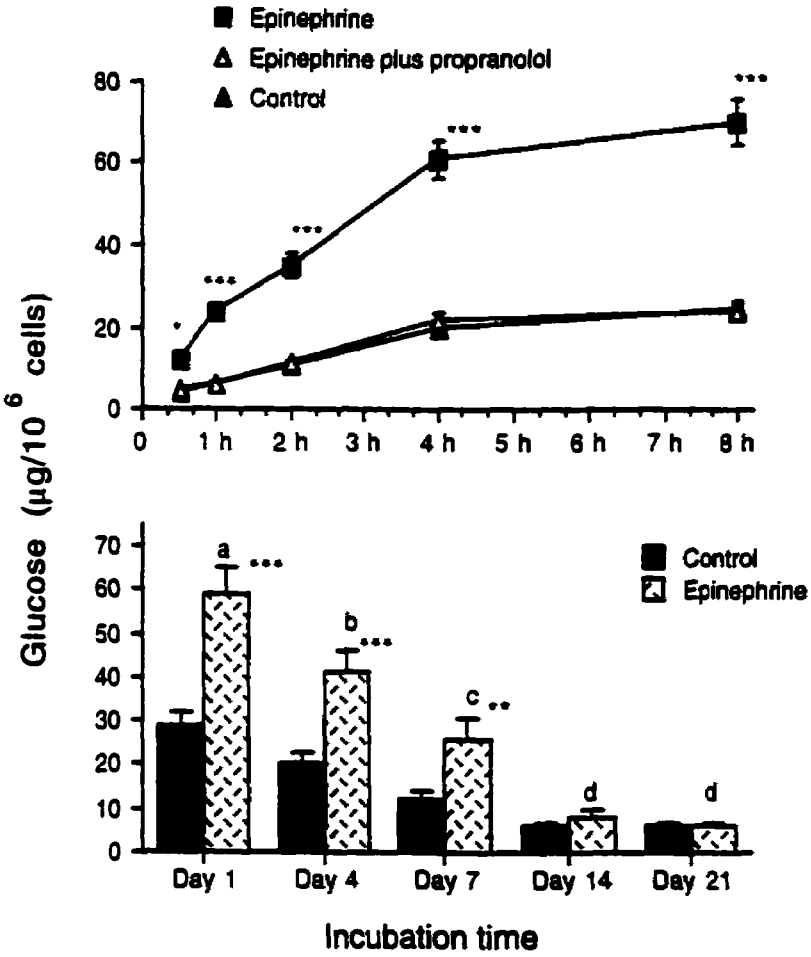

Figure 7.1 Effects of epinephrine on glucose production by freshly isolated goldfish hepatocytes (top panel) or cells cultured for various days (bottom panel). The amount of glucose produced by goldfish hepatocytes was significantly increased over time by 1 µM epinephrine (top panel, Student's t-test, *$p<0.05$, ***$p<0.001$). The effect of epinephrine was completely inhibited by addition of 1 µM of propranolol to the cultures (top panel). Significantly higher glucose levels resulting from epinephrine stimulation were found at day 1, day 4, and day 7 in cultured hepatocytes (bottom panel, Student's t-test, *$p<0.05$, ***$p<0.001$). Data were subjected to the Student-Newman-Keuls multiple comparison test ($p<0.05$); groups with the same superscript letter displayed a similar glucose level. Data, expressed as mean±SEM, were obtained from three experiments, each carried out in triplicate. All controls were treated with HEPES buffer only.

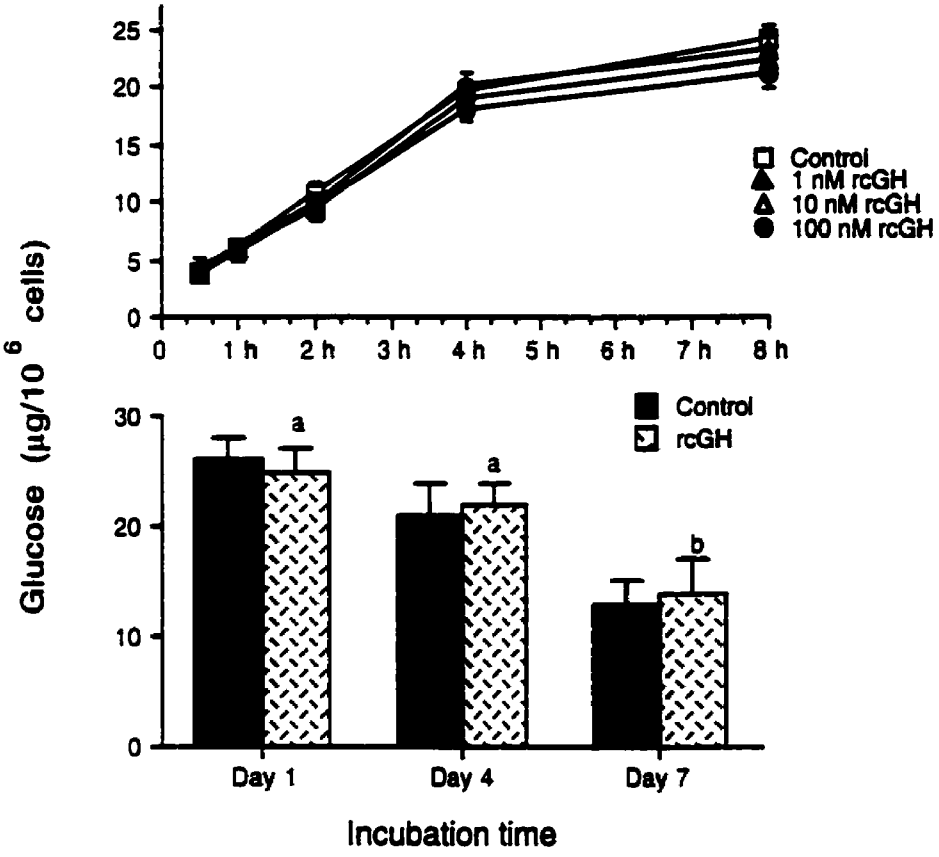

Figure 7.2 Effect of rcGH on glucose production by freshly isolated goldfish hepatocytes (top panel) and cells cultured for various days (bottom panel). The amount of glucose produced by goldfish hepatocytes was not altered over time by addition of rcGH. Data, expressed as mean±SEM, were obtained from three experiments, each carried out in triplicate. Data were subjected to the Student-Newman-Keuls multiple comparison test ($p<0.05$); groups with the same superscript letter displayed a similar glucose level. All controls were treated with medium only.

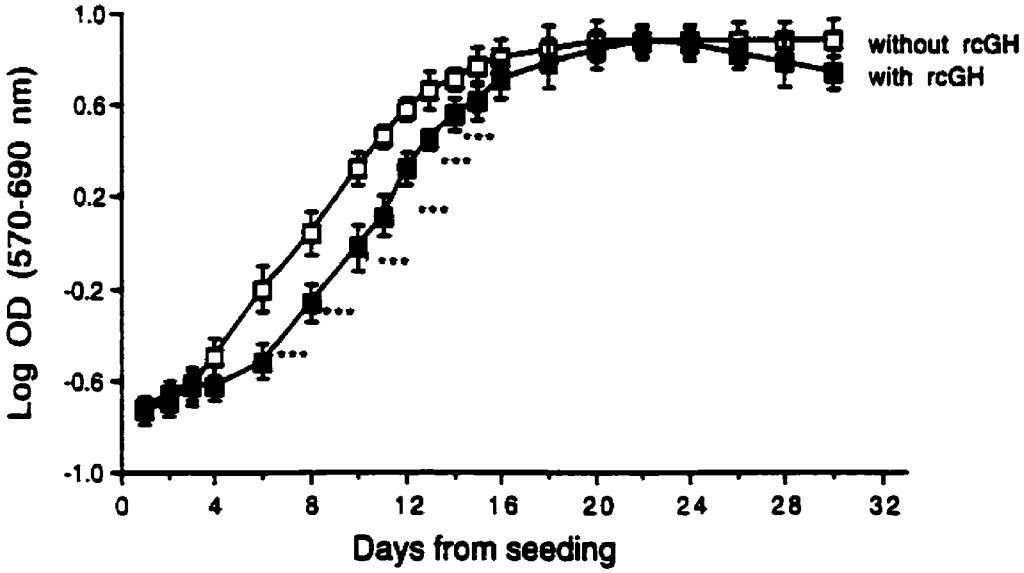

Figure 7.3 Growth curve of cultured goldfish hepatocytes. The cells were supplemented with DMEM/199 (3:1) containing rcGH (100 ng/well) or medium only. Incubation was performed for various days. The number of cells was determined using the MTT assay. The number of cells in culture between day 5 and day 13 without rcGH was significantly higher than that of cells treated with rcGH (Student's t-test, ***$p<0.001$). Data, expressed as mean±SEM, were obtained from three experiments, each carried out in triplicate.

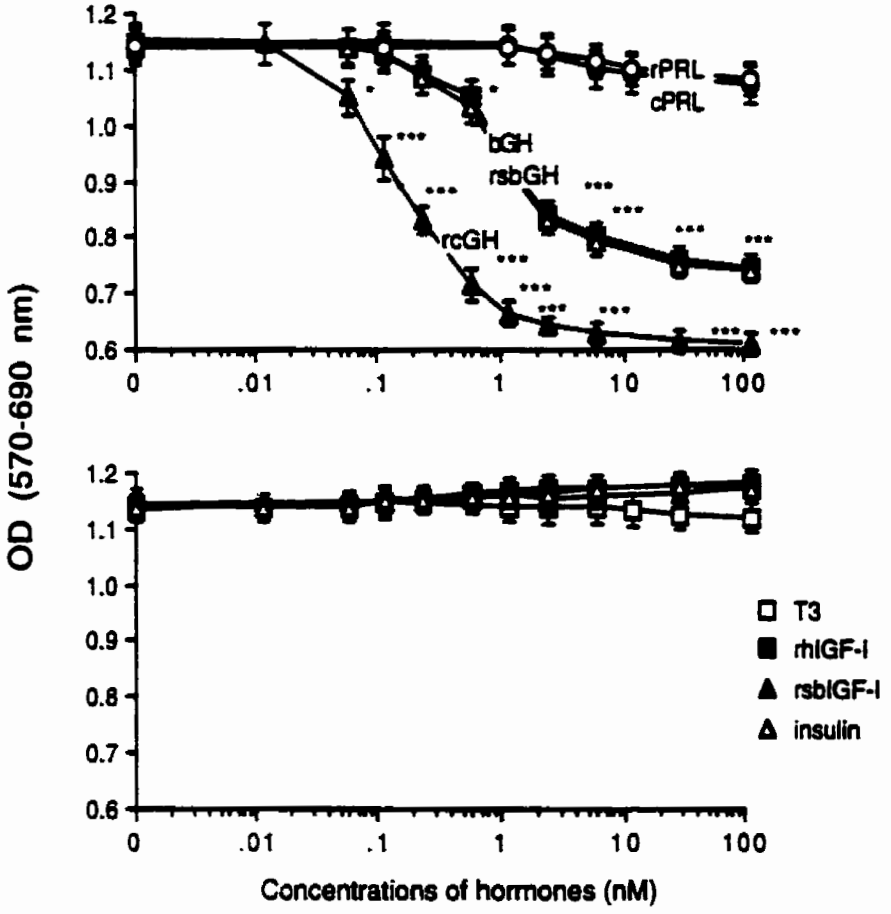

Figure 7.4 Effects of rcGH, bGH, rsbGH, cPRL and rPRL (top panel) and insulin, T_3, rhIGF-I, and rsbIGF-I (bottom panel) on *in vitro* growth of goldfish hepatocytes. At 5 days, cell number was determined using the MTT assay. All GH had antimitogenic effect on goldfish hepatocytes (top panel, Student's t-test, *$p<0.05$, ***$p<0.001$) whereas prolactins, T_3, rhIGF-I, rsbIGF-I, and insulin had little effect on cell growth. Data are presented as mean±SEM from three different experiments, each carried out in triplicate.

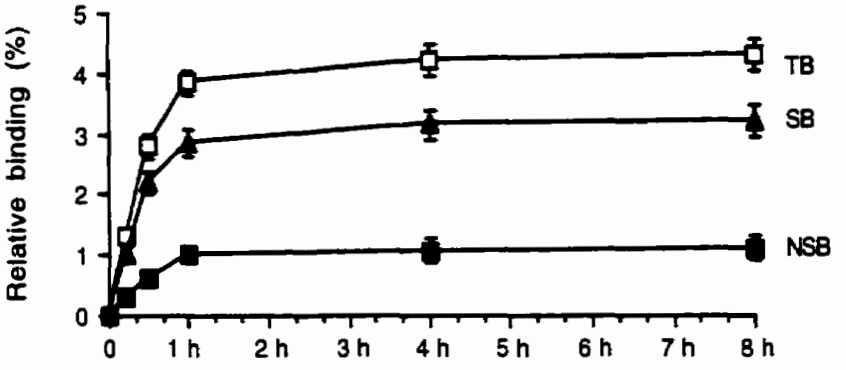

Figure 7.5 Total (TB), specific (SB) and non-specific (NSB) binding of ^{125}I-rcGH to cultured goldfish hepatocytes over time. Data are presented as mean±SEM from three different experiments, each carried out in triplicate.

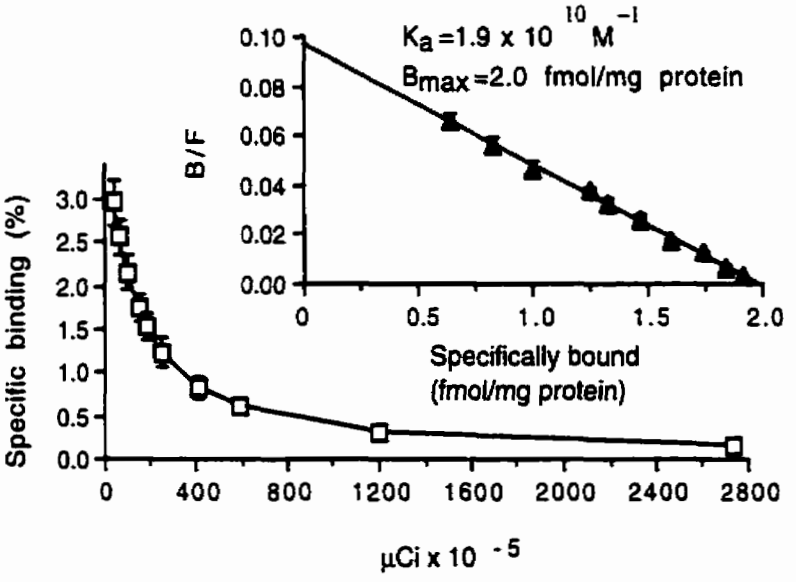

Figure 7.6 Displacement curve produced by incubating increasing amounts of ^{125}I-rcGH with cultured goldfish hepatocytes. Data are presented as mean±SEM from three different experiments, each carried out in triplicate. The inset graph represents the derived Scatchard plot. K_a and B_{max} were determined by LIGAND analysis of this plot.

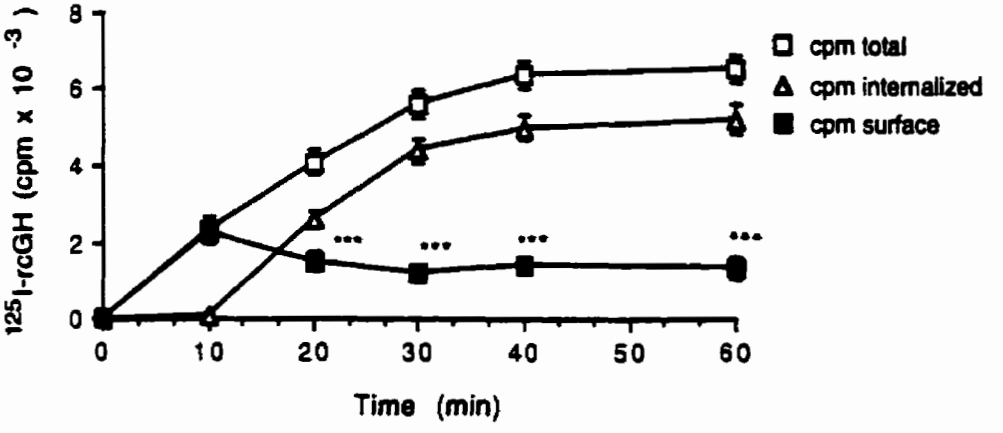

Figure 7.7 Binding of ^{125}I-rcGH to surface and internalized receptors on goldfish hepatocytes *in vitro*. ^{125}I-rcGH began to be internalized 10 min after addition of ^{125}I-rcGH. After 20 min of incubation, surface GHR number was significantly decreased compared to total ^{125}I-rcGH binding (Student's t-test, ***$p<0.001$). Data, expressed as mean±SEM, were obtained from three experiments, each carried out in triplicate.

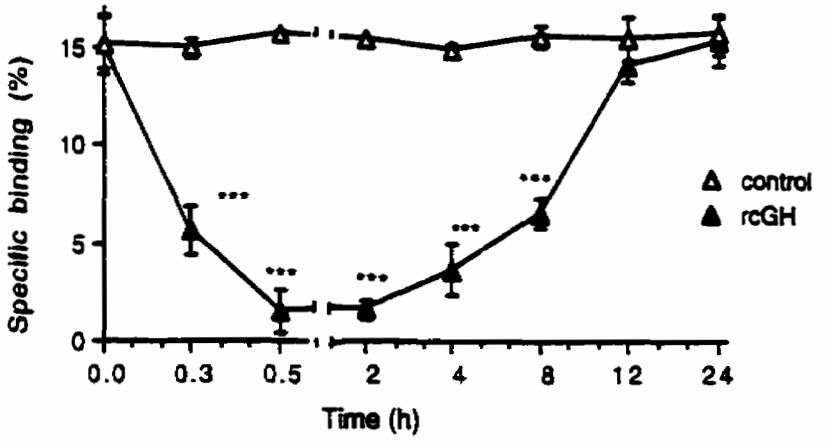

Figure 7.8 Effects of unlabeled rcGH over time on specific binding of ^{125}I-rcGH to goldfish hepatocyte microsomes. Data, expressed as mean±SEM, were obtained from three experiments, each carried out in triplicate (Student's t-test, ***, significantly different at the $p<0.001$ level compared to control).

CHAPTER 8 GENERAL DISCUSSION

Body growth in vertebrates ultimately results from cartilage growth and expansion, bone deposition, and the proliferation and differentiation of other somatic cells (Goffin *et al.* 1998). The endocrine regulation of somatic growth has been extensively studied in mammals (for review see Chapter 1); the major components of this endocrine pathway are summarized in Figure 8.1. According to this endocrine pathway, GH is secreted by the anterior pituitary into the circulation in response to stimulatory and inhibitory signals from the hypothalamus or peripheral feedback mechanisms. Circulating GH acts via hepatic GHR to stimulate the production and release of IGF-I, which then travels via the circulation to interact with its receptors in target tissues and promote cellular changes associated with somatic growth. GH also acts via GHR in other target tissues to produce IGF-I locally. The local IGF-I acts to stimulate somatic growth in mammals through a paracrine or autocrine mechanism. The interactions between GH and IGF-I described above are known as the "dual effector" model of GH action (Isaksson *et al.* 1987, Jones & Clemmons 1995). Both GH and IGF-I are present in the circulation complexed with specific binding proteins. These binding proteins act to reduce the clearance rate of the hormones and serve as a hormone reservoir in the circulation. The liver is the major source of circulating GHBP. Other variables such as circulating thyroid hormone levels and nutrition also have a profound influence on somatic growth. Thus, knowledge of changes that occur

simultaneously in each of these major components is needed to fully understand the endocrine regulation of somatic growth.

Several lines of evidence suggest that the endocrine regulation of somatic growth in teleosts is similar to that of mammals (Peter & Marchant 1995, Peng & Peter 1997). For example, studies in Japanese eel (Duan & Hirano 1990), coho salmon (McCormick *et al.* 1992, Tsai *et al.* 1995), long-jawed mudsucker (Gray & Kelley 1991), goldfish (Marchant & Moroz 1993), and common carp (Cheng & Chen 1995) indicate that IGF-I mediates at least some of the growth-promoting effects of GH. In addition to GH and IGF-I, thyroid hormones have been found to play a permissive role in somatic growth of teleosts (Eales 1988, Leatherland 1994). External factors which influence somatic growth in teleosts include nutrition (Pérez-Sánchez *et al.* 1994, Chapter 6), temperature (Marchant & Peter 1986, Marchant *et al.* 1989, Björnsson 1997) and photoperiod (Marchant & Peter 1986, Marchant *et al.* 1986, Björnsson *et al.* 1994). Although the mammalian endocrine model may be generally applicable, there is a lack of information about the nature and role of GHR in teleosts, and very little information is available about the relationship between teleost GHR and GHBP. The purpose of my research was to investigate more fully the involvement of these components in the endocrine regulation of somatic growth in the goldfish.

In order to study GHR in the goldfish, a sensitive GH receptor binding assay had to be established. I accomplished this task, as described in Chapter 2, through the use of very pure rcGH and the optimization of assay conditions, including a careful choice of enzyme inhibitors for microsomal preparation, buffer pH and composition, and incubation temperature. The findings presented in Chapter 2 represent the first time

that a GH receptor binding assay in a cyprinid species has been fully characterized. This GH receptor binding assay then allowed me to study the biochemical nature of goldfish GHR (Chapter 3), identify serum GHBP in the goldfish (Chapter 5), examine the *in vivo* (Chapter 6) and *in vitro* (Chapter 7) regulation of goldfish GHR, and compare binding characteristics between GH and GHR from various species (Chapter 4).

Goldfish GHR was found to be present as multiple forms following SDS-PAGE analysis of hepatic membranes (Chapter 3). Covalent cross-linking of rcGH to goldfish liver membrane proteins resulted in three specifically labeled bands, with M_r of 88, 142, and >200 KD (Chapter 3). The presence of reducing agents such as ß-mercaptoethanol or DTT did not alter the M_r of the bands. Similar findings were reported for other teleosts (Ng *et al.* 1991, Gray & Tsai 1994) and mammals (Hughes *et al.* 1983, Smith & Talamantes 1987, Ymer & Herington 1987, Husman *et al.* 1988, Orian *et al.* 1991), suggesting that the various bands observed following SDS-PAGE are not simply GH receptor subunits linked through disulfide bonds. Rather, the 88, 142, and > 200 KD bands observed in my study in goldfish appear to represent various GH-GHR complexes formed during GH binding and subsequent GHR dimerization.

The existence of specific GHBP in goldfish serum and cultured hepatocytes was confirmed by rcGH binding and ligand blotting studies (Chapter 5). To my knowledge there has only been one other study on GHBP in a teleost, the rainbow trout (Sohm *et al.* 1998). However, rainbow trout GHBP was found to bind to a preparation of GH purified from rainbow trout pituitaries with relatively low affinity. In contrast, goldfish GHBP displayed very high affinity and low capacity binding for rcGH

(Chapter 5). In goldfish, the affinity of the serum GHBP (Chapter 5) and liver membrane GHR (Chapter 2) for rcGH are very similar. This implies a close relationship between goldfish serum GHBP and the liver membrane GHR. Ligand blotting experiments indicated that goldfish GHBP was present as multiple forms in serum, and that small M_r GHBP were released from cultured goldfish hepatocytes, possibly via a mechanism involving proteolytic cleavage of membrane GHR (Chapter 5). Thus, the goldfish GHBP resembles GHBP from other vertebrates by displaying a high affinity and low-capacity binding for GH, presence of multiple forms of GHBP in the circulation, and a close relationship with liver membrane GHR.

In goldfish, GH was found to play an important role in regulating hepatic GHR (Chapter 6 & 7). Evidence from hypophysectomized goldfish indicated a down-regulation of hepatic GHR when the animals received repeated rcGH injections at 24 h intervals and were sampled 24 h after the last injection (Chapter 6). A similar GH-induced down-regulation of hepatic GHR has been reported in coho salmon (Gray *et al.* 1990, 1992) and long-jawed mudsucker (Gray & Kelley 1991). In contrast, administration of exogenous GH led to an up-regulation of hepatic GHR in Japanese eel (Mori *et al.* 1992). Several lines of evidence indicate that hypophysectomy reduces the number of total and free GH binding sites in mammals (Posner *et al.* 1980, Baxter & Zaltsman 1984, Sanchez-Jimenez *et al.* 1990) and teleosts (Gray & Kelley 1991, Mori *et al.* 1992). In the present study, hypophysectomy was found to induce an up-regulation of hepatic GHR in the goldfish (Chapter 6). These findings suggest that the role of GH in the regulation of its own receptors may vary according to species or with

experimental design, such as the use of Hx or intact animals or the frequency and duration of GH injection.

The role of GH in the regulation of its own receptors was also studied *in vitro* with goldfish hepatocytes (Chapter 7). Administration of rcGH to cultured goldfish hepatocytes induced a short-term down-regulation of cell surface GHR (Chapter 7) similar to that found in intact goldfish injected with rcGH (Chapter 6). In both *in vitro* and *in vivo* situations, the number of GHR recovered to the original level 12 h after GH treatment (Chapters 6 & 7). The acute down regulation of the GHR may involve multiple rapid and complex pathways, including dimerization of GHR, cellular internalization of the GH-GHR complex, and cellular degradation, recycling, or synthesis of GHR (Goffin *et al.* 1998).

Interactions between GH, GHR, GHBP, thyroid hormones, and nutrition in the regulation of somatic growth in goldfish were further studied in fasted animals. Food deprivation resulted in a significant decrease in somatic growth in goldfish (Chapter 6). The loss of tissue weight in fasted goldfish reflects a general pattern reported for other teleost species (Mommsen & Plisetskaya 1991). The significant reduction in the liver-somatic index during fasting appears to be caused by preferential utilization of liver components such as glycogen and lipid as an energy source in fasted teleosts (Pereira *et al.* 1995). Serum glucose concentrations are usually maintained in fasted fish either by reducing the rate of glucose use, increased gluconeogenesis, or increased tissue glycogen breakdown (Pereira *et al.* 1995). There is extensive evidence indicating that tissue glycogen is rapidly converted to glucose during the first week of fasting in some

teleosts including carp (Blasco *et al.* 1992a, b), dab (Zhang 1993), and Atlantic salmon (Soengas *et al.* 1996).

Evidence from mammals (Scanlon *et al.* 1996) and other teleosts (Björnsson 1997) indicates that GH has an anti-insulin-like effect and thus, may be responsible for the elevated serum glucose levels observed in the present study (Chapter 6). However, the exact mechanism by which GH alters blood glucose remains to be determined, as *in vitro* experiments (Chapter 7) found that GH did not directly stimulate glucose production from cultured goldfish hepatocytes. A similar finding was reported for rat hepatocytes treated with bGH (Blake & Clarke 1989), although bGH was found to stimulate glucose production by hepatocytes prepared from Hx rats, possibly via a gluconeogenesis pathway (Blake & Clarke 1989). In my study (Chapter 6), increased serum glucose levels were coincident with an elevation of serum GH levels in fasted goldfish. High levels of serum GH in fasted mammals (Ray & Melmed 1997) and teleosts (Björnsson 1997, Duan 1998) may be caused by a significant reduction in serum IGF-I which serves to regulate GH secretion via a negative feedback mechanism. However, other authors have proposed that an elevation in serum GH level during fasting may be a result of down-regulation of GHR in target tissues (Sumpter *et al.* 1991) or an artifact related to a metabolic role for GH (Leatherland & Farbridge 1992). Further study is obviously needed to clarify the mechanisms responsible for the elevation of serum GH level in fasted teleosts and to determine the precise physiological role for the elevated GH levels during fasting.

Food deprivation also resulted in a significant decrease in hepatic GHR, serum GHBP and T_4 levels, but not serum T_3 (Chapter 6). A significant finding from these

experiments is that the number of hepatic GHR correlates strongly with serum GHBP levels in goldfish, the first time that has been observed in a teleost. The decrease in serum T_4 levels suggest a suppression of the pituitary-thyroidal axis in fasted goldfish. Together, these results suggest that somatic growth of goldfish is determined not only by GH but also by other major regulators such as hepatic GHR, serum GHBP and thyroid hormones, and that the fasting goldfish is a good model to study interactions between these components.

The effects of GH and various other hormones on cell growth *in vitro* were studied using 3T3-F442A preadipocytes and goldfish hepatocytes. GH displayed an antimitogenic effect in 3T3-F442A preadipocytes (Chapter 4) and goldfish hepatocytes (Chapter 7). The antimitogenic effect of GH on the preadipocytes is related to cellular differentiation induced by GH treatment (Corin *et al.* 1990). Preadipocytes treated with GH exit the cell cycle at a discrete point in G_1 to enter a quiescent state, and do not proliferate further. The antimitogenic effect of GH observed in goldfish hepatocytes provides the possibility that a similar mechanism may be involved in GH actions in goldfish hepatocytes.

Perhaps the most novel finding in this thesis is that teleost GH highly cross-reacts with mammalian GHR (Chapter 4) and GHBP (Chapter 5), and that rcGH and rtGH show a biological activity equivalent to bGH in a mammalian *in vitro* GH bioassay (Chapter 4). The concept that fish GH is inactive in mammals was first proposed by Pickford (1957). In the 1950s, shortly after the production of the first purified bGH preparation (Li *et al.* 1945), Pickford used the ethanol precipitation method to purify GH from a variety of teleosts and tested the biological activities of

these GH (Pickford 1957). They found that the teleost GH preparations were inactive in mammals, whereas mammalian GH preparations were biologically active in teleosts. Later, Hayashida (1975) provided extensive immunological evidence indicating that teleost GH was structurally different from mammalian GH. Thus, teleost GH has traditionally been considered to be divergent from the main line of vertebrate GH evolution in terms of its structure-activity characteristics (Hayashida 1975, Nicoll *et al.* 1986, 1987).

Why can the concept that fish GH is inactive in mammals survive for almost half a century? First, there was little pure teleost GH available in the 1950s. All teleost GH preparations used at that time were crude pituitary extracts or were prepared using an ethanol precipitation method (Pickford 1957). Most of the immunological studies on the species-specificity of GH were performed using similar GH preparations (Hayashida 1975). Second, evidence obtained with antisera does not necessarily provide an accurate indication of structural differences that relate to receptor binding. Third, the relatively low sensitivity of the killifish (*Fundulus heteroclitus*) (Pickford 1957) and rat tibia (Hayashida 1975) bioassays requires that large amounts of GH are used to induce a biological effect *in vivo*. For example, in order to stimulate growth of killifish, fish were injected with bGH every week at 30 μg/g body weight for up to 28 days at 20°C (Pickford 1957). In contrast, less than 1 μg of GH is needed to induce an effect in the 3T3-F442A bioassay (Corin *et al.* 1990, Chapter 3) and goldfish hepatocytes (Chapter 6). Thus, the concept that fish GH is inactive in mammals was established based on cross-reactivity studies using impure GH preparations, imprecise immunological information, and insensitive bioassays.

Since the late 1970s, there has been some indication that teleost GH may be active in mammals. For example, GH from tilapia (Farmer *et al.* 1976) and chum salmon (*O. keta*) (Wagner *et al.* 1985) were found to be active in the rat tibia bioassay. Mammalian GH appeared to be more potent than tilapia or chum salmon GH, although the dose response relationship between mammalian and teleost GH was not fully investigated in these studies (Farmer *et al.* 1976, Wagner *et al.* 1985). In spite of these earlier findings, however, it appears no comprehensive study was conducted to verify these results or determine the species-specificity of GH from other teleosts. Results from my research clearly indicate that broad conclusions about species-specificity of teleost GH are not justifiable, and that the structure-activity relationship of GH from each teleost should be examined on an individual basis.

In order to fully understand the molecular basis for my findings on the species-specificity of the interaction between GH and GHR, detailed structural information about GH and its receptor is needed. Recent findings on the crystallization of the dimerized hGH-hGHBP complex have provided the molecular basis for interactions between hGH and hGHR/hGHBP (de *Vos et al.* 1992, Wells & de Vos 1996, Clackson *et al.* 1998). Such studies have made it possible for identification of individual amino acid interactions between the hormone and receptor that are responsible for binding affinity and the species-specificity of hGH.

hGH binds to its receptor in a 1:2 ratio through two regions in hGH called binding sites 1 and 2. Binding site 1 is formed by a pocket of amino acids in helix IV and the loop between helix II and III of hGH, whereas binding site 2 is formed by amino acids in helix I and III on the opposite side of the hormone. Binding site 1 in hGH consists of

amino acids in positions 60 to 68 (loop) and 168 to 179 (helix IV) (Clackson *et al.* 1998). According to Wells and de Vos (1996), binding site 2 in hGH consists of amino acids in positions 12 to 16 (helix I) and 115 to 120 (helix III). The binding affinity of hGH-hGHR is determined by aliphatic-aromatic stacking interactions between two tryptophan residues (Trp-104 and Trp-169) on hGHR and the residues forming the two binding sites on hGH (Clackson *et al.* 1998).

If these findings for hGH-hGHR are generally applicable to GH from other species, the molecular basis for my finding of a high affinity between teleost GH and mammalian GHR may be due to similarities between mammalian and teleost GH in residues 60 to 68 and 168 to 179 for binding site 1 and residues 12 to 16 and 115 to 120 for binding site 2. A comparison of the sequences of GH from several teleost and mammalian species in these key regions is shown in Figure 8.2. Within the 32 amino acid residues forming binding site 1 and 2, there are only 2 conserved substitutions in rat and rabbit GH compared to bGH, whereas hGH differs significantly from bGH by displaying 3 non-conserved and 9 conserved substitutions (Fig. 8.2). Comparison of bGH with goldfish and common carp GH reveals only 1 residue in sites 1 and 2 residues in site 2 that are non-conserved substitutions, although one of these substitutions (Val-173) is also found in hGH. In contrast, rainbow trout GH and gilthead seabream GH contain 9 non-conserved and 4 and 5 conserved amino acid substitutions in binding site 1 and 2, respectively, compared to those of bGH. Although the sea bream GH matches well with rainbow trout GH in both site 1 and 2, GH from gilthead seabream is a shorter molecule and lacks several residues immediately adjacent to helix III in binding site 2 (Funkenstein *et al.* 1991).

My results (Chapter 4) strongly support a hypothesis that the cross-reactivity of teleost GH with mammalian GHR is related to structural similarities of the teleost GH to mammalian GH. Both rcGH and rtGH were found to highly cross-react with rabbit and rat hepatic GHR and the biological activities of rcGH and rtGH were equivalent to bGH in the 3T3-F442A bioassay. However, rcGH displayed a higher K_a than rtGH in rabbit and rat GH receptor binding assays (Chapter 4). This suggests that rcGH is a slightly better agonist for these receptors, although rtGH was equivalent to rcGH in the bioassay. Discrepancies between receptor binding and bioactivity have been reported for other GH molecules (Vashdi *et al.* 1991) and are probably related to the hypothesis that only partial occupancy of GHR is required to elicit a full biological response. rsbGH was distinct from rcGH and rtGH in that rsbGH displayed little cross-reactivity with rabbit and rat hepatic GHR and a very low bioactivity in the 3T3-F442A bioassay. These results support my hypothesis that the cross-reactivity of teleost GH with mammalian GHR depends on the structural similarities between mammalian GH and teleost GH, especially in regions corresponding to binding site 1 and 2 of hGH.

A recent study found that teleost GH genes display a pattern of structural divergence related to teleost phylogenetic classification (Venkatesh & Brenner 1997). Within the subdivision Euteleostei, carp and goldfish belong to the superorder Ostariophysi, rainbow trout belongs to the superorder Protacanthopterygii, whereas gilthead seabream is a member of the superorder Acanthopterygii (Nelson 1994). Among Euteleostei, Acanthopterygii is the most divergent from the main line of vertebrate evolution. In my study, GH from the common carp highly cross-reacted

with mammalian GHR whereas GH from gilthead seabream displayed the lowest cross-reactivity with mammalian GHR. Thus, GH from teleost groups closer to the main line of vertebrate evolution appear to have higher cross-reactivity with mammalian GHR. This suggests that the pattern of cross-reactivity of teleost GH with mammalian GHR is also closely related to teleost phylogenetic classification.

Historically, the molecular basis for species-specificity of vertebrate GH-GHR interactions has been difficult to determine (Nicoll *et al.* 1986). It is now clear, however, that only a few residues in GH and GHR determine binding affinity and compatibility is needed within these contact residues between GH and GHR (Clackson *et al.* 1998). Recent evidence suggests that the low cross-reactivity of non-primate GH with hGHR results from the incompatibility of Arg-43 in the hGHR with His-171 in non-primate GH (Souza *et al.* 1995, Goodman *et al.* 1996, Behncken *et al.* 1997). Thus, changes in a single amino acid pair in hGH and hGHR confers a very high degree of species-specificity in this interaction.

In Chapter 4, rtGH was found to highly cross-react with goldfish GHR, whereas rsbGH, bGH and rhGH displayed little binding with goldfish GHR. In contrast, binding of all GH, except rtGH, to hepatic GHR from rainbow trout was too low to allow for LIGAND analysis. These results demonstrate that rainbow trout and, to a lesser extent, goldfish GHR, have very specific molecular requirements for GH binding. Although rcGH differs from rtGH within binding site 1 and 2 (Fig. 8.2) and other regions of the molecules (Koren *et al.* 1989), the precise molecular basis for the different pattern of hormone cross-reactivity observed with trout and goldfish GHR is not immediately evident. Additional information about the residues making contacts in

the teleost GH and GHR interaction will be required. To my knowledge, the structure of the GHR in any teleosts has not yet been determined; this information will be needed to fully understand the molecular basis for my findings.

Two distinct GH molecules encoded by two separate genes have been reported in carp (Chao *et al.* 1989, Chiou *et al.* 1990), goldfish (Law *et al.* 1996), and rainbow trout (Agellon *et al.* 1988). There are no differences in the amino acid residues in binding sites 1 and 2 between goldfish GH-I and GH-II (Fig. 8.2). Thus, the two GH variants in goldfish are likely to have identical receptor binding activity. There is only one conserved substitution at position 62 between common carp GH-I and GH-II, and these two variants should also display similar receptor binding. Overall, carp and goldfish GH are very similar to each other structurally and are likely to interact with receptors in a very similar manner. The two trout GH variants have been found to exhibit different biochemical and immunological characteristics in SDS-PAGE and RIA, but displayed similar receptor binding affinities (Björnsson 1997). Thus, the sequence variation in sites 1 and 2 of the trout GH also does not appear to result in a difference between rainbow trout GH-I and GH-II in terms of binding affinity.

In summary, my thesis provides new evidence about the characteristics of goldfish GHR, the differences and similarities between goldfish and mammalian GHR, the interactions of hepatic GHR with other components important in somatic growth, and the regulation of the hepatic GHR in goldfish. Together, these results suggest that the goldfish is a useful model to study the roles of GH, GHR and GHBP in endocrine regulation of somatic growth in teleosts. A significant finding also presented in this thesis is that GH from at least two teleosts highly cross-reacts with mammalian GHR

and GHBP. This finding challenges the long held dogma that fish GH is inactive in mammals (Pickford 1957, Hayashida 1975, Tarpey & Nicoll 1985, LeBail *et al.* 1989, Goodman *et al.* 1996) and I propose a new hypothesis to better explain the species-specificity of GH-GHR interactions in vertebrates.

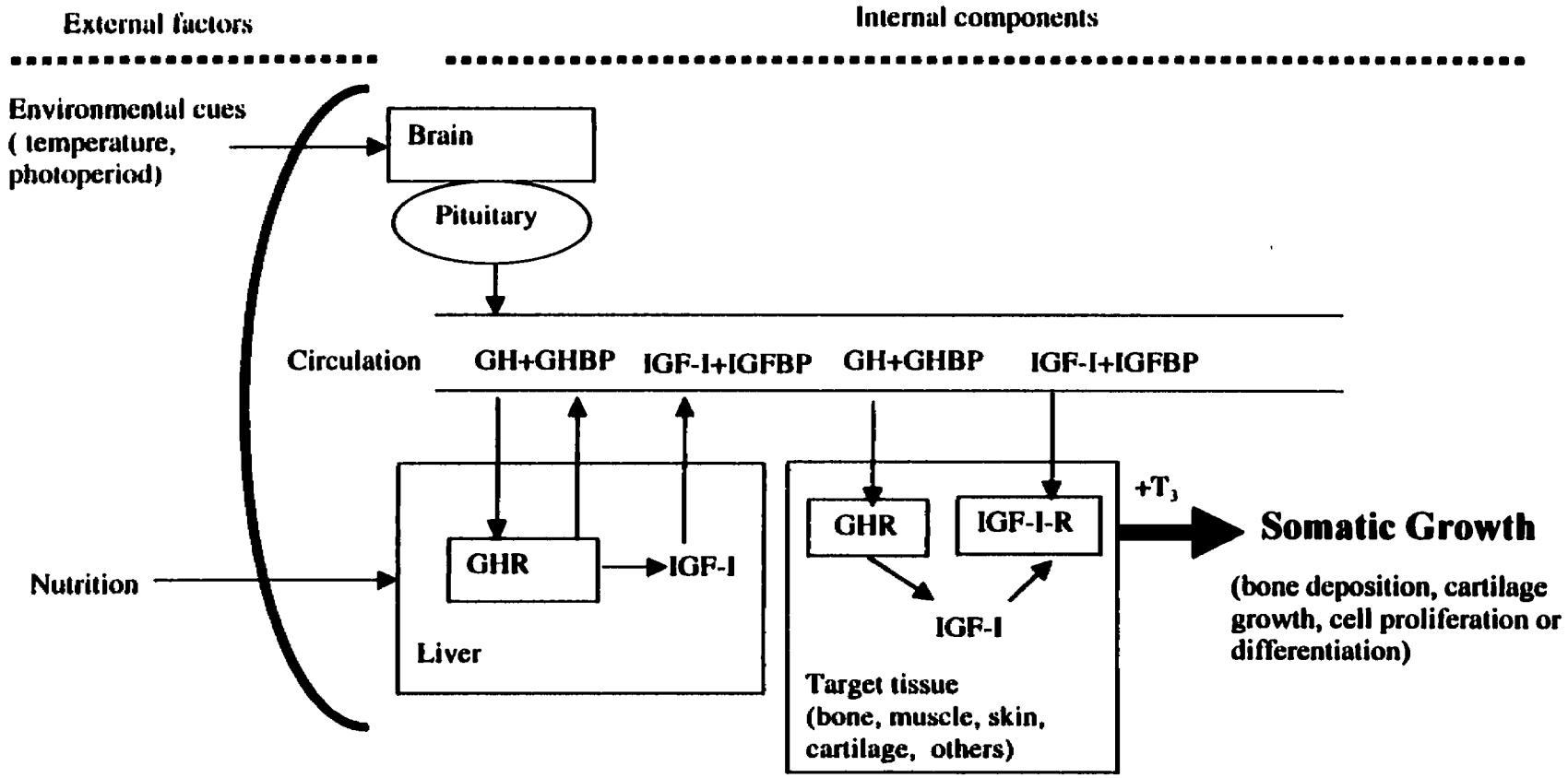

Figure 8.1 Major endocrine pathways involved in the regulation of somatic growth in vertebrates.

	Binding site 1				Binding site 2			
	60	68	168	179	12	16	115	120
Bovine	APTGKNEAQ		KDLHKTETYLRV		NAVLR		KDLEEG	
Human	t-sNre-t-		--mD-V--f--i		--m--		------	
Rat	-----e---		-----a------		-----		------	
Rabbit	-----d---		-----a------		-----		------	
Common carp-I	--a--d-t-		--m--V------		---i-		A--kM-	
Common carp-II	-----d-t-		--m--V------		---i-		A--kM-	
Goldfish-I	-----d-t-		--m--V------		---i-		A--kM-	
Goldfish-II	-----d-t-		--m--V------		---i-		A--kM-	
Rainbow trout-I	s-VD-H-t-		--m--V----T-		I--S-		S--kV-	
Rainbow trout-II	s-ID-Q-t-		--m--V----T-		I--N-		S--kV-	
Gilthead sea bream	s-ID-H-t-		--m--V----T-		I--S-		Se-kT-	
	Loop		Helix IV		Helix I		Helix III	

Figure 8.2 Amino acid sequences of GH from various mammalian and teleost species in regions corresponding to binding site 1 and binding site 2 of hGH. All hormones are aligned and the amino acid residues numbered relative to hGH. Conserved amino acid substitutions were assessed using the amino acid groupings of Wu and Brutlag (1996): hydrophobic amino acids (MIVLFWYH); charged amino acids (RKQEDN); small amino acids (AST); C; G; P. Residues in lower case letters represent conserved substitutions relative to those of bGH. Residues in capital letters are not conserved substitutions relative to those of bGH. Residues identical to those of bGH are indicated by a dash. All sequences were obtained from the Genbank database; accession numbers for the various GH are: bovine-P01246; human-P01241; rat-P01244; rabbit-P46407; common carp-I-P10298; common carp-II-S02764; goldfish-I-AAC19389; goldfish-II AAC19390; rainbow trout-I-A31363; rainbow trout-II-P20332; and gilthead sea bream-P29971. Common carp GH-I corresponds to rcGH and rainbow trout GH-I corresponds to rtGH used in the GH receptor binding assays in this thesis.

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APPENDIX A OPTIMIZATION OF GOLDFISH HEPATOCYTE CULTURE TECHNIQUES

A.1 Introduction

Barry and Friend (1969) first described the isolation of liver parenchymal cells by collagenase digestion in the rat. A similar technique has also been successfully employed to isolate teleost hepatocytes (Moon *et al.* 1985). Mammalian hepatocytes can survive *in vitro* for up to one month under an appropriate culture environment (Isom *et al.* 1985). In contrast, hepatocytes from carp and salmonids have been reported to survive for only a few days (Guguen-Guillouzo & Guillouzo 1986). The shorter life of teleost hepatocytes *in vitro* may be due to an inappropriate culture environment. Although high yield preparations of goldfish hepatocytes have been obtained (Birnbaum *et al.* 1976, Schwarzbaum *et al.* 1992, Krumschnabel *et al.* 1994, 1996), these cells were used for experiments immediately after preparation and were not continuously cultured.

In order to complete the *in vitro* study of GH receptors in Chapter 7, a goldfish hepatocyte culture had to be established and optimized for a longer period of *in vitro* hepatocyte survival. In this appendix, various factors that influence goldfish hepatocyte viability, yield and *in vitro* growth were examined experimentally. Optimization of these conditions then allowed large amounts of viable goldfish hepatocytes to be easily prepared and cultured *in vitro* for up to one month.

A.2 Materials and Methods

A.2.1 Enzymatic isolation of goldfish hepatocytes

Goldfish hepatocytes were routinely prepared using the method of Krumschnabel *et al.* (1994). Goldfish were sacrificed in excess anesthetic (0.05% MS222). Portions of liver were removed under sterile conditions and washed in HEPES buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 24 mM $NaHCO_3$, and 15 mM HEPES, pH 7.5) containing 0.1 mg ml^{-1} of kanamycin. The liver was minced and incubated with 0.1% collagenase for 30 min at 26°C in an atmosphere of 95% air/5% CO_2. Digested tissue was filtered through a 75 μm nylon mesh, centrifuged at 50 g for 45 seconds, and the supernatant removed by aspiration. Sedimented hepatocytes were washed three times in HEPES buffer and resuspended in a serum-free culture medium consisting of 3:1 of mixture of Dulbecco's modification of Eagle's medium plus medium 199 containing 0.1 mg ml^{-1} of kanamycin (DMEM/199).

A.2.2 Dye exclusion method to determine hepatocyte viability

Viable cells are impermeable to trypan blue, nigrosine, and a number of other dyes whereas dead cells are stained by the dyes (Freshney 1994). In this study, cells were exposed to 0.5% (wt/vol) trypan blue for several minutes and then counted on a hemocytometer. The viability of cells was determined as the percentage of cells present which were not stained by trypan blue. The yield of cells was calculated as the total number of viable cells obtained per gram of liver tissue.

A.2.3 *In vitro* culture of hepatocytes

Prior to seeding, the number of viable cells was counted on a hemocytometer using the dye exclusion method described above. Only cell preparations with a viability greater than 98% were used for the subsequent experiments. Approximately 1 x 10^7 cells were plated in 2.5 cm regular polystyrene culture dishes (Falcon brand, Becton Dickinson Co., NJ) and incubated at 26°C in a humidified atmosphere of 95% air/5% CO_2. Attachment efficiency was determined as the number of cells attached to the surface over the total number of viable cells plated. After cell attachment, fresh DMEM/199 (3:1) without antibiotics was added and renewed every 2 days thereafter.

A.2.4 Subculture of hepatocytes

Freshly isolated goldfish hepatocytes were seeded in 2.5 cm Primaria dishes (Becton Dickinson Co., NJ) at a density of 1 x 10^7 cells/dish. After 3 days in culture, the cells were detached by 0.25% (wt/vol) trypsin or by the rub stick method (Freshney 1994) and removed into new culture dishes for subculture. Attachment efficiency was determined as described above.

Significantly low levels of attachment efficiency were found in goldfish hepatocytes following subculture (Fig. A.1). After subculture, the attachment efficiency of the cells was only 15% whereas over 80% of the freshly isolated cells (control) were found to be attached. The extremely low attachment efficiency in the first passage of goldfish hepatocyte culture made it impossible for further subculture or testing of these cells.

A.2.5 Effect of collagenase concentration on viability and yield of hepatocytes

Incubation of minced liver was performed with various concentrations (0, 0.025, 0.05, and 0.1%) of type IV collagenase for various time periods (1, 2, and 4 h). The viability and yield of goldfish hepatocytes were determined as described above.

A.2.6 Effect of substrate on attachment efficiency

Two types of 24-well plates were tested for optimal culture of goldfish hepatocytes; one was made of normal polystyrene (regular Falcon brand, Becton Dickinson Co., NJ), the other is made of modified polystyrene (Primaria Falcon brand, Becton Dickinson Co., NJ). Incubation was performed for various time periods (1, 4, 12, and 24 h). Cell number was determined using the MTT assay described previously (Chapter 4).

A.2.7 Effect of rcGH, insulin and goldfish serum on attachment efficiency

Freshly isolated goldfish hepatocytes were seeded in 24-well Primaria plates at a density of 1×10^5 cells/well. Cells were supplemented with medium only (DMEM/199 3:1) or with medium containing 1 μg rcGH/well, 1 μg bovine insulin/well, or 10% (vol/vol) goldfish serum. Attachment efficiency under each treatment was determined as described above.

A.2.8 Effect of culture medium on *in vitro* growth of goldfish hepatocytes

Freshly isolated goldfish hepatocytes were seeded in 24-well Primaria plates at a density of 5 x 10^4 cells/well. After 2 to 3 h, living cells were attached to the plastic surface and began to spread. After cell attachment, various types of fresh media were added including DMEM, medium 199, or mixture of DMEM plus medium 199 (1:1 or 3:1). Cells were incubated for a period of 4 days. Fresh medium was added and renewed every 2 days. At the end of incubation periods, cell number was determined using the MTT assay described previously (Chapter 4).

A.3 Results

A.3.1 Effect of collagenase concentration on viability and yield of hepatocytes

Collagenase disaggregation resulted in the release of large number of intact goldfish hepatocytes with a viability of more than 95%. Incubation of minced goldfish liver with various concentrations of collagenase yielded various amounts of viable cells. A high yield of goldfish hepatocytes was obtained using 0.1% collagenase disaggregation, with a yield of $6.7 \pm 0.5 \times 10^7$ cells g^{-1} liver, a viability of more than 97% and a protein content of 2.5 mg/10^6 cells (Fig. A.2). All subsequent experiments were performed using 0.1% collagenase disaggregation. Longer incubation with collagenase resulted in a higher yield of cells but a lower viability (Fig. A.3). For a viability of more than 95%, 2 h of incubation with 0.1% collagenase resulted in the highest yield of viable hepatocytes (Fig. A.3).

A.3.2 Effect of rcGH, insulin and goldfish serum on attachment efficiency

The effects of rcGH, insulin, and goldfish serum on attachment efficiency of goldfish hepatocytes are illustrated in Figure A.4. Significantly higher attachment efficiency was found in cells treated with rcGH incubated less than 4 h compared to that of the control cells (Fig. A.4 top panel). However, by 24 h, attachment efficiency was similar in all treatments. Thus, hormone and serum were not used for cell attachment in all subsequent experiments.

A.3.3 Effect of substrate on attachment efficiency

Cells cultured on a modified polystyrene substrate (Primaria) had higher attachment efficiency than cells cultured on regular polystyrene substrate (Fig. A.4 bottom panel). Therefore, all the subsequent experiments were conducted using modified polystyrene as a substrate (Primaria culture plates).

A.3.4 Effect of culture medium on *in vitro* growth of goldfish hepatocytes

The effects of culture medium on *in vitro* growth of goldfish hepatocytes are shown in Figure A.5. There were no significant differences in cell growth cultured in the different media (Fig. A.5). DMEM/199 (3:1) medium was used for all subsequent goldfish hepatocyte cultures.

A.3.5 Morphological changes of cultured goldfish hepatocytes

Freshly isolated goldfish hepatocytes displayed a highly rounded morphology (Fig. A.6a). Goldfish hepatocytes began to proliferate after seeding (Fig. A.6b), gradually aggregated to form short cords by day 4 (Fig. A.6c), and maintained a similar structure for up to one week (Fig. A.6d). At two weeks, large cord-like structures developed with many processes (Fig. A.6e). Such structures were maintained until the end of goldfish hepatocyte culture (Fig. A.6f).

A.4 Discussion

A rapid and effective method for isolation of goldfish hepatocytes by collagenase dissaggregation was established in this study. These hepatocytes display a long period of survival *in vitro* and maintain their responsiveness to epinephrine stimulation (Chapter 7). The two-step *in situ* collagenase perfusion is the best method for preparation of mammalian hepatocytes (Freshney 1994). However, this technique is very time consuming, and is not efficient or economical for small-sized and anatomically dispersed livers such as that found in goldfish. Collagenase disaggregation is of greatest benefit for tissues including liver, which are either too fibrous or too sensitive to allow for the successful use of trypsin (Kralovansky *et al.* 1990).

Separation of the parenchymal cells from non-parenchymal liver cells may be achieved on the basis of size and density, using either gravity sedimentation, low speed centrifugation or to best advantage, centrifugal elutriation (Hayner *et al.* 1984). In the present study, a low speed centrifugation was also selected for isolation of goldfish hepatocytes. The goldfish hepatocytes selected by low speed centrifugation have been previously reported to have a large portion of parenchymal liver cells which display responsiveness to epinephrine similar to those of *in vivo* hepatocytes (Birnbaum *et al.* 1976, Van Waarde & Kesbeke 1981, Schwarzbaum *et al.* 1992, Krumschnabel *et al.* 1994, 1996). The use of an arginine-free culture medium has also been reported for selection of parenchymal liver cells based on the unique capacity of hepatocytes to synthesize arginine from ornithine via the urea cycle (Leffert & Paul 1973). In addition to the manipulation of arginine, the absence of serum inhibits the growth of fibroblasts in hepatocyte cultures (Enat *et al.* 1984). In the present study, DMEM/199 (3:1)

culture medium was used for goldfish hepatocytes. This culture medium contains a low concentration of arginine and is free of serum.

Hepatocytes require certain substances and suitable substrates for attachment, spreading, survival and growth. Serum has been reported to play an important role in cell attachment of mammalian hepatocytes (Horiuti *et al.* 1982). However, heterologous sera are less effective in stimulating DNA synthesis in rat hepatocytes than either a serum-free medium supplemented with insulin and epidermal growth factor or medium containing rat serum (Strain *et al.* 1982). In the present study, rcGH was found to be the most effective factor in stimulating attachment of goldfish hepatocytes. The use of rcGH instead of goldfish serum for cell attachment has advantages in the biochemical and physiological studies of goldfish hepatocytes since serum contains many unknown substances which complicates *in vitro* studies. However, rcGH has also an antimitogenic effect on goldfish hepatocytes (Chapter 7). Therefore, rcGH was not used to improve attachment efficiency of goldfish hepatocytes.

Potential substrates for cell attachment include collagens (type I, III, IV, V, and VI), noncollagenous glycoproteins such as fibronectin and laminin, heparan sulfate proteoglygan, or plastics such as polystyrene (Freshney 1994). Previous studies indicate that collagen substrate provides a condition most resembling the *in vivo* situation for mammalian hepatocytes (Dunn *et al.* 1989). In the present study, a polystyrene culture substrate was selected on the basis of simplicity and economy. Goldfish hepatocytes were found to have a higher attachment efficiency on a modified polystyrene substrate than that of regular polystyrene. The cultures of goldfish

hepatocytes were also found to proliferate and survive for a long period of time on the modified polystyrene substrate (Chapter 7).

There were no significant differences in the *in vitro* growth of goldfish hepatocytes cultured in the different media. This result suggests that the choice of basal medium is of little importance for *in vitro* growth of goldfish hepatocytes. Similar findings were reported for mammalian hepatocytes (Guguen-Guillouzo 1992). It appears that the importance of culture medium for cell survival is supplement of nutrients, metabolites, and minerals, and maintenance of pH, osmolality, and humidity, rather than direct stimulation of *in vitro* growth (Freshney 1994).

The present study developed a simple, rapid and economical method for preparation of goldfish hepatocytes with high viability and yield. The goldfish hepatocytes cultured *in vitro* displayed high attachment efficiency, proliferation over time, and morphological changes similar to mammalian hepatocytes (Guguen-Guillouzo 1992). Additional characteristics of goldfish hepatocytes prepared using these techniques were further studied in Chapter 7.

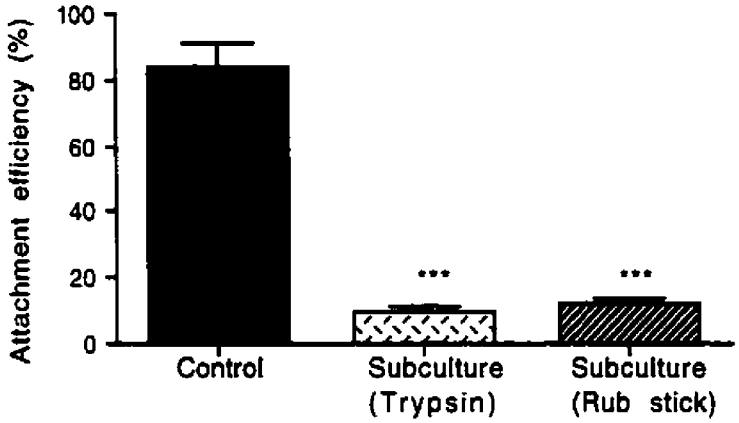

Figure A.1 Effects of subculture on attachment efficiency of goldfish hepatocytes. Freshly isolated goldfish hepatocytes (control) had significantly higher attachment efficiency than cells cultured for 3 days and subcultured following detachment by trypsin or the rub stick method (Student's t-test, *** $p<0.001$). Data, expressed as mean±SEM, were obtained from three experiments, each carried out in triplicate.

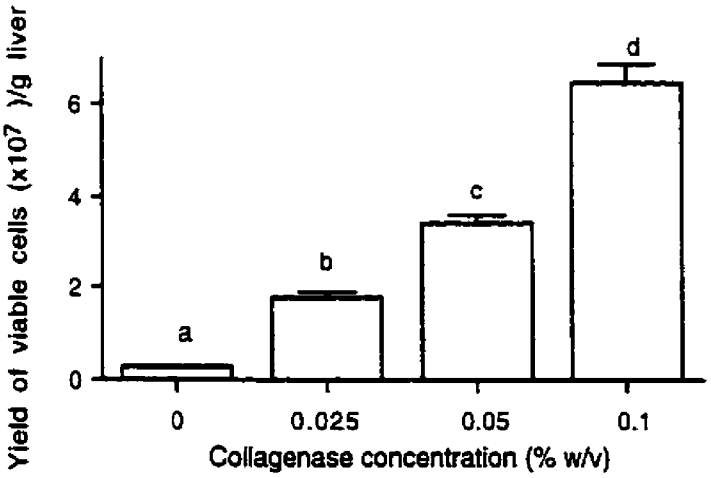

Figure A.2 Yield of viable goldfish hepatocytes obtained using various concentrations of collagenase. Liver tissue was incubated with collagenase at 26°C in a humidified atmosphere (5% CO_2/95% air) for 2 h. Data, expressed as mean±SEM, were obtained from three experiments, each carried out in triplicate. Groups with different superscripts are significantly different (Student-Newman-Keuls test, $p<0.05$).

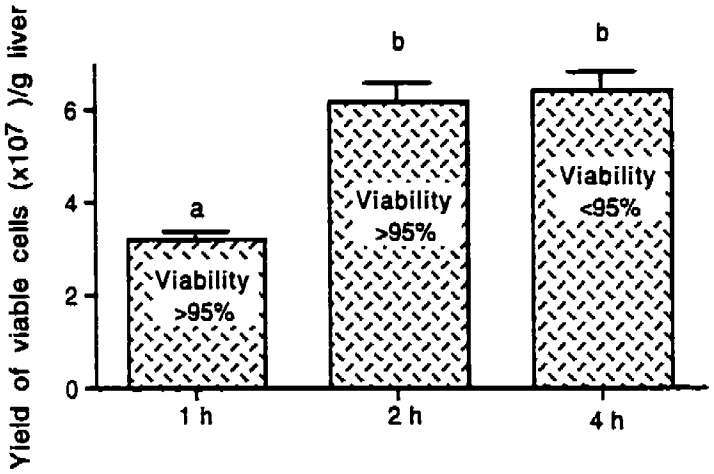

Figure A.3 Yield of viable goldfish hepatocytes obtained following digestion of liver tissue with 0.1% collagenase at 26°C in a humidified atmosphere (5% CO_2/95% air) for various time periods. Data, expressed as mean±SEM, were obtained from three experiments, each carried out in triplicate. Groups with different superscripts are significantly different (Student-Newman-Keuls test, $p<0.05$).

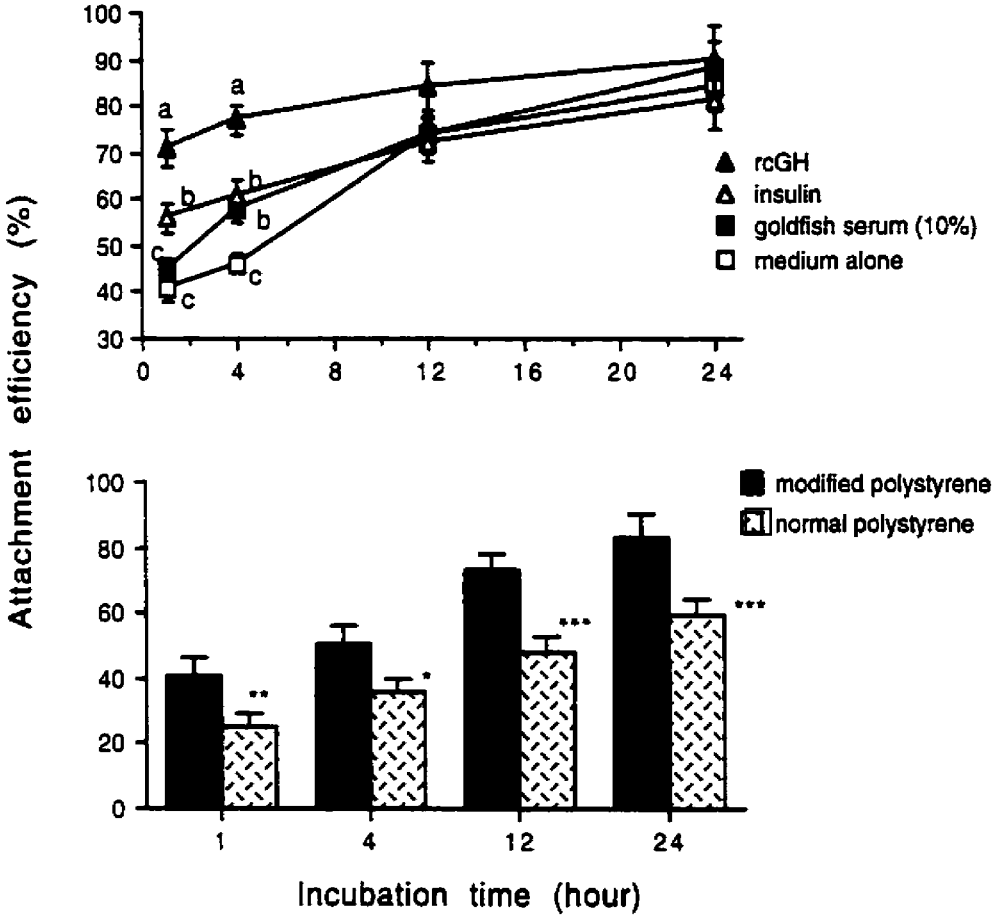

Figure A.4 Effects of rcGH, insulin, goldfish serum, and substrate on attachment efficiency of goldfish hepatocytes. Cells were cultured with DMEM/199 (3:1) containing rcGH, insulin or 10% goldfish serum on modified polystyrene substrate (top panel). Cells supplemented without hormones or serum were cultured on either on modified polystyrene substrate or normal polystyrene substrate (bottom panel). Incubation of cells was performed at 26°C in a humidified atmosphere (5% CO_2/95% air) for various time periods. Cells cultured on modified polystyrene had a significantly higher attachment efficiency than those cultured on normal polystyrene (Student's t-test, * $p<0.05$, *** $p<0.001$). Data, expressed as mean±SEM, were obtained from three experiments, each carried out in triplicate. Groups with different superscripts are significantly different (Student-Newman-Keuls test, $p<0.05$).

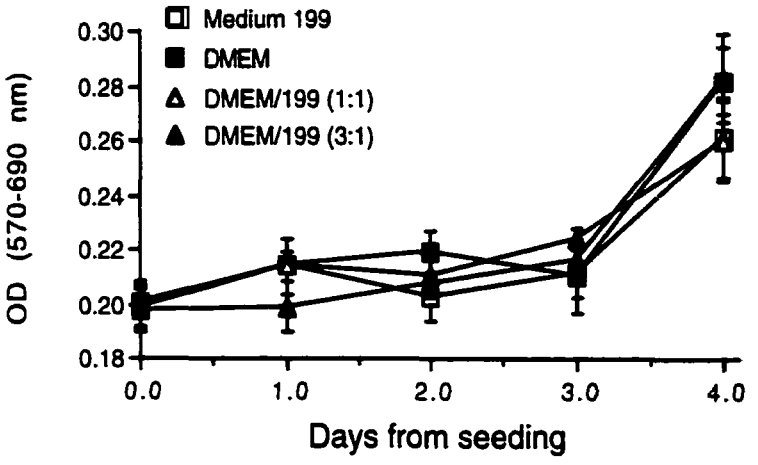

Figure A.5 Effects of various culture media on *in vitro* growth of goldfish hepatocytes. The number of cells was determined using the MTT assay. Data, expressed as mean±SEM, were obtained from three experiments, each carried out in triplicate. There were no significant differences in cell number between the various culture media.

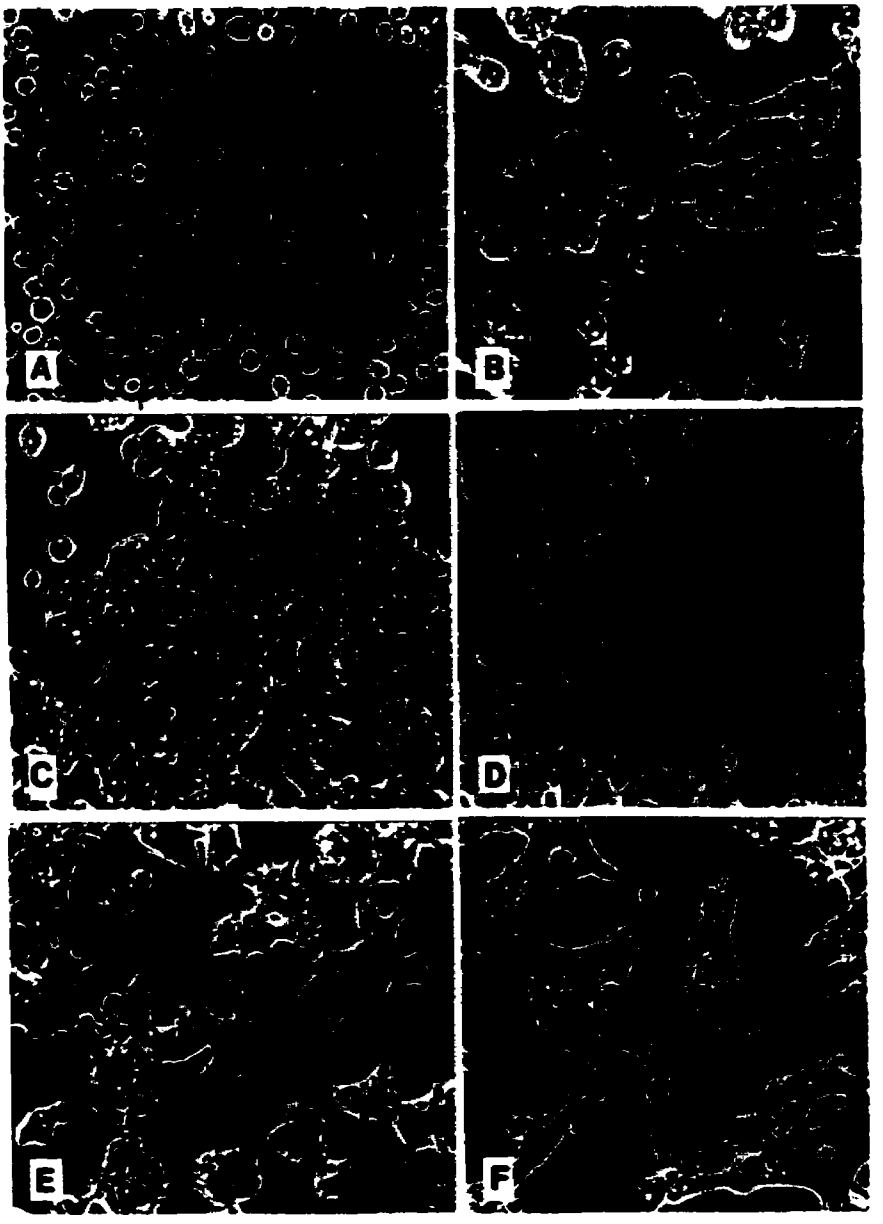

Figure A.6 Morphological changes of goldfish hepatocytes *in vitro*. Freshly isolated goldfish hepatocytes displayed a highly rounded morphology (Fig. A.6a). Goldfish hepatocytes began to proliferate after seeding (Fig. A.6b), gradually aggregatedto form short cords by day 4 (Fig. A.6c), and maintained similar structure for up to one week (Fig. A.6d). At two weeks, large cord-like structures developed with many processes (Fig. A.6e). Such structures were maintained until 30 days of goldfish hepatocyte culture (Fig. A.6f).