EVOLUTION OF THE GROWTH HORMONE RECEPTOR: INSIGHTS INTO THE MOLECULAR BASIS OD THE PHYSIOLOGICALLY PLEIOTROPIC NATURE OF THE GROWTH HORMONE RECEPTOR

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Evolution of the growth hormone receptor: insights into the molecular basis of the physiologically pleiotropic nature of the growth hormone-family

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ABSTRACT

One of the oldest, extant, lineages of vertebrates, the sea lamprey, was used to clarify the evolutionary origin and divergence of the growth hormone receptor (GHR) family. A single, full-length, cDNA, and a second, partial, cDNA were identified and shown to encode proteins that share amino acid identity with GHRs and prolactin receptors (PRLR s) previously identified. The complexity of the dynamic signaling system, with special emphasis on this system in fish and in the context of the evolution of this system, is discussed in the first chapter. The second chapter integrates the new insights gained by these studies. Included is a newly proposed phylogenetic analysis and revised nomenclature-system for vertebrate GHRs that better represents the evolutionary history of the receptor family. The molecular evolution of the receptors is, furthermore, highlighted as the backdrop for the continued discussion regarding how the GH-family of hormones exhibit such coordinated and pleiotropic actions.

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I must also give thanks to those that gave me guidance and encouragement to become a better scientist. I must start by thanking all that were a teacher to me, whether that was formally, as a professor, or informally, as a colleague. The work of Jeffrey Kittilson is gratefully acknowledged. The technical support and an experienced hand in much of this work was, not only, valuable to the research, but was also valuable to me as I learned. I would also like to acknowledge the fellow graduate students and undergraduate students that I worked along side with over the course of this project. Working alongside such talented students (young scientists) was truly an honor. I must also thank Dr. Mark Sheridan for the opportunity to work in his lab and learn, not just about science, but how I, as an individual, can bring a unique set of skills and talents to the table to contribute to science as part of a community.

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LIST OF ABBREVIATIONS

1R.................... first round of genome duplication

2R.................... second round of genome duplication

3R.................... third round of genome duplication

4R.................... fourth round of genome duplication

Akt .... protein kinase B/phosphokinase B

ANOVA.......... analysis of variance

Bp.................... base pairs

cDNA.............. complementary deoxyribonucleic acid

CoAA.............. co-activator activator, also known as pantothenate kinase

CHO-K1.......... Chinese hamster ovary cell line

c-raf................ protooncogene serine/threonine-protein kinase

E2.................... 17β estradiol

ED_{50} .... median effective dose

EPO .... erythropoietin

EPOR.............. erythropoietin receptor

ERK .... extracellular regulated kinase

ERK1................extracellular regulated kinase 1; synonymous with mitogen-activated protein kinase 3 (MAPK3)

ERK2 ....extracellular regulated kinase 2; synonymous with mitogen-activated protein kinase 1 (MAPK1)

FSGD .... fish specific genome duplication; synonymous with 3R

FW .... fresh water

GH...................growth hormone; variations (e.g., GH1, GH2, rtGH, hGH, etc.)

GHBP............. growth hormone binding protein

GHR .... growth hormone receptor; variations (e.g., GHR1, hGHR, etc.)

^{125}I .... radioisotope of iodine

IGF .... insulin-like growth factor; variations (e.g., IGF-1, etc.) are seen

IGFBP .... insulin-like growth factor binding protein

IGFR .... insulin-like growth factor receptor

ILs .... interleukins

INS .... insulin

JAK .... Janus kinase; variations (e.g., Jak2, et.) are seen

kDa................. kilo Dalton

LY294002 .... 2-morpholin-4-yl-8-phenylchromen-4-one; P13K inhibitor

MAPK............ mitogen-activated protein kinase

mRNA .... messenger ribonucleic acid

MEK...............mitogen-activated protein/extracellular signal-regulated kinase kinase

NMR .... nuclear magnetic resonance

μM.................. micromolar

PCR................. polymerase chain reaction

PI3K................ phosphatidylinositol 3-kinase

PKC................. protein kinase C

PL.................... placental lactogen

PLC .... phospholipase C

PRL .... prolactin; variations (e.g., PRL1, hPRL, etc.) are seen

PRLR .... prolactin receptor

RT-PCR .... reverse transcription polymerase chain reaction

S.E.M. .... standard error of the mean

SIE .... sis inducib;e element

SFK .... Src-family tyrosine kinase

SL.................... somatolactin; variations (e.g., SLα, etc.) are seen

SLR .... somatolactin receptor (arguably the type-1 GHR)

STAT .... signal transducer and activator activator

SS .... somatostatin; variations (e.g., SS-14) are seen

SW .... seawater

TG .... triglyceride

U0126.............. MEK inhibitor

UbE .... ubiqitin-depenendent endocytosis

Zn^{2+} .... zinc ion

$ZnCl_2$ .... zinc chloride

CHAPTER 1. MOLECULAR EVOLUTION AND REGULATION OF GROWTH HORMONE SIGNALING: TOWARD A HIGHLY INTEGRATED CONTROL SYSTEM OF GROWTH

Introduction

Although growth hormone (GH) regulates numerous processes in vertebrates, including feeding, metabolism, reproduction, osmoregulation, immune function, and behavior, perhaps the best studied actions of GH are those related to promotion of organismal growth (Forsyth and Wallis, 2002; Bjornsson *et al.,* 2004; Norrelund, 2005; Norbeck *et al.,* 2007; Moller and Jorgensen, 2009). GH results in increased amino acid uptake, increased RNA synthesis, increased protein synthesis, increased cartilage synthesis, and increased muscle growth (Sheridan, 2011). Many of the actions of the growth-promoting effects of GH in fish and other vertebrates are mediated by insulin-like growth factor (IGF). The GH-IGF system has been well studied in mammals and fish, and the main elements appear to be highly conserved (Baumann *et al.,* 1988; Reinecke *et al.,* 2005). Circulating GH stimulates the synthesis and secretion of IGF-1 from the liver, and IGF-1, in turn, stimulates cell growth and differentiation in a variety of target tissues via distinct IGF receptors (Wood *et al.,* 2005; Laviola *et al.,* 2007). The availability and actions of GH and IGF-1 are influenced by GH binding proteins (GHBPs) and IGF binding proteins (IGFBPs), respectively (Baumann *et al.,* 1988; Duan and Xu, 2005). In addition, local production of IGF-1 is important, and GH and IGFBPs have direct, non-

IGF-dependent effects (Butler and LeRoith, 2001; Duan and Xu, 2005; Wood *et al.,* 2005). The GH-IGF system of fish is particularly complex, and consists of multiple isoforms of GHs, GHRs, IGFs, IGFBPs, and IGF receptors (IGFR) (Reinecke *et al.,* 2005; Reindl and Sheridan, 2012).

Despite extensive knowledge of the GH-IGF system, the molecular basis of the pleiotropic nature of GH is not fully understood. Recent work on the multifaceted GH signaling system of rainbow trout *Oncorhynchus mykiss* and other fish hold promise for providing such understanding. In this chapter, we will review recent findings regarding the molecular evolution and structure of GH family peptides and their receptors and describe how hormone-receptor interactions selectively activate biological processes, with special emphasis on activation and regulation of growth-related processes.

Molecular evolution and structure of GH signaling elements

The GH signaling system of the rainbow trout and other species of fish is multifaceted, consisting of numerous types of GH-family peptides and a variety of GHR subtypes. In this section, the evolutionary origins and the bases of the structural heterogeneity of GH-family peptides and the GHR subtypes are described.

Evolution by gene duplication

The ancestral salmonids are believed to have gone through several genome duplication events. In each case, with a doubling in the chromosome number, salmonid species were left with two copies of each gene, some of which could have been subsequently lost (Ohno, 1970; Allendorf and Thorgaard, 1984; Hurley *et al*., 2007;

Kuraku *et al.,* 2009; Van de Peer *et al.,* 2009). Over time, however, mutations of the remaining duplicated genes would have resulted in structural and functional divergence (Konrad *et al.,* 2011). Since these events were the duplication of the entire genome, any ligands and receptors present at the time of the event would have been duplicated. Indeed, both the GH ligand and GH receptors are believed to have been duplicated during these whole genome duplication events (McKay *et al.,* 2004; Liongue and Ward, 2007). Furthermore, the family of ligands and receptors, to which GH and GHR belong, are both believed to have, long ago, diverged from each other in this duplication-dependent way.

In the evolutionary history leading up to actinopterygians (ray-finned fish), three whole genome duplication events (1R-3R) are believed to have occurred. The 2R event (two successive rounds of whole genome duplication occurred) is believed to have occurred early in chordate evolution, perhaps even before the cyclostome-gnathostome split (Kuraku *et al.,* 2009). The third of the whole-genome duplication events (3R), also known as the fish-specific genome duplication (FSGD) event, is believed to have occurred 226–316 MYA (Hurley, 2007; Van de Peer *et al.*, 2009). The 3R event occurred in the actinopterygian lineage, which had already diverged from the sarcopterygian (lobe-finned fish) linage, which included the common ancestor of tetrapods (Meyer and Van de Peer, 2005). Thus, for this reason, many fish have two copies of a gene, whereas the homologous gene in tetrapods only is found as a single copy. Salmonids, descendants of a post-3R common ancestor, underwent an additional duplication event, 4R, which is estimated to have occurred 25–100 MYA (Ohno, 1970; Allendorf and Thorgaard 1984). In each case of genome duplication, duplicate copies of a gene would result and, at least initially, would function fully despite the redundancy. Thereafter, the duplicated copies

may diverge resulting from non- (pseudo-), neo-, or subfunctionalization (Ohno 1970, Innan and Kondrashov, 2010; Konrad *et al.,* 2011). Other models such as Dosage Balance may explain the maintenance of both paralogues (Innan and Kondrashov, 2010). Alternatively, over time, genes may have been lost by various events, such as the deletion of chromosomal segments containing one or more genes or through gene-by gene events such as epigenetic silencing (Sankoff *et al.,* 2012).

Molecular evolution and structure of the GH-family hormones

GH is a protein hormone produced by the somatotroph cells of the adenohypophysis and is part of the class-I cytokine superfamily, which includes prolactin (PRL), the fish-specific somatolactin (SL), and the mammal-specific placental lactogen (PL). Additionally, the extra-pituitary production of GH in fish and other vertebrates is now well supported, and the extra-pituitary expression of GH genes has been reported in trout (Yang *et al.,* 1997a; Harvey, 2010). This protein hormone is evolutionary conserved, as it has been isolated from the pituitary of representatives of every extant class of vertebrate (Yamaguchi *et al.,* 1989; Kawauchi *et al.,* 2002; Moriyama *et al.,* 2008). GH is a single-chain polypeptide roughly 21-22 kDa in size, and shares structural similarities with the 22-25 kDa PRL-protein and the 24-28kDa SL-protein (Law *et al.,*1996; Yang *et al*., 1997a; Li *et al*., 1997; Wang *et al*., 2010; Cavari *et al*., 1995; Benedet *et al*., 2008; Yang *et al*., 1997b; Yang and Chen, 2003). While GH genes are known to contain five exons and four introns in tetrapods, both GH1 and GH2 genes from salmonids contain an additional intron, intron E, which interrupts what would otherwise be the last, continuously translated exon and thus separates the exon into two exons

(Yang *et al.,* 1997a). In general, hormones of this family have four alpha-helical bundles, corresponding to four conserved sequence domains that arrange in an anti-parallel manner. In order from the N terminal to the COOH-terminal, helices 1-2-3-4 of the hGH (human GH) run in an up-up-down-down fashion (de Vos *et al.,* 1992). Members of this family of protein hormones bind with members of a distinct family of single-transmembrane receptors and do so in a somewhat promiscuous way, as these receptors have been shown to have varying degrees of affinity among members of this family. The actions of these peptides occur upon binding to a dimerized receptor. The presence of GHR dimers on the plasma membrane offers an advantage for rapid signaling. Upon the binding of a ligand to a receptor dimer, no time is lost as it would be as if a bound monomeric-receptor were to require the recruitment of a second receptor for dimerization; thus, there is greater potential for the initiation of receptor signaling in response to low ligand concentrations, even in cells expressing GHRs at low levels (Gent *et al.,* 2002).

Evidence suggests that overall salmonid species have two functional, non-allelic GH paralogues, GH1 and GH2, that have been retained and their genes are differentially expressed at various developmental stages and between sexes (McKay *et al.,* 2004). The coding regions of rainbow trout (rt)GH1 and rtGH2 differ by 22 nucleotides, which results in 11 amino acid substitutions in the protein (Yang *et al.,* 1997a). Interestingly, the GH1 and GH2 genes are differentially expressed in pituitary glands of trout of different ages and sexes (Yang *et al.,* 1997; Chen *et al.,* 1989). Two PRL subtypes, PRL1 and PRL2, are believed to have been duplicated in the FSGD, although teleosts appear to have retained only a single copy from both PRL1 and PRL2 (Huang *et al.,* 2009). Indeed,

two PRLs have been characterized in zebrafish (*Danio rerio*) although only one has been characterized in trout, to date, thus suggesting the possibility that at least one other PRL has yet to be characterized in trout (Mercier *et al*., 1989). Two distinct SLs, with a 56% sequence identity, have been characterized in rainbow trout, and these two SL genes display a differential expression pattern (Yang and Chen, 2003). Due to the structural similarities among GHs, PRLs, and SL(s), binding of PRLs and SLs to the GHR has been widely documented in many species of fish, including salmonid species (Zhu *et al.,* 2004; Reindl *et al.,* 2009).

These ligands are known to have two locations on which they bind to the receptor complex, termed binding sites 1 and 2. Extensive structural analysis has been accomplished, especially with GH. With a high conservation of structure, a known crystal structure of the GH-GHR2 complex from human GH and GHR, and mutational studies, a comparative approach can be employed to delineate structural features that are particularly important to function. The current model of GHR activation includes the high affinity binding of the site 1 of GH to the first molecule of GHR initially, which is followed by the binding of site 2 by GH to the second GHR of the dimer (Brooks *et al.,* 2008). A mutational analysis of GH using domain swapping of goldfish (*Carassius auratus*) GH (gfGH) and goldfish prolactin (gfPRL) also supports a model for a single GH with two binding sites bound to a dimerized receptor. The analysis and mutational study of goldfish GH supports a model whereby three discontinuous regions on the first helix and the region in the middle of the fourth helix comprises binding site 1; the third helix appears to be associated with binding site 2 (Chan *et al.,* 2007). While it is believed that binding site 1 is involved in the initial, high affinity binding of the first GHR, site 2

could be considered more as the functional site that binds the other GHR and determines the degree of the rotation of the receptors, relative to each other (Chan *et al.,* 2007; Brooks and Waters, 2010; Broutin *et al.,* 2010). This could explain, for example, why a lower site 1 affinity for 20-kDa hGH, compared to the site 1 affinity for 22-kDa hGH, had nearly the same ability to activate the gfGHR as the 22-kDa hGH (Tsunekawa *et al.,* 2000), whereas a single steric mutation within binding site 2 creates antagonists, able to bind to the receptor, but induce little or no biological activity (Tsunekawa *et al.,* 2000; Langerheim *et al.,* 2006). Previous studies with hGH and hPRL have shown that mutations in helix 3 of GH and PRL disrupt the integrity of binding site 2, resulting in mutants with little or no biological activity but show little difference in ED_{50} values from competitive radioreceptor binding assays (Langerheim *et al.,* 2006; Chen *et al.,*1994a, b). Binding site 2 has been described as the glycine cavity and highlights the Gly and surrounding large amino acid residues that, together, create a pocket that a Trp (Trp72PRLR or Trp104GHR) from the second of the two receptors in the dimer fill, upon binding (Broutin *et al.,* 2010). Variants of hPRL showed that N-terminal mutations (which would affect the 3rd helix) also had only a minimal impact on the global affinity for membrane receptors (Broutin *et al.,* 2010).

Interestingly, for the ligand to bind to the dimerized receptor, it appears that the locations of the two binding sites on the ligand are not necessarily as critical as simply having two sites with which the two receptors can bind. Dimers and oligomers of GH, PRL, and PL which occur through interchain disulfide linkages, linkages between glycosylated monomers, or through noncovalent interactions, are found in a relatively small portion of the total plasma levels of these ligands in humans; interestingly, dimeric

forms of these ligands have been known to exhibit biological activities through the normal receptor (Langenheim, 2006). A natural dimeric form of hGH has been shown to bind to GHR and PRLR with high affinity and cause unique biological activity *in vitro* (Bustamante *et al.,* 2010; Langenheim, 2006). Interestingly, site-directed mutagenesis at the functional binding site 2 of GH creates a GH antagonist with little to no biological activity, and when recombinantly engineered into a homodimer does not only maintain the ability to bind, but does so through the use of two site 1's, one from each of the two GH antagonists involved in the homodimer. Furthermore, this homodimer, created using two antagonists, did not share in the antagonistic effects of the monomers, but instead acted as an agonist (Langerheim *et al.,* 2006).

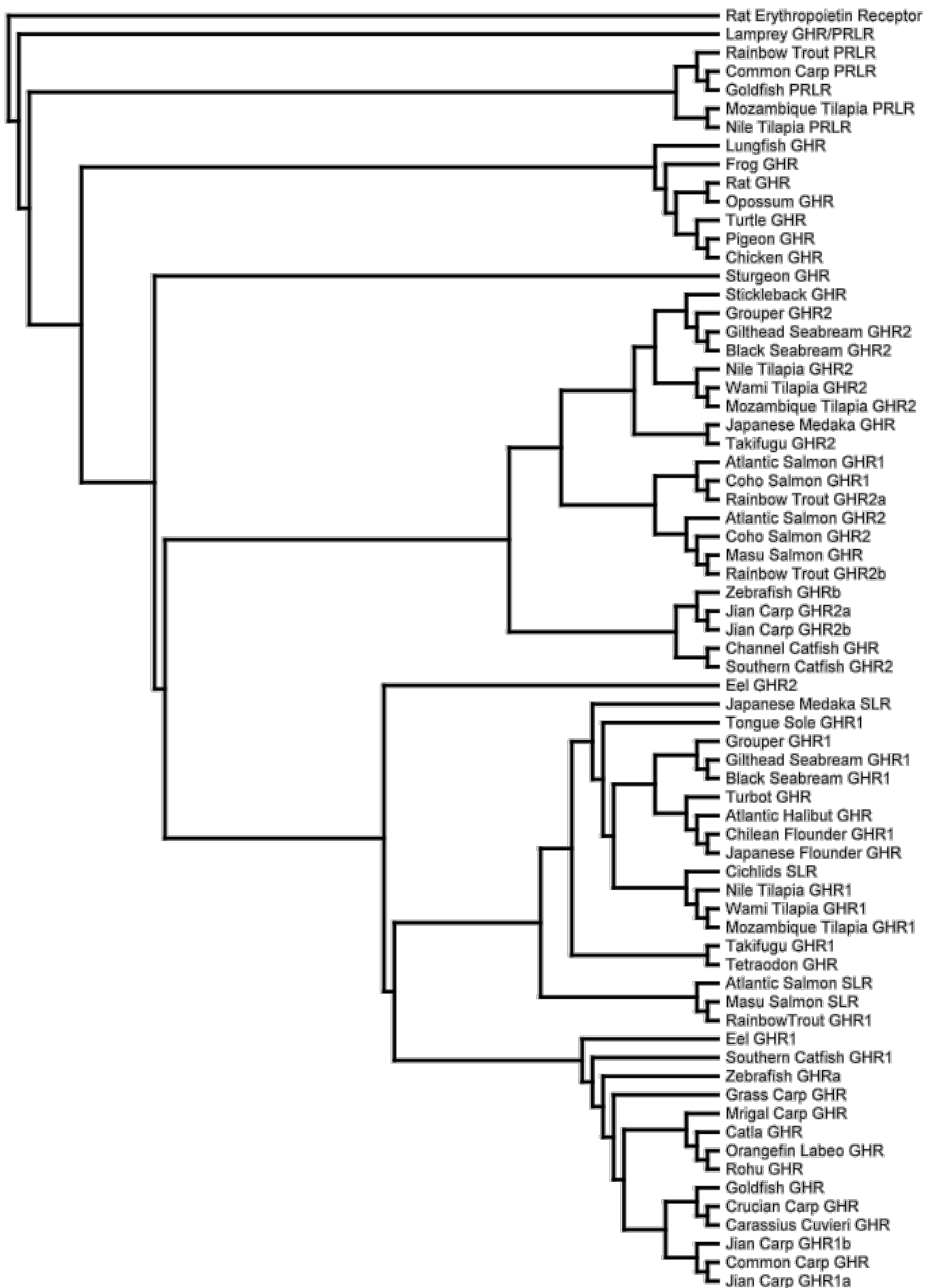
Together these studies support that the closely related cognate receptors exhibit considerable plasticity in their ability to accommodate various ligands of varying size and that, furthermore, the binding characteristics and biological functions of ligands and receptors do not necessarily go hand in hand. The receptors accommodate a ligand through two binding sites on the ligand, one which interacts with the first receptor in the dimer pair and the second that interacts with the second receptor of the dimer pair; tendencies of disruptions between the first ligand binding site and the first receptor affect global binding affinities of the receptor for the ligand, while the tendencies of disruptions between the second ligand binding site and second receptor affect the functional nature of the interaction and may not necessarily show differences in the global binding affinity as measured by competitive inhibition studies. Structural features of GH, and comparatively of PRL and SL, have important implications regarding the functions of these hormones - functions that hinge upon the interactions with their receptors

Molecular evolution of GH receptors

Multiple GHRs derived from distinct mRNAs, believed to be a result of a series of gene duplication events, have been described in many species of fish, including salmonids species such as masu salmon (*O. masou*), Atlantic salmon (*Salmo salar*), coho salmon (*O. kisutch*), and rainbow trout (Fukada *et al.*, 2004; Very *et al.,* 2005; Benedet *et al.,* 2008). The use of the terms "GHR1" and "GHR2" for the naming of the multiple GHR subtypes was first adopted in the tetraploid salmonids, and this convention continued for other teleosts (e.g., Ozaki *et al.,* 2006; Li *et al.,* 2007; Ma *et al.,* 2007). Two distinct clades have emerged: GHR type 1 (sometimes written type I) and GHR type 2 (sometimes written type II) (cf. Saera-Vila *et al.,* 2005, Ozaki *et al.,* 2006), with both of the original salmonid GHR1 and GHR2 subtypes contained within the type 2 clade. The nomenclature became more complex following the characterization of what appeared to be a distinct SL receptor (SLR) from masu salmon based on ^{125}I-SL binding (Fukada *et al.,* 2005) that fell within the clade with type 1 GHRs. Recently, Fukamachi and Meyer (2007) suggested that all of the teleost type GHR1s should be referred to as SLRs, and that the other major clade (type 2 GHRs, which includes the GHR1 and GHR2 of salmonids) be referred to as GHRs; they also noted that SLR is a teleost-specific paralogue of GHR that arose during the FSGD. The two GHRs of salmonids most likely arose during the more recent tetraploidization (4R) event associated with the evolution of this group. Reindl *et al.* (2009) suggested that the binding characteristics observed in masu salmon (Fukada *et al.,* 2005) may be a derived trait and that it may be premature to assign the label of "SLR" to all type 1 GHRs. Indeed, as will be discussed below, several type 1 GHRs retain GH binding characteristics.

Given the confused state of GHR nomenclature, it is clear that a community-wide movement towards a simplified nomenclature system that better represents the evolutionary history of this receptor family is needed. To this end, we suggest a change to a system similar to that already adopted for instances of multiple genes. This system utilizes different numbers to designate genes derived from one duplication event, then different letters to designate paralogues derived from a subsequent round of duplication. In application to GHRs, such a system would use numbers to designate the different GHR types that arose in the actinopterygian lineage (associated with 3R or FSGD); hence, in the teleosts there would be GHR1s (we urge abandonment of the term SLR to avoid confusion) and GHR2s. The addition of different letters would be added to distinguish paralogues associated with 4R duplication events (e.g., salmonids). This will necessitate changes to existing names (and some temporary confusion), but we have already done so for our GeneBank designations for trout GHRs. So, what were previously referred to as rainbow trout GHR1 and GHR2 (which were both in the type 2 GHR clade), are now GHR2a and GHR2b, respectively (cf. GenBank accession nos. NM001124535 and NM001124731). A similar scheme is proposed for the GHR1s. Whereas salmonids appear to have lost a gene following their 4R event and possess a single GHR1 (GHR 1 is proposed to be used in preference to SLR so as to avoid confusion and to better represent the evolutionary origins of this gene), other species (e.g., Jian carp, *Cyprinus carpio var. Jian*) retained both GHR1 paralogues, designated GHR1a and GHR1b. For clarity, we will use this new nomenclature system for the remainder of this chapter.

Figure 1. Phylogentic tree of receptors for prolactin, growth hormone, and somatolactin, from fish; GHRs and PRLRs from other vertebrates are included for comparison. The tree exemplifies two well-supported receptor clades across the fish taxa. The tree was based on the alignment of amino acid sequences using the N–J bootstrap method in Clustal X and rooted using the erythropoietin receptor as an out group; the tree was visualized with TreeView. Sequences were obtained from either GenBank (accession numbers in parentheses) or e! Ensembl (protein ID numbers in parentheses) as follows: Atlantic halibut GHR (DQ062814), Atlantic salmon SLR (NM001141617), Atlantic salmon GHR1 (NM001123576), Atlantic salmon GHR2 (NM001123594), black seabream GHR1 (AF502071), black seabream GHR2 (AY662334), *Carassius cuvieri* GHR (ADZ13485), catla GHR(AY691178), channel catfish GHR (DQ103502), chicken GHR (NM_001001293), Chilean flounder (EU004149), cichlid SLR (FJ208943), coho salmon GHR1 (AF403539), coho salmon GHR2 (AF403540), common carp PRLR (AY044448), common carp GHR (AY741100), eel GHR1 (AB180476), eel GHR2 (AB180477), frog GHR(AF193799), gilthead seabream GHR1 (AF438176), gilthead seabream GHR2 (AY573601), goldfish PRLR (AF144012), goldfish GHR (AF293417), grass carp GHR (AY283778), grouper GHR1 (EF052273), grouper GHR2 (EF052274), Japanese flounder (AB058418), Japanese medaka SLR (NP_001098560), Japanese medaka GHR (NM_001122905), Jian carp GHR1a (ADC35573), Jian carp GHR1b (ADC35574), Jian carp GHR2a (ADC35576), Jian carp GHR2b (ADC35577), lamprey GHR/PRLR (Ellens, unpublished) lungfish GHR (EF158850), masu salmon SLR (AB121047), masu salmon GHR (AB071216), Mozambique tilapia PRLR (EU999785), Mozambique tilapia GHR1 (AB115179), Mozambique tilapia GHR2 (EF452496), mrigal Carp (AY691179), Nile tilapia PRLR (L34783), Nile tilapia GHR1 (AY973232), Nile tilapia GHR2 (AY973233), opossum GHR (NM001032976), orangefin labeo GHR (EU147276), pigeon GHR (D84308), rainbow trout GHR1 (JQ408978), rainbow trout GHR2a (NM001124535), rainbow trout GHR2b (NM001124731), rainbow trout PRLR (AF229197), rat erythropoietin receptor (AAH89810), rat GHR (NM017094), red crucian Carp (ADZ13484), rohu GHR (AY691177), southern catfish GHR1(AY336104), southern catfish GHR2 (AY973231), stickleback GHR (ENSGACP00000023686), sturgeon GHR (EF158851), takifugu GHR1 (BAK86396), takifugu GHR2 (BAK86397), *Tetraodon* GHR (ENSTNIP00000004152), tongue sole GHR1 (FJ608664), turbot GHR (AF352396), turtle GHR (AF211173), wami tilapia GHR1 (EF371466), wami tilapia GHR2 (EF371467), zebrafish GHRa (EU649774), zebrafish GHRb (EU649775).


Rat Erythropoietin Receptor
Lamprey GHR/PRLR
Rainbow Trout PRLR
Common Carp PRLR
Goldfish PRLR
Mozambique Tilapia PRLR
Nile Tilapia PRLR
Lungfish GHR
Frog GHR
Rat GHR
Opossum GHR
Turtle GHR
Pigeon GHR
Chicken GHR
Sturgeon GHR
Stickleback GHR
Grouper GHR2
Gilthead Seabream GHR2
Black Seabream GHR2
Nile Tilapia GHR2
Wami Tilapia GHR2
Mozambique Tilapia GHR2
Japanese Medaka GHR
Takifugu GHR2
Atlantic Salmon GHR1
Coho Salmon GHR1
Rainbow Trout GHR2a
Atlantic Salmon GHR2
Coho Salmon GHR2
Masu Salmon GHR
Rainbow Trout GHR2b
Zebrafish GHRb
Jian Carp GHR2a
Jian Carp GHR2b
Channel Catfish GHR
Southern Catfish GHR2
Eel GHR2
Japanese Medaka SLR
Tongue Sole GHR1
Grouper GHR1
Gilthead Seabream GHR1
Black Seabream GHR1
Turbot GHR
Atlantic Halibut GHR
Chilean Flounder GHR1
Japanese Flounder GHR
Cichlids SLR
Nile Tilapia GHR1
Wami Tilapia GHR1
Mozambique Tilapia GHR1
Takifugu GHR1
Tetraodon GHR
Atlantic Salmon SLR
Masu Salmon SLR
RainbowTrout GHR1
Eel GHR1
Southern Catfish GHR1
Zebrafish GHRa
Grass Carp GHR
Mrigal Carp GHR
Catla GHR
Orangefin Labeo GHR
Rohu GHR
Goldfish GHR
Crucian Carp GHR
Carassius Cuvieri GHR
Jian Carp GHR1b
Common Carp GHR
Jian Carp GHR1a

The phylogenetic relationships of known GHRs are depicted in Figure 1. GHRs and PRLRs are believed to have diverged from the erythropoietin receptor (EPOR), thus this receptor is used to root the tree. A single GHR/PRLR has been isolated in lamprey (*Petromyzon marinus*) and appears to be the ancestral receptor of both GHRs and PRLRs (Ellens *et al.,* 2012). After divergence from the PRLR, the common ancestral GHR for the actinopterygian and sarcopterygian lineages would eventually be duplicated at the time of the FSGD event. The current hypothesis regarding the phylogenetic timing of the FSGD event is suggested to be sometime after the divergence of the Acipenseriformes (represented by sturgeon in Fig. 1) and the Semionotiformes (together considered a monophyletic group) from the lineage leading to teleost fish, but sometime before the divergence of Osteoglossiformes, the most basal teleost group (Hoegg *et al.,* 2004). To date, there is no sequence information for GHR in species representing Seminontiforms (e.g., gar) or Osteoglossiformes (e.g., bony tongue). As mentioned earlier, the GHRs of salmonids most likely arose during a more recent tetraploidization (4R) event associated with the evolution of this group. It appears that many species of salmonids have retained both copies of the duplicated GHR2 gene (i.e., GHR2a and GHR2b have both been characterized in rainbow trout, coho salmon, and Atlantic salmon). To date, only the Jian carp has had two GHR1s characterized, GHR1a and GHR1b (Yuan *et al.,* 2010). Interestingly, this variety of carp was artificially developed through integrated genetic breeding techniques, using two subspecies of common carp (using Hebao red common carp, *Cyprinus carpio var. Wuyuanensis,* as the original maternal parent and Yuanjiang common carp, *Cyprinus carpio Yuanjiang,* as the original paternal parent). The common carp (*Cyprinus carpio*) is one of two cyprinid species that is believed to have gone

through a tetraploidization event, likely independent of the salmonids (Yuan *et al.,* 2010). Presumably, one of the copies that resulted from the 4R duplication event in salmonids either has been lost or has yet to be characterized.

Molecular structure of the GH receptor family

As a member of the type-1 cytokine receptor superfamily, the GHRs have several evolutionarily conserved features characteristic of the superfamily: an extracellular domain, a single-chain transmembrane domain, and an intracellular domain that consists of at least two regions recognized to be important in the mediation of cellular effects that occur as a result of the receptor being bound by a ligand (Fig. 2). Besides the receptors for GH (including the fish-specific SL), members of this family include the receptors for erythropoietin (Epo), PRL, and the mammal-specific PL, as well as for thrombopoietin, leptin, ciliary neurotrophic factor, leukemia inhibitory factor, granulocyte colony-stimulating factor, and several of the interleukins (ILs) (Bazan, 1990; Carter-Su *et al.,* 1996). Within this superfamily, GHR is most closely related to the PRLR, EPOR, and the PL receptor (PLR). The same duplication event that resulted in paraloguous GHs in trout also resulted in duplicated GHR genes. The situation in rainbow trout has been complicated by the presence of two paralogous GHRs (GHR2a and GHR2b) as well as the one known orthologous GHR1 that has been retained (Very *et al.,* 2005; Walock, Kittilson, and Sheridan, unpublished observation). A single PRLR has been characterized in trout (Rouzic *et al.,* 2001). A second PRLR may remain to be characterized. Considering the mechanism by which these receptors are activated, and the degree of structural plasticity exhibited by these receptors that allows for such a wide

degree of binding capabilities, the structure of these receptors is inherently important to the resulting biological effects.

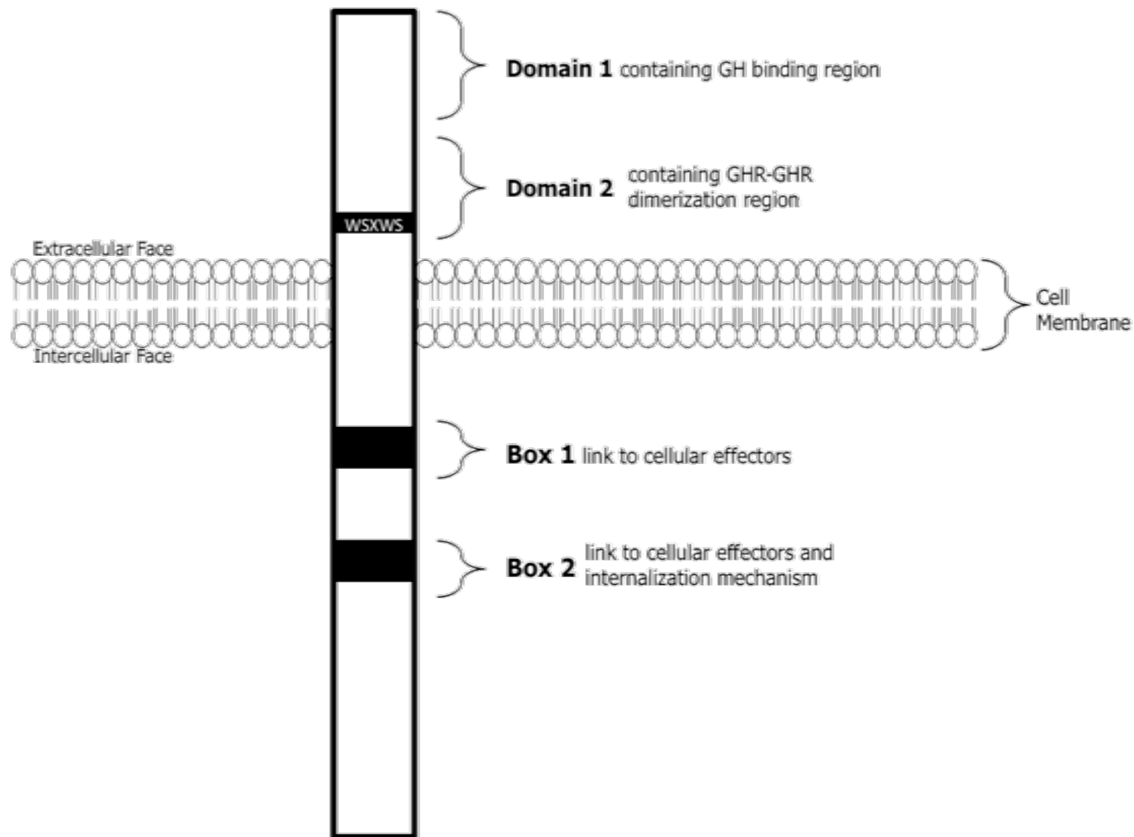


Figure 2. Schematic of the GHR family of receptors: the receptor includes a single extracellular domain that can be divided into two structural regions, (sub)domain 1 and (sub)domain 2, a single transmembrane domain transverses the cell membrane, and the intracellular domain contains two well recognized and conserved domains named box 1 and box 2.

Within the extracellular domain, there are numerous features, including conserved cysteine residues, a FGEFS motif (the WSXWS motif in other type-1 cytokine receptors), and potential N-linked glycosylation sites, which are conserved among these receptors and have been recognized to be important for receptor functionality (Fig. 2). The overall topology of the extracellular portion of type-I cytokine receptors includes a pair of

common beta sandwich motifs resembling fibronectin III/immunoglobulin domains, and is referred to by some as a cytokine receptor homology domain (Liongue and Alister, 2007). The beta sandwich motifs are titled domain 1 and domain 2 by some, and are linked by a short segment of the polypeptide chain (Conway-Campbell *et al*., 2008). These two domains of the extracellular GHRs contain roughly 100 residues each. Each of the two domains contain seven β strands that together form a sandwich of two antiparallel β sheets, one β sheet with four strands and one with three (de Vos *et al*., 1992).

Using the available sequences for the PRLR and GHRs of rainbow trout, we constructed the three-dimensional models shown in Figure 3. Based on these models, the corresponding extracellular domains (domain 1 and domain 2) of the rainbow trout GHR1 and GHR2s, contain six β strands that form the two β sheets. Trout PRLR contains 6 β strands in domain 1 and 7 β strands in domain 2 (Fig. 3).

Cysteine residues in the extracellular domain are involved in disulfide bonds that have numerous important roles including involvement in the folding of the protein, the efficiency in the dimerization of the units within the receptor, and the impact the overall rigidity of the receptor, as well as the ability of the receptor to bind the ligand. Ultimately, the three dimensional shape of the receptor and its rigidity will affect the conformational changes that take place to induce cellular effects. In hGHR, there are 7 cysteine residues, six of which are found in domain 1 of the extracellular portion of the receptor (de Vos *et al*., 1992). Similar to the pattern seen in hGHR, rainbow trout GHR1 have 7 cysteine residues in the extracellular domain, 6 of which are found in domain 1 with the last being found in domain 2. In rainbow trout, GHR2a and GHR2b have 5

cysteines that are conserved, 4 of which are found in domain 1 with the last, then, found in domain 2 (Very *et al*., 2005).

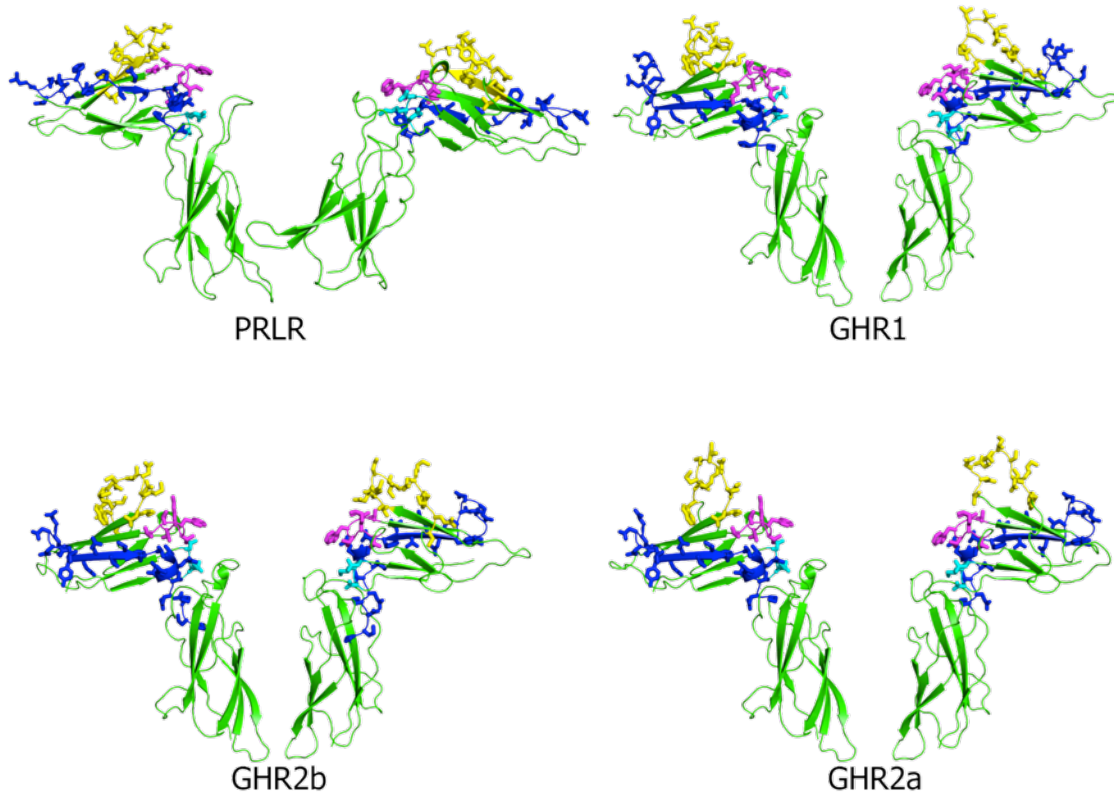


Figure 3. Receptor models were created using personalized pdb files based on the protein sequence of trout GHRs and PRLR. Models were made using SWISS-MODEL workspace (Bordoll et. al. 2006; Bordoll et. al. 2009) based on the crystal structures of GH-GHR_2 (PBD ID: 3hhr; de Vos et. al. 1992) and PRL-$PRLR_2$ (PDB ID: 3ew3; Broutin et. al. 2010) and visualized using POLYVIEW-3D (Porollo et. al. 2007).

Also conserved in the extracellular domain are potential N-glycosylation sites, which may be involved in cell surface targeting (Buteau *et al.,*1998). Five potential N-glycosylation sites exist in the extracellular domain of each rainbow trout GHR2, two of which are highly conserved in vertebrates; these highly conserved sites, in trout GHR2a/GHR2b, are found, respectively, at amino acids 140/144 and 188/184 (Very *et al*., 2005). A third potential N-glycosylation site, at amino acid 58/61, is conserved in all

fish examined, frog, turtle, and some birds, but is absent in higher vertebrates. Finally, located at amino acids 97/101 of trout GHR2s is the last potential N-glycosylation site, which is less conserved in fish (found only in salmonids, carp, and goldfish) but is conserved in all non-fish vertebrates with the exception of the frog. In the GHR1 (previously SLR) of Atlantic salmon, only 4 potential N-glycosylation sites are present (Benedet *et al.,* 2008); there are 5 potential sites in masu salmon GHR1 (previously SLR) (Fukada *et al*., 2005). Additional information on the single GHR1 that has been isolated in trout (Walock, Kittilson, and Sheridan, unpublished observation), will be available upon the finalization of its characterization.

Domain 1 of the extracellular domain includes the contact region for ligand binding. Both of the receptors involved in the dimerized complex, whether in GHR or PRLR, use the same regions to bind GH or PRL (Brooks and Waters, 2010; Broutin *et al.,* 2010). These regions of each receptor that in proximity to the ligand is encoded by four stretches of sequential amino acids, with each stretch found intermittently on the mRNA strand. The correct folding of the protein brings these four regions together to create the face that would directly face the ligand and would be directly involved in the receptor-ligand interaction. Based on our three-dimensional models of PRLR, GHR1, GHR2a, and GHR2b of rainbow trout shown in Figure 3, four sequence stretches that make up that face also are present and are indicated by the four colors, cyan, yellow, pink, and blue (in order, encoded from the 5' end of the respective mRNA to the 3'end; cf. Rouzic *et al*., 2001; Very *et al*., 2005; Walock, Kittislon, and Sheridan, unpublished observation).

Upon binding, the resulting conformational structure of each receptor of the dimer pair differ, as they would need to in order to adjust their conformation for the binding of the diverse and asymmetrically located sites 1 and 2 of GH. Depending on which receptor in the dimer receives the binding site 1 of the ligand and which receives the binding site 2 of the ligand determines which of the receptors in the dimer will take on the differing conformational changes, a concept that particularly important if heterodimerization were occurring between the GHR types or between the GHR types and PRLR. Indeed, preliminary evidence that a functional hPRLR-hGHR heterodimer either exists, or may be formed, has been shown (Langenheim and Chen, 2009). The existence and extent of heterodimerization between these receptors in fish have not been explored. Heterodimerization may certainly influence the relative receptor rotation and consequently the alignment of the intracellular domains and have important implications in the multi-functionality of GH signaling.

Among the different GHRs of rainbow trout, slight differences can be seen, especially when comparing the four contact regions of one receptor amongst the corresponding four contact regions in the other receptors (see Fig. 3). Although each of the receptors in the dimer pair have been modeled individually for this figure, they each have been modeled in accordance with one of the two receptors in the dimer of the 1:2 GH-GHR2 crystal structure (PDB ID: 3hhr). The trout PRLR has been modeled based on the 1:2 PRL:PRLR2 crystal structure (PDB ID: 3ew3) Thus, since the receptor is essentially able to reconfigure to interact appropriately with the ligand, the conformational configurations of the receptors in each dimer pair are reflective of the conformational changes that occur upon ligand binding, except that the two receptors

would, in reality, be in closer proximity to each other. In hGHR, both fibronectin III domains are believed to contribute a key tryptophan residue, in a precise orientation, that allows growth hormone to be locked into place through strong hydrophobic interactions (Brooks and Waters, 2010).

The second of the fibronectin-III motifs, found proximal to the cell membrane, is the extracellular domain 2 and contains the receptor–receptor dimerization area. Upon the binding of a ligand to the dimer-complex, there is a rotation of receptor subunits relative to one other which results in the locking between the receptor units at the receptor-receptor dimerization/interaction domain (Brooks and Waters, 2010). Mutations in the extracellular dimerization domain disrupt cell surface targeting and signaling without altering the binding capacity (Brooks and Waters, 2010; Van Agthoven *et al*., 2010).

Also found in the extracellular domain 2 of these receptors in trout is the FGEFS motif; this motif is the homologous WSXWS sequence-motif common of all cytokine receptors, which has been implicated to have numerous roles. In humans, this motif is seen as YGEFS, whereas in fish this motif is seen as conservative variations of FGEFS (Brooks and Waters, 2010; . In salmonids, the FGEFS motif in GHR2s and GHR1s is conserved as FGEFS (Fukada *et al*., 2004; Very *et al*., 2005; Benedet *et al*., 2008). It is interesting to note that there are some substitutions between GHR paralogues in certain species. For example, the first amino acid of this motif in Japanese eel (*Anguilla japonica*) GHR1 is the conserved phenylalanine, whereas the first amino acid of this motif in eel GHR2 is alanine (Ozaki *et al.,* 2006). This motif may be involved in maintaining the structural integrity of the extracellular domain, and may do so by playing a role in the proper folding of the fibronectin motif (Brooks and Waters, 2010). This

notion was supported by observations that three separate mutations (Tyr222His, Glu224Asp, Ser226Ile) in the YGEFS motif of humans resulted in Laron syndrome, a condition of growth hormone insensitivity due to the loss growth hormone receptor function (Brooks and Waters, 2010). This motif also may be important for receptor signal transduction, a possibility supported by a study by Baumgartner *et al.* (1994) that showed that a mutation at the first or final amino acid of the motif affected signal transduction. Furthermore, recent work has indicated that this motif is involved in transactivation. The mutation S226A was shown to be transcriptionally inactive (Conway-Campbell *et al.,* 2008). This serine is conserved as part of the FGEFS motif in the GHRs of fish. Two other residues in this consensus motif appear to be unnecessary for transcriptional activity because the Y222A (YGEFS) and the E224A (YGEFS) mutants retain full transcriptional activity (Conway-Campbell *et al.,* 2008). The significance of these residues may just simply fall elsewhere. This motif has also been shown to bind a co-activator and splicing protein, co-activator activator (CoAA), in response to the binding of GH; there is growing evidence to support that CoAA acts as a chaperone to promote nuclear localization of the full-length growth hormone receptor (Brooks and Waters, 2010; Conway-Campbell *et. al.,* 2008). Each of these observations supports the notion that the WSXWS motif in cytokine receptors is important for receptor activation (Dagil *et al.,* 2012).

There is tremendous plasticity in these receptors considering the flexibility of the conformational adjustments that occur to accommodate various ligands and the ligands' asymmetrical binding sites, and this certainly has implications in the proper alignments that are necessary to produce intracellular effects. The single transmembrane domain, a

characteristic of receptors belonging to the type-I cytokine receptor superfamily, is short (19 amino acids long in rainbow trout GHR2a and GHR2b) and plays a major role in initiating the presumed ligand-induced realignment of the intracellular domains, which leads to the differing intracellular signaling effects. An investigation and modeling of the undefined linker region between domain 2 and the transmembrane domain by using Pro and Gly substitutions within the linker sequence (13 amino acid long in hGHR) in mammals showed that alterations in this linker sequence alters the change in orientation of the two linker regions, relative to each other, upon ligand binding; a change in orientation would inadvertently change the orientation of the transmembrane helices (Rowlinson *et al.,* 2008). Furthermore, the investigators showed that differing ligand-induced conformational changes in the growth hormone receptors determines the choice of signaling pathway. Thus, it seems that the degree with which the ligand can alter the extracellular domains of the two GHRs of the dimer thus determines the relative movements of these linker regions and consequently, the alterations in the orientation of the transmembrane domains and presumably the intracellular domains.

Also characteristic of this family of receptors is the presence of numerous tyrosine residues, as well as the cytoplasmic Box 1, Box 2, and, more recently revealed, Box 3 regions in the intracellular domain. Truncation of the membrane-bound receptor, below the cell membrane, would presumably alter cell signaling, as well as other functions that may be associated with the intracellular domain, such as receptor internalization or nuclear translocation as displayed in mammals (Conway-Campbell *et al.*, 2008; Lobie *et al*., 1992); although, no such studies have been conducted in fish.

Intracellular tyrosine residues have been reported to be involved in the GH-dependent phosphorylation of signal transducer and activator of transcription (STAT) proteins (Kopchick and Andry, 2000; Gorisson *et al.,* 2011). The Janus kinase (JAK) family of protein kinases is known to phosphorylate STAT proteins. Like the GH-family ligands and receptors, the genes encoding these signaling elements have been duplicated in the same whole genome duplication events discussed earlier, and a phylogenetic analysis by Gorisson *et al.* (2011) showed that teleostean STATs are orthologues of the mammalian STATs, and the teleostean genes that encode them have somehow been scattered over their genome. STAT3 and STAT5 and the other STAT family members, as most of the classical hormones within the class-I helical cytokine family, such as GH, PRL, and EPO, signal predominantly via STAT3 and STAT5, whereas the other members of the STAT family serve predominantly in the immune response (Horvath *et al.,*1995; Gorisson *et al.,* 2011). Jak2 in mammals is constitutively associated with the GHR and PRLR and is activated by ligands that induce the phosphorylation and activation of STAT5 (Kelly *et al.,* 1994; Kopchick and Andry, 2000; Cesena *et al.,* 2007; O'Sullivan *et al*., 2007; Gorisson *et al.,* 2011). The phosphorylated tyrosines in the N-terminal half of the cytoplasmic domain of rat GHR are believed to be involved in the maximal activation of STAT1 and STAT3, in response to GH stimulation and GH-induced tyrosyl phosphorylation of STAT1 and STAT3 in mammalian GHR is followed by the binding of these STATs to the sis inducible element (SIE) of c-fos (Herrington *et al.,* 2000). Interestingly, a heterodimer of ovine GHR and ovine PRLR in living cells was demonstrated by fluorescence resonance energy transfer (FRET) microscopy and was shown to have prolonged phosphorylation of STAT1 and STAT3 (Biener *et al*.,

2003). Surely, the differences in the intracellular domains, including the number and location of the intracellular tyrosine residues among the GHRs and PRLs in fish would affect signaling although such studies have yet to be reported.

The sequence of Box 1 in both trout GHRs is LLPPIPGP. Fish GHRs show only a few conservative amino acid substitutions in Box 1. The proline-rich Box 1 motif has been shown to be involved in the JAK2-STAT signaling pathway in mammals (Brooks *et al*., 2008; Brooks and Waters, 2010). Until recently, JAK2 was believed to exclusively mediate the signaling by the GHR. However, a recent study using transgenic mice with mutations in the Box 1 region that disabled 4 proline residues required for JAK2 activation, found that an inability to activate JAK2 by the GHR does not block its ability to activate SFK or pathways that can be initiated by SFK (notably the ERK pathway in liver) (Barclay *et al.,* 2010). In Atlantic salmon GHR1, Box 1 shows slight differences; LLPPVPAPKIKGI versus the LLPPIPGPKIKGI as seen in the trout GHR2s (Very *et al.,* 2005; Benedet *et al.,* 2008).

The Box 2 motif is a highly acidic/hydrophobic motif that has been recognized to be involved in both cell signaling and signal degradation by receptor internalization. In addition to the binding of JAK2 to Box 1, JAK2 was shown to bind to Box 2 in mammals (Liongue and Alister, 2007). In addition to being involved in signaling, this motif is also recognized as the site of recruitment for an ubiquitin conjugation system to the receptor, necessary for the ubiquitin-dependent mechanism for GHR internalization (Grovers *et al.,* 1999). This motif, DSWVEFIELD in humans, is also known as the ubiquitin-dependent endocytosis (UbE)-motif for this reason. Upon binding of an active ubiquitin-conjugation system to the receptor, the ligand-receptor complex is internalized by the actions of

clathrin-coated vesicles; the receptor complex is subsequently transported to lysosomes by way of endosomes (Gent *et al.,* 2002). This motif is homologous to sequences in other proteins, several of which are known to be ubiquitinated, including PRLR (Grovers *et al.*1999). The Box 2 motif in trout is DPWVEFIELD (Very *et al.,* 2005); therefore, it likely plays roles similar to those in mammals.

With the characterization of numerous GHR receptors from a number of species of fish, the presence of a third conserved region in the intracellular domain of the GHR in fish is now apparent and was pointed out by Di Prinzio *et al.* (2010) and named Box 3. This domain in zebrafish GHR1 (called ghra by the investigators) has the sequence DDDSGRASCYDPE, whereas zebrafish GHR2 (ghrb) has the sequence DDDSGWASCCDPD. In rainbow trout, the sequence is DDDSGRASCCDPD for both GHR2a and GHR2b (Very *et al.*, 2005). The functional relevance of this domain has not been investigated, but the fact that it is a highly conserved, acidic motif of the intracellular domain, distal to the cell membrane, suggests a possible role in signal transduction.

Sources of variation in GH receptor structure

A number of alternatively spliced variants of membrane-bound GHRs have been identified in fish and mammals. To date, the presence of alternative transcripts have not been examined in trout; however, the presence of fish GHRs with truncated and long isoforms of membrane-bound GHRs have been characterized in turbot (*Scophthalmus maximus*) (Calduch-Giner *et al.,* 2001), black seabream (*Spondyliosoma cantharus*) (Tse *et al.,* 2003), and gilthead seabream (*Sparus aurata*)(Calduch-Giner *et al.,*2003). In adult

zebrafish, southern blots revealed the existence of two shorter amplification products (~500bp and ~700bp) in addition to the 898bp, full-length, GHR1 product. The zGHR2 also appears to have a shorter, alternative, amplification product; the full-length GHR2 southern blot fragment is 701bp while this alternative is 450bp. In Jian Carp, different jcGHR transcripts were found in the liver; jcGHR 1b' appears to have lost exon 4, jcGHR2a' appears to retain intron 3, which is normally spliced out, and jcGHR 2as has lost a portion of exon 8 (Yu *et al.,* 2011).

Besides the presence of multiple genes, alternate transcripts or alternative splicing of the duplicated GHR genes may be a principal source of differential functioning of these receptors as a means to modulate GH actions. In the adult zebrafish transcripts just mentioned, the 500bp GHR1-product was expressed in all tissues where the full-length GHR1 was amplified, whereas the 700bp GHR1-product was only detected in liver. The shorter amplification product of GHR2 was amplified in all tissues where the full-length GHR2 product was amplified (Di Prinzio *et al*., 2010). Interestingly, the presence of alternative transcripts of the GHR1 and GHR2 receptors differed during embryonic and early larval development, in zebrafish; while there were no observed alternative transcripts of GHR1 detected during development, three smaller GHR2 transcripts, in addition to the full-length GHR2 transcript, were differentially detected during various developmental stages. Southern blot analysis indicated these fragments to be 650, 470, and 420bp in size; when recalling that the single alternative GHR2 transcript was 450pb, it appears that GHR2 has a total of 4 alternative transcripts that are shorter than the full length GHR2 transcript and are differentially expressed throughout the life cycle of the zebrafish.

The hGHR is known to have up to 7 different promoters in exon 1 (Waters and Brooks, 2011). To date, the genomic organization of GHRs, in fish, have been investigated in turbot, gilthead sea bream (Perez-Sanchez *et al*., 2002), Japanese flounder (*Paralichthys olivaceus*) (Nakao *et al.,* 2004), zebrafish (Di Prinzio *et al.,* 2010), torafugu (*Takifugu rubripes*) (Hirano *et al.,* 2011), and Atlantic halibut (*Hippoglossus hippoglossus*) (Hildahl *et al.,* 2007), although the regulatory elements have not been extensively examined in fish. Furthermore, alternative splicing adds to the potential for dynamic, tissue-specific, receptor expression; diverse patterns of expression in response to variable factors are likely numerable for these receptors. In mammals, for example, the expression of exon 1A is known to be "liver specific" in humans, mouse, and rat and shows significant differences in expression patterns between the sexes. Besides the membrane bound receptor in the liver, exon 1A is expressed in the GHBP transcript (Waters and Brooks, 2011). Further studies to characterize the promoter and regulatory elements involved in variable receptor expression, as well as establishing the patterns of transcript expression and alternative splicing of GHRs and PRLR, in fish and in particular trout, are warranted.

The presence of a truncated form of GHR known as growth hormone binding protein (GHBP), has not been characterized in trout or any other salmonids to date. However, previous work by Sohm *et al.* (1998) showed that a monoclonal antibody to the extracellular region of hGHR was able to precipitate a GH complex from the serum of rainbow trout, indicating that a truncated mRNA resulting from alternative splicing, a proteolytic cleavage event, or a combination of both mechanisms, remains to be identified. The production of GHBP by both of these mechanisms has been observed in a

wide variety of vertebrate species. The GHBP is well conserved and the development of two mechanisms to produce GHBP infers the soluble binding protein is important. The production of GHBP by alternative splicing of the full-length GHR gene was reported in fish and mammals, including in turbot (Calduch-Giner *et al.,* 2001), Chinese sturgeon (*Acipenser sinensis*) (Liao *et al.,* 2004), and rodents (Smith *et al.,*1989). The production of GHBP by both mechanisms has been reported mammals, including monkey (Martini *et al.,*1997). In these alternatively spliced GHBPs, the sequence encoding the extracellular portion of the receptor differs in just a few base pairs before the hydrophobic transmembrane domain; after this divergence in the sequence a short (17-27bp) hydrophilic sequence and a divergent 3'UTR, replaces the transmembrane, intracellular domain, and 3'UTR of the full length receptor. The hydrophilic tail is encoded by an extra exon found between exon 7 (extracellular) and exon 8 (transmembrane), called exon 8A. Exon 8A is not known to be present in GHBPs produced by proteolytic cleavage (Zhang *et al*., 2000). The GHBP may have several important roles in the modulation of growth. GH bound to GHBP has an increased half-life compared to the free hormone (Baumann, 1991a), thus GHBP may represents a hormone-reservoir in serum (Baumann, 1991b; Sohm *et. al.,* 1998). GHBP may also act to negatively regulate GH actions in several ways: 1) by competing with membrane bound GHRs for ligands, 2) by forming GHR-GHBP dimers, resulting in an altered or ablated signaling response, or 3) by simply down-regulating membrane bound GHR abundance. The production of GHBP could be controlled in a tissue specific manner as a means of altering sensitivity to circulating GH in a localized manner and, furthermore, could be done in response to certain physiological conditions (Sohm *et al.,* 1998). A fourfold increase in the binding

capacity of GHBP has been noted to occur 48 h following seawater transfer, suggesting a potential role during seawater adaptation in salmonids (Sohm *et al.,* 1998).

GHBP has also been shown to be a transcriptional activator. The presence of both the full GHR and the alternatively spliced GHBP in the cell nucleus has been reported in mammals (Lobie *et al.,* 1992; Graichen *et al.,* 2003; Conway-Campbell *et al.,* 2008). Several proteins, relevant to transcriptional activation in the cell nucleus, have been found to bind the transcriptionally active GHBP. Two of these proteins include a nucleoporin and the coactivator activator (CoAA), which is a transcriptional regulator, in addition to an RNA binding-splicing protein (Conway-Campbell *et al*., 2008). The knowledge of the presence of an alternatively spliced GHBP in the nucleus is relatively new and has not, thus far, been explored in fish. The presence of an alternatively spliced or proteolytically cleaved GHBP in the circulation and in the nucleus of trout warrants further studies.

Interestingly, numerous reports of metal ion interactions with the type-1 cytokine receptors and their ligands have been reported, most notably for zinc. Although zinc is not required for the binding of GH or PRL to their receptors, the binding affinity between hGH and hPRLBP (extracellular binding domain of the hPRL receptor) was increased about 8000-fold by addition of 50 μM $ZnCl_2$ (Cunningham *et al.,* 1990). In this particular investigation, the hPRLBP and hGHBP were expressed and secreted into the periplasm of *Escherichia coli*, and binding determined with hGH and hPRL were analyzed. This study indicated that [Zn^{2+}] was required for tight binding of hGH to the hPRL receptor but not for binding to the hGH receptor. Furthermore, the binding of hGH to hPRLBP, under these conditions, is nearly 100-fold stronger than for hPRL and more than 10-fold

stronger than the affinity of hGH for one hGHBP (Cunningham *et al.,*1990). These results have been shown to be due to the direct interaction between Zn^{2+}, the hormone, and the extracellular receptor (Cunningham *et al.,*1990;Voorhees *et al.,* 2011). To date, the interaction of Zn^{2+} or any other metal ion with these GH-family receptors has not been examined in fish.

Overall, there are numerous sources of structural variation in these receptors. Structural features of GHR, and comparatively of PRLR, have important implications regarding its function, a function that is dependent upon the interactions with ligands in the extracellular domain and signaling proteins in the intracellular domain. Modification of the structure results in modification of the function and the mechanisms by which this occurs are beginning to be defined by accumulating evidence on many fronts.

Differential and overlapping aspects of GH signaling

The mechanistic basis that underlies the multifunctionality of GH and the means by which target cells adjust their responsiveness to GH to adapt to new environmental conditions is unknown. However, information from studies in trout and other species of fish suggests that differential expression of GHR types as well as differential linkages of GHR types to cell signaling pathways may play a role.

Differential expression

GHR mRNA and protein are expressed in numerous tissues, and although the receptor types are often expressed together in these tissues, distinct and differential expression patterns are evident. In rainbow trout, GHR2a is more abundant in the brain

while GHR2b is more abundant in spleen and pancreas (Very *et al.,* 2005). It is interesting to note that there have emerged distinct expression patterns that are apparently species specific. In Mozambique tilapia (*Oreochromis mossambicus*), GHR2 expression is highest in muscle, followed by the heart, testes, and liver; whereas, GHR1 expression is highest in adipose tissue, liver, muscle, and skin (Pierce *et al.,* 2007). In Nile tilapia (*O. niloticus*), expression of GHR2 is higher, overall, than GHR1 in all tissues except muscle; both GHR1 and GHR2 are most highly expressed in liver and muscle. GHR2 expression was also high in kidney and stomach, while there was little expression of GHR1 in these tissues. In Zanzibar tilapia (*O. urolepis hornorum*), GHR2 expression was overall higher than GHR1 (Ma *et al.,* 2007). GHR2 expression was highest in liver, muscle, and intestine while GHR1 expression was high in liver, muscle, and brain. Interestingly, GHR1 expression in tissues taken from females was higher than that in tissues from male Zanzibar tilapia (Gao *et al.,* 2011). In black seabream, the expression of GHR2 is higher, overall, than the expression of GHR1 in many tissues including gonad, kidney, muscle, pituitary, and spleen (Jiao *et al.,* 2006). In gilthead seabream, GHR1 mRNA was highly abundant in liver and adipose tissue (Saera-Vila *et al.,* 2005). In zebrafish, both receptors, GHR1 (ghra) and GHR2 (ghrb), were predominantly expressed in intestine and liver, while also being expressed in kidney, spleen, and ovary; GHR2, but not GHR1, was expressed in muscle (Di Prinzio *et al.,* 2010). Expression levels of the four GHRs in Jian carp (jcGHRs) were reported and the expression of all four were highest in muscle. Differential expression was seen, however; except in brain, jcGHR2b expression was higher than the other 3 genes in the remainder of the tissues that were investigated, which included liver, heart, head kidney, kidney, intestines, spleen and muscle (Yu *et al.,* 2011).

The differential expression of GHRs occurs in a tissue and species-specific manner, which may be indicative of specific adaptations for coordinating growth with other physiological processes that are reflective of particular developmental stages in unique life histories and in response to variable environmental factors. For example, the pattern of expression of GHR types changes over the course of embryonic development (Ma *et al.*, 2007; Raine *et al.*, 2007; Malkuch *et al.*, 2008) and in the face of altered nutritional states or osmotic environments (Saera-Vila *et al*., 2005; Norbeck *et al*., 2007; Pierce *et al.*, 2007; Poppinga *et al.*, 2007).

Differential receptor binding characteristics

As mentioned earlier, although trout GHRs bind GH, they also can bind PRL and SL. Competitive ligand binding using ^{125}I-GH with CHO-K1 cells expressing rainbow trout GHR2a or rainbow trout GHR2b showed that both receptors preferentially bind GH over PRL or SL, but GHR2b does so to a greater degree (Reindl *et al.,* 2009). This ligand selective binding of trout GHR is diagrammatically represented in Figure 4. To date, there have been few other GHR binding studies in fish. In masu salmon, SL could not displace ^{125}I-GH from GHR 2 (Fukada *et al.*, 2004), whereas GH and PRL were less effective than SL in displacing ^{125}I-SL from GHR1 (Fukada *et al*., 2005), findings that prompted the name SLR in salmon instead of GHR1. There are at least two reasons for not using the term SLR to describe GHR1, especially in species other than salmon. First, the binding of GH to eel GHR1 could not be displaced by SL (Ozaki *et al*., 2006). Second, GH, but not SL, activated both seabream GHR1 and GHR2 transcription reporter systems (Jiao *et al*., 2006; discussed in greater detail below). In the end, it

appears that the ability for these ligands and receptors to cross-bind (e.g., GH with PRLR or PRL with GHR) and elicit cellular effects in response to cross-binding may be species specific.

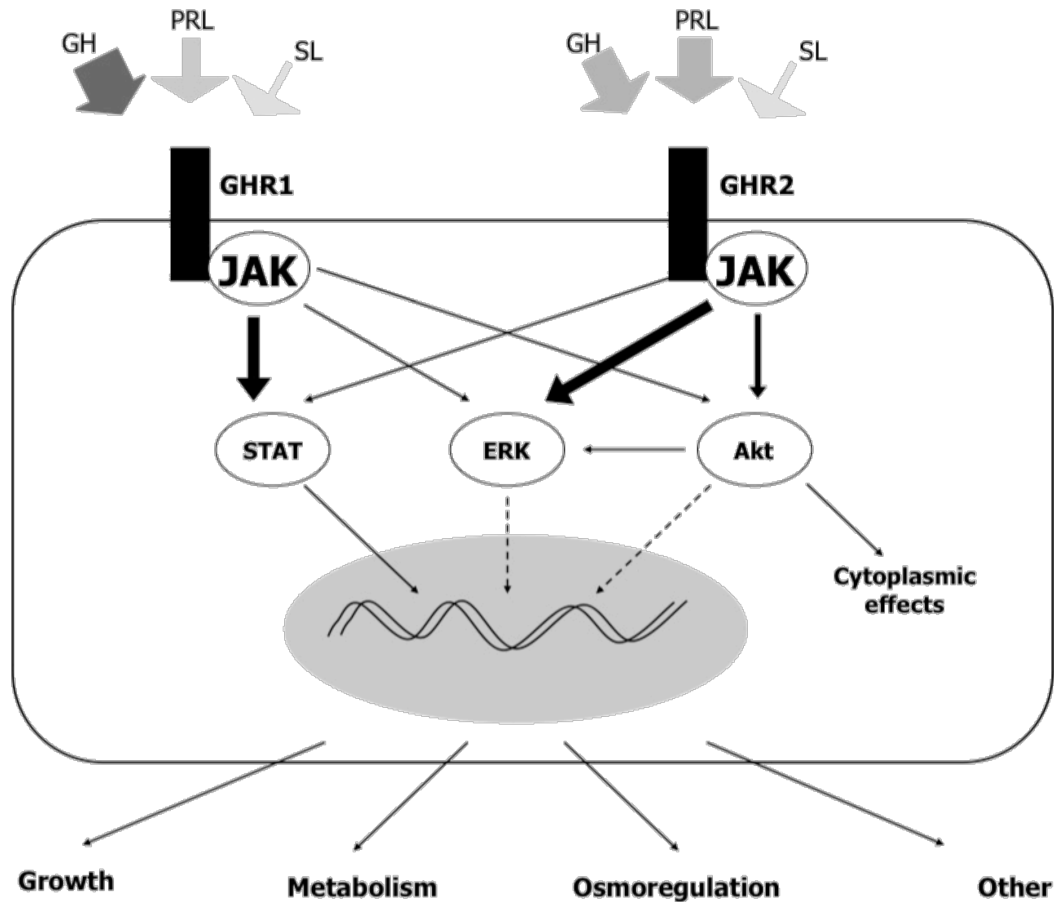


Figure 4. Model of differential activation of signaling pathways by growth hormone receptor (GHR) subtypes: JAK2 activation is essential for propagation of signaling from both GHR1 and GHR2 to the ERK, PI3K/Akt, and STAT5 pathways (details of pathway elements are omitted for simplicity). Cross-talk occurs between the ERK and PI3K/Akt pathways, possibly through Akt activation of c-Raf in the ERK pathway.

GH signal transduction and differential linkage to effector pathways

In mammals, GHR is well recognized as utilizing the JAK/STAT signaling pathway (Carter-Su *et al*., 1996; Brooks *et al*., 2008). More recently, evidence has

indicated that GHR also signals via Src-family tyrosine kinases (SFKs), independent of JAK2, resulting in the activation of linked extracellular-regulated kinases (ERKs) 1 and 2 (p44/42) (Brooks and Waters, 2010). The prolactin receptor is also known to utilize both the JAK/STAT and the SFK/ERK signaling pathways (Fresno-Vara *et al.,* 2001). SFK activation by GH has been shown to be linked to ERK1 (mitogen-activated protein kinase 3) and ERK2 (mitogen-activated protein kinase 1) through a phospholipase C pathway (Brooks and Waters, 2010).

Using hepatocytes isolated from rainbow trout, Reindl *et al.* (2011) examined the cellular signaling pathways activated by GH. GH directly stimulated the phosphorylation of ERK, protein kinase B (Akt), a downstream target of phosphatidylinositol 3-kinase (PI3K), JAK2, and STAT 5. Selective inhibitors for JAK2 (1,2,3,4,5,6-hexabromocyclohexane), MEK (U0126), and PI3K (LY294002), showed that the activation of the ERK and PI3K/Akt pathways were depended on JAK2 and that there was crosstalk between the ERK and PI3K/Akt pathways (Reindl *et al*., 2011). Selective inhibitors also were used to demonstrate that GH-stimulated IGF-1 synthesis and secretion is mediated through the JAK-STAT, ERK, and PI3K/Akt pathways (Reindl *et al*., 2011).

The possibility that the trout GHR types differentially link to cellular effector pathways was examined in CHO cells individually transfected with plasmids containing the recombinant rainbow trout GHR DNAs. GHR2a preferentially activated STAT5, whereas GHR2b preferentially activated the ERK and Akt signaling pathways (Kittilson *et al.,* 2011a). A model depicting the differential linkage is shown in Figure 4. Taken together with the binding data mentioned above, the activation of a particular signaling

pathway appears to depend on the form of GH-family peptide present and on the particular GHR subtype (which differs in its affinity to the various ligands) expressed on a target cell.

Differential cellular signaling by the multiple receptors has also been shown in other species, and there appears to be specific effector system-biological response linkages. The biological activities of GHR1 and GHR2 in black seabream (bsb) were examined in the presence of seabream (sbGH), salmon GH (sGH), salmon prolactin (sPRL), and salmon somatolactin (sSL). In CHO cells expressing bsbGHR1 and bsbGHR2, several transcription factors, known in the mammalian GHR gene to be activated upon GH stimulation, were shown to be differentially activated in this system. The first of these transcription factors, the Spi 2.1 promoter, is activated in response to signaling events involving JAK2 (Goujon *et al.,*1994; Sotiropoulos *et al.,*1994; Dinerstein *et al.,*1995; Gong *et al.,*1998). The β-casein promoter, the second of these transcription factors, is known to be activated in response to events involving STAT1 and STAT5. Finally, the c-fos promoter is believed to be activated upon the induction of events involving STAT1 and STAT3 (Smit *et al.,*1997; Gerland *et al.,* 2000; Jiao *et al.,* 2006).

In cells expressing bsbGHR1, sbGH and sGH could stimulate all three promoters (Spi 2.1 promoter, β-casein promoter, and c-fos promoter), whereas sSL and sPRL did not induce the activation of any of these promoters (Jiao *et al.,* 2006). In bsb2GHR2, sbGH activated the Spi 2.1 promoter and β-casein promoter, but not the c-fos promoter. sGH only activated the β-casein promoter, while sSL and sPRL did not activate any of the promoters (Jiao *et al.,* 2006). More recently, Chen *et al*. (2011) showed that neither

zebrafish SLα nor zebrafish SLβ could promote GHR1- or GHR2-mediated phosphorylation of ERK and Akt. In addition, whereas zebrafish GH could induce promoter activity of the Spi 2.1 promoter, β-casein promoter, and c-fos promoter in cells independently expressing zebrafish GHR1(GHRa) and GHR2(GHRb), neither zebrafish SLα nor zebrafish SLβ affected promoter activity in GHR-expressing cells. These results indicate that interactions of the GHRs with a ligand could induce different biological activities, and that this may be the case despite the affinity of the receptor for the ligand. However, ligand specificity or affinity could not be deduced by using this approach, since binding affinity may not always correlate with the degree of signaling the binding of a ligand may induce.

GH signaling and the regulation of growth

Fish are the most diverse group of vertebrates. They occur in a wide range of aquatic habitats including ephemeral streams and lakes, desert springs, open ocean and deep ocean trenches up to 9000 m deep), coastal estuaries, and mountain streams and lakes (up to 4500 m), and have evolved elegant and often elaborate life history patterns to adjust to the conditions present in their environment. Thus, it is not surprising that intrinsic (e.g., stage of life history, including sexual maturation) and extrinsic factors (nutritional status, temperature, photoperiod, salinity, etc.) modulate the growth of fish (Reinecke, 2010; Sheridan, 2011). The integration of such factors in concert with the genetic program of the fish, which for many species including rainbow trout enables indeterminate growth (Mommsen, 2001), leads to the coordination of growth through the interplay of numerous hormones.

As mentioned within the introduction, the primary hormonal control of growth in vertebrates is the GH-IGF system. Much of the research on the regulation of growth has focused on the production and release of GH, and there are several recent reviews that amply describe the dual antagonistic hypothalamic control of GH release (Canosa *et al*., 2007; Chang and Wong, 2009; see chapter by Canosa *et al.*, this volume). Factors produced by the peripheral organs and delivered to the pituitary through the systemic circulation also can affect GH release. For example, cortisol, thyroxin, 17β-estradiol, ghrelin (which is produced in the stomach as well as the hypothalamus), and leptin can stimulate GH release, whereas IGF-1 and somatostatin (which is produced in the gut and pancreas as well as in the hypothalamus) inhibit GH release (Sheridan, 2011). Increasing evidence suggests that regulation of growth also involves peripheral controls, including "tuning" GH sensitivity and post GHR events as well as modulation of downstream events such as IGF sensitivity and action.

A model of peripheral regulation

The membrane-bound GHR is a major means whereby growth can be modulated, and its actions and regulation in trout have become well documented in recent years. Extra-pituitary actions of GH, upon binding to the GHR, can control growth in several ways, as shown in Figure 5. GH can indirectly stimulate growth by binding to GHR on the liver, inducing the synthesis and secretion of IGF-1 from the liver. IGF-1, in turn, stimulates cell growth and differentiation in a variety of target tissues upon binding to distinct IGF-1 receptors (IGFR1) (Laviola *et al.,* 2007, Wood *et al.,* 2005, Reindl *et al.,* 2011). GH may also bind to GHR located on the pancreas, and in this way indirectly

modulate growth by the controlled/conditional induction of insulin (INS) (Caruso *et al.,* 2010; Caruso and Sheridan*,* 2011). INS works in concert with GH to promote growth in fish; INS deficiency results in growth retardation (Plisetskaya and Duan, 1994). The actions of INS to promote growth are many fold. INS synergizes with GH to stimulate hepatic IGF-1 production; and INS deficiency reduces hepatic IGF-1 mRNA levels (Duan *et al.*, 1994, Duan and Plisetskaya, 1994). INS and GH also increase peripheral sensitivity to IGF by increasing expression of IGF receptors (Very *et al*., 2008). Counter regulatory feedback mechanisms also operate to inhibit GH-stimulated growth. These include GH-stimulated expression of somatostatin (SS) expression from islet cells (Melroe *et al.,* 2004), as well as SS-induced reductions in the expression of INS, GHRs, IGFs, and IGFRs (Sheridan and Kittilson, 2004; Hagemeister and Sheridan, 2008; Caruso and Sheridan, 2011, 2012). This system does not monotonically proceed without regard to the physiological/developmental state of the organisms, for a constant and simultaneous induction of these hormones and their receptors would be futile. In response to extrinsic and intrinsic factors, feedback regulatory mechanisms are becoming apparent, and seem to be a means of integrating growth with other processes in order to appropriately respond to variable factors. The differential expression of GHRs, as well as the differential cellular functioning of GHRs, are becoming increasingly clear and are emerging as major mechanisms by which this integration can occur, as will be discussed .

GH regulated GHR expression

GH has been shown to promote growth, but with somewhat varying efficacy. Increased growth responses have been reported *in vivo* and *in vitro* for salmonid species,

but these seem to vary in degree depending on the species and even upon the strain of species. These increased growth responses have been reported in GH-transgenic coho salmon and wild-strain, GH-transgenic trout (Ahrens and Devlin, 2011). Since the effects of GH are dependent upon the binding of GH receptors, the effect of GH on the expression of GHRs is potentially a major mechanism by which these increased growth responses occur. Isolated rainbow trout hepatocytes show a 2.5- to 3-fold increase in transcription rates of both GHR2a and GHR2b in response to GH treatment (Very and Sheridan, 2007). However, it is important to note that the growth response to GH is dynamic and species-specific. In comparison to the wild-strain of the GH-transgenic trout that showed increased growth responses, the domestic strain of the same species of trout, under the same treatment, displayed only a marginal increase in growth (Devlin *et al.,* 2001). Likewise, a study with GH-transgenic zebrafish, using hemizygous and homozygous genotypes, revealed that body mass, GHR and IGF-1 mRNA expression, and condition factor were increased in hemizygous fish compared to homozygous fish (Figueiredo *et al.,* 2007). In a study with GH-transgenic common carp, there were no significant differences in total length, body weight, or condition factor between the GH-transgenic and non-transgenic genotypes when fed to satiation and reared separately (Duan *et al.,* 2011). Also, *in vivo* treatment of black seabream with seabream GH did not alter hepatic GHR2a or GHR2b mRNA expression (Jiao *et al.,* 2006).

The differential responses of various organisms to GH may ultimately be due to several factors. Certainly, the phenotypic and genetic character of the species or strain will put limitations on the responses to GH, a conclusion that logically coincides with the

variable structures of the GHRs discussed earlier in this chapter. The extra disulfide bond in the extracellular region of trout GHR1, for example, could reasonably limit conformational changes necessary for both ligand interactions and subsequent activation of cellular activities.

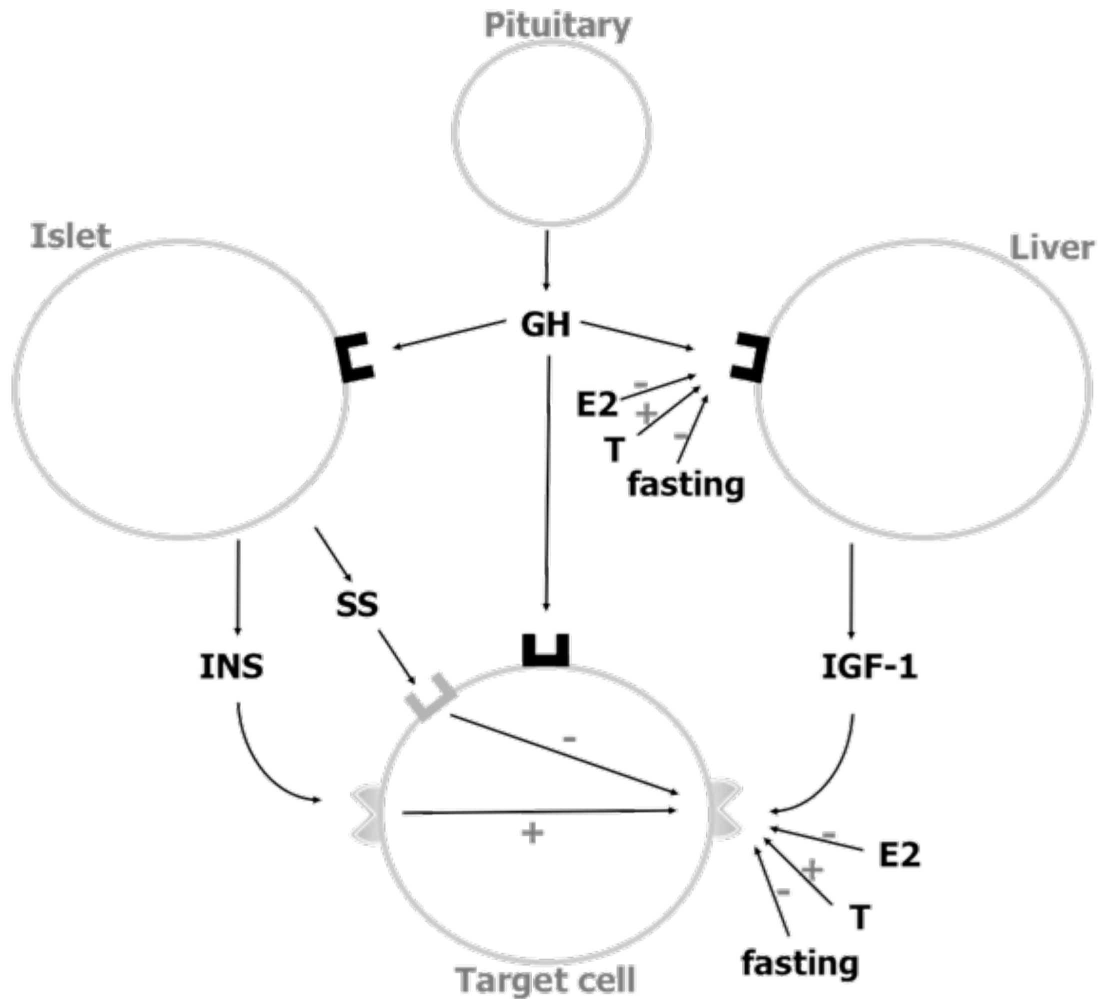


Figure 5. The peripheral-endocrine control of growth in trout, highlights the importance of tissue specific responses, which are essentially the key to coordinating growth in conjunction with all of the other vital physiological processes. The tissue specific expression of GHRs modulates tissue sensitivity to GH, and is a major means of coordinating other aspects of the GH-IGF axis.

In a comparative approach, the general growth pattern of a species is genetically determined and thus would be a factor to consider when evaluating the effects of GH on receptor expression. A slow growing species, such as the wild-strain rainbow trout, and a fast-growing species, such as the Jian carp (a common carp variety), would likely respond to GH differently. Likewise, GHR expression in response to GH treatment would likely not show identical patterns in indeterminate growers, like rainbow trout, and determinate growers, like zebrafish. In reality, the expression of the GHRs is not simply dependent on the presence or absence of GH at any one moment in time. For example, chronic versus acute effects of GH may modulate GHR expression and function. Likewise, the simple presence or absence of GH is not exactly expressive of the *in vivo* situation; in reality, variations in the levels of GH, as well as the levels of the GH-family ligands which compete with GH for receptor binding, are seen. Differences in the evolutionary history of the receptor subtypes and which receptor subtypes have been lost or maintained in that species' evolutionary history is the root determinate of the current characteristics of the receptor; the binding affinity and capacity of the receptor subtype for the ligand, the presence of the soluble GHBP, and the rate of receptor turnover, and receptor interaction with metal ions may all be variables determined by the structure of the receptor and are at play in the ultimate control of GH signaling. With unique life histories among each species, the genetic background of a species ultimately determines the characteristics of all of these factors and their influence on the expression patterns of the receptors subtypes, thereby modulating growth.

GH as a means of integrating metabolism with growth

GH as well as INS and SS have highly defined roles in maintaining metabolism. Growth hormone has been shown to be hyperglycemic in several species of fish and to increase glycogenolytic, glycolytic, and gluconeogenic flux in several tissues, including liver, brain, and gill; these protein-sparing actions of GH are consistent with the soft tissue and skeletal growth promoting-actions of GH that prevail when food is available (Norbeck *et al.,* 2007). The availability of food, however, is not a constant and guaranteed condition for fish in their natural environment. Growth, in situations of fasting is stunted, as resources would be shunted towards vital processes and thus, the ability to coordinate growth and metabolism in variable conditions is imperative for survival. Furthermore, the developmental pattern and life history of an individual species reflects natural periods of feeding and fasting. For example, during development the transition from alevin to fry is characterized by the movement to the surface of the water where they begin to feed. There are also natural differences in food availability patterns between species of fish. For example, salmonid fish in northern latitudes experience limited food availability during the cold winter months (Pottinger *et al.,* 2003; see chapter by Vargas-Chacoff *et al.*, this volume). Likely the most well-known example of natural periods of fasting is the home-stream return of salmonids

Times of fasting are typically associated with the cessation of growth; interestingly, however, plasma levels of GH have been shown to increase in fish and other vertebrates during periods of food deprivation. During periods when food is unavailable, energy is diverted away from growth to sustain essential metabolic processes. During fasting, fish mobilize stored lipid and carbohydrate, and activate

gluconeogenesis (Sheridan and Mommsen, 1991). The mechanisms by which GH promotes growth (an anabolic process) during periods of food availability yet retarded growth and depletion of energy reserves (catabolic process) is yet to be elucidated.

Plasma levels of INS increase following a meal and, in turn, INS promotes peripheral tissue uptake of glucose and amino acids, and activates synthesis of glycogen and triacylglycerols (TG). The postprandial increase in blood glucose is a potent stimulator of SS synthesis and secretion in trout (Caruso and Sheridan, 2011). Insulin has been shown to increase the expression of GHR2a and GHR2b (Sheridan, 2010), while SS-14 reduces sensitivity to GH in rainbow trout hepatocytes by internalizing surface GH receptors (GHRs) and by decreasing transcription of GHR mRNAs (Very and Sheridan, 2007).

Thus, when glucose levels rise in response to feeding, stimulating pancreatic islets to secrete INS, an INS-stimulated uptake of glucose into peripheral cells follows. To counter the growth-enabling response to INS, SS is simultaneously secreted in response to the rising levels of blood-glucose to inhibit INS secretion at which point SS-induced glycogenolysis is seen in peripheral tissues (Sheridan and Kittilson, 2004; Nelson and Sheridan, 2006). Therefore, the immediate effect of INS to stimulate glycogenesis is followed by a counter regulatory-mechanism that together, serves as a homeostatic switch between glycogenesis and glycogenolysis.

In addition to the regulation of GHR expression, the expression of SS receptors (SSTR), IR (INS receptor), and IGFR represent additional important components of this peripheral model for the control of growth and coordination of growth with metabolism (Fig. 5). An increase of SSTR expression in peripheral target cells occurs in response to

INS and GH (Nelson and Sheridan, 2006). Glucose levels further modulate this system by regulating both islet and peripheral SSTR expression; increasing concentrations of glucose resulted in increased expression of SSTR 2 mRNA in both Brockmann bodies and liver (Kittilson *et al.,* 2011b). In the islets, promotion of SSTR expression by glucose would enhance islet responsiveness to SS, thereby retarding INS production and blunting the growth-promoting and anabolic effects of INS (Caruso and Sheridan, 2011). Furthermore, SS-14 reduces hepatic basal and GH-stimulated IGF-I production of rainbow trout, which with increased sensitivity to SS (Very *et al*., 2008; Hagemeister and Sheridan, 2008) would lead to the cessation of growth and activation of observed SS-stimulated carbohydrate and lipid catabolic processes (Sheridan and Kittilson, 2004) . Thus, energy can be partitioned away from growth to meet metabolic demands when needed.

Integration of growth with stress and osmoregulation

The effects of cortisol on growth in trout and other species of fish remains somewhat elusive, as species-specific and condition-specific effects have been observed. The duration of post-stress cortisol levels, more so than the levels of post-stress cortisol, is correlated with growth rates in rainbow trout; trout with a relatively rapid decrease in post-stress cortisol concentrations had a higher rate of growth compared with those that had a slower rate of decrease in cortisol concentrations (Weil *et al.,* 2001). However, the direct effects of cortisol on gill and liver tissues from rainbow trout were examined *in vitro* and results showed that cortisol increased in both gill and liver tissues, steady-state levels of mRNAs encoding GHR2a and GHR2b (Norbeck and Sheridan, 2010). Cortisol

also increased hepatic expression of both IGF-1 and IGF-2 mRNAs thus supporting that direct effects of cortisol lead to endogenous conditions that would support growth. In gill, cortisol also increased levels of IGFR1A and IGFR1B mRNAs thus increasing the sensitivity to the cortisol-induced increase in IGF levels (Norbeck and Sheridan, 2010). It appears as though these direct effects of cortisol act through the differential modulation of GHR expression and, in a theme consistent with growth modulation in this chapter, is species specific. *In vivo* cortisol treatment of black seabream resulted in an increase of hepatic GHR1 but not of GHR2 (Jiao *et al.,* 2006). These results indicate that cortisol affects growth by modulating the GH-IGF system at several levels, including increasing sensitivity to GH, increasing IGF production, and increasing peripheral sensitivity to IGF.

The reported cases of both positive and negative effects on growth in response to cortisol likely reflect the plethora of environmental conditions (crowding, changes in salinity, etc.) that are considered to be “stressors,” as well as numerous physiological conditions that could be present at the onset of stress-induced cortisol secretion. However, other peripheral hormones reflect these physiological conditions. For example, GH, as well as SL and PRL have been reported to be elevated in response to stress (Avella *et al*., 1991; Pottinger *et al*., 1992; Rand-Weaver *et al.*, 1993; Kakizawa *et al*., 1995).

Some negative effects on growth in response to cortisol could, potentially, be due to indirect effects, such as the modulation of feeding behavior (Volkoff *et al.,* 2009; Sheridan, 2011). GH, however, is also known to affect feeding behavior; GH-treated trout foraged closer to the water surface, resumed feeding earlier, and ate more food than did control trout (Jonsson *et al.,* 1996). These hormones may be dynamically coordinating a

balance between feeding behavior and an antipreditor response. Certain developmental stages may be set to promote a certain behavior over the other. For example, a situation like this would occur in the natural development of trout, as young fry move to the surface of the water to feed on aquatic insects.

Cortisol and the GH family of ligands dynamically coordinate the transition from fresh water (FW) to seawater (SW) of euryhaline fish (McCormick, 2001). The transition of juvenile salmonids from FW streams to the open ocean is a natural part of the their life history, and this transfer is associated with increased plasma cortisol and glucose levels in cutthroat trout (*O. clarkia*) parr following a 24 h seawater challenge test (Morgan and Iwama, 1996). It is not known if the interaction of cortisol and GH during SW adaptation allows for the channeling of energy away from growth toward osmoregulation, but the pronounced stimulatory effects of cortisol on GHR, IGF-1 and IGFR expression in gill filaments of trout (Norbeck and Sheridan, 2010) are suggestive.

Furthermore, during the marked growth associated with development from fry to smolt, physiological preparation for the adjustment to a saltwater environment is occurring (Bjornsson *et al*., 2011). Also during this phase there is a change in coloration as the par marks and the brown/green to blue/green colors that were once advantageous in the rocky FW streams, fade into a silvery color that would be more advantageous in the open ocean (Hoar, 1988). Interestingly, in addition to possible roles in osmoregulation and growth, SL is believed to be involved in the development of chromatophores and the regulation of pigment (Cánepa *et al.,* 2012). Thus, such dynamic regulation of GH, PRL, SL, and their cognate receptors during this life phase of trout is likely the source of difficulty in characterizing the roles that each of these hormones play in a single specific

process, such as smoltification; it is the coordination of *multiple* processes that is crucial for organismal survival.

Integration of growth with reproduction

In rainbow trout, 17β-estradiol (E2) decreased GHR2a and GHR2b expression in both liver and muscle, but not in gill, while testosterone increased stead-state levels of GHR2a and GHR2b in all three tissues, in a time and concentration-dependent manner (Norbeck and Sheridan, 2011). Furthermore, E2 significantly decreased steady-state levels of IGF-1 mRNAs in liver and gill, while testosterone significantly increased levels of IGF-1 and IGF-2 in liver, and IGF-1 in muscle; there were no significant effects of E2 on IGFR1 mRNAs in muscle or gill, while IGFR1A and IGFR1B mRNA increased in gill, and IGFR1B mRNA increased in muscle, in response to testosterone. Functional expression of GHRs, as assessed by ^{125}I–GH binding capacity, was reduced by E2 in liver and muscle; however, E2 did not affect ^{125}I–IGF-1 binding capacity in muscle or ^{125}I–GH and ^{125}I–IGF-1 binding capacity in gill. In contrast, binding capacity of ^{125}I–GH in liver and of ^{125}I–GH and ^{125}I–IGF-1 in both muscle and gill also was increased by testosterone (Norbeck and Sheridan, 2011).

Again, as a reoccurring theme of this chapter, the modulation of growth appears to occur in a species-specific manner. Unlike what is seen in juvenile trout, testosterone significantly decreased gene expression of sbGHR2, but not sbGHR1, in liver, whereas expression of both sb-GHR1 and sbGHR2 was suppressed by E2 (Jiao *et al.,* 2006). Even among salmonids, the modulation of growth is likely to differ to some degree and most likely does so in a way that reflects the differences in life history patterns. For example,

semelparous species that typically die after a single spawning event, divert substantially more energy into the production of gametes, compared to iteroparous salmonids species that may reproduce several times and thus need to energetically maintain physiologically processes necessary for survival and for a lifespan that may include several long migrations that would include transitions between freshwater and seawater (Johnston and Post, 2009). Unfortunately, comparable data on growth-reproduction interactions for semelparous and iteroparous salmonids are limited. Although the prevailing hypothesis is that sex steroids are the major regulator of programmed death in salmon, hormones involved in growth go hand-in-hand with the regulation of sex steroids. In the year before spawning, a period of rapid body growth and gonadal development takes place during which plasma IGF positively correlates with body size and E2 levels (Campbell *et al*., 2006). Increased plasma IGF levels were found in salmon with more advanced gonadal development, suggesting that IGF may be involved in linking the timing of body growth and sexual maturation in preparation for spawning (Onuma *et al*., 2010). The integration between growth, stress responses and reproduction are integrated and reflect life history, and a study by Johnston and Post (2009) exemplifies this well and suggests that life-history trade-offs differ between the sexes.

Summary and conclusions

GH coordinates a vast array of physiological processes, including regulation of feeding, metabolism, reproduction, osmoregulation, immune function, behavior, and growth. The multifunctional nature of the hormone arises from a multifaceted signaling system consisting of the GH ligand, dimerized type-1 cytokine receptors, and cellular

effector pathways. Interestingly, the GH signaling system of fish displays substantial diversity at every level. For instance, there are multiple forms of GH ligands as well as multiple types/subtypes of GHRs. The structural heterogeneity of the ligands and the receptors arises from the existence of multiple genes derived from genome duplication events during the course of teleost evolution (the FSGD event ca. 300 MYA; more recent independent tetraploidization events in the case of salmoinds and other groups); additional heterogeneity of the receptors results from post-translational processing of encoded mRNAs. In addition, the GH family of peptides consists of several structurally related hormones (e.g., GH. PRL, SL), and in many cases the receptor subtypes display promiscuity in binding. Furthermore, the dimerized receptor complexes expressed on the surface of cells may be homodimers (e.g., GHR-GHR) or heterodimers (e.g., GHR-PRLR). Moreover, GHRs can interact with several cell signaling cascades (e.g., JAK-STAT, ERK, PI3K/Akt).

We suggest that the multifunctionality of GH arises from the diverse, multifaceted nature of the GH signaling system. Ultimately, a particular response in a given target cell will be determined by the specific interactions between and among the various elements of the signaling systems. For example, by producing and releasing one form of GH over another (or by adjusting the relative amounts of other members of the GH-family of peptides), the organism can target specific cells which display receptors that are selective for that peptide. The response of the target cell can be modulated by regulating the presentation of receptor subtypes presented on the surface of the cell and /or by regulating the presence of effect pathway elements to which the receptors link.

Evidence is emerging from studies in rainbow trout and other species regarding how some of the elements of the GH signaling system are modulated and how such modulation affects growth and serves to coordinate growth with other processes such as metabolism and reproduction. For example, nutritional state, insulin, thyroid hormones, steroids, including sex steroids, can modulate the sensitivity of targets cells to GH and IGFs by adjusting the expression/localization of GHRs and IGFRs and by adjusting the actions of GH and IGF-1 in target cells (Figure 5).

Our understanding of the GH signaling system, however, is far from complete. Although data regarding the differential activation of signaling pathways by some GHR subtypes in trout exists (Kittilson *et al*., 2011a), additional work is needed to gain a comprehensive understanding of all GHRs subtypes and whether or not such GHR-effector pathway linkages are tissue-specific or vary with developmental/physiological state. Fully understanding the multifunctional nature of GH also will require establishing the linkages between particular signaling pathways and specific biological responses (Figure 4). Recent work showing that during periods of feeding of rainbow trout, STAT and Akt are activated and PLC and PKC are deactivated in tissues, whereas during periods of food deprivation, STAT and Akt are deactivated and PLC and PKC are activated, hold promise for resolving the growth-promoting and lipid-catabolic actions of GH (Bergan *et al*., 2012). Lastly, it also will be important to identify how variations in the life histories of fish are reflected in the differences in the GH signaling systems as such information will be essential not only for understanding the diversity and evolution of GH signaling but for providing insight into agricultural production or conservation of species of fish that inhabit diverse aquatic habitats.

Objectives of this thesis

In the previous decade, receptors for GH, PRL, and SL, had been sequenced in numerous vertebrate species, including a plethora of piscine species. As it was found, there were multiple genes encoding these receptors, and the naming of these receptors has continued to be arbitrary, usually being numbered based upon the order of discovery of the homologues in a particular species. This made for a confusing system when using comparative approaches because GHR1 in one species and GHR1 of another species were not necessarily of the same clade. With the discovery of SL without the discovery of a distinct SLR, a change in name of numerous GHRs to SLR was suggested, based on the binding characteristics of the receptors in a single species. Thus, the entire evolutionary scheme of these receptors was obscured, which inadvertently complicated the study of this signaling system and especially complicated the study of this system using comparative approaches. A major goal of this thesis, then, was *to clarify the evolutionary history of these receptors, in vertebrates*.

Furthermore, with extensive information (e.g., binding characteristics, signaling effects) have become available from numerous species, another major goal of this thesis was to do an extensive review of the GH signaling system, in fish, to identify the unifying themes in the functionality of these receptors, as well as to identify current or potential caveats that may be a source for confusion or misguidance that would potentially obscure the overall understanding of the physiological effects of this signaling system.

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CHAPTER 2. EVOLUTIONARY ORIGIN AND DIVERGENCE OF THE GROWTH HORMONE/ PROLACTIN/ SOMATOLACTIN RECEPTOR FAMILY: INSIGHTS FROM STUDIES IN SEA LAMPREY

Introduction

Growth hormone (GH) and the closely related prolactin (PRL) and somatolactin (SL) proteins are hormones that have become well known for their multifunctional natures. GH, for example, regulates numerous processes in vertebrates, including growth, metabolism, reproduction, osmoregulation, immune function, and behavior (Forsyth and Wallis, 2002; Bjornsson *et al.,* 2004; Norrelund, 2005; Norbeck *et al.,* 2007; Moller and Jorgensen, 2009). Approximately 300 biological actions have been reported for prolactin, in vertebrates, with the most well known actions being those involved with the regulation of calcium transport in several organs, including gill, intestine, and kidney, and as the hormone responsible for the stimulation of milk production in mammals, as well as the development of the epithelial lining of the crop-sac that leads to the development of crop-milk in some types of birds (Wongdee and Charoenphandhu, 2012 ; Kaneko and Hirano, 1993). The actions of somatolactin, a protein hormone only found in fish, to date, is believed to share a relatively recent common ancestor with GH and PRL, and its actions also appear to be somewhat diverse. Evidence indicates that SL has involvements in metabolism/energy homeostasis and sexual maturation (Vega-Rubín et al. 2004; Rand Weaver et al., 1992; Benedet et al., 2008). More recently, evidence has accumulated to

give a well-established role, for SL, in the regulation of chromatophore development and the movement of pigments in chromatophores; events necessary for environmental background color adaptation in fish (Canepa et al., 2012). More work will be needed to delineate the pleiotropic nature of these hormones.

These hormones are produced by the cells of the adenohypophysis; GH from somatotrophs and PRL from lactotrophs of the pars distalis, and SL from distinct cells in the pars intermedia; although there is no physical distinction between these areas of the adenohypophesis in salmonids. Additionally, the extra-pituitary production of GH in fish, including salmonids, as well as in other vertebrates is now well reported (Harvey, 2010). Extra-pituitary production of PRL has been reported in vertebrates, including fish (Imaoka et al., 2010; Ben-Jonathan et al., 1996. Extra-pituitary production of SL has been reported in a few species of fish, including rainbow trout (Yang et al., 1997b). GH is a single-chain polypeptide roughly 21-22 kDa in size, and shares structural similarities with the 22-25 kDa PRL-protein and the roughly 23-24kDa SL-protein (Law *et al.,*1996; Yang *et al*., 1997a; Li *et al*., 1997; Wang *et al*., 2010; Cavari *et al*., 1995; Benedet *et al*., 2008; Yang *et al*., 1997b; Yang and Chen, 2003). The crystal structures of GH and PRL show that the overall structure of the hormones are a four α-helical bundle, and although a crystal structure of somatolactin has not been obtained, sequence comparison supports a structural model similar to that of GH and PRL.

The biological actions of these hormones occur upon interaction with their receptors of which belong to the type-1 cytokine receptor superfamily. Two receptors dimerize (receptor$_1$-receptor$_2$) to become the functional receptor complex with which these hormones then bind. The membrane-bound receptors of this family are single-

spanning transmembrane protein, which includes an extracellular domain involved in receptor$_1$-receptor$_2$ dimerization and hormone-binding, a single transmembrane domain, and an intracellular domain that connects the receptor to downstream effector pathways. There are several points of interest regarding the involvement of the receptors in the multi-functional nature of these hormones. First, Multiple GHRs derived from distinct genes, believed to be a result of a series of gene duplication events, have been described in many species of fish, including salmonids species such as masu salmon (*O. masou*), Atlantic salmon (*Salmo salar*), coho salmon (*O. kisutch*), and rainbow trout Fukada et al., 2004, Very et al., 2005, Benedet et al., 2008. For example, three distinct GHRs, able to bind GH with resulting receptor activation, have been isolated in rainbow trout Reindle et al., 2009). The consequences of the differential structure of these GHRs on the resulting cellular response to hormone stimulation must, reasonably, be explored. Furthermore, alternative splicing of any of these GHR genes would result in additional structural variants that could, potentially, diversify the GH-GHR signaling response, and indeed, the presence of multiple transcripts have been reported in a number of species (Baumbach et al., 1989; Calduch-Giner et al., 2001; Edens and Talamantes et. al., 1998; Martini et al., 1997; Tse et al., 2003; Di Prinzio et al., 2010). These structural variants could modify the physiological response of these hormones in several ways: 1) by altering the receptor-ligand affinity, 2) by altering the overall structure of the receptor enough that the conformational-change that occurs as part of the mechanism of receptor activation is altered, 3) by altering the intracellular domain to result in altered cell signaling, 4) by creating competition between expressed receptors, for the ligand, and 5) by, theoretically, creating heterodimers with divergent functional properties from

homodimerized receptors of which these receptors are assumed to be . Besides multiple structural variants for which GH can bind, a second important point of interest regarding the receptors' involvement in the multi-functionality of GH, would be the differential expression of these multiple structural variants in a single cell/tissue, as well as the differential expression of the structural variants among different tissues.

In the past decade, thanks to advancements in sequencing, numerous GHR-family mRNAs and several GHR-family genes have been characterized in a plethora of fish species as well as in numerous species of tetrapods, while additionally, the publication of several genomes databases allowing for predictions regarding genes and proteins, has occurred. Furthermore, the crystal structures of several complexes between GH and PRL bound to monomeric and dimeric forms of their receptors have been solved. Combined, this valuable information now provides a means by which various comparative strategies to explore the origins of the multi-functionality of these hormone-receptor systems can be employed. The overall objective of this study was to enhance our understanding of the polygenic origins of GHRs and to provide insight into the linkage between GHR subtype and physiological response. To this end, we have characterized a protein with characteristic features of a type-1 cytokine receptor from sea lamprey (*Petromyzon marinus*); we believe the gene encoding this protein is the evolutionary-precursor to both the GHRs and PRLRs thus far characterized in the vertebrate lineage.

Materials and methods

Experimental animals

Liver tissue for an adult lamprey was used to obtain and characterize the GHR mRNA. Three sexually mature adult male lampreys were used to obtain RNA used in the tissue distribution study. Finally, cDNAs were obtained from S.A.S., as pools of samples, of various tissues, from both male and female lampreys. These cDNAs were used in the tissue distribution study.

RNA extraction

Tissue was homogenized using RNAzol RT (Molecular Research Center, Inc., Cincinnati, OH, USA). Water was added to the homogenate and protein and DNA precipitation was accomplished by centrifugation of the contents at 12,000g; DNA precipitate was discarded. The remaining supernatant containing the RNA was isolated and transferred to a new microcentrifuge tube. mRNA precipitate was isolated by adding 75% Ethanol followed by centrifugation at 12,000g for 8 minutes. The supernatant was removed and discarded and the pellet containing the precipitated RNA was washed twice with 75% ethanol. Ethanol was removed and the pellet was dried. The RNA was re-hydrated in RNase-free water. Immediately after isolation of total-RNA, oligo dT beads from the GenElute™ mRNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA) were used to isolate mRNA.

Oligonucleotide primers and probes

Gene-specific primers used for isolation of cDNAs were designed by examining known GHR sequences using GeneTool software (BioTools, Inc., Edmonton, AB) and custom synthesized by Sigma-Genosys (The Woodlands, TX, USA). Additional primers for reverse transcription were provided in the SMARTER™ RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA, USA). Gene-specific oligonucleotide primers and probes used for real-time PCR of GHR were designed using ABI Primer Express® Version 2 software, based upon the sequence of our determined GHR sequence. Primers and probes were used for reverse transcription and PCR without further purification.

Isolation and characterization of putative GHR-like mRNA

A three-phase approach was adopted for the isolation of a GHR-encoding cDNA using reverse-transcription (RT)-PCR and rapid amplification of cDNA ends (RACE)-PCR. Isolation of the cDNA sequence was accomplished using the SMARTER™ RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA, USA) following the manufacturer's protocol. In phase I, RNA from rainbow trout liver was reverse transcribed into cDNA containing the sequence tags necessary for SMARTER™ chemistry, and remaining cycles of PCR were carried out using a gene-specific (5'-GCCCGGATTACGTCACTGC-3') primer designed for 3'RACE. Products amplified were then identified by electrophoresis on an agarose gel containing 1% of each OmniPur (EMD chemicals, Gibbstown, NF, USA) and NuSieve GTG agarose (Bio-Wittaker Molecular Applications, Rockland, ME, USA) in 1 x Tris-borate-EDTA (TBE) buffer

followed by ethidium bromide staining. The product of this first reaction was combined with a nested primer (5'-CGGCAAACACGTCCTTCGAG-3'), designed internally from the original 3'RACE primer, in second reaction to reamplify the correct fragment of interest and reduce the background and nonspecific amplification seen with the first primer. The products of this second reaction were then visualized as described previously. The PCR products were then cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) and inserted into JM109 cells. Purified plasmids (75 fmol) were sequenced using the CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's protocol.

After determining that the initial PCR reaction resulted in a unique cDNA with reasonable sequence identity with known GHR-encoding cDNAs, phase 2 consisted of two independent PCR reactions, the first with a unique set of primers (Forward, 5'-GTGCCGGACGGTGGACAT- 3'; Reverse, 5'-TGCGCTCGTCGTCCGTCTCTCACC-3') and the second with another unique set of primers (Forward, 5'-CTGCTGGCGTGATGACATT- 3'; Reverse, 5'-GGCCCCGCAGTGACGTAAT-3') Initial attempts at 5'RACE, immediately after obtaining sequence from phase 1, were unsuccessful; thus, these PCR reactions (phase 2) allowed us to obtain more sequence, extending off the known 3'end of the mRNA, so that new gene-specific primers could be designed for 5'RACE. The PCR products were visualized, cloned into the pGEM-T Easy Vector, and sequenced as described previously. To confirm that the sequence gathered from phase 1 and phase 2 could, indeed, be assembled as a single product, a forward primer (5'-CTGCTGGCGTGATGACATT- 3') and a newly designed reverse primer (5'-TTGCACCATATCGACATTCAGAA-3') were used for a "confirmation PCR" reaction.

The PCR products were visualized, cloned into the pGEM-T Easy Vector, and sequenced as described previously.

After obtaining the additional sequence, in phase 2, new gene-specific primers were designed and used in phase 3, 5'RACE. 5'-RACE was performed using a SMARTER™ RACE cDNA Amplification Kit under manufacturer's suggested conditions with the GHR gene-specific primer (5'-GCAGACTCGTTCGCCAGGG-3') designed for 5'RACE. The resulting PCR products were visualized, cloned into the pGEM-T Easy Vector, and sequenced as described previously.

Real-time PCR assay; quantification of GHR-encoding mRNA

Preparation of cDNA standards:

cDNA standards for were synthesized by PCR. Approximately 1 μg of the RACE-ready cDNA product was used as template for PCR with forward and reverse gene-specific primers under the same conditions as described previously (Slagter *et al*., 2004). The resulting PCR products were visualized under ultraviolet light, cloned into the pGEM-T Easy Vector, and their sequences verified, as described previously.

Real-time reverse transcription PCR:

Previously purified mRNA, the endogenous poly(A)$^+$ RNA was reverse transcribed in a 10 μL reaction using AffinityScript QPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA) to the manufacturer's instructions. Reactions without reverse transcriptase were included as negative controls; no amplification was detected in negative controls.

Real-time PCR reactions were carried out for samples, standards, and no-template controls in a 10 μL reaction; each reaction contained 1 μL cDNA, 1 μL each of forward primer, reverse primer and probe at concentrations optimized for the mRNA species to be measured, 1 μL RNase-free deionized water, and 5 μL Brilliant II SYBR® Green Low ROX QPCR Master Mix (Agilent Technologies®, Santa Clara, California, U.S.A.) The primers used were, (Forward, 5'-TGCACGCAAGTGTTTCTTCCT-3'; Reverse, 5'-GGGAGGCTGCAAATGTCATC-3') were designed to be gene specific. Cycling parameters for real-time PCR were as follows: 95 ^{o}C for 10 min, and 45 cycles for 95 ^{o}C for 30 s plus 59 ^{o}C for 1 min.

Copy number calculations were based on threshold cycle number (C_T). The C_T for each sample was determined by the MX3000P™ real time analysis detection software after manually setting the threshold. Sample mRNA expression (copy number) was determined by relating C_T to a standard curve comprised of serial dilutions of known amounts of lamprey GHR/PRLR cDNA. Copy numbers of mRNA were considered non-significant if C_T exceeded 45 cycles; this value corresponds to a detection limit of less than 100 mRNA copies.

Data analyses

The nucleotide and associated protein sequences were aligned and analyzed with GeneTool and PepTool sequence analysis programs, respectively (BioTools Inc., Edmonton, Alberta, Canada). TM-COFFEE, a program specifically designed to align transmembrane proteins using homology extension, was used (with default settings) to produce the alignment necessary for determining the conserved regions of the protein, as

well as the phylogenetic tree; the tree was visualized with TreeView and rooted to the rat erythropoietin receptor. Potential phosphorylation sites (either protein kinase A or protein kinase C) were predicted by NetPhos 2.0 (default settings). A signal peptide was predicted using the SingnalP 4.0 server, set a default settings for eukaryotes.

Quantitative data are expressed as means ± S.E.M. Statistical differences were estimated by a cell means model ANOVA followed by Duncan's multiple range test; a probability level of 0.05 was used to indicate significance. All statistics were performed using SigmaStat v. 1.0 (SPSS, Chicago, IL, USA).

To produce a predicted 3-dementional depiction of the protein, predicted from the obtained mRNA sequence, SWISS-MODEL (a protein structure homology-modeling server) was used to produce unique protein data bank files (.pdb files), based upon the amino-acid sequence of interest and upon a chosen target protein. human GHR (pdb ID: 3hhrB and 3hhrC) was used as the template structure for the homology modeling of the extracellular domain of the lamprey GHR-like protein, as well as for the trout GHRs. Rat PRLR (pdb ID: 3npzB and 3npzC) was used for the predication of the trout PRLR model.

Results and discussion

Characterization of GHR-like mRNA

A cDNA fragment, 1115 bp in length (including the poly-A tail), was amplified by phase 1 (3'RACE) of the previously described approach. Sequence analysis suggested the successful isolation of a fragment of a GHR/PRLR-like protein. Initial attempts at 5'RACE with primers designed from this cDNA fragment, however, were unsuccessful.

With the lamprey genome database available, we were able to circumvent the problem by designing forward primer based on a location of the scaffold that was predicted to be coding (exon) region. The reverse primer for PCR was designed based on the initial cDNA fragment we had obtained. The resulting cDNA obtained was 365 bp in length and overlapped with the cDNA fragment obtained in phase 1. A second PCR reaction using this approach (a forward primer based on predicted exon-region and a reverse primer based on isolated sequence) resulted in a second fragment that was 230 bp in length and overlapped, considerably, with the first of the PCR fragments from phase 2. The final phase, phase 3, of our approach consisted of 5'RACE PCR, using a gene-specific primer designed from sequence previously obtained. Figure 1 illustrates the 3-phase approach and the assembly of resulting fragments.

The three-phase approach, utilizing both RACE-PCR and traditional PCR, yielded a 1729 bp cDNA with an open reading frame encoding a coding region 249-amino acids in length (Fig. 6). Sequence analysis of the coding region revealed the successful isolation of the extracellular domain, but showed an absence of both the transmembrane domain and intracellular domain, explaining a shorter mRNA length than predicted (GHR mRNAs are typically about 2500-3000bp in length, in most fish). Upon further investigation, it appeared that the isolated protein was consistent with a soluble hormone binding protein produced by alternative splicing of the gene. The protein has a single signal peptide cleavage site was estimated to be between pos. 48 and 49. Furthermore, examination of the structure reveals a protein that includes an intact hormone-binding interface, which presents as being homologous to the hormone-binding interface of full-length GH receptors. An additional result of 5'RACE was a second mRNA fragment that

was nearly identical to the other 5'RACE fragment except that this second fragment included additional sequence that did not assemble with the fragments from phase 1 and phase 2. The additional fragment is identical to DNA sequence that is part of the same contig as the other fragments isolated, thus supporting the notion that this additional segment is alternatively spliced (Fig. 6e shows the alignment of this alternative fragment, with the other fragments isolated in the 3-phase approach).

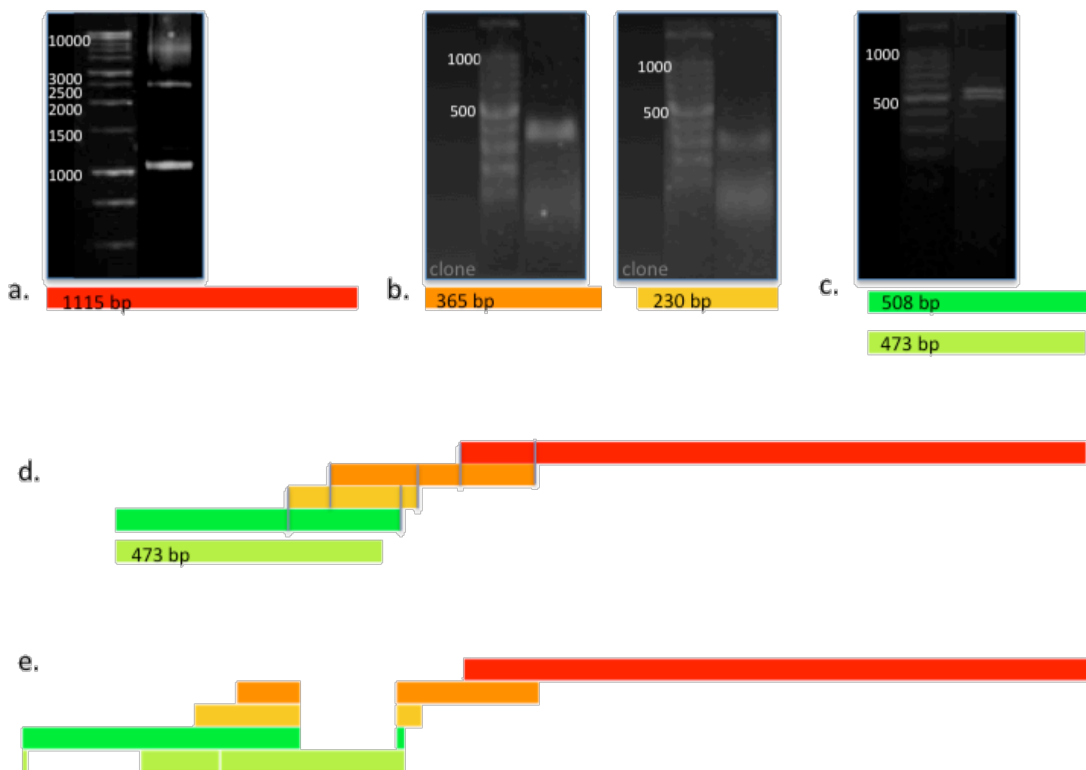


Figure 6. Three-phase approach to the characterization of sea lamprey GHR/PRLR mRNA. Agarose-gel electrophoresis, a. phase-1, b. phase-2, c. phase-3, and schematic representations, d. assembly of overlapping PCR-fragments yeilds a full-length, 1711-bp, transcript encoding a truncated GHR/PRLR and a separate, 473-bp, GHR/PRLR splice variant. e. alignment of the sequences obtained, illustrating sequence-gaps that distinguish the two obtained variants.

The sequence, overall, is similar to the sequences of both characterized GHRs and PRLRs, and exhibit conserved features that are characteristic of this family of receptors. This family of receptors has several conserved cysteine residues, two of which are

conserved in GHRs, but are not present in PRLRs or in the type-2 GHRs. Indeed, the characterized lamprey sequence exhibits these cysteine residues, except for the two that are not present in PRLRs nor in the type-2 GHRs, but are present in the type-1 GHRs. A modeled prediction of the lamprey GHBP, which will be discussed in more detail in a later section, reveals the structural conservation of subdomain 1 of the extracellular domain; this subdomain contains the interface for hormone binding. Subdomain 2 of the extracellular domain of the lamprey protein is truncated just before the FGEFS motif that is characteristic of GHRs in fish (homologous to the WSXWS motif in other type-1 cytokine receptors). However, much of the area of subdomain 2, is present in the lamprey sequence. The WSXWS domain is believed to be involved in the mechanism that transduces activity resulting in cell signaling involving the intracellular domain; thus, the exclusion of this motif in a soluble binding protein would be inconsequential to this protein's ability to function as a binding protein.

Subdomain 2, of the extracellular domain contains the dimerization domain, or stem-stem interface (region of interaction between the two receptors of the dimer), of the receptor. Furthermore, this area is also important in the rotation of the receptor-pair, relative to eachother, that occurs during receptor activation (van Agthoven *et al*., 2010). It is thought that the conformational rotation that occurs to activate the receptor results in downstream signal transduction by shifting the alignment of the "linker region," the short region found in the extracellular domain, just before the transmembrane domain, which subsequently realigns the transmembrane domain thus altering the alignment and conformation of the intracellular domain (Rowlinson *et al.,* 2008). Mutations in the linker region support this theory, with altered ratios of ratio of STAT5 to ERK1/2 signaling

(Rowlinson *et al*., 2008). Recently, the characterization of the dimerization interface, also known as the stem-stem interface (or site 3), of the PRLR has been completed, and upon comparison with the GHR, even though the general region of the dimerization domain is common between the two, the actual interface is different in in PRLR and GHR (van Agthoven *et al*., 2010).

Tissue expression of the characterized lamprey mRNA was surveyed in numerous tissues and its expression quantified. Results show (Figure 8) that the characterized mRNA is widely distributed among tissues, a characteristic consistent with the pleiotropic nature of the GH/PRL signaling system that is transduced through their receptors. Furthermore, differential expression is seen among the tissues, with the highest expressions seen in the pituitary and liver, a result that reflects GHR expression patterns, reported for liver and pituitary, in other species. High expression of the GHR is typically also seen in muscle; however, liver has been reported to be the major source of GHBP in other species (Ross *et al*., 1997; Carlsson *et al*., 1009). Thus, this result further supports the prediction that the characterized protein is indeed the GHBP.

Figure 7. The cDNA and deduced amino acid sequences of truncated GHR/PRLR (seq 1) and a GHR/PRLR 5' splice variant (seq 2) isolated from sea lamprey. The predicted hormone-binding regions are boxed in color (region 1=magenta, region 2=cyan, region 3=green, region 4=blue); the predicted dimerization domain is boxed in gray; and possible phosphorylation sites, denoted by ▲. The position of putative splice sites is indicated by ○.

```
seq 1  acatgggctccccctgccgtctgtctcaccacccctggcccttccctccctcctctccgcggagccgccctctcc   < -137
seq 2  acatggg--------------------------------------------------------------------   < -7

seq 1  tctcatctcccctctctctctctcccccagcctcctcctcctcctgcccccaccactcccgcaactttgtcgggc   < -62
seq 2  ---------------------------------------------------------------------------   < -7

seq 1  cgcggggggggcctctcggctcagtgagtgacgccgcgaggagtgagggagccgaggacggcATG CGA GGG GGG CAC   < 15
seq 2  --------------------------------------------------------------ATG CGA GGG GGG CAC   < 15
                                                                        M   R   G   G   H

seq 1  GGC CGC GCG GAG CGG CCC ACA CAG CGT GGC GGA GGA GGC GGC ACG GAC AGC ATG GTG CCT GCA CGC AAG TGT TTC   < 90
seq 2  GGC CGC GCG GAG CGG CCC ACA CAG CGT GGC GGA GGA GGC GGC ACG GAC AGC ATG GTG CCT GCA CGC AAG TGT TTC   < 90
        G   R   A   E   R   P   T   Q   R   G   G   G   G   G   T   D   S   M   V   P   A   R   K   C   F

seq 1  TTC CTG CTG CTG GCG CTG ATG ACA TTT GCA GCC TCC CCA AGC CCC GTT GCA GGA AAG TTT CCG GGC CGG CCG GTC   < 165
seq 2  TTC CTG CTG CTG GCG CTG ATG ACA TTT GCA GCC TCC CCA AGC CCC GTT --- GGA AAG TTT CCG GGC CGG CCG GTC   < 162
        F   L   L   L   A   L   M   T   F   A   A   S   P   S   P   V  A/-  G   K   F   P   G   R   P   V

seq 1  GTG TGG TGC CGG ACG GTG GAC ATG CTG ACG CTG ACA TGC TGG TGG CAA CCA GTG AAG GGA GAT GAC CCC AGC ACT   < 240
seq 2  GTG TGG TGC CGG ACG GTG GAC ATG CTG ACG CTG ACA TGC TGG TGG CAA CCA GTG AAG GGA GAT GAC CCC AGC ACT   < 237
        V   W   C   R   T   V   D   M   L   T   L   T   C   W   W   Q   P   V   K   G   D   D   P   S   T

seq 1  AAC TAC ACC CTC TTC TAC ACA ACG CAG TCC GGA TCC ACC CT- --- --- --- --- --- --- --- --- --- --- ---   < 281
seq 2  AAC TAC ACC CTC TTC TAC ACA ACG CAG TCC GGA TCC ACC CTA GCT CCA GGC GGC ACT CGT CCA GCT TCC GAC AAA   < 312
        N   Y   T   L   F   Y   T   T   Q   S   G   S   T   L   A   P   G   G   T   R   P   A   S   D   K

seq 1  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---   < 281
seq 2  GGA ATG GCA GCC TCT CCG GCG CAG TTA ACA TTT TGG ACA TTC CAC GTG GCC AGC CGC AGT GGT TTT CTG CTA CTT   < 387
        G   M   A   A   S   P   A   Q   L   T   F   W   T   F   H   V   A   S   R   S   G   F   L   L   L

seq 1  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --G GCG AAC GAG TCT   < 294
seq 2  CTG GAG GAG CCT GAG GGG GGG TGG AAG AGG GGG GAT TCA GTA TCC CAG CTG CAC CAA TTG AGG GCG AAC GAG TCT   < 462
        L   E   E   P   E   G   G   W   K   R   G   D   S   V   S   Q   L   H   Q   L  L/R  A   N   E   S

seq 1  GCC GAG TGC CCG GAT TAC GTC ACT GCG GGG CCC AAC AGC TGC TTC TTC GAC CAG GAG CAC ACC TCG ATG TGG GTG   < 369
seq 2  gc                                                                                                  < 464
        A   E   C   P   D   Y   V   T   A   G   P   N   S   C   F   F   D   Q   E   H   T   S   M   W   V

seq 1  ATG CAC TGC GTG AGA GTG GTG GCG AGC TCG CCC ACG GCA AAC ACG TCC TTC GAG AAG CAC TGC ATC AAG CTG CTG   < 444
        M   H   C   V   R   V   V   A   S   S   P   T   A   N   T   S   F   E   K   H   C   I   K   L   L

seq 1  GAC TAT GTG GAG CCG GAC GTT CCC CCC GTC AAC GTG AAC GTG ACG CTG CGG AAC GTC AGC GAC CCA GAT CCT GTG   < 519
        D   Y   V   E   P   D   V   P   V   N   V   N   V   T   L   R   N   V   S   D   P   D   P   V   V

seq 1  GTT CTG GTC ACT TGG GCG CCA CCT CCG TCC GCC AAC GTC AAA TAC GGG CTG GTG GTG CTG GAG TAC GAG GTG GAG   < 594
        V   L   V   T   W   A   P   P   P   S   A   N   V   K   Y   G   L   V   V   L   E   Y   E   V   E

seq 1  TAC CGG GCC GAA CAC CAG ACG AGT GGC ACG GTG AGA GAC GGA CGA GCG CAC ATG TAA aaccaggcccccggcgt   < 668
        Y   R   A   E   H   Q   T   S   G   T   V   R   D   G   R   A   H   M   *

seq 1  aaaaactaacttttctaaacgaaattacttcatcagctaaggccgaccgagctatatatcgctctttttcataag   < 743
seq 1  cgtacctggacgcaaatgatcgctaaactttagtggctcatcacgcccaaatgtatttgcagccaaatattttaa   < 818
seq 1  gcgcgtgtattctaagcgtagaacgcagggagttattctcaaatgagcatcgaggctttgtgcttatcccctgtt   < 893
seq 1  ctcagccctaagtcagtggtggaaattccacatttctctaacgggcgggggcggaggggtgaggctttccatatg   < 968
seq 1  acaagtgctgttcacttcccccaacaaagtggtactaatttgtatggaccccggggaaggtagggatagatgggc   < 1043
seq 1  taagtcaacccccctccaaccaagatttgaactcgcaatctcgtgattgcgagtcgctcgctcgactgctgagcag   < 1118
seq 1  ccaccgggccatttgcataatgagcggttacgccggcaaggctggcgtggcacggcgcttgcatctctggctcgc   < 1193
seq 1  gtaattttccaaataattcctgccaataggcttattaatgttacttcatgggcagggtcatcggtcggcagattt   < 1268
seq 1  ggaccgtaatgtcaaaaaataaccggagtgttagttttggcaggcaaatgccaaatgataaatgcataaataatg   < 1343
seq 1  tgtgatgcagtttatgttaatcgtccggagcttcatctccctggcaacgattctgaatgtcgatatggtgcaata   < 1418
seq 1  atcgtttccatttttttttgcctcccttgtaatttttttattggctttttttttacttcttagtacggcttcaataca   < 1493
seq 1  gaacac(a)n                                                                   < 1500
```

In general, GHBP is well conserved, as the production of GHBP by multiple mechanisms has been observed in a wide variety of vertebrate species, inferring that the soluble binding protein is, physiologically, important. The first-discovered GHBPs were found to be produced through proteolytic cleavage of the extracellular domain from the membrane-bound receptor (Leung *et al*., 1987; Baumann *et al*., 1988). Later, it was found that an alternative means of production GH binding proteins can also occur through the alternative splicing of the gene that, also, encodes the full-length receptor (Edens *et al*., 1994). The production of GHBP by alternative splicing of the full-length GHR gene was reported in fish and mammals, including in turbot (Calduch-Giner *et al*., 2001), Chinese sturgeon (Acipenser sinensis) (Lioa *et al*., 2004), and rodents (Smith *et al*., 1989; Edens *et al*., 1994; Edens *et al*., 1998; Baumbach *et al*., 1989; Talamantes and Ortiz, 2002), to name a few. The production of GHBP by both mechanisms, in a single species, has been reported in mammals (Martini *et al*., 1997).

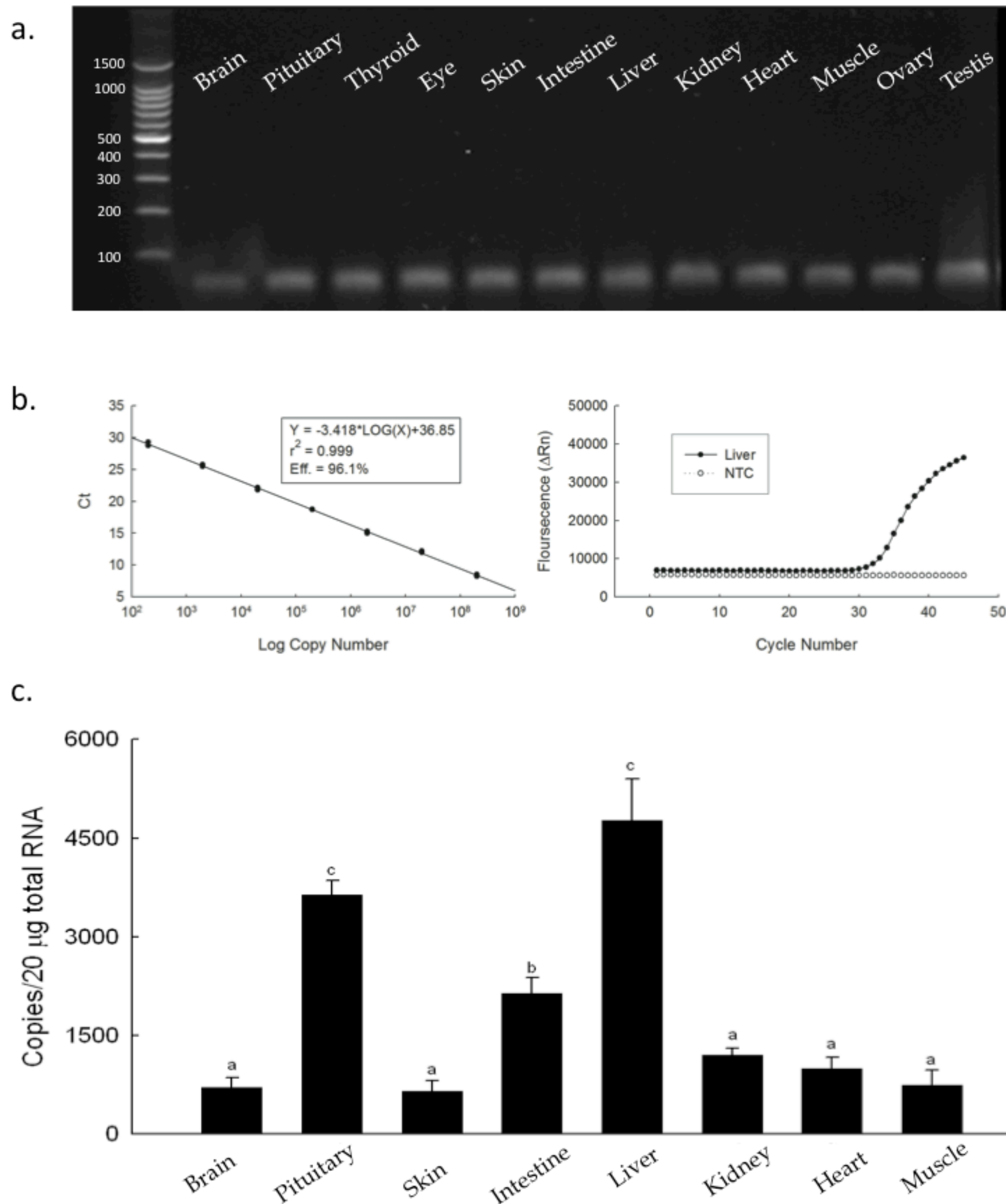


Figure 8. Distribution of GHR/PRLR mRNA among tissues in adult sea lamprey. Expression of mRNA was evaluated by real-time PCR. (A) Qualitative expression was assessed by separating PCR amplicons (after 45 PCR cycles) on agarose visualized by ethidium bromide staining and transillumination. mRNA expression was quantified by determining the threshold cycle number (C_T) for each sample from its amplification plot, as exemplified for liver (B). Sample copy number was then determined by relating C_T to a standard curve (C) comprised of a serial dilution of a known amount of lamprey GHR/PRL cDNA, then normalized to β-actin and (D) expressed as mean±SEM (n=4-6). Groups with different letters are significantly different ($p<0.05$).

Interestingly, alternative splicing of the GHR gene have been found to produce distinct mRNAs that encode other "truncated receptors," besides the soluble GHBP. In humans, alternative splicing of the GHR can produce a membrane bound receptor lacking the entire intracellular domain that is seen in the full-length receptor, and instead exhibits 6 intracellular amino acids that are novel, after the transmembrane domain and before the stop codon; another truncated receptor that is still membrane-bound, in humans, was shown to maintain the majority of the full-length intracellular domain, but lacks the intracellular portion encoded by exon 9; the joining of exon 8 and 10, then, creates a frame shift that results divergent sequence and an alternative stop codon (Ross *et al*., 1997). A second truncated hGHR was found to be missing 26 amino acids of exon 9 in which case a frameshift encoding an alternative amino acid sequence results in a premature stop codon, thus truncating the majority of the intracellular domain (Ross *et al*.,1997; Dastot et. al, 1996). The partial deletion of exon 9, in this truncated receptor, resulted from a cryptic splice acceptor consensus sequence (Ross *et al*, 1997; Dastot *et al* 1996). Besides the soluble GHBPs produced by alternative splicing, it appears that most of the truncated receptors that have been characterized result in a receptor with an altered intracellular domain. However, in several human prostate cancer cell lines, an expressed truncated receptor was found to be missing exon 3, which encodes the N-terminal portion of the extracellular domain to result in a receptor that is 22 amino acids shorter in length and that has an unknown physiological relevance (Chopin *et al*., 2002). Alternative splicing has also been found to be responsible for a long form of the growth hormone receptor, in Black Seabream, that includes an additional alternative splice site not characterized in any other organism (Tse *et al*., 2003). This long form of GHR includes

an additional 33 amino acid segment in the intracellular domain that does not result in a frameshift to cause any other differences in the intracellular domain. Alternative splicing of the PRLR gene has also been found to produce multiple transcripts, including both truncated and long-forms of membrane bound PRLRs (Tanika *et al*., 200)

Although evidence of GHBPs and alternative transcripts of membrane-bound receptors have been noted in a few fish species, complete genome organization and the actual characterization of GHBPs, or other truncated GHRs that are produced by alternative splicing, are generally lacking in fish. This is potentially important information that would need to be obtained for a full understanding of numerous processes, such as growth. Alternative transcripts can work in numerous ways to affect physiology. The soluble binding proteins, for example, can increase the half-life of the hormone, act as a hormone-reservoir, or create competition with the membrane-bound receptors, for the hormone. The alternative membrane-bound receptors may also have various functions. For example, truncated GHR, even those that have had the majority of the intracellular domain lost can still dimerize with a full length receptor inhibit cell signaling (Ross *et al*., 1997). Furthermore, an understanding of alternative splicing, especially since it appears to be highly conserved in the GHR system, would be an important in the understanding of disease. The altered expression of alternative transcripts, in tumorigenic or carcinogenic tissues, have been reported in numerous studies (Tan *et al*., 2011). To note, it has been concluded that a significant fraction of point mutations that result in human genetic disease disrupt splicing (Krawczak *et al*. 1992, Faustino and Cooper, 2003).

Evolution of GHRs

Figure 9 depicts the phylogenetic relationships of known members of the GHR/PRLR family, with emphasis on the GHRs characterized in fish, and includes the characterized GHR-like protein characterized in this paper. Although we understand that any analyses that come from this phylogenetic tree are tentative, as only the extracellular portion of the lamprey receptor could be included, the tree is, non-the-less, helpful in predictions. As before, the analysis reveals two distinct clades, for the GHRs: type-1 and type-2. Furthermore, the lamprey appears as an intermediary, between the PRLR clade and GHR clades, which is consistent with our prediction that Lamprey GHR/PRLR-like receptor is the evolutionary precursor that gave rise to both PRLRs and GHRs. The pattern that emerges from this analysis is consistent with the structural features of these receptors, which will be discussed in the next section.

First, it is important to clearly identify the evolutionary relationship of these receptors to ease comparative approaches to the understanding of receptor functioning at the molecular level. To further explore the relationships of these GHRs, in fish, a qualitative microsyntany survey (Figure 10) was performed. While the complete assembly of sequence and chromosome mapping has yet to have occurred in most species of fish, several sequence databases and BAC libraries have made it possible to search for genes and manually align contigs in a small number of fish species. Unfortunately, there is insufficient information in the lamprey genome to include this species in the analysis. In the analysis, a selection of candidate genes, known to be within close physical proximity to the GHR genes, were mapped in a number of species. As seen in Figure 10, there is differential patterning of these syntenic regions that is consistent with the two-

clade (type-1 and type-2 GHRs) conclusion of the phylogenetic analysis and that, furthermore, supports an orthologous relationship between the type-1 GHR and hGHR. The descent of GHRs in fish and Sarcopterygians from a common ancestor is supported by the juxtaposition of C7 near the GHR locus (except Coelacanth, the genome of which is incomplete and data on the location of C7 is not available). The duplication and divergence of teleost GHRs also is reflected in their synteny maps. Multiple genes retain their position near the GHR locus, including PLCXD3, C5orf51, FBX04, SEPP1, and ZNF131, in humans as compared to the Type 1 GHR genes of teleosts; however, other genes near the locus of GHR1 in teleosts, such as IP011 and OXTCT2, were moved to other locations in humans. The transposition of C6 and C7 in humans also is interesting to note. Genes such as PIP5K1B and TJP2 are only near the Type 2 GHR locus in teleosts and were moved to other locations in humans. Interestingly, the CCDC152 is positioned near the Type 2 GHR locus of teleosts and retained its juxtaposition near the GHR locus in humans. For some of these species, especially those that have sequencing projects in early stages (e.g., salmon) the absence of any one of these candidate genes, is not necessarily indicative of its true absence in the species, but is likely a gene we could not annotate by the manual alignment of the contigs/scaffolds. Despite some of the limitations, the global analysis still supports a conclusion consistent with that of the phylogenetic tree and with the previous analysis by Fukamachi and Meyer: teleost GHR1 and GHR2 are paralogs that arose through gene duplication, and that teleost GHR 1 and GHR2 are orthologs to Sarcopterygian GHR (as evidenced by humans). In summary, two distinct clades have emerged among the GHRs, type-1 and type-2, with the type-1 GHRs

having and orthologous relationship to the linage of GHR that gave rise to tetrapod GHRs.

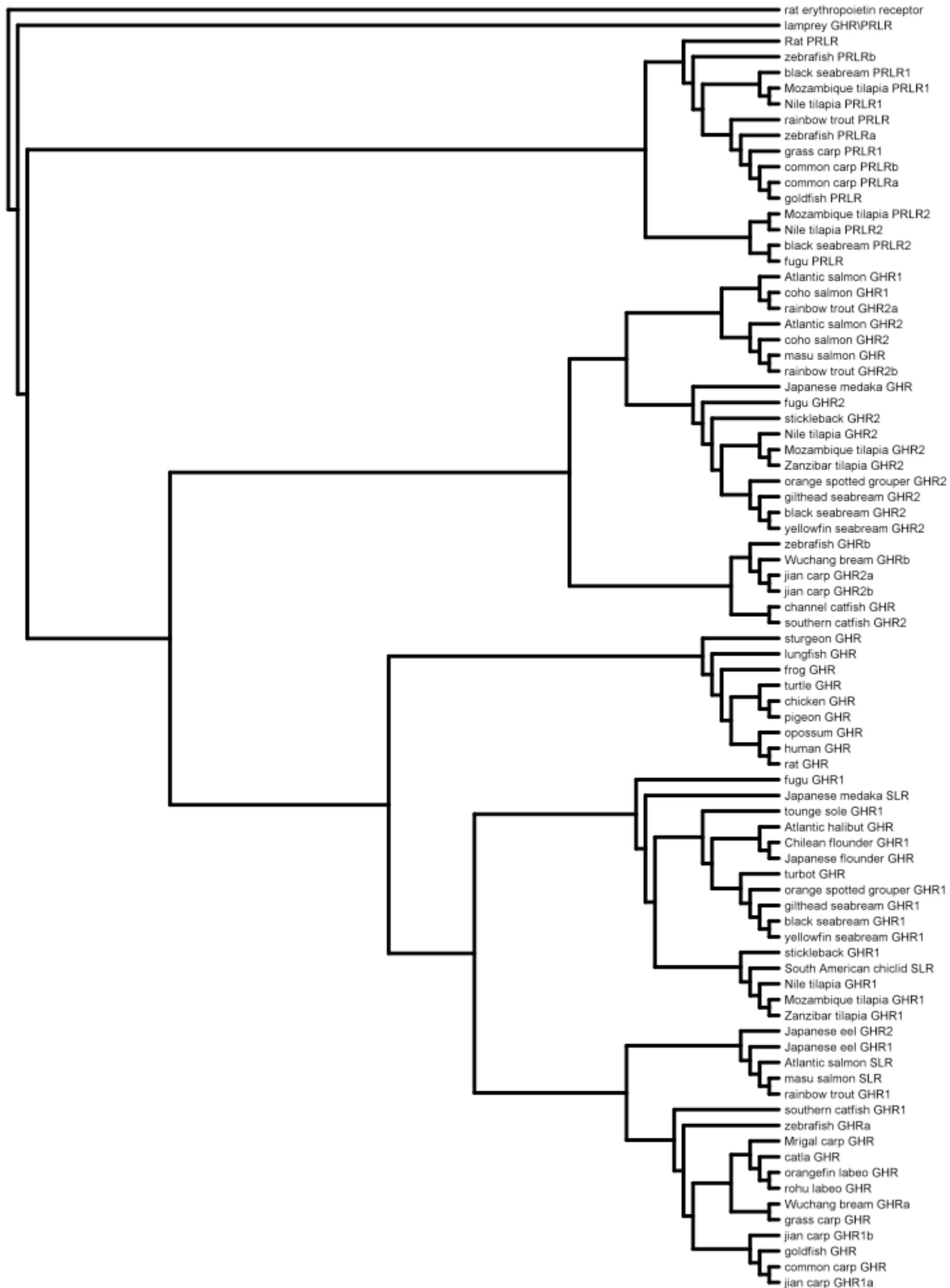
Structural assessment of the family of receptors for GH, PRL, and SL

With a clearer understanding of the evolutionary relationships between these receptors, comparative approaches to the assessment of the molecular basis of functionality can more easily be employed. Two regions of these receptors will be examined in detail: the dimerization domain, and the hormone-binding domain. Sequence alignments and 3-dimential predictions of structures will be used to support a deeper understanding of the molecular basis of functionality.

Since an altered alignment of GHR dimerization domain residues could alter degree of rotation of the GHR transmembrane domains to result in altered cell signaling, as discussed earlier, the dimerization domain is clearly an important region to examine the molecular basis of the receptor functionality and how that may be transduced into the physiological functions of these species. Figure 11 shows an alignment comparing the homologous regions of the dimerization domain from characterized PRLR and GHR, in fish species, as well as in a handful of tetrapod species. Indeed, clear patterns are seen between the receptor types (clades) and can even be seen between groups of closely related species. In the GHR complex, most of the interacting residues are part of the initial Leu-Gln172 region (human GHRas the reference), while most of the interacting residues in the PRLR interface are part of the latter domain region, which in human PRLR stretches from Lys-His197. Although the characterized regions of the crystal structures for GHR and PRLR used as the reference are from human (GHR) and rat

(PRLR) frame, with no structural reference frame available from a species of fish, conservation of several residues, found to be interacting residues in the human and rat receptor interfaces, is seen. For example, Asn161, Ser163 and Asp170 (hGHR) are involved in hydrogen bonding at the hGHR dimerization interface. These residues are highly conserved among the GHRs, in the species shown; while Asp is perfectly conserved in all GHRs, Asn and Ser are almost perfectly conserved in the type-1 GHR clade, while some substitutions are seen in the type-2 clade. To note, these Ser and Asp residues are also present in the characterized lamprey sequence. Looking, again, at the initial stretch that is highly involved in the GHR-GHR interface, and comparing the equivalent area for PRLR, the characterized rPRLR is two residues shorter and is therefore unable to get involved in the PRLR-PRLR interaction. This absence at the interface allows the two PRLR subdomain 2s to get closer than the two GHR subdomains (van Agthoven *et al*., 2010). The structural differences between the two receptors in a ligand-bound dimer, and between the receptor types (GHR1a, GHR1b, GHBP, PRLR) can be seen in Figure 12. Although it is not possible to completely predict the dimerization interfaces in these receptors without a crystal structure, the presence of a residue gap in the PRLRs, and not in the GHRs, is a conserved feature and suggests that, like in the hGHR, this stretch of the dimerization domain is likely involved in the GHR-GHR interactions. Interestingly, this stretch of the lamprey sequence has features similar to both the GHRs and the PRLRs; a two-residue gap is present, however, the sequence Asn-Val-Ser (Asn and Ser being involved in H-bonding at the GHR-GHR interface) that is present in hGHR, but not PRLR, is present in the lamprey sequence.

Figure 9. Phylogenetic tree of the known growth hormone receptors (GHR) of fish and selected other vertebrates. Prolactin receptors (PRLR) from selected teleosts are included for comparison. The tree was based on the alignment of amino acid sequences using the N–J bootstrap method in Clustal X and considered only completely overlapping segments greater than 300 nt in length. The tree was rooted using the erythropoietin receptor as an out group and was visualized with TreeView. The branch lengths represent amino acid substitutions per site from a common ancestor and are proportional to the estimated time since divergence occurred. The nomenclature for a particular receptor reflects that given by the authors originally or that which appears in databases; if the sequence was not annotated or the receptor type/subtype was not specified, the designation on the tree is ours and was chosen for consistency with the phylogenetic analysis and our proposed nomenclature. We recommend abandonment of the term somatolactin receptor (SLR), and the use of letters to designate GHR subtypes in teleosts. Sequences were obtained from either GenBank (accession numbers in parentheses) or Ensembl (protein ID numbers in parentheses) as follows: Atlantic halibut GHR (DQ062814), Atlantic salmon GHR1 (NM001123576), Atlantic salmon GHR2 (NM001123594), Atlantic salmon SLR (NM001141617), black seabream GHR1 (AF502071), black seabream GHR2 (AY662334), Catla GHR (AY691178), Channel catfish GHR (DQ103502), chicken GHR (NM_001001293), Chilean flounder GHR1 (EU004149), Coelacanth GHR (ENSLACG00000005546), coho salmon GHR1 (AF403539), coho salmon GHR2 (AF403540), common carp GHR (AY741100), common carp PRLR (AY044448), frog GHR (AF193799), gilthead seabream GHR1 (AF438176), gilthead seabream GHR2 (AY573601), goldfish GHR (AF293417), goldfish PRLR (AF144012), grass carp GHR (AY283778), Japanese crucian carp GHR (ADZ13485), Japanese eel GHR1 (AB180476), Japanese eel GHR2 (AB180477), Japanese flounder GHR (AB058418), Japanese medaka GHR (NM_001122905), Japanese medaka SLR (NP_001098560), jian carp GHR1a (ADC35573), jian carp GHR1b (ADC35574), jian carp GHR2a (ADC35576), jian carp GHR2b (ADC35577), lamprey GHR/PRLR (this sequence), lungfish GHR (EF158850), masu salmon GHR (AB071216), masu salmon SLR (AB121047), Mozambique tilapia GHR1(AB115179), Mozambique tilapia GHR2 (EF452496), Mozambique tilapia PRLR (EU999785), Mrigal carp GHR (AY691179), Nile tilapia GHR1 (AY973232), Nile tilapia GHR2 (AY973233), Nile tilapia PRLR(L34783), opossum GHR (NM001032976), orange spotted grouper GHR1 (EF052273), orange spotted grouper GHR2 (EF052274), orangefin labeo GHR (EU147276), pigeon GHR (D84308), rainbow trout GHR1 (JQ408978), rainbow trout GHR2a (NM001124535), rainbow trout GHR2b (NM001124731), rainbow trout PRLR (AF229197), rat erythropoietin receptor (AAH89810), rat GHR GHR (NM017094), rohu labeo GHR (AY691177), South American cichlid SLR (FJ208943), southern catfish GHR1 (AY336104), southern catfish GHR2 (AY973231), stickleback GHR (ENSGACT00000023732), sturgeon GHR (EF158851), Takifugu GHR1 (BAK86396), Takifugu GHR2 (BAK86397), Tetraodon GHR (ENSTNIP00000004152), tongue sole GHR1 (FJ608664), turbot GHR (AF352396), turtle GHR (AF211173), wami tilapia GHR1 (EF371466), wami tilapia GHR2 (EF371467), Wuchang bream GHRa (AFC38427), Wuchang bream GHRb (AFC38428), yellowfin seabream GHR2 (AEW29012), zebrafish GHRa (EU649774), zebrafish GHRb (EU649775).


rat erythropoietin receptor
lamprey GHR\PRLR
Rat PRLR
zebrafish PRLRb
black seabream PRLR1
Mozambique tilapia PRLR1
Nile tilapia PRLR1
rainbow trout PRLR
zebrafish PRLRa
grass carp PRLR1
common carp PRLRb
common carp PRLRa
goldfish PRLR
Mozambique tilapia PRLR2
Nile tilapia PRLR2
black seabream PRLR2
fugu PRLR
Atlantic salmon GHR1
coho salmon GHR1
rainbow trout GHR2a
Atlantic salmon GHR2
coho salmon GHR2
masu salmon GHR
rainbow trout GHR2b
Japanese medaka GHR
fugu GHR2
stickleback GHR2
Nile tilapia GHR2
Mozambique tilapia GHR2
Zanzibar tilapia GHR2
orange spotted grouper GHR2
gilthead seabream GHR2
black seabream GHR2
yellowfin seabream GHR2
zebrafish GHRb
Wuchang bream GHRb
jian carp GHR2a
jian carp GHR2b
channel catfish GHR
southern catfish GHR2
sturgeon GHR
lungfish GHR
frog GHR
turtle GHR
chicken GHR
pigeon GHR
opossum GHR
human GHR
rat GHR
fugu GHR1
Japanese medaka SLR
tounge sole GHR1
Atlantic halibut GHR
Chilean flounder GHR1
Japanese flounder GHR
turbot GHR
orange spotted grouper GHR1
gilthead seabream GHR1
black seabream GHR1
yellowfin seabream GHR1
stickleback GHR1
South American chiclid SLR
Nile tilapia GHR1
Mozambique tilapia GHR1
Zanzibar tilapia GHR1
Japanese eel GHR2
Japanese eel GHR1
Atlantic salmon SLR
masu salmon SLR
rainbow trout GHR1
southern catfish GHR1
zebrafish GHRa
Mrigal carp GHR
catla GHR
orangefin labeo GHR
rohu labeo GHR
Wuchang bream GHRa
grass carp GHR
jian carp GHR1b
goldfish GHR
common carp GHR
jian carp GHR1a

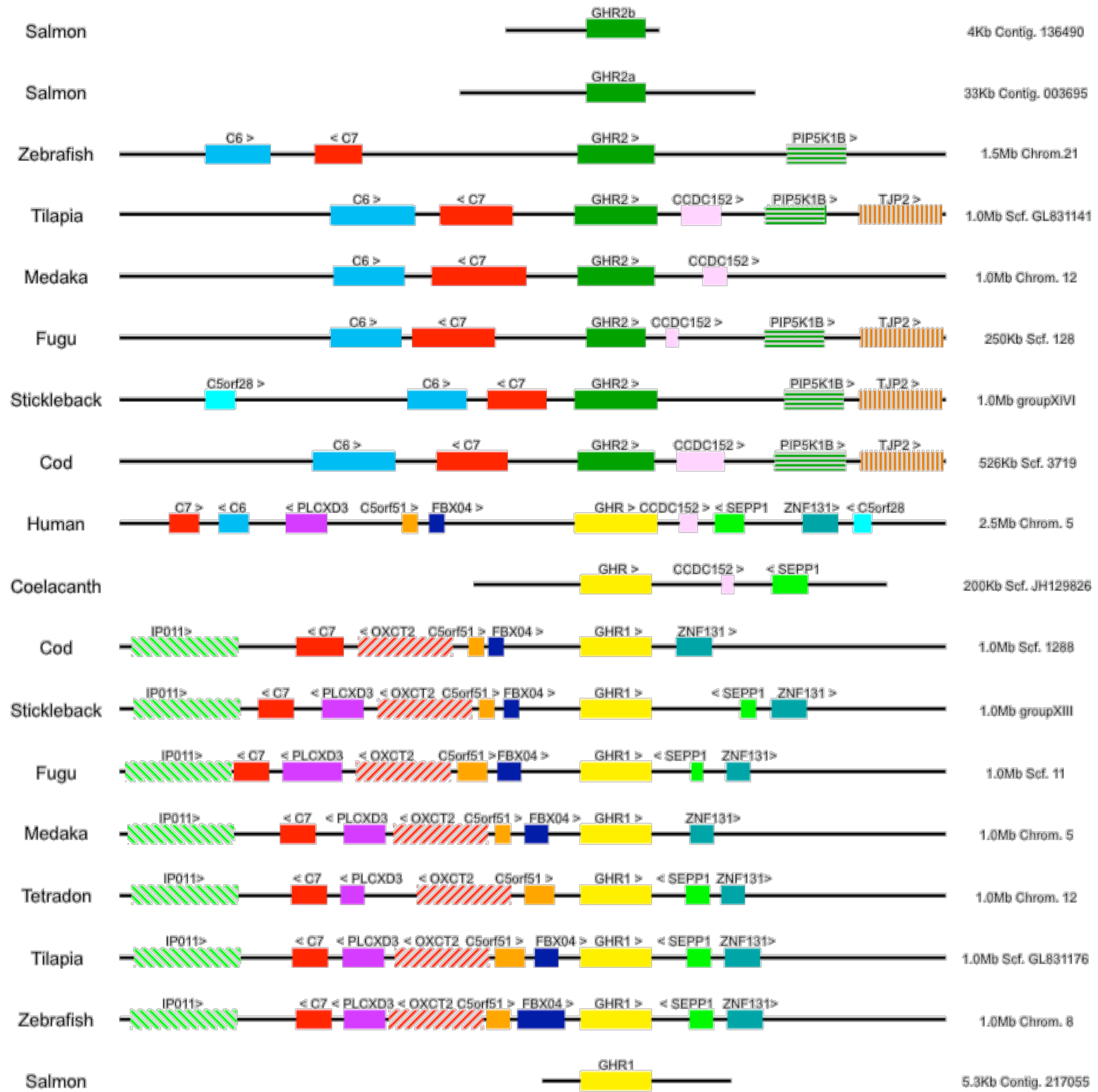


Figure 10. Synteny maps of growth hormone receptor (GHR) loci and the genes flanking them in humans and fish. Horizontal lines represent partial chromosomes/ scaffolds/ groups/ contigs with species name listed on the left and the chromosome/ scaffold/ group/ contig number and size listed on the right for each line; gene positions are relative and are omitted for clarity; The 5'-3' orientation of each gene, when known, is indicated by >. Colored boxes on the lines represent genes that were manually annotated as follows: GHRs and teleost type 1 GHRs (yellow), teleost type-2 GHRs (dark green), C5orf28 (cyan), C6 (blue), C7 (red) CCDC152 (pink), PIP5K1B (green horizontal strip), TJP2 (red vertical strip), IP011 (green diagonal strip), OXCT (red vertical strip), PLCXD3 (purple), C5orf51 (orange), FBX04 (dark blue) SEPP1 (bright green), ZNF131 (teal).

Another region that can be examined at the molecular level to reveal trends generally consistent with the phylogenetic analysis is the ligand-binding interface, also termed site 1 (site of binding between the first receptor and hormone) and site 2 (site of binding between the second receptor and hormone). This region is important considering the current model of GHR activation, which includes the high affinity binding of GH to the first of the receptors of the GHR dimer, initially, and is followed by the low-affinity binding of GH to the second GHR of the dimer (Brooks *et al*., 2008). A mutational analysis of GH using domain swapping of goldfish (Carassius auratus) GH (gfGH) and goldfish prolactin (gfPRL) also supports a model for a single GH with two binding sites bound to a dimerized receptor. The analysis and mutational study of goldfish GH supports a model whereby three discontinuous regions on the first helix and the region in the middle of the fourth helix comprises binding site 1; the third helix appears to be associated with binding site 2 (38). While it is believed that binding site 1 of the hormone is involved in the initial binding of the first GHR (GHR1), in which case the hormone has a conformational change while GHR1 does not have a major conformational change, site 2 of the hormone could be considered more as the functional site that binds the second GHR (GHR2), determinging the degree of the rotation of the receptors, relative to each other (Chan *et al* 2007; Brooks *et al* 2008; Broutin *et al*., 2010); GHR2 goes through a conformational change to, essentially, accommodate the binding of GH. Thus, since this binding induces this conformational change (rotation of receptors, relative to eachother) that results in signaling, differing degrees of conformational change which could happen if different ligands are binding (e.g., SL binds GHR versus GH binding GHR) may likely result in differential signaling in the cell.

Figure 11. An alignment of regions known to be involved in the dimerization of GHRs or of PRLRs. Human GHR and human PRLR are the reference sequences; lines appearing below or above resdues indicate strict conservation of the reference-residue in the non-human GHR or non-human PRLR sequence, respectively. The symbols and indicate a residue that has been conserved between the lamprey sequence and human GHR or the lamprey sequence and the human PRLR, respectively. Dashes (-) represent gaps that were inserted to maximize alignment; * denote missing sequence.

```
                                 Dimerization Domain                                              Conserved Cysteine Residues
Mozambique tilapia PRLR2    MKDQG---W|F|R      ||I      EES|||N|-A|||KM|N|F||R        |   |   |   |   T    T    C
Nile tilapia PRLR2          SI-G|---N|H|T      ||V      |SKI|KNY-TSDT||H|T|F||R       |   |   |   |   T    T    C
black seabream PRLR2        VETG|---S|||N      ||V      NNNK|KAY-MS|T||HFSLY|II       |   |   |   |   Y    K    C
zebrafish PRLRb             M|SQN---SQ|VL      ||V      EDSS||SY-S||K|L||S|Y|P|       |   |   |   |   N    K    C
rainbow trout PRLR          LED||---G|F|R      ||I      E|E||E|-R||KM|NNF||           |   |   |   |   N    D    C
Mozambique tilapia PRLR1    MKDQG---W|F|R      ||I      EES||N|-A||KM|N|F||R          |   |   |   |   N    D    C
Nile tilapia PRLR1          LED||---G|F|R      ||I      E|E||E|-R||KM|NNF||           |   |   |   |   N    D    C
black seabream PRLR1        MEDKG---W|F|R      ||I      EEND||T|-L||KM N|F||R         |   |   |   |   N    D    C
grass carp PRLR             VHEDQ---G|FVK      ||I      |DK||VEY-D||R|KYYNVF||        |   |   |   |   N    E    C
zebrafish PRLRa             IHEDR---D|FVS      ||I      EDK||VEY-D|ER|KNYNVF||        |   |   |   |   N    E    C
fugu PRLR                   ES-DE---I|N|H      ||I      NNSK|REY-LS|K||H|SLY||T       |   |   |   |   S    K    C
rat PRLR                    ||LK|K--|T|||      |||      E|E|||||-|T|H||Q||VFD|Y       |   |   |   |   I    Y    C
human PRLR                  KQPEDR--KPYLW148   GWF164   KAAEWEIH-FAGQQTEFKILSLH197    C36 C46 C75 C86 I100 Y118 C20

LAMPREY GHR/PRLR            RNVSDPD--PVVL      GLV      HQTSGTVR-D*************       C   C   C   C   C    C    *

human GHR                   LNVSLTGIHADIQ172   GWM188   NETKWKMM-DPILTTSVPVYSLK221    C56 C66 C101 C112 C126 C140 S21
rat GHR                     ||I||P||RG|||      ||I      |||||T|-S||WS|||||A|R         |   |   |   |   |    |    |
frog GHR                    ||I|||RMRI||       ||I      ||SQ|TIL-|KVQ|KYL||||         |   |   |   |   |    |    C
lungfish GHR                ||I|RS|LY|||L      ||I      |||Q|EQV-EHTVMTKLS|G|V        |   |   |   |   |    |    C
sturgeon GHR                ||I|Q||Q|F||L      ||I      |MSD||PL-EVERARYQS|G||        |   |   |   |   |    |    C
Japanese eel GHR2           ||||QSAL|F|AV      |||      TSPH|NTL-|WEKSS|HSL|G||       |   |   |   |   |    |    C
Japanese eel GHR1           ||||RS|LYF||M      |||      |ASQ|HTL-|QEKG|QQSI|G|L       |   |   |   |   |    |    |
southern catfish GHR        ||I|RSRL|F||M      |||      |SSH|DKL-|LESG|QQSI|G|H       |   |   |   |   |    |    C
zebrafish ghra              ||||RS|L|F|VL      |||      N|Y|E|Q-ELESG|QQSI|G|H        |   |   |   |   |    |    C
migral carp                 ||||RS|L|F|VL      |||      A|H|E|L-|LESG|QQSL|G|H        |   |   |   |   |    |    C
catla                       ||||RS|L|F|VL      |||      V|H|E|Q-|LESG|QQSL|G|H        |   |   |   |   |    |    C
orange-fin labeo            ||||RS|L|F|VL      |||      A|H|E|L-|LESG|QQSL|G|H        |   |   |   |   |    |    C
rohu labeo                  ||||RS|L|F|VL      |||      A|H|E|L-|LESG|QQSL|G|H        |   |   |   |   |    |    C
Wuchang bream GHRa          ||||RS|L|F||L      |||      TSQ|E|L-|LESG|QQSI|G|H        |   |   |   |   |    |    C
grass carp                  ||||Ls|L|F||L      |||      NSQ|E|L-|SESGTQQSI|G|H        |   |   |   |   |    |    C
Jian carp GHR1b             ||||RS|L|F|VL      |||      ASH|E|L-|LETG|QQSI|G|Y        |   |   |   |   |    |    C
Jian carp GHR1a             ||||RS|L|F|VM      |||      NSH|E|L-|LESG|QQSI|G|H        |   |   |   |   |    |    C
goldfish GHR                ||||RS|L|Y||L      |||      NSH|E|L-|LESG|QQSI|G|H        |   |   |   |   |    |    C
common carp GHR             ||||RS|L|F|VL      |||      NSH|E|L-|LESG|QQSI|G|H        |   |   |   |   |    |    C
Fugu GHR1                   ||I|PS|LSY|VI      |||      S|N|EAL-EVQTI|GLTI|G|T        |   |   |   |   |    |    C
Japanese medaka SLR         ||||PS|LSY|VM      |||      T|N|EAL-EMQPH|QQTI|G|Q        |   |   |   |   |    |    C
tonge sole GHR1             |SI|PS|VSF|VR      ||I      T|||EAL-EMQPQHQQTIFG|H        |   |   |   |   |    |    C
Atlantic halibut            ||S|PS|LSY|VM      |||      T|N||SL-AIQPHTQQAI|G|H        |   |   |   |   |    |    C
Chilean flounder GHR1       ||I|PS|LSY|VM      |||      A|N||SL-AIQPHTHQGI|G|H        |   |   |   |   |    |    C
Japanese flounder GHR       ||I|PS|LSY|VM      |||      A|N||SL-AIQPHTHQGI|G|H        |   |   |   |   |    |    C
turbot GHR                  ||I|PS|LSY|VM      |||      T|||EAL-EMQPQSQQTIFG|H        |   |   |   |   |    |    C
orange spotted grouper GHR  ||I|PS|LSY|VM      |||      T|N|EAL-EMQPH|LRTI|G|H        |   |   |   |   |    |    C
gilthead seabream GHR       ||I|PS|LSY|VM      |||      T|N|EAL-EMQPH|QQTI|G|Q        |   |   |   |   |    |    C
black seabream GHR          ||I|PS|LSY|VM      |||      TSN|EAL-EMQPH|QQTI|G|Q        |   |   |   |   |    |    C
yellow-fin seabream GHR     ||I|PS|LSY|VM      |||      TSN|EAL-EMQPH|QQTI|G|Q        |   |   |   |   |    |    C
stickleback GHR1            ||I|PS|LNY|VM      |||      T|||DAAMG|CTGSYSTI|G|H        |   |   |   |   |    |    C
South American cichlid SLR  ||I|PS|LNY|VK      |||      T|N|EAL-EMQPQHQQTIFG|H        |   |   |   |   |    |    C
Nile tilapia GHR            ||T|PS|LNY|VM      |||      T|N|EAL-|IQRQSHQTI|G|R        |   |   |   |   |    |    C
Mozambique tilapia GHR1     ||I|PS|LNY|VM      |||      T|N|EAL-|IQRQSHQTI|G|R        |   |   |   |   |    |    C
Zanzibar tilapia GHR        ||I|PS|LNY|VM      |||      T|N|EAL-|IQRQSHQTI|G|R        |   |   |   |   |    |    C
Atlantic salmon SLR         ||I|LS|LNY||M      ||L      |SSH||VL-EHEFG|QQSI|G|Q       |   |   |   |   |    |    C
Masu salmon GHR             ||I|RS|LNY||M      ||L      |SSH||VL-EHEFG|QQSI|G|Q       |   |   |   |   |    |    C
rainbow trout GHR1          ||I|RS|LNY||M      ||L      |SSH||VL-EHEFG|QQSI|G|Q       |   |   |   |   |    |    C
channel catfish GHR         SL|PT|LYC|VM       |||      GSEQ||SL-|SGKD|QANI|G|R       |   |   |   |   A    F    |
southern catfish GHR2       SLGP||LFC|VM       |||      GSDE||SL-|NGKD|QANI|G|R       |   |   |   |   A    Y    |
zebrafish ghrb              KMASS|L|C|VV       ||I      DSDQ|NTL-ESDKN|QAYI|G|E       |   |   |   |   V    S    A
Wuchang bream GHRb          SMGS||LIC|VV       |||      GSDQ|NSL-|NGKD|QAY||G|R       |   |   |   |   V    S    |
Jian carp GHR2b             SMGS||LIN|VV       ||I      GSDH|NSL-|NGKD|QAYI|G|H       |   |   |   |   V    S    |
Jian carp GHR2a             SMGS||LIY|VV       ||I      GSDQ|NSL-|NGKD|QAHI|G|R       |   |   |   |   V    S    |
Fugu GHR2                   ||Q|VTSTYS||M      ||L      DIEQ|QVT-|LVKS||RTL|G||       |   |   |   |   T    I    C
Japanese medaka GHR         ||||||T|F||M       |||      SSDH|EVT-|LVSS|HRTL|G|Q       |   |   |   |   A    F    C
Nile tilapia GHR2           ||||S||Y||L        |||      SSSV|E|V-||VSS|QCSL|G|Q       |   |   |   |   N    G    C
Mozambique tilapia GHR2     ||||S||Y||L        |||      SSSV|E|V-||VSS|QCSL|G|Q       |   |   |   |   N    G    C
Zanzibar tilapia GHR2       ||||S||Y||L        |||      SSSV|E|V-||VSS|QCSL|G|Q       |   |   |   |   N    G    C
orange spotted grouper GHR  |||||T|Y||I        |||      |SDL|EVL-|LVTS|YRSIFG|Q       |   |   |   |   L    S    C
gilthead seabream GHR2      ||E||S|TYY||I      |||      SSDL|HAV-E|VTV|QRSLFG||       |   |   |   |   K    T    C
black seabream GHR2         ||E||S|AYY|VL      |||      SSDQ||V|-||VIS|HRSL|G||       |   |   |   |   R    K    C
yellow-fin seabream GHR2    ||E||S|TYY|VL      |||      SSDQ||V|-||VIS|HRSL|G||       |   |   |   |   R    K    C
stickleback GHR2            |||||T|Y||M        |||      ESDL|EQT-|LGKE|HRSL|G|R       |   |   |   |   S    L    C
Atlantic salmon GHR1        |||G||S|F||M       |||      |S|L|RTV-|LEKGMQRSL|G|R       |   |   |   |   S    I    C
coho salmon GHR1            |||G||S|F||M       |||      |S|L|RTV-|LEKGMQRSL|G|R       |   |   |   |   S    I    C
rainbow trout GHR2a         |||G||S|F||M       |||      |S|L|RTV-|LDKGMQRSL|G|R       |   |   |   |   S    I    C
Atlantic salmon GHR2        |||G||S|F||M       |||      |S|L|R|V-|LEKGMQRSL|G|R       |   |   |   |   S    I    C
coho salmon GHR2            |||G||S|F||M       |||      |S|L|R|V-NLEKGMQRSL|G|R       |   |   |   |   S    I    C
masu salmon GHR             |||G||S|F||M       |||      |S|L|R|V-NLEKGMQRSL|G|R       |   |   |   |   S    I    C
rainbow trout GHR2b         |||G||S|F||M       |||      |S|L|R|VN-LEKGMQRSL|G|R       |   |   |   |   S    I    C
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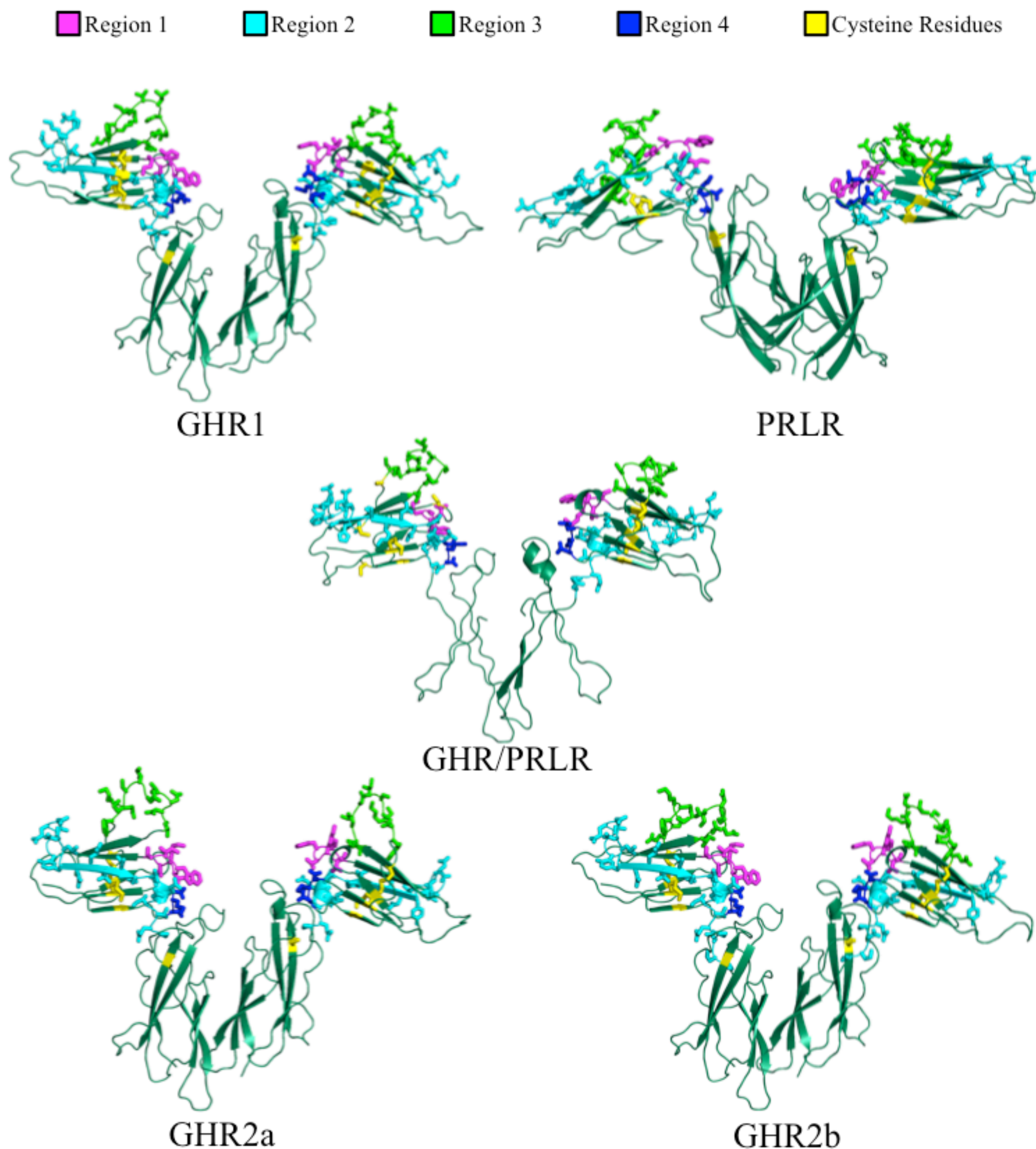

Figure 12. Three-dimensional models of the extracellular domains of the truncated growth hormone receptor (GHR)/prolactin receptor (PRLR) from sea lamprey (center) and of the GHRs and PRLR from rainbow trout. Receptor models were created using personalized pdb files based on the protein sequence of trout GHRs and PRLR; these were made using SWISS-MODEL workspace (Bordoll et. al. 2006; Bordoll et. al. 2009) based on the crystal structures of GH-GHR$_2$ (PBD ID: 3hhr; de Vos et. al. 1992) and PRL-PRLR$_2$ (PDB ID: 3ew3; Broutin et. al. 2010). 3-D models were generated, based on the created pdb files, using POLYVIEW-3D (Porollo et. al. 2007).

Figure 13. Comparison of the hormone binding regions in the extracellular domain of known teleost growth hormone receptors (GHR) and of selected prolactin receptors (PRLR). Human GHR and human PRLR are used as reference sequences; vertical lines extending below or above residues indicate strict conservation of the reference-residue in the non-human GHR or non-human PRLR sequences, respectively. The symbols ▼ and ▲ indicate a residue that has been conserved between the lamprey sequence and human GHR or between the lamprey sequence and human PRLR, respectively. Dashes (-) represent gaps that were inserted to maximize alignment; * denote missing sequence.

```
                              Region 1     Region 2                            Region 3                   Region 4
Mozambique tilapia PRLR2      || I |VE     S |--AL|NTS-||||DF|LMD||K|Y         T ||---------RETVRQ        D ||
Nile tilapia PRLR2            || I |VE     S |--AL|NTS-||||DF|LMD||K|Y         T ||---------RETVRQ        D ||
black seabream PRLR2          || I |VE     S |--AL|NAT-||VFDM||MQ||K|N         | R---------LEGV||         E ||
zebrafish PRLRb               AI |II       S |--AL|KAY-|E|VD||MVD|| |H         K N---------SLEVF|         E ||
rainbow trout PRLR            |I |VN         |--AL|NN -||VD||V|| ||H           K N---------SETVY          D ||
Mozambique tilapia PRLR1      LI |VS         |--AL|KTY-|||DI|V||K|H            K |---------SDVV|          E ||
Nile tilapia PRLR1            LI |VS         |--AL|KTY-||QDI|V|||H             K |---------SDVV|          E ||
black seabream PRLR1          |I |VN         |--AL|RT -||VDI|V||K|N            K S---------SDTVN          E ||
grass carp PRLR               |L |VN         |--AL|KNI-||VD||V|| |K            L H---------SETVY          E ||
zebrafish PRLRa               |L |VN         |--AL|RNI-|E|VD||V|| |N           L N---------SETVK          E ||
fugu PRLR                     |I |VD       F |--AL|NAT-|||DI|ME||K|N           | |---------L|GLQ          E ||
rat PRLR                      |I |KI       | |--|||||S-|||||||||E|E            K |---------|KTTY          D ||
human PRLR                    TSMWRT 98    ATN--QMGSSF-SDELYVDVTYIVQPD 128     REG---------ETLMHE 74      NKE 62

LAMPREY GHR/PRLR              TSMWVM       ASSPTA--NTS-FEKHCIKLLDYVEPD         TQSGS---TL--ANESAE         DML

human GHR                     TSIWIP 124   SNG------GT-VDEKCTSVDEIVQPD 150     RRNTQ---EW--TQEWKE 100     ERE 62
rat GHR                       ||||VS       T||------DL-L|||FT|||||             ||IAH---||--|P|||          |L|
frog GHR                      |||VS        |ED------TE-F|DY|F||D|E|            KKT---------EAN|TD         QQ|
lungfish GHR                  CNV||        |KE-----DVE-Y|KS|F|||||             KGS---------NDD|LD         |QV
sturgeon GHR                  ||||         |QI--Q--DIV-YHQM|FT|||V|            KN----------RDV|Q|         |M|
Japanese eel GHR2             |||TT        |KD--R--GV|-Y|NT|L||EN|H            KNG------S--PKD||          |Q
Japanese eel GHR1             |V|TQ        GRA--Q--DVI-YNQL|FQ|ED|H            TSS------L--PSD|Q          |M
southern catfish GHR          R||TS        |TLHTH--NI|-Y||Q|FN|EN|Y            MKII-----N--PS||Y          |LV
zebrafish ghra                H||TS        |VP--Q--NT|-Y||A|FT|EN|Y            TKAL--------SSD|Q          |QV
migral carp                   H||TS        |VP--Q--NI|-Y|DD|FT|EN|H            TK|A---------S||Q          |Q
catla                         H||TS        |VP--Q--NI|-Y|DT|FT|EN|H            TK|A---------S||R          |Q
orange-fin labeo              H||TS        |VP--Q--NI|-Y|DT|FT|EN|H            TK|A---------S||R          |Q
rohu labeo                    H||TS        |VP--Q--NI|-Y|DT|FT|EN|H            TK|A---------S||           |Q
Wuchang bream GHRa            H||TS        |VP--Q--NI|-Y||A|FT|EN|EH           LEKDS---KKS-SS||           |Q
grass carp                    R||TS        |VP--Q--NI|-Y||A|FT|EN|H            TK|A----L---SS||Q          |Q
Jian carp GHR1b               Q||TS        |VP--Q--NI|-Y||A|FT|EN|H            TK|A--------LS||Q          |Q
Jian carp GHR1a               Q||TS        |VR--E--NI|-Y||A|FT|EN|H            TK|F--------LS||Q          |Q
goldfish GHR                  Q||TS        |VR--E--NI|-Y||A|FT|EN|H            TKHA--------LS||Q          |Q
common carp GHR               |||TS        |VR--E--NI|-Y|DA|FT|EN|H            TK|F--------LS||           |Q
Fugu GHR1                     |V|TN        |HN--V--TYS-DQ|Y|FF|EN|R            K|ES-----GSLVN||           NQ
Japanese medaka SLR           |V|L|        |QN--N--VTYFNEDD|FT|EN|R            KKDP-----P--LS||           DL
tonge sole GHR1               |V||T        GEN--N--VTFYNEND|F||ED|R            KKES-----P--NS||           DQ
Atlantic halibut              |V||A        |QN--A--TFF-NEDD|FT|EN|R            KKDS-----P--|S||           |Q
Chilean flounder GHR1         ||||         |QN--A--TFI-NEDD|FT|EN|R            KKDS-----P--|S||           |Q
Japanese flounder GHR         ||||         |QN--A--TFI-NEDD|FT|EN|R            KKDS-----P--|S||           |Q
turbot GHR                    ||||         |QN--T--TFY-N|DD|FT|EN|R            KKES-----P--|S||           |Q
orange spotted grouper GHR    |V|V         GQN--N--ITYFNEDD|FT|EN|R            KK|L-----P--|S||           DL
gilthead seabream GHR         |V||         GQN--N--VTYLDEDY|FT|EN|R            KKDS-----P--NS||           |Q
black seabream GHR            |V|V         GQN--N--VTYLDEDY|FT|EN|R            KKDS-----P--|S||           |Q
yellow-fin seabream GHR       |V|V         GQN--N--VTYLDEDY|FT|EN|R            KKDS-----P--|S||           |Q
stickleback GHR1              ||||         GQN--N--VTYYNE|D|FR|EN|R            NSL---------AS||           DQA
South American cichlid SLR    |||T         TQN--N--VTYFNEDD|FT|EN|R            KKDS-----H--ASQ|           DQA
Nile tilapia GHR              |||T         TQN--N--ITYFNEDD|FT|EN|R            KKFP-----P--|SQ|           DQ
Mozambique tilapia GHR1       ||||         TQN--N--ITYFNEDD|F||EN|R            KKES-----P--|SQ|           DQ
Zanzibar tilapia GHR          |||VT        TQN--N--IT|FNEDD|F||EN|R            KKES-----P--|SQ|           DQ
Atlantic salmon SLR           V||K         |E|--Q--NK|-Y|TL|FELQD|H            KK|D-----L--|K||           |Q
Masu salmon GHR               V||K         |E|--Q--NK|-Y|TL|FELQD|H            KK|D-----L--|K||           |Q
rainbow trout GHR1            V||K         |E|--Q--NKV-Y|TL|FELQD|H            KK|D-----L--|K||           |Q
channel catfish GHR           |||LH        |QT--N---DV-Y|MFFT|E||F             LKDSK----S--D|T|HN         DLM
southern catfish GHR2         AV|FQ        |RT--N---DV-Y|MYF|LE||F             HKE|K----N--DCK|Q          |FV
zebrafish ghrb                FV|YT        |VD------NV-YE|MSFNIEN|F            LEKDPK--KT--YGK|I          DMI
Wuchang bream GHRb            V||Y|        |QA--L--GIV-Y|MSFN|ED|F             LEKDSK--KS--ERK|S          |LM
Jian carp GHR2b               I||F|        |VP--Q--NI|-Y|ACFT|EN|H             LEK------D--SKK|M          |LM
Jian carp GHR2a               I||FS        |VR--R--NI|-Y|ACFT|EN|H             LEK------D--SKK|M          |LM
Fugu GHR2                     T||TF        |GD--Q--SII-Y|NIID|AD||             NKKHPQ--TP--PN||R          NM|
Japanese medaka GHR           T||TQ        |GD--G--AVV-Y|VFFN|QD|E             NQ|SLS--AA--PA||R          NM|
Nile tilapia GHR2             ||||TY       |RD--E--TIL-Y||GFF|ND|              |HVPPP--LS--SKN|T          NMM
Mozambique tilapia GHR2       ||||TY       |RD--E--TIL-Y||GFF|ND|              |HVPPP--LA--SKN|T          NMM
Zanzibar tilapia GHR2         ||||TY       |RD--E--TIL-Y||GFF|ND|              |HVPPP--LA--SKN|T          NMM
orange spotted grouper GHR    ||||TS       |RD--Q--ATL-Y|NSFN|QD|              NKKSPH--AP--PK||S          NM|
gilthead seabream GHR2        |V|||        |RD--E--STL-Y|NTFT||A|              NKLSPL--DP--PK||T          NM|
black seabream GHR2           |V|V|        |RD--E--STV-Y|NKFT|EA|              NKLSPL--DP--PK||T          NM|
yellow-fin seabream GHR2      |V|V|        |RD--E--STV-Y|NKFT|EA|              NKLSPL--DS--PK||S          NM|
stickleback GHR2              ||||NT       |RD--Q--ATL-Y|NLFH|ED||             TIKPPANFPG--VEV|S          NT|
Atlantic salmon GHR1          KV|MT        |GD--Q--DIL-Y|VIFT|ED|E            INDKN---IS--PK||D          DMN
coho salmon GHR1              KV|MT        |GD--Q--DIL-Y|VIFT|ED|E            INDRN---IS--PK||G          DMN
rainbow trout GHR2a           KV||T        |GD--Q--DIL-Y|VIFT|ED|E            INDRN---IS--PK||G          DMN
Atlantic salmon GHR2          KV|MT        |GD--Q--DNL-Y|VIFT|ED|E            IN|KN---IS--PK||S          DMN
coho salmon GHR2              KV||T        |GD--Q--DNL-Y|VIFT|ED|E            IN|KN---IS--PK||S          DMN
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The structural characteristics of the dimerization domain that allows for the rotation of the receptors involved in the dimer, relative to eachother, has been discussed; however, the area involved in triggering that rotation is critical to these receptors' biological activity as well. In general, these hormones bind both receptors of the dimer in the same general areas of the receptor; these areas, region 1-4, will be examined more closely in the context of Figure 9, to be found later in this section. Focusing on binding site 2, considering its functional importance, the actual interface between the hormone and receptor involves the N-terminus of the hormone and the Gly cavity of the hormone, which is essentially a pocket with Gly as the bottom and large amino acids surrounding to create the walls of the pocket, and Trp122 (Trp72) of the receptor with the Trp essentially filling the pocket. Figure 8 shows strict conservation of this Trp in all species. Comparisons of the GHR and PRLR by Broutin *et al* (2010) indicated that the walls of the cavity involve residues of a-helicies 1 and 3 that form a hydrogen bond network with residues from the receptor and that these residues in the receptors (GHR or PRLR) are topologically similar, but not strictly conserved by amino acid, and not all interacting residues are equivalent in the alignment. For example, Ser142 (Asp120 in PRLR) and Asp144 (Thr122 in PRLR) are residues involved in this network that are in an equivalent position in the alignment. However, Trp122 (GHR) is involved in hydrogen bonding, while at a different location in the alignment Glu of PRLR interacts with the same residue of the hormone.

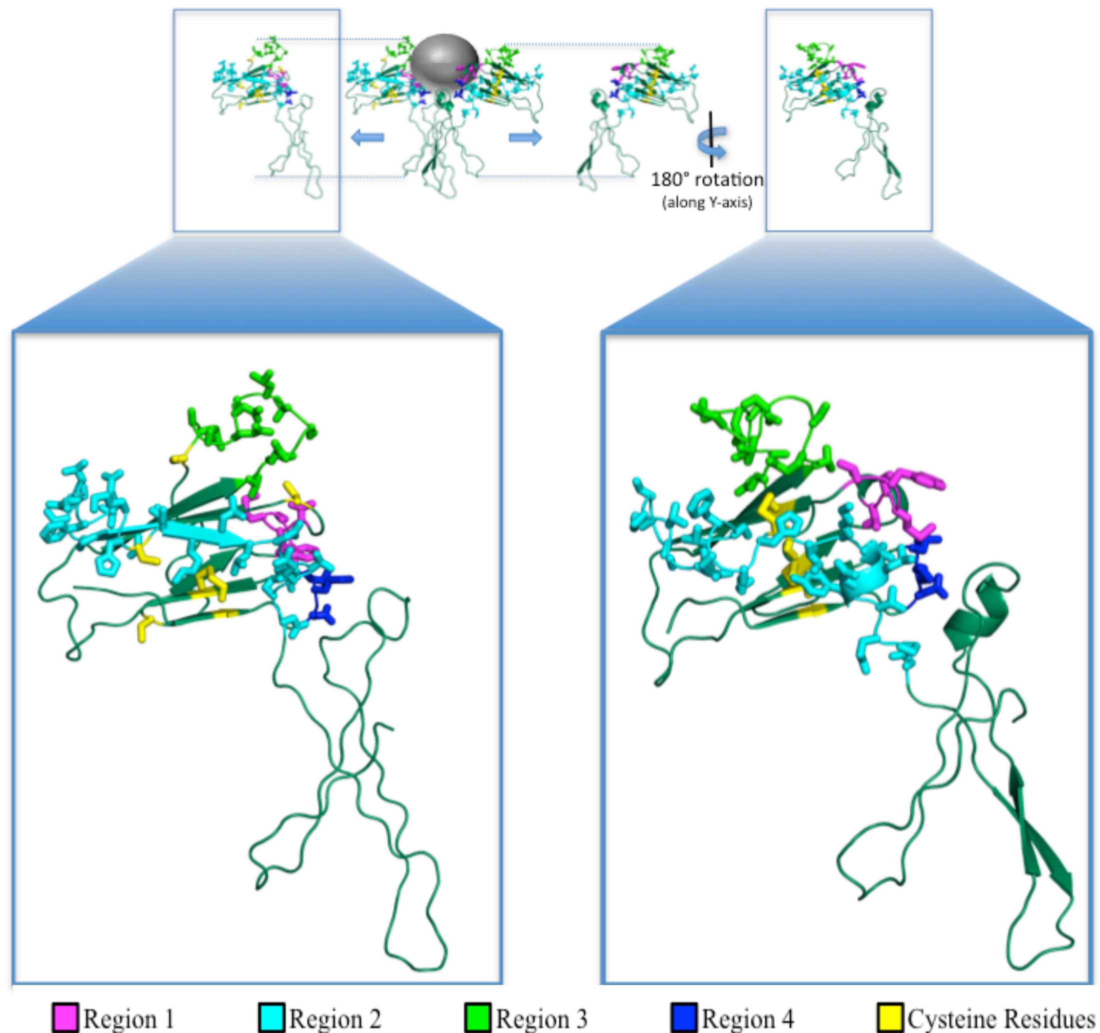

Figure 14. Three-dimensional models of the extracellular domains of the truncated growth hormone receptor (GHR)/prolactin receptor (PRLR), from sea lamprey, oriented to view the binding face of the receptors to illustrate that dimerization and ligand binding alter the conformation of the receptors. In the Upper Panel, center left, the GHR homodimer (GHR_2) is shown bound to growth hormone (GH; represented, simply, by a sphere). For illustrative purposes, the right-hand monomer is separated (Upper Panel, center right) and rotated 90° along the Y-axis (Upper Panel, right) to be in the same orientation, in space, as the left-hand monomer (Upper Panel, left). In the Lower Panel, the left-hand and right-hand monomers are enlarged to show that differences in the conformations of the receptors are apparent, particularly the orientation of the binding regions (regions 1-4; denoted in blue, green, cyan, magenta, respectively) and cysteine residues (denoted in yellow). The models were based on deduced protein sequences using the SWISS-MODEL workspace (Bordoli and Schwede, 2012) to produce coordinates based on the crystal structure of human GH-GHR2 (PBD ID: 3hhr) as template; images were generated with POLYVIEW-3D (Porollo and Meller, 2007).

The N-terminus of the hormone and the residue of the receptor with which it interacts with, is the other section that makes up site 2. This region of the hormone has several interactions with its receptor. Although the N-terminus of both GH and PRL are both involved in binding GHR, the amino acids, which are responsible for binding, are different between the GH-GHR and PRL-PRLR structures. Asp120 is suspected to be involved in the interaction, in PRLR. Pro124, Arg89, Cys140, and Ser142 are believed to be involved in binding, in GHR. While some of these interactions show conservation (Fig. 13), there is a great deal of difference, in general, when examining regions 1-4. For example, Cys140, which is within van der Waals' distance to Ile of hGh, is strictly conserved in the type-1 GHRs but is not seen in any of the type-2 GHRs, just as it is not seen in the PRLRs. Indeed, a complete picture of binding can not been see unless considering the molecular makeup of the hormones as well, but as evidence seems to indicate, the actual residues involved in the interactions that result in binding may be variable. Interestingly, site-directed mutagenesis at the functional binding site 2 of GH creates a GH antagonist with little to no biological activity, and when recombinantly engineered into a homodimer does not only maintain the ability to bind, but does so through the use of two site 1's, one from each of the two GH antagonists involved in the homodimer. Furthermore, this homodimer, created using two antagonists, did not share in the antagonistic effects of the monomers, but instead acted as an agonist (Langerheim *et al*., 2006). With the ability of GH, PRL, and SL to interchangeably bind with the GHR homologues as well as with PRLR homologues, the most notable characteristic of the hormone-binding interface on these receptors, then, is not necessarily any particular residues, but is the global ability to be versatile in its binding. The hormone binding

domain of the lamprey GHBP is shown in Figure 14; with both receptors in the dimer in a position to examine the binding domain, the differences between the receptor that binds the hormone first and the receptor that binds the hormone second becomes quite apparent, supporting the notion that these receptors are plastic in their ability to bind. Returning to examine the alignment (Figure 13), some of the most conserved residues in region 1-4 are not residues that were found to be involved in interactions with the hormones, they are residues such as Ala and Val that are intermittently dispersed in these regions (e.g., V142/V121, V146/V125) which would be necessary to allow various residues, that could potentially interact with differing ligands, to be free of steric hindrance. Even in region 1, where Val is not seen in human, numerous Val residues are intermittently present in this part of GHR, in fish species. In summary, both receptors in the dimer bind the hormone with the same regions (regions 1-4), but because the binding sites on the hormone are located asymmetrically, a major conformational change occurs in the receptor, not upon hormone-binding with the initial receptor of the dimer, but upon binding with the second as the receptor basically "reaches" to where it can interact with site 2 of the hormone; it is this conformational change in the receptor that allows for downstream signaling effects to occur and differential degrees of conformational change results in differential signaling.

Summary and conclusions

We have isolated a fragment of mRNA from sea lamprey, Petromyzon marinus, that we believe is transcribed from the gene that is the evolutionary-precursor to both the GHRs and PRLRs thus far characterized in the vertebrate lineage. The structural heterogeneity of GHRs results from the existence of multiple genes that arose through a

series of gene duplication events during the course of teleost evolution, as well as alternative transcripts of a single gene, and the alternative splicing of a single gene appears to be conserved in this ancient linage. The evolutionary history of these receptors was explored through a phylogenetic analysis that included the characterized protein in lamprey, as well as through a microsynteny analysis of candidate genes, in several species of fish; evidence supports an orthologous relationship between the Type-1 GHRs and GHR lineage that gave rise to humans GHR. Figure 15 illustrates the divergence of the GHRs, based on current information. Conclusions of the evolutionary relationships are consistent with differences in the structural characteristics of these receptors. We suggest that it is plausible, based on our structural assessment, that the lamprey GH and PRL both bind to the lamprey GHR/PRLR. Further studies will need to be done to confirm that the putative GHBP isolated in this study is, indeed, a soluble binding protein able to bind GH.

In the past several years, the question of the existence of a distinct "SLR" has resided. In an already-unstructured nomenclature system, the system became more complex following the characterization of what appeared to be a distinct SL receptor (SLR) from masu salmon, based on 125I-SL binding (Fukada *et al*., 2005) that fell within the clade with type 1 GHRs. Recently, Fukamachi and Meyer (2007) suggested that all of the teleost type GHR1s should be referred to as SLRs, and that the other major clade (type 2 GHRs, which includes the GHR1 and GHR2 of salmonids) be referred to as GHRs. The two GHRs of salmonids most likely arose during the more recent tetraploidization (4R) event associated with the evolution of this group. Reindl *et al*. (2009) suggested that the binding characteristics observed in masu salmon (Fukada *et al*.,

2005) may be a derived trait and that it may be premature to assign the label of "SLR" to all type 1 GHRs. Indeed, as will be discussed below, several type 1 GHRs retain GH binding characteristics.

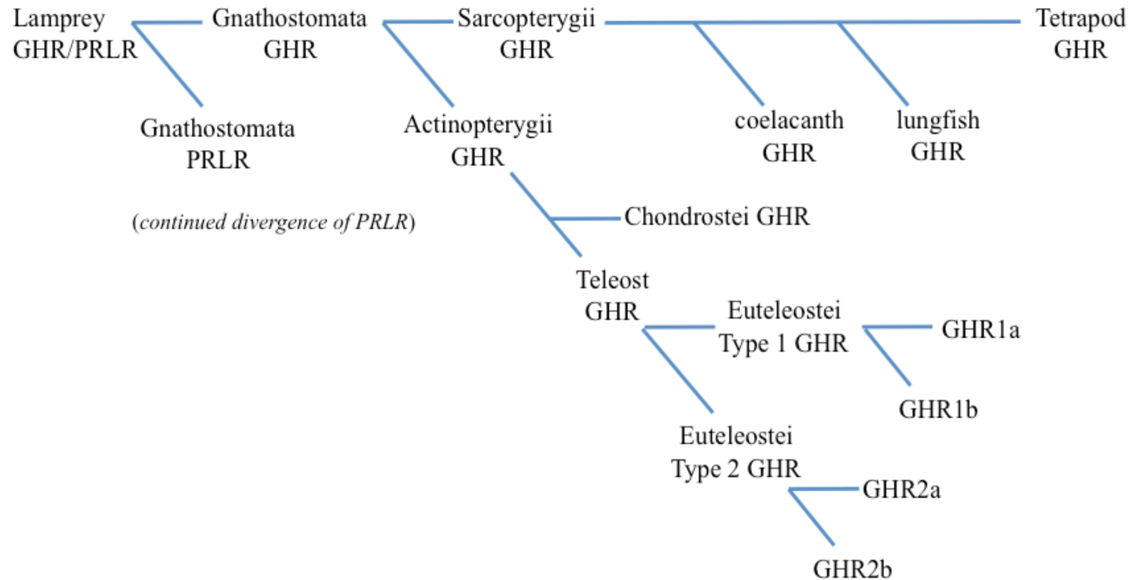

Figure 15. Proposed phylogeny of the growth hormone receptor (GHR) family in vertebrates. The divergence of GHRs and prolactin receptors (PRLR) results from a series of gene duplication events over the course of vertebrate evolution. Subsequent duplication events in teleosts results in multiple types and subtypes of GHRs in this lineage.

Given the confused state of GHR nomenclature, it is clear that a community-wide movement towards a simplified nomenclature system that better represents the evolutionary history of this receptor family is needed. To this end, we suggest a change to a system similar to that already adopted for instances of multiple genes. This system utilizes different numbers to designate genes derived from one duplication event, then different letters to designate paralogues derived from a subsequent round of duplication. In application to GHRs, such a system would use numbers to designate the different GHR

types that arose in the actinopterygian lineage (associated with 3R or FSGD); hence, in the teleosts there would be GHR1s (we urge abandonment of the term SLR to avoid confusion) and GHR2s. The addition of different letters would be added to distinguish paralogues associated with 4R duplication events (e.g., salmonids). This will necessitate changes to existing names (and some temporary confusion), but we have already done so for our GeneBank designations for trout GHRs. So, what were previously referred to as rainbow trout GHR1 and GHR2 (which were both in the type 2 GHR clade), are now GHR2a and GHR2b, respectively (*cf.* GenBank accession nos. NM_001124535 and NM_001124731). A similar scheme is proposed for the GHR1s. Whereas salmonids appear to have lost a gene following their 4R event and possess a single GHR1 (GHR 1 is proposed to be used in preference to SLR so as to avoid confusion and to better represent the evolutionary origins of this gene), other species (e.g., Jian carp, *Cyprinus carpio* var. Jian) retained both GHR1 paralogues, designated GHR1a and GHR1b.

There are at least two reasons for not using the term SLR to describe GHR1, especially in species other than salmon. First, the binding of GH to eel GHR1 could not be displaced by SL (Ozaki *et al*., 2006). Second, GH, but not SL, activated both seabream GHR1 and GHR2 transcription reporter systems (Jiao *et al*., 2006); discussed in greater detail below). In the end, it appears that the ability for these ligands and receptors to cross-bind (e.g., GH with PRLR or PRL with GHR) and elicit cellular effects in response to cross-binding may be species specific; thus, a nomenclature scheme based of the evolutionary relationships of these receptors would be most consistent and would ease the learning of any scientist or student new to the conversation of GH biology, which is something that should be encouraged.

Through an extensive review of literature on the receptors for GH, PRL, and Sl, and the multi-functionality of these hormone (see Ellens and Sheridan, 2012), it is theoretically possible to have the divergence of a unique ligand (e.g., somatolactin) by neofunctionalization following a gene duplication event, without neofunctionalization of a novel/distinct receptor (e.g., somatolactin receptor) if that ligand is able to work through an already-functionalized receptor to elicit differential effects; indeed, the versatile design of the family of receptors for GH, PRL, and SL, allows for plastic binding characteristics and differential downstream signaling effects. Another possibility which would allow for neofunctionalization that results in a new and distinct hormone, without a new and distinct receptor with which it operates through, would be a situation of herterodimerization of receptors within this family (e.g., a PRLR1-GHR1 dimer). To our knowledge, the possibility of herterodimerization of these receptors has never been researched in fish, to date. However, placental lactogen (PL), a mammalian-specific relative of GH/PRL/SL was shown to have prolonged STAT-1 and STAT-3 phosphorylation, and thus unique signaling, through binding of a GHR-PRLR heterodimer (Biener *et al* 2003). Similarly, herterodimerization between alternatively-spliced forms of receptors could, also, serve as a unique receptor for which unique signaling events may be transduced.

Especially in light of the possibility of novel functions through alternative transcripts of a gene or through herterodimerization between different homologues in this family of receptors (or herterodimerization between different transcripts of the same or different homologue(s), for that matter), hopefully this clarified understanding of the evolutionary history of these receptors which reflects the molecular basis of these

receptors, will support the continued conversation regarding the molecular basis of functionality and how that functionality is transduced to diverse and integrated physiological effects in an organism.

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CONCLUDING REMARKS AND FUTURE DIRECTIONS

General remarks and comments on the future direction of this research

With the information gained in this study, there are numerous objectives that could yet be done. First and foremost, the presence of this protein, and any alternative transcripts, must be confirmed. Gene expression could be determined for this protein, as well as any alternative transcripts, through Northern blotting. Western blotting could also be used to confirm translation of these transcripts into protein. Another way to biologically confirm that the binding protein is indeed a soluble binding protein would be through the precipitation of the soluble extracellular domain, while keeping in mind that there may be two different binding proteins in existence (one produced by alternative splice, the other produced by proteolytic cleavage). Furthermore, the putative GHBP, and any full-length receptors that may exist, would have to be confirmed to bind these ligands, in order to properly dub these proteins GH-binding protein or GH-receptors. A radioreceptor assay, with ^{125}I-labeled hormone, could be done to assess binding and establish the kinetics of binding.

Through reviewing literature on the topic of GH and GHR, additional points of interest, pertinent to the research that has already been done, were discovered which should be looked into. First, numerous reports of metal ion interactions with the type-1 cytokine receptors and their ligands have been reported, most notably for zinc. Although zinc is not required for the binding of GH or PRL to their receptors, the binding affinity between hGH and hPRLBP was increased about 8000-fold by addition of 50 μM $ZnCl_2$ (Cunningham *et al*., 1990). In this particular investigation, the hPRLBP and hGHBP were expressed and secreted into the periplasm of Escherichia coli, and binding determined

with hGH and hPRL were analyzed. This study indicated that [Zn2+] was required for tight binding of hGH to the hPRL receptor but not for binding to the hGH receptor. Furthermore, the binding of hGH to hPRLBP, under these conditions, is nearly 100-fold stronger than for hPRL and more than 10-fold stronger than the affinity of hGH for one hGHBP (Cunningham *et al*., 1990). These results have been shown to be due to the direct interaction between Zn2+, the hormone, and the extracellular receptor (Cunningham *et al*., 1990; Voorhees *et al*., 2011). To date, the interaction of Zn2+ or any other metal ion with these GH-family receptors has not been examined in fish. Finally, the differential expression of these ligands, and how multiple genes for GH, PRLR, and SL, affect binding and subsequent intracellular signaling must be investigated. To date, most of the binding studies on these receptors have not considered that multiple GH genes may be differentially expressed in a species-specific manner. With a new understanding of how the GHR (and PRLR) can differentially bind a ligand, due to the plastic nature of ligand binding in these receptors, to result in differential signaling, it can be concluded that different GHs (GH1 and GH2) and the relative amounts of those GHs would likely effect results, both in binding assays and in cell signaling studies. Furthermore, other molecular variants of hGH have been reported, and summarized by Bustamante *et al*., (2010); these variants include a 24 kDa and 12 kDa glycosylated hGHs, as well as a deamidated hGH and phosphorylated hGHs. Small hGH isoforms (5-kDa and 17-kDa), produced by fragmentation of the predominate form of hGH (22 kDa) have also been discovered in humans, and stable hGH dimers (a 35 kDa dimer and 45 kDa hGH dimer), as well as hGH oligomers have been reported. Differing biological activities were reported in response to these GH additional GH variants; some of these variants had increased

biological activity, when compared to the 22 kDa hGH form, and others had reduced biological activity or were biologically inactive. However, distinct biological activities, which regulated only a limited number of metabolic and physiological processes was also reported with some of the hGH variants (Bustamante *et al*., 2010). Thus, in reviewing recent literature on the physiological actions of these hormones, is clear that the use of hormone from other species in binding assays or studies assessing biological activity should be avoided when possible. Not only could differences in the proteins, from one species to another, cause differences in binding characteristics or biological activity, but the presence of structural variants of these hormones and the relative amounts of the variants, if present, could be different from species to species, and could be differentially regulated in a species specific manner.

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