General and Comparative Endocrinology 246 (2017) 309–320

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Research paper

Evolution of specificity in cartilaginous fish glycoprotein hormones and receptors

Hanna B. Buechi, Jamie T. Bridgham *

Institute of Ecology and Evolution, University of Oregon, Eugene, OR 97403, USA

ARTICLE INFO

Article history:
Received 30 August 2016
Revised 1 December 2016
Accepted 2 January 2017
Available online 4 January 2017

Keywords:
Glycoprotein hormone
Glycoprotein receptor
Thyroid-stimulating hormone
Follicle-stimulating hormone
Luteinizing hormone
Evolution
Phylogeny
Elephant shark

ABSTRACT

Glycoprotein hormones (GpH) interact very specifically with their receptors to mediate hypothalamic-pituitary-peripheral gland endocrine signaling. Vertebrates typically have three functionally distinct GpH endocrine signaling complexes: follicle-stimulating hormone, luteinizing hormone, and thyroid-stimulating hormone, and their receptors. Each hormone consists of a common α subunit bound to one of three different β subunits. Individual hormone subunits and receptors are present in genomes of early metazoans, and a subset of hormone subunits and receptors has been recently characterized in sea lamprey. However, it remains unclear when the full complement of hormone and receptor protein families first appeared, and when specificity of interactions between GpH hormones and receptors first evolved. Here we present phylogenetic analyses showing that the elephant shark (*Callorhinchus milii*) genome contains sequences representing the current diversity of all hormone subunits and receptors in these co-evolving protein families. We examined specificity of hormone and receptor interactions using functional assays testing reporter gene activation by elephant shark follicle-stimulating hormone, luteinizing hormone, and thyroid-stimulating hormone receptors. We show highly specific, dose-responsive hormone interactions for all three complexes. Our results suggest that co-evolution of specificity between proteins in these endocrine signaling complexes occurred prior to the divergence of Chondrichthyes from the chordate lineage.

1. Introduction

Glycoprotein hormones (GpH) and receptors (GpHR) are classic examples of co-evolution between protein families because they have evolved well-characterized, highly specific interactions in vertebrate model systems (Moyle et al., 1994; Freamat et al., 2006). They are crucial to endocrine signaling and regulate both reproduction and thyroid function through the hypothalamic-pituitary-gonadal (HPG) and -thyroidal axes (HPT) (Campbell et al., 2004). Despite having evolved important regulatory functions in vertebrates, little is known about the origin of these hormone-receptor complexes or their functioning in non-vertebrates (Rocco and Paluzzi, 2016).

GpH signaling complexes consist of three proteins: a receptor, and a heterodimeric hormone containing a common α subunit and a unique β subunit that determines specific receptor interactions. Previous studies have shown that most vertebrates have at least three well-characterized receptors: follicle-stimulating hormone receptor (FSHR) activated by follicle-stimulating hormone (FSH), luteinizing hormone receptor (LHR) activated by luteinizing hormone (LH), and thyroid-stimulating hormone receptor (TSHR) activated by thyroid-stimulating hormone (TSH) and thyrostimulin (Dos Santos et al., 2011; Kubokawa et al., 2010; Szkudlinski et al., 2002).

Both α and β hormone subunits are cysteine knot proteins and share an ancient common ancestor (Heyland et al., 2012; Roch and Sherwood, 2014) that duplicated in tandem, resulting in both subunits being encoded on the same chromosome (Dos Santos et al., 2009). Synteny analyses show that subsequent tandem and whole genome duplications followed by gene losses resulted in multiple α and β subunits (Dos Santos et al., 2011). Most vertebrates have two α subunit paralogs (GpHA2 and GpHα) and four β paralogs (GpHB5, TSHβ, FSHβ, and LHβ). Primate-specific duplications of the LHβ gene created several tandem genes encoding chorionic gonadotropins (CG) that activate LHR and are expressed in the placenta to maintain pregnancy (Maston and Ruvolo, 2002). GpHB5 heterodimerizes with GpHA2 to form thyrostimulin which has been shown to activate TSHR (Nakabayashi et al., 2002), although its physiological function is less clear. GpHα heterodimerizes with

* Corresponding author at: Institute of Ecology and Evolution, 5289 University of Oregon, Eugene, OR 97403, USA.
E-mail address: jamieb@uoregon.edu (J.T. Bridgham).

http://dx.doi.org/10.1016/j.ygcen.2017.01.007
0016-6480/

all other β subunits to form endocrine hormones that activate their specific receptors.

GpHRs belong to a large family of G-protein-coupled receptors with characteristic transmembrane (TM) and extracellular leucine-rich repeat domains (LRR) that determine specificity of hormone interactions (Vassart et al., 2004). They fall into two categories, gonadotropin receptors and thyrotropin receptors, which diverged from a common ancestor (Roch and Sherwood, 2014). A subsequent tandem duplication of the gonadotropin receptor gene gave rise to FSHR and LHR (Maugars and Dufour, 2015; Rousseau-Merck et al., 1993).

Both GpH hormone subunit and GpHR protein families are evolutionarily ancient. Single proteins belonging to these families have been identified in many lophotrochozoans, including the nematode *Caenorhabditis* (Kudo et al., 2000), and mollusk *Aplysia* (Heyland et al., 2012), and ecdysozoans, including the arthropod *Drosophila* (Sudo et al., 2005). The cephalochordate, *Branchiostoma*, has two α and one β hormone subunits (Dos Santos et al., 2009; Kubokawa et al., 2010), and a single receptor (Dong et al., 2013). The agnathan, *Petromyzon marinus*, has a single α subunit most similar to GpHA2, two β subunits called GpHB5 and GpHβ two receptors called GpHR1 and GpHR2 (Sower et al., 2015). Subsequent diversification of the protein families resulted in FSH, LH, and TSH hormones and receptors that have been isolated and characterized from various vertebrate species, including many teleosts, birds, and mammals (Cahoreau et al., 2015; Levavi-Sivan et al., 2010; Maugars et al., 2014).

Elephant sharks (*Callorhinchus milii*) were chosen for genome sequencing because of their relatively small genome size, and evolutionarily important position as basally-branching holocephalans within cartilaginous fish (Venkatesh et al., 2014). Elephant sharks are known to have all three receptors (TSHR, FSHR and LHR) as well as an α subunit and two thyrotropin β subunits (TSHβ1 and TSHβ2) (Maugars and Dufour, 2015; Maugars et al., 2014), but other hormone subunits have not yet been described. Here we use phylogenetic relationships to identify all elephant shark GpH hormone and receptor proteins, and cell culture reporter gene assays to show the specificity of glycoprotein hormone and receptor interactions.

2. Materials and methods

2.1. Molecular phylogenetics

Protein sequences of α and β hormone subunits and receptors used for phylogenetic analyses were identified through keyword and BLAST searches of the NCBI non-redundant protein sequences database (nr) and GenBank protein and genome databases. Elephant shark (*Callorhinchus milii*) sequences were obtained from the Elephant Shark Genome Database: http://esharkgenome.imcb.a-star.edu.sg/ (Venkatesh et al., 2014), refSeq genomic and transcriptomic resources available through GenBank, skatebase.org, and previously published information (Wyffels et al., 2014; Dos Santos et al., 2011; Maugars et al., 2014). The Elephant Shark Genome Database covers 937 Mb of the 1 Gb genome of *Callorhinchus milii*. This genome was first described with a 1.4× coverage, but with continued sequencing it reached a depth of 19.25× when we performed our search (Wyffels et al., 2014). Some accessions were mislabeled (i.e. XP_007903189 labeled LHR, but actually FSHR) as discovered through a reverse-BLAST verification step. For a full list of sequences and accessions including elephant shark data, see Supplemental Tables S1–S3 (α subunits, β subunits and receptors).

Each of the protein families were aligned using Multiple Sequence Alignment by Log-Expectation (MUSCLE) (Edgar, 2004) with a total of 107 α subunits, 149 β subunits, and 116 receptor sequences. The alignments were manually inspected for appropriate placement of lineage-specific indels, especially in the receptor hinge region between the LRR and transmembrane domains, and the N- and C-terminal domains that were difficult to align between paralogous proteins.

ProtTest analyses (Abascal et al., 2005) to determine the best-fit evolutionary model resulted in using the Jones-Taylor-Thornton model with a four-category gamma-distributed among-site rate variation and estimated proportion of invariant sites. Phylogenetic analyses were inferred using maximum likelihood with PhyML version 2.4.5 (Guindon and Gascuel, 2003). Statistical support for each node was evaluated by obtaining the approximate likelihood ratio (likelihood of the best tree with the node divided by the likelihood of the best tree without the node) (Anisimova and Gascuel, 2006). The trees were not species-constrained.

2.2. Elephant shark clones

The full-length coding region of each elephant shark hormone subunit was synthesized by GENEWIZ (South Plainfield, NJ) using codon optimization for expression in mammalian tissue culture cells and preceded by Kozak sequences. Each coding region was then subcloned into pcDNA3 vector using HindIII and EcoRI restriction digests. Final clones were verified by Sanger sequencing (GENEWIZ). Full-length FSHR and TSHR were synthesized by GenScript (Piscataway, NJ) using the same strategy. All sequences available in the elephant shark genome for LHR were lacking the first exon, including the signal peptide, as determined by sequence alignments. The first 36 amino acids, including the signal peptide, from the elephant shark FSHR receptor were fused to the N-terminus of the LHR sequence using a BamHI restriction digest and a mutagenesis reaction to correct coding sequence.

Fusions of all possible combinations of α and β hormone subunits were cloned into pcDNA3 expression vector. The β subunit was cloned 5′ of the α subunit and they were separated by an 8-residue Ser-Gly repeat linker as described previously (Heikoop et al., 1997). A Kozak sequence was added 5′ of the β subunit start codon and the stop codon of the β subunit and the signal peptide of the α subunit were both removed.

2.3. Fusion hormone synthesis in CHO-K1 cells

Chinese hamster ovary (CHO-K1, ATCC# CCL-61) cells were grown in α-MEM supplemented with 10% fetal bovine serum in the presence of antibiotics. They were passaged twice a week via trypsinization. At 80–90% confluency, cells were transferred to a 6-well plate and grown for 18 h in α-MEM supplemented with 10% fetal bovine serum. They were transiently transfected with Opti-MEM transfection media, Plus and Lipofectamine transfection reagents (ThermoFisher, Waltham, MA), 2400 ng pUC19 plasmid, and 300 ng fusion hormone plasmid. Transfection media was replaced after 4 h with 2.25 ml serum-free α-MEM. After 72 h, the media was collected, centrifuged, and concentrated at 4 °C in 0.5-mL centrifugal filters, Amicon Ultracel 3 K, per the manufacturer's instructions, to approximately 100 μl. Individual subunits are 16–18 kD and fusion hormones are ~32 kD. Concentrated hormones were stored in aliquots at −20 °C.

2.4. Functional hormone-receptor interactions

Reporter gene assays were used to test for receptor activation by hormone protein subunits. Transient transfections were done in human embryonic kidney cells (HEK293T/17, ATCC# CRL-11268) that were grown in DMEM supplemented with 10% fetal bovine serum. They were passaged three times a week via trypsinization. At 80–90% confluency, cells were transfected in a