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Minireview

Comparative genomics of leucine-rich repeats containing G protein-coupled receptors and their ligands

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Abstract

Leucine-rich repeats containing G protein-coupled receptors (LGRs) constitute a unique cluster of transmembrane proteins sharing a large leucine-rich extracellular domain for hormone binding. In mammals, LGRs steer important developmental, metabolic and reproductive processes as receptors for glycoprotein hormones and insulin/relaxin-related proteins. In insects, a receptor structurally related to human LGRs mediates the activity of the neurohormone bursicon thereby regulating wing expansion behaviour and remodelling of the newly synthesized exoskeleton. In the past decade, novel insights into the molecular evolution of LGR encoding genes accumulated rapidly due to comparative genome analyses indicating that the endocrine LGR signalling system likely emerged before the radiation of metazoan phyla and expanded throughout evolution. Here, we present a short survey on the evolution of LGRs and the hormones they interact with.

Keywords: Leucine-rich repeats containing G protein-coupled receptor; Glycoprotein hormone; Genomics; Development; Evolution; Bursicon

1. Introduction

G protein-coupled receptors (GPCRs) are transmembrane proteins that respond to a diverse array of stimuli [e.g., light, odorants, taste molecules, lipid analogous, amines, nucleotides, peptides and protein hormones] and transmit these signals intracellularly through activation of heterotrimeric G proteins (Joost and Methner, 2002). Genome analyses identified GPCRs as one of the largest functional protein "superfamilies" with about eight hundred human GPCRs (Bjarnadottir et al. 2006), one thousand in the nematode, *Caenorhabditis elegans* (Bargmann, 1998) and two hundred in the fruit fly, *Drosophila melanogaster* (Brody and Cravchik, 2000; Vanden Broeck, 2001a). The majority of GPCRs is believed to act as olfactory receptors, while a distinct subset regulates developmental processes as receptors for peptide or protein hormones.

GPCRs share several key structural features, i.e., seven α-helical membrane-spanning segments interconnected by three intra- and extracellular loops (the "serpentine region") and an intracellular C-terminal tail. Within the large rhodopsin-like receptor family ("family A GPCRs"), leucine-rich repeats containing GPCRs (LGRs) constitute a distinct subgroup characterized by a peculiar protein architecture. A true hallmark for LGRs is the large N-terminal extracellular (ecto-)domain involved in selective hormone binding (Braun et al. 1991) that is composed of tandem arrays of leucine-rich repeat (LRR) amino acid motifs, N- and C-terminally flanked by cysteine-rich sequences (Kajava, 1998). A so-called hinge region connects this ectodomain with the serpentine region.

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In mammals, LGRs mediate the activity of large glycoprotein hormones, i.e., gonadotropins [follicle stimulating hormone (FSH), luteinizing hormone (LH), choriogonadotropin (CG)] and thyroid stimulating hormone (TSH) and, in consequence, have long been known to act as key players in the endocrine regulation of reproduction (gonadotropins) and metabolism (TSH) (Grossmann et al. 1997; Themmen and Huhtaniemi, 2000). Recently, genomic approaches forced us to revise the evolution of the LGR signalling system. Novel insights into the molecular evolution of LGR encoding genes emerged and demonstrated that LGRs predate the divergence of major animal phyla and expanded throughout metazoan evolution. Here, we present an overview of recent accomplishments illustrating the molecular evolution of the LGR/hormone endocrine system in metazoans.

2. Leucine-rich repeats containing GPCRs exist in distinct animal phyla and can be classified into three subtypes

Multiple LGR encoding genes from diverse vertebrates and invertebrates have been identified *in silico* from genomic and/or complementary DNA resources, mainly based on the presence of the N-terminal ectodomain. Based on overall sequence similarity and the architecture of the ectodomain, i.e., the number of leucine-rich repeats and the presence or absence of a low density lipoprotein motif, LGRs can be classified into three subtypes (Fig. 1).

3. Subtype A contains receptors for glycoprotein hormones

A first LGR subclass comprises vertebrate receptors for cystine knot-forming gonadotropins (FSH, LH, CG) and TSH, all ~30 kDa dimeric proteins composed of a common α-subunit (glycoprotein hormone subunit alpha 1, GPA1) that non-covalently associates with a hormone-specific β-subunit (FSHβ, LHβ, CGβ or TSHβ). Apart from these "classic" glycoprotein hormone subunits, two additional cystine knot-containing proteins, related to the common α- and hormone-specific β-subunit, respectively, are encoded in the human genome and that of several other vertebrates (Hsu et al. 2002a; Park et al. 2005). In mammals, a "new hormone" consisting of this second α-subunit (glycoprotein hormone subunit α2, GPA2) and the fifth β-subunit (glycoprotein hormone subunit β5, GPB5) is capable of activating the TSH receptor both *in vitro* and *in vivo* and is therefore referred to as thyrostimulin (Nakabayashi et al. 2002). Although the exact physiological function(s) of thyrostimulin is yet to be determined, GPA2/GPB5 is expressed in multiple tissues including anterior pituitary, nervous system, skin, retina, testis and intestine where it might participate in paracrine signalling via non-thyroidal TSH receptors (Nakabayashi et al. 2002; Li et al. 2004; Okada et al. 2006; Nagasaki et al. 2006).

The sea anemone, *Anthopleura elegantissima*, LGR not only shares remarkable homology with vertebrate glycoprotein hormone receptors (44–48% sequence identity with the serpentine region of rat subtype A LGRs), but also shares a

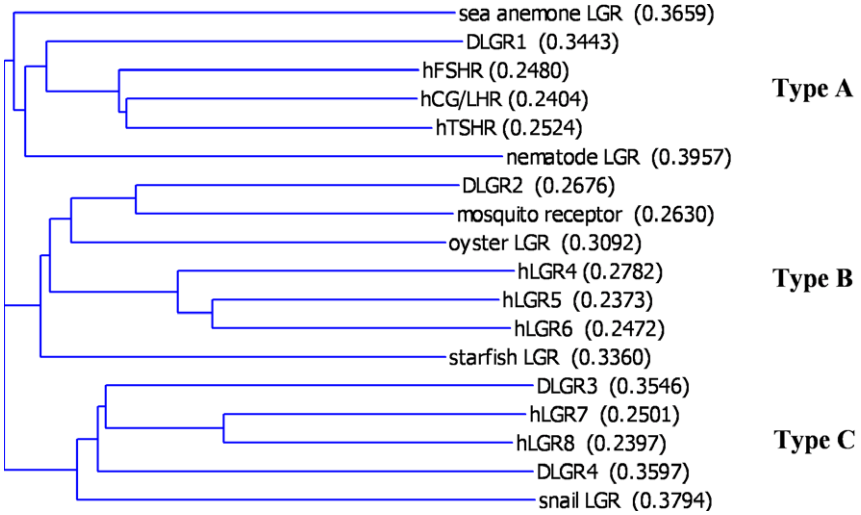


Fig. 1. Dendrogram of a sequence comparison of leucine-rich repeats containing GPCRs from vertebrates and invertebrates resulting from CLUSTALW analysis. The GenBank accession number for each of the used amino acid sequence is indicated between brackets in the legend. LGRs are phylogenetically classified into three different subtypes, i.e., type A, B and C (see text for more details). Subtype A LGRs contain a cluster of human glycoprotein hormone receptors (hFSHR, hLH/CGR and hTSHR) [AAB26480, AAB19917 and CAA02195, respectively] that are related to a nematode (*C. elegans*) LGR [AAF82248], fruit fly (*D. melanogaster*) DLGR1 [NP_524393] and a LGR from sea anemone (*A. elegantissima*) [P35409]. The fruit fly bursicon receptor (DLGR2) [NP_476702] belongs to subtype B LGRs and branches with a predicted receptor from the malaria mosquito (*A. gambiae*) and orphan receptors from oyster (*C. gigas* LGR) [CAD71143] and humans (hLGRs 4–6) [NP_060960, NP_003658 and NP_001017403, respectively]. Also a LGR from the echinoderm starfish (*A. pectinifera*) [BAB68208] belongs to subtype B receptors. Finally, subtype C LGRs contains receptors harbouring a N-terminal low density lipoprotein (LDL) motif. Here, human receptors LGR7 and LGR8 [Q9HBX9 and AAL69324, respectively], two predicted *D. melanogaster* receptors for unknown ligands (DLGR3 and DLGR4) [NP_733115 and ABI34171] and snail (*L. stagnalis*) LGR [CAA80651] are represented.

partially conserved gene organization, indicating a common evolutionary origin (Nothacker and Grimmelikhuijzen, 1993; Vibede et al. 1998). The fruit fly, *D. melanogaster*, receptor DLGR1 and the only LGR receptor encoded in the genome of the nematode, *C. elegans*, also branch into subtype A LGRs (Hauser et al. 1997; Nishi et al. 2000; Kudo et al. 2000). GenBank searches in these latter organisms resulted in the prediction of single pairs of putative glycoprotein hormone α- and β-subunit homologues, indicating that the glycoprotein hormone family likely emerged before the radiation of bilateral metazoans (Hsu et al. 2002a; Park et al. 2005). A heterodimer of recombinant fruit fly GPA2/GPB5-related proteins stimulates cAMP-production mediated by fly DLGR1, demonstrating the conserved intracellular coupling mechanisms (mainly through G_s-proteins) between fly and vertebrate subtype A LGRs (Sudo et al. 2005). In addition, also nematode LGR likely functions by activating G_s-proteins, given that recombinant expression of this receptor in mammalian cells results in an increased basal production of intracellular cAMP (Kudo et al. 2000).

Invertebrate glycoprotein hormone-related α- and β-subunits appear to most closely resemble human GPA2/GPB5. Despite the modest sequence similarity between fly and human GPA2/GPB5 (about 20–25%, Figs. 2a and b), all cysteine residues essential for the cystine knot-structure are conserved. In addition, fly and human GPB5 contain only 10 cysteine residues in their mature form, instead of 12 cysteines found in human gonadotropin and TSH β-subunits (Fig. 2b) and therefore appear to represent more ancient forms of glycoprotein hormone subunits. Although fly GPB5 contains an extended C-terminal tail, it appears to lack the C-terminal cysteine residue necessary for the formation of the "seat belt" structural motif present in human TSH and gonadotropin β-subunits (Fig. 2b). Therefore, fly and other invertebrate glycoprotein heterodimers, as well as thyrostimulin, likely are depleted of a C-terminal "seat belt" region that is implicated in dimer assembly and stability of vertebrate glycoprotein hormones (Lapthorn et al. 1994). Nevertheless, LGR stimulation by human and fly GPA2/GPB5 suggests that this region might not be crucial for bioactivity (Nakabayashi et al. 2002; Sudo et al. 2005). Similarly, in human FSH a deletion of the β-subunit "seat belt" region does not significantly affect hormone activity, although it hampers dimer formation (Hiro'oka et al. 2000). Clearly, comparative protein modelling approaches are needed to elucidate the three-dimensional structure of thyrostimulin and novel invertebrate glycoprotein hormone homologues, and to verify whether the possible interaction between both subunits occurs in a similar way as in vertebrate glycoprotein hormones.

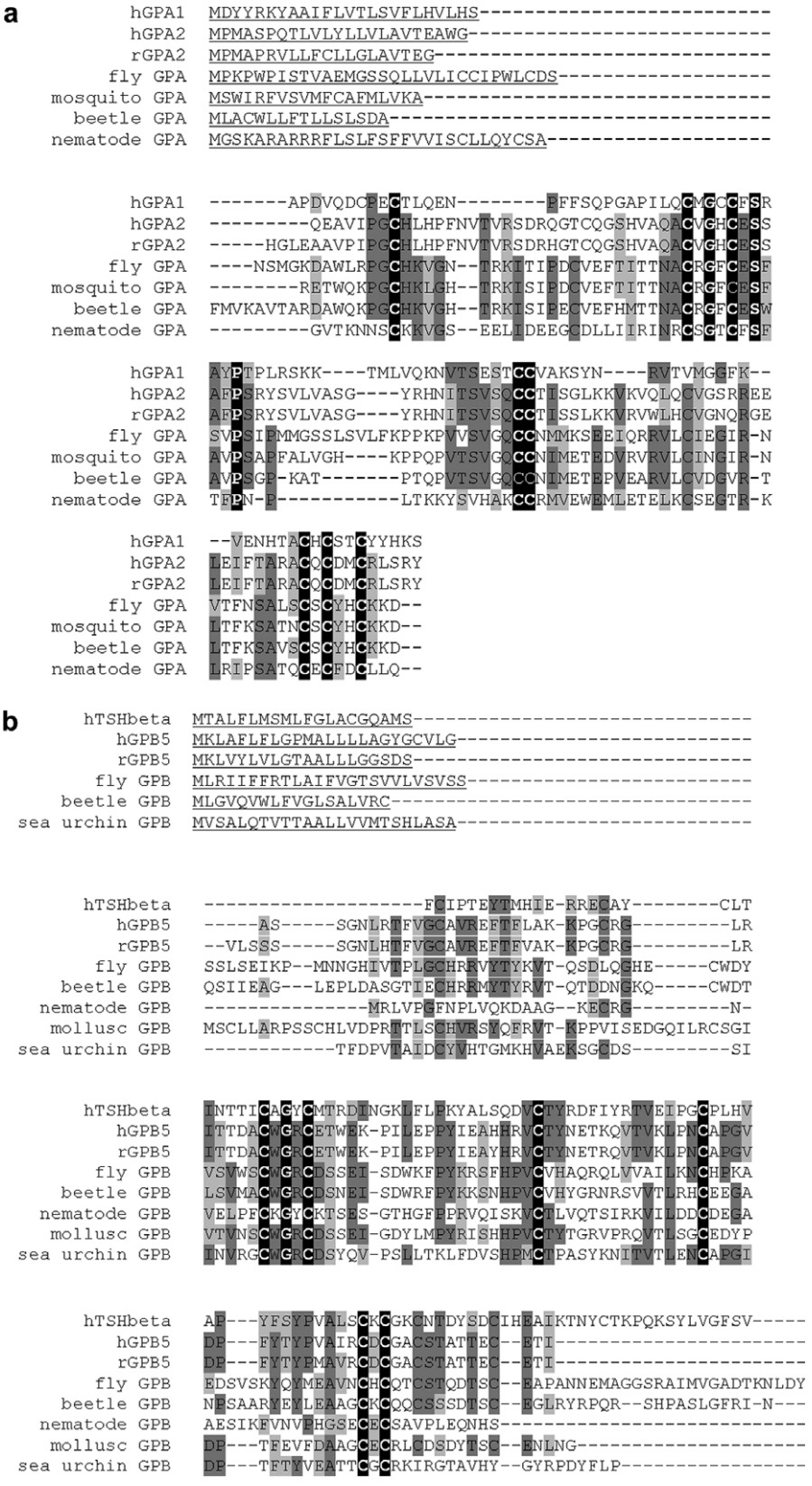
4. Subtype B contains human orphan receptors with essential roles in development and receptors for the arthropod bursicon hormone

LGRs of subtype B are characterized by the presence of 13–18 LRRs. Here, three paraloguous mammalian receptors for unknown ligands (LGRs 4–6) form a cluster (McDonald et al. 1998; Hsu et al. 1998, 2000) (Fig. 1). Taking advantage of gene trapping approaches and immunohistochemical analyses, a detailed atlas of mouse LGR4 expression was realized, indicating expression in a broad range of tissues including kidney, bone/cartilage, heart, stomach and nervous cells (Mazerbourg et al. 2004; Van Schoore et al. 2005). Studies with knock out mice implicated LGR4 signalling in perinatal growth, development and morphogenesis of the male reproductive tract (Mazerbourg et al. 2004; Mendive et al. 2006). LGR5 null mice exhibit neonatal lethality associated with ankyloglossia, a phenotype characterized by fusion of the tongue to the floor of the mouth (Morita et al. 2004).

Mammalian LGRs 4–6 exhibit structural homology with a LGR from *Drosophila* (fly DLGR2 or rickets) (Eriksen et al. 2000; Nishi et al. 2000) and predicted receptors from other insect species such as the mosquito, *Anopheles gambiae*, and the honeybee, *Apis mellifera* (Hill et al. 2002; Hauser et al. 2006). Fly DLGR2 has shown to be sensitive for the insect neurohormone bursicon, thereby being the first invertebrate LGR for which the ligand was characterized. Bursicon bioactivity has long been known to control the sclerotization (hardening) and melanization of the newly synthesized cuticle in insects (Fraenkel and Hsiao 1962; Fraenkel et al. 1966). In addition, genetic studies revealed its regulatory role in wing expansion behaviour and wing development in adult insects emerging from the pupal case (Baker and Truman, 2002; Kimura et al. 2004; Dewey et al.

Fig. 2. Amino acid sequence comparisons of glycoprotein hormone subunits. The GenBank/EMBL accession number of each protein is indicated between brackets. Fully conserved amino acids are displayed in white bold, similar residues are indicated by grey shading. Predicted signal peptides for each of the proteins are underlined. In the particular case of nematode and mollusc GPB (b) no convincing signal peptide could be predicted using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). (a) The common human gonadotropin and TSH α-subunit (hGPA1) [P01215] displays similarity with the thyrostimulin α-subunit of human (hGPA2) [NP_570125] and rat (rGPA2) [Q925Q4]. Putative α-subunit homologues have been identified in invertebrates: fruit fly (*D. melanogaster*) GPA (fly GPA) [AAX38184], malaria mosquito (*A. gambiae*) GPA [amino acid sequence derived from cDNA-clone AGAFX75TR], a GPA sequence from the red flour beetle (*T. castaneum*) (beetle GPA) [predicted from the genome data] and nematode (*C. briggsae*) GPA [WormBase protein CBP24452]. (b) The human TSHβ subunit [EAW56626] shows moderate sequence similarity with the thyrostimulin β-subunit from human (hGPB5) [Q86YW7] and rat GPB5 (rGPB5) [NP_001007014]. In insects, fruit fly (*D. melanogaster*) GPB (fly GPB) [AJ440748] and beetle (*T. castaneum*) GPB [predicted from the genome data] display relatively high sequence similarity with each other. Other putative β-subunits exist in phylogenetically diverse animals like nematodes, echinoderms and molluscs: nematode (*C. brigssae*) GPB [WormBase protein CBP00374], sea urchin (*S. purpuratus*) GPB [XM_793126] and sea slug (*A. californica*) (mollusc GPB) [AAX35673].

2004). Bursicon is a ~30 kDa dimeric protein hormone composed of two subunit proteins (Bursα and Bursβ) that are distantly related to each other and to members of the cystine knot-containing superfamily of developmental hormones and growth factors [e.g., glycoprotein hormone subunits, bone morphogenetic proteins



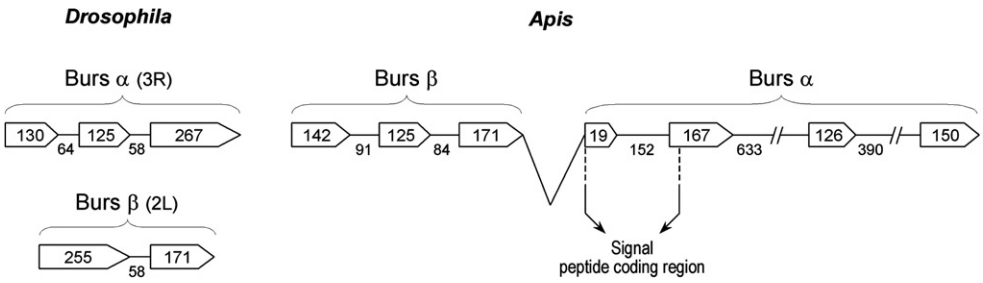


Fig. 3. Genomic organization of fly and honeybee bursicon encoding genes. Left panel: In the fruit fly (*Drosophila melanogaster*) Bursα and Bursβ genes are encoded on separate chromosomal arms (3R and 2L, respectively). The fly Bursα gene contains three exons (arrows) connected by two short introns (thin lines) while the fly Bursβ gene contains two exons separated by one intron. The number of nucleotides for each exon/intron is indicated. The similar positioning of the second intron of fly Bursα and the intron of Bursβ suggests a common ancestral relationship between both fly bursicon genes. Right panel: In the honeybee (*Apis mellifera*) both bursicon subunit encoding genes are positioned in a genomic locus of approximately 3.5 kb. The bee Bursβ gene harbours three exons and two introns and is encoded just upstream of the Bursα gene with an intergenic space of 1.3 kb. The signal sequence of bee Bursα is disrupted by the first intron and continues in the second exon. Honeybee bursicon gene organization also supports the hypothesis that both bursicon subunit genes originated from a duplication event of an ancestral cystine knot encoding gene.

(BMPs), transforming growth factor beta (TGFβ) family members] (Mendive et al. 2005; Luo et al. 2005). In *Drosophila*, the Bursα and Bursβ genes display a partially conserved gene structure suggesting that the bursicon heterodimer might have arisen from an early duplication event of an ancestral gene encoding a cystine knot-containing protein (Fig. 3). Interestingly, analysis of the genomic organization of bursicon encoding genes in the honeybee, *A. mellifera* (Fig. 3) and the red flour beetle, *Tribolium castaneum* seem to support this hypothesis. Although *in silico* screenings of genome and "expressed sequence tag" database (dbEST) resources point at the exceptional preservation of both bursicon subunits in arthropods, no convincing vertebrate bursicon orthologues have so far been identified which frustratingly leaves LGRs 4–6 in the state of orphan receptors (Van Loy et al. 2007).

Phylogenetic analysis reveals that the fly bursicon receptor is closely related to a LGR receptor from the bivalve mollusc, *Crassostrea gigas* (Fig. 1). Although no direct functional data are available for this mollusc receptor, transcript profiling demonstrates high expression in the adult digestive gland and only moderate expression in other organs and developmental stages (Herpin et al. 2004).

5. Subtype C contains receptors for human relaxin-related proteins

In addition to 7–9 LRRs in the ectodomain, all subtype C LGRs contain a N-terminal cysteine-rich low density lipoprotein (LDL) motif not found in other LGRs. The first member of this subtype was cloned from the central nervous system of the mollusc, *Lymnea stagnalis* (snail) and is predominantly expressed in a small subset of neurons within the central nervous system and to a lesser extent in the heart (Tensen et al. 1994). At present, this mollusc receptor still has the status of an orphan receptor, meaning that its corresponding hormone is yet to be identified.

Bio-informatic approaches led to the discovery of two paraloguous human receptors, LGR7 and LGR8, that share the structural motifs of snail LGR (Fig. 1) and that are expressed in diverse tissues such as the brain, kidney, testis, uterus and others (Hsu et al. 2002b). In *Drosophila*, two predicted genes (CG5046/DLGR3 and CG4187/DLGR4) display sequential and structural similarity with human LGRs 7–8. In addition, this receptor type has also been identified in the genome of other insect species (e.g., honey bee, malaria mosquito) (Vanden Broeck 2001a; Hill et al. 2002; Hauser et al. 2006).

Unlike glycoprotein hormone and bursicon receptors, human LGRs 7–8 are not stimulated by large dimeric protein hormones, but mediate the action of relaxin-related peptides (Hsu et al. 2002b). Whereas both LGR7 and LGR8 can be activated by relaxin, insulin-like peptide 3 (INSL3)/Leydig insulin-like peptide specifically stimulates LGR8 (Kumagai et al. 2002). Both INSL3 and LGR8 mutant mice display a similar phenotype characterized by a defective testicular descent (Zimmermann et al. 1999; Kumagai et al. 2002).

Insulin/relaxin-related peptides form an ancient category of small signalling proteins that have not solely evolved in vertebrates, but also exist in insects, nematodes, molluscs, and echinoderms (Claeys et al. 2002; Hummon et al. 2006; Burke et al. 2006). Usually, they initiate an evolutionary conserved signal transduction pathway by activating membrane-spanning receptor tyrosine kinases (RTKs) thereby playing pivotal roles in the control of metabolism, growth, reproduction and ageing (Claeys et al. 2002; Wu and Brown, 2006; Baumeister et al. 2006). At present, it is not clear whether some invertebrate insulin-related proteins also may participate in LGR signalling.

6. General discussion

Leucine-rich repeats containing GPCRs set an interesting example of how a receptor subfamily "expanded" through comparative genomics. Genome analysis and

molecular cloning efforts revealed the exceptional preservation of LGRs in metazoa, both structurally and at the amino acid sequence level. The early origin of LGRs is illustrated by the existence of a receptor related to human glycoprotein hormone receptors in cnidarians [the sea anemone (*A. elegantissima*)], one of the most primitive animal groups with a sensory system (Nothacker and Grimmelikhuijzen, 1993). Since the cloning of this first invertebrate LGR, additional homologous LGR encoding genes have been identified in nematodes, arthropods, molluscs and echinoderms while *in silico* screenings, mainly based on these invertebrate LGR sequences, revealed the existence of two additional clusters of previously unknown mammalian LGRs, i.e., LGRs 4–6 and LGRs 7–8.

Phylogenetic analysis demonstrated the existence of three distinct LGR types (subtype A, B and C). Whereas in arthropods, mammals and echinoderms all LGR subtypes exist, in molluscs only receptors belonging to subtypes B and C have been cloned so far. The relative lack of molluscan genomic data obviously is a drawback for LGR identification, implicating that the occurrence of subtype A receptor(s) cannot be excluded here. Moreover, glycoprotein hormone subunit β-related proteins appear to exist in molluscs (Fig. 2b) and a partial amino acid sequence originating from the central nervous system of *Aplysia californica* (GenBank Accession No. EB326753) displays similarity with glycoprotein hormone α-subunits. From an evolutionary point of view, the characterization of LGR stimulating hormones in molluscs bears particular interest as they belong to the large phylogenetic branch of Lophotrochozoa that has until now been largely neglected in genome analyses.

In nematodes, one sole LGR, homologous to glycoprotein hormone receptors, appears to be encoded in the genome. Like arthropods, nematodes periodically need to perform molting to permit growth and development. Molting culminates into ecdysis behaviour that allows the escape from the old exoskeleton and the subsequent remodelling (sclerotization and melanization) of the new one. In insects, ecdysis is regulated by a cascade of peptides, i.e., ecdysis triggering hormone (ETH), eclosion hormone (EH) and crustacean cardioactive peptide (CCAP), while bursicon controls post-ecdysis maturation of the exoskeleton (Truman, 2005). In the nematode *C. elegans*, a genome-wide RNA interference-based screen for worms unable to shed their old culticle resulted in a set of genes that likely participate in molting, but, interestingly, none of the corresponding peptides showed obvious sequence similarity to ETH and EH (Frand et al. 2005). In addition, nematodes do not seem to express a homologous bursicon/LGR B signalling system (Van Loy et al. 2007) making it tempting to hypothesize that nematodes and arthropods must have acquired, at least to some extent, different mechanisms to initiate and regulate ecdysis and post-ecdysial exoskeleton modelling.

Gene duplication events led to the expansion of subtype A LGRs and glycoprotein hormone subunits in vertebrates whereas the genome of the fruit fly and nematode most likely encode only one pair of putative hormone subunit homologues, as well as a single subtype A LGR. The pronounced ligand-independent activity displayed by fly DLGR1 and nematode LGR (Kudo et al. 2000; Sudo et al. 2005) suggests that ancestral subtype A LGRs may have been constitutively active receptors, a situation that still exists in flies and nematodes. During evolution in the chordate lineage, however, some LGRs have become more intramolecularly constrained, resulting in less basal activity. For instance, in the case of human FSH receptor, five naturally occurring mutations in the serpentine region lead to an increased basal receptor activity and, in addition, cause an increase in the sensitivity of FSHR to CG and TSH. It therefore has been suggested that an intramolecular constraint acquired during the evolution of primates contributed to the protection of the FSH receptor against promiscuous activation by the primate-specific chorionic gonadotropin hormone (Vassart et al. 2004; Costagliola et al. 2005).

The biological processes in which invertebrate LGRs, other than the bursicon receptor, participate are not yet understood. Fruit fly DLGR1 expression starts 8–16 h after the onset of embryonic development, and stays high until after pupariation, suggesting a role in development (Hauser et al. 1997). In addition, adult male flies express higher levels of DLGR1 mRNA than females, indicating potential gender-specific functional differences (Hauser et al. 1997). Accordingly, fly GPA2/GPB5, the endogenous ligand for DLGR1, is also expressed in different developmental stages (Sudo et al. 2005). It is clear that future tissue localizations and genetic studies are necessary to reveal molecular components that participate in invertebrate LGR signalling.

Current and future genome and EST sequencing projects will further improve our understanding of LGR/ligand (co-)evolution. For instance, the recently sequenced genome of the sea urchin, *Stronglycentrotus purpuratus*, a marine echinoderm, encodes putative new members of glycoprotein hormone subunit family, as well as LGR receptors (Burke et al. 2006). Two of these cystine knot-containing proteins were predicted to be the orthologues of arthropod bursicon subunit proteins (Burke et al. 2006; Van Loy et al. 2007). Also the generation of genomic and EST resources from Lophotrochozoa will shed more light on the comparative study of LGR/hormone signalling as the mere comparison of nematodes, arthropods and deuterostomes experiences its limits (Tessmar-Raible and Arendt, 2003).

7. Conclusion

Comparative genomics has proven a valuable tool for deciphering co-evolution of GPCRs and their (putative) ligands leading to novel insights in the evolutionary conservation of signalling systems between vertebrates and invertebrates (Bargmann, 1998; Vanden Broeck, 2001a,b; Hewes

and Taghert, 2001; Claeys et al. 2005) and which has been described here for leucine-rich repeats containing GPCRs. Where initially it was assumed that LGRs existed exclusively in vertebrates, new insights in the molecular evolution of LGR encoding genes and their (putative) ligands emerged and point at their exceptional preservation throughout the animal kingdom. This observation suggests fundamental role(s) for LGRs in endocrinology, a hypothesis that has so far been experimentally supported. Future research will likely result in the identification of LGR/hormone couples in an even wider range of animals while analyses of mutant phenotypes will contribute to our understanding of LGR signalling in phylogenetically diverse animal phyla.

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