Ambulacrarian insulin-related peptides and their putative receptors suggest how insulin and similar peptides may have evolved from Insulin-like Growth Factor

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Abstract

Background

Insulin is evolutionarily related to the insulin-like growth factors (IGFs) and like the latter stimulates a receptor tyrosine kinase (RTK) that transfers the extracellular hormonal signal into an intracellular response. Other hormones related to insulin, such as relaxin, do not use an RTK, but a G-protein coupled receptor (GPCR). This is unusual since evolutionarily related hormones typically either use the same or paralogous receptors. In arthropods three different IGF-related peptides likely evolved from a gene triplication, as in several species genes coding these three peptides are located next to one another on the same chromosomal fragment. Of these three hormones one, an IGF-like hormone, acts through an RTK, while the other two use a GPCR. This suggests that the ancestral IGF-like peptide may have used both types of receptors. These arthropod insulin-like peptides have homologs in vertebrates, which suggests that the initial gene triplication was perhaps already present in the last common ancestor of deuterostomes and protostomes. It would be interesting to know whether this is indeed so and to establish how insulin and other insulin-like peptides might be related to this trio of IGF-related hormones.

Methodology

Genes coding insulin and related peptides as well as their putative receptors were identified in genomes and transcriptomes from echinoderms and hemichordates.

Results

A similar triplet of genes coding insulin-like peptides is also found in some hemichordates and echinoderms. Two of the three ambulacrarian peptides are orthologs of arthropod IGF and *Drosophila* insulin-like peptide 7 (dilp7), while the third one looks like an ortholog of the arthropod peptide gonadulin. In echinoderms two novel insulin-like peptides emerged, gonad stimulating substance (GSS) and multinsulin, likely from gene duplications of the IGF and dilp7-like genes respectively. However, no novel receptors for insulin-like peptides evolved. If IGF were to act through both a GPCR and an RTK it would suggest that GSS acts on only one of the two receptors, possibly the RTK. The evolution of GSS from IGF may represent a pattern, where IGF gene duplications lead to novel genes coding shorter peptides that have lost their ability to

activate a GPCR. It is likely this is how insulin and the insect neuroendocrine insulin-like peptides evolved independently from IGF.

Conclusion

The local gene triplication previously described from arthropods that yielded three genes coding IGF-related peptides was already present in the last common ancestor of protostomes and deuterostomes. It seems plausible that insulin and other insulin-like peptides, such as those produced by neuroendocrine cells in the brain of insects and echinoderm GSS evolved independently from IGF and thus are not true orthologs, but the result of convergent evolution.

Key words: insulin; relaxin; receptor tyrosine kinase; G-protein coupled receptor; evolution; gonadulin; octinsulin; multinsulin; dilp7

Introduction

Many protein hormone and neuropeptide signaling pathways have orthologs in both protostomes and deutertostomes showing that these pathways were already present in their last common bilaterian ancestor. In some cases the orthologs of the ligands show only limited sequence similarity, but their receptors contain protein domains that are sufficiently conserved to establish homology. Virtually all ligands employ either a single receptor or a number of closely related receptors that evolved by gene duplication. Co-evolution of ligands and receptors insures that related protein hormones or neuropeptides use receptors akin to those of their orthologs (Mirabeau & Joly, 2013; Hsueh & Feng, 2020).

Insulin-related peptides (irps) are an exception to this rule. Whereas insulin acts through a receptor tyrosine kinase (RTK), relaxin uses a leucine-rich repeat G-protein coupuled receptor (LGR). This raises the interesting question as how this apparent jump from one type of receptor to another may have come about. In cockroaches, termites and stick insects three different irp genes, gonadulin, arthropod insulin-like growth factor (aIGF) and arthropod relaxin, are located next to one another in the genome and thus likely originated from a local gene triplication (Veenstra, 2020b). To avoid confusion with the vertebrate relaxins and related peptides, the arthropod relaxins will be referred to as *Drosophila* ilp7 (dilp7) in this manuscript. One of these, aIGF, is known to use an insulin RTK, while gonadulin acts through insect LGR3 (Vallejo et al., 2015; Garelli et al., 2015; Colombani et al., 2015). Bioinformatic evidence suggested that dilp7 must be the ligand for insect LGR4 and this has now been confirmed experimentally in *Drosophila* (Veenstra, Rombauts & Grbić, 2012; Imambocus et al., 2020), but dilp7 may also activate an RTK (Linneweber et al., 2014). This suggests that the archtype arthropod IGF-related peptide acted through both an RTK and an LGR and that after a likely gene triplication, some of the ligands may have lost one of the two original receptors. Although it is possible that the gene triplication of the ancestral insulin gene occurred in an early arthropod or protostomian, it may well have occurred in a bilaterian ancestor, as homologs of both aIGF and dilp7 are also present in deuterostomes.

Brain neuroendocrine insect irps are more closely related to IGF than either dilp7 or gonadulin and a gene duplication that gave rise to separate genes coding these peptides is therefore likely to have occurred after the triplication that gave rise to the ancestor genes of gonadulin and dilp7. Yet in insect genomes irp genes are not located near the IGF gene, while

the IGF gene is in the middle of the triplet. Thus the particular organization of these genes suggests that whereas the gonadulin and dilp7 genes likely originated by two successive local gene duplications, the IGF gene duplication that gave rise to an initial arthropod neuroendocrine brain irp must have materialized in a different fashion. If the earlier mentioned gene triplication was already present in the last common ancestor of the deuterostomes then a similar argument can also be made for the evolution of insulin. Given the importance of insulin as a human hormone and the inherent interest of its evolutionary origin, I explored the evolution of bilaterian insulin-related peptides in more detail and here report on the genes coding such peptides and their receptors in the Ambulacraria that suggest how insulin may have evolved from IGF.

Materials and Methods

Nomenclature

Hormones have often been discovered independently by different groups using different bioassays. The vertebrate insulin-like growth factors are a good example of that. Predicted protostomian peptides and their receptors have sometimes been given names that refer to similar deuterostomian proteins. In some cases this is very confusing, *e.g.* vertebrate LGR-3, -4 and -5 are not the orthologs of arthropod receptors that have been given the same names. A similar problem occurs with arthropod relaxin that is not an ortholog of vertebrate relaxin. This peptide will therefore be called dilp7 (*Drosophila* insulin-like peptide 7). I will refer to arthropod LGR3 as the gonadulin receptor, arthropod LGR4 as the dilp7 receptor and arthropod LGR5 as GRL101, a GPCR initially identified from the pond snail *Lymnaea stagnalis* (Tensen et al., 1994) that is an ortholog of arthropod LGR5 (Veenstra, 2020b).

Sequence analysis

Sequences for insulin related peptides and their likely receptors were identified from a number of Ambulacraria. This was done using using the Artemis program (Rutherford et al., 2000) and the BLAST+ program (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/) on publicly available genome sequences from the feather star *Anneissia japonica*, the sea urchins *Lytechinus variegatus* (Davidson et al., 2000) and *Strongylocentrus purpuratus* (Sea Urchin Genome Sequencing Consortium, 2006), the sea cucumbers *Apostichopus japonicus* (Jo et al., 2017; Zhang et al., 2017) and *Holothuria glaberrima*, the sea stars *Acanthaster planci* (Hall et

al., 2017), *Pisaster ochraceus* (Ruiz-Ramos et al., 2020) and *Patiria miniata*, the brittle star *Ophiothrix spiculata* and the hemichordates *Saccoglossus kowalevskii* and *Ptychodera flava* (Simakov et al., 2015). The genomes were downloaded from https://www.ncbi.nlm.nih.gov/genome. For many of these species there are also significant amounts of RNAseq data and these were analyzed using the sratoolkit (https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=software) in combination with Trinity (Grabherr et al., 2011) using methods described in detail elsewhere (Veenstra, 2020b). Some protein sequences were found in the NCBI database, but several of them contain errors or are incomplete. Where possible these were corrected and/or completed using the methods described above. As there is only a single crinoid genome assembly available, transcriptome data from *Antedon mediterranea, Florometra serratissima* and *Oligometra serripinna* were also included. For the same reason transcriptome data from the brittle star *Amphiura filliformis, Ophioderma brevispina* and the hemichordate *Schizocardium californicum* were likewise analyzed. Obviously, transcriptome data can only demonstrate the presence of gene but not its absence and their usefulness depends largely on the variety of tissues sampled and the expression levels of the genes of interest. Nevertheless, such data often provide additional sequences that even if they are incomplete increase the robustness of sequence comparisons. Genomic and transcriptomic RNAseq short read archives (SRAs) were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/sra/); a list of the SRAs analyzed is provided in the supplementary data.

As queries for the insulin-like peptides a number of such peptides from a variety of species was used. Insulin RTKs are easily identified in genome and transcriptome assemblies, as their kinase domains are very well conserved. The LGRs that could function as insulin receptors are more variable. Vertebrate RXFP1 and RXFP2 are LGRs are known receptors for relaxin and Ins3 and *Drosophila* LGR3 and LGR4 for gonadulin, and dilp7 respectively. Other LGRs function as receptors for the various glycoprotein hormones, GPA2/GPB5, bursicon, TSH, FSH and LH. These GPCRs cluster on phylogenetic trees with another protostomian LGR, GRL101. This GPCR was initially identified from the pond snail *Lymnaea stagnalis* and was the first GPCR discovered to have in addition to six leucine-rich repeats also twelve repeats of a sequence that was known to exist in the low density lipoprotein receptor and are now called

LDLa repeats (Tensen et al., 1994). I have suggested previously (Veenstra, 2020b) that this receptor might be an IGF receptor.

Both the RTK and LGR receptors have large ectodomains. Those of the insulin RTKs are very similar from one receptor to another, while those of the LGRs differ between different types. The latter all contain numerous Leucine-rich repeats (LRRs) and some also have LDL-receptor class A (LDLa) repeats. Both LRRs and LDLa's are present in many other proteins. Initial searches for orthologs receptors were therefore done using the transmembrane regions of various insect and vertebrate LGRs and the protein kinase domain of RTK. Once partial sequences of putative receptors were identified, the coding sequences of these domains were then used to complete the cDNA sequences as best as possible, using either Trinity on RNAseq SRAs or Artemis on genome sequences.

Sequence similarity and phylogenetic trees

Peptides having the characteristic insulin signature are notoriously variable in their primary amino acid sequences. Although the various residues allows one to align those sequences, such alignments will not always yield reliable phylogenetic trees as the basic tenet of such analyses is often not met. As an alternative I have proposed to use "sequence similarity trees". Such trees are constructed using the same methods but do not pretend to illustrate phylogenetic relations, rather similarities between the different proteins. Both phylogenetic and sequence similarity trees use Clustal omega (Sievers et al., 2011) to produce alignments and Fasttree (Price, Dehal & Arkin, 2010) to construct trees and estimate probabilities.

In order to identify putative receptors for the various irps, LGRs that show homology to various arthropod and vertebrate LGRs were identified and a phylogenetic tree based exclusively on the transmembrane regions of these receptors was constructed.

Precursor processing

Precursors of insulin-like peptides contain signal peptides that are removed on entry into endoplasmatic reticulum. Signal P 5.0 (Almagro Armenteros et al., 2019) was used online (http://www.cbs.dtu.dk/services/SignalP/) to predict where this cleavage would most likely occur. Some, but not all precursors are further processed by convertases. Of these furin is ubiquitously present in all cell types and can thus potentially cleave any secreted protein. Its

consensus cleavage site is K/R-X-K/R-R, the two human IGF precursors are processed at KSAR and KSER respectively (Humbel, 1990). Precursors that are produced in cells with a regulated pathway, such as neuroendocrine and enteroendocrine cells, are also exposed to other convertases like PC1/3 and PC2. Their consensus cleavages site is KR. However, effective proteolytic processing by convertases is strongly influenced by amino acid residues surrounding these consensus cleavage sites. For example bulky residues immediately following the arginine residue, a proline residue before the consensus site or disulfide bridges nearby can cause sufficient steric hindrance to inhibit cleavage. Using rules proposed to predict cleavage by PC1/3 and PC2 in both vertebrates and insects (Devi, 1991; Rholam et al., 1995; Veenstra, 2000) I have tried to indicate in Figs. 2-5 where the various precursors might be cleaved. It must be noted though that there is no certainty that these site will be cleaved nor can it be excluded that proteolytic processing occurs at sites that have not been indicated as such.

Expression

With a few notable exceptions (*e.g.* Lin et al., 2017), little is known about the expression of the various insulin-like peptides in either echinoderms or hemichordates and except for the GSS our knowledge of their functions is also very limited. Expression data may reveal some preliminary clues as to where and when they are expressed and thus provide a hint as to their function. For this reason the number of reads corresponding to the various insulin-related peptides and their putative receptors was determined in a number of SRAs that might provide evidence as to the time and tissue specific expression of these proteins. The analysis was performed as described previously (Veenstra, 2020b) and the data are supplied in Spreadsheet S2.

Results

Peptides related to insulin

Insulin-like peptide precursors are typically characterized as having A, B and C domains that correspond to the A- and B-chains of insulin and the connecting peptide respectively. In IGF D and E domains are also recognized, in which the D domain refers to the extension of the A chain and the E domain to part of the precursor after the D domain that is cleaved from IGF in the

Golgi apparatus. For dilp7 orthologs it is appropriate to add an F (front) domain for the sequence in N-terminal of the B-chain that in some peptides is not only larger, but also well conserved.

Previous work on insulin-related peptides in in echinoderms have identified two different types of insulin-like peptides, gonad-stimulating substances (GSS) and insulin-like growth factors (Mita et al., 2009; Perillo & Arnone, 2014; Semmens et al., 2016; Smith et al., 2019). The insulin-like growth factors, but not GSS, are also present in hemichordates. While only a single IGF gene was found in the crinoids and hemichordates, other echinoderms have two such genes (Figs. 1, S1, S2; Spreadsheet S1). These proteins have large C-terminal extension that are rich in charged amino acid residues, especially arginine and lysine, but also aspartic and glutamic acid residues. A comparison of the protein sequences and cDNAs from human IGFs identifies the exact separation between the D and E domains in these proteins (Humbel, 1990). However, although the corresponding sequences of the hemichordate and echinoderm IGFs contain numerous arginine and lysine residues (Figs. 1, S1, S2), there are no obvious convertase cleavages sites as many potential arginine residues are succeeded by residues known to inhibit such enzymes in vertebrates. It is thus not impossible that the D domains of these proteins are much larger than in the vertebrate IGFs and if so likely contain numerous positively charged amino acid residues. There are few transcriptome SRAs for specific tissues, the data that is available suggest that the IGFs are expressed by many tissues, with the ovary showing significant expression. *Patiria pectinifera* is the only species with follicle cell specific SRAs and IGF-1 is strongly expressed by these cells and is probably transferred to the oocyte (Spreadsheet S2).

The GSS are known to induce oocyte maturation and ovulation in a two step process, where GSS stimulates the follicle cells to produce 1-methyladenine which subsequently induces resumption of meiosis in the oocyte and about 30 minutes later this is followed by ovulation (Chiba, 2020). Interestingly, GSS was not found in either the genome nor the extensive transcriptome data from the feather star *Anneissia japonica* and was similarly not encountered in the transcriptomes of three other crinoids (Suppl data). Transcriptomes may miss expression of some genes and large genome assemblies are never perfect. The short sequence reads in the genomic SRAs from *Anneissia* were therefore also analyzed for the presence of GSS, but again no evidence for such a gene was found. This peptide is thus likely absent from *Anneissia* and perhaps all Crinoidea. In the Holothuroidea and the Asterozoa, but not the Echinoidea, this gene

is duplicated with the two paralogous peptides showing significant sequence variability (Figs. 2, S3, S4; Spreadsheet S1). As for all these peptides and their putative receptors expression data is very limited, but in *Apostichopus* the two GSSs are differentially expressed, with GSS-1 being expressed at specific stages during embryonic development as well as by muscle and GSS-2 strongly expressed by both the ovary and the testes. Interestingly, it is the ortholog of GSS-1 that in *Holothuria scabra* has been tested for biological activity and induces ovulation (Chieu et al., 2019). This makes one wonder what the effects of GSS-2 on ovulation might be in this species. However, *Apostichopus* was the only species where a significant GSS expression was found in the gonads (Spreadsheet S2).

Two other insulin-like peptides are commonly present in both hemichordates and echinoderms, including the Crinoidea. The first is an ortholog dilp7 which has a very characteristic F domain while its A chain is also remarkably well conserved (Figs. 3, S5, S6; Spreadsheet S1). The precursors of this peptide contain typical neuroendocrine KR convertase sites and seems to have its highest expression in the nervous system, although it is also found in other tissues. During embryogenesis its expression occurs relatively late (Spreadsheet S2). The second peptide present in all ambulacrarians has been called octinsulin as it has eight cysteine residues and is thus predicted to have four rather than three disulfide bridges. In echinoderms octinsulin are single copy genes, but hemichordates have several such genes (Fig. 4, S7, S8; Spreadsheet S1). Octinsulin expression levels are the highest in nervous tissue, and significant expression is also found in the gut and stomach of *Strongylocentrotus* and *P. pectinifera* respectively. Although virtually absent from normal gut in *Apostichopus*, it has significant expression during gut regenerating in this species (Spreadsheet S2).

The Asterozoa have genes coding for a fifth type of insulin, that is usually present in multiple copies and that are referred to as multinsulins. The predicted peptides share structural similarity with the dilp7 orthologs and their genes have typically four coding exons rather than the two or three of the other irp genes. The sprawl of these peptides is perhaps best illustrated by a phylogenetic tree that suggest independent multiplication of these genes in several species (Fig. S10). Within a single species the various multinsulins thus often seem more closely related to one another than to their putative orthologs of other Asterozoa. Some of the multinsulins, like the octinsulins, have acquired two additional cysteine residues and are thus predicted to have four disulfide bridges, but the location of these additional cysteine residues differs from that in

octinsulins (Figs. 5, 6, S9, S10; Spreadsheet S1). Like dilp7 the multinsulins have typical neuroendocrine KR convertase cleavage sites and can thus be expected to be expressed in neuroendocrine and/or enteroendocrine cells but expression data on *P. pectinifera* suggest a relatively ubiquitous expression in several tissues.

The genome assemblies of *A. planci* and *Pisaster ochraceus* shows these genes to be clustered in the genome and some RNAseq sequences suggests that at least on occasion coding exons from different genes may be combined (Fig. S10). This and the large numbers of SNPs typically present in animals caught in nature and used for RNAseq preparation make it impossible to reliably determine their exact numbers.

Genome assemblies allow identification of the introns in these genes. All insulin genes have a characteristic phase 1 intron somewhere in the conceptual C domain of these molecules. This is the only intron in the coding sequences of the octinsulin and GSS genes. The IGF genes have a phase 0 intron near the end of the coding sequence and at least some of them have another phase 1 intron just after the transcription start site. The genes coding the dilp7 orthologs and multinsulins share an additional phase 2 intron and the multinsulin genes have yet another phase 1 intron. All these introns appear perfectly conserved (Fig. 6).

Synteny of genes producing insulin-like peptides

In the *Strongylocentrotus* genome all five genes are located on the same chromosome, with the two IGF genes and those coding octinsulin and dilp7 orthologs next to one another and GSS at a distance of 6,000,000 bp (base pairs). At least the *Anneissia* octinsulin and IGF genes are likely located next to one another on the same chromosome also, as in the current genome assembly two of the three coding exons of IGF and one of the two octinsulin coding exons are located within about 10,000 bp. The three missing exons of these two genes are all located on minicontigs of less than 2,000 bp, as is one of the coding exons for the dilp7 ortholog. The contigs of the *Lytechinus variegatus* genome assembly are smaller and this may explain why in this species the genes are located on three different scaffolds, with the two ILGF-like peptides and the octinsulin together on a single contig. However in the recently published genome of the closely related *L. pictus* (Warner et al., 2021) the dilp7 ortholog is also closely associated with the other three genes. The GSS gene is on the same chromosome but at a distance of 28,000,000 bp. In the *Apostichus japonicus* genome assembly the genes coding the octinsulin and the two

IGF genes are located on the same contig, and the other genes each on a different one. In the draft *Holothuria glaberrima* genome assembly only the two IGF genes are located on the same contig, however in a single Oxford nanopore read (SRR9125585.2851.1) from *H. scabra* the octinsulin, dilp7 and two IGF genes are located next to one another as well (Fig. 7).

Whereas the various Echinozoa genome assemblies suggest a certain degree of synteny with regard to the various irp genes, the Asterozoa genome show that such syntenty is disintegrating. This is most clearly demonstrated in the genome assemblies from *Pisaster ochraceus* and *Acanthaster planci,* were the scaffolds are much larger than from *Patiria miniata.* In these species synteny is largely lost (Fig. 7). Interestingly the various multinsulin genes are present in small clusters on different chromosomes in those species.

Sequence similarity tree peptides related to insulin

A sequence similarity tree (Fig. 8) of the various irps from echinoderms and hemichordates show that the structures of the multinsulins are most similar to the dilp7 orthologs. This is not surprising as they look indeed similar (Fig. 5) and they share the position of an intron with the dilp7 orthologs (Fig. 6). The tree also illustrates significant sequence similarity between GSS and the IGF.

Orthologs of receptors for insulin-like peptides: Receptor tyrosine kinase

A single insulin RTK gene was found in all species analyzed here. An alternatively spliced form is present in *Acanthaster* and is likely commonly present in echinoderms (Spreadsheet S1). Hundreds of ambulacrarian protein sequences were identified at NBCI using a BLAST search with the *S. kovalevskii* protein kinase domain as a query. After aligning them with Clustal omega the protein kinase domains were used to make a phylogenetic tree. Results revealed no other known or predicted proteins with a similar protein kinase domain (data not shown). The insulin RTK is ubiquitously expressed (Spreadsheet S2).

Orthologs of receptors for insulin-like peptides: LGRs

LGR sequences were obtained using the combination of genomic sequences and, where available, transcriptome shotgun sequences and RNAseq SRAs. The latter were used to produce contigs using Trinity (Spreadsheet S1). Short read assemblers are good in combining sequences

into larger continuous ones, but they do produce artifacts, which are more easily obtained when very similar sequences are present in mulitple copies, such as the multinsulins, or the numerous LDLa and LRR repeats. These repeats are usually individually coded by single exons that are sometimes skipped and when such skipped individual reads enter in the RNAseq SRA, incorrect constructs are obtained. Furthermore, these repeats are present in numerous proteins, and from to time this leads to assembled constructs that are from mRNA species from different genes. It is therefore to be expected that not all assembled transcripts, both those in the databank and those produced here, will be correct. Some errors were corrected by challenging divergent sequences that were discovered on comparing putative orthologs with one another. Other differences could be confirmed as true differences, but it is not impossible that some errors remain, particular for those sequences that are incomplete. LGRs that might function as receptors for the various irps were identified by their homology with such receptors from vertebrates and arthropods. The transmembrane regions of the GPCRs don't have the assembly problems of the LDLa and LRR repeats and are the most characteristic domain of the GPCRs. This makes it easier to construct a phylogenetic trees for these receptors based on their transmembrane regions than that it is to produce complete LGR transcripts.

Results show a surprisingly similar distribution of LGRs in the species studied. The tree resolves two major branches, one for the glycoprotein hormone receptors, which itself is divided in two subbranches one for orthologs of the GPA2/GPB5 receptor - containing the receptors for human TSH, FSH and LH - and a second one for the bursicon receptor orthologs. All species studied are represented by one member on each of these two subbranches, except for *Ophiothrix*, where the draft genome reveals two orthologs each for the bursicon and GPA2/GPB5 receptors (Fig. 9). These are likely receptors for the bursicon and GPA2/GPB5 orthologs identified from various echinoderm species (Semmens et al., 2016). It is interesting to see that whereas vertebrates have different receptors for TSH, FSH and LH, most echinoderms have only one GPA2/GPB5-receptor ortholog, even though *A. rubens* has two GPA2 and three GPB5 orthologs (Semmens et al., 2016). The LGRs for the glycoproteins were included in the search for putative receptors for the ambulacrarian irp LGRs in order to be sure that no such receptors would be missed.

The second branch of the LGR phylogenetic tree is the one of interest as it contains receptors with irp ligands. It consists of three subbranches, that are characterized by *Drosophila* LGR3 and

LGR4 – the receptors for gonadulin and dilp7 respectively - and *Periplaneta* LGR5, an ortholog of *Lymnaea* GRL101. Here in all ambulacrarian species studied only one ortholog was found for each of them, despite extensive attempts to find additional LGRs in the various genomes and transcriptomes.

The GRL101 transmembrane regions puts it very close to vertebrate glycoprotein hormone and relaxin LGRs. LRRs are present in many different proteins, but when the LRR part of the *Anneissia* GRL101 (amino acid residues 576-717) are used as query in a protein BLAST against human proteins, it are the glycoprotein hormone and relaxin receptors that are identified as most similar to this ectodomain of GRL101 suggesting that similarity of the GRL101 receptors with vertebrate LGRs is not limited to the transmembrane region of this GPCR.

Sequence alignments of these GPCRs show strong sequence similarity (Figs. S11-S13), however the dil7 receptor ortholog varies more between species. A schematic representation of the the ectodomains of the LGRs on this second branch is drawn in Fig. 10. The orthologs of the dilp7 and gonadulin receptors each have a single LDLa repeat, except for the *Patiria* and *Acanthaster* orthologs of the dilp7 receptor which both have two LDLa repeats (Fig. S12). This additional LDLa is likely due to a relatively recent duplication of the LDLa since the two LDLa repeats have very similar amino acid sequences (Spreadsheet S1). All three receptors are expressed in the nervous system and the gonadulin receptor is well expressed in the gonads, both testis and ovary, and strongly so in the follicle cells of *P. pectinifera* (Spreadsheet 2).

Discussion

The genomic and transcriptomic data from both the hemichordates and the echinodermates show that these two groups share three irps, octinsulin, IGF and a dilp7 orthologs, that are present in both echinodermates and hemichordates. IGF and dilp7 are orthologs of the arthropod peptides that together with gonadulin originated from a gene triplication. Evolutionary pressure on gonadulin appears weak, as shown by the variable structure of this peptide and its loss in many arthropod lineages. This may well be the reason that its structure is so poorly maintained, even within insects (Veenstra, 2020b). This may explain why the amino acid sequence of gonadulin looks significantly different from octinsulin. Nevertheless, there are two lines of evidence that suggests that these peptides must be orthologs as well. For one, synteny of the

chromosome fragment containing these genes is conserved between the sea urchin *Strongylocentrotus purpuratus*, the hemichordate *Saccoglossus kowalewski* and the cockroach *Blattella germanica*, suggesting that these peptides are likely orthologs. More importantly, all ambulacrarians have an ortholog of the gonadulin receptor and the only plausible ligand for such a receptor encoded by their genomes is octinsulin. Thus the gene triplication previously reported from arthropods must have occurred in a common bilaterian ancestor of the deuterostomes and protostomes.

Crinoids have the simplest irp signaling system, one gene each for IGF, octinsulin and the dilp7 ortholog. Their putative receptors - insulin RTK, GRL101, and the orthologs of the dilp7 and gonadulin receptors – similarly are also each coded by a single gene. The hemichordates have a very similar repertoire, except that the octinsulin gene is systematically amplified and in some species the dilp7 ortholog as well. It thus appears likely that the first deuterostome had a single copy of each of these genes.

Within the echinoderms the irp genes evolved considerably, as shown both by an increase in the number of irp genes and the loss of synteny. Whereas the feather stars appear to have only a single IGF gene, all other echinoderms have two such genes and two novel insulin-like peptides, GSS and multinsulin, appeared. The GSS sequences are most similar to those of IGF, suggesting that they evolved from a gene duplication event from the IGF gene. Although some GSS genes are located on the same chromosome as the other irps, they are not close to the IGF genes, indicating that the IGF-GSS split was not a local duplication but may have been the result of an incorrectly repaired chromosome break.

In the Asterozoa a fifth type of irp gene emerged, those that code for the multinsulins which share significant sequence similarity with the dilp7 orthologs. The initial multinsulin gene must thus have its origin in a gene duplication of the dilp7 ortholog gene, with which they furthermore share a characteristic intron. Later the multinsulin gene seems to have undergone several additional gene duplications in this respect the multinsulins resemble the insect neuroendocrine irps.

The co-evolution of ligands and receptors allows one to assign the putative receptors for gonadulin, the dilp7 ortholog and IGF as the orthologs of the receptors of their arthropod orthologs. This allows the identification of the ambulacrarian LGRs that are the orthologs of the

gonadulin and dilp7 receptors as likely receptors for octinsulin and the dilp7 respectively, as well as the insulin RTK as a receptor for IGF.

The appearance of the multinsulins is not accompanied with the evolution of a novel insulin-receptor. Some animals have multiple insulin RTKs, *e.g.* some arthropods have up to four such genes (Veenstra, 2020a,b), however, in spite of extensive searches for a second insulin RTK in ambulacrarian genomes, none were found. Searches for an additional LGR that might function as a receptor for the GSS and/or multinsulin were neither successful and this raises the question which receptors are activated by these peptides.

I have previously argued that the close chromosomal association of the IGF, gonadulin and dilp7 ortholog genes in basal insects suggest that they derived from a gene triplication and that since IGF and gonadulin respectively activate an RTK and an LGR it is likely that the original IGF-like peptide - and possibly its descendants as well - acted on both a GPCR and an RTK (Veenstra, 2020b). Some of the descendant peptides might still do so, while others may have lost the ability to interact with one of the two original receptors. If the ancestral echinoderm dilp7 were still to use both a GPCR and an RTK, then the very different multinsulin sequences might be explained by the requirement that dip7 binds both a GPCR and the RTK while multinsulin is a ligand for only one of those two.

The binding of insulin and relaxin to their respective receptors has gotten resolved in much detail in the last couple of years. The effective binding and stimulation of insulin RTK by the small insulin-like peptide from the snail *Conus* to the RTK shows that a small insulin-like peptide can be an effective ligand for this receptor (Menting et al., 2015). On the other hand the complex interaction of relaxin to its LGR makes it more difficult to imagine a smaller peptide as an effective ligand (Hoare et al., 2019). Furthermore, considering the well conserved F-domain of the dilp7 receptor orthologs it is likely that it is necessary for interaction with its LGR receptor. The loss of this structure in multinsulin suggests that it is unlikely to be a dilp7 receptor agonist. On the other hand, the poor sequence conservation in the various *Drosophila* irps that activate a single RTK is reminiscent of the large structural variability of the multinsulins. This seems to suggest that the multinsulins are RTK ligands rather than activate the LGR.

The emergence of the GSS is neither accompanied with the evolution of a novel receptor for these irps. This can also be explained by assuming that IGF acts on both the RTK and an LGR

and that the GSS have lost their affinity for the LGR. This raises the question whether an IGF LGR might exist.

If there were an IGF LGR, one would expect it to be related to the gonadulin and dilp7 receptors. GRL101 appears a plausible candidate as its transmembrane regions are closely related to the receptors for gonadulin and dilp7. The ectodomain of GRL101 consists of two parts, a series of LRRs and a second series of LDLa's. In the related GPCRs the LRRs are expected to bind with the insulin core of gonadulin and dilp7 orthologs, just like the human relaxin receptors (Hoare et al., 2019). When the LRR part of the *Anneissia* GRL101, the most basal echinoderm, was used as query for similar human proteins in a BLAST search, it were the glycoprotein hormone and relaxin receptors that were identified as the most similar proteins. This shows that the resemblance of GRL101 to the other LGRs is not limited to the transmembrane regions and reinforces the hypothesis that the ligand of GRL101 has an insulin-like structure. GRL101 has a large number of LDLa's, the ligands of which are typically positively charged surfaces, which in the case of proteins consist of Lys and Arg residues (Daly et al., 1995; Prévost & Raussens, 2004; Fisher, Beglova & Blacklow, 2006; Yasui, Nogi & Takagi, 2010; Dagil et al., 2013). Thus the ligand of GRL101 may consist of two parts, an insulin-like structure and a piece with several positive charges that interact with the LDLa's. The C-terminal tails of the IGFs, whether from arthropods, echinoderms or hemichordates, are all rich in charged amino acid residues. The C-terminal tail of IGF with its numerous positively charged amino acid residue might interact with the LDLa's of GRL101. I therefore posit that in those species that have a GRL101 it functions as the second receptor for IGF. The absence of such a tail in GSS would make it likely that it acts on the RTK rather than an IGF GPCR.

The suggestion that GSS activates the RTK goes against the hypothesis that these peptides act through GPCRs. Indeed it has recently been proposed that it is the ortholog of the dilp7 receptor that would be activated by the gonad stimulator in *P. miniata* (Mita et al., 2020). Given the clear orthology of both the dilp7 echinoderm orthologs with the *Drosophila* peptide and the similar orthology between the dilp7 receptor and the echinoderm receptor, the conclusion that the two constitute a function ligand receptor combination seems inescapable. It was impossible to find a GSS in either the genome assembly or the individual reads of all the genomic SRAs of *Anneissia japonica*, yet it does have a dilp7 receptor ortholog, thus if the dilp7 receptor were to function as a GSS receptor, it most likely would not be an exclusive receptor. *A priori* this does

not exclude the possibility that GSS could function as a ligand for the same receptor. As mentioned above, since the dilp7 orthologs have well conserved F domains, one has to assume that it is important for binding to its receptor. Since this domain is absent from RTK ligands, it is difficult to understand how GSS that similarly lacks this domain would be able to bind the dilp7 receptor. It would thus seem unlikely that peptides as different as GSS and dilp7 would be effective ligands of the same LGR. Furthermore, the GSS genes have been duplicated and their structures have evolved considerably. Those duplicate gonad stimulators are present in many species and have not been selected against. Hence they must be phyisologically relevant and able to interact with a receptor. Sharing a common evolutionary origin the two gonad stimulators should be expected to act either on the same or paralogous receptors, but the number of putative echinoderm receptors for irps is limited, so it must be the same one. The same arguments that were used to argue that the multinsulins are likely RTK agonists but not LGR ligands, are therefore equally valid here and suggest that GSS is an RTK ligand.

Furhermore, the experimental evidence that GSS stimulates the ortholog of the dilp7 receptor is not convincing. The reported response to the dilp7 receptor when expressed in Sf9 cells is very weak and does not represent a typical response seen in this type of assay. Although the authors have shown high affinity binding of GSS to the follicle cells, such high affinity binding should also have been present in the Sf9 cells expressing the putative GSS receptor, but this was not reported. The follicle cell SRAs from which the putative GSS receptor was identified contains large amounts of RNAseq reads for the gonadulin receptor, a receptor that is more closely related to the vertebrate relaxin receptors than the dilp7 receptor, but surprisingly the authors do not mention this receptor, which they must have found (Mita et al., 2020).

I suggest that initially there was an IGF-like hormone that activated both a GPCR and an RTK, after two gene duplications some of the descendant ligands either lost their C-terminal tails or one acquired a larger one and this allowed all three ligands to activate, at least initially, the RTK while each acquired its own LGR. Later, some of the ligands may have lost their affinity for one receptor. Since the primary amino acid sequence of gonadulin is very different from that of the other insulin-like peptides, it likely lost its capacity to activate the RTK. Holometabolous insect species have lost GRL101 and hence in those species IGF can only act on the RTK. Under this hypothesis the arginine-rich C-terminal tail would be useless in such insect species and in higher flies, such as *Drosophila,* it was indeed lost (Veenstra, 2020b). In vertebrates, there is no

GRL101 and so IGF can only activate the two RTKs, while the relaxin related peptides are not known to interact with RTK. The presence of a similar arginine-rich E domain of the vertebrate IGF precursors might thus be an evolutionary relict.

This scheme raises the question as to how the functions of these two receptors activated by IGF might differ. IGF and the drosophila insulin-like peptides stimulate growth, the echinoderm GSS stimulates oocyte maturation and ovulation (Mita et al., 2009), relaxin and INSL3 affet various developmental and reproductive processes (Ivell et al., 2020; Esteban-Lopez & Agoulnik, 2020), gonadulin is expressed by the gonads as well as the imaginal in flies (Garelli et al., 2012; Liao & Nässel, 2020; Veenstra, 2020b) and dilp7 is expressed in a sex specific manner (Miguel-Aliaga, Thor & Gould, 2008; Yang et al., 2008; Castellanos, Tang & Allan, 2013). These hormones stimulate growth, development and reproduction, processes that are intimately linked; without growth and development reproduction is impossible and growth without reproduction is useless in sexually reproducing species. On the other hand, resources used for growth and development can not be used for reproduction or *vice versa*.

Growth is rarely a linear process independent of development; animals are not only getting bigger, but they also mature into adults. Metamorphosis is markedly different between hemi- and holo-metabolous insect species. Every time a cockroach nymph molts, it becomes a little more adult, however during the first molts of a caterpillar the insects mainly become bigger, it is only when it molts into a pupa that it significantly changes its morphology. Cockroaches have GRL101, caterpillars don't. This suggests that the RTK might be more directed toward linear growth, or allowing growth by increasing uptake of resources, such as glucose and amino acids, while the LGRs might be more important for insuring that the animal develops into an adult and becomes sexually competent. Both holometabolous insects and vertebrates have lost GRL101 and use steroid hormones to induce sexual maturation. Interestingly, in vertebrates the production of steroid hormones is controlled by glycoprotein hormones, the second group of ligands for LGRs.

It is plausible that IGF in an early bilaterian was produced by the tissue that stored energy and perhaps even protein as insects do in the form of storage proteins (Haunerland, 1996). Production and release of IGF might have happened when the animal had sufficient resources to allow for growth and/or reproduction. In arthropods growth has become a discontinuous process in which a new cuticle needs to be made before molting can take place. In those species IGF

produced by the fat body may well be the essential growth hormone. However, if the animal is suddenly starved, IGF would no longer be released. If formation of a new cuticle is too advanced to be interrupted, this become problematic. It may have obliged the brain to take at least partial control of growth away from the fat body by releasing one or more of the neuroendocrine insulin-like peptides to force growth and molting to proceed. It is possible that this achieved by simultaneously reducing growth of organs that are needed for (sexual) maturation but not essential for immediate survival, like the gonads. This could be how the neuroendocrine insect irps initially evolved. In echinoderms IGF probably stimulates growth of the follicles and oocytes, but the final growth spurt, the one that permits resumption of meiosis in the oocytes and subsequent ovulation, is delayed until optimal conditions to do so prevail. When the time and place are right the nervous system releases GSS likely in large amounts to finish the maturation process and induce ovulation. In vertebrates, growth and the release of IGF has also been brought under control of the brain but more forcefully by bringing IGF secretion by the liver under control of growth hormone. Whereas in an early ancestor high plasma concentrations of insulin might have led to secretion of IGF, this is no longer the case. Here insulin may have evolved to insure that plasma concentrations of glucose are kept sufficiently low by insuring its absorption by tissues in order to avoid it loss by excretion. In the three cases insulin and insulin-like peptides have very different functions, ovulation in echinoderms, sparing glucose in vertebrates and rescuing interrupted growth in insects. It is plausible then that these hormones each evolved from a non-local IGF gene duplication and that they are thus not proper orthologs but evolved by convergent evolution. This hypothesis would explain, why there is no insulin gene located near the IGF, octinsulin/gonadulin and dilp7 triplet in cockroaches, echinoderms and hemichordates, even though insulin – and other peptides such as the insect neuroendocrine insulin-like peptides and GSS - almost certainly evolved from IGF much later.

Conclusions

The gene triplication previously reported from arthropods must have occurred in a common bilaterian ancestor of the deuterostomes and protostomes. The hypothesis that IGF in an ancestral bilaterian used both a GPCR and an RTK may explain the combination of echinoderm irps and putative insulin receptors. This hypothesis implies that insulin is not a hormone that evolved before the split between protostomes and deuterostomes, but that insulin-like peptides evolved

579 independently in different metazoan clades as miniature copies of IGF capable to activate the
580 RTK but unable to stimulate the LGR.
581

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Figure 1

Sequences of selected ambulacrarian IGF.

Partial IGF sequences from selected ambulacrarians are illustrated to show their sequence similarity. The A-, B- and C-domains of the insulin core are aligned, but not the putative D- and E- domains, as their amino acid sequence is only conserved in closely related species (Fig. S1). Not aligning D- and E- domains allows the visualization the context of putative convertase cleavage sites. None of the arginine or lysine residues conform to a typical arthropod or vertebrate convertase cleavage site. Although the sequence of the latter part of the IGF precursors is not well conserved, all of them are rich in positively charged amino acid residues. Conserved cysteine residues are indicated in red, conserved amino acid residues are highlighted in black and conserved substitutions in grey. The arginine and lysine residues in the D- and E- domains are highlighted in blue.

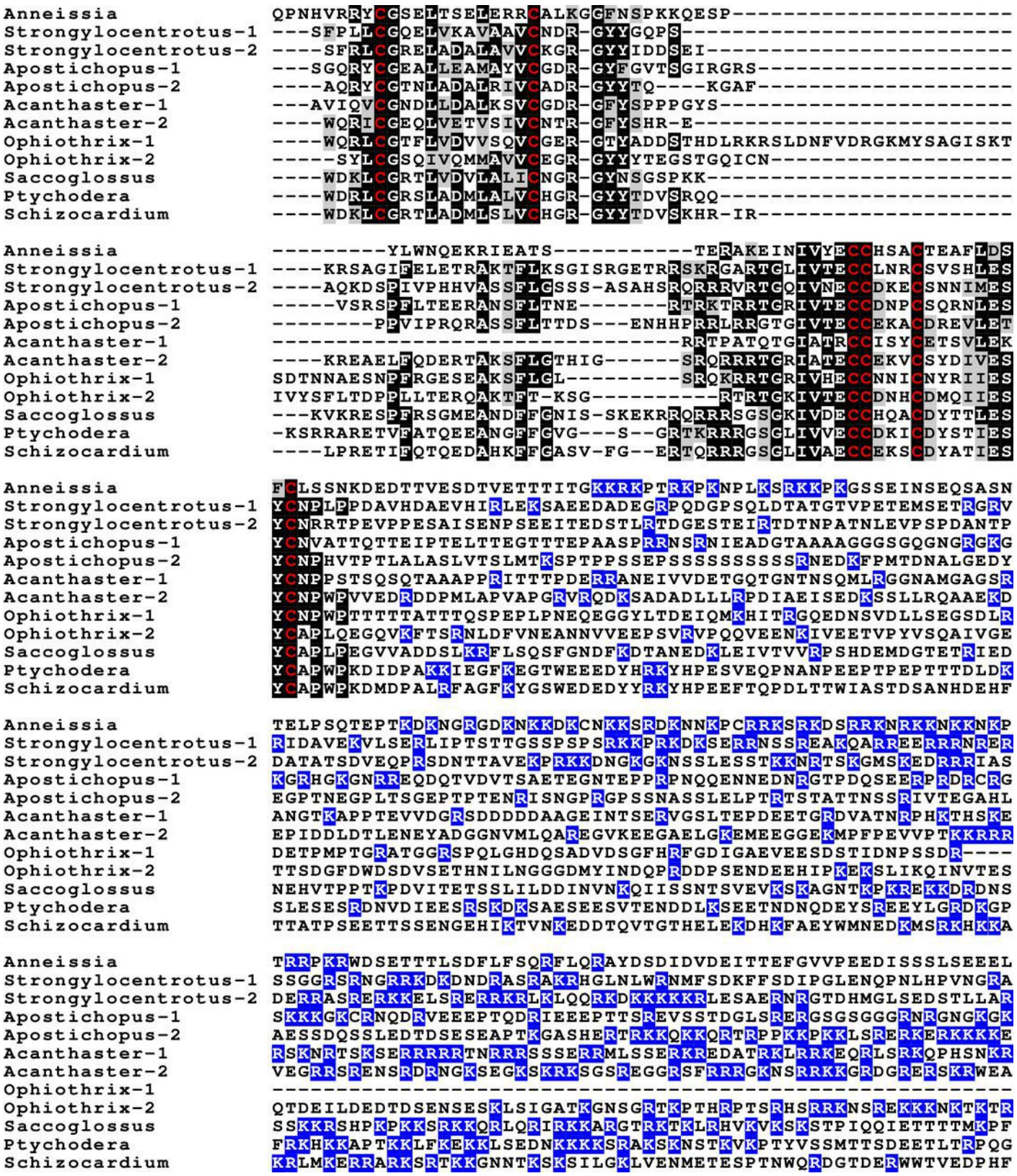

Figure 2

Sequences of selected echinoderm GSS.

Sequences of selected echinoderm GSS. Sequence alignment of a few echinoderm GSS showing relatively conserved A- and B- domains of the insulin core sequence and likely KR convertase cleavage sites that can be expected to be cleaved by neuroendocrine convertase as well as a few potential furin sites. Conserved cysteine residues are indicated in red, conserved amino acid residues are highlighted in black and conserved substitutions in grey. The arginine and lysine residues that form likely – or possibly in the case of *Apostichopus* GSS-2 - part of a convertase site are highlighted in blue. For the alignment of a larger number or echinoderm GSS sequences see Fig. S3.

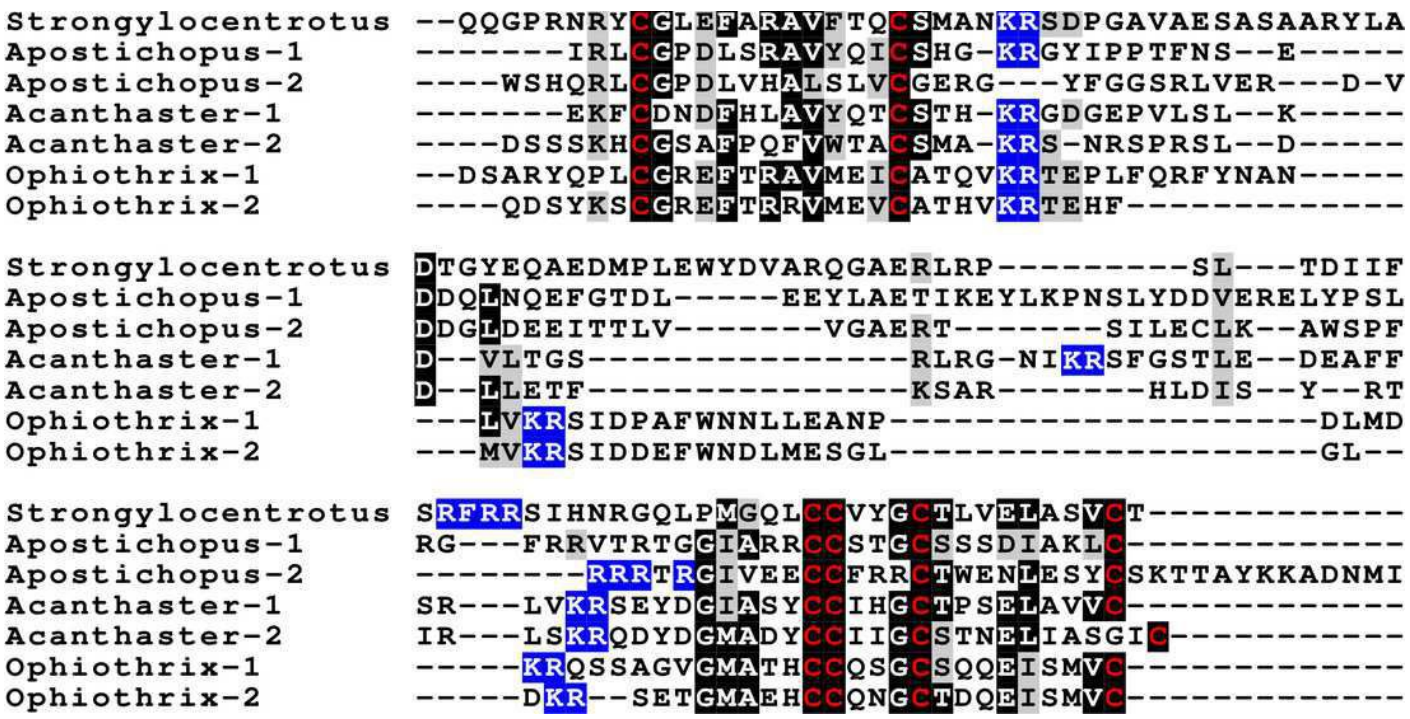

Figure 3

Sequences of selected dilp7 orthologs.

Sequences of selected dilp7 orthologs. Sequences of *Drosophila* dilp7 and several ambulacrarian orthologs illustrating well conserved sequences, not only in typical insulin core of the peptides, but also in the F-domain (underlined in blue). Note that the sequence conservation of these peptides is stronger than in the IGFs or GSSs (Figs. 1,2). Conserved cysteine residues are indicated in red, conserved amino acid residues are highlighted in black and conserved substitutions in grey. Likely convertase cleavage sites have been highlighted in blue. Sequences are from Spreadsheet S1 and Veenstra 2020b, a comparison of a larger number of sequences is presented in Figs. S5 and S6.

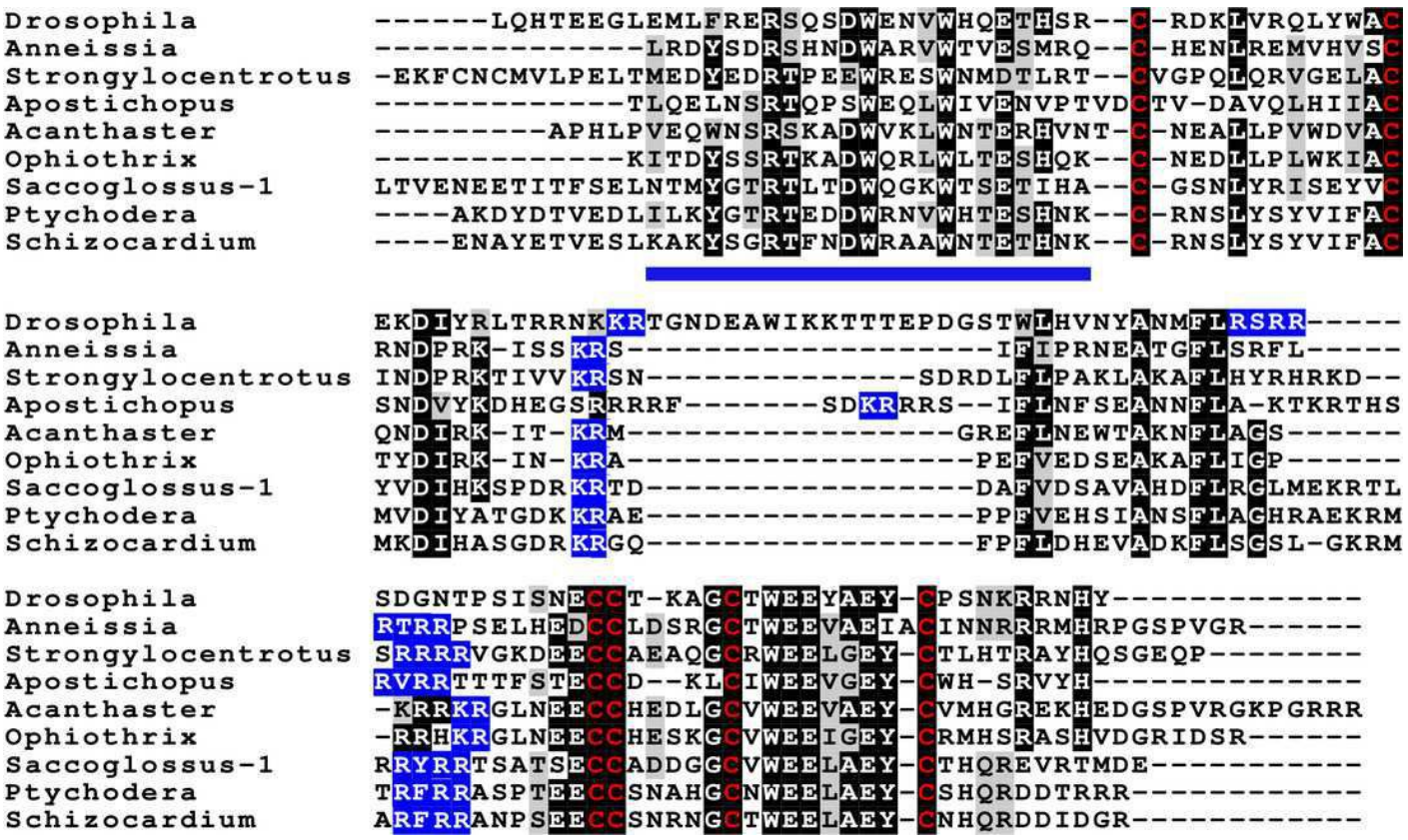

Figure 4

Sequences of selected ambulacrarian octinsulins.

Sequences of selected ambulacrarian octinsulins. Sequence alignment of a number of octinsulin sequences show that these sequences all have typical neuroendocrine convertase KR cleavage sites, suggesting these precursors are processed by enteroendocrine and/or neuroendocrine cells. Conserved cysteine residues are indicated in red, conserved amino acid residues are highlighted in black and conserved substitutions in grey. Likely convertase cleavage sites have been highlighted in blue. Sequences are from Spreadsheet S1, a comparison of a larger number of sequences is presented in Figs. S7 and S8.

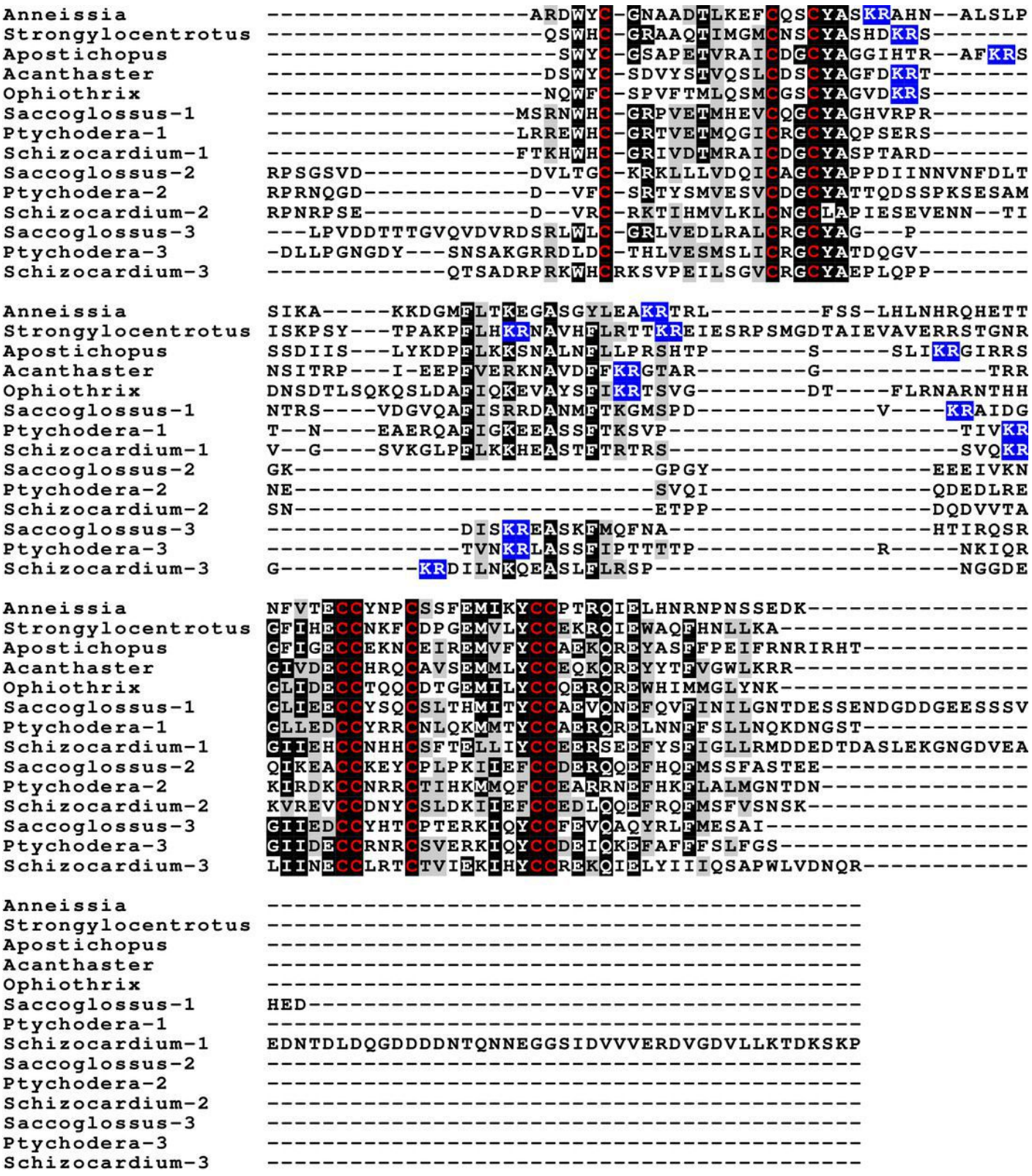

Figure 5

Sequence comparison of selected ambulacrarian multinsulins and dilp7 orthologs.

Sequence comparison of selected ambulacrarian multinsulins and dilp7 orthologs. Three different sets of sequences are compared. The top five sequences are dilp7 orthologs, the next five are multinsulins having three disulfide bridges and the last five multinsulins having four disulfide bridges. Note that although the multinsulins and the dilp7 orthologs share some sequences similarity this does not include the F-domain. Like the octinsulins these sequences all have typical neuroendocrine convertase KR cleavage sites, suggesting they are processed by enteroendocrine and/or neuroendocrine cells. Conserved cysteine residues are indicated in red, conserved amino acid residues are highlighted in black and conserved substitutions in grey. Likely convertase cleavage sites have been highlighted in blue. Sequences are from Spreadsheet S1, a comparison of a larger number of sequences is presented in Figs. S9 and S10.

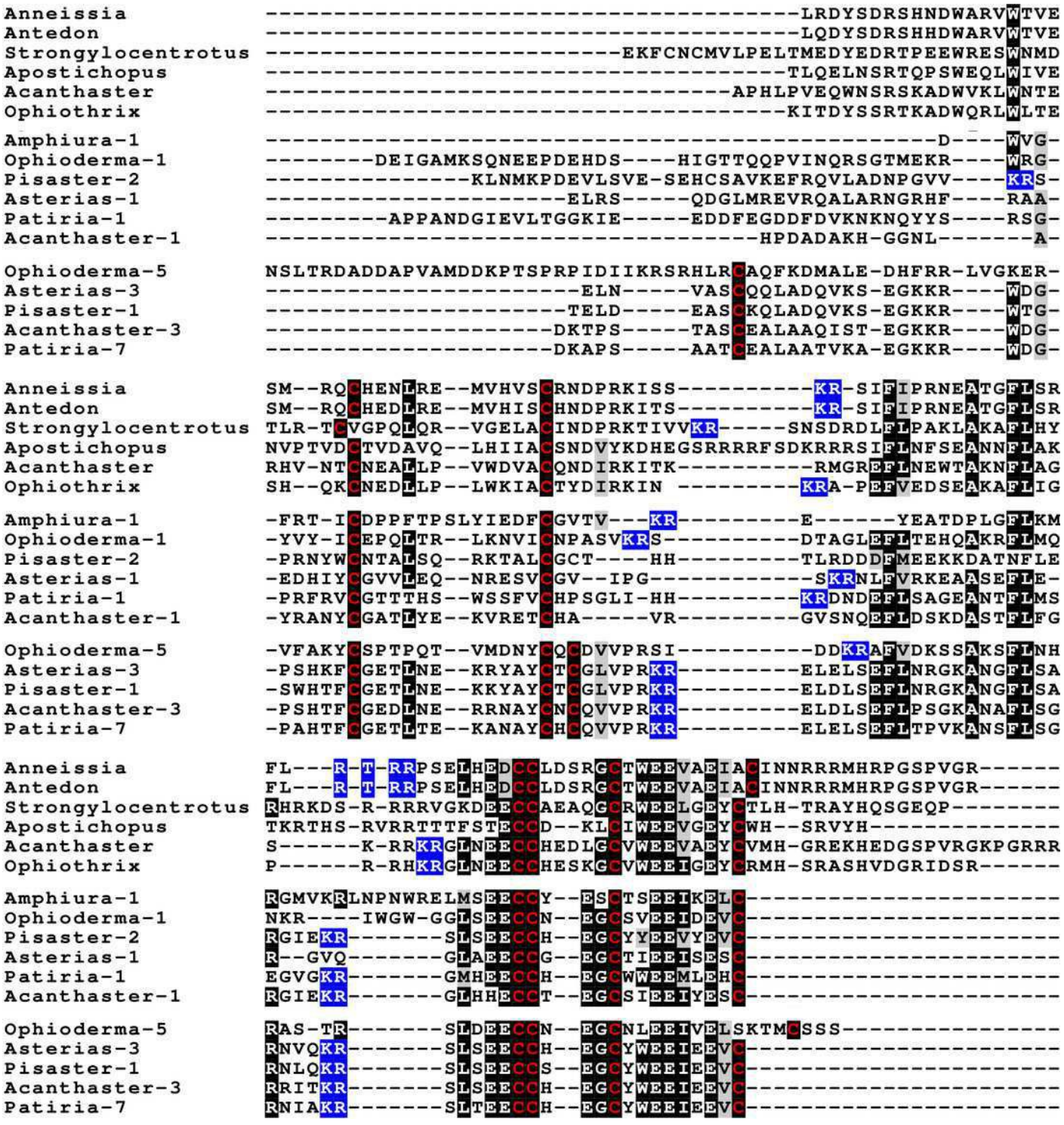

Figure 6

Position of introns in ambulacrarian insulin-like genes.

Schematic representation of the location of the cysteine residues, indicated as purple rectangles, and introns, represented by green T's, in the coding sequences of the various types of ambulacrarian insulin-like genes. Numbers indicate the phase of each intron. All genes share the typical phase 1 intron present in insulin-like genes, whereas dilp7 and multinsulin genes also share a phase 2 intron.

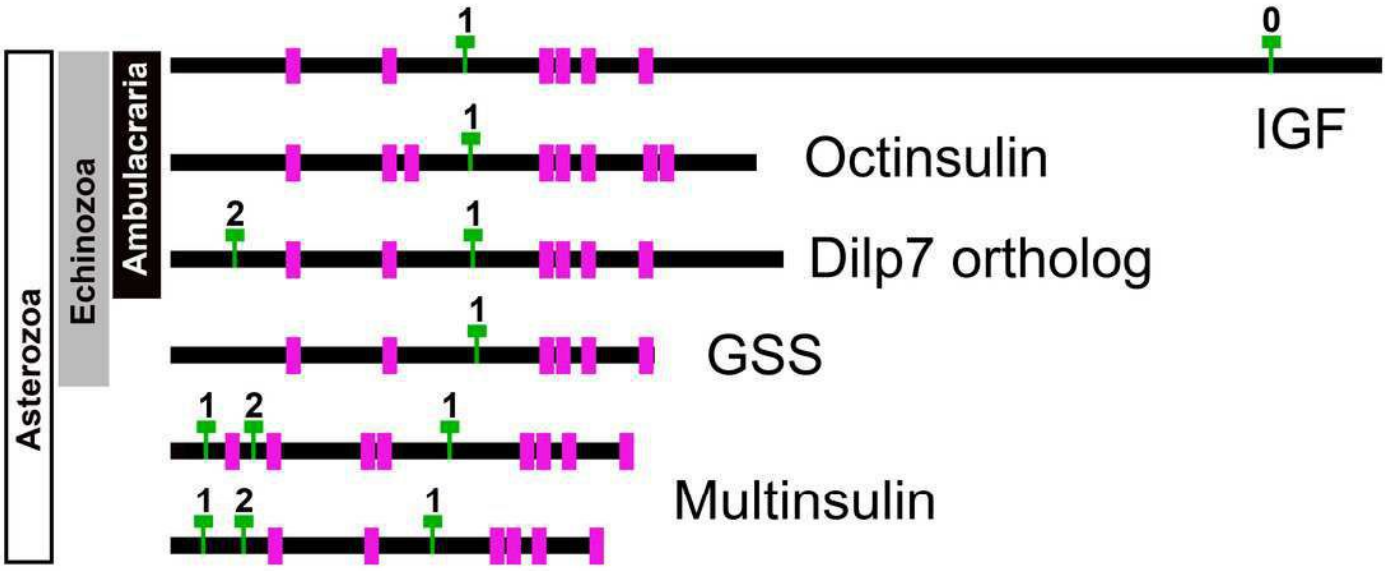

Figure 7

Synteny of ambulacrarian insulin-like genes.

Schematic representation of the relative localization of different IGF and IGF-related genes in several arthropod and ambulacrarian genomes. Arrow heads indicate transcription direction of the various genes, the numbers below the line indicate the number of nucleotides between the coding regions of adjacent genes in kilo base pairs. Note that the relative organization of the two insects – the cockroach *Blattella germanica* and the stick insect *Timema crisitinae* – is the same as in the hemichordate *Saccoglossus kovalewskii* and remarkably similar to that of the sea urchin *Strongylocentrotus purpuratus* and the sea cucumber *Holothuria scabra*. In the spider *Pardosa pseudoannulata* and the sea cucumber *Apostichopus japonicus* some of the genes are also next to one another. However, in the sea stars *Acanthaster planci* and *Pisaster ochraceus* synteny has been lost. Arthropod data from Veenstra, 2020b.

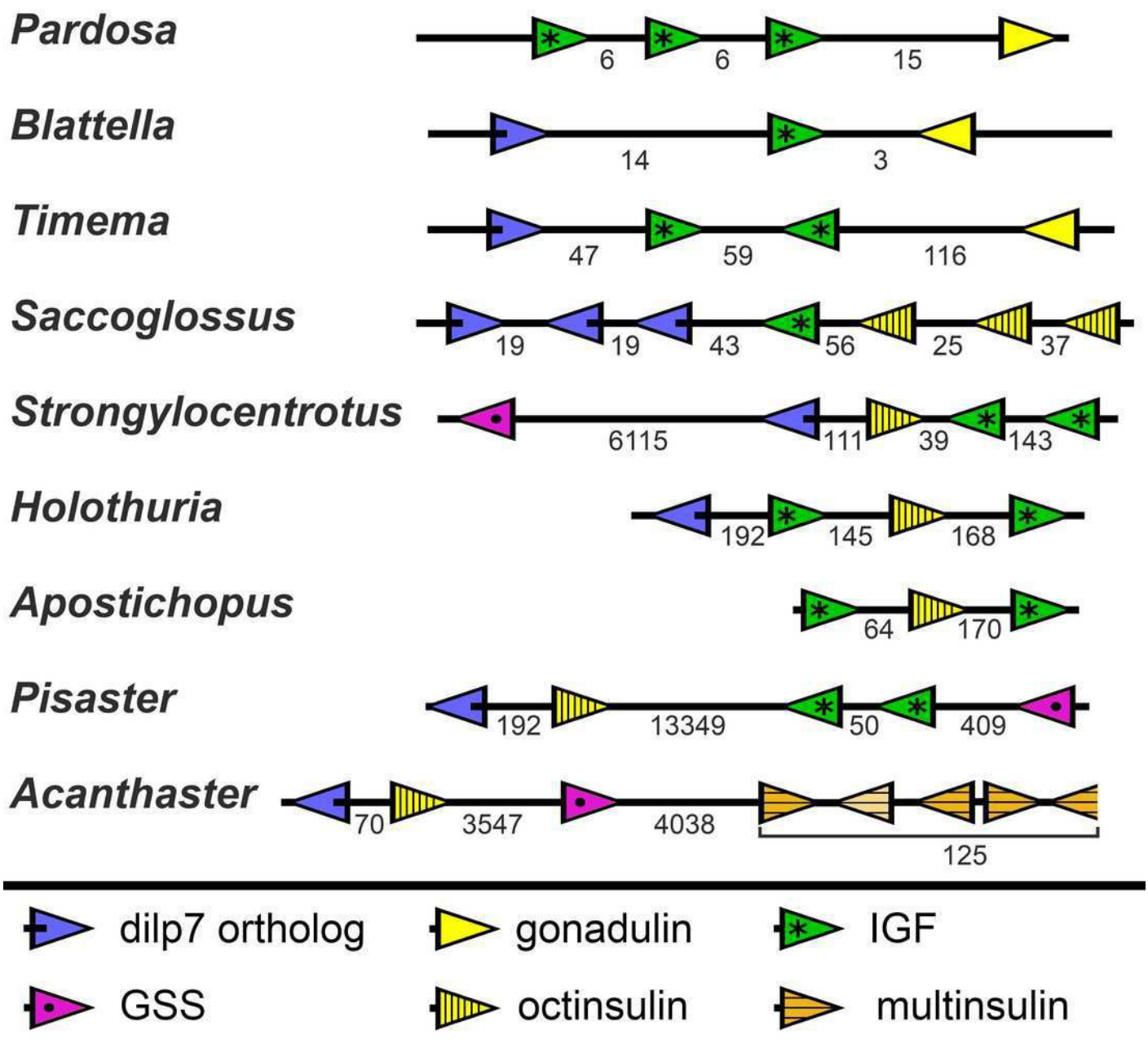

Figure 8

Radial sequence similarity tree of ambulacrarian insulin-like peptides.

The five different types of insulin-related peptides are clearly separated from one another. Note that the GSSs are similar to IGFs and seem to be related to them, while the multinsulins are most similar to the dilp7 orthologs. Echinoderm branches are in black, hemichordate branches in red. More extensive sequence comparisons and sequence trees are the supplementary data (Figs. S1-S10). All sequences are from Spreadsheet S1.

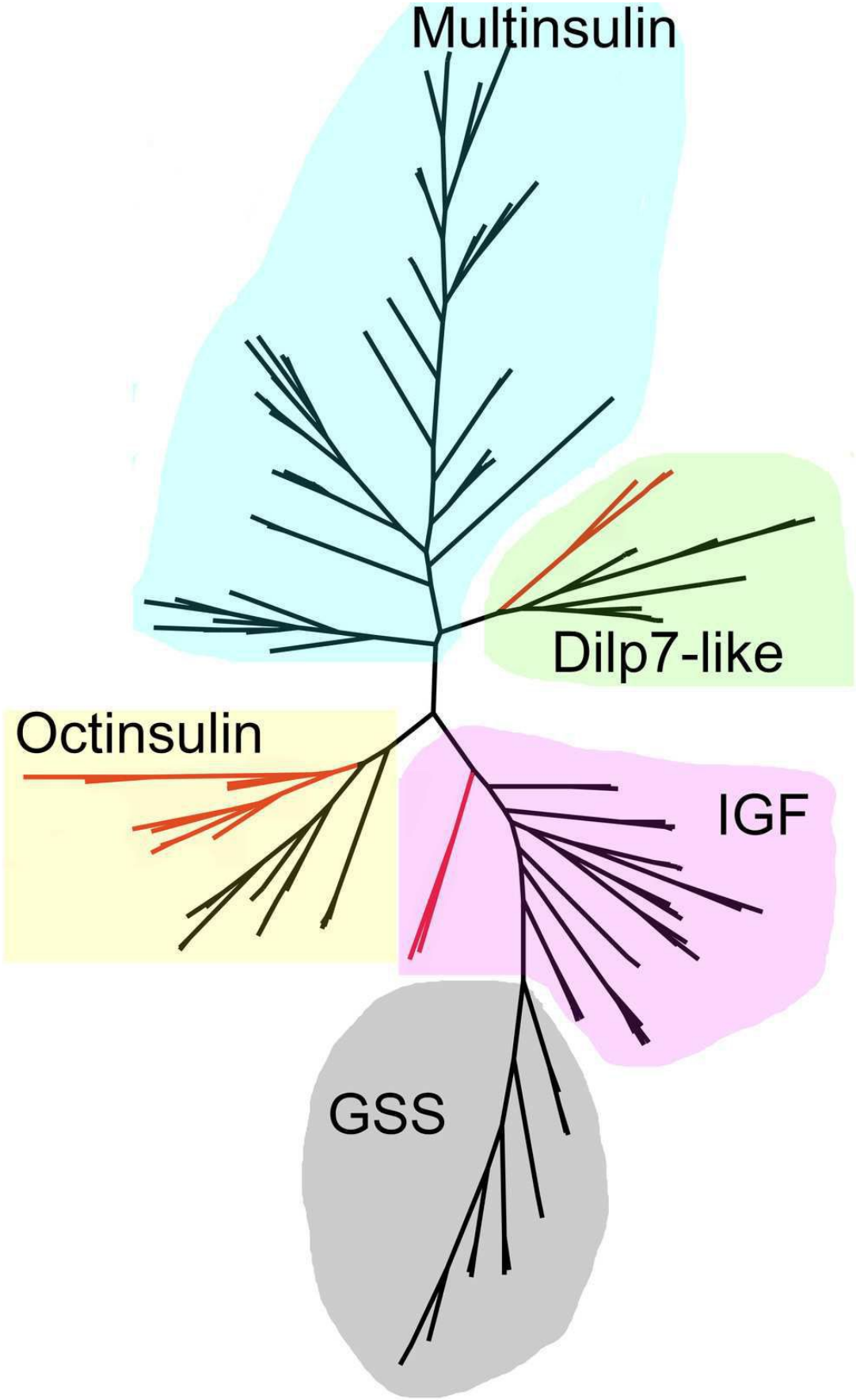

Figure 9

Phylogenetic tree of LGRs.

Phylogenetic tree constructed from the transmembrane regions of ambulacrarian LGRs that are putative receptors for insulin-related peptides. A few human and insect sequences have been added for comparison. The insert at the top shows the same data to which the glycoprotein LGRs have been added and where characteristic ligands for each branch have been identified. Numbers in blue indicate the apparent probabilities as determined by Fasttree.

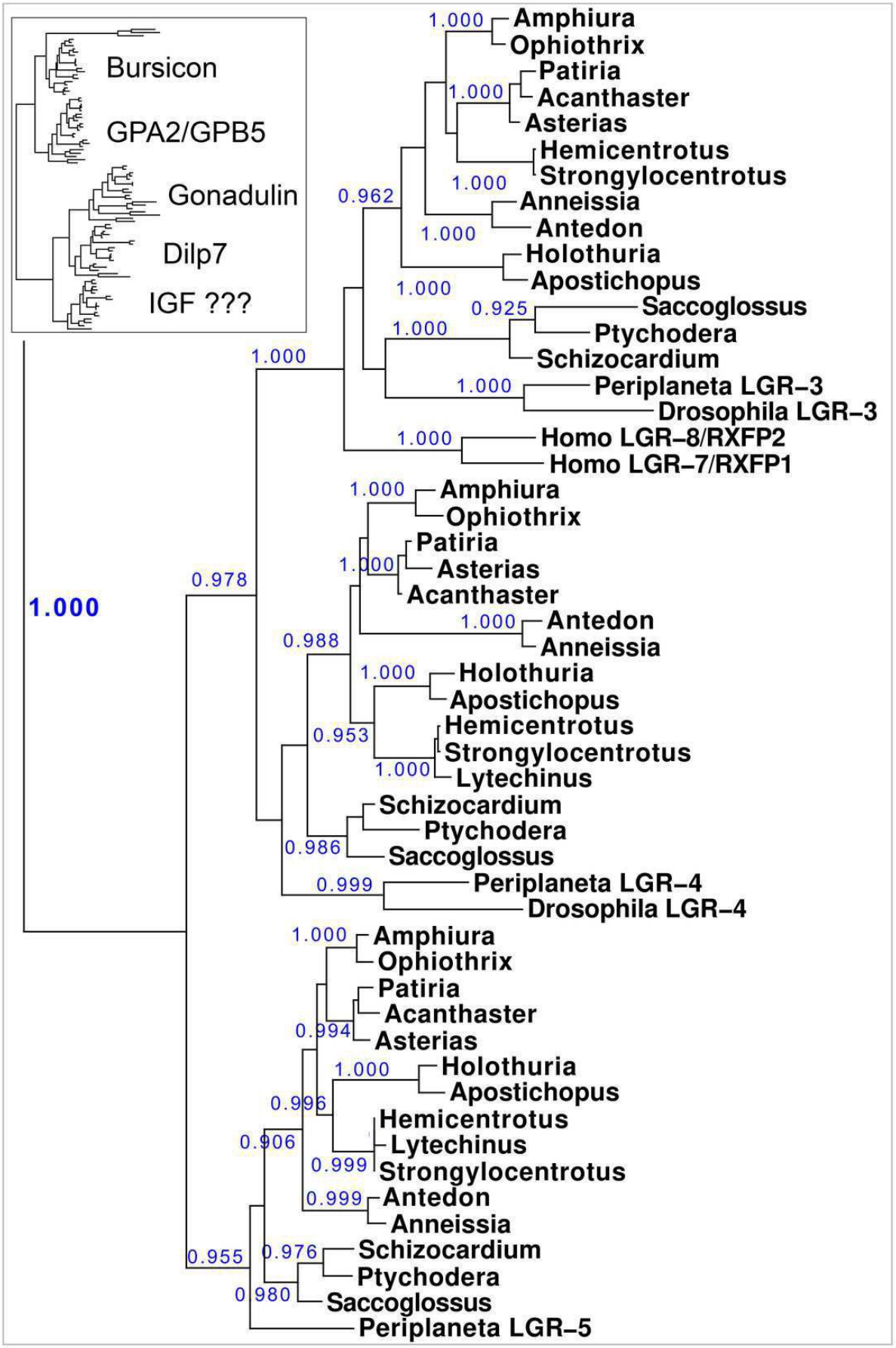

Figure 10

Ectodomains of ambulacrarian LGRs.

Ectodomains of ambulacrarian LGRs. Schematic representation of the various domains of the putative receptors for ambulacrarian insulin-related peptides. Each green circle symbolizes an LDLa repeat and each purple rectangle an LRR repeat, while the yellow oval indicates the seven transmembrane regions. The top representation corresponds to the gonadulin and dilp7 receptors (Figs. S11,S12). Note though, that the latter are somewhat variable, notably in the sea stars of two species of the *Patiria* genus and *Acanthaster planci* those receptors have two LDLa repeats (for details see Fig. S12). The bottom representation corresponds to the GRL101 receptors (Fig. S13).