Discovery of a second SALMFamide gene in the sea urchin *Strongylocentrotus purpuratus* reveals that L-type and F-type SALMFamide neuropeptides coexist in an echinoderm species.

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Abstract

The SALMFamides are a family of neuropeptides that act as muscle relaxants in the phylum Echinodermata. Two types of SALMFamides have been identified in echinoderms: firstly, the prototypical L-type SALMFamide peptides with the C-terminal sequence Leu-X-Phe-NH_2 (where X is variable), which have been identified in several starfish species and in the sea cucumber *Holothuria glaberrima*; secondly, F-type SALMFamide peptides with the C-terminal sequence Phe-X-Phe-NH_2, which have been identified in the sea cucumber *Apostichopus japonicus*. However, the genetic basis and functional significance of the occurrence of these two types of SALMFamides in echinoderms are unknown. Here we have obtained a new insight on this issue with the discovery that in the sea urchin *Strongylocentrotus purpuratus* there are two SALMFamide genes. In addition to a gene encoding seven putative F-type SALMFamide neuropeptides with the C-terminal sequence Phe-X-Phe-NH_2 (SpurS1-SpurS7), which has been reported previously (Elphick and Thorndyke, 2005; *J. Exp. Biol*., 208, 4273-4282 [1]), we have identified a gene that is expressed in the nervous system and that encodes a precursor of two putative L-type SALMFamide neuropeptides with the C-terminal sequences Ile-X-Phe-NH_2 (SpurS8) and Leu-X-Phe-NH_2 (SpurS9). Our discovery has revealed for the first time that L-type and F-type SALMFamide neuropeptides can coexist in an echinoderm species but are encoded by different genes. We speculate that this feature of *Strongylocentrotus purpuratus* may apply to other echinoderms and further insights on this issue will be possible if genomic and/or neural cDNA sequence data are obtained for other echinoderm species.

Keywords

neuropeptide precursor; FMRFamide; relaxant; echinoid; Echinodermata; radial nerve

Abbreviations

BLAST, basic local alignment search tool; cDNA, complementary deoxyribonucleic acid; EST, expressed sequence tag; GSS, gonad stimulating substance; MagS2, *Marthasterias glacialis* SALMFamide 2; MagS3, *Marthasterias glacialis* SALMFamide 3; MagS4, *Marthasterias glacialis* SALMFamide 4; NCBI, National Center for Biotechnology Information; S1, SALMFamide 1; S2, SALMFamide 2; SpurS1, *Strongylocentrotus purpuratus* SALMFamide 1; SpurS2, *Strongylocentrotus purpuratus* SALMFamide 2; SpurS3, *Strongylocentrotus purpuratus* SALMFamide 3; SpurS4, *Strongylocentrotus purpuratus* SALMFamide 4; SpurS5, *Strongylocentrotus purpuratus* SALMFamide 5; SpurS6, *Strongylocentrotus purpuratus* SALMFamide 6; SpurS7, *Strongylocentrotus purpuratus* SALMFamide 7; SpurS8, *Strongylocentrotus purpuratus* SALMFamide 8; SpurS9, *Strongylocentrotus purpuratus* SALMFamide 9; tBLASTn, translated nucleotide basic local alignment search tool

1. Introduction

SALMFamides are a family of neuropeptides that have been identified in species belonging to the phylum Echinodermata. The prototypes for the family, S1 and S2, were both isolated from the starfish species *Asterias rubens* and *Asterias forbesi* on account of their cross-reactivity with antibodies to the molluscan FMRFamide-related neuropeptide pQDPFLRFamide [2, 3]. S1 is an amidated octapeptide with the amino acid sequence Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-NH_2 and S2 is an amidated dodecapeptide with the amino acid sequence Ser-Gly-Pro-Tyr-Ser-Phe-Asn-Ser-Gly-Leu-Thr-Phe-NH_2 [2, 4, 5].

Following the discovery of S1 and S2, antibodies to pQDPFLRFamide were used to monitor purification of immunoreactive peptides from extracts of the sea cucumber *Holothuria glaberrima.* Two related peptides that share sequence similarity with S1 and S2 were identified, Gly-Phe-Ser-Lys-Leu-Tyr-Phe-NH_2 (GFSKLYFamide) and Ser-Gly-Tyr-Ser-Val-Leu-Tyr-Phe-NH_2 (SGYSVLYFamide) [6]. Thus, the concept of a SALMFamide neuropeptide family in echinoderms emerged, with the C-terminal motif Ser-X-Leu-X-Phe-NH_2 (where X is variable) apparently a characteristic feature of this family.

Antibodies to S1 and S2 have been developed and used to investigate the pattern of expression of these peptides in starfish. This has revealed that S1 and/or S2 are widely expressed by neurons in larval and adult nervous systems and in association with visceral organs (e.g. digestive system) and skeletal systems (e.g. the locomotory organs, tube feet) [7-11]. Likewise, antibodies to the holothurian neuropeptide GFSKLYFamide revealed a widespread pattern of expression in

Holothuria glaberrima [12], indicating that SALMFamide neuropeptides may have general roles as neurotransmitters or neuromodulators in echinoderms.

Investigation of the physiological roles of SALMFamide neuropeptides in starfish has revealed that they act as muscle relaxants [13]. Thus, both S1 and S2 cause relaxation of cardiac stomach, tube feet and apical muscle preparations from *Asterias rubens* [7, 14, 15]. Interestingly, injection of S1 or S2 causes cardiac stomach eversion in starfish, suggesting that these peptides may be involved in physiological mechanisms that mediate stomach eversion associated with feeding in these animals [14]. Moreover, it appears that SALMFamides may have a general role as muscle relaxants in echinoderms because GFSKLYFamide causes relaxation of intestine and body wall muscle preparations in *Holothuria glaberrima* [16] and S1 and S2 cause relaxation of tube foot preparations from the sea urchin *Echinus esculentus* [1]. Furthermore, other roles of SALMFamide neuropeptides in echinoderms have also been reported. For example, S1-like and S2-like immunoreactive peptides have been detected in ophiuroids (brittle stars) [17, 18] and S1 modulates luminescence in the ophiuroid *Amphipholis squamata* [19].

In 1999 a new insight on the structural diversity of SALMFamide neuropeptides emerged from a study characterising myoactive peptides in body wall extracts from the sea cucumber *Apostichopus japonicus* [20]. Two peptides were identified that had direct relaxing effects on muscle preparations from this species and both of these peptides were found to be novel members of the SALMFamide family: Gly-Tyr-Ser-Pro-Phe-Met-Phe-NH_2 and Phe-Lys-Ser-Pro-Phe-Met-Phe-NH_2 [20]. Interestingly, discovery of these SALMFamide neuropeptides in *Apostichopus japonicus* revealed structural variability in the SALMFamide family that was hitherto unknown. Thus, the presence of a phenylalanine residue in the position occupied by a

leucine residue in S1, S2 and the two SALMFamides identified in *Holothuria glaberrima* broadened the structural criteria for membership of the SALMFamide family to Ser-X-(Leu or Phe)-X-Phe-NH_2 [13].

Opportunities for gaining new insights on the genetic basis for the diversity of SALMFamide neuropeptides in echinoderms have emerged recently with the sequencing of the genome of the sea urchin *Strongylocentrotus purpuratus* [21, 22]. Analysis of genome sequence data using the Basic Local Alignment Search Tool (BLAST) revealed the presence of a gene encoding a protein comprising seven putative SALMFamide neuropeptides: SpurS1 (Pro-Pro-Val-Thr-Thr-Arg-Ser-Lys-Phe-Thr-Phe-NH_2), SpurS2 Asp-Ala-Tyr-Ser-Ala-Phe-Ser-Phe-NH_2), SpurS3 (Gly-Met-Ser-Ala-Phe-Ser-Phe-NH_2), SpurS4 (Ala-Gln-Pro-Ser-Phe-Ala-Phe-NH_2), SpurS5 (Gly-Leu-Met-Pro-Ser-Phe-Ala-Phe-NH_2), SpurS6 (Pro-His-Gly-Gly-Ser-Ala-Phe-Val-Phe-NH_2) and SpurS7 (Gly-Asp-Leu-Ala-Phe-Ala-Phe-NH_2) [1]. Interestingly, all seven of these putative SALMFamide neuropeptides have a C-terminal Phe-X-Phe-NH_2 sequence, like the two SALMFamides identified in the sea cucumber *Apostichopus japonicus*. This contrasts with SALMFamide neuropeptides thus far identified in starfish species [4, 5, 23] and in the sea cucumber *Holothuria glaberrima* [6], which all have a C-terminal Leu-X-Phe-NH_2 sequence.

The evolutionary and functional significance of the occurrence of SALMFamide neuropeptides in echinoderms with either Leu-X-Phe-NH_2 (L-type) or Phe-X-Phe-NH_2 type (F-type) C-terminal motifs is unclear. In particular, it is not known whether L-type and F-type SALMFamides coexist in echinoderm species. Here we provide a new insight on this issue with the discovery of a second SALMFamide gene in the sea urchin *Strongylocentrotus purpuratus* encoding a protein that comprises two putative L-type SALMFamide neuropeptides with the C-

terminal sequences Leu-X-Phe-NH_2 or Ile-X-Phe-NH_2.

2. Materials and Methods

A collection of 2026 expressed sequence tags (ESTs) derived from a *Strongylocentrotus purpuratus* radial nerve cDNA library generated by Eric Davidson's lab at Caltech was analysed to identify novel putative sea urchin neuropeptide precursors. The EST collection comprised 1027 3' ESTs and 999 5' ESTs, which were sequenced by the Human Genome Sequencing Center at Baylor College of Medicine and submitted to the National Center for Biotechnology Information (NCBI) EST database (dbEST). The 3' ESTs are GI:109401590 – GI109402616 and the 5' ESTs are GI:109402617 – GI109403615.

To assess the utility of this EST collection as a resource for identification of cDNAs encoding neuropeptide precursors, known sea urchin neuropeptide precursor sequences were submitted as queries in tBLASTn searches of dbEST. Using this approach a cDNA (RNSP-5L15) encoding the precursor of the myoactive neuropeptide NGFFFamide was identified based upon both 5' (GI: 109403168) and 3' (GI: 109402129) EST data, as reported previously [24]. In addition, a cDNA (RNSP-5H7) encoding the precursor of the F-type SALMFamides SpurS1-SpurS7 [1] was identified based upon both 5' (GI:109402985) and 3' (GI: 109402020) EST data. Finding that cDNAs encoding known sea urchin neuropeptide precursors are represented in the radial nerve EST dataset indicated that this EST collection might also contain the sequences of cDNAs encoding other neuropeptide precursors.

A variety of strategies were employed to identify ESTs encoding putative neuropeptide precursors. Initially, we restricted our analysis to the 5' EST dataset and this was done for two reasons. Firstly, 5' ESTs are more likely to contain coding nucleotide sequences because 5' untranslated regions (UTRs) are typically shorter

than the 3' UTRs [25]. Secondly, the 5' coding region of neuropeptide precursors and other secreted proteins encodes an N-terminal signal peptide sequence, which is essential for directing the translated precursor protein to the lumen of the endoplasmic reticulum. Therefore, using the online signal peptide prediction tool SignalP (http://www.cbs.dtu.dk/services/SignalP/) [26], it was possible to identify a subset of the 5' ESTs predicted to encode proteins with a N-terminal signal peptide.

The set of 5' ESTs were analysed by submission as queries against the GenBank protein database using BLASTx and ESTs encoding proteins that were clearly identifiable as homologs of known proteins that are not neuropeptide precursors were eliminated. Then, after having established the presence of a predicted N-terminal signal peptide using SignalP, other structural characteristics were used to identify ESTs encoding putative neuropeptide precursors. These included being comprised of less than 500 amino acid residues and the presence of peptide sequences bounded by putative monobasic and dibasic residue endopeptidase cleavage sites (KR, RK, RR, KK, R and K). Nonessential criteria, which are nonetheless characteristic of many neuropeptide precursors, were used as supporting evidence for the identification of ESTs encoding putative neuropeptide precursors. These included the repetition of homologous variations of a putative neuropeptide sequence within the protein and the presence of a glycine residue at the C-terminal of the putative neuropeptide(s), a structural prerequisite for C-terminal amidation that is a characteristic feature of many neuropeptides [27].

Having a identified a subset of 5' ESTs encoding putative neuropeptide precursors, the 3' EST dataset was analysed for corresponding overlapping 3' ESTs so that full-length cDNA sequences could be determined. Finally, predicted neuropeptide precursor cDNA sequences were submitted as BLASTn queries against

the *Strongylocentrotus purpuratus* genome sequence database (http://blast.hgsc.bcm.tmc.edu/blast.hgsc?organism=11), which enabled validation of the predicted cDNA sequence and correction of any EST sequencing errors. Furthermore, validation of putative precursor sequences with genome sequence data also enabled determination of the structure of the genes encoding the putative neuropeptide precursors and in particular the position and length of introns by identification of 5' (gt) and 3' (ag) consensus sites for intron splicing.

3. Results

3.1. Identification of cDNAs encoding the F-type SALMFamide precursor in Strongylocentrotus purpuratus

A gene encoding a precursor of F-type SALMFamide neuropeptides (SpurS1 – SpurrS7) was identified in 2005 by analysis of *Strongylocentrotus purpuratus* genome sequence data [1] and has been assigned the official gene identification number SPU_021555 on the Sea Urchin Genome Project gene annotation website (http://www.spbase.org/SpBase/search/). This gene was also predicted by both the Gnomon gene prediction algorithm (GI: 115650747) and the Glean3 gene prediction algorithm (Glean3_21555), which were used to identify genes during annotation of the *Strongylocentrotus purpuratus* genome sequence [22]. However, the gene predictions differ with respect to the position of the start codon, and therefore cDNA sequence data are needed to clarify this issue.

BLAST analysis of the *Strongylocentrotus purpuratus* radial nerve EST data revealed a cDNA (RNSP-5H7) encoding a 266 residue F-type SALMFamide precursor, confirming the protein predicted by Elphick and Thorndyke [1]. However, analysis of the 5' EST (GI: 109402985) for RNSP-5H7 revealed the presence of a 5' non-coding exon, the existence of which had not been predicted by analysis of genomic sequence data. Furthermore, analysis of the 3' EST (GI: 109402020) for RNSP-5H7 revealed overlapping sequence identity with the 3' EST (GI: 109401655) of a *Strongylocentrotus purpuratus* radial nerve cDNA (RNSP-5N22) that has a 3' extension with respect to RNSP-5H7.

Based on these EST data and genomic sequence data, Fig. 1 shows the deduced sequence of a 2222 base transcript encoding the *Strongylocentrotus purpuratus* F-type SALMFamide precursor. Bases 1-393 correspond to the first exon, a non-coding exon, which in the genome is followed by an intron that is 81051 bases in length. Bases 394 – 552 correspond to the second exon encoding residues 1-53 of the F-type SALMFamide precursor protein, which includes the N-terminal signal peptide. Bases 553 – 2222 correspond to the third exon, which in the genome is preceded by an intron that is 1195 bases in length. Bases 553 – 1191 encode residues 54 – 266 of the F-type SALMFamide precursor, which incorporates the seven putative SALMFamide neuropeptides – SpurS1 to SpurS7, bases 1192-1194 encode a stop codon and bases 1195 – 2222 encode a 3' non-coding region.

3.2. Identification of cDNAs encoding a novel L-type SALMFamide precursor in Strongylocentrotus purpuratus

Analysis of the *Strongylocentrotus purpuratus* radial nerve EST data revealed cDNAs encoding a 130 amino acid residue precursor protein containing two putative L-type SALMFamide neuropeptides (SpurS8 and SpurS9) (Fig. 2). The 2002 base transcript shown in Fig. 2 was deduced from the 5' EST (GI: 109403124) and 3'EST (GI:109402032) of cDNA RNSP-5H19 and the 5' ESTs of cDNAs RNSP-1D16 (GI:109402899) and RNSP-1K4 (GI:109403449) and is derived from a gene comprising two exons. The first exon is 487 bases in length and comprises a 307 base 5' non-coding region followed by a 180 base coding region, which encodes the first 60 residues of the precursor protein. Analysis of genome sequence data revealed that

the two exons are separated by an intron that is 45382 bases in length. The second exon (1515 bases) encodes the C-terminal 70 residues of the 130 residue precursor protein followed a stop codon and a 1299 base 3' non-coding region. Moreover, it is the C-terminal region of the precursor that contains two putative L-type SALMFamide neuropeptides: Asn-Met-Gly-Ser-Ile-His-Ser-His-Ser-Gly-Ile-His-Phe-NH_2 (SpurS8) and Met-Arg-Leu-His-Pro-Gly-Leu-Leu-Phe-NH_2 (SpurS9). Both peptides have the C-terminal consensus sequence Ile/Leu-X-Phe-NH_2, which contrasts with Phe-X-Phe- consensus sequence that is characteristic of F-type SALMFamides SpurS1 – SpurS7 (Fig. 1).

The L-type SALMFamide precursor cDNA was not predicted by the Glean3 gene prediction algorithm used for annotation of the *Strongylocentrotus purpuratus* genome [22], but it was predicted by the Gnomon gene prediction algorithm and it has been assigned the gene identification numbers GI:115946154 and GI:115711932 in the NCBI GenBank database.

4. Discussion

We report here the identification of two cDNAs encoding SALMFamide neuropeptides in the sea urchin *Strongylocentrotus purpuratus*. Both cDNAs were identified in a radial nerve cord cDNA library, consistent with notion that the putative SALMFamide peptides encoded by these cDNAs act as neuropeptide signalling molecules in sea urchins.

The first cDNA identified encodes seven putative F-type SALMFamide neuropeptides (SpurS1 - SpurS7) with the C-terminal consensus sequence Phe-X-Phe-NH_2, which confirms a predicted gene product reported previously based on analysis of genomic sequence data [1]. However, the cDNA sequence data has revealed that, in addition to the two protein-coding exons reported previously, the F-type SALMFamide gene has a 5' non-coding exon. Furthermore, the cDNA data has also revealed that the third exon of the gene has a 1028 base 3' non-coding region.

The second cDNA identified is completely novel and encodes two putative L-type SALMFamide neuropeptides (SpurS8 and SpurS9) with the C-terminal consensus sequence Leu/Ile-X-Phe-NH_2 (Fig. 3A). Hitherto L-type SALMFamide neuropeptides had been identified in several starfish species [4-6, 23] and in the sea cucumber *Holothuria glaberrima* [6] but not in sea urchins. The discovery of a gene encoding putative L-type neuropeptides in *Strongylocentrotus purpuratus* demonstrates for the first time that L-type and F-type SALMFamide neuropeptides coexist in an echinoderm species but are encoded on separate genes.

Evidence that L-type SALMFamide neuropeptides may exist in sea urchins has been reported previously, however. Antibodies to the starfish L-type SALMFamide neuropeptide S2 were used in a radioimmunoassay to monitor

purification of immunoreactive peptides in extracts of the sea urchin *Echinus esculentus*. Four S2-like immunoreactive peaks were detected and one of these (peak 3) was purified and sequenced but only the partial N-terminal sequence of peak 3 was determined: Met-Arg-Tyr-His [1]. Without the full-length sequence of this peptide, however, the structural basis for its immunoreactivity with antibodies to the starfish L-type SALMFamide neuropeptide S2 was unclear. Identification of a gene in *Strongylocentrotus purpuratus* encoding the putative Ile/Leu-X-Phe-NH_2 type SALMFamide neuropeptides SpurS8 and SpurS9 has now provided a new insight on this issue. The predicted structure of SpurS9 is Met-Arg-Leu-His-Pro-Gly-Leu-Leu-Phe-NH_2, which clearly shares structural similarity with the partial sequence obtained for peak 3 from *Echinus esculentus* (Met-Arg-Tyr-His). Furthermore, it seems likely that the structural similarity between peak 3 from *Echinus esculentus* and SpurS9 extends to the C-terminal regions of these peptides. Consistent with this notion, the presence of the glycine residue in SpurS9 is interesting because a glycine residue is present in an equivalent position in the C-terminal region of the starfish peptide S2 (Ser-Gly-Leu-Thr-Phe-NH_2) (Fig. 3A). Thus, if a glycine residue is present in this position in the *Echinus* peak 3 peptide then this may have contributed to its immunoreactivity with antibodies to S2.

Discovery of both a L-type SALMFamide gene and a F-type SALMFamide gene in the sea urchin *Strongylocentrotus purpuratus* provides a new insight on the evolution and diversity of SALMFamide neuropeptides in echinoderms. In particular, it suggests that both L-type SALMFamides and F-type SALMFamides may also coexist in other echinoderm species. If this is correct, then why has this not been revealed by previous studies on echinoderms? A possible explanation may reside in the methods that have been used to monitor purification and identification of

SALMFamide neuropeptides. The first SALMFamides to be identified, S1 and S2 in the starfish species *Asterias rubens* and *Asterias forbesi* and GFSKLYFamide and SGYSVLYFamide in the sea cucumber species *Holothuria glaberrima*, were purified based on their cross-reactivity with an antibody to the molluscan neuropeptide pQDPFLRFamide [4, 6]. As pQDPFLRFamide has a C-terminal Leu-X-Phe-NH_2 motif, antibodies to this peptide may therefore bind Leu-X-Phe-NH_2 type (L-type) SALMFamide neuropeptides whilst exhibiting little or no cross-reactivity with Phe-X-Phe-NH_2 type (F-type) SALMFamide neuropeptides, which might explain why F-type SALMFamides were not isolated from starfish and sea cucumbers by Elphick et al. [4] and by Diaz-Miranda et al. [6], respectively.

Antibodies to the starfish L-type SALMFamides S1 and S2 have been generated and used to monitor purification of SALMFamide neuropeptides from other echinoderm species. For example, S1 was identified in the starfish *Pycnopodia helianthoides* following purification using antibodies to S1, but other SALMFamide peptides were not detected in this species using these antibodies [5]. More recently, antibodies to S1 or S2 have been used to monitor purification of SALMFamide neuropeptides from extracts of radial nerve cords from the starfish species *Marthasterias glacialis*. Interestingly, four SALMFamide neuropeptides were identified in this species: S1, MagS2 (a S2-like peptide with the sequence Ser-Gly-Pro-Tyr-Ser-Met-Thr-Ser-Gly-Leu-Thr-Phe-NH_2), MagS3 (Ala-Tyr-His-Ser-Ala-Leu-Pro-Phe-NH_2) and MagS4 (Ala-Tyr-Gln-Thr-Gly-Leu-Pro-Phe-NH_2) [23] (Fig. 3A). Identification of these peptides revealed a hitherto unknown diversity in the number of SALMFamide neuropeptides that can exist in a starfish species. However, it is noteworthy that all of the peptides identified were L-type SALMFamides with a C-terminal Leu-X-Phe-NH_2 consensus sequence. Thus, if F-type SALMFamide

neuropeptides exist in starfish it is possible that they have remained undetected because analysis of SALMFamide neuropeptides in starfish has utilised antibodies to the molluscan Leu-X-Phe-NH_2 neuropeptide pQDPFLRFamide or antibodies to L-type SALMFamides.

As discussed above, antibodies to the starfish L-type SALMFamide neuropeptide S2 have been used to monitor purification of cross-reactive peptides from the sea urchin *Echinus esculentus* [1]. Four immunoreactive peaks were detected (Peaks 1-4) but only peak 3 was purified and sequenced and, as suggested above, it is likely that peak 3 is a homolog of the L-type SALMFamide neuropeptide SpurS9 identified here in *Strongylocentrotus purpuratus*. The identity of peaks 1, 2 and 4 remain unknown, although we can speculate that perhaps one of them is a homolog of the SpurS8 peptide identified here in *Strongylocentrotus purpuratus*. The possibility remains, however, that one or more of the peaks detected in *Echnius esculentus* are in fact F-type SALMFamide neuropeptides. Of particular interest in this regard is the putative F-type SALMFamide SpurS1 identified in *Strongylocentrotus purpuratus*, which has the C-terminal sequence Phe-Thr-Phe-NH_2 (Fig. 3B). The presence of the threonine residue confers on this peptide structural similarity with the C-terminal sequence of the starfish L-type SALMFamide S2 (Leu-Thr-Phe-NH_2). Thus, if a SpurS1-like peptide exists in *Echinus esculentus* then it is possible that it would be recognised by antibodies to S2 and one of the unidentified S2-like immunoreactive peaks detected in *Echinus esculentus* may therefore be a homolog of the F-type SALMFamide neuropeptide SpurS1.

If the use of antibodies to Leu-X-Phe-NH_2 type neuropeptides explains why Phe-X-Phe-NH_2 type SALMFamides have not been identified in starfish or hitherto in sea urchins, how then were F-type SALMFamides identified in other echinoderms

prior the discovery of a gene encoding F-type SALMFamides in the sea urchin *Strongylocentrotus purpuratus*? F-type SALMFamides were first identified in the sea cucumber species *Apostichopus japonicus* (Gly-Tyr-Ser-Pro-Phe-Met-Phe-NH_2 and Phe-Lys-Ser-Pro-Phe-Met-Phe-NH_2) on account of their ability to cause relaxation of muscle preparations (intestine) from this species [20]. However, L-type SALMFamide neuropeptides can also cause muscle relaxation in sea cucumbers; thus, the peptide GFSKLYFamide causes relaxation of intestine preparations from the sea cucumber *Holothuria glaberrima* [16]. Why then were L-type SALMFamide neuropeptides not identified in *Apostichopus japonicus* when intestinal preparations were used as a bioassay to monitor purification of myoactive peptides from this species. One possible explanation for this may be differences in the relative potency or efficacy of L-type and F-type SALMFamide neuropeptides in causing relaxation of *in vitro* intestine preparations from different sea cucumber species. Thus, F-type SALMFamide neuropeptides may be more potent/effective as relaxants of intestinal preparations from *Apostichopus japonicus* and L-type SALMFamides may be more potent/effective as relaxants of intestinal preparations from *Holothuria glaberrima*. Consistent with this notion, there is evidence of striking differences in the responsiveness of other sea cucumber muscle preparations to L-type and F-type SALMFamides. Thus, L-type SALMFamides cause relaxation of longitudinal body wall preparations from *Holothuria glaberrima* [16] but F-type SALMFamides have no effect on longitudinal body wall preparations from *Apostichopus japonicus* [20]. Such differences in the activity of F-type and L-type SALMFamides could presumably be accounted for by the existence and differential expression of receptors that preferentially bind either F-type or L-type SALMFamides.

Further evidence of tissue-specific differences in the relative expression of SALMFamide receptors comes from pharmacological studies comparing the responsiveness of three different muscle preparations from the starfish *Asterias rubens* to the L-type SALMFamide neuropeptides S1 and S2. Thus, when compared to the relaxing effect of a nitric oxide donor, S1 and S2 were found to be much more effective as relaxants of a gut (cardiac stomach) preparation than as relaxants of body wall associated preparations (apical muscle and tube feet) [15].

Based on the data presented in this paper and other published data discussed above, we speculate that both L-type and F-type SALMFamides may coexist in species throughout the phylum Echinodermata. The possibility remains, however, that F-type SALMFamides have a more restricted phylogenetic distribution in echinoderms than L-type SALMFamides because F-type SALMFamides have thus far only been identified in the sea cucumbers and sea urchins, whereas L-type SALMFamides have been identified in starfish, sea cucumbers and sea urchins. Further insights on this issue will be possible if genome sequences are obtained for other echinoderm species.

The discovery of genes encoding L-type and F-type SALMFamide neuropeptides in the sea urchin *Strongylocentrotus purpuratus* now paves the way for detailed comparative analysis expression and physiological roles on these two types SALMFamides in a model echinoderm species. In particular, it will be interesting to investigate if L-type and F-type SALMFamides have other roles in addition to their now well-characterised ability to cause relaxation of echinoderm muscle preparations. Evidence that SALMFamide neuropeptides may have a variety of roles in echinoderms has been reported. For example, the L-type SALMFamide S1 has been found to cause inhibition of potassium-induced release of the gonadotropic hormone

gonad stimulating substance (GSS) from radial nerves of the starfish *Asterina pectinifera* [28, 29]. Likewise, the L-type SALMFamides identified in this study could have a similar role in sea urchins and investigation of this hypothesis will be facilitated if the molecular identity of GSS in sea urchins is determined.

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Figure legends

Fig. 1.

The *Strongylocentrotus purpuratus* F-type SALMFamide precursor. The cDNA sequence (lowercase, 2222 bases) encoding the F-type SALMFamide precursor protein (bold uppercase, 266 amino acid residues) is shown. The DNA sequence was derived from genomic sequence data but EST data were used to determine the length of 5' and 3' non-coding regions and the positions of introns. The positions of introns in the gene encoding the F-type SALMFamide precursor are shown by highlighting the pairs of bases (bold and underline) in the cDNA sequence that are separated by an intron in the corresponding genomic sequence. The predicted signal peptide is shown in blue and the seven F-type SALMFamides, which are named sequentially as SpurS1 – SpurS7, are shown in red, flanked by putative dibasic cleavage sites (KR) shown in green. The asterisk shows the position of the stop codon.

Fig. 2.

The *Strongylocentrotus purpuratus* L-type SALMFamide precursor. The cDNA sequence (lowercase, 2002 bases) encoding the L-type SALMFamide precursor protein (bold uppercase, 130 amino acid residues) is shown. The DNA sequence was derived from genomic sequence data but EST data were used to determine the length of 5' and 3' non-coding regions and the position of the single intron, which is shown by highlighting the pairs of bases (bold and underline) in the cDNA sequence that are separated by the intron in the corresponding genomic sequence. The predicted signal peptide is shown in blue and the two F-type SALMFamides, which are named sequentially as SpurS8 and SpurS9, are shown in red, flanked by putative dibasic

cleavage sites (KR or KK) shown in green. The asterisk shows the position of the stop codon.

Fig. 3.

L-type and F-type SALMFamides in echinoderms. A. The sequences of the two *Strongylocentrotus purpuratus* L-type SALMFamide neuropeptides, SpurS8 and SpurS9, are shown aligned with L-type SALMFamide neuropeptides derived starfish and sea cucumber species and conserved structural features are underlined. Note the sequence similarity between the N-terminal region of SpurS9 and the partial sequence of a SALMFamide-like immunoreactive peptide isolated from the sea urchin *Echinus esculentus*. B. The sequences of the seven *Strongylocentrotus purpuratus* F-type SALMFamide neuropeptides, SpurS1 – SpurS7, are shown aligned with F-type SALMFamide neuropeptides derived from the sea cucumber *Apostichopus japonicus* and conserved structural features are underlined. References: a). This paper; b). [1]; c). [4]; d). [5]; e). [23]; f). [6]; g). [20].

Figure 1

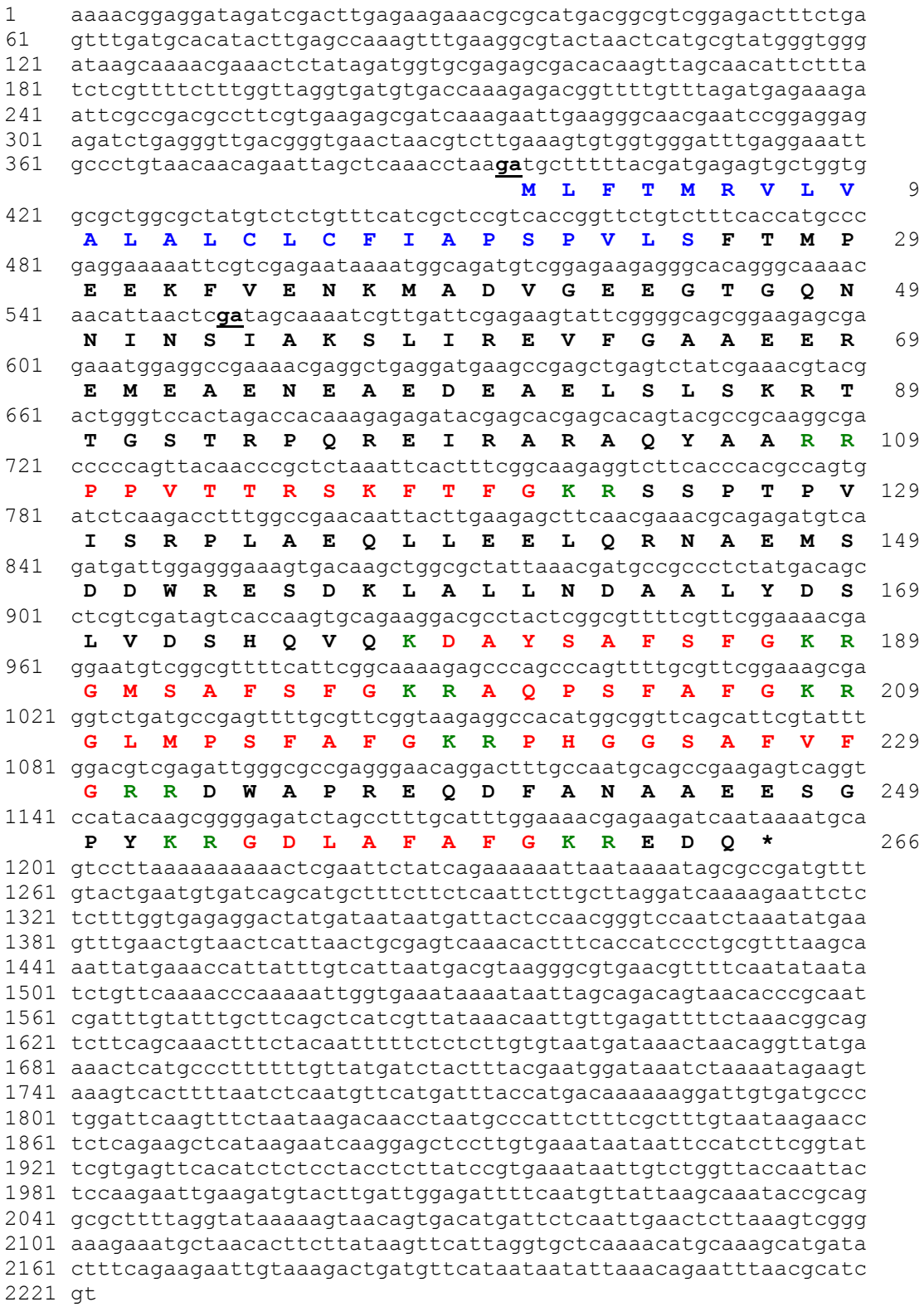
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Figure 2

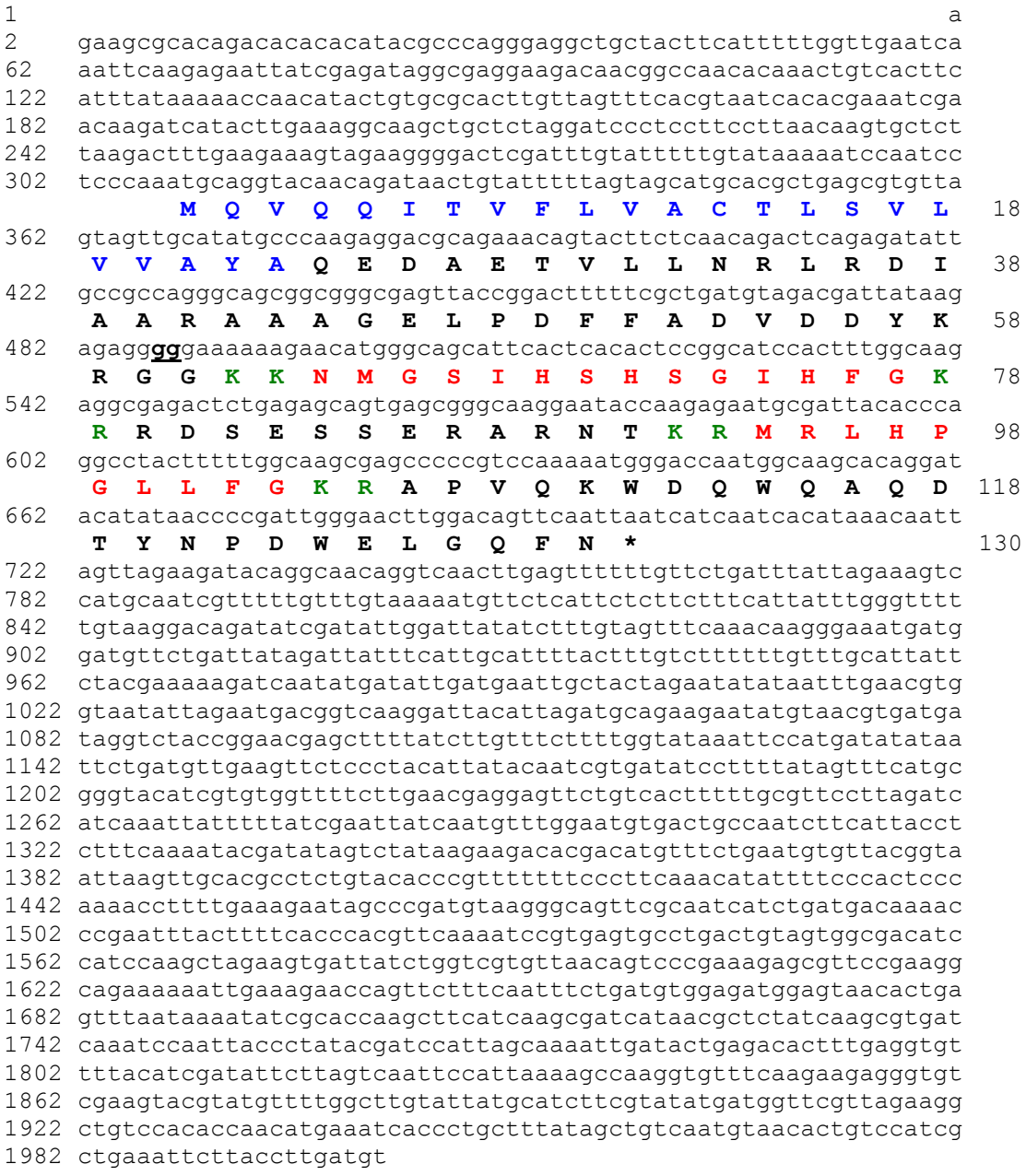
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Figure 3

A. Leu-X-Phe-NH_2 type (L-type) SALMFamides

Peptide	Sequence	Source	Ref.
SpurS8	Asn-Met-Gly-Ser-Ile-His-Ser-His-Ser-Gly-Ile-His-Phe-NH_2	*S. purpuratus* (Echinoidea)	a
SpurS9	Met-Arg-Leu-His-Pro-Gly-Leu-Leu-Phe-NH_2	*S. purpuratus* (Echinoidea)	a
SpurS9-like peptide (partial)	Met-Arg-Tyr-His-...	*E. esculentus* (Echinoidea)	b
S1	Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-NH_2	*A. rubens; A. forbesi; M. glacialis* *P. helianthoides* (Asteroidea)	c,d,e
S2	Ser-Gly-Pro-Tyr-Ser-Phe-Asn-Ser-Gly-Leu-Thr-Phe-NH_2	*A. rubens; A. forbesi* (Asteroidea)	c
MagS2	Ser-Gly-Pro-Tyr-Ser-Met-Thr-Ser-Gly-Leu-Thr-Phe-NH_2	*M. glacialis* (Asteroidea)	e
MagS3	Ala-Tyr-His-Ser-Ala-Leu-Pro-Phe-NH_2	*M. glacialis* (Asteroidea)	e
MagS4	Ala-Tyr-Gln-Thr-Gly-Leu-Pro-Phe-NH_2	*M. glacialis* (Asteroidea)	e
GFSKLYFamide	Gly-Phe-Ser-Lys-Leu-Tyr-Phe-NH_2	*H. glaberrima* (Holothuroidea)	f
SGYSVLYFamide	Ser-Gly-Tyr-Ser-Val-Leu-Tyr-Phe-NH_2	*H. glaberrima* (Holothuroidea)	f

B. Phe-X-Phe-NH_2 type (F-type) SALMFamides

Peptide	Sequence	Source	Ref.
SpurS1	Pro-Pro-Val-Thr-Thr-Arg-Ser-Lys-Phe-Thr-Phe-NH_2	*S. purpuratus* (Echinoidea)	a,b
SpurS2	Asp-Ala-Tyr-Ser-Ala-Phe-Ser-Phe-NH_2	*S. purpuratus* (Echinoidea)	a,b
SpurS3	Gly-Met-Ser-Ala-Phe-Ser-Phe-NH_2	*S. purpuratus* (Echinoidea)	a,b
SpurS4	Ala-Gln-Pro-Ser-Phe-Ala-Phe-NH_2	*S. purpuratus* (Echinoidea)	a,b
SpurS5	Gly-Leu-Met-Pro-Ser-Phe-Ala-Phe-NH_2	*S. purpuratus* (Echinoidea)	a,b
SpurS6	Pro-His-Gly-Gly-Ser-Ala-Phe-Val-Phe-NH_2	*S. purpuratus* (Echinoidea)	a,b
SpurS7	Gly-Asp-Leu-Ala-Phe-Ala-Phe-NH_2	*S. purpuratus* (Echinoidea)	a,b
GYSPFMFamide	Gly-Tyr-Ser-Pro-Phe-Met-Phe-NH_2	*A. japonicus* (Holothuroidea)	g
FKSPFMFamide	Phe-Lys-Ser-Pro-Phe-Met-Phe-NH_2	*A. japonicus* (Holothuroidea)	g