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Molecular characterisation of SALMFamide neuropeptides in sea urchins

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Summary

The SALMFamides are a family of neuropeptides found in species belonging to the phylum Echinodermata. Members of this family have been identified in starfish (class Asteroidea) and in sea cucumbers (class Holothuroidea) but not in other echinoderms. Our aim here was to characterise SALMFamide neuropeptides in sea urchins (class Echinoidea). Radioimmunoassays for the starfish SALMFamides S1 and S2 were used to test for related peptides in whole-body acetone extracts of the sea urchin *Echinus esculentus*. Fractionation of extracts using high performance liquid chromatography (HPLC) revealed several peaks of SALMFamide-like immunoreactivity, with two S2-like immunoreactive peaks (3 and 4) being the most prominent. However, peak 4 could not be purified to homogeneity and although peak 3 was purified, only a partial sequence (MRYH) could be obtained.

An alternative strategy for identification of echinoid SALMFamides was provided by sequencing the genome of the sea urchin *Strongylocentrotus purpuratus*. Analysis of whole-genome shotgun sequence data using the Basic Local Alignment Search Tool (BLAST) identified a contig (347664) that contains a coding region for seven putative SALMFamide neuropeptides (PPVTTRSKFTFamide, DAYSAFSFamide, GMSAFSFamide, AQPSFAFamide, GLMPSFAFamide, PHGGSAFVFamide and GDLAFAFamide), which we have named SpurS1–SpurS7, respectively. Three of these peptides (SpurS1–3) have the C-terminal sequences TFamide or SFamide, which are identical or similar to the C-terminal region of the starfish SALMFamide S2. This may explain the occurrence of several S2-like immunoreactive peptides in extracts of *Echinus esculentus*.

Detailed analysis of the sequence of contig 347664 indicated that the SALMFamide gene in *Strongylocentrotus purpuratus* comprises two exons, with the first exon encoding a signal peptide sequence and the second exon encoding SpurS1–SpurS7. Characterisation of this gene is important because it is the first echinoderm neuropeptide precursor sequence to be identified and, more specifically, it provides our first insight into the structure and organisation of a SALMFamide gene in an echinoderm. In particular, it has revealed a hitherto unknown complexity in the diversity of SALMFamide neuropeptides that may occur in an echinoderm species because all previous studies, which relied on peptide purification and sequencing, revealed only two SALMFamide neuropeptides in each species examined. It now remains to be established whether or not the occurrence of more than two SALMFamides in *Strongylocentrotus purpuratus* is a feature that is peculiar to this species and to echinoids in general or is more widespread across the phylum Echinodermata. Identification of SpurS1–SpurS7 provides the basis for comparative analysis of the physiological actions of these peptides in sea urchins and for exploitation of the sea urchin genome sequence to identify the receptor(s) that mediate effects of SALMFamides in echinoderms.

Key words: echinoderm, Echinoidea, *Strongylocentrotus purpuratus*, *Echinus esculentus*, neuropeptide, SALMFamide, genome.

Introduction

SALMFamide neuropeptides are a family of peptides that are present in the nervous systems of species belonging to the phylum Echinodermata. The first members of this family to be identified were isolated from the radial nerve cords of the starfish species *Asterias rubens* and *Asterias forbesi* (class Asteroidea) and are known as SALMFamide-1 (S1) and SALMFamide-2 (S2; Elphick et al., 1991a,b). S1 is an octapeptide with the amino acid sequence Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-NH_2 (GFNSALMF-NH_2 in single letter code) and S2 is a dodecapeptide with the sequence Ser-Gly-Pro-Tyr-Ser-Phe-Asn-Ser-Gly-Leu-Thr-Phe-NH_2 (SGPYSFNSGLTF-NH_2). Subsequent studies have investigated the distribution and/or functions of S1 and S2 in the adult and larval stages of *Asterias rubens* and other starfish species (Elphick et al., 1995; Moore and Thorndyke, 1993; Moss et al., 1994; Newman et al., 1995a,b; Byrne and Cisternas, 2002). Both peptides are present in the innervation of a variety of starfish organs, including the cardiac stomach and tube feet.

Moreover, S1 and S2 cause relaxation of cardiac stomach and tube foot preparations *in vitro*, with S2 being about ten times more potent than S1 (Melarange et al., 1999; Elphick and Melarange, 2001; Melarange and Elphick, 2003). Interestingly, SALMFamides may have an important behavioural role in starfish by causing relaxation-dependent eversion of the cardiac stomach during feeding (Melarange et al., 1999; Elphick and Melarange, 2001). Collectively, these data indicate that SALMFamide neuropeptides act as inhibitory neuromuscular transmitters in starfish (Elphick and Melarange, 2001). Furthermore, SALMFamides may act as inhibitory transmitters not only in the starfish neuromuscular system but also more generally in other parts of their nervous systems because S1 inhibits secretion of the hormone gonad-stimulating substance from the radial nerves in the starfish *Asterina pectinifera* (Mita et al., 2004).

The starfish SALMFamides S1 and S2 were the first echinoderm neuropeptides to be sequenced and therefore it was of interest to investigate the occurrence of SALMFamide neuropeptides in other echinoderms. Using the same strategy that was used to isolate S1 and S2 from starfish species (Elphick et al., 1991a), Díaz-Miranda et al. (1992) succeeded in identifying two SALMFamide neuropeptides in the sea cucumber *Holothuria glaberrima* (class Holothuroidea): Gly-Phe-Ser-Lys-Leu-Tyr-Phe-NH_2 (GFSKLYFamide) and Ser-Gly-Tyr-Ser-Val-Leu-Tyr-Phe-NH_2 (SGYSVLYFamide). The anatomical distribution and pharmacological actions of GFSKLYFamide have been examined in *Holothuria glaberrima* (Díaz-Miranda et al., 1995, Díaz-Miranda and García-Arrarás, 1995) and these studies indicate that, as in starfish, SALMFamides act as muscle relaxants in holothurians. Thus, it appears that SALMFamides have a general physiological role in echinoderms as inhibitory neuromuscular transmitters (Elphick and Melarange, 2001). Consistent with this notion, Ohtani et al. (1999) identified two SALMFamides in the sea cucumber *Stichopus japonicus* (GYSPFMFamide and FKSPFMFamide) using muscle contractility as a bioassay. Twenty peptides that influence muscle activity in *Stichopus* were identified (Iwakoshi et al., 1995; Ohtani et al., 1999) but GYSPFMFamide and FKSPFMFamide were the only peptides that were found to have a direct inhibitory (relaxing) effect on muscle.

Discovery of two starfish SALMFamides (S1 and S2) and four holothurian SALMFamides has provided an opportunity to identify structural features that may be characteristic of this neuropeptide family. Sequence comparison indicates that a conserved feature of these peptides is the C-terminal motif S*x*(L/F)*x*Famide, where *x* is variable. It remains to be determined, however, whether or not this motif is a feature of SALMFamide neuropeptides in other echinoderm classes (e.g. Ophiuroidea and Echinoidea). A number of studies have investigated the occurrence and distribution of SALMFamide-like peptides in ophiuroids and echinoids. For example, SALMFamide-like immunoreactivity is present in the adult nervous system of the brittle star species *Ophiura ophiura* and *Amphipholis squamata* (Ghyoot et al., 1994; De Bremaeker et al., 1997) and pharmacological tests using the starfish peptide S1 indicate that SALMFamides may regulate luminescence in *Amphipholis squamata* (De Bremaeker et al., 1999). SALMFamide-like immunoreactivity has also been described in the larval nervous system of echinoids, including the sand dollar *Dendraster excentricus* and the sea urchin *Psammechinus miliaris* (Thorndyke et al., 1992; Beer et al., 2001).

To facilitate comparative analysis of SALMFamide neuropeptide structure and function in echinoderms, it is important that SALMFamides are identified in species belonging to the class Ophiuroidea and/or the class Echinoidea. Therefore, in the present study we used two complementary approaches to characterise SALMFamide neuropeptides in sea urchins. SALMFamide radioimmunoassays in combination with high performance liquid chromatography (HPLC) were used to characterise and purify SALMFamides from whole-body extracts of the sea urchin *Echinus esculentus*. Then genomic sequence data for the sea urchin *Strongylocentrotus purpuratus* was analysed and a gene encoding a family of seven putative SALMFamide neuropeptides was identified. The data presented here provide the first description of an echinoderm neuropeptide precursor gene and the first insight into the structure of SALMFamide genes in echinoderms.

Materials and methods

Biochemical characterisation of SALMFamide neuropeptides in Echinus esculentus

Sixteen adult specimens of *Echinus esculentus* L. (1131 g dry mass) were obtained from the Plymouth Marine Laboratory (UK). Animals were broken into pieces and kept in 14 l of acetone for 1 week at –20°C to extract peptides. The acetone was removed by rotary evaporation and the remaining aqueous fraction (1.35 l) collected and frozen. 350 ml of this extract was loaded onto a series of five C_{18} Sep-Paks (Waters, Milford, MA, USA) at a flow rate of 0.5–1.0 ml min^{-1} using a peristaltic pump (P-3, Pharmacia, now Amersham Biosciences, Piscataway, NJ, USA). The Sep-Paks were washed with 0.1% trifluoroacetic acid (TFA) in HPLC grade water and then material retained on the Sep-Paks was eluted successively with 25 ml of 20%, 40%, 60% and 80% acetonitrile (ACN) in 0.1% TFA. 50 μl samples of the 20%, 40%, 60% and 80% ACN/TFA eluates were then assayed for SALMFamide peptides using radioimmunoassay (see below), following lyophilisation and resuspension in radioimmunoassay buffer. Sep-Pak eluates that contained SALMFamide-like immunoreactivity were further fractionated using HPLC. A Waters HPLC system was used and after dilution in ×4 0.1% TFA, material was loaded onto a C_8 column (Brownlee RP300, Perkin-Elmer, Boston, MA, USA; 2.1 mm×220 mm) and eluted with 0–80% ACN/TFA at 0.5 ml min^{-1} over a 30 min period. 0.5 min fractions were then collected and assayed for SALMFamide-like immunoreactivity.

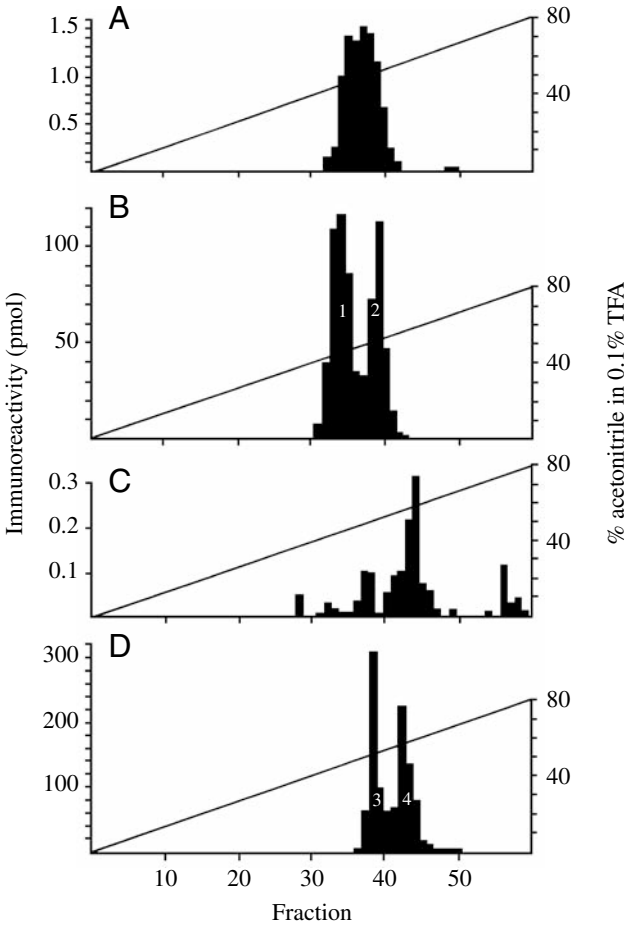

Fig. 1. SALMFamide-like immunoreactivity in whole-body extracts of *Echinus esculentus*. The graphs show SALMFamide-like immunoreactivity in a 40% ACN/TFA Sep-Pak eluate (A,B) and a 60% ACN/TFA eluate (C,D) after HPLC fractionation using a gradient of ACN/TFA indicated by the line. HPLC fractions were radioimmunoassayed for both S1- (A,C) and S2- (B,D) immunoreactivity using the antisera SLII and BGI, respectively. The 40% Sep-Pak eluate contains two S2-immunoreactive peaks, 1 and 2 (B), which are weakly immunoreactive with the S1 antiserum SLII. The 60% Sep-Pak eluate also contains two S2-immunoreactive peaks, 3 and 4 (D), which are weakly immunoreactive with the S1 antiserum SLII. The elution times of peaks 2 and 3 are the same so they may contain identical immunoreactive peptides. The SLII antiserum also detects a few minor immunoreactive peaks, which elute before or after the major peaks 1, 2, 3 and 4.

The radioimmunoassay protocol used has been described previously (Elphick et al., 1991b). Five antisera were used to test for the presence of SALMFamide neuropeptides in the four Sep-Pak eluate samples. Two (BLII and SLII) are S1 antisera raised to the C-terminal pentapeptide sequence of S1 (SALMFamide) and three (BGI, CLII, TRII) are S2 antisera raised to the C-terminal pentapeptide sequence of S2 (SGLTFamide). The cross-reactivity profiles of all five antisera have been characterised extensively (Elphick et al., 1991b; Elphick et al., 1995). BLII and CLII are highly specific for their respective antigens, S1 and S2, and have low cross-reactivity with other structurally related peptides. In contrast, SLII, BGI and TRII are less specific antisera and recognise variously both S1 and S2 and also FMRFamide-related peptides such as FLRFamide and LPLRFamide.

Analysis of Strongylocentrotus purpuratus *genomic sequence data*

The genome of the sea urchin *Strongylocentrotus purpuratus* has been sequenced by the Baylor College of Medicine Human Genome Sequencing Center (BCMHGSC) using Clone-Array Pooled Shotgun Sequencing (CAPSS; Cai et al., 2001) in response to a proposal by Eric Davidson and colleagues at the California Institute of Technology (Cameron et al., 2000). To search the *Strongylocentrotus purpuratus* genome for genes encoding putative sea urchin SALMFamide neuropeptides, the Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990) facility on the BCMHGSC website was used (http://www.hgsc.bcm.tmc.edu/blast/blast.cgi?organism=Spurpuratus). As a query for a tBLASTn search of sea urchin genomic sequence data, a hypothetical SALMFamide neuropeptide precursor sequence was used. This query sequence comprised repeating copies of a peptide incorporating the amino acid sequence of the starfish SALMFamide S1 followed by a glycine residue as a C-terminal substrate for amidation and a lysine–arginine

Table 1. *SALMFamide-like immunoreactivity in a whole-body extract of* Echinus *fractionated on C_8 Sep-Paks by elution with 25 ml 20%, 40%, 60% and 80% acetonitrile in 0.1% trifluoroacetic acid*

Sep-Pak eluate (%ACN/TFA)	S1-like immunoreactivity (pmol)		S2-like immunoreactivity (pmol)		
	BLII	SLII	BGI	CLII	TRII
20	9	1	54	65	55
40	35	32	903	1293	443
60	12	1	799	364	544
80	2	0	48	6	90

ACN, acetonitrile; TFA, trifluoroacetic acid.

Radioimmunoassay with two S1 antisera (BLII, SLII) and three S2 antisera (BGI, CLII, TRII) reveals the majority of the SALMFamide-like immunoreactivity in the 40% and 60% ACN/TFA eluates. Note that the *Echinus* material is much more immunoreactive with the S2 antisera than the S1 antisera.

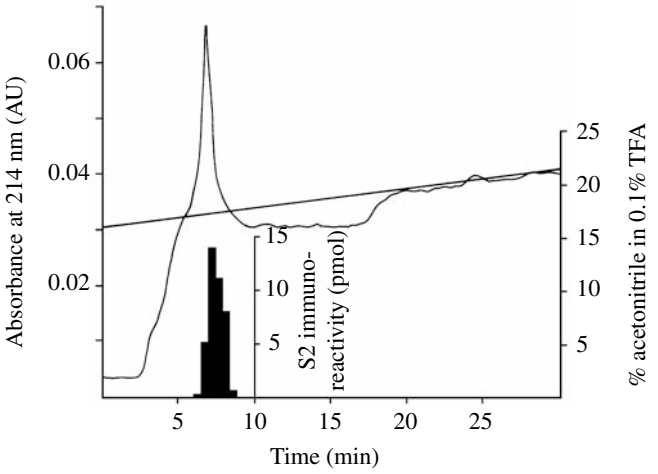

Fig. 2. HPLC purification of the S2-like immunoreactive peak 3 from *Echinus esculentus*. Seven HPLC steps were required to purify this peak and the final chromatogram is shown here. The S2-like immunoreactivity elutes in four fractions corresponding to a single peak of absorbance at 210 nm and amino acid sequencing of these fractions yielded the partial sequence MRYH.

dipeptide sequence as a dibasic cleavage site for endopeptidases (i.e. GFNSALMFGKRGFNSALMFGKRG-FNSALMFGKRGFNSALMFGKRGFNSALMFGKRGFNSA-LMFGKR). This BLAST analysis of the sea urchin genome was performed during January 2005 using the dataset of *Strongylocentrotus purpuratus* contigs available as of 23/11/2004.

Results

Characterisation of SALMFamide neuropeptides in Echinus esculentus

The results of the radioimmunoassay analysis of SALMFamide-like immunoreactivity in the *Echinus* Sep-Pak eluates are summarised in Table 1. SALMFamide-like immunoreactivity was detected in all four Sep-Pak eluates but the majority was present in the 40% and 60% ACN/TFA eluates. The immunoreactive material in these two eluates was detected by both S1 and S2 antisera but it was much more immunoreactive with the S2 antisera. Both the 40% and 60% ACN/TFA eluates were subjected to further HPLC fractionation followed by radioimmunoassay using the S1 antiserum SLII and the S2 antiserum BGI. These two antisera were selected from the panel of five available antisera because they both have high sensitivity and low selectivity in the radioimmunoassay. These characteristics are important if antisera are to be used to detect small amounts of peptides whose amino acid sequences are different but related to that of the antigen peptide, as expected for the *Echinus* SALMFamides.

Two S2-like immunoreactive peaks (1 and 2) were detected in the HPLC fractionated 40% ACN/TFA Sep-Pak eluate (Fig. 1B) and two S2-like immunoreactive peaks (3 and 4) were detected in the HPLC fractionated 60% ACN/TFA Sep-Pak eluate (Fig. 1D). Peaks 1, 2, 3 and 4 were also detected by the S1 antiserum SLII (Fig. 1A,C) but were much less immunoreactive with this antiserum than the S2-antiserum BGI (note the differences in the scales of the *y* axes in Fig. 1). This observation is consistent with the lower levels of S1-like immunoreactivity detected in the Sep-Pak eluates (Table 1). SLII did, however,

A

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>gnl|Spur20041123|Contig347664
Length = 16425
Score = 42.7 bits (99), Expect = 8e-04
Identities = 27/53 (50%), Positives = 31/53 (57%), Gaps = 1/53 (1%)
Frame = +3

Query: 15     SALMFGKRGFNSALMFGKRGFNSALMFGKRGFNSALMFGKRGF-NSALMFGKR 66
               SA  FGKRG  SA  FGKR    +  FGKRG   +  FGKR    SA +FG+R
Sbjct: 13566 SAFSFGKRGM-SAFSFGKRA-QPSFAFGKRGLMPSFAFGKRPHGGSAFVFGRR 13718
```

B

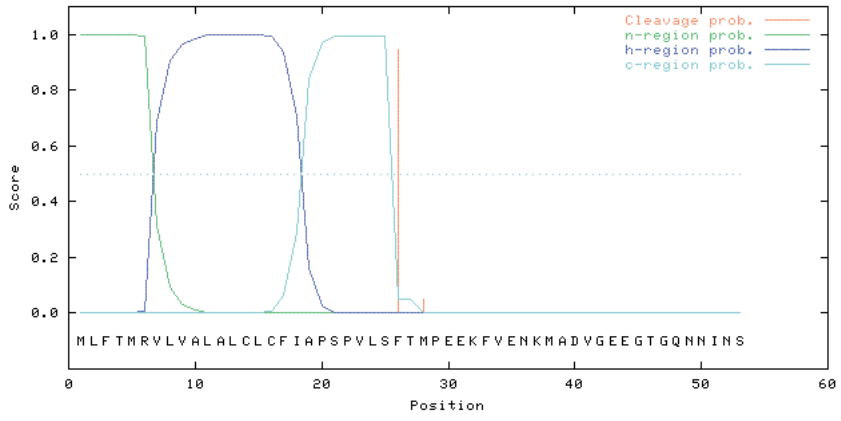

Fig. 3. Identification of a SALMFamide precusor gene in *Strongylocentrotus purpuratus*. (A) Output data from a tBLASTn analysis of *Strongylocentrotus purpuratus* genomic sequence data using a putative starfish SALMFamide-1 (S1) precursor sequence (GFNSALMFGKR × 6) as the query. A region of contig 347664 was identified as the sea urchin sequence with the highest level of sequence identity/similarity with the query sequence (E=8e-04). The translated sequence of bases 13566–13718 from contig 347664 is shown aligned with the query sequence and four putative sea urchin SALMFamide neuropeptides are underlined. (B) Diagram showing data obtained from SignalP 3.0 analysis of a 53 amino acid residue sequence encoded by bases 11827–11986 of contig 347664, using hidden Markov models based on eukaryotic data for signal peptide prediction. The red line shows cleavage site probability, indicating that cleavage occurs between residues 25 (S) and 26 (F). The green, dark blue and light blue lines indicate that the first 25 residues of the sequence conform with high probability to n-terminal (n), hydrophobic (h) and c-terminal (c) regions, respectively, that are characteristic of eukaryotic signal peptides. Collectively, these data provide strong evidence that the first 25 amino acids of the 53 residue sequence are likely to function as a signal peptide, which is a characteristic feature of all neuropeptide precursors.

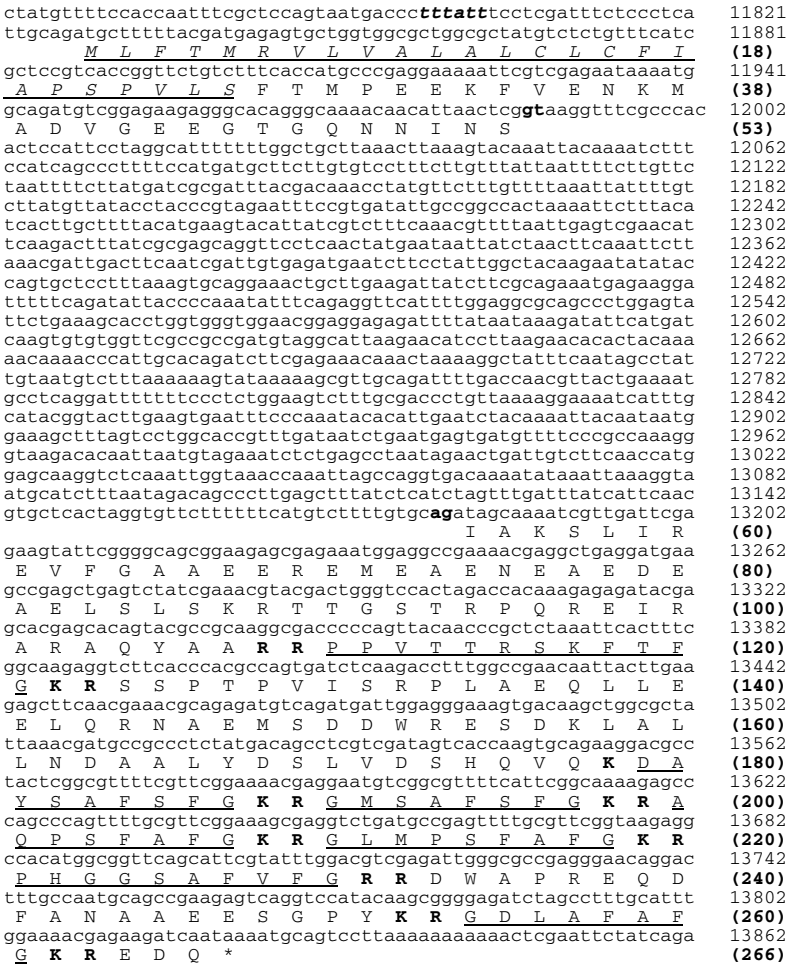

Fig. 4. Structure of the SALMFamide precursor gene in *Strongylocentrotus purpuratus.* The DNA sequence of the gene is shown in lowercase letters with base positions in contig 347664 shown in the column on the right (11762–13920). The predicted precursor sequence is shown in uppercase letters with amino acid positions shown in bold and in brackets in the column on the right (1–266). The gene comprises two exons, whose positions are indicated by the underlying amino acid sequence in uppercase letters, with the start and end of the intervening intron labelled in bold letters (gt/ag). 24 bases upstream of the start codon (atg) in the first exon is a putative TATA-box-like promoter sequence (tttatt), which is shown in bold. The predicted signal peptide sequence encoded by the first exon is shown in underlined italics, based on the analysis shown in Fig. 3B. Amino acid sequences corresponding to putative SALMFamide neuropeptides encoded by the second exon are shown underlined, with monobasic and dibasic (K, KR, RR) cleavage sites shown in bold. In the 3′ region of the gene there is consensus polyadenylation signal sequence (aataaa) located 47 bases downstream from the stop codon (*).

detect some additional immunoreactive peaks, which eluted before and after peaks 1–4 but were not detected by BGI (Fig. 1).

Peaks 2 and 3 were derived from different Sep-Pak eluates, the 40% and 60% ACN/TFA eluates, respectively. Following HPLC fractionation, however, they eluted in identical fractions with about 50% ACN/TFA. This suggests that the immunoreactive peptide in peaks 2 and 3 may be identical. It is also possible, however, that peaks 2 and 3 comprise more than one immunoreative peptide, which were not resolved by the first HPLC step.

As peak 3 contained more SALMFamide-immunoreactivity than any of the other immunoreactive fractions it was selected for further purification by HPLC. Moreover, if, as suggested above, peaks 2 and 3 contain an identical immunoreactive peptide then it is likely that this is by far the most abundant SALMFamide neuropeptide in *Echinus*. Peak 3 was purified by repeated HPLC fractionation on the C_8 column using ACN/TFA or ACN/30 mmol l^{-1} sodium phosphate (pH 7.0; ACN/PO_4) as elutants. A single peak of immunoreactivity was detected throughout purification, indicating that peak 3 contains only one immunoreactive peptide. Seven HPLC steps were required to purify this immunoreactive peptide and the final HPLC chromatogram is shown in Fig. 2.

Purified peak 3 was subjected to automated Edman degradation sequencing using an Applied Biosystems sequencer. Peak 3 eluted in four fractions in HPLC step 7 (Fig. 2) and was sequenced twice; first the second major immunoreactive fraction alone, and then the first, third and fourth fractions combined. Both samples were subjected to seven cleavage cycles and the amino acid sequence Met-Arg-Tyr-His could be clearly resolved from both sets of sequencing data (not shown). The yield of the predominant (cleaved) amino acid fell from about 7 pmol in the first three cycles to about 2 pmol in the fourth cycle and no further sequence data could be obtained from later cycles.

Peak 4 was also subjected to further purification; however, it was not possible to purify this peak to homogeneity (data not shown). In view of this inherent difficulty in purifying neuropeptides from whole-body extracts of sea urchins, we adopted an alternative *in silico* approach by analysing DNA sequence data made available by the recent sequencing of the genome of the sea urchin *Strongylocentrotus purpuratus.*

Identification of a gene encoding SALMFamide neuropeptides in Strongylocentrotus purpuratus

Analysis of *Strongylocentrotus purpuratus* genomic sequence data using the tBLASTn method with the query

A

MLFTMRVLVALALCLCFIAPSPVLSFTMPEEKFVENKMADVGEEGTGQNNINSIAKSLIREVFGAAEEREMEAENEAEDEAELS
SpurS1
LSKRTTGSTRPQREIRARAQYAARRPPVTTRSKFTFGKRSSPTPVISRPLAEQLLEELQRNAEMSDDWRESDKLALLNDAALYD
SpurS2 SpurS3 SpurS4 SpurS5 SpurS6
SLVDSHQVQKDAYSAFSFGKRGMSAFSFGKRAQPSFAFGKRGLMPSFAFGKRPHGGSAFVFGRRDWAPREQDFANAAEESGPYK
SpurS7
RGDLAFAFGKREDQ

B

Peptide	Sequence	Source	Ref.
S1	Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-NH_2	*Asterias rubens* (Asteroidea)	1
S2	Ser-Gly-Pro-Tyr-Ser-Phe-Asn-Ser-Gly-Leu-Thr-Phe-NH_2	*Asterias rubens* (Asteroidea)	1
GFSKLYFamide	Gly-Phe-Ser-Lys-Leu-Tyr-Phe-NH_2	*Holothuria glaberrima* (Holothuroidea)	2
SGYSVLYFamide	Ser-Gly-Tyr-Ser-Val-Leu-Tyr-Phe-NH_2	*Holothuria glaberrima* (Holothuroidea)	2
GYSPFMFamide	Gly-Tyr-Ser-Pro-Phe-Met-Phe-NH_2	*Stichopus japonicus* (Holothuroidea)	3
FKSPFMFamide	Phe-Lys-Ser-Pro-Phe-Met-Phe-NH_2	*Stichopus japonicus* (Holothuroidea)	3
SpurS1	Pro-Pro-Val-Thr-Thr-Arg-Ser-Lys-Phe-Thr-Phe-NH_2	*S. purpuratus* (Echinoidea)	4
SpurS2	Asp-Ala-Tyr-Ser-Ala-Phe-Ser-Phe-NH_2	*S. purpuratus* (Echinoidea)	4
SpurS3	Gly-Met-Ser-Ala-Phe-Ser-Phe-NH_2	*S. purpuratus* (Echinoidea)	4
SpurS4	Ala-Gln-Pro-Ser-Phe-Ala-Phe-NH_2	*S. purpuratus* (Echinoidea)	4
SpurS5	Gly-Leu-Met-Pro-Ser-Phe-Ala-Phe-NH_2	*S. purpuratus* (Echinoidea)	4
SpurS6	Pro-His-Gly-Gly-Ser-Ala-Phe-Val-Phe-NH_2	*S. purpuratus* (Echinoidea)	4
SpurS7	Gly-Asp-Leu-Ala-Phe-Ala-Phe-NH_2	*S. purpuratus* (Echinoidea)	4

Fig. 5. Nomenclature for *Strongylocentrotus purpuratus* SALMFamide neuropeptides and comparison with the sequences of other echinoderm SALMFamide neuropeptides. (A) The predicted amino acid sequence of the *Strongylocentrotus purpuratus* SALMFamide precursor showing the positions of putative SALMFamide neuropeptides (red), which we have named SpurS1–SpurS7 based on their relative position in the precursor. The signal peptide is shown in blue and putative monobasic and dibasic (K, KR, RR) cleavage sites are shown in green. (B). The sequences of starfish and sea cucumber SALMFamides are shown aligned with the seven putative sea urchin SALMFamides (SpurS1–SpurS7) identified in this study. Four of the putative neuropeptides in *Strongylocentrotus purpuratus* (SpurS1, SpurS2, SpurS3, SpurS6) have the same consensus sequence as the starfish and sea cucumber SALMFamides (i.e. S*x*L/F*x*Famide, where *x* is variable). SpurS4, SpurS5 and SpurS7 have a proline or leucine residue substituted for the serine residue in the consensus sequence but share the sequence F*x*Famide with SpurS1, SpurS2, SpurS3, SpurS6 and the *Stichopus* SALMFamides GYSPFMFamide and FKSPFMFamide. References: [1]Elphick et al., 1991a,b; [2]Díaz-Miranda et al., 1992; [3]Ohtani et al., 1999; [4]present study.

GFNSALMFGKRGFNSALMFGKRGFNSALMFGKRGFNSALMFGKRGFNSALMFGKR identified a 16 425 base contig (347664) containing a sequence of 153 bases encoding a polypeptide that shared significant similarity with the query sequence (E value=8e-04; Fig. 3A). Moreover, the polypeptide sequence contained four putative SALMFamide neuropeptides: GMSAFSFamide, AQPSFAFamide, GLMPSFAFamide, PHGGSAFVFamide (Fig. 3A). Two of these peptides have the motif S*x*F/L*x*Famide (where *x* is variable), which is a characteristic of the SALMFamide neuropeptides that have been identified in other echinoderms (see Introduction). These data indicated, therefore, that contig 347664 contains a gene encoding a sea urchin SALMFamide neuropeptide precursor.

As a first step towards to determining the full-length sequence of the putative SALMFamide precursor protein, contig 347664 was translated into amino acid sequence in all six possible frames. A putative polypeptide sequence of 232 residues coded for by bases 13125–13820 was identified in the +3 frame of contig 347664. Analysis of this sequence revealed three additional putative SALMFamide neuropeptides located on both the N-terminal side (PPVTTRSKFTFamide, DAYSAFSFamide) and the C-terminal side (GDLAFAFamide) of the four peptides identified above. Then the 232 residue polypeptide sequence was scanned for the presence of a signal peptide sequence, which is a characteristic feature of neuropeptide precursors. To do this the signal peptide prediction server SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) was used (Bendtsen et al., 2004). This analysis indicated that the 232 residue polypeptide containing seven putative SALMFamide neuropeptides does not have a signal peptide sequence (e.g. using hidden Markov models the signal peptide probability was 0.073). This suggested, therefore, that the putative sea urchin SALMFamide precursor is encoded by a gene comprising more than one exon, with the signal peptide presumably encoded by an exon located 5′ to the exon encoding SALMFamide neuropeptides.

To facilitate identification of a 5′ exon(s), the sequence of contig 347664 was analysed using GenScan 1.0, an online tool for predicting the locations and exon–intron structures of genes in genomic DNA sequences (http://genes.mit.edu/GENSCAN.html; Burge and Karlin, 1997). This analysis revealed the presence of a putative exon located between bases 11828 and 11986 of contig 347664 encoding a polypeptide sequence of 53 residues, which was then analysed for the presence of a signal peptide sequence using SignalP 3.0. The results provided very strong evidence for the presence of a signal peptide in the N-terminal region of the 53 residue

sequence, with hidden Markov models giving a signal peptide probability of 1.0 (Fig. 3B). Furthermore, SignalP 3.0 predicts that the signal peptide would be cleaved during precursor processing between residues 25 and 26 (probability=0.945, using hidden Markov models; Fig. 3B).

Collectively, these data indicate that SALMFamide neuropeptides in *Strongylocentrotus purpuratus* are encoded by a gene comprising two exons, with exon 1 encoding the N-terminal signal peptide of the putative precursor protein and exon 2 encoding seven structurally related SALMFamide neuropeptides. The sequence of this gene is shown in Fig. 4, with the locations of the two exons indicated by the underlying predicted 266 amino acid residue precursor protein sequence. The exon–intron boundaries are based on predictions from analysis of the sequence of contig 347664 using GenScan 1.0 and that conform to the classical 5′-donor and 3′-acceptor consensus rule (gt/ag). Analysis of genomic DNA sequence 5′ to the predicted start codon (atg) of exon 1 revealed a putative TATA-box-like promoter (tttatt). The tttatt sequence and the start codon are separated by 24 bases, which is within the normal range for TATA-box containing promoters (Arkhipova, 1995). At the 3′ end of the gene there is consensus polyadenylation signal sequence (aataaa) located 47 bases downstream from the stop codon.

The predicted sequence of the 266 amino acid residue SALMFamide precursor protein is shown in Fig. 5A. Seven putative SALMFamide neuropeptides are predicted to be generated from this precursor following cleavage at monobasic (K) or dibasic (KR, RR) cleavage sites by endopeptidases and C-terminal amidation by peptidyl-glycine alpha-amidating monooxygenase. Based on their relative positions in the *Strongylocentrotus purpuratus* SALMFamide precursor, we have named these putative neuropeptides: SpurS1 (PPVTTRSKFTFamide), SpurS2 (DAYSAFSFamide), SpurS3 (GMSAFSFamide), SpurS4 (AQPSFAFamide), SpurS5 (GLMPSFAFamide), SpurS6 (PHGGSAFVFamide) and SpurS7 (GDLAFAFamide; see Fig. 5A,B).

Discussion

Previous studies have identified SALMFamide neuropeptides in echinoderm species belonging to the classes Asteroidea and Holothuroidea, and in the present study we have investigated the occurrence and characteristics of SALMFamides in two species of sea urchin (class Echinoidea), *Echinus esculentus* and *Strongylocentrotus purpuratus*.

Our attempts to purify and sequence SALMFamide neuropeptides from whole-body acetone extracts of the sea urchin *Echinus esculentus* had limited success, with only a partial N-terminal sequence (MRYH) being obtained for one of the SALMFamide-like immunoreactive peptides present in this species. The difficulty we had in obtaining pure samples of sea urchin SALMFamides in sufficient quantities to determine full-length sequences is probably a consequence of using whole-body extracts. In our previous studies on starfish, radial nerve cord extracts were used, providing a highly enriched source of neuropeptides (Elphick et al., 1991a,b). However, this approach was not feasible for *Echinus* because, unlike in starfish where the nerve cords are located accessibly along the external midline of the ambulacrum in each arm, in sea urchins the nerve cords are embedded within a calcareous exoskeleton, making dissection a much more difficult and time-consuming procedure. Therefore, we decided that alternative strategies were more appropriate for identification of SALMFamide neuropeptides in sea urchins. By analysing whole-genome shotgun sequence data for the sea urchin *Strongylocentrotus purpuratus* we have succeeded in identifying a gene encoding a SALMFamide neuropeptide precursor. This is the first neuropeptide precursor gene to be characterised in the phylum Echinodermata and our data provide the first insight into the structural organisation of echinoderm SALMFamide genes.

The SALMFamide precursor gene in *Strongylocentrotus purpuratus* appears to comprise two exons, with the first exon encoding an N-terminal signal peptide sequence and the second exon encoding seven putative SALMFamide neuropeptides, which we have named SpurS1–SpurS7 (Fig. 5). The occurrence of a signal peptide sequence that is encoded by a different exon to the exon encoding putative neuropeptides is of particular interest because this is a feature that has been reported before in some, but not all, neuropeptide precursor genes. For example, in the mollusc *Lymnaea stagnalis* the precursor for the neuropeptide FMRFamide is encoded by an mRNA derived from two exons, with exon 1 encoding the signal peptide and exon 2 encoding multiple copies of FMRFamide (Kellett et al., 1994). In contrast, in *Drosophila melanogaster* the precursor for FMRFamide-like peptides is encoded by a single exon (Schneider and Taghert, 1990). These differences in the structural organisation of neuropeptide precursor genes invite functional explanations. In the case of the *Lymnaea* FMRFamide gene, an explanation for the presence of a separate exon that encodes the signal peptide is provided by the occurrence of additional neuropeptide-encoding exons (3,4,5) located downstream from the FMRFamide-encoding exon (2). Consequently, transcripts of this gene can be alternatively spliced to give rise to two different mRNAs: one encoding the signal peptide (exon 1) and the FMRFamide products of exon 2 and the other encoding the signal peptide (exon 1) and the alternative neuropeptides encoded by exons 3, 4 and 5 (Kellett et al., 1994). Although the occurrence of a separate signal peptide-encoding exon does not in itself necessarily imply the existence of multiple neuropeptide-encoding exons that are alternatively spliced, it is possible that in *Strongylocentrotus purpuratus* there are additional neuropeptide-encoding exon(s) located downstream of the exon encoding SpurS1–SpurS7. Therefore, we analysed contig 347664 for the presence of additional neuropeptide-encoding exons but we did not find evidence of any such coding regions in the ~2.5 kb sequence located downstream of the exon encoding SpurS1–SpurS7.

In addition to the seven neuropeptides (SpurS1–SpurS7) that are likely to be generated by proteolytic processing of the

SALMFamide precursor protein in *Strongylocentrotus purpuratus*, other peptides need to be considered as potential biologically active molecules that may be co-released with the SALMFamide peptides (Fig. 5A). Firstly, located between the N-terminal signal peptide and the dibasic cleavage site that precedes SpurS1, there is an 82 amino acid residue sequence. A noteworthy feature of this sequence is that it comprises a high proportion of acidic residues (18× E or D, i.e. ~22%), indicating that this part of the precursor protein probably functions as an acidic spacer. Similarly, located between SpurS1 and SpurS2 there is a 54 amino acid residue sequence comprised of ~19% acidic residues and located between SpurS6 and SpurS7 there is a 19 amino acid residue sequence, which contains 5 acidic residues (i.e. ~26%). Although the primary functions of these parts of the precursor protein may be to simply function as acidic spacers, all three regions do also contain potential monobasic and/or dibasic cleavage sites, which if targeted by endopeptidases may generate smaller peptide fragments that could have biological activity as secreted molecules.

Discovery of the SALMFamide precursor gene in *Strongylocentrotus purpuratus* has provided an opportunity to analyse the full complement of SALMFamide neuropeptides that occurs in an echinoderm species. Previous studies, which have relied on biochemical purification of SALMFamide neuropeptides, have revealed only two SALMFamides in each species examined (Elphick et al., 1991a,b; Díaz-Miranda et al., 1992; Ohtani et al., 1999). Therefore, the discovery of a gene encoding seven putative SALMFamide neuropeptides in *Strongylocentrotus purpuratus* is indicative of an unprecedented level of SALMFamide diversity in this species. One noteworthy feature of the SALMFamide precursor in *Strongylocentrotus purpuratus* is that SpurS2–SpurS6 are positioned in tandem without spacers and separated only by dibasic cleavage sites. In contrast SpurS1 and SpurS7, located N-terminally and C-terminally with respect to SpurS2–SpurS6, are separated from SpurS2–SpurS6 by spacer sequences. One possible explanation for this pattern in precursor structure is that SpurS2–SpurS6 arose more recently than SpurS1 and SpurS7 as a result of a series of intragenic tandem duplications. If this is correct, then the number of SALMFamides in *Strongylocentrotus purpuratus* may not necessarily be representative of all sea urchins. Nevertheless, our biochemical analysis of *Echinus esculentus* extracts indicates the presence of at least three SALMFamides in this species (Fig. 1). Further studies are now required to address this issue. In particular, it will be interesting to determine the sequences of SALMFamide precursor genes in other echinoids and in species belonging to other echinoderm classes.

A striking feature of our analysis of SALMFamide-like peptides in *Echinus esculentus* was that the immunoreactive peptides detected in this species exhibited much greater reactivity with antisera to the starfish SALMFamide neuropeptide S2 than with antisera to the starfish SALMFamide neuropeptide S1 (Table 1, Fig. 1). Identification of a SALMFamide precursor gene in *Strongylocentrotus purpuratus* may facilitate determination of a possible structural basis for this feature of sea urchin SALMFamides. In particular, it is noteworthy that SpurS1 shares the C-terminal sequence TFamide with S2, whilst SpurS2 and SpurS3 have the C-terminal sequence SFamide, where the penultimate threonine residue of S2 and SpurS1 is replaced with a structurally similar amino acid, serine. The presence of these three putative peptides in *Strongylocentrotus purpuratus*, which share more C-terminal structural similarity with S2 (TFamide) than with S1 (MFamide), may explain why SALMFamide-like peptides in *Echinus esculentus* are more immunoreactive with S2 antisera than with S1 antisera.

Discovery of the SALMFamide precursor gene in *Strongylocentrotus purpuratus* has also enabled further evaluation of the structural features that are characteristic of members of the SALMFamide neuropeptide family. Four of the putative neuropeptides in *Strongylocentrotus purpuratus* (SpurS1, SpurS2, SpurS3, SpurS6) have the same consensus sequence as the starfish and sea cucumber SALMFamides (i.e. S*x*L/F*x*Famide, where *x* is variable; Fig. 5B). However, SpurS4, SpurS5 and SpurS7 have a proline or leucine residue substituted for the serine residue in the consensus sequence, although they do share the sequence F*x*Famide with SpurS1, SpurS2, SpurS3, SpurS6 and the *Stichopus* SALMFamides GYSPFMFamide and FKSPFMFamide. These data indicate that S*x*L/F*x*Famide should continue to be recognised as the characteristic motif for SALMFamide neuropeptides in echinoderms. However, identification of the SALMFamide precursor in *Strongylocentrotus purpuratus* has revealed that deviations from this consensus sequence can occur in at least some of the SALMFamides that are found in any one species of echinoderm.

Based on the sequence conservation that occurs in the C-terminal region of SALMFamide neuropeptides and cross-reactivity with antibodies to the C-terminal region of S2, it is likely that SALMFamide neuropeptides in *Echinus* share C-terminal sequence similarity with *Strongylocentrotus* SALMFamides and with other echinoderm SALMFamides. In contrast, the partial N-terminal sequence MRYH obtained for peak 3 from *Echinus* does not share any obvious sequence similarity with SpurS1–SpurS7 or with other echinoderm SALMFamides. This is not surprising because this region of SALMFamide neuropeptides in *Strongylocentrotus* and in other echinoderms is highly variable in sequence (see Fig. 5B).

The diversity of putative SALMFamide neuropeptides encoded by the SALMFamide precursor gene in *Strongylocentrotus purpuratus* raises questions about their functions and relative activities. Previous studies on starfish and sea cucumbers (Elphick et al., 1995; Díaz-Miranda and García-Arrarás, 1995; Melarange et al., 1999; Ohtani et al., 1999) suggest that at least some of the SALMFamides present in sea urchins may act as muscle relaxants. To address this issue, we have performed preliminary experiments in which the starfish SALMFamides S1 and S2 were tested on tube feet from *Echinus esculentus*. Both peptides caused tube foot relaxation when tested at a concentration of 10 μmol l^{-1} (N=3;

data not shown), consistent with the relaxing effects that S1 and S2 have on starfish tube feet (Melarange and Elphick, 2003). These data provide further evidence that SALMFamide neuropeptides act as muscle relaxants throughout the phylum Echinodermata, as discussed previously by Elphick and Melarange (2001).

It will be interesting to determine whether or not all seven of the putative *Strongylocentrotus* SALMFamide neuropeptides (SpurS1–SpurS7) act as muscle relaxants and if they do, to determine the relative potencies of these peptides. Unfortunately, this issue has not been addressed for the two pairs of SALMFamides identified in sea cucumbers (Díaz-Miranda et al., 1992; Ohtani et al., 1999 but it has been found that the starfish SALMFamide S2 is approximately ten times more potent than S1 as a muscle relaxant in *Asterias rubens* (Melarange et al., 1999; Elphick and Melarange, 2001). Ongoing studies are investigating the structural basis for this difference in potency of S1 and S2 (Otara et al., 2004, 2005) and there now exists an opportunity to extend these studies to the much larger repertoire of SALMFamide neuropeptides that appear to be present in *Strongylocentrotus purpuratus*.

One important aspect of SALMFamide neuropeptides about which little is known is their mode of action in target tissues (Elphick and Melarange, 2001). A series of tests with the starfish SALMFamides S1 and S2 have investigated cyclic-AMP (cAMP) and cyclic-GMP (cGMP) as potential mediators of the effects of these peptides; however, no changes in the levels of cAMP or cGMP in target tissues were observed when exposed to S1 or S2 at concentrations that exert physiological effects (Melarange and Elphick, 2003). Indeed, nothing is known about the molecular properties of the receptor(s) that mediate effects of SALMFamides in echinoderms. Identification of the SALMFamide gene in *Strongylocentrotus purpuratus* now provides a basis for addressing this issue. In particular, by analogy with neuropeptides in other animal phyla, it seems likely that SALMFamides exert at least some of their physiological effects by activating one or more G-protein coupled receptors (GPCRs). For example, by exploiting data provided by genome sequencing, a GPCR that is activated by FMRFamide-like peptides was recently identified in *Drosophila* (Cazzamali and Grimmelikhuijzen, 2002). Similarly, it may therefore be possible to exploit the complete genome sequence of *Strongylocentrotus purpuratus* to identify a SALMFamide receptor(s) in this species.

Finally, our exploitation of *Strongylocentrotus purpuratus* genomic sequence data to identify a SALMFamide gene paves the way for identification of other neuropeptide precursor genes in this species. In other invertebrate species where genomes have been sequenced it has been possible to identify large, and possibly complete, complements of genes encoding neuropeptides. However, these studies have so far been focused on species belonging to protostomian phyla (Hewes and Taghert, 2001; Li et al., 1999; Nathoo et al., 2001; Riehe et al., 2002; Vanden Broeck, 2001). The genome sequence of *Strongylocentrotus purpuratus* provides an opportunity for genome-wide analysis of neuropeptide structure and function in a deuterostomian invertebrate belonging to the phylum Echinodermata. For example, there exists the intriguing prospect of identifying genes encoding neuropeptides such as gonad-stimulating substance, an echinoderm hormone that was first discovered in starfish more than 45 years ago (Chaet and McConnaughy, 1959) but whose molecular identity still remains unknown.

We are grateful to the Baylor College of Medicine Human Genome Sequencing Center for providing free access to *Strongylocentrotus purpuratus* genome sequence data, which can be used in scientific papers analyzing particular genes and regions (see http://www.hgsc.bcm.tmc.edu/projects/seaurchin/).

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