

## MOLECULAR ANALYSIS OF FMRFamide- AND FMRFamide-RELATED PEPTIDES (FaRPs) IN THE CUTTLEFISH *SEPIA OFFICINALIS*

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### Summary

The display of complex color patterns of the cuttlefish *Sepia officinalis* is under the regulation of the FMRFamide-related peptide (FaRP) family, but their exact identities are unknown. We report the isolation and characterization of a full-length FaRP cDNA from the brain of *S. officinalis*. This cDNA is 1850 base pairs long, including an open reading frame of 996 base pairs. The cDNA encodes a precursor protein containing four FaRPs: ALSGDAFLRF, FIRF, FLRF and FMRF. Each propeptide has a C-terminal glycine residue that is presumably converted post-translationally to an amide. Every FaRP propeptide is also flanked by basic amino acid residues at the amino and

carboxy termini, indicative of putative cleavage sites during post-translational processing. Each of the four FaRPs encoded by this cDNA causes chromatophore expansion when assayed in an *in vitro* chromatophore bioassay. Thus, it is likely that one or more of the FaRPs identified in this study are involved in controlling chromatophore activity in cuttlefish.

Key words: FMRFamide, FMRFamide-related peptide, FMRFamide cDNA, neuropeptide, invertebrate neuropeptide, cephalopod, cuttlefish, *Sepia officinalis*.

### Introduction

Cephalopods exhibit a wide variety of intricate and fascinating body patterns used for inter- and intraspecific communication as well as for camouflage (Holmes, 1940; Hanlon and Messenger, 1988). The primary component underlying the intricate detail of these patterns is the chromatophore system, which in adult cephalopods consists of millions of individual chromatophores (Hanlon and Messenger, 1988). In cephalopods, in contrast to other animals, the chromatophores are under neuromuscular control (Florey, 1966; Cloney and Florey, 1968; Florey and Kriebel, 1969; Reed, 1995). The muscles controlling chromatophore size, the chromatophore muscles, are capable of operating in phasic and/or tonic modes, properties that allow complex body patterns to be formed in less than a second and maintained for several hours.

Studies on the neural control of cephalopod chromatophore muscles suggest that they are under the regulation of multiple neurotransmitters and modulators (Cornwell and Messenger, 1995; Loi *et al.* 1996; Reed, 1995). Physiological and immunocytochemical studies have shown that glutamate is likely to be one of the neurotransmitters acting at the chromatophore neuromuscular junction (NMJ; Florey *et al.* 1985; Cornwell and Messenger, 1995). Acetylcholine and serotonin have also been implicated as possible transmitters at the chromatophore NMJ (Florey and Kriebel, 1969; Cornwell and Messenger, 1995).

In addition to classical transmitters, other factors are involved in regulating chromatophore function. Previous work has shown that one or more members of the FMRFamide-related peptide family (FaRPs) probably act as excitatory neurotransmitters at the chromatophore NMJ in the European cuttlefish *Sepia officinalis* (Loi *et al.* 1996). This conclusion was based on several lines of evidence: (1) several different FaRPs each cause chromatophore expansion when applied to an *in vitro* chromatophore bioassay; (2) FMRFamide-like immunoreactivity is present in cell bodies of putative chromatophore motoneurons and in peripheral axons closely associated with the chromatophore muscles; and (3) biochemical extraction of native FaRP-like peptides from the dermal chromatophore layer reveals the presence of several peptides that are both bioactive in the *in vitro* chromatophore bioassay and FMRF-immunopositive on a dot-immunoblot assay. Thus, one or more FaRPs probably play an important regulatory role in chromatophore function. However, the precise number, identity and primary structures of the FaRPs are still unknown. This issue is further exacerbated by the very large number of FaRPs already identified in other species (Price and Greenberg, 1994; Saunders *et al.* 1991; Schneider and Taghert, 1988).

Originally identified in molluscs, the FaRP family of peptides includes FMRFamide, FLRFamide and others that are structurally related to FMRFamide by virtue of their C-

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terminal RFamide (Price *et al.* 1994). FaRPs have been found in every major metazoan phylum, from coelenterates to chordates (e.g. Grimmelikhuijzen and Westfall, 1995; Aarnisalo and Panula, 1995; Saunders *et al.* 1992; Schneider and Taghert, 1988). Many species contain multiple FaRPs; for example, molecular studies in the fruit fly *Drosophila melanogaster* predict the presence of at least 10 FaRPs (Schneider *et al.* 1991; Schneider and Taghert, 1988). Studies on gastropod molluscs have identified several classes of FaRPs classified according to their length (Saunders *et al.* 1991; Price *et al.* 1994). These include tetrapeptides (FMRFamide and FLRFamide), heptapeptides (SDPFLRFamide and GDPFLRFamide; Saunders *et al.* 1991) and decapeptides (ALAGDHFFRFamide and ALTNDHELRFamide; Kuroki *et al.* 1993; Fujisawa *et al.* 1992). Despite the wealth of information about FaRPs in gastropods and other molluscan classes, little is known about the number and identity of FaRPs in cephalopods. The only published molecular study of FaRPs in cephalopods used biochemical techniques to identify the presence of FMRFamide, FLRFamide and two pentapeptides (AFLRFamide and TFLRFamide) from octopus optic lobe and vena cava extracts (Martin and Voigt, 1987). More detailed information has come from unpublished work using the squid *Loligo opalescens* (D. A. Price, personal communication). Price isolated partial sequences from two cDNAs, one coding for two tetrapeptides and a decapeptide (FMRFamide, FLRFamide and ALSGDAFLRFamide) and a second containing a pentapeptide and three hexapeptides (TIFRFamide, GNLFRLFamide, GSLFRFamide and NSLFRFamide).

As a first step in determining which FaRPs are acting at the cuttlefish chromatophore NMJ, we used molecular biological techniques to identify the FaRPs present in *Sepia officinalis*. A full-length FaRP-coding cDNA from the cuttlefish brain was isolated and sequenced. This cDNA encodes a protein with 11 copies of FMRFamide and one copy each of FIRFamide, FLRFamide and the decapeptide ALSGDAFLRFamide. Each of the four FaRPs encoded in the cDNA elicited chromatophore expansion when assayed in an *in vitro* chromatophore bioassay. Thus, it is likely that one or more of these four is the endogenous FaRP at the chromatophore neuromuscular junction.

## Materials and methods

### Materials

A *Sepia officinalis*  $\lambda$  Zap cDNA library from an adult male brain (excluding the optic lobes) was produced in collaboration with Stratagene Corp. Synthetic oligonucleotides used as primers in polymerase chain reaction (PCR) and sequencing were synthesized using DNA Express. Taq DNA polymerase was obtained from Perkin Elmer Corporation and the DNA sequencing kit was supplied by U.S. Biochemicals. PCRscript cloning kit, Klenow fragments, random octamer primers and stop buffer were supplied by Life Technologies.  $\alpha$ -[<sup>35</sup>S]dATP and  $\alpha$ -[<sup>32</sup>P]dCTP were obtained from Amersham.

FIRFamide, FLRFamide and ALSGDAFLRFamide were synthesized by Research Genetics, and FMRFamide was purchased from Bachem Corporation.

### PCR analysis of the *Sepia officinalis* brain cDNA library

The following were used for each 100  $\mu$ l PCR sample: DNA template (200 ng), sense and antisense primers (250 ng each), Taq DNA polymerase (2.5 units), 10 $\times$  PCR buffer (10  $\mu$ l of buffer containing 100 mmol l<sup>-1</sup> Tris-HCl, pH 9.0, 15 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 500 mmol l<sup>-1</sup> KCl and 1% Triton X-100), dNTPs (dGTP, dATP, dTTP, dCTP; 3 mmol l<sup>-1</sup> of each) and sufficient double-distilled water to bring the total volume up to 100  $\mu$ l. All PCRs were carried out in a Perkin Elmer 9600 thermal cycler under the following conditions: each cycle consisted of denaturing at 94 °C for 2 min; annealing at 60 °C for 2 min; and extension at 72 °C for 2 min. This was repeated for 35 cycles, after which the reaction mixture was held at 4 °C until further analysis.

The DNA template for the PCRs was obtained by making a 50 ml liquid culture from 50  $\mu$ l of unamplified  $\lambda$  Zap *Sepia officinalis* brain cDNA library using the protocol described by Sambrook *et al.* (1989). DNA from the library was extracted using a phenol/chloroform (three times) and ethanol precipitation (three times) method, and the precipitated DNA was then re-suspended in double distilled water.

Degenerate oligonucleotides containing deoxyinosine (dI) were used as primers. Primer sequences were based on specific regions of the FaRP-encoding cDNA from the squid *Loligo vulgaris*, kindly provided by Dr D. A. Price. The sense primer was complementary to the cDNA sequence that encodes the peptide sequence KRFRFG, and the antisense primer was derived from the cDNA sequence that encodes for the N-terminal region of the decapeptide ALSGDAFL. The sequence of the sense primer was 5'-d[TTICCAAGAA-(CT)CTCAT(AA)AAIC(GT)TTT]-3'. The sequence of the antisense primer was 5'-d[GCI(CT)TI(AT)(GC)IGGIGA-(CT)GCITT(CT)]-3'.

### Subcloning and transformation

The products from the first PCR were purified by phenol/chloroform extraction, precipitated with ethanol and electrophoresed on a 0.8% agarose gel. The most intensely staining DNA band (approximately 500 base pairs) was cut out of the gel, eluted by freezing and centrifuging the frozen gel, and subcloned into a PCRscript vector following the procedure specified by Stratagene Corp.

### DNA sequencing

Cloned PCR products were extracted and purified using a Wizard miniprep column (Promega) following the manufacturer's instructions. The DNA obtained was used as templates for both manual and automated sequencing. For manual sequencing, a sequencing kit was used with  $\alpha$ -[<sup>35</sup>S]dATP, following the protocol supplied by the manufacturer. DNA was sequenced in both directions using T7 and M13 reverse primers. For automated sequencing, DNA

samples were analyzed using sequencing facilities at the University of Georgia, Oregon State University, and the in-house facilities at the University of Oregon. As with the manual sequencing, the cloned PCR product was sequenced in both directions using T7 and M13 reverse primers.

#### *cDNA library screening*

Prior to screening the *Sepia officinalis* cDNA library, the amplified library was reamplified to increase the titer, using the methods described above. The excised insert of the cloned first PCR product was used as a DNA template to generate the radioactive probe used for screening the library. This reaction used 140 ng of template DNA, 20  $\mu$ l of random octamer primers (750  $\mu$ g ml<sup>-1</sup> oligodeoxyribonucleotide primers, 125 mmol l<sup>-1</sup> Tris, pH 6.8, 12.5 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 25 mmol l<sup>-1</sup> 2-mercaptoethanol),  $\alpha$ -[<sup>32</sup>P]dCTP (1.8  $\times$  10<sup>3</sup> MBq), 5  $\mu$ l of a mixture of dATP, dTTP and dGTP (2 mmol l<sup>-1</sup> each) and Klenow fragment (2  $\mu$ l). The mixture was incubated for 60 min at 37 °C and the reaction was terminated by adding 5  $\mu$ l of stop buffer (0.2 mol l<sup>-1</sup> Na<sub>2</sub>EDTA, pH 7.5).

The library was screened according to the instructions supplied by Stratagene with some minor changes. RNA (100  $\mu$ g ml<sup>-1</sup>) was used instead of salmon sperm DNA, and all prehybridization, hybridization and wash steps were carried out in a minihybridization oven. Prehybridization (2 h; 20 ml per vial) and hybridization (overnight; 20 ml per vial with radiolabeled probes at 5  $\times$  10<sup>6</sup> cts min<sup>-1</sup>) were carried out at 42 °C. Washes after hybridization were performed at 52 °C (0.1 % SSC; 0.1 % SDS; 40 ml per vial), three times for 15 min each and once for 60 min. Film (Fuji) was exposed to the plaque lifts for 2 days before processing. Plaques hybridizing to the probe in the first screen were subjected to a second screen using the same probe. Phage suspensions of individual positive plaques from the second screen were subjected to PCR with the two degenerate oligonucleotide primers described above. Four clones from the second screen generated PCR products, and one was sequenced using automated techniques. The clone was sequenced in both directions using synthetic oligonucleotides derived from sequences obtained in earlier steps. The DNA template used for sequencing was either generated by PCR of the positive clone with two universal primers (M13 forward and M13 reverse) or extracted from phage.

#### *In vitro chromatophore bioassay*

The *in vitro* chromatophore bioassay has been previously described in detail (Loi *et al.* 1996). In brief, a small piece of *Sepia officinalis* fin dermis containing approximately 100 chromatophores was removed from the animal and pinned into a perfusion chamber. The animal was anesthetized in 0.1 %

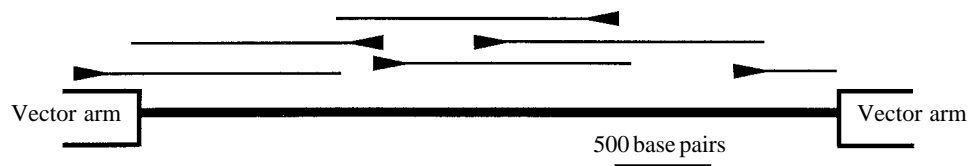
ethanol in artificial sea water (ASW) before removal of the piece of fin. The response of an individual chromatophore to application of test substances was detected using a photocell. The test substance was diluted in ASW and applied directly to the chamber for 5 min, followed by an extended wash-out period with ASW. Each of the four FaRPs was tested separately in a dilution series from 10<sup>-10</sup> to 10<sup>-6</sup> mol l<sup>-1</sup>.

### Results

As a first step towards obtaining an FaRP-coding cDNA, PCR was performed on a *Sepia officinalis* brain cDNA library using primers described in the Materials and methods section. This procedure yielded several products ranging in size between 400 and 700 bp. The presence of numerous PCR products was probably the result of primer binding to multiple FMRF-coding regions in the *S. officinalis* FMRFamide gene, an assumption based on the presence of multiple FMRFamide-coding regions in the FMRFamide genes of other molluscs (Linacre *et al.* 1990; Schaefer *et al.* 1985). A 500 bp band was isolated, subcloned into a vector (PCRscript) and manually sequenced using a sequencing kit with  $\alpha$ -[<sup>35</sup>S]dATP. The sequence obtained encoded for an FaRP decapeptide, followed by one copy of FLRFamide and four copies of FMRFamide. The exact sequence of the decapeptide could not be determined because of several missing base pairs corresponding to the deoxyinosine residues in the primers. The PCR product was also sequenced using automated sequencing techniques, and the results were identical with that obtained with manual sequencing except for five base positions. As before, there were also several missing base pairs corresponding to the deoxyinosine residues in the primers. This sequence very closely resembled a portion of an FaRP-coding cDNA from the squid *Loligo opalescens* (D. A. Price, personal communication). On the basis of this similarity, the PCR product was used to generate a probe to screen the *S. officinalis* library for a full-length cDNA.

The first library screen using this probe generated 36 positive plaques, eight of which were again positive in a second screen with the same probe. To verify that these clones contained FaRP-coding regions, the eight were subjected to PCR using the same two degenerate oligonucleotide primers used in the initial PCR step. Of the eight clones, four generated PCR products similar to those from the initial PCR performed on the whole cDNA library (data not shown), and one of these was randomly selected for sequencing. Fig. 1 illustrates the strategy used to obtain the full sequence. The complete nucleotide sequence of the FaRP-coding cDNA is presented in Fig. 2. The full-length cDNA is 1850 bp in length. The AUG

Fig. 1. Schematic diagram showing the strategy used in obtaining the sequence of the full-length cDNA. Arrowheads indicate primer sites and direction of sequencing. Line length represents fragment size.



1	GCACGAGAAGATCCCACGTTTCAATCAACGCTAAGGCGAAGAGCACACAGCTAAAAGCAACAACAACAACAACA	
77	TCACTCCCCATAAAGAAAAAAGCAGACACTCATTGGACTGAAGAGAAAAACAACGCACGTTGGTGCCTGTTGTT	
153	TTCTTCTTGCCCGTAATCTCAGCGTGTCCCTTCGATCTTTTTTGTCTGTGTGCGGCTCCATCACCGTACCTCG	
229	TCTTTGCTAGTGTCCCCTACCTCTGTAGTTCGACCTAAGTCATCTTGTTCCTGTAGGAGACCCATTACCCCTGG	
		MET ARG CYS TRP SER PRO CYS SER LEU LEU VAL VAL
305	CAGTTTAGAAGAATAACGGGACCCGTG ATG CGT TGT TGG AGT CCC TGT AGC CTA CTT GTC GTC	12
		ILE ALA ILE TYR CYS LEU SER SER HIS THR SER GLU ALA PHE ASP LEU ALA GLN ALA
369	ATC GCC ATC TAC TGT CTC TCT TCG CAT ACG TCT GAA GCC TTT GAT TTG GCC CAG GCA	31
		CYS VAL GLU SER GLN ARG LEU SER LEU LEU PRO ILE CYS ASP THR ILE PHE ALA VAL
426	TGC GTC GAG TCG CAG CGA CTG AGT CTT CTT CCG ATC TGT GAT ACA ATC TTT GCT GTA	50
		GLN GLN GLU GLY ALA GLN GLN SER ALA ASP ASP GLY LEU ARG SER ARG PHE ILE
483	CAG CAA GAA GGA GCT CAG CAA AGT GCT GAT GAT GGA CTG AGA AGC AAG CGT TTC ATC	69
		* * * * *
540	<u>ARG PHE</u> GLY ARG <u>ALA LEU SER GLY ASP ALA PHE LEU ARG PHE</u> GLY LYS ASN VAL PRO	88
	<u>CGA TTC GGA CGA GCC CTG TCA GGA GAT GCT TTC TTG CGT TTT</u> GGG AAA AAT GTA CCA	
		* * * * *
597	ASP LEU PRO PHE GLU ASP LYS ARG PHE LEU ARG PHE GLY ARG ALA ALA PRO GLN LEU	107
	GAC CTT CCT TTC GAG GAT AAA AGA TTC CTT CGA TTT GGA CGA GCA GCT CCT CAA TTG	
		* * * * *
654	ASP ASP LEU LEU LYS GLN ALA LEU GLN ARG VAL GLU SER LEU GLN LYS SER ASP ASP	126
	GAT GAC TTG CTC AAA CAA GCC CTT CAG AGA GTT GAA AGC CTG CAA AAG TCC GAC GAC	
		* * * * *
711	THR SER ILE ARG ARG LYS ARG SER THR ASP ALA ALA PRO GLN SER ASN THR ASP SER	145
	ACC AGT GTT CGA CGA AAG CGC TCC ACA GAC GCA GCT CCT CAA AGC AAC ACA GAC AGT	
		* * * * *
768	ALA GLU GLN LYS ASN ASP SER ALA LYS ILE THR LYS ARG TYR VAL ASP ASP VAL GLU	164
	GCC GAG CAG AAA AAC GAC AGC GCC AAA ATC ACA AAA AGA TAC GTC GAT GAC GTC GAA	
		* * * * *
825	ASP SER ASP VAL LYS ARG PHE MET ARG PHE GLY LYS ARG PHE MET ARG PHE GLY ARG	183
	GAT TCT GAT GTG AAG AGA TTC ATG CGT TTC GGA AAG AGG TTC ATG CGT TTT GGC CGC	
		* * * * *
882	ASN PRO SER ASP VAL GLY SER LYS LEU THR GLU LYS ARG PHE MET ARG PHE GLY ARG	202
	AAC CCG AGC GAC GTT GGC AGC AAA CTG ACC GAA AAG CGA TTC ATG AGA TTC GGA CGC	
		* * * * *
939	ASP PRO GLU LYS ARG PHE MET ARG PHE GLY LYS SER ASP ASP LYS LYS PHE MET ARG	221
	GAT CCC GAA AAA AGG TTC ATG CGT TTT GGC AAA TCA GAC GAC AAG AAG TTC ATG AGA	
		* * * * *
996	<u>PHE</u> GLY ARG ASN PRO GLY ASP ALA GLU ASP GLU LEU GLU GLU ASP LYS ARG <u>PHE MET</u>	240
	TTC GGT CGA AAC CCC GGC GAT GCT GAA GAT GAA CTG GAA GAA GAC AAA CGT TTC ATG	
		* * * * *
1053	<u>ARG PHE</u> GLY ARG GLY ASP GLU GLU ASP GLU GLU GLU ALA GLU LYS ARG <u>PHE MET ARG</u>	259
	<u>AGA TTC GGA CGG GGA GAC GAA GAG GAT GAG GAA GAA GCC GAA AAA CGA TTT ATG AGA</u>	
		* * * * *
1110	<u>PHE</u> GLY ARG ASP PRO GLU LYS LYS <u>PHE MET ARG PHE</u> GLY LYS ASN GLY GLU GLU LYS	278
	TTT GGA CGT GAC CCT GAA AAG AAA TTC ATG AGA TTT GGG AAG AAC GGC GAA GAG AAG	
		* * * * *
1167	ARG PHE MET ARG PHE GLY ARG ASN PRO GLU GLU PRO GLU ALA ASP LYS ARG PHE MET	297
	AGG TTC ATG CGT TTT GGT CGA AAC CCT GAG GAG CCA GAA GCC GAC AAG AGG TTC ATG	
		* * * * *
1224	<u>ARG PHE</u> GLY ARG GLY GLY GLU GLU ASP ASP VAL ASN THR GLU GLU LYS ARG PHE MET	316
	<u>AGA TTT GGA CGC GGC GGT GAA GAG GAT GAC GTG AAC ACA GAG GAA AAA CGT TTC ATG</u>	
		* * * * *
1281	<u>ARG PHE</u> GLY ARG SER ALA GLU LYS CYS LYS GLY CYS LEU GLU GLY STOP	331
1342	AGA TTT GGT CGA TCT GCA GAA AAA TGC AAA GGG TGT CTG GAA GGT TAATGCTAATGTGCGC	
1418	TAGCTTGCTTCCTTCTTTTTTGTGCTGAACAGAGAAAAAAGAATAACAAATTAAGCTATCCTCTCTGATTAAT	
1494	ACTTTACATTTTTTAAATACTAAAAGAAAGTTATTAGTATTTGGACTGGGACACACACGCATACATACACACA	
1570	TACATTCCTCTCTCACTCTCTCAATACTTTGGACAGTATGTTTCGTTAAACCTGCTTTTTCCGGTGGCTG	
1646	TGTTACATCCTAACCTCACTTCAGTTGTTCAATTTATGAGATTGGTGTATACCTGGCTCATTCAGTTATTTGA	
1722	ATACGCATGCGCAGTAAACCTGGATGCTTCTCTAAGGACTTTCAGCATTATGAAAATCTAAAAAAAATAAAAATA	
1798	AAAAATACAAACAAAAATCTCCATAAAAAGCCACCTTGAAACAGATGTTGATTTTCATGAAAATGATTGTAATTT	
	TTGTAGGAAATTAATTTTAAATATATATATCCTTAAAAAAAATAAAAAAAAT	

Fig. 2. Nucleotide sequence of a full-length *Sepia officinalis* FMRFamide cDNA (1850 bp, numbered on the left). The predicted amino acid sequence is shown above the nucleotide sequence (331 amino acid residues, numbered on the right). The 21 amino acid residues that may form the hydrophobic domain of a signal peptide are indicated by an overscored line. The monobasic and dibasic cleavage sites are identified with an asterisk, and the tetrabasic putative furin cleavage site is indicated with double asterisks. There are 14 FaRPs (underlined amino acid residues), two potential *N*-linked glycosylation sites (▼ above the amino acid residues) and six putative polyadenylation sites (underlined nucleotides).

triplet coding for the methionine residue that initiates translation is located at nucleotides 333–335, and the stop codon is located at nucleotides 1326–1328. Both start and stop codons are in frame with the peptide sequence. There are 522 untranslated base pairs at the 3' end between the stop codon and the poly(A) tail. This region contains six AMUAAA polyadenylation signals (where *N*=G, A, U, C; Loh and Gainer, 1983).

The single open reading frame of this cDNA encodes a predicted precursor protein of 331 amino acid residues containing 14 FaRPs ending with RFamide at the C terminus (Fig. 2). These include one copy of the peptide FIRFamide,

one copy of the novel decapeptide ALSGDAFLRFamide, one copy of FLRFamide and 11 copies of FMRFamide (Fig. 3). Each FaRP propeptide is flanked by lysine–arginine, by lysine–lysine or by a single arginine at the amino terminus, and an arginine or lysine at the carboxy terminus. These basic amino acid residues serve as internal proteolytic cleavage sites during post-translational processing of the proprotein (Loh and Gainer, 1983). Each FaRP propeptide has a glycine residue at the carboxy terminus, presumed to be post-translationally converted to an amide (Loh and Gainer, 1983; Eipper and Mains, 1988). This prepropeptide possesses a tetrabasic putative furin cleavage site between FLRF and the first FMRF,



Fig. 3. Schematic diagram of the FMRFamide precursor protein showing the arrangement of the 14 encoded FaRPs. ↓, Met; \*, basic amino acid residues; I, FIRF; A, ALSGDAFLRF; L, FLRF; M, FMRF.

which potentially splits the prepropeptide into two fragments. There are also six putative *N*-linked glycosylation sites. The spacer peptides between the FaRPs are mostly acidic. The deduced amino acid sequence and organization of the cuttlefish FMRFamide cDNA are very similar to those of the squid *Loligo opalescens* (D. A. Price, personal communication). Like the *S. officinalis* FMRFamide cDNA, the squid FMRFamide cDNA also encodes single copies of FLRFamide, the decapeptide ALSGDAFLRFamide, FIRFamide and multiple copies of FMRFamide.

To determine whether any of the four FaRPs on the cDNA affected chromatophore function, each was tested individually on an *in vitro* chromatophore bioassay. All four FaRPs elicited a similar response by the brown chromatophores in terms of rate of expansion during peptide application and rate of condensation during the wash-out period, but they had different thresholds (Fig. 4; Table 1). FLRFamide was the most potent (threshold concentration  $10^{-9}$  mol l<sup>-1</sup>), followed by the decapeptide ( $10^{-8}$  mol l<sup>-1</sup>) and then FMRFamide and FIRFamide ( $10^{-7}$  mol l<sup>-1</sup>).

## Discussion

### FaRPs in the cuttlefish *Sepia officinalis*

The aim of this study was to determine the number and structure of FaRP family members expressed in cuttlefish as a first step towards identifying which FaRPs are involved in regulating chromatophore function. Towards this goal, we have isolated and characterized what we believe is the first cDNA to be sequenced from *S. officinalis*, one that encodes four FaRPs (ALSGDAFLRFamide, FLRFamide, FMRFamide and FIRFamide). This result provides molecular confirmation of a previous study which proposed that FaRPs were probably

Table 1. The effect on the *in vitro* chromatophore bioassay of the four FaRPs identified in this study

Neuronal factors tested in the <i>in vitro</i> chromatophore bioassay	Response	Threshold concentration (mol l <sup>-1</sup> )
FMRFamide	Expansion	$10^{-7}$
FLRFamide	Expansion	$10^{-9}$
FIRFamide	Expansion	$10^{-7}$
ALSGDAFLRFamide	Expansion	$10^{-8}$

present in cuttlefish on the basis of immunocytochemical, biochemical and pharmacological evidence (Loi *et al.* 1996). That study predicted the presence of at least three FaRPs, ALSGDAFLRFamide, FMRFamide and an unidentified FaRP. The current molecular results reveal two additional FaRPs, FLRFamide and FIRFamide. Found in several species (Salzet *et al.* 1994; Linacre *et al.* 1990; Taussig and Scheller, 1986), FLRFamide probably evaded detection in the previous study because of its low molecular mass and low copy number. FIRFamide was not tested in that study because it was unknown at that time, although similar peptides ending in -IRFamide have been found in the flatworm *Dugesia tigrina* (GYIRFamide; Johnston *et al.* 1995) and in the locust (AFIRFamide; Lange *et al.* 1994).

### The role of FaRPs in chromatophore function

Previous work implicated the FaRP peptide family as putative regulators of chromatophore function in *S. officinalis* (Loi *et al.* 1996). However, because all FaRPs tested in that study caused chromatophore expansion in the *in vitro* chromatophore bioassay, the possibility was raised that the bioassay responded to all FaRPs and thus would not be useful

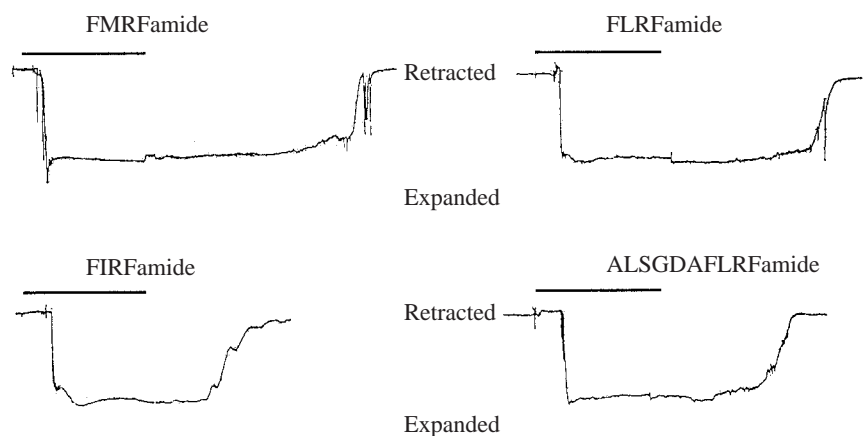


Fig. 4. The effect of FMRFamide ( $10^{-6}$  mol l<sup>-1</sup>), FLRFamide ( $10^{-6}$  mol l<sup>-1</sup>), FIRFamide ( $10^{-7}$  mol l<sup>-1</sup>) and ALSGDAFLRFamide ( $10^{-8}$  mol l<sup>-1</sup>) on the *in vitro* chromatophore bioassay. The bars above each trace indicate the duration of peptide application (5 min).

in identifying individual FaRPs endogenous to *S. officinalis*. The present study was performed to circumvent that difficulty and has identified four FaRPs expressed in the *S. officinalis* brain, each of which elicits chromatophore expansion *in vitro* (Fig. 4). These data are also consistent with previous immunocytochemical data showing the presence of FMRFamide-like immunostaining in the cell bodies and terminals of putative chromatophore motoneurons (Loi *et al.* 1996). The results of that study and the data presented here raise the strong possibility that the four peptides identified here are the endogenous FaRPs in the chromatophore system in *S. officinalis*. This conclusion must be viewed as tentative since we have not excluded the possibility that other as yet unidentified FaRPs are the actual neuroregulators in this system. If the four FaRPs are involved in this system, then they must be present in chromatophore motoneurons and terminals, and this hypothesis is currently being tested using a combination of more specific antibodies and *in situ* hybridization.

#### *FaRPs in other cephalopods and their physiological roles*

The results of this study, when added to previous investigations (Martin and Voigt, 1987; D. A. Price, personal communication), bring the number of FaRPs identified to date in cephalopods to ten. These include three tetrapeptides (FIRFamide, FLRFamide and FMRFamide), three pentapeptides (TIFRFamide, TFLRFamide and AFLRFamide), three hexapeptides (GNLFRFamide, NSLFRFamide and GSLFRFamide) and a decapeptide (ALSGDAFLRFamide).

It is highly probable that these FaRPs serve different physiological roles in cephalopods. As described above, ALSGDAFLRFamide, FMRFamide, FIRFamide and FLRFamide probably regulate chromatophore activity. FLRFamide and FMRFamide also modulate the heart activity of *Sepia officinalis* (Jakobs and Schipp, 1992). In octopus, four FaRPs (FMRFamide, FLRFamide, TFLRFamide and AFLRFamide) have been localized to the optic lobes and the heart, suggesting possible of cardioregulatory and visual processing roles (Martin and Voigt, 1987). The physiological roles of the three hexapeptides and the pentapeptide TIFRFamide have not yet been explored.

#### *FaRPs in invertebrates*

Within the last decade, the definition of FaRPs has been expanded to include all peptides with -FRFamide or -IRFamide at the C terminus. Using this broad definition, FaRPs are found in nearly all invertebrate phyla, including annelids (GYIRFamide; Johnston *et al.* 1995), insects (AFIRFamide; Lange *et al.* 1994) and molluscs (e.g. FMRFamide and FLRFamide; Price *et al.* 1994). FaRPs have also been reported in mammals (Neuropeptide FF; Aarnisalo and Panula, 1995). FaRPs can be classified either by the structure of their C-terminal region or by their length. Using the latter classification system, molluscs contain five FaRP classes: tetrapeptides (e.g. FMRFamide and FLRFamide; Price *et al.* 1994); pentapeptides (TIFRFamide, AFLRFamide and TFLRFamide; Martin and Voigt, 1987; D. A. Price, personal communication);

hexapeptides (e.g. GNLFRFamide, NSLFRFamide; Price *et al.* 1994); heptapeptides (e.g. GDPFLRFamide and SDPFLRFamide; Saunders *et al.* 1991); and decapeptides (e.g. ALAGDHFFRFamide and ALSGDAFLRFamide; Kuroki *et al.* 1993; D. A. Price, personal communication). The present data suggest that heptapeptides are restricted to pulmonates, whereas the decapeptides and peptides ending with -LFRFamide are found only in non-pulmonate gastropods and other molluscs. Intriguingly, insects appear to have a completely different complement of FaRPs, ranging in length from hexapeptides to decapeptides (Schneider and Taghert, 1988; Nichols, 1992). It is interesting and somewhat perplexing that insects appear to lack any tetrapeptides (i.e. FMRFamide, FLRFamide, FIRFamide).

#### *FaRP gene organization*

Our understanding of FaRP coding genes and their structure comes mainly from studies in insects and molluscs. The fruit fly *Drosophila melanogaster* has at least two FaRP genes, one that encodes ten different N-terminally extended FMRFamides (Schneider and Taghert, 1988) and a second that codes for FaRPs ending in FRFamide (Nichols *et al.* 1988). However, there is probably at least one other FaRP gene in *Drosophila* because one biochemically isolated FaRP (TDVDHVFLRFamide) is not encoded by either known gene (Nichols, 1992).

The gene organization of FaRPs in molluscs is very different from that of insects. Pulmonates have a single FaRP gene that is alternatively spliced into two transcripts, one that codes for tetrapeptides and a second that generates heptapeptides ending in FLRFamide (Price and Greenberg, 1994). In contrast, non-pulmonate gastropods and other molluscs such as *Loligo opalescens*, *Fusinus ferrugineus* and *Aplysia californica* have two separate FaRP genes, one that codes for tetrapeptides, plus in some cases a single decapeptide, and a second that encodes hexapeptides with LFRFamide at the C terminus (Price and Greenberg, 1994). If cuttlefish express the same classes of FaRPs as other non-pulmonates, then they should contain a second FaRP gene that codes for LFRFamide hexapeptides. Cuttlefish may also, as in octopus, contain one or more pentapeptides, but whether they are found on the hexapeptide gene or in a separate gene is a matter for future investigation.

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