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Purification and sequencing of neuropeptides contained in neuron R15 of *Aplysia californica*

(invertebrate/mollusc/water balance)

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ABSTRACT **R15 is a large identified neuron present in the abdominal ganglion of the mollusc *Aplysia*. Previous studies have indicated that this neuron may play a role in water balance and possibly renovascular functions. A peptidic factor contained in the neuron R15 has been shown to increase the water content of *Aplysia*. To determine the structure of the peptides contained in R15, we purified the extracts of 820 R15 cells by means of two steps of reverse-phase HPLC. The purification yielded a number of peptides, only one of which, R15α1, resulted in water uptake when injected into animals. Determination of the amino acid content and sequence analysis of the R15α1 peptide demonstrated that this peptide contains 38 residues, including two cysteines. The peptide failed to react with iodoacetate, indicating that the two cysteines are connected by a disulfide bridge. To confirm the assigned structure, the peptide was synthesized with a disulfide bridge. The chromatographic properties and bioactivity of the synthetic material were identical to those of the native peptide. Several other R15 peptides were inactive in the bioassay for water uptake. The sequence of one of these peptides (R15β) was determined, and it was established that the peptide contains 28 residues. Amino acid analysis of three other peaks was performed. One of these peaks contained a peptide (R15βf) whose amino acid composition suggests that it is a fragment of the R15β peptide. The other two peaks contained peptides with identical amino acid compositions, suggesting that they are variants of a single peptide (R15γ). The amino acid sequences of all the peptides identified in neuron R15 correspond to stretches of a polyprotein encoded by a recently sequenced R15 cDNA.**

Over the past decade, numerous small peptides have been described in neurons of both vertebrates and invertebrates. The large neurons of *Aplysia* have proven particularly well suited for the analysis of peptide function. One such peptidergic neuron (1–3), R15, has been the subject of many biophysical (4) and biochemical studies (2, 3, 5–8). The wealth of these studies contrasts with the paucity of studies of the physiological role of R15. The limited data that exist suggest that R15 may be involved in the regulation of ionic balance or maintenance of body volume (9–13). For example, injection of a peptidic extract of R15 causes animals to increase their body weight, due to an increase of body water content (12).

Recently, Buck *et al.* (14) isolated a cDNA that represents an abundant mRNA present in R15 as well as in some other neurons in *Aplysia*. This cDNA encodes a low molecular weight protein, which contains five consensus pairs of basic residues that are potential cleavage sites for the processing of this molecule into a number of peptides. The mRNA encoding the R15 polyprotein appears to be alternatively spliced in different *Aplysia* neurons. The R15 alternative splice choice affects only a small region in the center of the polyprotein. Thus, alternative splicing of a single R15 transcript in different *Aplysia* neurons may generate overlapping sets of peptides. Since it is not possible to unequivocally predict peptide products on the basis of the amino acid sequence of the precursor polyprotein, we attempted to determine what peptides are actually synthesized in R15. We obtained the amino acid sequence for two of the R15 peptides. These sequences correspond to segments of the R15 polyprotein encoded by the R15 cDNA. One of these peptides, the R15α1 peptide, exhibits the potent osmoregulatory activity previously described for extracts of R15. Interestingly, this peptide is encoded by the region of the mRNA that appears to be alternatively spliced in different neurons of *Aplysia*. We have also obtained the amino acid composition of two other R15 peptides, which also appear to be encoded by the R15 polyprotein.

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METHODS

Extraction and Purification of Peptides from R15 Neurons. Peptides were purified from 820 R15 neurons of *Aplysia californica*. Pooled cells were extracted by boiling (in groups of 10) for 5 min in polypropylene microvials containing 50 μl of 0.1 M acetic acid. The combined extracts were applied to a C18 cartridge (Sep-Pak, Waters). The cartridge was washed with 5 ml of 0.01 M trifluoroacetic acid. The peptides were eluted with 5 ml of 0.01 M trifluoroacetic acid containing 60% CH_3CN and the eluate was then lyophilized.

Peptides were purified from this material in two steps of reverse-phase high-pressure liquid chromatography (HPLC) performed on a DuPont high-pressure liquid chromatograph 850. In the first step, a Zorbax C8 column (4.6 × 250 mm) was eluted with a linear gradient from 20% CH_3CN/80% H_2O/0.01 M HFBA to 65% CH_3CN/35% H_2O/0.01 M HFBA in 45 min at a rate of 1 ml/min. Fractions absorbing at 215 nm were collected. In the second step, individual fractions from step one were applied to an Aquapore RP300 column (4.6 × 220 mm) and eluted with a linear gradient from 10% CH_3CN/90% H_2O/0.01 M trifluoroacetic acid to 50% CH_3CN/50% H_2O/0.01 M trifluoroacetic acid in 40 min at 1 ml/min.

Bioassay. *Aplysia* (90–160 g) were maintained in individual 1.5-liter cages suspended in tanks filled with cooled (15°C) artificial seawater. Fractions to be bioassayed were lyophilized and then reconstituted in artificial seawater supplemented with 1 μM bovine serum albumin, a carrier to prevent peptide losses. For each fraction tested, five animals were each injected with 1% of the fraction. Animals were given a 1-ml injection and weighed immediately (12). The weighing

was repeated 45 and 90 min later. Control animals that were injected with artificial seawater alone or that containing 1 μM bovine serum albumin did not show any significant changes of body weight.

Peptide Structure Determination. Amino acid analysis and gas-phase sequencing were performed at the HHMI Protein Center Core Facility of Columbia University. For amino acid analysis, 1–2 μg of peptide was hydrolyzed under vacuum with 6 M HCl containing 0.1% phenol at 110°C for 24 hr. The samples were then derivatized with phenylisothiocyanate and analyzed with equipment, reagents, and methodology supplied by Waters for their Picotag system.

Automated Edman degradation of peptides was performed on an Applied Biosystems 470A gas-phase sequencer. Samples were loaded onto a glass fiber filter that had been pretreated with 3 mg of Polybrene and 0.2 mg of NaCl and precycled. Phenylthiohydantoin-derivatized amino acids were analyzed on an Applied Biosystems 120A PTH analyzer.

For reduction and carboxymethylation, native or synthetic peptides (0.5 nmol) were dissolved in 1.0 M Tris·HCl/1 mM EDTA, pH 8.6, saturated with nitrogen. The same buffer was used as a solvent for the reagents for subsequent additions, which were 0.02 M tyramine (10 μl; an iodine scavenger), 0.10 M dithiothreitol (10 μl) followed by an incubation at 50°C for 30 min, 0.19 M iodoacetic acid (10 μl) followed by an incubation in the dark at room temperature for 20 min, and finally a further portion of 0.10 M dithiothreitol (10 μl). The modified peptide was frozen until analyzed on HPLC using the Aquapore RP300 column (4.6 × 250 mm). A linear gradient was developed from 15% solvent A [0.1 M triethylamine acetate (TEAA), pH 5.5/0.01% $HSCH_2CH_2OH$] to 45% solvent B (0.1 M TEAA/0.01% CH_3SH/80% $HSCH_2CH_2OH$/20% H_2O) in 30 min at 1 ml/min.

Structure Verification. The proposed sequence for the bioactive R15 peptide (peak IVa, R15α1 peptide) was verified by determining whether the bioactivity of synthetic material (Peninsula Laboratories), containing a disulfide bridge, was quantitatively similar to the native peptide. In addition, the chromatographic characteristics of the synthetic peptide were compared to those of the native R15 peptide labeled *in situ* with [^{35}S]methionine as described (15), except that the incubation period with radioactivity was 30 hr and the chase period was 12 hr. Four cells were dissected and extracted by boiling for 5 min in 50 μl of 0.1 M acetic acid containing 1 nmol of synthetic R15α1 peptide. The identities of the synthetic and radiolabeled R15 peptides were compared by HPLC. The mixture was applied first to the C8 Zorbax column, which was eluted with a linear gradient over 40 min from 20% CH_3CN/80% H_2O/0.01 M trifluoroacetic acid to 60% CH_3CN/40% H_2O/0.01 M trifluoroacetic acid at 1 ml/min. Synthetic peptide was detected by absorbance measurements at 215 nm. The radiolabeled native peptide was detected by scintillation counting of portions of eluate. The peak containing the synthetic peptide, which included the radiolabeled R15 peptide, was applied to the Aquapore RP300 column and eluted as described for the purification of native material.

RESULTS

Peptides present in R15 neurons were purified by two steps of sequential HPLC. The first step (Fig. 1*A*) revealed four major peaks of significant absorbance (peaks I, II, IV, and V) and one minor peak (peak III). Peaks I–V and the breakthrough material were tested for bioactivity by weighing animals after injection of given peaks. Only peak IV was bioactive—i.e., it significantly ($P < 0.05$) increased the weight of the animals. The increase was 6.4% (SEM = 0.61) at 45 min and 10.4% (SEM = 0.97) at 90 min.

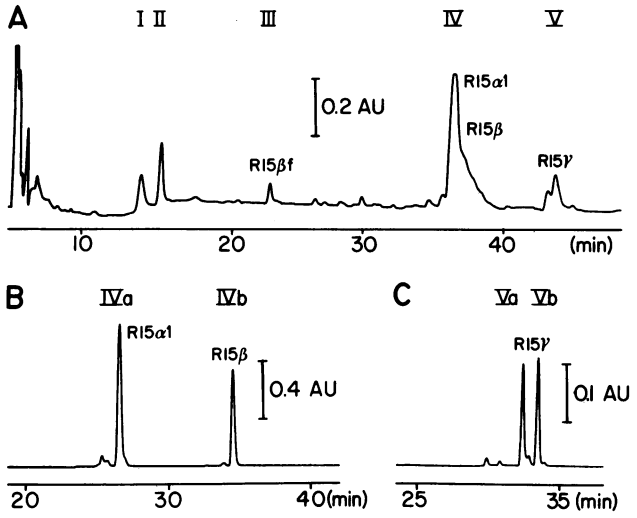

FIG. 1. Purification of peptides from cell R15. (*A*) The first step of purification yielded five significant peaks of absorbance (I–V). (*B*) Rechromatography of peak IV yielded two separate peaks of absorbance (IVa and IVb). (*C*) Rechromatography of peak V yielded two separate peaks of absorbance (Va and Vb). The peptides shown to be in the various peaks are indicated. AU, absorbance unit.

Each of the absorbance peaks identified in the first step of HPLC (Fig. 1*A*) was subjected to a second step of chromatography on an Aquapore RP300 column. Peaks I, II, and III yielded single peaks of absorbance, while peaks IV and V each split into two major peaks (IVa, IVb and Va, Vb; Fig. 1 *B* and *C*). Aliquots (1%) of the two peaks resulting from rechromatography of the bioactive peak IV (namely, peaks IVa and IVb) were tested for bioactivity. Only peak IVa significantly increased ($P < 0.05$) the weight of the animals [3.9% (SEM = 0.3) increase at 45 min and 8.1% (SEM = 0.85) increase at 90 min].

Aliquots of each peak from the second step of HPLC separation were subjected to amino acid analysis (Table 1). Material from peaks I and II did not contain any amino acids.

Amino acid analysis of the material in peaks Va and Vb (derived from peak V) gave identical results, suggesting that these two peaks may represent slightly modified variants of a single peptide (R15γ; Fig. 2). The amino acid ratios of this material match those of a peptide predicted (14) from the R15 cDNA sequence, which extends from the fourth pair of basic residues to the carboxyl terminus of the R15 polyprotein (Fig. 2 *Lower*).

Based on the calculated amino acid ratios (Table 1), peak IVb corresponds to the sequence predicted for a 27-residue peptide (R15β peptide; Fig. 2) produced by cleavage at the third and the fourth pair of basic residues present in the predicted R15 polyprotein. This was confirmed by sequence analysis. Listed below are the residues and their picomolar amounts (in parentheses). Residue: 1, Ser (163); 2, Asp (894); 3, Leu (1334); 4, Leu (1334); 5, Gly (1105); 6, Ala (1354); 7, Leu (1176); 8, Leu (1265); 9, Ser (95); 10, Arg (240); 11, Asn (619); 12, Ser (67); 13, Pro (583); 14, Ser (55); 15, Ser (59); 16, Tyr (403); 17, Gly (304); 18, Leu (337); 19, Pro (293); 20, Ser (26); 21, Arg (99); 22, Asp (130); 23, Met (219); 24, Ser (16); 25, Thr (14); 26, Ala (90); 27, Tyr (56).

Amino acid composition (data not shown) of the material derived from the minor peak (peak III) indicated that it is a fragment of the above peptide, which extends from amino acid residue 11 to the carboxyl terminus of the peptide (R15βf peptide; Fig. 2).

The amino acid ratios (Table 1) of the material present in the bioactive peptide in peak IVa also agree with that of the peptide encoded by the R15 cDNA. This peptide is flanked by the first and third pairs of basic residues of the polyprotein

Table 1. Amino acid composition of peaks from the second step of HPLC purification

Amino acid	Peak Va			Peak Vb			Peak IVb			Peak IVa		
	nmol	Ratio	Ratio for R15γ	nmol	Ratio	Ratio for R15γ	nmol	Ratio	Ratio for R15β	nmol	Ratio	Ratio for R15α1
Asx	1.4	4.9	5.0	1.3	4.9	5.0	1.2	3.1	3.0	1.5	3.1	3.0
Glx	2.4	8.3	8.0	2.2	8.6	8.0	0.1	0.3	0.0	1.2	2.4	2.0
Ser	0.4	1.5	2.0	0.4	1.6	2.0	2.0	5.4	7.0	1.2	2.4	3.0
Gly	0.2	0.7	0.0	0.2	0.6	0.0	0.9	2.4	2.0	3.2	6.4	6.0
His	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0	1.0	1.9	2.0
Arg	1.2	4.2	4.0	1.0	4.1	4.0	0.8	2.1	2.0	1.5	3.0	3.0
Thr	0.3	1.0	1.0	0.3	1.0	1.0	0.4	1.0	1.0	0.9	1.8	2.0
Ala	0.3	1.2	1.0	0.3	1.1	1.0	0.8	2.2	2.0	1.0	2.1	2.0
Pro	0.6	2.2	2.0	0.5	2.1	2.0	0.8	2.1	2.0	1.5	3.0	3.0
Tyr	0.5	1.7	2.0	0.4	1.7	2.0	0.5	1.4	2.0	0.4	0.8	1.0
Val	0.8	3.0	3.0	0.8	3.0	3.0	0.0	0.0	0.0	1.4	2.9	3.0
Met	0.2	0.8	1.0	0.2	0.8	1.0	0.2	0.7	1.0	0.6	1.3	2.0
Cys	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.1	0.0	1.0	0.2	2.0
Ile	0.3	1.0	1.0	0.3	1.0	1.0	0.0	0.1	0.0	0.0	0.1	0.0
Leu	1.3	4.7	5.0	1.2	4.7	5.0	1.9	5.0	5.0	1.6	3.1	3.0
Phe	0.3	1.0	1.0	0.3	1.0	1.0	0.0	0.0	0.0	0.0	0.1	0.0
Lys	0.3	1.0	1.0	0.3	1.0	1.0	0.0	0.1	0.0	0.5	1.0	1.0

Expected ratios for R15γ, -β, and -α peptides were calculated by using the data of Buck *et al.* (14).

predicted by the sequence of the R15-2 cDNA (R15α1; Fig. 2). However, the match was imperfect since only small amounts of the two cysteines predicted from the cDNA sequence were found. Since the phenylisothiocyanate methodology is insensitive to unmodified cysteines, the peptide was subjected to reduction and carboxymethylation and then analyzed for amino acid content. As predicted by the cDNA, the ratio for carboxymethyl cysteines became 2.

To unequivocally determine the structure of the bioactive R15α1 peptide, the purified material (2.5 nmol) was subjected to sequence analysis of Edman degradation. Listed below are the residues and their picomolar amounts (in parentheses). Residue: 1, Asp (1600); 2, Val (1704); 3, Ser (576); 4, Asp (850); 5, Gly (1140); 6, Ser (303); 7, Ala (1199); 8, Glu (783); 9, Arg (623); 10, Arg (815); 11, Pro (839); 12, Tyr (769); 13, Thr (769); 14, Arg (379); 15, Met (666); 16, Gly (498); 17, Ser (114); 18, Gly (413); 19, Gly (469); 20, Leu (322); 21, Lys (281); 22, Leu (336); 23, His (94); 24, unassigned; 25, Gln (148); 26, Val (144); 27, His (49); 28, Pro (190); 29, Ala (124); 30, Asn (80); 31, unassigned; 32, Pro (75); 33, Gly (69); 34, Gly (89); 35, Leu (48); 36, Met (41); 37, Val (30); 38, Thr (11). The amino acids at positions 24 and 31 can be inferred on the basis of the amino acid analysis and the structure deduced from the cDNA (14) to be occupied by cysteines.

We wished to determine whether the two cysteines are connected by a disulfide bridge. The carboxymethylation procedure applied to the native peptide without prior reduction yielded a peptide with a retention time identical to the native peptide (Fig. 3, traces A and B), indicating that no reduced sulfhydryl groups were present in the native peptide. This was confirmed by carboxymethylation with prior reduction of the native peptide, which yielded a peptide with

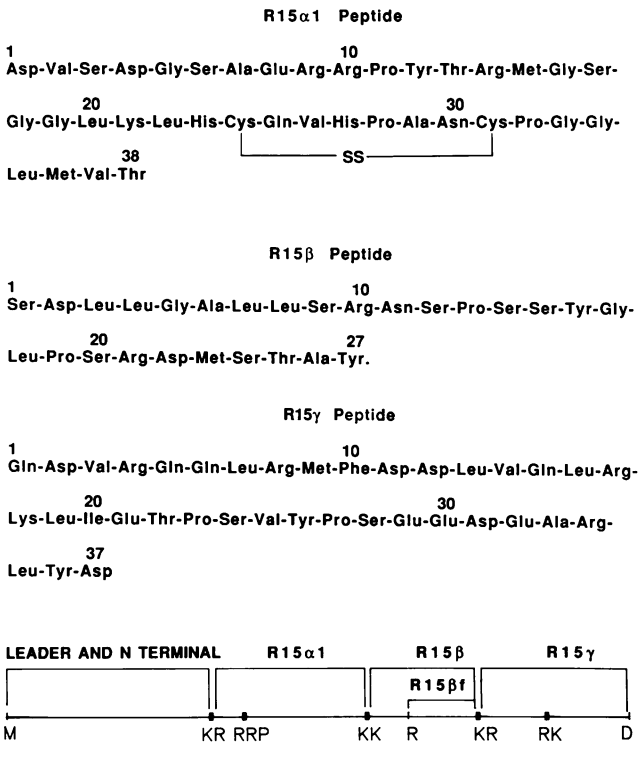

FIG. 2. (*Upper*) Amino acid sequence of R15α1, R15β, and R15γ peptides. The disulfide bridge in the R15α1 peptide is indicated. (*Lower*) Schematic representation of the R15 precursor peptide, encoded by the R15-2 cDNA (14). Positions of dibasic residues are indicated by thick vertical lines, and other key amino acids are indicated (R, arginine; D, aspartic acid; K, lysine; M, methionine; P, proline).

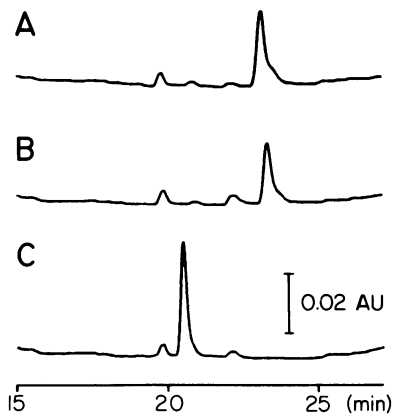

FIG. 3. Effects of peptide reduction and carboxymethylation on the chromatographic properties of native R15α1 peptide. Trace A, elution of the untreated R15α1. Trace B, elution of R15α1, which underwent carboxymethylation without a prior reduction. Trace C, elution of R15α1, which was reduced and then carboxymethylated. AU, absorbance unit.

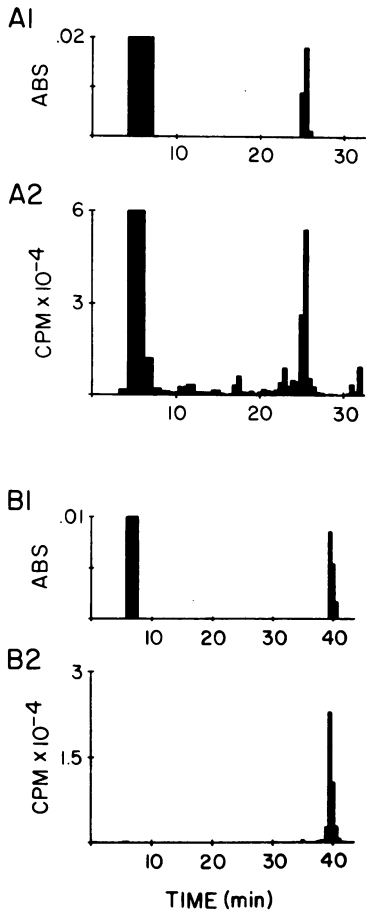

FIG. 4. Chromatographic properties of native and synthetic peptide R15α1. (*A*) Synthetic (*A1*) and native (*A2*) R15α1 coeluted when trifluoroacetic acid was used as a counterion. (*B*) When the peak containing R15α1 was rechromatographed with HFBA as a counterion, the synthetic (*B1*) and native (*B2*) peptides again coeluted.

shorter retention time. Thus, the two cysteines are linked by a disulfide bridge in the native peptide (Fig. 3, trace C).

To confirm the structure of the R15α1 peptide we compared the chromatographic and biological properties of synthetic R15α1 with those of the native peptide. Radiolabeled peptide R15α1 extracted from R15 neurons that were incubated in the presence of [^{35}S]methionine comigrated with synthetic peptide R15α1 in the chromatographic systems that were used in the original purification (Fig. 4). The native and synthetic R15α1 peptides were also equally effective in causing weight increases when injected into animals (Fig. 5).

DISCUSSION

In this study, we have isolated and characterized a set of peptides synthesized by R15, an identified neuron in *Aplysia*. All of the major peptides that we have isolated from R15 extracts are contained within a single polyprotein, which is encoded by a cDNA cloned from neuron R15 (14). The peptides described account for most, but not all, of the polyprotein deduced from the R15 cDNA sequence. Lacking is an amino-terminal peptide of ≈48 amino acids that includes a presumptive leader sequence (Fig. 2). This peptide may be rapidly degraded during processing of the precursor polyprotein. The three major peptides that we have identified we term the α1, β, and γ peptides. The finding of a peptide fragment (βf peptide) that lacks residues 1–10 of the β peptide suggests that the β peptide may be further cleaved to yield two peptides of 9 and 17 amino acids. Our results therefore

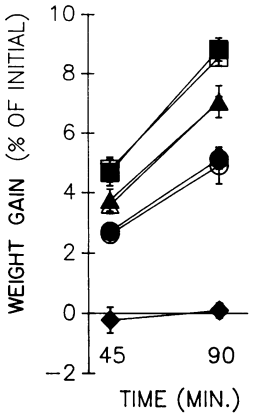

FIG. 5. Bioactivity of synthetic and native R15α1 peptide 45 and 90 min after injections. ◆, Bovine serum albumin; open symbols, native peptide; solid symbols, synthetic peptides; ○ and ●, 100 pmol/kg; △ and ▲, 500 pmol/kg; □ and ■, 1 nmol/kg. Bars represent SEM.

indicate the existence of four peptides that are released by endopeptidase cleavage of the R15 polyprotein. The data do not preclude the possibility that we failed to detect additional peptides that may also be released from the polyprotein, including an amino-terminal fragment and an amino-terminal sequence of the R15β peptide. Based on the recoveries of synthetic peptides, we estimated that a single R15 neuron contains 7–8 pmol of R15α and R15β peptides and ≈0.6 pmol of the R15βf peptide.

The R15α1 peptide that is present in neuron R15 is encoded by a region of the R15 mRNA that undergoes alternative RNA splicing to yield two different mature mRNA species, R15-1 and R15-2 (14). Both R15-1 and R15-2 encode the β peptide, β fragment, and γ peptide. Different individual *Aplysia* neurons appear to process the R15 transcript in different ways. In the polyprotein encoded by the R15-1 mRNA, the 38-amino acid α1 peptide is replaced by a 24-amino acid peptide (putative α2 peptide) in which 16 amino acids of the α1 peptide (residues 3–18) have been replaced by a pair of amino acids not present in the α1 peptide. We find no evidence for the expression of the α2 peptide in neuron R15. This is consistent with previous experiments that demonstrated that neuron R15 makes the alternative splice choice that generates the R15-2 mRNA species (14).

Our data indicate that the α1 peptide is responsible for the previously described osmoregulatory properties of R15 extracts (12). Although the other R15 peptides isolated were not active in the water uptake bioassay, it is possible that these may mediate other effects that are produced by the firing of neuron R15. R15 has been shown to induce changes in the ionic content of the blood (10) and to alter the activity of neurons in the abdominal ganglion (16).

Interestingly, the R15α1 peptide includes a dibasic sequence that may be cleaved in other cells or perhaps during transport in the axon of neuron R15. Thus, in addition to alternative RNA splicing, the R15 gene may produce cell-specific products as a function of posttranslational processing (17). Consistent with this possibility is the finding of the alternative γ peptides and the β fragment. The β fragment is unlikely to be an artifact of the extraction procedure, since acid heat extraction disrupts aspartyl bonds, especially aspartyl-prolyl bonds, but not bonds formed by basic residues.

Substantial evidence indicates that neuron R15 is involved in the regulation of extracellular water volume or ionic composition (9–13). Regulation of water or ionic composition may be needed during a variety of different conditions,

including egg laying, feeding (18), and desiccation during low tides (19), and such regulation could involve a coordinated response involving locomotor behavior, renal, cardiac, and gill function, among others. Our evidence suggests that the R15 neuron may produce several different peptides, and this is consistent with its performing a coordinating role as suggested for the bag cells of *Aplysia* (20) and indicated for other peptidergic neurons such as those that secrete products of the POMC gene (21). The availability of the sequences of peptides synthesized by R15 should facilitate further functional studies of this neuron.

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