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A novel peptide designated PYL[a] and its precursor as predicted from cloned mRNA of *Xenopus laevis* skin

W. Hoffmann*, K. Richter and G. Kreil

Institut für Molekularbiologie, Österreichische Akademie der Wissenschaften, Billrothstrasse 11, A-5020 Salzburg, Austria

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A variety of peptides closely related to mammalian hormones and neurotransmitters are secreted from amphibian skin. Using cDNA clones of mRNA isolated from skin of *Xenopus laevis*, we have been searching for precursors of some of these constituents. Here we present the sequences of parts of cloned mRNAs which code for precursors of a novel peptide. In the predicted polypeptides, pairs of basic residues flank a sequence of 25 amino acids terminating with glycine, the signal for the formation of a terminal amide. The predicted final product liberated from these precursors would be a peptide comprised of 24 amino acids starting with tyrosine and ending with leucine amide, which has therefore been designated PYL[a]. This peptide can form an amphipathic helix similar to that found in peptides with cytotoxic, bacteriostatic and/or lytic properties.

Key words: amphiphathic helix/cDNA cloning/frog skin peptides/precursor processing/PYL[a]

Introduction

The skin secretions of amphibia contain a variety of biogenic amines and polypeptides of diverse physiological properties. From skin of different species of frogs, peptides similar or identical to thyrotropin-releasing hormone, substance P, gastrin/cholecystokinin, neurotensin and bradykinin have been isolated and characterized (for review, see Erspamer, 1971; Nakajima, 1980). Since no systematic, large-scale studies on these secretions have yet been undertaken, it appears quite likely that additional peptides might also be present. This is borne out by the recent finding that extracts from the skin of *Xenopus laevis* contain a peptide which is very similar to the head activator from *Hydra* (H.C. Schaller, personal communication).

We are interested in the biosynthesis of these frog skin peptides which we consider to be a model for the formation of homologous peptides serving as hormones and/or neurotransmitters in mammals. Using total mRNA from the skin of *X. laevis*, a cDNA library has been constructed and this library has been screened with radioactive cDNA prepared with the synthetic oligonucleotide d(AGTCCATCCA) as primer. This primer is complementary to part of the mRNA of caerulein, a decapeptide homologous to the mammalian hormone gastrin and cholecystokinin. Among the clones strongly hybridizing with this cDNA, several were identified with inserts derived from parts of mRNAs for caerulein precursors (Hoffmann *et al.*, 1983). In the course of these experiments, we also detected three other clones which did not contain genetic information for caerulein but for an unknown polypeptide which might be the precursor of a novel constituent of frog skin secretion.

*To whom reprint requests should be sent. Present address: ZYMOS Corporation, 1212 N, 35th Street, Seattle, WA 98103, USA

Results

Poly(A)-containing RNA was isolated from the skin of *X. laevis* and transcribed into cDNA with oligo(dT) as primer. Double-stranded cDNA was then prepared and inserted *via* poly(dG)-poly(dC) homopolymeric extensions into the unique *PstI* site of the plasmid pUC8 (Vieira and Messing, 1982) as described previously (Hoffmann *et al.*, 1983). From several thousand ampicillin-resistant clones, 300 were selected at random (named pUF1 – 300) and screened with radioactively labelled cDNA prepared from skin mRNA with the synthetic primer d(AGTCCATCCA). This oligonucleotide is complementary to the segment of caerulein mRNA coding for the peptide Trp-Met-Asp-Phe. Of the 300 clones, nine were found to strongly hybridize with this radioactive cDNA and three of these have been shown to contain inserts which code for parts of caerulein precursors (Hoffmann *et al.*, 1983).

We have now determined the nucleotide sequence of the inserts of three more clones from this group, numbered pUF38, 47 and 81 (see Figure 1). These sequences show a high degree of homology, yet none contains genetic information related to caerulein.

The largest insert is present in clone pUF38. It has a poly(A) tail and two polyadenylation signals AATAAA (Proudfoot and Brownlee, 1976) at a distance of 20 and 43 nucleotides from this end. Two such sites have also been observed in, for example, the mRNAs coding for parathyroid hormone (Hendy *et al.*, 1981) and somatostatin (Hobart *et al.*, 1980). Clone pUF81 is very similar to pUF38 and only differs by three deletions of two or three base pairs near the poly(A) end and 12 point mutations. The shortest insert found in clone pUF47 has no poly(A) tail and no polyadenylation signals. Furthermore, it differs from the two other clones by a deletion of five nucleotides near the 3′ end and by several point mutations.

All three clones have only one open reading frame in their inserts, which can be translated into almost identical amino acid sequences. The differences in the nucleotide sequence of these clones are either located after the stop codon of the unique reading frame or involve only third bases of codons which do not alter the genetic information. The only exceptions are two point mutations which change a phenylalanine to leucine and a glutamic to aspartic acid in the postulated amino acid sequences.

It is clear that these clones are derived from parts of mRNAs coding for closely related precursor polypeptides. The amino acid sequences deduced from these clones contain two pairs of basic residues which could function as recognition sites in a typical prohormone-hormone conversion. The resulting peptide of 25 amino acids would then contain a COOH-terminal glycine which could in turn serve as a signal for the formation of a terminal amide, as has been found for precursors of several other amidated peptides (see Discus-

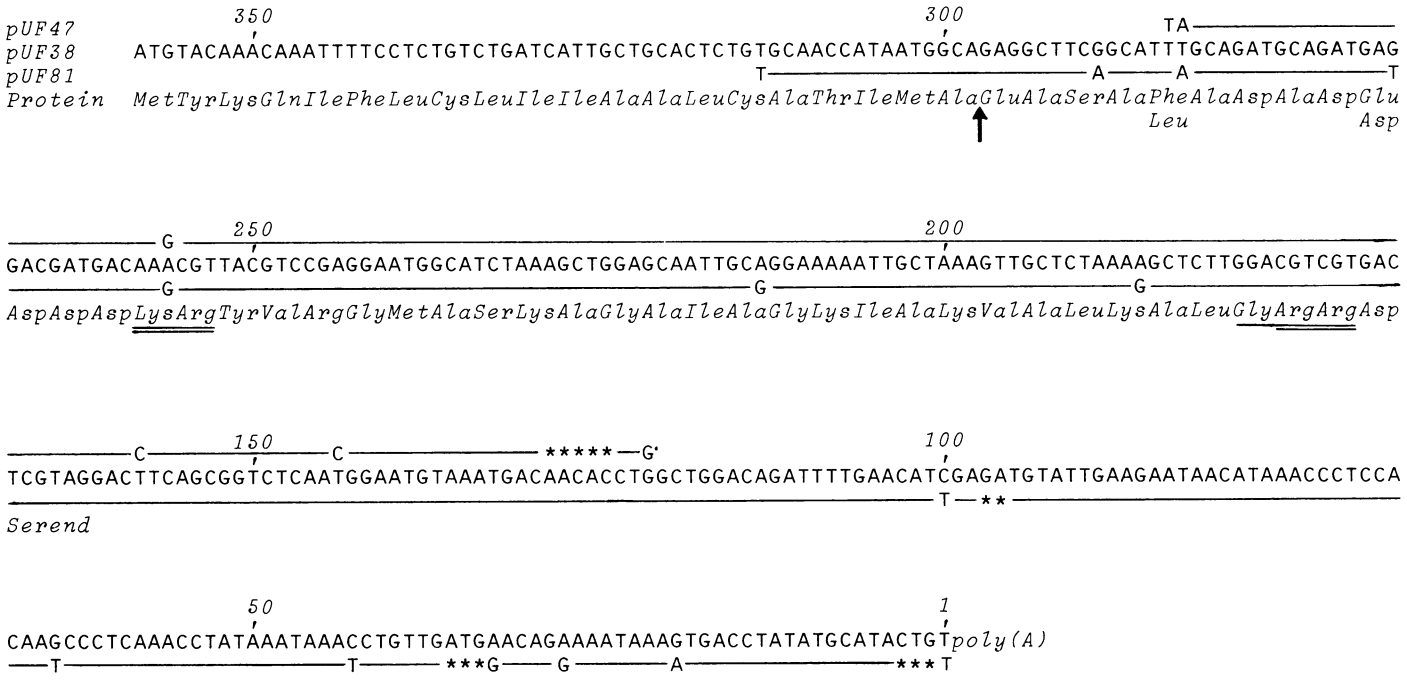

Fig. 1. Partial structures of different mRNAs coding for precursors of PYL[a]. The sequences were obtained from the inserts of the cDNA clones pUF47, 38 and 81. Deletions are indicated by stars. Pairs of basic residues and the terminal glycine are underlined.

sion). The final product of the postulated processing would be a peptide, comprised of 24 amino acids, with NH_2-terminal tyrosine, COOH-terminal leucine-amide and a net charge of +6. The amino acid composition is Lys_4, Arg_1, Ser_1, Gly_3, Ala_7, Met_1, Val_2, Leu_2, Ile_2, Tyr_1. This unusual composition with a large proportion of apolar and basic amino acids suggests that we are dealing with a membrane-active peptide.

In order to test whether these clones are derived from an mRNA of reasonable abundance, hybrid selected translation experiments were performed. Clone pUF81 was bound to nitrocellulose filters and used for hybridization with total skin mRNA as described by Cochet *et al.* (1979). An *in vitro* translation of the mRNA bound to the filter and of total frog skin mRNA are shown in Figure 2. The clone pUF81 clearly selects an mRNA which codes for a major polypeptide of mol. wt. ~9000. Both in the translate of total mRNA and in the hybrid selected translation, this band is somewhat diffuse and may represent two components with very similar migration. As a control, the clone pUF262 which contains part of the mRNA for a caerulein precursor in its insert (Hoffmann *et al.*, 1983) was included. As can be seen in Figure 2, this clone selects mainly an mRNA which codes for a polypeptide with a mol. wt. of ~25 000.

Discussion

Nucleic acid sequences of different mRNAs

The sequences presented in Figure 1 represent parts of different mRNAs which apparently code for very similar polypeptides and which could be the precursors of a novel peptide not previously observed in frog skin secretions. This information is present in at least three out of 300 randomly selected clones from a cDNA library prepared from skin of *X. laevis*. As already indicated, these three clones were detected using radioactive cDNA primed with an oligonucleotide complementary to part of the sequence of caerulein mRNA. It is not clear why the clones pUF38, 47 and 81 hybridize with this cDNA. There is a possible primer site at position 113 – 122 of the inserts of these clones (see Figure 1) which, however, included two mismatches. It is conceivable that for the preparation of cDNA with a small oligonucleotide, the exact fit between primer and the mRNA may not be of overriding importance (see for example Panabières *et al.*, 1982), and other factors, like secondary structure at putative binding sites, may also have a strong influence on the yield of a particular cDNA. Alternatively, these clones may hybridize with a caerulein cDNA because of a fortuitous similarity between the mRNAs coding for caerulein and this novel peptide. Indeed, pUF38 is remarkably similar to pUF262, one of the caerulein clones described previously (Hoffmann *et al.*, 1983). As shown in Figure 3, this similarity extends over most parts of the sequence and is particularly high, ~70%, prior to the respective polyadenylation sites. This latter homology is in fact quite striking and may have some biological significance.

Amino acid sequences and postulated processing of the precursors

It is noteworthy that the inserts of clones pUF38, 47 and 81 contain only a single open reading frame and that the observed differences in the nucleotide sequences are confined either to the putative 3′ non-coding regions or to the third positions of codons. The largest insert present in clone pUF38 can be translated into a polypeptide comprised of 64 amino acids. This starts with a methionine and contains an amino-terminal sequence of ~20 residues with the characteristics of signal sequences of secreted polypeptides. The possible cleavage site for signal peptidase is indicated by an arrow in Figure 1. It is thus possible that these 64 amino acids represent the complete pre-proform of this novel peptide, which could be the shortest precursor of this type hitherto observed in nature. The experiments using hybrid selected translation support this notion. As shown in Figure 2, the clone pUF81 binds an

mRNA which codes for a polypeptide with a mol. wt. of ~9000. Under these conditions, prepromelittin which is comprised of 70 amino acids migrates like cytochrome c. SDS-polyacrylamide gel electrophoresis is obviously quite unreliable for determing the mol. wt. of prepropeptides of this size. However, it appears quite likely that the primary translation product of the mRNA hybridizing with clone pUF81 is smaller than prepromelittin. An analysis of the amino-terminal sequence of this *in vitro* product will be necessary to establish the exact size of the precursor of PYL[a].

The two other clones, pUF47 and pUF81 contain inserts which code for shorter peptides than the one found in clone pUF38. These are however very similar and only two point mutations, namely leucine instead of phenylalanine and aspartic instead of glutamic acid, have been found (see Figure 1).

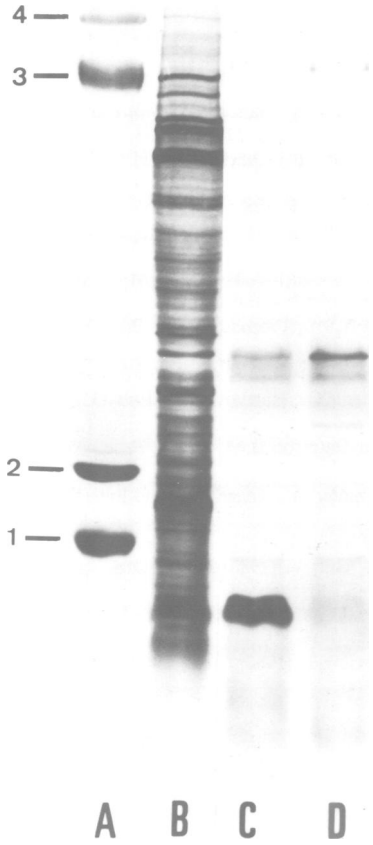

Fig. 2. Hybridization selection and translation of mRNAs. Translation products were separated by SDS-polyacrylamide gel electrophoresis. **Lane A**: mol. wt. markers labeled with succinimidyl [2,3-^{3}H]propionate. **(1)** Cytochrome c (12 500), **(2)** myoglobin (17 000), **(3)** bovine serum albumin (66 000), **(4)** phosphorylase B (97 000). **Lanes B,C,D**: translation products of frog skin mRNA. **Lane B**: total poly(A)$^+$ RNA; **lane C**: mRNA selected with plasmid DNA of clone pUF81; **lane D**: mRNA selected with plasmid DNA of clone pUF262.

The analysis of proinsulin and subsequently of many other pro-hormones and other pro-peptides has shown that in these precursors the sequences of the final product are mostly flanked by pairs of arginine/lysine residues (Steiner *et al.*, 1974). Moreover, in precursors of peptides terminating with an amide rather than with a free carboxyl group, a glycine residue is found adjacent to the COOH-terminal amino acid of the mature product (Suchanek and Kreil, 1977; Amara *et al.*, 1980). Both characteristics are present in the amino acid sequences deduced from the structure of the cDNA clones pUF38, 47 and 81. A segment of 24 amino acids is flanked by an Lys-Arg sequence at the amino terminus and by a Gly-Arg-Arg sequence at the other end. This leads us to the assumption that we have cloned parts of mRNAs for a prepropeptide which can be processed to an end product of 24 amino acids starting with tyrosine and ending with leucine-amide. According to a suggestion by Tatemoto and Mutt (1980), this postulated peptide is named PYL[a]. As two peptides from frog skin secretions, namely caerulein and a bradykinin-like component, have been shown to contain tyrosine-O-sulfate, it appears quite possible that the amino-terminal tyrosine of PYL[a] could also be modified *in vivo* in this way.

In the course of the processing of these putative precursors, other fragments would also be liberated. We do not know the precise location of the pre-pro-junction in this precursor, but it is noteworthy that in the amino-terminal part, possibly beginning with the pro-region (see arrow in Figure 1), a periodicity is present in the sequence in that every other residue is alanine. This suggests further processing of the pro-region by a dipeptidylaminopeptidase, as has been suggested for the caerulein precursor (Hoffmann *et al.*, 1983). Such an enzyme has in fact been detected in the skin secretion of *X. laevis* (A. Hutticher, unpublished experiments). Activation of precursors by stepwise cleavage of dipeptides has previously been demonstrated for honeybee promelittin (Kreil *et al.*, 1980) and yeast α-mating factor (Thorner *et al.*, 1982). In the present case, processing by a dipeptidylaminopeptidase would, however, still be incomplete as it would be expected to stop at the sequence Asp-$\overset{\text{Asp}}{\underset{\text{Glu}}{}}$-Asp-Asp, which precedes the first pair of arginine residues.

Possible biological action

At present, nothing is known about the possible physiological properties of a peptide with the sequence deduced from these cDNA clones. If one arranges PYL[a] into an α-helical configuration (see Figure 4), it is striking that this yields a highly amphipathic structure (Segrest *et al.*, 1974).

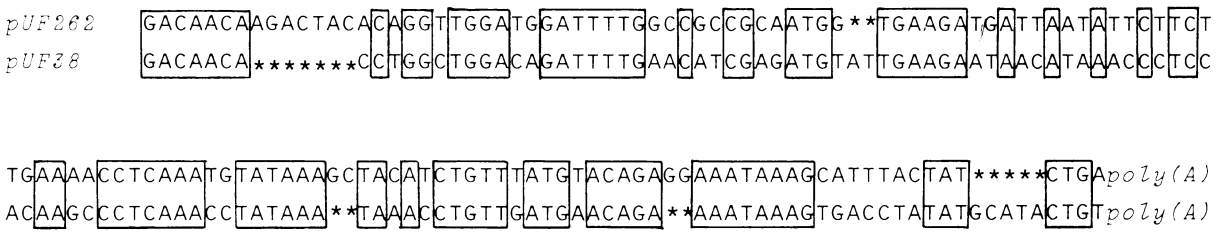

Fig. 3. Comparison of the 3′ ends of cDNA clones pUF38 and pUF262 (data from Hoffmann *et al.*, 1983). Homologous sequences are enclosed in boxes, deletions are indicated by stars.

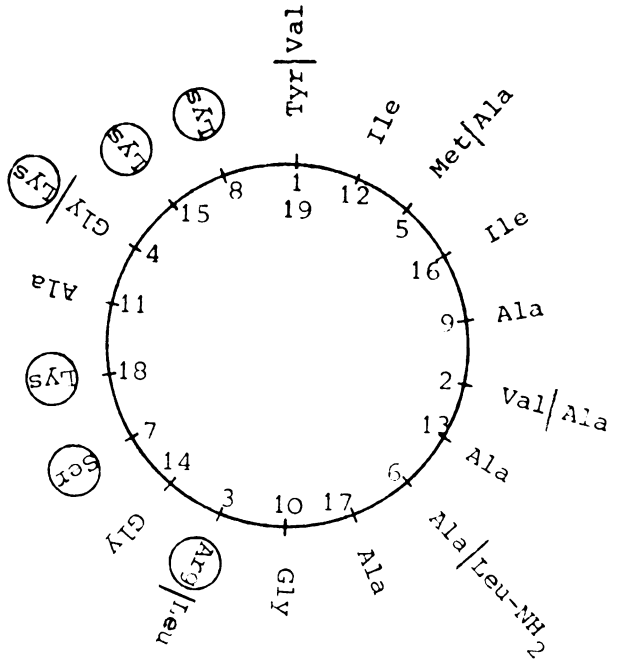

Fig. 4. Axial projection of the α-helical conformation from PYL[a]. Hydrophilic amino acids are circled to show the highly amphipathic structure.

Several examples are known where peptides capable of forming amphipathic helices have been shown to have a high affinity for cell membranes and to have cytotoxic, bactericidal or lytic effects. These include mellitin (Terwilliger and Eisenberg, 1982), a melittin-like synthetic peptide (DeGrado *et al.*, 1981), δ-haemolysin from *Staphyloccocus aureus* (Fitton *et al.*, 1980) and cecropins, a group of insect hemolymph peptides with antibacterial properties (Boman and Hultmark, 1981; Merrifield *et al.*, 1982). In view of this, we would predict that PYL[a] is a peptide with affinity for phospholipid bilayers which may have any of the above-mentioned effects. It will be interesting to check whether such a peptide predicted from a cDNA sequence can be isolated from the skin secretion of *X. laevis* and what, if any, physiological action it may have.

Materials and methods

The decanucleotide d(AGTCCATCCA) was synthesized in solution by a modified triester method (Narang *et al.*, 1980). All experimental procedures, including construction of the cDNA library, detection and sequence analysis of the clones, etc. have been described in a previous publication (Hoffmann *et al.*, 1983).

For hybrid selected translation, plasmid DNAs from clones pUF81 and clone pUF262 (Hoffmann *et al.*, 1983) were immobilized on nitrocellulose filters. Filters were hybridized with poly(A)$^+$ RNA from frog skin and washed as described by Cochet *et al.* (1979). Extracted mRNA was precipitated once with ethanol in the presence of 10 μg carrier tRNA. The mRNA was translated in the wheat germ system (Roberts and Paterson, 1973) with some modifications (Richter *et al.*, 1980) in the presence of [^{35}S]methionine (Amersham International, sp. act. 600 Ci/mmol). Translation products were separated by electrophoresis in 15% polyacrylamide gels (Laemmli, 1970) and treated for fluorography (Bonner and Laskey, 1974).

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