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26. We gratefully acknowledge the technical suggestions contributed by B. I. Hillman, J. C. Carrington, and D. G. Garrott. Supported in part by USDA/ARS grant 12-14-5001-272 and grants from the California Cling Peach Association.

3 June 1987; accepted 7 August 1987

D-Alanine in the Frog Skin Peptide Dermorphin is Derived from L-Alanine in the Precursor

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A D-alanine–containing peptide termed dermorphin, with potent opiate-like activity, has been isolated from skin of the frog *Phyllomedusa sauvagei*. Complementary DNA (cDNA) libraries were constructed from frog skin messenger RNA and screened with a mixture of oligonucleotides that contained the codons complementary to five amino acids of dermorphin. Clones were detected with inserts coding for different dermorphin precursors. The predicted amino acid sequences of these precursors contained homologous repeats of 35 amino acids that included one copy of the heptapeptide dermorphin. In these cloned cDNAs, the alanine codon GCG occurred at the position where D-alanine is present in the end product. This suggests the existence of a novel post-translational reaction for the conversion of an L-amino acid to its D-isomer.

AMPHIBIAN SKIN IS A RICH SOURCE of biologically active peptides, many of them related to mammalian hormones or neurotransmitters (*1*). The skin of the South American tree frog *Phyllomedusa sauvagei* contains several different peptides in impressive amounts, up to 700 μg per gram of tissue (*2*), of which phyllocaerulein, phyllokinin, sauvagine, and dermorphin have been isolated and studied in some detail. Dermorphin has opioid activity, particularly on the central nervous system, that is up to 1000-fold greater than that of morphine (*2, 3*). This heptapeptide, which has the sequence Tyr-Ala-Phe-Gly-Tyr-Pro-Ser amide (*4*), contains an alanine in position 2 that is in the D-configuration. A synthetic dermorphin analog with the D- replaced with L-alanine was found to be devoid of biological activity (*5*). To our knowledge, this was the first case of a D-amino acid being detected in a peptide of animal origin. Material cross-reacting with antibodies against dermorphin could also be detected in mammalian tissues (*6*).

By analogy with the biosynthesis of D-amino acid–containing peptide antibiotics (such as gramicidins, tyrocidines, and bacitracins) in microorganisms, one might expect dermorphin to be synthesized by a multisubunit enzyme that binds individual amino acids as thioesters (*7*). Alternatively, dermorphin might be excised from a larger precursor polypeptide after synthesis on ribosomes, as has been demonstrated for numerous peptides of diverse function. By an unknown post-translational reaction, the D-alanine may then originate from an L-amino acid present in the precursor polypeptide.

In an effort to understand the mechanism of dermorphin synthesis, we have twice obtained 20 live specimens of *Ph. sauvagei*, caught near Tucuman (Argentina). For preparation of messenger RNA (mRNA) from the tough, waxy skin of this frog, a combination of methods was used (*8*). Only about 1 μg of polyadenylated RNA could be obtained per gram of *Phyllomedusa* skin, which was less than 10% of the amount previously obtained from skin of *Xenopus laevis* (*9*). Double-stranded (ds) complementary DNA (cDNA) prepared from skin mRNA was inserted into plasmid pUC18 (*10*) and transformed into *Escherichia coli* RR1; 50,000 clones were then screened with the oligonucleotide d(GANGGPTANCCPAA) (*11*). Four clones contained inserts coding for fragments of dermorphin precursors (D-9, D-12, D-43, and D-44) and each of these contained two or more cleavage sites for the restriction endonuclease Alu I separated by 105 base pairs (bp).

A second library was then constructed in λ-gt11 (*12*). The library was amplified and screened in *E. coli* Y1090r⁻ (Promega Biotec). As hybridization probes, the synthetic oligonucleotide as well as a nick-translated 105-bp Alu I fragment obtained from the clones mentioned above were used. After additional subcloning of strongly hybridizing phages, clones D-1/2, D-2/2, D-4/1, D-6/3, and D-8/1 (which had inserts of more than 400 bp and the 105-bp Alu I fragment) were sequenced (*13*).

The nucleotide sequence of the insert present in clone D-1/2 is shown in Fig. 1. This sequence of 700 bases contains a single open reading frame that starts with an ATG codon at position 43 and ends with a TAA stop codon at position 634. The 3′ untranslated region has a modified polyadenylation signal AATATA and it ends with a poly(A) tract. In Fig. 1, we also show the sequence of the clones D-4/1 and D-43, which were incomplete at the 3′ and 5′ end, respectively. Except for a single point mutation, D-43 is identical to the 3′ half of D-1/2. On the other hand, D-4/1 differs markedly in its middle region from D-1/2, while at both ends long stretches of sequence identity exist. The two types of mRNA represented by clones D-1/2 and D-4/1 could be generated through alternative splicing of a common pre-mRNA, as has been observed for other precursors of peptide hormones (*14*). Clone D-8/1 has an insert which starts with a sequence of 149 nucleotides almost identical to the 3′ ends of clones D-1/2 and D-43. Of the two point mutations, one is in the polyadenylation signal (from AATATA to AATACA), and this cDNA has a 3′ untranslated region longer by at least 100 nucleotides (*15*). It thus appears likely that several types of mRNAs coding for dermorphin precursors are present in the skin of *Ph. sauvagei*.

The cloned nucleotide sequences contained up to five very similar copies of the 105-bp repeat; each repeat had one site for Alu I and contained approximately 50 adenines and 30 guanines. Apparently, the high adenine content caused wrong priming by oligo(dT) in the reverse transcriptase reaction. In the insert present in clone D-4/1 (Fig. 1) and in several of the clones shown in Fig. 2, reverse transcription had started at an AAAAAAATAAAAA sequence present once in each repeat. While this explains the internal priming during cDNA synthesis, we do not know why many of the cloned inserts were also incomplete at the 5′ end. One reason for this could be instability of the repetitive sequences in the host bacteria.

The open reading frame present in the insert of clone D-1/2 can be translated into a 197–amino acid polypeptide that starts with an initiating methionine followed by a typical signal sequence. In this precursor polypeptide, four copies of a 35–amino acid repeat are present; three are complete, and one is incomplete, but each contains one dermorphin sequence. An additional incomplete repeat contains a different heptapeptide. Clone D-4/1 yields a similar polypep-

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tide with a total of five dermorphin copies (see Fig. 1).

Several additional clones with incomplete inserts have been detected. The open reading frames present in these cDNAs can be translated into polypeptides containing one or more copies of the 35–amino acid repeat (Fig. 2). However, some of the clones, as for example D-12 and D-9, are probably derived from different dermorphin mRNAs as they differ by one or more point mutations from the other cDNAs. In these clones, two main variants of the 35–amino acid repeat have been detected, which strictly alternate with each other. One type is characterized by the presence of two asparagines and a TTC codon for phenylalanine in the dermorphin sequence, while the other has lysine and isoleucine and a TTT codon in the respective positions. Except for the few point mutations shown in Fig. 2, the two types of repeats are identical in their nucleotide sequences. This close similarity also with respect to third positions of codons is surprising. Possibly each repeat or pair of repeats is encoded by a separate exon and these exons are kept homologous by gene conversions (*16*). Homologous repeats have also been observed in the caerulein precursors of *X. laevis* (*9*).

Each of these 35–amino acid repeats contains one dermorphin sequence flanked by the typical pro-hormone processing signal Lys-Arg at the amino end and by Gly-Glu-Ala-Lys-Lys at the carboxyl end (Fig. 3). Excision of the paired lysine/arginine residues would yield an intermediate with an extra tripeptide at the carboxyl end. Cleavage of the dipeptide Glu-Ala by a carboxy- or a dipeptidyl carboxypeptidase would then expose a glycine required for formation of the terminal amide (*17*). In the insert present in clone D-1/2, one dermorphin copy is replaced by a different heptapeptide which is, however, flanked by the same processing signals. Besides dermorphin, this precursor should therefore also yield the peptide Tyr-Met-Phe-His-Leu-Met-Asp-NH_2 as one of the end products (Fig. 3). This peptide has not been observed during analysis of peptides present in the skin of *Ph. sauvagei*; moreover, it is apparently not related to any known peptide from another source.

The second codon in the dermorphin sequence, which is present more than 20 times in the different cloned cDNAs, is always the alanine codon GCG. This implies the presence of a normal L-alanine in the primary products of translation. At some stage in the processing cascade this has to be converted to the D-isomer. At present, nothing is known about an enzymatic mechanism whereby an L-amino acid could be converted to the D-isomer in the peptide linkage. By analogy with the two-base mechanism proposed for some amino acid racemases (*18*), one could hypothesize that removal of a proton from the α-carbon of the particular alanine yields a planar intermediate which can then isomerize to the D-configuration. However, contrary to the reaction catalyzed by racemases, formation of the D-isomer should go to completion as

Fig. 1. Sequence of cloned prepro-dermorphin cDNAs are presented. Clone D-1/2 is compared with clone D-4/1 with differences in the nucleotide sequence marked above and in amino acid sequence below the D-1/2 sequences. Identities are marked (. . .), the stop codon is (///). Two gaps (***) have been introduced into the sequence of D-1/2 to maximize homology. Dermorphin sequences are underlined. Clone D-43 has an identical nucleotide sequence to D-1/2 from (●) to the poly(A) tail.

Fig. 2. Internal 35–amino acid repeats in dermorphin precursors. Identical amino acids (and corresponding codons) are marked by (−), stop codons by (/). Dermorphin sequences at the beginning of each line are emphasized, the (=) stands for the codon TTT. Gaps have been introduced to mark the end of the dermorphin sequence and of the carboxyl-terminal processing sequence. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

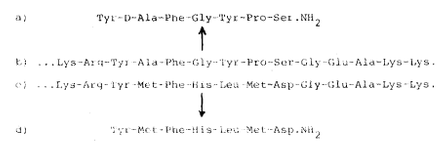

Fig. 3. Sequences of end products and of the corresponding segments in the precursors. (a) Sequence of dermorphin; (b) dermorphin and flanking sequences deduced from the cloned cDNAs; (c) homologous region from insert of clone D-1/2 (see Fig. 1); (d) sequence of predicted peptide, assuming the same processing cascade as for the release of dermorphin. Arrows indicate post-translational processing.

only dermorphin with a D-alanine has been detected in *Phyllomedusa* skin (*4*). The reaction may thus require energy.

The presence of a D-amino acid in other animal peptides has not been considered in recent decades. Minute quantities of D-aspartic acid have been detected in proteins of aging erythrocytes (*19*), but these apparently arise at random via chemical isomerization. The postulated existence of a mechanism in an amphibian species which converts a particular L-alanine to its D-isomer raises the question whether this may also occur elsewhere. It will now be possible to use the cDNAs cloned from skin of *Ph. sauvagei* to search for dermorphin-like sequences in genomic libraries from other sources. The detection of such sequences in a more accessible species is a prerequisite to the study of epimerization of an amino acid during processing of a peptide precursor.

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20. We thank S. Burgschwaiger for help with the sequencing of cDNA clones and J. Greco and F. Barbieri (National University of Tucuman) for supplying the frogs. Supported by grant S29T4 from the Austrian Fonds zur Förderung der Wissenschaftlichen Forschung.

14 April 1987; accepted 1 July 1987

Transformation by Oncogenes Encoding Protein Kinases Induces the Metastatic Phenotype

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Oncogenes encoding serine/threonine or tyrosine kinases were introduced into the established rodent fibroblast cell line NIH 3T3 and tested for tumorigenic and metastatic behavior in T cell–deficient nude mice. Transforming oncogenes of the *ras* family were capable of converting fibroblast cell lines to fully metastatic tumors. Cell lines transformed by the kinase oncogenes *mos, raf, src, fes*, and *fms* formed experimental metastases and (in some cases) these genes were more efficient at metastatic conversion than a mutant *ras* gene. In contrast, cells transformed by either of two nuclear oncogenes, *myc* or p53, were tumorigenic when injected subcutaneously but were virtually nonmetastatic after intravenous injection. These data demonstrate that, in addition to *ras*, a structurally divergent group of kinase oncogenes can induce the metastatic phenotype.

METASTASIS IS THE PROCESS BY which tumor cells spread and colonize secondary sites throughout an organism. For a cell to be able to metastasize, many specialized characteristics are required, including the ability to invade through host barriers into the vasculature, survive in the circulation, extravasate, and subsequently establish and grow. Recent work has firmly established that NIH 3T3 and 10T1/2 cells transformed by *ras* genes are capable of forming metastases (*1, 2*), and this appears to be a direct result of *ras* function (*2*). Also, transfection of activated *ras* into poorly metastatic murine adenocarcinoma cells significantly enhances metastatic potential (*3*). However, it is unlikely that aberrant *ras* function is essential for all tumor dissemination since not all metastases contain activated *ras* sequences (*4*), and correlative studies have implicated amplification of non–*ras* oncogenes in progression of specific tumors (*5, 6*). To directly test the hypothesis that other oncogenes may be involved in regulating or inducing metastatic activity, we have assessed the ability of NIH 3T3 (clone 7) cells transformed by a wide variety of oncogenes to form experimental and spontaneous metastases. We report here that transformation by all kinase-encoding oncogenes tested results in the metastatic conversion of this fibroblast line.

Representative oncogenes of the cytoplasmic serine/threonine kinases were evaluated for their ability to induce experimental metastases. Three lines of NIH 3T3 cells transformed by v-*mos* were isolated and cloned from transformed foci after Moloney murine sarcoma virus infection (*7*). Southern blot analysis revealed the presence of v-*mos* sequences in each clone (*7*). These freshly transformed cell lines (which were not releasing

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