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Characterization of Novel mRNAs Encoding Enzymes Involved in Peptide α-Amidation*

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The COOH-terminal α-amidation of bioactive peptides is a 2-step process catalyzed by two separable enzymatic activities both derived from the peptidylglycine α-amidating monooxygenase (PAM) precursor. Two forms of PAM mRNA (rPAM-1 and -2), differing by the presence or absence of optional Exon A, were previously characterized; both encode precursors predicted to have an NH_2-terminal signal sequence, an intragranular domain containing both enzymatic activities, and a single transmembrane domain followed by a short, cytoplasmic COOH-terminal domain. In this report, two novel types of PAM mRNA were identified in adult rat atrium. A cDNA of each type was sequenced, and the results indicate that rPAM-3 and -4 could be related to each other and to the previously characterized rat PAM cDNAs by alternative mRNA splicing. Deletion of a 258-nucleotide segment (optional Exon B) encoding the transmembrane domain from rPAM-3 and the presence of a novel 3′-exon in rPAM-4 mean that both rPAM-3 and -4 mRNAs encode precursor proteins that have an NH_2-terminal signal peptide but lack a transmembrane domain. The rPAM-4 precursor protein lacks the region of the PAM precursor catalyzing the second step in the α-amidation reaction. Low levels of rPAM-3 and -4 type mRNA were detected in atrium. Utilizing the polymerase chain reaction, two major patterns of distribution of forms of PAM mRNA were found. In the heart and central nervous system, PAM mRNAs both containing and lacking optional Exon A were prevalent and almost all of the PAM mRNAs detected contained optional Exon B. In the pituitary and submaxillary glands, PAM mRNAs lacking optimal Exon A were prevalent, as were PAM mRNAs lacking all or part of optional Exon B. Since the distribution of PAM activity between soluble and membrane fractions is tissue-specific and developmentally regulated and since rPAM-4 lacks an enzymatic portion of the PAM precursor, the tissue-specific expression of these forms of rat PAM mRNA is expected to be of functional significance.

More than one-half of all known bioactive peptides terminate with an essential carboxyl-terminal α-amide. Recent studies have demonstrated that conversion of peptidylglycine substrates into α-amidated products is a 2-step process involving the copper- and ascorbate-dependent production of peptidyl-α-hydroxyglycine; at very alkaline pH, α-amidated peptides can form spontaneously, but at neutral to acidic pH values a separate enzymatic activity catalyzes the subsequent formation of α-amidated product peptides (1–6). The enzyme catalyzing the first step is derived from the NH_2-terminal third of the peptidylglycine α-amidating monooxygenase (PAM)[1] (EC 1.14.17.3) precursor and is a peptidylglycine α-hydroxylating monooxygenase, while the second step is catalyzed by a separable peptidyl-α-hydroxyglycine α-amidating lyase whose activity derives from the remaining intragranular region of the PAM precursor (5).

PAM cDNAs isolated from bovine intermediate pituitary (bPAM-1 (formerly λPAM-1) and -2 (formerly λPAM-5), frog skin (AE-II), and rat atrium (rPAM-1 and -2) cDNA libraries (7–9) encode approximately 100-kDa proteins with a single putative transmembrane domain (Fig. 1). The precursors are predicted to contain an NH_2-terminal signal sequence followed by a short propeptide. The monooxygenase domain situated in the NH_2-terminal third of the precursor is followed by the lyase domain, a transmembrane domain, and a cytoplasmic tail. The bovine and rat PAM cDNAs appear to represent alternatively spliced mRNAs (7, 9), and preliminary analysis of genomic clones is consistent with the presence of one large gene encoding PAM in the rat.[2] bPAM-2 lacks a 54-nt segment encoding 18 amino acids in the putative cytoplasmic tail (Fig. 1) (7). rPAM-1 and -2 are identical with each other except for the deletion from rPAM-2 of a 315-nt segment (optional Exon A) which encodes a 105-amino acid peptide situated between the monooxygenase and lyase domains (Fig. 1) (9). Frog skin AE-II is homologous to rPAM-2. In contrast, frog skin AE-I encodes a 39-kDa soluble protein that includes the monooxygenase domain but lacks the peptide encoded by optional Exon A and the lyase domain (10). AE-I and -II are highly homologous but not identical and must arise by transcription of separate genes (8, 10); in the rat, there is no evidence for multiple genes.[2]

Northern blot analysis of RNA from a variety of rat tissues suggested the presence of additional forms of PAM mRNA (11). To search for additional mammalian mRNAs encoding PAM, we rescreened our atrial cDNA library. We now report the characterization of two novel types of PAM mRNA encoding soluble proteins. rPAM-3 encodes a soluble protein containing both enzymatic activities (peptidylglycine α-hy-

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[1] The abbreviations used are: PAM, peptidylglycine α-amidating monooxygenase; AE, amidating enzyme; RT, reverse transcription; nt, nucleotide(s); bp, base pair(s); kb, kilobase(s); PCR, polymerase chain reaction.

[2] L'H. Ouafik, D. A. Stoffers, B. T. Bloomquist, T. A. Campbell, and B. A. Eipper, manuscript in preparation. Genomic clones spanning 95 kb of the PAM gene have been mapped, and the intron-exon junctions reported here have been sequenced. The PAM gene in the rat appears to be greater than 95 kb in length with no evidence for multiple genes.

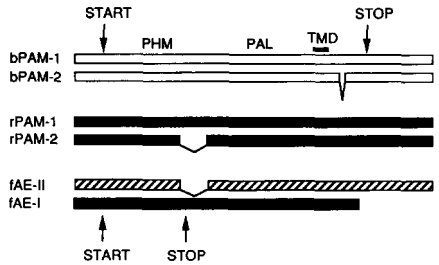

FIG. 1. **PAM cDNAs previously isolated from bovine (7), rat (9), and frog (8, 10) cDNA libraries.** Regions of complete nucleotide identity are indicated by identical *bar shadings* in the *scale drawings*. *Breaks* indicate missing segments and are connected by *solid lines*. The positions of the translation initiation and termination codons (fAE-I is shown at the *bottom*) are shown as well as the location of the monooxygenase (*PHM*), lyase (*PAL*), and transmembrane (*TMD*) domains.

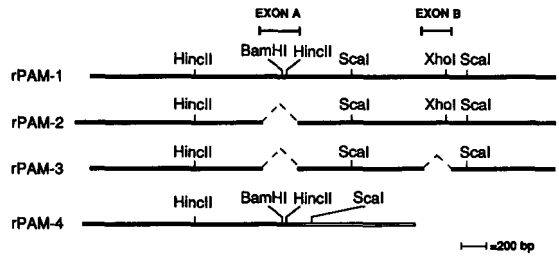

FIG. 2. **Four forms of rat atrium PAM cDNA.** rPAM-1 and -2 were previously described (9). *Solid bars* indicate regions of complete identity among the clones. *Dashed lines* demarcate missing segments. The *open bar* indicates the unique 3′ end of rPAM-4. Shown above the lines are selected restriction endonuclease cleavage sites used to map and distinguish among the four types of PAM cDNA.

droxylating monooxygenase and peptidyl-α-hydroxyglycine α-amidating lyase), while rPAM-4 encodes a soluble protein containing only the monooxygenase domain. The corresponding mRNAs were confirmed by Northern analysis and combined reverse transcription-polymerase chain reaction (RT-PCR). Identification of rPAM-3 has been reported in preliminary form (12).

MATERIALS AND METHODS

Screening of Adult Rat Atrium cDNA Library and Sequencing of cDNA Clones—The synthesis of the adult rat atrial cDNA library using the λZAP vector was described previously (9). Clone Z9 (rPAM-3 type cDNA) was isolated during the initial screening and was subsequently distinguished from rPAM-2 type inserts by restriction enzyme analysis with *Xho*I and *Sca*I (Fig. 2). The unamplified library was rescreened (200,000 phage) using shorter cDNA probes derived from rPAM-1: 5′-untranslated region (*Eco*RI site of vector/*Pst*I at bp 355), optional Exon A (*Xmn*I/*Hin*cII; bp 1517–1708), and optional Exon B (*Sfa*NI/*Bst*NI; bp 2854–3034). Of 267 plaques hybridizing with the 5′-untranslated region probe, 24 containing potentially novel PAM cDNA inserts based on their hybridization patterns with probes A and B were plaque-purified, rescued as Bluescript plasmids by automatic excision using R408 helper phage, and characterized by restriction endonuclease mapping. Of eight phage hybridizing with both optional Exon A and B probes, six had restriction maps compatible with rPAM-1 type cDNAs and two had unique restriction maps. Upon sequencing, these two were found to diverge from rPAM-1 at *Eco*RI sites (used in cloning) and are presumed to be cloning artifacts resulting from multiple insertion. Of three plaques hybridizing with probe A but not probe B, two were very short (1.5 kb) and one had a unique restriction map (clone Z2-20) and was further characterized. All five plaques hybridizing with probe B and not probe A had restriction maps compatible with rPAM-2 type cDNAs. Of six plaques hybridizing with neither probe A nor probe B, one had a restriction map compatible with rPAM-3, three were very short (<1.5 kb), and two had unique restriction maps but were subsequently shown to be cloning artifacts as above.

Sequencing was by the dideoxy chain termination method (13) using the T7 Sequencing Kit (Pharmacia LKB Biotechnology Inc.). The insert in Z9 was sequenced on one strand in its entirety and on both strands across presumed splice junctions using synthetic oligonucleotide primers. The insert in Z2-20 was sequenced on one strand over the region in common with rPAM-1 using synthetic oligonucleotide primers; the 1.2-kb 3′ region unique to Z2-20 was sequenced on both strands and across the *Sca*I restriction site used in subcloning.

Northern Blot Analysis—Total RNA was prepared from adult male rat (Sprague-Dawley) atria using the acid guanidinium isothiocyanate/phenol/chloroform procedure (14). RNA was fractionated on a 1.0% formaldehyde agarose gel and transferred to Nytran as previously described (7). The riboprobe was synthesized as a T3 RNA polymerase transcript of Bluescript plasmid pA6 containing the 0.98-kb *Sca*I-*Eco*RI fragment derived from the unique 3′-untranslated region of rPAM-4 (plasmid Z2-20) using [^{32}P]CTP. An RNA ladder (Bethesda Research Laboratories) was fractionated in an adjacent lane. Prehybridization, hybridization, and washing conditions were also as previously described except that prehybridization and hybridization were carried out in the presence of 5% dextran sulfate, and all steps were carried out at 65 °C (7).

Combined Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)—Total RNA was prepared from adult male rat tissues as above or with the guanidinium isothiocyanate/cesium chloride procedure (15). RNA (5 μg) from rat atrium, ventricle, anterior pituitary, neurointermediate pituitary, hypothalamus, submaxillary gland, and cerebral cortex was reverse-transcribed into cDNA using 1 μg of oligo(dT)$_{12-18}$ (Pharmacia) as primer in a 20-μl reaction volume containing 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 50 μg/ml bovine serum albumin, 1.25 μl RNasin (Promega Biotec), 0.5 mM concentration of each dNTP (Pharmacia), and 10 units of avian myeloblastosis virus reverse transcriptase (Life Sciences) at 42 °C for 60 min. The amount of reverse-transcribed RNA used as substrate in the PCR was adjusted to represent similar amounts of total PAM RNA as previously determined by quantitative Northern blot analysis (11). Minor adjustments were subsequently made to result in similar amounts of PCR product. Synthetic oligonucleotide primers utilized in the PCR were all 17-mers. Primers yielding sense cDNA were (all base pair numbers are for rPAM-1): 3 (2517–2533), 4 (1359–1375), 5 (366–382), 9 (3124–3140), and 11 (1060–1076). Primers yielding antisense cDNA were: 10 (3188–3172), 16 (3418–3140), 17 (2290–2274), 19 (1455–1439), and 21 (3834–3816). Antisense primer 14 (2238–2222 of Z2-20) was specific to rPAM-4. PCRs were typically carried out in a 50-μl volume according to Cetus specifications: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM concentration of each dNTP, 1 μM concentration of each primer, varying amounts of cDNA or plasmid standard, and 1.25 units of Amplitaq DNA polymerase (Cetus). Samples were overlaid with 1 drop of light mineral oil (Sigma) and subjected to 24 to 36 cycles in the Perkin-Elmer Cetus thermal cycler. Cycling parameters were generally as follows: the initial denaturation step was at 94 °C for 4 min; the repeat cycle consisted of annealing at 45 to 52 °C for 2 min followed by extension at 72 °C for 2 or 3 min and denaturation at 94 °C for 1 min; the last extension time was lengthened to 5 min. Annealing temperatures and number of cycles depended on the particular pair of primers used and are indicated in the figure legends. After thermal cycling, most of the oil was manually removed and the remaining oil was extracted with chloroform. The aqueous phase was treated with Gene Clean (Bio101, Inc.) according to the manufacturer's instructions and resuspended in 10 μl of sterile water. Samples were fractionated on agarose gels in 89 mM Tris, 89 mM borate, 2.5 mM EDTA, pH 8.0 buffer. For gels containing more than 2.0% agarose, a 1:1 mixture of agarose and NuSieve agarose (FMC Bioproducts) was employed. After staining with ethidium bromide, gels were photographed and prepared for Southern transfer by soaking for 10 min in 1.5 M NaCl, 0.5 N NaOH and then for 30 min in 1 M Tris-HCl, pH 8.0, 1.5 M NaCl and transferred to Nytran as described (7). The filters were hybridized with random-primed rPAM-1 cDNA insert, washed as previously described (7), and exposed to film to verify the identity of the bands.

RESULTS

Identification and Characterization of Additional PAM cDNA Clones—In order to determine whether a form of PAM analogous to bPAM-2 (Fig. 1) existed in the rat atrium, the

18 PAM cDNAs obtained in the initial library screen (9) were examined by restriction analysis with *Xho*I whose unique site is in the middle of the 54-nt segment missing in bPAM-2. One clone (Z9; rPAM-3 type) out of the set of 18 was missing this *Xho*I site (Fig. 2). Further restriction mapping with *Sca*I, which generates an internal restriction fragment bracketing this *Xho*I site, showed that approximately 250 bp, not the anticipated 54 bp, were missing from Z9.

The insert in Z9 is 3096 bp long and extends from base 175 to base 3863 of the previously reported rPAM-1 cDNA sequence (9) (Fig. 3). Like rPAM-2, the insert in Z9 is missing nucleotides 1474–1788; until the sequence of the gene encoding PAM is available, we will refer to this 315-nt segment as optional Exon A (Fig. 2). The distinguishing feature of the insert in Z9 is that it does not contain nucleotides 2791–3048 (optional Exon B). The 3′ end of optional Exon B corresponds exactly to the 3′ end of the 54-nt deletion distinguishing bPAM-2 from bPAM-1 (Fig. 3). This 258-bp segment encodes an 86-amino acid domain (amino acids 832–917) containing the entire putative transmembrane domain as well as 35 amino acids of the intragranular domain and 27 amino acids of the cytoplasmic domain (Fig. 3); PAM cDNAs lacking both optional Exon A and optional Exon B are referred to as rPAM-3 type.

To search for cDNAs derived from additional forms of PAM mRNA, the adult rat atrium cDNA library was rescreened using cDNA probes capable of distinguishing cDNAs of the rPAM-1, -2, and -3 type. Of 200,000 plaques screened, 267 phage were recognized by the 5′-untranslated region probe. Twenty-four of these (see "Materials and Methods") were characterized by restriction mapping; 19 exhibited patterns consistent with their identification as rPAM-1, -2, or -3 type cDNAs. Four had unique restriction patterns but were subsequently shown to be cloning artifacts. One clone (Z2-20) was selected for further analysis based on the pattern obtained by restriction digestion with *Sca*I (Fig. 2), which resulted in a slightly longer than expected fragment from the 3′ end of the insert.

The cDNA insert in Z2-20 is 2995 bp long and is identical with rPAM-1 from nucleotides 1–1788; the entire sequence of optional Exon A is present. The sequence of clone Z2-20 diverges after bp 1788, the 3′ border of optional Exon A (Figs. 2 and 4). The 1207 bp following this junction bear no resemblance to any region of rPAM-1 or -2. The open reading frame Z2-20 continues for only 20 amino acids beyond optional Exon A before an in-frame stop codon is encountered (Fig. 4); the protein encoded by Z2-20 has a predicted molecular weight of 57,809. The coding region is followed by a 1,148-bp AT-rich 3′-untranslated region containing a consensus poly(A) addition site 39 nucleotides upstream from a string of 12 As at the end of the cDNA (Fig. 4). PAM cDNAs of this type are referred to as rPAM-4 type. The 20-amino acid segment that distinguishes the protein encoded by rPAM-4 from the other PAM proteins is very positively charged (1 Arg, 4 Lys, 2 His) and includes 2 Cys residues. rPAM-4 and frog skin AE-I, while both encoding short soluble forms of PAM, exhibit key differences. Frog AE-I does not contain a region homologous to optional Exon A and the divergent 10 amino acids at the COOH terminus of AE-I are not homologous to the 20 COOH-terminal amino acids of rPAM-4. In addition, no significant homology could be detected between the 3′-untranslated regions of rPAM-4 and frog AE-1; this is in striking contrast to the high degree of homology found in an 86-nucleotide segment of the 3′-untranslated regions of frog AE-II, rPAM (1/2/3), and bPAM (1/2) (7–9).

Detection of rPAM-3 and -4 mRNAs—Since rPAM-3 appeared to be of low abundance in the adult rat atrium (based on its 1/18 abundance in the atrial cDNA library) and differed from rPAM-1 and -2 only by deletions, reverse transcription followed by PCR utilizing oligonucleotide primers flanking optional Exon B was used to identify mRNA of the rPAM-3 type in adult rat atrium and ventricle. As expected, the major product detected on the ethidium bromide-stained gel and after blotting and hybridization was the size of the 671-bp fragment generated from mRNAs of the rPAM-1 and -2 type. A fragment of the size predicted for mRNAs of the rPAM-3 type (413 bp) was barely visible on the ethidium bromide-stained gel (Fig. 5*a*). Overexposure of the Southern blot demonstrated a distinct product of the size expected for rPAM-3 in addition to two other cross-hybridizing species of intermediate size.

When adult atrial RNA was assessed for the presence of rPAM-4 mRNA on a Northern blot using a riboprobe derived from the unique 3′ untranslated region of rPAM-4, a broad band of 3.4 ± 0.1 kb was detected (Fig. 5*b*). Reverse transcription-PCR utilizing a sense primer upstream of optional Exon A and an antisense primer in the unique 3′-untranslated region of Z2-20 was also utilized to detect mRNA of the rPAM-4 type in adult rat atrium (Fig. 5*c*). Small amounts of a product of the correct size (882 bp) could be detected on the

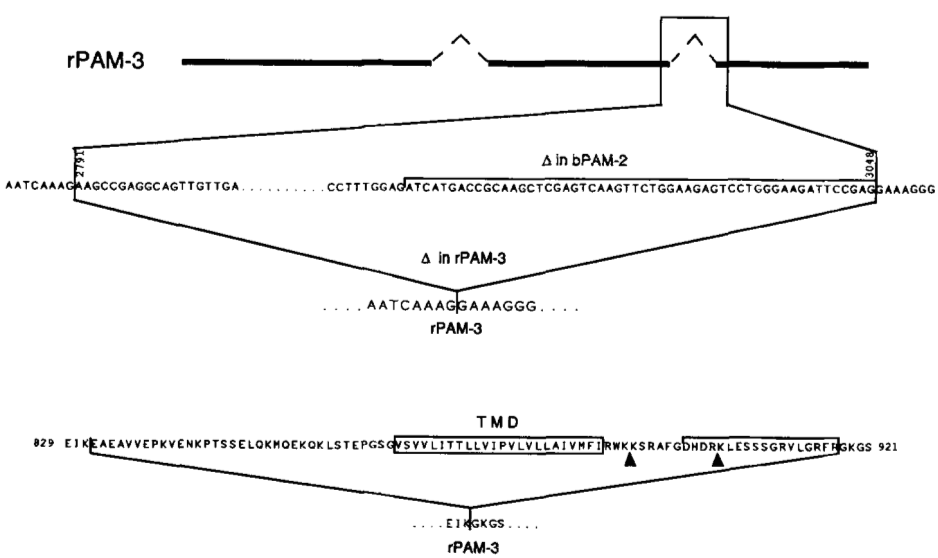

FIG. 3. **Features distinguishing rPAM-3 from rPAM-2.** The nucleotide sequence of rPAM-1 (and rPAM-2) in the region where rPAM-3 diverges is shown along with the borders of the segment missing in Z9 (rPAM-3 type; bp 2791–3048). The region absent from bPAM-2 (Δ in bPAM-2) is also indicated (7). Shown *below* is the corresponding amino acid sequence (*single-letter code*) with the same borders marked off (amino acids 832–917). The amino acids in the transmembrane domain (*TMD*) are *boxed*. Pairs of basic amino acid residues are marked by a *solid arrowhead*. In the course of analyzing Z9 and additional cDNA clones, the following corrections to the original published sequence (9) were noted: one additional C should be inserted after the G at position 257 and the C at position 3230 should be deleted.

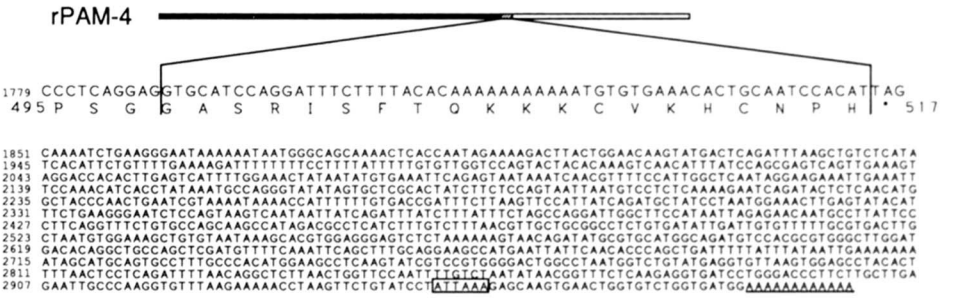

FIG. 4. **Features distinguishing rPAM-4.** The nucleotide sequence of Z2-20 (rPAM-4 type) is given beginning just before the point at which it diverges from rPAM-1 (*filled bar*). At the point of divergence, the coding region continues with a unique 20-amino acid segment (*shaded bar above*; marked off on sequence *below*); the stop codon is indicated by an *asterisk*(*). The novel 3′ region of 1207 bp (*open bar*) terminates with a poly(A) tail (*underlined*). The putative polyadenylation signal is *boxed*.

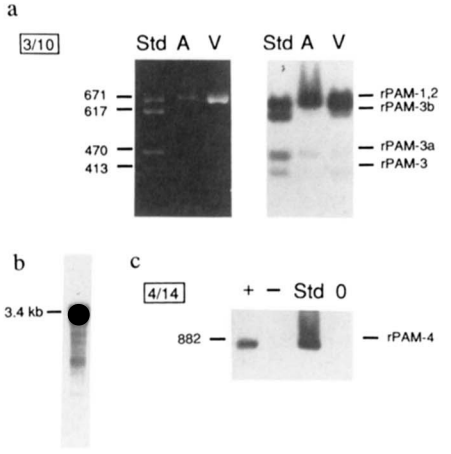

FIG. 5. **rPAM-3 and -4 type PAM mRNAs in adult rat atrium and ventricle.** *a*, cDNA prepared from atrial (*A*) and ventricular (*V*) total RNA (13 and 89 ng, respectively) was subjected to 30 cycles of PCR utilizing primers 3 and 10 (see Fig. 6*g*) as described under "Materials and Methods"; annealing was carried out at 48 °C followed by extension at 72 °C for 2 min. The products were fractionated on a 2.5% agarose gel and prepared for Southern blot analysis using a full-length rPAM-1 cDNA probe as described under "Materials and Methods." For standards, plasmids containing rPAM cDNA inserts (Z6, Z9, Int12[3], and Int22[3]) were individually subjected to the same PCR conditions, mixed, and fractionated in an adjacent lane (*Std*). *b*, Northern blot of total atrial RNA (15 μg) hybridized with a riboprobe spanning the unique 3′-untranslated region of rPAM-4. An RNA ladder fractionated in parallel was used to determine the molecular weight of the hybridizing species. *c*, cDNA was prepared from total atrial RNA (50 ng) by reverse transcription (+); as a control, avian myeloblastosis virus reverse transcriptase was omitted from the reaction (−). A control lacking cDNA was subjected to PCR at the same time (0). All samples were subjected to 31 cycles of PCR using primers 4 and 14 (see Fig. 6*g*). The annealing temperature was 45 °C, and the extension time was 3 min. The standard was prepared from plasmid Z2-20.

ethidium bromide-stained gel (not shown) and after hybridization (Fig. 5*c*). Omission of the reverse transcriptase eliminated this product, indicating that the signal was not due to contaminating genomic DNA. This control is important since analysis of rat genomic clones demonstrates that optional Exon A is contiguous with the unique 3′ region of rPAM-4 in the rat PAM gene.[2] The presence of transcripts with the unique 3′ region of rPAM-4 and lacking optional Exon A would generate a fragment of 561 bp upon RT-PCR using these primers; no fragment of this size was detected in rat atrium, even on prolonged exposure of the blot to film. Thus, removal of optional Exon A does not occur when the unique 3′ exon(s) of rPAM-4 are included in the processed RNA transcript.

Tissue Specificity of PAM Expression—Northern blot analysis revealed a preponderance of slightly smaller PAM mRNAs in pituitary and submaxillary gland (11). Utilizing pairs of primers that span the coding region of rPAM-1 and rPAM-4, RT-PCR was used to compare the prevalence of rPAM-1, -2, -3, and -4 mRNAs in various tissues, and to identify additional alternatively spliced forms of PAM mRNA. The positions of these primers are indicated in Fig. 6*g*. Forms of PAM mRNA with novel exonic regions in the 5′- or 3′-untranslated regions would not be identified using these primers.

When a pair of primers spanning the portion of the PAM mRNA encoding the monooxygenase domain were utilized, PCR of cDNA from all of the tissues examined yielded a single product of the expected size (1089 bp; Fig. 6*a*). No other hybridizing bands suggestive of alternative splicing in this region were detected. A similarly simple pattern was obtained when primers spanning the 3′-end common to the rPAM-1, -2, and -3 cDNAs were utilized; a single band of 710 bp was visualized in all tissues (Fig. 6*e*).

Examination of the Exon A region revealed a tissue-specific pattern of expression of PAM mRNAs containing optional Exon A (1230-bp fragment) and lacking optional Exon A (915-bp fragment). PAM mRNAs containing optional Exon A were prevalent in atrium, ventricle, hypothalamus, and cerebral cortex (Fig. 6*b*); significant amounts of PAM mRNAs lacking optional Exon A were also present. In the anterior and neurointermediate lobes of the pituitary, PAM mRNAs lacking optional Exon A predominated; in the submaxillary gland, PAM mRNAs containing optional Exon A were undetectable. These results are consistent with the results of Northern blot analysis of these tissues using an optional Exon A-specific cDNA fragment as probe (11). No additional variants across optional Exon A were detected.

Examination of the optional Exon B region also revealed a tissue-specific pattern of expression of PAM mRNAs. Although detectable in the adult atrium, ventricle, hypothalamus, and cerebral cortex, PAM mRNAs of the rPAM-3 type (643-bp fragment) comprise a larger proportion of the total PAM mRNA in the anterior and neurointermediate lobes of the pituitary and the submaxillary gland (Fig. 6*c*). This result is supported by the greater relative abundance of rPAM-3 type cDNAs in rat anterior and neurointermediate pituitary cDNA libraries.[3] As in atrium and ventricle, the 901-bp fragment corresponding to mRNAs of the rPAM-1 and -2 type predominated in the hypothalamus and cerebral cortex (Fig. 6*c*). The 847-bp and 697-bp fragments seen clearly in the pituitary and submaxillary gland samples represent mRNAs (rPAM-3a and -3b type) containing smaller segments of optional Exon B.[3]

None of the PAM cDNA clones examined contained optional Exon B but lacked optional Exon A. In order to determine whether PAM mRNAs of this type were present, RT-PCR was carried out using a pair of primers that span both

[3] B. A. Eipper, C. B.-R. Green, D. A. Stoffers, R. E. Mains, H. T. Keutmann, and L'H. Ouafik, manuscript in preparation.

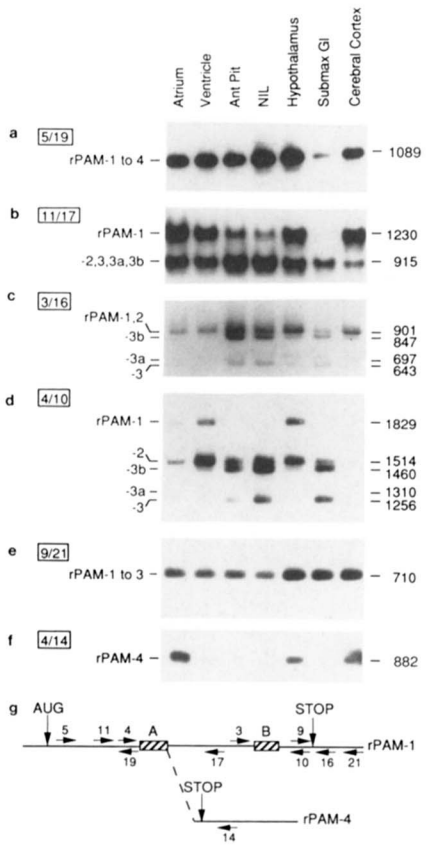

FIG. 6. **Tissue-specific expression of PAM mRNA forms.** *a–f*, Total RNA from a series of adult rat tissues was subjected to RT-PCR utilizing the six pairs of primers indicated by the *boxed numbers* at the *left*. The amounts of total RNA used to synthesize the reverse-transcribed cDNA used as input for amplification were adjusted based on Northern blot analysis (11) to result in similar amounts of amplified product, and quantitative comparisons are not valid. The PCR products were fractionated on agarose gels, prepared for Southern blot analysis, and hybridized with full length rPAM-1 cDNA insert as described under "Materials and Methods." The number of base pairs present in PCR products derived from plasmid controls are indicated on the *right*; the forms of PAM to which these bands correspond are indicated on the *left*. PCR conditions were as described under "Materials and Methods" unless otherwise stated. Similar results were obtained with a set of RNA samples prepared from a separate set of rats. *a*, primers 5 and 19, 26 cycles with annealing temperature (T_{ann}) = 45 °C, 1.5% gel. *b*, primers 11 and 17, 31 cycles with T_{ann} = 52 °C, 1.8% gel. *c*, primers 3 and 16, 24 cycles with T_{ann} = 52 °C, 2.0% gel. *d*, primers 4 and 10, 28 cycles with T_{ann} = 52 °C, 1.5% gel. *e*, primers 9 and 21, 26 cycles with T_{ann} = 45 °C, 1.0% gel. *f*, primers 4 and 14, 36 cycles with T_{ann} = 45 °C, 1.2% gel. *g*, schematic of rPAM-1 and rPAM-4 cDNAs depicting the positions and orientations of oligonucleotide primers utilized in the experiments shown in Figs. 5 and 6. The initiation (*AUG*) and termination (*STOP*) codons as well as the positions of optional Exons A and B (*hatched boxes*) are indicated. The primers utilized in each *panel* are indicated in *boxes* along the *left side*.

optional Exons A and B (Fig. 6*d*). Reverse-transcribed cDNA from atrium, ventricle, hypothalamus, and cerebral cortex (data not shown) yielded primarily fragments amplified from rPAM-1 (1829 bp) and rPAM-2 (1514 bp) type PAM mRNAs. Fragments corresponding to rPAM-3 type and related mRNAs (rPAM-3a and -3b) could be clearly detected in anterior and neurointermediate pituitary and submaxillary gland (1256-, 1310-, and 1460-bp fragments, respectively). None of the tissues yielded fragments between 1514 bp and 1829 bp, even on prolonged exposures of the Southern blot, indicating that removal of optional Exon B does not occur to any appreciable extent unless optional Exon A has also been removed.

Finally, when similar relative amounts of reverse-transcribed cDNA from these tissues were utilized to amplify fragments from rPAM-4 type mRNA, rPAM-4 specific product (882 bp) was detected in atrium, hypothalamus, cerebral cortex, and to a much lesser extent in ventricle and anterior pituitary (Fig. 6*f*). To visualize these products, 36 cycles of PCR were necessary. As in Fig. 5*c*, no additional fragments suggestive of alternative splicing in the region between these primers were detected.

DISCUSSION

Two novel types of PAM cDNA were identified in an adult rat atrium cDNA library. rPAM-3 differs from rPAM-2 by the deletion of a 258-nucleotide segment (optional Exon B) and encodes a protein precursor lacking a transmembrane domain. Both the monooxygenase and lyase domains are predicted to be encoded by rPAM-3. Amidating enzyme purified from medullary thyroid carcinoma cells is somewhat smaller than the protein encoded by rPAM-3 (16) and contains both monooxygenase and lyase activity (1). rPAM-4 is identical with the 5′ half of rPAM-1 through the 3′ border of optional Exon A, after which an additional novel 1.2-kb sequence is observed. The protein encoded by rPAM-4 thus contains the monooxygenase domain and the 105-amino acid segment encoded by optional Exon A followed by a novel hydrophilic COOH-terminal peptide; the rPAM-4 protein lacks a transmembrane domain as well as the lyase domain of the PAM precursor. The sequence at the COOH terminus of the rPAM-4 protein (-Cys-Asn-Pro-His) resembles the consensus sequence for isoprenylation (17); however, rPAM-4 has a signal sequence and its synthesis should be directed into the lumen of the endoplasmic reticulum. Based upon the results of transfection of truncated cDNAs encoding only the monooxygenase domain of bovine PAM into AtT-20 cells (18), the protein encoded by rPAM-4 would be expected to have monooxygenase activity. The complete identity in nucleotide sequence observed in overlapping regions of the four forms of rat atrial PAM mRNA along with preliminary analysis of genomic DNA clones suggest that the four forms of mRNA derive from a single large gene via alternative RNA splicing.[2] The structures of additional types of PAM cDNA isolated from rat pituitary cDNA libraries[3] (Figs. 5*a* and 6, *c* and *d*) are also consistent with this hypothesis.

The distribution of PAM mRNA among the different types is tissue-specific (11) and developmentally regulated (19). Combined RT-PCR utilizing several pairs of primers flanking segments along the known forms of PAM mRNA revealed two major patterns. In the heart (atrium and ventricle) and the central nervous system (hypothalamus and cerebral cortex), both mRNAs containing and missing optional Exon A are found. Since optional Exon A separates the monooxygenase and lyase domains and encodes a paired basic endoproteolytic cleavage site, its presence or absence may play a role in determining whether the two enzymatic activities are largely separated (as in the bovine neurointermediate pituitary) (2, 5) or remain as parts of one protein (as in medullary thyroid carcinoma cells) (1, 16). The major PAM mRNAs in the heart and central nervous system contain optional Exon B (rPAM-1 and -2) and only minor amounts of PAM mRNA without optional Exon B (rPAM-3) are found. In contrast, in the anterior and neurointermediate pituitary and the submaxillary gland, PAM mRNAs containing optional Exon A are found at much lower levels and forms of PAM in which all or part of optional Exon B is absent are prevalent.

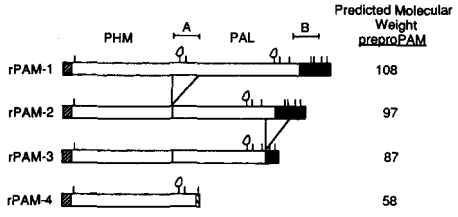

FIG. 7. **Key features of the proteins encoded by rPAM-1 to -4.** The predicted molecular masses of the preproproteins are indicated; the signal peptide (*fine hatching*) has a predicted molecular mass of 2.5 kDa, and the propeptide has a predicted mass of 2.0 kDa. The *open box* designates the intragranular portion (including the peptidylglycine α-hydroxylating monooxygenase and peptidyl-α-hydroxyglycine α-amidating lyase catalytic domains). The *filled box* designates the transmembrane domain. The *stippled box* designates the peptide predicted to be cytoplasmic in rPAM-1 and -2. The unique COOH-terminal peptide of rPAM-4 is designated by *thick hatching*. *Vertical lines* indicate paired basic amino acid sequences (potential endoproteolytic cleavage sites); *irregular closed shapes* indicate potential glycosylation sites. The positions of the monooxygenase (*PHM*) and lyase (*PAL*) domains and the peptides encoded by optional Exons A and B are indicated *above*.

Through Northern analysis and RT-PCR, rPAM-4 was detected in atrium and ventricle as well as in anterior pituitary, hypothalamus, and cerebral cortex. Of note is the absence of any forms related to rPAM-4 but lacking optional Exon A. This is consistent with analysis of a rat genomic clone spanning this region; optional Exon A is contiguous with the unique 3′ region of rPAM-4, and the nucleotide sequence at the junction $(T/C)_{6/11}$GGAG:G (Fig. 4) between them does not optimally satisfy the consensus for a splice acceptor site $(T/C)_{11}$NCAG:G (20).[3] Although present in each of these tissues, rPAM-4 mRNA does not account for a large percentage of the PAM mRNA in any of the adult tissues.

The protein encoded by rPAM-3 would be predicted to have a distinctly different molecular weight (Fig. 7) and subcellular localization than the proteins encoded by rPAM-1 and rPAM-2. Western blot analysis of soluble proteins from the anterior pituitary demonstrated a 95-kDa protein cross-reactive with antibodies raised against synthetic peptides from the monooxygenase and lyase domains of PAM; this protein was not visualized with an antibody raised against a synthetic peptide from within optional Exon B and could represent the protein encoded by rPAM-3.[3] The COOH-terminal region of the PAM protein, which is predicted to reside in the cytoplasm when expressed as part of rPAM-1 or rPAM-2, is predicted to reside inside an intracellular compartment when expressed as part of rPAM-3 (Fig. 7). It will be interesting to determine whether this region and its position play a role in the targeting of different forms of PAM to specific subcellular locations. Like rPAM-3, rPAM-4 is predicted to encode a soluble protein residing inside an intracellular compartment and available for co-secretion with peptide and other soluble components of the secretory granule (Fig. 7). Measurement of PAM activity in rat and human serum (21, 22), human cerebrospinal fluid (23), and spent medium from primary heart cultures (24) and several endocrine cell lines (25, 26) indicates that soluble PAM activity is secreted.

The distribution of PAM activity between soluble and crude particulate fractions is tissue-specific (27) and developmentally regulated (19) suggesting a biological function for this distinction. At least two mechanisms for the generation of soluble PAM activity appear to be operative. First, endoproteolysis of membrane-associated PAM precursors (*e.g.* rPAM-1 and -2) has been demonstrated in AtT-20 cells transfected with bPAM-1 cDNA (18) and in CA-77 cells (28). Second, translation of mRNAs from which the transmembrane domain has been deleted by alternative splicing could yield soluble proteins (*e.g.* rPAM-3 and -4). The use of alternative splicing to generate distinct mRNAs encoding soluble and membrane-associated forms of a protein is emerging as a common theme. As in the case of rPAM-4, soluble forms of the immunoglobulin μ heavy chain (29), the growth hormone receptor (30), and the mouse amyloid peptide precursor protein (31) are produced when a novel terminal 3′ exon replaces exons encoding the COOH-terminal transmembrane domain of the protein. For the neural cell adhesion molecule (32) and the interleukin-4 receptor (33), an exon that disrupts the reading frame is inserted and leads to translation termination before the transmembrane domain; downstream exons encoding the transmembrane domain are, however, retained in the mRNA. To our knowledge, rPAM-3 represents a novel way in which alternative splicing leads to a message encoding a soluble form of a membrane protein, in that the exon encoding the transmembrane domain is removed in a manner that preserves the reading frame, leaving a portion of the cytoplasmic tail intact and presumably residing in a different intracellular location.

An understanding of the biological significance of the multiple mRNAs will require assessment of the ability of the individual proteins to produce amidated peptides in test tube assays and in various cellular environments. Since most tissues express several forms of PAM mRNA simultaneously, any role the different domains might play in subcellular localization will require generation of specific antisera and expression of individual cDNAs in isolation. The consequences of expressing a form such as rPAM-4, which encodes only one of two separable enzymatic activities involved in peptide α-amidation, warrants further investigation.

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