Alternative Splicing and Endoproteolytic Processing Generate Tissue-specific Forms of Pituitary Peptidylglycine α-Amidating Monoxygenase (PAM)*

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The pituitary is a rich source of peptidylglycine α-amidating monoxygenase (PAM). This bifunctional protein contains peptidylglycine α-hydroxylating monoxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL) catalytic domains necessary for the two-step formation of α-amidated peptides from their peptidylglycine precursors. In addition to the four forms of PAM mRNA identified previously, three novel forms of PAM mRNA were identified by examining anterior and neurointermediate pituitary cDNA libraries. None of the PAM cDNAs found in pituitary cDNA libraries contained exon A, the 315-nucleotide (nt) segment situated between the PHM and PAL domains and present in rPAM-1 but absent from rPAM-2. Although mRNAs of the rPAM-3a and -3b type encode bifunctional PAM precursors, the proteins differ significantly. rPAM-3b lacks a 54-nt segment encoding an 18-amino acid peptide predicted to occur in the cytoplasmic domain of this integral membrane protein; rPAM-3a lacks a 204-nt segment including the transmembrane domain and encodes a soluble protein. rPAM-5 is identical to rPAM-1 through nt 1217 in the PHM domain; alternative splicing generates a novel 3′-region encoding a COOH-terminal pentapeptide followed by 1.1 kb of 3′-untranslated region. The soluble rPAM-5 protein lacks PAL, transmembrane, and cytoplasmic domains. These three forms of PAM mRNA can be generated by alternative splicing. The major forms of PAM mRNA in both lobes of the pituitary are rPAM-3b and rPAM-2. Despite the fact that anterior and neurointermediate pituitary contain a similar distribution of forms of PAM mRNA, the distribution of PAM proteins in the two lobes of the pituitary is quite different. Although integral membrane proteins similar to rPAM-2 and rPAM-3b are major components of anterior pituitary granules, the PAM proteins in the neurointermediate lobe have undergone more extensive endoproteolytic processing, and a 75-kDa protein containing both PHM and PAL domains predominates. The bifunctional PAM precursor undergoes tissue-specific endoproteolytic cleavage reminiscent of the processing of prohormones.

The pituitary is one of the richest sources of peptidylglycine α-amidating monoxygenase (PAM; EC 1.14.17.3) in the adult rat (1, 2). Immunocytochemical studies indicate that the highest levels of PAM protein are found in gonadotropes, but detectable levels of PAM protein are found in each of the major pituitary cell types (3). Although none of the major anterior pituitary hormones is α-amidated, several amidated peptides are synthesized in the anterior pituitary (4, 5). Following thyroidectomy, levels of PAM mRNA in the anterior pituitary rise severalfold, along with levels of the mRNAs encoding several α-amidated peptides (5, 6). Intermediate pituitary melanotropes produce large amounts of two α-amidated products from proopiomelanocortin (α-melanocyte stimulating hormone and joining peptide) (4, 7), and the major peptide products stored in the neural lobe (oxytocin and vasopressin) are α-amidated.

Peptide α-amidation involves a two-step reaction with a peptidyl-α-hydroxyglycine intermediate (8–12). The PAM precursor protein encodes both of the enzymatic activities involved in peptide α-amidation (13–15). The first enzyme, peptidylglycine α-hydroxylating monoxygenase (PHM), is contained within the NH2-terminal third of the rat PAM-1 precursor (Fig. 1) and catalyzes the copper, molecular oxygen, and ascorbate-dependent formation of peptidyl-α-hydroxyglycine. The second enzyme, peptidyl-α-hydroxyglycine α-amidating lyase (PAL), follows the PHM domain and precedes the putative transmembrane domain; although spontaneous conversion of the α-hydroxyglycine intermediate into α-amidated product occurs at high pH, conversion at physiological pH values requires the action of PAL.

By screening an adult rat atrium cDNA library, we previously identified four forms of PAM mRNA that arise from the single copy PAM gene by alternative splicing (Fig. 1) (16–18). PAM mRNAs of the rPAM-1 type are the longest; removal of exon A gives rise to rPAM-2, and removal of exons A and B gives rise to rPAM-3. In rPAM-4, a unique 3′-region replaces the sequence of rPAM-1 following exon A; as a result, rPAM-4 encodes only the PHM domain. Based on Northern blot analysis, the anterior and neurointermediate lobes of the rat pituitary lack large amounts of rPAM-1 type mRNA and

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The abbreviations used are: PAM, peptidylglycine α-amidating monoxygenase; PHM, peptidylglycine α-hydroxylating monoxygenase; PAL, peptidyl-α-hydroxyglycine α-amidating lyase; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; nt, nucleotide(s); bp, base pair(s); kb, kilobase pair(s).
The nucleotide and amino acid sequences of rPAM-3a and -3b in the region previously referred to as exon B (Fig. 2). Reverse transcription coupled to the polymerase chain reaction (Fig. 4). Levels of the amplified rPAM-5 specific product generated with cDNA from the various tissues mirrored total levels of PAM activity (2). Based on its prevalence in atrial and pituitary cDNA libraries, rPAM-5 is not a major transcript and attempts to visualize rPAM-5 mRNA on Northern blots were unsuccessful. To eliminate the possibility that Ant67 was the result of a cloning artifact, the structure of this region of the PAM gene was investigated using the polymerase chain reaction (Fig. 5). The PAM exon terminating with nt 1217 is separated from the exon containing the unique 3'-end of rPAM-5 by an approximately 550-nt intron. Thus mRNAs of the rPAM-5 type are the product of alternative splicing and are not generated by failure to remove the intron contiguous with nt 1217.

Comparison of PAM Proteins Found in Anterior and Neu-

3 L'H. Ouafik, D. A. Stoffers, T. A. Campbell, R. C. Johnson, B. T. Bloomquist, and B. A. Eipper, unpublished observation.

7 Portions of this paper (including "Materials and Methods," part of "Results," and Figs. 1, 4, 5, 7, and 8) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Polyclonal antibody to a peptide within the PAL catalytic domain of PAM mRNA present and the co- or post-translational forms of PAM mRNA in the anterior and neurointermediate lobes of the rat pituitary, previous studies indicated that the location of the initiator Met and the first in-frame Stop codon (*) are indicated. Characteristic restriction sites are shown: H, HindIII; X, XbaI. A cDNA probe specific for rPAM-5 was generated by using the fragment between the HindIII sites at 1001 and 1200 bp. The complete nucleotide sequence of the novel 1.1 kb 3′-region of Ant67 is shown along with the pentapeptide sequence forming the novel COOH terminus of rPAM-5; the four consensus poly(A) addition signals are underlined.

**Fig. 3.** Sequence of rPAM-5 type cDNA. The cDNA insert in Ant67 is drawn to scale with the region of identity to rPAM-1 shown by the filled bar and the new sequence shown by the open bar. The location of the PAM proteins found in any tissue reflect both the forms of PAM proteins were visualized with affinity-purified rabbit polyclonal antibody to a peptide within the PAL catalytic domain. Granules from each tissue contained a distinctive collection of proteins recognized by the PAL antibody. As expected, atrial granules contained primarily intact rPAM-1 (120 kDa) and rPAM-2 (105 kDa) (28). Almost no protein the size of rPAM-1 was detected in anterior or neurointermediate pituitary granules. Both anterior and neurointermediate pituitary granules contained a protein the size of rPAM-2. A characteristic set of smaller PAL proteins (95, 84, and 75 kDa) were present in both anterior and neurointermediate pituitary granules. The 75-kDa PAL protein was a major component of neurointermediate pituitary granules, whereas the 105-kDa PAL protein was more prevalent in anterior pituitary granules. The 95-kDa and 84-kDa PAL proteins (28) were present in both anterior and neurointermediate pituitary granules. The 75-kDa PAL protein was a major component of neurointermediate pituitary granules, whereas the 105-kDa PAL protein was more prevalent in anterior pituitary granules.

In order to investigate further the PAM proteins in pituitary granules, much more concentrated aliquots of a different preparation of anterior and neurointermediate pituitary granules were fractionated and visualized with antisera to synthetic peptides from within the PHM, PAL, and COOH-terminal domains of rPAM-1 (Fig. 6, B and C). A complex

**Fig. 6.** Comparison of PAM proteins in atrial, anterior, and neurointermediate pituitary secretory granules by Western blot analysis. A, a crude secretory granule fraction was prepared from adult rat anterior and neurointermediate pituitary and from adult rat atrium by differential centrifugation. The atrial and anterior pituitary samples each contained 20 pmol/h PAM activity (8.4 and 19.6 μg of protein, respectively); the neurointermediate pituitary sample contained 97 pg of protein (420 pmol/h PHM activity; 2900 pmol/h PAL activity). Each sample of neurointermediate pituitary granules contained 100 μg of protein (1150 pmol/h PHM activity; 3100 pmol/h PAL activity). Each sample of neurointermediate pituitary granules contained 97 μg of protein (420 pmol/h PHM activity; 2900 pmol/h PAL activity). Following transfer to Immobilon-P, blots were exposed to the PAM antibodies indicated; exposure times were adjusted to give bands of comparable intensity. The exon B antibody (Ab26) was applied to blots that had been stripped; this antibody cross-reacts with several lower molecular weight proteins not thought to be related to PAM. The location of molecular weight markers analyzed in a separate lane are indicated on the right; apparent molecular weights of the various PAM proteins are indicated on the left.
pattern of PAM proteins was identified in secretory granules from both tissues. Although PAM proteins of similar apparent molecular weight were detected in both anterior and neurointermediate pituitary granules, different forms of PAM protein predominated in anterior and neurointermediate pituitary granules.

Anterior pituitary granules contain a set of PAM proteins cross-reactive with antisera to peptides within both the PHM and PAL catalytic domains (Fig. 6B). The most prominent bands had molecular masses of 105 ± 5 kDa, 95 ± 3 kDa, and 75 ± 3 kDa; minor and somewhat variable amounts of an 84 ± 3 kDa protein detected by PHM and PAL antisera were also present. In contrast, a 75 kDa PAM protein was the major PAM protein found in neurointermediate pituitary granules; only minor amounts of a 105-kDa PAM protein were present. The same set of higher molecular weight proteins were visualized by antiserum to a peptide closer to the NH2-terminus of PHM (rPAM(116–131)) (Fig. 6B). Although a number of smaller PHM and PAL proteins were visualized in secretory granules from both regions of the pituitary, no proteins smaller than 75 kDa were visualized by antiserum to both PHM and PAL (Fig. 6B). PHM proteins of 44–45 kDa were visualized with varying intensity by both PHM antisera. The 42-kDa protein visualized by antibody to rPAM(116–131) was not visualized as well by antibody to rPAM(293–315); the 59-kDa protein visualized by antibody to rPAM(293–315) was not visualized by the other PHM antibody, and its relationship to PAM is unclear. Small amounts of PAL proteins of approximately 50 kDa were visualized in both anterior and neurointermediate pituitary granules.

In order to aid in identification of the various PAM proteins, the same samples were visualized with antiserum to peptides contained within exon B, and the COOH-terminal cytoplasmic domain of rPAM-1 (Fig. 6C). Antibody to exon B, visualized the 105-kDa PAM protein, but not the 75-kDa PAM protein in the anterior pituitary granule preparation; adequate evaluation of the cross-reactivity of the 95-kDa PAM protein(s) requires separation of soluble and membrane proteins. The COOH-terminal domain antibody detected 105- and 95-kDa PAM proteins, but not the 75-kDa PAM protein. Since all of the PAM mRNAs encoding both PHM and PAL also encode this COOH-terminal determinant, the 75-kDa PAM protein must arise from precursor forms of PAM.

The 105-kDa PAM protein is found in anterior pituitary membranes washed with 0.1 M Na2C03 to remove peripheral proteins and is thought to represent rPAM-2 and -3b (Fig. 7). Small amounts of a 95-kDa PAM protein lacking COOH-terminal antigenic determinants are also found associated with the membranes and may represent a processed form of these proteins. Neurointermediate pituitary membranes also contain small amounts of a 105-kDa PAM protein (Fig. 8B). The soluble fraction of anterior pituitary secretory granules contains large amounts of both the 75-kDa PAM protein recognized by antiserum to PHM and PAL and the monofunctional 44–45 kDa PHM protein (Fig. 8A). The 95-kDa PAM protein found in the soluble fraction is recognized by antiserum to the COOH-terminal domain and is thought to represent intact rPAM-3/3a. Very little monofunctional PAL protein is found in the soluble fraction of anterior pituitary granules.

A single complex gene encodes PAM in the rat (25, 29). The functional consequences of expressing the seven different forms of PAM mRNA identified in the Sprague-Dawley rat (16–18) are significant. Five of the mRNAs encode bifunctional PAM proteins, with three of the mRNAs encoding PAM proteins with a transmembrane domain and two encoding soluble bifunctional proteins (Fig. 9). Rat PAM-4 mRNA encodes a soluble form of PHM (18). Rat PAM-5 mRNA encodes only part of the PHM domain and current studies indicate that rPAM-5 is inactive; this observation is consistent with the fact that the protein encoded by rPAM-5 does not include the entire region of homology to dopamine β-monooxygenase (30). The importance of synthesizing an inactive truncated PHM protein is unclear; alternative splicing generates an inactive form of glutamic acid decarboxylase that is expressed at high levels early in embryonic brain development (31).

We previously demonstrated the tissue-specific expression of different forms of PAM mRNA; thus atrium contains primarily rPAM-1 and -2 mRNA, whereas little rPAM-1 mRNA is found in the pituitary (2, 18). In this study, the anterior and neurointermediate lobes of the Sprague-Dawley rat pituitary were found to contain a very similar collection of PAM mRNAs; mRNAs of the rPAM-2 and -3b type were the most prevalent, with less rPAM-3, -3a, and -1 and very small amounts of rPAM-4 and -5. Despite the presence of similar forms of PAM mRNA, the PAM proteins found in the anterior and neurointermediate lobes of the pituitary differ. Thus both alternative splicing and post-translational processing contribute to the tissue specific production of proteins derived from PAM.

When exon A is absent, no paired basic potential endoproteolytic cleavage site separates the PHM and PAL domains. Exons B, and B, separate the PAL catalytic domain from the COOH-terminal region; this COOH-terminal region forms the cytoplasmic domain of rPAM-1 and -2. Exon B, contains the transmembrane domain and its stop transfer signal, whereas exon B, contains a pair of basic amino acids (Arg56-Lys564). The fact that exon B, corresponds exactly to the 54-n region distinguishing two forms of bovine PAM mRNA (24) suggests that it may have functional significance. The peptide encoded...
by exon B, could perform different functions when expressed as part of rPAM-2 and rPAM-3a. In rPAM-2 this peptide forms part of the cytoplasmic domain and might affect intracellular routing of PAM. For example, the ligand-mediated internalization of the FeR receptor is governed by the presence or absence of a 47-amino segment in its COOH-terminal domain (32). In rPAM-3a the exon B peptide should be situated within the secretory granule; in this location it could serve as a paired basic endopeptidase processing site.

Several other laboratories have characterized PAM mRNAs from other tissues or other species. Type A and Type B PAM cDNAs were isolated from a rat medullary thyroid carcinoma cDNA library (33); except for minor differences, the type A cDNA is identical to rPAM-3b (it lacks the 315 bp of exon A and the 54 bp of exon B). The Type B cDNA is essentially identical to rPAM-1 until a point close to the 3'-end of exon B; a 3-bp insertion is followed by 47 bp of exon B. The sequence of the Type B cDNA then diverges completely from rPAM-1; a stop codon is reached 55 amino acids after the transmembrane domain and the 3'-untranslated region is extremely purine-rich (23). No PAM cDNAs with a 3'-end resembling that of Type B PAM cDNA were identified in the PAM cDNAs examined from our atrial and pituitary libraries.

Five types of PAM cDNA were identified in libraries prepared from the pituitaries of adult Wistar rats (29); the five forms arise via alternative splicing at the regions referred to in this study as exons A, B, and B,. Unexpectedly, different forms of PAM mRNA were found to be prevalent in Sprague-Dawley and Wistar rat pituitaries. As found previously in bovine pituitary (24), PAM cDNAs retaining exon A were prevalent in libraries prepared from Wistar rat pituitary (29); in contrast, PAM cDNAs retaining exon A were rare in libraries prepared from Sprague-Dawley pituitary. The scarcity of PAM mRNAs of the rPAM-1 type in Sprague-Dawley and Wistar rat pituitaries. As found previously in the anterior lobe of the pituitary. Given that the extent of PAM processing correlates with the extent of pro-ACTH and pro-POMC processing, the PAM precursor must occur (35, 36).

One of the 58- and 44-45-kDa PHM products from rPAM-2, 3-3a, or -3b, the cleavages must occur at nonpaired basic sites. Alternatively, the smaller PHM proteins could represent products of rPAM-4 and rPAM-5 mRNAs. No major membrane-associated processing products were detected with antisera to exon B, or the COOH-terminal domain of rPAM-1. Biosynthetic labeling of cells expressing individual forms of PAM mRNA will be required to delineate the steps involved in processing.

We have information on the endoproteolytic processing of PAM in several tissues. In the brain, both PAM and pro-ACTH are active as independent soluble forms; primary cultures of neonatal atrial myocytes cleave proANF at the time of secretion and also secrete large amounts of pro-HAB and POMC. Alternatively, the smaller PHM proteins could represent products of rPAM-4 and rPAM-5 mRNAs. No major membrane-associated processing products were detected with antisera to exon B, or the COOH-terminal domain of rPAM-1. Biosynthetic labeling of cells expressing individual forms of PAM mRNA will be required to delineate the steps involved in processing.

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SUPPLEMENTARY MATERIAL TO: Alternative Splicing and Endopeptidolytic Processing Generates These Specific Forms of Pituitary PAM

**MATERIALS AND METHODS**

**Construction and Screening of Neurointermediate and Anterior Pituitary cDNA Libraries**

Total RNA (170 µg) was prepared from the neurointermediate lobes of 60 adult male Sprague-Dawley rats pituitaries. Poly(A)- RNA (3.2 µg) was isolated and used to synthesize cDNA with Eppri adapters using the Pharnacia mRNA isolation and cDNA synthesis kit. Up to 40% of the cDNA to 1 µg Eppri-digested Lambda ZAP II virus (Stratagene) and packaging with the Gigapack Gold helper packaging kit (Stratagene) yielded 1.6 x 10⁶ pfu. For screening, 300,000 pfu from the unamplified library were plated at a density of 50 pfu per 150-mm plate. Triplicate filters were screened with 3 random primed restriction fragments spanning the sequence of PAM-1 (Fig. 1).

**SIG**

![SIG](image)

**PAM-1**

![PAM-1](image)

**PAM-2**

![PAM-2](image)

**PAM-3**

![PAM-3](image)

**PAM-4**

![PAM-4](image)

**Pal**

![Pal](image)

**Stu**

![Stu](image)

**HpaII**

![HpaII](image)

**EcoRI**

![EcoRI](image)

**EcoRV**

![EcoRV](image)

**BamHI**

![BamHI](image)

**SalI**

![SalI](image)

**SpeI**

![SpeI](image)

**Fig. 1. cDNA Library Screened in Adult Pituitary**

The structures of the 14 types of PAM cDNA characterized from an adult pituitary library are shown (25-36). Epsri A, B, and C are indicated, as are the PAM-1 cDNA fragments used as probes to screen the pituitary libraries (37, 38) (cloning sites used in construction of library). In the first round of screening with the 1.2 kb x fragment (306/3618) cDNA-PAM probe (Fig.1), 110 positive plaques were identified in 300,000 pfu of 30 of these remained positive through a second round of screening. Eighteen were selected for further characterization; 7 were in the rescue step and only 8 of the remaining 11 had cDNA inserts. Restriction mapping indicated that 8 of the PAM-1 type, 2 were of the PAM-2 type, 2 were of the PAM-3 type, and 1 new class described by HpaII (PAM-4). One fragment (3510) contained a new 3' end in the PAM cDNA that could not be hybridized with a cDNA probe from the 2nd region of PAM-1 (Sphi/SstI fragment; Fig. 1); restriction enzyme analysis indicated that the insert was different from any of the previously characterized PAM cDNAs. At least was sequenced on a single strand in the region identical to PAM-1 and on both strands throughout its novel 3'-end.

The greater prevalence of PAM positive phage isolated in the anterior pituitary lobe compared to the neurointermediate pituitary lobe is consistent with the higher levels of PAM mRNA detected in the rat anterior pituitary by Northern blot analysis (29). Similar screenings of anterior pituitary libraries yielded approximately 4 times as many positive plaques as in the anterior pituitary library. In both the anterior and neurointermediate pituitary libraries, almost half of the PAM positive cDNA inserts are the type of PAM-3b type; CNA inserts corresponding to PAM-3b were recovered less often. No cDNA inserts lacking exon 1 were isolated from either library.
The polyol bulb antibodies used in this study include several related to P(PAM)(5-310) conjugated to soybean trypsin inhibitor by its Cys residues (Ab605, Ab606, Ab608, Ab100); P(PAM)(38-310) lies in the PIM domain and differs from P(PAM)295-310 at 1 residue. Two antibodies were generated to P(PAM)551-579 conjugated to hemoglobin with glutaraldehyde (Ab58, Ab68); this peptide lies in the PAL domain and differs from P(PAM)646-682 at 2 residues. Antibodies specific to exon B were generated to P(PAM)844-859 conjugated to hemoglobin with glutaraldehyde (Ab56, Ab68). Reduction of non-specific background with Abs required replacement of the normal blocking solution with 100 mM Na phosphate, pH 7.4, 5% non-fat dry milk, 0.2% Tween-20, 0.05% NaN3, (the turid solution was clarified by centrifugation before use). Antisera to the COOH-terminal domain of P(PAM)-1 were generated to P(PAM)932-946 linked to keyhole limpet hemocyanin with glutaraldehyde. Anti-peptide antisera were affinity purified using the appropriate peptide-resin (23). Preparation and affinity purification of antibody to purified bovine PAM plus B (Ab30) was as described before (24). Affinity purified antibodies were used at a dilution of 1:100 except for Ab100, which was used at a dilution of 1:300. Specificity was established by including the appropriate peptide (10 µg/ml) during incubation of the blots with antibody; using [3H]-Protein A for visualization, the appearance of all bands was blocked in this manner. Blots were stripped by incubation in 0.2 M glycine HCl, 0.05% Triton X-100 at 80° for two 30 min periods and were then hybridized with another primary antibody as above.

RESULTS
Pharyngeal PAM mRNA. PAM-5 mRNA could not be detected by Northern blot analysis of pharyngeal RNA. Therefore reverse transcription-PCR was used to detect PAM-5 mRNA. Duplicate samples of cDNA prepared from various tissues were subjected to amplification using primers common to PAM-1,2,3,6,7- and bo mRNA or using primers specific to mRNA of the PAM-5 type. Amplified products derived from PAM mRNA of the PAM-5 type were found in each tissue. Fig. 4. Detection of P(PAM)-5 mRNA by RT-PCR. Reverse transcribed cDNA from the tissues indicated (Ar, atrium; Sub, submaxillary gland; Str, striatum; Hypo, hypothalamus; NIL, neurointermediate pituitary; Ant, anterior pituitary) was amplified using a pair of oligonucleotides common to P(PAM)-1,2,3,6,7- and bo mRNA (BES/BE19) or a pair of oligonucleotides specific to P(PAM)-5 (BES11/BE22); extension time was 3 min and number of cycles was 30. Appropriate plasmid controls (10 pg pZAP for BES/BE19 and 10 pg Am7 for BES11/BE22) served as standards along with the blanks. The same amount of input cDNA was used for both primer pairs; amounts were selected based on the prevalence of total PAM mRNA in each tissue (Braas et al., 1989). Upper. The location of the oligonucleotide primers used is indicated. Middle. Ethidium bromide stained gels. Lower. Southern blots; the 1.3 kb probe derived from the PAM domain (Fig.1) was used to detect the products amplified with BES11/BE19 and the 0.2 kb probe derived from Am7 (Fig.3) was used to detect products amplified with BES11/BE22.

Transcripts of the P(PAM)-5 type could arise by alternative splicing or by retention of an intron. These possibilities were distinguished by amplification of genomic DNA prepared from Sprague Dawley rat liver with a sense oligonucleotide primer situated immediately preceding the point at which P(PAM)-5 diverges (BE21) paired with either of two antisense oligonucleotides primers situated within the novel 3′-sequence of each of the following 3′-ends of P(PAM): (BES/BE22) (Fig.5). While both pairs of primers generated the expected products when used to analyze the Am7 plasmid (334 and 1170 bp, respectively), analysis of genomic DNA yielded larger fragments (8.67 and 1.75 kb fragments, respectively). These data indicate that an approximately 550 bp intron separates the PAM exon containing BE23 from the exon containing the unique 3′-end of P(PAM).
Characterization of soluble and membrane-associated PAM proteins. Of the features distinguishing the p-antigens encoded by the different PAM mRNAs in the absence or presence of a bystander membrane domain (Fig. 7). Anterior and intermediate pituitary fractions differing in which amino-terminal activity remains membrane associated following extraction of membranes with 0.1 M NaCO₃. While 34% of the total PAM activity in anterior pituitary homogenates remained membrane associated, only 6% of the total PAM activity in an intermediate pituitary homogenate did so. For comparison, 59% of the total PAM activity in a pituitary granule was 7% of the total PAM activity in an anterior pituitary homogenate, but 0.1 M NaCO₃ washed membranes. Similar results were obtained when granule enriched fractions were separated into soluble proteins and carbonate-washed membranes and assayed for PAM and PAL activity. In anterior pituitary granules, 27% of the PAM and 49% of the PAL activity remained with the carbonate washed membranes; in an intermediate pituitary granule, only 11% of the PAM and PAL activity remained with the carbonate washed membranes.

Anterior pituitary membranes were washed with 0.1 M NaCO₃ to remove peripheral proteins and the membranes were subjected to Western blot analysis (Fig. 7). In contrast to the complex pattern observed when whole secretory granules were examined, antisera to purified rPAM-A/B as well as antisera to peptides derived from PAM, PAL, exon B, or the COOH-terminal domain all visualized a prominent 105 kDa protein in carbonate washed anterior pituitary membranes. This 105 kDa PAM protein is likely to include proteins derived from rPAM-A and rPAM-B. Consistent with the low abundance of rPAM-A mRNA in the pituitary, a 120 kDa PAM protein corresponding to rPAM-A was not a major component of anterior pituitary membranes. The minor 95 kDa PAM protein visualized by PHM and PAL antisera in carbonate washed anterior pituitary membranes was recognized by antisera to exon B, but not by antisera to the COOH-terminal domain and may represent a form of PAM that has undergone endoproteolytic cleavage in the COOH-terminal domain. No prominent low molecular weight cross-reactive antibodies were detected in anterior pituitary membranes.

The soluble proteins present in anterior pituitary secretory granules were separated by gel filtration and analyzed by Western blotting (Fig. 8A). In the soluble fraction, the 75 kDa protein detected by both the PHM and PAL antibodies predominated; most of the 105 kDa rPAM protein was removed along with the membranes. The 105 kDa PAM protein recovered from the soluble fraction differed from the 95 kDa protein recovered in the carbonate washed membranes and was recognized by the COOH-terminal antisera but not by the exon B, anti-AM (data not shown).

Fig. 5. Structure of the PAM gene in the region probed by rPAM-5. Sprague-Dawley rat liver DNA (0.5 μg) was amplified using a sense oligonucleotide primer within the PHM domain (1B, bp 1172-1193 of rPAM-1) and antisense oligonucleotides from the 3′ untranslated region of rPAM-5 (BE23, bp 1491-1512, or BE 22, bp 2323-2342) of rPAM-5; numbers for rPAM-5 have been adjusted to coincide with rPAM-1. Primase (20 μg 106 mg) was used as a control. Amplified fragments were separated or a 1% agarose gel and the ethidium bromide stained gel is shown. Apparent molecular weights are shown to the side of each panel.

Fig. 6. Western blot analysis of carbonate washed membrane fraction prepared from anterior pituitary. The crude particulate fraction from a homogenate of anterior pituitary was subjected to a series of washes with 1 M NaCl and 0.1 M NaCO₃, pH 11.5 as described in Methods. Samples contained 35 μg protein and 39 pmol PAM activity. Antibodies were used as indicated.

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Fig. 8A. Western blot analysis of soluble and particulate fractions. The same preparation of anterior pituitary secretory granules analyzed in Fig. 6 was frozen, diluted with an equal volume of water and granule membranes were removed by ultracentrifugation. Proteins in the soluble fraction

(25 μg protein) were fractionated and stained with the antibodies indicated. B. Soluble and carbonate washed crude particulate fractions were prepared from intermediate pituitary extracts. Seventy μg of soluble protein and 115 μg of membrane protein were analyzed.

Several smaller proteins ranging in size from 37 to 58 kDa were detected in the soluble fraction by various PHM antibodies (Fig. 8A). A doublet of 44-45 kDa PHM proteins along with lesser and variable amounts of 78, 42 and 37 kDa PHM protein were visualized by antibody to purified rPAM-A/B, rPAM(116-131) and rPAM(293-315). Since no paired basic potential endoproteolytic cleavage sites separate the PHM and PAL domains in PAM mRNAs lacking exon A, separation of a PHM domain from this size from the PAL domain requires endoproteolytic cleavage at a different type of site. Peptides encoded by mRNAs of the rPAM-4 and rPAM-5 type could generate proteins of this size without any endoproteolytic cleavage events subsequent to removal of the signal peptide. Relatively small amounts of soluble, monofunctional PAL (54 and 50 kDa) were detected in anterior pituitary granules. Definitive identification of these proteins will require purification and sequence analysis.

The PHM and PAL proteins in soluble and membrane fractions prepared from intermediate pituitary extracts were compared (Fig. 8B). Like anterior pituitary membranes, intermediate pituitary membranes contained a 105 kDa protein detected by antisera to PHM and PAL; some 75 kDa PAM protein along with a 53 kDa protein detected by this PHM antisem remained membrane associated. The major bifunctional PAM protein in the soluble fraction was 75 kDa; lesser amounts of 55 and 44 kDa PAL proteins were present. Soluble soluble PAM proteins of 45, 44 and 37 kDa were observed. Only small amounts of 52 and 50 kDa PAL were present in the soluble fraction prepared from intermediate pituitary extracts.