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Betty A. Eipper, C Green, T Campbell, D Stoffers, H Keutmann, R Mains, L'Houcine Ouafik

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

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Betty A. Eipper‡, Cristina B.-R. Green, Tracey A. Campbell, Doris A. Stoffers, Henry T. Keutmann, Richard E. Mains, and L'Houcine Ouafik

From the Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

The pituitary is a rich source of peptidylglycine α-amidating monooxygenase (PAM). This bifunctional protein contains peptidylglycine α-hydroxylating monooxygenase (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 monooxygenase (PAM; EC 1.14.17.3)[1] 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 monooxygenase (PHM), is contained within the NH_2-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 nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M82845.

‡ To whom correspondence should be addressed: Rm. 902, Wood Basic Science Bldg., Dept. of Neuroscience, The Johns Hopkins University School of Medicine, 725 North Wolfe St., Baltimore, MD 21205. Tel.: 410-955-6937; Fax: 410-955-0681.

[1] The abbreviations used are: PAM, peptidylglycine α-amidating monooxygenase; PHM, peptidylglycine α-hydroxylating monooxygenase; 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).

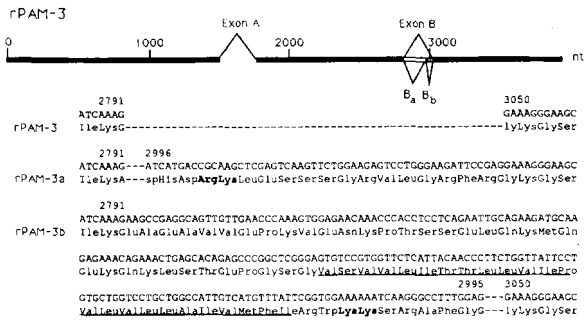

FIG. 2. **Alternative splicing in the exon B region: rPAM -3a and -3b.** The nucleotide and amino acid sequences of rPAM-3a and -3b in the region previously referred to as exon B are shown; the remainder of the sequence is identical to that of rPAM-2 (16, 18). Paired basic amino acids are *boldface*; amino acids in the putative transmembrane domain are *underlined*.

instead contain PAM mRNAs smaller than those found in the atrium (2). Reverse transcription coupled to the polymerase chain reaction using oligonucleotide primers spanning most of the protein coding region of rPAM-1 demonstrated that the forms of PAM mRNA in the anterior and neurointermediate lobes of the pituitary were similar to each other and included forms in addition to those characterized in adult atrium (18). By constructing and screening anterior and neurointermediate pituitary cDNA libraries, we were able to identify three additional novel forms of PAM mRNA.

While rPAM-1 and -2 mRNA encode PAM proteins with a signal sequence and a putative transmembrane domain near their COOH terminus, the proteins encoded by rPAM-3 and -4 mRNA lack a transmembrane domain (17, 18). Despite a similar distribution of forms of PAM mRNA in the anterior and neurointermediate lobes of the pituitary, the majority of the PAM activity in the neurointermediate lobe is soluble, whereas equivalent amounts of soluble and membrane-associated PAM activity are found in the anterior pituitary (2, 19). In this study, the major forms of PAM protein in the anterior and neurointermediate lobes of the pituitary were identified using antipeptide antibodies. In addition to tissue-specific alternative mRNA splicing, PAM proteins are subject to tissue-specific endoproteolytic processing.

MATERIALS AND METHODS AND RESULTS[2]

Identification of Novel rPAM cDNAs in Pituitary cDNA Libraries—cDNA libraries prepared from rat neurointermediate and anterior pituitary poly(A)$^+$ RNA were screened with cDNA probes spanning the sequence of rPAM-1 (Fig. 1). Two novel types of PAM cDNA, rPAM-3a and rPAM-3b, were identified by characterizing PAM cDNA inserts from the neurointermediate pituitary library (Fig. 2). Inserts of each type were sequenced and found to represent additional splicing variants. cDNAs of the rPAM-3b type (Int12) differed from rPAM-2 only by the deletion of a 54-nt segment corresponding to the final 54 nt of the 258-nt region referred to as exon B. cDNAs of the rPAM-3a type (Int22) differed from rPAM-2 only by the deletion of the first 204 nt of exon B. Analysis of the gene encoding rat PAM indicates that the region previously referred to as exon B is composed of two exons: a 204-nt exon (exon B_a) is situated approximately 3000 nt upstream of a 54-nt exon (exon B_b) (25). Exon B_b corresponds exactly to the 54 nt deleted from bPAM-1 (originally called λPAM-1) to form bPAM-2 (λPAM-5) (24).

The 853-amino acid rPAM-3b preproprotein has a predicted molecular weight of 94,721 (pI 5.76) and lacks an 18-amino acid peptide predicted to reside in the cytoplasmic domain of rPAM-2 (Fig. 2). The deleted 2115-dalton peptide (pI 10.83) is extremely hydrophilic and includes a pair of basic amino acids (Arg^{903}-Lys^{904}; amino acid positions are all numbered as for the protein encoded by rPAM-1). The 803-amino acid rPAM-3a preproprotein has a predicted molecular weight of 89,344 (pI 5.78) and lacks a 68-amino acid peptide present in rPAM-1 and -2; the deleted peptide consists of a hydrophilic domain followed by the hydrophobic 24-amino acid putative transmembrane domain and the subsequent Arg-Trp-Lys_2-Lys^{894} putative stop transfer signal. This 7492-dalton peptide has a pI of 9.06 and lacks Cys residues or potential *N*-glycosylation sites. Thus mRNAs of the rPAM-3b type would be expected to encode an integral membrane protein, whereas mRNAs of the rPAM-3a type would be expected to encode a soluble protein.

When the PAM containing cDNA inserts in the anterior pituitary library were subsequently examined, a novel type of PAM cDNA (Ant67, rPAM-5 type) was identified by virtue of its failure to hybridize with the cDNA probe from the 3′-third of rPAM-1 (Fig. 1). Upon sequence analysis, Ant67 was found to be identical to rPAM-1 from bp 247 through bp 1217 (bp 971 of Ant67) (Fig. 3). A novel 1.1-kb sequence followed; this relatively AT-rich region (61% AT) did not terminate with a poly(A) tail but contained four consensus poly(A) addition signals (26, 27). The novel 3′-domain of Ant67 bore no resemblance to the novel 3′-domain of rPAM-4 (18). The point of divergence of Ant67 follows amino acid Gly^{307} of rPAM-1; Ant67 encodes an additional pentapeptide before an in-frame stop codon is reached.

The 312-amino acid preproprotein encoded by Ant67 has a predicted molecular weight of 34,674 and a pI of 8.93. Following removal of the signal peptide, the protein encoded by Ant67 would have a molecular weight of 32,151; the Lys_2-Arg^{35} sequence following the putative propeptide is the only paired basic amino acid sequence in the protein encoded by rPAM-5. Two cysteine residues and the -His-Gly-His-His^{367} sequence postulated to play a role in the interaction of the PHM domain with copper are absent from the rPAM-5 protein. Transient expression of cDNA encoding rPAM-5 in human fibroblasts (hEK293) yielded expression of foreign protein but failed to yield PHM activity under the same conditions yielding PHM activity upon expression of cDNA encoding rPAM-4.

The presence of mRNAs of the rPAM-5 type in several tissues was demonstrated using reverse transcription and 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 PHM exon terminating with nt 1217 is separated from the exon containing the unique 3′-end of rPAM-5 by an approximately 550-nt intron.[3] 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-

[2] 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.

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

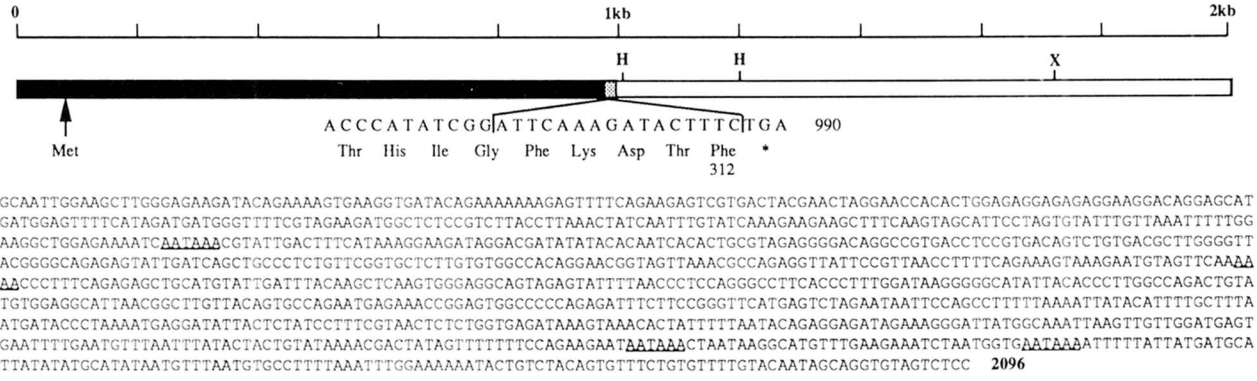

FIG. 3. **Sequence of rPAM-5 type cDNA.** The cDNA insert in Ant67 is drawn to scale with the region of identity to rPAM-1 shown by the *filled bar* and the new sequence shown by the *open bar*. The location of the initiator Met and the first in-frame Stop codon (*) are indicated. Characteristic restriction sites are shown: *H*, *Hin*dIII; *X*, *Xba*I. A cDNA probe specific for rPAM-5 was generated by using the fragment between the *Hin*dIII 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*.

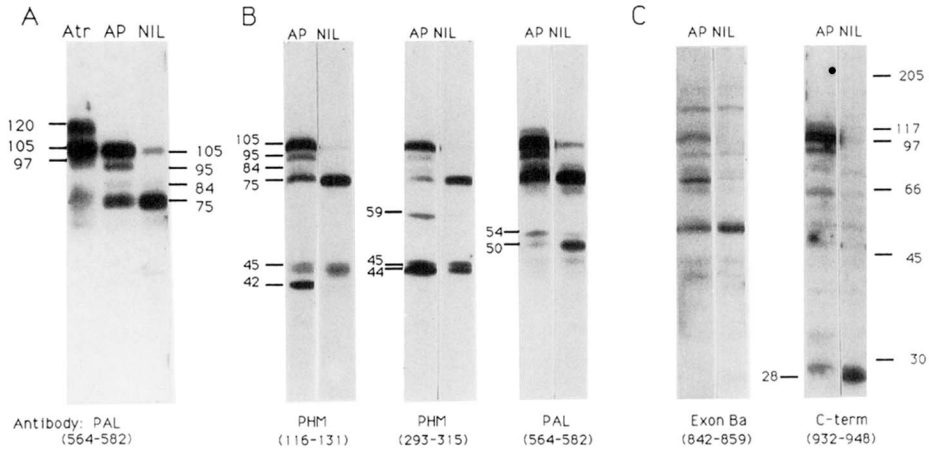

FIG. 6. **Comparison of PAM proteins in atrial, anterior, and neurointermediate pituitary secretory granules by Western blot analysis.** *A*, a crude secretory granule fraction was prepared from adult rat anterior and neurointermediate pituitary and from adult rat atrium by differential centrifugation. 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 4 pmol/h PAM activity (15 μg of protein). The samples were visualized with PAL antibody (Ab69). *B* and *C*, a more concentrated preparation of crude pituitary granules was analyzed. Each sample of anterior 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_a 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*.

rointermediate Pituitary—Despite the presence of similar forms of PAM mRNA in the anterior and neurointermediate lobes of the rat pituitary, previous studies indicated that membrane associated PAM was more prevalent in anterior pituitary than in neurointermediate pituitary extracts (2). The PAM proteins found in any tissue reflect both the forms of PAM mRNA present and the co- or post-translational modifications that occur. The higher molecular weight PAM proteins in secretory granule-enriched fractions prepared from rat anterior pituitary, neurointermediate pituitary, and atrium were compared by Western blot analysis (Fig. 6*A*). 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.

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

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. 6*B*). The most prominent bands had molecular masses of 105 ± 3 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 NH_2 terminus of PHM (rPAM(116–131)) (Fig. 6*B*). 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 antisera to both PHM and PAL (Fig. 6*B*). 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 antisera to peptides contained within exon B_a and the COOH-terminal cytoplasmic domain of rPAM-1 (Fig. 6*C*). Antibody to exon B_a 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 (rPAM-1, -2, -3a, -3b, or -3) by endoproteolytic cleavage. In the neurointermediate pituitary granules, an approximately 28-kDa protein was visualized by the COOH-terminal domain antiserum; it is not yet clear whether this protein is derived from PAM.

The 105-kDa PAM protein is found in anterior pituitary membranes washed with 0.1 M Na_2CO_3 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. 8*B*). The soluble fraction of anterior pituitary secretory granules contains large amounts of both the 75-kDa PAM protein recognized by antisera to PHM and PAL and the monofunctional 44–45 kDa PHM protein (Fig. 8*A*). The 95-kDa PAM protein found in the soluble fraction is recognized by antisera 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.

DISCUSSION

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_a and B_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_a contains the transmembrane domain and its stop transfer signal, whereas exon B_b contains a pair of basic amino acids (Arg^{903}-Lys^{904}). The fact that exon B_b corresponds exactly to the 54-nt region distinguishing two forms of bovine PAM mRNA (24) suggests that it may have functional significance. The peptide encoded

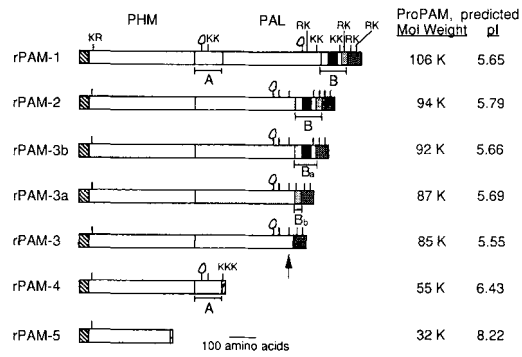

FIG. 9. **PAM proteins.** The proteins encoded by each type of PAM mRNA are drawn to scale with the signal peptide (*cross-hatched*), putative transmembrane domain (*filled box*), exon B_b (*lightly stippled*), remainder of the COOH-terminal domain (*darkly stippled*), and potential paired basic amino acid endoproteolytic cleavage sites (K = Lys, R = Arg) and *N*-glycosylation sites (*irregular enclosed shape*) indicated. Predicted molecular weights and isoelectric points (pI) of the nonglycosylated proproteins are shown. *Arrows*, endoproteolytic cleavage site thought to be used in the production of the 75-kDa PAM protein from rPAM-2, -3b, -3a, and -3.

by exon B_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 FcR_{II} 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_b peptide should be situated within the secretory granule; in this location it could serve as a paired basic endoproteolytic 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_b). The Type B cDNA is essentially identical to rPAM-1 until a point close to the 3′-end of exon B_a; a 3-bp insertion is followed by 47 bp of exon B_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 (33). 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_a, and B_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 rat pituitary was confirmed by Northern blot analysis (2), reverse transcription coupled with polymerase chain reaction (18), and Western blot analysis of the PAM proteins present (Figs. 6 and 7). Differences between Sprague-Dawley and Wistar rats were not confined to the exon A region. Using an RNase protection assay the most prevalent form of PAM mRNA in the Wistar rat pituitary was found to lack exon B_a and retain exon B_b (29); in the Sprague-Dawley rat this was the least prevalent splicing pattern. Forms of PAM mRNA retaining exon B_a and lacking exon B_b were prevalent in the Sprague-Dawley rat and rare in the Wistar rat. Although PAM mRNAs encoding the transmembrane domain predominate in Sprague-Dawley rat pituitary (rPAM-2 and -3b), the major form of PAM mRNA in the Wistar rat pituitary lacks a transmembrane domain (29). Although we searched for forms of PAM mRNA retaining exon A and lacking exon B_a and/or exon B_b, none were found. Comparison of the genes encoding PAM in Sprague-Dawley and Wistar rats may clarify the reasons for these differences.

Although PHM and PAL are active as independent soluble enzymes, and also active when part of a bifunctional membrane-associated protein (28), secretion of PHM and PAL along with the secretory granule content requires an endoproteolytic cleavage to separate the PAL domain from the transmembrane domain in forms retaining exon B_a. Secretion of the proteins encoded by rPAM-3, -3a, -4, and -5 requires no endoproteolytic cleavages subsequent to signal peptide removal.

PAM undergoes tissue-specific endoproteolytic processing, with more extensive endoproteolytic processing of PAM occurring in the neurointermediate lobe. The only major integral membrane protein form of PAM seen in the anterior pituitary is a 105-kDa doublet likely to represent rPAM-2 and -3b; very little of this 105-kDa PAM protein is found in the neurointermediate lobe. Small amounts of a 95-kDa PAM protein detected in anterior pituitary membranes appear to lack at least part of the COOH-terminal cytoplasmic domain. The 95-kDa PAM protein found in the soluble fraction is visualized by antisera to the COOH-terminal domain of rPAM-1 and is likely a combination of rPAM-3/3a. The major soluble 75-kDa PAM protein observed in both anterior and neurointermediate pituitary granules lacks antigenic determinants for exon B_a and the COOH terminus of rPAM-1 and could be derived from rPAM-2, -3, -3a, or -3b by endoproteolytic cleavage at one of the two paired basic amino acid sequences following the PAL domain. In CA-77 cells, processing at $Lys\text{-}Lys^{822}$ is thought to generate a soluble 75-kDa PAM protein from rPAM-3b (Type A); the PAM protein containing the peptide encoded by exon A (Type B mRNA) is cleaved to form 41- and 43-kDa proteins (33, 34). If endoproteolytic processing events generate 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_a 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 now have information on the endoproteolytic processing of PAM in several tissues. In the atrium, both PAM and pro-atrial natriuretic factor are stored in a largely unprocessed form; primary cultures of neonatal atrial myocytes cleave proANF at the time of secretion and also secrete large amounts of PHM and PAL activity, indicating that endoproteolytic cleavage of the PAM precursor must occur (35, 36). The endoproteolytic processing of PAM in the neurointermediate lobe of the pituitary is more extensive than that observed in the anterior lobe. The endoproteolytic processing of proopiomelanocortin is also much more extensive in the intermediate than in the anterior lobe of the pituitary. Given that the extent of PAM processing correlates with the extent of endogenous preprohormone processing, one wonders whether similar endoproteases are involved in processing PAM and its prohormone substrates. The tissue-specific endoproteolytic processing of PAM resembles the tissue-specific processing of prohormones; instead of generating sets of bioactive peptides, various forms of active enzyme are created.

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SUPPLEMENTARY MATERIAL TO:

Alternative Splicing and Endoproteolytic Processing Generate Tissue Specific Forms of Pituitary PAM

Betty A. Eipper, Cristina B.-R. Green, Tracey A. Campbell, Doris A. Stoffers, Henry T. Keutmann, Richard E. Mains, L'Houcine Ouafik

MATERIALS AND METHODS

Construction and Screening of Neurointermediate and Anterior Pituitary cDNA Libraries. Total RNA (173 μg) was prepared from the neurointermediate lobes of 65 adult male Sprague Dawley rat pituitaries. Poly(A)+ RNA (3.2 μg) was isolated and used to synthesize cDNA with EcoRI adaptors using the Pharmacia mRNA isolation and cDNA synthesis kits. Ligation of 40% of the cDNA to 1 μg EcoRI-digested Lambda ZAP II arms (Stratagene) and packaging with the Gigapack Gold In vitro packaging kit (Stratagene) yielded 1.6×10^6 pfu. For screening, 300,000 phage from the unamplified library were plated at a density of 50,000 phage per 150-mm plate. Triplicate lifts were screened with 3 random primed restriction fragments spanning the sequence of rPAM-1 (Fig. 1).

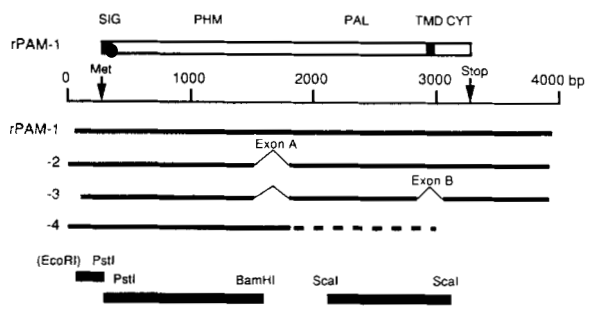

Fig. 1. PAM cDNAs Identified in Adult Rat Atrium. The structures of the 4 types of rPAM cDNA characterized from an atrial library are shown (16-18). Exons A and B are indicated, as are the rPAM-1 cDNA fragments used as probes to screen the pituitary libraries: EcoRI (cloning site used in construction of library)/PstI, bp 117-356; PstI/BamHI fragment, bp356-1682; ScaI/ScaI fragment, bp 2229-3190. Key features of the PAM protein are indicated for orientation: Met, initiator methionine; SIG, signal peptide; PHM; PAL; TMD, transmembrane domain; CYT, cytoplasmic domain. Stop, termination codon.

Of the 63 positive plaques, the 38 yielding the most intense signal were plaque purified and 34 remained positive. Twenty seven of these phage were rescued using R408 helper phage (Stratagene); 8 of the resultant plasmids contained inserts less than 1 kb in length and were not further characterized, and 6 lacked identifiable inserts. The 13 PAM positive plasmids were compared by restriction analysis and by PCR using pairs of oligonucleotide primers spanning the sequence of rPAM-1. Two inserts were of the rPAM-2 type and 4 were of the rPAM-3 type. Seven cDNA inserts gave patterns differing from any of the known forms of rPAM and were subdivided into 2 types, rPAM-3a (1 insert) and 3b (6 inserts). Using oligonucleotides derived from rPAM-1 and the Sanger dideoxy chain termination method (20), sequence information was obtained for a cDNA representing the rPAM-3a (int22) and rPAM-3b (int12) type. int12 was sequenced on 1 strand in its entirety and on both strands over splice junctions. int22 and int26 (rPAM-3 type) were sequenced on both strands over splice junctions and at both ends.

Poly(A)+ RNA (11.2 μg) was prepared from 400 μg total RNA from adult male Sprague Dawley rat anterior pituitary and 3 μg poly(A)+ RNA was used as above to synthesize cDNA; packaging of 36% of the material yielded a Lambda ZAP cDNA library containing 3.6×10^6 pfu. In the first round of screening with the 1.3 kb PstI/BamHI (356/1682) rPAM cDNA probe (Fig.1), 110 positive phage were identified in 300,000 pfu; 53 of these remained positive through a second round of screening. Eighteen were selected for further characterization; 7 were lost in the rescue step and only 8 of the remaining 11 had cDNA inserts. Restriction mapping indicated that 1 was of the rPAM-2 type, 2 were of the rPAM-3 type; 3 fell into the new class defined by int12 (rPAM-3b) and 1 into the new class defined by int22 (rPAM-3a). One plasmid (Ant67) contained a 2.1kb cDNA insert that failed to hybridize with a cDNA probe from the 3'-region of rPAM-1 (ScaI/ScaI fragment; Fig.1); restriction enzyme analysis indicated that the insert was distinct from any of the previously characterized rPAM cDNAs. Ant67 was sequenced on a single strand in the region identical to rPAM-1 and on both strands throughout its novel 3'-domain.

The greater prevalence of PAM positive phage observed in the anterior pituitary library compared to the neurointermediate pituitary library is consistent with the higher levels of PAM mRNA detected in the rat anterior pituitary by Northern blot analysis (2). Similar screenings of atrial cDNA libraries yielded approximately 4 times as many positive plaques as in the anterior pituitary (16). In both the anterior and neurointermediate pituitary libraries, almost half of the PAM positive cDNA inserts examined were of the rPAM-3b type; cDNA inserts corresponding to rPAM-3a were recovered least often. No cDNA inserts retaining exon A were isolated from either library.

Polymerase chain reaction. PCR was utilized to characterize the cDNA inserts in phage or plasmids and to evaluate the representation of forms of PAM cDNA in the libraries. Aliquots of phage were heat denatured (5 min, 95°) and subjected to 20 - 25 cycles of PCR using 1 μM primer (all were 17-mers) as described (18). Annealing temperatures were generally 52° and extension times ranged from 2 min for pieces smaller than 1 kb to 4 min for the entire insert. Products were analyzed after visualization with ethidium bromide or after transfer to Nytran and hybridization with appropriate random primed cDNA probes. Insert length was determined using T_3 and T_7 primers and the forms of rPAM cDNA were determined with primers spanning exons A and B (18). cDNA was synthesized from 2 - 5 μg total RNA using 1 μg oligo$(dT)_{12-18}$ (Pharmacia) as primer and AMV Reverse Transcriptase (Life Sciences) as described previously (18). The amounts of cDNA from different tissues utilized for PCR were adjusted to yield signals of comparable intensity (2).

Amplification of aliquots of the cDNA libraries using the polymerase chain reaction and primer pairs spanning exon A or exon B also indicated that cDNA inserts containing exon A were absent from the neurointermediate pituitary library and represented only a minor component of the anterior pituitary library. With primers spanning exon B, cDNA inserts containing all of exon B (rPAM-1, rPAM-2) or corresponding to rPAM-3b predominated; cDNA inserts of the rPAM-3 type were considerably less prevalent, with cDNA inserts of the rPAM-3a type the least common. Reverse transcription and amplification by polymerase chain reaction using primer pairs spanning exon A, exon B or both exon A and exon B also indicated a predominance of mRNAs of the rPAM-2 and rPAM-3b type, with small amounts of rPAM-3 and very little rPAM-3a. The various ways of estimating the prevalence of different PAM mRNAs all indicated that the anterior and neurointermediate lobes of the pituitary contain a similar collection of forms.

Preparation of Subcellular Fractions. For preparation of crude particulate and soluble fractions, fresh or frozen tissues were homogenized in 20 mM Na TES, pH 7.0, 10 mM mannitol (TES/mannitol) containing protease inhibitors (16 μg/ml benzamidine, 10 μg/ml lima bean trypsin inhibitor, 2 μg/ml leupeptin, 3 μg/ml phenylmethylsulfonyl fluoride); crude particulate and soluble fractions were prepared after removal of nuclei and debris as described (2). Peripheral membrane proteins were removed by washing the membranes sequentially with TES/mannitol, TES/mannitol containing 1 M NaCl, TES/mannitol, 0.1 M Na_2CO_3, pH 11.5, and TES/mannitol (21). Triton X-100 (1%) was added to all particulate fractions before enzyme assays were carried out.

A secretory granule enriched fraction was prepared from adult rat anterior and neurointermediate pituitaries by homogenization in 20 mM Na TES, pH 7.0, 0.25 M sucrose containing protease inhibitors as above and 20 μg/ml DNase I. A crude nuclear pellet was removed by centrifugation at 1700 x g_{av} for 10 min; crude mitochondria were pelleted by centrifugation at 1700 x g_{av} for 10 min; crude granules/microsomes were pelleted together by centrifugation at 356,000 x g_{av} for 15 min. Where indicated, the granule and/or microsome contents were separated from membranes by resuspension in 0.1 M Na_2CO_3 (21); following a 30 min incubation on ice, carbonate washed granule/microsome membranes were pelleted and soluble contents were neutralized by addition of one tenth volume 2 M TES.

Assays of enzyme activity. PAM activity was measured as described using [^{125}I]-D-Tyr-Val-Gly and 0.5 μM D-Tyr-Val-Gly at pH 8.5 in the presence of 0.5 mM ascorbate and 5 μM $CuSO_4$ (2,22); at pH 8.5 the peptidyl-α-hydroxyglycine intermediate created by PHM is converted into α-amidated product non-enzymatically. PHM activity was measured using [^{125}I]-α-N-acetyl-Tyr-Val-Gly; the α-N-acetyl-Tyr-Val-α-hydroxyglycine created by PHM was converted into α-amidated product by the addition of base at the end of the incubation (14). PAL activity was measured using [^{125}I]-α-N-acetyl-Tyr-Val-α-hydroxyglycine as substrate (14). Samples were assayed in duplicate at two or more concentrations of protein. The protein content of samples was determined with the bicinchoninic acid assay using bovine serum albumin as standard (Pierce Chemical Co., Rockford, IL). Duplicates agreed within ± 5% and reaction rates were linear in protein.

Western Blot Analysis. Aliquots containing known amounts of protein and enzyme activity were fractionated on 10% polyacrylamide, 0.25% N,N'-methylene-bis-acrylamide SDS gels as described (22). Proteins were transferred (200 mAmp for 4 h) to Immobilon-P membranes (Millipore) and visualized with Coomassie Brilliant Blue R-250. Following destaining in methanol, the membrane was blocked by incubation with 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.2% Tween-20, 10 mg/ml bovine serum albumin and then incubated with affinity purified antibody diluted in blocking buffer either overnight at 4° or for 2-3 h at room temperature. After washing, bound antibody was visualized by incubation with 10^6 cpm/ml [^{125}I]-Protein A (ICN) in 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.2% Tween-20, 2.5 mg/ml bovine serum albumin for 2 h at room temperature. In earlier experiments, bound antibody was visualized with alkaline phosphatase coupled to goat anti-rabbit immunoglobulin as described (22). Molecular weight standards (myosin, β-galactosidase, phosphorylase b, bovine albumin, ovalbumin, carbonic anhydrase; Sigma Chemical Co.) were analyzed in a separate lane. Prestained α_2-macroglobulin and fumarase (Sigma Chemical Co.) were included in the standard to allow immediate evaluation of transfer efficiency; use of prestained standards (Rainbow, Amersham Corp.) for molecular weight determination gave slightly different molecular weight estimates. All molecular weight data presented were derived by comparison to the unlabeled protein standards.

The polyclonal rabbit antibodies used in this study include several raised to bPAM(288-310) conjugated to soybean trypsin inhibitor through its Cys residues (Ab59, Ab60, Ab98, Ab100) (23); bPAM(288-310) lies in the PHM domain and differs from rPAM(293-315) at 1 residue. Two antibodies were generated to bPAM(561-579) conjugated to hemocyanin with glutaraldehyde (Ab68, Ab69); this peptide lies in the PAL domain and differs from rPAM(564-582) at 2 residues. Antisera specific to exon B_s were generated to rPAM(842-859) conjugated to hemocyanin with glutaraldehyde (Ab25, Ab26). Reduction of non-specific background with Ab26 required replacement of the normal blocking solution with 100 mM Na phosphate, pH 7.4, 5% non-fat dry milk, 0.2% Tween-20, 0.02% NaN_3 (the turbid solution was clarified by centrifugation before use). Antisera to the COOH-terminal domain of rPAM-1 were generated to rPAM(932-948) linked to keyhole limpet hemocyanin with glutaraldehyde. Anti-peptide antisera were affinity purified using the appropriate peptide-resin (22). Preparation and affinity purification of antibody to purified bovine PAM A plus PAM B (Ab36) 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 blot with antibody; using [^{125}I]-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

Pituitary PAM mRNAs. PAM-5 mRNA could not be detected by Northern blot analysis of pituitary 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,-3a and -3b mRNAs or using primers specific to mRNAs of the rPAM-5 type. Amplified products derived from PAM mRNAs of the rPAM-5 type were found in each tissue.

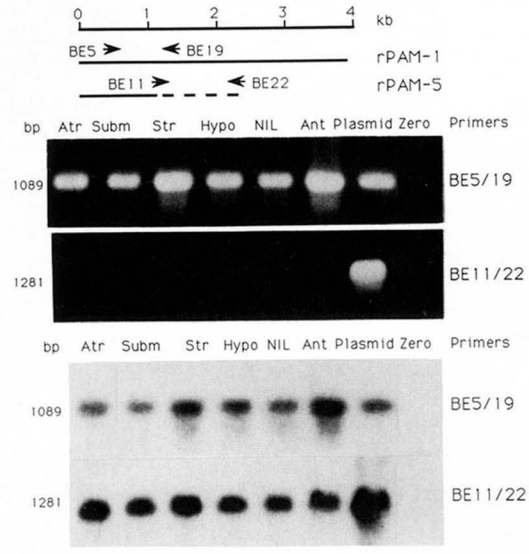

Fig.4. Detection of rPAM-5 mRNA by RT-PCR. Reverse transcribed cDNA from the tissues indicated (Atr, atrium; Subm, submaxillary gland; Str, striatum; Hypo, hypothalamus; NIL, neurointermediate pituitary; Ant, anterior pituitary) was amplified using a pair of oligonucleotides common to rPAM-1,-2,-3,-3a,-3b and -4 (BE5/BE19) or a pair of oligonucleotides specific to rPAM-5 (BE11/BE22); extension time was 3 min and number of cycles was 30. Appropriate plasmid controls (10 pg ZAP8 for BE5/BE19 and 10 pg Ant67 for BE11/BE22) served as standards along with the blank. 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 PHM domain (Fig.1) was used to detect the products amplified with BE5/19 and the 0.2 kb probe derived from Ant67 (Fig.3) was used to detect products amplified with BE11/22.

Transcripts of the rPAM-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 rPAM-5 diverges (BE39) paired with either of two antisense oligonucleotide primers situated within the novel 3'-sequence of rPAM-5 (BE23, BE22) (Fig.5). While both pairs of primers generated the expected products when used to analyze the Ant67 plasmid (334 and 1170 bp, respectively), analysis of genomic DNA yielded larger fragments (0.87 and 1.75 kb fragments, respectively). These data indicate that an approximately 550 bp intron separates the PHM exon containing BE39 from the exon containing the unique 3'-end of rPAM-5.

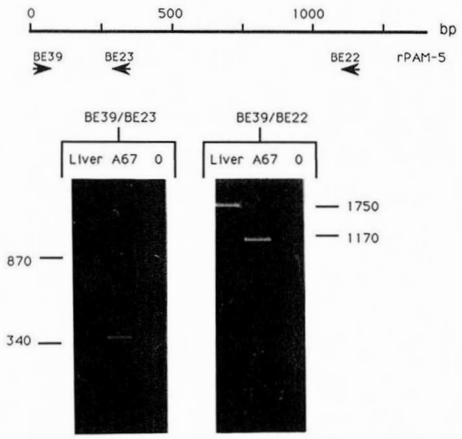

Fig.5. Structure of the PAM gene in the region preceding rPAM-5. Sprague-Dawley rat liver DNA (0.5 μg) was amplified using a sense oligonucleotide primer within the PHM domain (BE 39, bp 1172-1193 of rPAM-1) and antisense oligonucleotides from the 3'-untranslated region of rPAM-5 (BE23, bp 1496-1512, or BE 22, bp 2323-2342 of rPAM-5; numbers for rPAM-5 have been adjusted to coincide with rPAM-1). Plasmid DNA (10 pg A67) was used as a control. Amplified fragments were separated on a 1 % agarose gel and the ethidium bromide stained gel is shown. Apparent molecular weights are shown to the side of each panel.

Characterization of soluble and membrane-associated PAM proteins. One of the features distinguishing the proteins encoded by the different PAM mRNAs is the presence or absence of a transmembrane domain (Fig.2). Anterior and neurointermediate pituitary differ in the extent to which amidation activity remains membrane associated following extraction of membranes with 0.1 M Na_2CO_3. While 34% of the total PAM activity in an anterior pituitary homogenate remained membrane associated, only 6% of the total PAM activity in a neurointermediate pituitary homogenate did so. For comparison, 59% of the total PAM activity in atrial granules and 78% of the total PAM activity in atrial microsomes remained membrane associated following extraction with carbonate. Similar results were obtained when granule enriched fractions were separated into soluble proteins and carbonate-washed membranes and assayed for PHM and PAL activity. In anterior pituitary granules, 27% of the PHM and 49% of the PAL activity remained with the carbonate washed membranes; in neurointermediate pituitary granules, only 11% of the PHM and PAL activity remained with the carbonate washed membranes.

Anterior pituitary membranes were washed with 0.1 M Na_2CO_3 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 bPAM-A/B as well as antisera to peptides derived from PHM, PAL, exon B_a and 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-2 and rPAM-3b. Consistent with the low abundance of rPAM-1 mRNA in the pituitary, a 120 kDa PAM protein corresponding to rPAM-1 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_a, 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 proteins cross-reactive with the COOH-terminal domain antibody were detected in anterior pituitary membranes.

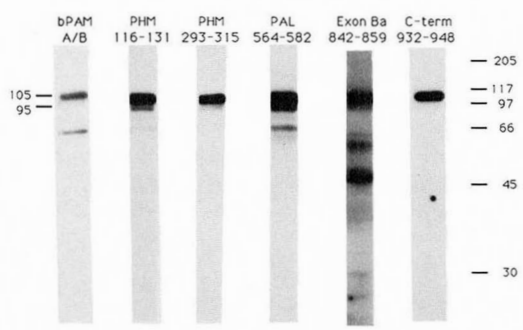

Fig.7. Western blot analysis of carbonate washed membrane fraction prepared from anterior pituitary. The crude particulate fraction from a homogenate of anterior pituitary was subjected to a series of washes with 1 M NaCl and 0.1 M Na_2CO_3, pH11.5 as described in Methods. Samples contained 35 μg protein and 39 pmol/h PAM activity. Antibodies were used as indicated.

The soluble proteins present in anterior pituitary secretory granules were separated from secretory granule membranes and analyzed (Fig. 8A). In the soluble fraction, the 75 kDa protein detected by both the PHM and PAL antibodies predominated; most of the 105 kDa PAM protein was removed along with the membranes. The 95 kDa PAM protein recovered in the soluble fraction differed from the 95 kDa protein recovered in the carbonate washed membranes and was recognized by the COOH-terminal antiserum but not by the exon B_a antiserum (data not shown); absence of the exon B_a antigenic determinant is consistent with identification of the soluble 95 kDa PAM protein as a product of rPAM-3/3a. The 75 kDa PAM protein was not visualized by antisera to the COOH-terminus or to exon B_a, indicating that it is a product of an endoproteolytic event that occurs at a site preceding these antigenic determinants. The small amounts of 84 kDa PAM protein in anterior and neurointermediate pituitary granules precluded evaluation of its recognition by antisera to the COOH-terminus or exon B_a.

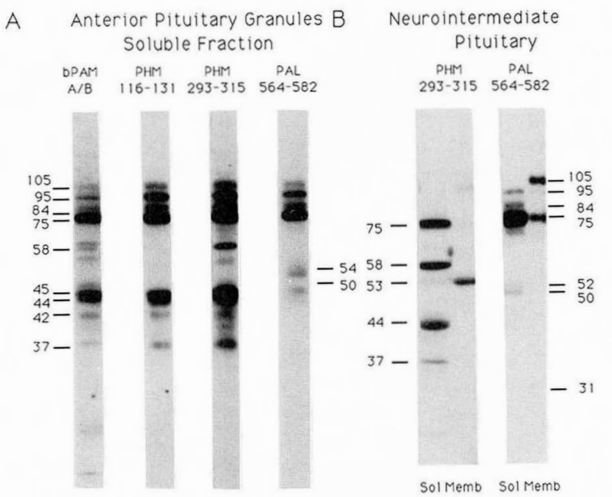

Fig.8. Western blot analysis of soluble and particulate fractions. The same preparation of anterior pituitary secretory granules analyzed in Fig.6 was frozen, diluted with an equal volume of water nd granule membranes were removed by ultracentrifugation. Proteins in the soluble fraction (25 μg protein) were fractionated and analyzed with the antibodies indicated. B. Soluble and carbonate washed crude particulate fractions were prepared from neurointermediate pituitary extracts. Seventy μg of soluble protein and 110 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 58, 42 and 37 kDa PHM protein were visualized by antibody to purified bPAM-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 of this size from the PAL domain requires endoproteolytic cleavage at a different type of site. Proteins 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 neurointermediate pituitary extracts were compared (Fig. 8B). Like anterior pituitary membranes, neurointermediate 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 antiserum remained membrane associated. The major bifunctional PAM protein in the soluble fraction was 75 kDa; lesser amounts of 95 and 84 kDa PAM proteins were present. Smaller soluble PHM proteins of 58, 44 and 37 kDa were observed. Only small amounts of 52 and 50 kDa PAL were present in the soluble fraction prepared from neurointermediate pituitary extracts.