# Expression of Individual Forms of Peptidylglycine α-Amidating Monooxygenase in AtT-20 Cells: Endoproteolytic Processing and Routing to Secretory Granules

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*Abstract.* Peptidylglycine α-amidating monooxygenase (PAM: EC 1.14.17.3) is a bifunctional protein which catalyzes the COOH-terminal amidation of bioactive peptides; the $NH_2$-terminal monooxygenase and mid-region lyase act in sequence to perform the peptide α-amidation reaction. Alternative splicing of the single PAM gene gives rise to mRNAs generating PAM proteins with and without a putative transmembrane domain, with and without a linker region between the two enzymes, and forms containing only the monooxygenase domain. The expression, endoproteolytic processing, storage, and secretion of this secretory granule–associated protein were examined after stable transfection of AtT-20 mouse pituitary cells with naturally occurring and truncated PAM proteins. The transfected proteins were examined using enzyme assays, subcellular fractionation, Western blotting, and immunocytochemistry.

Western blots of crude membrane and soluble fractions of transfected cells demonstrated that all PAM proteins were endoproteolytically processed. When the linker region was present between the monooxygenase and lyase domains, monofunctional soluble enzymes were generated from bifunctional PAM proteins; without the linker region, bifunctional enzymes were generated. Soluble forms of PAM expressed in AtT-20 cells and soluble proteins generated through selective endoproteolysis of membrane-associated PAM were secreted in an active form into the medium; secretion of the transfected proteins and endogenous hormone were stimulated in parallel by secretagogues. PAM proteins were localized by immunocytochemistry in the perinuclear region near the Golgi apparatus and in secretory granules, with the greatest intensity of staining in the perinuclear region in cell lines expressing integral membrane forms of PAM. Monofunctional and bifunctional PAM proteins that were soluble or membrane-associated were all packaged into regulated secretory granules in AtT-20 cells.

**B**IOLOGICALLY active peptides are generated through endoproteolytic cleavage of precursors which are largely inactive (Mains et al., 1990; Steiner, 1991; Dickerson and Noel, 1991). The enzymes involved in post-translational modification of peptide hormones must be packaged together with their substrates in the same cellular compartments. Some of the putative posttranslational processing enzymes including PC1, PC2, and CPH are soluble proteins (Hutton, 1990; Guest et al., 1991); others like furin and Kex 2 are integral membrane proteins (Fuller et al., 1989; Mains and Eipper, 1990), while peptidylglycine α-amidating monooxygenase (PAM)[1] occurs in both membrane-associated and soluble forms (Stoffers and Eipper, 1990). While the maturation of hormone precursors into the regulated secretory pathway has been extensively studied in

neuroendocrine cell lines, trafficking of posttranslational processing enzymes has been much less thoroughly examined.

As demonstrated by the presence of immunoreactive protein in dense-core vesicles or the ability to stimulate the secretion of the expressed protein by secretagogues, prohormones expressed in heterologous neuroendocrine cell lines are usually correctly processed and sorted into secretory granules (Moore et al., 1983; Lapps et al., 1988; Devi et al., 1989; MacDonald et al., 1989; Sevarino et al., 1989; Chevrier et al., 1991). One hypothesis is that a sorting signal similar to KDEL for resident ER proteins (Munro and Pelham, 1986; Pelham, 1988) or mannose-6-phosphate for lysosomal proteins (Kornfeld and Mellman, 1989) might be responsible for the routing of secretory granule-associated proteins; however, such a signal has not been identified. It has also been suggested that protein aggregation within the lumen of the *trans*-Golgi compartment is involved in the correct sorting of proteins to the regulated pathway (Tooze et al., 1989; Reaves and Dannies, 1991). The mechanisms responsible for the routing of integral membrane proteins destined

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1. *Abbreviations used in this paper:* CSFM, complete serum-free medium; PAL, peptidyl-α-hydroxyglycine α-amidating lyase; PAM, peptidylglycine α-amidating monooxygenase; PHM, peptidylglycine α-hydroxylating monooxygenase; POMC, proopiomelanocortin; TES, (*N*-tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid.

for secretory granules have not been thoroughly studied. Since the posttranslational processing enzyme PAM occurs naturally in both membrane-associated and soluble forms, expression of its individual forms in stable cell lines provides a unique opportunity to compare the maturation of membrane-associated and soluble proteins into secretory granules. Furthermore, since PAM is a bifunctional enzyme, expression of one enzymatic domain independent of the other facilitates the identification of routing signals in the full-length PAM precursor.

PAM is a key posttranslational processing enzyme which catalyzes the conversion of glycine-extended biosynthetic intermediates into bioactive amidated peptides (Eipper et al., 1992). Since amidation occurs at a late stage in peptide processing, PAM must be localized in secretory granules together with the prohormone-derived peptides. In the pituitary and AtT-20 cells, PAM activity cosediments with the granule-associated hormone fraction (Eipper et al., 1983; Mains et al., 1984). PAM is a bifunctional enzyme consisting of monooxygenase (PHM) and lyase (PAL) domains; the activities of PHM and then PAL are required in sequence to produce $\alpha$-amidated peptides (Perkins et al., 1990; Takahashi et al., 1990; Katapodis et al., 1990). Alternative splicing of a single gene generates mRNAs encoding at least seven PAM proteins in a tissue-specific fashion (Eipper et al., 1992) (Fig. 1). In PAM-1, PHM and PAL are separated by 105 amino acids encoded by optional exon A; PAL is followed by putative transmembrane (encoded by exon B) and COOH-terminal domains (Fig. 1 A). Deletion of exon A generates PAM-2, which includes the putative transmembrane and COOH-terminal domains whereas deletion of both exons A and B generates soluble, bifunctional PAM-3 (Fig. 1 A). PAM-4 consists of PHM, exon A, and a unique region of 20 amino acids (Fig. 1 A). Membrane-associated PAM is endoproteolytically processed in a tissue-specific fashion to yield soluble proteins containing one or both of the enzymatic activities found in the full-length PAM precursor (Beaudry and Bertelsen, 1989; Perkins et al., 1990; Stoffers and Eipper, 1990; Eipper et al., 1991). The pattern and significance of this endoproteolytic processing is not understood.

To study the posttranslational processing and routing of this secretory granule–associated protein, we have generated a panel of stable AtT-20 cell lines expressing cDNAs encoding naturally occurring PAM proteins (Fig. 1 A). For comparison, we have also generated stable cell lines expressing monofunctional truncated, soluble PHM (PHM-s) and PAL (PAL-s) as well as membrane-associated PAL (PAL-m, Fig. 1 B). AtT-20 corticotrope tumor cells were chosen for these studies because their processing and regulated secretion of endogenous proopiomelanocortin (POMC) has been well characterized. AtT-20 cells store processed POMC peptides in dense-core secretory granules and secretion can be regulated by secretagogues including corticotropin releasing factor, cAMP, and PMA (Tooze and Tooze, 1986; Miller and Moore, 1990; Thiele and Eipper, 1990). AtT-20 cells normally synthesize and secrete PAM, but the endogenous level of PAM expression is at most 5% of the level observed in the transfected cell lines. We have examined the localization of soluble and membrane-associated forms of PAM in AtT-20 cells using biochemical and morphological criteria. Additionally, we have used Western blots of whole cell extracts,
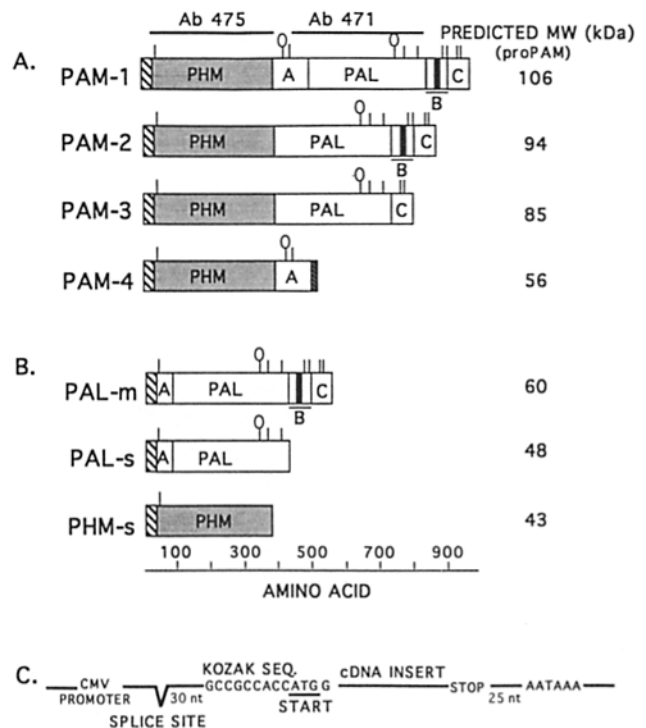


*Figure 1.* Natural (*A*) and truncated (*B*) forms of PAM expressed in AtT-20 cell lines and linearized diagram of pCIS vector used in PAM cDNA transfections (*C*). PHM (*stippled*) and PAL (*clear*) domains and exons A and B are labeled; *C* refers to the COOH-terminal domain which is proposed to be extragranular in PAM-1 and -2 but intragranular in PAM-3. The crosshatched box represents the signal sequence (amino acids 1–25) present in all constructions; the black box within exon B marks the putative transmembrane domain. Potential dibasic cleavage sites are indicated by vertical lines and ovals mark putative *N*-glycosylation sites. The first potential dibasic cleavage site represents the end of the PAM propeptide (amino acids 26–35) present in all constructions. The unique region of rPAM-4 is denoted by the darkly crosshatched box. In *A* the bacterially expressed PHM and PAL domains used to generate antisera are marked above rPAM-1. In *C* the splice site in the pCIS vector occurs 30 nt upstream of the consensus ribosome binding site (Kozak sequence) present in all constructions. The stop codon is followed within a short stretch of nucleotides by a poly(A) addition site.

soluble and membrane fractions, and spent medium to investigate the patterns of endoproteolytic cleavage for the individual forms of PAM.

## Materials and Methods

### Construction of Expression Vectors

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into the larger PpuMI/HindIII fragment to create a plasmid which encodes a protein with rPAM residues 1–36 fused in reading frame with residues 438–976 (rPAL-m). To delete the transmembrane and COOH-terminal domains and create a presumably soluble form of the PAL enzymatic domain, pBS.KrPAL-m was subjected to cleavage with AvaI at nt 2889, digestion with S1 nuclease and then alkaline phosphatase, ligation of an XbaI linker (CTCTAGAG; New England Biolabs, Beverly, MA), and finally cleavage with XbaI and religation. The resultant pBS.KrPAL-s plasmid encodes a protein with the COOH-terminal sequence Pro[863]-Gly[864]-Leu, followed by a stop codon. The pBS.KrPAM-4 plasmid was created from pBS.PAM-4 and pBS.KrPAM-1. The long 3'-untranslated region of PAM-4 was deleted and an XbaI site was inserted. The polymerase chain reaction was performed with an antisense primer (5'-CCTCTAGATTGCTAATGTGGA-TTGC-3') composed on an artificial XbaI site (underlined) followed by nt 1854 to 1838 of rPAM-4 (the normal stop codon is at nt 1849–1851) and the BE-4 sense primer (nt 1359–1375). The polymerase chain reaction fragment and pBS.KrPAM-1 were digested with BamHI (nt 1682) and XbaI. The BamHI/XbaI fragment from the polymerase chain reaction (nt 1682–1854) was then inserted into the larger fragment of the KrPAM-1 digest to create pBS.KrPAM-4. For PAM-2 and PAM-3, pBS.rPAM-2 and -3 were digested with EcoRV (nt 822) and XmaI (nt 3242; stop codon is nt 3229) and the two fragments inserted into the corresponding sites in pBS.Kr-PAM-1, to create plasmids lacking exon A (pBS.KrPAM-2) or both exons A and B (pBS.KrPAM-3). The resultant pBluescript plasmids all contain the consensus ribosome binding site 5'-GCCGCCACC-3' (Kozak, 1991) right before the ATG encoding Met[1] and all have 10-25-nt 3'-untranslated regions (Fig. 1 C). All polymerase chain reaction products and blunt ligations were verified by DNA sequencing. All of the KrPAM cDNAs were inserted as ClaI/NotI fragments into the pCIS.2CXXNH expression vector kindly provided by Dr. Cornelia Gorman (Genentech Inc., San Francisco, CA) as described (Eipper et al., 1991; Gorman et al., 1990).

## Transfection and Cell Culture

AtT-20/D-16v cells were grown in DMEM-F12 with 10% NuSerum, 10% FCS, and antibiotics as described (Perkins et al., 1990). Transfected cells were cultured in medium containing 0.25 mg/ml G418 (Gibco Laboratories, Gaithersburg, MD); all cells were passaged weekly. To establish stable cell lines overexpressing the different forms of PAM, 3 μg of pMt.Neo (Dickerson et al., 1989) and 30–45 μg of pCIS.KrPAM containing the desired cDNA insert downstream of the cytomegalovirus promoter were cotransfected using lipofection as described (Eipper et al., 1991). After transfection, cells were subcultured in G418-containing medium for 3 wk. Drug-resistant lines were selected and passed; PHM and PAL activity were measured as described below. In most transfections, 2–6 positive clones were chosen for further analysis and eventually a single clone expressing the highest enzyme activity was used in all experiments. When necessary, cell lines were subcloned by limiting dilution into 96-well plates in medium containing 0.5 mg/ml G418, using a feeder layer of wild-type GH3-L cells. After 3 wk, wells containing single colonies of AtT-20 cells were expanded and tested; a single clone was chosen for further analysis. Cell lines have expressed stable levels of enzyme activity for up to 8 mo.

## Northern Blots

Total RNA was isolated using the Promega RNagent Isolation kit and fractionated using denaturing formaldehyde gels (Thiele and Eipper, 1990). RNA was transferred to Nytran and crosslinked using the Ultraviolet Cross-Linker (Stratagene Inc., La Jolla, CA). Blots were hybridized with cDNA probes labeled with [$^{32}$P]dCTP by random priming. The PHM probe used was a 1.3-kb PstI-BamHI fragment of rPAM-1 (nt 356–1682) and the PAL probe was a 0.4 kb DraII fragment of rPAM-1 (nt 1761–2139). Since exon A extends from nt 1475 to nt 1789, both probes hybridize with mRNAs containing exon A. Hybridization was carried out in SDS-Pipes buffer (Virca et al., 1990).

## Cell Extracts and Enzyme Assays

To measure PHM or PAL activity in whole cell extracts, cells were scraped into ice cold 20 mM Na(N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid) (Na TES)/10 mM mannitol, pH 7.40, 1% Triton X-100, 30 μg/ml PMSF (Eipper et al., 1991). After three cycles of freezing and thawing, extracts were diluted in TES/mannitol, 1% Triton X-100, 1.0 mg/ml BSA. PHM and PAL assays were performed at pH 5.0 in 150 mM Na MES using acetyl-Tyr-Val-Gly and acetyl-Tyr-Val-OH-Gly substrates, respectively

(Husten and Eipper, 1991). The substrate concentration was 0.5 μM in a final volume of 40 μl; 10,000–20,000 cpm of radiolabeled substrate was included in each assay tube. Samples were assayed in duplicate for 1 h at 37°C and reaction velocities increased linearly with protein (typically, 0.1–1 μg of protein was assayed). For measuring PHM activity in cell extracts and spent medium, 1 μM CuSO$_4$, 500 μM ascorbate, and 0.18 mg/ml catalase were included in the assay. Protein concentrations of the undiluted extract samples were determined using the bicinchoninic acid protein reagent kit from Pierce Chem. Co., Rockford, IL.

To determine the percentage of PHM or PAL activity which was membrane-associated in each of the transfected cell lines, cells were gently scraped from 60-mm culture dishes into 37°C DMEM-AIR (May and Eipper, 1986) and pelleted by centrifugation. Cell pellets were resuspended in ice-cold detergent-free TES/mannitol containing 30 μg/ml PMSF, 2 μg/ml leupeptin, 10 μg/ml α2-macroglobulin, and 16 μg/ml benzamidine, and then disrupted with a polytron (Brinkman Instrs., Inc., Westbury, NY). After an initial centrifugation at 1,000 g for 5 min to pellet nuclei and cell debris, crude soluble and membrane fractions were separated by centrifugation at 430,000 g for 15 min in a TL-100 (Beckman Instrs., Inc., Fullerton, CA). Detergent was then added to membrane fractions; soluble and membrane fractions were assayed for PHM and PAL activity and protein as described above. Proteins in crude membrane and soluble fractions were fractionated on SDS–polyacrylamide gels containing 10% acrylamide (0.27% N,N'-methylene-bisacrylamide), transferred to Immobilon-P, and analyzed as described below.

## Secretion of PAM Activity

Cells were incubated in complete serum-free medium (CSFM; May and Eipper, 1986) with bacitracin (50 μg/ml), lima bean trypsin inhibitor (50 μg/ml), and BSA (200 μg/ml) from 1–6 h and spent medium was assayed for PHM and PAL activity. Basal secretion rates of PHM and PAL were expressed as a percent of the corresponding total enzyme activity within the cell extract (% total cell content of PHM or PAL activity secreted/h).

To determine whether the secretion of PAM proteins in transfected cells could be stimulated by secretagogues, cells in 6-well plates were rinsed with prewarmed CSFM and then incubated in 1 ml CSFM for two sequential 1-h periods. The medium from an additional 1- or 2-h incubation in basal medium was collected, nonadherent cells were pelleted, and the supernatant was stored at −20°C after the addition of 30 μg/ml PMSF. After the basal period, cells were incubated an additional 1 or 2 h in CSFM containing 10 nM PMA (Calbiochem Corp., La Jolla, CA) diluted from a 100× stock in DMSO. After the challenge period, the media were collected and the cells extracted for measurement of total enzyme activity. In all experiments the basal and challenge periods were of equal length. PHM and PAL activity secreted during each period were determined as described above; enzyme activity secreted during the challenge period was expressed as a fold of basal activity which was defined as 1. ACTH secretion was measured by RIA using antibody Kathy (final dilution of 1:10,000) and 10,000 cpm/tube [$^{125}$I]ACTH (18–39) (Schnabel et al., 1989). To visualize the secreted PAM proteins in basal and challenge media, incubation times of 2 h were used. Equal volumes of basal and challenge media were fractionated by PAGE, transferred to Immobilon-P, and probed with PHM and PAL antisera.

## Western Blots

Cell extracts or conditioned media were analyzed by Western blotting as described previously (Husten and Eipper, 1991). Polyclonal rabbit antisera (Hazleton Laboratories, Denver, PA) raised against bacterially expressed PHM (rPAM-1 [37–432]; Ab 475) and PAL (rPAM-1 [464–864]; Ab 471) were used to probe proteins transferred to Immobilon-P membranes. The generation and purification of the antigens used to immunize rabbits will be described (Yun, H.-Y., R. J. Johnson, R. E. Mains, manuscript in preparation). Qualitatively similar results were obtained using affinity-purified antibodies directed against synthetic peptides in the PHM and PAL domains (residues 117–136 in PHM and 564–582 in PAL; Husten and Eipper, 1991). Proteins were visualized using $^{125}$I-labeled protein A and blots were exposed to Kodak XAR film for 1–24 h. In some experiments, membranes were blocked in 10% nonfat dry milk diluted in 50 mM Tris HCl, 150 mM NaCl, pH 7.5, containing 0.05% Tween-20 (TTBS), incubated in primary antibody (1:1,500 in TTBS for 1 h at room temperature or overnight at 4°C), and rinsed. Blots were then incubated for 20 min at room temperature with HRP-linked donkey anti–rabbit IgG antibody (1:10,000, Amersham Corp., Arlington Heights, IL) and visualized using the Enhanced chemiluminescence kit (Amersham Corp.). Exposure times ranged from 1 to 20 min.

## Immunofluorescence

Cells grown on chamber slides (Lab-Tek Div. Miles Laboratories Inc., Naperville, IL) for 24–72 h were rinsed once in PBS (50 mM phosphate, 150 mM NaCl, pH 7.4) and fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. Cells were then sequentially incubated with 0.1% Triton X-100 in PBS for 20 min at room temperature, 0.5% gelatin in PBS at room temperature, and primary antibody overnight at 4°C. Antibodies were diluted in PBS containing 0.1% gelatin and used at a final dilution of 1:2,000 (PHM and PAL antisera) or 1:50,000 (COOH-terminal ACTH antibody Kathy). After rinsing with PBS, the cells were incubated for 1 h at room temperature with FITC-conjugated goat anti-rabbit IgG (CalTag) diluted 1:200 or 1:400. In some experiments, rhodamine-labeled wheat germ agglutinin (Vector Labs, Inc., Burlingame, CA) diluted 1:2,000 was included as a marker of the Golgi complex (Meiniel et al., 1988). After rinsing, the cell chambers were removed and the slides mounted in Permount (Lipshaw, Detroit, MI) containing 0.03 M 1,4-diazobicyclo-[2.2.2]-octane (Sigma Chem. Co.). Samples were viewed under epifluorescence optics with an Axioskop microscope (Carl Zeiss Inc., Thornwood, MT) using FITC (BP 485/20, barrier filter 520–560) and rhodamine (BP 546/12, LP 590) filters. Controls included omission of primary antibody, omission of secondary antibody, staining of wild-type cells, and staining of cells expressing a single enzymatic activity with antibodies to the other enzyme.

## Results

### Generation of Stable Cell Lines

AtT-20 cell lines expressing each of the forms of PAM illustrated in Fig. 1 were generated. Three of these proteins are integral membrane proteins (PAM-1, PAM-2, and PAL-m) and four are soluble proteins (PHM-s, PAL-s, PAM-3, and PAM-4). PAM expression in each of the cell lines was compared with wild type by evaluating levels of mRNA and enzyme activity. Total RNA was isolated from wild-type and transfected cell lines and fractionated on denaturing agarose gels. Northern analysis demonstrated the presence of a major RNA transcript of the appropriate size in each of the PAM cell lines; the level of expression varied among the cell lines, with the PAL-m cells expressing the lowest mRNA level and the PAM-2 cells expressing the highest level (Fig. 2 A). The levels of PHM and PAL activity in transfected cells correlated with the expression levels observed by Northern analysis and ranged from 20- to 200-fold over the level in wild-type cells (Fig. 2 B). In cells where only the PHM or PAL domain was expressed, no change in the activity of the other enzyme was observed, indicating that foreign expression of PAM does not significantly alter expression of endogenous enzyme activity. The fact that enzyme assays and Northern blot analysis give similar estimates of expression levels for the seven cell lines suggests that there is not a great difference in the stability of the proteins expressed.

### PAM Proteins in Transfected AtT-20 Cells

PAM proteins purified from different tissues are produced by endoproteolytic cleavage of larger precursors (Eipper et al., 1991, 1992); since these tissue contain multiple forms of PAM mRNA, it has not been possible to identify the products derived from the specific precursors. The transfected rat PAM proteins are subjected to endoproteolytic cleavage by the resident AtT-20 processing enzymes. To determine the major endoproteolytic cleavage products of the individual forms of PAM in AtT-20 cells, crude soluble and membrane fractions were prepared from wild-type and transfected cells. As expected, over 90% of the PHM or PAL activity in cells expressing soluble monofunctional (PHM-s, PAL-s, and

PAM-4) or bifunctional (PAM-3) PAM proteins was in the soluble fraction. Endoproteolytic processing of membrane-associated PAM proteins released PHM and PAL activity into the crude soluble fraction of PAM-1, PAM-2, and PAL-m cells. In PAL-m and PAM-1 cells, only ~50% of the PAL activity was recovered in the soluble fraction, suggesting that endoproteolytic processing between the lyase and transmembrane domains occurred in these cells but did not proceed to completion. In contrast, ~80% of the PHM activity in PAM-1 cells was found in the soluble fraction. In PAM-2 cells, 70% of the PHM and PAL activity was recovered in the soluble fraction, while 30% of the activity was membrane associated.

Endoproteolytic cleavage can alter the enzymatic activity of integral membrane protein forms of PAM (Husten and Eipper, 1991). Therefore, PAM proteins present in crude soluble and membrane fractions were visualized on Western blots with PHM and PAL antibodies (Fig. 3). All of the PAM proteins were endoproteolytically processed in AtT-20 cells. Consistent with the activity measurements, the majority of PHM and PAL protein in cells expressing soluble proteins was in the soluble fraction. PHM-s cells contained two proteins migrating as a doublet with apparent molecular masses of 40 and 38 kD. The soluble fraction of PAM-4 cells contained PHM proteins of 46 ± 3 and 44 ± 2 kD. The predominant PAL protein in the soluble fraction of PAL-s cells had a mass of 59 ± 1 kD; a 50 ± 4 kD cleavage product was also visualized. Proteins of 97 ± 3 and 75 ± 5 kD were visualized with PHM and PAL antibodies in the soluble fraction of PAM-3 cells.

While a portion of the membrane-associated PAM was processed into soluble proteins in AtT-20 cells, PAM proteins were also seen in crude membranes from cells expressing PAM-1, PAM-2, and PAL-m. The membrane fraction of PAL-m cells contained a 70 ± 3-kD protein which represents the full-length PAL-m protein. PAL-m is endoproteolytically processed to generate small amounts of soluble PAL proteins which migrated with apparent molecular masses of 56 and 50 kD.

In PAM-1 cells, there were 120 ± 5-kD and 70 ± 3-kD PAL proteins present in the crude membrane fraction; while the larger protein was also visualized by the PHM antibody, the 70-kD protein was only visualized by the PAL antibody. The soluble fraction from PAM-1 cells was enriched in 110-, 56-, and 50-kD PAL proteins and a doublet of 46- and 44-kD PHM proteins. The 110-kD protein was also visualized with the PHM antibody upon longer exposure of the autoradiogram. The major soluble proteins generated through selective proteolysis of the PAM-1 precursor are monofunctional PHM and PAL. The PAL domain is poorly cleaved from the transmembrane domain, leaving monofunctional membrane-associated PAL as a major product. The soluble PHM proteins generated through endoproteolytic processing of membrane-associated PAM-1 are the same size as the PHM proteins produced from PAM-4.

In PAM-2 cells, the PHM and PAL domains were not separated (Fig. 3). Both PHM and PAL antisera visualized full-length PAM-2, a 105-kD protein present in the crude membrane fraction. Two soluble proteins (93 and 75 kD) also containing antigenic determinants for both PHM and PAL represented major products. Therefore, while soluble proteins are generated by the endoproteolytic processing of
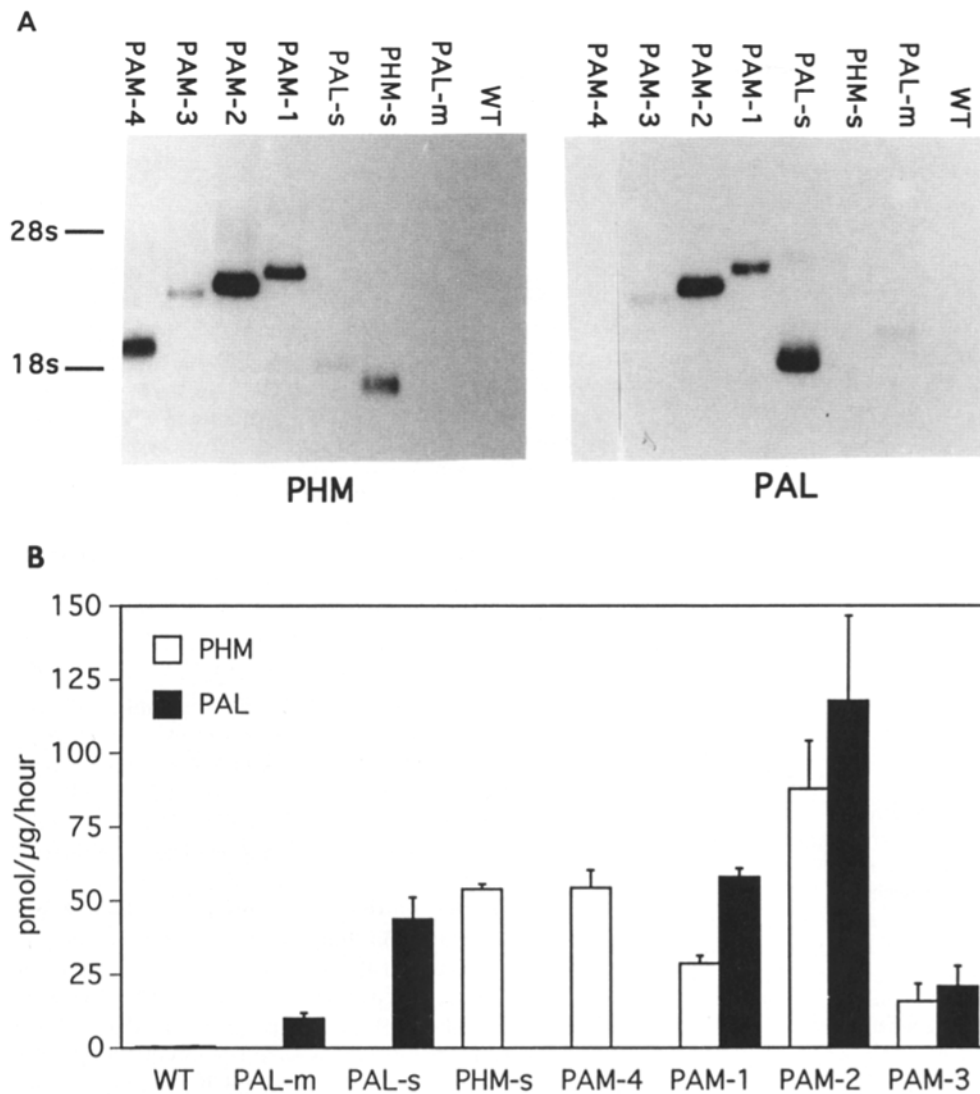
Figure 2. PAM expression in wild-type and transfected AtT-20 cells: Northern blots (A) and enzyme activity (B). (A) Total RNA (10 μg) from wild-type (WT) and transfected cells was fractionated on a denaturing agarose gel, blotted to Nytran, and visualized with PHM and PAL cDNA probes. All lanes were exposed to film for an equal length of time to allow direct comparison of expression levels in the transfected cells. (B) Wild-type and transfected cells were extracted in TES/mannitol, Triton X-100, and then assayed for total PHM and PAL activity as described in Materials and Methods. Total extract proteins was determined and data are expressed as specific PHM and PAL activity (pmol/μg per h) and represent the mean ± SEM of three observations. Wild-type levels of PHM and PAL activity are 0.2 pmol/μg per h and 0.3 pmol/μg per h, respectively.

PAM-1 and PAM-2, monofunctional proteins are generated from PAM-1 and bifunctional proteins are produced from PAM-2.

### Basal Secretion of Enzyme Activity and PAM Proteins

Wild-type AtT-20 cells exhibit a measurable basal rate of secretion of PAM activity and hormone (Mains et al., 1984; Noel and Mains, 1991). The basal rate of secretion was determined for each of the expressed forms of PAM and compared with the rate for the endogenous hormone. Cells were incubated in CSFM for 1–4 h; PHM and PAL activity in spent medium were assayed and compared with the activity in whole cell extracts (Fig. 4). In preliminary experiments, media collected from the same cells at selected time points from 1 to 24 h demonstrated that the amount of enzyme activity secreted was linear with time (not shown). Soluble forms of PAM were secreted from AtT-20 cells at rates between 9 and 15% of the cell content per hour; no significant differences were noted between cell lines expressing soluble PHM (PHM-s and PAM-4), soluble PAL, and soluble, bifunctional PAM-3 (Fig. 4). This basal rate of secretion is similar to the basal rate of secretion of POMC-derived prod-

ucts by AtT-20 cells (Noel and Mains, 1991; Noel et al., 1991).

In all cell lines expressing membrane-associated PAM (PAM-1, PAM-2, and PAL-m) only ~4% of the total cellular PAL activity was secreted per hour. The rate of secretion was not simply a reflection of the level of expression since the PAL-m and PAM-2 lines, respectively, exhibit the lowest and highest levels of expression. When the secretion of PHM activity was compared in PAM-1 and PAM-2 cells, it became evident that the presence of exon A in PAM-1 significantly altered the rate of secretion of PHM activity. PHM activity from PAM-1 cells was secreted at an approximately threefold greater rate than the secretion of PAL activity from the same cells (12%/h vs. 4%/h). In contrast, in PAM-2 cells, where exon A is absent, PHM and PAL activities were secreted at equal rates ~4%/h).

AtT-20 cells process and store their endogenous hormone, POMC, and the POMC product peptides secreted under basal conditions are not a simple reflection of the POMC products in cell extracts (Mains and Eipper, 1981). Therefore, the forms of PAM protein secreted by each of the cell lines under basal conditions were determined by probing
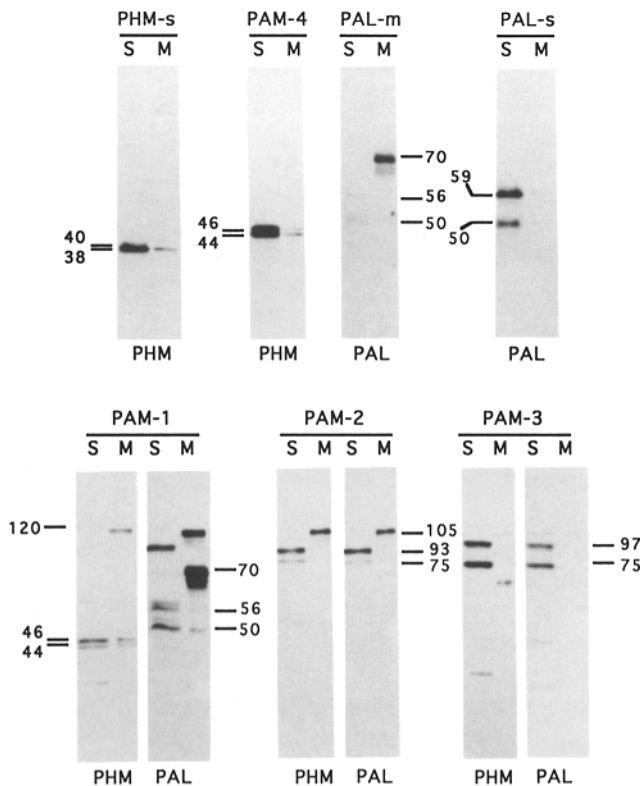
Figure 3. Western blot of crude soluble and membrane fractions from transfected cells. Cells were extracted in TES/mannitol and fractionated into crude soluble (S) and membrane (M) fractions as described in Materials and Methods. For each cell line, an equal percentage of the soluble and membrane fractions (from 2.5 to 10% of the total volume) were separated by PAGE, transferred to Immobilon-P, and probed with PHM antibody (antibody 475). The blots were stripped as described (Husten and Eipper, 1991) and reprobed with PAL antibody (471). Protein bands were visualized using HRP-linked secondary antibody and Enhanced Chemiluminescent reagents (Amersham Corp.). Similar results on forms of protein in soluble and membrane fractions were obtained in three other analyses.
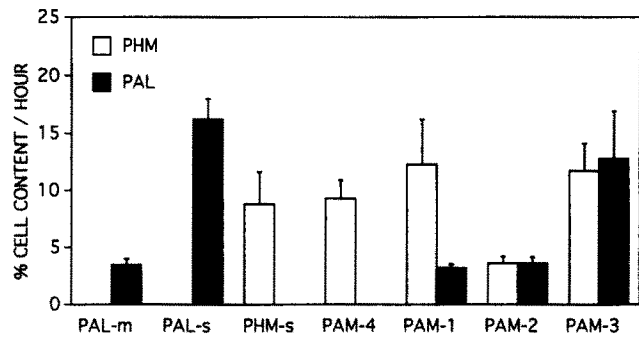


Figure 4. Basal secretion of PHM and PAL activity from transfected AtT-20 cells. Cells were incubated in CSFM for 1–6 h, spent medium was collected, and the cells were extracted in TES/mannitol, Triton X-100 as described. PHM and PAL activity in spent medium and whole cell extracts were determined; the activity in the medium was expressed as the percentage of the cell content secreted per hour. Each point represents the mean ± SEM of three observations. PHM and PAL activity secreted by wild-type AtT-20 cells accounts for <1% of the activity measured in the medium. The basal rate of secretion of PHM and PAL from wild-type AtT-20 cells ranged from 12 to 17% in four observations.

blots of whole cell extracts and spent medium with PHM and PAL antibodies (Fig. 5). As expected, PAM proteins present in the soluble fraction of the whole cell extracts (Fig. 3) were found in spent medium. However, the PAM proteins in basal medium were not a simple reflection of the PAM proteins present in the soluble fraction of transfected cells. In general the larger, less processed soluble PAM proteins were the predominant forms in basal medium. Thus, the 97-kD bifunctional PAM protein was the major component in spent medium from PAM-3 cells while cell extracts contained similar amounts of bifunctional 97- and 75-kD PAM proteins. In conditioned medium from PAL-s cells, the 59-kD PAL protein was more abundant, while in cell extracts both 59- and 50-kD proteins were visualized. Similarly, in PAM-4 cell extracts (Fig. 5), PHM antisera visualized proteins of 56, 46, and 44 kD; the 56-kD form was selectively secreted into basal medium from PAM-4 cells. This 56-kD PAM-4 protein was also visualized by PAL (Ab 471) antibodies while the 46- and 44-kD PAM-4 proteins were not recognized by the PAL antibody (Fig. 5). Since the PAL antibody was generated against a PAL protein which contained a portion

of exon A (Fig. 1), the 56-kD PAM-4 protein contained antigenic determinants within exon A and was processed to generate smaller PHM proteins lacking these determinants.

PHM and PAL proteins were also secreted from cells expressing integral membrane PAM proteins. PHM in conditioned medium from PAM-1 cells migrated as a doublet of 46- and 44-kD proteins. The endoproteolytic cleavage between PAL and the transmembrane domain in PAM-1 and PAL-m cells occurred to a limited extent to release soluble PAL into the medium of these cells. Longer collections of basal media were required to visualize a 56-kD PAL protein secreted from PAM-1 or PAL-m cells (data not shown). Consistent with the observation that endoproteolytic cleavage of PAM-2 generated a bifunctional soluble enzyme, spent medium from PAM-2 cells contained 93- and 75-kD proteins with antigenic determinants for both PHM and PAL.

## Localization of PAM Protein in AtT-20 Cells: Immunofluorescence Studies

To determine the localization of the expressed proteins in AtT-20 cells, transfected cells were fixed, permeabilized, and stained with antibodies to PHM or PAL (Fig. 6). PAM proteins were visualized using indirect immunofluorescent techniques with fluorescein-labeled secondary antisera. The staining patterns observed with PAM antibodies were compared with the patterns obtained when transfected cells were stained with an antibody to ACTH. The ACTH antibody preferentially stains secretory granules at the periphery of AtT-20 cells since it is specific for the COOH terminus of ACTH and does not detect the intact POMC precursor (Schnabel et al., 1989). Wild-type or transfected AtT-20 cells stained with the ACTH antiserum gave similar patterns, with intense punctate immunofluorescence observed throughout the cell. ACTH immunoreactivity was especially prominent in small discrete spots at the periphery of both wild-type and transfected cells; less intense staining was also seen in the perinuclear region (Fig. 6 A). When cells expressing soluble forms of PAM (PHM-s, PAL-s, PAM-3, and
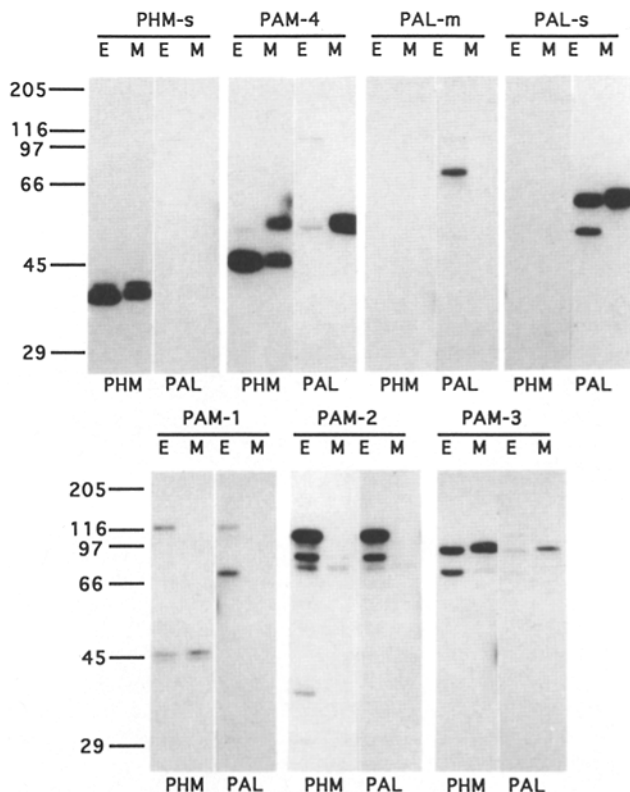
*Figure 5.* Basal secretion of PAM proteins from transfected AtT-20 cells. Cells were incubated in 60-mm dishes in 1 ml of CSFM for 6 h. The spent medium was collected and the cells were extracted in 1 ml TES/mannitol, Triton X-100 + 30 μg PMSF. Equal volumes of cell extract (*E*) and spent medium (*M*) were separated by PAGE. The amount analyzed corresponded to 60 μg of whole cell protein. The proteins were transferred to Immobilon-P, probed with PHM and PAL antisera, and visualized using $^{125}$I-protein A. Similar results were obtained in another Western blot of extract and medium samples.

PAM-4) were stained with PHM or PAL antisera (6 B-C) the staining pattern observed was similar to that observed with the ACTH antibody. There was punctate staining throughout the cell body with an accumulation of immunofluorescence in peripheral processes of transfected cells. In addition there was staining in the perinuclear region of the transfected cells. Cells expressing membrane forms of PAM (PAM-1, PAM-2, and PAL-m) exhibited a staining pattern for ACTH that was identical to that observed in wild-type cells and transfected cells expressing soluble PAM proteins (Fig. 6 *A*). In contrast, when antisera to PHM and PAL were used, cells expressing membrane-bound PAM exhibited a greater intensity of immunofluorescence in the perinuclear region of the cell compared with that observed in discrete spots in the periphery (Fig. 6, *D* and *E*). PHM and/or PAL immunoreactivity was also accumulated in peripheral processes of cells expressing PAM-1, PAM-2, and PAL-m (Fig. 6, *D* and *E*), suggesting that some of the expressed proteins were correctly routed to the exocytotic pathway in AtT-20 cells. The Golgi apparatus is enriched in glycoproteins containing *N*-acetyl-glucosamine; therefore rhodamine-labeled wheat germ agglutinin can be used to visualize the Golgi apparatus (Meiniel et al., 1988). In double-labeled cells, the fluorescein immunofluorescence (PAM proteins) in the perinuclear

area overlapped with the WGA-rhodamine fluorescence (Fig. 6, *E* and *F*) indicating that PAM was localized in or near the Golgi compartment of transfected cells.
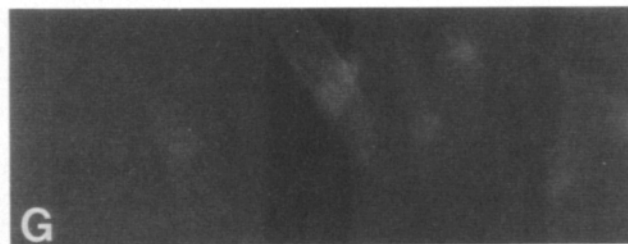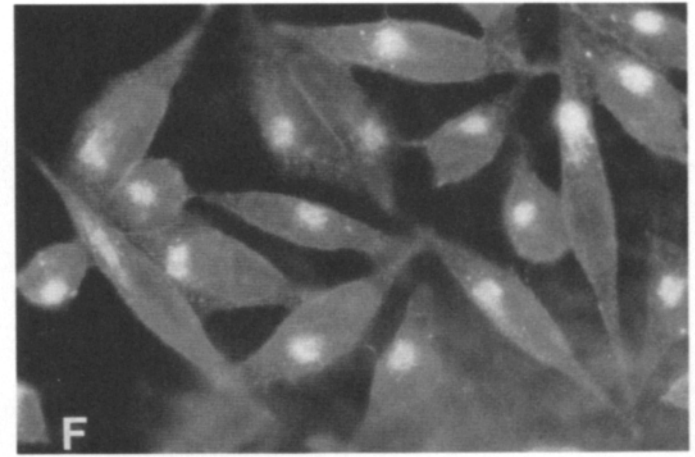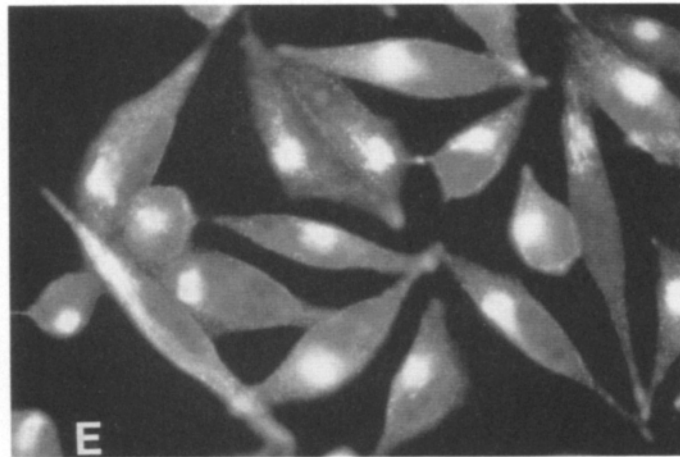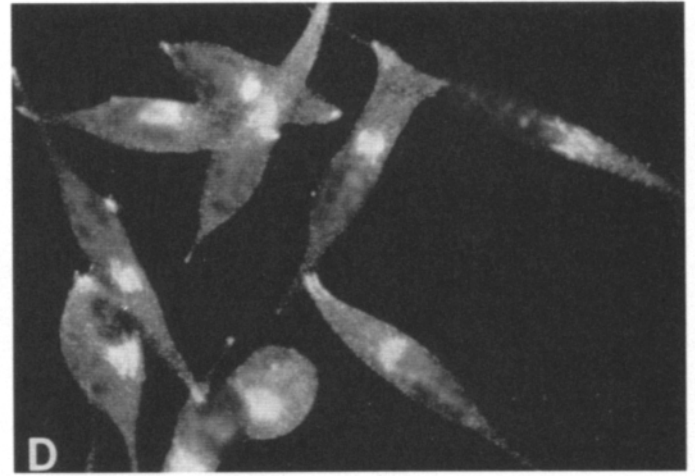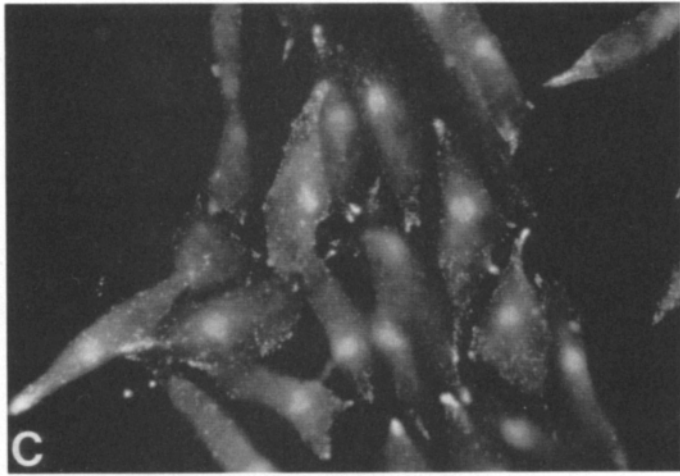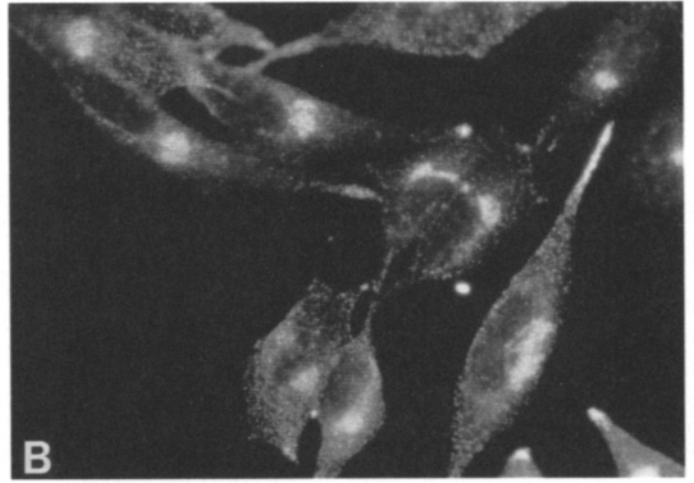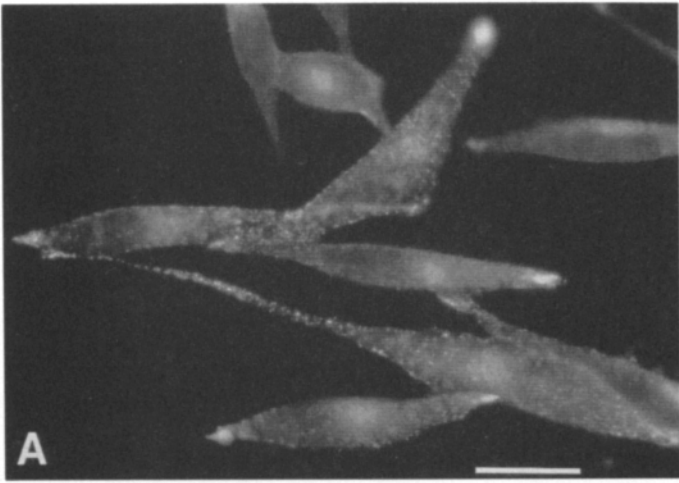
### Stimulated Secretion of PHM and PAL Activity and PAM Proteins in Transfected Cells

Further evidence of correct routing of PAM proteins to the regulated secretory pathway was obtained by determining whether secretagogues regulated the secretion of the PAM proteins expressed in the various cell lines. The secretion of enzyme activity in response to secretagogues was compared with the secretion of ACTH, a POMC product whose release is known to be stimulated by phorbol esters (Thiele and Eipper, 1990). In cells expressing membrane-associated or soluble PAM proteins, secretion of enzyme activity was stimulated in parallel with secretion of POMC products (Fig. 7). Although the magnitude of stimulation by PMA differed substantially among the various cell lines, there was a good correlation between the magnitude of the stimulation of enzyme secretion and hormone secretion, providing strong evidence that the transfected PAM proteins entered the regulated pathway in AtT-20 cells along with endogenous hormone.

To identify which PAM proteins were secreted upon stimulation with PMA, equal volumes of spent medium from basal and challenge periods were fractionated on SDS–polyacrylamide gels and visualized on Western blots using PHM and PAL antisera (Fig. 8). Consistent with the results of the enzyme assays, 10 nM PMA stimulated the secretion of the expressed PAM proteins. As seen with the enzyme assays, the cell lines differed in the magnitude of their response to PMA as assessed by Western blot analysis of secreted PAM proteins. The differences appeared to represent clonal variations. The PAM proteins in the challenge medium were not identical to the PAM proteins in the basal medium (Fig. 8). In general, the secretion of smaller, more highly processed PAM proteins was stimulated to a greater extent. For example, the 75-kD cleavage product of PAM-3 and PAM-2 and the 49-kD cleavage product of PAL-s were more prevalent in challenge medium than in basal medium. Preferential stimulation of secretion of more highly processed forms is also observed for POMC products (Mains and Eipper, 1981).

### Discussion

Endocrine cells and neurons are characterized by the presence of several distinct secretory pathways (Miller and Moore, 1990; Sossin et al., 1990; Huttner and Tooze, 1989; Arvan et al., 1991). When expressed in neuroendocrine cell lines, foreign prohormones are almost always correctly routed to the regulated secretory pathway (Lapps et al., 1988; Devi et al., 1989; MacDonald et al., 1989; Sevarino et al., 1989; Chevrier et al., 1991). Investigators have also reported that when proteins normally secreted from cells in a constitutive manner are expressed in endocrine cells, they are not automatically routed to the regulated pathway (Matsuuchi et al., 1988; Rivas and Moore, 1989). However, chimeric proteins containing domains from both regulated and constitutively secreted proteins can be routed to regulated granules (Moore and Kelly, 1986). One interpretation of these data is that the sorting of constitutive and regulated proteins is an active process requiring the presence of a sort-
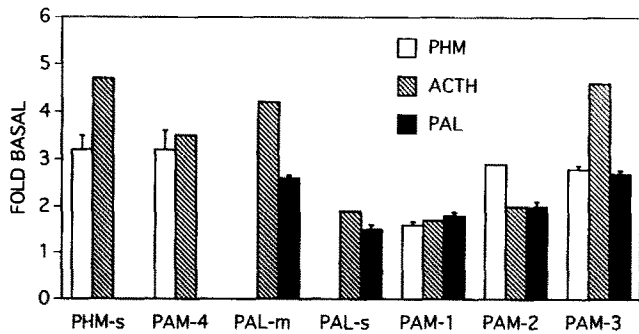
*Figure 7.* Stimulated secretion of enzyme activity and ACTH immunoreactivity in transfected cells. Cells were incubated in basal medium followed by incubation in medium containing 10 nM PMA. Enzyme activity and hormone secreted during the challenge period was expressed as fold stimulation over basal (set at 1). Each point for enzyme activity represents the mean ± the range of two observations; ACTH secretion was measured in a single experiment. Similar results were obtained when the secretion of β-endorphin and enzyme activity was stimulated with 8-Br-cAMP (not shown).

ing signal on proteins destined for secretory granules (Miller and Moore, 1990). Alternatively, the condensation of soluble proteins within the lumen of the *trans*-Golgi compartment may exclude proteins not destined for secretory granules (Tooze et al., 1989; Huttner and Tooze, 1989; Reaves and Dannies, 1991).

To begin to examine the question of how secretory granule enzymes are routed, processed, and stored in neuroendocrine cells, we have stably expressed natural and truncated PAM proteins in AtT-20 cells. PAM occurs naturally in both integral membrane protein and soluble forms making it an ideal model protein for these studies. By comparing stable cell lines expressing individual forms of PAM it is possible to determine whether integral membrane proteins destined for secretory granules are processed or stored differently from soluble proteins destined for secretory granules. The question of PAM processing is in fact not a separate question from how PAM is routed in AtT-20 cells, since proteins found in regulated secretory granules are endoproteolytically processed en route through the secretory pathway. The
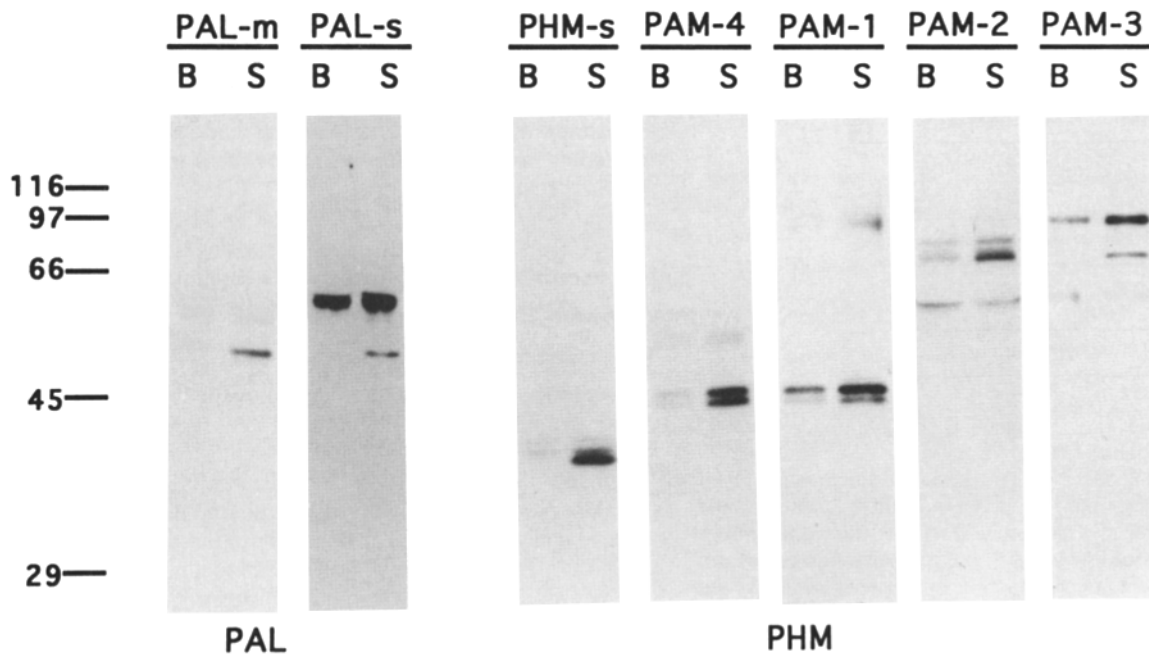


*Figure 8.* Proteins secreted from transfected cells in response to 10 nM PMA. Equal amounts of basal and challenge medium were fractionated on SDS–polyacrylamide gels and probed with PHM or PAL antibodies. The amount of medium fractionated was set to equal between 20 and 50 pmol/h of activity secreted during the basal period. An equal volume of challenge medium was fractionated. Proteins were visualized with the Enhanced Chemiluminescent Kit (Amersham Corp.). Similar results were obtained in four other Western blots using Ab 471 and 475 and in a single Western blot using $^{125}$I-protein A and antipeptide antibodies.

*Figure 6.* PHM, PAL, and ACTH immunoreactivity in transfected AtT-20 cells. (*A*) ACTH immunoreactivity is found in discrete spots at the periphery of PAM-1 cells and also in the perinuclear region. Identical staining patterns were observed in wild-type cells. (*B*) PHM-s cells stained with PHM antibody demonstrate a similar staining pattern as observed in *A*. (*C*) PAL immunoreactivity in PAL-s cells displayed a pattern similar to that observed for soluble PHM and ACTH. (*D* and *E*) PAL immunoreactivity in cells expressing PAL-m (*D*) or PAM-1 (*E*). While there is immunoreactive PAL in discrete spots at the periphery of these cells, a greater accumulation of immunofluorescence is observed in the perinuclear region. (*F*) Wheat germ agglutinin– rhodamine staining of the same PAM-1 cells shown in *E* demonstrates that the staining in the perinuclear region overlaps with the Golgi-selective marker. (*G*) PAL-m cells stained with PHM antibody demonstrate the low level of background staining. Bar, 10 μm.
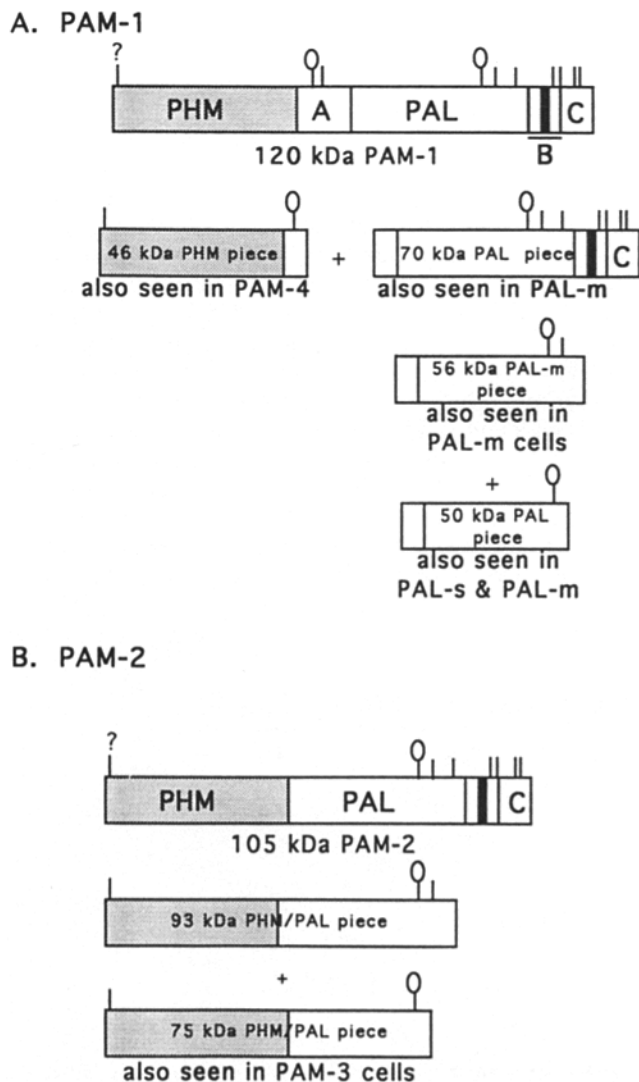
## A. PAM-1



## B. PAM-2



*Figure 9.* Schematic diagram showing possible endoproteolytic cleavages in PAM-1 and -2. Potential endoproteolytic cleavages at dibasic amino acid residues in PAM-1 and -2 which would result in the generation of proteins observed in Western blots of whole cells extracts, soluble and membrane fractions, and medium from AtT-20 cells expressing PAM-1 or -2. For comparison, similar sized PHM and/or PAL fragments which occur in other cell lines are noted under each protein form. The question mark above the first potential dibasic site represents that it is unclear whether the propeptide is removed or retained in the mature PAM protein.

same enzymes which process prohormones may also process PAM.

Proteolytic processing of prohormones frequently occurs at pairs of basic amino acids, although cleavages at monobasic and tetrabasic sites have also been identified (Mains et al., 1990; Steiner, 1991; Dickerson and Noel, 1991). There are eight potential dibasic cleavage sites in rat PAM-1 (Fig. 1); none of these sites fits the rules established for cleavage by furin (Hosaka et al., 1991; Barr, 1991; Steiner, 1991; Lindberg, 1991). The forms of PHM and PAL observed on Western blots are consistent with the occurrence of endoproteolytic processing of PAM at some of these dibasic amino acid residues. Comparison of Western blots of crude membrane and soluble fractions from PAM-1 cells indicates that endo-

proteolytic cleavage of PAM-1 at the Lys–Lys site in exon A would account for both the 44- and 46-kD PHM proteins visualized in the crude soluble fraction and in spent medium and the membrane-associated 70-kD PAL protein (Figs. 3, 5, and 9). Microsequence analysis of PAL purified from bovine neurointermediate pituitary identified this Lys–Lys site as the cleavage site yielding soluble 50-kD PAL (Eipper et al., 1991). Partial removal of the propeptide from the NH2 terminus of PHM could account for the 44–46-kD doublet. A 70-kD integral membrane protein form of PAL is also a major product in PAL-m cells (Figs. 3 and 5). The molecular mass predicted for PAL-m without its signal sequence is 60 kD; the fact that it migrates as a 70-73-kD protein on SDS–polyacrylamide gels strongly suggests that the potential N-linked glycosylation site within PAL was glycosylated in PAM-1 and PAL-m cells; this prediction has been confirmed by Western blot analysis of cell extracts and medium digested with N-glycanase (not shown). The soluble fraction from PAM-1 and PAL-m cells contained soluble, 50- and 56-kD monofunctional PAL suggesting that both of the potential dibasic cleavage sites near the COOH-terminal of PAL may be used (Figs. 3 and 9). Consistent with the importance of endoproteolytic cleavage within exon A, PAM-4 (which contains exon A) was processed to generate smaller soluble PHM proteins lacking antigenic determinants within exon A.

The only paired basic amino acid site separating the PHM and PAL domains of rPAM-1 occurs in exon A. Bovine PAM-1 has an additional paired basic amino acid site immediately preceding exon A and cleavage at this site is thought to yield bovine PHM-B (Eipper et al., 1987). PHM-s represents the rat equivalent of bovine PHM-B. Rat PAM-1 is not cleaved to produce a product of this size, suggesting that the Lys–Lys site in exon A is the only site used to separate the two catalytic domains. Consistent with this, PAM-2 and -3, which lack the dibasic site in exon A, encode proteins containing both enzymatic activities. Soluble 93- and 75-kD proteins generated by proteolytic cleavage following the PAL domain were seen in the crude soluble fraction of PAM-2 cells; both proteins stained with PHM and PAL antisera. Based on the predicted sizes, both dibasic cleavage sites following the PAL domain and preceding the transmembrane domain may be used to generate soluble bifunctional proteins in PAM-2 cells (Fig. 9); further analysis will be needed to identify the actual site of cleavage. Likewise soluble, bifunctional PAM-3 was not processed to generate monofunctional PHM and PAL, although PAM-3 was processed to generate a smaller bifunctional protein presumably lacking the COOH-terminal domain, and corresponding in size to the smaller soluble bifunctional PAM protein produced from PAM-2 (Figs. 3 and 5).

The Western blots and secretion data clearly demonstrate that monofunctional PHM and PAL proteins are generated by endoproteolytic cleavage of membrane-associated PAM-1, while a bifunctional enzyme is generated through processing of membrane-associated PAM-2. The intracellular site of these cleavages is unknown and will only be resolved with biosynthetic labeling and subcellular fractionation of the various cell lines. Perhaps in cells expressing predominantly PAM-1, the 70-kD PAL membrane protein remains within the cell or at the cell surface to serve an as yet unidentified function. Alternatively, the consequence of generating monofunctional or bifunctional enzyme might be related to the catalytic activity or co-factor requirements of the enzymes.

Our results indicate that monofunctional and bifunctional PAM proteins expressed in AtT-20 cells are correctly routed to the regulated secretory pathway. The low basal secretion rate of PAM-4, PHM-s, and PAL-s suggests that AtT-20 cells store soluble PAM proteins as they do POMC-derived peptides. In addition, the immunofluorescent staining pattern of cells expressing soluble PAM proteins was similar to the pattern observed when wild-type or transfected cells were stained with ACTH antisera (Fig. 6). The punctate staining of POMC products especially concentrated in the peripheral processes of AtT-20 cells has been well documented (Mains and May, 1988; Rivas and Moore, 1989; Schnabel et al., 1989). Furthermore, the secretion of these soluble proteins was stimulated in parallel with POMC-derived peptides by secretagogue (Figs. 7 and 8). The major PAM proteins whose release was stimulated by secretagogues were the more highly processed form of PAL from PAL-s cells, PHM from PAM-4 cells, and bifunctional PAM from PAM-3 cells (Fig. 8). In pulse–chase experiments, only smaller POMC-derived products show an increase in secretion rate in response to corticotropin releasing factor or cAMP (Mains and Eipper, 1981). This preferential stimulation of secretion of more highly processed proteins and peptides may be related to the degree of maturation of secretory granules storing PAM and POMC proteins.

Expression of soluble monofunctional PHM or PAL proteins led to storage of the transfected proteins within a stimulatable compartment in AtT-20 cells. The simplest explanation of these results is that in endocrine cells, soluble proteins lacking retention signals for other intracellular organelles are packaged into secretory granules (Huttner and Tooze, 1989). Alternatively, similarities in the secondary structure of PHM and PAL might provide routing information; no similarities in the primary sequences of PHM and PAL are apparent, however (Eipper et al., 1992). A caveat in interpreting the results of these studies is that both PHM-s and PAL-s contained the signal peptide and "pro" sequence of full-length PAM (Fig. 1). This short "pro" region might mediate the correct routing of soluble PAM proteins into the regulated secretory pathway. Other investigators have shown that the amino-terminal sequence of different forms of prosomatostatin strongly affects the intracellular disposition of these proteins in endocrine cells (Sevarino et al., 1989; Stoller and Shields, 1989; Sevarino and Stork, 1991). In contrast, deletion of the "pro" sequence from trypsinogen or renin did not disrupt the routing of these proteins into regulated secretory granules when expressed in AtT-20 cells (Burgess et al., 1987; Nagahama et al., 1990; Chu et al., 1990).

Expression of natural and truncated forms of PAM containing the putative transmembrane domain (PAM-1, PAM-2, and PAL-m) also led to storage of PAM proteins in the regulated pathway of AtT-20 cells. The secretion of PHM and/or PAL activity from PAM-1, PAM-2, and PAL-m cells was stimulated by cAMP and PMA (Figs. 7 and 8). Immunofluorescent staining of PAL-m, PAM-1, and PAM-2 cells with ACTH, PHM, or PAL antibodies demonstrated the presence of punctate staining in the peripheral processes of the transfected cells (Fig. 6). Secretion of PHM and PAL from cells expressing integral membrane forms of PAM requires endoproteolytic cleavage; determination of the subcellular site at which these cleavages occur will require biosynthetic labeling or Western blot analysis coupled with subcellular

fractionation. Interestingly, the perinuclear region and the area around the Golgi compartment were intensely stained with PHM or PAL antisera in cells expressing membrane-associated PAM, while the same areas was less intensely stained in cells expressing soluble PAM proteins (Fig. 6). The fact that membrane-associated and soluble PAM proteins exhibit different patterns of localization suggests a role for the transmembrane domain and/or the COOH terminus in routing. Cytosolic adaptor proteins identified in the region of the *trans*-Golgi are thought to mediate the interaction of clathrin with proteins destined for clathrin-coated vesicles (Brodsky, 1988; Pearse and Robinson, 1990). Clathrin has been shown to play a role in retention of yeast Kex 2 in the Golgi apparatus of yeast (Fuller et al., 1989; Payne and Sheckman, 1989). Experiments are currently under way to determine whether the cytoplasmic domain of membrane-associated PAM is involved in mediating the routing of this protein into secretory granules.

## References

Arvan, P., R. Kuliawat, D. Prabakaran, A.-M. Zavacki, D. Elahi, S. Wang, and D. Pilkey. 1991. Protein discharge from immature secretory granules displays both regulated and constitutive characteristics. *J. Biol. Chem.* 266:14171–14174.

Barr, P. J. 1991. Mammalian subtilisen: the long-sought dibasic processing endoproteases. *Cell.* 66:1–3.

Beaudry, G. A., and A. H. Bertelsen. 1989. Secreted alpha amidating enzymes are generated by specific posttranslational processing of precursors containing transmembrane domains. *Biochem. Biophys. Res. Commun.* 163:959–966.

Brodsky, F. M. 1988. Living with clathrin: its role in intracellular membrane traffic. *Science (Wash. DC).* 242:1396–1402.

Burgess, T. L., C. S. Craik, L. Matsuuchi, and R. B. Kelly. 1987. In vitro mutagenesis of trypsinogen: role of the amino terminus in intracellular protein targeting to secretory granules. *J. Cell Biol.* 105:659–668.

Chevrier, D., H. Fournier, C. Nault, M. Zollinger, P. Crine, and G. Boileau. 1991. Expression of porcine pro-opiomelanocortin in mouse neuroblastoma (Neuro2A) cells: targeting of the foreign neuropeptide to dense-core vesicles. *Mol. Cell. Endocrinol.* 79:109–118.

Chu, W. N., J. D. Baxter, and T. L. Reudelhuber. 1990. A targeting sequence for dense secretory granules resides in the active renin protein moiety of human prorenin. *Mol. Endocrinol.* 4:1905–1913.

Devi, L., P. Gupta, and J. Douglass. 1989. Expression and posttranslational processing of preprodynorphin complementary DNA in the mouse anterior pituitary cell line AtT-20. *Mol. Endocrinol.* 3:1852–1860.

Dickerson, I. M., and G. Noel. 1991. Tissue-specific peptide processing. *In* Peptide Biosynthesis and Processing. L. D. Fricker, editor. CRC Press, Boston. 71–109.

Dickerson, I. M., K. W. C. Peden, and R. E. Mains. 1989. Metallothionein-I promoter-directed expression of foreign proteins in a mouse pituitary corticotrope tumor cell line. *Mol. Cell. Endocrinol.* 64:205–212.

Eipper, B. A., R. E. Mains, and C. C. Glembotski. 1983. Identification in pituitary tissue of a peptide α-amidation activity that acts on glycine-extended peptides and requires molecular oxygen, copper, and ascorbic acid. *Proc. Natl. Acad. Sci. USA.* 80:5144–5148.

Eipper, B. A., L. P. Park, I. M. Dickerson, H. T. Keutmann, E. A. Thiele, H. Rodriguez, P. R. Schofield, and R. E. Mains. 1987. Structure of the precursor to an enzyme mediating COOH-terminal amidation in peptide biosynthesis. *Mol. Endocrinol.* 1:777–790.

Eipper, B. A., S. N. Perkins, E. J. Husten, R. C. Johnson, H. T. Keutmann, and R. E. Mains. 1991. Peptidyl-α-hydroxyglycine α-amidating lyase: purification, characterization, and expression. *J. Biol. Chem.* 266:7827–7833.

Eipper, B. A., C. B.-R. Green, T. A. Campbell, D. A. Stoffers, H. T. Keutmann, R. E. Mains, and L.'H. Ouafik. 1992. Alternative splicing and endoproteolytic processing generate tissue-specific forms of pituitary PAM. *J. Biol. Chem.* 267:4008–4015.

Eipper, B. A., D. A. Stoffers, and R. E. Mains. 1992. The biosynthesis of neuropeptides: peptide α-amidation. *Annu. Rev. Neurosci.* 15:57-85.

Fuller, R. S., A. J. Brake, and J. Thorner. 1989. Intracellular targeting and structural conservation of a prohormone-processing endoproteases. *Science (Wash. DC).* 246:482-486.

Gorman, C. M., D. R. Gies, and G. McCray. 1990. Transient production of proteins using an adenovirus transformed cell line. *DNA and Protein Engineering Technology.* 2:3-10.

Guest, P. C., M. Ravazzola, H. W. Davidson, L. Orci, and J. C. Hutton. 1991. Molecular heterogeneity and cellular localization of carboxypeptidase H in the islets of Langerhans. *Endocrinology.* 129:734-740.

Hosaka, M. M., M. Nagahama, W.-S. Kim, T. Watanabe, K. Hatsuzawa, J. Ikemizu, K. Murakami, and K. Nakayama. 1991. Arg-X-Lys/Arg-Arg motif as a signal for precursor cleavage catalyzed by furin within the constitutive pathway. *J. Biol. Chem.* 266:12127-12130.

Husten, E. J., and B. A. Eipper. 1991. The membrane-bound bifunctional peptidylglycine α-amidating monooxygenase protein: exploration of its domain structure through limited proteolysis. *J. Biol. Chem.* 266:17004-17010.

Huttner, W. B., and S. A. Tooze. 1989. Biosynthetic protein transport in the secretory pathway. *Curr. Opin. Cell Biol.* 1:648-654.

Hutton, J. C. 1990. Subtilisin-like proteinases involved in the activation of proproteins of the eukaryotic secretory pathway. *Curr. Opin. Cell Biol.* 2:1131-1142.

Katapodis, A. G., D. Ping, and S. W. May. 1990. A novel enzyme from bovine neurointermediate pituitary catalyzes dealkylation of α-hydroxyglycine derivatives, thereby functioning sequentially with peptidylglycine α-amidating monooxygenase in peptide amidation. *Biochemistry.* 29:6115-6120.

Kornfeld, S., and I. Mellman. 1989. The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* 5:483-525.

Kozak, M. 1991. An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell Biol.* 115:887-903.

Lapps, W., J. Eng, A. S. Stern, and U. Gubler. 1988. Expression of porcine cholecystokinin cDNA in a murine neuroendocrine cell line. *J. Biol. Chem.* 263:13456-13462.

Lindberg, I. 1991. The new eukaryotic precursor processing proteinases. *Mol. Endocrinol.* 5:1361-1365.

MacDonald, M. R., J. Takeda, C. M. Rice, and J. E. Krause. 1989. Multiple tachykinins are produced and secreted upon post-translational processing of the three substance P precursor proteins, α-, β-, and γ-preprotachykinin. *J. Biol. Chem.* 264:15578-15592.

Mains, R. E., and B. A. Eipper. 1981. Coordinate, equimolar section of smaller peptide products derived from pro-ACTH/endorphin by mouse pituitary tumor cells. *J. Cell Biol.* 89:21-28.

Mains, R. E., and B. A. Eipper. 1990. The tissue-specific processing of pro-ACTH/endorphin recent advances and unsolved problems. *Trends Endocrinol. Metab.* 1:388-394.

Mains, R. E., and V. May. 1988. The role of a low pH intracellular compartment in the processing, storage, and secretion of ACTH and endorphin. *J. Biol. Chem.* 263:7887-7894.

Mains, R. E., C. C. Glembotski, and B. A. Eipper. 1984. Peptide α-amidation activity in mouse anterior pituitary AtT-20 cell granules: properties and secretion. *Endocrinology.* 114:1522-1530.

Mains, R. E., I. M. Dickerson, V. May, D. A. Stoffers, S. N. Perkins, L'H. Ouafik, E. J. Husten, and B. A. Eipper. 1990. Cellular and molecular aspects of peptide hormone biosynthesis. *Front. Neuroendocrinol.* 11:52-59.

Matsuuchi, L., K. M. Buckley, A. W. Lowe, and R. B. Kelly. 1988. Targeting of secretory vesicles to cytoplasmic domains in AtT-20 and PC-12 cells. *J. Cell Biol.* 106:239-251.

May, V., and B. A. Eipper. 1986. Long term culture of primary rat pituitary adrenocorticotropin/endorphin-producing cells in serum-free medium. *Endocrinology.* 118:1284-1295.

Meiniel, A., J. L. Molat, and R. Meiniel. 1988. Complex-type glycoproteins synthesized in the subcommissural organ of mammals: electron-microscopic investigations using lectins. *Cell Tissue Res.* 253:383-395.

Miller, S. G., and H-P. Moore. 1990. Regulated secretion. *Curr. Opin. Cell Biol.* 2:642-647.

Moore, H-P. H., and R. B. Kelly. 1986. Re-routing of a secretory protein by fusion with human growth hormone sequences. *Science (Wash. DC).* 321:443-446.

Moore, H-P. H., M. D. Walker, F. Lee, and R. B. Kelly. 1983. Expressing a human proinsulin cDNA in a mouse ACTH-secreting cell. Intracellular storage, proteolytic processing, and secretion on stimulation. *Cell.* 35:531-538.

Munro, S., and H. R. B. Pelham. 1986. A C-terminal signal prevents secretion of luminal ER proteins. *Cell.* 48:899-907.

Nagahama, M., K. Nakayama, and K. Murakami. 1990. Effects of propeptide deletion on human renin secretion from mouse pituitary AtT-20 cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 264:67-70.

Noel, G., and R. E. Mains. 1991. The ordered secretion of bioactive peptides: oldest or newest first? *Mol. Endocrinol.* 5:787-794.

Noel, G., H. T. Keutmann, and R. E. Mains. 1991. Investigation of the structural requirements for peptide precursor processing in AtT-20 cells using site directed mutagenesis of proadrenocorticotropin/endorphin. *Mol. Endocrinol.* 5:404-413.

Payne, G. S., and R. Schekman. 1989. Clathrin: a role in the intracellular retention of a Golgi membrane protein. *Science (Wash. DC).* 245:1358-1365.

Pearse, B. M., and M. S. Robinson. 1990. Clathrin, adaptors, and sorting. *Annu. Rev. Cell Biol.* 6:151-171.

Pelham, H. R. B. 1988. Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:913-918.

Perkins, S. N., B. A. Eipper, and R. E. Mains. 1990. Stable expression of full-length and truncated bovine peptidylglycine α-amidating monooxygenase complementary DNAs in cultured cells. *Mol. Endocrinol.* 4:132-139.

Perkins, S. N., E. J. Husten, and B. A. Eipper. 1990. The 108-kDA peptidylglycine α-amidating monooxygenase precursor contains two separable enzymatic activities involved in peptide amidation. *Biochem. Biophys. Res. Commun.* 171:926-932.

Reaves, B. J., and P. S. Dannies. 1991. Is a sorting signal necessary to package proteins into secretory granules? *Mol. Cell. Endocrinol.* 79:C141-C145.

Rivas, R. J., and H-P. H. Moore. 1989. Spatial segregation of the regulated and constitutive secretory pathways. *J. Cell Biol.* 109:51-60.

Schnabel, E., R. E. Mains, and M. G. Farquhar. 1989. Proteolytic processing of pro-ACTH/endorphin begins in the Golgi complex of pituitary corticotropes and AtT-20 cells. *Mol. Endocrinol.* 3:1223-1235.

Sevarino, K. A., and P. Stork. 1991. Multiple preprosomatostatin sorting signals mediate secretion via discrete cAMP- and tetradecanoylphorbolacetate-responsive pathways. *J. Biol. Chem.* 266:18507-18513.

Sevarino, K. A., P. Stork, and R. Ventimiglia. 1989. Amino-terminal sequences of prosomatostatin direct intracellular targeting but not processing specificity. *Cell.* 57:11-19.

Sossin, W. S., J. M. Fisher, and R. H. Scheller. 1990. Sorting within the regulated secretory pathway occurs in the *trans*-Golgi network. *J. Cell Biol.* 110:1-12.

Steiner, D. F. 1991. The biosynthesis of biologically active peptides, a perspective. *In* Peptide Biosynthesis and Processing. L. D. Fricker, editor. CRC Press, Boston. 1-15.

Stoffers, D. A., and B. A. Eipper. 1990. Tissue-specific expression of membrane and soluble forms of peptidyl-glycine α-amidating monooxygenase. *Alfred Benzon Symp.* 29:152-159.

Stoffers, D. A., C. B-R. Green, and B. A. Eipper. 1989. Alternative mRNA splicing generates multiple forms of peptidyl-glycine α-amidating monooxygenase in rat atrium. *Proc. Natl. Acad. Sci. USA.* 86:735-739.

Stoffers, D. A., L'H. Ouafik, and B. A. Eipper. 1991. Characterization of novel mRNAs encoding enzymes involved in peptide α-amidation. *J. Biol. Chem.* 266:1701-1707.

Stoller, T. J., and D. Shields. 1989. The propeptide of preprosomatostatin mediates intracellular transport and secretion of alpha-globin from mammalian cells. *J. Cell Biol.* 108:1647-1655.

Takahashi, K., H. Okamoto, H. Seino, and M. Noguchi. 1990. Peptidylglycine α-amidating reaction: evidence for a two-step mechanism involving a stable intermediate at neutral pH. *Biochem. Biophys. Res. Commun.* 169:524-530.

Thiele, E. A., and B. A. Eipper. 1990. Effect of secretagogues on components of the secretory system in AtT-20 cells. *Endocrinology.* 126:809-817.

Tooze, J., and S. A. Tooze. 1986. Clathrin-coated vesicular transport of secretory proteins during the formation of ACTH-containing secretory granules in AtT-20 cells. *J. Cell Biol.* 103:839-850.

Tooze, J., H. F. Kern, and S. D. Fuller. 1989. Condensation-sorting events in the rough endoplasmic reticulum of exocrine pancreatic cells. *J. Cell Biol.* 109:35-50.

Virca, G. D., W. Northemann, and B. R. Shiels. 1990. Simplified Northern blot hybridization using 5% sodium dodecyl sulfate. *Biotechnol. Tech.* 8:370-371.