COOH-terminal Signals Mediate the Trafficking of a Peptide Processing Enzyme in Endocrine Cells

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Abstract. Peptidylglycine α-amidating monooxygenase (PAM) catalyzes the COOH-terminal amidation of bioactive peptides through a two step reaction catalyzed by separate enzymes contained within the PAM precursor. To characterize the trafficking of integral membrane PAM proteins in neuroendocrine cells, we have generated stable AtT-20 cell lines expressing full length and COOH-terminally truncated integral membrane PAM proteins. Full length integral membrane PAM was present on the cell surface in low but detectable amounts and PAM proteins which reached the cell surface were rapidly internalized but not immediately degraded in lysosomes. Internalized PAM complexed with PAM antibody was found in a subcellular compartment which overlapped with internalized transferrin and with structures binding WGA. Thus the punctate juxtanuclear staining of full length PAM represents PAM in endosomes. Endoproteolytic processing of full length PAM-1 and PAM-2 resulted in the secretion of soluble PAM proteins; the secretion of these soluble PAM proteins was stimulus dependent. Although some of the truncated PAM protein was also processed and stored in AtT-20 cells, much of the expressed protein was redistributed to the plasma membrane. Soluble proteins not observed in large amounts in cells expressing full length PAM were released from the surface of cells expressing truncated PAM and little internalization of truncated integral membrane PAM was observed. Thus, the COOH-terminal domain of PAM contains information important for its trafficking within the regulated secretory pathway as well as information necessary for its retrieval from the cell surface.

In neuroendocrine cells, peptide hormones are stored in regulated granules until an external stimulus triggers calcium-mediated exocytosis. Preprohormones are synthesized on membrane-bound ribosomes and undergo a series of posttranslational modifications enroute through the secretory pathway. These modifications may include proteolytic cleavage, glycosylation, tyrosine sulfation, phosphorylation, and amidation (Shields, 1991; Steiner, 1991; Eipper et al., 1992). For over half of the peptides produced from prohormones, removal of basic residues by a carboxypeptidase exposes a COOH-terminal glycine which is then converted to the COOH-terminal α-amide in the final bioactive peptide (Eipper et al., 1992). Understanding the synthesis, processing, and storage of posttranslational processing enzymes is essential for our understanding of peptide hormone biosynthesis and secretion. The mechanisms responsible for the trafficking of posttranslational processing enzymes in neuroendocrine cells have not been elucidated.

Posttranslational processing enzymes which function late in the secretory pathway must be routed together with their substrates into secretory granules. If soluble, these enzymes could be secreted upon exocytosis. Carboxypeptidase E, peptidylglycine α-amidating monooxygenase (PAM),1 and prohormone convertase 1 are found in conditioned medium from AtT-20 cells and the secretion of these enzymes can be stimulated by secretagogues (Mains and Eipper, 1984; Thiele and Eipper, 1990; Klein et al., 1992; Vindrola and Lindberg, 1992). Integral membrane posttranslational processing enzymes would be inserted into the plasma membrane upon granule fusion; their fate following exocytosis is unknown.

With the goal of understanding how secretory granule-associated posttranslational processing enzymes are routed in neuroendocrine cells, we previously overexpressed PAM in the neuroendocrine AtT-20 cell line (Milgram et al., 1992), a well characterized cell line which secretes proopiomelanocortin (POMC)-derived peptides in a stimulus-dependent manner. PAM catalyzes the conversion of glycine-extended precursors into mature α-amidated products; since amidation occurs after proteolytic processing of prohormones, PAM must function in a late secretory compartment. In AtT-20 cells and pituitary tissue, much of the PAM activity co-sediments with the hormone containing fractions (Eipper et al., 1992).

A single gene encodes several alternatively spliced PAM proteins; PAM-1 and PAM-2 are type 1a integral membrane proteins (see Fig. 1) while other PAM proteins lack the α-amidating monooxygenase; PHM, peptidylglycine α-hydroxylating monooxygenase; POMC, proopiomelanocortin.

1 Abbreviations used in this paper: CSFM, complete serum-free medium; PAL, peptidyl α-hydroxyglycine α-amidating lyase; PAM, peptidylglycine

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membrane domain and are soluble (Eipper et al., 1992). Two catalytic domains are contained within the PAM precursor; peptidylglycine α-hydroxylation monoxygenase (PHM) and peptidyl α-hydroxyglycine α-amidating lyase (PAL) act in sequence to catalyze the α-amination reaction (Perkins et al., 1990; Takahashi et al., 1990; Katopodis et al., 1990). In PAM-1, PHM and PAL are separated by the 105–amino acid peptide encoded by optional exon A; PAL is followed by putative transmembrane and COOH-terminal domains (see Fig. 1). Deletion of exon A generates PAM-2, which includes the transmembrane and COOH-terminal domains whereas deletion of both exons A and B generates soluble PAM-3.

We previously demonstrated that expression of monofunctional and bifunctional PAM proteins which were soluble or membrane-associated led to storage of PAM-derived proteins in regulated secretory granules in AtT-20 cells (Milgram et al., 1992). Using immunofluorescent staining with PHM or PAL antisera, we demonstrated that PAM proteins were accumulated in the peripheral processes of cells in a pattern similar to hormone staining. PAM proteins were also heavily concentrated in discrete vesicles in the vicinity of the Golgi apparatus in cells expressing integral membrane but not soluble forms of PAM. Therefore, we hypothesized that the COOH-terminal domain of PAM contains signals which strongly influenced the intracellular distribution of integral membrane PAM proteins.

To determine whether the COOH-terminal domain plays a role in the routing of integral membrane PAM in neuroendocrine cells, we expressed cDNAs encoding mutant forms of PAM-1 and -2 truncated nine amino acids after the transmembrane domain (see Fig. 1). We have used a combination of biosynthetic labeling and immunofluorescent staining to study the maturation and localization of wild-type and mutant PAM proteins. These studies demonstrate that the COOH-terminal domain of PAM contains information important for the routing of this peptide processing enzyme into the regulated secretory pathway plus information necessary for the retrieval of PAM from the cell surface after exocytosis.

Materials and Methods

Generation of Stable Cell Lines

All pBluescript plasmids encoding full length or truncated PAM proteins contain the consensus ribosome binding site 5′-GCCGCCACC (Kozak, 1992). The construction of pBluescript plasmids encoding PAM-1, PAM-2, PAM-1/899, and PAM-2/899 has been described (Milgram et al., 1992; Taut et al., 1992). All of the KrPAM cDNAs were inserted into the pCIS vector by the manufacturer (Pierce, Rockford, IL); the purity of the Fab fraction of IgG prepared from PHM antiserum with immobilized papain as described by the manufacturer (Pierce, Rockford, IL) was verified by SDS-PAGE. Parallel wells of PAM-2 cells were incubated with FITC-conjugated goat anti-rabbit IgG (1:500, CalTag, San Francisco, CA) to visualize PAM proteins present on the surface of the transfected cells. To determine whether PAM antibodies were internalized together with PAM proteins present on the surface of transfected cells, cells in an identical well were permeabilized by incubation with 0.075% Triton X-100 in PBS for 20 min before the addition of secondary antibody. In control experiments, cells expressing soluble PAM-3 were incubated with PHM or PAL antisera as above; in addition, cells expressing integral membrane PAM were incubated with antisera to adrenocorticotropin, a soluble peptide synthesized by AtT-20 cells and stored in dense-core secretory granules. To determine whether the endocytosis of PAM were induced or stimulated by the binding of bivalent PAM antibodies, control experiments were performed using nonimmune Fab fragments. Fab fragments were prepared by digestion of IgG prepared from PHM antiserum with immobilized papain as described by the manufacturer (Pierce, Rockford, IL); the purity of the Fab fragment was verified by SDS-PAGE. Parallel wells of PAM-2 cells were incubated with PHM antiserum or Fab fragments prepared from the same serum for 30 min at 37°C and then chased in the absence of antibody for an additional 30 min at 37°C.

Immunofluorescence

The localization of PAM proteins in transfected cell lines was determined using indirect immunofluorescence as described previously except that incubations with PHM or PAL antisera were performed for 4 h at room temperature (Milgram et al., 1992). Samples were viewed under epifluorescence optics with a Zeiss Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY) using FITC (BP 485/20, barrier filter 520–560) and rhodamine (BP 546/12, LP 590) filters. Cells were photographed under identical conditions using Kodak T-Max 400 film (Eastman Kodak Co., Rochester, NY).

Secretion of PAM Activity and Expression of PAL on the Surface of Transfected Cells

Previously described in vitro assays to measure PAL activity in cell extracts and conditioned medium were modified to allow the measurement of PAL activity on the surface of live cells. Cells were grown in 96-well culture dishes coated with 0.1 mg/ml poly-l-lysine for 48–72 h in complete medium. At the outset of experiments, cells were incubated for 30 min at 37°C in an air atmosphere in complete serum-free medium (CSFM-AIR), pH 7.0 (Milgram et al., 1992); CSFM-AIR contained 20 mM Hepes but no NaHCO3. To quantitate the secretion of PAL activity, the rinse medium was discarded and replaced with 40 μl of fresh CSFM-AIR. After 30 min, the spent medium was collected, centrifuged at 300 g to remove cell debris and assayed for secreted PAL activity at pH 7.0. An additional 30-min incubation was then performed to quantitate PAL activity on the surface of transfected cells; cells were incubated with CSFM-AIR which contained 0.5 μM α-N-acetyl-Tyr-Val-α-hydroxyglycine and 3,000 cpm of the same radiola-beled PAL substrate. Media were collected and the assay terminated by the addition of 240 μl 1 M Tris-HCl, pH 7.0; PAL activity measured during this incubation period represented PAL on the surface of live cells as well as PAL activity secreted during the incubation. Surface activity was determined by subtracting the contribution of secreted PAL activity; PAL activity secreted during the 30-min incubation of live cells with substrate was calculated using the fact that secretion proceeded linearly (Milgram et al., 1992). To measure steady-state levels of PAL activity in whole cell extracts, cells were scraped into ice cold 20 mM Na/(tris)hydroxymethyl)methyl-2-aminoethanesulfonic acid (NTrES)/10 mM mannitol, pH 7.40, 1% Triton X-100, 30 μg/ml PMSF. After three cycles of freezing and thawing, extracts were assayed for PAL activity at pH 7.0. The data for surface and secreted PAL were expressed as a percent of the corresponding total enzyme activity within the cell extract.

Internalization Experiments

Incubation of live cells with antibodies to luminal epitopes can be used to study the internalization of proteins exposed at the cell surface (Patzak and Winkler, 1986; Matteoli et al., 1992). Cells were grown to 50% confluence on Lab-Tek chamber slides coated with poly-l-lysine (0.1 mg/ml). At the outset of experiments, cells were frozen by incubation for 15 min at 37°C in prewarmed DME-F12 containing 0.2 mg/ml BSA (DME-F12/BSA) and then incubated at 37°C for 10 min in DME-F12/BSA containing 10 or 20 μl of PHM or PAL antiserum. At the end of the incubation with antibody, cells were rinsed three times in DME-F12/BSA and either immediately fixed with 4% paraformaldehyde in PBS or chased for varying periods of time in DME-F12/BSA before fixation; duplicate wells were examined for all time points. Fixed cells were incubated with FITC-conjugated goat anti-rabbit IgG (1:500, CalTag, San Francisco, CA) to visualize PAM proteins present on the surface of the transfected cells. To determine whether PAM antibodies were internalized together with PAM proteins present on the surface of transfected cell, cells in an identical well were permeabilized by incubation with 0.075% Triton X-100 in PBS, 0.2% gelatin for 20 min before the addition of secondary antibody. In control experiments, cells expressing soluble PAM-3 were incubated with PHM or PAL antisera as above; in addition, cells expressing integral membrane PAM were incubated with antisera to adrenocorticotropin, a soluble peptide synthesized by AtT-20 cells and stored in dense-core secretory granules. To determine whether the endocytosis of PAM were induced or stimulated by the binding of bivalent PAM antibodies, control experiments were performed using nonimmune Fab fragments. Fab fragments were prepared by digestion of IgG prepared from PHM antiserum with immobilized papain as described by the manufacturer (Pierce, Rockford, IL); the purity of the Fab fragment was verified by SDS-PAGE. Parallel wells of PAM-2 cells were incubated with PHM antiserum or Fab fragments prepared from the same serum for 30 min at 37°C and then chased in the absence of antibody for an additional
30 min at 37°C; internalization of PAM proteins present on the surface of transfected cells was determined as described above.

In a series of experiments, rhodamine-conjugated transferrin (50 μg/ml; Molecular Probes, Inc., Eugene, OR) was included in the 10-min incubation with PHM or PAL antibody; cells were then analyzed as above. The localization of internalized PHM or PAL antibody was also compared with the localization of rhodamine-labeled WGA (1:5000) which served as a marker of the Golgi complex (Molecular Probes, Inc.). To visualize lysosomes, cells were incubated with rat anti-mouse LAMP-1 kindly provided by Dr. Thomas August (The Johns Hopkins University, Baltimore, Maryland) diluted 1:40 in PBS, 0.2% gelatin. Fluorescein-conjugated goat anti-rabbit IgG (1:500; CalTag) was used to visualize PAM proteins internalized from the surface of cells expressing full length PAM and rhodamine-conjugated goat anti-rat IgG (1:100) was used to visualize LAMP-1.

To determine whether PAM proteins were internalized from the surface of primary anterior pituitary cell cultures, adult male rat anterior pituitaries were dispersed and cultured as described previously (May and Epper, 1986). At the outset of the internalization assay, cells were rinsed in DME-F12/BSA and then incubated for 1 h in DME-F12/BSA containing 10 nM phorbol, 12-myristate-13-acetate (PMA; Calbiochem-Novabiochem Corp., La Jolla, CA) and 10 μM of PHM or PAL antibody; cells were rinsed and incubated an additional 1 h in basal medium before fixation. Control experiments included incubation of parallel wells with preimmune serum or antibody to the COOH-terminal domain of PAM. Cells were processed for immunofluorescence after permeabilization with Triton X-100 as described above.

**Biosynthetic Labeling**

Cells were plated on 12-mm culture dishes coated with 0.1 mg/ml poly-L-lysine and grown a minimum of 36 h before experiments were begun; cultures were used when they were between 50 and 75% confluent. Cells were rinsed once for 5 min in methionine-free CSFM and then incubated in 250–300 μl of the same medium with 0.4–0.6 μCi [35S]methionine (Amersham Corp., Arlington Heights, IL) for 30 min; the concentration of methionine was adjusted to 5 μM (final specific activity 575 Ci/mmol). At the end of the incubation with [35S]methionine, the cells were rinsed once with CSFM containing 10 mM methionine and then either extracted and measured as described below, or low or incubated for varying periods of time in 250 or 300 μl CSFM containing the normal level of methionine. In one series of experiments, cells were chased for two successive 1-h periods in CSFM and then incubated for an additional 1 h in CSFM containing 10 nM phorbol, 12-myristate-13-acetate diluted from a 1 mg/ml stock in DMSO. Chase media were collected, centrifuged at 300 g to pellet nonadherent cells and stored at −80°C until used for immunoprecipitation and Western blot analysis.

**Immunoprecipitation**

Aliquots of medium (50 μl) were diluted fourfold in 50 mM sodium phosphate, pH 7.4, 1% Triton X-100, 30 μg/ml PMSE, 1 mM unlabeled methionine and 10% v/v methanol. Aliquots were added to 20 μl preimmune serum or anti-serum at 4°C. Aliquots of whole cell extracts were made 1% in SDS and boiled for 5 min after the addition of 30 μg/ml PMSE. Extract samples were then diluted fourfold in 50 mM sodium phosphate, pH 7.4, 1% Triton X-100, 30 μg/ml PMSE, 1 mM unlabeled methionine, 0.5% NP-40 and incubated with domain-specific antibodies overnight at 4°C. After an initial centrifugation to pellet any insoluble material, immune complexes were isolated by incubation for 2 h at room temperature with 20 μl Protein A-Sepharose (Sigma Immunochemicals, St. Louis, MO) diluted in a final volume of 880 μl 50 mM sodium phosphate, pH 7.4, 1% Triton X-100 (Super E). The resin was washed four times in 800 μl of Super E, twice in 50 mM sodium phosphate, pH 7.4, and bound proteins were released by boiling the resin in 50 μl SDS-PAGE sample buffer. Boiled samples were fractionated by SDS-PAGE on 10% polyacrylamide (0.27% N,N'-methylene-bisacrylamide) gels. Gels were fixed for 30 min in 30% isopropanol, 10% acetic acid and then impregnated for 30 min with Amplify (Amersham Corp.) before drying and fluorography. Gels were exposed to film at −80°C with intensifying screens; exposure times ranged from 12 h to 5 d. The apparent molecular weights of the immunoprecipitated proteins were determined by comparison with proteins of known molecular weight standards (Rainbow Standards; Amersham Corp.). Densitometric analyses were performed using an Abatron Scan 300/GS linked to an Apple Macintosh Ilci and NIH Image 1.35 software (National Institute of Mental Health); band intensities were corrected for the predicted number of methionines in each PAM proteins. For each experiment several exposure times were analyzed and exposures within the linear range were used for the calculations.

**Immunoprecipitation of Cell Surface-associated PAM Proteins**

Duplicate wells of PAM-1 and PAM-1/899 cells were incubated with [35S]methionine for 30 min as described above and then chased for 2 h in basal medium containing the normal concentration of methionine. To immunoprecipitate cell surface-associated PAM proteins, cells were rinsed twice with ice-cold CSFM and incubated for 2 h at 4°C with 25 μl/μl PHM or PAL antiserum. An additional well of each cell type was incubated at 4°C with PHM or PAL antiserum in CSFM containing 1% Triton X-100. For immunoprecipitation of cell surface PAM proteins, the medium containing the corresponding full length integral membrane PAM to remove unbound antibodies. The cells were then permeabilized with 1% Triton X-100 in CSFM and the samples were spun in a microfuge to pellet insoluble material. The supernatants were incubated with Protein A-Sepharose as described above and the samples were analyzed by SDS-PAGE.

**Results**

We have generated stable AtT-20 cell lines expressing PAM-1 and -2 truncated 9 amino acids following the putative transmembrane domain at Gly999 (Fig. 1). Northern blot analysis of the transfected cell lines demonstrated the presence of RNA transcripts migrating with the expected size (not shown). Our previous studies demonstrated that AtT-20 cells transfected with cDNAs encoding soluble and integral membrane PAM proteins produced active PHM and PAL enzymes (Milgram et al., 1992). To determine whether the truncated PAM proteins were also enzymatically active, PHM and PAL activities were measured in conditioned medium and whole cell extracts from the transfected cell lines. PHM and PAL activities in transfected cells expressing PAM-1/899 and PAM-2/899 were 50–100-fold greater than the corresponding endogenous activity in wild-type AtT-20 cells (Milgram et al., 1992; not shown). The fact that the intensity of the signals obtained on Northern blots correlated with the PHM and PAL specific activities demonstrates that the truncated PAM proteins were not misfolded. Based on measuring PHM and PAL activity in whole cell extracts, the levels of expression of the full length and truncated PAM proteins were comparable, differing by less than a factor of two. The processing, routing, and secretion of the truncated PAM proteins (PAM-1/899, PAM-2/899) were compared with the corresponding full length integral membrane PAM proteins.

**Localization of Transfected PAM Proteins**

Cell lines expressing full length (PAM-1, PAM-2) and truncated (PAM-1/899 and PAM-2/899) PAM proteins were fixed, permeabilized, and visualized with PHM or PAL antibody.
Figure 2. Immunofluorescent staining of permeabilized and non-permeabilized cells expressing full length or truncated PAM. After fixation, permeabilized (A, C, E, and G) or nonpermeabilized (B, D, F, and H) cells were incubated with PHM antibodies and processed for indirect immunofluorescence as described in Materials and Methods. A and B, PAM-1; C and D, PAM-1/899; E and F, PAM-2; G and H, PAM-2/899. Similar results were obtained when transfected cells were stained with PAL antibodies. All cells were photographed under identical conditions. Bar, 20 \( \mu \text{m} \).
tisera using indirect immunofluorescence. As was observed previously, a strong PHM or PAL signal was detected in the perinuclear region in cell lines expressing PAM-1 or PAM-2 (Fig. 2, A and E). Punctate staining was also observed in the peripheral processes of these cells, indicating that some of the PAM proteins were localized in discrete vesicles in AtT-20 cells; the peripheral processes of AtT-20 cells contain dense-core secretory granules which stain positively for POMC-derived peptides (Rivas and Moore, 1989; Schnabel et al., 1989; Kreis et al., 1989). Staining of permeabilized PAM-1/899 and PAM-2/899 cells revealed a different steady-state distribution of PAM proteins. There was a marked decrease in staining intensity in the perinuclear region; staining for PHM and PAL was observed along the margins of the transfected cells, suggesting that PAM proteins were accumulating at the plasma membrane of cells expressing these truncated PAM proteins (Fig. 2, C and G). Staining was also observed in the peripheral processes of cells expressing truncated PAM proteins. Cell lines expressing full length or truncated integral membrane PAM all exhibited intense punctate staining in peripheral processes when visualized with antibodies to adrenocorticotropicin, a peptide product stored in secretory granules (as in Milgram et al., 1992; not shown).

To determine whether PAM proteins were localized on the surface of transfected cells, cells were fixed and stained with PHM or PAL antisera without prior permeabilization with Triton X-100. A much stronger signal was observed with PAM-1/899 and PAM-2/899 cells (Fig. 2, D and H) than with cell lines expressing full length PAM-1 or PAM-2 (Fig. 2, B and F). In control experiments, when cells expressing soluble PAM-3 were stained without the addition of Triton X-100, no surface labeling with PAM antisera was detected; a strong signal was detected when PAM-3 cells were permeabilized (see below). These data demonstrate that removal of the COOH terminus of integral membrane forms of PAM resulted in their accumulation on the surface of AtT-20 cells.

**Surface Expression and Basal Secretion of PAL Activity**

To quantify the surface expression of PAM in cell lines expressing full length and truncated PAM proteins, cells were incubated in serum-free medium in the absence and then in the presence of PAL substrate. When live cells were incubated with PAL substrate, product was generated by PAL on the cell surface and by PAL secreted into the medium; surface PAL activity was calculated by subtracting the contribution of secreted PAL.

Consistent with the immunofluorescent staining, a small amount of the total PAL activity in transfected cells expressing PAM-1 and PAM-2 was detected on the cell surface. Cells expressing truncated PAM proteins displayed significant increases in surface expression of PAL activity when compared with cells expressing full length integral membrane PAM; PAM-1/899 and PAM-2/899 cells had >30% of their total PAL activity exposed on the cell surface (Fig. 3). In contrast, 5 and 11% of the total cellular PAL activity was on the surface of cells expressing PAM-1 and PAM-2, respectively. The basal secretion rates for PAL activity from cell lines expressing truncated integral membrane PAM-1/899 and PAM-2/899 were approximately eight- and fivefold higher than the basal secretion rates of PAL activity from PAM-1 and PAM-2 cells, respectively (Fig. 3).

**Internalization of PAM Proteins**

To determine the fate of integral membrane PAM proteins expressed on the surface of AtT-20 cells, and to determine whether the COOH terminus controls the disposition of PAM proteins that have reached the cell surface, live cells expressing full length or truncated PAM proteins were incubated at 37°C with PHM or PAL antisera. After 10 min the medium containing PAM antiserum was removed; the cells were rinsed several times and immediately fixed. After fixation, cells were incubated with fluorescein-labeled goat anti-rabbit IgG to visualize PAM proteins on the surface of the transfected cells; an identical well of cells was permeabilized with detergent before the addition of secondary antibody.

In cells expressing full length PAM proteins, there was faint staining visualized on the surface of nonpermeabilized cells after the 10-min incubation with PAL antibody (Fig. 4 A); however, distinct punctate staining of much greater intensity was evident in permeabilized cells (Fig. 4 B), indicating that integral membrane PAM-1 was rapidly internalized together with PAM antibody (subsequently referred to as PAM/PAM Ab) in AtT-20 cells. Although PAM-1/899 cells showed greater expression of integral membrane PAM on the cell surface than cell lines expressing full length PAM, no internalization of truncated PAM/PAM Ab was evident during the 10-min incubation with antibody (Fig. 4, C-D). When PAM-2 and PAM-2/899 were incubated in an identical manner, similar results were obtained (Fig. 5, left column). When antibody binding to the surface of transfected cells was carried out at 4°C, which blocks receptor-mediated endocytosis (Steinman et al., 1983), no internalization of PAM proteins was observed; upon warming, there was rapid internalization of PAM/PAM Ab in cell lines expressing full length PAM but not in cells expressing truncated PAM (not shown). Internalization of PAM proteins was also observed when as-

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**Figure 3.** Surface expression and basal secretion (per hour) of PAL enzyme activity in transfected cells. Secreted (●) and surface (□) PAL activity were measured as described in Materials and Methods and expressed as a percentage of the total PAL activity measured at pH 7.0 in whole cell extracts. Data represent the mean ± SD of six observations in two separate experiments. The PAL specific activities were 58 ± 3, 46 ± 11, 100 ± 29, and 46 ± 7.9 pmol/µg/hr (mean ± SD, n = 3) for PAM-1, PAM-1/899, PAM-2, and PAM-2/899, respectively.
Figure 4. Internalization of PAM from the surface of PAM-1 (A and B), PAM-1/899 cells (C and D), and PAM-3 (E and F) cells. Cells were incubated with 10 μl of PAL antibody in DME-F12/BSA for 10 min, rinsed and then fixed and processed for indirect immunofluorescence without (A, C, and E) or with (B, D, and F) prior permeabilization with Triton X-100. Bar, 20 μm.

says were performed using monovalent Fab fragments prepared from an antiserum to PHM. Thus the internalization of PAM from the surface of AtT-20 cells was not caused by the binding of bivalent antibodies. When cells expressing soluble PAM-3 were analyzed for surface expression and internalization of PAM, no specific signal was detected on the surface nor within intracellular organelles in cells permeabilized with Triton X-100 (Fig. 4, E–F). These data demonstrate that PAM antibody was bound specifically to PAM proteins on the surface of cells expressing integral membrane PAM. Thus, we conclude that the rapid internalization of PAM-1 and PAM-2 was dependent upon a signal present in the COOH-terminal domain of this secretory granule-associated enzyme.

To determine the fate of the internalized PAM proteins, live cells were incubated with antiserum for 10 min at 37°C and chased for varying periods of time at 37°C in basal medium. At all time points, PAM present on the cell surface and internalized PAM/PAM Ab were visualized with FITC-conjugated secondary antibody used in the absence or presence of detergent, respectively. The results of such a time course comparing PAM-2 and PAM-2/899 cells are presented in Fig. 5; similar results were obtained when PAM-1 and PAM-1/899 cells were compared (not shown). We observed rapid internalization of antisera bound to PAM-2 proteins from the surface of AtT-20 cells; at short times of chase, discrete punctate immunofluorescence was observed throughout the cells. With increasing times of chase there was a decrease in the amount of PAM/PAM Ab which could be visualized on the cell surface (Fig. 5, top row) with an increase in the intensity of staining in the perinuclear region of permeabilized cells (Fig. 5, second row). By 30 min of chase, the majority of the internalized protein was present in a juxtanuclear position and little staining was observed on the cell surface. When cells expressing PAM-2/899 were analyzed in the same way, a dramatic decrease in the rate and extent of internalization of PAM-2/899 proteins present on the cell surface was evident, and antibody remained on the surface at all time points examined (Fig. 5, third row). Internalized PAM/PAM Ab was observed in cells expressing PAM-2/899 only after a 30-min chase (Fig. 5, bottom row).

The accumulation of internalized PAM in a perinuclear location suggested that PAM might be retrieved from the cell surface and reused in neuroendocrine cells. Therefore, a series of experiments were performed to compare the location of internalized PAM/PAM Ab with the location of internalized transferrin. After binding to its cell surface receptor and endocytosis, transferrin bound to its receptor is recycled to the cell surface in endosomes (van Rensburg et al., 1982; Lamb et al., 1983; Yamashiro et al., 1984). Cells were in-
Figure 5. Internalization of PAM from the surface of PAM-2 and PAM-2/899 cells after 0, 5, and 30 min of chase. Cells were incubated with 20 μl/ml of PHM antiserum in DME-F12/BSA for 10 min, rinsed and incubated at 37°C in basal medium for the times indicated. Cells were then fixed and incubated with fluorescein-conjugated secondary antibodies with or without prior permeabilization as indicated. Bar, 20 μm.
Figure 6. Colocalization of internalized PAM with internalized transferrin, WGA, or LAMP-1 in PAM-2 cells. Three wells of PAM-2 cells were incubated with 10-20 μl/ml PHM antiserum for 10 min. In one well, the cells were simultaneously incubated with 50 μg/ml rhodamine-conjugated transferrin. Cells from all three wells were chased for 30 min in basal medium, rinsed, fixed, and incubated with Triton X-100 as described in Materials and Methods. The second well was incubated with rhodamine-WGA and the third well was incubated with rat LAMP-1 mAbs and rhodamine-conjugated goat anti-rat secondary antiserum. PAM/PAM Ab in all three wells was visualized by incubation with fluorescein-conjugated goat anti-rabbit IgG as described in Materials and Methods. A, C, and E show the rhodamine fluorescence corresponding to internalized transferrin, WGA, and LAMP-1, respectively. B, D, and F show the corresponding fluorescein fluorescence demonstrating the localization of internalized PAM/PAM Ab in each well of cells. Bar, 20 μm.

cubated simultaneously with PAM antibody and rhodamine-conjugated transferrin; by visualizing the PAM antibody with fluorescein-conjugated secondary antibody, the localization of PAM and transferrin could be compared. After 30 min of chase, the staining pattern for PAM (Fig. 6 B) overlapped with the staining pattern for rhodamine-transferrin (Fig. 6 A). The distribution of internalized PAM/PAM Ab also overlapped with the distribution of WGA indicating that the internalized PAM/PAM Ab complex was localized in endosomes in a juxtanuclear position (Fig. 6 C).

Proteins internalized by receptor-mediated endocytosis can travel to lysosomes (Steinman et al., 1983). To determine whether a major pathway for internalized PAM/PAM Ab involved lysosomes, the localization of internalized

Figure 7. Internalization of PAM from the surface of primary anterior pituitary cell cultures. Anterior pituitaries from six adult male rats were dispersed and cultured as described previously (May and Eipper, 1986). Cells were incubated in DME-F12/BSA containing 10 nM phorbol ester and 10 μl/ml of PHM antiserum (A) or preimmune serum (B) for 1 h and then incubated in the absence of antibody for an additional 1 h. Cells were fixed, permeabilized, and stained as described in Materials and Methods. Bar, 20 μm.
Figure 8. Basal secretion, endoproteolytic processing, and surface expression of PAM proteins. To identify PAM proteins secreted from transfected cells (A and C), the indicated cells were incubated for 30 min in medium containing 400 μCi [35S]methionine and then chased for 6 h in basal medium. Cell extract and medium samples were immunoprecipitated and analyzed by SDS-PAGE as described in Materials and Methods. For cells expressing PAM-2 and PAM-2/899, (C), PHM and PAL immunoprecipitates yielded identical results and the data shown were obtained using the PHM antibody. The right panel in C shows medium samples from PAM-2 and PAM-2/899 cells analyzed in adjacent lanes of a 6% SDS-PAGE gel. Apparent molecular weights are indicated in kD and standard molecular weights are on the left. Similar results were obtained in four separate experiments. To identify PAM proteins present on the surface of cells expressing PAM-1 and PAM-1/899 (B), cells were incubated for 30 min in medium containing 400 μCi [35S]methionine and then chased for 2 h in basal medium. Cell surface-associated PAM proteins (SUR) and total PAM proteins (TOT) were immunoprecipitated from parallel wells using PHM antiserum as described in Materials and Methods.

Endoproteolytic Processing and Basal Secretion of PAM Proteins

In our previous study we used Western blot analysis to demonstrate that PAM-1 and PAM-2 proteins were subjected to limited endoproteolytic cleavage by resident AtT-20 processing enzymes resulting in the secretion of monofunctional or bifunctional PAM proteins, respectively (Milgram et al., 1992). The processing and secretion of full length and truncated PAM-1 and -2 were compared using biosynthetic labeling and immunoprecipitation with domain specific antisera. Cell lines were compared using a pulse/chase paradigm; after incubation with medium containing [35S]methionine for 30 min, cells were either harvested (pulse) or incubated in unlabeled medium for 6 h (chase). After the pulse incubation with [35S]methionine, a 113-kD bifunctional protein was detected in extracts of cells expressing PAM-1 (Fig. 8 A). After the chase, the intact protein increased in size by ~6 kD (Fig. 8 A) due at least in part to modifications in the glycosylation of PAM-1 (not shown). During the chase, almost all of the PAM-1 precursor underwent endoproteolytic cleavage to generate 44–46-kD PHM proteins visualized in both cell extracts and conditioned medium and a 70-kD PAL protein visualized only in cell extracts. The predominant protein present in conditioned medium from PAM-1 cells was monofunctional 44–46-kD PHM; however, trace amounts of 105- and 107-kD bifunctional soluble PAM proteins were also visualized (Fig. 8). Monofunctional PAL proteins of 56 and 50 kD, representing cleavage products of the 70 kD integral membrane PAL protein, were also visualized in conditioned medium upon longer exposure of the autoradiograms (not shown). Monofunctional PAL proteins were difficult to visualize since PAL contains only four methionine residues while 20 methionine residues are present in PHM.

When corresponding cell extracts and conditioned medium from PAM-1/899 cells were analyzed, important differ-
ences in the processing and secretion of PAM proteins were evident. After the pulse labeling period, PAM-1/899 cells contained a 101-kD PAM protein; this protein also increased \( \sim 6 \text{ kD} \) in size during the chase (Fig. 8 A). Conditioned medium from PAM-1/899 cells contained significant amounts of a doublet of 105- and 107-kD bifunctional PAM proteins; these proteins were not visualized in immunoprecipitates of PAM-1/899 whole cell extracts and do not appear to be stored in the cells in significant amounts (Fig. 8 A). In addition to the bifunctional PAM proteins present in conditioned medium, some of the PAM-1/899 was endoproteolytically processed to generate monofunctional PHM (Fig. 8 A); the 44-46-kD PHM was visualized in both cell extracts and chase medium. Prolonged exposure of the autoradiograms also demonstrated monofunctional integral membrane PAL (58 kD) in cell extracts after the 6-h chase. Therefore, a portion of the PAM-1/899 protein was processed in a manner similar to PAM-1 to generate monofunctional PHM and PAL; however, the majority of the PAM-1/899 protein was processed in a different manner to generate bifunctional PAM proteins.

Bio synthetic labeling followed by cell surface immunoprecipitation was used to compare the amount and forms of PAM on the cell surface. PAM-1 and PAM-1/899 cells were incubated with \( [\text{\textsuperscript{35}}S] \) methionine for 30 min followed by a 2-h chase in basal medium. Intact cells were incubated with PHM or PAL antiserum at 4°C and antibody bound to PAM proteins on the cell surface was isolated using protein A-Sepharose. For comparison, a parallel well of each cell type was permeabilized with detergent before incubation with antiserum. Surface-associated and total PAM proteins were visualized after SDS-PAGE (Fig. 8 B). After a 2-h chase, extracts of PAM-1 cells contain full length bifunctional PAM (120 kD) as well as monofunctional 44–46-kD PHM and 70-kD PAL proteins. A small fraction of the full length PAM was visualized in immunoprecipitates of surface-associated PAM proteins; longer exposures of the autoradiogram demonstrated that 70-kD monofunctional PAL was also present on the surface of cells expressing PAM-1. Extracts of PAM-1/899 cells contained full length PAM and monofunctional PHM. Consistent with the immunofluorescent staining of surface-associated PAM and the measurements of PAL enzyme activity on the surface of the transfected cells, cells expressing PAM-1/899 had significantly greater amount of their total PAM protein present on the cell surface.

The endoproteolytic processing and secretion of PAM-2 and PAM-2/899 were also compared. In PAM-2 and PAM-2/899 cells, the PHM and PAL domains were not separated by endoproteolytic processing. After the pulse incubation, PHM and PAL antiserum immunoprecipitated a 105-kD protein from PAM-2 cell extracts and a 93-kD protein from PAM-2/899 cell extracts (Fig. 8 C). PAM-2 was processed at a slower rate than PAM-1 to generate 83- and 75-kD proteins containing antigenic determinants for both PHM and PAL; these proteins were visualized in small amounts in immunoprecipitates of cell extracts after 6 h of chase and were secreted from PAM-2 cells (Fig. 8 C). When conditioned medium from PAM-2/899 cells was examined by immunoprecipitation with PHM or PAL antiserum; two proteins were visualized; each of these proteins migrated with apparent molecular masses 2–3 kD larger than the bifunctional proteins observed in cells expressing full length PAM-2 (Fig. 8 C, right). The 85- and 78-kD proteins present in conditioned medium from cells expressing PAM-2/899 were not visible in cell extracts; however, a bifunctional PAM protein which migrated with an apparent molecular mass of 75 kD was visible in immunoprecipitates from PAM-2/899 cell extracts (Fig. 8 C). This 75-kD bifunctional protein may be the same protein which was observed in whole cell extracts of cells expressing PAM-2. Thus deletion of the COOH-terminal domain alters the endoproteolytic processing and/or glycosylation of some, but not all, of the PAM-1 and PAM-2.

**Kinetic Analysis of the Secretion of PAM-1 and PAM-1/899**

Since the novel products generated in cells expressing PAM-1/899 were easier to distinguish from the wild-type products than were products generated from PAM-2/899 cells, further comparisons were performed using PAM-1 and PAM-1/899 cell lines. To compare the kinetics of secretion in PAM-1 and PAM-1/899 cell lines, cells were labeled with \( [\text{\textsuperscript{35}}S] \) methionine for 15 min and chased in basal medium for 10 h. At intervals throughout the chase period, conditioned medium was collected and replaced with fresh medium; samples were analyzed by immunoprecipitation using PHM antiserum. In cells expressing PAM-1, secretion of newly synthesized monofunctional PHM was highest during the 2-4 and 4-6-h chase periods (Fig. 9). By comparison, PAM-1/899 cells secreted large amounts of newly synthesized bifunctional PAM proteins during the 1-2 h chase period, while PAM-1 cells secreted little or no bifunctional PAM (Fig. 9). Release of newly synthesized bifunctional PAM proteins was largely complete by the end of the 2-4-h chase period. Monofunctional PHM was also secreted from cells expressing PAM-1/899. Densitometric analyses of autoradiograms showed that 20–40% of the secreted PAM was 44–46-kD PHM while the remainder represented bifunctional PAM. In cells expressing PAM-1/899, equal amounts of newly synthesized monofunctional PHM were secreted during the 1-2- and 2-4-h chase periods; this pattern differs from the pattern ob-

![Figure 9. Kinetics of secretion of PHM from PAM-1 and PAM-1/899 cell lines.](image-url)
secretion of monofunctional PHM from cells expressing full length PAM-1.

When PAM-1/899 cells were labeled with [35S]methionine for 15 min and chased for 30, 45, 60, or 90 min in basal medium, it was evident that newly synthesized bifunctional PAM appeared in the medium before monofunctional PHM. Small amounts of bifunctional PAM were present in conditioned medium following 30–45 min of chase while mono-functional PHM was not visualized in significant amounts until 90 min of chase (not shown). Thus the basal secretion of monofunctional PHM from PAM-1 and PAM-1/899 cells and the secretion of bifunctional PAM from PAM-1/899 cells occurred with different but overlapping kinetics.

**Stimulated Secretion of PAM Proteins**

To determine which of the PAM proteins generated through cleavage of the truncated PAM were stored in regulated granules, cells expressing full length and truncated PAM proteins were incubated in medium containing [35S]methionine for 30 min, chased for two consecutive 1-h periods in basal medium, and then chased an additional 1 h in medium containing 10 nM phorbol, 12-myristate-13-acetate, a known secretagogue of POMC peptides in AtT-20 cells (Thiele and Eipper, 1990). The proteins present in basal and stimulated media were compared after immunoprecipitation with PHM antisera (Fig. 10). Phorbol ester stimulated the secretion of monofunctional PHM from cells expressing PAM-1 and from cells expressing PAM-1/899; however, secretion of the 105- and 107-kD bifunctional PAM proteins from cells expressing PAM-1/899 was not significantly stimulated. In cells expressing PAM-1/899, the stimulated secretion of monofunctional PHM varied in magnitude; however, when compared with the secretion of the bifunctional PAM in each experiment, a consistent stimulation was observed. Secretagogues also stimulated the secretion of bifunctional 75- and 83-kD PAM from cells expressing PAM-2 (Fig. 10). Conditioned medium from cells expressing PAM-2/899 contained 78- and 85-kD bifunctional PAM proteins; secretion of the

![Figure 11. Schematic diagram of trafficking of full length and truncated PAM-1 in AtT-20 cells. Full length PAM-1 is routed into secretory granules where processing into monofunctional PHM and PAL may occur. Full length bifunctional PAM and integral membrane PAL which reach the cell surface are rapidly internalized and routed to late endosomes in the vicinity of the Golgi apparatus. In contrast, most of the PAM-1/899 reaches the cell surface in either immature secretory granules or constitutive vesicles. Unprocessed PAM-1/899 proteins at the cell surface are not efficiently internalized but are cleared from the surface through the action of cell surface proteases. In addition, 20–40% of the PAM-1/899 is exposed to proteases associated with the secretory pathway; these proteins undergo endoproteolytic cleavage to generate monofunctional PHM and PAL which are secreted in a stimulus-dependent fashion.](image-url)
produced in PAM-1/899 and PAM-2/899 cells do not appear present on the cell surface (Fuller et al., 1989); this differ-
al., 1991; Miettinin et al., 1992), a signal necessary for the yeast posttranslational processing enzyme 
basal conditions in low but detectable amounts (Figs. 2, 3, 2/899 produced active PHM and PAL enzymes; this fact and PAM-2 (Fig. 11). Ceils expressing PAM-1/899 and PAM-
protein was routed to secretory granules. However, much of the protein was found by immunofluorescent staining in discrete vesicular structures in the vicinity of the Golgi appar-
ment in cells expressing truncated PAM.

Discussion

When integral membrane PAM proteins were expressed in the neuroendocrine AtT-20 cell line, some of the expressed protein was routed to secretory granules. However, much of the protein was found by immunofluorescent staining in discrete vesicular structures in the vicinity of the Golgi appar-
assays clearly establish a role for the COOH-terminal tail of PAM proteins in biosynthetic labeling experiments (Fig. 8), argues against the possibility that internalized PAM was immediately degraded in lysosomes. The stability of newly synthesized PAM was not altered by the addition of 10 mM ammonium chloride, which disrupts the pH gradient necessary for the function of lysosomal proteases, or by the addition of the permeant lysosomal protease inhibitor leupeptin (not shown).

Bulk endocytic tracers and labeling of cell surface glycoproteins have been used to demonstrate that in secretory cells, internalization from the cell surface to the Golgi apparatus represents a major trafficking pathway (Herzog and Farquhar, 1977; Winkler, 1977; Orci et al., 1986; Green and Kelly, 1990 and 1992); our data indicate that PAM may be a component of this recycling pathway. The internalization of PAM/PAM Ab and its routing to a juxtanuclear position raise the possibility that integral membrane peptide processing enzymes are reused in endocrine cells as was observed for glycoprotein III in chromaffin cells (Patzak and Winkler, 1986). It is well accepted that after exocytosis, synaptic vesicle membrane proteins are internalized and empty synaptic vesicles are refilled with neurotransmitter so that the integral membrane proteins found in these vesicles are reutilized in multiple cycles of exo- and endocytosis (Sudhof and Jahn, 1991; Matteoli et al., 1992). Our data indicate that integral membrane secretory granule proteins may be internalized in a similar manner. However, in contrast to synaptic vesicles which are filled and refilled with neurotransmitter at the cell periphery, reuse of secretory granule membrane components would require their return to a much earlier stage of the secretory pathway.

While the immunofluorescent staining and internalization assays clearly establish a role for the COOH-terminal tail of PAM in the internalization of PAM proteins after exocytosis, these data do not indicate whether full length and truncated PAM proteins reach the cell surface in the same or different vesicle populations. A complication in the analysis of PAM trafficking in neuroendocrine cells comes from the fact that posttranslational processing enzymes undergo endoproteolytic processing enroute through the secretory pathway, as seen with prohormones (Fricker, 1991; Milgram et al., 1992; Vindrola and Lindberg, 1992). We used pulse chase paradigms and immunoprecipitation to compare the proteins generated by proteolytic cleavage of wild-type and mutant integral membrane PAM. Integral membrane PAM-1 and -2

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are processed to generate soluble monofunctional and bifunctional proteins, respectively (Milgram et al., 1992 and Fig. 8 in this paper). Removal of the COOH-terminal domain of integral membrane PAM altered the endoproteolytic processing pattern of the expressed proteins, indicating that the truncated proteins were exposed to additional endoproteases not usually acting upon the full length PAM proteins. The novel proteins produced by PAM-1/899 and PAM-2/899 cells were released rapidly from cells and were not visualized in immunoprecipitates of cell extracts (Fig. 8), suggesting that the endoprotease(s) responsible for generating the 105-107-kD PAM-1 proteins and the 85- and 78-kD PAM-2 proteins were located at or near the cell surface. The intense immunofluorescent staining of cells expressing truncated PAM proteins in the absence of permeabilization (Fig. 2) and our measurements of cell surface enzyme activity (Fig. 3) are consistent with the idea that the cleavage of truncated PAM occurred at the cell surface (Fig. 11).

When PAM-1 was expressed in hEK-293 cells, a kidney fibroblast cell line which lacks a regulated secretory pathway, no endoproteolytic cleavage occurred within exon A to generate soluble monofunctional PHM (Tausk et al., 1993). The complete absence of this cleavage in cells lacking the regulated secretory pathway, as well as our ability to stimulate the secretion of soluble monofunctional PHM from AtT-20 cells expressing PAM-1 (Milgram et al., 1992 and Fig. 10, this paper), suggest that the enzyme(s) responsible for generating monofunctional PHM are localized within regulated secretory granules. Thus, sorting of PAM-1 proteins into the regulated secretory pathway can be assessed by the generation of monofunctional PHM and by the demonstration of stimulus-induced secretion of PAM proteins.

Based on these two criteria, almost all of the full length PAM-1 protein was sorted to the regulated secretory pathway; after 6 h of chase most of the PAM-1 precursor had been processed to monofunctional PHM and PAL and secretagogues stimulated the secretion of these proteins (Figs. 8-10). Some of the PAM-1 protein reaches the plasma membrane intact since bifunctional PAM-1 was immunoprecipitated from the surface of [35S]methionine-labeled cells following a 2-h chase in basal medium (Fig. 8 B). Internalization and targeting of the full length integral membrane PAM-1 to secretory granules may be necessary for complete processing of the full length PAM-1 into monofunctional PHM and PAL proteins.

Approximately one third of the PAM-1/899 was processed to generate monofunctional PHM and PAL (Figs. 8-10); the secretion of monofunctional PHM was stimulated by secretagogues, indicating that some of the PAM-1/899 protein was sorted into the regulated pathway. However, the predominant PAM protein generated in cells expressing PAM-1/899 was bifunctional 105-107-kD PAM (Fig. 8). Newly synthesized bifunctional PAM was first visualized in conditioned medium from PAM-1/899 cells within 1 h after synthesis and ~50% of the newly synthesized bifunctional PAM-1/899 protein was released within 2 h of chase (Fig. 9); the inability of secretagogues to stimulate the secretion of these proteins suggests that they are not routed to the regulated secretory pathway (Fig. 10). The appearance of bifunctional PAM proteins in conditioned medium follows shortly after the incorporation of sialic acid into N- and O-linked oligosaccharides in PAM (not shown) indicating that these proteins are not stored in AtT-20 cells. Therefore, we conclude that the COOH-terminal domain of PAM contains information important for the trafficking of this protein within the secretory pathway.

When expressed in AtT-20 cells, the α-granule protein P-selectin, a plasma membrane receptor for monocytes and neutrophils whose topology is identical to that of PAM, was sorted into storage granules which also contained peptide hormone (Disdier et al., 1992; Koedam et al., 1992). Removal of the COOH-terminal domain of P-selectin resulted in its accumulation at the plasma membrane and transfer of the COOH-terminal of P-selectin to a plasma membrane protein redirected the fusion protein to granules (Disdier et al., 1992). In contrast to PAM, which was localized in endosomes when expressed in hEK-293 and CHO cells, expression of P-selectin in COS and CHO cells led to its presence on the plasma membrane (Disdier et al., 1992; Koedam et al., 1992; Tausk et al., 1993). While P-selectin and PAM share no sequence homology, both proteins contain targeting information for the regulated secretory pathway within their COOH-terminal domains.

One hypothesis to explain the incomplete routing of truncated PAM into the regulated secretory pathway is that a COOH-terminal signal functions to increase the efficiency of targeting via intraluminal interactions. Based on this model, some truncated PAM would be targeted to dense-core granules for storage while some truncated PAM would reach the surface in constitutive vesicles and be rapidly released following cleavage from the cell surface. Alternatively, all of the integral membrane PAM might be routed into immature secretory granules regardless of its COOH-terminal sequence. A signal within the COOH-terminal domain might then be required for the retention of integral membrane PAM in granules during granule maturation. Based on this model truncated PAM proteins would reach the cell surface in the immature secretory granules which are responsible for the basal or constitutive-like secretion observed in endocrine cells (Matsubuchi and Kelly, 1991; Kulliawat and Arvan, 1992). In support of this model is our observation that monofunctional PHM is secreted from PAM-1/899 cells with different kinetics than was observed in cells expressing PAM-1 (Fig. 9). A comparison of the secretion of peptide hormone and PAM may indicate which of these two models is more appropriate.

Based on these data, the COOH-terminal domain of integral membrane PAM may contain two signals: one signal mediates the maturation of PAM into the regulated secretory pathway while another mediates the internalization of PAM after exocytosis. Alternatively, PAM may reach secretory granules via the plasma membrane as has been observed for synaptophysin routing to synaptic-like microvesicles in PC12 cells (Regnier-Vigouroux et al., 1991) and lysosomal associated protein routing to lysosomes (Braun et al., 1989). AtT-20 cells expressing COOH-terminal domain mutants should serve as a useful model for defining the rules governing the trafficking of PAM in neuroendocrine cells. It will be interesting to determine whether there is a relationship between the endosomal and exocytic pathways involved in the trafficking of secretory granule-associated integral membrane proteins.

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