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REVIEW

Peptidylglycine α-amidating monooxygenase: A multifunctional protein with catalytic, processing, and routing domains

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Abstract

Peptide α-amidation is a widespread, often essential posttranslational modification shared by many bioactive peptides and accomplished by the products of a single gene encoding a multifunctional protein, peptidylglycine α-amidating monooxygenase (PAM). PAM has two catalytic domains that work sequentially to produce the final α-amidated product peptide. Tissue-specific alternative splicing can generate forms of PAM retaining or lacking a domain required for the posttranslational separation of the two catalytic activities by endoproteases found in neuroendocrine tissue. Tissue-specific alternative splicing also governs the presence of a transmembrane domain and generation of integral membrane or soluble forms of PAM. The COOH-terminal domain of the integral membrane PAM proteins contains routing information essential for the retrieval of PAM from the surface of endocrine and nonendocrine cells. Tissue-specific endoproteolytic processing can generate soluble PAM proteins from integral membrane precursors. Soluble PAM proteins are rapidly secreted from stably transfected nonneuroendocrine cells but are stored in the regulated secretory granules characteristic of neurons and endocrine cells.

Keywords: hormones; neuroendocrine cells; peptides; peptidylglycine α-amidating monooxygenase

Biologically active peptides are important as hormones for signalling at great distances in the body and as neurotransmitters and paracrine agents for signalling to nearby cells. Most bioactive peptides are synthesized from large, inactive precursors by a set of co- and posttranslational modifications including signal peptide cleavage, disulfide bond formation, and the addition of *N*- and *O*-linked oligosaccharide chains (Mains et al., 1990; Dickerson & Noel, 1991; Jung & Scheller, 1991; Steiner, 1991; Eipper et al., 1992b). The propeptides are cleaved by specific endopeptidases, often at pairs of basic amino acid residues and occasionally at single arginine residues. Several mammalian candidate endoproteases structurally related to bacterial subtilisins and yeast *Kex2*p have recently been cloned, and other proteases are under study (Barr, 1991; Devi, 1991; Lindberg, 1991; Nakayama et al., 1991; Steiner, 1991; Bloomquist & Mains, 1992; Seidah & Chretien, 1992). After endoproteolysis, basic residues are removed by carboxypeptidase H (EC 3.4.17.10; also carboxypeptidase E; Fricker, 1991).

For over half of these bioactive peptides, formation of a COOH-terminal α-amide group is required to complete the biosynthesis of active peptide. The α-amidation reaction is performed by peptidylglycine α-amidating monooxygenase (PAM; EC 1.14.17.3), a bifunctional enzyme catalyzing the conversion of peptidylglycine substrates into α-amidated products. Unlike the endoproteases mentioned above, peptide α-amidation generally appears to be carried out by the products of a single gene.

Peptide α-amidation — A widespread modification

Peptides terminating with an α-amide are found widely in vertebrates, invertebrates, and even in plants; examples include substance P, neuropeptide Y, thyrotropin and gonadotropin releasing hormones, oxytocin and vasopres-

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sin, cholecystokinin and gastrin, calcitonin, many of the snail conotoxins, locust adipokinetic hormone, *Aplysia* egg-laying hormone, and pyro-Glu-Tyr-Pro-NH_2 from alfalfa (Lackey, 1992; reviewed in Eipper et al., 1992b). The α-amides of neutral amino acids predominate, but cDNAs encoding peptides predicted to terminate with the α-amides of all 20 amino acids have been identified. It is often speculated that the key role of α-amidation is prevention of the ionization of the COOH-terminus of the peptide, rendering it more hydrophobic and thus better able to bind to its receptor. For nearly all α-amidated peptides, the α-amide group is required for full biological activity.

Current data indicate that all peptide α-amidation is catalyzed by a single bifunctional enzyme, which requires copper ions and uses molecular oxygen and reduced ascoracid as substrates along with peptidylglycine. These are the same cofactor requirements exhibited by dopamine β-monooxygenase (DβM, EC 1.14.17.1), the enzyme that converts dopamine to norepinephrine during catecholamine biosynthesis (Stewart & Klinman, 1991). Both in vivo and in vitro it has been demonstrated that copper chelators or ascorbic acid deprivation result in a decrease in peptide α-amidation (Hilsted, 1990a,b; Marchand et al., 1990; Singh et al., 1990; Mueller et al., 1991; Eipper et al., 1992b). Although expression of PAM is high in many neurons and endocrine cells, PAM is also expressed at high levels in atrial myocytes and at lower levels in brain ependymal cells and astrocytes (Rhodes et al., 1991; Klein & Fricker, 1992; Maltese & Eipper, 1992; Schafer et al., 1992). Consistent with the presence of PAM in several nonneuroendocrine cell lines (Eipper et al., 1992a), expression of proneuropeptide Y-Gly-Lys-Arg in Chinese hamster ovary (CHO) cells resulted in the secretion of α-amidated neuropeptide Y (Johansen et al., 1991). The presence of PAM in unexpected places may point to a novel role for known or new peptides or to additional functions for PAM.

The close coupling of levels of PAM enzyme activity and peptide α-amidation indicates that this step can be a rate-limiting one in the production of bioactive peptides. Raising or lowering the level of PAM protein by transfection of sense or antisense PAM vectors results in a corresponding increase or decrease in peptide α-amidation (Mains et al., 1991). As might be expected for a rate-limiting enzyme, the levels of PAM activity in different neuroendocrine tissues are regulated in a tissue-specific manner, often in parallel with the propeptide substrate (Grino et al., 1990; Bloomquist et al., 1991; reviewed in Eipper et al., 1992b).

The peptide α-amidation reaction

Figure 1 outlines the two-step reaction involved in the production of most α-amidated peptides. The peptidylglycine substrate is generally the end product of the actions of PC

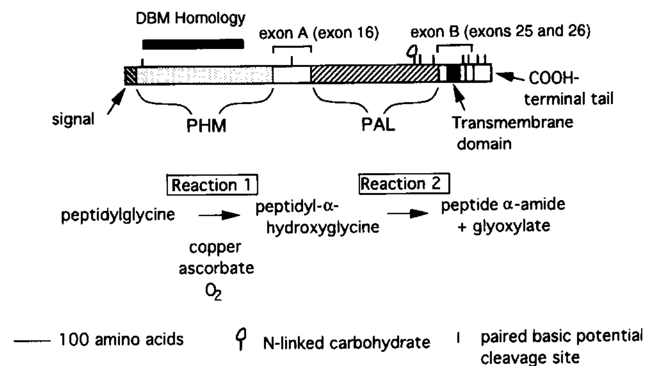

Fig. 1. The two-step α-amidation reaction and the bifunctional PAM enzyme. **Upper:** Key features of the largest rat PAM protein are shown; alternative splicing generates PAM proteins lacking exons A and B. **Lower:** The two steps of the reaction are indicated. PHM has also been called peptidylglycine hydroxylase (Bradbury et al., 1990); PAL has also been called hydroxyglycine amidating dealkylase (HGAD; Katopodis et al., 1990), peptidylamidoglycolate lyase (PGL; Katopodis et al., 1991), and peptidylhydroxyglycine N-C lyase (PHL; Iwasaki et al., 1991).

endoproteases and carboxypeptidase H. The enzyme catalyzing Reaction 1 is peptidylglycine α-hydroxylating monooxygenase (PHM); the first step is rate limiting and is the one requiring copper, molecular oxygen, and ascorbate (Eipper et al., 1992b). The peptidyl-α-hydroxyglycine intermediate is actually quite stable at the pH found in secretory granules (pH ~5), where the bulk of the reaction occurs (Young & Tamburini, 1989; Tajima et al., 1990; Takahashi et al., 1990). Reaction 2 is catalyzed by peptidyl-α-hydroxyglycine α-amidating lyase (PAL). This step proceeds spontaneously at alkaline pH, a fortuitous simplification in the enzyme assay that contributed to the successful purification of PHM by several groups. PAL was identified as an activity that enabled the PHM reaction to produce a fully α-amidated peptide at pH 5 (Perkins et al., 1990; Takahashi et al., 1990; Eipper et al., 1991) and as the enzyme converting the stable reaction intermediate into an α-amidated product (Katopodis et al., 1990, 1991; Suzuki et al., 1990).

Although peptide α-amides can be formed through other reactions in vitro (Reddy et al., 1990; Ranganathan & Saini, 1991; Henriksen et al., 1992), the two α-amidated peptides derived from myelin basic protein represent the first examples of α-amidated peptides purified from natural sources yet lacking a potential peptidylglycine precursor (Takamatsu & Tatemoto, 1992).

In their initial description of peptide amidation, Bradbury et al. (1982) demonstrated that the nitrogen in the peptide-NH_2 came from the Gly residue in the original peptidylglycine substrate and that glyoxylate was one of the products. One mole of ascorbic acid is consumed for each mole of peptidylglycine converted to product (Murthy et al., 1987). The source of the single oxygen atom in the α-hydroxyglycine intermediate has been identified as

molecular oxygen, making the enzyme a monooxygenase (Zabriskie et al., 1991; Noguchi et al., 1992). If PHM functions in a manner analogous to that described for dopamine β-monooxygenase, copper bound to PHM cycles from Cu^{2+} to Cu^{+} during the reaction. PAL has simpler cofactor requirements than PHM; PAL presumably has a tightly bound divalent cation, since the PAL reaction is abolished by EDTA and is restored by a slight excess of several divalent cations (Eipper et al., 1991). Although much work remains to be done to elucidate the actual reaction mechanism, it is clear that the stereochemistry of the two enzymes is matched and useful mechanism-based inhibitors are being designed (Bradbury et al., 1990; Ping et al., 1992; Zabriskie et al., 1992).

The PAM precursor proteins

Antibodies to PHM and partial amino acid sequence data for PHM led to its cloning from bovine, frog, rat, and human sources (reviewed in Eipper et al., 1992b). The structure of the bifunctional protein precursor for PHM and PAL is similar in all species and is shown at the top of Figure 1. The PAM protein has an NH_2-terminal signal sequence, followed by a short proregion predicted by the cDNA sequence but absent from the PHM proteins purified from bovine pituitary and frog skin. The PHM domain has significant amino acid sequence homology to DβM, suggesting an evolutionary relationship of these two enzymes catalyzing similar reactions in different biosynthetic pathways (Southan & Kruse, 1989). The PAL domain is located COOH-terminal to the PHM domain and is separated from PHM by a well-conserved segment called exon A. Naturally occurring co-expression of the two catalytic activities, PHM and PAL, contributed to the initial realization that both might be derived from a common precursor. The PAL domain contains the single site for *N*-glycosylation in mammalian PAM and is followed by a transmembrane domain and a short COOH-terminal region.

In the rat, alternative RNA splicing generates mRNAs encoding at least seven forms of PAM protein. The major splicing events result in deletion of exon A (exon 16) and deletion of exon B (exon 25, which includes the transmembrane domain, and exon 26) (Fig. 2). Minor mRNAs encode soluble PHM proteins (Fig. 2). Soluble PHM and PAL proteins can also be generated from membrane precursors by tissue-specific endoproteolytic processing. The soluble PHM and PAL proteins that were purified from bovine neurointermediate pituitary were generated by endoproteolytic cleavage at the paired basic amino acid sites preceding PHM and in exon A and by another cleavage near the transmembrane domain, possibly at a paired basic amino acid site (Eipper et al., 1991; Katopodis et al., 1991). The enzyme purified from rat medullary thyroid carcinoma cells was bifunctional, containing both PHM and PAL (Bertelsen et al., 1990). Endoproteolytic cleavage of the bifunctional PAM protein at pairs of basic amino acids is reminiscent of the endoproteolytic cleavages seen in preprohormone processing; it is not yet clear whether the same enzymes are involved in cleaving both substrates.

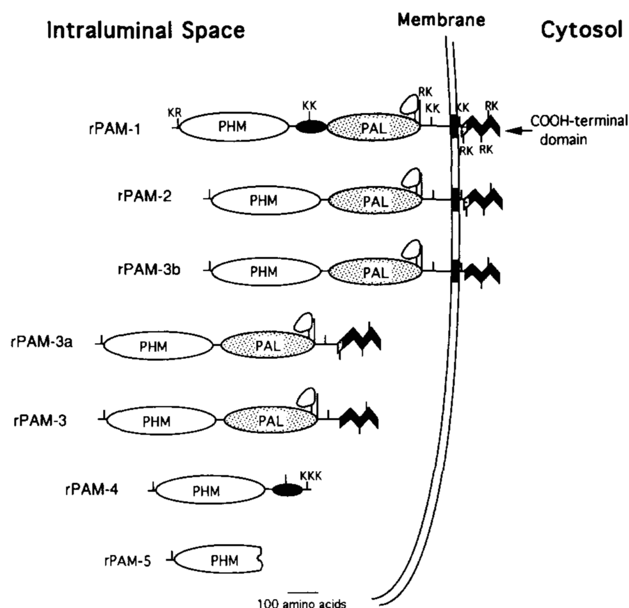

Fig. 2. PAM proteins produced by alternative splicing. The PAM proteins encoded by the seven different PAM cDNAs characterized in our laboratory are drawn to scale. The orientation of each protein with respect to the membranes separating the lumen of the secretory pathway from the cytosol is indicated. Expression of the various mRNAs is tissue specific and developmentally regulated. PAM-4 and PAM-5 have not been identified as major transcripts in any tissues examined to date.

In order to determine whether the putative transmembrane domain indeed functions as predicted and to examine the state of glycosylation of various PAM proteins, cell-free translation of in vitro-transcribed PAM mRNAs was employed (Yun et al., 1993). Cell-free translation of PAM-1, -2, and -3 mRNAs, in the presence and absence of endoplasmic reticulum (ER) membranes, demonstrated directly that the COOH-terminal domain is situated outside the ER membranes when expressed as part of PAM-1 or PAM-2 and inside the lumen of the ER when expressed as part of PAM-3. The topological switching of the COOH-terminal domain of PAM has important consequences for the intracellular routing of PAM, since the COOH-terminal domain is essential for the retrieval of membrane-bound forms of PAM from the cell surface (see below). Topological switching also means that potential phosphorylation and sulfation sequences plus a PEST (Pro-Glu-Ser-Thr rich) sequence in the COOH-terminal domain will be exposed to very different sets of enzymes, depending on the presence or absence of the transmembrane domain. Cell-free translation also demonstrated that the only *N*-linked glycosylation site utilized is the one shown in the PAL domain in Figures 1 and 2.

What are the consequences of cleaving the PAM precursor into separate enzymes?

For preprohormones, the answer to such a question is easy – most preprohormones are inactive and even many of the biosynthetic intermediates are inactive. Most neuroendocrine tissues express more than one form of PAM mRNA and cleave each PAM precursor into several products, making it difficult to address questions about PAM structure and function (Bertelsen et al., 1990; Eipper et al., 1992b; Milgram et al., 1992; Oyarce & Eipper, 1993). To overcome these problems, individual forms of PAM cDNA were expressed at high levels in stably transfected hEK-293 embryonic kidney cells. These cells lack the endoproteases characteristic of neuroendocrine cells and secrete soluble forms of PAM that have been glycosylated but undergo very little endoproteolytic cleavage (Tausk et al., 1992).

We have used these stable cell lines to determine whether the activity of the two catalytic domains might be altered in the bifunctional protein and whether routing through the cell might be governed by the COOH-terminal domain of the protein. The simplest experiments of this type established that both the PHM and PAL catalytic domains could be separately expressed in a fully active form; there is no need to produce the entire PAM precursor in order to produce active PHM or PAL (Kato et al., 1990a; Eipper et al., 1991).

PAM-3 is the simplest natural form of PAM that has both enzymatic domains and was therefore selected to address questions about the catalytic activity of PHM and PAL functioning separately and as part of a bifunctional protein. PAM-3 lacks both exon A and the transmembrane domain (Fig. 3). Consistent with its lack of a transmembrane domain, PAM-3 is rapidly secreted from transfected hEK-293 cells, and a simple purification scheme was devised to prepare milligram amounts of homogeneous PAM-3 from spent medium (Husten et al., 1992). Purified monofunctional PHM and PAL proteins were also prepared from the spent medium of transfected hEK-293 cells expressing truncated cDNAs encoding each enzyme individually.

Experiments with atrial membranes had demonstrated that the PHM and PAL activity of integral membrane PAM were remarkably resistant to a wide variety of endoproteases and that both enzymes could be released from the membranes in good yield as stable, monofunctional catalytic domains following digestion with trypsin or endoproteinase Lys-C (Husten & Eipper, 1991). Digestion of purified PAM-3 with the same enzymes resulted in the separation of PHM from PAL (Husten et al., 1992). Sequence analysis identified a region immediately following the end of the PHM/DβM homology domain as the site of protease sensitivity; the propeptide and the entire COOH-terminal domain were degraded. Treatment of PAM-3 with endoproteinase Arg-C resulted in degradation of the COOH-terminus and removal of the propeptide, but the PHM and PAL domains were not separated from each other. Digestion of PAM-3 with any of the three endoproteases increased the V_{max}/K_m for the PHM reaction fourfold and shifted its pH optimum from 4.5 to 5.5 without altering the kinetic parameters of PAL. Comparison to the properties of purified monofunctional PHM and PAL indicates that removal of the COOH-terminal domain, not separation of PHM from PAL, is responsible for the enhanced kinetic parameters of PHM. Addition of a peptide corresponding to the COOH-terminal domain to PAM-3 or monofunctional PHM had no effect on PHM activity (E.J. Husten, unpubl.). Thus the increase in PHM activity after protease digestion was the result of removal of the COOH-terminal domain from the other end of the molecule.

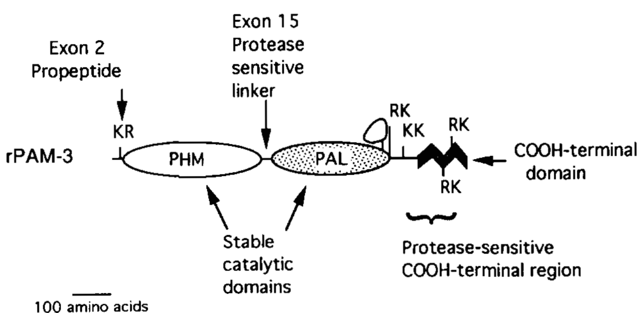

Fig. 3. Structure of the bifunctional PAM-3 protein. Many of the structural features deduced by studying PAM-3 purified to homogeneity from the spent medium of stably transfected hEK-293 cells are summarized. Proteolytic digests of PAM-3 were assayed for PHM and PAL activity and fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Western blot analysis using a battery of antibodies or by NH_2-terminal sequence analysis (Husten et al., 1992).

The structure of the rat PAM gene

Southern blot analyses consistently indicated the presence of a single gene encoding PAM in beef and rat (Kato et al., 1990b; Ouafik et al., 1992); at least two genes for PAM have been identified in *Xenopus laevis* (Iwasaki et al., 1991), a tetraploid species. As shown in Figure 4, the PAM gene consists of at least 27 exons and encompasses over 160 kb of genomic DNA (Ouafik et al., 1992). The PAM gene is unusual in the extraordinarily large size of many of the introns in the PHM region. The 12 exons encoding PHM are of average size, ranging from 49 to 185 bp, but 4 of the introns are over 10 kb long. The lyase domain is more compact, being encoded by only eight exons separated by much smaller introns. The protease-sensitive linker region identified by endoprotease treatment of purified PAM-3 (Fig. 3) corresponds exactly to exon 15, a highly species-specific region immediately following the region homologous to DβM.

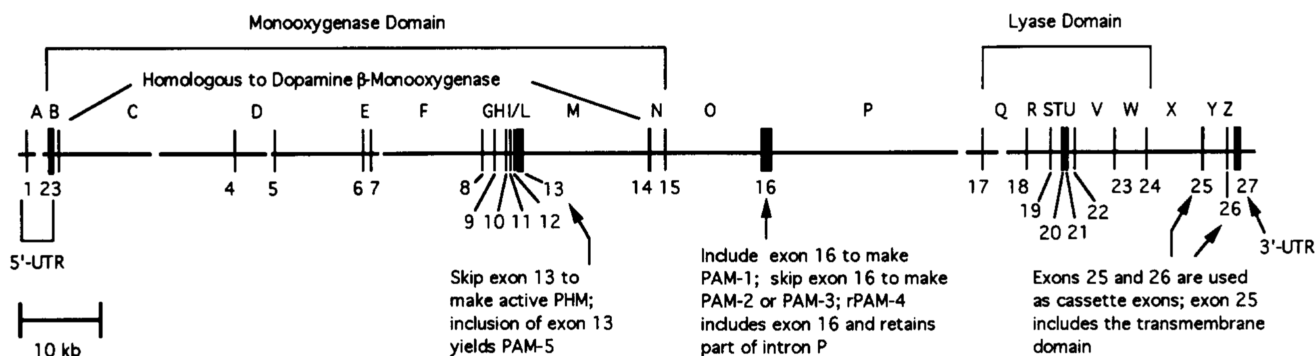

Fig. 4. The exon/intron structure of the gene encoding rat PAM. Exons, indicated by vertical lines, are numbered below the line and are drawn to scale. The size of exon 1 is inferred from the size of the primer extension product (Ouafik et al., 1992). Introns are identified by letter above the line and are drawn to scale with gaps indicated. Exons comprising the monooxygenase and lyase domains are bracketed. The region with homology to DβM is indicated. Sites of alternative splicing are indicated. UTR, untranslated region.

When the exon/intron structures of PHM and DβM are compared, the evolutionary relationship suggested by the similarity in amino acid sequence is confirmed. The introns in PHM are longer than the corresponding introns in DβM, and two exons in PHM often correspond to a single exon in DβM, but the peptide biosynthetic enzyme and the catecholamine biosynthetic enzyme are clearly related (Kobayashi et al., 1989; Southan & Kruse, 1989; Glauder et al., 1990). The gene for human PAM is located on the long arm of chromosome 5 (5q) (Ouafik et al., 1992).

Functionally significant alternative splicing focuses on exon 16 and exons 25 and 26. Exon 13 must be skipped in order to produce an active PHM protein; when exon 13 is included, a short transcript encoding the inactive PAM-5 protein results. Exon 16 (Fig. 4) corresponds to the region originally called exon A (Fig. 1). Inclusion of exon 16 generates the longest PAM protein, PAM-1, while deletion of exon 16 yields PAM-2. The presence of the peptide encoded by exon 16 is essential if neurons and AtT-20 cells are to separate the PHM domain from the PAL domain (Milgram et al., 1992; Oyarce & Eipper, 1993). Although exogenously added endoproteases cleave readily between PHM and PAL in the region encoded by exon 15 (noted in Fig. 3), endogenous biosynthetic endoproteases do not separate PHM from PAL in secretory granules unless exon 16 is present.

Exons 25 and 26 function as cassette exons; because exon 25 includes the transmembrane domain, the presence or absence of this exon determines the topology of the PAM protein in the membrane. Exon 27 encodes the COOH-terminal domain of all bifunctional PAM proteins. When exon 25 is present, the peptide encoded by exon 27 plays a critical role in the localization of integral membrane PAM proteins in both neuroendocrine and nonneuroendocrine cells (see below). When exon 25 is absent, the peptide encoded by exon 27 resides within the lumen of the secretory system.

Predicted and experimentally determined features of the PAM-1 protein have been combined in Figure 5. Exon 15, a region known to be sensitive to exogenous endoproteases, is predicted to form a hydrophilic α-helix. The sites at which endoproteolysis is thought to occur in neuroendocrine cells, exon 16, exon 24, and the part of exon 25 preceding the transmembrane domain are all predicted to form hydrophilic regions rich in α-helix. The protease-sensitive COOH-terminal domain is hydrophilic but not predicted to be rich in α-helix, sheet, or turn structures.

Trafficking of PAM in neuroendocrine and nonneuroendocrine cells

No consensus has been reached on the mechanisms involved in routing soluble proteins into peptide storage granules, and very little work has even addressed the routing of membrane proteins to secretory granules. PAM is one of the few integral membrane proteins associated with peptide-containing secretory granules, and PAM has been used as a tool to investigate the routing of soluble and membrane proteins into peptide-containing granules (Milgram et al., 1992).

The intracellular routing of PAM proteins in nonneuroendocrine cells (hEK-293 human embryonic kidney) and endocrine cells (AtT-20 mouse pituitary corticotropes) was compared. The results are summarized in Figure 6. When soluble forms of PAM are expressed in hEK-293 cells, the proteins are secreted rapidly without endoproteolytic cleavage; even the propeptide remains attached to the NH_2-terminus (E.J. Husten, unpubl.). Similar results were obtained when soluble, truncated PAM proteins were expressed in C127 cells (Beaudry et al., 1990). The kinetics of secretion are consistent with release via the constitutive pathway; soluble PAM proteins are not stored, and newly synthesized soluble PAM proteins are released from hEK-293 cells within a few hours (Tausk et al., 1992).

Fig. 5. PAM protein—Correlation of exon organization with predicted and observed structural and functional domains. **A:** The exons comprising the protein-coding part of PAM-1 are drawn to scale (Ouafik et al., 1992). **B:** A model indicating the functional domains of the PAM-1 protein; hydrophobic domains (signal and TMD [transmembrane domain]) are indicated by filled rectangles. The two protease-resistant catalytic domains are indicated by ovoids. Regions sensitive to proteolytic attack either in vivo or in a test tube are indicated by a coil. Vertical tic marks, pairs of basic amino acids; irregular closed curve, *N*-glycosylation site that is utilized; dot, cysteine residue. **C:** Secondary structure profile predictions (Yun et al., 1993). Regions predicted to be rich in α-helix, β-pleated sheet and turns are shown. The protease-sensitive regions between PHM and PAL and between PAL and the transmembrane domain are rich in α-helix.

By contrast, when PAM-3 is expressed in AtT-20 cells, much of the PAM-3 protein enters regulated secretory granules, is subjected to endoproteolytic cleavage, and is stored along with the peptide hormone for regulated or stimulated secretion at a later time (Milgram et al., 1992). Because soluble monofunctional PHM and PAL proteins expressed in AtT-20 cells both exhibit efficient routing to regulated secretory granules, a single routing domain cannot be crucial to the localization of PAM to regulated granules.

Fig. 6. Routing of integral membrane forms of PAM in endocrine and nonneuroendocrine cells. Data obtained by examining stably transfected hEK-293 and AtT-20 cells are summarized; hEK-293 cells lack the regulated secretory granules shown to the right. The catalytic domains of integral membrane PAM are indicated by rectangles; the cytosolic COOH-terminal domain is represented by a triangle. The proteolytic cleavage of integral membrane PAM proteins in AtT-20 cells is depicted by the appearance of monofunctional PHM and PAL proteins in the regulated secretory granules. PAM/antibody internalization experiments revealed the existence of the endocytic pathway. Lysosomes are not thought to represent a major destination for PAM following internalization.

When integral membrane forms of PAM are expressed in hEK-293 cells and AtT-20 cells, different patterns are again observed in the two cell types (Tausk et al., 1992; Milgram et al., 1993). As expected, hEK-293 cells do not store smaller proteins derived from integral membrane PAM. However, integral membrane PAM proteins do not accumulate on the surface of transfected hEK-293 cells, as would be expected for membrane proteins lacking routing information. Instead, integral membrane PAM proteins accumulate in hEK-293 cells in a perinuclear position coincident with markers for the Golgi apparatus. Enzyme assays carried out on live cells indicate that at steady state a small amount of PAM protein is exposed on the cell surface. Upon incubation with antisera to PHM or PAL at 37 °C, hEK-293 cells expressing integral membrane forms of PAM rapidly internalize the PAM/antibody complex, suggesting that much of the PAM protein observed in a perinuclear position resides in endosomes. The stability of integral membrane PAM proteins expressed in hEK-293 cells argues against their rapid degradation in lysosomes. The sizes of the released bifunctional PAM proteins suggest that endoproteolytic cleavage occurs quite close to the transmembrane domain (Tausk et al., 1992).

When PAM-1 is expressed in AtT-20 cells, an endoproteolytic cleavage separates PHM from PAL so that solu-

ble monofunctional PHM is stored in regulated secretory granules. There is also a slow cleavage of the PAL domain from the membrane, but much of the PAL remains membrane bound (Milgram et al., 1992). AtT-20 cells expressing PAM-1 show a major accumulation of PAL in a perinuclear location and express a small amount of enzymatically active protein on the cell surface. AtT-20 cells expressing integral membrane forms of PAM exhibit rapid internalization of PAM/antibody complex from the cell surface followed by accumulation of the complex in endosomes (Fig. 6) (Milgram et al., 1993). Based on immunostaining and stability, little of the internalized PAM protein goes directly to lysosomes. It is quite possible that integral membrane PAM proteins may have repeated access to regulated secretory granules (Milgram et al., 1993).

Since the cytoplasmic tails of a wide variety of integral membrane proteins contain signals governing routing and endocytosis, mutant forms of PAM were constructed in which various portions of the COOH-terminal domain were deleted (Tausk et al., 1992; Milgram et al., 1993). The 18 amino acids in exon 26 are removed by alternate RNA splicing in one naturally occurring form of PAM, but deletion of these 18 residues from the COOH-terminal domain had no effect on routing or internalization from the surface. When most of the COOH-terminal domain (exons 26 plus 27) was deleted, however, a very different picture emerged. In both endocrine and nonneuroendocrine cells, integral membrane forms of PAM retaining only nine amino acids of the COOH-terminal domain proceeded rapidly to the cell surface. Only 20–40% of the truncated integral membrane PAM protein found its way into regulated secretory granules in AtT-20 cells (Milgram et al., 1993). In both cell types, a large fraction of the COOH-terminally truncated PAM protein was found on the cell surface at any one time, and the rate of release of PAM from the cells was greatly increased compared to the rate of release of PAM from cells expressing wild-type integral membrane PAM. Thus rapid endocytosis of PAM, which occurs in neuroendocrine and nonneuroendocrine cells, requires the presence of an intact COOH-terminal domain in the cytosol. AtT-20 cells expressing truncated integral membrane PAM proteins exhibit additional alterations in routing.

Prospects for the future

Comparison of the posttranslational modifications affecting soluble and integral membrane forms of PAM in endocrine and nonneuroendocrine cells should identify functionally important modifications. Purification of full-length integral membrane PAM and its reconstitution into membranes should make it possible to determine whether the enzyme functions differently when in the membrane. Identification of targetting signals in the COOH-terminal domain of full-length PAM should facilitate identification of the cytosolic proteins that interact with this domain and offer significant insight into the routing of integral membrane proteins associated with secretory granules.

Acknowledgments

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