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Original Article

Distribution of Peptidyl-Glycine α-Amidating Mono-oxygenase (PAM) Enzymes in Normal Human Lung and in Lung Epithelial Tumors[1]

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C-terminal α-amidation is a post-translational modification necessary for the biological activity of many regulatory peptides produced in the respiratory tract. This modification is a two-step process catalyzed by two separate enzyme activities, both derived from the peptidyl-glycine α-amidating mono-oxygenase (PAM) precursor. The distribution of these two enzymes, peptidyl-glycine α-hydroxylating mono-oxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL), was studied in the normal lung and in lung tumors using immunocytochemical methods and in situ hybridization. In normal lung the enzymes were located in some cells of the airway epithelium and glands, the endothelium of blood vessels, some chondrocytes of the bronchial cartilage, the alveolar macrophages, smooth muscle cells, neurons of the intrinsic ganglia, and in myelinated nerves. A total of 24 lung tumors of seven different histological types were studied. All cases contained PAM-immunoreactive cells with various patterns of distribution. All immunoreactive cells were positive for the PHM antiserum but only some of them for the PAL antiserum. The distribution of PAM co-localizes with some other previously described amidated peptides, suggesting that amidation is an important physiological process taking place in the normal and malignant human lung tissue. (*J Histochem Cytochem 44:3–12, 1996*)
KEY WORDS: Human lung; Lung tumors; Amidation; PAM; PHM; PAL.

Introduction

Peptide hormone production is a multistep process involving synthesis of large, biologically inert prohormones that acquire activity through a specific sequence of post-translational modifications. For production of α-amidated peptides, the first step consists of an endoproteolytic cleavage at basic amino acids that define the amino- and carboxy-terminal ends of the mature peptide sequence, carried out by a member of the subtilisin-like serine endoprotease family (Barr, 1991). The second step is exoproteolytic cleavage at the carboxy terminus to remove basic residues and expose a glycine that is the substrate for the subsequent amidating process. This cleavage step is believed to be catalyzed by carboxypeptidase E (Fricker, 1988). The final step is the amidation reaction, in which the terminal glycine residue is cleaved to form the amide of the C-terminal amino acid. This α-amidation is a two-step process catalyzed by two separable enzymatic activities, both derived from the peptidyl-glycine α-amidating mono-oxygenase (PAM) precursor, a 108-KD polypeptide (Milgram et al., 1992; Glauder et al., 1990; Stoffers et al., 1990). The amino-terminal third of the PAM precursor contains the first enzyme, peptidylglycine α-hydroxylating mono-oxygenase (PHM) that catalyzes the conversion of glycine-extended peptides into peptidyl-α-hydroxyglycine intermediates. The second enzyme, peptidyl-α-hydroxyglycine α-amidating lyase (PAL) is contained in the middle third of the PAM precursor. The COOH-terminal third of the molecule encodes a transmembrane domain and a hydrophilic cytoplasmic tail (Figure 1) (Eipper et al., 1993; Milgram et al., 1993).

Despite the suggested relevance of the amidating process in lung cancer (Quinn et al., 1991) and the finding of the corresponding enzymes in cultured lung tumor cells (Treston et al., 1993) the ex-

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pression of PAM in normal lung and in lung tumors has not been studied. It has been suggested that PAM may be a target for early lung tumor detection, and that it may be possible to neutralize the mitogenic activity of some peptides produced by the tumor cells by interfering with their post-translational processing mechanisms (Mulshine et al., 1993; Treston et al., 1992). In fact, a recent study demonstrated that anti-sense expression of PAM in tumor cell lines very efficiently inhibits tumor growth (Martínez et al., 1995a).

The main aim of the present investigation was to study: (a) the distribution of PAM enzymes in normal human lung; (b) whether they are present in tumor cells derived from different epithelial cell types of the lung; and (c) the distribution of the mRNA of PAM in these tissues.

Materials and Methods

Human lungs were dissected into three parts: hilus, medial lung, and peripheral lung. Lung epithelial tumors were classified as shown in Table 1, together with the sex and the age of the patient. Tumors with endocrine differentiation were classified according to the criteria of Gosney (1992). All of the specimens were obtained at surgery or from autopsy cases from the University Clinic of Navarra. Normal lungs were obtained from trauma accident patients. The lung from a 20-week-old fetus, product of a natural miscarriage, was used to investigate neuroendocrine cells. Autopsy specimens were collected within 5–10 hr postmortem. All samples were fixed for 8–12 hr in 10% formalin or 4% paraformaldehyde in phosphate buffer and were paraffin-embedded.

Immunocytochemistry. Sections (4 μm thick) were mounted on glass slides. After dewaxing, immunocytochemical staining was performed by the avidin–biotin method (Hsu et al., 1981). To prevent nonspecific background the sections were pre-treated with 3% H_2O_2 in methanol for 30 min and with normal swine serum (NP1, 1:20; University of Navarra) for 30 min, and were incubated overnight at 4°C with the primary antisera.

The antisera were raised against synthetic fragments of the predicted sequence of human PAM; CC (PAM 288–310 with a Val for Ala conservative substitution at position 11) and PAL2 (PAM 527–546) (Martínez et al., 1993a,b) (Figure 1). The antisera have been shown to specifically immunoprecipitate PAM from transfected cells (Tateishi et al., 1994). To show neuroendocrine features, antisera to PGP 9.5 and GRP (Department of Histochemistry, Hammersmith Hospital; antisera nos. 1648 and 593, respectively) were used.

The sections were incubated with biotinylated swine anti-rabbit immunoglobulin (K353; Dakopatts, Glostrup, Denmark) and then with avidin–biotin peroxidase complex (K355; Dakopatts). The bound antibodies were visualized with 3–3′-diaminobenzidine tetrahydrochloride (D-5637; Sigma, St Louis, MO) with nickel enhancement (Shu et al., 1988).

Table 1. *Characteristics of the cases used in this study and immunocytochemical and in situ hybridization (ISH) results*

Case	Type of tumor	Age/Sex	Immunocytochemical results[a] PHM	PAL	ISH
1	Tumorlet	60/M	*	*	***
2	Carcinoid	60/F	***	*	nt
3	Carcinoid	58/M	***	*	***
4	Carcinoid	64/F	***	–	nt
5	Carcinoid	64/M	**	*	nt
6	AC[b]	61/F	***	–	***
7	AC	64/M	***	–	***
8	AC	65/M	***	*	nt
9	AC	78/F	***	–	***
10	Squamous	70/M	***	*	nt
11	Squamous	72/M	***	*/–	nt
12	Squamous	72/M	*	*/–	nt
13	Squamous	55/M	**	–	***
14	SCLC[c]	70/M	*	–	***
15	SCLC	69/M	*/–	–	***
16	SCLC	59/M	*/–	–	nt
17	SCLC	54/M	*/–	–	nt
18	LCC[d]	62/M	***	**	nt
19	LCC	57/M	***	*	nt
20	LCC	84/F	***	–	***
21	LCC	43/M	**	–	***
22	WDNC[e]	47/M	***	–	***
23	WDNC	53/M	***	***	nt
24	WDNC	73/M	***	**	nt

[a] ***, Large number of positive cells (>60%); **, moderate number (30–60%); *, few immunoreactive cells (<30%); */–, few positive cells, but not in all the sections; –, negative result; nt, not tested.
[b] Adenocarcinoma.
[c] Small-cell lung carcinoma.
[d] Large-cell carcinoma.
[e] Well-differentiated neuroendocrine carcinoma.

Specificity Controls. Antisera to PHM and PAL were pre-incubated with their respective synthetic antigens at a concentration of 1–10 nmol of peptide per ml of optimally diluted antiserum before application to tissue sections.

In Situ Hybridization. Sections 4 μm thick were mounted on slides coated with Vectabond (SP-1800; Vector, Burlingame, CA), dewaxed, and prepared for hybridization with RNA probes as described by Gibson and Polak (1990). A plasmid named hPAM1200 containing 1200 BP of the human PAM gene (Glauder et al., 1990) (Figure 1), kindly donated by Prof. Engels (Frankfurt, Germany), was used to generate riboprobes. Briefly, the

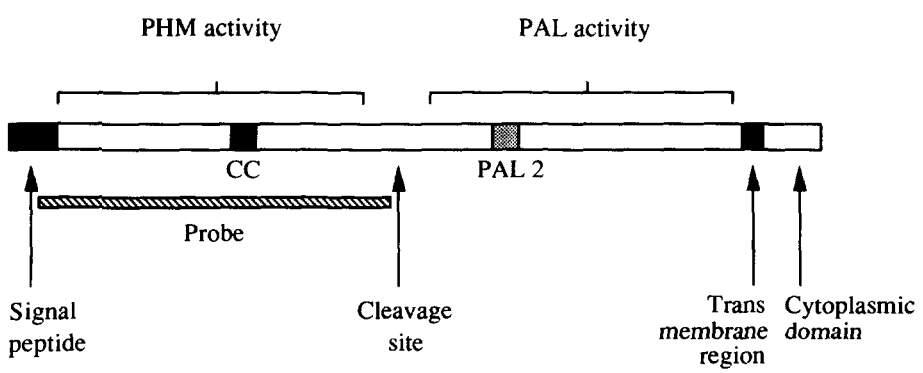
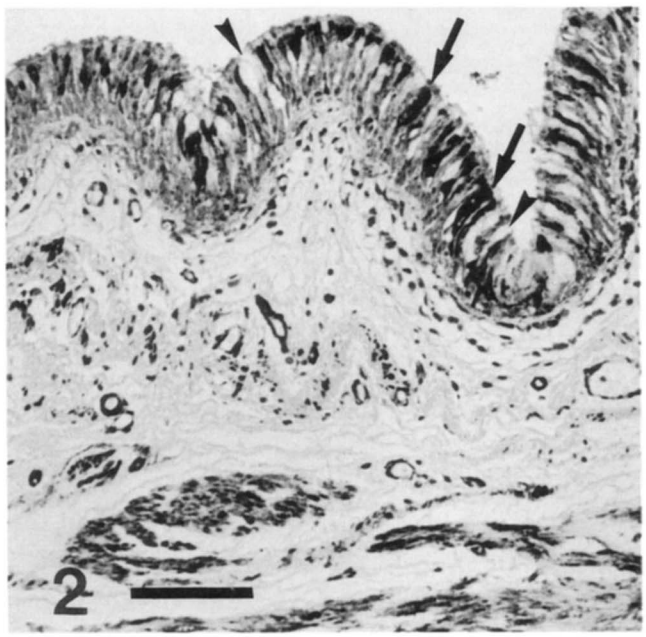
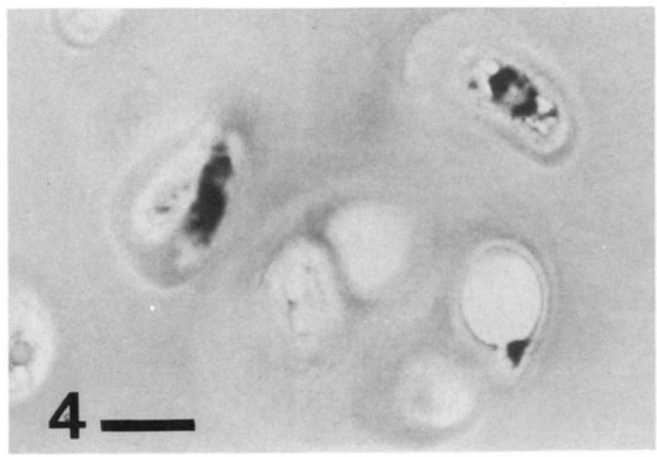
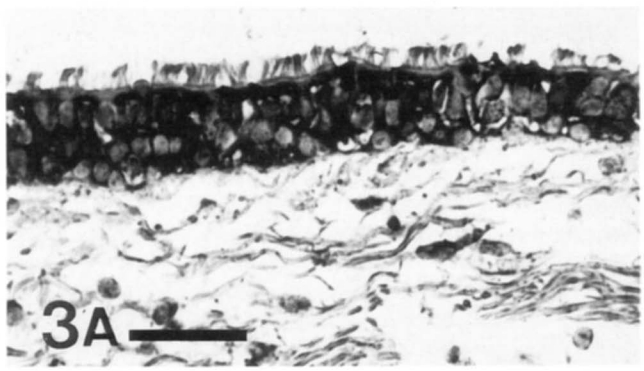
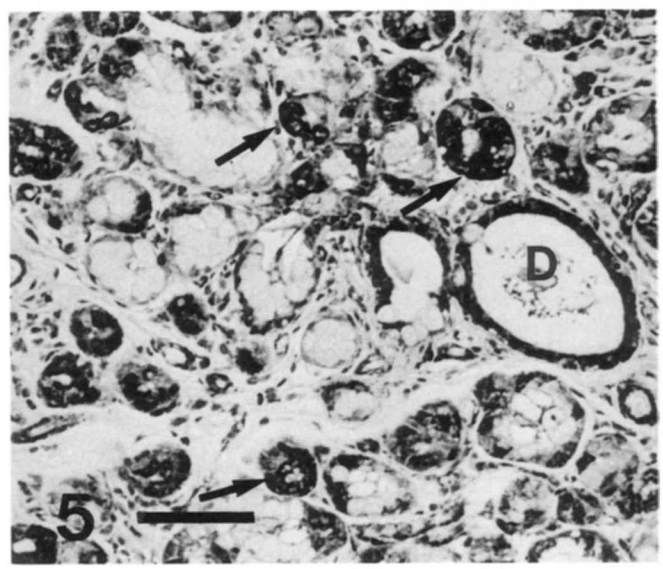


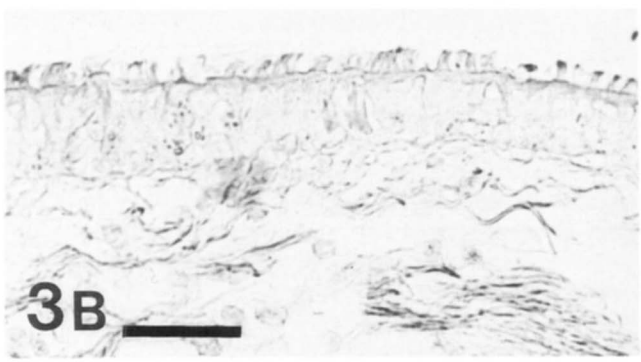
Figure 1. Structure of the PAM pre-pro-enzyme, showing the localization of the two regions (CC and PAL 2) against which antisera were raised and the probe used in this study. The main functional regions of the molecule are labeled.











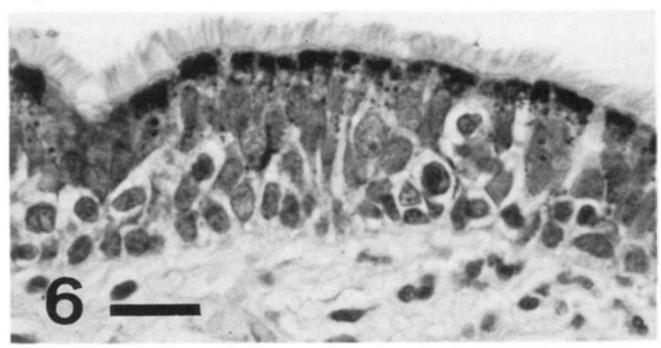


Figure 2. Low-power microphotograph of a bronchial wall showing immunoreactivity to PHM in epithelial ciliated cells (arrows), in blood vessels, and in the smooth muscle cells of the bronchial wall. Mucous cells are negative (arrowheads). Original magnification × 150. Bar = 100 μm.

Figure 3. Localization of PAM mRNA in the bronchial epithelium after applying the hybridization technique with the (**A**) anti-sense and (**B**) sense probes. Original magnification × 375. Bars = 40 μm.

Figure 4. Immunocytochemical localization of PHM in some of the chondrocytes of the bronchial cartilage. Original magnification × 600. Bar = 20 μm.

Figure 5. Distribution of PHM immunoreactivity in the exocrine glands. Positivity is located in the serous (arrows) and duct (D) cells. Original magnification × 150. Bar = 100 μm.

Figure 6. Paraffin section showing cells immunoreactive for anti-PHM in the bronchiolar epithelium. Immunostaining is preferentially found in the apical region of the ciliated cells. Original magnification × 600. Bar = 20 μm.

DNA fragment was subcloned into Bluescript II SK(+) (Stratagene; La Jolla, CA) and linearized with the appropriate restriction enzymes. Labeled probes were prepared with digoxigenin-11-UTP (1277 073; Boehringer, Barcelona, Spain) and T7 (881 767; Boehringer) or T3 RNA polymerases (1031 163; Boehringer) to synthesize sense and anti-sense RNA transcripts, respectively. Hybridization was performed in a moist chamber at 46°C for 20 hr in a 15-µl volume containing 0.5 ng/µl of probe for each section. Stringency washes included treatments with 150 mM NaCl, 15 mM sodium citrate, pH 7.0 (SSC), and sodium dodecyl sulfate (SDS); four washes in 2 × SSC/0.1% SDS; two washes in 0.1 × SSC/0.1%SDS at 46°C; brief rinses in 2 × SSC; incubation in 2 × SSC containing 10 µg/ml RNAse at 37°C for 15 min; and further rinses in 2 × SSC.

Visualization of digoxigenin was performed with a monoclonal antibody coupled to alkaline phosphatase (1093 274; Boehringer) 1:500 acting for 2 hr at room temperature. Nitroblue tetrazolium chloride (N-5514; Sigma) and 5-bromo-4-chloro-3-indolyl-phosphate (B-8503; Sigma) were used as substrates for the alkaline phosphatase. Controls included the use of the sense probe and treatment of the sections with RNAse before the hybridization.

Results

Normal Lung

Immunoreactivity for PHM was widely distributed in several cell types of the intrapulmonary bronchi. The epithelium was heterogeneously stained, some cells intensely so, whereas others were negative (Figure 2). The immunoreactivity was concentrated mainly in the apical region of the ciliated epithelial cells (Figure 2). Goblet (mucous) cells were clearly negative (Figure 2). The in situ hybridization technique confirmed that the immunoreactive cells also were producing PAM mRNA, and showed that PAM mRNA is more homogeneously distributed throughout the cytoplasm of the epithelial cells (Figures 3A and 3B). In the hyaline bronchial cartilage, some chondrocytes were also positive for PHM (Figure 4). The cells of the bronchial exocrine glands showed a heterogeneous staining pattern: only some of the serous secretory cells were immunoreactive for the PAM antibodies (Figure 5). Some ductal cells were also stained (Figure 5). Moderate immunoreactivity was observed in the smooth muscle cells of the bronchial wall (Figure 2).

In bronchioli, the epithelium was strongly immunostained, mainly in the apical region of the ciliated cells (Figure 6); the Clara cells of the terminal bronchioli were also positive (results not shown). Smooth muscle cells were also mildly stained, as in the bronchi. No immunoreactivity or in situ hybridization signal was found in the alveolar epithelium. The study on the 20-week-old fetal lung showed a normal histology for the canalicular phase and confirmed an intriguing fact we had observed before in the adult lung, i.e., the total lack of co-localization between GRP, which is generally considered to be an amidated peptide, and the peptide-amidating enzyme PAM (Figure 7).

Some of the blood vessels expressed the enzymes both in the endothelium (Figures 8, 9A, and 9B) and in some of the smooth muscle cells (Figures 8 and 10). Immunoreactive muscle cells appeared both in arteries and veins. The number of positive cells depended on vessel size, being higher in the smaller vessels. Several neuron cell bodies of the neural ganglia present in the bronchial wall were also stained. The pattern of immunostaining for PHM in nerve trunks and the comparison of serial sections stained respectively with antibodies against PHM and the general neural marker PGP 9.5 support the view that the stained cells within the nerves are mostly Schwann cells (Figure 10). The intraepithelial nerve fibers shown clearly by PGP 9.5 immunoreactivity were not positive when PHM immunocytochemistry was performed. Some alveolar macrophages were stained with PHM with variable intensity (Figures 11A and 11B). In some cases a faint immunoreactivity was associated with the perinuclear cisterna.

The results obtained with the antibody directed against PAL matched those described above for the anti-PHM antibody. In general, the immunostaining with the PAL antibody was fainter. The results obtained with in situ hybridization showed a distribution similar to the immunocytochemistry.

Tumors

Table 1 summarizes the results obtained in the different tumors used in this study. In addition to the immunoreactivity observed in normal structures, as described above, some of the tumor cells were immunoreactive for the PAM antibodies and the same cells were also positive by in situ hybridization.

The single case of carcinoid-type tumorlet studied consisted of some foci of regular cells bordering air spaces. Both PHM and PAL antibodies stained most of the tumorlet cells (Figure 12), which were also positive for PAM mRNA by in situ hybridization.

The four cases of carcinoid tumor studied were well-demarcated, extending into the lumen of the bronchus in a dumbell-like fashion. The tumor cells were arranged in a mosaic pattern or as a combination of cords, nests, trabeculae, and ribbons. They were Grimelius-positive. The peripheral cells of the tumor masses were immunoreactive for both PHM and PAL antibodies (Figure 13). In situ hybridization showed a similar pattern.

Adenocarcinomas showed tubular, acinar, or papillary differentiation. In all four cases, tumor cells were strongly stained with the PHM antiserum but only one of the cases had staining for PAL (Figure 14). All cases analyzed for PAM mRNA were strongly positive.

The squamous tumors studied were centrally located, with tumor cells growing in cords or sheets in which keratin and/or intercellular bridges were detected. Most of the cells, but especially those of the basal layer, showed strong immunoreactivity for the PHM antiserum (Figures 15A and 15B).

Small-cell lung carcinomas consisted of dense, sheet-like masses, composed of cells with a high nuclear/cytoplasmic ratio. Although scattered cells positive for PHM could be found in the inner areas of the tumor, the bulk of the immunoreactivity was located in the periphery (Figure 16A). The reactivity for the PHM antiserum was mild in all the cases (Figure 16A) but none of them was immunoreactive for PAL. The in situ hybridization technique showed a strong reactivity for the PAM mRNA in most of the cells of the two cases studied (Figure 16B).

The large-cell carcinomas were composed of large cells, often arranged in sheets. Most of the neoplastic cells, in two of the four cases studied, were strongly labeled for both PHM and PAL antisera (Figures 17A and 17B), whereas in the other two cases only immunoreactivity for PHM was found.

Three neoplasms were classified as well-differentiated neuroendocrine carcinoma (Gosney, 1992). These tumors gave a strong posi-

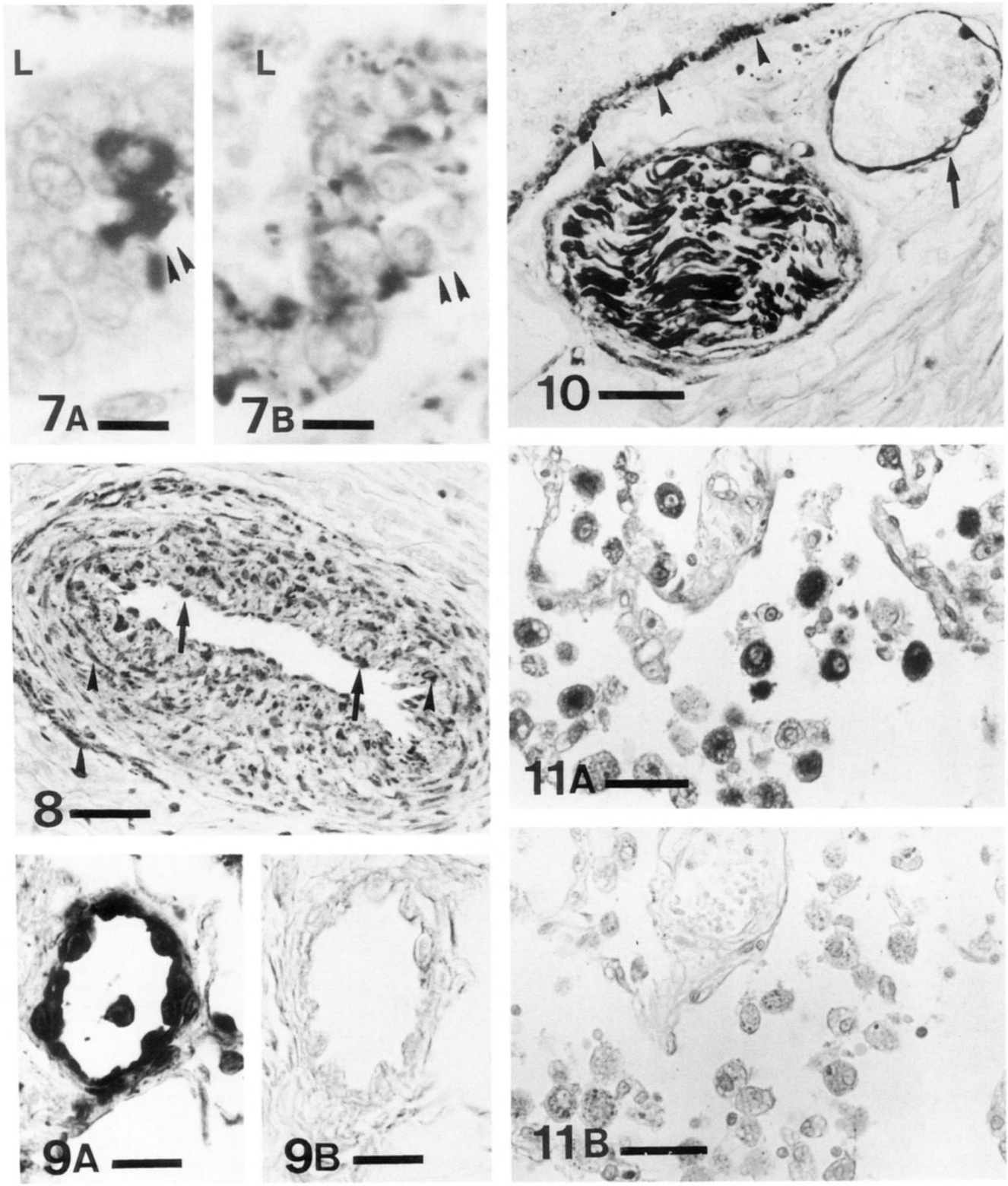


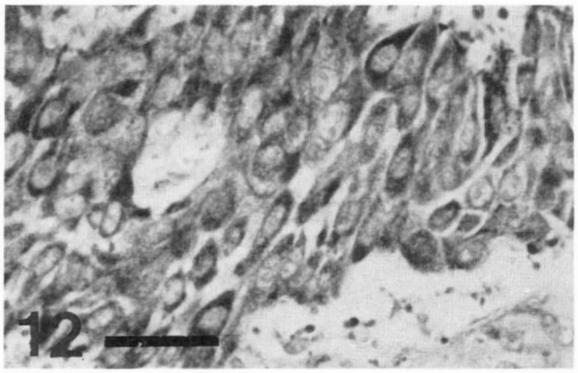
Figure 7. Serial sections of a 20-week-old fetal lung stained with (**A**) anti-GRP and (**B**) anti-PHM. Note the lack of PAM immunoreactivity in the GRP-positive endocrine cells (arrowheads). Original magnification × 600. Bar = 20 μm.

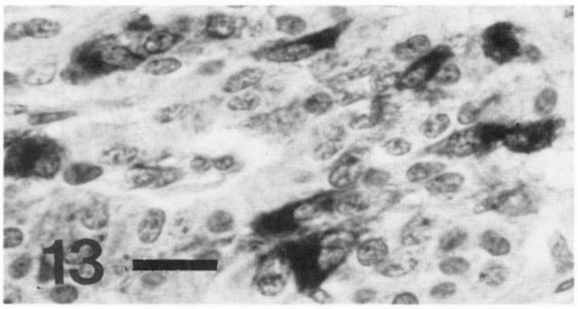
Figure 8. A medium-sized artery showing positive reaction for the PHM antiserum in the endothelium (arrows) and the wall smooth muscle cells (arrowheads). Original magnification × 460. Bar = 30 μm.

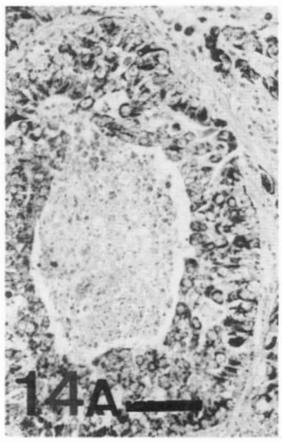
Figure 9. In situ hybridization for PAM in serial sections showing the endothelium of a venule with the (**A**) anti-sense and (**B**) sense probes. Original magnifications × 600. Bars = 20 μm.

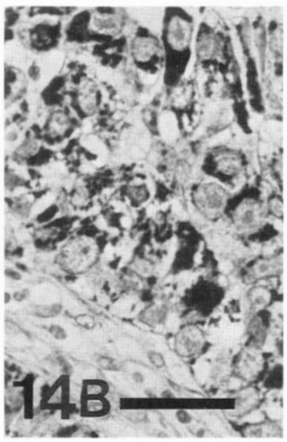
Figure 10. Section of airway wall showing a nerve trunk immunoreactive for PHM. Schwann cells are positive for PHM. Endothelium in a transverse section of a vein (arrow) and smooth muscle cells of a longitudinally sectioned vein (arrowheads) are also stained. Original magnification × 375. Bar = 40 μm.

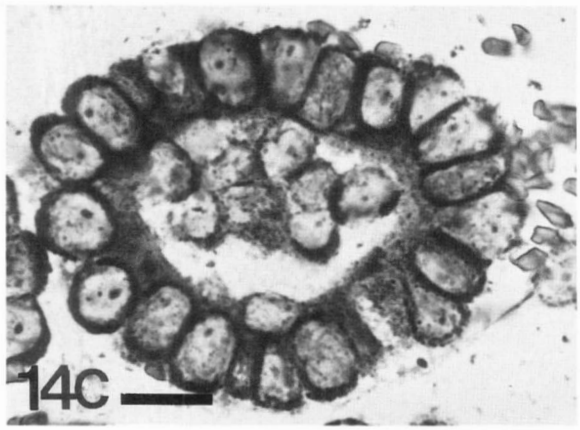
Figure 11. (**A**) Alveolar macrophages immunostained for PHM. (**B**) Pre-absorption of the antiserum with the peptide abolishes staining. Original magnifications × 375. Bars = 40 μm.

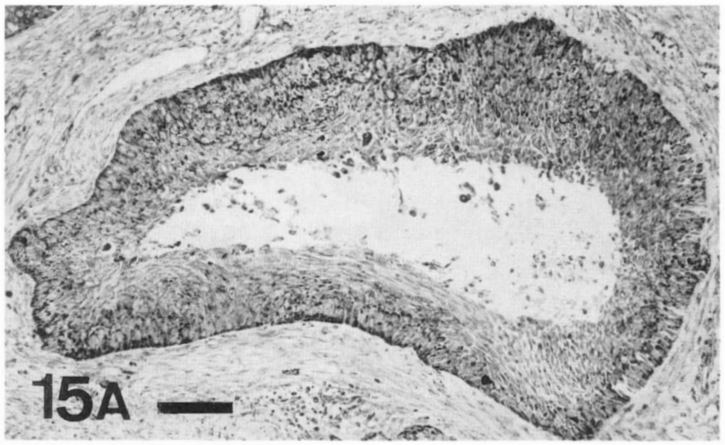


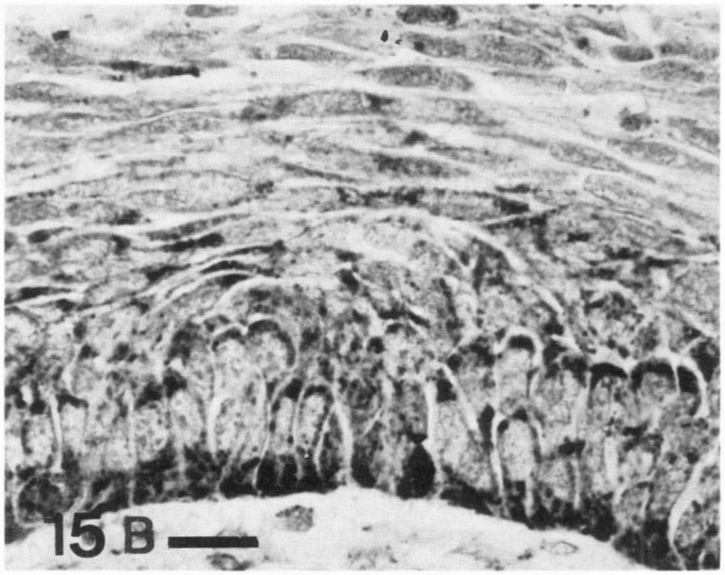


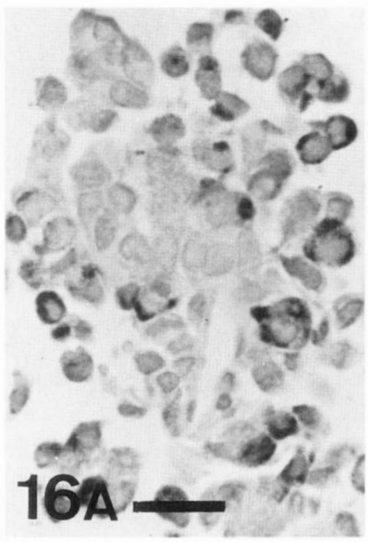












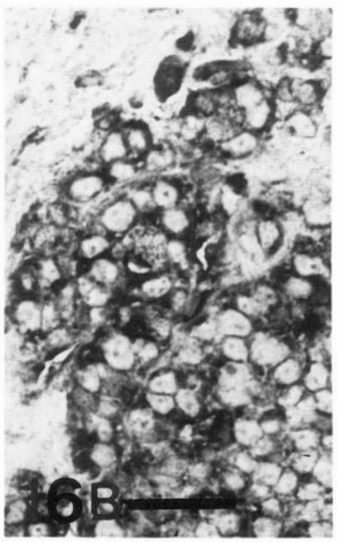


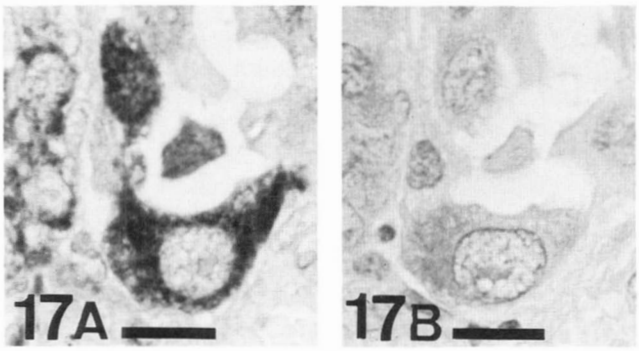
Figure 12. Cells immunoreactive to PHM in a tumorlet. Original magnification × 375. Bar = 40 μm.

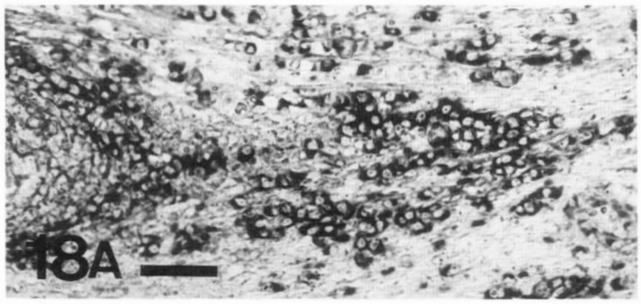
Figure 13. Carcinoid showing immunoreactivity for the PAL antiserum. Original magnification × 600. Bar = 20 μm.

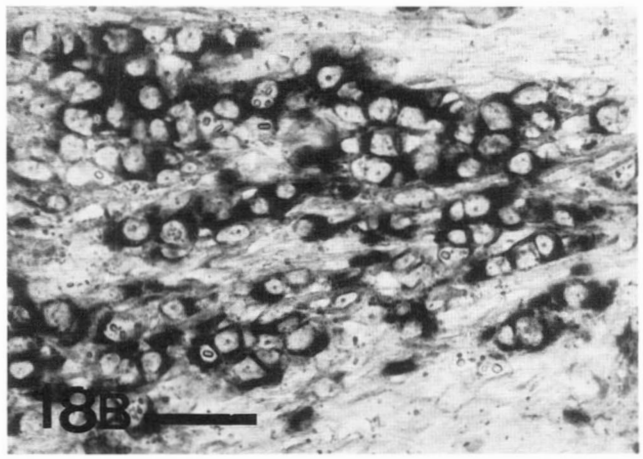
Figure 14. (**A**) Positive cells for the PHM antiserum in an adenocarcinoma. (**B**) Detail of the stained cells. (**C**) The same type of tumor after applying in situ hybridization. Original magnifications: **A** × 95; **B** × 375; **C** × 600. Bars: **A** = 100 μm; **B** = 40 μm; **C** = 20 μm.

Figure 15. (**A**) Cord of a very well-differentiated squamous cell carcinoma stained with PHM. (**B**) Detail of the tumor epithelium. The staining is stronger in the basal region. Original magnifications: **A** × 95; **B** × 600. Bars: **A** = 100 μm; **B** = 20 μm.

Figure 16. (**A**) Cells immunoreactive to PHM antiserum in a small cell lung carcinoma. (**B**) In situ hybridization of the same case. Original magnifications: **A** × 600; **B** × 375. Bars: **A** = 20 μm; **B** = 40 μm.







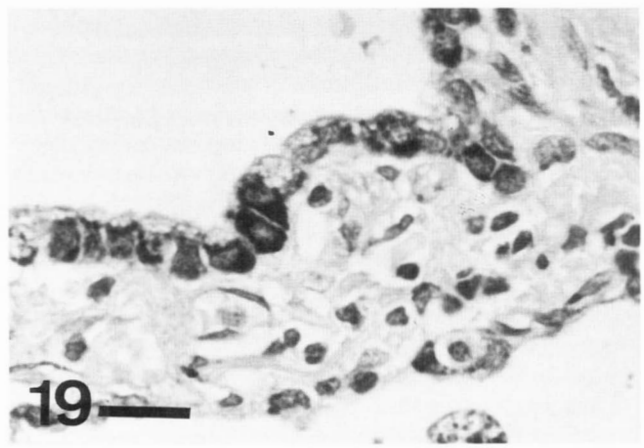


Figure 17. (**A**) Large-cell carcinoma stained with anti-PHM and (**B**) a serial section incubated with the pre-absorbed antiserum. Original magnification × 600. Bars = 20 μm.

tive reaction for both PHM and PAL antisera, and the in situ hybridization for PAM mRNA was also strongly positive (Figures 18A and 18B).

In some of the cases studied, belonging to several classes of tumors, the normal flattened epithelial cells of the alveoli and alveolar ducts adjacent to the tumor masses were replaced by cuboidal cells. This is known as metaplasia of Type II cells (Type II pneumocyte hyperplasia). All of these metaplastic cuboidal cells gave a strong immunoreactivity for PHM and PAL (Figure 19).

Discussion

This study shows the distribution of peptide-amidating enzymes in the human lung, both in normal and in tumor tissue. The techniques used, immunocytochemistry and in situ hybridization, allowed the localization of the PAM enzymes and their messenger RNA. As expected, both techniques demonstrated consistent patterns, specially when immunoreactivity for PHM was compared with in situ hybridization.

In normal lung, several structures, including airway epithelium, exocrine glands, chondrocytes, alveolar macrophages, endothelium of the blood vessels, smooth muscle cells (both of the airways and of the blood vessels), neurons, and Schwann cells were immunoreactive for the PAM enzymes and expressed the corresponding mRNA, as demonstrated by in situ hybridization. Most of these localizations were expected because amidated peptides have been previously reported in these cell types.

The PHM and PAL in non-endocrine epithelial cells of bronchi and bronchioli found in this study could be necessary for post-translational processing of peptides present in the airway epithelium, such as endothelin (Springall et al., 1991b; Giaid et al., 1990) and adrenomedullin (Martínez et al., 1995b). Endothelin itself is not an amidated peptide but the function of the PAM enzymes could be related to the amidation of some small peptides of the endothelin precursor which contain predicted amidation sites. The biological action of these small molecules is as yet unknown (Cuttitta, 1993). Adrenomedullin is a recently described C-terminally-amidated peptide with hypotensive activity (Kitamura et al., 1993). It has been demonstrated that amidation is required for binding to its receptor (Eguchi et al., 1994) and its possible functions in the lung include defense and growth stimulation (Martínez et al., 1995b).

Immunocytochemistry demonstrated that in the airway epithelial cells the PAM enzymes appear to have a predominantly apical distribution, whereas in situ hybridization showed that the mRNA of the proenzyme displays a more homogeneous distribution throughout the cytoplasm. The localization of PAM in the airway

Figure 18. (**A,B**) In situ hybridization for PAM mRNA in a well differentiated endocrine tumor. Original magnifications: **A** × 95; **B** × 375. Bars: **A** = 100 μm; **B** = 40 μm.

Figure 19. Part of the alveolar wall lined by metaplasic cuboidal cells. Strong immunoreactivity for PHM is clearly observed. Original magnification × 600. Bar = 20 μm.

epithelial cells could be related to the processing and secretion of endothelin or adrenomedullin into the airway lumen. Bronchial epithelial cells in culture are known to secrete an endothelin-like material (Mattoli et al., 1990; Black et al., 1989) and endothelin-I has been shown to be present in bronchoalveolar lavage fluid from pig and human lungs (Aarnio et al., 1994; Sofia et al., 1993). Furthermore, PHM and PAL enzymatic activities can be found in human bronchoalveolar lavage fluid (Scott et al., 1993). The possibility of secretion of the amidating enzymes by the epithelial cells is further supported by the finding of these enzymes in the pulmonary exocrine glands. This is consistent with the description of PAM in the secretory granules of other exocrine glands (Kato et al., 1992; von Zastrow et al., 1986). The presence of the amidating enzymes in exocrine secretory cells suggests the existence of amidated peptides involved in the regulation of secretion or acting as luminal messengers or growth factors on epithelial cells. The immunoreactivity in the perinuclear cisterna is in agreement with previous reports (Milgram et al., 1993; Tausk et al., 1992).

We expected to find PAM expression in pulmonary endocrine cells because it has been demonstrated that they contain peptides that are usually amidated. Among others, the best known peptides produced by endocrine cells of the lung are gastrin-releasing peptide (GRP), which is the human analogue of bombesin (Wharton et al., 1978), calcitonin gene-related peptide (CGRP), and calcitonin (Springall et al., 1991a). The antibodies used in those references were not selective for the amidated forms. Bombesin appears to be an important growth factor for human bronchial epithelial cells, both in the adult (Baraniuk et al., 1992; Willey et al., 1984) and during development (Sunday et al., 1990). It has been shown that amidated GRP is 100–1000-fold more powerful in receptor binding and in the induction of physiological responses than its corresponding free acid or glycine-extended analogues (Moody et al., 1985,1988). In our case, no co-localization between GRP and PAM was found, and similar results have been reported in the epithelium of the mouse lung between CGRP-immunoreactive cells and PAM (Guembe et al., 1994). Because PAM is not produced in the pulmonary endocrine cells, these peptides may be amidated in a paracrine fashion after release, or they may act through unidentified receptors that recognize the glycine-extended precursors, as has been described for gastrin (Seva et al., 1994).

The endothelium of the blood vessels has been previously described as a site of PAM expression (Oldham et al., 1992; May and Braas, 1991). The above-mentioned presence of small amidated peptides related to the endothelin precursor could explain the finding of PAM in the endothelial cells. May and Braas (1991) also described mild immunoreactivity for PAM in deep smooth muscle cells of hypophysial arteries. This is the first report of blood vessel smooth muscle PAM immunoreactivity in human tissues, and the first time that it is shown in the muscle layers of the airways. The expression of amidating enzymes in these cells implies production of amidated regulatory peptides in smooth muscle cells. The differences in staining intensity among muscle cells from one blood vessel to another suggest some degree of heterogeneity among individual cells and vessels, possibly related to physiological differences. Adrenomedullin has also been found in endothelial and muscle cells (Martínez et al., 1995b).

The localization of PAM in neurons was expected because of the many amidated peptides found in lung intrinsic neurons. Among them, VIP and galanin (Springall et al., 1991a) are the more abundant. Neurons immunoreactive for PAM have been found in the central (Schafer et al., 1992; Rhodes et al., 1990) and peripheral (Martínez et al., 1993b; Tozawa et al., 1990) nervous systems. Immunoreactivity for these enzymes has also been described in Schwann and glial cells (Rhodes et al., 1990) in keeping with our results of positive cells in nerve trunks.

The localization of PAM in human alveolar macrophages could be related to the reported production of IGF-I (Rom et al., 1988) and adrenomedullin (Martínez et al., 1995b) by these cells. It is known that at least one C-terminally-amidated peptide is potentially released from the IGF-I precursor during the maturation process (Siegfried et al., 1992).

Many lung epithelial tumor cell lines have been investigated in culture and expression of amidating enzymes has been shown (Treston et al., 1993), but to the best of our knowledge this is the first time that the PAM enzymes are demonstrated in resected lung tumors. PAM immunoreactivity has been also described in pituitary tumors (Steel et al., 1994). The cells of lung carcinomas originate from the normal epithelium by anaplasia. They conserve all the abilities of the epithelial cells, including the production of peptides and, at least in culture, their amidating machinery. Nevertheless, they lack growth regulation and the expression of these peptides is altered (Rehfeld et al., 1994). Intense labeling for PAM is therefore expected in tumors derived from cell types that normally express the amidating enzymes, such as those of the bronchial epithelium, even if they are not usually classified as endocrine tumors. In non-small-cell lung carcinomas approximately 20% of cases express some aspects of neuroendocrine differentiation (Linnoila et al., 1994). In this study every case was positive for PAM, suggesting a much broader distribution than other neuroendocrine markers.

All of the cases studied were immunoreactive for PHM, but only a subset of them were also positive for PAL (Table 1). Differences in the staining pattern with antibodies directed against PHM and PAL were not expected because the two enzymes are derived from the same mRNA precursor. Tissue-related differences in the immunoreactivity for PHM and PAL have been reported in endocrine cells of the digestive tract of the rat. Whereas in the stomach most of the gastrin cells were immunoreactive for PAL and only some were positive for PHM, in the colon only immunoreactivity for PHM was found (Martínez et al., 1993b). Tissue specific expression of alternate mRNA splice forms and post-translational proteolysis and secretion of PAM proteins have been extensively studied (Oyarce and Eipper, 1993; Stoffers et al., 1989) and could explain some of the differences found. In addition, possible differences in sensitivity between the antibodies could also explain this discrepancy.

Typical endocrine tumors, i.e. the carcinoid tumors and the small-cell lung carcinomas, showed the lowest immunoreactivity for the amidating enzymes. This finding might be explained by the high secretion rate of the amidating enzymes in these types of tumors (Black et al., 1993; Treston et al., 1993). It is well known that these types of tumor cells have few granules accumulated in the cytoplasm. Because our previous work has shown that in the endocrine pancreas PAM immunoreactivity appears to be mainly located in granules in endocrine cells (Martínez et al., 1993a), it is logical that lung endocrine tumors with high rates of secretion would be poorly

immunoreactive for PAM. The strong expression of PAM mRNA supports this view. Interestingly, the well-differentiated neuroendocrine carcinomas show an immunoreactivity for PHM intermediate in intensity between carcinoids and small-cell lung carcinomas, supporting the idea stressed by Gould et al. (1983) that they represent an intermediate category.

An interesting finding concerning the expression of PAM in epithelial cells was the PHM immunoreactivity observed in the cuboidal metaplastic epithelium lining alveoli and alveolar ducts found in the neighborhood of the tumors. This finding contrasts with the negativity of the normal flattened epithelial cells. This fact could be explained by an endocrine differentiation of the cuboidal hyperplastic epithelium, as discussed below. It might also be that these hyperplastic cells are involved in the production of amidated growth factors. We are in the process of studying PAM expression in a larger number of morphological atypias that may be pre-malignant lesions.

The regional distribution of pulmonary PAM and its co-localization with amidated regulatory peptides such as adrenomedullin in cell types and tumors historically classified as nonendocrine led us to reconsider the concept of "endocrine." As new regulatory peptides and their processing enzymes are discovered and characterized, new cell types enter the arena of endocrinology. Similar trends have previously occurred with the gastrointestinal tract (Bloom and Polak, 1980) and endothelial cells (Vane and Botting, 1992). The present study challenges previous notions of restricted expression of neuroendocrine features to the Kulchinsky cells in the lung, and highlights the pluripotency of the airway epithelium and other pulmonary cell types. On the other hand, the lack of PAM immunoreactivity in the pulmonary neuroendocrine cells suggests that the regulation of pulmonary physiology is more complex than previously suspected.

In summary, the amidating enzymes appear in normal structures of the lung and in many types of tumor cells, indicating that amidation of regulatory peptides is a common function in lung biology. Peptide amidation in tumor cells could contribute to tumor growth and may be an important target for new therapeutic strategies.

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