Journal of Cellular Biochemistry Supplement 24:257–268 (1996)

Expression in Human Lung Cancer Cell Lines of Genes of Prohormone Processing and the Neuroendocrine Phenotype

Michele D. Vos, Frank M. Scott, Naomichi Iwai, and Anthony M. Treston

Biomarkers and Prevention Research Branch, National Cancer Institute, Rockville, Maryland 20850-3300

Abstract Lung tumor cells and cell lines, principally the histologically classified small cell lung cancer, are characterized by the expression of neuroendocrine (NE) features including AADC (aromatic amino acid decarboxylase, previously called DOPA decarboxylase) and the production of many peptide hormones. The general mechanisms by which most aspects of the NE phenotype affect the clinical behavior of lung tumor cells are unknown, but it is well recognized that peptide hormones can have systemic effects (paraneoplastic syndromes) and several have been shown to be autocrine growth factors for cancer cells. In order to determine the relationship between expression of different aspects of the NE phenotype in lung cancer cell lines, we have compared expression of a gene required for biosynthesis of some active peptide hormones (PAM, peptidylglycine α-amidating monooxygenase) to the gene for AADC in 32 lung cancer cell lines. Expression of these genes was quantified by both steady state Northern blot analysis and radiochemical enzymatic activity measurements. To ensure a range of expression of NE markers, non-small cell lung cancer (NSCLC) cell lines were chosen to include several which had previously been shown to express NE markers, and several small cell lung cancer (SCLC) cell lines with previous low levels of AADC were included. PAM enzyme activity and Northern blot analysis showed a two to three log variation in levels of expression in both the small cell and non-small cell lines. A smaller range was found for AADC expression. Using the highly sensitive PAM enzyme assays, all cell lines were found to express detectable PAM. PAM activities were secreted into the growth medium of all cell lines.

There was no simple correlation apparent between AADC and PAM gene expression in the lung cancer cell lines. However, classic small cell lines demonstrated high levels of expression of both PAM and AADC genes, as did the carcinoid subset of the NSCLC lines. NSCLC lines expressed levels of PAM mRNA and enzyme activities equivalent to those of SCLC, but had infrequent expression of AADC (principally only carcinoid NSCLC expressed AADC). These data demonstrate that separate aspects of the NE phenotype can be differentially expressed in lung cancer histological sub-types. Expression of PAM enzymes in all sub-types of lung cancer suggests that peptide prohormone activation may be a common mechanism for autocrine growth stimulation even in non-NE NSCLC cell lines, or may reflect maintenance in cell lines of a common pathway of lung tumor promotion.

Key words: small cell lung cancer, non-small cell lung cancer, peptidylglycine α-amidating monooxygenase, lung tumor cell lines

A number of reports have explored the hypothesis that neuroendocrine (NE) differentiation in tumors, for example small cell lung cancer (SCLC) and a NE subset of non-small cell lung cancer (NSCLC), can affect the clinical course of the tumors and their response to therapy [1,2]. NE differentiation in advanced tumors may reflect not only the phenotype of the non-transformed cell of origin, but also maintenance of expression of functions involved in the early steps of carcinogenesis. NE cells in normal tissues, for example the lung, control fetal organ growth, chemoreception, and promote cellular hyperplasia after damage including cigarette smoking [3]. These normal cellular NE functions, particularly the repair processes resulting in mitogenesis and hyperplasia, are potentially subsumed in the carcinogenic process.

Abbreviations used: AADC, aromatic amino acid decarboxylase (previously called DOPA decarboxylase); GRP, gastrin-releasing peptide; NE, neuroendocrine; NSCLC, non-small cell lung cancer; PAL, peptidylamidoglycolate lyase; PAM, peptidylglycine α-amidating monooxygenase; PHM, peptidylglycine α-hydroxylating monooxygenase; SCLC, small cell lung cancer.

Received January 19, 1996.

Address reprint requests to Anthony M. Treston, Biomarkers and Prevention Research Branch, National Cancer Institute, Suite 300, 9610 Medical Center Drive, Rockville, MD 20850-3300.

The NE phenotype is best described by frequent expression of a panel of NE markers. Expression of aromatic amino acid decarboxylase (AADC), which is involved in synthesis of bioactive amines such as dopamine, norepinephrine, and epinephrine, is frequently assumed to be the best single general marker for the NE phenotype. This reflects an early classification of pulmonary NE cells as APUD (amine precursor uptake and decarboxylation) cells [reviewed in 4]. Other reported general markers of NE differentiation include synaptophysin, chromogranin, neuron-specific enolase, several neural cell adhesion molecules, the BB isoenzyme of creatine kinase, and presence of dense core secretory granules [5–7]. There are also specific markers of NE differentiation, many of which are peptide hormones, for example bombesin/gastrin releasing peptide (GRP) [8–10]. NE peptides reported in lung tumors include GRP, calcitonin, calcitonin gene-related peptide, oxytocin, vasopressin, cholecystokinin, gastrin, tachykinins such as substance P, and the proopiomelanocortin peptides alpha-MSH, beta-endorphin, and ACTH [4]. Cells that express individual peptide hormones frequently express general NE markers. However, there are many peptide hormones which are expressed by subsets of NE cells, and so any individual peptide hormone is not an appropriate general marker for the NE phenotype. In tumor cells, the biological relevance of peptide hormones is their role in growth regulation. This is a corollary of their roles in control of proliferation, differentiation, and wound repair in the normal epithelium, which are principally mediated by NE peptide hormones [3,8]. For example, GRP is a mitogen for normal fetal lung cells and an autocrine growth factor for SCLC [11,12]. Peptide hormones are also responsible for many of the reported paraneoplastic syndromes caused by neuroendocrine tumors [4,8].

Peptide hormones when first translated as precursor prohormones are inactive or weakly active, and only become fully bioactive when the prohormone has been correctly post-translationally processed [13,14]. As post-translational modifications are necessary for complete bioactivity of all peptide hormones, the enzymes which carry out the steps between synthesis of the inactive prohormone and production of the active form are central to all facets of cell biology which involve peptide neurohormones [15,16]. Post-translational processing enzymes may therefore constitute a strategic indicator of activation of NE peptide hormone biology in epithelial cells.

The most common post-translational modification of peptide hormones, present on half of all gastrointestinal and NE peptide hormones, is α-amidation [14]. α-Amidation is necessary for both high affinity peptide-receptor binding and/or appropriate half-life of action of peptide hormones [17]. For example, GRP has an absolute dependence on α-amidation for receptor binding and activation. Two enzyme activities (a peptidylglycine α-hydroxylating monooxygenase, PHM, and a peptidylamidoglycolate lyase, PAL) are required to produce this post-translational modification in vivo (see Fig. 1A). PHM and PAL are separable enzymes encoded by a single gene, PAM (peptidylglycine α-amidating mono-oxygenase) (Fig. 1B). PAM has been identified in cell populations known to produce α-amidated peptides, and in NE tumors [18,19]. The translated PAM protein is itself subject to cell-specific post-translational processing, and several protein isoforms differing by alternative splicing of the pre-mRNA are known (Fig. 1B) [14,20]. In vitro, PHM and PAL can amidate peptides either as the bifunctional PAM or as separate monofunctional enzymes. We have previously identified high levels of PHM and PAL enzyme activities in NE lung tumor cell lines [21]. The variation in levels with respect to cell type suggested that PAM could potentially be a marker of the NE phenotype. However, recently we have immunohistochemically identified PAM expression in both NE and non-NE lung tumor histological types, and in the bronchial and bronchiolar ciliated epithelial cells which are presumably the progenitor cells for lung tumors [22].

To determine whether PAM expression is coordinately linked to expression of other markers of the neuroendocrine phenotype, we have extended our previous cell line study to a wider range of both SCLC and NSCLC cell lines. Our aim is to explore this aspect of the biology of the NE phenotype, and to elucidate aspects of the biochemistry of peptide post-translational processing which may be developed into targets for pre-neoplastic diagnosis and intervention in epithelial carcinogenesis. To complement our previous study and ensure a range of expression of NE phenotype markers, we selected several SCLC cell lines which had low reported levels of AADC activity, and NSCLC lines with both high and low frequency of expression of a panel of NE

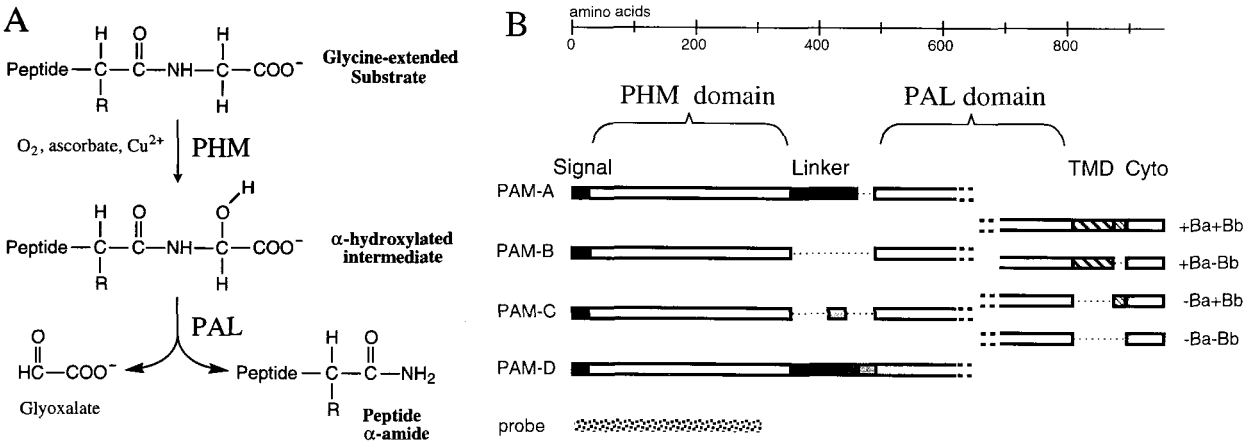

Fig. 1. **A:** The two-step peptidylglycine α-amidation (PAM) reaction, catalyzed by sequential action of PHM and PAL enzymes. **B:** The structure of human PAM mRNAs and proteins. The enzymatic PHM and PAL domains are indicated, as are the signal peptide, linker region, trans-membrane domain (TMD), and the cytoplasmic tail (Cyto). The probe used for Northern analysis is common to all forms. PAM-A and PAM-B differ by the presence of exon A (dark stippling) and were identified by Glauder et al. [39]. PAM-C and PAM-D differ from PAM-B and PAM-A by the presence of exon C (light stippling) [20]. The presence of four splice forms in the TMD (exons Ba and Bb, dark and light hatching, respectively) was identified by pcr in a pancreatic tumor cell line [33] and in lung tumor cell lines [34].

markers. The cell lines were principally developed at the NCI-VA Medical Oncology Branch and the NCI-Navy Medical Oncology Branch and are described elsewhere in this supplement.

MATERIALS AND METHODS

Cell Lines and Extracts

All cell lines except AtT 20, A549, Molt 4, and U937 were developed by the NCI-VA and NCI-Navy Medical Oncology Branch. Cell lines were generally maintained in RPMI-1640 with 5% fetal bovine serum in T-175 flasks, passaged as necessary to maintain exponential growth conditions, and fed 3–4 days before extraction. At the time of harvest, the conditioned medium was subjected to centrifugation (3,000*g*, 5 min) to pellet cell debris and aliquots were stored for analysis. For extraction of the cells, one to four flasks of cells were pooled (adherent cell lines were collected by scraping; floater lines by centrifugation at 800*g*) and washed with Dulbecco's phosphate buffered saline. The cell pellet was then split into two aliquots and extracted for either RNA or protein. Total RNA was purified by standard methods. In brief, GIT buffer (4M guanidine isothiocyanate with 2-mercaptoethanol, 8 mL) was added to the cell pellet, mixed to lyse cells, then layered over 3 mL of 5.7 M CsCl buffer and centrifuged overnight (22 h) at 32,000 rpm (174,000*g*) at 22°C. Further purification of mRNA was accomplished using the FastTrack mRNA isolation kit according to the manufacturer's instructions (Invitrogen, La Jolla, CA). For protein, the pellet was sonicated in 4–5 mL of 20 mM TES, pH 7.4, 10 mM mannitol, 1% Triton X-100 containing an antiprotease cocktail of PMSF (150 μM), aprotinin (50 μg/mL), bacitracin (150 μg/mL), and leupeptin (25 μg/mL). This detergent extraction is necessary to release all forms of PAM, some of which are membrane-bound. The extract was spun in an ultracentrifuge at 100,000*g* for 30 min. Aliquots were stored at −20°C, and a sample was kept at 4°C for the PAM enzyme assays performed within 24 h.

PHM and PAL Enzyme Assays

α-N-acetyl-Tyr-Phe-Gly (acYFG) is converted to the C-terminal α-carboxyamidated analogue (N-acetyl-Tyr-Phe-amide : acYFamide) by sequential PHM and PAL activity. The assays were carried out in microfuge tubes with 35 μL of assay mix and 5 μL of enzyme preparation. Reactions were shown to be linear with time (1–2 h) and amount of protein (up to 5 μg per assay) over the ranges used here. For both assays, the reaction was stopped by the addition of 250 μL 1.0 M, pH 7.5 HEPES buffer saturated with ethyl acetate, and 650 μL ethyl acetate saturated with the same HEPES buffer. The tubes were capped and mixed to extract the α-amidated product into the organic phase. Specific activities were calculated from enzyme activities and protein concentrations determined using the Pierce bicinchoninic acid reagent in

the microtiter plate format. PHM assays on conditioned medium containing 5% fetal bovine serum were carried out at a single copper concentration (2 μM) shown in preliminary tests to result in maximal activity. PAL activity is independent of copper.

PHM Assay

The standard assay mix contained approximately 150 mM NaMES buffer, pH 6.25, with 20,000 cpm [^{125}I-Tyr]-acYFG, 0.2 μM acYFG, 5 μL bovine catalase, 1 μM Cu^{2+} (as $CuSO_4$) for each 0.5 μg of analyte protein, and 0.5 mM ascorbate. After 1 h reaction, any α-hydroxyglycine intermediate [N-acetyl-Tyr-Phe-α-hydroxyglycine, acYF(aHO)G] remaining in the pH 6.25 reaction mix was converted to acYFamide by addition of 50 μL 0.2 M NaOH for 5 min at 37°C. This base-catalyzed conversion step ensures that the assay is independent of PAL, and so is a measure of PHM activity only. Both acYFG and acYF(aHO)G are acidic and do not extract from the aqueous phase at pH 7.5 into the organic solvent.

PAL Assay

An aliquot of purified bovine PHM with no PAL activity was used with a PHM reaction mix at reduced pH (150 mM NaMES, pH 5.5) to produce acYF(aHO)G. After 5 h at 37°C the PHM reaction was terminated by reaction for 30 min with 100 μM of a covalent PHM inhibitor. The PAL reaction was carried out with 35 μL of this mix added to 5 μL of enzyme preparation for 1–2 h at 37°C.

Aromatic Amino Acid Decarboxylase Assay

The method used is a variation of that of Beaven [23], as modified by Edward Russell of the NCI-Navy Medical Oncology Branch, using DOPA as a substrate. Cell extract (80 μL) was mixed in a microfuge tube with 120 μL of a reaction mix yielding final concentrations of 0.5 mM DOPA, 0.4 mM pyridoxal phosphate, and 200,000 cpm ^{14}C-DOPA in 100 mM phosphate buffer, pH 6.8, with 2.5 mM dithiothreitol. The mix was allowed to react at 37°C inside a capped scintillation vial containing 25 μL of 1 M methylbenzethonium hydroxide to trap any released $^{14}CO_2$. After 1 h, 100 μL of 0.2 M perchloric acid was added to acidify the mix and release $^{14}CO_2$. The microfuge tube was then removed from the scintillation vial, 3 mL of scintillant was added, and samples were counted.

Agarose Gel Electrophoresis

Ten micrograms of total RNA or 2 μg mRNA was fractionated on a 1.0% agarose gel containing formaldehyde, at 150 V for 2 h. Before transfer, gels were visualized by staining with ethidium bromide and photographed under UV light (302 nm) to determine the quality of the RNA and the equivalency in loading. Standard methods for passive capillary transfer of the fractionated RNA onto a nitrocellulose membrane were performed.

Northern Analysis

Northern blots were hybridized with an EcoR1 800 bp fragment of human PAM cDNA (from the λPAM-1 cDNA in the pUC plasmid [24]). The filter was then stripped and reprobed with an 1,850 bp EcoR1 fragment of the human AADC cDNA (Bluescript KS+ vector [25]). Both inserts were gel purified before use. Radiolabeled (random primed) probe was added to the hybridization buffer at a specific activity of 10^6 cpm per mL and allowed to hybridize overnight at 42°C. Molecular weights were estimated by comparison with both an RNA ladder (BRL) and positions of 18S and 28S ribosomal RNA bands. Filters were washed twice at room temperature in 2 × SSC and 0.05% SDS and then twice at 58°C in 0.1 × SSC and 0.05% SDS. For quantification, the filters were exposed to the phosphor storage plates of a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Results were printed directly from the Molecular Dynamics ImageQuant software on a laser printer modified to print grayscale images. Quantification was carried out by summing the individual pixel intensities in a user-defined area. Data was exported to Excel and corrected for background determined from a neighboring identical-sized area.

RESULTS

Expression of PAM and AADC Genes

We determined expression of the PAM and AADC genes by quantitative radiochemical enzyme assays and Northern blot analysis (Table I and Fig. 2). Data presented are from a single enzyme analysis and Northern analysis, but are representative of 2–10 repeated analyses on independent extracts for most cell lines. PHM and PAL enzyme activities measured in these lines varied over more than two orders of magnitude (approximately 2–600 fmol/h/μL cell extract). The lowest levels of activity detected were in the variant SCLC lines and a subset of NSCLC cell

TABLE I. Expression of PAM and AADC Genes

Cell line	Type	Extract PHM (pmol/h/mg)	Extract PAL (pmol/h/mg)	Extract AADC (pmol/h/mg)	PAM RNA (intensity)	AADC RNA (intensity)	PHM medium (pmol/h/mL)	PAL medium (pmol/h/mL)	Total PHM (pmol/h)	Total PAL (pmol/h)
AtT-20	pituit	438	489	93	+ve	+ve	286	107	9,581	4,314
U937	hemat	17	16	<0.5	24,186	bgd	−2	18	23	558
H60c	sclc-c	65	65	<0.5	nd	nd	−45	10	118	630
H69c	sclc-c	712	959	72	239,187	64,163	12	228	2,982	25,233
H146	sclc-c	540	716	18	284,126	55,143	14	302	2,564	11,912
H187	sclc-c	37	52	nd	43,939	5,005	−40	40	158	1,407
H209	sclc-c	188	138	42	25,636	28,657	−62	35	867	3,411
H345	sclc-c	517	412	17	137,175	36,418	148	189	15,027	17,660
H510	sclc-c	299	445	25	204,487	61,986	177	208	6,356	7,796
H735	sclc-c	831	795	3	10,609	1,713	298	192	15,690	10,358
H774	sclc-c	757	540	27	16,401	22,594	98	122	5,757	6,696
H847b	sclc-c	29	45	<0.5	75,715	2,445	101	90	3,196	2,954
H889	sclc-c	1,663	1,907	91	206,966	42,619	98	74	6,669	5,767
H1062	sclc-c	<1	<1	<0.5	bgd	1,354	−5	6	0	300
H1092	sclc-c	478	300	179	nd	nd	4	38	1,333	2,627
H82	sclc-v	7	4	<0.5	12,939	bgd	−53	14	28	441
N417	sclc-v	34	83	<0.5	63,291	bgd	−28	74	155	2,619
H446	sclc-v	147	263	<0.5	186,872	bgd	94	16	7,869	13,805
H23	adeno	18	48	<0.5	26,210	bgd	−6	32	63	2,760
A549	adeno	8	24	<0.5	31,034	bgd	−16	29	51	2,500
H157	squam	150	224	<0.5	47,282	bgd	48	20	1,702	776
H820	bac	322	546	<0.5	336,120	17,732	186	87	18,696	8,931
H1404	bac	124	260	<0.5	133,908	2,017	151	208	9,461	13,361
H2087	bac	71	159	<0.5	185,323	1,089	431	143	26,085	9,049
H2228	bac	122	199	<0.5	245,625	209	49	43	2,103	1,912
H720	carc	331	293	23	58,671	18,192	196	121	9,386	6,207
H727	carc	156	256	16	63,632	14,072	28	60	2,504	5,249

Cell lines and assay of enzyme activities are described in the text. Tumor types are: pituit = mouse pituitary, hemat = hematopoietic; SCLC subtypes are sclc-c = classic, sclc-v = variant; NSCLC histologies are: adeno = adenocarcinoma, squam = squamous carcinoma, bac = bronchoalveolar carcinoma, carc = carcinoid. nd = not done. For cell extracts the values are reported as specific activities, that is, adjusted for protein concentration. Specific activities lower than the limit of detection are dependent on the protein concentration in the extract but are approximately <1 for PAM enzymes and <0.5 for AADC activity. RNA intensity values are in arbitrary units determined using a direct-imaging densitometer as described in the text. Intensity of RNA signals for AtT-20 was not calculated as the efficiency of cross-species hybridization was not known. Both probes yielded a positive signal (+ve). For cell lines where no band could be discerned, the value is reported as background (bgd). Cell-derived PHM and PAL activities in cell-conditioned medium were corrected by subtracting the activities present in unconditioned medium, and so can be less than zero. For calculation of the total PHM and PAL activities, activities in conditioned medium less than the unconditioned medium were defined as zero, activities in cell extracts lower than the limit of detection were defined as zero, and the totals take into account the volume of medium and the volume of the cell extract.

lines. For comparison, Table I and Figure 2 include data on two non-NE hematopoietic cell lines, which as expected express no AADC and have low levels of PAM mRNA and enzyme activities, and a mouse NE pituitary tumor cell line AtT-20 which as expected expresses both PAM and AADC activities and mRNA. In a majority of cell lines, a significant proportion of both PHM and PAL enzyme activities is released into the culture medium (Table I and Discussion). No cell line tested was negative for both PHM and PAL activity, although the presence of both enzymes in fetal calf serum used for maintaining the cell cultures makes it difficult to exclude contamination by bovine PAM enzymes for the low-PAM lines (typical analyses of RPMI-1640 with 5% fetal calf serum: PHM 60–90 pmol/h/mL, PAL 30–60 pmol/h/mL). Several cell lines appear to be negative for PAM mRNA expression using Northern analysis with the 800 bp probe obtained from λPAM-1 [24] (Fig. 2A). However, reverse transcriptase-polymerase chain reaction (RT-PCR) analysis confirms the results of the radiometric enzyme assay. All cell lines in this report express PAM mRNA (RT-PCR results not shown).

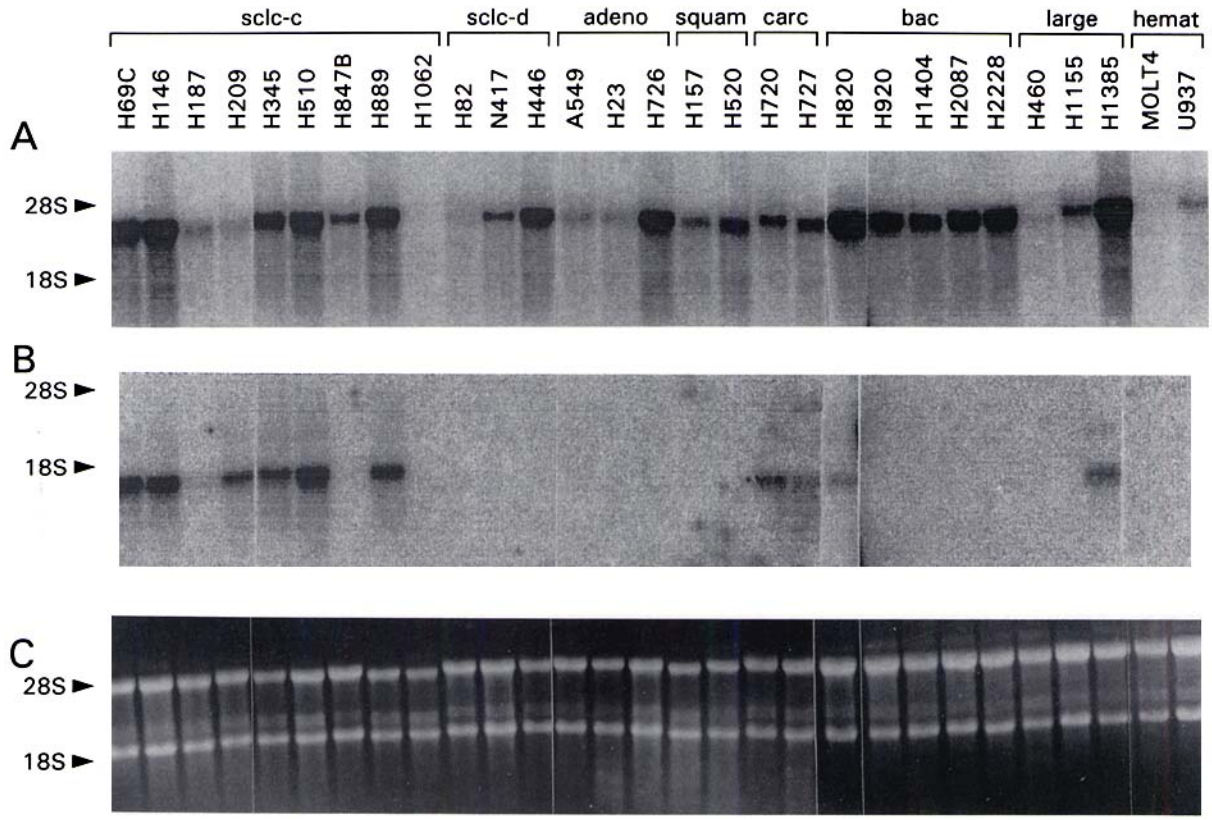

Fig. 2. Northern analysis of PAM and AADC gene expression. Cell lines are as described in the legend to Table I. Several NSCLC of large cell histology and the T-cell lymphoma MOLT 4 were also analyzed. **A:** Northern analysis of PAM mRNA, probed using a 800 bp EcoR1 PAM DNA fragment common to all splice forms. PAM mRNA is approximately 4.1 kb. **B:** The membrane was stripped and reprobed with a 1.85 kb EcoR1 AADC DNA fragment. AADC mRNA is approximately 2.1 kb. **C:** Photograph of the 28S and 18S rRNAs visualized with ethidium bromide under UV illumination to demonstrate RNA integrity and equivalence of loading.

A 1.85 kb AADC cDNA was used as a probe for levels of this NE marker on Northern blots (Fig. 2B) [25]. The relatively poor detection limit for the decarboxylase assay, relative to the PAM assays, is obvious from Table I and Figure 2B where several lines with detectable AADC signals on Northern analysis had DOPA decarboxylase enzyme levels below the assay background (listed as <0.5 in Table I). The relative lack of sensitivity of the AADC assay could be due to several reasons including competition from endogenous nonradioactive substrate, post-transcriptional control of expression, or abnormal protein processing. To confirm that a low enzyme level in a cell extract does not reflect secretion of decarboxylase activity into the cell-conditioned medium, we assayed medium from six cell lines selected to cover the range of extract activities (H1062, H2087, H820, H735, H720, H889). No decarboxylase enzyme activity could be measured in conditioned medium for any of these lines.

PAM Enzymes Are Secreted Into Culture Medium But Are Not Stable

As expected for two enzyme activities co-translated on a single pro-enzyme, there was a good correlation between PHM and PAL activity measured in the cell extracts (Fig. 3A, $r^2 = 0.924$). However, high levels of both PAM enzymes are secreted into the conditioned culture medium by both SCLC and NSCLC cell lines. After 3–4 days, PAM activity levels in medium account for generally greater than 90% of the total cell-derived PAM activities. To compare the total cell line-derived PAM activities, we added the activities in the cell extract to that in the medium after correction for the background levels of PHM and PAL activities in un-conditioned medium (Table I). As shown in Figure 3B, the correlation between total cell-derived PHM and PAL activities is poor ($r^2 = 0.227$). The PHM activity in several of the conditioned media is less than that of the base-

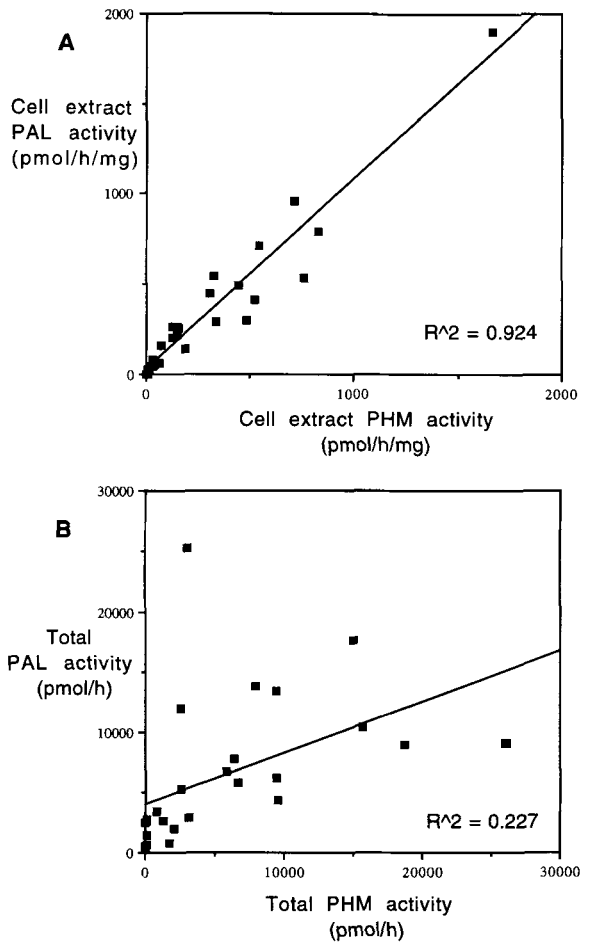

Fig. 3. Graphical representation of PHM and PAL activity data from Table I. **A:** Comparison of PAL and PHM cell extract specific activities. **B:** Comparison of total PAL and PHM activities (extract plus cell-conditioned medium) calculated as described in the legend to Table I.

line level of un-conditioned medium (PHM of 5% fetal calf serum in RPMI-1640: 60–90 pmol/h/mL), for example H209 and N417 (Table I). All assays were carried out under conditions where the metal co-factor of PHM (Cu^{2+}) was not limiting to prevent PHM inhibition by copper-binding proteins present in fetal calf serum. The loss of activity of PHM or PAL could be due to secretion of a specific inhibitor from the cells, or to proteolytic digestion of either enzyme caused by cell-derived protease/s.

Correlation of AADC and PAM Enzyme Activities and mRNA Levels

Expression of the PAM gene measured by Northern blot analysis generally parallels total PHM and PAL enzyme activity levels although the correlation coefficients are low (Fig. 4A and B). PAM mRNA is transcribed into as many as 16 alternatively-spliced forms [20], resulting in translated enzymes that are differentially packaged within and secreted from the cell [26], and the translated protein of each splice form studied to date has different kinetic constants. Furthermore, as described above, PAM activities are subject to inhibition or catabolism after se-

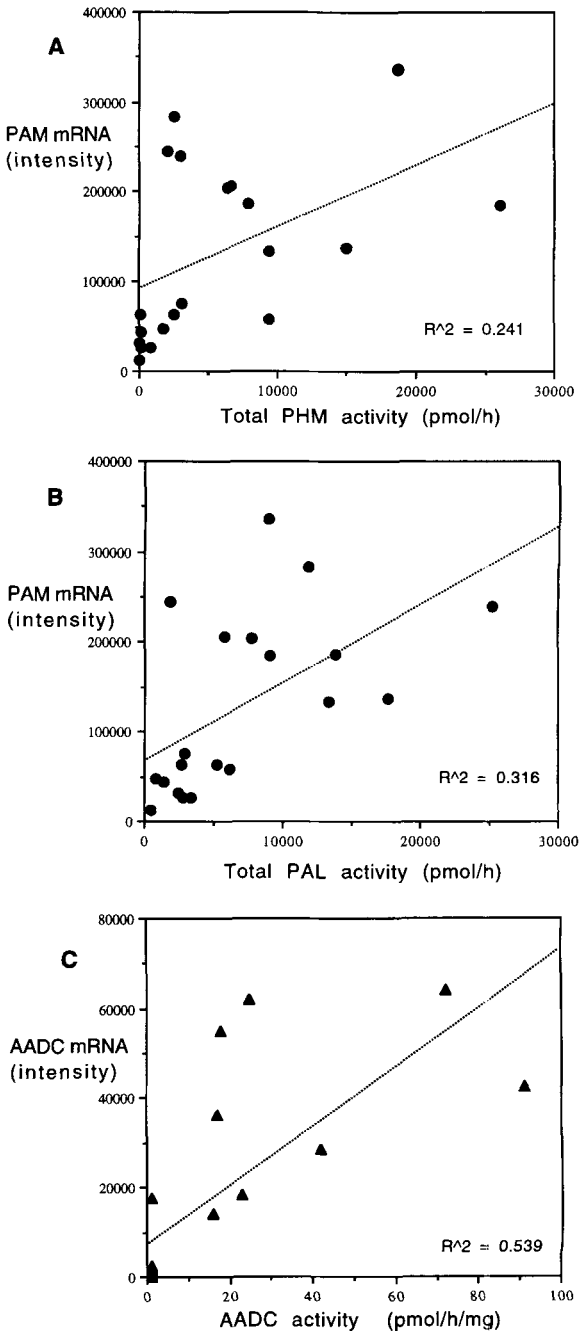

Fig. 4. Comparison of mRNA intensity and enzyme activities. **A:** PAM mRNA compared with total (extract + medium) PHM activity. **B:** PAM mRNA compared with total (extract + medium) PAL activity. **C:** AADC mRNA compared with DOPA decarboxylase activity.

cretion into the growth medium. Northern analysis cannot distinguish between the different alternatively spliced forms of human PAM, thus preventing direct comparison of mRNA levels and enzyme activities. For the panel of cell lines in Table I, signal intensity on Northern blot for AADC mRNA generally paralleled AADC enzyme activity in cell extracts, measured with DOPA as a substrate (Fig. 4C, $r^2 = 0.539$).

AADC and PAM Are Expressed in Classic SCLC and Carcinoid NSCLC Cell Lines

The data from Table I and Figure 2, and further Northern analysis data from several more classic SCLC and carcinoid NSCLC cell lines, are presented graphically in Figure 5. Only Northern analysis data was used for this comparison due to the inhibition or catabolism of PAM enzymes released into cell-conditioned media. Classic small cell and carcinoid NSCLC cell lines analyzed showed expression of both PAM and AADC (Fig. 5A), as expected from previous reports that these tumor cell types express NE markers including peptide hormones. The data points for the carcinoid lines appear to fit onto a regression line distinct from that of the classic SCLC lines, although as the number of samples is small (n = 5) the regression line is not shown.

PAM Is Frequently and Highly Expressed in NSCLC Cell Lines

In contrast to the expression seen with classic SCLC cell lines, variant SCLC and NSCLC cell lines aside from the carcinoid type generally did not show co-expression of AADC with PAM (Figs. 2 and 5B). With a few exceptions, all non-carcinoid NSCLC cell lines analyzed by Northern analysis were negative for or had low levels of AADC RNA (note the different axis scales used for Fig. 5A and B). However, all NSCLC cell lines expressed PAM, in many cases at levels similar to or higher than those found in the classic SCLC and carcinoid cell lines. All non-carcinoid NSCLC cell lines tested had levels of decarboxylase activity below the level of detection of the assay (see Table I). Although this data demonstrates frequent expression of PAM in NSCLC cells, this data cannot be used to estimate frequency of PAM expression in NSCLC tumor cell lines generally, as the lines studied here were not randomly selected. Immunohistochemical and mRNA/mRNA in situ hybridization data reported by us supports this report

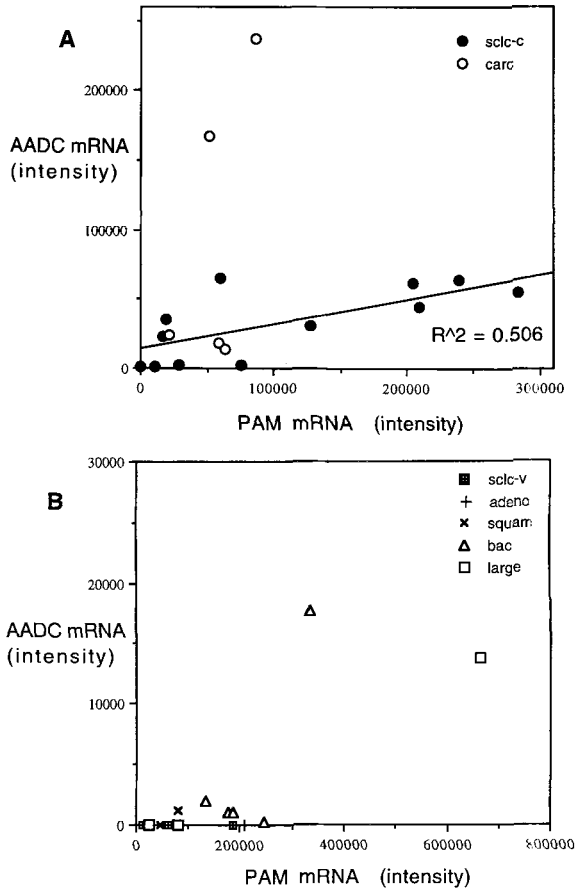

Fig. 5. Co-expression of AADC and PAM genes. Abbreviations used to distinguish tumor cell histologies are as explained in Table I. Note the different scales for both AADC and PAM which had to be used between Figures 5A and B. **A:** NE histologies (classic SCLC and carcinoid NSCLC). **B:** Non-NE histologies (variant SCLC and other NSCLC).

that most or all NSCLC tumors express PAM [22].

DISCUSSION

The group of cell lines developed by the NCI Medical Oncology Branch presents a unique resource for elucidation of aspects of cell biology which may be shared to varying extents amongst the range of cell types represented in the archive. Here we demonstrate that these cell lines can be used to study expression of markers of the NE phenotype, with a goal of identifying disparate cellular mechanisms for control of different aspects of the NE phenotype.

Certain facets of lung tumor clinical characteristics, such as initial response to radiation or chemotherapy and clinical course including propensity for early metastasis, may be related to expression of the NE phenotype [2,7,27]. How-

ever, it is not yet well established what exactly defines the NE phenotype. Most reports of clinical correlations of the NE phenotype have studied general NE markers [1,2]. Of these, AADC (DOPA decarboxylase) is accepted as being the best single marker, although in most reports a panel of general NE markers is studied [several studies are reviewed in 1]. In this report we have studied the link between expression of the NE marker AADC with a marker for a specific NE characteristic, that is, an enzyme required for synthesis of bioactive peptide hormones.

We have chosen to study the prohormone processing enzymes which are responsible for synthesis of many active hormones as an economical alternative to identifying all peptide hormones relevant to lung tumor biology. The steps in post-translational processing vary between peptides, but include processes which do not alter the amino acids (removal of signal sequences, proteolytic cleavage to smaller forms, trimming of either terminus), and processes which result in post-translational amino acid modifications [13]. Post-translational modifications of peptide hormones include N-terminal acetylation, sulfation of tyrosine, and C-terminal amidation [28]. The most common modification, and the one most closely linked to prohormone activation, is C-terminal α-amidation [15,17]. As these modifications are in all cases necessary for production of bioactive peptide hormones, post-translational processing enzymes are surrogates for peptide hormone activation.

Our previous report on PAM expression in lung cancer cell lines suggested that increased levels of PAM were associated with increased expression of a panel of NE markers, including synaptophysin, chromogranin A, neurosecretory granules, creatine kinase BB isoenzyme, neuron-specific enolase, AADC (measured as DOPA decarboxylase), and GRP levels [21]. This present study extends our initial report. The lung cancer lines studied here were not intended to represent a random sample of the indicated tumor histologies. The principal aim of this project was to demonstrate whether there is a simple relationship between expression of different markers of the NE phenotype in lung cancer cell lines. A more-detailed statistical analysis of PAM expression principally in NE- and non-NE-NSCLC cell lines is being carried out in our group (Scott et al., manuscript in preparation).

As PHM and PAL are expressed as a single bifunctional precursor, we had expected good correlation between the levels of the two activities. The data here show that although PHM and PAL activities are highly correlated with each other in cell extracts (Fig. 3A), there is a poor correlation between total PHM and PAL activities (Fig. 3B), or between total activities and mRNA levels (Fig. 4A, B). This poor correlation is partly due to inhibition or catabolism of PAM activities released from the cells into their growth medium. Although non-NE cell lines might not be expected to actively secrete proteins into their growth medium, several reports in the literature support our finding that non-NE cell types express significant levels of PAM proteins, and that these proteins can be exported from the cell via a non-regulated secretion pathway [26,29,30]. Furthermore, the PAM gene in a variety of species is expressed as cell- and tissue-specific splice variants [30–32]. Our recent report of expression of an apparently human-specific splice form containing a novel uncharacterized exon [20], and demonstration of four splice forms in the transmembrane domain [33], allow for 16 human PAM forms (Fig. 1B). We have presented preliminary data which suggest particular splice variants are expressed by different histological classes of lung tumor cells [34]. Northern analysis cannot distinguish between these forms. The protein variants which have been studied to date have different characteristics, including cellular localization and routing, and biochemical enzyme kinetics. A principal difference is that the PHM enzymatic rate is dependent on splicing of the transmembrane region, and on separation of the bifunctional PAM into monofunctional PHM and PAL enzymes [35]. These differences would also contribute to the poor correlation between PHM and PAL activities secreted from tumor cell lines (Fig. 3B) and between PAM mRNA expression and either PHM or PAL activity (Fig. 4A and B).

Compared to the data for PAM mRNA and activity expression, AADC mRNA expression and decarboxylase enzyme activity using DOPA as a substrate are reasonably well correlated over several orders of magnitude (Fig. 4C). This finding suggests that Northern blot or quantitative PCR analysis might be an appropriate technique to replace the tedious and technically difficult radiochemical and HPLC assays currently used for determination of AADC enzyme activity. We have commenced a study to investigate in situ

hybridization for detection of AADC expression in tumor cells in clinical specimens.

These data demonstrate that classic SCLC and carcinoid NSCLC cell lines do show co-expression of the prohormone processing gene PAM and NE phenotype marker AADC (Figs. 2 and 5A). However, the finding that most NSCLC lines and the variant SCLC sub-group also express PAM but do not express AADC activity demonstrates that different aspects of the NE phenotype can be controlled discordantly in these lung tumor histologies (Figs. 2 and 5B). This analysis is strengthened by the quantitative nature of the assays performed. Unlike many NE markers which are measured using relatively non-quantitative immunohistochemical staining, the quantitative nature of this data shows that expression of PAM at both the RNA and the total enzyme activity levels is similar in NSCLC to that of the highest of the SCLC lines (Table I). In contrast, NSCLC cell lines generally do not express high levels of AADC as either RNA or enzyme activity. Frequent expression of PAM in NSCLC cell lines differs from previous reports suggesting that NE-NSCLC represents 10–20% of NSCLC [1]. However, our data agree with several reports that the percentage of identified NE-NSCLC varies significantly depending on the markers studied. Proportions as high as 70% for neuron-specific enolase and 68.5% for carcinoembryonic antigen have been reported [2].

Co-expression of PAM and AADC in classic SCLC and carcinoid NSCLC cell lines suggests common mechanism/s for regulation of these aspects of the NE phenotype in these tumor cell histologies. Presumably the transcription factors expressed in these cell types exert control over both aspects of the NE phenotype, although the determination of the mechanism/s will depend on cloning of the promoter elements of these genes. However, the NE phenotype is difficult to define for NSCLC cells. The NSCLC lines studied here would not be considered neuroendocrine using the criterion of the NE marker AADC. However, high levels of PAM enzymes (equivalent to those found in SCLC cell lines) suggests that as yet unidentified autocrine amidated peptide hormones are relevant to the biology of these cells, and therefore these histologies could be considered NE. We have preliminary data supporting an autocrine role for amidated peptides in these cell types, as inhibition of PHM activity using chemical inhibitors or antisense technologies results in growth suppression in cell lines not previously known to express any α-amidated peptide [36,37]. A newly reported peptide, adrenomedullin, has recently been reported in human lung and lung tumors, and may be an autocrine growth factor for cell lines currently considered to be non-NE [38]. Presumably in these non-NE tumor histologies there are different transcription factors controlling separate aspects of the NE phenotype.

These data support the identification of peptidylglycine amidation enzymes as a novel NE marker, and broaden the definition of the NE phenotype. This analysis will assist in clarification of the role of NE processes, particularly autocrine effects involving peptide hormones, in tumor cell growth. Expression of the prohormone processing gene PAM in both NE and non-NE tumor cell lines, and in all tumor histologies in situ [22], suggests that autocrine biology involving α-amidated peptide hormones may be common in the promotion and proliferation stages of carcinogenesis, and so might provide targets for novel early detection and intervention approaches.

ACKNOWLEDGMENTS

Dr. Nagatsu provided the cloned AADC cDNA and Prof. Engels provided the cloned hPAM cDNA used for the Northern analyses. Thanks to Mr. Ed Russell of the NCI-Navy Medical Oncology Branch for help with setting up the decarboxylase assays.

REFERENCES

1. Linnoila RI, Piantadosi S, Ruckdeschel JC (1994): Impact of neuroendocrine differentiation in non-small cell lung cancer. Chest 106:367S–371S.
2. Graziano SL, Tatum AH, Newman NB, Oler A, Kohman LJ, Veit LJ, Gamble GP, Coleman MJ, Barmada S, O'Lear S (1994): The prognostic significance of neuroendocrine markers and carcinoembryonic antigen in patients with resected Stage I and Stage II non-small cell lung cancer. Cancer Res 54:2908–2913.
3. Sorokin SP, Hoyt RF (1993): Workshop on pulmonary neuroendocrine cells in health and disease. Anat Rec 236:167.
4. Abe K, Kameya T, Yamaguchi K, Kikuchi K, Adachi I, Tanaka M, Kimura S, Kodama T, Shimosato Y, Ishikawa S (1984): Hormone producing lung cancers; endocrinologic and morphologic studies. In Becker KL, Gazadar AF (eds): "The Endocrine Lung in Health and Disease." Philadelphia: W.B. Saunders Co., pp 549–593.

5. Gazdar AF, Helman LJ, Israel MA, Russell EK, Linnoila RI, Mulshine JL, Schuller HM, Park J-G (1988): Expression of neuroendocrine cell markers L-dopa decarboxylase, chromogranin A, and dense core granules in human tumors of endocrine and nonendocrine origin. Cancer Res 48:4078–4082.
6. Jensen SM, Gazdar AF, Cuttitta F, Russell EK, Linnoila RI (1989): A comparison of synaptophysin, chromogranin, and L-dopa decarboxylase as markers for neuroendocrine differentiation in lung cancer cell lines. Cancer Res 50:6068–6074.
7. Linnoila RI (1994): Pulmonary endocrine cells in vivo and in vitro. In Kaliner MA, Barnes PJ, Hunkel GHH, Baranuik JN (eds): "Neuropeptides in Respiratory Medicine." New York: Marcel Dekker, Inc., pp 197–224.
8. Becker KL (1984): The endocrine lung. In: Becker KL, Gazdar AF (eds): "The Endocrine Lung in Health and Disease." Philadelphia: W.B. Saunders Co., pp 3–41.
9. Aguayo SM, Miller YE, Waldron JA, Bogin RM, Sunday ME, Staton GW, Beam WR, King TE (1992): Idiopathic diffuse hyperplasia of pulmonary neuroendocrine cells and airways disease. New Engl J Med 327:1285–1288.
10. Moody TW, Pert CB, Gazdar AF, Carney DN, Minna JD (1981): High levels of intracellular bombesin characterize human small-cell lung carcinoma. Science 214:1246–1248.
11. Cuttitta F, Carney DN, Mulshine J, Moody TW, Fedorko J, Fischler A, Minna JD (1985): Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. Nature 316:823–826.
12. Carney DN, Cuttitta F, Moody TW, Minna JD (1987): Selective stimulation of small cell lung cancer clonal growth by bombesin and gastrin-releasing peptide. Cancer Res 47:821–825.
13. Mains RE, Dickerson IM, May V, Stoffers DA, Perkins SN, Ouafik L, Husten EJ, Eipper BA (1990): Cellular and molecular aspects of peptide hormone biosynthesis. Frontiers Neuroendocrinol 11:52–89.
14. Eipper BA, Stoffers DS, Mains RE (1992): The biosynthesis of neuropeptides: Peptide α-amidation. Annu Rev Neurosci 15:57–85.
15. Quinn KA, Treston AM, Scott FM, Kasprzyk PG, Avis I, Siegfried JM, Mulshine JL, Cuttitta F (1992): α-Amidation of peptide hormones in lung cancer. Cancer Cells 3:504–510.
16. Treston AM, Mulshine JL, Cuttitta F (1992): Control of tumor cell biology through regulation of peptide hormone processing. J Natl Cancer Inst Monogr 13:169–175.
17. Merkler DS (1994): C-terminal amidated peptides: Production by the in vitro enzymatic amidation of glycine-extended peptides and the importance of the amide to bioactivity. Enzyme Microb Technol 16:450–456.
18. Martínez A, Montuenga LM, Springall DR, Treston A, Cuttitta F, Polak JM (1993): Immunocytochemical localization of peptidylglycine alpha-amidating monooxogenase enzymes (PAM) in human endocrine pancreas. J Histochem Cytochem 41:375–380.
19. Steel JH, Martínez A, Springall DR, Treston AM, Cuttitta F, Polak JM (1994): Peptidylglycine α-amidating monooxygenase (PAM) immunoreactivity and messanger RNA in human pituitary and increased expression in pituitary tumors. Cell Tissue Res 276:197–207.
20. Vos MD, Jones JE, Treston AM (1995): Human peptidylglycine α-amidating monooxygenase transcripts derived by alternative mRNA splicing of an unreported exon. Gene 163:307–311.
21. Treston A, Scott F, Vos M, Iwai N, Mains R, Eipper B, Cuttitta F, Mulshine J (1993): Biochemical characterization of peptide α-amidation enzyme activities of human neuroendocrine lung cancer cell lines. Cell Growth Differ 4:911–920.
22. Saldise L, Martínez A, Montuenga LM, Treston A, Springall D, Polak JM, Vázquez JJ (1995): Distribution of peptidyl-glycine α-amidating monooxygenase (PAM) enzymes in normal lung and in lung epithelial tumors. J Histochem Cytochem 44:3–12.
23. Beaven M, Wilcox G, Terpstra G (1978): A microprocedure for the measurement of $^{14}CO_2$ release from [^{14}C]carboxyl-labelled amino acids. Anal Biochem 84:638–641.
24. Glauder J, Ragg H, Rauch J, Engels JW (1990): Human peptidylglycine α-amidating monooxygenase: cDNA, cloning and functional expression of a truncated form in cos cells. Biochem Biophys Res Commun 169:551–558.
25. Ichinose H, Kurosawa Y, Titani K, Fujita K, Nagatsu T (1989): Isolation and characterization of a cDNA clone encoding human aromatic L-amino acid decarboxylase. Biochem Biophys Res Commun 164:1024–1030.
26. Yun H-Y, Keutmann HT, Eipper BA (1994): Alternative splicing governs sulfation of tyrosine or oligosaccharide on peptidylglycine α-amidating monooxygenase. J Biol Chem 269:10946–10955.
27. Kalemkerian GP, Mabry M (1993): Cellular and molecular biology of small cell lung cancer. In Roth JA, Cox JD, Hong WK (eds): "Lung Cancer." Boston: Blackwell Scientific Publications, pp 57–84.
28. Treston AM, Yergey A, Kasprzyk PG, Cuttitta F, Mulshine JL (1988): Application of mass spectrometry to the identification of novel peptide hormones involved with lung cancer biology. "The Biology of Lung Cancer: Diagnosis and Treatment." In Rosen S, Mulshine J, Cuttita F, Abrams P (eds): New York, NY: Marcel Dekker, pp 91–119.
29. Tausk FA, Milgram SL, Mains RE, Eipper BA (1992): Expression of a peptide processing enzyme in cultured cells: Truncation mutants reveal a routing domain. Mol Endocrinol 6:2185–2196.
30. Eipper BA, Green CB-R, Mains RE (1992): Expression of prohormone processing enzymes in neuroendocrine and non-neuroendocrine cells. J Natl Cancer Inst Monogr 13:163–168.
31. Stoffers DA, Green CB-R, Eipper BA (1989): Alternative mRNA splicing generates multiple forms of peptidylglycine α-amidating monooxygenase in rat atrium. Proc Natl Acad Sci USA 86:735–739.
32. Stoffers DA, Ouafik L, A EB (1991): Characterization of novel mRNAs encoding enzymes involved in peptide α-amidation. J Biol Chem 266:1701–1707.
33. Tateishi K, Arakawa F, Misumi Y, Treston AM, Vos MD, Matsuoka Y (1994): Isolation and functional expression of human pancreatic peptidylglycine α-amidating monooxygenase. Biochem Biophys Res Commun 205: 282–290.
34. Vos MD, Treston AM (1995): Alternatively spliced forms of peptide amidating enzyme are differentially expressed in neuroendocrine and non-neuroendocrine hu-

man lung cancer cell lines. Presented at Endocrine Society annual meeting, Washington, DC.

35. Husten EJ, Eipper BA (1994): Purification and characterization of PAM-1, an integral membrane protein involved in peptide processing. Arch Biochem Biophys 312:487–492.
36. Iwai N, Avis I, Scott F, Quinn K, Cuttitta F, Mulshine J, Treston A (1992): Growth inhibition of neuroendocrine lung cancer cell lines via inhibition of peptide α-amidating enzyme. Presented at Am. Assoc. Cancer Res. Annual Meeting, San Diego, CA.
37. Martínez A, Vos M, Treston AM (1995): Antisense expression of peptide amidating enzyme RNA reduces growth in tumor cells. Presented at Endocrine Society Annual Meeting, Washington, DC.
38. Martínez A, Miller MJ, Unsworth EJ, Siegfried JM, Cuttitta F (1995): Expression of Adrenomedullin in normal human lung and in pulmonary tumors. Endocrinology 136:4099–4105.
39. Glauder J, Ragg H, Rauch J, Engels JW (1990): Human peptidylglycine α-amidating monooxygenase: cDNA, cloning and functional expression of a truncated form in cos cells. Biochem Biophys Res Commun 169:551–558.