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Immunology

Expression of neurophysin-related precursor in cell membranes of a small-cell lung carcinoma

(Northern blots/*in situ* hybridization/Western blots/tumor antigen/lung neoplasms)

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ABSTRACT **A monoclonal antibody (mAb L6) to a small-cell lung carcinoma surface antigen recognizes a common epitope of vasopressin–neurophysin and oxytocin–neurophysin in hypothalamic nuclei. We now report on the identification of a neurophysin-like precursor in human lung carcinoma (LX-1) cell membrane. mAb L6 immunoaffinity chromatography of solubilized membranes resulted in a single band of ≈45 kDa. Western blot analysis demonstrated immunoreactivity of this band with mAb L6, anti-vasopressin, and an antibody to the vasopressin precursor, pro-pressophysin. N-terminal sequencing of this band demonstrated a 21-amino acid homology with the N terminus of human pro-pressophysin, and substitution of a Cys^{33} residue in the tumor antigen with Arg^{33}. Absence of immunoreactivity with the antibodies described above in cytosolic extracts and culture medium suggests nonsecretion of processed or intact pro-pressophysin-like peptide. Northern analysis of LX-1 mRNA with a 30-mer to the C terminus of rat pro-pressophysin resulted in a band of ≈1000 base pairs, 250 base pairs larger than hypothalamic message. *In situ* hybridization of LX-1 tumor-bearing nude rat brain with the same probe demonstrated specific hybridization in rat hypothalamus and xenografted tumor. These findings suggest expression of a pro-pressophysin-like protein in this tumor cell line that is preferentially targeted to the cell membrane.**

Lung carcinomas are well known for their association with paraneoplastic syndromes, often through production and secretion of peptide hormones. Hormones such as vasopressin (VP) (1), neurophysin (NP) (2), and bombesin (3) can stimulate growth of tumor cells by functioning as autocrine growth factors. Elevated plasma levels of tumor-secreted hormones have been documented in patients with small-cell lung carcinomas and thus may allow early detection of tumor and monitor efficacy of treatment (4).

We have been characterizing a monoclonal antibody generated against a human lung adenocarcinoma (mAb L6) that binds to a surface epitope of human lung, colon, breast, and ovarian carcinomas (5). Our studies demonstrated that mAb L6 specifically recognizes a common domain within vasopressin-neurophysin (VP-NP) and oxytocin-neurophysin (OT-NP) (6). Since mAb L6 is immunoreactive with a membrane-bound protein, it was of interest to determine whether the mAb L6-reactive surface antigen was NP or NP related.

We now report on the identification of a NP-like precursor isolated from the human lung cancer cell line, LX-1 (7). This tumor cell line does not appear to process or secrete this precursor, being targeted instead to the cell membrane. To our knowledge no one else has reported a neuroendocrine hormone precursor being preferentially expressed in cell membranes.

MATERIALS AND METHODS

Materials. ^{125}I (140 mCi/ml; 1 Ci = 37 GBq), [^{35}S]cysteine (1064 Ci/mmol), deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate (1300 Ci/mmol), and [α-^{32}P]dATP (300 Ci/mmol) were from New England Nuclear. Dimethylpimelimidate and porcine pancreatic carboxypeptidase B (type II) were from Sigma. Immobilon polyvinylidene difluoride (PVDF) membranes were obtained from Millipore. Synthetic pro-pressophysin (PPYsin) and pro-oxyphysin (POYsin) 30-mer oligonucleotides corresponding to the last 10 amino acids in VP-NP (exon 3; ref. 8) and the last 10 amino acids in OT-NP (exon 3; ref. 9), respectively, were from Midland Reagent (Midland, TX). Oligonucleotide 50-mer probes to human PPYsin and POYsin corresponding to the 16 amino acids of the C-terminal glycopeptide (exon 3; ref. 10) and the last 9 amino acids of OT-NP (exon 3; ref. 10), respectively, were synthesized on an Applied Biosystems model 380A synthesizer. Bovine pituitary glands were obtained locally, and PPYsin was extracted as described (11).

Antibodies. mAb L6 is an IgG_{2a} mAb that recognizes a cell-surface antigen in human lung, breast, colon, and ovarian carcinomas (5) as well as specifically recognizing a common domain of both VP-NP and OT-NP (6). P1.17, an IgG2a mAb, was used as a control in the blotting experiments. YL-3 is a polyclonal antibody to a synthetic peptide fragment bridging the Lys-Arg cleavage site between the VP and VP-NP sequences of human PPYsin (see Table 1). This antibody specifically labels the paraventricular (PVN) and supraoptic (SON) nuclei of rat hypothalamus by immunohistochemistry and PPYsin in rat and mouse hypothalamus, as well as human, bovine, and mouse pituitary by immunoblotting (12). Polyclonal antibody to VP has been characterized (13).

LX-1 Cells and Tumors. Lung carcinoma cell line LX-1, established from a human small-cell carcinoma (7), was grown in spinner flasks and intracerebrally inoculated in the right cerebral hemisphere of nude rats as described (14). In addition to NP-related immunoreactivity (6), these cells demonstrate significant cytoplasmic reactivity for neuron-specific enolase and bombesin; moderate levels of somatostatin; and no reactivity with OT, motilin, vasoactive intes-

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Abbreviations: mAb, monoclonal antibody; NP, neurophysin; OT, oxytocin; PVN, paraventricular nucleus; SON, supraoptic nucleus; VP, vasopressin; POYsin, pro-oxyphysin; PPYsin, pro-pressophysin; PPLP, pro-pressophysin-like protein; PVDF, poly(vinylidene difluoride).

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tinal peptide, corticotropin, calcitonin, and β-endorphin (G.N. and L.C.R., unpublished observations). Since these cells were screened by immunohistochemistry, it is not possible to determine whether these peptides are being processed and/or secreted.

Immunoaffinity Isolation of LX-1 Antigen. mAb L6 was cross-linked to protein A-Sepharose (12 mg of mAb per ml of gel) with 20 mM dimethylpimelimidate (16). LX-1 cells (5×10^8 cells per ml) were solubilized in a lysis buffer containing 10 mM Tris·HCl (pH 8.2), 0.15 M NaCl, 1 mM EDTA, 10^{-4} M phenylmethylsulfonyl fluoride, and 0.5% (vol/vol) Nonidet P-40 (buffer A). After a 15-min incubation on ice, the suspension was centrifuged at $3000 \times g$ for 10 min, and the supernatant was centrifuged at $100,000 \times g$ for 1 hr. Nonspecific antibody binding was eliminated with formalin-fixed *Staphylococcus aureus* (16). This was also done prior to immunoprecipitation (see below). The supernatant was added to the mAb L6/protein A affinity matrix, rotated overnight at 4°C, and pelleted at $500 \times g$ (2 min). The pellet was washed with buffer A, followed by washes in 50 mM Tris·HCl (pH 8.2) containing 0.15 M NaCl, until the A_{280} was background. Specifically bound antigen was eluted with 50 mM diethylamine (pH 11.5) and neutralized with 0.5 M NaH_2PO_4 (16).

LX-1 Cell Labeling with [^{35}S]Cysteine and ^{125}I. Two days after passaging, [^{35}S]cysteine was added (1 mCi per 10^8 LX-1 cells) and incubated for 20 hr. Cells were surface iodinated with lactoperoxidase (50 μg/ml), glucose oxidase (25 μg/ml), and $Na^{125}I$ (1 mCi per 10^8 cells). The reaction was initiated with glucose (250 μg/ml), incubated for 20 min at 25°C, and quenched with KI (0.4 mg/ml). Both ^{35}S- and ^{125}I-labeled cells were washed three times in 50 mM NaH_2PO_4 (pH 7.5) containing 0.15 M NaCl and solubilized as described above.

Immunoprecipitation of Cell Extracts. Solubilized LX-1 cells (5×10^6 cells per tube) were incubated with YL-3 antibody (1:500) overnight at 4°C. Protein A-Sepharose (50 μl) was added and equilibrated for 30 min at 4°C. The beads were pelleted and washed sequentially in 50 mM Tris·HCl (pH 8.2) containing 0.5 M NaCl, in 50 mM Tris·HCl (pH 8.2) containing 0.1% (vol/vol) SDS, and in 10 mM Tris·HCl (pH 7.4) containing 0.1% (vol/vol) Nonidet P-40. The pellet was heated (100°C; 2 min) in Laemmli sample buffer (15) and centrifuged, and the supernatants were electrophoresed.

Gel Electrophoresis and Immunoblotting. The discontinuous buffer system of Laemmli (15) was used for SDS/polyacrylamide gel electrophoresis with 12.5% acrylamide gels. In some experiments, purified antigen was deglycosylated with glycopeptidase F before electrophoresis (17). Gels were stained with Coomassie blue or silver (18). Immunoblotting was done as described by Rosenbaum *et al.* (19). Gels containing ^{35}S and ^{125}I samples were dried and exposed to Hyperfilm for 48 hr.

Amino Acid Sequencing. Nondenatured purified LX-1 antigen (150 pmol) was digested with carboxypeptidase B (0.6 μg) in 0.3 mM *N*-ethylmorpholine acetate (pH 8.5) for 5 hr at 37°C. The reaction was quenched with acetic acid (pH 3.0). Samples were electrophoresed as described above and electroblotted onto Immobilon PVDF membranes (20). Membranes were stained in 0.1% (wt/vol) Coomassie blue R-250 and destained, and the band was excised (20). The band was sequenced by automated Edman degradation in a gas-phase sequencer (model 470A; Applied Biosystems) equipped with an Applied Biosystems 120A PTH analyzer. Approximately 60 pmol of LX-1 antigen was sequenced based on the yield of identified alanine.

Northern Blot Analysis. Total RNA was prepared as described (21). Poly $(A)^+$ RNAs were isolated with oligo(dT)-cellulose (22). Samples were fractionated on 1.5% agarose/2.2 M formaldehyde gels and transferred to nylon membranes as described (23). Membranes were prehybridized overnight at 45°C in 5× SSC/20 mM NaH_2PO_4, pH 7.5, containing 20% (vol/vol) formamide, 5× Denhardt's solution, 0.1% (vol/vol) SDS, and 10 μg of salmon sperm DNA per ml. (1× SSC = 0.15 M NaCl/0.015 M sodium citrate; 1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone). Filters were then hybridized (45°C for 24 hr) in a similar buffer containing 1× Denhardt's solution, 1.0 μg of salmon sperm DNA per ml, and 4 nM 30-mer oligonucleotide (VP specific) ^{32}P labeled at the 3′ end to a specific activity of $5–8 \times 10^8$ dpm/nmol. Oligonucleotides were labeled on the 3′ end with terminal deoxynucleotidyltransferase (23). Membranes were washed in 2× SSC containing 1% (vol/vol) SDS at 45°C (22) before autoradiography.

***In Situ* Hybridization.** Rats were anesthetized with sodium pentobarbital (50 mg per kg of body wt, i.p.) and perfused through transcardiac puncture with saline followed by buffered 10% formalin. The brain was removed and cryoprotected with 30% (wt/vol) sucrose containing 0.02% (vol/vol) diethylpyrocarbonate. Cryostat sections (10 μm) were cut in the coronal plane to include the tumor-bearing region and hypothalamus and mounted on silane-treated glass slides. *In situ* hybridization was performed with oligonucleotide probes as described (34). Sections were delipidated (progressively graded alcohols and chloroform) and rehydrated to 2× SSC. Sections were then prehybridized (1 hr, 25°C) with 2× SSC containing 50% (vol/vol) formamide, 10× Denhardt's solution, 0.1% (vol/vol) SDS, and 0.1% (wt/vol) salmon sperm DNA (hybridization buffer). Sections were then overlaid with hybridization buffer containing oligonucleotide probes ^{35}S-labeled at the 3′ end (2.0×10^5 cpm in 30 μl) and 0.1 M dithiothreitol (24 hr; 25°C). Sections were rinsed in 2× SSC (4 hr; 25°C; with 15-min changes), air dried, exposed to Hyperfilm or dipped in Kodak emulsion (NTB-3). After 2 weeks, the sections were developed, counterstained, dehydrated, and coverslipped for microscopic analysis.

Sequence homology between the rat and human PPYsin in the 30-mer region of the rat probe allows detection of both rat and human PPYsin mRNA. The 50-mer human oligonucleotide probe recognizes a unique domain in the human sequence and, consequently, should only hybridize with the human PPYsin. The following controls were also performed to minimize the possibility of a false-positive signal; (*i*) prehybridization RNase treatment of tissue; (*ii*) addition of excess unlabeled probe.

Radioimmunoassay. Radioimmunoassay for plasma VP in nude rats was performed as described (24).

RESULTS

Immunological Characterization of the LX-1 Antigen. The isolation and immunologic characterization of LX-1 cell-surface antigen are shown in Fig. 1. Solubilized membranes that were electrophoresed and stained with Coomassie blue (Fig. 1, lane 1) demonstrated weak immunoreactivity when blotted and probed with various antibodies. Specific tumor antigen was then isolated by mAb L6 immunoaffinity chromatography, resulting in a band of ≈45 kDa by silver staining (lane 2). A weak 42-kDa band appears to copurify with the 45-kDa protein and may be a proteolytic fragment, although it is not immunoreactive with the antibodies used in this study. The 45-kDa fragment is highly immunoreactive with antibody YL-3 (lane 3), strongly suggesting the mAb L6-isolated LX-1 surface antigen is related to PPYsin. Lane 4 demonstrates the ability of the antigen to bind mAb L6, while mAb P1.17 fails to show immunoreactivity (lane 5). The ability of the 45-kDa band to bind anti-VP (lane 6) confirms its identity as a PPYsin-like protein (PPLP). Polyclonal antibodies to processed NP and OT failed to demonstrate immunoreactivity with the purified antigen (data not shown),

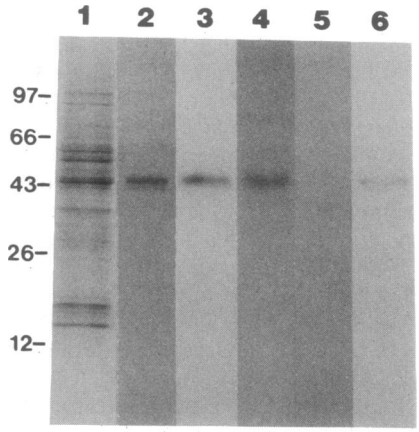

FIG. 1. Isolation and immunological characterization of LX-1 cell-surface antigen. LX-1 cell membranes were solubilized, purified, electrophoresed, blotted, and probed with antibody as described in *Materials and Methods*. Lanes: 1, Coomassie-stained gel pattern of solubilized LX-1 cell membranes (20 μg); 2, silver-stained gel pattern of mAb L6 immunoaffinity-purified LX-1 antigen (0.5 μg); 3–6, immunoblots of purified LX-1 antigen (3.0 μg) probed with YL-3 (1:500; lane 3), mAb L6 (20 μg/ml; lane 4), mAb P1.17 (20 μg/ml; lane 5), and polyclonal anti-VP (1:500; lane 6). Molecular weight markers were phosphorylase b (M_r 97,111), bovine serum albumin (M_r 66,296), ovalbumin (M_r 42,807), chymotrypsinogen A (M_r 25,666), and cytochrome *c* (M_r 11,761).

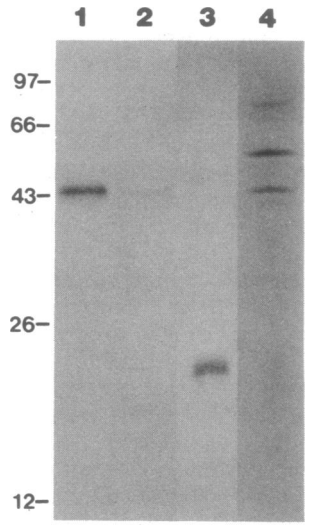

FIG. 2. Western blot analysis and immunoprecipitation of LX-1 antigen with anti-human PPYsin. Samples were electrophoresed, blotted, and probed as described in Fig. 1. LX-1 cells were incubated with [^{35}S]cysteine, solubilized, and immunoprecipitated with YL-3 antibody as described in *Materials and Methods*. Lanes: 1 and 2, immunoblots of immunoaffinity-purified LX-1 cell membrane (0.5 μg; lane 1) and LX-1 cytosolic extract (50 μg; lane 2) probed with YL-3 (1:500); 3, immunoblot of bovine PPYsin (2.5 μg) probed with YL-3 (1:500); 4, autoradiogram of [^{35}S]cysteine incubated LX-1 cells immunoprecipitated with YL-3. Molecular weight markers are the same as in Fig. 1.

whereas L6 immunoreacts with both processed NP and PPYsin (6).

Since previous studies (5, 6) suggested mAb L6 immunoreactivity to be confined to the surface of LX-1 tumor cells, it was of interest to confirm this. Fig. 2 shows purified LX-1 antigen (lane 1), LX-1 cytosolic extract (lane 2), and bovine pituitary PPYsin (lane 3) probed with YL-3. As demonstrated above, LX-1 membrane antigen reacts strongly with YL-3, resulting in a 45-kDa band (lane 1). The cytosolic extract shows a faint band at 45 kDa and a slight band at 23 kDa when probed with YL-3 (lane 2). The position of the band in lane 3 confirms the reactivity of YL-3 with PPYsin (23 kDa). Since the protein loaded in lane 2 was 100 times more than that in lane 1, it appears the antigen is found predominantly in the cell membrane. The culture medium was negative for immunoreactivity with anti-VP, anti-NP, YL-3, and mAb L6 by immunoblotting, and the serum from tumor-bearing nude rats showed no increase in VP levels by radioimmunoassay when compared to controls (6). Finally, ^{125}I surface labeling of LX-1 cells demonstrated membrane localization of antigen (data not shown). All of these results confirm the nonsecretory nature of LX-1 cells and the preferential targeting of PPLP to LX-1 cell membrane.

A potential explanation for the high molecular weight PPLP LX-1 antigen is extensive glycosylation, altering mobility on the gel, or a dimerization of PPYsin. Purified tumor antigen was digested with glycopeptidase F, blotted, and probed with YL-3 antibody. This resulted in an immunoreactive 35-kDa band, and the complete elimination of the 45-kDa band (data not shown). When cultured LX-1 tumor cells were incubated with [^{35}S]cysteine and immunoprecipitated with YL-3, a 45-kDa band was seen when electrophoresed under reducing and nonreducing conditions, corresponding to the purified antigen (Fig. 2, lane 4). A band of ≈57 kDa (lane 4) was also detected, which could represent a larger, less processed 45-kDa protein. These studies suggest that the 45-kDa band is not the result of dimerization or excessive glycosylation but, rather, a unique form of PPYsin.

N-Terminal Sequencing of LX-1 Tumor Antigen. Table 1 summarizes the results of the sequence analysis of LX-1 tumor antigen. Initial attempts to sequence the purified antigen from HPLC fractions in acetonitrile or directly from protein electroblotted onto PVDF membranes were unsuccessful due to N-terminal blockage. To circumvent this, the protein was subjected to limited proteolysis with non-N^{α}-(*p*-tosyl-L-lysine chloromethyl ketone-treated carboxypeptidase B containing a trace amount of trypsin. Automated Edman degradation of the cleaved antigen electroblotted onto PVDF membranes revealed a 21-amino acid sequence homology with the N terminus of human PPYsin (see Table 1). A deviation between the two sequences is the substitution

Table 1. Comparison of N-terminal sequences of PPYsin and LX-1 cell-surface antigen

Normal human PPYsin*																															
				5					10					15					20					25					30			
C	Y	F	Q	N	C	P	R	G	G	K	R	A	M	S	D	L	E	L	R	Q	C	L	P	C	G	P	G	G	K	G	R	C	F
LX-1 tumor antigen†																															
														15					20					25					30			
													A	M	S	D	L	E	L	R	Q(C)L			P(C)G			P(G)(G)		K(G)R		R	F
Immunogen for YL-3 antibody‡																															
									10					15																		
						Y	P	R	G	G	K	R	A	M	S	D	L															

Parentheses indicate tentative amino acid assignment.
*Deduced amino acid sequence from human pre-PPYsin gene (10).
†N-terminal sequence determined by automated Edman degradation.
‡Antibody YL-3 was generated to a synthetic peptide that includes the Lys^{11}-Arg^{12} cleavage site between VP and VP-NP, with a tyrosine residue attached to the N terminus to allow iodination of the synthetic peptide.

of an Arg^{33} residue in the tumor antigen in place of Cys^{33} in human PPYsin. Our sequence analysis confirms the identity of the mAb L6-isolated tumor antigen as PPLP.

Molecular Characterization of the PPLP. To characterize the expression of PPLP in LX-1 tumor cells, oligonucleotides directed to the C terminus of human and rat PPYsin were used in Northern and *in situ* hybridization analyses. *In situ* hybridization of LX-1 tumor xenografts in nude rat brain with the 30-mer probe to rat PPYsin demonstrated specific hybridization within VPergic neurons in the SON and PVN nuclei of rat hypothalamus (Fig. 3*Bi*). Autoradiographic grains were localized in the intracerebral xenograft confirming expression of VP-like mRNA in the tumor cells. When serial adjacent sections of tumor-bearing nude rat brain were hybridized with the 50-mer probe to human PPYsin (Fig. 3*Bii*), hybridization was confined to the tumor xenograft, with no labeling detected within VPergic neurons of the rat hypothalamus. These studies confirmed the stringency of hybridization as well as the specificity of the human and rat PPYsin probes used in the experiments. Parallel experiments with the 30-mer rat and 50-mer human synthetic POYsin probes performed on adjacent sections of tumor-bearing nude rat brain failed to demonstrate the presence of OT-like mRNA in the LX-1 tumor xenografts (Fig. 3*B iii* and *iv*). Hybridization within OTergic neurons of the rat SON and PVN in sections probed with the 30-mer rat probe (Fig. 3*Biii*) confirmed the stability of tissue mRNAs and the specificity of the probe. Absence of signal in corresponding hypothalamic regions of sections probed with the 50-mer human probe (Fig. 3*Biv*) further validated specificity of hybridization.

Size fractionation of rat hypothalamic and LX-1 RNA by Northern analysis is shown in Fig. 4. When rat hypothalamic total RNA was probed with the ^{32}P-labeled rat 30-mer, a strong signal was observed corresponding to the reported size (9) of 750 base pairs (lane 1). Total RNA from rat cerebellum failed to hybridize with the rat 30-mer probe (lane 2). When LX-1 tumor poly(A)$^+$ RNA was probed with the rat 30-mer, a band corresponding to ≈1 kilobase was detected (lane 3). This is ≈250 base pairs larger than the reported size of normal PPYsin mRNA. Both *in situ* hybridization and Northern analysis demonstrate the expression of PPLP mRNA in LX-1 human tumor xenografts and in cultured cells.

DISCUSSION

We have previously demonstrated that mAb L6 specifically labels both VP-NP and OT-NP in the cytoplasm of rat and human hypothalamic neurons, in addition to binding to a membrane-bound antigen in LX-1 lung carcinoma (6). Although it was initially felt that mAb L6 identified a ganglioside antigen (5), we now report on the characterization of a PPLP isolated from LX-1 cells with mAb L6. This 45-kDa antigen shares a 21-amino acid homology with the N terminus of human PPYsin as demonstrated by sequence analysis. Hybridization with oligonucleotides corresponding to the C terminus of PPYsin with LX-1 mRNA suggests additional homology at this region. The fact that PPLP is almost twice the reported size of PPYsin from bovine, human, or rat (see ref. 25 for review) raises some interesting issues.

High molecular weight forms of NP have been reported, which appear to cross-react with antibodies to both VP and VP-NP (26). Pulse–chase experiments have demonstrated a series of high molecular weight VP-NP precursors (27). Although some of these data may be attributed to high molecular weight aggregates due to dimerization or hydrophobic interactions between PPYsin and NP (28), we have shown this is not the case with PPLP. Electrophoresis of purified PPLP or immunoprecipitated ^{35}S-labeled LX-1 cells demonstrated a band of identical molecular weight under both reducing and nonreducing conditions. Our studies with glycopeptidase F show that glycosylation alone cannot account for this difference. The 1-kilobase message detected in the LX-1 mRNA could potentially encode a primary sequence corresponding to the deglycosylated 35-kDa protein. The demonstration of amino acid homology at the N terminus and nucleotide homology at the C terminus between PPLP and PPYsin leads us to speculate that the size difference between these two proteins resides in the middle domain of VP-NP. Whether PPLP is the product of alternative splicing

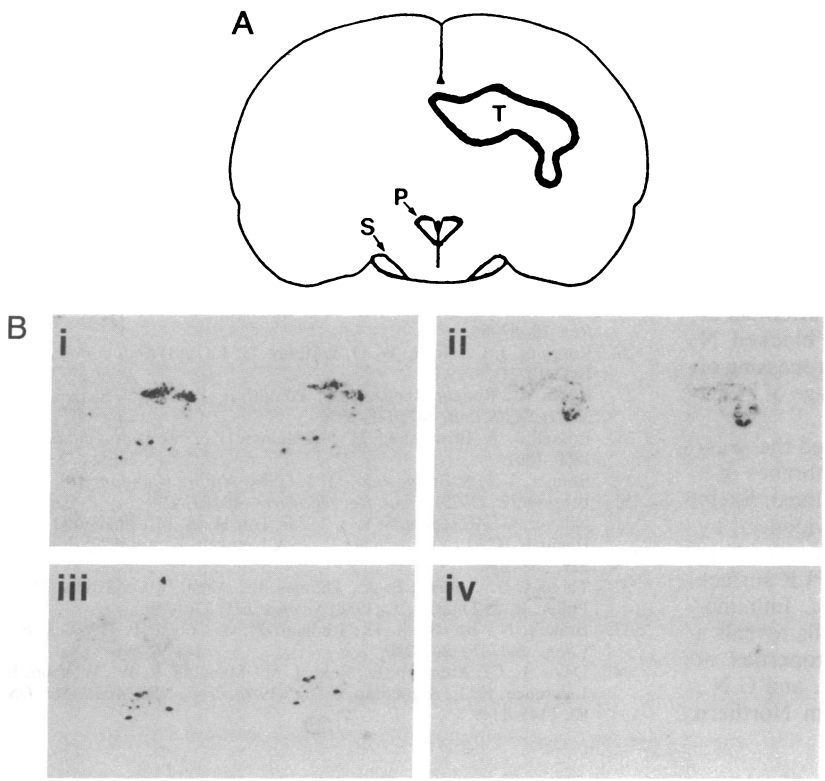

FIG. 3. *In situ* hybridization of LX-1 tumor-bearing nude rat brain. (*A*) Diagrammatic representation of LX-1 tumor-bearing nude rat brain section used for the *in situ* hybridization as shown below. Intracerebrally xenografted LX-1 tumor (T) is located dorsolateral to the hypothalamus, in the left cerebral hemisphere. The paraventricular nuclei (P) are located on each side of the third ventricle (line), and the supraoptic nuclei (S) are dorsal to the optic tracts. (*B i–iv*) Whole brain autoradiograms (two whole brain sections per panel) of *in situ* hybridization histochemistry on adjacent (coronal) sections of LX-1 tumor-bearing nude rat brain. Sections were probed with (*i*) ^{35}S-labeled 30-mer oligonucleotide to the C terminus of rat PPYsin; (*ii*) ^{35}S-labeled 50-mer oligonucleotide to the C terminus of human PPYsin; (*iii*) ^{35}S-labeled 30-mer oligonucleotide to the C terminus of rat POYsin; and (*iv*) ^{35}S-labeled 50-mer to the C terminus of human POYsin.

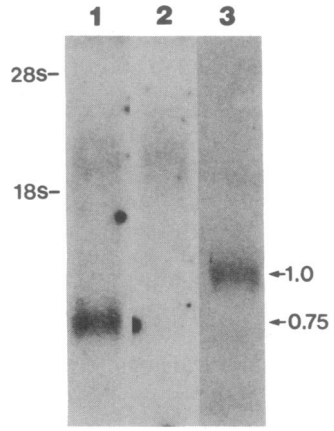

FIG. 4. Northern blot analysis of LX-1 lung tumor RNA. RNA was extracted and purified as described in *Materials and Methods*. Lanes: 1, rat hypothalamic total RNA (20 μg); 2, rat cerebellar total RNA (20 μg); 3, poly(A)$^+$ RNA from LX-1 cells (2 μg). All lanes were probed with a 3′ ^{32}P-labeled 30-mer synthetic oligonucleotide to the C terminus of rat PPYsin. The positions of 28S and 18S ribosomal RNAs from lymphoma total RNA are shown. Normal rat hypothalamic message (750 base pairs) and the 1.0-kilobase band from LX-1 mRNA are noted.

of the reported VP gene or is an entirely new gene product remains to be seen. The possibility of a second gene for VP has been proposed (29), although this has yet to be confirmed.

In addition to finding differences in molecular mass of the translated protein and the size of the mRNA, we have identified an Arg^{33} residue in PPLP in place of Cys^{33} in normal PPYsin. Since the tertiary conformation of VP-NP is dependent on seven disulfide bonds (30), this substitution could affect the antigenicity of NP. Since reduction of the disulfide bonds in NP can result in either decreased imunoreactivity with polyclonal antibodies (11) or increased reactivity with mAb L6 (31), replacement of Cys^{33} in PPLP with Arg^{33} likely contributes to a unique conformation.

Several lines of evidence suggest that PPLP is targeted unprocessed to LX-1 cell membrane, and in this report we confirm that PPLP is localized in LX-1 membranes. There are several potential explanations for the expression of unprocessed PPLP in LX-1 membranes. The first is absence of processing enzyme (32) in the LX-1 cell line. However, LX-1 cells incubated with a synthetic fluorogenic substrate containing a Lys-Arg cleavage site cleave at this dibasic site (L.C.R. and G.N., unpublished observations). A second possibility is the lack of a Lys-Arg cleavage site in PPLP. However, antibody YL-3, which only reacts with an intact Lys-Arg (Table 1), demonstrates strong immunoreactivity with PPLP. A third alternative is posttranslational modification of PPLP preventing processing. Our initial attempts at N-terminal sequencing of PPLP resulted in a blocked N terminus. Since the N terminus is critical in the processing of POYsin synthetic analogs (33), N-terminal blockage of PPLP could potentially prevent its processing.

We have isolated, characterized, and confirmed the presence of an alternative form of PPLP in cell membranes of a small-cell lung carcinoma. PPLP expression most likely extends to other lung carcinomas as well, as evidenced by mAb L6 surface immunoreactivity in many types of lung tumors (5). We have recently demonstrated PPLP surface immunoreactivity in a breast and colon cell line. Immunoblotting of solubilized membranes from these cells reveals a 45-kDa band with identical immunological properties of PPLP described in this report (L.C.R., E.A.N., and G.N., unpublished observations). We need to perform Northern and amino acid sequence analyses on these cells to definitively establish the presence of PPLP. Such studies should provide additional information to better define the function and significance of NP (and/or its precursor) expression on the cell surface of these tumors. Once the complete structure of these antigens is elucidated, it may be possible to develop more effective antibodies against these surface antigens for immunodiagnosis and/or immunotherapy.

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