# BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF THE NEUROPHYSINS IN NORMAL AND NEOPLASTIC TISSUE

by

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## LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| ABC | avidin-biotin-peroxidase |
| ANF | atrial natriuretic factor |
| BCIP | 5-bromo-4-chloro-3-indolyl-phosphate |
| BSA | bovine serum albumin |
| CRF | corticotropin releasing factor |
| HoDI | homozygous Brattleboro rats with diabetes insipidus |
| HRP | horseradish peroxidase |
| mAb | monoclonal antibody |
| NBT | *p*-nitroblue tetrazolium chloride |
| NC | nitrocellulose membranes |
| NP | neurophysin |
| OT | oxytocin |
| OT NP | oxytocin-neurophysin |
| pAb | polyclonal antibody |
| PPLP | pro-pressophysin-like protein |
| PPYsin | pro-pressophysin |
| PVDF | polyvinylidene difluoride |
| PVN | paraventricular nucleus |
| RIA | radioimmunoassay |
| SDS | sodium dodecyl sulfate |
| SON | supraoptic nucleus |
| TTBS | 20 m<u>M</u> Tris, with 0.5 <u>M</u> NaCl, 0.1% Tween-20 and 0.02% $NaN_3$ |
| VP | vasopressin |
| VP NP | vasopressin-neurophysin |

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ABSTRACT

While studying the binding activity of a mouse monoclonal antibody (mAb L6) to LX-1 intracerebral tumor xenografts in nude rats we observed that L6 can also bind to a cytoplasmic antigen expressed in rat hypothalamus. In order to identify the nature of this binding, an immunoblotting method was developed to detect low-molecular-weight peptides with monoclonal antibodies that normally fail to demonstrate immunoreactivity using conventional blotting techniques. Detection of neurophysin, insulin, calcitonin, vasopressin, and $\beta$-endorphin electroblotted on nitrocellulose membranes were optimized after introducing four modifications into the conventional procedure. These include renaturing the gels after SDS electrophoresis, electroblotting the renatured gels in basic transfer buffer, heating the blots, and the use of avidin/alkaline phosphatase conjugates. This technique likely enables the denatured peptides to regain their native conformation and, therefore, restore antigenicity and recognition by highly structural specific monoclonal antibodies.

This immunoblotting method was used to characterize a mouse monoclonal antibody L6 (mAb L6) which identifies a cell surface antigen of many different human carcinomas. Immunohistological techniques demonstrated the binding of mAb L6 to a cytoplasmic antigen expressed in the magnocellular component of the hypothalamo-neurohypophysial system. Double-labeling experiments with antisera to vasopressin and oxytocin confirmed the localization L6 immunoreactivity within both peptide-containing cell groups. Oxytocin and vasopressin failed to block L6 staining which suggested that its target epitope resides within the

neurophysin sequence. Western blot analysis of bovine neurophysin and human pituitary extracts identified L6-immunoreactive bands that corresponded to the position of neurophysin and its precursor, propressophysin. mAb L6 thus immunoreacts with a cytoplasmic epitope in hypothalamic neurons and a membrane antigen of human lung cancer cell line LX-1.

LX-1 membrane antigen was analyzed for neurophysin-like domains. mAb L6 immunoaffinity chromatography of solubilized membranes resulted in a single band of approximately 45 kd. Western blot analysis demonstrated immunoreactivity of this band with mAb L6, antivasopressin and anti-pro-pressophysin. Amino-terminal sequencing of this band demonstrated a 21-amino acid homology with the N-terminus of human pro-pressophysin and substitution of an $Arg^{33}$ residue in the tumor in place of $Cys^{33}$. Absence of immunoreactivity in cytosolic extracts and culture medium suggests nonsecretion of processed or intact pro-pressophysin-like protein (PPLP). Northern analysis of LX-1 mRNA with a 30-mer to the C-terminus of rat pro-pressophysin resulted in a band of approximately 1 kb, 250 bp 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 demonstrate expression of PPLP in LX-1 cells.

In order to evaluate the problem of cross-reactivity the distribution of atrial natriuretic factor (ANF)-like reactivity was examined in rat heart and brain. Comparison of sequence data between rat ANF-28 and bovine neurophysins revealed three regions of three amino acid homology between these peptides. Preabsorption of ANF antiserum

with bovine neuorphysin I resulted in complete elimination of "ANF-immunoreactivity" in both atrium and hypothalamus. Cross-reactivity of ANF antiserum with bovine neuorphysin I and II was further confirmed by Western blotting. These findings suggest cross-reactivity can be an inherent problem when two proteins have shared antigenic epitopes, which in the case of neurophysin is conformationally dependent.

Changes in neurophysin conformation and thus antigenicity were demonstrated with the ability of mAb L6 to specifically label magnocellular VP and OT neurons, but not the parvicellular system. This was confirmed by immunohistochemistry, immunoprecipitation and Western blotting. These results imply the existence of neurophysin in a unique conformational form in hypothalamic parvicellular systems.

# INTRODUCTION

The neurophysins, although first identified over 30 years ago, have only recently begun to be fully characterized at both the biochemical and molecular level. With the recent cloning and sequencing of neurophysins from rat (Schmale and Richter, 1984), bovine (Land et al., 1982, 1983) and human (Sausville et al., 1985) much progress has been made in structurally defining these proteins. However, aside from functioning as carrier proteins for the nonapeptides vasopressin (VP) and oxytocin (OT), little is known about the physiological significance of the neurophysins.

One potential biological role of the neurophysins may be associated with the onset and development of carcinomas. It has been known for many years that a clear relationship exists between oat cell carcinoma for example (Schwartz et al., 1957; Lippscomb et al., 1968), and the production and secretion of both VP and neurophysin. These findings have suggested the possibility of monitoring neurophysin levels in the plasma as an indicator of tumor activity (North et al., 1988). The question of the physiological function of neurophysin in these tumors remains unclear, although studies have suggested that neurophysin may modulate growth and DNA synthesis in non-neuronal cells (Worley and Pickering, 1984). After a more thorough introduction on the structural characterization of neurophysin and the relationship to paraneoplastic syndromes, the rationale for these present studies will be discussed.

## A. Structural Characterization of the Neurophysins

In most mammalian systems there are two types of neurophysin; vasopressin-neurophysin (VP-NP) and oxytocin-neurophysin (OT-NP).

Single copy genes for OT and VP are contained in a single DNA fragment in all species studied to date. In the rat, the OT and VP genes are separated by 11 kb of intervening sequence on an 18 kb fragment and are transcribed from opposite DNA strands (Mohr et al., 1988). The human OT and VP genes are similarly organized on a single DNA fragment with a 12 kb intervening sequence (Sausville et al., 1985).

A schematic representation of the vasopressin and oxytocin genes and their translation products is shown in Fig. 1. The VP gene contains three exons and 2 introns in both rat (Schmale et al., 1983) and human (Sausville et al., 1985), and is 1.85 and 2.59 kb in length, respectively. Exon A codes for the signal sequence, VP, and the first nine amino acids of the N-terminus of VP-NP. The signal sequence, consisting of 19 amino acids including the methionine initiation site, is typical for hydrophobic secretory proteins (Blobel et al., 1975). Exon B codes for the middle portion of VP-NP and exon C for the last 17 amino acids at the C-terminus of VP-NP as well as the C-terminal glycopeptide (CPP). Extensive interspecies homology in the 5' flanking region of the VP gene, which contain promotor and enhancer sequences involved in binding RNA polymerase and initiation of transcription, suggest this region may be important in the control of gene expression.

Transcription of the VP gene and splicing, and polyadenylation of the VP precursor results in a mature mRNA species of approximately 750 bp in the rat (Ivell et al., 1984) and approximately 700 bp in the human (Sausville et al., 1985). VP mRNA codes for a 20 kd precursor in rat and 17 kd in human, termed pre-propressophysin. Following translation,

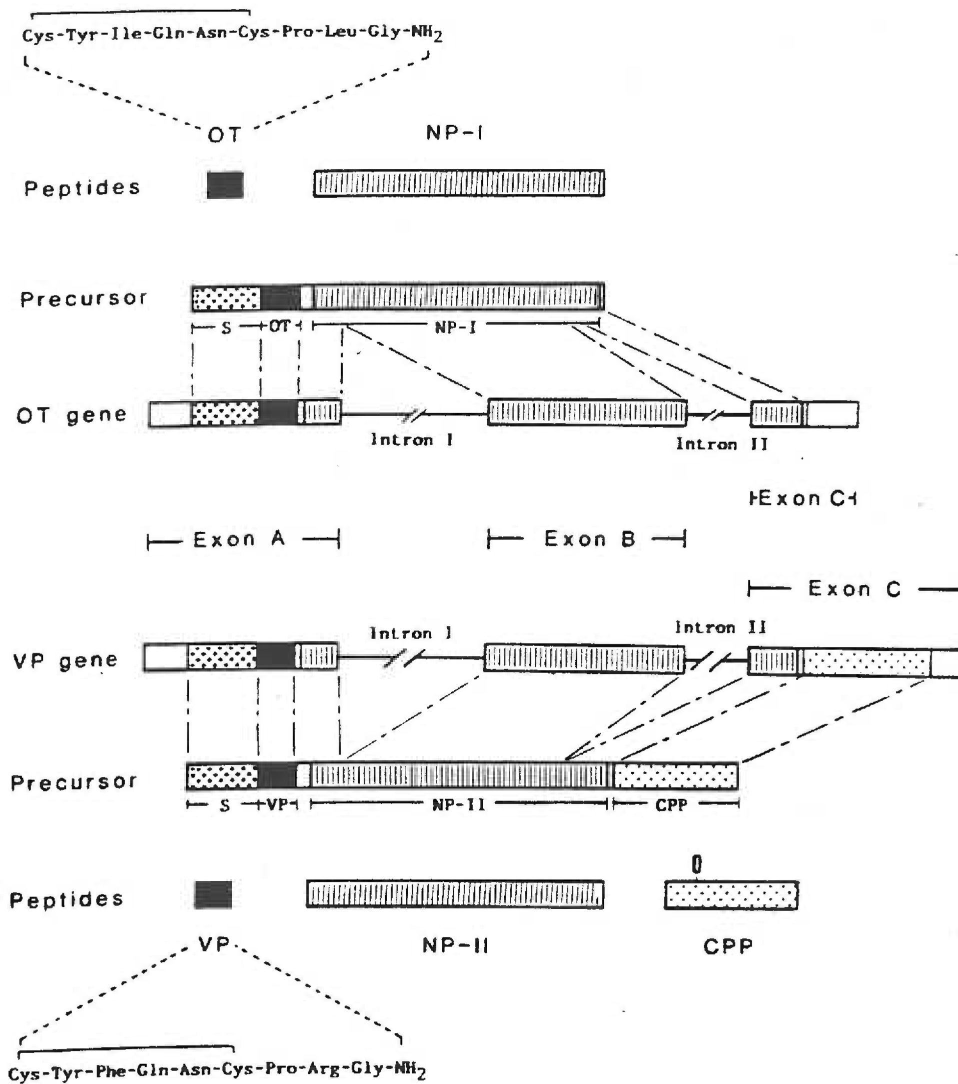


Figure 1: Structural organization of the vasopressin (VP) and oxytocin (OT) genes. S: signal peptide; VP-NP: vasopressin-neurophysin; OT-NP: oxytocin-neurophysin; O: asparagine glycosylation site on CPP. Both genes contain exons A, B, and C. The introns have been abbreviated. Adapted from Liu, 1988.

the signal sequence is removed (Schmale et al., 1981) to give propressophysin. The C-terminal peptide of propressophysin likely becomes glycosylated at the asparagine residue prior to further proteolytic processing in the secretory vesicles. Final processed polypeptides include VP, VP-NP and CPP.

A species comparison of propressophysin amino acid sequence is shown in Figure 2. VP is conserved in all species. There is 88% homology between rat VP-NP and human VP-NP and 90% homology between bovine VP-NP and human VP-NP. There are significant stretches (up to 23 amino acids) of homology between all three species. VP-NP is extremely cysteine rich, containing 14 cysteines and 7 disulfide bonds (Breslow, 1979). Interestingly, the positions of the cysteines are conserved in all three species. In the CPP sequence, there is a 65% homology between rat and human, and an 85% homology between bovine and human. The carbohydrate moiety in human CPP is thought to consist of a fucosylated, bisected complex oligosaccharide (Lambert, 1986) tentatively depicted in Figure 3.

The OT gene is considerably shorter than the VP gene, although it also contains three exons and two introns (Fig. 1). In the rat, the OT gene is approximately 850 bp (Ivell et al., 1984) and in the human is approximately 1.4 kb (Sausville et al., 1985). Exon A codes for the signal sequence, OT, and the first nine amino acids of OT-NP. Exon B codes for the middle portion of OT-NP and exon C for the last 18 amino acids of OT-NP. There is no C-terminal peptide coded for by the OT gene. As with the VP gene, there appears to be conserved promoter and enhancer sequences at the 5′ flanking regions, which may be involved in

```
Human    C Y F Q N C P R G   G K R   A M S D L E L
Bovine   C Y F Q N C P R G   G K R   A M S D L E L
Rat      C Y F Q N C P R G   G K R   A T S D M E L
         |_______VP________|         |___VP - NP___|
                                       *     *

         R Q C L P C G P G G K G R C F G P S I C C
         R Q C L P C G P G G K G R C F G P S I C C
         R Q C L P C G P G G K G R C F G P S I C C
         |________________VP - NP__________________|

         A D E L G C F V G T A E A L R C Q E E N Y
         G D E L G C F V G T A E A L R C Q E E N Y
         A D E L G C F L G T A E A L R C Q E E N Y
         |________________VP - NP__________________|
         †             †

         L P S P C Q S G Q K A C G S G G R C A A F
         L P S P C Q S G Q K P C G S G G R C A A A
         L P S P C Q S G Q K P C G S G G R C A A A
         |________________VP - NP__________________|
                             #                   #

         G V C C N D E S C V T E P E C R E G F H R R A
         G I C C N D E S C V T E P E C R E G V G F P R
         G I C C S D E S C V A E P E C R E G F F R L T
         |_________________VP - NP_____________________|
           #     *         *               † # † # #

         R A S D R S N A T Q L D G P A G A L L L R L V Q
         R A N D R S N A T L L D G P S G A L L L R L V Q
         R A R E Q S N A T Q L D G P A R E L L L R L V Q
         |____________________CPP________________________|
             # * *         †         † * *

         L A G A P E P F E P A Q P D A Y
         L A G A P E P A E P A Q P G V Y
         L A G T Q E S V D S A L P R V Y
         |_____________CPP_______________|
             * *   * # * *   *   # #
```

Figure 2: Comparison of propressophysin amino acid sequence in human, bovine, and rat. Differences in sequence homology are noted at the bottom of each bracket as follows: *: deviation between rat and human; †: deviation between bovine and human; #: deviation between rat and bovine with human. VP: vasopressin; VP-NP: vasopressin-neurophysin; CPP: C-terminal glycopeptide.

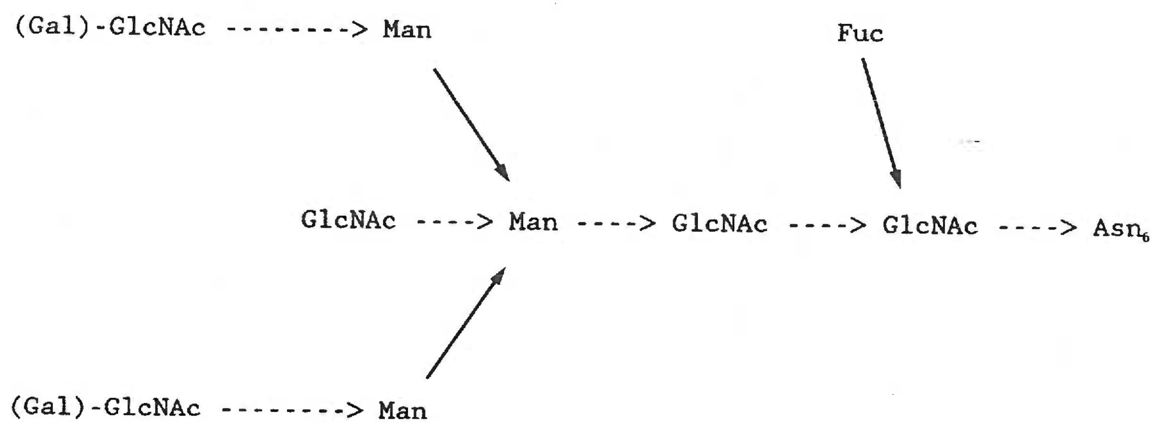


Figure 3: Tentative structure of human CPP asparagine linked oligosaccharide. Gal: galactose; GlcNAc: N-acetylglucoseamine; Man: mannose; Fuc: fucosamine (from Lambert, 1986).

gene expression and regulation.

Mature OT mRNA is approximately 650 bp in the rat (Ivell et al., 1984) and is transcribed, spliced, and polyadenylated as described for VP mRNA. OT mRNA codes for a 14 kd precursor in rat and 13 kd in human, called pre-prooxyphysin. Removal of the signal sequence, giving prooxyphysin and proteolytic processing in the secretory vesicles proceeds as with propressophysin, with the exception of no glycosylation. Final processed polypeptides include OT and OT NP.

A species comparison of prooxyphysin amino acid sequence is shown in Figure 4. As with VP, OT is conserved throughout the species. In the OT-NP sequence there is 44% homology between rat and human and 65% homology between bovine and human. In rat OT-NP, the last 46 amino acids of the sequence share almost no homology with human, and in bovine OT-NP, there is virtually no homology to the human sequence in the last 26 amino acids. As in the case of VP-NP, OT-NP contains 14 cysteines and 7 disulfide bridges. There is considerable homology between the VP and OT genes in all species studied. As mentioned earlier, the genes are located closely to each other on opposite strands of a single DNA fragment. This has led many to speculate that both genes may originate from a single common ancestral gene by duplication and inversion (Ruppert et al., 1984; Sausville et al., 1985). Although there is a high degree of homology between OT-NP and VP-NP (81% in bovine) x-ray crystallographic studies have shown significant differences in conformation between the two (Breslow, 1979). This suggests specific functions of each NP. A summary of the molecular weights and homologies between rat, bovine, and human precursors and neurophysins is shown in Table 1.

Human C Y I Q N C P L G G K R A A P D L D
Bovine C Y I Q N C P L G G K R A V L D L D
Rat C Y I Q N C P L G G K R A V L D L D
OT OT - NP
# #

V R K C L P C G P G G K G R C F G P N I
V R T C L P C G P G G K G R C F G P S I
V R T C L P C G P G G K G R C F G P S I
OT - NP
# #

C C A E E L G C F V G T A E A L R C Q E N Y L
C C G D E L G C F V G T A E A L R C Q E N Y L
C C G D E L G C F V G T A E A L R C Q E E N Y
OT - NP
# # * * *

P S P C Q S G Q K A C G S G G R C A L G L C C S
P S P C Q S G Q K P C G S G G R C A A A G I C C
L P S P C Q S G Q K P C G S G G R C A A A G I C
OT - NP
* * * * * * * * * # * * * * * * * # # # # * #

P D G C H A D P A C D A E A T F S Q R
S P D G C H E D P A C D P E A A F S Q H
C S P D G C H E D P A C D P E A A F S Q
OT - NP
# # # # # # # # # # # # # # # # # # # #

Figure 4: Comparison of prooxyphysin amino acid sequence in human, bovine, and rat. Differences in sequence homology are noted at the bottom of the brackets as follows: *: deviation between rat and human; †: deviation between bovine and human; #: deviation between rat and bovine with human. OT: oxytocin, OT-NP: neurophysin.

Table 1 Summary of Neurophysin Molecular Weight and Homology

| MW of Peptide/Precursor * | Species | | |
|---|---|---|---|
| | Rat | Bovine | Human |
| Pre-propressophysin | 17,887 | 16,990 | 17,332 |
| Pre-prooxyphysin | 11,082 | 11,427 | 12,742 |
| Propressophysin | 15,489 | 15,040 | 15,381 |
| Prooxyphysin | 10,710 | 10,852 | 10,956 |
| VP | 1,067 | 1,067 | 1,067 |
| OT | 990 | 990 | 990 |
| VP-NP | 9,661 | 9,707 | 9,755 |
| OT-NP | 9,378 | 9,521 | 9,625 |
| CPP | 4,264 | 3,925 | 4,062 |
| Carbohydrate | ND | ND | 2,171# |
| % Carbohydrate | ND | ND | 14 |
| % Homology between OT-NP and VP-NP | 75 | 81 | 72 |

* Molecular weight is expressed in daltons; calculated from deduced a.a. sequence.

\# Determined from tentative carbohydrate structure

ND Not determined

## B. The Brattleboro Rat

An example of a VP gene defect in an animal model which has been extremely useful in studies of both VP and OT was discovered in the early 60s. The Brattleboro rat, first identified by Henry Schroeder in West Brattleboro, Vermont (Valtin and Schroeder, 1964), contains a genetic defect resulting in hypothalamic diabetes insipidus. In homozygous Brattleboro rats, with diabetes insipidus (HoDI) plasma VP levels are not detectable by radioimmunoassay, whereas OT levels are normal (Valtin et al., 1978). Plasma VP in heterozygous Brattleboro DI rats (HzDI) demonstrate levels of 50-70% of normal rat VP values (Valtin et al., 1978).

The molecular defect in the HoDI rat has been identified as a single base deletion (Schmale and Richter, 1984). As shown in Figure 5, a single G is deleted following serine, amino acid number 63 of VP-NP. The mutant gene thus contains VP and the first 63 amino acids of VP-NP. The single base deletion in the triplet coding for $Gly^{64}$ results in a frame shift and a subsequent change in the amino acid sequence for the remainder of VP-NP as well as CPP. Of note, is the loss of the Asn in CPP and the absence of glycosylation. Although transcription can occur, as recently demonstrated both by *in situ* hybridization and Northern blot analysis of HoDI hypothalamic tissue (McCabe et al., 1988), it is thought the mRNA is poorly translated, possibly due to the absence of a STOP codon (Schmale and Richter, 1984).

Recently, studies have additionally shown the presence of vasopressin and glycopeptide (CPP) immunoreactivity in solitary magnocellular neurons (Richards et al., 1985) and supraoptic nuclei

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Normal | 60 | Pro | Cys | Gly | Ser | Gly | Arg | . . . | |
| | | C C T | T G C | G G A | A G C | G G A | G G C | C G C . . . | |
| Brattleboro | 60 | Pro | Cys | Gly | Ser | | Glu | Ala | Ala . . |
| | | C C T | T G C | G G A | A G C * | | G A G | G C C | G C T . . |

Figure 5: Comparison of nucleotide and amino acid sequence of normal and Brattleboro rat. The animo acid sequence comparison starts at number 60 in the polypeptide with proline. The position of a single base deletion is noted (*).

(Guldenaar et al., 1986) of HoDI rats. This appears to conflict with the above data on the absence of VP and propressophysin in HoDI rats. Some attempts have been made to explain this finding, such as a "crossing over" of the OT and VP genes with the subsequent "repair" of the VP gene defect (van Leeuwen et al., 1987). Both van Leeuwen and Ivell (1987) speculate that gene conversion may take place by the transfer of information in a nonreciprocal fashion between the VP and OT genes in the HoDI rat. van Leeuwen et al. (1989) have very recently demonstrated that increased VP immunoreactivity in HoDI rats is linear with respect to age. They further speculate that once mitotic division has ceased, somatic intrachromosomal gene conversion between the homologous exons of the VP and OT genes may account for this.

It must be noted, however, that some of the variations in the data generated with the HoDI rats, may be due to the animals not being truly homozygous for the gene defect. It is imperative this is established before any data can be properly interpreted. Thus, it remains to be seen what the significance is of VP immunoreactivity that is detected in documented HoDI animals.

C. Neuroanatomy of the Neurophysins

Propressophysin and prooxyphysin are synthesized in two groups of cells within the brain, which can be differentiated on the basis of size. These include magnocellular neurons, which are large perikarya and the parvicellular neurons, which are of a smaller or intermediate size. The magnocellular VP and OT neurons are found in the supraoptic nucleus (SON), paraventricular nucleus (PVN) and accessory nuclei, such as the nucleus circularis and other intranuclear cells that form a

discontinuous plane between the PVN and SON (Kozlowski and Nilaver, 1986). OT-containing cells are localized in the dorsal aspect of the SON, while VP-containing cells are found in the ventral portion of the SON (Defendini and Zimmerman, 1978). The magnocellular neurons in the SON, PVN, and accessory nuclei are typically termed the hypothalamo-neurohypophyseal system (Kozlowski and Nilaver, 1986).

Parvicellular VP neurons are localized in the PVN, the suprachiasmatic nucleus (SCN) and extra-hypothalamic regions such as the locus coeruleus and the medial amygdaloid nucleus (Caffe et al., 1985). In the PVN, the parvicellular VP neurons are located more medial than the magnocellular VP neurons. The SCN does not contain any OT neurons or magnocellular VP neurons (Kozlowski and Nilaver, 1986).

Projections of the VP neurons of the PVN and SON are illustrated in Figure 6. The predominant projection of the SON is to the neural lobe of the pituitary, while only some of the fibers of the PVN project to the neural lobe. Processed neurophysin as well as OT and VP are stored in the posterior pituitary and released into the systemic circulation. Remaining PVN neurons project to the zona externa of the median eminence (Kozlowski and Nilaver, 1986). VP released from PVN and SON neurons into the portal capillaries of the median eminence appears to function as a co-corticotropin releasing factor (CRF), necessary for the action of CRF (Lamberts et al., 1984).

There is no evidence to date of parvicellular neurons projecting to the posterior pituitary. Parvicellular neurons from the PVN project to the zona externa of the median eminence (Valiquette, 1987). In the

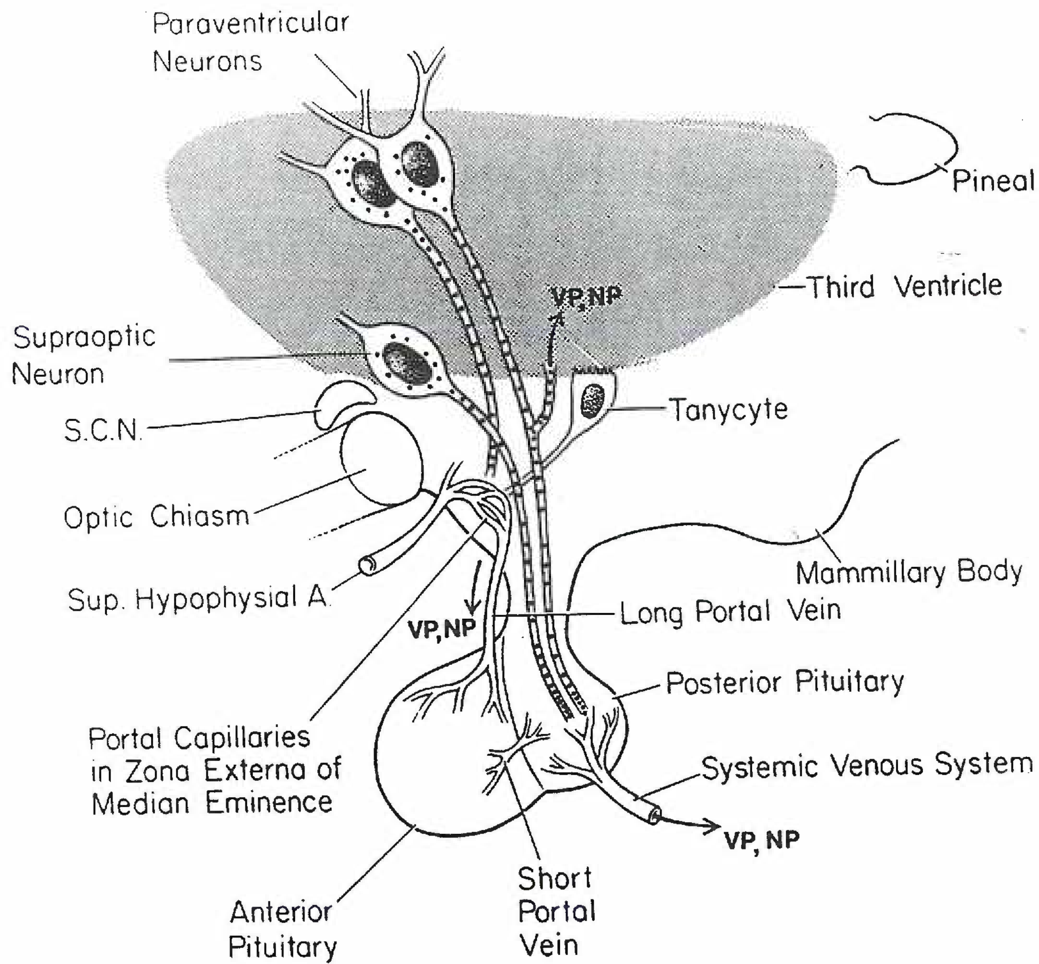


Figure 6: Schematic representation of the hypothalamo-neurohypophyseal system. SCN: Suprachiasmatic nucleus; VP: vasopressin; NP: neurophysin. Drawing of system from Earl Zimmerman (unpublished).

SCN, parvicellular neurons project to the dorso-medial nucleus of the hypothalamus and the organum vasculosum of the lamina terminalis in the forebrain (Hoorneman and Buijs, 1982).

D. Vasopressin and the Syndrome of Inappropriate Secretion of Antidiuretic Hormone

The syndrome of inappropriate antidiuretic hormone release (SIADH) also known as Bartter-Schwartz syndrome was first described in 1957 in two patients diagnosed with bronchogenic carcinoma (Schwartz et al., 1957). The clinical description of SIADH includes hyponatremia, sustained urinary sodium excretion, elevated urinary osmolality and normal renal and adrenal function. There appears to be four major types of SIADH, depending on plasma VP levels (Robertson et al., 1976). The first, type A, produces varying plasma levels of VP with no apparent relationship to plasma osmolality. In the second class of SIADH, type B, the plasma VP levels can be suppressed with the introduction of hypertonic saline, whereas, type C patients fail to suppress completely with hypertonic saline. In the fourth class, type D, patients demonstrate normal VP levels in the plasma and show normal response to plasma osmolality. The precise mechanism for production of SIADH in these patients is unclear.

There are many pathophysiological conditions which ultimately can result in SIADH (Bunn and Minna, 1985). These include malignancies, central nervous system disease such as encephalitis and head trauma, pulmonary and cardiovascular disease, and drug induced (morphine, tetracyclines, etc.). Of particular interest is the production of SIADH by tumor secretion of VP. Although SIADH is most often associated with

Table 2: Tumors Associated with SIADH

| Class of Tumor | Reference |
| --- | --- |
| Prostatic carcinoma | Matzkin and Graf, 1987<br>Ghandur-Mnaymneh et al., 1986 |
| Pyriform squamous cell carcinoma | Kandylis et al., 1986 |
| Head and neck squamous cell carcinoma | Hayes et al., 1986 |
| Chronic lymphocytic leukemic meningitis | Stagg and Gumbart, 1987 |
| Olfactory neuroblastoma | Cullen et al., 1986 |
| Tongue carcinoma | Abdi and Bishop, 1988 |
| Bronchogenic small cell carcinoma | Pederson et al., 1985 |
| Bronchogenic oat cell carcinoma | Spruce and Baylis, 1983 |
| Lung small cell carcinoma | North et al., 1988<br>Hansen and Pederson, 1986 |
| Lung oat cell carcinoma | Yamaji et al., 1981 |

small cell lung carcinomas, a number of tumors have been documented to produce SIADH in patients, as shown in Table 2. In addition to vasopressin being released into the plasma by these tumors, it has been shown that neurophysin levels (North et al., 1988) and propressophysin levels (Yamaji et al., 1983) are elevated.

The finding of elevated circulating levels of VP, neurophysin, and propressophysin in many of these tumor patients, suggests this may be a means to diagnose the presence of these tumors in the early stages. Indeed, North et al. (1988) conclude that plasma neurophysin levels may provide adequate sensitivity to not only detect early tumor formation, but also to monitor the efficacy of treatment. However, in patients with type D SIADH, this would clearly not be of use.

The ability of malignant tissue to produce and secrete peptides is not limited to VP. In many lung tumors, a number of peptides were found to be elevated in the patient's plasma. Table 3 shows some of the peptides and hormones produced and released by small cell, epidermoid, adenocarcinoma, and large cell lung tumors. Although the highest levels appear to be in small cell carcinomas, other tumor types can produce large amounts of peptides/hormones and release them into the plasma. This is particularly apparent with neurophysin and ACTH production.

The precise role and significance of this peptide production by various tumors is not entirely clear. As mentioned earlier, neurophysin may indeed function as a growth factor in non-neuronal cells. It has, additionally, been reported that VP functions as a potent growth factor in adrenal glomerulas cells (Payet et al., 1984), stimulates mouse 3T3 cell growth (Rozengurt et al., 1979) and stimulates acid-base transport

Table 3 Secretion of Peptides/Hormones in Lung Tumors

| Peptide/Hormone | Small Cell | Epider-moid | Adeno-carcinoma | Large Cell |
|---|---|---|---|---|
| ACTH | +++ | +++ | +++ | ++ |
| Lipotropic Pituitary Hormone | +++ | ++ | ++ | ND |
| Calcitonin | +++ | + | 0 | + |
| Antidiuretic Hormone | ++ | ND | ND | ND |
| Parathyroid Hormone | ++ | ++ | 0 | ++ |
| Beta-human chorionic gonadotropin | ++ | ++ | ++ | ++ |
| Growth hormone | 0 | + | 0 | 0 |
| Gastrin-releasing peptide | +++ | ++ | ++ | + |
| Somatostatin | ++ | ++ | ND | ND |
| Neuron-specific enolase | +++ | ND | ND | ND |
| Neurophysins | +++ | ++ | +++ | ++ |

Adapted from Ihde, 1987. +++ = significantly elevated levels; ++ = moderately elevated levels; + = minimally elevated levels; 0 = no detectable levels; ND = not done.

systems (Ganz et al., 1989). Recently, it has been shown that bombesin (Ruff and Pert, 1984) and bombesin-like peptides (Cuttitta et al., 1985) such as gastrin-releasing peptide may function as autocrine growth factors in human small cell lung carcinoma.

E. Biochemical and Molecular Characterization of the Neurophysins in Normal and Neoplastic Tissue

The first phase of this dissertation investigates the detection of low molecular weight peptides by monoclonal antibodies using Western blotting. Conventional electrophoretic and immunoblotting protocols are limited both in their ability to resolve low molecular weight peptides and maintain antigenicity due to SDS denaturation. The methodology used in this study allows the resolution of very low molecular weight peptides and their recognition by monoclonal antibodies. This is critical to the following studies in immunologically characterizing the neurophysins.

Chapter II of this study is the identification of neurophysin immunoreactivity in hypothalamus by a monoclonal antibody to a lung carcinoma surface antigen. Since this antibody (mAb L6) was raised against a lung carcinoma and immunoreacts strongly with a human variant oat cell carcinoma cell line, it was clearly of interest to identify and characterize the mAb L6 antigen in these cells and determine whether or not it is neurophysin or neurophysin related. These characterization studies are described in Chapter III.

The potential problem of antibody cross-reactivity is studied in Chapter IV. This involves the characterization of an antiserum to atrial natriuretic factor and its cross-reactivity to the neurophysins in the

hypothalamo-neurohypophysial system of the rat. This is a crucial point, since immunohistochemistry and absorption studies alone cannot unequivocally confirm the presence or identity of a specific antigen.

Finally, in Chapter V, differences in conformation of neurophysin in magnocellular neurons vs. parvicellular neurons is investigated. Since it is known that the secretory vesicles in parvicellular neurons are much smaller than those in magnocellular neurons, there is a potential for variation in pH within these granules, leading to different conformations of neurophysin. This possibility is explored in this study. These proposed studies should help address the question of mAb L6 immunoreactivity in the hypothalamus, and the nature of the L6 surface antigen on LX-1 lung carcinoma cells.

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Chapter I

# Detection of Low-Molecular-Weight Polypeptides on Nitrocellulose with Monoclonal Antibodies

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ABSTRACT

An immunoblotting method is described to detect low-molecular-weight peptides with monoclonal antibodies that normally fail to demonstrate immunoreactivity using conventional blotting techniques. Detection of neurophysin, insulin, calcitonin, vasopressin and $\beta$-endorphin electroblotted on nitrocellulose membranes were optimized after introducing four modifications into the conventional procedure. These include renaturing the gels after sodium dodecyl sulphate electrophoresis, electroblotting the renatured gels in basic transfer buffer, fixing and/or heating the blots, and the use of avidin/alkaline phosphatase conjugates for antigen/antibody detection. This technique likely enables the denatured peptides to regain their native conformation and, therefore, restore antigenicity and recognition by highly-structural specific monoclonal antibodies. Although the most dramatic improvement with this technique is with monoclonal antibodies, a modest improvement in sensitivity can be obtained when immunoblots are probed with polyclonal antibodies. The high resolution of this system will be useful in probing blots of partial proteolytic digests of proteins with both monoclonal and polyclonal antibodies.

# INTRODUCTION

The development of protein transfer to nitrocellulose membranes, first described by Towbin et al. (1979) has led to the characterization of many biologically important molecules. Protein or Western blotting has been used: to purify proteins (Korth et al., 1988), purify monospecific antibodies (Olmsted, 1981), in competitive ligand-binding assays (Gershoni et al., 1983) and in detecting proteins and enzymes using various ligands (Hirano et al., 1988), or antibodies (Tobin and Gordon, 1984; Burnette, 1981; Gershoni, 1988). While polyclonal antibodies generally demonstrate a high degree of specificity and affinity for protein immobilized on nitrocellulose (Towbin and Gordon, 1984; Bers and Garfin, 1985; Gershoni, 1988), it is often difficult to detect transferred protein when monoclonal antibodies (mAbs[1]) are used in conventional blotting techniques (Towbin and Gordon, 1984; Bers and Garfin, 1985; Gershoni, 1988). Since mAbs are directed against a single antigenic epitope, and are usually dependent on the three-dimensional structure of the protein for binding, changes in conformation by SDS denaturation may block or inhibit immunoreactivity (Bers and Garfin, 1985; Benjamin et al., 1984). When the protein of interest is of a very low molecular weight ($M_r$ <12,000), detection by mAb may be further inhibited due to the poor retention of small polypeptides on nitrocellulose (Towbin and Gordon, 1984; Bers and Garfin, 1985).

Modification of conventional blotting techniques to improve immunoreactivity and detection by mAb include the use of avidin/biotin conjugates (Ogata et al., 1983), alkaline phosphatase conjugated secondary detection reagents (Turner, 1983), renaturation of SDS gels

prior to transfer (Dunn, 1986), use of different blocking agents (Hauri and Bucher, 1986), and varying transfer buffer composition (Dunn, 1986; Szewczyk and Kozloff, 1985). Eliminating the use of SDS as in native gel electrophoresis often results in a poor resolution of antigens (Tovey et al., 1987). Although many of these improvements have increased mAb immunoreactivity and sensitivity, the proteins studied have been of moderate to high molecular weight ($M_r$ >15,000) or include urea in the gel (Sheng et al., 1988). Unfortunately, there is no report of a general method incorporating the many individual modifications for effective mAb probing of low-molecular-weight proteins transferred to nitrocellulose. This study represents a single method of detecting a number of low-molecular-weight peptides (1-23 kd) with mAbs. This technique, which uses a high resolution, relatively low acrylamide gel system, gel renaturation, alkaline transfer buffer, fixation/heating of the nitrocellulose blots, and immunodetection with avidin-alkaline phosphatase conjugates, is applicable to detecting a variety of low-molecular-weight peptides.

## MATERIALS AND METHODS

Materials: Polyclonal antibodies (pAbs) to human insulin, human calcitonin, porcine neurophysin and vasopressin, and mAbs to human insulin were from ICN Immunobiologicals (Lisle, IL). Monoclonal antibodies to human $\beta$-endorphin and human calcitonin were from Boehringer Manheim (Indianapolis, IN). L6 mAb, an $IgG_{2a}$ raised to a human lung adenocarcinoma cell line, and demonstrating immunoreactivity with neurophysin (manuscript in preparation) was the generous gift of Drs.

[1]Abbreviations used: SDS, sodium dodecyl sulfate; mAb, monoclonal antibody; pAb, polyclonal antibody; NC, nitrocellulose membranes; BSA, bovine serum albumin; HRP, horseradish peroxidase; NP neurophysin; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; NBT, *p*-nitroblue tetrazolium chloride.

Hellström (Oncogen, Seattle, WA). Monoclonal antibody to human vasopressin has been previously characterized (Hou-Yu et al., 1982). ExtrAvidin alkaline phosphatase conjugate, protein G, Ponceau S concentrate, chloroauric acid, silver nitrate, and myoglobin peptide molecular weight standards were obtained from Sigma (St. Louis, MO). Protein G was biotinylated according to the procedure of Hsu et al. (Hsu and Soban, 1981). Streptavidin-horseradish peroxidase and streptavidin-$\beta$-galactosidase conjugates were from Bethesda Research Laboratories (Gaithersburg, MD). Biotinylated protein A was from Vector Laboratories (Burlingame, CA). Human calcitonin, human insulin and porcine neurophysin II were from Calbiochem (San Diego, CA). Arginine$^8$-vasopressin and human $\beta$-endorphin were from Serva Fine Biochemicals (Westbury, NY). Nitrocellulose membranes, acrylamide, N-N'-methylenebis(acrylamide), gelatin, and SDS were from Bio-Rad Laboratories (Richmond, CA).

<u>Gel Electrophoresis</u>: All protein and polypeptide samples were solubilized in 1X Laemmli sample buffer (Laemmli, 1970) containing 20 mM DTT and heated to 100°C for 5 min except for the insulin samples which were not reduced in order to maintain the intact molecule. Electrophoresis was performed in 16 x 14 x 0.75 cm small-pore gels using the tricine-SDS-PAGE system described by Schägger and von Jagow (1987). The separating gels were prepared from a stock solution of 46.5% (w/v) acrylamide plus 3% (w/v) bisacrylamide to give a final acrylamide concentration of 14.5% (%T, acrylamide plus bisacrylamide). Glycerol was added to a final concentration of 13.3% (w/v). The 3 cm spacer gel and the stacking gel were prepared from a stock solution of 48% (w/v)

acrylamide plus 1.5% (w/v) bisacrylamide to give final concentrations of 10% T and 5% T, respectively. Samples were electrophoresed initially at 30 V for 1 hr until they exited the stacking gel and then run at 150 V for 16 hr at 4°C. Gels not used for protein blotting were fixed for 1 hr in MeOH:$H_2O$:HOAc (5:4:1), stained for 2 hr with 0.2% (w/v) coomassie blue in 20% acetic acid and 0.1 M picric acid (30:70) and destained for 1 hr in several changes of water followed by 5% MeOH/7.5% acetic acid (personal communication from Dr. Malencik, Corvallis, OR).

Gel Renaturation and Protein Transfer: After electrophoresis and prior to electroblotting, gels were treated in two different ways. In the first, Method A, gels were washed 3 x 10 min in 50 mM Tris pH 7.4 containing 20% (v/v) glycerol (Dunn, 1986). In Method B, gels were briefly immersed in 25 mM Tris/192 mM glycine pH 8.3 prior to transfer, as described by Towbin et al. (1979). Protein transfer was performed with a TE-42 transfer unit (Hoefer, San Francisco, CA). In Method A, gels were electroblotted onto 0.2 $\mu$m nitrocellulose (NC) paper for 45 min at 1 amp in transfer buffer consisting of 10 mM $NaHCO_3$/3 mM $Na_2CO_3$ pH 10.0 with 20% (v/v) methanol (Dunn, 1986). In Method B, gels were transferred to 0.45 $\mu$m NC for 45 min at 1 amp in 25 mM Tris/192 mM glycine pH 8.3 containing 20% (v/v) methanol. Decreasing the methanol concentration led to a concomitant loss of protein from the NC membranes. All buffers were used only once and the temperature was maintained at 4°C during transfer. After transfer, nitrocellulose membranes were allowed to air dry overnight and the gels stained as described above or with silver according to the method of Heukeshoven and Dernick (1985). Lanes containing molecular weight standards were cut

out, stained in 0.2% (v/v) Ponceau S for 10 min and destained in 2% (v/v) acetic acid. In order to monitor transfer efficiency of total protein to the NC membranes, blots were stained with colloidal gold for 2 hr and washed with distilled $H_2O$ as described by Yamaguchi and Asakawa (1988).

Immunodetection of Transferred Peptides: In Method A, the air dried blots were fixed in paraformaldehyde vapor at 70°C for 90 min in order to immobilize the transferred proteins (Larsson, 1981; Scopsi et al., 1986). A large desiccator with 7 g of paraformaldehyde at the bottom was used to fix the nitrocellulose membranes which were typically cut into strips corresponding to the appropriate lanes. Alternatively, blots were heated without paraformaldehyde fixation, for 90 min at 70°C. In Method B, blots were not fixed or heated unless otherwise indicated. From this point on, Methods A and B were identical. Nonspecific binding sites on the blots were blocked with 3% (w/v) gelatin in 20 mM Tris pH 7.5 containing 0.5 M NaCl, 0.1% (v/v) Tween-20 and 0.02% (w/v) $NaN_3$ (TTBS) for 1 hr followed by 3 x 5 min washes in TTBS. The gelatin used in this blocking step was dissolved at 37°C, added to the blots at that temperature, and allowed to cool to 25°C during the 1 hr incubation. Blots were then incubated in primary antibody (concentration as indicated in the figure legends). All mAb incubations were performed for 3 hr at room temperature (RT) with gentle rocking. Polyclonal antibody incubations were done for 1 hr at room temperature unless otherwise indicated. Antibody solutions were freshly prepared in TTBS containing 0.1% (w/v) recrystallized BSA. Reutilization of the antibodies for up to two or three times did not appear to significantly

reduce immunoreactivity.

Following primary antibody incubation, blots were incubated with biotinylated protein A (3 μg/ml) in TTBS containing 0.1% (w/v) BSA for 1 hr at RT. Biotinylated protein G (3 μg/ml) was substituted for protein A when the primary antibody used was polyclonal antiinsulin raised in sheep, since some subclasses of sheep IgG are known to react poorly with protein A (Goding, 1978). After washing 3 x 5 min in TTBS, the blots were incubated in avidin/enzyme conjugates as indicated in the figure legends. The blots were incubated with ExtrAvidin alkaline phosphatase (1.7 μg/ml), streptavidin-horseradish peroxidase (1.7 μg/ml) or streptavidin-β-galactosidase (1.7 μg/ml) in TTBS for 45 min at RT. Blots were then washed 3 x 5 min in TTBS, transferred to clean trays and reacted with the appropriate substrate. Alkaline phosphatase activity was detected by developing the blots in 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; 0.165 mg/ml)/*p*-nitro-blue tetrazolium chloride (NBT; 0.33 mg/ml) in 0.1 M Tris pH 9.5 containing 0.1 M NaCl, 5 mM $MgCl_2$ and 0.5 % (v/v) Tween-20. BCIP was dissolved in N,N-dimethylformamide (DMF) if the toluidine salt is used. The sodium salt can be dissolved in distilled $H_2O$. NBT was dissolved in 70% (v/v) DMF. Horseradish peroxidase activity was detected by incubating blots in 3,3'diaminobenzidine tetrahydrochloride dihydrate (0.8 mg/ml) in 0.1 M Tris pH 7.5 containing 0.1 M NaCl, 0.5% (v/v) Tween-20 and 0.01% (v/v) $H_2O_2$. The sensitivity of the peroxidase reaction was increased by the addition of $Ni^{+2}$ to the above solution to a final concentration of 0.04% (v/v) (Hsu et al., 1982). β-Galactosidase activity was detected by incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BCIG; 1.2 mM;

dissolved in DMF) in 20 mM $Na_2HPO_4$ pH 7.4 containing 0.1 M NaCl, 0.5 % (v/v) Tween-20, 1 mM $MgCl_2$, 3 mM potassium ferricyanide and 3 mM potassium ferrocyanide. Unless otherwise noted, all substrate reactions were allowed to develop for a maximum of 3 min followed by quenching in distilled water.

Protein Determination Peptide samples were resuspended in 25 mM phosphate pH 7.4 and the protein determined by the Folin phenol method described by Peterson (1983). BSA was used as the protein standard.

RESULTS

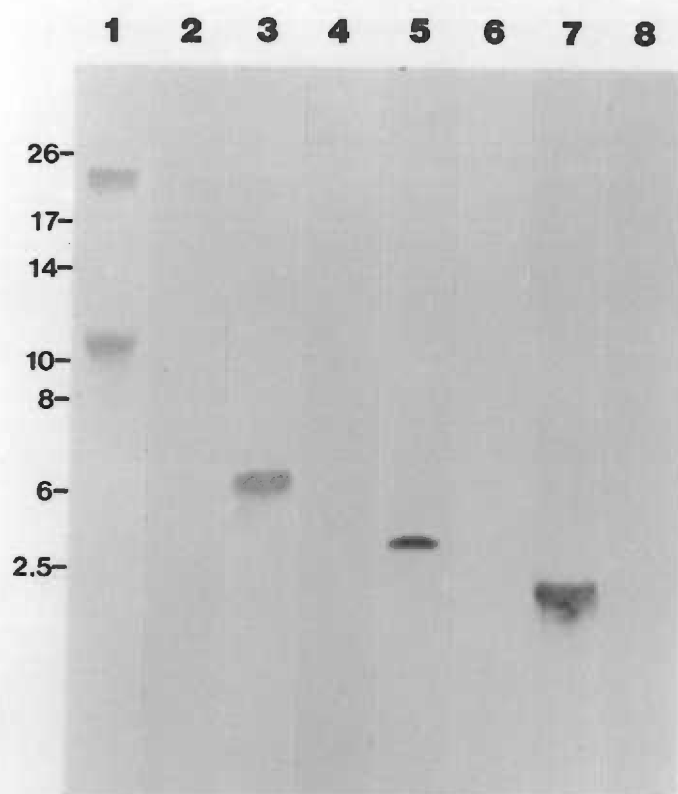
Immunodetection of low-molecular-weight peptides with mAbs using the technique described in this paper (Method A) and conventional blotting techniques (Method B) is shown in Figure 1. Porcine neurophysin II, consisting of processed neurophysin ($M_r$ 11,000) and a glycosylated precursor (propressophysin, $M_r$ 23,000) demonstrated immunoreactivity when probed with mAb L6 using Method A (Lane 1), but was not detectable with Method B (Lane 2). Similar results were obtained when porcine neurophysin I, bovine neurophysin I and II, and human neurophysins were probed with mAb L6 using Methods A and B. Human insulin ($M_r$ 6000) was incubated with an antiinsulin mAb and demonstrated immunoreactivity when Method A was used (Lane 3) but failed to show a detectable band when Method B was used (Lane 4). This mAb appears to be very sensitive to conformation, failing to recognize individual insulin A and B chains by either Method A or B (data not shown). Human calcitonin ($M_r$ 3425) showed immunoreactivity with an anticalcitonin mAb with Method A (Lane 5) and virtually no immunodetection with Method B (Lane 6). Using Method A, peptides as small as vasopressin ($M_r$ 1067) can be detected

with a mAb (Lane 7) whereas Method B fails to demonstrate an immunoreactive band (Lane 8). Oxytocin ($M_r$ 990), and 2.5S nerve growth factor ($M_r$ 13,000) were also probed with mAbs using both transfer techniques, with only Method A demonstrating immunoreactivity for oxytocin and significantly improving immunodetection of nerve growth factor (data not shown). Increasing either the antigen or mAb concentration by as much as fivefold still failed to demonstrate immunoreactivity with Method B.

One potential explanation for the absence of immunoreactivity with mAbs using Method B, is a less efficient transfer of peptide to the NC membranes. To address this possibility, peptide blots by both Methods A and B were stained for total protein with colloidal gold. Figure 2 shows gold staining of NP II and vasopressin (arrowhead) with Method A (Lane 1) and Method B (Lane 2). The staining intensity of propressophysin and processed NP II was virtually identical with Methods A and B, indicating equal transfer efficiency. Vasopressin however, appears to be more intensely stained with gold in Method A when compared to Method B, suggesting an improvement of transfer efficiency. Calcitonin staining intensity with Method A (Lane 3) and Method B (Lane 4) appear to be equal. Gold staining of insulin blots by Method A (Lane 5) and Method B (Lane 6) were generally similar, although insulin B-chain ($M_r$ 3500) was more intense in Method A and pro-insulin ($M_r$ 9020) slightly more intense in Method B.

Figure I-1 Immunodetection of low-molecular-weight peptides with monoclonal antibodies (mAb). Peptides were electrophoresed, blotted and probed with antibodies as described under Materials and Methods. Lanes 1 and 2 show immunoblots of porcine NP II (2.5 μg) probed with mAb L6 (75 μg/ml) using Methods A and B, respectively. Lanes 3 and 4 are immunoblots of human insulin (10 μg) probed with a mAb to insulin (1:200) using Methods A and B, respectively. Lanes 5 (Method A) and 6 (Method B) show immunoblots of human calcitonin (0.5 μg) probed with a mAb to calcitonin (2 μg/ml). Lanes 7 and 8 are vasopressin (10 μg) blots probed with a mAb to vasopressin (1:100) using Methods A and B, respectively. Molecular weight markers were chymotrypsinogen A ($M_r$ 25,666), myoglobin ($M_r$ 16,950), myoglobin fragments I and II ($M_r$ 10,670), myoglobin fragment I ($M_r$ 8160), myoglobin fragment II ($M_r$ 6210), and myoglobin fragment III ($M_r$ 2510).

Figure I-1



Polyclonal antibodies were next used to probe the blots in order to determine whether the absence of immunoreactivity for neurophysin, insulin, calcitonin and vasopressin (using Method B in Figure 1) was because of loss of protein from the NC blots or the inability of mAbs to recognize the peptides. Figure 3 demonstrates immunoreactivity of porcine NP II as detected by a NP pAb using Method A (Lane 1) and Method B (Lane 2). Although protein load, antibody dilution and incubation times were identical, the sensitivity of detection is significantly improved with Method A. This improved detection with pAbs is even more pronounced with human insulin. When insulin was probed with a pAb using Method A (Lane 3) pro-insulin and insulin B-chain are detected along with processed insulin. The remaining bands at approximately 7800 Da and 6500 Da are probable proteolytic fragments. When the insulin was probed with pAb using Method B (Lane 4), pro-insulin and processed insulin were visualized with less intensity than with Method A. Insulin B-chain and the proteolytic fragments were not detectable. Polyclonal Abs to human calcitonin demonstrated a slight increase in immunoreactivity with Method A (Lane 5) when compared to Method B (Lane 6). A similar improvement in immunodetection was seen with antivasopressin pAbs when vasopressin NC blots were probed using Method A (Lane 7) as compared with Method B (Lane 8). This, however, may be due to the increased vasopressin transfer efficiency using Method A mentioned above. When pAb incubations were increased to 3 hr (to compare with mAb incubations) identical results were obtained, although background was

Figure I-2 Colloidal gold staining of Total Transferred Protein on nitrocellulose. After electrophoresis, blots were transferred and stained as described under Material and Methods. Lanes 1 and 2 show gold staining of porcine NP II (2.5 μg) and vasopressin (arrowhead; 10 μg) using Methods A and B, respectively. Human calcitonin (0.5 μg) staining with Method A and B are shown in Lanes 3 and 4, respectively. Lanes 5 and 6 are gold stained blots of human insulin (10 μg) using Methods A and B, respectively. Molecular weight markers were the same as in Fig. 1 with the addition of ovalbumin ($M_r$ 42,807).

Figure I-2

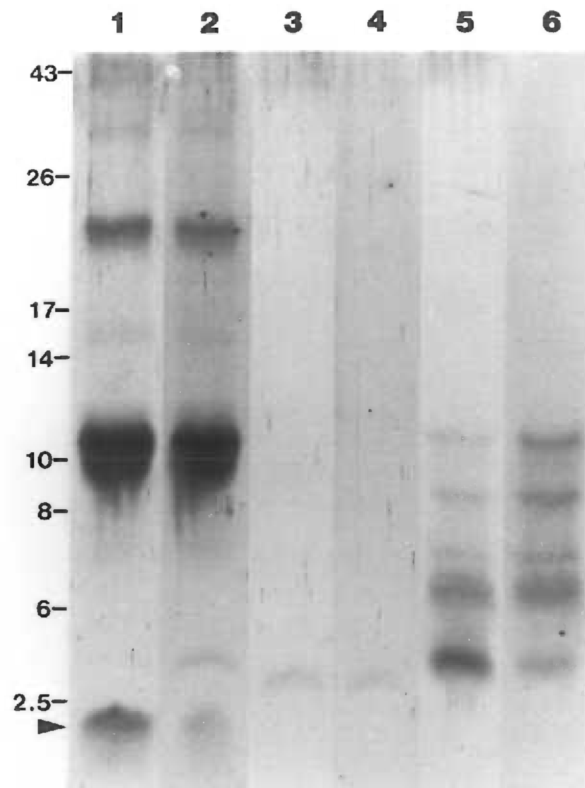
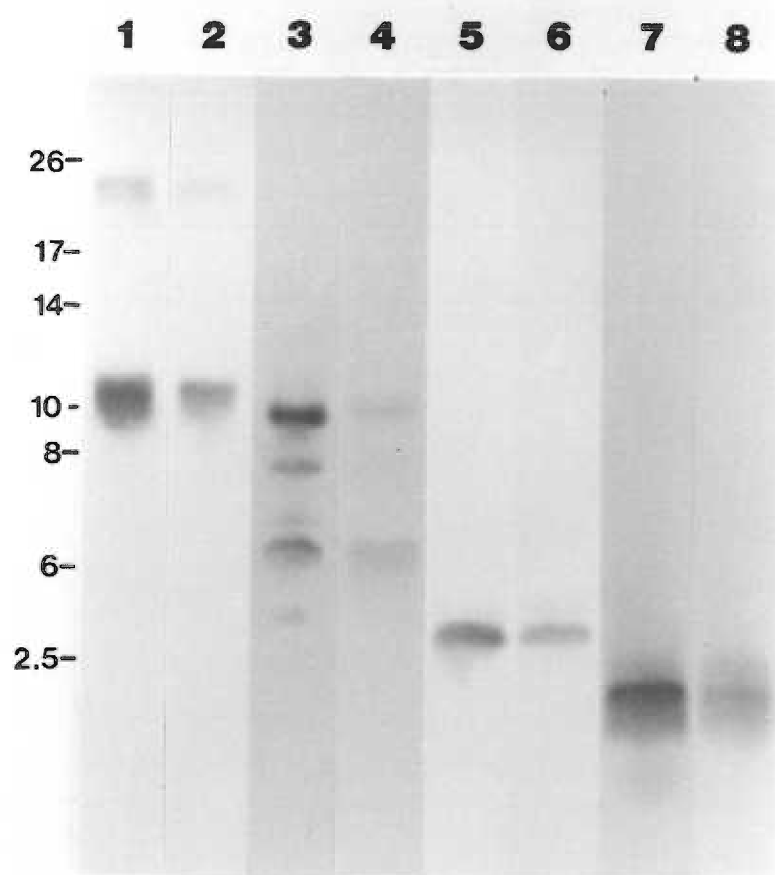


Figure I-3 Immunodetection of low-molecular-weight peptides with polyclonal antibodies. Peptides were electrophoresed, blotted and probed as in Fig. 1. Lanes 1 and 2 show porcine NP II (2.5 μg) blots probed with polyclonal antiporcine neurophysin (1:5000) using Methods A and B, respectively. Lanes 3 and 4 are human insulin (5 μg) immunoblots probed with a polyclonal antihuman insulin (1:3000) with Methods A and B, respectively. Lanes 5 (Method A) and 6 (Method B) are human calcitonin (0.1 μg) probed with a polyclonal antihuman calcitonin (1:4000). Lanes 7 and 8 are immunoblots of vasopressin (1 μg) probed with a polyclonal antivasopressin (1:5000) using Methods A and B, respectively. Molecular weight markers were the same as in Fig. 1.

Figure I-3

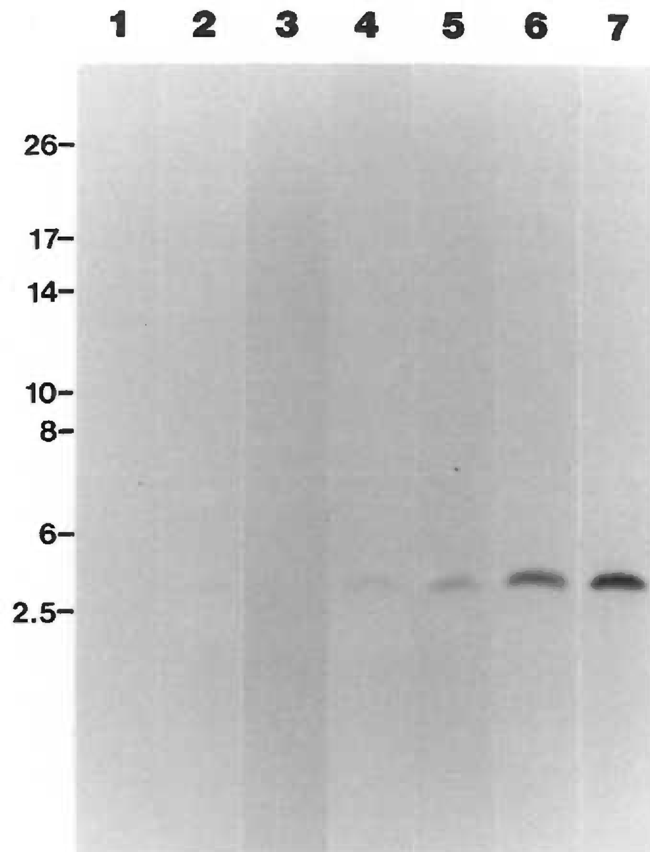


generally higher. This suggests that the 3-hr incubation with mAbs in Figure 1 did not result in a loss of protein from the NC membranes when Method B was used.

In order to determine if any one aspect of Method A is mainly responsible for the improved immunodetection with mAbs, human calcitonin was probed with anticalcitonin mAb introducing one variation at a time prior to antibody incubation. Figure 4 (Lane 1), shows that when calcitonin was electroblotted using Method B and then fixing the strips at 70°C with paraformaldehyde vapor, virtually no immunoreactivity is seen. When the gel is renatured and then blotted according to Method B a very faint band can be visualized (Lane 2). Introducing both heating/ fixation and renaturation into Method B, again failed to demonstrate any significant immunoreactivity (Lane 3). When the basic bicarbonate/ carbonate transfer buffer was used with Method B, a weak band was detected (Lane 4). Combining the basic transfer buffer with heating/ fixation of the NC blots results in a moderate amount of mAb reactivity with calcitonin (Lane 5). Immunodetection was significantly increased when the gels were renatured prior to transfer and electroblotted in basic buffer (Lane 6). When heating/fixation was combined with renaturation and basic transfer buffer, a slight increase in sensitivity was noted (Lane 7). This last combination is essentially Method A with 0.45 μm NC instead of 0.2 μm. There is essentially no difference in sensitivity between Lane 5 in Figure 1 and Lane 7 in Figure 3. This suggests that using 0.2 μm NC offers no significant advantage over 0.45 μm NC. While this may be true for calcitonin, very low-molecular-weight

Figure I-4 Effect of renaturation, transfer buffer composition and fixation/heating on calcitonin immunoblots probed with monoclonal antibody (mAb). After electrophoresis, blots were renatured, transferred, and fixed as described under Materials and Methods. Lanes 1-7 are immunoblots of human calcitonin (0.5 $\mu$g) probed with antihuman calcitonin mAb (2 $\mu$g/ml) using Method B plus fixation (Lane 1), Method B plus renaturation (Lane 2), Method B plus fixation and renaturation (Lane 3), Method B plus basic transfer buffer (Lane 4), Method B plus basic transfer buffer and fixation (Lane 5), Method B plus basic transfer buffer and renaturation (Lane 6), or Method B plus fixation, renaturation, and basic transfer buffer (Lane 7). All immunoblots were on 0.45 $\mu$m nitrocellulose membranes. Molecular weight markers were the same as in Fig. 1.

Figure I-4



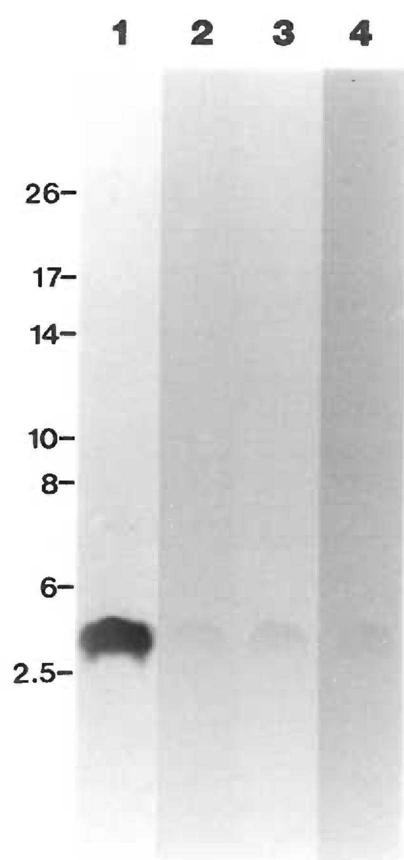
peptides such as vasopressin and oxytocin appear to be retained better on 0.2 $\mu$m NC (data not shown).

The potential advantage of using avidin alkaline phosphatase conjugates over horseradish peroxidase (HRP) and $\beta$-galactosidase ($\beta$-Gal) conjugates was also investigated. Figure 5 shows an immunoblot of human $\beta$-endorphin ($M_r$ 3534) probed with a mAb to $\beta$-endorphin and detected with various enzyme-avidin conjugates. ExtrAvidin alkaline phosphatase incubation (Lane 1) resulted in an intense immunoreactive band when developed with BCIP/NBT as the substrate. By contrast, streptavidin HRP used at the same concentration gave an almost undetectable band when developed with diaminobenzidine (Lane 2). ExtrAvidin HRP also demonstrated a very low level of detection. In an attempt to increase the sensitivity of detection by streptavidin HRP, $Ni^{+2}$ was added to the diaminobenzidine substrate (Lane 3), resulting in a slight increase in immunoreactivity. Streptavidin $\beta$-Gal also gave a very weak signal when developed in BCIG (Lane 4). The immunoreactivity detected with ExtrAvidin alkaline phosphatase (Lane 1) was of such intensity when compared with streptavidin HRP or $\beta$-Gal, that the concentration of $\beta$-endorphin had to be decreased by twofold and mAb concentration by fivefold in order to obtain a blot that was not over developed. Interestingly, $\beta$-endorphin demonstrated weak immunoreactivity with high concentrations (1 $\mu$g/ml) of anti-$\beta$-endorphin mAb using Method B (data not shown).

In all the immunoblots described, biotinylated protein A (biotinylated protein G in the case of insulin) was used as the

Figure I-5 Detection of human β-endorphin immunoblots with avidin enzyme conjugates. Samples were electrophoresed, blotted and probed with antibody using Method A as described in Materials and Methods. Lane 1 is an immunoblot of human β-endorphin (1 μg) probed with anti-β-endorphin mAb (0.04 μg/ml) and incubated in ExtrAvidin alkaline phosphatase (1.7 μg/ml). Lanes 2-4 are immunoblots of human β-endorphin (2 μg) probed with anti-β-endorphin mAb (0.2 μg/ml) and incubated in streptavidin/horseradish peroxidase (1.7 μg/ml; Lane 2), streptavidin/horseradish peroxidase (1.7 μg/ml) plus $Ni^{+2}$ (Lane 3), or streptavidin-β-galactosidase (1.7 μg/ml; Lane 4). Molecular weight markers were the same as in Figure 1.

Figure I-5



secondary detection reagent instead of a biotinylated antispecies antibody. In our experience protein A gives a much lower background on the blots when substituted for antispecies antibody. Except in the very few examples wherein protein A and G bind weakly to Fc regions, such as with rat $IgG_{2a}$ and mouse $IgG_1$ (Goding, 1978), they are preferred over antispecies antibody. We have also noted a significant increase in sensitivity by using avidin enzyme conjugates to biotinylated protein A as opposed to protein A conjugated directly to the enzyme or with [$^{125}$I]labeled protein A (data not shown).

## DISCUSSION

We have presented an improved method of detecting very low-molecular-weight peptides electrophoretically separated on SDS gels, using mAbs. All the peptides tested for immunoreactivity, except for human β-endorphin, failed to demonstrate reactivity with conventional blotting techniques (even when protein load and antibody concentration were increased). With the exception of vasopressin, transfer efficiency of peptides was essentially equal with Methods A and B. Although retention of low-molecular-weight proteins on NC during or after transfer can pose a problem (Bers and Garfin, 1985), similar detection of peptides with either Method A or B using pAbs in this study suggests protein is not being lost from NC membranes during the incubation steps. We have thus shown, in agreement with others (Mandrell and Zollinger, 1984), that the absence of immunoreactivity with mAbs is likely because of changes in protein conformation upon denaturation in SDS gels. Since the binding of mAbs to a specific epitope is highly dependent on conformation (Benjamin et al., 1984), particularly with a protein such

as neurophysin with seven disulfide bridges, this method should be applicable to other electroblotted low-molecular-weight proteins previously undetectable by mAbs.

The use of the gel electrophoresis system described by Schägger and von Jagow (1987) to separate the low-molecular-weight peptides in this report is advantageous in two respects. First, due to the substitution of the faster migrating tricine in place of glycine for the cathode-running buffer, a medium concentration of acrylamide can be used. Since the efficiency of protein transfer depends both on the acrylamide concentration and cross-linking of the gel (Gershoni et al., 1983), this becomes an important issue. Although 14.5% T gels were used in this study, acrylamide concentrations up to 16.5% T can be used for Western blotting without significant decrease in transfer efficiency. Second, this system obviates the inclusion of urea in the gels. Although Sheng et al. (1988) recently described the use of urea gels to separate myelin basic protein for subsequent transfer to NC and probing with mAbs, urea can present problems in resolution and calibration of some proteins. For example, the $bc_1$ complex of beef heart mitochondria is resolved poorly in urea (Schägger and von Jagow, 1987) and small-molecular-weight proteins such as insulin and bradykinin were found to deviate from standard curves when electrophoresed in urea gels (Schägger and von Jagow, 1987). We have, therefore, found it preferable to omit urea from the gels.

The effect of renaturing the gels prior to transfer and the use of a basic buffer for electroblotting has been described by Szewczyk and Kozloff (1985), Dunn (1986) and more recently by Bestagno et al. (1987).

Although this method was originally conceived for the transfer of very basic proteins such as histones and heterogeneous ribonuclear proteins, it appears to dramatically improve transfer and subsequent detection of nonbasic proteins with mAbs. Dunn (1986) was able to demonstrate significant improvement in the detection of the 15 KDa $\epsilon$-subunit of *E. coli* $F_1$-ATPase when gels were renatured and then electroblotted in basic transfer buffer. We have shown that the most dramatic improvement in mAb immunodetection of calcitonin, neurophysin, vasopressin, insulin and other low-molecular-weight peptides results from renaturing the gels and then electroblotting in basic transfer buffer, findings that agree with Dunn (1986). While it is clear that renaturing the gels in the Tris/glycerol buffer prior to transfer is allowing the protein to approximate its original native conformation, and, therefore, enhanced mAb binding, the effect of basic transfer buffer is not so straightforward. Although modifying the charge on the amino acid side chains at the protein surface may not normally affect conformation, if these amino acids are contributing to salt bridges and thus folding of the protein, charge may be important (Hollecker and Creighton, 1982; Creighton, 1978). Therefore, at pH 10, the transfer buffer may be causing a refolding of the protein or stabilizing its conformation, once the SDS is removed in the renaturation step.

It is evident from our study that heating the NC blots at 70°C in paraformaldehyde vapor results in a slight increase in sensitivity. Paraformaldehyde fixation, described by Larsson (1981), was used in our study to immobilize transferred peptides to NC in an attempt to improve retention. Additionally, since formaldehyde inactivates the $\epsilon$-amino

group on lysine residues (Hougaard and Larsson, 1981), nonspecific antibody binding because of electrostatic interaction via the Fc region was reduced. However, our data, and those of others, show that heating of the blots may be the critical factor in increasing sensitivity. Swerdlow et al. (1986) reported that the heating of dry or hydrated NC membranes resulted in increased immunodetection for some antibody/antigen reactions. Additionally, when discussing the mechanism of protein and nucleic acid binding to NC membranes, Van Oss et al. (1987) conclude proteins should be baked on the membranes for maximum retention. There, however, does not appear to be any significant decrease in detection sensitivity of protein blots when paraformaldehyde is omitted from the heating step in our procedure. The reduction of electrostatic interaction is evidenced by a significant decrease of Ponceau S staining of peptide molecular weight standards on paraformaldehyde-fixed NC blots.

The importance of enzyme conjugates in the detection of transferred low-molecular-weight peptides by mAbs is dramatically demonstrated in this report. The use of alkaline phosphatase conjugated to avidin led to an increase in sensitivity by over tenfold when compared with HRP and β-Gal avidin conjugates. There appears to be very little difference between ExtrAvidin and Streptavidin in terms of sensitivity. Turner (1983) and Ey and Ashman (1986) have also reported the increased sensitivity of alkaline phosphatase conjugates in the visualization of blotted proteins probed with mAbs. However, when probing crude extracts, particularly from brain, higher backgrounds or nonspecific staining may result from endogenous alkaline phosphatase

activity. This endogenous activity can be greatly reduced by incubating the blots in 0.1 M EDTA for several hours at RT (Ey and Ashman, 1986).

Although antibody/antigen binding is dependent on several factors, particularly when using mAbs, the method described in this report for detecting low-molecular-weight peptides with mAbs should be of value for visualizing other antibody/antigen pairs by Western blot analysis, particularly partial proteolytic digests. We have additionally shown that this method can improve the sensitivity of low-molecular-weight NC blots probed with pAbs, possibly because of increased transfer efficiency (as seen with vasopressin) and conformational change. Minor variations in the procedure such as length of electrotransfer, peptide and antibody concentrations, and pH of the transfer buffer may be introduced in order to optimize immunodetection. For example, decreasing the pH of the transfer buffer to 9.5 for example, results in a more efficient transfer of a peptide fragment of atrial natriuretic factor to NC (Nilaver et al., 1989).

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Chapter II

# Identification of Neurophysin Immunoreactivity in Hypothalamus by a Monoclonal Antibody to a Carcinoma Cell Surface Antigen

(neurophysin/immunohistochemistry/Western blots/tumor antigen/lung neoplasms)

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Abbreviations: mAb, monoclonal antibody; ABC, avidin-biotin-peroxidase; HoDI, homozygous Brattleboro rats with diabetes insipidus; OT, oxytocin; PVN, paraventricular nucleus; SON, supraoptic nucleus; TTBS, 20 mM Tris, with 0.5 M NaCl, 0.1% Tween-20 and 0.02% $NaN_3$; VP, vasopressin.

## STATEMENT OF CO-AUTHORSHIP

My contribution to this manuscript included affinity purification of porcine neurophysin, extraction and immunologic characterization of human neurophysin from pituitary glands, all gel electrophoresis and Western blotting of bovine and human neurophysin samples and affinity preabsorption experiments of mAb L6. Dr. Gajanan Nilaver, who made the initial finding that mAb L6 immunoreacted with rat hypothalamic nuclei, performed all the immunohistochemistry experiments. I had an equal share in the writing and preparation of this manuscript along with Dr. Nilaver.

## ABSTRACT

Mouse monoclonal antibody (mAb) L6 identifies an antigen expressed on the cell surface of many different human carcinomas. While studying the binding activity of mAb L6 to LX-1 intracerebral tumor xenografts in nude rats using immunohistological techniques we observed that L6 can also bind to a cytoplasmic antigen expressed in the magnocellular component of the hypothalamo-neurohypophysial system. Double-labeling experiments with antisera to vasopressin and oxytocin confirmed the localization of L6 immunoreactivity within both peptide-containing cell groups. L6 immunoreactivity in Brattleboro rats (with genetic deletion in the vasopressin gene) was exclusively localized within oxytocin neurons. Oxytocin and vasopressin failed to block L6 staining which suggested that its target epitope resides within the neurophysin sequence, and this explanation was supported by the finding that absorption of L6 with porcine neurophysin completely eliminated hypothalamic immunoreactivity. Western blot analysis of bovine neurophysin and human pituitary extracts identified L6-immunoreactive bands which corresponded to the position of neurophysin and propressophysin, confirming that L6 immunoreactivity is related to neurophysin. Thus, monoclonal antibody L6, which is highly reactive with a *membrane* antigen of human lung cancer cell line LX-1, recognizes a *cytoplasmic* epitope in hypothalamic neurons identified as neurophysin by immunohistochemistry and Western analysis.

# INTRODUCTION

Monoclonal antibodies (mAbs) to tumor-associated cell surface antigens offer promise for cancer diagnosis and therapy (Hellström and Hellström, 1985; Reisfeld and Sell, 1985; Reithmuller et al., 1984). Mouse mAbs of the $IgG_{2a}$ and $IgG_3$ subclasses can have the added advantage of mediating antibody-dependent cellular cytotoxicity (ADCC) as well as complement-dependent cytotoxicity (CDC). A therapeutic potential of mAbs has been suggested by experiments in nude mice and rats bearing human tumors (Hellström et al., 1985; Powe et al., 1984; Lee et al., 1988) as well as in human cancer patients treated with antitumor mAbs (Houghton et al., 1985; Sears et al., 1982).

mAb L6, an $IgG_{2a}$ generated by immunizing mice with human lung adenocarcinoma cells (Hellström et al., 1986), has been shown to bind to a surface epitope of many different human carcinomas, including those of the lung, colon, breast, and ovary (Hellström et al., 1986). Labeled mAb L6, and its $F(ab')_2$ fragments demonstrate specific binding to human LX-1 small-cell lung carcinoma tumor xenografts in nude mice and rats. Intact L6 can also manifest ADCC- and CDC-mediated oncolytic activity (Hellström et al., 1986). The antibody does not internalize when tumor cells are exposed to it (I.H., unpublished data).

We have been investigating the efficacy of CNS delivery of systemically administered antibodies following transient blood-brain barrier disruption (BBB-D) as an approach to open the "tight junctions" between brain tumor capillary endothelial cells, which normally preclude the entry of antibodies into brain and brain tumor (Neuwelt et al., 1987; Neuwelt et al., 1980). While studying the delivery of mAb L6 in

LX-1 tumor-bearing nude rats by immunohistochemistry we observed its specific labeling of neurophysin-containing hypothalamic neurons. This paper reports on our immunohistochemical and immunoblot studies demonstrating mAb L6 binding to rat and human oxytocin-neurophysin (OT-NP) and vasopressin-neurophysin (VP-NP).

MATERIALS AND METHODS

Materials: Bovine oxytocin-neurophysin (OT-NP), vasopressin-neurophysin (VP-NP), ExtrAvidin alkaline phosphatase, diaminobenzidine and benzidine dihydrochloride were from Sigma (St. Louis, MO). Biotinylated protein A and the ABC kit were obtained from Vector laboratories (Burlingame, CA). Alkaline phosphatase-conjugated goat antimouse IgG $F(ab')_2$ was from ICN Immunobiologicals (Lisle, IL). Oxytocin (OT) and $Arg^8$ vasopressin (VP) were from Serva (Westbury, NY). Affigel-10 was obtained from Bio-Rad (Richmond, CA). Human pituitary glands were obtained from the National Pituitary Agency, and a crude NP extract was prepared using the protocol of Verbalis and Robinson (1983), without the column chromatography step. Porcine neurophysin extract, provided by Earl Zimmerman and Alan Robinson, was used in the absorption experiments.

Antibodies: Monoclonal antibody L6 is an $IgG_{2a}$ produced by a hybridoma derived by fusing spleen cells from a BALB/c mouse immunized with live cultured cells from a human lung adenocarcinoma, using NS-1 mouse myeloma cells as the fusion partner (Hellström et al., 1986). This antibody has previously been shown to recognize a cell surface antigen expressed in human lung, breast, colon, and ovarian carcinomas (Hellström et al., 1986) and to lyse L6-antigen positive human tumor

cells in the presence of human mononuclear cells and/or complement (Hellström et al., 1986). P1.17, an $IgG_{2a}$ myeloma protein (American Type Culture Collection), was employed as a negative control for immunohistochemistry and the Western blots. A polyclonal antiserum to porcine NPs was obtained from ICN Immunobiologicals (Lisle, IL). This antiserum recognizes both OT-NP and VP-NP, and was used at a dilution of 1:1000 in immunocytochemical procedures. The antihuman VP-NP was provided by Dr. Alan G. Robinson (University of Pennsylvania). Its specificity has been previously established (Robinson, 1975). The VP and OT antisera used in the immunocytochemical procedures were generated in rabbits, and have been previously characterized (Watson et al., 1982; Bodnar et al., 1985).

Target cells: Lung carcinoma cell line LX-1 was established from a human oat cell carcinoma (Ovejera and Houchens, 1981) and was obtained from Mason Laboratories (Worcester, MA). The tumor cells were grown in RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with 10-15% heat-inactivated fetal bovine serum.

Animals: Nude rats were obtained by breeding from rats which had been originally obtained from the National Cancer Institute. Twelve-week-old males were used for our experiments. They were placed in filter top cages and maintained in condominium units in a pathogen free room. Rats were intracerebrally inoculated with 10 $\mu l$ (8 x $10^7$ ml) of LX-1 tumor cells in the right cerebral hemisphere (Chambers et al., 1981). Ten adult male Long-Evans rats and five adult male homozygous Brattleboro rats (250-300 g) with diabetes insipidus (HoDI) were obtained from Blue Spruce Farms (Altamont, NY) and also used for

immunohistochemical analysis.

Rats were anesthetized with sodium pentobarbital (60 mg/kg body weight, i.p.) and perfused through transcardiac puncture with normal saline followed by 4% (w/v) ice cold buffered paraformaldehyde. The brains were removed and blocked in the coronal plane to include both the tumor bearing region and hypothalamus (nude rats) or the hypothalamic region (Long-Evans and HoDI rats). The pituitary glands were also removed for immunocytochemical analysis. Brain and pituitary tissue were postfixed in 4% (w/v) buffered paraformaldehyde for 24 hr. Human hypothalamic tissue, obtained at postmortem, was blocked in the coronal plane to include the regions of the supraoptic (SON) and paraventricular (PVN) nuclei, and immersion fixed in ice cold buffered 4% (w/v) buffered paraformaldehyde for several days.

Immunocytochemistry: Cytocentrifuged samples of cultured LX-1 tumor cells, smeared on silanated glass slides, and Vibratome cut sections of rat and human brain were used for the immunohistochemical analysis. Tumor cells on slides were fixed by immersion (10 min) in 4% (w/v) buffered paraformaldehyde. Tumor bearing regions of nude rat brain, and rat and human hypothalamus were sectioned serially at 100 μm with a Vibratome (Oxford Instruments, Bedford, MA) following fixation. Tumor cells on slides, and free floating tissue sections were rinsed in 50 mM Tris buffer, pH 7.6 containing 0.9% NaCl, and immunocytochemically labeled with mAb L6, employing biotinylated protein A and avidin-biotin-peroxidase (ABC) in the preembedding staining technique (Kozlowski and Nilaver, 1983; Nilaver and Kozlowski, 1989). In brief, the tissues were incubated with mAb L6 (50 μg/ml, overnight at 4°C) and

then sequentially reacted with biotinylated Protein A (3 μg/ml, 45 min at room temperature) and the ABC complex (1:1000, 1 hr at room temperature). The ABC complex was prepared 5 min prior to use by mixing together equal parts of 1:1000 dilutions of the stock Avidin DH and biotinylated peroxidase reagents provided in the Vectastain ABC kit (Vector Labs, Burlingame, CA). Reaction products were then formed with 15 mg% 3,3'diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO). Following development of the brown reaction product, tumor cells on slides were dehydrated, cleared with xylene and coverslipped for histological analysis. Tissue sections were mounted on gelatin coated slides, dehydrated, cleared with xylene and permanently mounted under cover slips.

Double staining immunohistochemistry: A few selected sections of hypothalamus from nude, normal and HoDI rats were immunoreacted with mAb L6 to yield a brown reaction product with diaminobenzidine after which they were also immunoreacted for VP, OT or their carrier proteins, NPs. The sections were incubated with polyclonal antisera specific for these hormones as described above, employing benzidine dihydrochloride as the alternate chromogen to produce blue labeling for the second antigen (Lakos and Basbaum, 1986; Levey et al., 1986).

Controls: Methods controls included replacement of mAb L6 with Tris buffer or P-1.17 ($IgG_{2a}$ myeloma protein of the same class as L6). Controls for specificity included overnight preincubation of mAb L6 (50 μg/ml) with Affigel-10 coupled to porcine NP, synthetic VP or OT (see section on preabsorption of L6). The gel-antibody suspension was centrifuged (1200 x g; 15 min) and the supernatant (absorbed antibody)

used for the absorption control in immunohistochemistry.

Gel Electrophoresis and Western Blotting: Gel electrophoresis and Western blotting was performed according to the procedure of Rosenbaum et al. (1989). Protein and polypeptide samples were solubilized in 1X Laemmli sample buffer (Laemmli, 1970) and heated to 100°C for 5 min. Samples were electrophoresed on 12.5% total acrylamide (% T; acrylamide:bisacrylamide; 1:15.5) using the tricine-SDS-PAGE system. After electrophoresis, gels were renatured by washing 3 times for 10 min in 50 mM Tris, pH 7.4, containing 20% (v/v) glycerol. The proteins were then electrophoretically transferred to 0.2 $\mu$m nitrocellulose in 10 mM $NaHCO_3$/3 mM $Na_2CO_3$, pH 10.0, with 20% (v/v) methanol for 1 hr at 1 amp employing a TE-42 Transphor unit (Hoefer, San Francisco, CA).

After air drying overnight, nitrocellulose blots were fixed for 4 hr in 0.5% (v/v) paraformaldehyde vapor at 70°C to immobilize the peptides. Nonspecific binding sites on the blots were blocked with 3% (w/v) gelatin in 20 mM Tris, pH 7.5, containing 0.5 M NaCl, 0.1% (v/v) Tween-20 and 0.02% (w/v) $NaN_3$ (TTBS) for 1 hr. This was followed by washing 3 times for 5 min each in TTBS. Blots were then incubated with primary antibody (concentration indicated in figure legend) in TTBS with 0.1% (w/v) recrystallized bovine serum albumin (BSA) for 1 hr (polyclonal antibodies) or 3 hr (mAbs) at room temperature and washed 3 times for 5 min in TTBS. Blots were then incubated with biotinylated protein A (3 $\mu$g/ml) in TTBS with 0.1% (w/v) BSA for 1 hr. After washing 3 times for 5 min in TTBS, the blots were incubated in ExtrAvidin-alkaline phosphatase (1.7 $\mu$g/ml) in TTBS for 45 min, again washed 3 times for 5 min in TTBS, and transferred to clean trays. Immobilized

polypeptides were detected by developing the blots in a mixture of *p*-nitro blue tetrazolium chloride (NBT; 0.33 mg/ml) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; 0.165 mg/ml) in 0.1 M Tris, pH 9.5, containing 0.1 M NaCl, 5 mM $MgCl_2$ and 0.5% (v/v) Tween-20. Blots incubated with $F(ab')_2$ fragments prepared from mAb L6 were reacted with alkaline phosphatase-conjugated goat antimouse IgG $F(ab')_2$ (1:500) for 90 min, washed 3 times for 5 min in TTBS and developed in NBT/BCIP as above.

Preabsorption of L6: Ten milliliters (ml) of Affigel-10 washed with 6 volumes of cold distilled water was incubated with porcine NP (final concentration of 10 mg/ml), VP (1 mg/ml) or OT (1 mg/ml) in 10 ml of 0.1 M HEPES, pH 7.5, containing 80 mM $CaCl_2$ (buffer A) for 4 hr (end on end stirring; 4°C). Any remaining active sites on the gel were blocked by the addition of 1 M ethanolamine-HCl, pH 8.0 (0.1 ml/ml gel) for 1 hr while rotating at 4°C. The gel was then washed with buffer A until the $OD_{280}$ was background. Coupling efficiency was determined by quantitating protein in the wash by the method of Bradford (1976).

Coupled Affigel-10 was centrifuged at 1200 x g in a Sorvall SS-34 rotor for 15 min and the supernatant decanted and discarded. The gel was then incubated with mAb L6 (200 µg/ml) overnight while rotating at 4°C. The gel-antibody suspension was then recentrifuged at 1200 x g as above for 15 min and the supernatant (absorbed antibody) decanted for use in Western analysis and immunohistochemistry. The coupled Affigel-10 was regenerated by washing with 10 volumes each of 0.1 M glycine (pH 3.0), 25 mM $Na_2HPO_4$ (pH 8.5), each containing 0.5 M NaCl, and finally with buffer A.

In order to eliminate the possibility of nonspecific binding of mAb L6 to the coupled Affigel-10 matrix through electrostatic interaction, a few experiments were performed in which mAb L6 was preincubated with poly-l-lysine (2 mg/ml), according to the procedure of Scopsi et al. (1986), prior to absorption with coupled Affigel-10.

## RESULTS

Immunohistochemical detection of L6 immunoreactivity: Studies on vibratome sections of tumor-bearing nude rat brain demonstrated L6 immunoreactivity with intracerebrally xenografted LX-1 tumor cells (Fig. 1A), and within cells in the region of the PVN and SON nuclei of the hypothalamus (Fig. 1C).

The immunoreactivity with tumor (Fig. 1B) was exclusively confined to the surface of the LX-1 tumors cells. A similar pattern of surface staining was seen with cultured LX-1 cells that had been cytocentrifuged onto silanated glass slides prior to immunohistochemical staining (data not shown).

L6 immunoreactivity of hypothalamic PVN and SON neurons was localized to the cytoplasmic compartment, and extended into the proximal dendrites and axonal processes with no detectable binding to cell membranes (Fig. 1,E and F). The axonal staining had a typical "beaded" appearance, and could be traced in its entirety to terminate in the posterior lobe of the pituitary gland. An identical pattern of cytoplasmic staining of PVN (Fig. 1H) and SON neurons and their axonal processes was noted in the human hypothalamus.

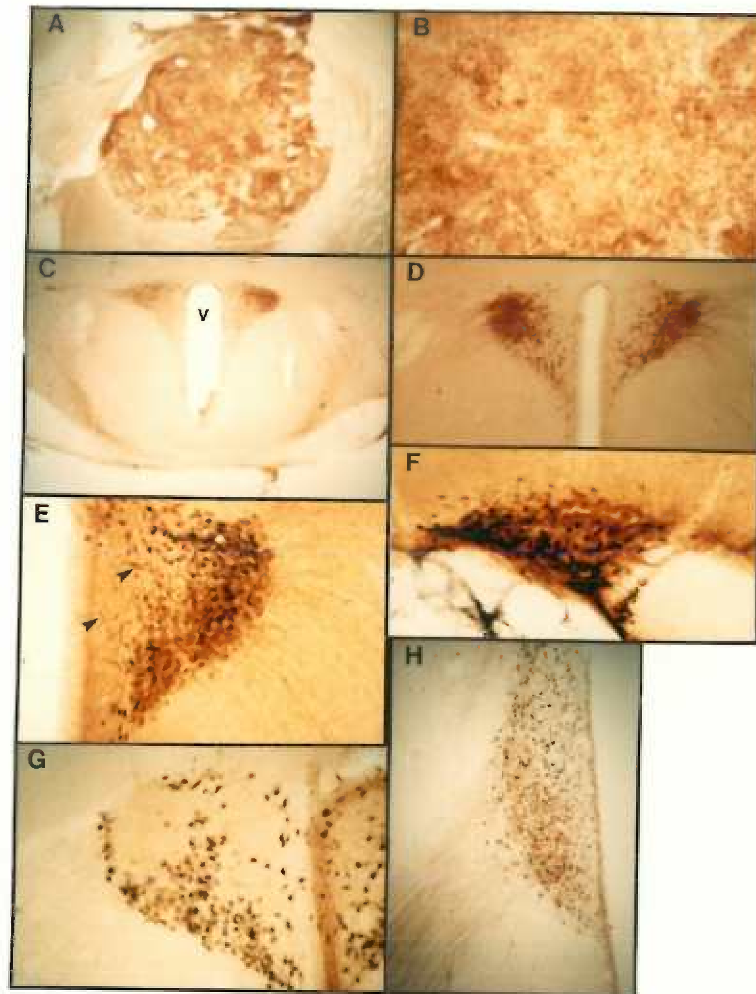
A similar pattern of staining in rat PVN (Fig. 1D) and SON neurons was demonstrated using an antiserum to porcine NPs which suggested that

mAb L6 recognized an antigenic epitope common to both OT- and VP-neurophysins. This was further supported by the observation that L6-labeled perikarya in rat and human hypothalamus were distributed in both the ventral and the dorsal parts of the SON, and the central and peripheral regions of the PVN, respectively. This corresponds to the distribution of both OT and VP neurons (Swanson and Sawchenco, 1983). Double-staining immunohistochemistry with mAb L6 and polyclonal antibodies to VP, OT or NPs was, therefore, performed on a few selected sections of nude, normal and HoDI rat hypothalamus to determine the precise relationship of L6 immunoreactive neurons to the VP and OT neuronal systems. As shown in Figure 1E, mAb L6 labels neurons in both the central and peripheral parts of the PVN (brown) and approximately half of the L6-positive neurons also expressed VP immunoreactivity (blue). In the SON (Fig. 1F), the L6-reactive neurons occupied the dorsal and ventral regions of the nucleus (brown), with only the ventral neurons showing additional immunoreactivity for VP (blue). When adjacent, L6-stained, hypothalamic sections were incubated with the OT antiserum as the second reactant of the double-labeling technique, the L6-reactive neurons not visualized with the VP antibody were found to express OT immunoreactivity.

Figure II-1 L6 immunoreactivity in LX-1 tumor, and rat and human hypothalamus. (A) Low power photomicrograph of a Vibratome section through LX-1 tumor-bearing region of nude rat brain immunoreacted with mAb L6. L6 selectively outlines the tumor. (B) At this higher magnification, L6-immunoreactivity within the tumor graft can be seen to be exclusively confined to the surface of LX-1 tumor cells. (C) Low power photomicrograph of LX-1 tumor-bearing nude rat hypothalamus demonstrating L6-immunoreactivity in the region of the PVN, located on either side of the third ventricle (v). (D) Vibratome section through normal rat hypothalamus immunoreacted with a polyclonal antiserum to porcine NPs demonstrates a similar pattern of staining in the PVN. (E) Vibratome section of normal rat PVN double-labeled with mAb L6 (brown) and a polyclonal antiserum to VP (blue). L6 labels cytoplasm, proximal dendrites (arrowheads) and "beaded" axonal processes of PVN neurons in both the central and peripheral regions of the nucleus. Approximately half of the L6-positive neurons can also be seen to express VP-immunoreactivity (blue). (F) SON region of same section demonstrates L6-reactive neurons in both the dorsal and ventral parts of the nucleus (brown). VP-immunoreactivity (blue) is coexpressed in only the ventral SON neurons. (G). Double-staining immunohistochemistry through HoDI PVN employing mAb L6 (brown) and a polyclonal antiserum to OT (blue). Only about 50% of the HoDI PVN neurons express L6-

immunoreactivity (brown) when compared to normal rat PVN (as seen in E). The L6-immunoreactivity (brown) appears to exclusively reside within OT neurons (blue). (H). Vibratome section through human hypothalamus demonstrates cytoplasmic L6-immunoreactivity of PVN neurons and their axonal processes. (original magnifications = A, C and H: x 13; B, E-G: x 52; D: x 16).

Figure II-1



Immunostaining of HoDI rat hypothalamus showed L6-immunoreactivity in only 50% of PVN (Fig. 1G) and SON neurons. This was expected since a single base deletion in the NP coding domain of the HoDI propressophysin gene has been shown to preclude translation of VP and its associated NP (Schmale and Richter, 1984). The L6-immunolabeled neurons in HoDI hypothalamus were selectively localized in the peripheral region of the PVN (Fig. 1G) and the dorsal aspect of the SON. Furthermore, double-label experiments showed the L6-immunoreactivity to reside exclusively within the OT-ergic subpopulation of PVN (Fig. 1G) and SON neurons (data not shown).

Solid-phase absorption of mAb L6 (50 $\mu$g/ml) with synthetic arginine$^8$ VP-or OT-conjugated Affigel-10 (1 mg/ml) had no effect on the hypothalamic immunoreactivity. Preabsorption of L6 with equimolar amounts of Affigel-10 conjugated porcine NPs, however, completely eliminated all hypothalamic immunoreactivity.

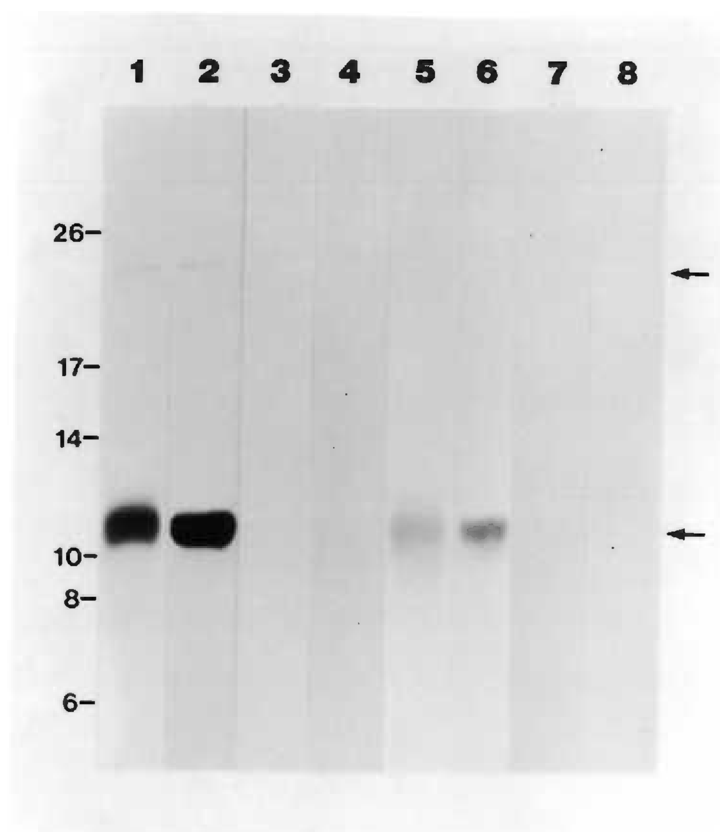
Western blot analysis: L6 immunoreactivity with purified bovine VP-NP and OT-NP is shown in Figure 2. The polyclonal antiporcine NP, which recognizes both NPs by immunohistochemistry (see above), shows immunoreactivity with processed VP-NP (Lane 1) and OT-NP (Lane 5) (corresponding to the lower arrow in Figure 2), as well as with precursor (upper arrow in Figure 2) present in the VP-NP preparation (Lane 1). mAb L6 appeared to recognize a shared sequence in the two NPs as demonstrated by its immunoreactivity with VP-NP (Lane 2) and OT-NP (Lane 6). These data are in agreement with the immunohistochemical observations, and indicate that mAb L6 recognizes a common sequence within the NP molecules. A control mouse immunoglobulin (P1.17) of the

same class as L6 ($IgG_{2a}$), did not bind to either VP-NP (Lane 3) or OT-NP (Lane 7), indicating that the L6 binding to NPs was antigen specific. Preincubation of mAb L6 with NP-bound Affigel 10, using the supernatant to probe VP-NP and OT-NP in the immunoblots, eliminated virtually all immunoreactivity for both VP-NP (Fig. 2, Lane 4) and OT-NP (Fig. 2, Lane 8), further validating the specificity of the binding of L6. Preincubation of mAb L6 with poly-l-lysine prior to absorption with NP showed identical elimination of immunoreactivity.

The binding of L6 to NP extracted from human pituitary glands is shown in Figure 3. Lane 2 shows the relative positions of processed NP (lower arrow) and its precursor (upper arrow) as determined by a polyclonal antiserum to human VP-NP. The binding of a polyclonal arginine$^{8}$ VP antibody to the upper band (Lane 5) confirmed its identification as propressophysin. L6 immunoreactivity (Lane 3) is demonstrated in both processed NP ($M_r$ 11,000) and propressophysin ($M_r$ 21,000). The blot was also probed with $F(ab')_2$ fragments prepared from mAb L6 in order to

Figure II-2 Immunoreactivity of mAb L6 with bovine NP I and II. NPs were electrophoresed, blotted and probed with antibodies as described in Materials and Methods. Lanes 1-4 show immunoblots of bovine NP II (5 μg) probed with porcine anti-NPs (1:5000; Lane 1), mAb L6 (200 μg/ml; Lane 2), mAb P1.17 (200 μg/ml; Lane 3), and mAb L6 preabsorbed with porcine NPs (200 μg/ml prior to preabsorption; Lane 4). Protein was slightly overloaded in order to facilitate detection of the small amounts of propressophysin in the preparation. Lanes 5-8 are immunoblots of bovine NP I (5 μg) probed with anti-NPs (1:5000; Lane 5), mAb L6 (200 μg/ml; Lane 6), mAb P1.17 (200 μg/ml; Lane 7), and mAb L6 preabsorbed with porcine NPs (200 μg/ml prior to preabsorption; Lane 8). Molecular weight markers were: chymotrypsinogen A ($M_r$ 25,666), myoglobin ($M_r$ 16,950), myoglobin fragments I + II( ($M_r$ 10,670), myoglobin fragment I ($M_r$ 8,160), myoglobin fragment II ($M_r$ 6,210), and myoglobin fragment III ($M_r$ 2,510). The relative positions of propressophysin and processed NPs are indicated by the upper and lower arrows, respectively.

Figure II-2



determine that the immunoreactive bands in Lane 3 represented specific antigen binding via the Fab region. As shown in Lane 4, L6 $F(ab')_2$ demonstrates a similar pattern of reactivity with human NP as the intact antibody molecule, suggesting the immunoreactivity is highly specific. Blots were additionally probed with antihuman OT-NP, producing similar immunoreactivity (data not shown), again suggesting L6 is recognizing a common epitope shared by VP-NP and OT-NP.

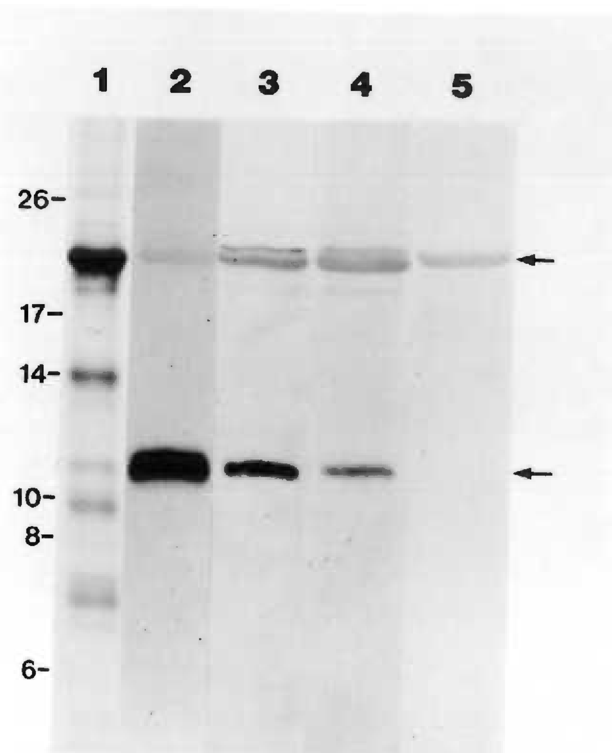
DISCUSSION

We have demonstrated the binding of mAb L6 to the surface of grafted LX-1 tumor cells using immunohistochemistry, which is in agreement with published results (Hellström et al., 1986). We have also shown L6 immunoreactivity within neurons of the SON and PVN in rat and human hypothalamus. The hypothalamic immunoreactivity, however, is exclusively cytoplasmic, being confined to neuronal perikarya, their proximal dendrites, and the entire length of their axonal processes. Our findings, thus, imply that L6 can bind to an axonally transported secretory protein of the hypothalamo-neurohypophysial system.

The hypothalamic nonapeptides, OT and VP, are synthesized together with their carrier proteins, the NPs, within perikarya of the SON and PVN. The hormones and NPs are packaged within neurosecretory granules and transported to nerve terminals in the posterior pituitary gland. Two distinct NPs have been identified, one associated with OT (termed OT-NP)

Figure II-3 Immunoreactivity of mAb L6 with human pituitary extract. NPs from which human pituitary was extracted, electrophoresed, blotted and probed with antibody as described in Materials and Methods. Lane 1 shows a Coomassie-blue stained gel pattern of the pituitary extract (15 μg) prior to electroblotting and probing with antibody. Lanes 2-5 are immunoblots of the pituitary extract (15 μg) probed with antihuman NPs (1:5000; Lane 2), mAb L6 (200 μg/ml; Lane 3), mAb L6, $F(ab')_2$ (200 μg/ml; Lane 4), and rabbit anti-VP (1:500; Lane 5). Molecular weight markers were the same as in Fig. 2. The relative positions of propressophysin and processed NPs are indicated by the upper and lower arrows, respectively.

Figure II-3



and another with VP (called VP-NP). Each nonapeptide and its corresponding NP are synthesized from large molecular weight common precursors within distinct neuronal populations of the SON and PVN. The common precursor for VP and its NP is termed propressophysin, and a similar common precursor for OT and its NP is called prooxyphysin.

As seen in Figure 1D, a polyclonal antiserum that recognizes both NPs demonstrates a pattern of immunohistochemical staining similar to that obtained with mAb L6, suggesting that L6 recognizes an antigenic epitope common to VP-NP and OT-NP. The staining by L6 of VP and OT neurons within the SON and PVN supports this view. The inability of OT and VP to block hypothalamic L6 immunoreactivity implies that L6 does not recognize either of these nonapeptides, an important issue since a seven amino acid domain is shared by these two hormones. This was further substantiated by Western blot analysis using mAb L6 to probe synthetic VP and OT (data not shown). A continuous 62 amino acid stretch of sequence homology (region 10-72) is known to exist between the two NPs (Schmale and Richter, 1984). L6 immunoreactivity could reside in this shared NP domain. The selective binding of L6 to OT neurons of HoDI rat, which cannot express VP or VP-NP due to a deletion in the propressophysin gene (Schmale and Richter, 1984), further suggests that L6 immunoreacts with NP. This conclusion is supported by the complete elimination of L6 immunoreactivity in the hypothalamus by preabsorption with Affigel-conjugated porcine NP.

Since immunohistochemistry does not suffice to definitively establish the nature of tissue antigens, we performed an electrophoretic size-separation of hypothalamic peptides on acrylamide gels.

Immunoreactivity of L6 was demonstrated to protein bands corresponding to the positions of NPs and/or their precursors. Western analysis with purified bovine and human NPs showed that L6 can bind to bands corresponding to processed VP-NP, OT-NP, and propressophysin; the identity of proteins within these bands were confirmed by using antisera to porcine NP, human NP and VP.

An inherent limitation of many immunological studies is that antibodies can form nonspecific complexes with proteins. Nonspecific binding of IgG can in large part occur from electrostatic interactions (Scopsi et al., 1986). They can also result from Van der Waals and/or hydrophobic interactions (Scopsi et al., 1986). Strongly basic molecules such as histones, ACTH, secretin and dynorphin/enkephalin can demonstrate nonspecific binding to IgG as well as to protein A and streptavidin-conjugated secondary detection agents (Scopsi et al., 1986). Both human OT-NP and VP-NP are moderately acidic with pIs of 5.15 and 5.55, respectively (North et al., 1980). The possibility of their nonspecific binding to mAb L6 IgG is consequently greatly reduced.

Several lines of evidence make it unlikely that nonspecific binding of mAb L6 to NPs account for our findings. (i) a mAb identical in subclass ($IgG_{2a}$) to L6 (P1.17) did not bind to NP blots and when tested in parallel with mAb L6 and did not stain LX-1 cells, human or rat hypothalamus; (ii) $F(ab')_2$ fragments of L6, used to probe human and bovine NP immunoblots, demonstrated immunoreactivity of an intensity similar to that of intact mouse L6, indicating specific binding of the variable region of the L6 molecule to NP. (iii) Preabsorption of L6 to Affigel-bound NP eliminated all L6 immunoreactivity for bovine VP-NP and

OT-NP; (iv) to ensure the binding of L6 to Affigel-NP was mediated by specific antigen-antibody reaction, rather than electrostatic interaction, L6 was also preabsorbed with poly-l-lysine prior to incubation with the NP-bound Affigel resin. Elimination of electrostatic charge with the basic peptide poly-l-lysine failed to affect L6-NP binding; (v) finally, the nitrocellulose blots were exposed to paraformaldehyde vapor prior to immunoblotting, a procedure which inactivates the $\epsilon$-amino group on lysine residues (Hougaard and Larsson, 1981) and significantly reduces electrostatic interaction, as evidenced by the reduced ability of protein stains to bind to paraformaldehyde fixed blots (unpublished observation).

Our findings may have relevance to the pathogenesis of ectopic VP production by human lung carcinomas. The biosynthesis of propressophysin by human oat cell tumors has been described (Yamaji et al., 1983) and several studies have reported on the ectopic production of NP (North et al., 1988) and VP (Spruce and Baylis, 1983) by small cell carcinomas of the lung. The detection of biologically active VP in pleural exudates and blood, and the associated inappropriate antidiuresis (Spruce and Baylis, 1983) point to the neurosecretory nature of some of these tumors. Several findings imply, however, that the L6 positive LX-1 tumor cells used in the present study are not quite analogous to the small cell carcinomas associated with VP secretion and inappropriate antidiuresis. We have found no evidence of fluid retention (inappropriate antidiuresis) or elevated VP levels in nude rats bearing intracerebral or subcutaneous LX-1 tumors, (see Appendix V)

and have not detected NP or VP immunoreactivity in the cytoplasm of LX-1 tumor cells by immunohistochemistry.

We have presented several lines of histological and biochemical evidence that suggests that mAb L6, in addition to binding to human oat cell (LX-1) tumor membranes, specifically recognizes NP in the cytoplasm of rat and human hypothalamic neurons. North et al. (1983) have previously shown that small-cell carcinoma cells demonstrate patchy intensity of cell membrane labeling when reacted with an antiserum to human VP-NP. It remains to be investigated whether binding of L6 to the surface of carcinoma cells is to a normal or mutant form of neurophysin, or whether it relates to an entirely different molecule. We have recently demonstrated expression of propressophysin mRNA in these cells by Northern analysis and *in situ* hybridization (Rosenbaum et al, 1989). We are currently in the process of extracting the membrane antigen from LX-1 tumors for immunobiochemical studies and sequence analysis. If the antigen is neurophysin it will be of interest to determine why it is preferentially expressed on the cell surface of this tumor.

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# Chapter III

## Expression of Neurophysin-related Precursor in Cell Membranes of a Small-Cell Lung Carcinoma

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

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Abbreviations: mAb, monoclonal antibody; NP, neurophysin; OT, oxytocin; PVN, paraventricular nucleus; SON, supraoptic nucleus; TTBS, 20 mM Tris, with 0.5 M NaCl, 0.1% Tween-20 and 0.02% $NaN_3$; VP, vasopressin; PPYsin, pro-pressophysin; PPLP, pro-pressophysin-like protein; PVDF, polyvinylidene difluoride; RIA, radioimmunoassay.

ABSTRACT

A monoclonal antibody (mAb L6), raised to a small-cell lung carcinoma surface antigen, recognizes a common epitope of vasopressin-neurophysin and oxytocin-neurophysin in hypothalamic nuclei (Nilaver et al., 1990a). We 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 approximately 45 Kd. Western blot analysis demonstrated immunoreactivity of this band with mAb L6, antivasopressin and an antibody to the vasopressin-neurophysin precursor, pro-pressophysin. Amino-terminal sequencing of this band demonstrated a 21-amino acid homology with the N-terminus of human pro-pressophysin, and substitution of an $Arg^{33}$ residue in the tumor antigen with $Cys^{33}$. Absence of immunoreactivity with the above antibodies 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 approximately 1000 bp, 250 bp 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 rats also did not have elevated serum vasopressin levels. These findings suggest expression of a pro-pressophysin-like protein in this tumor cell line which is preferentially targeted to the cell membrane. The nonsecretory nature of the LX-1 cell line suggests lack of a cleavage site in the precursor or a posttranslational modification preventing precursor processing and secretion.

# INTRODUCTION

Lung carcinomas are notorious for their association with paraneoplastic syndromes, often through production and secretion of peptide hormones. Hormones such as vasopressin (Payet et al., 1984; Rozengurt et al., 1979), neurophysin (NP) (Worley and Pickering, 1984), bombesin (Ruff and Pert, 1984) and bombesin-like peptides (Cuttitta et al., 1985) 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 (SCLC) and thus may allow early detection of tumor formation and monitor efficacy of treatment (North et al., 1988).

We have been characterizing a monoclonal antibody generated against a human lung adenocarcinoma (mAb L6) which binds to a surface epitope of human lung, colon, breast, and ovarian carcinomas (Hellström et al., 1986a). Our studies have demonstrated that mAb L6 specifically recognizes a common domain within vasopressin-neurophysin (VP-NP) and oxytocin-neurophysin (OT-NP) (Nilaver et al., 1990a). Since mAb L6 clearly is immunoreactive with a membrane-bound antigen, it was of interest to determine if the mAb L6-identifiable material on the surface of these tumors 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 (Ovejera and Houchens, 1981). This tumor cell line does not appear to process or secrete this precursor, instead being targeted to the cell membrane. To our knowledge this is the first report of a neuroendocrine hormone precursor being preferentially expressed in cell membranes.

# MATERIALS AND METHODS

## Materials

$^{125}$I (140 mCi/ml), [$^{35}$S]cysteine (1064 Ci/mmol), [$^{35}$S]$\alpha$-dATP (1300 Ci/mmol), [$^{32}$P]$\alpha$-dATP (300 Ci/mmol) and [$^{125}$I]vasopressin (2200 Ci/mmol) were from New England Nuclear (Wilmington, DE). Dimethylpimelimidate, ExtrAvidin alkaline phosphatase, nitroblue tetrazolium chloride, carboxypeptidase B (Type II), and 5-bromo-4-chloro-3-indolyl phosphate were from Sigma (St. Louis, MO). Oligo (dT)-cellulose Type 3 was from Collaborative Research (Bedford, MA). Terminal deoxynucleotidyl transferase was from Bethesda Research laboratories (Gaithersburg, MD). Protein A-Sepharose and concanavalin A-Sepharose were from Pharmacia (Piscataway, NJ). Biotinylated protein A was from Vector Laboratories (Burlingame, CA). Immobilon polyvinylidene difluoride (PVDF) membranes were obtained from Millipore (Bedford, MA). Synthetic pro-pressophysin (PPYsin) and pro-oxyphysin 30-mer oligonucleotides corresponding to the last 10 amino acids in VP-NP (exon 3; Schmale et al., 1983) and the last 10 amino acids in OT-NP (Exon 3; Ivell and Richter, 1984) respectively, were from Midland Reagent (Midland, TX). Hyperfilm was from Amersham (Arlington Heights, IL). Oligonucleotide 50 mer probes to human PPYsin and pro-oxyphysin corresponding to the 16 amino acids of the C-terminal glycopeptide (Exon 3; Sausville et al., 1985) and the last 9 amino acids of OT-NP (Exon 3; Sausville et al., 1985) respectively were synthesized on an Applied Biosystem Model 380A (Foster City, CA). Bovine pituitary glands were obtained locally, and PPYsin extracted using the protocol of Verbalis and Robinson (1983), followed by concanavalin A-affinity chromatography.

Antibodies

mAb L6 is an $IgG_{2a}$ produced by a hybridoma derived by fusing spleen cells from a BALB/c mouse immunized with cultured human lung adenocarcinoma cells using NS-1 mouse myeloma cells as the fusion partner (provided by Oncogen, Seattle, WA). This monoclonal antibody has been shown to recognize a cell surface antigen expressed in human lung, breast, colon and ovarian carcinomas (Hellström et al., 1986a) as well as specifically recognizing a common domain of both VP-NP and OT-NP (Nilaver et al., 1990a). P1.17, an $IgG_{2a}$ monoclonal antibody was from Oncogen (Seattle, WA) and employed as a negative control in the blotting experiments. YL-3 is a polyclonal antibody raised to the decapeptide sequence of human PPYsin as shown in Table I. This antibody has been shown to specifically label paraventricular (PVN) and supraoptic (SON) nuclei of monkey and rat hypothalamus by immunohistochemistry and NP precursor in human and bovine pituitary by Western blot analysis (manuscript in preparation). A polyclonal antibody to vasopressin was generated in rabbits and has been previously characterized (Bodnar et al., 1985).

LX-1 Cells and Tumors

Lung carcinoma cell line LX-1 was established from a human small cell carcinoma (Ovejera and Houchens, 1981) and was obtained from Mason Laboratories (Worcester, MA). Tumor cells were grown in spinner flasks with RPMI-1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum. Nude rats were bred from rats originally obtained from the National Cancer Institute. The rats were then intracerebrally inoculated with 10 $\mu l$ ($8 \times 10^7$ cells/ml) of LX-1 tumor cells in the right cerebral

hemisphere (Chambers et al., 1981; Neuwelt et al., 1985).

Immunoaffinity Isolation of LX-1 Antigen

mAb L6 was covalently cross-linked to protein A-Sepharose (12 mg antibody/ml gel) with dimethylpimelimidate (20 mM) essentially as described by Schneider et al. (1982). LX-1 cells ($5 \times 10^8$ cells/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 PMSF and 0.5% (v/v) Nonidet P-40. After a 15 min incubation on ice, the suspension was centrifuged at 3000 x g for 10 min to remove debris and the supernatant centrifuged at 100,000 x g for 1 hr. After adjusting the supernatant to 0.5 M NaCl, 1/50 volume of 10% formalin-fixed *staphylococcus aureus* suspension was added (30 min at 4°C) to remove nonspecific binding. This elimination of nonspecific binding was also done prior to immunoprecipitation (see below). This was followed by centrifugation at 100,000 x g for 30 min and the supernatant added to the mAb L6/protein A affinity matrix. After rotating gently overnight at 4°C, the protein A-Sepharose was pelleted at 500 x g for 2 min and washed with 50 mM Tris HCl, pH 8.2 containing 0.5 M NaCl, 1 mM EDTA and 0.5% (v/v) Nonidet P-40, 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 twice with an equal volume of 50 mM diethylamine, pH 11.5. The pooled elutions were immediately neutralized by adding 1/10 volume 0.5 M $NaH_2PO_4$.

LX-1 cell labeling with [$^{35}$S]cysteine and $^{125}$I

Two days after passaging, [$^{35}$S]cysteine was added (1 mCi/$10^8$ cells) to the LX-1 cells and allowed to incubate for 20 hr under normal culture conditions (37°C; 5% $CO_2$). Cells were surface iodinated using

lactoperoxidase (50 $\mu$g/ml), glucose oxidase (25 $\mu$g/ml), and Na $^{125}$I (1 mCi/$10^8$ cells). The reaction was initiated with the addition of glucose (250 $\mu$g/ml), allowed to incubate for 20 min at room temperature, and terminated 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 cell extracts (containing approximately 5 x $10^6$ cells/tube) were incubated with YL-3 antibody to PPYsin (at a dilution of 1:500) overnight at 4°C. Protein A-Sepharose (50 $\mu$l) was then added and allowed to incubate for 30 min at 4°C. After centrifugation in an Eppendorf Microfuge (2 min) to sediment the beads, the pellet was washed sequentially in 50 mM Tris HCl pH 8.2 containing 0.5 M NaCl, 50 mM Tris HCl pH 8.2 containing 0.1% (v/v) SDS, and 10 mM Tris HCl pH 7.4 containing 0.1 % (v/v) Nonidet P-40. The washed pellet was then boiled for 2 min in 1X Laemmli (1970) sample buffer, centrifuged, and the supernatants electrophoresed.

Gel electrophoresis and immunoblotting

SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970), using 12.5% fixed acrylamide (acrylamide:bisacrylamide; 1:30). Protein samples were solubilized in 1X Laemmli sample buffer containing 25 mM DTT, and heated to 100°C for 5 mins. In some experiments, purified antigen was deglycosylated with glycopeptidase F prior to electrophoresis, according to the procedure of Tarentino et al. (1985). Gels not used for blotting or autoradiography were stained with Coomassie blue or with silver, according to the method of Heukenshoven

and Dernick (1985). Immunoblotting was done as previously described (Rosenbaum et al., 1989). Gels containing $^{35}S$ and $^{125}I$ samples were dried and exposed to emulsion coated Hyperfilm $\beta$-max for 48 hr.

## Amino acid sequencing

Nondenatured immunoaffinity purified-LX 1 antigen (150 pmols) was subjected to limited proteolysis with carboxypeptidase B (0.6  g) in 0.3 mM N-ethylmorpholine acetate (pH 8.5) for 5 hrs at 37°C. The reaction was quenched by adding acetic acid to pH 3.0. Samples were then electrophoresed as described above, and electroblotted onto Immobilon PVDF membranes (Matsudaira, 1987). Membranes were stained briefly in 0.1% (w/v) Coomassie blue R-250, destained and the band excised. The stained protein band was sequenced by automated Edman degradation in a gas-phase sequencer (Model 470A, Applied Biosystems, Inc., Foster City, CA) equipped with a Applied Biosystems, Inc. 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 by homogenizing tissue or cells in 5 M guanidine isothiocyanate and precipitated with 4 M LiCl as described by Cathala et al. (1986). Poly($A^+$) RNAs were isolated using the oligo(dT)-cellulose batch method described by Sherman et al. (1986). Samples were fractionated on standard 1.5% agarose formaldehyde gels and passively transferred to nylon membranes in 20 x SSC. Membranes were prehybridized overnight at 45°C in 5 x SSC/20 mM $NaH_2PO_4$, pH 7.5 containing 20% (v/v) deionized formamide, 5 x Denhardt's (1% [w/v] polyvinylpyrrolidone, $M_r$ 40,000; 1% [w/v] Ficoll, $M_r$ 400,000; and 1% [w/v] BSA), 0.1 % (v/v) SDS

and 10 μg/ml sonicated, heat denatured salmon sperm DNA. Filters were then hybridized (45°C; 24 hr) in a similar buffer containing 1 x Denhardt's 1.0 μg/ml salmon sperm DNA and 4 nM 30-mer oligonucleotide $^{32}P$ labeled at the 3' end to a specific activity of 5-8 x $10^8$ dpm/nmol. Oligonucleotides were labeled on the 3' end with terminal deoxynucleotidyl transferase, and purified as described by Davis et al. (1986a). Membranes were washed to a stringency of 2 x SSC containing 1% (v/v) SDS at 45°C and exposed to x-ray film.

*In situ* hybridization

Rats were anesthetized with sodium pentobarbital (50 mg/kg body wt, i.p.) and perfused transcardially with saline followed by buffered 10% formalin. The brain was removed, and cryoprotected with 30% (w/v) sucrose containing 0.02% (v/v) diethylpyrocarbonate (DEPC). Cryostat sections (10 m) where cut in the coronal plane to include the tumor bearing region and hypothalamus, and were mounted on silanated glass slides. *In situ* hybridization was performed with oligonucleotide probes employing the technique of Davis et al. (1986b). Sections were delipidated (progressively graded alcohols and chloroform) and rehydrated (through regressively graded alcohols) to 2 x SSC. Sections were then prehybridized (1 hr, 25°C) with 2 x SSC containing 50% (v/v) deionized formamide, 10 x Denhardt's, 0.1% (v/v) SDS, and 0.1% (w/v) salmon sperm DNA (hybridization buffer). Sections are then overlayed with hybridization buffer containing oligonucleotide probes $^{35}S$-labeled at the 3' end (2.0 x $10^5$ cpm in 30 1) and 0.1 M of DTT (24 hrs, 25°C). The sections were then rinsed in 2 x SSC (4 hrs, 25°C, with 15 min changes), air dried, exposed to Hyperfilm β-max or dipped in Kodak

emulsion (NTB-3). After two weeks, the sections were developed, counter-stained, dehydrated, and coverslipped for microscopic analysis.

Specificity of the oligonucleotide probes used in the study were previously confirmed by Northern analysis (see above). 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, in contrast, recognizes a unique domain in the human sequence, and consequently should only hybridize with the human PPYsin. The following additional controls were performed to minimize the possibility of a false-positive signal: (i) prehybridization RNAse treatment of tissue; (ii) blocking cDNA-mRNA hybridization by addition of excess unlabeled probe.

Radioimmunoassay:

Radioimmunoassay (RIA) for plasma vasopressin in both normal nude rats and tumor bearing nude rats were performed essentially as previously described (Morton et al., 1975).

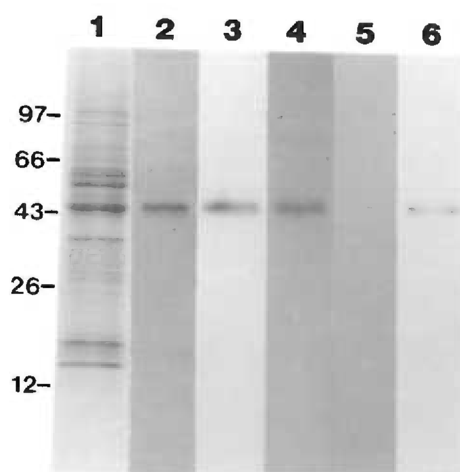
## RESULTS

Immunological characterization of the LX-1 antigen:

The isolation and immunologic characterization of LX-1 cell surface antigen is shown in Fig.1. Cell membranes solubilized in 0.5% (w/v) Nonidet P-40 resulted in many bands when electrophoresed and stained with Coomassie blue (Fig. 1; Lane 1). Since this extract demonstrated relatively weak immunoreactivity when blotted and probed with various antibodies, specific tumor antigen was isolated using an mAb L6-immunoaffinity column and eluted with 50 mM diethylamine at pH 11.5. The neutralized sample was then electrophoresed, resulting in a

Figure III-1 Isolation and immunological characterization of LX-1 cell surface antigen. LX-1 cell membranes were solubilized, immunoaffinity purified with mAb L6, electrophoresed, blotted and probed with antibody as described in Materials and Methods. Lane 1 shows a Coomassie-stained gel pattern of solubilized LX-1 cell membranes (20 $\mu$g). Lane 2 is a silver-stained gel pattern of mAb L6 immunoaffinity purified LX-1 antigen (0.5 $\mu$g). Lanes 3-6 are immunoblots of purified LX-1 antigen (3.0 $\mu$g) probed with YL-3 (1:500; Lane 3), mAb L6 (20 $\mu$g/ml; Lane 4), mAb P1.17 (20 $\mu$g/ml; Lane 5), and polyclonal anti-vasopressin (1:500; Lane 6). Molecular weight markers were phosphorylase b ($M_r$ 97111), 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).

Figure III-1

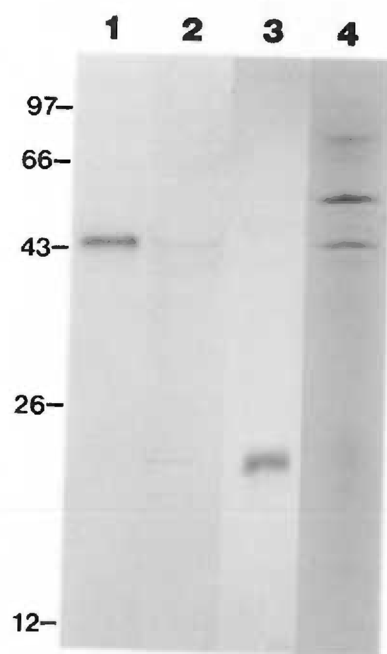


band corresponding to a $M_r$ of approximately 45,000 by silver staining (Fig. 1; Lane 2). A much less intense band with an apparent $M_r$ of approximately 42,000 appears to co-purify with the 45 kd protein. This band may be a proteolytic fragment of the $M_r$ 45,000 polypeptide, although it does not appear to be immunoreactive with the antibodies used in this study. The 45 kd fragment is highly immunoreactive with antibody YL-3, raised against human PPYsin (Fig. 1; Lane 3). Since this antibody only reacts with an intact Lys-Arg cleavage site in PPYsin, this strongly suggests the mAb L6-isolated LX-1 surface antigen to be related to PPYsin. Lane 4 (Fig. 1) demonstrates the ability of the antigen to bind mAb L6, while a monoclonal antibody of the same subtype ($IgG_{2a}$), P1.17, fails to show any immunoreactivity (Fig 1; Lane 5). The ability of the 45 kd band to bind a polyclonal antibody to vasopressin (Fig. 1; Lane 6) confirms its identity as a pro-pressophysin-related protein. Polyclonal antibodies to processed NP and oxytocin (OT) failed to demonstrate immunoreactivity with the purified antigen (data not shown).

Since previous studies (Hellström et al., 1986a; Nilaver et al., 1990a) demonstrated mAb L6 immunoreactivity to be exclusively confined to the surface of LX-1 tumor cells, it was of interest to further characterize the cells. Fig 2 shows LX-1 purified cell membrane 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 band with an approximate $M_r$ of 45,000 (Fig 2; Lane 1). The cytosolic extract shows only a very faint band at 45 kd and a slight band at 23 kd when probed with YL-3 (Fig 2;

Figure III-2 Western blot analysis and immunoprecipitation of LX-1 antigen with anti-human pro-pressophysin. Samples were electrophoresed, blotted, and probed as in Fig. I. LX-1 cells were incubated with [$^{35}$S] cysteine, solubilized, and immunoprecpitated with YL-3 antibody (see <u>Methods</u>). Lanes 1 and 2 are immunoblots of immunoaffinity purified LX-1 cell membrane (0.5 $\mu$g; Lane 1) and LX-1 cytosolic extract (50 $\mu$g; Lane 2) probed with YL-3 (1:500). Lane 3 is an immunoblot of bovine pro-pressophysin (2.5 $\mu$g) probed with YL-3 (1:500). Lane 4 is an autoradiogram of [$^{35}$S] cysteine incubated LX-1 cells immunoprecipitated with YL-3. Molecular weight markers are the same as in Fig. 1.

Figure III-2



Lane 2). The position of the band in Lane 3 confirms the reactivity of YL-3 with normal PPYsin ($M_r$ 23,000). Since the total protein loaded in Lane 2 was 100 times more than that in Lane 1, it appears the vast majority of antigen is found in the cell membrane. The culture medium, furthermore, was negative for immunoreactivity with anti-VP, anti-NP, YL-3, and mAb L6 by Western blot analysis. The serum from tumor-bearing nude rats also showed no increase in VP levels by RIA when compared to control nude rats (data not shown). Finally, [$^{125}$I] surface labeling of LX-1 cells and their subsequent immunoprecipitation with YL-3 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 propressophysin-related protein to LX-1 cell membrane.

A potential explanation for the observed high molecular weight of the PPYsin-like LX-1 antigen is a high degree of glycosylation, altering mobility on the gel or dimerization of the normal 23 kd protein (Fig. 2, Lane 3). To address these possibilities, purified tumor antigen was digested with glycopeptidase F, blotted and probed with YL-3 antibody. This resulted in a weak immunoreactive band with a $M_r$ of approximately 35,000 and the complete elimination of the 45 kd band (data not shown). Additionally, when cultured LX-1 tumor cells were incubated with [$^{35}$S] cysteine and immunoprecipitated with YL-3, a 45 kd band was seen, corresponding to the purified antigen (Fig 2, Lane 4). A higher molecular weight band of approximately 57 kd (Fig 2, Lane 4) was also detected, which could represent a pre-pro form of the polypeptide, or a larger, less processed 45 kd protein. These studies suggest that the 45 kd band

is not the result of dimerization or excessive glycosylation,

TABLE III-1. COMPARISON OF AMINO-TERMINAL SEQUENCES OF PRO-PRESSOPHYSIN AND LX-1 CELL SURFACE ANTIGEN.

Normal Human Pro-pressophysin[a]

C - Y - F - Q - $N^{5}$ - C - P - R - G - $G^{10}$ - K - R - A - M - $S^{15}$ - D - L - E - L - $R^{20}$ - Q - C - L - P - $C^{25}$ - G - P - G - G - $K^{30}$ - G - R - **C** - F

LX-1 Tumor Antigen[b]

A - M - $S^{15}$ - D - L - E - L - $R^{20}$ - Q - (C) - L - P - $(C)^{25}$ - G - P - (G) - (G) - $K^{30}$ - (G) - R - **R** - F

Immunogen for YL-3 Antibody[c]

P - R - G - $G^{10}$ - K - R - A - M - $S^{15}$ - D

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[a]Deduced amino acid sequence from human pre-pro-AVP-NP gene (Sausville et al., 1985).

[b]Amino-terminal sequence determined by automated Edman degradation as described in Materials and Methods.

[c]Antibody YL-3 was generated to the shown decapeptide which includes the $Lys^{11}$-$Arg^{12}$ cleavage site between vasopressin and vasopressin-neurophysin.

Parentheses indicate tentative amino acid assignments.

but rather, a unique form of pro-propressophysin.

Amino terminal sequencing of LX-1 tumor antigen:

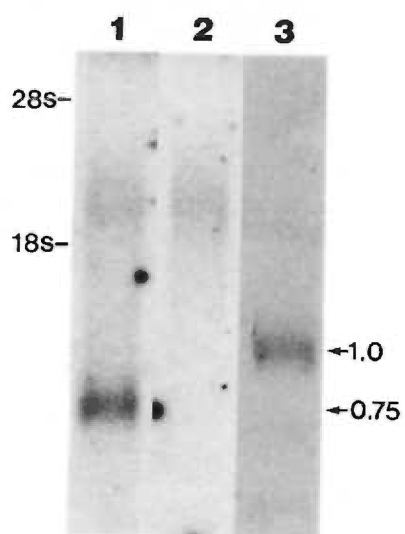
Table I summarizes the results of the sequence analysis of LX-1 tumor antigen, and compares this sequence to normal human PPYsin and the immunogen for the YL-3 antibody. Initial attempts to sequence the immunoaffinity purified antigen from HPLC fractions in acetonitrile or directly from protein electroblotted onto PVDF membranes were unsuccessful due to N-terminal blockage. In order to circumvent this, the protein was subjected to limited proteolysis with carboxypeptidase B containing a trace amount of trypsin. Automated Edman degradation of the cleaved antigen electroblotted onto PVDF membranes revealed a sequence with a 21 amino acid homology (including tentative assignments) with the N-terminal portion of human PPYsin (see Table I). A major deviation between the two sequences is the substitution of an $Arg^{33}$ residue in the tumor antigen in place of $Cys^{33}$ in human PPYsin. This change may have profound effects on the three dimensional structure of the protein. Our sequence analysis, nevertheless, confirms the identity of the mAb L6-isolated tumor antigen as PPYsin-like.

Molecular characterization of the pro-pressophysin-like protein:

In order to further characterize the expression of pro-pressophysin-like protein (PPLP) in LX-1 tumor cells, oligonucleotides directed to the C-terminal region of human and rat PPYsin were used in Northern and *in situ* hybridization analyses. *In situ* hybridization of nude rat brain bearing LX-1 tumor xenografts with the 30-mer oligonucleotide probe to rat PPYsin demonstrated specific hybridization signal within vasopressin-ergic neurons in the SON and PVN nuclei of rat

Figure III-3 Northern blot analysis of LX-1 lung tumor RNA. RNA was extracted and purified with oligo (dT) cellulose as described in Materials and Methods. Lane 1 is rat hypothalamic total RNA (20 $\mu$g); Lane 2 is rat cerebellar total RNA (20 $\mu$g); and Lane 3 is poly ($A^+$) RNA from LX-1 cells (2 $\mu$g). All lanes were probed with a $^{32}P$ 3′-labeled 30 mer synthetic oligonucleotide to the C-terminus of rat pro-pressophysin. The positions of 28s and 18s ribosomal RNA from lymphoma total RNA are shown. Normal rat hypothalamic message (750 bp) and the 1.0 kb band from LX-1 mRNA are noted.

Figure III-3



hypothalamus (Fig. 4 B-i). Autoradiographic grains were also localized in the intracerebral human tumor xenograft confirming expression of vasopressin mRNA in the tumor cells. When serial adjacent sections of tumor-bearing nude rat brain were hybridized with the 50-mer oligonucleotide probe to human PPYsin (Fig. 4 B-ii), the hybridization signal was confined to the human tumor xenograft, with no labeling being detected within vasopressin-ergic 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 pro-oxyphysin probes, performed on adjacent sections of tumor bearing nude rat brain failed to demonstrate presence of OT mRNA in the LX-1 tumor xenografts (Figs. 4 B-iii and B-iv). The presence of *in situ* signal within OT-ergic neurons of the rat SON and PVN in sections probed with the 30 mer rat probe (Fig. 4 B-iii) confirmed the stability of tissue mRNAs and the hybridizing specificity of the probe used. The absence of signal in corresponding hypothalamic regions of sections probed with the 50-mer human probe (Fig. 4 B-iv) served to further validate the specificity of hybridization.

Size fractionation of rat hypothalamic and LX-1 RNA by Northern analysis is shown in Figure 3. When rat hypothalamic total RNA was probed with the [$^{32}$P] labeled rat 30-mer, a strong signal was observed corresponding to a message size of 750 bp (Fig. 3, Lane 1), in agreement with previous reports (Ivell et al., 1984). Total RNA from rat cerebellum, which should not contain any vasopressin message, did not show hybridization signal with the rat 30-mer probe (Fig. 3, Lane 2).

When poly $(A^{+})$RNA isolated from LX-1 tumor cells was probed with the rat 30-mer, a band corresponding to a message size of approximately 1000 bp was observed (Fig. 3, Lane 3). This is approximately 250 bp larger than the reported size of normal PPYsin mRNA. This may, in part, account for the high molecular weight of PPLP in the tumor membrane. Both the *in situ* hybridization and Northern analysis suggest the selective expression of VP mRNA in LX-1 human tumor xenografts and in cultured cells.

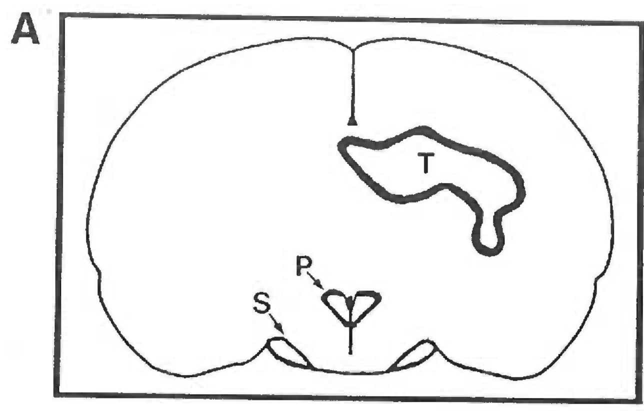
## DISCUSSION

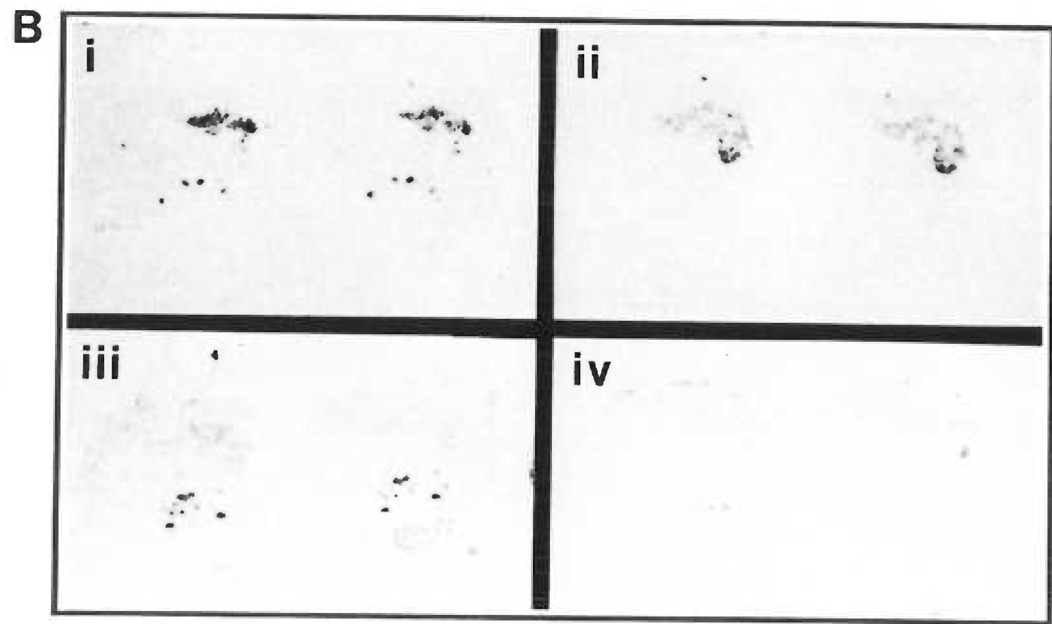
We have previously demonstrated that mAb L6 specifically labels both VP-NP and OT-NP in the cytoplasm of rat and human hypothalamic neurons, in addition to binding to a membrane bound-antigen in LX-1 lung carcinoma (Nilaver et al., 1990a). Although it was initially felt that mAb L6 identified a ganglioside antigen (Hellström et al., 1986a; Hellström et al., 1986b), we now report on the characterization of a PPLP, isolated from solubilized LX-1 tumor cell membranes with mAb L6. This 45 kd antigen shares a 21 amino acid homology with the N-terminal domain of human PPYsin as demonstrated by sequence analysis. Furthermore, 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 Nilaver et al., 1990b for review), raises some interesting issues.

High molecular weight forms of NP have been reported by many groups, which appear to cross-react with antibodies to both VP and VP-NP (Nicholas et al., 1980; Beguin et al., 1981; Rosenior et al., 1981).

Figure III-4 *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 xeno-grafted LX-1 tumor (T) is located dorso-lateral 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 (see Methods). Sections were probed with: (i) $^{35}S$ labeled 30-mer oligonucleotide to the C-terminus of rat pro-pressophysin; (ii) a $^{35}S$ labeled 50-mer oligonucleotide to the C-terminus of human pro-pressophysin; (iii) a $^{35}S$ labeled 30-mer oligonucleotide to the C-terminus of rat pro-oxyphysin; and (iv) a $^{35}S$ labeled 50-mer to the C-terminus of human pro-oxyphysin.

Figure III-4



Injection of [$^{35}$S] cysteine in the SON of rat brain has additionally demonstrated a series of high molecular weight VP-NP precursors (Cupo et al., 1982). Although some of these data may be attributed to high molecular aggregates, due to intra- or intermolecular disulfide bonding, or due to hydrophobic interactions between PPYsin and NP (Russell et al., 1980), we have shown this is not the case with PPLP. Gel electrophoresis of immunoaffinity purified PPLP or immunoprecipitated [$^{35}$S]-labeled LX-1 cells demonstrated the same relative mobility under both reducing and nonreducing conditions. When PPLP was subjected to glycopeptidase F digestion, the 45 kd band was eliminated, and a weak immunoreactive band of approximately 35 kd was seen. Since the molecular weight of this band is still higher than that normally found in hypothalamus, glycosylation alone cannot account for this difference. The 1 kb message detected in the LX-1 mRNA could potentially code for a primary sequence of this size. While it seems unlikely that either of these possibilities could individually account for PPLP having a $M_r$ of 45,000, they both may contribute to the larger size. 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 of the reported VP gene or is an entirely new product remains to be seen. Tracer and Loh (1989) have reported the existence of a high molecular weight mRNA (950-1000 bp) in the rat pituitary, and speculate that this may be the result of alternative splicing or differential upstream initiation. The possibility of a second gene for vasopressin and/or

related proteins has also been proposed (Lim et al., 1984; Lauber et al., 1981; Bonner and Brownstein, 1984), although this has yet to be confirmed. The cloning and sequencing of PPLP from LX-1 tumor will be necessary to address these possibilities.

In addition to finding differences in molecular weight 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 the normal precursor. Since the highly structured configuration of VP-NP is dependent on seven disulfide bonds (Breslow, 1979) this substitution could have a major impact on the tertiary structure of PPLP. It is well documented that the antigenicity of NP can often be a function of conformation. For example, depending on the antibodies used, reduction of the disulfide bonds in NP can result in either decreased immunoreactivity with polyclonal antibodies (Verbalis and Robinson, 1983) or increased reactivity with mAb L6 (Nilaver et al., 1990c), presumably by exposing "buried" antigenic determinants. The replacement of $Cys^{33}$ in PPLP with $Arg^{33}$ likely contributes to a unique conformation, allowing recognition by mAb L6, YL-3 and anti-VP but not by other antibodies directed to various epitopes of NP (which fail to immunoreact with the tumor antigen).

We have presented several lines of evidence suggesting that PPLP is targeted unprocessed to LX-1 cell membrane. Our earlier studies showed mAb L6 immunoreactivity to be exclusively confined to the cell surface of xenografted tumor (Nilaver et al., 1990a). In this report we have demonstrated PPLP is almost exclusively in the membrane, as evidenced by surface iodination and Western blot analysis. The trace amount of PPLP-reactivity in the cytosolic extract is not surprising,

since one would expect to "trap" some of the protein on its way to the cell surface at any point in time. Analysis of culture medium showed no VP, NP, or PPYsin immunoreactivity. This was further substantiated by the lack of increased serum vasopressin levels in LX-1 tumor bearing-nude rats, as compared to control nude rats. There are several potential explanations for the expression of unprocessed PPLP in LX-1 tumor cell membrane. The first is a lack of processing enzyme in the LX-1 cell line. Thomas et al. (1988) suggest that a lack of prohormone processing in any cell line is likely due to absence of endogenous enzyme. However, initial experiments with fluorogenic artificial substrates containing a dibasic cleavage site indicate endogenous processing in these cells (LCR and GN, unpublished data).

A second possibility is the lack of an intact Lys-Arg cleavage site in PPLP. This, however, seems unlikely as the antibody YL-3, which will only react with an intact dibasic cleavage site (Table I), demonstrates strong immunoreactivity with PPLP. A third alternative is a posttranslational modification of PPLP preventing its proper processing. As mentioned earlier, initial attempts at amino-terminal sequencing of PPLP resulted in a blocked N-terminus. If this naturally occurs in LX-1 tumor, and is not artifactual, it could partially explain the lack of processing. Brakch et al. (1989) have recently defined a number of structural criteria for the proper processing of pro-oxyphysin synthetic derivatives. A consistent finding was the critical role of the N-terminus in prohormone processing even with an intact Lys-Arg cleavage site. An N-terminally blocked precursor could, therefore, prevent processing.

We have isolated, identified and confirmed the presence of an alternate form of pro-pressophysin (PPLP) in the cell membranes of a small-cell lung carcinoma (LX-1). Since mAb L6 also demonstrates surface immunoreactivity with breast, colon and ovarian carcinomas (Hellström et al., 1986a) we are currently analyzing these cell lines for expression of PPLP. Information obtained from these studies should shed more light on the function and significance of NP (and/or its precursor) expression on the surface of these tumor cells. Once the complete structure of these antigens are elucidated it will be possible to define more precise roles for the NPs other than "carrier proteins", and to raise more effective antibodies against these surface antigens for the radioimmunotherapy of carcinomas.

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# Chapter IV

# AN ANTISERUM TO ATRIAL NATRIURETIC FACTOR (ANF) CROSS-REACTS WITH NEUROPHYSINS IN THE HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM OF RAT BRAIN

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# STATEMENT OF CO-AUTHORSHIP

My contribution to this manuscript included all gel electrophoresis and Western blotting experiments, preabsorption of the ANF antiserum with neurophysin and analysis of cross-reacting sequences between ANF and neurophysin. The manuscript was written and prepared equally by Dr. Nilaver and myself.

## ABSTRACT

The distribution of atrial natriuretic factor (ANF)-like reactivity was examined in rat brain and heart by immunohistochemistry. Immunostaining in heart was confined to atrial myocytes. In the hypothalamus, ANF-absorbable immunoreactivity was observed in magnocellular perikarya of the paraventricular and supraoptic nuclei, and in their projections to the neural lobe of the pituitary gland. No staining was seen in the preoptic or arcuate hypothalamic nuclei or in brain stem nuclei as previously reported by other investigators. The pattern of reactivity for ANF reported here is similar to that observed for neurophysins (NPs). Comparison of sequence data between rat ANF-28 and bovine NPs revealed three regions of 3 amino acid homology between these hypothalamic peptides. Preabsorption of the ANF antiserum with Affigel-coupled bovine NP I also resulted in complete elimination of all "ANF-immunoreactivity" in both atrium and hypothalamus. Cross-reactivity of the ANF antiserum with bovine NP I and II was further confirmed by Western blot analysis. Our findings suggest that ANF antisera can cross-react with NPs if they are directed against the shared antigenic epitopes; complete elimination of staining by preabsorption of the antibody with the immunogen, therefore, does not guarantee authenticity of localization. These observations may have relevance to an earlier study which reported on the existence of ANF-immunoreactivity in oxytocin neurons of the hypothalamus.

## INTRODUCTION

Atrial natriuretic factor (ANF), a novel peptide isolated from mammalian cardiac atria [Atlas and Laragh, 1986; Currie et al., 1983],

exhibits potent natriuretic and diuretic activity [Bold et al., 1981]. ANF also inhibits the synthesis of aldosterone [Atarashi et al., 1984] and relaxes vascular smooth muscles [Currie et al., 1983]. These activities suggest important roles for ANF in the regulation of water balance and blood pressure [Atlas and Laragh, 1986]. The cloning and sequence analysis of rat pre-pro ANF cDNA from heart [Yamanaka et al., 1984] has led to the identification of a group of structurally related peptides [Atlas and Laragh, 1986]. ANF-immunoreactive material has been identified in several extra-cardiac regions including the kidney, adrenal medulla, and pituitary gland [McKenzie et al., 1985]. Radioimmunoassay [Glembotski et al., 1985; Gutkowska et al., 1984; Kawata et al., 1985; Samson, 1985; Tanaka et al., 1984] and immunohistochemical studies [Kawata et al., 1985; Jacobowitz et al, 1985; Netchitalio et al., 1986; Saper et al., 1985, Skofitsch et al., 1985, Standaert et al., 1986] using antisera to ANF have also shown the existence of ANF-like material in brain.

While mapping the distribution of ANF in rat brain and atrium, employing an antiserum generated against rat ANF-28, we detected ANF-like immunoreactivity in the hypothalamo-neurohypophysial system. Magnocellular neurons of the supraoptic (SON) and paraventricular (PVN) nuclei were found to contain ANF-absorbable staining. These findings are in agreement with a previous study which reported on the existence of ANF-immunoreactivity in oxytocin (OT) neurons of the SON and PVN [Jirikowski et al., 1986]. Additional ANF-immunoreactivity was also detected within neurons of the dorso-medial suprachiasmatic nucleus, an area known to contain vasopressin (VP) neurons [Swaab et al., 1975],

suggesting co-localization of ANF and VP immunoreactivities. The overall pattern of hypothalamic ANF-immunoreactivity, furthermore, was quite identical to that reported for neurophysins (NPs) [Swanson and Sawchenco, 1983].

The present study was therefore undertaken to evaluate potential cross-reactivity of the ANF antiserum with NPs, employing immunohistochemistry and Western analysis, taking into account homologies in published amino acid sequence data for these hypothalamic peptides

## MATERIALS AND METHODS

Synthetic rat ANF-28 was obtained from Bachem (Torrance, CA). Atriopeptin 3, human ANF, ANF peptide fragment 1-11, arginine$^{8}$ VP, OT and corticotropin releasing factor (CRF) were purchased from Peninsula Laboratories (Belmont, CA). ExtrAvidin alkaline phosphatase, diaminobenzidine, bovine oxytocin-neurophysin (OT-NP, NP I), vasopressin-neurophysin (VP-NP, NP II), thyroglobulin and myoglobin peptide molecular weight standards were from Sigma Chemical Co. (St. Louis, MO). Biotinylated protein A and the avidin-biotin-peroxidase (ABC) kit were obtained from Vector Laboratories (Burlingame, CA). Affigel-10 was obtained from Bio-Rad (Richmond, CA). p-Nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were from Promega (Madison, WI).

Antisera: The ANF antiserum (R1-3) was generated in a rabbit employing synthetic rat ANF-28 (Bachem, Torrance, CA) conjugated to bovine thyroglobulin. Cross-reactivity of this serum with rat ANF-28, and with atriopeptin 3 (100%), human ANF (3%), VP (<0.1%), OT (<0.1%) and CRF (<0.1%) has been previously described by radioimmunoassay

[Gibbs, 1987]. The rabbit anti-ANF was used at a final dilution of 1:2000; cross-reactivity of this antiserum with the same antigens was also examined by immunohistochemistry.

Animals: Six colchicine-treated (80 μg; intracerebroventricular) and 6 untreated adult male Sprague-Dawley rats (200-250 gm; Simonsen) were used for the immunohistochemical analysis. Animals were anesthetized with pentobarbital (60 mg/kg body weight, i.p.) and perfused through transcardiac puncture with 0.9% saline followed by ice-cold fixative containing 4% paraformaldehyde and saturated picric acid in 0.1 M phosphate buffer (pH 7.4) [Kawata et al., 1985]. Brains were immediately dissected, postfixed for 48 hr in the same fixative at 4°C and processed for immunohistochemistry.

Immunohistochemistry was performed employing biotinylated protein A and avidin-biotin-peroxidase (ABC) in the preembedding staining technique as previously described [Nilaver and Kozlowski, 1989]. In brief, 50 μm Vibratome-cut sections incubated with rabbit anti-rat ANF (R1-3, 1:2000, overnight, 4°C), were sequentially reacted with biotinylated protein A (3 μg/ml, 45 min, 37°C) and avidin biotinylated-peroxidase complex (1:1000, 60 min, 37°C). The ABC complex was prepared 5 min prior to use by mixing together equal parts of 1:1000 dilutions of the stock Avidin DH and biotinylated peroxidase reagents provided in the Vectastain ABC kit. Reaction products were formed with 15 mg% 3,3'diaminobenzidine. The tissue sections were mounted on gelatin-coated slides, dehydrated, cleared with xylene and permanently mounted under cover slips.

Gel Electrophoresis and Western Blotting: Rat ANF-28 (2 μg) and

porcine NP (2 μg) were solubilized in 1X Laemmli sample buffer [Laemmli, 1970] and heated to 100°C for 5 min. Samples were electrophoresed on 14.5% total acrylamide (% T; acrylamide:bisacrylamide; 1:15.5) using the tricine-SDS-PAGE system as described by Schäger and von Jagow [1987]. After electrophoresis, the gels were renatured by washing 3 x 10 min in 50 mM Tris, pH 7.4, containing 20% (v/v) glycerol. The proteins were then electrophoretically transferred to 0.2 μm nitrocellulose in 10 mM $NaHCO_3$/3 mM $Na_2CO_3$, pH 10.0, with 20% (v/v) methanol for 1 hr at 1 amp employing a TE-42 Transphor unit (Hoefer, San Francisco, CA). ANF peptide fragment 1-11 was transferred in the same buffer as above, but with the pH decreased to 9.5.

The nitrocellulose blots were air-dried overnight, and fixed in 0.5% (v/v) para-formaldehyde vapor (1 hr, 70°C) to immobilize the peptides. The blots were incubated with 3% (w/v) gelatin in 20 mM Tris, pH 7.5, containing 0.5 M NaCl, 0.1% (v/v) Tween-20 and 0.02% (v/v) $NaN_3$ (TTBS for 1 hr) to block nonspecific binding sites. Following TTBS washes (3 x 5 min), the blots were incubated with primary antiserum diluted to 1:500 in TTBS containing 0.1% (w/v) recrystallized bovine serum albumin (TTBS-BSA) for 1 hr at 37°C. Excess antibody was removed by washing the blots in TTBS (3 x 5 min). Blots were then incubated with biotinylated protein A (3 μg/ml) in TTBS-BSA (1 hr, 37°C), washed in TTBS (3 x 5 min), followed by ExtrAvidin-alkaline phosphatase (1.7 μg/ml) in TTBS (45 min, 37°C). After a final wash in TTBS (3 x 5 min), the blots were transferred to clean trays, and the immobilized poly-peptides detected by developing the blots in a mixture of NBT (0.33 mg/ml) and BCIP (0.165 mg/ml) in 0.1 M Tris, pH 9.5, containing 0.1 M

NaCl, 5 mM $MgCl_2$ and 0.5% (v/v) Tween-20.

Preabsorption of the ANF antiserum with NP: Affigel-10 was washed with 6 volumes of cold distilled water and incubated with bovine NP I (final concentration of 0.5 mg/ml) in 2 ml of 0.1 M HEPES, pH 7.5, containing 80 mM $CaCl_2$ (buffer A) for 4 hr (end on end stirring; 4°C). Any remaining active sites on the gel were blocked by the addition of 1 M ethanolamine-HCl, pH 8.0 (0.1 ml/ml gel) for 1 hr while rotating at 4°C. The gel was then washed with buffer A until the $OD_{280}$ was reduced to background. Coupling efficiency was determined by quantitating protein in the wash by the method of Bradford [1976].

Coupled Affigel-10 was centrifuged at 1200 x g for 15 min and the supernatant decanted and discarded. The gel was then incubated with the antiserum to rat ANF-28 (1:500 dilution) overnight, while rotating at 4°C. The gel-antibody suspension was then recentrifuged at 1200 x g as above for 15 min and the supernatant (absorbed antibody) decanted for use in Western analysis and immunohistochemistry. The coupled Affigel-10 was regenerated by washing with 10 volumes each of 0.1 M glycine (pH 3.0), 25 mM $Na_2HPO_4$ (pH 8.5), each containing 0.5 M NaCl, and finally with buffer A.

## RESULTS

Immunostaining in heart was confined to the atrial myocytes (Fig. 2A). In the brain, immunoreactivity was observed in the hypothalamus of both normal and colchicine treated animals. Figure 1 is a schematic representation of the "ANF-immunoreactive" structures in the hypothalamus. ANF-positive perikarya were seen in the paraventricular (Fig. 2B) and supraoptic nuclei (Fig. 2C). Reactive perikarya in the paraven-

tricular nucleus were distributed in both the magnocellular and parvicellular components. A moderate number of perikarya were also found in areas ventral to the zona incerta, in the perifornical nucleus and the medial forebrain bundle. Reactive perikarya in the supraoptic nucleus could also be traced to the retrochiasmatic area. Additional ANF-positive perikarya were found in the periventricular nucleus, the suprachiasmatic nucleus, particularly in its dorso-medial aspects, (Fig. 2D) and the nucleus circularis. Colchicine treatment did not substantially change the pattern of perikaryal staining in all regions examined.

Axonal projections could be traced from paraventricular and supraoptic nuclei through the infundibular stalk and the median eminence, with densely stained fibers localized mainly in the zona inserta, to their termination in the neural lobe of the pituitary (Fig. 2E, F). A few fiber terminals were also observed in the zona externa (Fig. 2E). ANF staining was not detected in the anterior lobe of the pituitary gland or in extrahypothalamic fiber projections.

Immunostaining in the hypothalamus and atrium was completely eliminated by pre-absorption of 1 ml of the antiserum (1:2000 dilution) with 80 μg of rat ANF-28 (Bachem) or atriopeptin 3 (Peninsula), whereas equimolar amounts of arginine[8] VP, OT, CRF, (Peninsula) or bovine thyroglobulin had no effect. While the atrial staining and the complete elimination of both hypothalamic and atrial immunoreactivity suggested

Figure IV-1 Schematic drawings illustrating the distribution of ANF-immunoreactive perikarya in the rat hypothalamus. a, b, and c correspond to the planes 1.3 mm, 1.8 mm, and 2.3 mm caudal to bregma respectively, in the rat stereotaxic atlas [Paxinos et al., 1982]. F: fornix, me: median eminence, mfb: median forebrain bundle, oc: optic chiasma, opt: optic tract, pe: periventricular nucleus, pvm: paraventricular nucleus (pars magnocellularis), pvp: paraventricular nucleus (pars parvocellularis), rcn: retrochiasmatic area, scn: suprachiasmatic nucleus, so: supraoptic nucleus, 3 v: third ventricle, ZI: zona incerta.

Figure IV-1

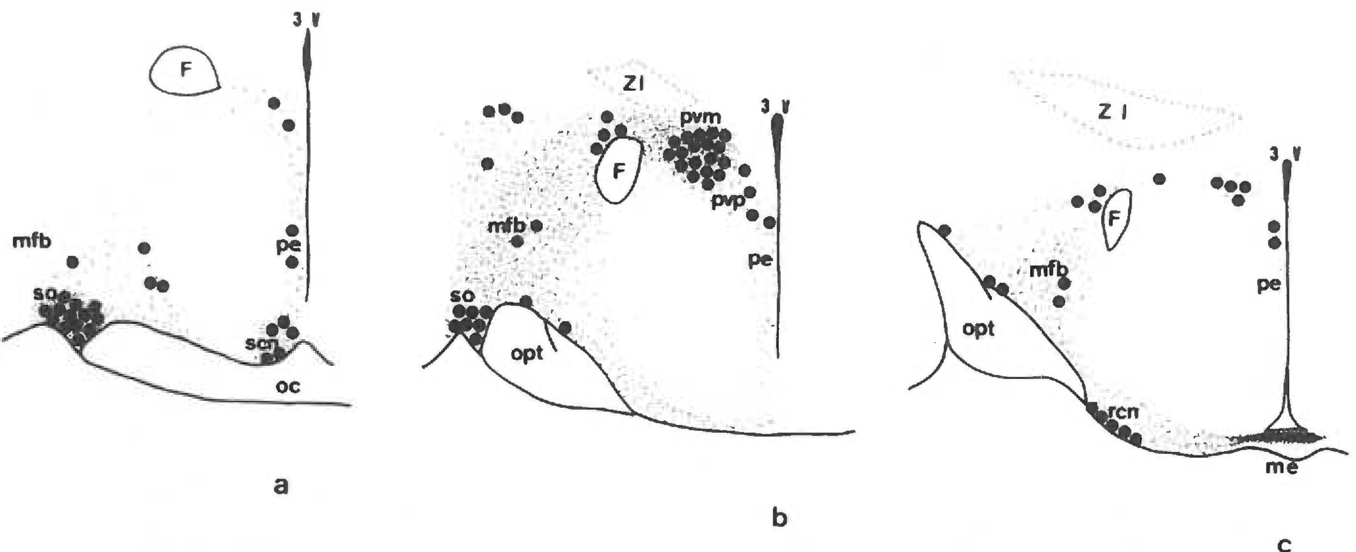
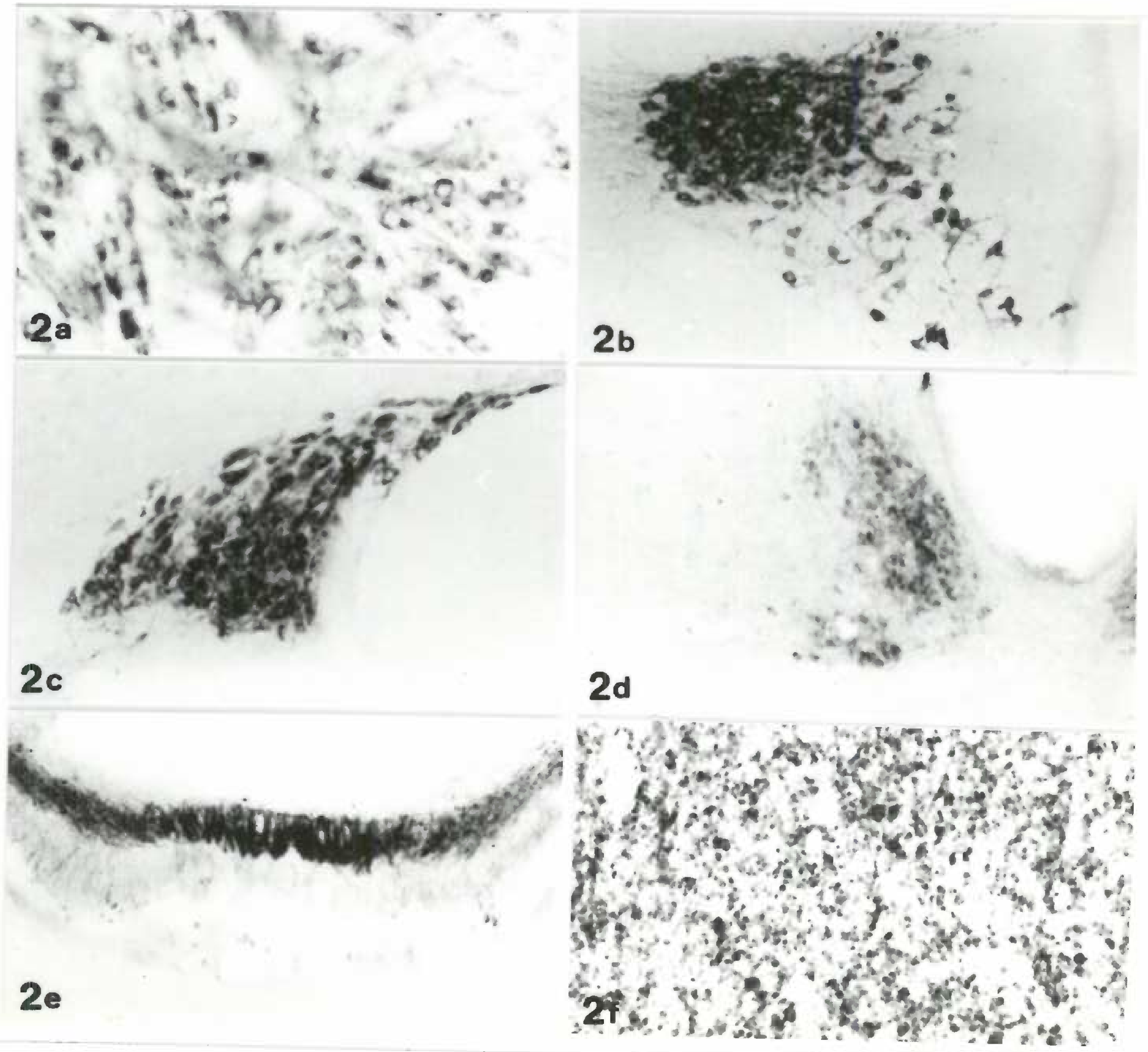


Figure IV-2 Immunohistochemical localization of ANF-like immunoreactivity within myocytes in the in rat atrium (a), within neuronal perikarya and fibers in the hypothalamus (b-e), and in fiber terminals in the posterior lobe of the pituitary gland (f). b: paraventricular nucleus, c: supraoptic nucleus, d: suprachiasmatic nucleus, e: median eminence. magnification a: x 150, b-f: x 100.

Figure IV-2

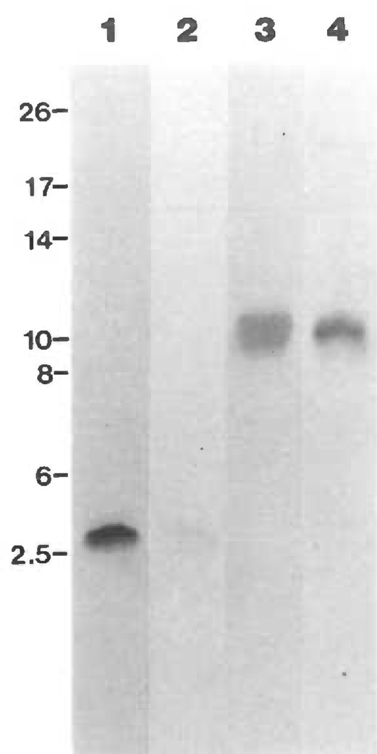


the immunostaining to be ANF-related, the overall pattern of hypothalamic staining was quite similar to that reported for the NPs [Swanson et al., 1983]. Additional immunostaining studies were therefore performed in which the ANF-antiserum was preabsorbed with Affigel-coupled bovine NP I. Preabsorption of the ANF antiserum with the Affigel-coupled NP also resulted in complete elimination of all "ANF-related" immunoreactivity in the atrium and the hypothalamus.

Western blot analysis: Immunoreactivity of rabbit anti-ANF with synthetic ANF-28 and purified bovine NP I and II is shown in Figure 3. The polyclonal antibody to ANF shows immunoreactivity with synthetic ANF (Lane 1). The antibody also appears to recognize a shared sequence in the two NPs as demonstrated by immunoreactivity with bovine NP I (Lane 3) and NP II (Lane 4). This cross reactivity was further validated by preabsorption of the ANF antibody with bovine NP I-bound Affigel, using the supernatant to probe synthetic ANF in the immunoblots. As seen in Lane 2, this resulted in elimination of virtually all immunoreactivity for synthetic ANF. These data are in agreement with the immunohistochemical observations, and indicate recognition of a shared antigenic domain in the two NPs by the polyclonal anti-ANF. As shown in Table I, comparison of the amino acid sequence of rat ANF-28 and bovine NP I and II [Land et al., 1983] shows 3 potential regions of cross-reactivity between shared antigenic domains (shaded areas) in ANF-28 and a common sequence in the two NPs. When ANF peptide fragment 1-11, which contains one of these potential domains, was immunoblotted and probed with the ANF antiserum, no immunoreactivity was observed (data not shown). This, however, does not exclude participation of this domain in the antibody's

Figure IV-3 Immunoblot analysis of ANF and neurophysins with the polyclonal anti-ANF antibody. Peptide samples were electrophoresed, blotted and probed with antibody as described in Materials and Methods. Lanes 1 and 2 are immunoblots of ANF (2 $\mu$g) probed with the anti-ANF polyclonal antibody (R1-3; 1:500, Lane 1) and the antibody preabsorbed with bovine NP I (Lane 2). Lanes 3 and 4 are immunoblots of bovine NP I (2 $\mu$g) and bovine NP II (2 $\mu$g) probed with the anti-ANF antibody R1-3 (1:500). Molecular weight markers employed were chymotrypsinogen A ($M_r$ 25,666), myoglobin ($M_r$ 16,950), myoglobin fragments I and II ($M_r$ 10,670), myoglobin fragment I ($M_r$ 8,160), myoglobin fragment II ($M_r$ 6,210), and myoglobin fragment III ($M_r$ 2,510).

Figure IV-3



immunoreactivity since the antigenicity of ANF may be dependent on conformation and consequently altered in the 1-11 fragment.

DISCUSSION

Our present study demonstrates ANF-like immunoreactivity in the hypothalamo- neurohypophysial system. The localization of immunoreactivity in the periventricular and parvicellular regions of the paraventricular nucleus and in the zona externa of the median eminence is in agreement with previous reports [Kawata et al., 1985; Jacobowitz et al., 1985; Skofitsch et al., 1985; Standaert et al., 1986]. Fiber terminals have also been previously described in the zona inserta [Netchitalio et al., 1986] and neural lobe [Netchitalio et al., 1986; Skofitsch et al., 1985]. In contrast to earlier reports, however, we detected additional immunoreactivity within magnocellular neurons of the paraventricular and supraoptic nuclei, as well as in the suprachiasmatic nucleus and nucleus circularis. This pattern of staining corresponds to the hypothalamic distribution of NP I and II [Swanson and Sawchenco, 1983]. Antibody R1-3, furthermore, did not detect ANF-reactive perikarya in the preoptic area and arcuate regions of the hypothalamus, or in brain stem nuclei as reported previously [Kawata et al., 1985; Jacobowitz et al., 1985; Netchitalio et al., 1986; Skofitsch et al., 1985; Standaert et al., 1986].

While the complete elimination of all atrial and hypothalamic immunoreactivity with the ANF antibody following preincubation with synthetic ANF-28 led us to initially believe the staining to be "ANF-related", comparison of sequence data between rat ANF-28 and the bovine NPs (Table I) alerted us to three regions of 3 amino acid homology

between these hypothalamic peptides. Additional immunohistochemical studies employing ANF antiserum immunoabsorbed with Affigel-conjugated NP I, and Western analysis of rat ANF-28, and bovine NP I and II with the ANF-antiserum were consequently performed, which confirmed cross reactivity of the ANF antiserum with some or all of these shared antigenic domains.

The present study serves to underscore a major inherent limitation in antigen localization employing immunohistochemical techniques. Antibody recognition of shared antigenic sequences in another protein, as encountered here, is the most common basis for false positive staining. Our findings also exemplify the admonition that complete elimination of staining by preabsorption of the antibody with the immunogen does not exclude the possibility of false-localization.

Immunoblotting procedures cannot assure the validity of immunohistochemical results because the information they yield is obtained by the same immunologic method. An extractable cross-reacting antigen in tissue will probably give the same false results in solution. This caveat not withstanding, the use of both immunohistochemistry and Western analysis in studying the anatomical distribution of an antigen provides useful information when the data are confirmatory and even possibly more useful information when the identity of the antigens can be further established on the basis of electrophoretic size-separation.

There are two important points to consider when analyzing potential cross-reactive epitopes in ANF and NPs. First, although we have found three regions of 3 amino acid homology between these peptide sequences (Table I), one or more of these regions may form a longer

assembled sequence in a folded state, thereby facilitating recognition by the heterologous antibody. Studies of myoglobin [Benjamin et al., 1984] have demonstrated the presence of several antigenic sites separated by stretches of nonantigenic sequences. When the protein is in a native conformation these sites form a contiguous antigenic determinant recognized by the antibody. The second aspect of cross-reactivity is the number and location of disulfide bridges in relation to the antigenic sequences. The antigenicity of lysozyme has been attributed to an intact conformational loop structure held in place by a disulfide bond [Benjamin et al., 1984]. Since both ANF and NPs have disulfide bridges critical for maintaining their conformation and structural integrity, this may also contribute to antibody cross-reactivity with the heterologous antigens. Given the highly structured state of NPs and their greater number of disulfide bonds, such cross-reactivity would be potentially greater for an ANF antibody than a NP antibody when these shared antigenic epitopes are involved. This could explain why ANF antibody R1-3 recognizes authentic ANF in the atrium (where NPs are not expressed), while preferentially binding to NPs in the hypothalamus. These observations have profound implications when evaluating the immunohistochemical results of ANF localization in the hypothalamus, and may have relevance to an earlier study which reported on the existence of ANF-immunoreactivity in OT neurons of the SON and PVN [Jirikowski et al., 1986].

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Table IV-1

Comparison of amino acid sequences between rat ANF-28 [Atlas and Laragh, 1986] and bovine NP I and NP II [Land et al., 1983]. The homologous sequences between the two NPs are underlined, and the three regions of 3 amino acid homology between NP I, NP II and ANF-28 are shaded. Since the shaded homologous areas (1, 2 & 3) in the NPs are adjacent to cysteines, regions 2 and 3 could form a continuous 6 amino acid antigenic determinant (Ile-Gly-Ala-Gln-Ser-Gly) in NPs in their native confirmation, which in turn is recognized by an anti-ANF directed to aa 15-20 of the ANF sequence (see text for details).

TABLE IV-1 COMPARISON OF AMINO ACID SEQUENCES BETWEEN ANF AND NEUROPHYSIN

**Rat Atriopeptin 28**

```
                                     1                             2
NH2-Ser Leu Arg Arg Ser Ser Cys Phe Gly Gly Arg Ile Asp Arg Ile Gly Ala
                                    ---------->             ---------->
Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr-OH
---------->
    3
```

**Neurophysin II (Upper case); Neurophysin I (Lower case)**

```
ALA MET SER ASP LEU GLU LEU ARG GLN CYS LEU PRO CYS GLY PRO GLY GLY LYS GLY
Ala Val Ile Asp Leu Asp Val Arg Thr Cys Leu Pro Cys Gly Pro Gly Gly Lys Gly

ARG CYS PHE GLY PRO SER ILE CYS CYS GLY ASP GLU LEU GLY CYS PHE VAL GLY THR
Arg Cys Phe Gly Pro Ser Ile Cys Cys Gly Asp Glu Leu Gly Cys Phe Val Gly Thr

ALA GLU ALA LEU ARG CYS GLN GLU GLU ASN TYR LEU PRO SER PRO CYS GLN SER GLY
Ala Glu Ala Leu Arg Cys Gln Glu Glu Asn Tyr Leu Pro Ser Pro Cys Gln Ser Gly
                                                                ---------->
                                                                     3

GLN LYS PRO CYS GLY SER GLY GLY ARG CYS ALA ALA ALA GLY ILE CYS CYS ASN ASP
Gln Lys Pro Cys Gly Ser Gly Gly Arg Cys Ala Ala Ala Gly Ile Cys Cys Ser Pro
                        <----------             ---------->
                             1                       2

GLU SER CYS VAL THR GLU PRO GLU CYS ARG GLU GLY VAL GLY PHE PRO ARG ARG VAL
Asp Gly Cys His Glu Asp Pro Ala Cys Asp Pro Glu Ala Ala Phe Ser Gln His
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Chapter V

# IMMUNOLOGICAL STUDIES WITH A MONOCLONAL ANTIBODY SUGGESTS NEUROPHYSIN EXISTS IN A DIFFERENT CONFORMATIONAL FORM IN PARVICELLULAR NEURONS OF RAT HYPOTHALAMUS

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Key Words: Hypothalamus; Immunohistochemistry; Monoclonal antibody; Neurophysin; Paraventricular nucleus; Supraoptic nucleus; Suprachiasmatic nucleus; Immunoprecipitation; Western blots.

# SUMMARY

Neurophysins are carrier proteins associated with oxytocin (OT) and vasopressin (VP) within mammalian hypothalamic neurons. We have previously demonstrated that a monoclonal antibody (mAb L6) to a human lung carcinoma surface antigen recognizes a shared antigenic epitope in oxytocin-neurophysin (OT-NP) and vasopressin-neurophysin (VP-NP) by immunohistochemistry and Western analysis (Nilaver et al., 1990). We now report on the distribution of L6-immunoreactivity in control rats, adrenalectomized rats, and in rats following intracerebroventricular injection of colchicine or tunicamycin. Control rat hypothalamus demonstrated L6 immunoreactivity, exclusively confined to magnocellular OT and VP neurons, and their projections to neural lobe. L6-staining was absent in the suprachiasmatic nucleus, and the parvicellular paraventricular nucleus and its projections to the median eminence. Similar findings in adrenalectomized, colchicine- and tunicamycin-treated rats suggest the existence of NP in a unique conformational form in these regions. The ability of mAb L6 to react with VP-NP in Western blots, only after gel-renaturation and electroblotting in basic buffer, also confirm these observations. mAb L6 also immunoprecipitated VP-NP only under reducing conditions. These findings suggest that mAb L6 is exquisitely sensitive to conformational changes in NP, and imply the existence of NP in a unique configurational form in hypothalamic parvicellular systems.

# INTRODUCTION

The nonapeptide hormones oxytocin (OT) and vasopressin (VP) are synthesized together with their corresponding carrier proteins, the neurophysins (NPs), within discrete neuronal cell groups in the mammalian hypothalamus (De Mey et al.; Dierickx and Vandesande, 1979; Zimmerman and Defendini, 1977; Zimmerman et al., 1978. Two distinct NPs have been identified, one associated with OT (termed OT-NP) and another with VP (called VP-NP). Each nonapeptide and its corresponding NP are synthesized from distinct large molecular weight common precursors (Brownstein et al. 1980; Gainer, 1983; Ivell et al., 1983; Land et al., 1982; Land et al., 1983; Schmale et al., 1983) and are co-packaged within same neurosecretory granules. The hypothalamo-neurohypophysial system containing these peptides includes neurons of the supraoptic (SON) and paraventricular (PVN) nuclei, as well as the internuclear groups of cells lying between the PVN and SON, the most prominent of which is the nucleus circularis. The term "magnocellular" generally refers to the large perikarya of these neuronal cell groups, whose fibers pass through the zona interna (ZI) of the median eminence, to terminate in the posterior pituitary gland (neurohypophysis). The medial (periventricular) region of the PVN also contains smaller or intermediate sized ("parvicellular") neurons whose axons terminate on portal capillaries in the zona externa (ZE) of the median eminence (hypothalmo-adenohypophysial system) Silverman and Zimmerman, 1975; Stillman et al., 1977; Zimmerman et al., 1977). Extrahypothalamic projections to the spinal cord, arising from the PVN, are also mainly derived from parvicellular neurons (Hosoya and Matsushita, 1979; Swanson

and Kuypers, 1980), although a few scattered magnocellular neurons in the posterior PVN also contribute to this projection (Sofroniew and Weindl, 1978). In contrast, the vast majority of neurons in the SON are magnocellular and primarily project to the neurohypophysis. The suprachiasmatic nucleus (SCN), on the other hand, is composed only of parvicellular neurons.

Several studies have established the localization of both OT and VP within separate neuronal subgroups in SON and PVN (Sofroniew et al., 1979; Vandesande and Diericks, 1975; Zimmerman and Antunes, 1976; Zimmerman and Defendini, 1977; Zimmerman et al., 1978). The SCN, however, is totally deficient in OT and OT-NP, the stainable neurosecretory material in this nucleus exclusively representing VP and its specific NP (Sofroniew and Weindl, 1978; Swaab et al., 1975).

Immunohistochemical studies, employing antisera to VP, OT and their NPs, have mapped the distribution of these peptides in hypothalamus and other brain regions (Nilaver et al., 1980). Antisera to the nonapeptides generally produce discrete labeling of specific hormonal cell groups. Although polyclonal antibodies against specific rat NPs, and showing low cross-reactivity in radioimmunoassays, have been produced (McPherson and Pickering, 1978; North, 1983), their use in immunohistochemistry has not been reported. Indeed, most polyclonal as well as monoclonal antibodies to the NPs recognize both OT-NP and VP-NP in immunohistological techniques (Ben-Barak et al., 1985; Nilaver et al., 1980; Nilaver et al., 1990). A continuous stretch of 64 amino acids with high sequence homology (region 10-74) that exists between the two disulfide-rich NPs (Land et al., 1982; Land et al., 1983), probably

accounts for this cross-reactivity. These polyclonal anti-NPs consequently identify both NPs in magnocellular and parvicellular hypophysial systems by immunohistochemistry.

Recently, while studying the binding activity of mAb L6 (a monoclonal antibody to a carcinoma surface antigen) (Hellström et al., 1986a; Hellström et al., 1986b) to human lung tumor xenografts in nude rat brain, we made the unexpected observation that mAb L6 also stained neurons in SON and PVN of the host hypothalamus (Nilaver et al., 1990). While mAb L6 only stained tumor cell membranes in the xenografts, the reactivity in host hypothalamus was cytoplasmic. Subsequent double-labeling experiments in normal and nude rat hypothalamus, performed with mAb L6 in conjunction with VP and OT antisera, confirmed the localization of L6-reactivity in both peptide-containing cell groups. These findings and the complete elimination of L6-hypothalamic immunoreactivity by solid phase absorption of the mAb with porcine NPs suggested that its target epitope also resided within the 10-74 amino acid shared domain of the two NPs. Western blot analysis of pituitary extracts also identified L6-immunoreactive bands corresponding to the position of NPs, providing additional evidence that the hypothalamic L6-immunoreactivity was related to these carrier proteins (Nilaver et al., 1990).

The L6-reactivity in rat hypothalamus appeared to be selectively confined to magnocellular neurons and their projections to the pituitary neural lobe. No staining was noted in the SCN, in parvicellular neurons of the PVN or their projections to ZE of the median eminence. The present studies involving immunohistochemistry, immunoprecipitation and

Western blotting procedures were undertaken in order to determine whether the selective absence of L6-staining within parvicellular neurons and their efferent projections in rat hypothalamus was due to quantitative changes in their NP content or whether it represented configurational differences in NP expression in this system that preclude its identification with mAb L6.

## MATERIALS AND METHODS

[$^{125}$I]Bolton-Hunter reagent (N-succinimidyl 3-[4-hydroxy, 3-[$^{125}$I]diiodo-phenyl] propionate; 4400 Ci/mmol) was from New England Nuclear (Boston, MA). Bovine VP-NP, colchicine, tunicamycin, ExtrAvidin alkaline phosphatase, 3,3'diaminobenzidine tetrahydrochloride, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), *p*-nitro blue tetrazolium chloride (NBT) and Ponceau S solution were from Sigma (St. Louis, MO). Biotinylated protein A and the ABC Kit were obtained from Vector laboratories (Burlingame, CA). Protein A-Sepharose was from Pharmacia (Piscataway, NJ). Hyperfilm $\beta$-max was obtained from Amersham (Arlington Heights, IL).

<u>Antibodies</u>: mAb L6 and P1.17, $IgG_{2a}$ monoclonal antibodies, were generous gifts of Drs. K.E. and I. Hellström (Oncogen, Seattle, WA). This antibody has been previously shown to recognize a common domain of vasopressin neurophysin and oxytocin-neurophysin (Nilaver et al., 1990). A polyclonal antiserum to porcine neurophysins was obtained from ICN Immunobiologicals (Lisle, IL.). This antiserum recognizes both neurophysins as well as pro-pressophysin (Nilaver et al., 1990) and was used at a dilution of 1:1000 in immunocytochemical procedures.

<u>Animals</u>: Male Long-Evans rats (250-300 g) were obtained from Blue

Spruce Farms (Altamont, NY). All animals were maintained on 12 h light/dark cycle (lights on 8 a.m.). Immunohistochemical studies were carried out in normal rats (n=5), in adrenalectomized rats (n=5), and in rats pretreated with intraventricular colchicine (n=5) or tunicamycin (n=5).

Adrenalectomy: Animals were bilaterally adrenalectomized under sodium pentobarbital anesthesia (60 mg/kg, i.p.) and received 0.9% NaCl and 5% dextrose in their drinking water. Adrenalectomized (ADX) rats were studied 3 weeks postsurgery. Effectiveness of adrenalectomy was confirmed at autopsy.

Colchicine and tunicamycin administration: 100 μg of colchicine or tunicamycin (in 50 μl of physiological saline) was injected intracisternally into the lateral ventricle of anesthetized rats 48 hrs prior to sacrifice.

Preparation of tissue for histological analysis: Rats were anesthetized with sodium pentobarbital (60 mg/kg body weight, i.p.) and perfused through transcardiac puncture with 0.9% (w/v) saline followed by 4% (w/v) ice cold buffered 4% (w/v) paraformaldehyde. The brains were removed and blocked in the coronal plane to include the regions of the supraoptic (SON), paraventricular (PVN) and suprachiasmatic (SCN) hypothalamic nuclei. Tissues were postfixed in buffered paraformaldehyde for an additional 48 hrs. Additional studies were performed in tissues fixed with 2% glutaraldehyde or lysine periodate paraformaldehyde (McLean and Nakane, 1974).

Immunohistochemistry: Tissue blocks were sectioned serially at 100 μm (coronal) with a Vibratome (Oxford Instruments, Bedford, MA)

following fixation. The sections were rinsed in 50 mM Tris buffer, pH 7.6 containing 0.9% (w/v) NaCl. Alternate sections were immunocytochemically labeled with mAb L6 or anti-NP, employing biotinylated protein A and avidin-biotin peroxidase (ABC) in the preembedding staining technique (Nilaver and Kozlowski, 1989). Briefly, the tissues were incubated with mAb L6 (50 μg/ml) or polyclonal anti-NP (1:2000) overnight at 4°C. Tissues were then sequentially reacted with biotinylated protein A (3 μg/ml; 45 min; room temperature) and the ABC complex (1:1000; 1 hr; room temperature). The ABC complex was prepared 5 min prior to use by mixing together equal parts of 1:1000 dilutions of the stock avidin DH and biotinylated horseradish peroxidase. Reaction products were then formed with 15 mg% 3,3'diaminobenzidine tetrahydrochloride. Following development of the brown reaction product, tissue sections were mounted on gelatin-coated slides, dehydrated, cleared with xylene and cover-slipped.

Gel Electrophoresis and Western Blotting: Bovine VP-NP was electrophoresed and blotted essentially as described by Rosenbaum et al. (1989). Briefly, samples were reduced and electrophoresed on 14.5% total acrylamide (%T; acrylamide: bisacrylamide; 1:15.5) followed by 3 x 10 min washes in 50 mM Tris-HCl, pH 7.4 containing 20% (v/v) glycerol. Gels were then electroblotted onto 0.2 μm nitrocellulose in transfer buffer consisting of 10 mM $NaHCO_3$/3 mM $Na_2CO_3$, pH 10.0 containing 20% (v/v) methanol. Filters were then air dried and baked at 70°C for 90 min. Nonspecific binding was blocked with 3% (w/v) gelatin in 20 mM Tris-HCl, pH 7.4 containing 0.5 M NaCl, 0.1% (v/v) Tween-20 and 0.02% (w/v) $NaN_3$ (TTBS). After washing in TTBS, blots were then sequentially

incubated in mAb (50 μg/ml; 4 hr room temperature) Biotinylated protein A (3 μg/ml; 1 hr room temperature) and ExtrAvidin alkaline phosphatase (1.7 μg/ml; 45 min room temperature). Alkaline phosphatase activity was detected by developing the blots in 5-bromo-4-chloro-3-indolyl phosphate (0.165 mg/ml)/*p*-nitro-blue tetrazolium chloride (0.33 mg/ml) in 0.1 M Tris-HCl, pH 9.5 containing 0.1 M NaCl, 5 mM $MgCl_2$ and 0.5% (v/v) Tween-20. Reactions were quenched by rinsing the blots in distilled water.

Neurophysin Immunoprecipitation: [$^{125}$I]Bolton-Hunter reagent (N-succinimidyl 3-[4-hydroxy, 3-[$^{125}$I]diiodophenyl] propionate; 250 μCi) in benzene was dried with a stream of nitrogen. Bovine VP-NP (25 μg) in 50 ml of 0.1 M borate buffer, pH 8.5 was added to the dried Bolton-Hunter reagent and gently rotated at 4°C for 24 hr (the preparation of VP-NP used contains both processed VP-NP and small amounts of pro-pressophysin). Any remaining active ester was reacted with 0.2 M glycine. The reaction mixture was then purified on a Sephadex G-10 column preequilibrated with 0.1% (w/v) gelatin in 0.1 M borate buffer, pH 8.5, and the radioactive fractions pooled. $^{125}$I-labeled VP-NP was then incubated with mAb L6 (50 μg/ml) or first reduced with 20 mM DTT prior to equilibration with L6. Control experiments were performed by incubating reduced or nonreduced [$^{125}$I] VP-NP with mAb P1.17 ($IgG_{2a}$ myeloma protein of the same class as L6). In parallel experiments $^{125}$I-labeled VP-NP was incubated with the polyclonal anti-NP. After incubating overnight at 4°C, 50 μl of protein-A Sepharose was added and allowed to react with the NP/L6 complexes for 30 min at 4°C. The beads were pelleted in an Eppendorf Microfuge for 3 min and washed 3 times with 10 mM Tris-HCl, pH 7.4 containing 0.15 M NaCl, 2 mM EDTA and 0.1%

(v/v) Nonidet P-40. Laemmli sample buffer (40 $\mu$l) was added and the pellets boiled for 2 min. Samples were centrifuged in a Microfuge for 5 min and the supernatant electrophoresed as above. The gels were then dried and subjected to autoradiography using Hyperfilm $\beta$-max.

RESULTS

Immunohistochemistry: In control rats, NP-immunoreactivity could be detected within both magnocellular and parvicellular neurons of the hypothalamus. Labeled magnocellular neurons were located in the SON, the PVN, and the nucleus circularis, corresponding to the distribution of OT-NP and VP-NP containing cells. Reactive axons could be traced from these cell groups to the zona interna of the median eminence in the hypothalamo-neurohypophysial tract. Immunoreactivity was also detected within parvicellular neurons of the SCN, and the medial (periventricular) region of the PVN. Axonal projections from the latter cell group could be traced to portal capillaries in the zona externa of the median eminence, corresponding to the hypothalamo-adenohypophysial projection. When adjacent sections of control rat brain were immunoreacted with mAb L6, selective labeling of magnocellular neurons and their axonal projections was seen. No staining was detected within parvicellular neurons of the SCN, or in parvicellular adenohypophysial projections to the median eminence.

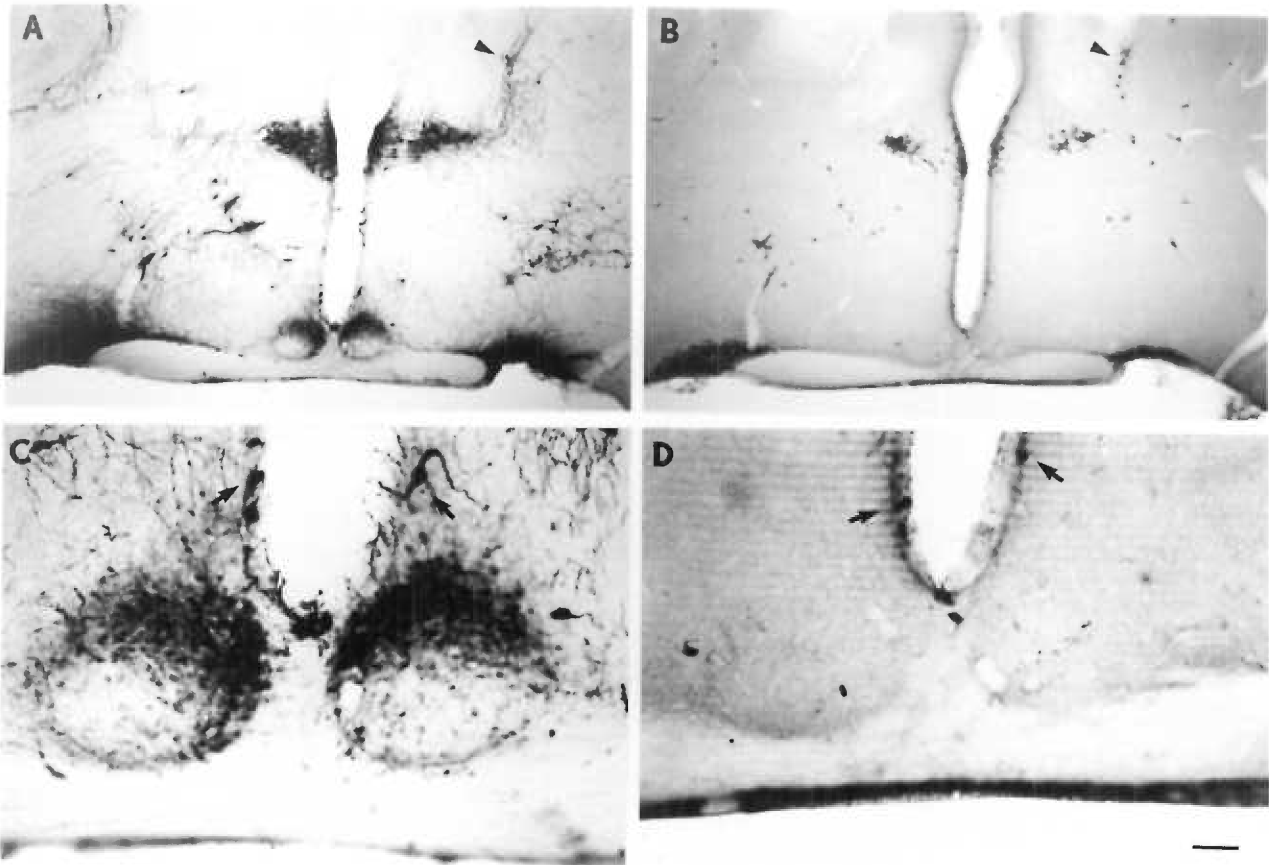
Variations in binding affinities between polyclonal and monoclonal antibodies, or in peptide content between magnocellular and parvicellular systems could well account for the divergent results obtained with polyclonal anti-NP and mAb L6 in normal rat hypothalamus. Additional immunohistochemical studies were therefore performed on

hypothalamic tissue fixed with 2% glutaraldehyde and lysine periodate paraformaldehyde. The use of these different fixatives, however, still produced the same discrepancy. Studies were then performed to determine if the inability of mAb L6 to label parvicellular neurons was secondary to reduced peptide content in these systems. Since both colchicine (Parish et al., 1981; Pickering, 1978) and tunicamycin (Gonzalez et al., 1981) have been shown to induce a build up of neurosecretory material in hypothalamic neurons, immunohistochemical studies were performed with anti-NP and mAb L6 on adjacent sections of colchicine- and tunicamycin-treated rats. Finally, the pattern of immunolabeling with these two antibodies was also evaluated in rats, two weeks postadrenalectomy, since this procedure has been shown to markedly enhance the content of VP and VP-NP in the hypothalamo-adenohypophysial system.

Sections of colchicine-treated rats reacted with polyclonal anti-NP showed an overall build up of immunoreactivity within magnocellular and parvicellular hypothalamic neurons. In addition to cell bodies in the SON, PVN and nucleus circularis (Fig. 1 A), scattered immunoreactive magnocellular perikarya were detected immediately adjacent to the ventral aspect of the third ventricle, dorsal to the SCN (arrows; Fig. 1 C). A few isolated immunoreactive neurons were also located in dorsolateral preoptic area and in the stria terminalis (arrow heads; Fig. 1 A). Enhancement of immunoreactivity was also noted within parvicellular neurons in the dorso-medial region of the SCN, corresponding to the distribution of VP and VP-NP (Fig 1A and IC). Adjacent sections, immunoreacted with mAb L6 however, still demonstrated selective labeling only in magnocellular hypothalamic neurons. The

Figure V-1 Neurophysin and L6-immunoreactivity in rat hypothalamus. Adjacent serial sections (coronal; 100 μm) of colchicine-treated rat hypothalamus stained with polyclonal anti-NP (1:2000; A and C) and mAb L6 (50 μg/ml; B and D). As seen in the low power photomicrograph, anti-NP stains SON, PVN and SCN neurons and their processes (Figure 1A). L6-staining is selectively confined to magnocellular neurons of the SON and PVN, with no staining of SCN (Figure 1 B). At a higher magnification, the total absence of L6-immunoreactivity in the parvicellular SCN is seen in Figure 1D, where a few magnocellular periventricular neurons are still reactive. Arrows: scattered immunoreactive magnocellular perikarya. Arrowheads: isolated immunoreactive neurons.

Figure V-1



pattern of L6-staining within neurons of the SON, PVN and nucleus circularis (Fig. 1 B) corresponded to that noted for the NPs; isolated magnocellular neurons in the ventral peri-third ventricular region also showed L6-staining (arrows; Fig. 1 B). L6-reactivity was similarly localized within magnocellular neurons in dorsolateral preoptic area and stria terminalis (arrow heads; Fig. 1 B). No staining was detected within parvicellular neurons of the SCN, despite clear morphological documentation of the presence of this nucleus in the tissue section (Fig. 1 B, D) or in neurons of the periventricular (parvicellular) region of the PVN. Similar discrepancies in the pattern of NP-and L6-perikaryal staining were noted in the hypothalamus of tunicamycin-treated rats (data not shown).

The relative number of immunoreactive magnocellular perikarya in the NP-(Fig. 1 A) and L6-stained (Fig. 1 B) sections of colchicine-treated rats were comparable. Perikaryal accumulation immunoreactivity, furthermore, was accompanied by reduction in the density of immunoreactive axonal profiles in both the hypothalamo-neurohypophysial and hypothalamo-adenohypophysial projections. As seen in Figs. 1 A and B, however, immunoreactive axons were better visualized with the polyclonal anti-NPs; fiber-staining was scarce in the mAb L6-reacted sections (Fig. 1 B).

Adrenalectomized rats showed a marked increase in NP-immunoreactivity in the zona externa of the median eminence (Fig. 2 A), confirming effectiveness of the surgical procedure. Immunoreactive fibers extended along the full expanse of the zona externa, with no appreciable difference being observed in the region of the zona interna.

When adjacent sections of adrenalectomized rats were immunoreacted with mAb L6, virtually no staining was detected in the zona externa (Fig. 2 B). Interestingly, the density of L6-reactivity in the zona interna of these animals was again less intense when compared to that obtained with polyclonal anti-NP.

Immunoprecipitation and Western Blotting:

The unique specificity of mAb L6, as compared to other polyclonal anti-NPs, in recognizing NP was further verified by immunoprecipitation and immunoblotting. As seen in Fig. 3, initial attempts at immunoprecipitating [$^{125}$I]-labeled VP-NP with mAb L6 were negative (Lane 2). Modifying concentrations of mAb L6, [$^{125}$I] VP-NP or increasing incubation times did not yield immunoprecipitation. Parallel experiments with polyclonal anti-NP consistently resulted in immunoprecipitation of bands corresponding to both pro-pressophysin ($M_r$ 23,000) and processed VP-VP ($M_r$ 10,000) (data not shown). When the [$^{125}$I] VP-NP was reduced with DTT prior to incubation with mAb L6, both pro-pressophysin and VP-NP were immunoprecipitated (Fig. 3, Lane 1). No immunoprecipitation was observed when either reduced or nonreduced [$^{125}$I] VP-NP were incubated with mAb P1.17 (data not shown).

We have recently shown that the inability of many monoclonal antibodies to recognize low molecular weight peptides by Western blotting is a function of electrophoresis and blotting conditions. The restoration of antigenicity is presumably due to conformational change induced by renaturation (removal of SDS from gels) and blotting in basic transfer buffer (pH 10.0) (Rosenbaum et al., 1989). In the present study, when mAb L6 was used to probe VP-NP in Western blots by the

Figure V-2 NP and L6-immunoreactivity in rat median eminence. Adjacent sections (coronal; 100 μm) through the median eminence of ADX rats were immunoreacted with polyclonal anti-NP (1:2000; A) and mAb L6 (50 μg/ml; B). In A, NP stains both parvicellular and magnocellular projections in zona externa and interna regions respectively, whereas mAb L6 (B) selectively labels the magnocellular projections (interna).

Figure V-2

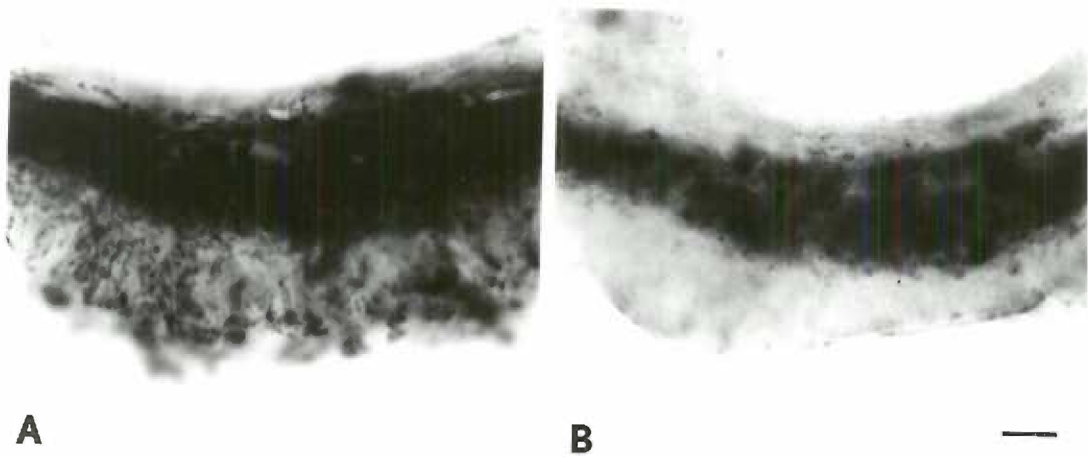
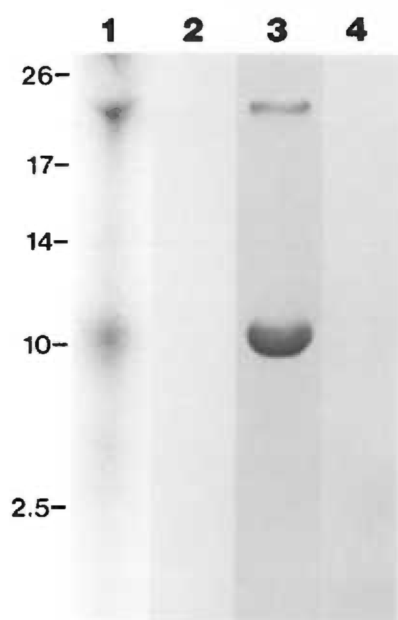


Figure V-3 Immunoprecipitation and immunoblotting of vasopressin-neurophysin (VP-NP). VP-NP was iodinated, immunoprecipitated, and/or immunoblotted as described in Materials and Methods. Lanes 1 and 2 are autoradiograms of iodinated bovine VP-NP (5 g) reduced with 20 mM DTT (Lane 1) or non-reduced (Lane 2), and immunoprecipitated with mAb L6 (50 μg/ml). Note the presence of both pro-pressophysin and processed VP-NP in Lane 1, and their corresponding absence in Lane 2. Lanes 3 and 4 are immunoblots of bovine VP-NP (5 μg) probed with mAb L6 (50 μg/ml), after gel renaturation and transfer in basic buffer (Lane 3) or with no renaturation and neutral transfer buffer (Lane 4). Immunoreactive bands corresponding to pro-pressophysin and processed VP-NP in again seen in Lane 3, but not in Lane 4. Molecular weight markers were: chymotrypsinogen A ($M_r$ 25,666), myoglobin ($M_r$ 16,950), myoglobin fragments I + II( ($M_r$ 14,370), myoglobin fragment I + III ($M_r$ 10,670), and myoglobin fragment III ($M_r$ 2,510).

Figure V-3



conventional procedure as described by Towbin et al. (1979) no detectable signal was observed (Fig. 3; Lane 4). Positive results were obtained when the same nitrocellulose blot was probed with polyclonal anti-NPs (Rosenbaum et al., 1989), indicating that the absence of immunoreactivity with mAb L6 was not due to loss of protein from the blot. Renaturation of the SDS-gels and electroblotting in basic transfer buffer resulted in the immunodetection of pro-pressophysin and processed VP-NP with mAb L6 (Fig. 3; Lane 3).

## DISCUSSION

We have previously shown that both mAb L6 and the polyclonal antiserum to NPs used in the present study recognize OT-NP and VP-NP in immunohistological techniques and Western blotting procedures (Nilaver et al., 1990). Their antigenic epitopes probably reside in the continuous 64 amino acid homologous domain (region 10-74) between the two NPs, thereby accounting for the cross-reactivity. Despite its ability to label both OT- and VP-ergic systems in the hypothalamus, our present study demonstrates that mAb L6 selectively labels (OT-NP and VP-NP containing) magnocellular neurons, and their axonal projections that constitute the hypothalamo-neurohypophysial system in the rat. Labeling was notably absent in (VP-NP containing) parvicellular neurons of the SCN, and in NP-containing projections from the parvicellular PVN to portal capillaries in the zona externa of the median eminence (hypothalamo-adenohypophysial projections).

The possibility that NP-containing parvicellular neuronal systems escaped detection by mAb L6 because of improper choice of fixative or due to their lower peptide content are issues that need to be addressed.

False negative results often encountered in immunostaining generally result from irreparable degradation of tissue antigens. Other factors, such as alteration of antigenicity by fixatives are also common sources of failure. The situation becomes particularly problematic when monoclonal antibodies, directed to conformationally restricted amino acid sequences in peptide molecules, are employed in immunohistochemical procedures. Our past immunohistochemical experience with mAb IIID-7 to vasopressin (Hou-Yu et al., 1982) is a case in point. The IIID-7 clone was found to be directed to the ring sequence of vasopressin. When brain sections fixed with 4% buffered paraformaldehyde, periodate-lysine-paraformaldehyde (PLP) or 2% glutaraldehyde were immunoreacted with mAb IIID-7, different results were obtained depending on the fixative used. Formaldehyde-fixed tissues demonstrated reactivity mainly in perinuclear portions of magnocellular perikarya in the SON and PVN. Weak reactivity was detected in parvicellular neurons of the SCN, and reactive fibers were scarce in the both the adenohypophysial and neurohypophysial projections. A rabbit antiserum to vasopressin, however, stained cell bodies as well as fibers in the same sections. PLP-fixed sections immunoreacted with IIID-7 demonstrated better visualization of both cell bodies and fibers. Glutaraldehyde fixation yielded maximal immunoreactivity, permitting visualization of all hypothalamic vasopressin-containing systems (magnocellular and parvicellular). The effects of fixatives, particularly formaldehyde, on aromatic amino acids in tissue antigens could well account for these divergent staining results with mAb IIID-7. Specifically, irreversible reaction of formaldehyde with hydrogen in aromatic amino acids, such as tyrosine or

phenylalanine in vasopressin, important determinants for mAb IIID-7 (as shown by absorption tests), probably accounts for the deleterious effect of this fixative on vasopressin recognition by IIID-7. PLP probably results in less tissue denaturation because of its lower concentration of formaldehyde (less than 2%), with fixation being maintained by cross-linking of lysine groups to carbohydrates oxidized by periodate (McLean and Nakane, 1974). Glutaraldehyde fixes tissues by reacting with α-amino groups (Pearse and Steward, 1973), and reacts poorly (if at all) with aromatic rings. It therefore does not affect the antigenic epitope in vasopressin recognized by mAb IIID-7, and thereby facilitates maximal preservation of immunoreactivity.

The deleterious effects of fixatives on tissue antigens can be particularly problematic when comparing results obtained with polyclonal and monoclonal antibodies. As seen in Figs. 1 A and B, immunoreactive axons are better visualized with the polyclonal anti-NPs than with mAb L-6. This discrepancy reflects the inherent limitation in employing single antibody populations (mAbs), directed to restricted amino acid sequences, for visualizing antigens in fixed tissue. However, it is unlikely that our inability to label parvicellular NP systems in rat hypothalamus with mAb L6 in the present study represents a technical artifact since parvicellular neurons were consistently negative for L6-reactivity, irrespective of the fixation protocol employed.

The possibility that NP-containing parvicellular neuronal systems escaped detection by mAb L6 due to their lowered peptide content also appears untenable. Despite the paucity of published data to support this premise, studies were carried out to augment the content of

neurosecretory material within hypothalamic neurons. Colchicine has previously been shown to inhibit granular transport, leading to a build up of immunostainable and immunoassayable NPs and hormones within hypothalamic perikarya (Parish et al., 1981; Pickering, 1978). Tunicamycin, an inhibitor of glycosylation, has also been shown to induce accumulation of neurosecretory product within neuronal cell bodies (Gonzalez et al., 1981). Both these procedures were employed in the present study, and resulted in a build up of NP-reactivity within parvicellular neurons of the SCN and the medial-PVN. Adjacent brain sections labeled with mAb L6, nevertheless, failed to demonstrate immunostaining in these hypothalamic nuclei.

The axonal pathway containing VP and its associated NP, arising from the parvicellular PVN and ending on the portal capillary bed in the zona externa of the median eminence has been shown to be extremely sensitive to glucocorticoids (Silverman et al., 1981). The content of VP and VP-NP in this pathway increases markedly in response to adrenalectomy (Stillman et al., 1982; Zimmerman et al., 1977). We therefore evaluated the ability of mAb L6 to label this projection in adrenalectomized rats. Adrenalectomy led to a marked increase in NP-immunoreactivity in the zona externa of the median eminence; mAb L6, however, still failed to label axonal fibers in this projection.

Our immunohistochemical data suggests the expression of NP in a unique conformational form within parvicellular neurons, that is not recognizable by mAb L6. Immunohistochemistry, however, as discussed earlier, cannot provide absolute validity to antigen localization studies, given the exquisite sensitivity of monoclonal antibodies to the

denaturing effects of tissue fixatives. Additional studies, involving immunoprecipitation and Western blotting procedures with mAb L6, were therefore performed to determine whether the tertiary structure of NP played a role in masking the antigenic epitope for mAb L6. As discussed in the Results section, radiolabeled VP-NP could not be immunoprecipitated with mAb L6. However, when the antigen preparation was reduced with DTT prior to incubation with mAb L6, immunoprecipitable bands corresponding to both pro-pressophysin and processed VP-NP were detected. This observation was also confirmed by Western analysis. When VP-NP was probed with mAb L6 using the conventional Towbin technique (Towbin et al., 1979), no reactivity was detected. Renaturation of the gel and electroblotting in basic transfer buffer, however, enabled the visualization of both pro-pressophysin and VP-NP. All of these results demonstrate that mAb L6 is exquisitively sensitive to the tertiary conformation of NP, with additional treatments being required to liberate it in an immunoreactive form.

Single copy genes for oxytocin and vasopressin are present in all species studied to date (Ivell et al., 1983). The VP mRNA is also identical in SON, PVN and SCN neurons (Sherman et al., 1988; Sherman and Watson, 1988), with the possible exception of its differential polyadenylation in the SCN (Carrazana et al., 1988; Robinson et al., 1988; Zingg et al., 1988). Our data would therefore suggest that the changes in NP configuration noted in the histological and biochemical studies are posttranslational. The highly structured tertiary state of NPs, determined by their seven disulfide bonds, is critical in determining which epitopes are available for antibody recognition.

Reduction of these bonds would expose previously inaccessible epitopes for recognition by mAbs, as evidenced by our immunoprecipitation studies. Polyclonal antibodies, in contrast, recognize several antigenic epitopes, and are consequently not configuration-sensitive. The situation with mAb L6 is particularly unique since it was generated to a surface antigen expressed in human lung cancer cells, with living cells being used for the immunization (Hellström et al., 1986a). We have recently characterized the nature of the tumor surface antigen recognized by mAb L6 by automated Edman degradation (Rosenbaum et al., 1990). Sequence analysis of the isolated protein while demonstrating a 21 amino acid homology with the N-terminus of human pro-pressophysin, has also revealed the absence of a cysteine at amino acid 33 corresponding to human pro-pressophysin. These data suggest the expression of an alternate form of the VP precursor in lung tumor cell membranes. Since the mAb L6 clone is directed to this alternate epitope (Rosenbaum et al., 1990), it fortuitously appears to be configuration specific. This exquisite sensitivity of mAb L6 to the tertiary configuration of NP precludes recognition of the form of NP that appears to be expressed within parvicellular hypothalamic systems.

While studying the immunocytochemical distribution of VP and NP in guinea pig median eminence and the posterior pituitary gland by electron microscopy, Silverman and Zimmerman (1975) made the observation that posterior pituitary NP was localized within large neurosecretory granules (1300-1500 Å), whereas the NP-immunoreactive granules within nerve terminals of the zona externa were much smaller (900-1100 Å). A unique feature of vesicles (both endocytotic and exocytotic) is their

low pH relative to the surrounding cytoplasm (Reeves et al., 1984; Rudnick, 1986). The use of pH sensitive dyes has allowed direct determination of intravesicular pH within both endocytotic (Ohkuma and Poole, 1978) and secretory (Orci et al., 1986) pathways. These studies have shown the pH of the vesicles to be dependent on the type of vesicle (Ohkuma and Poole, 1978; Yamashiro and Maxfield, 1984). Since intravesicular pH has also been found to play a critical role in the posttranslational processing of hormone precursors (North, 1987; Orci et al., 1986), it would be of interest to determine if the smaller neurosecretory vesicles of the parvicellular adenohypophysial projection have a different pH *milieu* than that found within the larger vesicles of magnocellular neurons. We are currently evaluating this possibility employing probes that permit visualization of acidic organelles in intact cells (Anderson et al., 1984). Should pH differences be found between neurosecretory vesicles of the magnocellular and parvicellular hypothalamic systems (which could alter conformation of proteins; Hollecker and Creighton, 1982), it may explain the existence of neurophysin in a different conformational form within parvicellular neurons of the rat hypothalamus.

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# DISCUSSION AND CONCLUSIONS

The work described in this dissertation contains several important findings that should continue to be relevant. Although this work was specifically focused on the characterization of the neurophysins, several aspects have general significance in both biochemical and cancer research. The first of these was the development of a technique to detect low-molecular-weight peptides on nitrocellulose with monoclonal antibodies. As described in Chapter I, conventional immunoblotting methods often were not able to detect SDS-denatured electroblotted proteins with monoclonal antibodies, particularly when the proteins were of very low molecular weight (<15 kd). By using a high resolution polyacrylamide gel electrophoresis system, "renaturing" the gels after electrophoresis to remove SDS, and electroblotting in a basic transfer buffer (pH 10.0), a number of monoclonal antibodies were successful in the detection of peptides as low as to 1 kd. The same antibodies produced negative results with conventional blotting protocols. The single most likely explanation for the ability of monoclonal antibodies to detect low-molecular-weight peptides in this system is the protein attaining a more "native" state during the renaturation step and blotting in basic transfer buffer. This technique should be useful for any investigator analyzing very low-molecular-weight peptides in tissue or cell extracts, or proteolytic digests of purified proteins.

Using this blotting technique it was possible to determine whether the immunoreactivity observed in rat hypothalamus with a monoclonal antibody to a human lung adenocarcinoma (mAb L6) was oxytocin (OT), vasopressin (VP), or neurophysin (NP)-related. Probing OT, VP, and NP

with mAb L6 demonstrated specific immunoreactivity within a common domain of both OT-NP and VP-NP, as well as immunoreacting with a surface antigen of a human small cell lung carcinoma cell line (LX-1). In order to determine whether or not the LX-1 antigen was neurophysin related, cell membranes were solubilized, immunoaffinity purified with mAb L6, and subjected to immunoblot and amino-terminal sequence analysis. These studies demonstrated and confirmed the expression of a 45 kd pro-pressophysin-like protein (PPLP) in LX-1 cell membrane. Although PPLP is most probably the mAb L6 antigen (which was originally thought to be a ganglioside), there are two more significant implications associated with these findings. This is, to our knowledge, the first documentation of a neuroendocrine precursor being preferentially expressed in cell membranes. Since neurophysin was first characterized, the only physiologically significant role assigned to this molecule has been that of a "carrier protein" for vasopressin or oxytocin. The findings in this study may finally assign a role for the neurophysins, possibly in the modulation and growth of tumors. The observations that mAb L6 directed to a PPLP epitope in LX-1 cells exhibits ADCC-mediated oncolytic activity also supports such a speculation. If it turns out that the PPLP immunoreactivity observed in breast and colon carcinoma (see Appendix V) is neurophysin-related, the role of neurophysins in tumor production would be quite relevant.

The second important finding is related to the potential early diagnosis of patients with cancer. As discussed earlier, since many forms of lung carcinoma can secrete a variety of peptides and/or hormones, elevated serum levels (by regular sampling of blood) could

allow early detection of disease in these patients. However, in the characterization of PPLP in LX-1 tumor cells, it was shown this antigen is not elevated in the serum, presumably due to lack of processing and secretion. There is no reason to believe this is an isolated example; it may indeed occur in many other carcinomas. This demonstrates the need for performing nucleic acid analysis to assess whether mRNA coding for the peptide and/or hormone is present in a tissue specimen. Since an abnormal VP message is produced in this particular tumor, this would be a useful diagnostic tool.

The exquisite sensitivity of mAb L6 towards its antigenic epitope has additionally suggested the existence of a conformationally different form of neurophysin in parvicellular neurons of the hypothalamus. This difference in conformation, which is likely due to differential vesicular pH in magnocellular versus parvicellular neurons, could have physiological significance in the functions of these two systems in the brain.

In conclusion, this work has produced some new techniques for studying the neurophysins and has provided much additional data on the precise role of these molecules. Many of these studies should provide the foundation for a better understanding of the relationship between neurophysin and tumor production and their role in the magnocellular/ parvicellular hypothalamic system in brain.

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# APPENDICES

# APPENDIX I

# APPENDIX I

## PURIFICATION OF PROPRESSOPHYSIN AND NEUROPHYSIN FROM BOVINE AND HUMAN PITUITARY

Neurophysin extraction and isolation from pituitary glands was first reported by Chauvet et al. (1960) and later refined by Hollenberg and Hope (1967). Although the early extraction procedures indicated there were as many as seven forms of neurophysin, many of these were thought to be the result of inappropriate extraction procedures. This common method of extraction in the early 1960s used acetic acid at a pH of 4.0, which is close to the pH optimum for proteases in the posterior pituitary (Dean and Hope, 1967). Extraction at a pH of 1.5 results in the isolation of two major neurophysin peptides (Robinson, 1975).

The experiments described below were done in order to extract both neurophysin and precursor (propressophysin) peptides for use in the various studies described in this dissertation. Many of the procedures described below are based on the method of Verbalis and Robinson (1983).

### Materials and Methods

[$^{125}$I]Bolton-Hunter reagent N-succinimidyl 3-(4-hydroxy,3-[$^{125}$I]diiodo-phenyl) propionate; 4400 (Ci/mmol) was from New England Nuclear. Concanavalin A-Sepharose and Sephacryl S-200 were from Pharmacia. Bovine neurophysin II was from Sigma. Bovine pituitary glands were obtained fresh locally and human pituitary glands were from the National Pituitary Agency. All other reagents were obtained as described in Chapter I.

#### Tissue Extraction

Five whole pituitary glands (approximately 10 g) were homogenized

in 50 ml 0.1 M HCl, pH 1.5 for 1 min at medium speed using a Polytron. Prior to homogenization frozen pituitaries were fragmented into smaller pieces with a mortar and pestle. Homogenized pituitaries were extracted for 24 hr at 4°C with stirring or rotating. The homogenates were then centrifuged at 2000 x g for 30 min. The supernatant was aspirated and saved, and the pellet reextracted for 24 hr with 0.1 M HCl pH 1.5. This was again centrifuged at 2000 x g for 30 min and the supernatant added to the first. The pooled supernatants were neutralized to pH 7.0 with 5 N NaOH. At this point, a large precipitate forms and the neutralized extract is centrifuged at 3000 x g for 45 min. The supernatant, containing neurophysins and precursors, are then concentrated on an Amicon PM-10 membrane and lyophilized, or used directly for lectin affinity chromatography.

Gel Filtration: Lyophilized acid extracts from above were resuspended in 2.0 ml of 0.1 M formic acid, pH 3.3, containing 6 M urea. A 100 x 2.5 cm Sephacryl S-200 gel filtration column was equilibrated for 48 hr with 0.2 M formic acid, pH 3.3, containing 6 M urea, at 4°C. The column was then calibrated with bovine liver catalase, chymotrypsinogen A, and cytochrome C.

Bovine NP II, which contained approximately 5-10% precursor, was iodinated by the method of Bolton and Hunter (1973; described in detail in Chapter V) and chromatographed to determine the elution positions of processed NP and precursor. The void volume and total volume of the column were determined with blue dextran 2000 and $^3H$.

After calibration of the column and reequilibration, the lyophilized acid extracts in formic acid/urea were chromatographed with

a trace amount of $^{125}$I labeled NP II. Fractions containing protein were determined by the Folin phenol method (Peterson, 1983) and the tubes corresponding to peaks 1 and 2 in Figure AI-1, pooled. Pooled peak fractions were then dialyzed against 0.1 M formic acid, pH 3.3, at 4°C to remove the urea, and finally dialyzed against 0.1 M Tris-HCl pH 7.0.

Concanavalin A Chromatography: Concanavalin A (Con A)-Sepharose (4 ml bed volume) was washed with (5 bed volumes) 25 mM $Na_2HPO_4$, pH 7.0 containing 5 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$ (buffer A). The column was then loaded at 0.35 ml/min with the supernatant from the neutralized pituitary acid extract above (either human or bovine). After washing with 5 column volumes of buffer A, the column was eluted at 0.35 ml/min sequentially with 8 ml 0.25 M NaCl, 8 ml of 10 mM $\alpha$-methyl glucoside, and 10 ml of 0.5 M $\alpha$-methyl glucoside, all in 25 mM $Na_2HPO_4$ pH 7.0. Fractions were assayed for total protein by the method of Bradford (1976), the peak fractions pooled, and dialyzed against 25 mM $Na_2HPO_4$ pH 7.0.

Gel Electrophoresis and Western Blotting: Samples from the crude acid extract, gel filtration column and the Con A column were subjected to SDS-PAGE and/or probed with antibody after transfer to nitrocellulose as described by Rosenbaum et al. (1989; Chapter I).

## RESULTS AND DISCUSSION

A representative gel elution profile of $^{125}$I labeled bovine NP II is shown in Figure AI-1. Both pro-pressophysin (23 kd; peak 1) and NP II (11 kd; peak 2) are resolved. Since this preparation of neurophysin contains only about 5 to 10% pro-pressophysin, peak 1 is correspondingly smaller. Once the positions of the eluted neurophysins were determined,

lyophilized pituitary acid extract in 2 ml of 0.1 M formic acid pH 3.3/ 6 M urea were chromatographed. The fractions corresponding to peaks 1 and 2 were assayed for total protein, individually pooled, dialyzed, and used for various experiments and controls throughout this dissertation.

In order to assess the molecular weight and immunoreactivity of the size fractionated pituitary extracts, samples from both peaks were subjected to gel electrophoresis and Western blotting. The results of these studies are shown in Figure AI-2. Coomassie blue staining of bovine pro-pressophysin/neurophysin pooled from the two Sephacryl S-200 peaks (Lane 1) resulted in an intense band at approximately 11 kd corresponding to processed neurophysin II and a weaker band at approximately 23 kd corresponding to pro-pressophysin. Both of these bands were highly immunoreactive with a polyclonal antibody to neurophysin (Lane 2). Additional immunoreactivity was demonstrated with mAb L6, polyclonal antivasopressin and antibody YL-3 raised to human pro-pressophysin (see Chapter 3; data not shown). Both the gel electrophoresis and Western blotting experiments confirm the identity of these bands as pro-pressophysin and vasopressin-neurophysin (NP II).

In some experiments, the neutralized pituitary acid extract was loaded directly onto a concanavalin A Sepharose column to specifically bind glycosylated pro-pressophysin. After loading the column with pituitary extract (typically 25 ml/4 ml bed volume) the column was eluted sequentially with 0.25 M NaCl to remove nonspecifically bound protein, 10 mM α-methyl glucoside (to elute weakly-bound glycoproteins)

Figure AI-1 Gel filtration of [$^{125}$I]labeled bovine neurophysin II (this preparation also contains propressophysin) on Sephacryl S-200. Neurophysin II (150 $\mu$g) was iodinated and chromatographed in 0.1 $\underline{M}$ formic acid-6$\underline{M}$ urea as described in Materials and Methods. Neurophysin was chromatographed together with the indicated standards showing their observed elution positions. The position of precursor ($M_r$ 23 kd) is in peak 1 and processed NP II ($M_r$ 11 kd) is in peak 2. S: bovine liver catalase ($M_r$ 57,471); K: chymotrypsinogen A ($M_r$ 25,666); cytochrome C ($M_r$ 11,761), Vo and Vt represent the elution positions of blue dextran 2000 and [$^3$H] water, respectively.

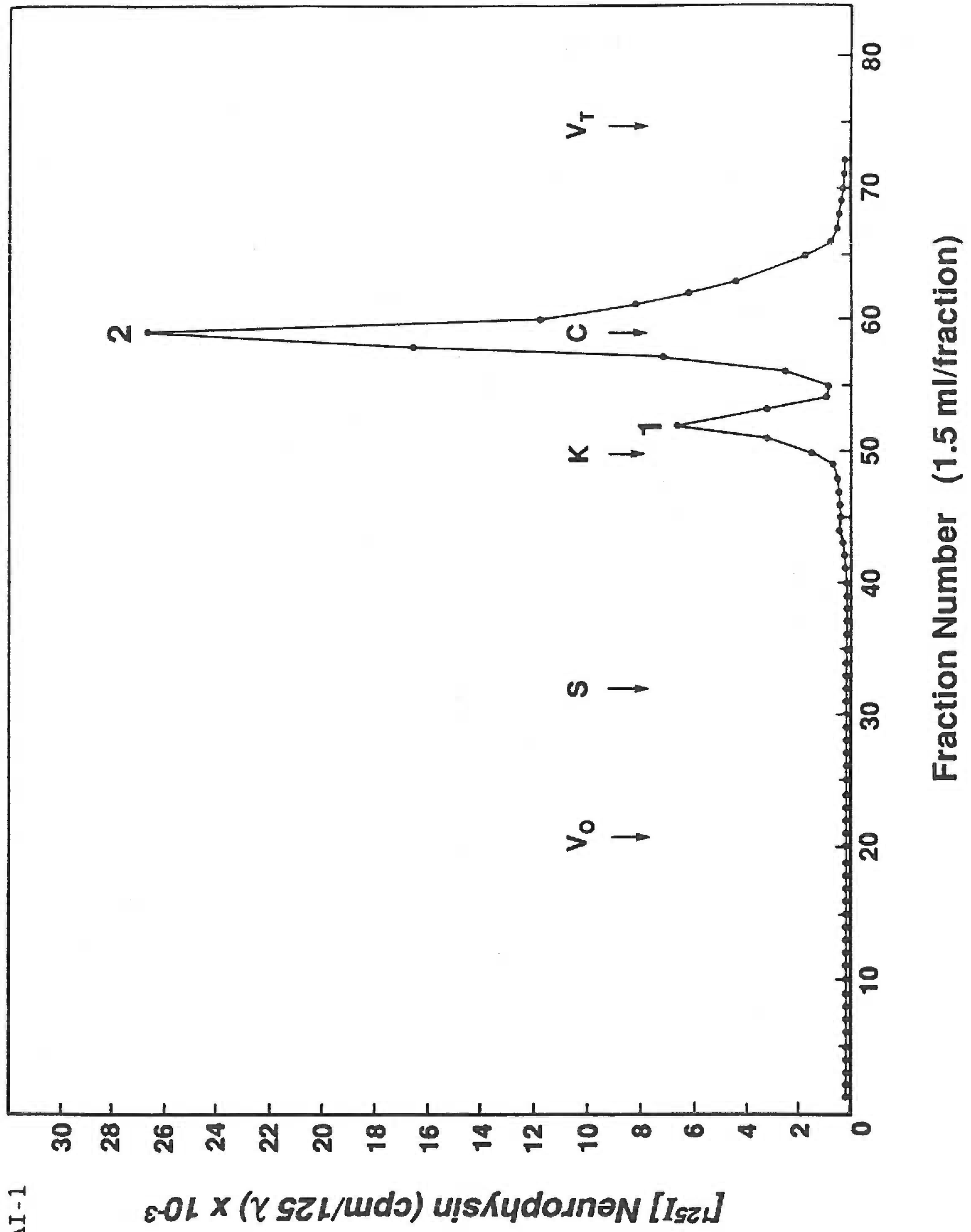
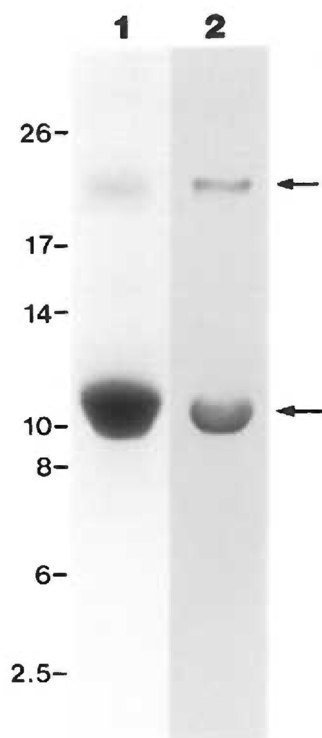



Figure AI-1

Figure AI-2 Gel electrophoresis and immunoblot analysis of gel filtration purified bovine propressophysin and neurophysin II. Electrophoresis and blotting procedures were as described in Materials and Methods of Chapter I. Lane 1 is a coomassie stained gel pattern of bovine neurophysins pooled from peak 1 (5 $\mu$g) and peak 2 (15 $\mu$g) of the Sephacryl S-200 column elution shown in Figure AI-1. Lane 2 is an immunoblot of the above peaks probed with antineurophysin (1:4000). Both neurophysin II (lower arrow) and propressophysin (upper arrow) are visible. Molecular weight markers were as detailed in Fig I-1 of Chapter I.

Figure AI-2



and finally 0.5 M α-methyl glucoside to elute remaining specifically-bound glycoproteins. A representative elution profile of the 0.5 M α-methyl glucoside elution is shown in Figure AI-3. The fractions within the bar were pooled, dialyzed and used for various experiments throughout the dissertation. The identity of pro-pressophysin from this step was confirmed by gel electrophoresis and Western blotting (data not shown).

Figure AI-3 Elution profile of concanavalin A purified bovine pituitary extract. Bovine pituitaries were extracted for neurophysins as described in Material and Methods. The pooled fractions are indicated by the bar (I——I). Pituitary extract was loaded onto the column, washed with buffer (see Methods) and the void material collected in bulk. The column was then eluted sequentially with 0.25 M NaCl, 10 mM α-methyl glucoside and finally 0.5 M α-methyl glucoside. The final high sugar elution profile is shown in this figure. Total glycosylated protein in each fraction was determined by the Bradford method (1976) at $A_{595}$.

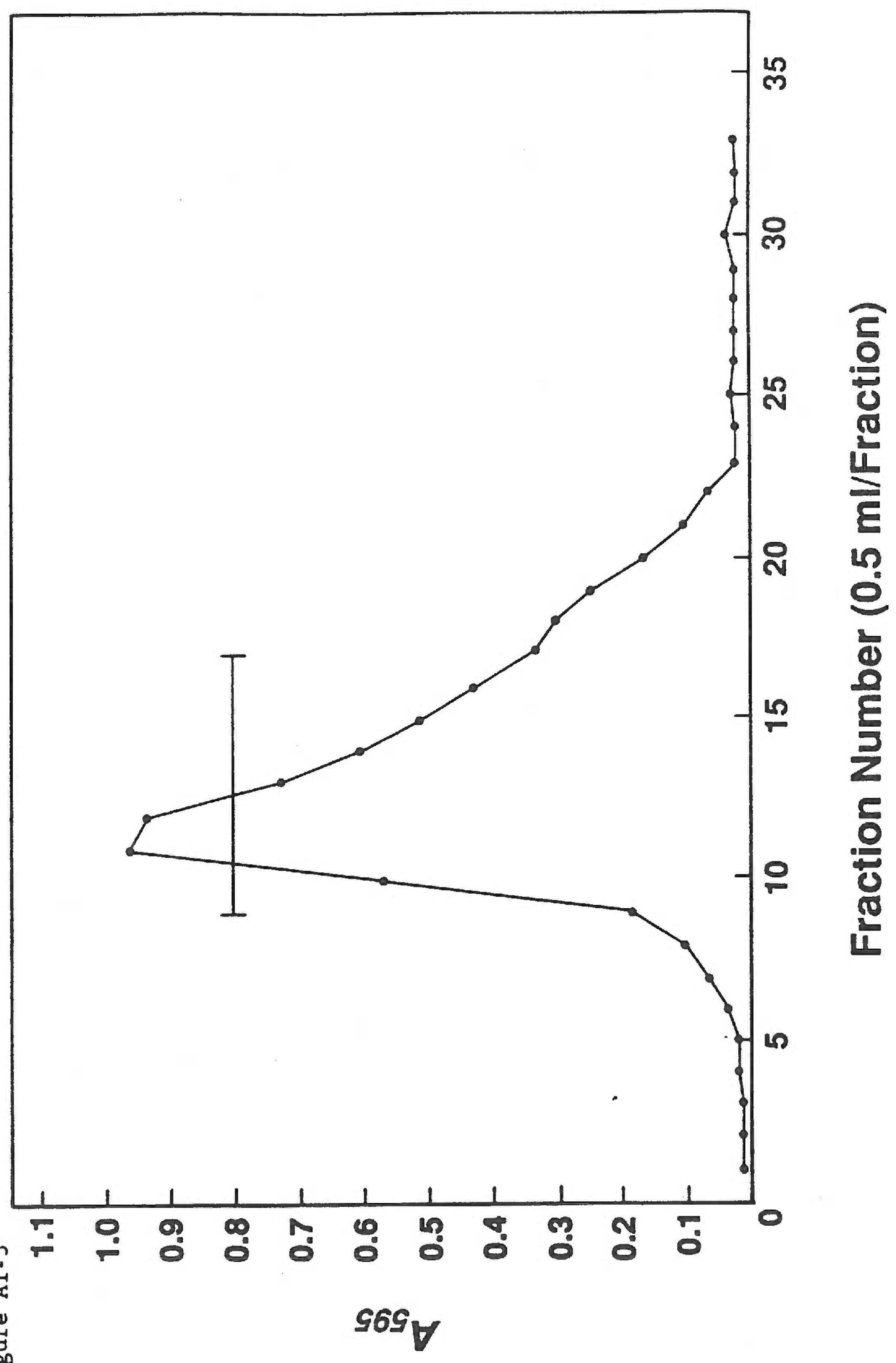



Figure AI-3

# APPENDIX II

# APPENDIX II

## RADIOIMMUNOASSAY OF VASOPRESSIN IN NORMAL AND TUMOR-BEARING NUDE RATS

Small cell lung carcinomas (SCLC) are capable of secreting a wide variety of neuroendocrine hormones. As discussed in the introduction of this dissertation, SCLCs and other types of lung carcinomas can result in elevated serum levels of vasopressin, neurophysin and other peptides and hormones. Biochemical and immunohistochemical studies of our LX-1 SCLC cell line, however, did not appear to indicate secretion of vasopressin, neurophysin or the precursor propressophysin. Since we have an animal model bearing intracerebrally xenografted LX-1 tumors, it was of interest to evaluate the serum vasopressin levels of these animals as compared with controls.

This appendix describes the details of serum radioimmunoassays of both normal and tumor-bearing nude rats. The results of these studies were mentioned briefly in Chapter III.

## MATERIALS AND METHODS

[$^{125}$I]vasopressin (2200 Ci/mmol) was from New England Nuclear. Protein A-Sepharose was obtained from Pharmacia. Polyclonal antivasopressin was from ICN Immunobiologicals.

### Radioimmunoassay of Vasopressin

Radioimmunoassay (RIA) for plasma vasopressin in both control nude rats and tumor-bearing nude rats were performed essentially as described by Morton et al. (1975). In order to determine the correct antibody dilution (60% binding of labeled antigen) [$^{125}$I]vasopressin (100 pg/tube) was incubated with varying dilutions of polyclonal antivasopressin

(1:3000 to 1:150,000) in RIA buffer consisting of 20 m$\underline{M}$ Tris, pH 7.5, 0.02% (w/v) $NaN_3$ and 0.1% (v/v) recrystallized BSA. Total incubation volume was 125 $\mu$l and allowed to equilibrate for 24 hr at 4°C. After equilibration, protein A-Sepharose (25 $\mu$l) was added to each tube and the tubes were gently agitated for 1 hr at 4°C. Protein A-Sepharose beads were pelleted in an Eppendorf Microfuge for 2 min, the supernatant aspirated and the pellet washed 3 times with RIA buffer. Specifically bound [$^{125}$I]vasopressin was determined by quantitating pellets in a gamma counter.

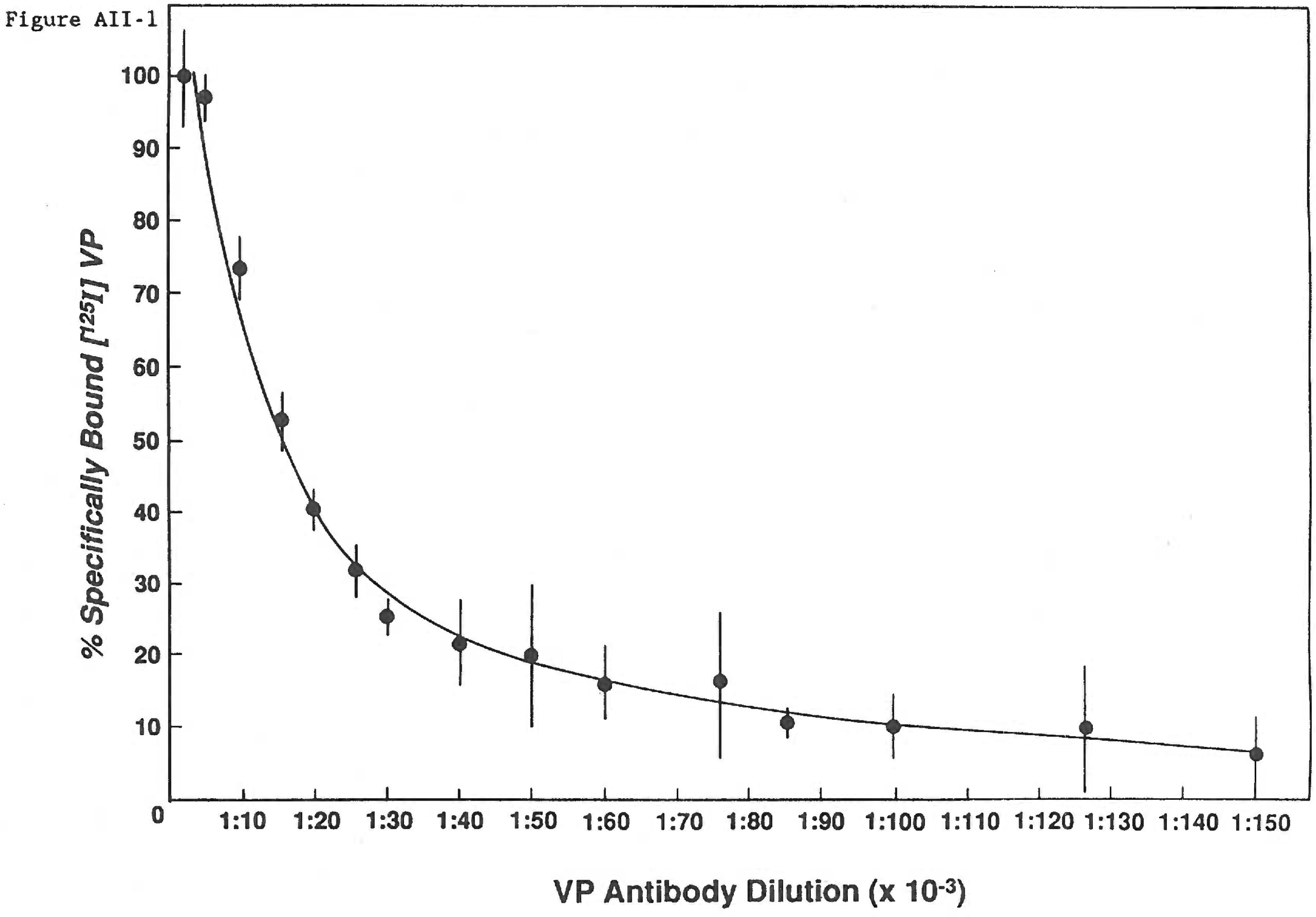
Standard competitive binding curves for unlabeled vasopressin were generated by incubating [$^{125}$I]vasopressin (100 pg/tube) with antivasopressin (1:12,000; determined from the antibody dilution curves above) for 24 hr at 4°C as above. After equilibration, varying amounts of unlabeled vasopressin (0.1 ng to 10 ng) were added to each tube and allowed to equilibrate for 24 hr at 4°C. Specifically bound [$^{125}$I]vasopressin was determined by the addition of protein A-Sepharose, and counting the pellets as described above. Serum vasopressin levels in normal and tumor-bearing nude rats were determined by adding varying amounts of serum normalized for total protein (50 $\mu$l to 250 $\mu$l) in place of unlabeled vasopressin. The data were fitted by linear least square regression.

## RESULTS AND DISCUSSION

Figure AII-1 shows the antibody dilution curve to determine 60% binding of [$^{125}$I]vasopressin. Antivasopressin dilutions of 1:3000 or lower resulted in total (100%) specific binding of [$^{125}$I]vasopressin. Approximately 60% binding of [$^{125}$I]vasopressin was achieved with an

Figure AII-1 Vasopressin antibody dilution curve for radioimmunoassay. Tubes containing 100 pg of [$^{125}$I]vasopressin were incubated with varying dilutions of polyclonal antivasopressin and specifically immunoprecipitated with protein-A Sepharose (see Methods). Specifically bound [$^{125}$I]vasopressin in the pellet was determined by gamma counting.

Figure AII-1



antibody dilution of 1:12,000. This dilution was used for all subsequent incubations.

A standard displacement curve with unlabeled vasopressin is shown in Figure AII-2. Varying concentrations of vasopressin from 0.1 to 10 ng resulted in a linear displacement of [$^{125}$I]vasopressin to 0.5 ng. This corresponds to approximately 30% binding of [$^{125}$I]vasopressin. The linear portion of this curve was used to estimate the relative amount of vasopressin in unknown samples.

In order to determine whether LX-1 tumor-bearing nude rats had elevated serum vasopressin levels as compared with control nude rats, varying amounts of serum from each group were incubated with [$^{125}$I]vasopressin (100 pg/tube) preequilibrated with antivasopressin (1:12,000). Both serum samples were normalized for total protein (Bradford, 1976) prior to incubation. When serum was added from both groups, specifically bound [$^{125}$I]vasopressin was displaced linearly in the range of 50 to 250 $\mu$l as shown in Figure AII-3. When a point from this line was compared with the standard displacement curve (Fig. AII-2) values of 1.7 pg/$\mu$l and 1.6 pg/$\mu$l were obtained for normal nude rat and LX-1 tumor-bearing nude rat, respectively. These results suggest there is no difference in plasma vasopressin levels in normal nude rat as compared with tumor-bearing nude rats and consequently no excess secretion by the tumor. However, there may not be increased serum VP levels due to the blood-brain barrier. If only a small amount of VP is secreted it may be detected only in the CSF.

Figure AII-2 Standard curve for radioimmunoassay of vasopressin. Tubes containing 100 pg of [$^{125}$I]vasopressin were incubated with antivasopressin at a dilution of 1:12,500, corresponding to approximately 60% specific binding (see Figure A II-1). After equilibration (24 hrs at 4°C), varying concentrations of unlabeled vasopressin were added. Specifically bound [$^{125}$I]vasopressin was then immunoprecipitated and counted as described in Materials and Methods.

Figure AII-2

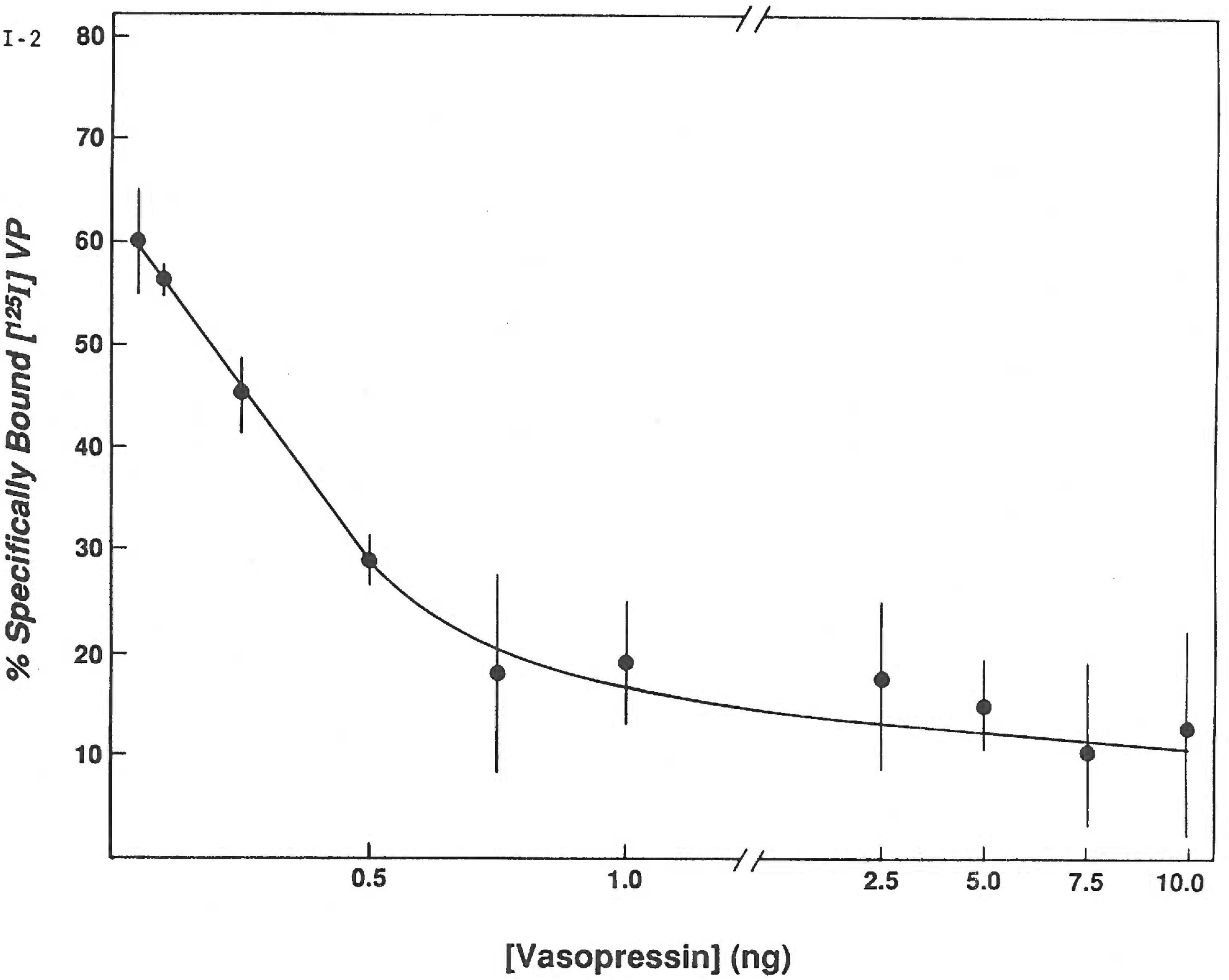
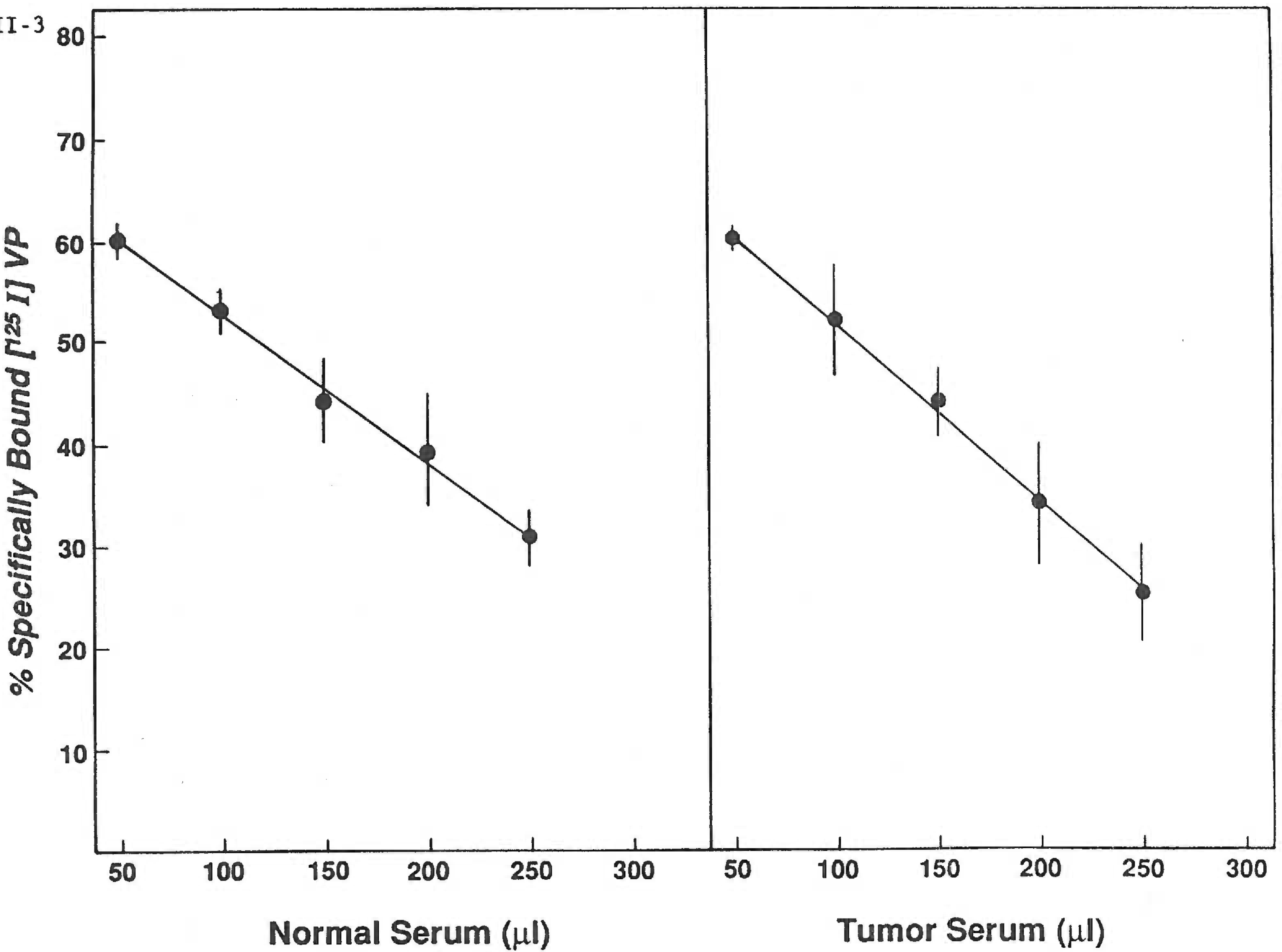


Figure AII-3 Vasopressin radioimmunoassays with normal and tumor-bearing nude rat serum. Each assay contained 100 pg of [$_{125}$I]vasopressin and was incubated with antivasopressin at a dilution of 1:12,500. After equilibration, varying amounts of serum were added from normal nude rats (A) or from tumor bearing nude rats (B). Specifically bound [$^{125}$I]vasopressin was determined as in Figure A II-1. Vasopressin serum levels in the animals were determined by matching specifically bound [$^{125}$I]vasopressin to the linear portion of the standard curve in Figure A II-2. The data were fitted by linear least square regression that gave $r^2 = 0.99$ for both normal and tumor-bearing nude rat serum.

Figure AII-3



# APPENDIX III

# APPENDIX III

## ANALYSIS OF ENDOGENOUS ENDOPEPTIDASE ACTIVITY IN LX-1 CELLS

The demonstration of pro-pressophysin-like protein (PPLP) immunoreactivity in LX-1 lung tumor cells have indicated targeting of unprocessed peptide to tumor cell membrane. This could potentially result from: (a) lack of an endogenous processing enzyme in LX-1 cells, (b) lack of an intact dibasic cleavage site within PPLP, or (c) a posttranslational modification preventing PPLP from being processed (i.e., a change in conformation resulting in the cleavage site being inaccessible to enzyme). In order to address the first of these possibilities, lack of endogenous processing enzyme, LX-1 cells lysates were incubated with synthetic fluorogenic substrates containing a paired basic residue. The details of these experiments are described in this appendix.

## MATERIALS AND METHODS

Artificial fluorogenic substrates were obtained form Peninsula Laboratories (Belmont, CA). All other materials were as described in Chapter III.

### Enzymatic assay of endopeptidase activity in LX-1 cells

This assay is based on the cleavage of 7-amino-4-methyl-coumarin (AMC) from a synthetic peptide containing a paired basic residue. Cleaved AMC is fluorogenic and thus allows the monitoring of enzymatic activity by fluorescence. LX-1 cells (~$10^6$) were pelleted and resuspended in 50 m*M* potassium acetate, pH 5.0 (0.5 ml). Cells were permeabilized by the addition of the nonionic detergent Brij 58 to a final concentration of 1% (v/v) and allowed to sit on ice for 30 min.

Cell lysates (25 μl) were then added to 200 μl of 0.2 M HEPES, pH 7.3 containing 1% (v/v) Triton t-100, 2 mM $C_aCl_2$, 1 mM PMSF and 50 μM Boc-Gln-Arg-Arg-AMC (added from a 10 mM stock in DMSO). As controls, LX-1 cell lysates were added to the HEPES buffer without fluorogenic substrate or with 50 mM BZ-Arg-AMC. After incubation at 37°C for 30 min, cell debris were removed by centrifugation in an Eppendorf microfuge. Fluorogenic product in the supernatant was determined spectrofluorometrically at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

## RESULTS AND DISCUSSION

The substrate specificity of LX-1 cell endopeptidase activity is shown in Table AIII-1. The synthetic substrate Box-Gln-Arg-Arg-MCA, containing a dibasic sequence, was cleaved by endogenous endopeptidase, whereas the single basic residue, Bz-Arg-MAC was only background. Although the amount of LX-1 cell lysate was not optimized in these experiments, a 10-fold increase in enzymatic activity was observed. These results indicate that the LX-1 tumor cell line has the capacity to enzymatically cleave dibasic residues. While this does not prove the existence of a specific pro-pressophysin processing enzyme in the cells, the preferential targeting of unprocessed PPLP to the LX-1 cell membrane is probably not due to lack of endogenous processing enzyme.

Table AIII-1 Substrate Specificity of Endopeptidase Activity in LX-1 Cell Lysates

| Substrate | Relative Fluorescence |
|---|---|
| Boc-Gln-Arg-Arg-MCA | 10.10 ± 1.56 |
| Bz-Arg-MCA | 0.90 ± 0.01 |

# APPENDIX IV

# APPENDIX IV

## DETECTION OF A POTENTIAL NEW VASOPRESSIN GENE TRANSCRIPT IN THE PITUITARY GLAND

Over the last decade, it has become increasingly apparent that the hypothalamus is not the sole domain of vasopressin (VP) and oxytocin (OT) synthesis. Vasopressin production has been demonstrated in extra hypothalamic brain by both immunohistochemistry (Van Leeuwen and Caffe, 1983) and *in situ* hybridization (Miller et al., 1988). Expression of pro-pressophysin mRNA (Ivell et al., 1985) and peptide (Caffe, 1985) has been reported in the rat cerebellum, and VP-immunoreactivity in rat sympathetic ganglia (Hanley et al., 1984), ovary (Lim et al., 1984), testis (Rehbein et al., 1986), adrenal gland (Nussey et al., 1984), and thymus (Gainer et al., 1977). These studies provide unambiguous verification of pro-pressophysin expression outside the hypothalamus.

Pro-pressophysin mRNA has recently been detected in the pituitary gland (Murphy et al., 1989). The demonstration of VP mRNA in the pituitary is particularly interesting, given VP's role in pituitary release of ACTH. In the course of the purification of bovine pituitary extract for use as controls in this dissertation, we detected a 45 kd pro-pressophysin-like protein, identical in molecular weight and immunoreactivity as the LX-1 PPLP described in Chapter III. Subsequent screening of a human anterior pituitary cDNA library revealed several vasopressin positive clones. This appendix describes these very preliminary studies.

## MATERIALS AND METHODS

A human anterior pituitary cDNA library in λgt10 was made

available to us by Dr. Oliver Civelli at the Vollum Institute of Advanced Biomedical Research (VIABR) here at the Oregon Health Sciences University. Approximately 5 x $10^5$ phage were plated, lifted with nitrocellulose, and hybridized overnight at 42°C employing a cocktail of rat $^{32}P$ labeled VP cDNA (Fig. AIV-1) and $^{32}P$ labeled rat 30-mer oligonucleotide (Fig. AIV-2) that recognizes both rat and human VP mRNA (see Chapter III). Filters were washed 2 x 10 min in 2 x SSC at 42°C followed by 2 x 10 min in 1 x SSC at 50°C, dried and exposed to x-ray film. Positive clones were picked, replated and rescreened with either the VP cDNA or the VP 30-mer.

RESULTS AND DISCUSSION

A representative autoradiogram of the initial screening of a human pituitary cDNA library is shown in Figure AIV-3. Out of approximately 6 x $10^5$ clones plated out 103 positive signals were identified. Secondary screening with the rat 30-mer VP probe has verified four of these positive clones (data not shown). Southern blotting of these clones has not yet been performed.

While it is possible that some (or all) of these positive clones represent normal pro-pressophysin message, it remains to be seen what the precise nature of these clones are. Since we have detected a high molecular weight pro-pressophysin-like protein in bovine pituitary and an alternative (1 kb) form of pro-pressophysin transcript in LX-1 tumor cells (comparable to a 1 kb message reported in pituitary [Tracer and Loh, 1989]) these clones may represent an alternate VP gene in human pituitary. Many additional studies are required to determine whether this 45 kd pituitary pro-pressophysin-like protein is the product of a

Figure AIV-1 Diagram of rat 30-mer pro-pressophysin probe. This probe corresponds to the last 10 amino acids in VP-NP (Exon 3), and hybridizes with both rat and human VP mRNA. Probes were 3' labeled with $^{32}P$ using terminal deoxynucleotidyl transferase.

Figure AIV-1

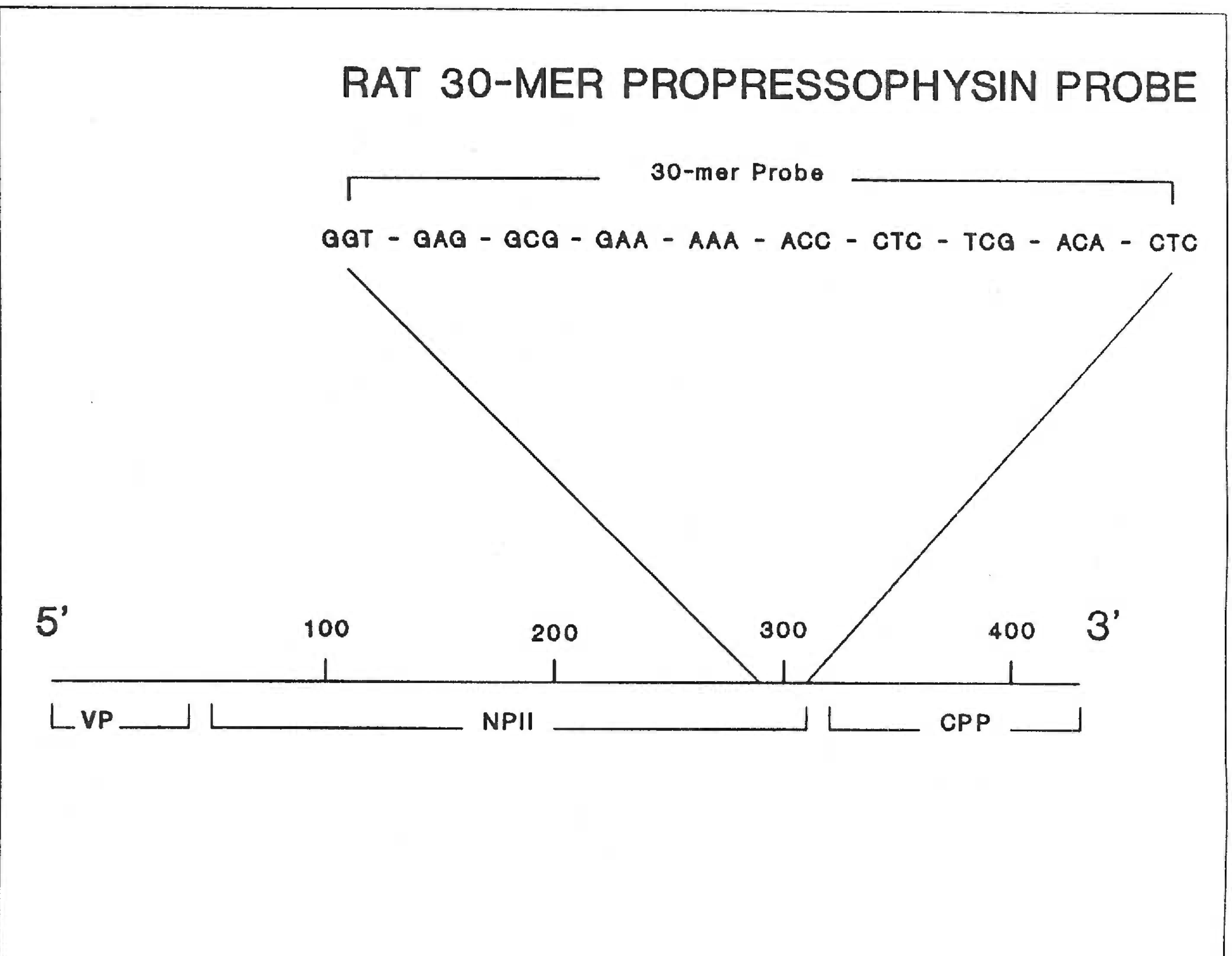


Figure AIV-2 Diagram of rat pro-pressophysin cDNA. This cDNA was provided by Dr. Tom Sherman and was subcloned into the EcoRI site of pGEM3 plasmid oriented as shown. The percentages shown refer to homology of that region with the corresponding rat pro-oxyphysin cDNA. Since the VP and OT cDNAs share a 176 bp midregion of almost 100% homology at the extreme 3' end of a Sac II restriction site, this cut makes a specific VP cDNA probe. A 215 bp VP specific cDNA was generated in some cases by restriction digests at the indicated Sac II site within the cDNA and at the Hind III site in the polylinker. cDNA probes were random hexamer labeled with $^{32}P$.

Figure AIV-2

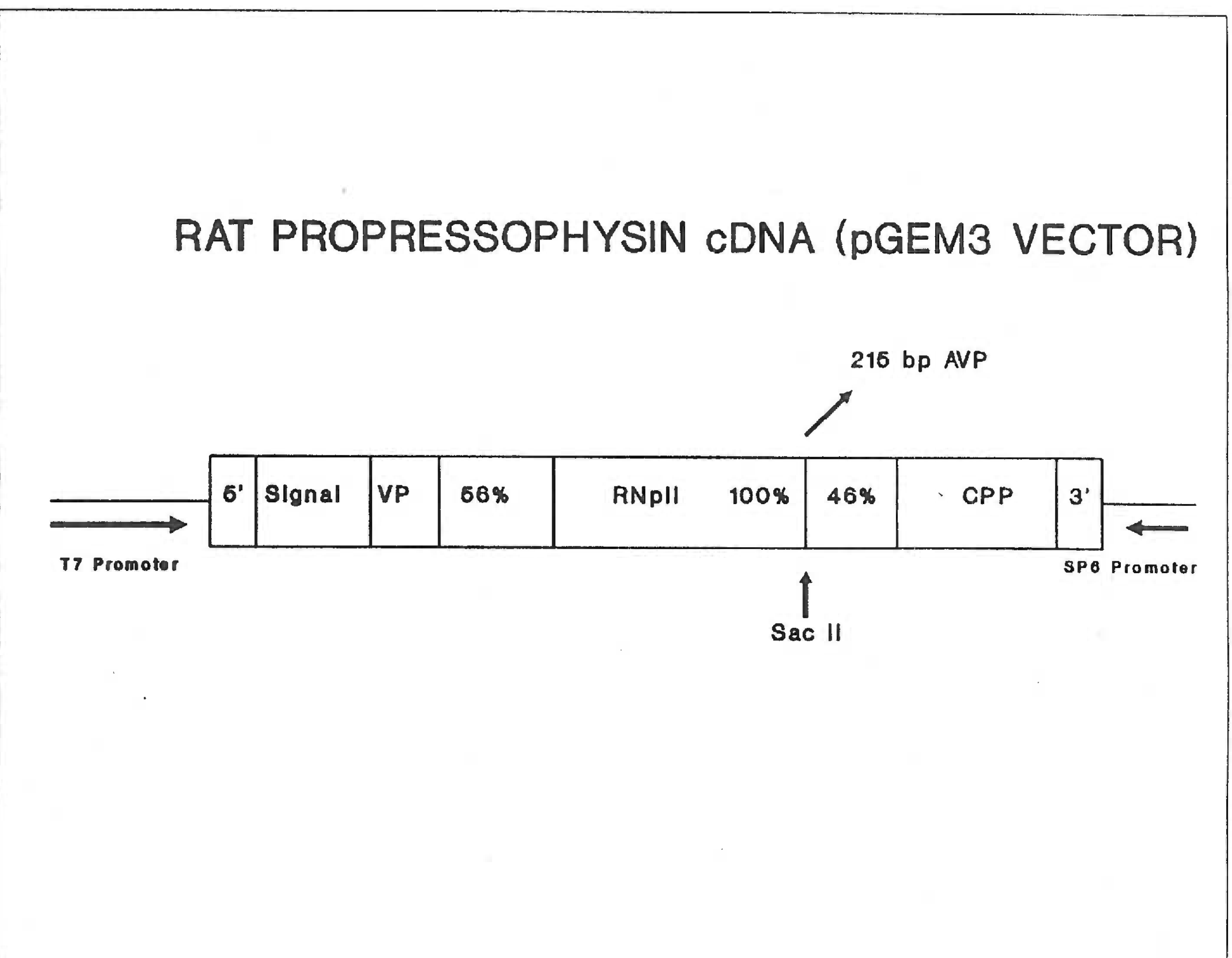
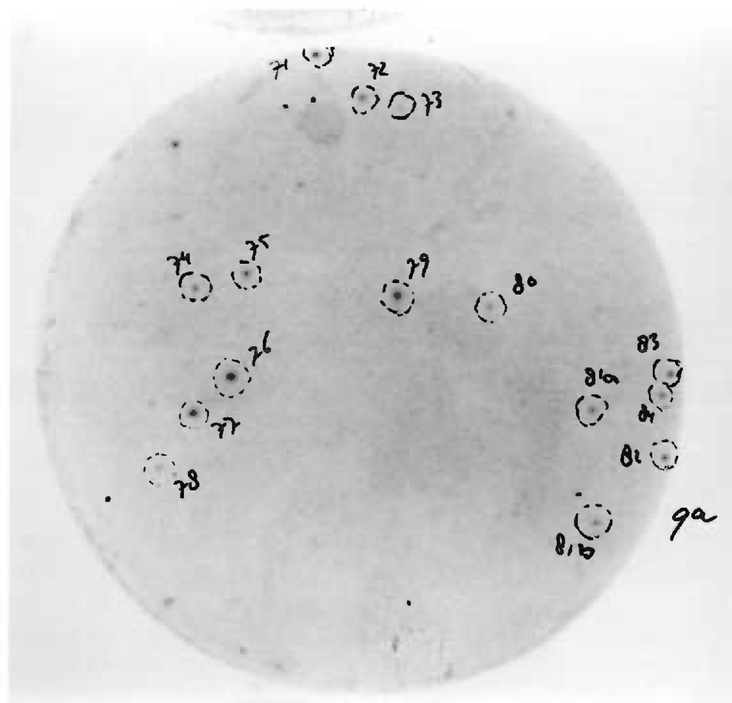


Figure AVI-3 Screening of human anterior pituitary cDNA library for positive VP clones. Approximately 6 x $10^5$ phage were plated, lifted and probed as described in Materials and Methods. This autoradiogram shows positive clones 71-84 in the initial screening.

Figure AIV-3



new gene transcript or is the result of alternative splicing or differential upstream initiation of the normal VP gene.

# APPENDIX V

# APPENDIX V

## IDENTIFICATION OF PRO-PRESSOPHYSIN-LIKE IMMUNOREACTIVITY IN BREAST AND COLON CARCINOMA

The monoclonal antibody L6 (mAb L6) that specifically recognizes a 45 kd pro-pressophysin-like protein (PPLP) in LX-1 human lung tumor cell membrane (Chapter III of this dissertation) has additionally been reported to recognize a surface antigen in a number of other carcinomas. Hellström et al. (1986a) have reported immunoreactivity of mAb L6 with lung adenocarcinoma, breast carcinoma, colon carcinoma, ovarian carcinoma and some melanomas. Since we have characterized the L6 positive antigen in LX-1 small cell lung carcinoma, it was clearly of interest to investigate some of these additional tumor cell lines for any potential similarity with the LX-1 PPLP. This appendix describes some preliminary characterization studies on a mAb L6 positive breast and colon cell line.

## MATERIALS AND METHODS

The breast carcinoma cell line HBT 3477, the colon carcinoma line C-3347 and mAb L6 were the generous gift of Drs. I. and K.E. Hellström (Oncogen, Seattle, WA). The tumor cells were solubilized in Nonidet P-40, electrophoresed, and immunoblotted as described for the LX-1 cells in Chapter III.

## RESULTS AND DISCUSSION

Immunoblots of solubilized breast and colon cells are shown in Figure AV-1. When breast (Lane 1) and colon (Lane 2) were probed with mAb L6, two prominent bands are seen. The first of these is a band of approximately 25 kd with equal intensity in both colon and breast. The

second band is approximately 45 kd, with comparable intensity in both cell lines. A much less intense band at approximately 28 kd is also present in breast and colon. Lanes 3 and 4 are breast and colon, respectively, probed with polyclonal antioxytocin were essentially negative with respect to the positive mAb L6 bands. Immunoreactive bands of approximately 55 kd and 65 kd are visible in colon (Lane 4) and to a lesser extent in breast (Lane 3). Antibody YL-3, raised against human pro-pressophysin demonstrates strong immunoreactivity with a 45 kd band in colon (Lane 6) and is weakly reactive with a 45 kd band in breast (Lane 5). The colon cell line additionally shows a YL-3 reactive band at approximately 34 kd. Polyclonal antivasopressin shows a highly immunoreactive band at 45 kd in colon carcinoma (Lane 8) and a weakly immunoreactive band at 45 kd in breast carcinoma (Lane 7). Lymphoma cells, which show no immunoreactivity with mAb L6 (Hellström et al., 1986a) were also negative with antibody YL-3, antivasopressin and antioxytocin (data not shown). The results of the immunoblots in Figure AV-1 are summarized in Table AV-1.

Although these experiments are very preliminary in nature, they clearly show an immunoreactive band at 45 kd in both breast and colon with mAb L6, YL-3 and anti-VP. This is highly suggestive of these cell lines expressing a PPLP, similar or identical to that identified on LX-1 cells. However, it will be critical to further isolate and sequence these bands in order to confirm this. The nature of the other immunoreactive bands is unclear. The very intense mAb L6 band at approximately 25 kd may be a cleavage product of the 45 kd band, containing a neurophysin domain. If this is the case, it would not be

Figure AV-1 Immunoblot analysis of breast and colon carcinoma. Samples were solubilized, electrophoresed and probed with antibodies as described in Chapters I and III. Lanes 1, 3, 5, and 7 are immunoblots of solubilized breast tumor cells (30 $\mu g$) probed with mAb L6 (50 $\mu g/ml$; Lane 1), polyclonal antioxytocin (1:2000; Lane 3), polyclonal antibody YL-3 (1:500; Lane 5), and polyclonal anti-VP (1:2000; Lane 7). Lanes 2, 4, 6, and 8 are solubilized colon tumor cells (30 $\mu g$) probed with mAb L6 (50 $\mu g/ml$; Lane 2), antioxytocin (1:2000; Lane 4), YL-3 (1:500; Lane 6), and anti-VP (1:2000; Lane 8). Molecular weight markers were bovine serum albumin ($M_r$ 66,296), ovalbumin ($M_r$ 42,807), bovine carbonic anhydrase B ($M_r$ 31,000), soybean trypsin inhibitor ($M_r$ 21,500), and hen egg white lysozyme ($M_r$ 14,400).

Figure AV-1

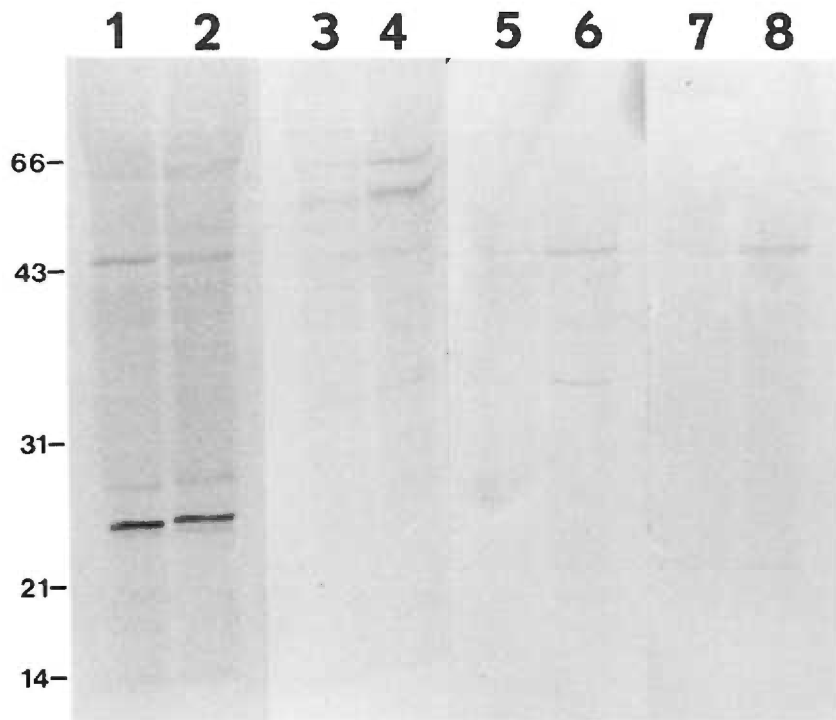


Table AV-1  Summary of Breast and Colon Immunoblotting

| Tumor/Reactive Band | | Antibody | | | |
|---|---|---|---|---|---|
| | | mAb L6 | YL-3 | Anti-VP | Anti-OT |
| Breast | 65 kd | 0 | 0 | 0 | ++ |
| | 55 kd | 0 | 0 | 0 | ++ |
| | 45 kd | ++ | + | + | 0 |
| | 28 kd | + | 0 | 0 | 0 |
| | 25 kd | +++ | 0 | 0 | 0 |
| Colon | 65 kd | 0 | 0 | 0 | + |
| | 55 kd | 0 | 0 | 0 | + |
| | 45 kd | ++ | ++ | ++ | 0 |
| | 34 kd | 0 | + | 0 | 0 |
| | 28 kd | + | 0 | 0 | 0 |
| | 25 kd | +++ | 0 | 0 | 0 |

+++, strongly immunoreactive signal; ++, moderate to strong immunoreactive signal; +, weakly immunoreactive signal; 0, essentially negative immunoreactive signal.

surprising that YL-3, which recognizes only intact prohormone, or anti-VP would be negative with this band.

It additionally appears that both the breast and colon tumor cell lines used in these experiments produce more immunoreactive protein than the LX-1 cells. Solubilized LX-1 cells had to be affinity purified in order to concentrate enough PPLP for visualization on electrophoresis or by Western blotting. These cell lines may thus be useful for expression studies of PPLP at the mRNA level.