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# Molecular Diagnostics of Cancer

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<sup>1</sup> Page on which contribution begins.

# Membrane Proteins as Markers for Normal and Neoplastic Endocrine Cells

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## Introduction

Neurons, endocrine cells and their neoplastic derivatives share a variety of similar or even identical characteristic proteins. The analysis of these cellular constituents provides valuable information on the nature, location and distribution of tumor cells in the body.

Neuron-specific enolase, a soluble cytoplasmic glycolytic enzyme, was the first widely used marker protein for neural and endocrine cells and serves as an excellent diagnostic tool in neurologic and endocrine disease [22]. Recently, specific secretory products of endocrine cells, the chromogranins/secretogranins, which are costored and coreleased with catecholamines and peptide hormones, have also attracted interest as neural or endocrine cell markers. These markers can be measured in tumor tissue or cells and also in serum [15, 36, 37, 41].

In addition to the proteins mentioned above, intracellular membrane proteins or constituents of the plasma membrane have very recently been accepted as diagnostic markers. One group of intracellular membrane proteins common to neural and endocrine cells is found in small translucent vesicles (SVs). In neurons SVs contain transmitters and are thus termed synaptic vesicles [6, 16]. In endocrine cells the function of SVs, which contain the same membrane proteins as synaptic vesicles, is unknown. If SVs in endocrine cells also contain transmitters, they could reflect the existence of a second secretory pathway in addition to the well-characterized release of peptide hormones from large, dense core vesicles (LVs) by exocytosis.

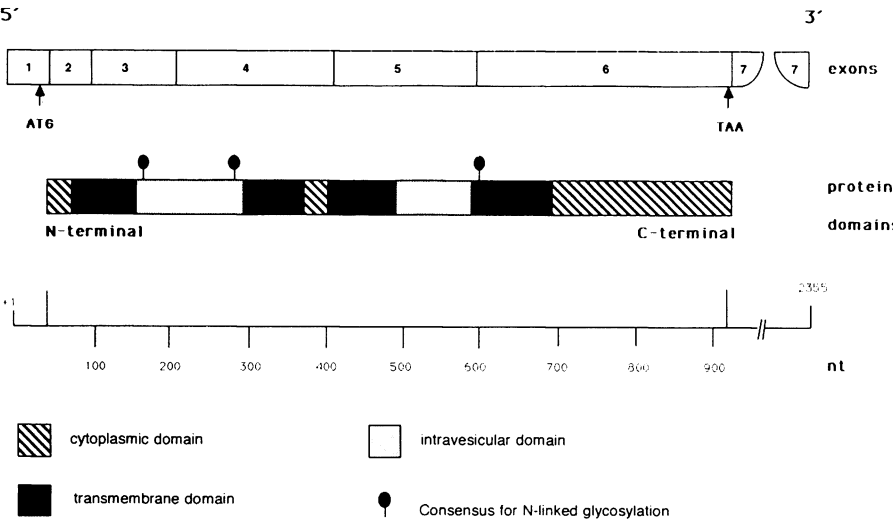
Cell surface antigens shared by neural and endocrine cells have also been identified. For example, the neural cell adhesion molecules (NCAMs), have been found in peptide hormone- as well as steroid hormone-producing endocrine cells. Moreover, NCAM expression characterizes the cell-cell adhesion specificities between these cells [18–21, 23, 27]. In addition,

NCAMs have been found in a variety of rodent and human endocrine tumor cells, suggesting a possible application as markers [1, 2, 9, 10, 13, 17–19, 21, 24, 26, 31].

In this chapter we will depict the structure and function of synaptophysin, as an example of a SV membrane protein already used as an endocrine tumor marker, and describe the molecular and cell biology of NCAMs, a recently elucidated group of membrane antigens present in normal and neoplastic endocrine cells.

**Structure and Function of Synaptophysin**

Neurotransmission between a presynaptic nerve terminal and postsynaptic target cell involves discharge of transmitter molecules. The neurotransmitters are stored in specialized organelles, the synaptic vesicles. The specific function of synaptic vesicles includes uptake and storage of neurotransmitters, interaction with the cytoskeleton, docking and fusion with the plasma membrane. Synaptophysin [6, 16] is one of the major integral membrane proteins of SVs (average diameter 50 nm). In the SV membrane it forms hexamers, composed of identical subunits of 38 kDa, whose primary structure has been elucidated by sequencing rat, cow and human cDNA. Synaptophysin is a highly conserved protein consisting of four transmembrane domains as well as short N-terminal and extended C-terminal cytoplasmic domains (Fig. 1).



**Fig. 1.** Schematic representation of the synaptophysin mRNA illustrating the positions of the protein domains coded by the different exons. Exons are represented by the *white boxes*. The start (*ATG*) and stop (*TAA*) codons for translation are also indicated. The other boxes are explained at the foot of the figure. The positions of nucleotides are given in the *lower panel*

Synaptophysin incorporated into lipid membranes exhibits voltage-dependent channel activity [39] and it may dock to a complementary protein, physophilin, a component of the presynaptic plasma membrane, possibly initiating formation of a fusion pore and subsequent neurotransmitter release [38].

Isolation and comparison of the complete rat and human synaptophysin genes showed that, despite the difference in molecular size (16 kb in rat vs 13 kb in man), intron/exon boundaries are precisely conserved [3]. Exon 7, because of a stop codon in exon 6, is not translated in either species (Fig. 1). The 5' upstream region is devoid of any TATA or CAAT boxes but is characterized instead by features typical of "housekeeping" genes. Specifically, GC-rich islands and four Sp1-binding motifs indicate that synaptophysin synthesis is regulated at the transcriptional level. Sequences more than 1.2 kb downstream from the immediate upstream region may be responsible for cell type-specific expression of synaptophysin.

### **Molecular and Cell Biology of Neural Cell Adhesion Molecules**

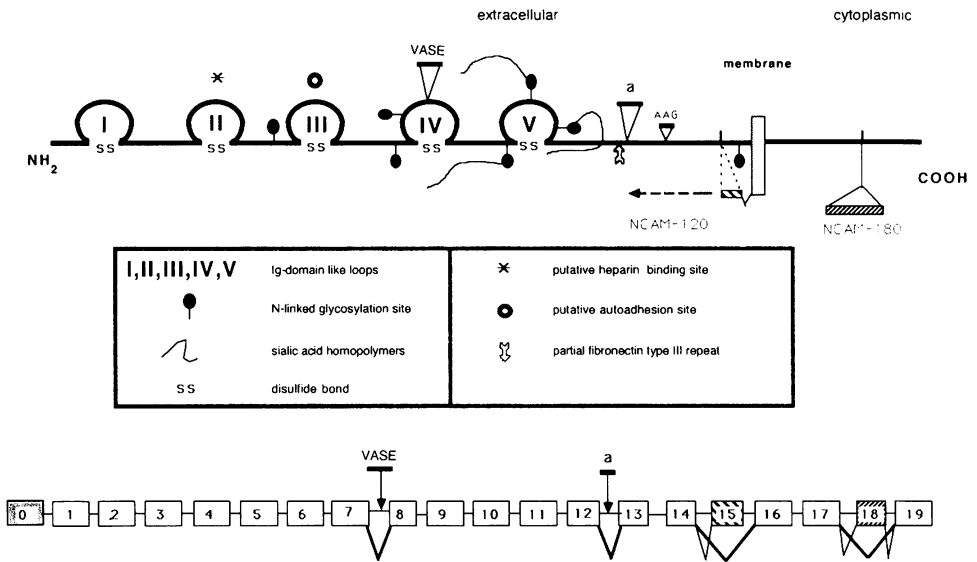
Cell-cell adhesion molecules (CAMs) – the ligands or receptors involved in cell-cell contact formation – are important regulators of cell assembly and maintenance of tissue architecture.

The NCAMs are among the most prevalent CAMs in vertebrates. They are glycoproteins anchored in different ways to the plasma membrane and promote cell-cell adhesion through a homophilic binding mechanism. The different NCAMs are primary translation products arising through alternative splicing of a single gene located on human chromosome 11 and mouse chromosome 9. They have similar extracellular domains but differ primarily in their plasma membrane associated and intracellular domains. For example, NCAM-180 (the isoform having a molecular weight of 180 kDa) contains a cytoplasmic domain which is larger than that of NCAM-140. By contrast, NCAM-120 consists only of extracellular domains and is linked to the plasma membrane by a phosphatidylinositol anchor (Fig. 2).

Alternative splicing can allow the fine modulation of gene expression such that protein isoforms with functional differences are expressed in the proper spatiotemporal fashion. It is a posttranscriptional process by which pre-mRNA transcribed from the gene can code a variety of protein isoforms that differ in function or localization. Twenty major exons in both mammals and birds code for the different NCAM isoforms. While exon O encodes the 5'-untranslated and leader sequences [33], exons 1–14 generate the extracellular sequences of all known NCAMs (Fig. 2). Exon 15 codes for the membrane-anchoring sequence of NCAM-120, and exon 18 codes for the additional cytoplasmic insert unique to NCAM-180 [4, 5, 25, 32].

Recently, additional exons in the NCAM gene have been discovered. They can be added at the exon 12/13, 13/14 junction, thereby coding for the





**Fig. 2.** Scheme of the protein and exon structure of the neural cell adhesion molecule with a molecular weight of 140 kDa (NCAM-140). The N- and C-termini of the protein are indicated. *Loops I-V* represent the disulfide-linked loop structures of the immunoglobulin-like domains. The *inset* explains the symbols used in the upper panel. The plasma membrane is indicated by the *dotted box*. NCAM-120 contains the translation product of exon 15 which is attached to the membrane via a phosphatidylinositol anchor. The sequence coded by exon 18 which is unique to NCAM-180 is shown at the right side of the schema. Minor exons are indicated by *VASE*, *a* and *AAG*. In the *lower panel* the exons of NCAM are represented as boxes. The characteristic exons of NCAM-120 and NCAM-180 are marked in addition to exon 0, which is not translated. The alternatively spliced minor “extra-exons” at exon junctions 7/8 and 12/13 are also shown. The *V* marks indicate alternative splice junctions

hinge region of the molecule, or at the exon 7/8 junction. Genomic cloning revealed that three smaller exons of 15, 48 and 42 bp could be positioned between exons 12 and 13 [8, 28, 40]. Furthermore, an exon named SEC, which contains a stop codon generating a secreted NCAM form, has been spliced into this position [11]. A 15-nt exon (a15) with or without an additional AAG triplet has also been detected between exon 12 and exon 13 [33]. It is not clear whether the 3'-terminal AAG triplet is encoded by a 3-nt exon [33]. An AAG triplet has also been found at the exon 13/14 junction (D. Barthels et al. 1991, personal communication). From the examples given above it is obvious that the hinge region of the NCAMs can be modified by alternative splicing in many ways. The functional consequences of these insertions are as yet unknown. The structure of the N-terminal extracellular regions of NCAMs contains five domains which are similar to each other and to the homology units of immunoglobulins.

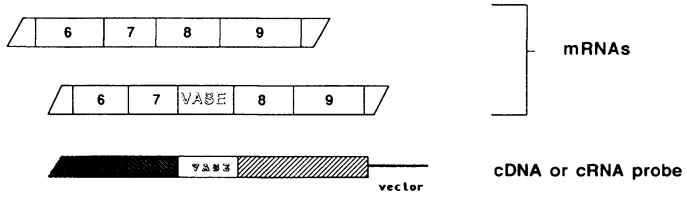
Thus NCAMs, like other cell adhesion molecules such as L1, F1, contactin, TAG-1 and MAG, are members of the immunoglobulin superfamily.

A heparin-binding domain has been identified in the second immunoglobulin-like domain of NCAMs. Additionally, an N-linked oligosaccharide has been mapped to the third immunoglobulin-like domain which is engaged in the autoadhesion between NCAMs of neighboring cells. It is the adjacent fourth immunoglobulin-like loop of NCAMs which can be varied by alternative splicing. A 30-nt exon, termed pi or VASE (variable alternatively spliced exon), is located between exons 7 and 8 [33, 35]; it has been suggested that VASE may modify binding affinity [34]. Taken together, 198 different mRNA species could code for NCAM polypeptides (D. Barthels et al. 1991, personal communication) [30, 33]. The presence of extra exons in cellular mRNA can be detected using S1 nuclease protection assays. This method allows direct comparison of mRNA and labeled DNA or RNA probe sequences. The S1 nuclease hydrolyzes only single-stranded DNA and RNA probes. Thus, if the DNA or RNA probe does not pair precisely, tails or loops not protected by hybridization to the mRNA will be excised. Resolution of the undigested S1-protected fragments of the probes on denaturing polyacrylamide gels yields detailed information about the regions of sequence homology between the probe and the mRNA (Fig. 3).

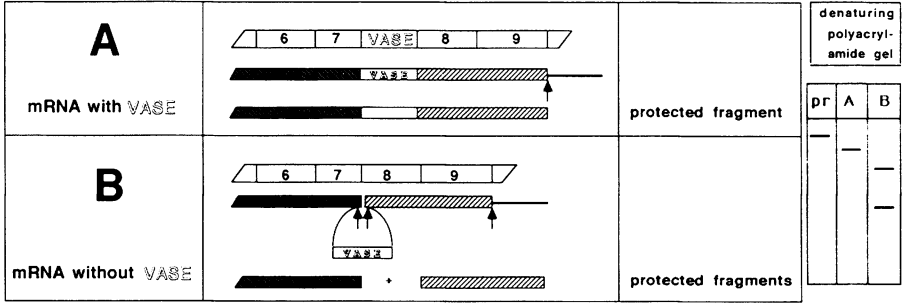
The fifth extracellular loop of NCAMs is subject to functionally important posttranslational modification. Though NCAMs have multiple carbohydrate attachment sites, modulation of adhesion specifically arises from differences in the length of homopolymers of alpha-2, 8-linked neuraminic acid units (polysialic acid, PSA) linked to NCAMs via a core carbohydrate. NCAMs occur in highly sialylated embryonic and less sialylated adult forms. The precise molecular mechanism whereby polysialic acid on NCAMs modulates the calcium-independent autoadhesion remains to be analyzed, but it is clear that enzymatic removal of PSA increases binding between NCAM-bearing liposomes and neuroblastoma cells [29].

Following transient expression in various embryonic structures, NCAMs are subsequently found predominantly in adult neural, endocrine, skeletal and cardiac muscle cells. Available data imply that NCAMs influence a number of developmental processes such as segregation of cells into discrete regions, axon guidance and formation and innervation of skeletal muscle [4, 5, 23, 25, 32].

Regulation of gene expression can occur at the transcriptional, post-transcriptional, translational and posttranslational levels as developmental processes unfold. Regulation at the transcription level is initiated by the promoter region of the gene. It should be noted that the NCAM promoter does not contain a typical TATA box, and therefore initiation of RNA transcription occurs at several sites on the gene; this initiation mechanism is often observed in genes that lack this sequence. Sequences for both promotion and inhibition of transcription reside within 840 bp upstream of the main transcription start site. In this upstream region a juxtaposition



Hybridization and S1 nuclease ( ↓ ) digestion



**Fig. 3.** Detection of additional exons in NCAM mRNA by S1 nuclease protection assays. The exons of two different mRNAs as well as the labeled cDNA or cRNA probe with additional vector sequences used are shown at the top. The probe is hybridized with the mRNAs with (A) or without (B) exon VASE followed by S1 nuclease digestion, which hydrolyzes all single-stranded regions (indicated by the arrows). In A only the vector sequences are removed, whereas in B the single strand coding for extra-exon VASE contained in the labeled probe will be totally digested by S1 nuclease, leaving two protected fragments. The samples are run on a denaturing polyacrylamide gel to determine the size of probe fragments protected from the S1 nuclease by hybridization

of a Sp1 factor-binding consensus site and a nuclear factor I-binding site has been mapped. Moreover, motifs, such as three A + T-rich segments containing ATTA motifs and an AGGA repeat which resembles negative regulatory elements in other promoters, were also mapped. It seems that negative and positive elements in the promoter, with features typical of “housekeeping” genes, interact to regulate the tissue-specific pattern of NCAM gene expression. Furthermore, a factor related to nuclear factor I is involved in transcriptional control of the NCAM gene [14].

### Expression of Synaptophysin and NCAMs in Normal and Neoplastic Endocrine Cells

The distribution of synaptophysin and NCAMs in normal and neoplastic endocrine cells is summarized in Table 1. In addition to their occurrence in

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**Table 1.** Occurrence of the antigen of small secretory vesicles, synaptophysin (SYN), and of the neural cell adhesion molecules (NCAMs) in normal and neoplastic endocrine cells and tissues

Tissues	Cells and tumors	SYN	NCAMs
Adenohypophysis	Normal endocrine cells	+	+
	Adenoma	+	+
Adrenal medulla	Normal chromaffin cells	+	+
	Pheochromocytoma	+	+
Adrenal cortex	Normal steroid-producing cells	—	+
	Cortical carcinoma	n.d.	+
Bronchial tract	Normal endocrine cells	+	n.d.
	Small cell carcinoma	+	+
	Carcinoid	+	n.d.
Gastrointestinal tract	Normal enteroendocrine cells	+	n.d.
	Carcinoid	+	n.d.
Pancreas	Normal islet cells	+	+
	Adenoma	+	n.d.
Parathyroid	Normal chief cells	+	n.d.
	Adenoma	+	n.d.
Thyroid	Normal C cells	+	n.d.
	C-cell carcinoma	+	n.d.
Testis	Leydig cells	n.d.	+
Ovary	Granulosa and luteal cells	n.d.	+

n.d., not determined.

catecholamine- and peptide hormone-containing cells, NCAMs have also been detected in steroid-producing cells of adrenal cortex [27] and ovarian granulosa and luteal cells [23]. The potential application of NCAM analysis in tumors derived from these tissues can be envisioned, based on a report of NCAM expression in an adrenocortical carcinoma [17]. Synaptophysin, on the other hand, occurs in all normal peptidergic endocrine cells and in neoplasms derived from these cells (Table 1).

A variety of different techniques has been applied in the analysis of synaptophysin and NCAMs in normal and neoplastic endocrine cells. These analytical techniques provide precise descriptions of the extent of transcription, translation and even posttranslational modification of cellular membrane proteins. For example, transcription is analysed using *in situ* hybridization and Northern blot techniques in addition to the more sophisticated S1 nuclease protection assays, described above, which can elucidate the sequence of a specific mRNA. Insights into translation of synaptophysin and NCAMs in particular cells are provided by light and ultrastructural immunocytochemistry. These morphological techniques, in conjunction with immunological techniques such as Western blotting, can be utilized to analyze the anatomical arrangements related to posttranslational processing or modification (e.g. proteolytic modification, presence of PSA and others). Taken together, these techniques have been used to define the nature of

certain primary tumors and, importantly, to detect their metastases. It is also possible to identify neoplastic cells which express endocrine marker proteins in mixed tumors. For example, cell heterogeneity is a prominent characteristic in most lung neoplasms. Yet, in mixed tumors as well as in homogeneous tumors immunocytochemical techniques reveal different cell types based on their expression of NCAM-140; specifically, small cell carcinoma cells express NCAM-140 while mesotheliomas and squamous cell carcinomas do not [2, 9, 21, 24, 26]. Differential diagnosis of the lung tumors based on their expression of cell markers provides important insights for prognosis and therapy.

Detection of synaptophysin (like neuron-specific enolase) can certainly be regarded as a general tool to distinguish endocrine from nonendocrine tumor cells. NCAMs share with other endocrine markers the property of differential expression in endocrine tumor cells. For example, all pituitary adenomas, characterized by hormone markers [2], have been found to contain synaptophysin and neuron-specific enolase, but NCAM-140, the prevalent isoform in normal and neoplastic endocrine tumor cells, was not detected by immunoblotting in prolactinomas, suggesting that it is minimally expressed in this cell type [2]. In like manner, chromogranin A, a protein costored and coreleased from a variety of peptide hormone-producing cells, has not been observed in all their neoplasms. While prolactinomas do not appear to contain chromogranin A [7], they can be identified by their hormone content and the expression of synaptophysin and neuron-specific enolase. Adenomas not expressing hormones, termed inactive adenomas, generally express the latter markers together with NCAM-140 and chromogranin A; on the other hand, analysis of the hormones in serum or by immunocytochemistry in these adenomas is not of diagnostic value [2, 7, 12].

The examples given above clearly demonstrate that analysis of membrane markers for endocrine tumor cells provides important contributions to a precise (differential) diagnosis and elucidation of the biological properties of the neoplastic cells even though they differ in their degrees of proliferation and their tendencies to form metastases. Thus, membrane marker characterizations form an important basis for therapeutic design and modulation of its effect.

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