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# Characterization of soluble forms of NCAM

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Neural cell adhesion molecule (NCAM) has been described as a family of membrane glycoproteins. However, soluble NCAM immunoreactivity has long been recognized. We here show that soluble NCAM is composed of two quantitatively major polypeptides of $M_r$ 180 000 and 115 000 and two minor components of $M_r$ 160 000 and 145 000. Soluble NCAM was immunochemically identical to membrane NCAM, was polysialylated and carried the HNK-1 epitope. It only constituted 0.8% of total NCAM in newborn rat brain. Soluble NCAM appeared in neuronal cell culture medium 15–30 min after the start of synthesis preceding accumulation of membrane-associated NCAM on the cell surface. This indicates that soluble NCAM contains a secreted component.

Neural cell adhesion molecule; Cell adhesion; Polysialylation; HNK-1 epitope

## 1. INTRODUCTION

Nearly all biochemical analyses on the neural cell adhesion molecule NCAM have been performed on membrane-bound forms of the molecule. However, soluble forms of NCAM have been observed in body fluids [1] and cell culture media [2].

The membrane-associated forms of NCAM consist of three polypeptides: A ($M_r$ ~190000), B ($M_r$ ~135000) and C ($M_r$ ~115000). The NCAM-A and -B polypeptides are integral membrane proteins, whereas -C lacks a transmembrane domain and is held in the membrane by a covalently bound phosphatidylinositol anchor. NCAM-C can be partly recovered in a membrane-free supernatant [3–5]. Membrane-associated NCAM polypeptides are subjected to several posttranslational modifications including polysialylation, which in vertebrates has so far been demonstrated only on NCAM [6]. Approx. 15–20% of membrane-bound NCAM carries a terminal 3-sulphoglucuronyl residue which is involved in adhesion and is recognized by the monoclonal antibodies HNK-1 and L2 [7].

The purpose of this study was to characterize the soluble NCAM forms in brain and determine their relationship with the membrane-associated NCAM polypeptides.

## 2. MATERIALS AND METHODS

Rabbit antibodies to rat NCAM were made as described by Rasmussen et al. [8]. Horse antiserum against *E. coli* polysialic acid (PSA) residues was a gift from Drs F.A. Troy (University of California) and J. Robbins (NIH). Monoclonal mouse antibody against the HNK-1 epitope was obtained from the culture supernatant of HNK-1 hybridoma

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cells purchased from the American Type Culture Collection (Rockville, MD).

Monolayer cultures of fetal rat cerebral neurons were prepared as in [9]. The cultures were maintained for 4–6 days before labelling experiments and immunoprecipitation in gels was performed as described [9].

For radioiodination [10] we used the supernatant of newborn rat brain homogenized in 100 mM potassium phosphate buffer (pH 7.1), 100 U/ml aprotinin, centrifuged at 100000 × *g* for 3 h. For quantitation by enzyme immunoassay (ELISA [11]) and preparation of material for immunosorbent chromatography homogenates were centrifuged at 45000 × *g* for 3 h. Soluble NCAM was isolated by an munosorbent technique using polyclonal rabbit antibodies to NCAM coupled to agarose.

To demonstrate PSA residues, immunoisolated soluble NCAM was incubated with anti-PSA antiserum or horse preimmune serum. After 30 min the fraction of unprecipitated NCAM was determined by rocket immunoelectrophoresis [12]. Similarly, for demonstration of the HNK-1 epitope, the NCAM preparation was incubated with the monoclonal antibody HNK-1 of the IgM class or with IgM antibody from an unrelated hybridoma clone. After incubation, antigen-antibody complexes were precipitated with a rabbit antibody to mouse immunoglobulins, and the fraction of unprecipitated NCAM was measured as above.

## 3. RESULTS AND DISCUSSION

The amount of soluble NCAM in newborn rat brain was determined by ELISA. The total NCAM concentration in solubilized brain homogenates was 950 ± 30 μg per g brain wet wt (means ± SE; $n = 4$). The soluble NCAM concentration was only 7.1 ± 0.1 μg per g brain ($n = 4$), corresponding to 0.8% of total NCAM. Assuming that the volume of the extracellular space is 15–20% of the tissue [13], it can be calculated that the concentration of soluble NCAM here is 35–50 μg/ml which is considerably higher than that in CSF (0.6 μg/ml [1]).

The immunochemical relationship between soluble and membrane-bound forms of NCAM was investigated by comparing conditioned media from neuronal cultures and supernatant from newborn rat brain with detergent extracts of membranes from newborn rat brain. The preparations were compared immunochemically using a polyclonal rabbit antibody raised against membrane NCAM by an immunotitration experiment using ELISA. Dilution curves of all three samples were found to be parallel, indicating immunochemical identity (not shown).

Membrane NCAM from newborn brain is polysialylated and a fraction of NCAM-A and -B carries the HNK-1 epitope [7]. We found that anti-PSA antiserum was able to precipitate ~65% of soluble NCAM from newborn rat brain (fig.1). HNK-1 antibody was able to precipitate ~30% of soluble NCAM (not shown). Thus, both modifications are present on a fraction of soluble NCAM, further substantiating the relationship with the membrane forms of the molecule.

The composition of soluble NCAM in brain was studied by radioiodination of supernatants obtained from homogenates of newborn rat brains. After labelling, NCAM was isolated by immunoprecipitation and submitted to SDS-PAGE (see fig.2, lane 1). For comparison the polypeptide composition of membrane-bound NCAM at the same age is shown in lane 2. It can be seen that NCAM in brain supernatant is composed of a major polypeptide of 180 kDa as well as two minor components of 160 and 145 kDa which all migrate distinctly differently from the membrane-bound forms. Occasionally, a 115 kDa polypeptide migrating as NCAM-C occurred.

A similar pattern was observed in radioiodinated media from cultured fetal rat cerebral neurons (not shown) or in chase media from biosynthetically labelled cells (fig.2, lane 3). No difference in polypeptide pattern was observed between media centrifuged for 3 h at 100000 × *g* or 30 min at 12000 × *g*. To determine whether soluble NCAM was secreted into the extracellular space or derived from the membrane-associated forms, we determined the rate of appearance of soluble NCAM in media from cultured fetal rat brain neurons (fig.3). In three out of four experiments the highest rate of secretion was found 15–30 min after synthesis had begun and only in one experiment was the highest rate of secretion found later (30–45 min after the start of synthesis). Since membrane-associated NCAM has been shown to appear at the cell sur-

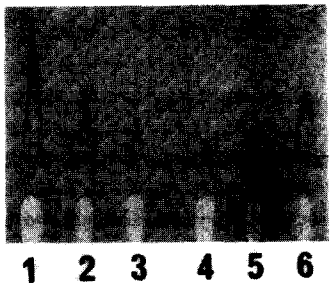


Fig.1. Demonstration of PSA residues in soluble NCAM. Rocket immunoelectrophoresis against polyclonal anti-NCAM of an NCAM standard (32 $\mu$g/ml) in dilutions 1:1, 1:2, 1:4 and 1:8 (wells 1–4) and soluble NCAM with anti-PSA (well 5) or preimmune horse serum (well 6).

face approx. 35 min after synthesis [6] these experiments indicate that at least some of the soluble NCAM polypeptides are secreted rather than derived from the membrane-associated forms. In contrast, similar experiments with another neural cell adhesion molecule, L1 [14], showed a low and constant release of L1 dpm (~150) during the whole experimental period. Soluble L1 is assumed to be derived from membrane L1 by degradation [15].

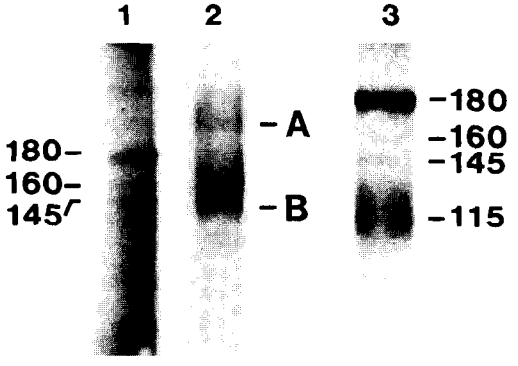


Fig.2. Polypeptide composition of soluble NCAM. NCAM was immunoisolated from radioiodinated supernatants (lane 1) or membranes (lane 2) from newborn rat brain and analysed by SDS-PAGE. (Lane 3) NCAM from chase medium of fetal rat neuronal cultures labelled for 5 h with [$^{35}$S]methionine followed by 19 h chase in the presence of 100 U/ml aprotinin. Molecular masses (in kDa) and the positions of NCAM-A and -B are indicated. Lane 3 was run on a separate gel.

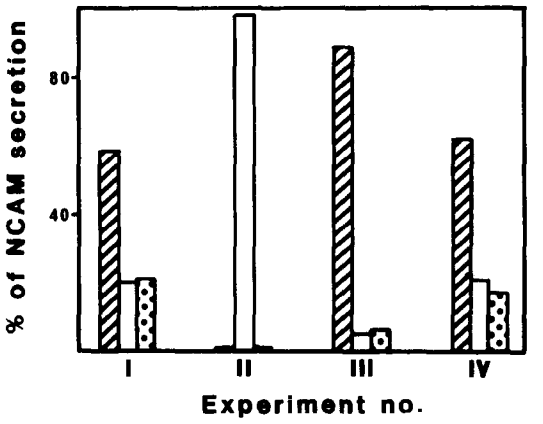


Fig.3. Secretion of NCAM. In four separate experiments a neuronal cell culture was labelled for 15 min with [$^{35}$S]methionine followed by three successive chase periods of 15 min. NCAM was immunoisolated from each of the three chase media and secretion determined setting the total amount of NCAM dpm (~500) isolated from the three chase media to 100%. (Hatched bars) Fraction secreted in the period 15–30 min after the start of synthesis; (unfilled bars) 30–45 min after start of synthesis; (dotted bars) 45–60 min.

The exact relationship between soluble and membrane-bound NCAM remains to be fully elucidated. We find it unlikely that the soluble 180 kDa NCAM is derived from NCAM-A as this polypeptide is a 190 kDa integral membrane protein with a cytoplasmic domain of ~30 kDa [16]. Furthermore, the secretion data suggest that at least some soluble NCAM is present in the media before NCAM appears on the cell surface. We therefore suggest that the 180 kDa form is a new member of the NCAM polypeptide family. Five species of NCAM mRNA have been detected in mouse brain [17]. Three of these give rise to the membrane-associated polypeptides A, B and C, whereas translation products of the remaining two are unknown so far. One of these may code for the 180 kDa polypeptide. The soluble 115 kDa polypeptide, on the other hand, may be derived from NCAM-C. The presence of the HNK-1 epitope on soluble NCAM, however, cannot be attributed to NCAM-C shed from the membrane, as the C polypeptide does not carry this epitope [7].

Further studies on the origin of the different soluble NCAM polypeptides are currently being performed in our laboratory.

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