Intact transmembrane isoforms of the neural cell adhesion molecule are released from the plasma membrane

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Three soluble neural cell adhesion molecule (NCAM) polypeptide classes of M_r values 190 000 (NCAM-s1), 135 000 (NCAM-s2) and 115 000–110 000 (NCAM-s3) have been demonstrated in rat brain and cerebrospinal fluid [Krog, Olsen, Dalseg, Roth and Bock (1992) *J. Neurochem.* **59**, 838–847]. NCAM-s3 is known to arise from released glycosylphosphatidylinositol (GPI)-linked NCAM [He, Finne and Goridis (1987) *J. Cell. Biol.* **105**, 2489–2500] as well as from extracellularly cleaved transmembrane NCAM isoforms [Nybroe, Linnemann and Bock (1989) *J. Neurochem.* **53**, 1372–1378]. In this study the origin of NCAM-s1 and NCAM-s2 and the function of soluble NCAM forms were investigated. It was shown that all three soluble forms could be released from brain membranes with M_r values identical to the three major membrane-associated forms: the large transmembrane 190 000- M_r form (NCAM-A), the smaller transmembrane 135 000- M_r form (NCAM-B) and the GPI-anchored 115 000–110 000- M_r form (NCAM-C). A polyclonal antibody,

directed against transmembrane and cytoplasmic epitopes common to NCAM-A and NCAM-B, was shown to react with NCAM-s1 and NCAM-s2. Furthermore, NCAM-B was shown to be shed in a presumably intact soluble form from membranes of cells transfected with this isoform. Thus, NCAM-s1 and NCAM-s2 probably represent intact released transmembrane NCAM-A and NCAM-B. The soluble transmembrane forms are likely to exist *in vivo*, as NCAM-s1 and NCAM-s2 were readily demonstrated in cerebrospinal fluid. By density-gradient centrifugation it was shown that shed transmembrane NCAM-B was present in fractions of high, as well as low, density, indicating that a fraction of the shed NCAM is associated with minor plasma membrane fragments. Finally, it was shown that isolated soluble NCAM inhibited cell binding to an immobilized NCAM substratum, attributing a pivotal role to soluble NCAM *in vivo* as a modulator of NCAM-mediated cell behaviour.

INTRODUCTION

The neural cell adhesion molecule (NCAM) is a heterogeneous glycoprotein which mediates cell–cell and cell–substratum interactions (for reviews, see Edelman and Crossin, 1991; Schachner, 1992; Krog and Bock, 1992). NCAM is a member of the immunoglobulin superfamily, the product of a single-copy gene, which consists of at least 24 exons (Owens et al., 1987; Small et al., 1987; Santoni et al., 1989; Thompson et al., 1989). Three major polypeptide classes are found in rat brain: the two transmembrane isoforms NCAM-A (M_r 190 000) and NCAM-B (M_r 135 000) (Lyles et al., 1984b), and the glycosylphosphatidylinositol (GPI)-anchored NCAM-C (M_r 115 000–110 000) (He et al., 1986, 1987; Sadoul et al., 1986). Transcription, translation and post-translational modifications of NCAM are regulated in a developmental and cell-specific fashion. NCAM binds in a homophilic manner to NCAM (Rutishauser et al., 1982; Moran and Bock, 1988) and in a heterophilic manner to heparin/heparan sulphate (Cole and Glaser, 1986; Nybroe et al., 1989b), to various collagens (Probstmeier et al., 1989, 1992), and to chondroitin sulphate proteoglycan (Grumet et al., 1993).

Although most studies on NCAM have focused on membrane-associated NCAM forms, it has, however, long been known that soluble forms of NCAM also exist. Soluble NCAM was demonstrated in cerebrospinal fluid (CSF) by Jørgensen and Bock (1975), and in cell culture media from retinal cells, astrocytes and neurons (Rutishauser et al., 1976; Nybroe et al., 1989a) and in amniotic fluid and serum (Ibsen et al., 1983). Furthermore,

NCAM has been demonstrated in the extracellular matrix of striated and smooth muscle and peripheral nerve (Rieger et al., 1985, 1988; Martini and Schachner, 1986; Gulbenkian et al., 1989). Soluble NCAM polypeptide bands of M_r 190 000, 135 000 and a duplet band of 115 000–110 000 have been demonstrated by immunoblotting of supernatant from high speed centrifugation of rat brain homogenate (Krog et al., 1992). Similar bands are observed by immunoisolation of radio-iodinated soluble brain NCAM (Bock et al., 1987; Dalseg et al., 1989) and these NCAM forms have been termed NCAM-s1, NCAM-s2 and NCAM-s3/s4 respectively (Krog et al., 1992; the duplet NCAM-s3/s4 will in the following text be referred to as NCAM-s3). Dalseg et al. (1989) found that soluble NCAM constitutes 1–2 % of the total amount of NCAM in rat brain and calculated the extracellular concentration in rat brain to vary from 25 to 100 $\mu\text{g/ml}$, depending on the developmental stage. All three soluble NCAM forms are also present in CSF and plasma (Krog et al., 1992; Linnemann et al., 1993) at a concentration of approx. 1 $\mu\text{g/ml}$ (Dalseg et al., 1989).

NCAM is not the only cell adhesion molecule that exists in both membrane-bound and soluble form. Intercellular adhesion molecule-1 (ICAM-1) is a cell-surface glycoprotein of M_r 90 000, mediating various cell–cell interactions in the immune system. A soluble counterpart to ICAM-1 of M_r 82 000 is released specifically from the surface of melanoma cells by certain cytokines (Becker et al., 1991; Giavazzi et al., 1992) and inhibits adhesion between cells of the immune system and target cells. Thus, soluble ICAM-1 is likely to play a functional role in the immune

Abbreviations used: CSF, cerebrospinal fluid; GPI, glycosylphosphatidylinositol; ICAM-1, intercellular adhesion molecule-1; NCAM, neural cell adhesion molecule; PI-PLC, phosphatidylinositol-specific phospholipase C; PMSF, phenylmethanesulphonyl fluoride; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum.

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system (Becker et al., 1991). It has been suggested that soluble ICAM-1 could be derived from alternatively spliced messenger RNA or from membrane-associated ICAM-1 (Rothlein et al., 1991; Giavazzi et al., 1992); however, its origin remains to be determined.

The origin of the soluble NCAM polypeptides is only partly elucidated. He et al. (1987) showed that GPI-linked NCAM-C was released into the extracellular milieu from cultured cerebellar cells and glioma cells, and Nybroe et al. (1989a) showed that a 115000- M_r soluble form was derived from NCAM-A and/or NCAM-B of cultured neurons. Thus, NCAM-s3 is a heterogeneous population originating from cleaved transmembrane isoforms as well as released GPI-anchored NCAM-C. NCAM-C may, however, not be the only GPI-linked NCAM isoform giving rise to soluble forms; e.g. in mouse muscle cell lines an NCAM form of M_r 155000, released from the membrane by phosphatidylinositol-specific phospholipase C (PI-PLC), has been reported by Moore et al. (1987). Several observations indicate strong similarity between soluble and membrane-associated NCAM forms: (1) soluble NCAM-s3 has the same N-terminal amino acid sequence as membrane NCAM (Krog et al., 1992); (2) soluble NCAM is polysialylated and a fraction carries the HNK-1 epitope, which is expressed also by a fraction of the transmembrane NCAM isoforms (Bock et al., 1987); (3) soluble NCAM polypeptides co-migrate in SDS/PAGE with membrane NCAM polypeptides (Thiery et al., 1977; Dalseg et al., 1989; Krog et al., 1992; Andersson et al., 1993); (4) a 110000- M_r soluble form can be isolated from crude brain membrane preparations after incubation in physiological buffer (Probstmeier et al., 1989).

The aim of this study was to elucidate the origin and function of soluble NCAM. Our results indicate that intact transmembrane NCAM isoforms are shed from membranes as soluble polypeptides, both with and without attached lipid. Furthermore, soluble NCAM is shown to inhibit binding of NCAM-expressing cells to immobilized NCAM, indicating that soluble NCAM is capable of modulating NCAM-mediated cell behaviour *in vivo*.

MATERIALS AND METHODS

Antibodies

Polyclonal rabbit antibodies raised against human and rat brain NCAM were prepared as described by Ibsen et al. (1983) and Rasmussen et al. (1982) respectively. For immunoblotting experiments rabbit antibodies against rat brain NCAM were affinity purified by retroblotting and characterized as described in Krog et al. (1992). Polyclonal rabbit antibodies were raised against a fusion protein consisting of β -galactosidase and the transmembrane and cytoplasmic domains of NCAM encoded by part of exon 16 and the entire exons 17 and 19. This sequence was prepared from a cDNA encoding the human NCAM-B isoform (Walsh et al., 1989). Briefly NCAM cDNA was cut with restriction enzymes *AatI* and *BamHI*. The fragment obtained by this treatment was subcloned in reading frame into the expression vector pUEX2 (Haymerle et al., 1986). Induction and purification of the fusion protein were performed as described by Doorbar et al. (1985). Rabbits were immunized according to Harboe and Ingild (1983) and the antibodies were affinity-purified: rat brain membranes were mixed with antibodies in 0.5 M NaCl at 4 °C for 25 min. Membranes were then pelleted and washed once in 0.5 M NaCl. Bound antibodies were eluted with 0.1 M sodium citrate buffer, pH 2.6, containing 0.5 M NaCl and neutralized with NaOH. Before use the antibodies were absorbed with rat liver membranes. The antibody was termed anti-NCAMcyt. For immunofluorescence studies a monoclonal antibody, T199

(CD56) (Dakopatts, Denmark), was used. CD56 is identical to human NCAM-B (Lanier et al., 1989). All other antibodies used were obtained from Dakopatts.

Cell cultures

Monolayer cultures of fetal neurons were prepared from embryonic day 15–16 rat forebrain as described by Lyles et al. (1984a). Mixed cultures of neurons and glial cells were prepared according to the same procedure using newborn rat forebrain, and cultured for 10 days in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, U.K.) containing 10 % fetal-calf serum (FCS). The rat glioma cells BT4C_n and BT4C were obtained from O. D. Laerum, Bergen, Norway (Laerum et al., 1977) and were grown in DMEM supplemented with 10 % (v/v) FCS.

Transfection

BT4C_n cells were transfected using the calcium phosphate coprecipitation method (Sambrook et al., 1989) with full-length human NCAM-B cDNA (Walsh et al., 1989) inserted in sense of the eukaryotic expression vector pH β APr-1-neo (Gunning et al., 1987). G418-resistant colonies were isolated by means of cloning cylinders and tested for NCAM expression by immunofluorescence and immunoblotting (see below). Positive cells were subcloned and used for further studies. Three clones termed B2, B6 and B8 were used. Control cells were transfected with the expression vector alone.

Immunofluorescence staining

Cells were grown on coverslips, fixed in 2 % (v/v) paraformaldehyde for 20 min, and incubated with the monoclonal antibody T199 diluted 1:50 in PBS. After washing in PBS, rhodamine-conjugated rabbit anti-(mouse immunoglobulin) antibodies suspended in PBS were added. The specimens were examined with a Leitz Ortholux microscope equipped with epillumination.

Preparation of biological materials

Rat brain

Wistar rat brain was homogenized in a 1:3 (w/v) ratio in PBS/0.1 mM phenylmethanesulphonyl fluoride (PMSF) 100 units/ml aprotinin and centrifuged for 15 min at 18000 g. The resulting supernatant was further centrifuged for 1 or 3 h at 100000 g. The membrane fractions were treated in two different ways: (1) Membrane proteins were extracted by sonication for 2 min in homogenization buffer containing 2 % (v/v) Triton X-100. (2) For the study of protein shedding, membranes were washed by resuspension 1:10 (v/v) in homogenization buffer followed by centrifugation for 15 min at 18000 g. Washings were repeated five times. The washed membranes were then incubated for 2 h at 37 °C in homogenization buffer, and the soluble fraction was obtained by an initial centrifugation for 15 min at 18000 g followed by a centrifugation for 1 or 3 h at 100000 g. In some experiments washed rat brain membranes were frozen at –20 °C (to lyse any membrane vesicles), thawed and washed additionally three times, before incubation for 2 h at 37 °C as described above.

NCAM-B transfected cells

A confluent monolayer of NCAM-B or control transfected cells were harvested in homogenization buffer, washed once and frozen overnight. Membranes were treated as above.

CSF

CSF was collected as described by Dalseg et al. (1989) and in some experiments centrifuged for 1 h at 100 000 *g*.

Enzyme treatment and [³H]ethanolamine labelling

PI-PLC treatment

Washed rat brain membranes corresponding to approx. 20 µg of protein were treated with 0.5 unit of PI-PLC (from *Bacillus cereus*, Boehringer-Mannheim Biochemica, Mannheim, Germany) in Tris/HCl, pH 7.4, in a total volume of 50 µl at 37 °C for 5 h (1 unit hydrolyses 1 µmol of phosphatidylinositol/min under the conditions used). Controls were incubated at 4 °C or 37 °C for 5 h without enzyme. After incubation, membranes and supernatant were separated by centrifugation for 1 h at 100 000 *g*.

Labelling

[³H]Ethanolamine labelling was performed according to He et al. (1987).

SDS/PAGE and immunoblotting

SDS/PAGE was performed on 7.5 % (w/v) polyacrylamide gels according to the method of Laemmli (1970). Separated proteins were electroblotted on poly(vinylidene difluoride) membrane (Millipore Corporation, Bedford, MA, U.S.A.) which had been activated in methanol. Non-specific binding was blocked by incubation with 2 % (v/v) Tween-20 in washing buffer (50 mM Tris/HCl, pH 10.2/150 mM NaCl/0.1 mM PMSF/0.05 % Tween-20). Blots were incubated with rabbit anti-(human NCAM) antibody diluted 1:1000, affinity-purified rabbit anti-(rat brain NCAM) (see above) or rabbit anti-NCAMcyt diluted 1:500. Before use anti-(human NCAM) and anti-NCAMcyt were absorbed with human keratin (Sigma, St. Louis, MO, U.S.A.) to remove any cytokeratin antibodies which occur naturally in many rabbits. Bound primary antibodies were visualized by incubation with alkaline phosphatase-conjugated pig anti-(rabbit immunoglobulin) antibodies followed by a chromogenic enzyme reaction.

Density-gradient centrifugation

The procedure was essentially as described in Krog et al. (1992). Human serum proteins and incubation medium (after centrifugation for 1 h or 3 h at 100 000 *g*) from NCAM-B-transfected cell membranes (see above) were adjusted to a density of 1.005 g/ml and placed on top of a salt solution of 1.21 g/ml. Centrifugation was performed at 200 000 *g* in swinging buckets (Beckman 18-70 ultracentrifuge, SW 60 rotor) at 10 °C for 48 h. Serum proteins were identified and localized by fused rocket immunoelectrophoresis (Harboe and Svendsen, 1983) using rabbit anti-(total human serum proteins) and anti-(human low-density lipoprotein) antibodies. Proteins in the NCAM-containing samples were precipitated using trichloroacetic acid and subjected to immunoblotting using rabbit anti-(human NCAM) antibody as described above.

Cell-binding assay

Microtitre wells (NUNC Immuno Plate Maxisorp) were coated with 0.8 µg of purified adult rat brain membrane NCAM

(Rasmussen et al., 1982) or BSA (Sigma) in 100 µl of water. Water was removed by evaporation overnight at room temperature. The wells were thereafter washed three times with PBS and incubated for 4 h at 4 °C with 0, 0.2, 0.4 or 0.8 µg of purified soluble NCAM in 50 µl of PBS in order to allow the soluble NCAM to bind to the immobilized NCAM. Soluble NCAM was affinity-purified from rat brain supernatant as described by Krog et al. (1992) and consisted mainly of NCAM-s3. The rat glioma line BT4C was harvested with 2 mM EDTA in PBS and brought into single-cell suspension. Cells (10⁵/50 µl of DMEM) were added to each microtitre well, still containing the 50 µl of PBS with various amounts of soluble NCAM, and incubated for 45 min at 37 °C. Unbound cells were removed by three gentle washes with PBS maintained at 4 °C. Attached cells were fixed and stained with 0.2 % (w/v) Fast Green (Sigma) and 0.5 % (w/v) Crystal Violet (Sigma) in 20 % (v/v) methanol. The amount of bound cells was determined by measurement of the absorbance at 600 nm. The median value of the absorbance measured in three wells was used as an estimate of cell number. In control experiments, up to 40 µg of BSA was added per well instead of soluble NCAM. Furthermore, the anti-NCAM antibody column, which was used for purification of soluble NCAM, was loaded with homogenization buffer instead of brain supernatant and eluted. Addition of this 'empty eluate', instead of soluble NCAM, was also used as a control and shown to have no effect.

RESULTS

NCAM release from brain membranes

Membrane NCAM appears by immunoblotting as three major polypeptide classes in brain, NCAM-A, NCAM-B and NCAM-C (Figure 1, lane 1). Soluble NCAM in supernatants from high-speed centrifuged brain homogenate appeared as three major polypeptide bands, NCAM-s1, NCAM-s2 and NCAM-s3 (Figure 1, lane 2), with *M_r* values identical to the three membrane NCAM polypeptide classes. The polypeptide pattern and the amount of soluble NCAM observed in immunoblots were independent of centrifugation time (1 h or 3 h). To examine whether soluble NCAM polypeptides originated from membrane-associated NCAM, washed rat brain membranes were incubated in PBS and NCAM released into the incubation

Figure 1 Immunoblotting of membrane and soluble fractions from brain, and CSF from adult rat

Lane 1, membrane fraction; lane 2, high-speed supernatant from brain homogenate; lane 3, membrane incubation medium prepared by incubation of brain membranes for 2 h at 37 °C in PBS followed by ultracentrifugation; lane 4, CSF. All blots were incubated with polyclonal rabbit antibodies against rat NCAM. A polypeptide band of *M_r* > 250 000 was occasionally observed in the incubation media (see e.g. this figure, lane 3). Bars on the left-hand side indicate the positions of NCAM-A (*M_r* 190 000), NCAM-B (*M_r* 135 000) and NCAM-C (*M_r* 115 000–110 000).

Figure 2 Immunoblotting of PI-PLC-treated rat brain

Lanes 1–4, rat brain membranes. Identical amounts of membrane proteins were incubated for 5 h at 37 °C with or without PI-PLC. Subsequently membranes and supernatant were separated by ultracentrifugation. Untreated rat brain membranes (lane 1) and supernatant (lane 2). PI-PLC treated rat brain membranes (lane 3) and supernatant (lane 4). The blots were incubated with polyclonal rabbit anti-(rat NCAM) antibodies. Bars on the left-hand side indicate the position of NCAM-A (M_r 190 000), NCAM-B (M_r 135 000) and NCAM-C (M_r 115 000–110 000).

Figure 3 Immunoblotting of brain membranes and incubation medium using an antibody against transmembrane and/or cytoplasmic epitopes of NCAM

Immunoblots of brain membranes (lane 1) and incubation medium (lane 2) prepared by incubation of brain membranes for 2 h at 37 °C in PBS followed by ultracentrifugation. The blot was incubated with rabbit anti-NCAMcyt. Bars on the left-hand side indicate the position of NCAM-A (M_r 190 000) and NCAM-B (M_r 135 000).

medium was identified by immunoblotting. All three soluble NCAM polypeptide bands were observed (see Figure 1, lane 3), indicating that they all were released from the membranes. Even after resuspension and incubation of the membranes had been repeated three times, membrane release of NCAM could be demonstrated.

To rule out the possibility that soluble NCAM in the incubation medium simply represented polypeptides trapped in membrane vesicles and released during incubation, washed membranes were frozen, thawed and washed repeatedly. This treatment did not affect the release of soluble NCAM from membranes.

NCAM-s1, NCAM-s2 and NCAM-s3 are also observed in CSF (Figure 1, lane 4), indicating that these polypeptides do not simply arise from homogenization of the brain or unphysiological treatment of the membranes. To determine whether NCAM in CSF originated from contaminating cells or cell debris, CSF from adult rats was centrifuged at 100 000 g for 1 h and compared with untreated CSF. The polypeptide patterns observed were identical and thus independent of whether centrifugation was

performed or not. Thus, NCAM-s1, NCAM-s2 and NCAM-s3 exist in an extracellular soluble form *in vivo*.

GPI-anchorage of NCAM isoforms

Demonstration of release of NCAM-s1 and NCAM-s2 from membranes raised the question whether these isoforms represented released, but not previously identified, high- M_r GPI-linked NCAM forms in brain. However, treatment of brain membranes with PI-PLC only released NCAM-C [Figure 2, compare lanes 3 and 4 (PI-PLC treated) with lanes 1 and 2 (controls)]. Fetal rat neurons express high amounts of 190 000- M_r and 135 000- M_r membrane-associated NCAM forms but not NCAM-C when analysed by immunoblotting (Lyles et al., 1984a; Nybroe et al., 1989a). To examine the presence of high- M_r GPI-anchored NCAM, fetal rat neurons were labelled with [3 H]ethanolamine; however, no labelling was observed, whereas NCAM-C was easily labelled in a mixed culture of neurons and glial cells expressing this isoform (not shown). In conclusion, even though a GPI-anchored form other than NCAM-C is reported in muscle, NCAM-s1 and NCAM-s2 did not seem to be derived from GPI-linked NCAM isoforms in brain.

Demonstration of cytoplasmic and/or transmembrane epitopes in NCAM-s1 and NCAM-s2

Since NCAM-s1 and NCAM-s2 did not represent GPI-linked isoforms, they might represent shed intact transmembrane forms and thus contain cytoplasmic and transmembrane domains. Therefore, a polyclonal antibody, anti-NCAMcyt, raised against a fusion protein containing the transmembrane and cytoplasmic domains present in both NCAM-A and NCAM-B, was prepared. By immunoblotting, it was shown that anti-NCAMcyt reacted as expected with the two transmembrane isoforms but not with the GPI-linked form (Figure 3, lane 1). Incubation media from rat brain membranes and CSF were subsequently subjected to immunoblotting using the anti-NCAMcyt antibody. The antibody reacted with NCAM-s1 and NCAM-s2 in membrane incubation media (Figure 3, lane 2) and in CSF (not shown), indicating that both NCAM-s1 and NCAM-s2 contained transmembrane and cytoplasmic epitopes and thus probably represent shed intact transmembrane NCAM-A and NCAM-B.

Shedding of soluble NCAM from NCAM-B-transfected cells

To substantiate further the contention that intact transmembrane NCAM is shed from membranes, a simple test system was established. A rat glioma cell line BT4C_n, which does not express NCAM (Andersson et al., 1991), was transfected with a full-length cDNA encoding the transmembrane human NCAM-B isoform, and NCAM-B was shown to be expressed on the cell surface by immunofluorescence staining (Figure 4a). No staining of cells transfected with vector alone was observed (Figure 4b).

Cell membranes derived from NCAM-B-expressing BT4C_n cells were incubated in PBS and release of NCAM was tested by immunoblotting. The membrane fraction contained NCAM-B (Figure 5, lane 1) and a polypeptide corresponding in size to NCAM-B was demonstrated in the incubation medium (Figure 5, lane 2). This polypeptide also reacted with anti-NCAMcyt (Figure 5, lane 3). Identical immunoblots were obtained using membranes of three independent transfectant clones. The identical M_r values of soluble NCAM in the incubation media and NCAM-B in the membrane fraction, and the binding of anti-NCAMcyt, indicated that transmembrane NCAM-B was shed from the plasma membrane in an intact soluble form. Ad-

Figure 4 Immunofluorescence staining of transfected glioma cells

Cell-surface immunofluorescence staining for NCAM of BT4C_n-cells transfected with a gene construct encoding human NCAM-B (a, clone B6) or with expression vector alone (b). A monoclonal anti-NCAM antibody, T199 (CD56), was used. Scale bar represents 25 μ m.

ditionally, a polypeptide of M_r approx. 115000 in the medium reacted with anti-NCAM but not with anti-NCAMcyt (Figure 5, lanes 2 and 3). This polypeptide must therefore be derived from the extracellular part of the 135000- M_r form. Using anti-NCAMcyt, an apparently unspecific polypeptide band of slightly higher M_r was observed in incubation media from control transfectants, as well as cells transfected with NCAM-B (Figure 5, lanes 4 and 3).

Association of lipid with soluble transmembrane NCAM

Shed transmembrane NCAM forms behaved as soluble molecules as they could not be pelleted by centrifugation at 100000 g for 3 h. To test whether soluble transmembrane NCAM forms were associated with minor membrane fragments when released from the plasma membrane, soluble NCAM shed from NCAM-B-transfected cells (see Figure 5, lane 2) was subjected to density-gradient ultracentrifugation, in which proteins associated with lipid bilayer fragments will be retrieved in fractions of low density. After ultracentrifugation shed NCAM-B and the 115000- M_r fragment of NCAM-B were detected in high-density, as well as low-density fractions, indicating that some, but not all, soluble NCAM was associated with lipid (Figure 6). The 115000- M_r

Figure 5 Immunoblotting of transfected glioma cells

Immunoblots of membranes (lane 1), incubation media obtained after incubation of membranes from NCAM-B-transfected BT4C_n cells for 2 h at 37 °C in PBS (lanes 2 and 3), and incubation media after incubation of membranes from control-transfected cells for 2 h at 37 °C in PBS (lane 4). All incubation media were ultracentrifuged. Lanes 1 and 2 were incubated with rabbit anti-(human NCAM) antibody. Lanes 3 and 4 were incubated with rabbit anti-NCAMcyt. A band of slightly higher M_r than NCAM-B reacted with anti-NCAMcyt (lane 3) but not with anti-NCAM (lane 2). This band was also found in the membrane incubation media from control cells (lane 4) and therefore presumably represents an unspecific reaction. The bars on the left-hand side indicate the position of NCAM-B (M_r 135000) and NCAM-C (M_r 115000–110000).

Figure 6 Fused rocket immunoelectrophoresis of samples from a density-gradient ultracentrifugation

Membrane incubation medium from NCAM-B-transfected BT4C_n cells (see Figure 5, lane 2) and human serum proteins were adjusted to a density of 1.005 g/ml, placed on top of a 1.21 g/ml salt solution and submitted to ultracentrifugation at 200000 g for 48 h. The positions of serum proteins after centrifugation were localized by immunoelectrophoresis using rabbit antibodies raised against total human serum proteins and human low-density lipoprotein respectively. The positions of low-density lipoprotein (LDL) and albumin (ALB) are indicated. The line illustrates the extent of the salt solution densities from bottom (1.21 = 1.21 g/ml) to top (1.0 = 1.005 g/ml) of the tube. The position of NCAM was determined by immunoblotting and is indicated below the line.

fragment in the low-density fraction may represent NCAM-B cleaved under or after the centrifugation. The distribution and polypeptide composition of NCAM in the density gradient were independent of whether NCAM was centrifuged for 1 h or 3 h at 100000 g before application to the gradient.

Figure 7 Interference of increasing concentrations of soluble NCAM with cell binding to immobilized membrane NCAM

Wells were coated with 0.8 µg of membrane NCAM or BSA (control substratum) per well. After washing, increasing amounts of soluble NCAM (0–0.8 µg in 100 µl) were added. Finally, after 4 h of incubation, BT4C cells were added. Each point represents the ratio between cells binding to NCAM and cells binding to BSA in one experiment. The geometric means of the ratio for cell binding in the presence of 0, 0.2, 0.4 and 0.8 µg of soluble NCAM respectively are connected with a line. The broken line at ratio NCAM/BSA = 1 indicates the situation in which binding to NCAM is identical to binding to BSA.

Effect of soluble NCAM on cell binding to immobilized NCAM

It has been shown previously that the NCAM-expressing cell line BT4C binds to immobilized soluble NCAM (Krog et al., 1992), indicating that soluble NCAM contains the cell-binding domain(s) in a functional conformation. Thus, soluble NCAM presumably is able to interfere with NCAM-mediated cell behaviour. Therefore binding of BT4C cells to wells coated with 0.8 µg of membrane NCAM/well and preincubated with increasing amounts of soluble NCAM in isotonic medium was investigated (Figure 7). Soluble NCAM was not removed before addition of cells, allowing soluble NCAM to bind to both the insolubilized NCAM substratum and NCAM ligands on the cell surface. Addition of increasing amounts of soluble NCAM to control substratum had no effect ($P > 0.1$, paired-signed-rank test on the logarithm of the slope). Binding of cells to insolubilized NCAM decreased significantly when 0.2 µg or more of soluble NCAM was added to the wells ($P < 0.05$, paired-signed-rank test on the logarithm of the slope). Quantities of 0.4 µg or more of soluble NCAM reduced cell binding to the level of binding to BSA ($P = 0.11$ for test of identical binding, paired-signed-rank test on the logarithm of the absorption values). Addition of up to 40 µg of BSA instead of soluble NCAM to the medium had no inhibiting effect.

DISCUSSION

In this study we have presented data indicating that soluble NCAM-s1 and NCAM-s2 represent intact shed transmembrane NCAM polypeptides and that soluble NCAM may be capable of affecting NCAM-mediated cell behaviour *in vivo*.

The soluble NCAM polypeptides, NCAM-s1, NCAM-s2 and NCAM-s3, were demonstrated in rat brain and CSF and co-

migrated with the three major membrane forms in SDS/PAGE. The soluble NCAM polypeptides were shown to be continuously released from brain membranes in suspension. NCAM-s1 and NCAM-s2 represented shed intact transmembrane forms, as judged from the following evidence: (1) a polyclonal antibody against transmembrane and/or cytoplasmic epitopes of NCAM reacted with NCAM-s1 and NCAM-s2, and (2) soluble NCAM with an M_r value identical to NCAM-B was shed from membranes derived from BT4C_n cells transfected with human NCAM-B cDNA. Release of NCAM-B was not a cell-specific phenomenon as it also has been observed from membranes derived from transfected mouse fibroblast L cells (M. Olsen, K. Edvardsen, unpublished work).

The shed transmembrane NCAM isoforms described in this study behaved as soluble molecules since they were found in the supernatant after high-speed centrifugation. However, density-gradient ultracentrifugation indicated that a fraction of the soluble polypeptides were associated with lipid. NCAM may thus be associated with minor membrane fragments attached to the transmembrane domain when shed from the plasma membrane. Presumably the size or composition of the attached membrane fragments do not allow sedimentation during high-speed centrifugation. Non-sedimentable lipid-protein microvesicles have been observed in extracts of beans (Yao et al., 1991) and were shown to be enriched in phospholipid degradation products and therefore differed in lipid composition from the plasma membrane, apparently resulting in the non-sedimentable nature of the vesicles. Whether soluble NCAM without attached lipid is stripped of lipid before or after release from the membrane remains to be determined. Release of proteins containing hydrophobic transmembrane domains has been described previously, e.g. a transmembrane precursor of epidermal growth factor is released from NIH 3T3 cells transfected with cDNA encoding this protein (Mroczkowski et al., 1989), and a fragment of the amyloid precursor protein, containing part of the extracellular domain and the entire transmembrane and cytoplasmic domain, is shed from PC-12 cells (Baskin et al., 1991). Several events may result in shedding of transmembrane proteins *in vivo*, e.g. processes involved in synaptic plasticity, cell death, active cell movement, where proteins adhering to the substratum are left behind, or as a consequence of plasma membrane protein turnover (Black, 1980).

NCAM-s3, which is the quantitatively major soluble polypeptide class, can be generated by cleavage of NCAM-B, but whether it also can be derived from NCAM-A remains to be determined. Furthermore, NCAM-s3 originates from spontaneous or specific release of GPI-linked NCAM-C (Gennarini et al., 1984; He et al., 1986; Sadoul et al., 1986). Spontaneously released NCAM-C may still be attached to the GPI-anchor, as is indicated by the presence in the extracellular matrix of NCAM susceptible to cleavage by PI-PLC (Théveniau et al., 1991). Release of GPI anchored NCAM-C and limited proteolysis of transmembrane NCAM with subsequent generation of NCAM-s3 is likely to be regulated *in vivo* (Ehlers and Riordan, 1991; Théveniau et al., 1992). No specific regulatory mechanism for release of the low amounts of transmembrane NCAM-isoforms can be envisaged at present. Down-regulation of soluble NCAM from the extracellular space may take place via catabolism, or by diffusion into the CSF (Dalseg et al., 1989). Studies on the origin of soluble NCAM in CSF have not been performed; however, extracellular proteins in brain may be transferred to CSF and NCAM-s1, NCAM-s2 and NCAM-s3 from CSF may thus also originate from released intact or cleaved transmembrane forms and from released GPI-linked NCAM.

Earlier studies have shown that NCAM-expressing rat glioma

cells bind to soluble NCAM (Krog et al., 1992), indicating that soluble NCAM contains the cell-binding domain(s) in a functional conformation. In this study we show that purified soluble NCAM (2 µg/ml or more) in isotonic buffer was able to block binding of glioma cells to immobilized NCAM, indicating that soluble NCAM has the capability of affecting NCAM-mediated cell behaviour. An inhibiting effect of soluble NCAM on cell binding *in vivo* will depend on the relative amount of NCAM to other cell-adhesion molecules expressed by the involved cells, and on the local concentrations of NCAM in solution and on the cells. In brain, soluble NCAM constitutes 1–2 % of total NCAM (Dalseg et al., 1989). However, release of soluble NCAM might be enhanced locally in the brain with a concomitant decrease of cell-surface-associated NCAM. Reduced amounts of NCAM on the cell surface is known to decrease NCAM binding non-linearly (Hoffman and Edelman, 1983) and simultaneously increased amounts of extracellular NCAM are likely to enhance this effect.

Binding of soluble NCAM to cell-surface NCAM may also trigger signal transduction to the cell interior. Binding of NCAM antibodies or extracellular NCAM fragments to neuronal cells leads to changes in the level of *myo*-inositol phosphates, intracellular Ca^{2+} and pH (Schuch et al., 1989; Frei et al., 1992). Regulated release of NCAM from the cell surface allows for rapid switching from a restricted, localized action of the membrane form to a regional action of the soluble form, thus making it possible to trigger transmembrane signalling in a larger area of cells. It has also been suggested that soluble NCAM, when bound to the extracellular matrix, may act as an insolubilized chemotactic factor guiding migrating cells or growing axons expressing NCAM and/or NCAM ligands on their surface, in specific directions (Dalseg et al., 1989). Rieger et al. (1988) detected NCAM in the basal lamina of frog muscle. Addition of NCAM antibodies after denervation resulted in abnormal patterns of re-innervation. Both old synaptic sites and extra-synaptic regions were re-innervated, suggesting that selective adhesivity due to NCAM in the basal lamina at synaptic sites may be decreased in the presence of anti-NCAM antibodies. Finally soluble NCAM retards collagen type-I fibril formation and increases the diameter of the fibrils formed, indicating that soluble NCAM is able to influence the configuration of extracellular matrix constituents (Probstmeier et al., 1992).

In conclusion, the present study has elucidated the origin of various soluble NCAM forms, and soluble NCAM was shown to inhibit NCAM-mediated cell binding, suggesting an important role of soluble NCAM in the extracellular space *in vivo*.

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