

Biosynthesis, Membrane Association, and Release of N-CAM-120, a Phosphatidylinositol-linked Form of the Neural Cell Adhesion Molecule

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Abstract. The neural cell adhesion molecule (N-CAM) of rodents comprises three distinct proteins of M_r 180,000, 140,000, and 120,000 (designated N-CAM-180, -140, and -120). They are expressed in different proportions by different tissues and cell types, but the individual contribution of each form to cell adhesion is presently unknown. Previous studies have shown that the two N-CAM species of higher relative molecular mass span the membrane whereas N-CAM-120 lacks a transmembrane domain and can be released from the cell surface by phosphatidylinositol-specific phospholipase C. In this report, we provided evidence that N-CAM-120 contained covalently bound phosphatidylinositol and studied N-CAM-120 from its biosynthesis to its membrane insertion and finally to its release from the cell surface. Evidence was presented showing that the lipid tail of N-CAM-120 contained ethanolamine as is the case for other lipid-linked molecules. The phospholipid anchor was attached to the protein during the first minutes after completion of the polypeptide chain. This process took place in the endoplasmic reticulum as judged

from endoglycosidase H digestion experiments. Immediately after a 2-min pulse with [35 S]methionine, we detected also a short-lived precursor that had not yet acquired the lipid tail. Pulse-chase studies established that N-CAM-120 was transported to the cell surface from which it was slowly released into the extracellular milieu. The molecules recovered in the incubation medium appeared to have lost all of their bound fatty acid but only around half of the ethanolamine. Upon fractionation of brain tissue, ~75% of N-CAM-120 was recovered with a membrane fraction and ~25% in a membrane-free supernatant. A small proportion (~6%) was found to be resistant to extraction by non-ionic detergent. A major posttranslational modification of N-CAM is polysialylation. Our results showed that also N-CAM-120 was polysialylated in the young postnatal brain and released in this form from cultured cerebellar cells. The presence of N-CAM in a form that can be released from the cell surface and accumulates in the extracellular fluid suggests a novel mechanism by which N-CAM-mediated adhesion may be modulated.

THE neural cell adhesion molecule (N-CAM)¹ is a cell surface glycoprotein that serves as a ligand in the formation of cell-cell bonds (see 12, 16, 44 for reviews) and may also be involved in cell-substrate interactions (8). N-CAM is expressed in a variety of structures during embryonic development but it is largely restricted to neurons and glial cells in the normal adult animal (see reference 44). N-CAM-mediated adhesion appears to play a central role in various developmental events including the orderly outgrowth of axons (21, 48, 50), cell pattern formation (6), and nerve-muscle interactions (46). N-CAM exists in several

molecular forms that differ at the carbohydrate and protein level. They are selectively expressed by different cell types and during different stages of development (reviewed in reference 45). The most striking variations in N-CAM glycosylation involve differences in the polymer length of polysialosyl residues. More highly sialylated forms are prevalent in immature tissues and are gradually replaced by less sialylated forms as development proceeds (19, 26). Three protein isoforms of N-CAM have been isolated from rodent tissues. In the mouse, their desialo-forms migrate in SDS-polyacrylamide gels with apparent M_r s of 180,000, 140,000 and 120,000 (25, 26, 31). The corresponding rat proteins migrate with slightly higher relative molecular masses (35). For the sake of clarity, however, we will hereafter call the three N-CAM protein isoforms N-CAM-180, -140, and -120 whether they are of rat or mouse origin. The expression of the different

1. *Abbreviations used in this paper:* endo H, endoglycosidase H; N-CAM, neural cell adhesion molecule; P, postnatal day; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; RIPA buffer, 50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS; VSG, variant surface glycoprotein of *Trypanosoma brucei*.

polypeptides depends on tissue origin and age. For example, N-CAM-180 is produced by neurones but not by muscle cells whereas N-CAM-140 is associated with both cell types (9, 40, 41). N-CAM-120 is expressed by astrocytes and muscle cells (9, 40, 41) and appears later in development than the other forms (24, 35). The analysis of N-CAM cDNA and genomic sequences indicates that these protein isoforms are coded for by mRNAs that are derived from a single gene by alternative splicing (2, 24, 30, 39).

Protein biochemical and cDNA data on rodent and chicken N-CAM have shown that the main difference between N-CAM-180 and -140 is the size of their cytoplasmic domains (13, 22, 23, 39, 41). In contrast to these N-CAM forms, N-CAM-120 lacks a transmembrane domain (2, 23, 30, 41). Until recently its mode of membrane insertion has been unclear. A source of confusion has been that while N-CAM-120 can be recovered in a membrane-free supernatant in soluble form (25) it can also be reconstituted into lipid vesicles (23). The most common mechanism of membrane insertion of cell surface proteins is via a stretch of hydrophobic amino acids. There are exceptions to this rule and some proteins are anchored to the membrane by phosphatidylinositol (PI) and can be released by PI-specific phospholipase C (PI-PLC) (see references 10, 34). The recent demonstration (29, 47) that PI-PLC solubilizes membrane-bound N-CAM-120 suggests but does not prove that this N-CAM species is also held in the membrane by covalently linked phospholipid.

In light of these results we set out to study the peculiar mode of membrane insertion of N-CAM-120 in more detail and to investigate its fate from its biosynthesis to its membrane insertion and finally to its release into the extracellular fluid. The results show that N-CAM-120 contained PI in covalently bound form and that the PI anchor was added during the first minutes after biosynthesis of the polypeptide. During biosynthesis, N-CAM-120 was first transported to the cell surface from which it was slowly released into the extracellular milieu. Upon fractionation of brain homogenates, most of N-CAM-120 was recovered in detergent extracts of membranes. Substantial amounts were found in soluble form and a small fraction was recovered in a nonionic detergent-insoluble compartment.

Materials and Methods

Cells and Antibodies

The C6 rat cell line of glial origin (3) was grown in DME supplemented with 10% FCS. Primary cultures from postnatal day-4 mouse cerebella were prepared as previously described (27); they were used after 2 d *in vitro*.

The preparation and specificity of the two monoclonal antibodies, H28 and P61, and of the rabbit anti-mouse N-CAM serum have been described (25). Whereas H28 reacts with mouse but not with rat N-CAM, P61 and the rabbit serum recognize N-CAM of both species. The mouse anti-rat kappa chain monoclonal antibody produced by a hybridoma (kindly provided by H. Bazin, University of Louvain, Belgium) was purified and coupled to Sepharose CL-4B.

Preparation of Tissue Extracts and Immune Blot Analysis

Mouse forebrains were homogenized in ice in 5 vol of 10% sucrose, 5 mM Hepes, pH 7.8 containing the following protease inhibitors (referred to as protease inhibitor cocktail): 5 mM iodoacetamide, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 U/ml aprotinin, 5 μ M pepstatin, 40 μ M leupeptin, and 5 μ g/ml α 2-macroglobulin (all from Boehringer Mannheim GmbH, Mannheim, FRG). The homogenate was centrifuged at 2,500 g for

20 min to eliminate cell debris, nuclei, and large mitochondria. The supernatant was centrifuged at 140,000 g for 1.5 h to separate the soluble and crude membrane fraction. The membrane fraction was extracted for 10 min with 1% NP-40 in 50 mM Tris-HCl buffer, pH 8.0, containing the protease inhibitors described above. Further centrifugation at 140,000 g for 1.5 h yielded the nonionic detergent-soluble fraction and an insoluble pellet. In some cases, the final pellet was extracted in RIPA buffer (8, 50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS) containing the protease inhibitor cocktail and again separated into a soluble and insoluble fraction.

For immune blotting, soluble fractions were mixed 1:1 with double-concentrated SDS-PAGE sample buffer and boiled for 2 min, pellets were boiled directly in sample buffer. The samples were fractionated on 6.7% gels and immunoreactive bands revealed after transfer to nitrocellulose as described (25) using 125 I-protein A or 125 I-rabbit (anti-rat Ig) antibodies as developing reagents for rabbit and rat antibodies, respectively.

Labeling Procedures and Immunoprecipitation

When pulse labeling with [35 S]methionine was to be done for 20 min, C6 cell monolayers were washed in methionine-free DME and incubated in the same medium containing 10% dialyzed FCS in the presence of 0.5 mCi/ml [35 S]methionine (800 Ci/mmol; Amersham International, Amersham, UK). Then chase medium (DME with 2 mM methionine and 10% FCS) was added and the incubation continued for the time periods specified. The medium was supplemented with the protease inhibitors described above and centrifuged at 12,000 g for 30 min to remove cells and debris. The cells were detached from the dish with 1 mM EDTA in PBS, collected by centrifugation, lysed in 1% NP-40 in 10 mM Tris HCl (pH 8.0) containing 2 mM MgCl₂ and protease inhibitors, and the lysate clarified by centrifugation (20 min in an Airfuge [Beckman Instruments, Inc., Palo Alto, CA] at 20 psi).

For 2-min pulse labeling with [35 S]methionine, the monolayers were deprived of endogenous methionine by incubation for 30 min in methionine-free DME with 10% dialyzed FCS before addition of 1.4 mCi/ml [35 S]methionine. After 2 min, the cell layer was either rinsed with ice-cold chase medium and the cells harvested as described below (0 time point), or the chase was initiated with a brief rinse with prewarmed chase medium and the incubation continued in the same medium. After the time periods specified, the culture dish was put on ice and the cells scraped off into PBS containing 3 mM EDTA and the protease inhibitor cocktail but omitting iodoacetamide and PMSF. The cells were pelleted, taken up in 50 mM Tris HCl (pH 7.5) containing 1 mM MgCl₂, and the protease inhibitor cocktail and disrupted by sonication (three times for 1 s in ice). The cells were lysed in 1% NP-40 (containing 4 mM EDTA to inhibit divalent cation-dependent processes). The lysate was further processed as in the case of the 20-min pulse experiments. Soluble and membrane fractions were prepared by centrifugation through a sucrose cushion. This procedure was adopted since, in the case of a 2-min pulse experiment, we encountered difficulties in cleanly separating soluble from membrane-associated labeled material by simple centrifugation. Accordingly, the sonicated cells (0.3 ml) were layered over 0.13 ml of 0.25 M sucrose containing 50 mM Tris HCl (pH 7.5), 2.5 mM EDTA, 0.2 mg/ml BSA and the protease inhibitor cocktail. After centrifugation (25 psi for 45 min in an Airfuge; Beckman Instruments, Inc.), the overlaid phase was withdrawn (soluble fraction) and the pellet taken up in 50 mM Tris HCl (pH 7.5) containing 1.5 mg/ml BSA and protease inhibitors. The sucrose layer, which contained between 20 and 40% of the TCA-precipitable counts, was discarded. Recovery of TCA-precipitable counts applied to the gradient was ~80%.

The incorporation of fatty acid residues into N-CAM was analysed using [3 H]palmitic acid. Palmitic acid (9,10- 3 H-; 30 Ci/mmol; New England Nuclear, Cambridge, MA) was dried under N₂ and redissolved in a small volume of dimethyl sulfoxide. C6 cell monolayers were washed with DME with 5% FCS and then incubated in the same medium in the presence of 1 mCi/ml of the tritiated fatty acid for 24 h. Then, the medium was removed, the cells scraped off the dish in PBS containing 3 mM EDTA, and lysed in boiling deoxycholate (18) to prevent enzymatic hydrolysis of a labile ester bond. The samples were supplemented with protease inhibitors and clarified by centrifugation (20 min in an Airfuge [Beckman Instruments, Inc.] at 25 psi).

For labeling with [3 H]ethanolamine, C6 cell monolayers were incubated in the presence of 0.18 mCi/ml of ethanolamine (1- 3 H-; 30 Ci/mmol; Amersham International) in DME with 10% dialyzed FCS for 22 h. The cells were processed as described for [3 H]palmitic acid labeling. When labeled N-CAM was to be isolated from the medium, the culture supernatant was first centrifuged at 12,000 g for 30 min to remove cells and debris.

Primary cultures of cerebellar cells were surface iodinated as described elsewhere (27). After iodination, the monolayer was washed three times with PBS containing 2% horse serum, 5 mM KI, and 100 U/ml aprotinin. The cells were then either scraped from the dish and lysed in 1% NP-40 or incubated for 12 h with serum-free DME containing 100 U/ml aprotinin and 0.2 mg/ml BSA. The medium was then harvested, supplemented with 0.5 mM PMSF and 5 μ M pepstatin as additional protease inhibitors, centrifuged for 1.5 h at 140,000 g, and the supernatant dialysed against PBS.

Radiolabeled N-CAM was isolated from cell lysates, culture media, or NP-40 lysates of membranes by immunoprecipitation (23). Rat N-CAM was precipitated by rabbit anti-mouse N-CAM antiserum (5 μ l), mouse N-CAM by the H28 monoclonal antibody (10 μ g). Antigen-antibody complexes were bound to either protein A coupled to Sepharose beads (Pharmacia, Uppsala, Sweden) or to mouse anti-rat kappa chain monoclonal antibody coupled to Sepharose, for rabbit and rat antibodies, respectively. The precipitations with H28 or the polyclonal antibodies were quantitative as judged from the observation that no specific bands were precipitated by the same antibody from supernatants of a first round of immunoprecipitations (results not shown). The immunoprecipitates were washed and dissolved in SDS-PAGE sample buffer as described (52). In this method, the beads are washed consecutively with RIPA buffer and 0.01% SDS to eliminate the risk of precipitating noncovalently associated material. The samples were fractionated on 6.7% gels, and the dried gels exposed to Fuji RX films in the presence of sensitizing screens. For ^3H - and ^{35}S -labeled samples the gels were impregnated with Enhance (New England Nuclear) before drying. Exposure times were 1 d for iodinated, 1 (20-min pulse) to 10 d (2-min pulse) for ^{35}S -labeled, and 2 wk for ^3H -labeled material.

Peptide Mapping

"Soluble" N-CAM-120 was isolated from a sucrose extract of adult mouse brain, "insoluble" N-CAM-120 from a RIPA buffer extract of the NP-40-insoluble pellet by immunoprecipitation with H28 antibodies coupled to Sepharose CL 4B. As control, immunoprecipitation with P61 antibodies was used since P61 does not recognize N-CAM-120 (25). The bound antigen was detached from the beads by incubation in 1% SDS for 20 min at 37°C and the proteins released into the supernatant were iodinated with chloramine T (23). The labeled material was fractionated on a 6.7% gel, the band corresponding to N-CAM-120 excised and subjected to limited proteolysis with *Staphylococcus aureus* V8 protease (Sigma Chemical Co., St. Louis, MO) according to Cleveland et al. (7) with the modifications described (52).

In a different type of experiment, a buffer extract or the material that remained insoluble after three extractions with NP-40 and which contained N-CAM-120 as the sole N-CAM species, were digested for 2 h at 20°C with 0.5 μ g/ml V8 protease. The reaction was stopped by adding 1% mercaptoethanol and the proteins fractionated on a 12% gel. Immunoreactive bands were revealed by immune blotting using rabbit anti-mouse N-CAM antiserum.

Enzymatic Treatments

PI-PLC was purified from *S. aureus* culture supernatants according to Low (33). The enzyme preparation used in the present study was the same that had been used in previous work (29). These enzyme preparations have been shown before to hydrolyze only PI and its derivatives (33) and, as shown in our previous work contain no detectable proteolytic activity (29). Conditions for treatment of cell lysates or culture media were identical to those used previously (29). Release from membrane fractions of pulse-labeled C6 cells was tested as follows. PI-PLC in 50 mM Tris HCl (pH 7.5) was added to the resuspended membrane fraction of C6 cells pulse labeled for 2 min to a final concentration of 25 μ g/ml, controls received buffer only. The samples were incubated for 2 h at 37°C. To facilitate access of the enzyme to material sequestered inside closed membrane vesicles, two cycles of freeze-thawing were done during the incubation followed by readdition of PI-PLC. After incubation, another freeze-thaw cycle was carried out. Then, 2.5 mM EDTA was added and soluble and particulate material separated by centrifugation through a sucrose cushion as described above.

Treatment with endoglycosidase H (endo H) was done on immunoisolated N-CAM. At the end of the wash steps, the protein A-Sepharose beads were taken up in 20 μ l 0.15 M Na citrate (pH 5.5) containing 0.1% SDS, 0.01% β -mercaptoethanol, 2 mM EDTA, 5 μ g BSA, and the protease inhibitor cocktail without iodoacetamide. The samples were then incubated in the presence of 8 μ g/ml endo H (from *Sireptomycetes plicatus*; New England Nuclear) for 4 h at 37°C. Controls were incubated in the absence of enzyme. The reaction was stopped by adding 10 μ l 0.2 M Tris HCl (pH 8) and 30 μ l double concentrated SDS-gel sample buffer.

An endosialidase associated with the bacteriophage PK1A specific for *Escherichia coli* of the capsular type K1 was purified as described (20). This enzyme cleaves polymers of α 2-8-linked *N*-acetylneuraminic acid with a minimum chain length of eight residues, leaving five to seven residues attached to the proximal (reducing end) side. Samples were incubated with 0.4 U/ml of enzyme for 4 h at 37°C in the presence of all protease inhibitors except iodoacetamide. One unit of endosialidase activity is defined as the amount needed to cleave 50 μ g of colominic acid in 24 h at 37°C.

Results

Evidence for Covalent Linkage of Phospholipid to N-CAM-120

We have shown before (29) that unlike N-CAM-180 or -140, N-CAM-120 can be solubilized from C6 cells by PI-PLC indicating that this N-CAM form is anchored in the surface membrane via PI. To provide evidence that N-CAM-120 indeed contains covalently bound PI, we labeled C6 cells with [^3H]palmitic acid and isolated N-CAM by immunoprecipitation. We initially labeled the cells for 6–8 h, but under these conditions label was incorporated only into N-CAM-180 and -140 (results not shown). When we extended the labeling period to 24 h, radioactive N-CAM-120 was precipitated along with N-CAM-180 and -140 (Fig. 1, lane a). To prove that the label in N-CAM-120 was present as PI-linked fatty acid, lysates of labeled cells were incubated in the presence of purified PI-PLC from *S. aureus*. This treatment re-

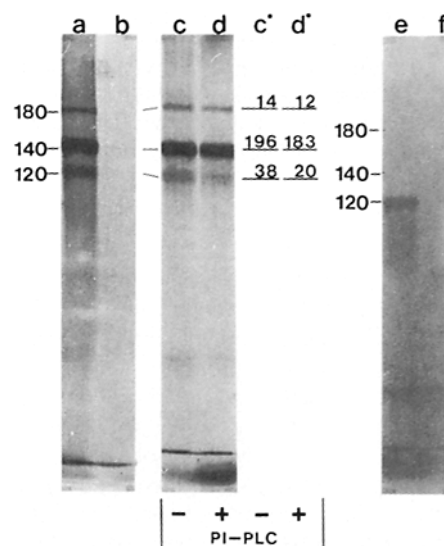


Figure 1. ^3H -Fatty acid and [^3H]ethanolamine labeling of N-CAM-120. C6 cells were labeled with [^3H]palmitic acid for 24 h (lanes a–c), or with [^3H]ethanolamine for 22 h (lanes e and f) and lysed in boiling deoxycholate. The lysates were either processed immediately (lanes a, b, e, and f) or incubated for 30 min at 37°C in the presence (lane d) or absence (lane c) of 50 μ g/ml PI-PLC. Immunoprecipitations were performed using rabbit anti-N-CAM serum (lanes a, c, d, and e) or preimmune serum (lanes b and f). Suitable exposures of lanes c and d were scanned with a densitometer under conditions in which the peak area was a linear function of the exposure time. The values, expressed in arbitrary units, are given in lanes c* and d* for treated and untreated samples, respectively, alongside the corresponding bands. The positions of N-CAM-180, -140, and -120 are indicated at the left. The diffuse band in lane d that migrates ahead of the dye front probably represents the diacylglycerol liberated by PI-PLC.

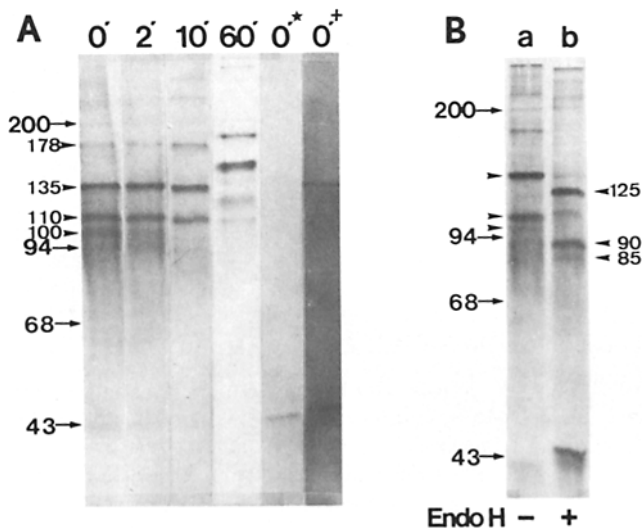


Figure 2. Early kinetics of the processing of pulse-labeled N-CAM. (A) C6 cells were pulsed with [³⁵S]methionine for 2 min and chased for up to 60 min. The cells were harvested immediately after the pulse (time 0) or after 2, 10, and 60 min. Immunoprecipitations were performed using rabbit anti-N-CAM serum (lanes 0', 2', 10', and 60'), and at 0 time also with preimmune serum (lane 0'*) or monoclonal P61 antibody (lane 0'+). A N-CAM-180 precursor band (M_r 178,000) becomes visible in lane 0'+ after a longer exposure time. Arrowheads mark the position of the M_r 178, 135, 110, and 100 $\times 10^3$ precursor bands. The positions of the relative molecular mass standards run in an adjacent lane are designated by the arrows, their relative molecular mass is given in kilodaltons. (B) Cells were harvested immediately after a 3-min pulse and immunoprecipitates prepared using rabbit anti-N-CAM serum incubated in the presence (lane b) or absence (lane a) of 8 μ g/ml endo H. Arrowheads mark the positions of the precursor bands; the relative molecular mass after endo H treatment is given at the right in kilodaltons.

moved about half of the label from N-CAM-120 without affecting the radioactivity in N-CAM-180 and -140 (Fig. 1, lanes c and d). These experiments strongly indicated that N-CAM-120 but not the other two forms contained covalently bound PI. The failure to detect fatty acid incorporation into N-CAM-120 after shorter labeling periods might be explained by the large pool size and/or slow turnover time of PI, whereas N-CAM-180 and -140 appeared to contain the fatty acid in a different type of linkage. Since removal of fatty acid label from N-CAM-120 was not quantitative in these experiments, we could not exclude that N-CAM-120 contained differently linked fatty acid residues in addition to those present in PI. Another possibility was that reutilization of the ³H during the 24-h labeling period has led to incorporation in the form of amino acids.

In the cases where this has been studied (variant surface glycoprotein of *T. brucei* [VSG] [18], Thy-1 [51], acetylcholine esterase, [28] and decay-accelerating factor [36]), the phospholipid anchor has been found to contain ethanolamine, part or all of which is amide linked to the COOH terminus of the protein. As shown in Fig. 1 (lane e), [³H]ethanolamine was readily incorporated into N-CAM-120 but not into the other two N-CAM proteins suggesting that the lipid tails bound to N-CAM-120 and the other proteins are structurally very similar.

Early Intermediates in N-CAM-120 Biosynthesis

Studies of the biosynthesis and processing of VSG have shown that, immediately after its synthesis on membrane-bound polysomes, a stretch of COOH-terminal hydrophobic amino acids is removed and replaced by a complex, PI-containing glycolipid. This process is completed within 1 min after translation (1, 17). A similar mechanism is assumed to take place during biosynthesis of mammalian proteins that contain covalently bound PI (10, 34). However, the early kinetics of their processing have never been described, probably, in part, because of the difficulties in incorporating sufficient amounts of radioactivity during a short pulse. To investigate this point, we pulse-labeled C6 cells with [³⁵S]methionine for 2 min followed by a chase for up to 60 min. Despite the short pulse time, we were able to specifically immunoprecipitate labeled bands with anti-N-CAM serum (Fig. 2 A). At 0 time of chase, three major labeled polypeptides were observed that migrated with M_r s of 135, 110, and 100 $\times 10^3$. Their immunoprecipitation appeared specific since they were absent when preimmune serum was used (Fig. 2 A, lane 0'*). C6 cells synthesized only small amounts of N-CAM-180; the band of weaker intensity of corresponding M_r (178,000) probably represents the precursor of this N-CAM form. A fuzzy band migrated in the M_r 94,000 region. Its relative intensity varied from experiment to experiment and it increased in intensity upon incubation of cell lysates at 37°C. It might thus represent a breakdown product of one of the other chains; however with our present data we cannot exclude the possibility that it represented a biosynthetic intermediate.

The most striking change observed at early times after initiation of the chase was the rapid disappearance of the M_r 100,000 precursor form, possibly as a result of conversion into the M_r 110,000 species. The M_r 100,000 band had already become faint after a chase period of 2 min and had nearly disappeared by 10 min (Fig. 2 A). Subsequently, between 10 and 60 min, the M_r 110 and 135 $\times 10^3$ precursors were apparently processed to the mature N-CAM-120 and -140 proteins (we found values of M_r 147,000 and 123,000 for the mature N-CAM-140 and -120 proteins synthesized by C6 cells). However, bands comigrating with the precursor forms persisted during the 60-min chase period. We had no explanation as to why precursor bands persisted after a short pulse for prolonged periods, whereas they had completely disappeared at comparable times after a longer pulse (compare with Fig. 4). The slight but reproducible increase in mobility of the M_r 110 and 135 $\times 10^3$ bands between 2 and 10 min, we assumed, represented trimming of the glucose and mannose residues from the core oligosaccharide. To obtain further evidence that the M_r 110 and 100 $\times 10^3$ bands were precursor forms of N-CAM-120 and the M_r 135,000 band of N-CAM-140, the monoclonal antibody P61 was used. This antibody recognizes a determinant located in the cytoplasmic domain of N-CAM-180 and -140, which is absent from N-CAM-120 (25). P61 did not precipitate the M_r 110,000 and 100,000 precursors (Fig. 2 A lane 0'+). The polypeptide band of M_r 178,000 became visible after prolonged exposure (not shown).

Newly synthesized N-glycosylated proteins bear only oligosaccharides of the high mannose type that are susceptible to cleavage by endo H (32) (O-linked sugars are believed to be attached during later processing in the Golgi appara-

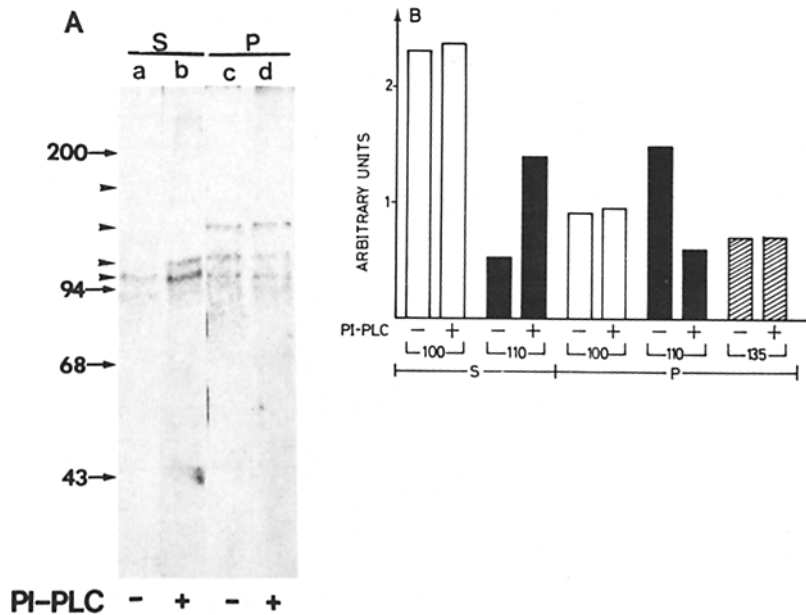


Figure 3. The effect of PI-PLC treatment on N-CAM precursor forms in membranes from pulse-labeled cells. (A) C6 cells pulse-labeled with [35 S]methionine for 2 min were disrupted by sonication and membranes prepared by centrifugation through a sucrose cushion. The membranes were incubated in the presence or absence of PI-PLC for 2 h at 37°C. After the incubation, soluble (lanes a and b) and particulate (lanes c and d) material were again separated by centrifugation through a sucrose cushion. Arrowheads mark the positions of the M_r 178, 135, 110, and 100 $\times 10^3$ precursors; the M_r 178,000 band became only visible after prolonged exposure times. The positions of relative molecular mass standards run in an adjacent lane are designated by the arrows, their relative molecular mass is given in kilodaltons. (B) Suitable exposures of the fluorograph were scanned with a densitometer under conditions in which the peak area was proportional to the exposure time. The crude values measured for the peak areas were divided by the total TCA-precipitable radioactivity recovered in the soluble and particulate fractions to correct for variable losses occurring during separation. The values obtained for the M_r 100, 110, and 135 $\times 10^3$ bands in soluble (S) and particulate (P) fractions are given in arbitrary units. The soluble fraction did not contain a measurable peak for the M_r 135,000 band.

tus). Digestion with endo H of immunisolated N-CAM pulse labeled for 3 min was used to exclude the possibility that the M_r 100,000 precursor might be a glycosylation intermediate. As illustrated in Fig. 2 B, treatment with endo H resulted in large shifts of the M_r 135, 110, and 100 $\times 10^3$ bands. However, the reduction in relative molecular mass was not the same for the three chains. It was smallest for the M_r 135,000 band ($\sim 10,000$), highest for the M_r 110,000 band ($\sim 20,000$), and intermediate for the M_r 100,000 band ($\sim 15,000$). Recent sequence data for mouse N-CAM show the presence of six potential sites for N-linked glycosylation (2). Cleavage of six $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ structures would result in a shift of M_r 10–15 $\times 10^3$ and it is unclear at present what causes the higher relative molecular mass reduction in apparent relative molecular mass of the 110,000 precursor. In any case, it became rather clear that the M_r 100,000 protein was not a glycosylation intermediate of one of the other chains. After endo H treatment, a fainter band appeared that migrated slightly behind the M_r 133,000 species. It might represent incompletely deglycosylated molecules. Together, the data indicated that the M_r 100 and 110 $\times 10^3$ forms were both precursors of N-CAM-120 and that the differences between these two components were not due to differences in glycosylation.

To investigate which of the precursors of N-CAM-120 had already acquired a phospholipid tail, experiments with PI-PLC were performed. Accordingly, a membrane fraction prepared from cells pulse labeled for 2 min was incubated in the presence or absence of *S. aureus* PI-PLC. To facilitate the access of the enzyme to material sequestered inside closed vesicles, two freeze-thaw cycles were performed during the incubation. After the incubation, soluble and

membrane-associated material were again separated by centrifugation through a sucrose cushion. In the absence of enzyme, part of the M_r 100,000 polypeptide, but very little of the other components, was released from the membranes (Fig. 3 A, lanes a and c). PI-PLC treatment increased the amount of the M_r 110,000 polypeptide recovered in the soluble fraction (Fig. 3 A, lanes b and d). To evaluate the distribution of the different forms between soluble and membrane-bound material in a more quantitative way, the fluorographs were scanned by densitometry. The results showed that PI-PLC released substantial amounts of the M_r 110,000 bands from the membranes without affecting the distribution of the M_r 100,000 component (Fig. 3 B). There was no difference in migration between the soluble and membrane-bound M_r 110,000 forms; as has been observed before for the mature N-CAM-120 protein (29) cleavage of the lipid component does not lead to a detectable relative molecular mass shift. As expected, PI-PLC did not solubilize the M_r 135,000 band, which should contain a transmembrane domain. These results thus showed that the M_r 110 but not the 100 $\times 10^3$ component had already acquired the phospholipid tail at the onset of the chase.

Cultured Cells Release N-CAM-120 into the Medium

The attachment of a cell surface protein via a phospholipid anchor may provide a mechanism for the release of plasma membrane proteins into the extracellular milieu by endogenous phospholipases. To detect release of N-CAM-120 from the surface membrane without addition of exogenous PLC, we conducted pulse-chase experiments using [35 S]methionine-labeled C6 cells. Immediately after the 20-min pulse,

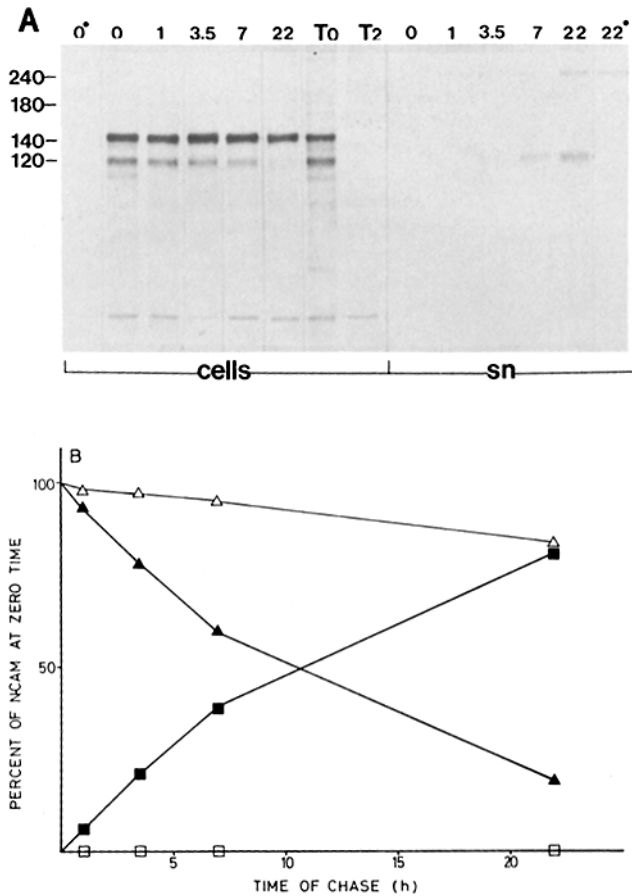


Figure 4. Kinetics of the release of N-CAM-120 from pulse-labeled C6 cells. (A) C6 cells were pulsed for 20 min with [³⁵S]methionine and chased for intervals ranging from 0 to 22 h. The chase media were centrifuged for 30 min at 12,000 g to remove cells and debris, the cells were lysed in NP-40, and the N-CAM forms immunoprecipitated with rabbit anti-N-CAM serum. The immunoprecipitates from cell lysates and chase media are marked *cells* and *sn*, respectively. Control precipitates with preimmune serum are designated by an asterisk (0* and 22*). Lanes T0 and T2 show the results of experiments in which intact cells were treated with trypsin. Labeled cells at 0 time and after 2 h of chase were detached from the culture dish with PBS/EDTA and incubated for 10 min at 37°C in the presence of 1 mg/ml trypsin in DME. The digestion was stopped by adding 4 mg/ml soybean trypsin inhibitor and 0.1 mg/ml PMSF. Cell viability was >98% after this treatment as judged from trypan blue exclusion. The positions of N-CAM-180, -140, and -120 are indicated. A high relative molecular mass contaminant which is also precipitated with preimmune serum is seen in the supernatants. (B) The radioactive bands corresponding to N-CAM-120 and -140 were excised from the gels and counted. The radioactivity in each band was expressed in percent of the radioactivity present at time 0. The solid triangles represent N-CAM-120 recovered with the cells, the solid squares N-CAM-120 recovered in the culture media, and the open triangles N-CAM-140 recovered with cells. There was no radioactivity above background at the position of the N-CAM-140 band in the supernatants (*open squares*).

labeled N-CAM-140 and -120 together with their respective precursors of slightly lower relative molecular mass (135,000 and 110,000) were immunoprecipitated from C6 cell extracts (Fig. 4 A). C6 cells produced relatively little N-CAM-180, and detection of this band required longer exposure times

(not shown). After 1 h of chase, the precursor bands had disappeared, and between 3.5 and 22 h a steady decline of cell-associated N-CAM-120 was observed. There was also some reduction in the amount of radioactive N-CAM-140 recovered from the cells, but this represented only ~15% of the radioactivity initially present (Fig. 4 B) (from this decline a $t_{1/2}$ of ~80 h could be calculated for N-CAM-140, a value that is in the normal range for membrane proteins [38]). By contrast, the amount of cell-bound N-CAM-120 declined by 80%. Over the same time period, labeled N-CAM-120 appeared in the cell-free supernatant, a faint band being first visible at 3.5 h. A high relative molecular mass band was also precipitated from the supernatants. It was precipitated nonspecifically since equivalent amounts were also found in control precipitates. This band might correspond to fibronectin, which has been shown to have affinity for protein A (15).

The regions corresponding to the N-CAM-120 and -140 bands were cut out from the dried gels and counted. The results obtained for N-CAM-120, expressed as percent of N-CAM-120 recovered at 0 time of chase are shown in Fig. 4 B. Labeled N-CAM-120 was lost from the cells with a half-life of ~11 h and appeared with identical kinetics in the supernatant. To confirm that N-CAM-120 was present at the cell surface before being released in the incubation medium, cells chased for 0 or 2 h were slightly trypsinized. Trypsin had little effect on the cellular content of N-CAM at 0 time, but N-CAM-120, as well as N-CAM-140, were completely lost from the cells after 2 h of chase (Fig. 4 A, lanes T0 and T2). These results thus showed that N-CAM-120 was externalized by C6 cells in a trypsin-sensitive location from which it was slowly released into the medium.

We considered the possibility that N-CAM-120 might be lost from the cell surface with the lipid tail still attached to it. To investigate this point and to obtain information on the mechanism of the spontaneous release process, the following experiment was carried out. Of six sister cultures of C6 cells, two were labeled with [³⁵S]methionine, two with [³H]ethanolamine, and two with [³H]palmitic acid. After 22 h, one

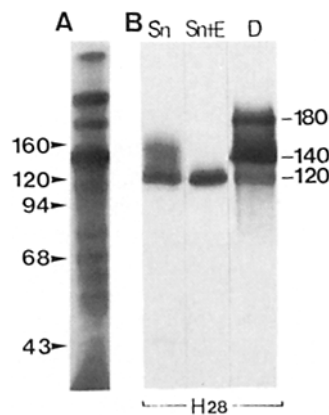


Figure 5. Cultured cerebellar cells release N-CAM-120 into the medium. Monolayer cultures prepared from P4 mouse cerebella were surface iodinated and the cells either lysed directly in NP-40 or incubated for 12 h in fresh medium. N-CAM proteins were isolated from 140,000 g supernatants of cell lysates and media by immunoprecipitation with antibody H28. The precipitates from the culture media were incubated for 4 h at 37°C in the presence or absence of 0.4 U/ml PK1A endosialidase. (A) The pattern of total iodinated proteins in the medium; (B) the immunoprecipitates. *Sn*, untreated immunoprecipitate from the medium supernatant; *Sn+E*, endosialidase-treated immunoprecipitate from the medium supernatant; *D*, immunoprecipitate from the detergent lysate. The positions of the relative molecular mass standards are indicated, their relative molecular mass is given in kilodaltons.

Table I. Release of N-CAM-120 Labeled with Different Precursors from C6 Cells

Label	Cells	Cells	Medium	Δ cells
	0 h	6 h	6 h	
$[^{35}\text{S}]$ Methionine	34.9	25.0	10.4	9.9
	100%	72%	30%	28%
$[^3\text{H}]$ Ethanolamine	34.2	25.7	4.8	8.6
	100%	75%	14%	25%
$[^3\text{H}]$ Palmitic acid	8.3	6.3	<0.3	2.0
	100%	76%	—	24%

Parallel cultures of C6 cells were labeled for 22 h with either 0.18 mCi/ml $[^{35}\text{S}]$ methionine, or with 0.18 mCi/ml $[^3\text{H}]$ ethanolamine, or with 0.6 mCi/ml $[^3\text{H}]$ palmitic acid. Then, the culture dishes were washed with complete medium with 10% FCS and incubated for 6 h in chase medium which consisted of DME with 10% FCS, to which had been added 130 $\mu\text{g}/\text{ml}$ cycloheximide (a concentration sufficient to reduce protein synthesis by 98%) and 5 $\mu\text{g}/\text{ml}$ α_2 -macroglobulin and 10 U/ml aprotinin as protease inhibitors. After the chase period, the cells were detached in PBS-EDTA and lysed immediately in boiling 1% deoxycholate. The medium was centrifuged for 30 min at 12,000 g to remove cells and debris. N-CAM proteins were isolated from cells and the medium supernatant by immunoprecipitation and fractionated by SDS-PAGE. Suitably exposed (i.e., in the linear range of the film response) autoradiographs were scanned with a densitometer, the results are expressed as arbitrary units. The zero time point (0 h) was done on sister cultures that were processed after the 22-h labeling period. Δ cells, difference between the radioactivity associated with cell-bound N-CAM-120 at zero time and after the 6-h chase. No detectable decrease in $[^{35}\text{S}]$ methionine or $[^3\text{H}]$ palmitic acid recovered with cell-bound N-CAM-140 was observed during the chase period.

of each pair of cultures was harvested. Cold chase medium was added to the remaining three and the incubation continued for another 6 h. N-CAM proteins were then isolated and the radioactivity associated with N-CAM-120 determined by scanning suitable exposures of the gels by densitometry. As shown in Table I, $\sim 30\%$ of $[^{35}\text{S}]$ methionine-labeled N-CAM-120 was lost from the cell surface during the chase period and equivalent amounts were recovered in the medium. Over the same time period, there was a 25% reduction in the amount of ^3H -fatty acid recovered with the cell-bound N-CAM-120, but no radioactivity could be detected in the released protein. There was also a $\sim 25\%$ reduction in $[^3\text{H}]$ ethanolamine-labeled cell-bound N-CAM-120, but surprisingly, only around half of it was recovered with the molecules released into the medium. It was thus clear that the N-CAM-120 recovered in the extracellular milieu had lost the lipid part of its COOH-terminal anchor, but additional hypotheses were needed to explain the partial loss of ethanolamine.

To test whether primary cultures of brain cells would also release N-CAM-120, surface-iodinated cultures of young postnatal mouse cerebella were used. After labeling, the cells were incubated for 12 h and the N-CAM proteins released into the medium isolated by immunoprecipitation (Fig. 5 B). The material recovered from the medium consisted of M_r 120,000 band plus some diffuse material of slower mobility. In contrast to C6 cells, these cultures might thus synthesize polysialylated N-CAM. Indeed, treatment with endosialidase (20) removed the diffuse zone leaving a well defined band that co-migrated with N-CAM-120. Clearly, cerebellar cells spontaneously released N-CAM-120, part of which was in polysialylated form, into the extracellular milieu. Since these cells do not proliferate, they incorporate low amounts of $[^{35}\text{S}]$ methionine and precise pulse-chase kinetics could not be established as in the case of C6 cells.

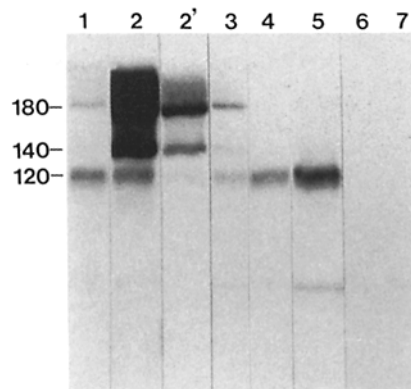


Figure 6. Sequential extraction of N-CAM-120 from mouse brain. A P21 mouse brain homogenate was separated into a soluble and a membrane fraction. The membranes were extracted twice with 1% NP-40 and once with RIPA buffer and the insoluble final pellet with either 10 mM Tris-HCl, pH 8.0, or with 1% NP-40. All extracts were centrifuged for 1.5 h at 140,000 g . N-CAM proteins were revealed by immune blotting using H28 antibody. Around 200 μg protein were applied to each lane. Lane 1, soluble fraction; lane 2, first NP-40 extract; lane 2', same as lane 2, but less protein was applied to reveal the bands; lane 3, second NP-40 extract; lane 4, RIPA buffer extract; lane 5, the pellet after RIPA buffer extraction; lane 6, the material released from the RIPA buffer-insoluble pellet with 10 mM Tris-HCl; lane 7, the material released from the RIPA buffer-insoluble pellet with 1% NP-40. The positions of N-CAM-180, -140, and -120 are indicated.

Solubility Characteristics of N-CAM-120 in Mouse Brain

Previous studies have shown that N-CAM-120 from rodent brain is partially recovered in a buffer extract in soluble form (25). When a brain homogenate from postnatal day-21 (P21) mouse brain was fractionated into a buffer-soluble, a NP-40-soluble and a NP-40-insoluble fraction, we again found a N-CAM-120 band in the buffer extract (Fig. 6, lane 1). The immunoreactive material of higher relative molecular mass might represent highly sialylated N-CAM which is still found at this age (19, 31). Surprisingly, however, N-CAM-120, but not the higher relative molecular mass chains, was still present in the material that remained insoluble, and only part of it could be extracted by a SDS-containing detergent mixture (Fig. 6, lanes 4 and 5). Equal amounts of protein were loaded on each lane to evaluate the relative enrichment of N-CAM-120 in the different fractions. Further treatment of the pellet with buffer or NP-40 did not release any immunoreactive material (Fig. 6, lanes 6 and 7). In Fig. 8, a quantitative estimation of the relative amounts of the N-CAM-120 forms with different solubilities is shown. This estimation was done by cutting the corresponding regions out of the nitrocellulose paper for counting. Control experiments with purified N-CAM established that within the range measured, the amount of radioactive second antibody bound was proportional to the amount of N-CAM present (not shown). In the adult mouse brain, $\sim 25\%$ of the total N-CAM recovered was found in the soluble fraction, slightly $>70\%$ in the NP-40 extract, and $<6\%$ in the insoluble pellet.

Conceivably, soluble and insoluble N-CAM-120 could represent quite distinct polypeptides. To approach this ques-

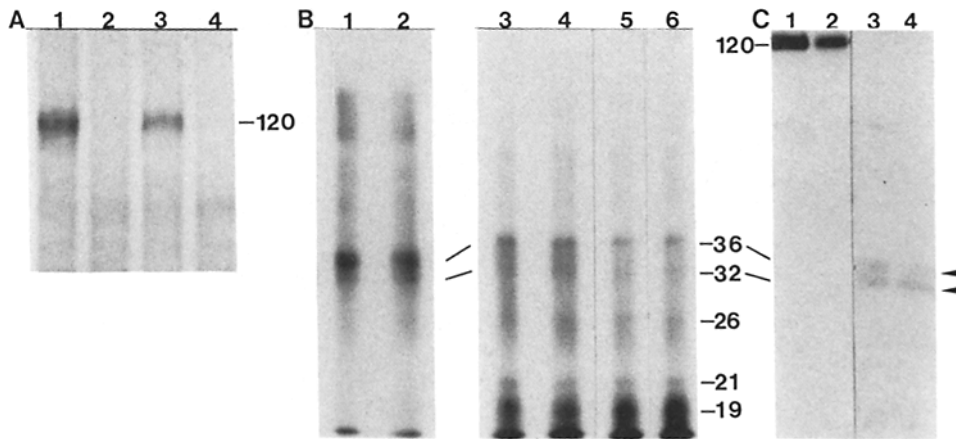


Figure 7. Comparative peptide mapping of soluble and insoluble N-CAM-120. (A) N-CAM proteins were immunoprecipitated with antibody H28 from a HEPES-buffered sucrose extract of adult mouse brain (lane 1) or from a RIPA buffer extract of the NP-40-insoluble material (lane 3) and radioiodinated. Precipitation with antibody P61 served as control (lanes 2 and 4), no labeled band was detected in the N-CAM-120 region. (B) The bands corresponding to N-CAM-120 were cut out from the gel shown in A and subjected to limited proteoly-

sis. Lanes 1, 3, and 5, soluble N-CAM-120; lanes 2, 4, and 6, insoluble N-CAM-120; lanes 1 and 2, 1 ng *S. aureus* V8 protease; lanes 3 and 4, 100 ng; lanes 5 and 6, 300 ng. (C) A HEPES-buffered sucrose extract and the material that remained insoluble after three NP-40 extraction was treated with 0.5 μ g/ml *S. aureus* V8 protease and the immunoreactive material revealed by immune blotting using rabbit anti-N-CAM serum. Lanes 1 and 3, soluble N-CAM-120; lanes 2 and 4, insoluble N-CAM-120; lanes 1 and 2, control without protease; lanes 3 and 4, digested samples. The arrowheads indicate the position of the immunoreactive bands in the protease-treated material.

tion, the peptide maps of the two types of molecules generated by limited proteolysis were compared. Buffer-soluble and NP-40-insoluble N-CAM-120 were immunoprecipitated, iodinated, and repurified by gel electrophoresis. Both molecular forms yielded identical patterns of iodinated peptides (Fig. 7 B). In a different type of experiment, soluble and insoluble N-CAM-120 were slightly digested with *S. aureus* V8 protease and the fragments revealed by immune blotting (Fig. 7 C). Again, fragments of identical sizes were generated.

To test whether some of the N-CAM-120 recovered with brain membranes was loosely bound and thus constituted a potential source of the form recovered in the buffer extracts, a microsomal pellet was resuspended in different media followed by high speed centrifugation. Of the various treatments tried, i.e., 3 M NaCl, pH 11, mercaptoethanol, and urea plus mercaptoethanol, only the latter released N-CAM-120 into the supernatant (results not shown). However, in the presence of urea N-CAM-180 and -140 were also recovered in the supernatant. In fact, only a very small amount of membrane protein was pelleted in 8 M urea; apparently small membrane vesicles were generated, which, due to the high density of the urea solution, were prevented from sedimenting. Clearly, the N-CAM-120 recovered in a membrane fraction could not be released by treatments that solubilized peripheral membrane proteins.

Forms of N-CAM-120 in the Developing Mouse Brain

In the embryonic mouse brain, N-CAM is present in a polysialylated form that migrates as a diffuse zone in which individual proteins cannot be resolved (26, 31). Distinct N-CAM-180, -140, and -120 bands gradually appear during postnatal development, but the diffusely migrating material is present up to P21. To reveal the underlying polypeptides we used an endosialidase, which rapidly and quantitatively cleaves the sialic acid polymers (20). As shown in Fig. 8 A for a P15 brain, which contains a mixture of highly and less sialylated N-CAM, treatment with this enzyme led to the disappearance of the diffusely migrating zone at the top of the

gel and enhanced the N-CAM-180, -140, and -120 bands to approximately the same extent. Incubation in the absence of enzyme had no effect. This experiment thus showed that N-CAM-120 was polysialylated in the brain. It was not surprising that polysialylated N-CAM-120 migrated above the N-CAM-180 band since previous results showed that a M_r 45,000 polysialylated N-CAM fragment was migrating with an apparent M_r of 170,000 (23).

To determine the timing of appearance and the relative proportions of buffer-soluble, NP-40-soluble, and NP-40-insoluble N-CAM-120, the corresponding fractions were prepared from mouse brains of different ages, endosialidase treated, and the N-CAM proteins were revealed by immune blotting (Fig. 8 B). We made two quantitative analyses of the data. The change in total N-CAM-120 concentration was estimated by adding the radioactivity bound by the N-CAM-120 band in each fraction (*solid triangles* in Fig. 8 C). There was a fourfold increase in total N-CAM-120 concentration in the brain between P8 and adult ages. To evaluate the relative amounts of each N-CAM-120 form of different solubility, the crude counts were corrected for differences in the volumes of the three fractions and expressed as percent of total N-CAM-120 (*open bars* in Fig. 8 C). A higher percentage of soluble N-CAM was found in the embryo, but also in the adult, as compared with young postnatal ages. Overall, it became clear that from P15 onwards substantial amounts of the different N-CAM forms were recovered in an apparently buffer-soluble form. Regardless of age, the bulk of N-CAM-120 was recovered in the NP-40-soluble form.

Discussion

In our initial report, we showed that PI-PLC solubilizes a substantial fraction of N-CAM-120 from brain microsomes and quantitatively releases N-CAM-120 present at the surface of C6 cells without affecting the other two N-CAM species (29). This data and similar results obtained by another laboratory (47) indicate that N-CAM-120 is a member of the group of membrane proteins that are anchored to the

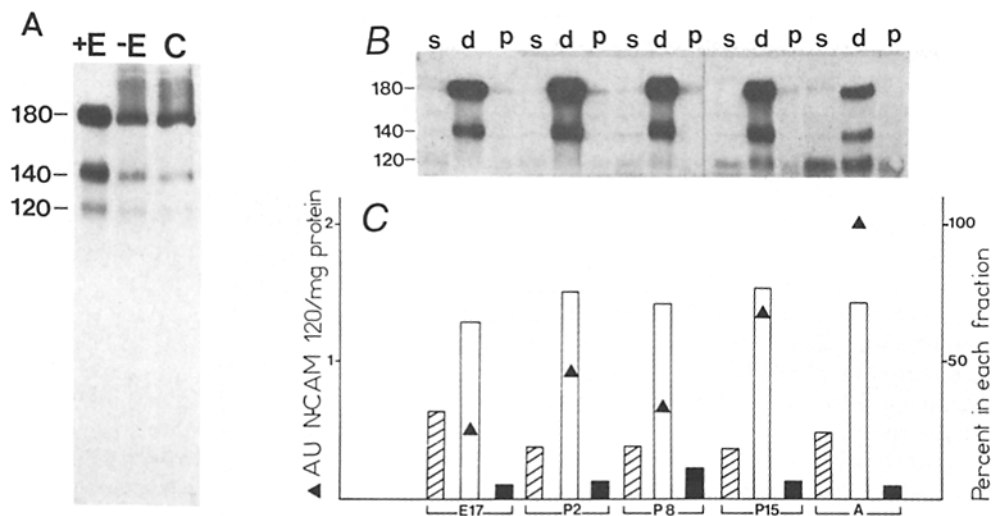


Figure 8. Developmental changes in N-CAM-120 expression in mouse brain and quantification of its distribution between different fractions. (A) The effect of endosialidase treatment on a NP-40 extract from a P15 forebrain. (Lane C) Control sample, boiled immediately in SDS-PAGE sample buffer; (lane +E) sample incubated in the presence, (lane -E) in the absence of PK1A endosialidase. N-CAM bands were revealed with H28 monoclonal antibody. (B) Immune blot analysis of N-CAM proteins in different fractions during development. Forebrains from

embryonic day-17 (E17), P2, P8, P15, and adult mice were homogenized in HEPES-buffered sucrose and separated into a soluble fraction (s), a NP-40 extract (d), and a NP-40-insoluble pellet (p). The different fractions were treated with endosialidase and the proteins separated by SDS-PAGE. Total volumes were 4.5 ml for the HEPES/sucrose extract, 13 ml for the NP-40 extract, and 3.4 ml for the insoluble material after addition of SDS-PAGE sample buffer. The blot was developed with H28 monoclonal antibody. (C) The quantifications of N-CAM-120 obtained by cutting out and counting the regions corresponding to the N-CAM-120 band in B. The values are expressed as percent of N-CAM-120 recovered at each age. These values are shown in the histograms (▨ soluble, □ NP-40, ■ insoluble fraction). The sum of the counts in the three fractions was then divided by the sum of the amounts of protein charged on the gel; these values are expressed in arbitrary units (AU) and allow assessment of the changes in N-CAM-120 concentration during development (▲).

membrane by PI. This group includes alkaline phosphatase, 5'-nucleotidase, acetylcholine esterase, Thy-1, T cell-activating protein, decay accelerating factor, and VSG (14, 18, 34, 36, 43, 51). N-CAM is already known to have several modes of posttranslational modifications, namely N-linked glycosylation, polysialylation, phosphorylation, and sulfation (11, 19, 25, 35, 37, 41, 49). The presence of PI in one N-CAM form not only adds yet another mode of posttranslational modification to this list but also raises the possibility that this N-CAM isoform may be selectively released from cell surfaces. In the present study we provide evidence that N-CAM-120 indeed contains covalently bound PI and that it is spontaneously released into the extracellular milieu. We further show that the PI anchor is added very early during biosynthesis and have investigated the solubility characteristics of this N-CAM species in brain tissue.

When C6 cells are labeled with [³H]palmitic acid, label is incorporated into all three N-CAM proteins. PI-PLC treatment removes the label only from N-CAM-120 demonstrating that in this N-CAM isoform the fatty acid, at least in part, is present in covalently bound phospholipid. Although detailed structural information is not yet available for N-CAM-120, it seems likely in analogy with rat Thy-1 (51) and *T. brucei* VSG (18) that the linkage between the protein and PI involves an ethanolamine- and glucosamine-containing glycan. This conclusion is supported by our observation that N-CAM-120 can be labeled with [³H]ethanolamine. Since neither N-CAM-180 nor -140 incorporate this precursor, these experiments rule out the possibility that the two larger N-CAM isoforms bear a similar kind of structure.

Short-time pulse-labeling experiments have been done to investigate the early kinetics of NCAM synthesis. Three major labeled polypeptides are seen after a 2-min pulse with

[³⁵S]methionine. Two of them (M_r 135 and 110×10^3) are relatively long lived and are assumed to correspond to the core-glycosylated precursors of N-CAM-140 and -120, respectively. The M_r 110,000 precursor form has already acquired the PI tail at the onset of the chase since it can be released from membranes by PI-PLC. VSG is initially synthesized with a hydrophobic COOH-terminal peptide which is subsequently cleaved and replaced by glycopospholipid within 1–2 min after completion of the polypeptide (1, 5, 17). A comparison of cDNA with protein sequences of the Thy-1 antigen suggests that a similar mechanism may take place in mammalian cells (51). Recent cDNA data for chicken (30) and mouse (2) N-CAM indicate that N-CAM-120 may also initially be synthesized with a COOH-terminal stretch of hydrophobic amino acids. It appears from our studies that addition of the phospholipid is also a very rapid step in mammalian cells. The enzymes involved would presumably reside close to the site of protein synthesis in the endoplasmic reticulum. This assumption is supported by our finding that the M_r 110,000 precursor, which is the first detectable form with a PI anchor, also bears oligosaccharides that are susceptible to endo H digestion. The specificity of this enzyme for high mannose oligosaccharides has been well established and susceptibility to endo H has been taken as indication that the protein has not yet reached the Golgi apparatus (32). It has already been shown in previous studies that mature rat and mouse N-CAM are resistant to endo H (31, 35).

In contrast to the studies on the VSG system (1, 17), we detect in our biosynthetic labeling experiments a short-lived intermediate of lower apparent relative molecular mass (100,000) which is not affected by PI-PLC treatment and hence, does not appear to bear a phospholipid anchor. This chain may correspond to one or the other of the two hypo-

thetical precursors that have not yet acquired the phospholipid tail: the primary translation product still possessing the hydrophobic amino acid extension, and the form from which this segment has been cleaved. The solubility characteristics of the M_r 100,000 species are compatible with both possibilities. It is recovered in a membrane fraction, but is spontaneously released upon incubation. It could be argued that the COOH-terminal segment of uncharged amino acids may interact more loosely with the membrane than a protein with a transmembrane domain and is thus released during the incubation. On the other hand, the precursor from which this segment has been cleaved may remain associated with the membrane by virtue of its interaction with a membrane-bound enzyme complex involved in its conversion. Consideration of relative molecular mass may be taken as argument that the M_r 100,000 form is the primary translation product; it migrates after endo H digestion with an apparent M_r of 85,000, a value close to the M_r of 79,000 predicted by the nucleotide sequence (2). The endo H digestion also shows that the key difference between the M_r 100 and 110×10^3 species is not due to N-linked oligosaccharides. The relative molecular mass difference of 5,000 that remains after endo H (plus $\sim 2,500$ if the M_r 100,000 chain should not possess the COOH-terminal peptide segment) may be due to the presence of sugars in the phospholipid. Antisera recognizing the COOH-terminal peptide or the putative oligosaccharide moiety of the lipid tail will be important tools for distinguishing these possibilities. Unfortunately, antisera recognizing the glycan-PI complex of VSG (anti-CRD) did not react with N-CAM-120 (He, H.-T., and C. Bordier, and He, H.-T., and G. Rougon, unpublished results).

As shown in the present study, a substantial fraction of brain N-CAM-120 is recovered in high speed supernatants after mechanical disruption of the tissue. Given the fact that N-CAM-120 is attached to the membrane by a phospholipid anchor, the soluble N-CAM-120 found in buffer extracts may be derived from the plasma membrane, perhaps by cleavage of the PI tail by endogenous phospholipases. To investigate this point further, we studied the sensitivity of N-CAM-120 to trypsin treatment of intact cells and the kinetics of its release in C6 cultures. The results establish that N-CAM-120 is present at the cell surface before it accumulates to a significant extent in the medium and show that the decline in cell-bound N-CAM-120 is exactly counterbalanced by the amounts that appear in the medium. The release is a rather slow process with a $t_{1/2}$ of 11 h. Spontaneous release of N-CAM-120 from the plasma membrane is not a special feature of C6 cells, since surface-iodinated cerebellar cells also release labeled N-CAM-120 (but neither N-CAM-140 nor -180) into the medium.

To gain further insight into the mechanism of the spontaneous release process we compared the release of N-CAM-120 labeled with either [35 S]methionine, 3 H-palmitic acid, or [3 H]ethanolamine. Whatever the precursor compound, 25–30% of labeled N-CAM-120 was lost from the cell surface over the time period studied. However, whereas recovery of the [35 S]methionine-labeled molecules in the medium was quantitative, [3 H]palmitic acid was not detectable in the released protein, and only half of the [3 H]ethanolamine label was recovered in the supernatant. These data suggest several possible mechanisms for the release. One is that most of the shedding from the cell surface is in fact due to proteo-

lytic attack. However, such a mechanism would require that N-CAM-120 is more sensitive to the release by proteases than the other two forms, and does not explain why around half of the ethanolamine is retained by soluble N-CAM-120. A more likely explanation is that an initial cleavage step within the lipid anchor, perhaps by a phospholipase which releases it from the cells, renders N-CAM-120 susceptible to proteolytic attack. Finally, a more far-fetched possibility is that the glycolipid tail of N-CAM-120 contains two ethanolamine residues, as has been shown for Thy-1 (51) and that the lipid part of it is removed by an enzyme, e.g., an endoglycosidase or a phosphodiesterase, in such a way that one of the two ethanolamine residues would remain attached to the protein. A precise chemical analysis of the COOH-terminal structure of soluble N-CAM-120 will be required to distinguish between these possibilities.

Upon fractionation of mouse brain homogenates N-CAM-120, but neither N-CAM-180 nor N-CAM-140, was found in a compartment which was insoluble in nonionic detergent. Quantification of the data shows that this insoluble form represents only 6% of total N-CAM-120, whereas 25% are recovered in soluble form, and 70% in detergent extracts of the membranes. We envisage that this insoluble N-CAM-120 is derived from extracellularly deposited N-CAM-120 which has become resistant to solubilization by detergent. This possibility is supported by the finding that detergent-insoluble N-CAM-120 is not found in C6 cell extracts and that a fraction of the N-CAM-120 released into the medium is refractory to detergent extraction (unpublished results). In brain tissue, the bulk of N-CAM-120 is present in a membrane-bound, nonionic detergent-soluble form. It cannot be released from the membranes by treatments known to solubilize peripheral membrane proteins, and, in contrast to Nybroe et al. (41), we find no evidence for a mature form of N-CAM-120 with the characteristics of a peripheral membrane protein. In our earlier study (29) we have found that PI-PLC solubilizes a maximum of 50% of N-CAM-120 from brain microsomes (whereas the release from the surface of C6 cells was quantitative). This result is to be expected if one assumes that 50% of the microsomal vesicles are inside out. It is thus likely that all N-CAM-120 recovered with brain membranes and soluble in detergent is held in the membrane by a lipid tail.

A striking posttranslational modification of N-CAM is polysialylation. Previous studies have shown that N-CAM-180 and -140 are polysialylated (19, 24). Our endosialidase digestion experiments show that N-CAM-120 is polysialylated at young postnatal ages and that it is released from cerebellar cells in that form. In essential agreement with previous studies (41), we observe an approximate fourfold increase in the concentration of N-CAM-120 during postnatal brain development. Over the same developmental time course, striking changes in its distribution between soluble, detergent-soluble, and -insoluble fractions are not revealed.

Based on these results we can propose the following chronology for N-CAM-120 synthesis and release. The first event in the processing is probably the co-translational addition of N-linked sugars. Attachment of the phospholipid anchor occurs immediately after synthesis of the polypeptide, when the protein is still in the endoplasmic reticulum. N-CAM-120 is then transported to an intracellular site where processing of the N-linked oligosaccharides occurs, including polysialy-

lation at early postnatal ages. Thereafter, it is transported to the cell surface from which it can be slowly released into the extracellular fluid, probably by cleavage of the lipid tail.

The biological significance of phospholipid-mediated membrane insertion is unknown at present. Possibly N-CAM-120 may serve a dual role both as cell-surface molecules and as extracellular proteins. Since phosphoinositide-specific phospholipases are known to be activated during cell stimulation (4), circumstances may exist in which release of N-CAM-120 from neural cell surfaces may be specifically triggered. The interest in extracellular N-CAM is heightened by the recent findings (8) that N-CAM is present in adherons, i.e., macromolecular complexes that are shed from cultured cells and mediate cell-extracellular matrix interactions. Another possibility is that the phospholipid tail is involved in transmembrane signaling. The cleavage of PI by PI-PLC liberates 1,2 diacylglycerol, a molecule of known second messenger function (see reference 4), and at least two lymphocyte surface proteins that are anchored in the membrane by phospholipid have been implicated in cellular activation (42, 43).

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