Biosynthesis of the Neural Cell Adhesion Molecule: Characterization of Polypeptide C

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ABSTRACT The biosynthesis of the neural cell adhesion molecule (N-CAM) was studied in primary cultures of rat cerebral glial cells, cerebellar granule neurons, and skeletal muscle cells. The three cell types produced different N-CAM polypeptide patterns. Glial cells synthesized a 135,000 M_r polypeptide B and a 115,000 M_r polypeptide C, whereas neurons expressed a 200,000 M_r polypeptide A as well as polypeptide B. Skeletal muscle cells produced polypeptide B. The polypeptides synthesized by the three cell types were immunochemically identical. The membrane association of polypeptide C was investigated with methods that distinguish peripheral and integral membrane proteins. Polypeptide C was found to be a peripheral membrane protein, whereas polypeptides A and B were integral membrane proteins with cytoplasmic domains of ~50,000 and ~25,000 M_r, respectively. The affinity of the membrane binding of polypeptide C increased during postnatal development. The posttranslational modifications of polypeptide C were investigated in glial cell cultures, and it was found to be N-linked glycosylated and sulfated.

The neural cell adhesion molecule (N-CAM)[1] is a cell surface glycoprotein that is involved in cell-cell adhesion (1). N-CAM has been shown to be identical to two molecules called the D2-cell adhesion molecule (D2-CAM; references 2–4) and brain surface protein-2 (BSP-2; reference 5). In postnatal tissues N-CAM has been demonstrated on three cell types: neurons, glial cells, and skeletal muscle cells (6, 7). The joining together of axons to form fascicles and the apposition of neurons to glial cells during early development suggest that neuron-neuron and neuron-glia interactions are important in defining nerve tracts. The addition of N-CAM antibodies to cultures of spinal ganglia disrupt the side to side adherence of growing neurites during fasciculation (2, 8, 9). In addition, antibodies to N-CAM interfere with the in vivo guidance of optic nerve axons by neuroepithelial endfeet (10). Likewise, the initial establishment of a physical contact between neurites and muscles depends on N-CAM-mediated adhesive interactions (11, 12).

N-CAM from postnatal rat brain consists of three distinct polypeptides: 190,000–200,000 M_r (polypeptide A), 135,000–140,000 M_r (polypeptide B), and 115,000–120,000 M_r (polypeptide C; 13). (Polypeptides A–C will be referred to by letter.) Biosynthetic studies have shown, that the N-CAM polypeptides are differentially expressed in different experimental systems. In cultured fetal rat brain neuronal cells A and B are synthesized (3, 14) whereas astrocytes in culture synthesize B and C (4). In studies of cell-free translation of membrane-bound polysomes from whole rat brain in a rabbit reticulocyte lysate system, the A-B-C triplet is observed (13). No interconversion between A, B, and C is seen during biosynthesis (3, 13, 14). These results and data obtained by recombinant DNA techniques (15, 16) suggests that there are at least three mRNAs coding for N-CAM.

Synthesis studies using neuronal cell cultures have shown that A and B undergo extensive co- and posttranslational modifications (3, 14). During postnatal development the pattern of N-CAM synthesis changes from an embryonic to an adult one. This conversion involves changes in the posttranslational modifications of the polypeptides as well as a change in the pattern of polypeptides synthesized (3, 13). The relative proportion of A and B polypeptides synthesized decreases, whereas that of C increases. These changes could reflect changes in the proportions of different cell types in the brain

[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; N-CAM, neural cell adhesion molecule; P4, P25, and P40, postnatal day 4, 25, and 40, respectively; TID, 3-(trifluoromethyl)-3-(m-iodophenyl) diazarine.

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or could be due to modulation of N-CAM expression within one cell type. Since the adhesiveness of N-CAM increases with age (17), the function of N-CAM in cell-cell adhesion may well be modulated by developmental changes in its synthesis.

The A and B polypeptides have been well characterized in fetal neuronal cultures. Less is known about the modulations of C, and the nature of its membrane association has so far not been conclusively determined (18). Therefore, the aim of the present work was to examine the biosynthesis and membrane association of C. We compared the N-CAM polypeptides synthesized in primary cultures of postnatal cerebellar granule neurons, cerebral astrocytes, and skeletal muscle cells. C was predominantly a glial product. C was found to be a peripheral membrane protein, whereas A and B were integral membrane proteins with large cytoplasmic domains. The membrane association of C increased during postnatal development, and C was found to be sulfated and N-linked glycosylated.

MATERIALS AND METHODS

Materials: Micrococcal nuclease (EC 3.1.31.1), creatine phosphokinase (EC 2.7.3.2), and creatine phosphate were obtained from Boehringer Mannheim (Federal Republic of Germany). ^{125}I-3-(trifluoromethyl)-3-(m[^{125}I]iodophenyl) diazarine (TID, 10 Ci/mmol), ^{14}C-labeled molecular weight markers, and RNasin were obtained from Amersham International (Amersham, U.K.). [^{125}I]NaI (carrier-free), [^{35}S]methionine (>800 Ci/mmol), and [^{35}S]sulfate (>350 Ci/mmol) were obtained from New England Nuclear (Braunschweig, FRG). Cell culture flasks were from NUNC (Roskilde, Denmark). Dulbecco's modified Eagle's medium (DME) and methionine-free DME were from Gibco Laboratories (Grand Island, NY) or Flow Laboratories, Inc. (McLean, VA). Horse serum and sulfate-free basal medium Eagle's were from Gibco Laboratories. Chloramine-T, glycine, ethylene-diamino-tetra acetate (EDTA), hydrogen peroxide, and 2,5 diphenyloxazole were obtained from Merck (Darmstadt, FRG). Dimethylsulfoxide was from Fluka (FRG). Aqualuma was purchased from Lumac Systems (Switzerland). Aprotinin (Trasylol) was from Bayer (FRG.), and agarose HSA was obtained from Litex (Denmark). Peroxidase-conjugated antibodies and antibodies to fibronectin were from DAKOPATTS (Copenhagen). X-Omat (LX-1) film was from Eastman-Kodak Co. (Rochester, NY). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Cell Cultures: Cell cultures were grown in 5% CO_2 humidified atmosphere at 37°C in DME containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Astrocyte cultures were prepared from newborn rat brain by mechanical dissociation as described by Chapman and Rumsby (19), with the following modifications: the growth medium contained 10% vol/vol horse serum instead of fetal calf serum, and media were changed twice a week. Cells from three brain hemispheres were seeded into each 25-cm^2 tissue culture flask. Glial cultures were labeled after from 2 to 4 wk in vitro.

Cultures of skeletal muscle cells were prepared from newborn rat thigh muscle. Cells were dissociated with trypsin, using 0.125% of trypsin in Ca^{2+}-free Krebs buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NAHCO_3$, and 13 mM glucose) for 1 h at 37°C. The tissue was triturated and cell clumps were allowed to settle. Cells from the supernatant were pelleted, resuspended in DME containing 10% horse serum, and seeded in gelatine-coated tissue culture flasks. The medium was changed after 24 h and subsequently twice a week. Muscle cultures were labeled after from 4 to 8 d in vitro.

Cultures of cerebellar granule neurons were prepared from 8-d-old rats as described by Wilkins et al. (20) with the following modifications: EDTA treatment and centrifugation through 4% bovine serum albumin (BSA) was omitted, and 10% horse serum was used instead of fetal calf serum. The medium was changed after 24 and 48 h, and subsequently twice a week. Cytosine arabinoside (0.05 mM) was present during the second day in vitro. Neuronal cultures were labeled after from 2 to 10 d in vitro.

The cellular composition of the cultures was investigated by light microscopy and immunocytochemical staining. Immunocytochemistry was performed as previously described (21). Granule neurones in the neuronal cultures were identified on the basis of cellular morphology as being small, round, phase-bright cells that extended long, thin processes. Contaminating astrocytes were identified by their positive reaction with a monoclonal antibody, anti-GFAP-3, which is specific for glial fibrillary acidic protein (21). The number of astrocytes in neuronal cultures amounted to at most 3% of the number of neurons. Fibroblasts were identified by staining with an antiserum against fibronectin. Fibroblast contamination in the neuronal cultures was found to be negligible. In muscle cultures, the fusion of myoblasts to form myotubes was evident after 3 d in vitro and almost completed 3 d later. Spontaneous contraction of myotubes could be observed as early as day four in vitro, with contractile activity becoming intense around day six. A low number of fibronectin-positive cells were present in muscle cultures. Approximately 95% of the cells in astrocyte cultures were labeled with monoclonal antibody GFAP-3, the remainder being fibronectin positive.

Biosynthetic Labelings of Cell Cultures: When labeling with [^{35}S]methionine was performed, cell cultures were preincubated with methionine-free DME for up to 30 min before [^{35}S]methionine was added. Labeling with [^{35}S]sulfate was performed similarly except that sulfate-free basal medium was the Eagle's medium used. Cultures labeled in the presence of tunicamycin were preincubated with 20 μg/ml of tunicamycin for 2 h before the addition of [^{35}S]methionine. Tunicamycin was present at 20 μg/ml during the labeling period. To avoid proteolytic breakdown of N-CAM, the protease inhibitor aprotinin was employed at 100 U/ml during labeling.

The cells were harvested in ice-cold phosphate-buffered saline containing 100 U/ml aprotinin and 0.4 mM phenylmethylsulfonyl fluoride, and solubilized in Tris-barbital buffer, pH 8.6 (70 mM with respect to Tris) containing 4% Triton X-100, 100 U/ml aprotinin, and 0.4 mM phenylmethylsulfonyl fluoride.

Preparation of Microsomes: Rough microsomes were prepared from brains of 4-d-old Wistar rats as described by Featherstone and Boime (22). The tissue was homogenized and centrifuged at 12,000 *g* for 10 min to remove nuclei and cell debris. The resulting supernatant was applied onto a 38% wt/vol sucrose cushion, and rough microsomes were pelleted by centrifugation at 100,000 *g* for 4 h.

In Vitro Translation: Rabbit reticulocyte lysates were prepared as described by Jackson and Hunt (23). Lysates were treated with micrococcal nuclease essentially according to Pelham and Jackson (24). Translations were performed as described previously (13). Assay mixtures were incubated for 60–120 min at 30°C. Each assay contained 100–150 μCi [^{35}S]methionine.

Carbonate Treatment of Microsomes: Microsomal membranes were isolated from translation assays by centrifugation at 10,000 *g* for 15 min. Pellets were resuspended in 100 mM sodium carbonate, pH 11.0, and incubated at 4°C for 30 min (25). The incubation mixtures were then neutralized by the addition of 1 M potassium phosphate buffer, pH 7.1. Membranes were pelleted by centrifugation for 1 h at 10,000 *g*, and N-CAM was immunoisolated from both the membrane fraction and the supernatant.

Posttranslational Proteolysis of Rat Brain Microsomes: After translation of microsomes in vitro for 60 min at 30°C, translation mixtures were incubated for 5 min at 20°C in the presence of 1.6 mM tetracaine to stabilize microsomal membranes. Assays were then adjusted to 10 mM calcium chloride. Trypsin was added to 25 μg/ml, and enzymatic digestion was carried out for 30 min at 4°C. The reaction was terminated by the addition of aprotinin (300 U/ml) and phenylmethylsulfonyl fluoride (2 mM). Some digestions were performed in the presence of 4% Triton X-100. After termination of the digestions the incubation mixtures were immediately submitted to immunoisolation.

^{125}I-Labeling by the Chloramine-T Procedure: Brains of rats aged 4, 25, or 40 d were homogenized 1:10 (wt/vol) in 116 mM phosphate buffer, pH 7.1, containing aprotinin (100 U/ml). Membranes were obtained by centrifuging the homogenate at 10,000 *g* for 15 min. In some cases, the pellet was washed twice in Tris-barbital buffer, 70 mM with respect to Tris, pH 8.6. Iodination by the Chloramine-T procedure was performed according to Greenwood et al. (26).

Labeling with ^{125}I-TID: Washed membranes were prepared as described above. Aliquots corresponding to 25 mg original tissue weight were suspended in 1 ml of 50 mM sodium phosphate buffer, pH 7.6. Membranes were iodinated by photoactivation for 30 s in a 1-ml cuvette with constant stirring using 50 μCi ^{125}I-TID per ml membrane suspension. The light source was a mercury lamp focused by a quartz lens. Samples were quenched by the addition of BSA (1% wt/vol) and cooling on ice (see reference 3).

Immunoisolation of N-CAM: Radiolabeled N-CAM was immunoisolated by crossed immunoelectrophoresis as described by Hansen et al. (13). The N-CAM content of cell-free translation assays, glial cell cultures, and muscle cell cultures was too low to produce visible immunoprecipitates. Therefore, solubilized rat brain membranes containing unlabeled N-CAM were used as carrier. Visible immunoprecipitates were excised from the agarose gels and submitted to SDS PAGE.

SDS PAGE: Immunoprecipitates containing labeled N-CAM were analyzed by SDS PAGE essentially according to Schäfer-Nielsen and Rose (27). Immunoprecipitates were boiled in SDS PAGE sample buffer containing 5% β-mercaptoethanol and electrophoresed on 4–20% gradient gels. The ^{14}C-

labeled molecular weight markers employed were myosin (200,000), phosphorylase b (92,500), BSA (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,300). Gels were either developed for autoradiography on X-Omat film at −70°C for 1–3 d, or prepared for fluorography as described by Bonner and Laskey (28) and exposed to X-Omat film at −70°C for 1–2 wk.

Enzyme-linked Immunosorbent Assay of N-CAM: Cultures were harvested and solubilized as described above, and N-CAM was quantified by an enzyme-linked immunosorbent assay (29). The enzyme immune assay employed was an inhibition assay in which solubilized rat brain membranes are attached to a polystyrene microtiter tray. Extracts of the cell cultures to be measured were mixed with a specific rabbit anti-rat N-CAM antibody, and this mixture was incubated with the sensitized solid phase. After washing was done, enzyme-labeled anti-rabbit immunoglobulin was added, and the immunosorbent assay was developed by the addition of enzyme substrate. The titration of neuronal, glial, and muscle cell extracts was performed using twofold serial dilutions.

RESULTS

N-CAM Polypeptide Composition in Neuronal, Glial, and Muscle Cells in Culture

When radioiodinated N-CAM was immunoisolated from postnatal rat brain and analyzed by SDS PAGE and autoradiography it was found to contain three distinct polypeptides, A, B, and C, of approximate M_r 190,000 (A), 140,000 (B), and 120,000 (C), (Fig. 1, lane *1*).

Cultures of cerebellar granule neurons obtained from 8-d-old rats produced two N-CAM polypeptides of M_r 200,000 (A) and 135,000 (B; Fig. 1, lane *2*). The A-B pattern was consistently obtained when labeling was performed after both 2 and 10 d in vitro. The N-CAM content of similar unlabeled cultures was determined by an immunosorbent assay and found to be 18 ± 2 μg/mg total protein (mean ± SD, n = 5).

Astrocytes in culture were found to produce mainly the B and C polypeptides of M_r of 135,000 and 115,000, respectively (Fig. 1, lane *3*). A very faint A band could occasionally be observed. Again the polypeptide pattern was unaffected by the in vitro age of the cultures during the period from 2 to 28 d. Glial cultures contained 0.8 ± 0.2 μg N-CAM/mg total protein (mean ± SD, n = 4), which is ~20 times less than found in the neuronal cultures.

Finally, N-CAM isolated from muscle cell cultures appeared as one predominant polypeptide of 135,000 M_r (B; Fig. 1, lane *4*). Trace amounts of A and C appeared inconsistently. The amount of N-CAM in five muscle cell cultures was determined to be 2.4 ± 0.3 μg/mg total protein.

In cell cultures the M_r's of the N-CAM polypeptides differ by ~5 kD from the M_r's of the polypeptides isolated from whole brain. This is probably due to differences in glycosylation.

The immunochemical relationship between the N-CAM polypeptides produced by the three cell types was investigated by an immunotitration experiment. In the immunosorbent assay employed, titration curves of N-CAM from all three cultures were found to be parallel (Fig. 2), indicating that neuronal, glial, and muscle N-CAM were immunochemically identical.

Membrane Insertion of Polypeptide C

Although the A and B polypeptides have been shown to be integral membrane proteins (3), the membrane association of the C polypeptide has not been conclusively determined (18). We have therefore determined the nature of the membrane association of C by techniques that distinguish integral and peripheral membrane proteins.

To study the membrane association of newly synthesized N-CAM polypeptides rat brain rough microsomes were translated in vitro in a rabbit reticulocyte lysate system. After translation, microsomal membranes were treated with carbonate buffer, pH 11.0, which opens the microsomal vesicles and converts them into membrane sheets. Proteins originally secreted into the microsomal lumen are thus released into the medium and, simultaneously, peripheral membrane proteins are detached from the membranes (25). After carbonate treatment membranes were isolated by centrifugation and N-CAM was immunoisolated from both the membrane and the supernatant fractions.

In control assays microsomes were translated but not treated with carbonate. A, B, and C could be immunoisolated from the whole microsomes (Fig. 3*a*, lane *1*). After carbonate treatment only A and B were found in the membrane fraction (Fig. 3*a*, lane *2*), whereas C was found exclusively in the supernatant (Fig. 3*a*, lane *3*). This demonstrates that C is not an integral membrane protein but a peripheral membrane protein or a secreted protein.

The membrane association of the mature N-CAM polypep-

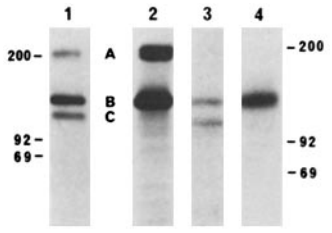

FIGURE 1 ^{125}I-labeled N-CAM from rat brain and biosynthetical labeling of cerebellar granule neurons, cerebral glial cells, and skeletal muscle cells with [^{35}S]methionine. Radiolabeled N-CAM was immunoisolated and analyzed by SDS PAGE and autoradiography or fluorography. Lane *1*, ^{125}I-labeled N-CAM from adult rat brain. Lane *2*, [^{35}S]methionine-labeled cerebellar granule neurons, pulse 60 min, chase 60 min. Lane *3*, [^{35}S]methionine-labeled cerebral glial cells, pulse 30 min, chase 30 min. Lane *4*, [^{35}S]methionine-labeled skeletal muscle cells, pulse 60 min, chase 60 min. The three N-CAM polypeptides, *A*, *B*, and *C*, are marked. The positions of standard proteins are indicated in the margin.

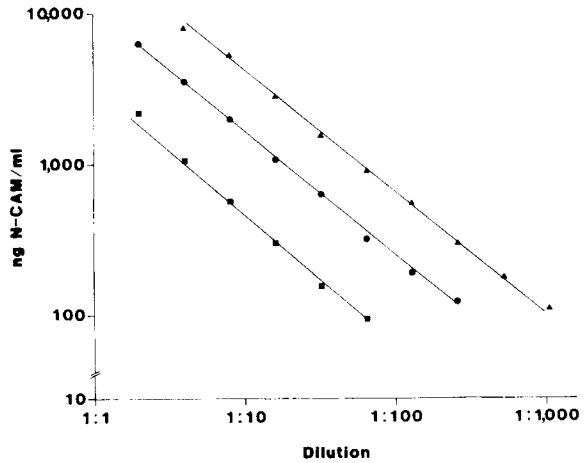

FIGURE 2 Enzyme-linked immunosorbent assay titration curve of N-CAM in cerebellar granule neurons (▲), skeletal muscle cells (●), and cerebral glial cells (■). The data represent mean values of from four to six determinations. See Materials and Methods for experimental details.

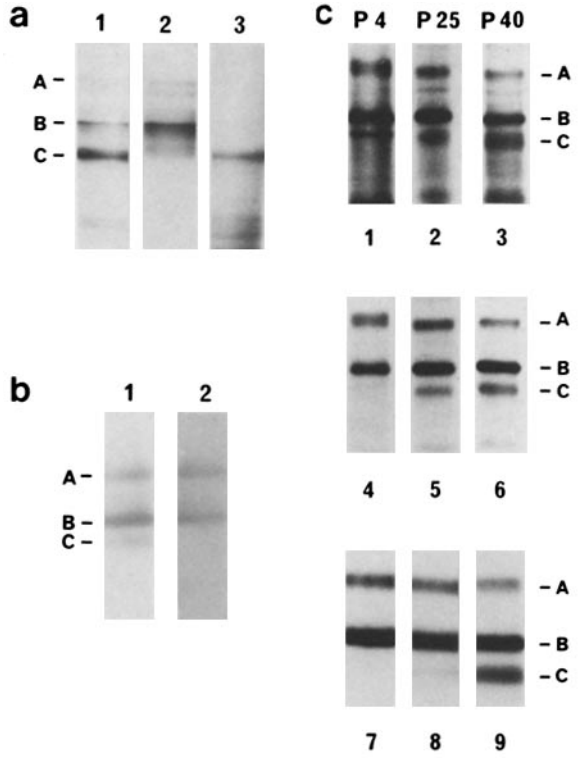

FIGURE 3 Membrane association of N-CAM polypeptides. (*a*) [^{35}S] methionine-labeled N-CAM synthesized in vitro. Lane *1*, translation of microsomes. Lane *2*, translation of microsomes; after translation microsomes were treated with carbonate buffer, and N-CAM was isolated from the membrane fraction. Lane *3*, translation of microsomes; after translation microsomes were treated with carbonate buffer, and N-CAM was isolated from the supernatant fraction. (*b*) Labeling of postnatal day 25 rat brain membranes with ^{125}I (lane *1*) or ^{125}I-TID (lane *2*) (*c*) ^{125}I-labeled N-CAM polypeptides. Total homogenates, membranes and pH 8.6 buffer-washed membranes from postnatal day 4 (*P4*), postnatal day 25 (*P25*), and postnatal day 40 (*P40*) rat brain were radioiodinated. ^{125}I-labeled N-CAM was immunoisolated and analyzed by SDS PAGE and autoradiography. Lanes *1–3*, P4, P25, and P40 total homogenates in phosphate buffer, pH 7.1. Lanes *4–6*, P4, P25, and P40 membranes in phosphate buffer, pH 7.1. Lanes *7–9*, P4, P25, and P40 pH 8.6 buffer-washed membranes. N-CAM polypeptides are indicated.

tides was studied by radioiodination of rat brain membranes. When membranes from adult rat brain were labeled with ^{125}I the A-B-C triplet was observed (Fig. 3*b*, lane *1*). When the same membrane preparation was labeled by the hydrophobic labeling reagent ^{125}I-TID, only A and B were identified (Fig. 3*b*, lane *2*). This further demonstrates that the mature A and B polypeptides are integral membrane proteins. Since C was not labeled by ^{125}I-TID, it is apparently not inserted into the lipid bilayer. However as C was labeled by Chloramine-T iodination of rat brain membranes it appears to be more loosely associated to the membrane as a peripheral membrane protein.

Age-dependent Changes in the Membrane Association of C

The possibility that C exists in a soluble form, in addition to the membrane associated form, was investigated. Total homogenates and membranes from postnatal day 4 (P4), day 25 (P25), and day 40 (P40) were radioiodinated. Some membrane aliquots were washed in Tris-barbital buffer, pH 8.6, before iodination (see Materials and Methods).

N-CAM isolated from total homogenates exhibited the A-B-C pattern at all ages investigated (Fig. 3*c*, lanes *1–3*). When membranes were separated by centrifugation, N-CAM from P4 membranes showed only the A-B pattern, whereas P25 and P40 membranes showed an A-B-C pattern (Fig. 3*c*, lanes *4–6*). This suggests that the membrane association of C became stronger between day P4 and day P25. Washing membranes with Tris-barbital buffer, pH 8.6, removed C from the membranes at P4 and P25, but not at P40 (Fig. 3*c*, lanes *7–9*). Thus, C is firmly attached to membranes at the adult stage (P40). That washing of membranes with 6 M urea could not detach C at this age (not shown) indicates a very strong membrane attachment of C.

To exclude the possibility that factors in the homogenate could convert A and B into a C-like component, control experiments were performed in which a radioiodinated P4 membrane preparation was incubated with an unlabeled P4 crude homogenate. This did not change the radioiodinated A-B pattern observed, which illustrates that no factors in the P4 homogenate produce a C-like component from A or B under the conditions employed.

Cytoplasmic Domains of N-CAM

Microsomes were treated with trypsin immediately after cell-free translation. In this procedure cytoplasmic domains are degraded, whereas domains destined to become extracellular are protected inside the microsomes (30). Fig. 4 shows that the M_r of C was not affected by trypsin digestion (compare lanes *1* and *2*). Since this polypeptide was completely protected from the action of trypsin, it was apparently located inside the microsomes corresponding to a final extracellular location. Translations performed for short periods, before the synthesis of A was completed, allowed the identification of the two remaining polypeptides seen in Fig. 4, lane *2*. The reduction in M_r effected by trypsin treatment was ~50,000 for A and ~25,000 for B, representing the minimum sizes of their cytoplasmic domains.

To verify that parts of the N-CAM polypeptides were not resistant to the action of trypsin, enzymatic degradation was performed in buffer containing 4% Triton X-100, which ensured solubilization of microsomal membranes. That no N-CAM polypeptides could be isolated after this treatment (not shown) indicates that the parts of N-CAM that are normally protected by the microsomal membranes are susceptible to the action of trypsin.

Posttranslational Modifications of C

Tunicamycin is a known inhibitor of N-linked glycosylation of proteins (31). When astrocyte cultures were labeled with

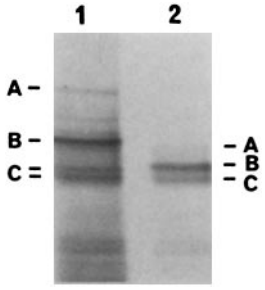

FIGURE 4 Posttranslational proteolysis of [^{35}S]methionine-labeled N-CAM synthesized in vitro. Rat brain rough microsomes were translated in vitro, and N-CAM was immunoisolated and analyzed by SDS PAGE and fluorography. Lane *1*, translation of microsomes. Lane *2*, translation of microsomes; after translation microsomes were treated with 25 μg/ml of trypsin for 30 min at 4°C. The major N-CAM polypeptides are indicated.

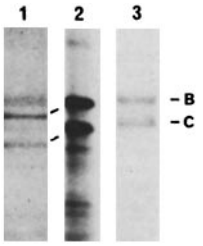

FIGURE 5 Posttranslational modifications of N-CAM in cultured cerebral glial cells. Cultures were labeled with [^{35}S]methionine in the presence or in the absence of 20 μg/ml tunicamycin, or were labeled with [^{35}S]sulfate. Lane *1*, 40-min pulse labeling with [^{35}S]methionine in the presence of tunicamycin. Lane *2*, 10-min pulse labeling with [^{35}S]methionine. Lane *3*, 4-h pulse labeling with [^{35}S]sulfate. N-CAM polypeptides are indicated by bars.

[^{35}S]methionine in the presence of tunicamycin, a decrease in M_r of ~10,000 was observed for B and C as compared to pulse-labeled control cultures (Fig. 5, lanes *1* and *2*). This demonstrated N-linked glycosylation of these polypeptides with four or five high mannose cores each. A similar number of high mannose cores are added to A and B in fetal neuronal cell cultures (14).

To demonstrate sulfation of the C polypeptide, astrocyte cultures were labeled with [^{35}S]sulfate for from 3 to 4 h. The glial cultures produced sulfate-labeled B and C (Fig. 5, lane *3*).

DISCUSSION

Primary cell cultures of astrocytes, muscle, and neuronal cells synthesize N-CAM polypeptides. Although N-CAM from these three cell types is immunochemically identical, differences have been noted in their polypeptide composition. Muscle cells synthesize mainly B, and glial cells produce B and C. Neuronal cells from postnatal brain express A and B. Thus, C seems to be predominantly a glial product. It has previously been shown that fetal neuronal cells synthesize A and B (3, 14).

Despite the differences in polypeptide pattern, the observed immunochemical identity of muscle, glial, and neuronal N-CAM indicates that the N-CAM polypeptides from these cell types are quite similar. The employed immunoassay used the immunoglobulin fraction of pooled sera from four animals that had been immunized for 6 mo. Such an antibody preparation would be expected to recognize at least 5 to 10 different epitopes, corresponding to 35 to 70 amino acid residues on the N-CAM polypeptides (32). Strong similarities between the N-CAM polypeptides A and B have been suggested by peptide mapping and by NH_2-terminal amino acid sequence analysis (3, 33).

Our results indicate that the expression of the individual N-CAM polypeptides can be regulated independently. Only one (15) or a few (16) N-CAM genes are presumed to exist, yet there appear to be three or more mRNAs producing the different N-CAM polypeptides (13, 15, 16). Hence, a modulation in N-CAM expression may occur that involves differential RNA splicing or differentiation control at the translational level.

The expression of C in vivo increases relative to that of A and B during postnatal development (3, 5, 13). C is not expressed by cultured neuronal cells but is synthesized by cultured astrocytes. The late expression of C in developing whole brain might therefore reflect the later proliferation of glial cells than of neurons in brain ontogeny.

In this report we show that C is N-linked glycosylated with about four high mannose cores. This finding has been confirmed by susceptibility to endoglycosidase H treatment of N-CAM synthesized by microsomes in cell-free translation assays (Nybroe, O., unpublished results). Furthermore, C is sulfated. These modifications are also found for A and B, which supports the suggestion that the three N-CAM polypeptides have quite similar extracellular regions (18, 34). The membrane association of C is different from that of A and B. Neither the newly synthesized nor the mature C polypeptide contains membrane-spanning regions. Since C was labeled by radioiodination of brain membranes at least part of the C population is assumed to be membrane associated as a peripheral membrane protein. It has previously been proposed that C occurs as two populations: one being soluble and another being an integral membrane protein (18).

The membrane affinity of C appeared to increase during postnatal development. A large proportion of the C polypeptide was found to be soluble at P4, whereas C could not be detached from the membranes at P40, even in the presence of urea. This increased affinity may be related to the increased affinity of N-CAM to N-CAM binding that occurs during development (17).

We determined the minimal size of the A and B cytoplasmic domains to be ~50,000 and ~25,000 M_r, respectively. Gennarini et al. (18) determined the maximal size of these domains using an array of tryptic fragments of mouse N-CAM incorporated into liposomes. They found domains of M_r ~90,000 (A) and M_r ~35,000 (B). Thus, our results, from the use of a different approach, confirm the presence of large cytoplasmic domains of the N-CAM polypeptides A and B. Since these domains seem to be phosphorylated (3, 34, 35), intracellular portions of N-CAM may be important sites of regulatory control of N-CAM function. Further work on the molecular structure of N-CAM and on the control of N-CAM expression will, we hope, make it possible to obtain a detailed understanding of the mechanism underlying N-CAM mediated cell-cell adhesion.

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