Biosynthesis of the D2 Cell Adhesion Molecule: Pulse-chase Studies in Cultured Fetal Rat Neuronal Cells

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ABSTRACT D2 is a membrane glycoprotein that is believed to function as a cell adhesion molecule (CAM) in neural cells. We have examined its biosynthesis in cultured fetal rat brain neurones. We found D2-CAM to be synthesized initially as two polypeptides: M_r 186,000 (A) and M_r 136,000 (B). With increasing chase times the M_r of both molecules increased to 187,000–201,000 (A) and 137,000–158,000 (B). These were similar to the sizes of D2-CAM labeled with [^{14}C]glucosamine, [^{3}H]fucose and [^{14}C]mannosamine, indicating that the higher M_r species are glycoproteins. In the presence of tunicamycin, which specifically blocks the synthesis of high mannose cores, M_r were reduced to 175,000 (A) and 124,000 (B). Newly synthesized A and B are susceptible to degradation by endo-β-*N*-acetyl-glucosaminidase H, which specifically degrades high mannose cores, but they are resistant to such degradation after 150 min of posttranslational processing. Hence, we deduce that A and B are initially synthesized with four to five high mannose cores which are later converted into N-linked complex oligosaccharides attached to asparagine residues. However, no shift of [^{35}S]methionine radioactivity between A and B was detected with different pulse or chase times, showing that these molecules are not interconverted. Thus, our data indicate that the neuronal D2-CAM glycoproteins are derived from two mRNAs.

D2 is a glycoprotein, originally demonstrated in rat neuronal plasma membranes, which is believed to function as a cell adhesion molecule (CAM)[1] (1–3). The D2 cell adhesion molecule (D2-CAM) has been shown to be immunochemically related, if not identical, to neural CAM (N-CAM; 3, 4) and BSP-2 (5). Results indicate that CAMs are present as three polypeptides of ~200,000–250,000, 140,000, and 120,000 M_r (5). The two larger forms predominate in fetal brain, whereas the 120,000 M_r form (in addition to the two higher M_r forms) has been detected in adult brain. We have, therefore, established a rat brain neuronal culture system to examine selectively the biosynthesis of D2-CAM in this cell type. We report here our findings on the biosynthetic relationship between the different D2-CAM molecules and an initial characterization of their carbohydrate moieties.

[1] *Abbreviations used in this paper:* CAM, cell adhesion molecule; DME, Dulbecco's modified Eagle's medium; Endo H, endo-β-acetyl-glycosaminidase H; GFAP, glial fibrillary acidic protein; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SSF, serum-substituting factors.

MATERIALS AND METHODS

Materials: Dulbecco's modified Eagle's medium (DME), methionine-free DME, basal minimal essential medium and mycoplasma-free horse serum were obtained from Gibco Laboratories (Grand Island, NY). Low glucose (200 μg/ml) DME was obtained from Statens Serum Institut (Copenhagen, Denmark). Poly-L-lysine (type 1B), putrescine, progesterone, insulin, selenium, and tunicamycin were obtained from Sigma Chemical Co. (St. Louis, MO). Purified human transferrin was a gift from Dr. Søren Blirup-Jensen, DAKO Patts, A/S. Aprotinin was obtained from Bayer. Endo-β-*N*-acetylglycosaminidase H, EC 3.2.1.30 (Endo H), was obtained from Miles Laboratories, Inc. (Elkhart, IN) [^{35}S]methionine (>800 Ci/mmol) and ^{14}C-labeled molecular weight markers were obtained from Amersham Corp. (Arlington Heights, IL). [^{14}C]glucosamine hydrochloride (35 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Aqualuma, Liposolve, and Lipoluma were obtained from Lumac (Basle, Switzerland). X-Omat (XL1) film was from Eastman-Kodak; Ultrofilm (^{3}H) was from LKB Instruments, Inc. (Gaithersburg, MD).

Tissue Culture: Tissue culture flasks (4 × 6.5 cm) were pretreated with poly-L-lysine (10 μg/ml H_2O, 5 ml/flask) for 4–6 h at room temperature. They were rinsed twice with phosphate-buffered saline (PBS), then incubated overnight at 37°C with DME containing 20% horse serum. Fetal rat brains from embryos aged 15–16 d were collected and extruded in serum-free DME through a 80 μm nylon gauze filter. All media contained L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 U/ml). Cells were seeded at 10^7 cells/flask in 5 ml DME with 10% horse serum. Cultures were incubated at 37°C in 5% CO_2. After 24 h, monolayers were rinsed once in PBS. Further culture was

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continued in 5 ml serum-free media that contained serum-substituting factors (SSF) as described by Bottenstein et al. (6): transferrin (5 μg/ml), progesterone (2×10^{-8} M), putrescine (100 μM), insulin (5 μg/ml), and selenium (3×10^{-8} M). This culture system yields nearly pure neuronal cultures after 6 d in vitro, as determined in our laboratory and by others (6–8). Immunocytochemical staining for neural cells was performed using rabbit polyclonal anti-rat D2-CAM antibody (9) and for glial cells using mouse monoclonal anti-human glial fibrillary acidic protein (GFAP) antibody (which cross reacts with rat GFAP; 10).

Radio-isotope Labeling: Cell monolayers were rinsed once with PBS and preincubated 30 min in methionine-free SSF media. Unless specified otherwise, [^{35}S]methionine (400 μCi/1.0 ml medium per flask) was added for various pulse periods. Monolayers were rinsed twice in 37°C PBS. SSF media were replaced for chase periods. A continuous pulse of 18 h was employed for labeling with [^{14}C]glucosamine (25 μCi/1.5 ml medium per flask). Continuous 5-h pulses were employed for labeling with [^{3}H]fucose (200 μCi/1.5 ml basal minimal essential medium with SSF per flask) and [^{14}C]mannosamine (2.5 μCi/1.5 ml low glucose, 200 μg/ml, SSF medium per flask). When radiolabeling was done in the presence of tunicamycin, the drug (5 μg/ml) was added 30 min before labeling in the methionine-free pre-incubation media; fresh drug was added at the same concentration to the chase medium. Radiolabeling was terminated by rinsing monolayers twice in ice-cold PBS with phenylmethylsulfonyl fluoride (PMSF, 2 mM) and aprotinin (100 U/ml).

Cell Harvest and D2-CAM Isolation: Monolayers were harvested by scraping cells in 1.2 ml ice-cold PBS (with PMSF-aprotinin); flasks were rinsed with 1.2 ml PBS-PMSF-aprotinin. Cells were centrifuged for 10 min at 10,000 *g*. Pellets were extracted 60 min at 4°C with intermittent vigorous shaking in Tris-barbital buffer (0.07 M, pH 8.6) containing 4% (vol/vol) Triton X-100, PMSF (2 mM), and aprotinin (100 U/ml). The lysates were clarified by centrifugation at 10,000 *g* for 15 min. Acid precipitable radioactive counts were determined: aliquots were hydrolyzed 15 min at 37°C in 0.5 M NaOH, 4 mg/ml casamino acids, and precipitated in 20% trichloroacetic acid for 5 min at 0°C; precipitates were filtered on Whatman glassfiber (GF/C) filters (Whatman Laboratory Products, Inc., Clifton, NJ) and counted in Aqualuma. Unlabeled carrier proteins extracted from perinatal rat brains in Tris-barbital extraction buffer were added to aliquots of radiolabeled culture lysates. D2-CAM was then isolated by precipitation in crossed immunoelectrophoresis at 14–16°C in 1% (wt/vol) agarose-Tris-barbital buffer with 0.5% (vol/vol) Triton X-100. The first dimension electrophoresis was run at 10 V/cm for 35 min; the second dimension was run at 1–1.5 V/cm for 15 h in agarose containing 12 μl/cm^2 purified anti-D2-CAM antibodies (9). Immunoprecipitates were directly visible in the agarose gel. The gels were alternately pressed and rinsed in distilled water two times before precipitates were excised and submitted to electrophoresis. In some instances precipitates were stored frozen (−20°C) a few days before electrophoresis.

SDS PAGE: D2-CAM immunoprecipitates were excised and boiled 5–10 min in electrophoresis sample buffer containing 5% β-mercaptoethanol. Aliquots were incubated with Liposolve for 30 min at 55°C and the radioactivity counted in Lipoluma. Immunoprecipitates were electrophoresed on 3.8–17% SDS polyacrylamide gradient gels in Tris-glycine buffer according to Laemmli (11). Gels were developed for autoradiography on Ultrofilm at −70°C for 1 to 5 wk. In certain instances gels were developed for fluorography with diphenyloxazol as described by Bonner and Lasky (12) before being exposed to X-Omat film. M_r was determined with reference to a range of ^{14}C-labeled standard proteins. The activity in individual SDS polyacrylamide gel bands was measured after eluting gel slices in Liposolve for 30 min at 55°C and then counting in Lipoluma.

Endo H Degradation: Immunoprecipitates were degraded with Endo-H (6 mU/100 μl) for 5 h at 37°C in acetate buffer (0.05 M, pH 5.0) with $CaCl_2$ (0.09 M) and 0.2% SDS.

RESULTS

Immunocytochemical Staining for D2-CAM and GFAP

Nearly all (>99%) of the cells in 6-d cultures were found to react positively with a rabbit anti-rat D2-CAM antiserum by the indirect immunoperoxidase technique. Virtually no GFAP positive (astroglial) cells (<1%) were detected. Fibroblasts were rarely detected and, furthermore, have been found not to react with the anti D2-CAM antibody (unpublished results).

Relative Electrophoretic Migration of D2-CAM from Cultured Brain Neurons

The relative electrophoretic migration of D2-CAM was estimated in crossed immunoelectrophoresis under conditions in which human hemoglobin migrated 10 mm in the first dimension agarose gel. The value determined for D2-CAM in 6-d cultured neurons was 22 mm, which is similar to the relative migration of D2-CAM from day 2 postnatal rat brain (23 mm). This contrasts with the slower electrophoretic migration of D2-CAM from adult (day 80) brain of 15 mm. The decrease in the electrophoretic mobility of D2-CAM with increasing age has been suggested as being due to decreased charge which in turn was presumed to be due to a decrease in sialation (13).

Polypeptide Sizes of D2-CAM

Fetal rat brain neurons grown in tissue culture initially synthesized D2-CAM during a short (3 or 10 min) pulse as two major polypeptides: A, with an M_r 186,000, and B, with an M_r 136,000 (Fig. 1, Table I). With increasing chase periods both bands broaden, yielding polypeptides with M_r ranges of 187,000–201,000 (A) and 137,000–158,000 (B). As the length of the chase period was increased (from 0 to 50 min), the widths of the A and B bands appeared to "grow" from lower to higher M_r. The M_r of bands A and B after a 150-min chase (see Fig. 4, lane *2*) or an 18-h continuous pulse (not shown) were similar to those found after a 50-min chase. Hence, the

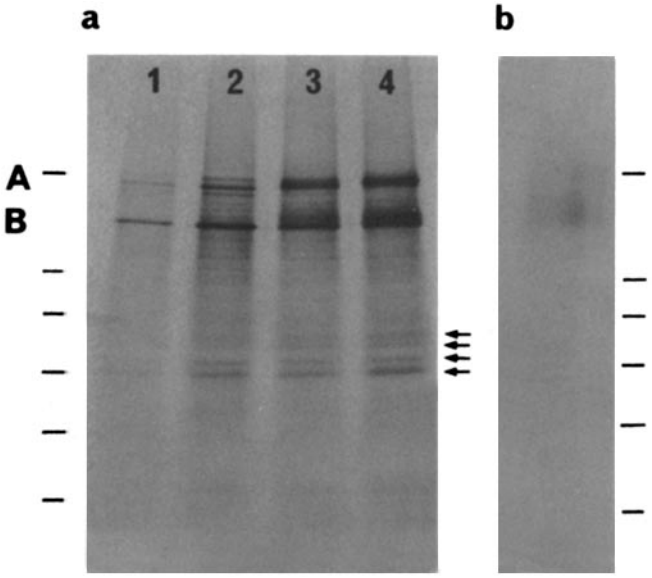

FIGURE 1 In vivo labeling of D2-CAM with [^{35}S]methionine (*a*) and [^{14}C]glucosamine (*b*). Autoradiographs (in this and subsequent figures) of radiolabeled D2-CAM isolated by immunoprecipitation in agarose gel and run on 3–17% SDS polyacrylamide gradient gels, as described in Materials and Methods. Pulse or pulse/chase periods were the following: (*a*) lane *1* (3-min pulse), lane *2* (10-min pulse), lane *3* (10-min pulse/20-min chase), lane *4* (10-min pulse/50-min chase); (*b*) 18 h pulse. The major species, *A* and *B*, are labeled. Arrows indicate the positions of bands of lesser intensity which were routinely detected. Note the set of four bands of low M_r which were seen with longer labeling periods and which appear to be standard degradation species in lane *2* (*a*). The positions of standard proteins are indicated by lines at the margin; M_r from top to bottom are: 200,000, 92,500, 69,000, 46,000, 30,000, and 14,300.

TABLE I

M_r of D2-CAM Molecules Synthesized in Cultured Neuronal Cells*

	D2-CAM	
	A	B
A. [^{35}S]Methionine labeling		
Pulse‡	186,000 ± 2,150	136,000 ± 2,250
(*n*)	(6)	(6)
Pulse + tunicamycin§	175,000	124,000
Difference from paired control	13,000	14,000
Pulse/chase‖	187,000–201,000	137,000–158,000
B. Radioactive sugar labeling¶		
^{14}C-*N*-Acetyl-D-glucosamine	188,000–230,000	140,000–178,000
[^{3}H]Fucose	188,000–208,000	138,000–155,000
[^{14}C]Mannosamine	188,000–208,000	138,000–155,000

* Mean M_r as determined from a series of ^{14}C-labeled standard proteins, ± SEM.
‡ Pulse periods were for 10 min (*n* = number of determinations)
§ Pulse (10 min) in the presence of 5 μg/ml tunicamycin, as in Fig. 2; paired control was a 3-min pulse sample run on the same SDS gel with M_r of A equal to 188,000 and M_r of B equal to 138,000.
‖ Values given represent the range of M_r determined for 10 samples chased for 50 or more min.
¶ Labeling period was 5 to 18 h.

posttranslational processing that increases the sizes of A and B appears to be nearly maximal by 50 min after translation. Lesser bands between A and B, at ~167,000 and 178,000 M_r, appeared inconstantly (see Fig. 1 *a*, lane *2*). Several characteristic low M_r species (<100,000) were also detected which were believed to be break down products (Fig. 1 *a*, lane *4*; also see Fig. 3, lane *4*), as previously shown for N-CAM (13). Between 0.1% and 0.25% of the acid precipitated [^{35}S]methionine activity was routinely recovered in the immunoprecipitated D2-CAM.

Labeling with Radioactive Sugars

Both A and B are glycoproteins as shown by their incorporation of [^{14}C]glucosamine (Fig. 1 *b*). The M_r of the glycosylated forms roughly corresponds to those of the larger methionine-labeled polypeptides: glycoprotein A, 188,000–230,000 M_r; glycoprotein B, 137,000–178,000 M_r. Both A and B were also labeled with [^{3}H]fucose and [^{14}C]mannosamine, a metabolic precursor of sialic acid, yielding glycoproteins with M_r of similar ranges for all sugar radiolabels (Table I, Pt. *B*).

Effect of Tunicamycin on Synthesis

Tunicamycin is a specific inhibitor of N-linked glycosylation of asparagine residues (14). This drug inhibits the synthesis of the high mannose-dolichol phosphate which functions as the high mannose core donor in the first step of N-linked glycosylation (15). Synthesis of D2-CAM in the presence of tunicamycin produced two polypeptides of lower M_r than those synthesized during a brief 3-min pulse (Fig. 2, Table II): the M_r of A was 175,000 and of B was 124,000, yielding a net difference in M_r from its paired (3-min pulse) control of 13,000 for A and 14,000 for B (Table I, Pt. *A*). Hence, both forms of D2-CAM contain high mannose cores that appear to be added co-translationally.

Endo H Sensitivity

Another means of testing for the presence of high mannose cores is by determining the sensitivity of a molecule to degradation with Endo H. This enzyme selectively degrades high mannose cores containing five to nine mannosyl groups which can later be processed into complex oligosaccharide chains (15). We found that both A and B were sensitive to Endo H degradation after a 10-min pulse, but were resistent after a 150-min chase (Fig. 3). The difference in M_r between the D2-CAM forms detected after a 10-min pulse with or without Endo H degradation is 11,000 for A and 9,000 for B. This is similar to the difference in M_r observed during pulses with and without tunicamycin. These results showing Endo H sensitivity of A and B, along with the data on the effect of tunicamycin on polypeptides size, indicate that both A and B contain high mannose cores that undergo further processing to yield complex glycosylated species.

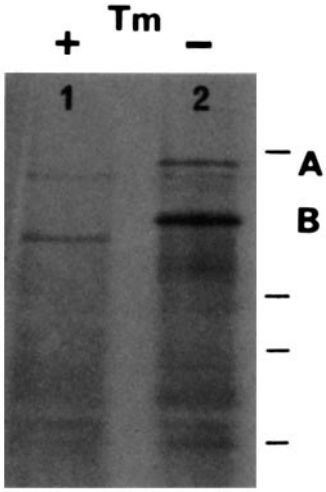

FIGURE 2 The effect of tunicamycin on the synthesis of D2-CAM. (Lane *1*) 10-min pulse with [^{35}S]methionine in the presence of 5 μg/ml tunicamycin. (Lane *2*) 3-min control pulse. See Table I for a comparison of the actual M_r.

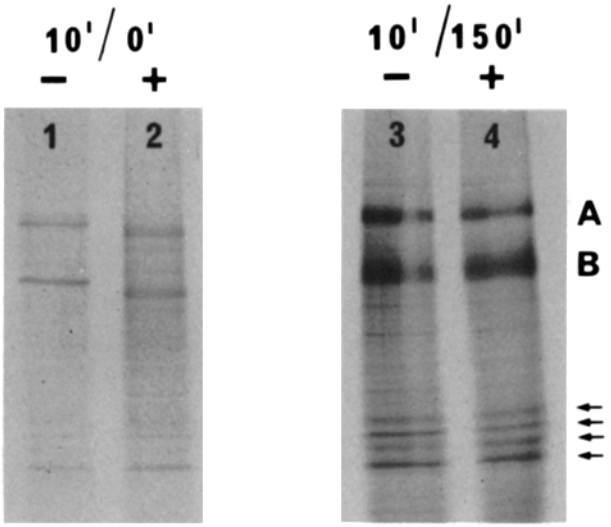

FIGURE 3 Sensitivity of D2-CAM A and B to Endo H degradation. (Lane *1*) 10-min pulse control. (Lane *2*) 10-min pulse with Endo H. (Lane *3*) 10-min pulse/150 min chase control. (Lane *4*) 10-min pulse/150 min chase with Endo H. All samples were incubated with Endo H for 5 h at 37°C as described in Materials and Methods.

Relative Incorporation of [^{35}S]Methionine into A and B

The relative [^{35}S]methionine activity incorporated into D2-CAM A and B, as isolated from SDS polyacrylamide gels, was determined for various pulse and pulse-chase periods (Table II). Chase periods were done in the presence of cycloheximide or excess cold methionine to block completely further labeling. Similar gel patterns were observed for 20- and 50-min chase periods in the presence of cycloheximide as those shown in Fig. 1 *a*, lanes *3* and *4*. For synthesis periods of up to 60 min, the relative distribution of isotope into A and B was ~40% and 60%, respectively. No significant shift of activity from one form to the other was observed with different lengths of pulse or chase periods up to 60 min. No significant processing of one D2-CAM polypeptide into the other was apparent during the initial period of synthesis. A chase period of 150 min was also examined (see Fig. 4). With the longer chase period there was slightly less relative radioactivity in A relative to B (30% vs. 70%) than with shorter labeling periods. After long (150 min) chase periods the decrease in absolute radioactivity recovered in the A band (−31%) was much greater than the minor increase in radioactivity found in the B band (+6%; see footnote, Table II). This indicates a higher rate of degradation of A than B during the chase period.

Contrary to previous reports for N-CAM (13), we did not observe any conversion of the A band into the B band with varying periods of boiling between 5 and 30 min. Also contrary to Rothbard et al. (13), we did not observe any marked autolytic activity when isolated D2-CAM (by immunoprecipitation in agarose gel) was incubated at 37°C for 6 or 24 h (see Fig. 4). We did, however, observe some increased general proteolysis of D2-CAM in the cell lysate when cell harvest was performed at 20°C and in the absence of protease inhibitors, rather than at 0°C in the presence of protease inhibitors, which was the usual procedure.

TABLE II

Incorporation of [^{35}S]Methionine into D2-CAM A and B during Pulse and Pulse/Chase Synthesis

	% of radioactivity in SDS gel bands*	
Pulse/chase period	A	B
A. Short		
10′/−	42	58
10′/20′ (cyc)‡	43	57
10′/50′ (cyc)‡	40	60
30′/−	40	60
30′/−	36	64
60′/−	42	58
Mean ± SD	40.5 ± 2.5	59.5 ± 2.5
B. Long		
10′/150′	32	68
10′/150′ (cyc)‡	32	68
10′/150′ (met)§	32	68
30′/150′	23	77
Mean ± SD	29.8 ± 4.5	70.3 ± 4.5

* Between 200 and 1,500 disintegrations per min (dpm)/band were counted. Percentages for each band represent the fraction of the total activity detected in (A + B). Total dpm recovered from the A and B bands from 10′ pulse samples with short (0′–50′) or long (150′) chases were determined. Total dpm recovered decreased 10% with long chase periods (418 vs. 378 dpm), after normalization of the data for equivalent initial dpm employed for immunoprecipitation. Dpm in the A band decreased by 31% (174 vs. 120), whereas dpm in the B band increased 6% (244 vs. 258).

‡ Cycloheximide (1 mg/ml) was added to the chase medium.

§ Excess cold methionine (2.5 mM) was added to the chase medium.

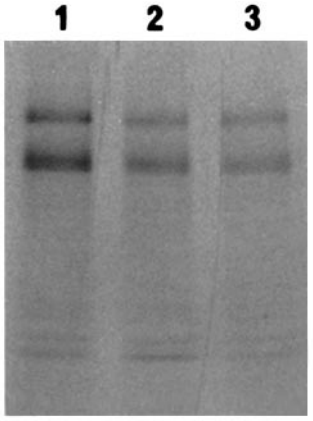

FIGURE 4 Posttranslational processing of D2-CAM A and B in the presence of cycloheximide and excess methionine. (Lane *1*) Control 10-min pulse/150-min chase. (Lane *2*) 10-min pulse/150-min chase in the presence of 1 mg/ml cycloheximide. (Lane *3*) 10-min pulse/150-min chase with 2.5 mM methionine.

DISCUSSION

Sizes of D2-CAM Synthesized in Cultured Neuronal Cells

D2-CAM is synthesized in vivo during a short pulse period in neurons from fetal brain as two distinct polypeptides of M_r 186,000 (A) and 136,000 (B). Within 20 to 50 min these are converted to more heterogeneous groups of larger molecules of M_r 187,000–201,000 (A) and 137,000–157,000 (B) which correspond to the sizes of two glycosylated molecules detected after longer labeling periods. The broader M_r ranges found for A and B, even during relatively short chase periods, may thus be attributed to oligosaccharide microheterogeneity.

Can One D2-CAM Polypeptide Be Designated a Specific "Fetal" Form?

It is apparent that fetal neurons produce two discrete D2-CAM polypeptides when grown in tissue culture. Other data (not shown) indicate that fetal neurons cultured for only 24 h also produce discrete A and B species having the same M_r as those synthesized on day 6. This contrasts with previous studies (17, 18) that did not clearly identify the B band in fetal brain, suggesting that A represents a "fetal form." Others, however, have shown that A and B are expressed both in adult and fetal brain (5). According to the present study, it does not seem appropriate to designate either A or B as being specific to fetal brain. In adult brain, a third polypeptide band with M_r lower than that of B (~120,000) was detected (5, 9). Such a lower M_r species was not obvious in our studies, although occasionally a diffuse band in this region was observed—but this was only detected after fluorography and prolonged exposure of the film (Fig. 2, lane *2*).

Carbohydrate Characterization of D2-CAM

The nature of D2-CAM glycosylation was explored in several ways. First, the higher M_r species of both A and B were found to be glycoproteins as determined by biosynthetic labeling with glucosamine, fucose, and mannosamine (the latter being a precursor of sialic acid). Fucosylation and sialation indicate the presence of complex oligosaccharides on A and B (20). Hence, the larger species of A and B represent complex glycoproteins. Second, synthesis in the presence of tunicamycin blocked the production of the higher M_r forms

of A and B confirming that complex oligosaccharides are N-linked via asparagine residues. Third, both of the lower M_r species of A and B contain high mannose cores as proven by their sensitivity to Endo H. The difference in M_r due to Endo H treatment is 11,000 for A and 9,000 for B, which are similar to the reductions in M_r caused by tunicamycin (13,000 less for A and 14,000 less for B). Thus, newly synthesized A and B both contain four to five high mannose cores (presuming a M_r of 2,600 for one high mannose core, $Glc_3Man_9GlcNAc_2$). Addition of high mannose cores to polypeptides is generally believed to be a co-translational event. This seems to be true in the case of the synthesis of D2-CAM A and B, since the M_r of both during a pulse as short as 3 min were higher than the M_r of those synthesized in the presence of tunicamycin. Several distinct, although weaker, SDS PAGE bands in the regions of A and B observed during short chase periods (see Fig. 1 *a*, lane *2*) may represent the D2-CAM species that contain different numbers of nascent complex oligosaccharides.

Is There a Biosynthetic Precursor Relationship between the A and B Glycoproteins?

Immunologically A and B appear to be identical, as seen by the identification of both with monoclonal antibodies against N-CAM (19) and BSP-2 (5). The two forms of N-CAM have, also, been found to have nearly identical peptide subunit compositions as determined by limited proteolysis, but small differences have been noted (16). Although it has been suggested that the A form of N-CAM can be degraded to yield the B form (17), no proof of a precursor relationship in vivo has been presented. In fact, our biosynthesis data indicate the opposite. The relative distribution of radioactivity between A and B remained constant during the initial biosynthetic period and there was no evidence of posttranslational interconversion.

The relative proportions of the A and B polypeptides are known to change during brain development: A decreases, whereas B increases along with the appearance of a third, smaller polypeptide (5, 21). In fetal neurons, as examined here, two discrete D2-CAM polypeptides are synthesized concurrently and both become complex glycosylated. Since no biosynthetic interconversion was observed, it appears that A and B are products of separate mRNAs. The developmental changes in the levels of A and B may, therefore, be due to differential regulation of gene expression or different rates of degradation, rather than differential posttranslational processing of a single polypeptide. This conclusion is in contrast to recent proposals of Cunningham et al. (16) and Hirn et al. (5) that suggest that CAM polypeptides are derived from a single precursor. We propose that D2-CAM A and B, although exhibiting similar epitopes and other structural features, are two discrete entities that lack a subunit-precursor relationship. Future investigation of their topological and functional relationship should, therefore, contribute to our understanding of their purported roles in cell adhesion phenomena.

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