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, 186,000 (A) and M, 136,000 (B). With increasing chase times the M, 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 [14C]glucosamine, [3H]fucose and [14C]mannosamine, indicating that the higher M, species are glycoproteins. In the presence of tunicamycin, which specifically blocks the synthesis of high mannose cores, M, were reduced to 175,000 (A) and 124,000 (B). Newly synthesized A and B are susceptible to degradation by endo-o-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 [35S]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) in neural cells. 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, (5). The two larger forms predominate in fetal brain, whereas the 120,000 M, form (in addition to the two higher M, 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-N-acetylglucosaminidase 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. Soren Blirup-Jensen, DAKO Patts, A/S. Aprotinin was obtained from Bayer. Endo-β-N-acetylglucosaminidase H, EC 3.2.1.30 (Endo H), was obtained from Miles Laboratories, Inc. (Elkhart, IN) [35S]methionine (>800 Ci/mmol) and [14C]labeled molecular weight markers were obtained from Amersham Corp. (Arlington Heights, IL). [14C]glucosamine hydrochloride (35 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Aqualuma, Liposolve, and Lipoluma were obtained from Lumac (Basel, Switzerland). X-Omat (XL1) film was from Eastman-Kodak; Ultrasolve (T) 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 H2O, 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 1-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 U/ml). Cells were seeded at 105 cells/flask in 5 ml DME with 10% horse serum. Cultures were incubated at 37°C in 5% CO2. After 24 h, monolayers were rinsed once in PBS. Further culture was...
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 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

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 antisera by the indirect immunoperoxidase technique. Virtually no GFAP positive (astrogial) cells (<1%) were detected. Fibroblasts were rarely detected and, furthermore, have been found not to react with the anti D2-CAM antibody (unpublished results).

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TABLE I

M, of D2-CAM Molecules Synthesized in Cultured Neuronal Cells

|                      | A            | B            |
|----------------------|--------------|--------------|
| **A. [35S]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** | 188,000–230,000 | 140,000–178,000 |
| 14C-N-Acetyl-d-glucosamine | 188,000–208,000 | 138,000–155,000 |
| [3H]Fucose           | 188,000–208,000 | 138,000–155,000 |
| [14C]Mannosamine     | 188,000–208,000 | 138,000–155,000 |

*Mean M, as determined from a series of 14C-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, of A equal to 188,000 and M, of B equal to 138,000.
+ Values given represent the range of M, 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, appeared inconstantly (see Fig. 1 a, lane 2). Several characteristic low M, 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 [35S]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 [14C]glucosamine (Fig. 1 b). The M, of the glycosylated forms roughly corresponds to those of the larger methionine-labeled polypeptides: glycoprotein A, 188,000–230,000 M,; glycoprotein B, 137,000–178,000 M,. Both A and B were also labeled with [3H]fucose and [14C]mannosamine, a metabolic precursor of sialic acid, yielding glycoproteins with M, 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, than those synthesized during a brief 3-min pulse (Fig. 2, Table II): the M, of A was 175,000 and of B was 124,000, yielding a net difference in M, 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 resistant after a 150-min chase (Fig. 3). The difference in M, 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, 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 [35S]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,.

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 [35S]Methionine into A and B

The relative [35S]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. 1a, 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.

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, 186,000 (A) and 136,000 (B). Within 20 to 30 min these are converted to more heterogeneous groups of larger molecules of M, 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, 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, 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, lower than that of B (~120,000) was detected (5, 9). Such a lower M, 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, 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, forms.
of A and B confirming that complex oligosaccharides are N-linked via asparagine residues. Third, both of the lower Mr species of A and B contain high mannose cores as proven by their sensitivity to Endo H. The difference in Mr due to Endo H treatment is 11,000 for A and 9,000 for B, which are similar to the reductions in Mr 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 Mr of 2,600 for one high mannose core, Glc3Man9GlcNAc2). 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 Mr of both during a pulse as short as 3 min were higher than the Mr 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. 1a, 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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