Biosynthesis of the D2-Cell Adhesion Molecule: Post-translational Modifications, Intracellular Transport, and Developmental Changes

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ABSTRACT Posttranslational modifications and intracellular transport of the D2-cell adhesion molecule (D2-CAM) were examined in cultured fetal rat neuronal cells. Developmental changes in biosynthesis were studied in rat forebrain explant cultures. Two D2-CAM polypeptides with Mr of 187,000–210,000 (A) and 131,000–158,000 (B) were synthesized using radiolabeled precursors in cultured neurons. A and B were found to contain only N-linked complex oligosaccharides, and both polypeptides appeared to be polysialated as determined by [14C]mannosamine incorporation and precipitation with anti-polysialic acid antibody. The two polypeptides were sulfated in the trans-Golgi compartment and phosphorylated at the plasma membrane. D2-CAM underwent rapid intracellular transport, appearing at the cell surface within 35 min of synthesis. A and B were shown to be integral membrane proteins as seen by radiiodination by photoactivation employing a hydrophobic labeling reagent. In rat forebrain explant cultures, D2-CAM was synthesized as four polypeptides: A (195,000 Mr), B (137,000 Mr), C (115,000 Mr), and a group of polypeptides in the high molecular weight region (HM) between 250,000 and 350,000. Peptide maps of the four polypeptides yielded similar patterns. Biosynthesis of C and HM increased with age, relative to A and B. A and B were sulfated in embryonic brain, however, sulfation was not noticeable at postnatal ages. Phosphorylation, on the other hand, of A and B was observed at all ages examined. We suggest that D2-CAM function may be modified during development by changes in the relative synthesis of the different polypeptides, as well as by changes in their glycosylation and sulfation.

The D2-cell adhesion molecule (D2-CAM) has been shown to be synthesized in cultured neuronal cells as two polypeptides, A and B (1). Both were shown to be co-translationally glycosylated with high mannose cores that were processed into complex N-linked oligosaccharides, yielding two groups of glycopeptides, with Mr of 187,000–210,000 (A) and 131,000–158,000 (B). D2-CAM, neuronal CAM (N-CAM; 2), and the brain specific protein (BSP-2) (3) have been shown to be closely related, if not identical, molecules (4, Noble, M., M. Albrechtsen, C. Mølle, J. M. Lyles, E. Bock, M. Watanabe, and U. Rutishauser, submitted for publication).

Glycosylation is an important modification of cell surface proteins (5, 6). N-CAM derived from fetal brain has been shown to contain higher levels of sialic acid than that from adult brain (7), and the developmental variation in sialation has been suggested to be an important factor in the regulation of its function (2). Hence, posttranslational modifications of D2-CAM glycosylation may be intimately connected with its functional role. After a preliminary report (1), we present additional studies concerning the glycosylation of D2-CAM. We have also examined two other posttranslational modifications of D2-CAM: sulfation and phosphorylation. Sulfation has been shown to be a widespread modification of proteins (8, 9). Sulfated glycosaminoglycans and proteoglycans have...
been shown to be important in the organization of the extracellular matrix of many cell types (10–12). Sulfated proteoglycans have been identified in the in vitro substrate adhesion sites of glial cells, fibroblasts, epithelial cells, and neurons (11, 13, 14). Phosphorylation is another common protein modification known to modulate enzyme activity and to regulate neurotransmission (15). Because D2-CAM is presumably involved in cell–cell interactions, these modifications could be highly significant to its functional role.

We also established a rat forebrain explant culture system to study age-dependent alterations in D2-CAM biosynthesis. One advantage of this culture system in contrast to monolayer cultures is that biosynthesis can be examined in a complex tissue at various ages. Furthermore, in this system different cell types are preserved with their intercellular connections intact.

Although D2-CAM is presumed to function in cell adhesion its mode of action has not been explained. A homotypic type of binding has been proposed (16), which implies that the molecule interacts with itself. However, more recent studies show that two to three discrete D2-CAM polypeptides can be synthesized at all ages (1, 17). Their association and possible interaction is at present unknown.

Our data allow the following conclusions: D2-CAM is N-linked glycosylated, including sialation and polysialation. Furthermore, it is shown that the A and B polypeptides of D2-CAM are sulfated in the trans-Golgi compartment and phosphorylated at the plasma membrane. D2-CAM is rapidly transported intracellularly and appears on the cell surface 35 min after synthesis. Both the A and B polypeptide are integral membrane proteins. Biosynthesis of the A and B polypeptide decreases with age, whereas biosynthesis of a polypeptide of M, 115,000 (C) and a group of polypeptides in the high molecular weight region (HMw) increase. Sulfation of A and B is only observed in embryonic brain. Phosphorylation, on the other hand, is observed at all ages examined.

**MATERIALS AND METHODS**

**Materials:** Dulbecco’s modified Eagle’s medium (DME), methionine-free DME, basal minimal essential medium, and mycophenolase-free horse serum were obtained from GIBCO Laboratories (Grand Island, NY). Low glucose (200 mg/ml) and phosphate-free DME were obtained from Statens Serum Institut, Copenhagen. Poly-l-lysine (type 1B), putrescine, progesterone, insulin, selenium, tumicamycin, trypsin (type III, E.C.3.4.21.4), soybean trypsin inhibitor (type II S), cycloheximide, monensin, N-acetyl-l-lysine chloromethyl ketone, bovine serum albumin, and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co., (St. Louis, MO). Tween-20 was obtained from Protex vulgaris. Proteus vulgaris chondroitinase ABC (chondroitin ABC lyase, E.C.4.2.2.4) and Flavobacterium heparinum heparinase (heparin lyase, E.C.4.2.2.7) were obtained from DAKO Immunoglobulins, Copenhagen. Eucalyptus globulus leaves were obtained from the Eastman Kodak Co., Rochester, NY. Trypsin (type II, E.C.3.4.21.4) was obtained from Miles Laboratories, Elkhart, IN. Protein A-Sepharose was from Pharmacia (Uppsala, Sweden). Rabbit immunoglobulins raised against rat brain D2-CAM were purified as described (18). The same drug concentrations were added to pulse and chase media.

**Biosynthetic Labeling of D2-CAM in Cultured Fetal Neuronal Cells:** Cell monolayers were rinsed once in PBS and preincubated for 30 min in the appropriate labeling medium without isotope; cultures labeled with 35PO4 were rinsed with phosphate-free DME. Radiotopes were added to labeling media on cell monolayers (26 cm2 per flask) for times indicated in the text. [3H]methionine (400 Ci/ml in 1 ml methionine-free SDF-DEME per flask), [14C]glucosamine (200 Ci/ml in 1 ml of low-glucose DME per flask), [3H]-mannosamine (5 Ci/ml in 1 ml of low-glucose DME per flask), [3H]fucose (200 Ci/ml in 2 ml low-glucose DME per flask), [35SO4] (400 Ci/ml in 1.5 ml of sulfate-free basal minimal essential medium per flask), and inorganic 35PO4 (1 mCi in 1 ml of phosphate-free DME per flask). Cells were rinsed twice with PBS at 4°C and kept pulse and chase media for 10 min. Cultures were then incubated with serum-substituting factor-DME. Radiolabeling was terminated by washing cells with ice-cold PBS-PMSF-aprotinin. In experiments employing drugs during labeling, drug preincubations were performed: tunicamycin (10 000 U/ml, 100 ng/ml) monensin (10–4 M, 45–60 min), and cycloheximide (1 mg/ml, 40 min). The same drug concentrations were used to pulse and chase media.

**Biosynthetic Labeling of D2-CAM in Explant Cultures:** Isotopes were used 400 Ci [35S]methionine, 400 Ci [35SO4] or 800 Ci [35PO4 per explant culture. Cultures were incubated for 5–5 h at 37°C in 5% CO2. Labeling was terminated by removing the medium and triturating the tissue in a glass syringe in 1 ml of PBS, 5 mM EDTA, 0.2 mM PMSF, 1 mM N-acetyl-l-lysine chloromethyl ketone, 100 mM iodoacetamide, and 20 mg/ml pepstatin.

**Isolation of Cell Surface Localized D2-CAM by Means of Antibodies:** After radiolabeling for various times, cell monolayers were

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Lyles et al.  Biosynthesis of D2-Cell Adhesion Molecule 2083
D2-CAM Glycosylation in Fetal Neurons in Culture

BIOSYNTHETIC LABELING WITH RADIOACTIVE SUGARS: Several radiolabeled sugars were employed to characterize D2-CAM glycosylation (Fig. 1a). Both polypeptides A and B were labeled with [3H]glucosamine, [3H]fucose, [3H]mannose, and [3H]mannotosamine, the latter being a metabolic precursor of sialic acid. The relative labeling of D2-CAM with the different sugars is shown in Table I as the fraction of min with [3S]methionine and chased 50 min, as described in Materials and Methods. Lane 1, control; lane 2, with monensin (+M). See Materials and Methods for further details.

RESULTS

D2-CAM Glycosylation in Fetal Neurons in Culture

D2-CAM Degradation: Endo H; immunoprecipitates were incubated with Endo H (6 mU/100 μl) for 5 h at 37°C in 0.25 M, pH 8.0, Tris-HCl containing 0.1 M sodium acetate and 0.25 M NaCl with 20 μl/100 μl chondroitinase ABC for 4 h at 37°C. Heparinase; immunoprecipitates were incubated with 0.05 M, pH 7.0, calcium acetate buffer with 20 μl/100 μl heparinase for 4 h at 37°C. β-Elimination; this was performed by incubating immunoprecipitates in 0.5 M NaOH for 18 h at 4°C. Subsequently, the samples were neutralized. Acid treatment; immunoprecipitates were submitted to mild acid treatment in 1 M HCl 30 min at 60°C and subsequently neutralized.

Peptide Mapping: Gel slices containing individual SDS PAGE bands were excised and boiled for 3–10 min in SDS-PAGE sample buffer. Chymotrypsin (20 μg/ml) was added and the samples were submitted to electrophoresis on a 7.5–17.5% SDS polyacrylamide gradient gel containing EDTA (1 mM) as described by Cleveland et al. (24).

Figure 1 Biosynthetic labeling of D2-CAM with radioactive sugars. (a) Neuronal monolayers were labeled for 18 h, except for lane 3, where [3S]methionine labeling was for 3 h before a 1-h chase. Lane 7, [3H]mannose; lane 2, [3H]glucosamine; lane 3, [3S]methionine; lane 4, [14C]mannosamine; lane 5, [3H]fucose. 200 μCi of each radiolabel was employed except for [14C]mannosamine, where 3 μCi was used. Bars indicate the positions of standard Mr markers (+M). See Materials and Methods for further details.

Materials and Methods. The H Pomatia samples were analyzed on a 7.5–17.5% SDS polyacrylamide gel fluorograms:

- Lane 1, [3H]mannose; lane 2, [3H]glucosamine; lane 3, [35S]methionine; lane 4, [14C]mannosamine; lane 5, [3H]fucose. 200 μCi of each radiolabel was employed except for [14C]mannosamine, where 3 μCi was used. Bars indicate the positions of standard Mr markers (+M).
- The binding of D2-CAM to lectins. The fluorogram shows the D2-CAM polypeptides that were bound (+) or not bound (−) to WGA, Con A, or H Pomatia. [3S]methionine labeled D2-CAM was incubated with lectin, as described in Materials and Methods. The H Pomatia samples were analyzed on a separate gel and a higher amount of radioactivity was employed that produced broader bands. (c) The effects of monensin on D2-CAM polypeptide synthesis. Neuronal monolayers were labeled 10 min on ice with 1.0 ml of chilled PBS-BSA-proteinase inhibitor solution, containing 20 μl of anti-D2-CAM antibodies and 200 μl swine serum (carrier protein). Antibody-containing PBS was removed and monolayers were rinsed three times with PBS-BSA-proteinase inhibitor solution at 4°C. Cells were harvested as described above. Cells from each flask were solubilized in 140 μl of extraction buffer, which contained 40 μl of perinatal forebrain extract (solubilized 1:1, wt/vol, in extraction buffer); this was done to block surplus anti-D2-CAM antibody from binding intracellular D2-CAM during solubilization. Solubilized complexes of anti-D2-CAM + surface localized D2-CAM were isolated on Protein A-Sepharose (21).

Trypsin Treatment of Surface D2-CAM on Cultured Cells: Cell monolayers were rinsed twice with PBS at 37°C and incubated with 1 ml of 0.5 mg/ml trypsin in PBS containing 0.2 mg/ml EDTA for 10 min at 37°C. Subsequently, cells were detached from the plastic surface of the culture flask by taping. 1 ml of soybean trypsin inhibitor (1.0 mg/ml PBS) was added to the cells, which were subsequently pelleted (500 g for 5 min) and rinsed in PBS.

Lectin Binding of D2-CAM: Con A, WGA, or H Pomatia bound to Sepharose beads were equilibrated in 10 mM Tris-HCl buffer, pH 7.4, containing 0.05% vol/vol Tween-20 and 1% wt/vol BSA. Aliquots of cell extract (15 μl) were incubated with 50–75 μl of beads for 18 h at 4°C. After extensive washing in equilibration buffer, bound glycoproteins were eluted with Tris-barbital buffer (see immunosolubion subsection above) containing 5% wt/vol sugar: α-D-galactopyranoside for Con A, N-acetyl-D-glucosamine for WGA, N-acetyl-D-galactosamine for H Pomatia. D2-CAM was immunosolubolated from both unbound and bound fractions as described above.

5′-32P-Labeling by the Chloramine T Method: Brains from rats postnatal day 4 were homogenized (1:10 wt/vol) in PBS-BSA-proteinase inhibitor at 4°C. Membranes were centrifuged for 30 min at 10,000 g. Pellets were washed twice. Aliquots corresponding to ~10 mg wet weight tissue were labeled with 1 mCi of [35S]methionine and BSA (1% wt/vol) and cooling on ice.
radioactivity recovered in immunoisolated D2-CAM relative to radioactivity incorporated into total protein. There was a relatively high incorporation of mannose and fucose, less incorporation of glucosamine, and a low incorporation of mannose. This agrees with our previous finding that both A and B polypeptides are synthesized with high-mannose cores. These are later modified during transport through the cis-Golgi compartment by the stepwise removal of glucose and mannose residues; complex glycosylation is completed in the trans-Golgi compartment by the addition of glucosamine, galactose, glucose, fucose, and sialic acid as described (25). Our data show that the characteristic sugars present in N-linked complex oligosaccharides are incorporated into D2-CAM A and B. The low incorporation of mannose probably indicates that neither A nor B, when fully modified, contains the high-mannose type of oligosaccharide chain. Approximately twice as much of each radiolabel was found to be incorporated into B, relative to A, which is similar to the distribution of [35S]methionine. Tunicamycin is a drug that prevents the formation of high-mannose cores and, hence, blocks complex N-linked glycosylation (26). No labeling of A or B with [3H]glucosamine or [14C]mannosamine was detected in the presence of tunicamycin (not shown), further indicating that the oligosaccharides on A and B are complex N-linked.

Affinity of D2-CAM for Various Lectins: The affinity of biosynthetically [35S]methionine labeled A and B polypeptides to Con A, WGA, and H Pomatia was determined (Fig. 1c). Bands were detected in two peptide bands were detected when biosynthetically radiolabeled anti-PSA immunoprecipitates were analyzed on SDS PAGE. Several polypeptide bands were detected when biosynthetically radiolabeled anti-PSA immunoprecipitates were analyzed on SDS PAGE. (See arrow, Fig. 2b). Thus, it appears that anti-PSA reacts with molecules other than D2-CAM, the system was reversed, placing the anti-D2-CAM in the intermediate gel and anti-PSA in the upper reference gel. A small precipitate was observed with anti-PSA, in addition to the anti-D2-CAM precipitate (see arrow, Fig. 2b). Thus, it appears that anti-PSA reacts with molecules other than D2-CAM, the system was reversed, placing the anti-D2-CAM immunoprecipitate in the reference gel, indicating that anti-PSA reacted with D2-CAM. To test whether anti-PSA reacted with molecules other than D2-CAM, the system was reversed, placing the anti-D2-CAM in the intermediate gel and anti-PSA in the upper reference gel (Fig. 2a). A small precipitate was observed with anti-PSA, in addition to the anti-D2-CAM precipitate (see arrow, Fig. 2b). Thus, it appears that anti-PSA reacts with molecules other than D2-CAM, the system was reversed, placing the anti-D2-CAM immunoprecipitate in the reference gel, indicating that anti-PSA reacted with D2-CAM. To test whether anti-PSA reacted with molecules other than D2-CAM, the system was reversed, placing the anti-D2-CAM in the intermediate gel and anti-PSA in the upper reference gel (Fig. 2a). A small precipitate was observed with anti-PSA, in addition to the anti-D2-CAM precipitate (see arrow, Fig. 2b).

**Table I**

| Radiolabel | Labeling period | Incorporation into D2-CAM* | Relative incorporation† |
|------------|-----------------|---------------------------|-------------------------|
|            | %               |                           |                         |
|            | A               | B                         | A/B                     |
| [35S]Methionine | 0.1–1           | 0.15 ± 0.002 (20)         | 40                      |
| [3H]Glucosamine   | 18              | 0.46 (3)                  | 33                      |
| [3H]Fucose              | 18              | 0.87 (2)                  | 38                      |
| [14C]Mannosamine        | 18              | 1.04 (1)                  | 37                      |
| [3H]Mannose                | 18              | <0.01 (1)                 | –                       |
| [35SO4]2-              | 2–6             | 3.5 ± 0.8 (5)             | 28                      |
| [35PO4]2-              | 2–6             | 0.24 ± 0.05 (4)           | 53                      |

* The radioactivity detected in the D2-CAM immunoprecipitate (1) is expressed as a percentage of the total trichloroacetic acid-precipitable radioactivity submitted to immunoelectrophoresis. Results are given as mean values ± SEM; number of experiments is given in parentheses.

† The radioactivity detected in the A or B bands eluted from SDS gels is expressed as the percentage of the total in (A + B). Results are given as mean values of one to three determinations.

Effects of Monensin on Intracellular Maturation of D2-CAM: The ionophore monensin causes vacuolization of the Golgi complex and prevents the transit of membrane proteins from the cis- to trans-Golgi compartments without interfering with other cellular events, such as protein synthesis (27). The effect of monensin on D2-CAM synthesis is shown in Fig. 1c. Cells were labeled by a 10-min pulse with [35S]methionine followed by a 50-min chase period. Synthesis in the presence of monensin (Fig. 1c, lane 2), yielded A and B polypeptides seen by SDS PAGE as narrow bands of lower molecular weight than those of the corresponding control (Fig. 1c, lane 1). Hence, the increase in band width observed with increasing time of posttranslational processing probably is due to complex glycosylation in the trans-Golgi compartment.

**Demonstration of Polysialic Acid in D2-CAM:** A polyspecific antiserum produced against polysialosyl chains purified from Escherichia coli (anti-PSA) (28) was employed to isolate polysialylated glycoproteins from [35S]methionine-labeled cell extracts. A comparison between anti-PSA and anti-D2-CAM was performed by immunoprecipitation in gel, using crossed immunoelectrophoresis with intermediate gel (Fig. 2a, a and b). Solubilized membranes from perinatal rat brain was the antigen tested against anti-PSA in the intermediate gel and anti-D2-CAM in the upper reference gel; a control experiment was performed in which the anti-PSA was omitted from the intermediate gel. Fig. 2a shows that anti-PSA was able to retract the D2-CAM precipitate from the reference gel, indicating that anti-PSA reacted with D2-CAM. To test whether anti-PSA reacted with molecules other than D2-CAM, the system was reversed, placing the anti-D2-CAM in the intermediate gel and anti-PSA in the upper reference gel (Fig. 2b). A small precipitate was observed with anti-PSA, in addition to the anti-D2-CAM precipitate (see arrow, Fig. 2b). Thus, it appears that anti-PSA reacts with D2-CAM and, also, with a population of other antigens that are unrelated to D2-CAM. Isolation of glycoproteins with polysialyl chains was performed by immunoelectrophoresis with anti-PSA or with protein A and anti-PSA. Several polypeptide bands were detected when biosynthetically radiolabeled anti-PSA immunoprecipitates were analyzed on SDS polyacrylamide gels (Fig. 2c). Bands were detected in two regions with molecular weights corresponding to D2-CAM polypeptides A and B, indicating that these represent D2-CAM with polysialyl immunoreactivity. The widths of the bands corresponded to the band patterns detected after prolonged biosynthetic labeling with [35S]methionine (cf. Fig. 1, lane 3). Anti-PSA also isolated other peptides, including four low-molecular-weight polypeptides (LMP). (Four similar [35S]methionine-labeled polypeptides with corresponding Mr of 42,000, 46,000, 52,000, and 58,000 were routinely observed previously (1) and in this study (Fig. 1a, lane 3), by immunosolubilization of anti-D2-CAM antibody. From these experiments it appears that D2-CAM A and B are polysialylated. However, other molecules from neurons are also apparently polysialylated, as revealed by the additional bands detected by LYLES ET AL.. Biosynthesis of D2-Cell Adhesion Molecule 2085
FIGURE 2. Identification of D2-CAM with an antibody against polysialic acid. (a) Comparison of anti-D2-CAM (a-D2-CAM) and anti-PSA (a-PSA) by crossed immunoelectrophoresis. The intermediate gel (I) contained no antibody (−) or anti-PSA as described in Materials and Methods. The reference gel (R) contained anti-D2-CAM. All D2-CAM-reactive species were removed from the reference gel and precipitated in the intermediate (+ a-PSA) gel. (b) Comparison of anti-D2-CAM and anti-PSA by crossed immunoelectrophoresis. In this experiment the intermediate gel (I) contained no antibody (−) or anti-D2-CAM. The reference gel (R) contained anti-PSA. Note the faint precipitate in the anti-PSA gel (arrow) indicating the presence of PSA-antigens unrelated to D2-CAM. (c) Analysis by SDS PAGE. [35S]methionine labeled D2-CAM from neuronal culture extracts was isolated by crossed immunoelectrophoresis employing anti-PSA; precipitates were submitted to SDS PAGE. Lane 1, 40 min pulse/60-min chase; lane 2, 18 h label. Bars indicate the positions of standard M, markers as in Fig. 1. Arrows indicate low Mr polypeptides similar to those identified by anti-D2-CAM.

SDS PAGE and by the immunoprecipitation experiment shown in Fig. 2b.

Sulfation of D2-CAM in Fetal Neurons in Culture

[35S]SO₄ was incorporated into polypeptides A and B (Fig. 3a). The fraction of radioactive [35S]SO₄ incorporated into D2-CAM compared with the amount incorporated into total protein was considerably higher than the incorporation of [35S]methionine into D2-CAM, Table I. The relative incorporation into B/A was 2.3. A broad band of [35S]SO₄ activity was observed at the top of the gel with M₆ of ca. 250,000-350,000. The amount of radioactivity detected in this high-molecular-weight component was 67% of the amount detected in A + B. This band was not observed after labeling with [35S]methionine or radioactive sugars.

To determine the subcellular site of D2-CAM sulfation, labeling was performed in the presence of monensin or tunicamycin (not shown). In the presence of monensin, hardly any [35S]SO₄ labeling of A and B was detected, whereas a slight labeling of the high molecular weight component was observed indicating that most of the sulfation of D2-CAM occurs in the trans-Golgi compartment. No sulfation of A or B was observed in the presence of tunicamycin, although a very slight labeling of the high molecular weight component was seen. Tunicamycin prevents the formation of complex N-linked oligosaccharides, suggesting that D2-CAM sulfation may occur on the oligosaccharide chains. However, tunicamycin has been reported to prevent transport of N-CAM to the cell surface (29). Hence, it may block D2-CAM sulfation by preventing transport through the Golgi apparatus.

To characterize further the sulfation of A and B, additional experiments were performed involving the digestion of [35S]SO₄-labeled D2-CAM with the proteoglycan-specific enzymes chondroitinase ABC and heparinase. Neither of the enzymes had any effect on the sulfation of A or B, indicating that they are not proteoglycans. [35S]SO₄-labeled D2-CAM was also submitted to mild acid treatment (1 M HCl for 30 min at 60°C), which has been shown to remove peptide-linked sulfate selec-
trypsin to remove surface-localized D2-CAM, tides. If however, neuronal monolayers were pretreated with correspond to those of the fully modified A and B polypep-

None of these inhibitors interfered concentration employed.) None of these inhibitors interfered the presence of tunicamycin, monensin, or cycloheximide.

The positions of A, B, and the high molecular weight component (H) are indicated.

tively, but not oligosaccharide-bound sulfate (30). This treatment removed all $^{32}$P$_{i}$ label from both A and B indicating that sulfation occurs on the polypeptide, rather than on the oligosaccharide, chains of A and B.

**Phosphorylation of D2-CAM in Fetal Neuronal Cultures**

Inorganic $^{32}$PO$_{4}$ was incorporated into D2-CAM A and B (see Table I and Fig. 3b). The ratio of relative radioactivity detected in B/A was 0.85, which is lower than the ratio of 1.5 obtained with $[^{35}S]$methionine. To determine the subcellular site of D2-CAM phosphorylation, labeling was performed in the presence of tunicamycin, monensin, or cycloheximide. (The latter reagent completely blocks protein synthesis in the concentration employed.) None of these inhibitors interfered with the overall incorporation of $^{32}$PO$_{4}$ into D2-CAM. Neither did they alter the relative labeling of B/A. The molecular weights of A and B were identical in all experiments and correspond to those of the fully modified A and B polypeptides. If however, neuronal monolayers were pretreated with trypsin to remove surface-localized D2-CAM, $^{32}$PO$_{4}$ incorporation was greatly diminished (Fig. 3b, lane 3). As a control to show that D2-CAM synthesis was unaffected by the trypsinization, cells were trypsinized and subsequently labeled for 2 h with $[^{35}S]$methionine. Total $[^{35}S]$methionine incorpora-

**Intracellular Transport of D2-CAM**

Endoplasmic reticulum to Golgi apparatus: D2-CAM has previously been shown to be initially synthesized with high-mannose cores, which can be detected by their sensitivity to degradation with the glycosidase, Endo H (1). This enzyme specifically degrades N-linked high-mannose cores with between 5 and 9 mannose residues; oligosaccharides with fewer or more mannose residues are not affected by Endo H. Because trimming of high-mannose cores to Man$_{3}$GlcNAc$_{2}$ occurs in the cis-Golgi compartment, we have examined the time course of the loss of sensitivity of D2-CAM to Endo H as a gauge of its transport from the endoplasmic reticulum to the Golgi complex (Fig. 4a). Almost all of the polypeptides in the A and B SDS gel bands were sensitive to degradation with Endo H after a 10-min pulse, whereas only part of each band was susceptible to degradation after a subsequent 10-min chase (i.e., 20 min after synthesis). It can be seen that the lower molecular weight regions of A and B in the 10-min pulse/10-min chase sample were sensitive to Endo H treatment, whereas the higher molecular weight regions of the bands were unaffected. Both A and B had lost their high-mannose cores, as indicated by their complete insensitivity to Endo H treatment, 20–30 min after synthesis. Therefore, D2-CAM polypeptides are apparently transported through the cis-Golgi compartment within 20 min of synthesis.

Appearance at the cell surface—Isolation of plasma membrane D2-CAM with antibody: Cell monolayers were $[^{35}S]$methionine radiolabeled and then incubated with anti-D2-CAM antibodies, which bound D2-CAM present on the cell surface. After cell solubilization, antibody–antigen complexes were isolated with Protein A-Sepharose. Radiolabeled A and B were detected on the surface 35 min after start of synthesis (Fig. 4b). The intensity of both increased with the length of the chase period.

Insertion of D2-CAM in the plasma membrane: Perinatal rat brain membranes were labeled with $^{125}$I by the chloramine T method and by photoactivation with TID. The former method labels proteins in general, whereas photoactivation with TID selectively labels integral membrane proteins in their lipophilic domains (23). Fig. 5a shows that both A and B were similarly labeled with $^{125}$I by both methods. The percentage of radioactivity incorporated into the D2-CAM immunoprecipitate, relative to total radioactive protein, was found to be 0.17% with chloramine T and 0.01% with TID. The lower relative radiolabeling of D2-CAM observed with TID may indicate that hydrophobic domains of A and B comprise a relatively small portion of the polypeptides.

Selective Extraction of LMP

The LMP may represent D2-CAM fragments or distinct polypeptides that are attached to D2-CAM. An aliquot of

LYLES ET AL. Biosynthesis of D2-Cell Adhesion Molecule 2087
Developmental Changes in the D2-CAM Biosynthesis

Explant cultures of forebrains from rats at embryonic day 17 and postnatal days 4, 8, and 25 were biosynthetically labeled with \([35S]\)methionine. A and B polypeptides were synthesized at all ages examined. In addition, a polypeptide of \(M_r\) 115,000 (C) and one to three discrete polypeptide bands with \(M_r\) between 2,500,000 and 3,500,000, the high-molecular-weight polypeptides (HMr), were observed at all ages. Peptide mapping of the individual HMr, A, B, and C polypeptides was performed according to Cleveland et al. (24). Similar chymotryptic degradation patterns for all four polypeptides were observed (Fig. 6b). No correlation was apparent between age and the size or number of different high-molecular-weight polypeptides. The molecular weight of polypeptides A, B, and C remained the same at all ages examined, although the widths of the A and B bands were found to decrease postnatally, which may reflect a decrease in glycosylation. The relative amounts of A and B decreased with age, while the relative proportion of C and HMr increased with age (Fig. 7).

Explant cultures of forebrains from rats at embryonic day 17 and postnatal days 4 and 8 were labeled with \([35S]\)sulfate. Sulfation of A and B polypeptides was detected in tissue from embryonic day 17; C and HMr polypeptides were faintly labeled. Almost no sulfation was detected at postnatal days 4 and 8.

Finally, explant cultures of forebrains from rats at embryonic day 17 and postnatal days 4, 8, and 15 were labeled with inorganic \([32P]\)phosphate. The A and B polypeptides were seen to be phosphorylated at all ages whereas no phosphorylation was detected of the C and HMr, polypeptides.

\(\text{\^{35S}}\)methionine-labeled cell extracts was treated with SDS for 3 min at 100°C; subsequently an excess Triton X-100 was added and D2-CAM was immunoisolated. The relative radioactivity in three of the four LMPs was reduced (Fig. 5b) by SDS treatment, therefore SDS treatment either detached these noncovalently linked polypeptides or, if they are D2-CAM fragments, destroyed their antigenicity. We also tried to separate the LMPs from D2-CAM under nondenaturing conditions: \(\text{\^{35S}}\)methionine-labeled cell monolayers were treated with 0.5 mM EGTA. The cells were partially lysed after the treatment. EGTA selectively extracted LMP, as shown in Fig. 3a, lanes 2 and 3.

\[\text{\^{35S}}\text{methionine-labeled cell extract was incubated with 2\% SDS at 100^\circ C for 3 min. Triton X-100 was added (final concentration 6\%). Carrier protein from perinatal brain extract was added to the sample and D2-CAM was immunoisolated. The positions of A, B, and LMP are indicated.}\]
after digestion with chymotrypsin (20 μg/ml): from the B polypeptide was not reproducibly detected. The extra fragment observed in the bands from CAM in explant cultures from rat forebrains. The general degradation pattern detected appeared similar to the general patterns observed for polypeptides A, B, and C. The extra fragment from the B polypeptide was not reproducibly detected.

**DISCUSSION**

In this report we have characterized the glycosylation of D2-CAM polypeptides A and B. In addition, we have shown that A and B undergo two other posttranslational modifications, sulfation and phosphorylation, that have not previously been reported for D2-CAM. A model illustrating D2-CAM biosynthesis and intracellular transport is shown in Fig. 8.

During D2-CAM synthesis in the endoplasmic reticulum, four to five high-mannose cores are co-translationally linked to asparagine residues on both polypeptides A and B (1). The high-mannose cores undergo a process of trimming in the cis-Golgi compartment. The loss of high-mannose cores, as judged by loss of sensitivity of A and B to Endo H, reflects the transport of both polypeptides through the cis-Golgi compartment within 20 min of translation.

After the trimming of high-mannose cores, the complex oligosaccharide chains on A and B are built up during their passage through the trans-Golgi compartment. This includes the incorporation of glucosamine, fucose, and terminal sugars, especially sialic acid. All polypeptides may not receive identical complex oligosaccharide chains, resulting in a final population of molecules with heterogenous sizes. This is observed in the synthesis of D2-CAM where broader molecular weight ranges are detected for A and B after 30 min of posttranslational processing. The partial binding of A and B to the Con A lectin may also be due to heterogeneity in glycosylation. Monensin, which inhibits the cis- to trans-Golgi transport of A and B, prevents their complex glycosylation and the resulting size heterogeneity. A novel type of oligosaccharide modification, that involves the sequential linkage of ~200 sialic acid residues has been described in E. coli. Recent evidence using an antibody raised against E. coli polysialic acid indicates that some proteins in rat brain contain up to four sialosyl residues in a similar polysialic α-2,8 ketosidic linkage (30, 31). We have found that the A and B polypeptides from rat neuronal cultures are also immunoisolated by this antibody which suggests that both polypeptides are polysialated. However, we cannot rule out the possibility that only one of the polypeptides might be polysialated, since both would be immunoisolated if they were joined by a noncovalent linkage. Additional evidence from biosynthetic labeling with mannoseamine indicates that both A and B are sialated. The relative labeling of A and B with the different radiolabeled sugars was similar to that seen with [35S]methionine, suggesting an equivalent level of glycosylation, including sialation, of A and B.

D2-CAM was found to be a highly sulfated protein, accounting for 2–4% of total sulfated proteins in cultured rat neurons. Sulfation occurred to A and B in the trans-Golgi compartment. The lability of the sulfate label under mild acid conditions (32), indicates that it is linked to the A and B peptide chains, rather than to oligosaccharides. A and B do not appear to be proteoglycans as indicated by their resistance to chondroitinase ABC and heparinase and the absence of O-linked oligosaccharides.

D2-CAM appears on the cell surface ~35 min after synthesis. Both A and B were shown to contain lipophilic domains with which they are inserted in the plasma membrane as integral membrane proteins. Phosphorylation occurs to both A and B when they are located at the plasma membrane. Polypeptide A was relatively more phosphorylated than B, as contrasted to amino acid or sugar incorporation. This suggests that A contains more sites for phosphorylation than B. Because phosphorylation is generally considered to occur on the

**FIGURE 6** (a) Biosynthetic labeling with [35S]methionine of D2-CAM in explant cultures from rat forebrains. HM, A, B, and C polypeptides are indicated. Lane 1, embryonic day 17; lane 2, postnatal day 8. (b) Peptide maps of the D2-CAM polypeptides after digestion with chymotrypsin (20 μg/ml): HM (lane 1), A (lane 2), B (lane 3), and C (lane 4). Only low levels of radioactivity were observed in the bands from HM (lane 1), however the general pattern detected appeared similar to the general patterns observed for polypeptides A, B, and C. The extra fragment from the B polypeptide was not reproducibly detected.

**FIGURE 7** Developmental changes in the biosynthesis of D2-CAM polypeptides in rat forebrains. The abscissa represents the animal age in days; embryonic day 17 is indicated (E17). The percentages of total disintegrations per minute in D2-CAM detected in the HM, A, B, and C polypeptides are shown on the ordinate (x ± SEM; n = 5 [E17], n = 7 [P4], n = 4 [P8 and P25], n = 1 [P15]).
cytoplasmic side of the plasma membrane, this may reflect a larger cytoplasmic domain on polypeptide A. Because phosphorylation plays a dynamic role in the regulation of several neuronal membrane proteins (33), the relatively higher phosphorylation of A may indicate different functional roles of the two polypeptides in cell–cell interactions.

A group of four LMPs were identified as being D2-CAM-associated. These polypeptides were invariably co-isolated with D2-CAM from [35S]methionine labeled neuronal cell extracts in similar relative amounts and have $M_r$ of 44,000, 48,000, 52,000, and 58,000. Previously these molecules were suggested as being degradation products of D2-CAM (1). However, LMP do not display several of the characteristic features of D2-CAM. They are not $N$-linked glycosylated, sulfated, or phosphorylated. They are not firmly attached to the plasma membrane, as indicated by their selective extraction with EGTA and lack of labeling with $^{125}$I-TID.

Trypsinization of intact cells did not alter the detection of these polypeptides (unpublished data). SDS treatment, however, did prevent their co-isolation with D2-CAM A and B. Our findings suggest that LMP are independent polypeptides that are noncovalently associated with D2-CAM and are thereby co-immunoprecipitated. This would explain their isolation by anti-PSA even though they apparently are not glycoproteins. Proteins with similar low molecular weight have been identified in neural tumor cell substrate adhesion sites and are thought to play a role in neural cell adhesion in vitro (13). Some of these proteins have been tentatively identified as cytoskeletal elements (e.g., actin and 10-nm filament subunit proteins). Fibronectin has also been proposed to interact with extracellular matrix and cytoskeletal components, thereby playing a role in both cell adhesion and cell motility (34, 35).

An explant culture system of rat forebrain was employed to study age-dependent changes in the biosynthesis of D2-CAM in tissue with preserved structure. Here D2-CAM was synthesized as several polypeptides: A ($195,000 M_r$), B ($137,000 M_r$), C ($115,000 M_r$), and one to three discrete HM polypeptides (between 250,000 and 350,000 $M_r$). Previous reports have indicated that a decreased sialylation of D2-CAM/N-CAM occurs with increasing age (7, 36). The reduced widths of the A and B polypeptide bands detected on SDS gels with increasing age probably reflect reduced glycosylation of these two polypeptides. The molecular weight of A synthesized in the fetal neuronal cultures was found to be 187,000 (1) which is significantly lower than the molecular weight of A determined in the explant culture system (using the lowest limit of SDS gel bands as a measure of molecular weight): the difference probably reflects further posttranslational modifications, especially glycosylation, of A in tissue with preserved structure. The molecular weight of B was found to be identical in the two systems. The C polypeptide was present in low amounts at embryonic and early postnatal ages; the amount of this polypeptide increased with age, relative to A and B. The C polypeptide is not observed in significant amounts in the fetal neuronal cultures. Neither is C detected in embryonic membrane extracts by immunoblotting, although C is detected by immunoblotting at later postnatal ages (3); D2-CAM has been demonstrated, not only in neurons, but also in astroglial cells (3, 37, Noble, M., M. Albrechtsen, C. Mølle, J. M. Lyles, E. Bock, M. Watanabe, and U. Rutishauser, submitted for publication). Explant cultures are composed of a variety of cell types, predominantly neurons and glial cells. In glial cells account for an increasing proportion with increasing postnatal age, the relative increase of C with age may reflect an increasing contribution of D2-CAM from glial cells. Alternatively, C may be derived also from neuronal cells and the relative increase of C could reflect an altering D2-CAM function during development.

HM, co-precipitates with the A, B, and C polypeptides during immunoprecipitation and exhibit similar peptide maps to A, B, and C, which indicates at least a partial identity of the different D2-CAM polypeptides. The relative biosynthesis of HM, was found to increase postnatally, comprising one third of immunoisolated D2-CAM at postnatal day 15. HM, thus accounts for a significant proportion of D2-CAM postnataIly.

The sulfation of D2-CAM decreased markedly during postnatal development. Decreased sulfation may be partially responsible for the age-related decrease in net charge observed as a decreased electrophoretic mobility of D2-CAM, which was previously only attributed to a decrease in sialylation (36). Because it has been proposed (16) that the net charge of D2-CAM plays a role in its ability to function in cell adhesion, sulfation may be important for the developmental regulation of D2-CAM function.

A and B were found to be phosphorylated in explant cultures at all ages examined with no apparent age-related changes, in contrast to glycosylation and sulfation. No phosphorylation of HM, and C polypeptides was observed, even at ages where these are predominant species of D2-CAM. The difference in the phosphorylation of the different D2-CAM polypeptides may reflect differences in their functional roles.

**Figure 8** A model of the posttranslational modifications and intracellular transport of D2-CAM.
Thus, the biosynthesis of the different D2-CAM polypeptides and their glycosylation and sulfation change with age. In contrast, phosphorylation does not appear to be altered with development indicating that this modification of D2-CAM, may be necessary for its functional role at all ages. We suggest that D2-CAM function may be regulated during development by variations in the relative amounts of the different D2-CAM polypeptides synthesized and by modifying their glycosylation and sulfation.

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REFERENCES

1. Lyles, J. M., B. Norrild, and E. Bock. 1984. Biosynthesis of the D2-cell adhesion molecule. Pulse chase studies in cultured fetal rat neuronal cells. J. Cell. Biol. 98:2077–2081.

2. Edelman, G. M. 1983. Cell adhesion molecules. Science (Wash. DC) 219:450–457.

3. Hirn, M., M. S. Ghandour, H. Deagostini-Bazin, and C. Goridis. 1983. Molecular heterogeneity and structural evolution during cerebellar ontogeny detected by monoclonal antibody of the mouse cell surface antigen B6/12. Brain Res. 265:87–100.

4. Jørgensen, O. S., A. Delouvec, J.-P. Thibery, and G. Edelman. 1980. The nervous system specific protein D2 is involved in adhesion among neurites from cultured rat ganglia. FEBS Lett. 111:39–42.

5. Olden, K. R., M. Pratt, and K. M. Yamada. 1978. Role of carbohydrates in protein secretion and turnover: effects of tunicamycin on the major cell surface glycoprotein of chick embryo fibroblasts. Cell. 13:461–473.

6. Raivala, H., W. G. Carter, and S. J. Hakomori. 1981. Studies on cell adhesion and recognition. 1. Extent and specificity of cell adhesion triggered by carbohydrate reactive proteins (glycosidases and lectins) and by fibronectin. J. Cell. Biol. 88:127–137.

7. Rothbard, J. B., R. Brackenbury, B. A. Cunningham, and G. M. Edelman. 1982. Differences in the carbohydrate structures of cell-cell adhesion molecules from adult and embryonic chicken brains. J. Biol. Chem. 257:1064–1069.

8. Huttner, W. B. 1982. Sulphation of tyrosine residues. A widespread modification of proteins. Nature (Lond.) 299:273–276.

9. Margolis, R. K., S. R. J. Salton, and R. U. Margolis. 1983. Structural features of the nerve growth factor inducible large external glycoprotein of PC 12 phaeochromocytoma cells. Cell. 32:1622–1629.

10. Yamada, K. M. 1983. Cell surface interactions with extracellular materials. Annu. Rev. Biochem. 52:761–799.

11. Silberstein, G. B., and C. W. Daniel. 1980. The nervous system role in locomotion and anchorage of primary chick fibroblasts and can promote entry into the division cycle. J. Cell. Biol. 91:403–410.

12. Gilbert, W. J., R. C. Couchman, R. A. Badley, H. J. Saunders, and C. G. Smith. 1983. Fibronectin in cultured rat keratinocytes: distribution, synthesis and relationship to cytoskeletal protein. Eur. J. Cell Biol. 34:205–213.

13. Culp, L. A., R. Ansbacher, and C. Domen. 1980. Adhesion sites of neural tumor cells: biochemical composition. Biochemistry. 19:5899–5907.

14. Harrison, D., and L. A. Culp. 1983. Adhesion responses of fibroblast and neuroblastoma cells to substrate coated with polyvalent or monovalent antibody to fibronectin. Exp. Cell Res. 146:29–42.

15. Greengard, P. 1978. Phosphorylated proteins as physiological effectors. Science (Wash. DC) 199:146–152.

16. Hoffmann, S., and G. M. Edelman. 1983. Kinetics of homophilic binding by embryonic and adult forms of the neural cell adhesion molecule. Proc. Natl. Acad. Sci. USA 80:5762–5766.

17. Hansen, O. C., O. Nybroe, and E. Bock. 1984. Cell-free synthesis of the D2-cell adhesion molecule: evidence for three primary translation products. J. Neurochem. In press.

18. Rasmussen, S., J. Ramlau, N. H. Axelsen, and E. Bock. 1982. Purification of the neural membrane glycoprotein D2 from rat brain. Scand. J. Immunol. 15:179–185.

19. Bottenstein, J. E., D. S. Skipper, S. S. Varon, and H. G. Sato. 1980. Selective survival of neurons from chick sensory ganglionic dissociates utilizing serum-free supplemented medium. Exp. Cell Res. 125:13–23.

20. Axelsen, N. H. 1983. Electromembranoprecipitation: principles and fundamental methods. Scand. J. Immunol. 17 (Suppl. 10):87–171.

21. Fitting, T., and D. Kabat. 1982. Evidence for a glycoprotein "signal" involved in transport between subcellular organelles. J. Biol. Chem. 257:14001–14007.

22. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1983. The preparation of [35S]-labelled human growth hormone of high specific radioactivity. Biochem. J. 209:14–123.

23. Brunner, J., and G. Semenza. 1981. Selective labeling of the hydrophobic core of membranes with 3-(methylmethotanyl)-3-(m-iodophenyl) diazirane, a carbene generating reagent. Biochemistry 20:774–1781.

24. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecylsulfate and analysis by gel electrophoresis. J. Biol. Chem. 252:102–106.

25. Kornfeld, R., and S. Kornfeld. 1980. Structure of glycoproteins and their oligosaccharide units. In The Biochemistry of Glycoproteins and Proteoglycans. W. J. Lessner, editor. Plenum Press, New York. 1–34.

26. Nakamura, K., and R. W. Compans. 1978. Effect of glucosamine, 2-deoxyglucose, and tunicamycin on glycosylation, sulfation and assembly of influenza and viral proteins. Virology 84:303–319.

27. Tartschakoff, A. M. 1983. Purification of vesicular traffic with the carbosylic isomerophore ammonium. Cell. 32:1026–1028.

28. Rube, T. E., and F. A. Troy. 1980. Structure and biosynthesis of surface polymers containing polysialic acid in Escherichia coli. J. Biol. Chem. 6:2332–2342.

29. Cunningham, B. A., S. H. Raff, U. Ruhstaller, J. J. H. Hemperly, and G. M. Edelman. 1983. Molecular topography of the neural cell adhesion molecule N-CAM: surface orientation and location of sialic acid-rich and binding regions. Proc. Natl. Acad. Sci. USA 80:3116–3120.

30. Vimr, E., R. D. McCoy, H. F. Volger, N. W. Wilkinson, and F. A. Troy. 1984. Use of prokaryotic-derived probes to identify poly (sialic acid) in neonatal neuronal membranes. Proc. Natl. Acad. Sci. USA. In press.

31. Freme, J. U., F. Finnet, H. Deagostini-Bazin, and C. Goridis. 1983. Occurrence of 2-4 linked poly saccharides in a neural cell adhesion molecule. Biochem. Biophys. Res. Commun. 112:482–487.

32. Stone, G. G., R. Hammerschlag, and J. A. Bobinski. 1983. Fast-transported glycoprotein and nonglycosylated proteins contain sulfate. J. Neurochem. 41:1085–1089.

33. Kennedy, M. B. 1983. Experimental approaches to understanding the role of protein phosphorylation in the regulation of neuronal function. Annu. Rev. Neurosci. 6:493–525.

34. Couchman, J. R., D. A. Rees, R. Green, and C. G. Smith. 1983. Fibronectin has a dual role in locomotion and anchorage of primary chick fibroblasts and can promote entry into the division cycle. J. Cell. Biol. 93:403–410.

35. Brown, W. J., R. C. Couchman, R. A. Badley, H. J. Saunders, and C. G. Smith. 1983. Fibronectin in cultured rat keratinocytes: distribution, synthesis and relationship to cytoskeletal protein. Eur. J. Cell Biol. 36:205–213.

36. Edelman, G. M., and C. M. Chuong. 1982. Embryonic to adult conversion of neural cell adhesion molecules in normal and staggerer mice. Proc. Natl. Acad. Sci. USA 79:7035–7040.

37. Langley, R. G., K. Gombos, M. Hirm, and C. Goridis. 1983. Distribution of the neural antigen B5BP2 in the cerebellum during development. Int. J. Dev. Neurosci. 1:393–401.