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ECTOGLYCOSYLTRANSFERASE ACTIVITIES AT THE SURFACE OF CULTURED NEURONS

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SUMMARY : Glycosyltransferase activities (ectogalactosyl, ectofucosyl and ectosialyl) were studied at the external surface of exclusively neuronal cultures. An appropriate methodology gave the possibility to eliminate sources of errors due to the hydrolysis of nucleotide sugar substrates or due to cellular uptake of free sugars. Ovomucoïd and asialofetuin coupled to Sepharose and Ultrogel beads were used as exogenous substrate to circumvent possible substrates pinocytosis. Ectoglycosyltransferase activities were studied as function of protein concentration, incubation time and amount of bead coupled exogenous acceptors. The data show that these enzymes are present at the external surface of the neuronal membrane ; their possible role in cell - cell interactions is suggested.

An increasing amount of evidence suggests that glycoconjugates (glycoproteins and glycolipids) may play an important role at the external surface of the cell membrane. In particular, sialoglycoconjugates and surface glycosyltransferases (ectoglycosyltransferases) seem to be involved in surface mechanisms such as electrochemical potentials (1), membrane permeability (2), cell to cell recognition and interactions (3), contact inhibition (4) and crypticity of immunogenic loci (5). In nerve cells a large amount of gangliosides (sialoglycolipids) and sialoglycoproteins are present in the plasma membranes, particularly at the synaptic junctions (for review, see 6 and 7). Glycoconjugates may also be involved in the formation of neuronal circuits and in the function and activity of synapses (2, 8). Gangliosides in particular seem to play an important role in cell differentiation and in carcinogenesis as suggested by the observed loss of polysialogangliosides in tumoral cells (9, 10).

The presence of ectoglycosyltransferase activities has been shown at the surface of different cell types such as nerve cell cultures from chick embryo retinas (11), lymphocytes (12, 13) and leukemic cells (14).

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However, an unequivocal demonstration of the existence of ectoglycosyltransferases is difficult to achieve and misleading conclusions can be drawn (15). In the present paper, we report the existence and the kinetics of ectogalactosyl-, ectofucosyl- and ectosialyltransferases at the surface of neuronal cells ; the methodology employed in this study excludes possible sources of errors.

MATERIALS AND METHODS

Neuronal cell cultures

Neurons of cerebral hemispheres from 8 day-old chick embryos were cultured on poly-L-lysine coated Petri dishes (10, 6 and 3.5 cm) as it has been previously described (16).

Materials

UDP-(^{14}C)-galactose (302 mCi/mmol), CMP-(^{14}C)-sialic acid (262 mCi/mmol), GDP-(^{14}C)-fucose (30 mCi/mmol), (^{14}C)-galactose (95 mCi/mmol), (^{14}C)-sialic acid (214 mCi/mmol), (^{14}C)-fucose (57 mCi/mmol) were purchased from Amersham. Cold UDP-galactose, CMP-sialic acid, galactose, fucose, sialic acid (NeuAc), fetuin and ovomucoid were obtained from Sigma. Ovomucoid and asialofetuin coupled to Sepharose 4B and Ultrogel beads were prepared as described by March *et al.* (17) and Weston and Avrameas (18).

Ectoglycosyltransferase assays

Cells were carefully washed 3 times with 10 ml serum free Dulbecco's Modified Eagle Medium (DMEM) and incubated in the Petri dish. For 10 cm diameter Petri dish, the incubation medium in 1 ml DMEM was the following :

- galactosyltransferase assay : 20 mM $MnCl_2$, 1 mM galactose, 1 mM UDP-(^{14}C)-galactose (1.5 µCi), 25 mg of bead coupled ovomucoid.
- fucosyltransferase assay : 20 mM $MnCl_2$, 1 mM fucose, 20 µM GDP-(^{14}C)-fucose (0.6 µCi), 2.5 mg of bead coupled asialofetuin.
- sialyltransferase assay : 20 mM $MnCl_2$, 1 mM NeuAc, 1 mM CMP-(^{14}C)-NeuAc (1.5 µCi), 2.5 mg of bead coupled asialofetuin.

After various periods of incubation at 37°C in a humidified atmosphere of 95 % air and 5 % CO_2, the medium containing the Sepharose beads was collected while the cell monolayer was washed with 0.147 M NaCl. The beads remaining in the washing eluates were added to the medium fraction. The beads contained in the pooled medium and washing solution were washed 10 times by centrifugation (1 500 xg, 5 min), using 6 ml of a solution of saturated NaCl, collected on glass filters (Whatman GF/B) and finally dried with absolute ethanol.

Neurons were collected in 1.5 ml of 0.147 M NaCl. Aliquots were used for protein determination (19) and the remaining was precipitated with 10 ml of 10 % TCA and washed on GF/B glass filters 10 times using 10 ml of 5 % TCA and 10 times using 10 ml of water and finally by absolute ethanol.

The filters containing the beads and the cell precipitates were then dried under an infrared lamp, introduced in a vial containing 10 ml of Rotiszint 22 (Roth), and the radioactivity was determined with an Intertechnique SL 30 scintillation spectrometer.

Measurement of the sugar-nucleotide hydrolysis

After the incubation performed as described in the glycosyltransferase assays, the medium was collected ; the remaining (^{14}C)-sugar-nucleotides and the free (^{14}C)-sugars obtained from the hydrolysis of (^{14}C)-sugar nucleo-

tides were analysed by paper chromatography (Whatman 3) developed in pyridine-ethyl acetate-acetic acid-water (5:5:1:3 by vol.). The radioactivity contained in each compound was determined with a TLC scanner (LB 2820 Berthold).

Uptake of the liberated free sugars into neurons

In order to determine the possible entry into the cells of sugars resulting from sugar nucleotide hydrolysis, cells were incubated in the Petri dishes with 1 ml DMEM containing 20 mM $MnCl_2$ and 1 mM (^{14}C)-galactose or (^{14}C)-fucose or (^{14}C)-NeuAc for 60 min as described in the glycosyltransferase assays. After incubation and washing with 0.147 M NaCl, the radioactivity associated to the cells was determined as described above.

RESULTS

After 60 min of incubation the analysis of the residual sugar nucleotides and the liberated free sugars in the medium by paper chromatography indicated that 40 %, 12 % and 31 % of the substrates UDP-(^{14}C)-Gal, GDP-(^{14}C)-fucose and CMP-(^{14}C)-NeuAc were metabolized respectively. 0.5 %, 7.4 % and 5.4 % of these metabolized nucleotide-sugars led to the formation of respectively free (^{14}C)-galactose, (^{14}C)-fucose and (^{14}C)-NeuAc which might have been then entered into the cells and might be used for the glycosylation of endocellular acceptors. In order to measure this possible source of error cells were incubated with free (^{14}C)-galactose, (^{14}C)-fucose and (^{14}C)-NeuAc (in amounts corresponding to the 0.5 %, 7.4 % and 5.4 %) after addition of cold galactose, fucose and NeuAc (up to a total concentration of 1 mM, see enzyme assays) which were used in order to reduce the specific activity of labelled sugars which enter into the cells. The results showed that 0.12 %, 0.006 % and 0.006 % of the free sugars were incorporated respectively into cellular glycoconjugates, and that these values represented at a maximum 12.7 %, 1.4 % and 8.3 % of the respective total ectogalactosyl-, ectofucosyl- and ectosialyltransferase activities measured with intact cells in the experimental conditions defined in this study.

Table 1 shows the activities (per dish of 60 mm Ø) of the three glycosyltransferases towards cellular endogenous and exogenous bead coupled acceptors. In each case, the glycosylation of exogenous acceptors were strikingly higher than that observed for endogenous acceptors.

In order to determine the transglycosylation effect due to enzymes eventually released from intact and/or endommaged cells, the enzymatic

TABLE 1. ECTOGLYCOSYLTRANSFERASE ACTIVITIES AT THE SURFACE OF NEURONAL CELLS

	GALACTOSYLTRANSFERASE		FUCOSYLTRANSFERASE		SIALYLTRANSFERASE	
	Cellular acceptors	Bead coupled ovomucoïd	Cellular acceptors	Bead coupled asialofetuin	Cellular acceptors	Bead coupled asialofetuin
Standard assay Incubation 60 min	161	487	215	1073	151	304
(A) Incubation 0 min (control)	61	108	63	132	75	77
(B) Incubation 60 min without cells (control radioactivity adsorbed)	-	118	-	121	-	75
(C) Preincubation 60 min and incubation 60 min without cells*	-	136	-	110	-	75

Glycosyltransferase assays were performed as described in Materials and Methods. Activities towards endogenous and exogenous acceptors are expressed as cpm (^{14}C)-sugar incorporated/dish and are the means of two experiments.

*In this assay, intact cells were incubated 60 min with 1 ml DMEM containing 20 mM $MnCl_2$ and 1 mM free sugar; the medium was then reincubated with the labelled sugar nucleotide and the bead coupled acceptors, in order to determine the activity due to some intracellular glycosyltransferase eventually released into the medium.

assays were performed in the media in which we preincubated with neuronal cultures for 60 min (Table 1,C). The activities attached to the bead coupled acceptors were, under these conditions, about 20 %, 10 % and 25 % of those obtained in presence of intact cells for galactosyl-, fucosyl-, and sialyl-transferase respectively. These values were close to those found with the exogenous acceptors when the incubation was performed the closest to zero time (Table 1,A) and when the incubation was performed without cells (Table 1, B). Thus it appears that extremely low level of glycosylation, if any, may be due to the release of enzymes from the cells.

Figure 1 shows the kinetics of enzymatic activities varying the protein concentration (A), incubation time (B) and the amount of bead coupled exogenous acceptors (C). The activities of ectogalactosyltransferase (GT), ectofucosyltransferase (FT) and ectosialyltransferase (ST) measured with the bead coupled exogenous acceptors are higher than those measured with endogenous acceptors of the cell surface. The activities increased linearly as a function of protein concentration (A). The activities of GT,

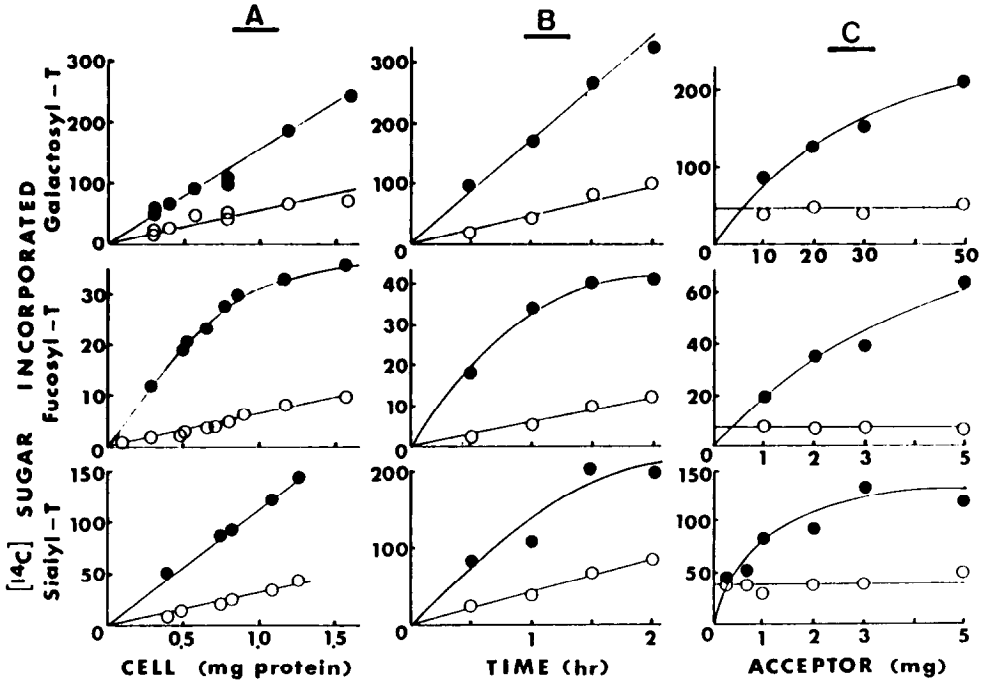

FIGURE 1

Kinetics of ectogalactosyltransferase (galactosyl-T), ectofucosyltransferase (fucosyl-T), and ectosialyltransferase (sialyl-T).
o——o : transfer of glycosyl units onto surface endogenous cellular acceptors
●——● : transfer of glycosyl units onto exogenous insoluble acceptors (ovomucoïd linked to Sepharose 4B beads for galactosyl-T and asialofetuin linked to Ultrogel beads for fucosyl-T and sialyl-T).

Left column (A) : effect of protein concentration. The cells (7 day-old cultures) were incubated 60 min in 3 different sized dishes (3.5, 6 and 10 cm diameter) with similar cell density in order to have different protein concentration using 25 mg (galactosyl-T) and 2.5 mg (fucosyl-T and sialyl-T) or bead coupled acceptors. The assays were performed as described in Materials and Methods and the activities (in ordinate) are given in 10^{-12} moles/h.

Center column (B) : Effect of incubation time. The cells (7 day-old cultures) in 10 cm diameter dishes were incubated in the same conditions as in (A) for different periods. The assays were performed as described in Materials and Methods and the activities (in ordinate) are the means of two experiments given in 10^{-12} moles/mg protein.

Right column (C) : Effect of concentration of bead coupled exogenous acceptors. The cells (7 day-old cultures) in 10 cm diameter dishes were incubated 60 min with increasing concentrations of ovomucoïd (galactosyl-T) and asialofetuin (fucosyl-T and sialyl-T). The assays were performed as described in Materials and Methods and the activities (in ordinate) are the means of two experiments given in 10^{-12} moles/mg protein/h).

FT and ST showed also a linear increase towards endogenous acceptors as a function of the incubation time (0-120 min) (B). With the exogenous acceptor, this linearity was observed until 120 min for GT and until 90 min and 60 min for ST and FT respectively. After 60 min of incubation and with 25 mg of bead coupled ovomucoïd and 2.5 mg of bead coupled asialofetuin,

the ectoglycosyltransferase activities of GT and ST with exogenous acceptors were 3 fold higher while, in the case of FT, 5 fold higher than those measured with the endogenous acceptors. The transfer of the labelled sugar on exogenous acceptors increased with increasing amounts of the substrates. A plateau was reached at 3 mg/ml of bead coupled asialofetuin for ST. Concerning FT and GT, the plateau was not yet reached with 5 mg/ml of bead coupled asialofetuin and 50 mg/ml of bead coupled-ovomucoid. Higher amounts of exogenous substrates however were not tested because the amounts used were already maximal to cover the cell monolayer at the bottom of the Petri dish.

DISCUSSION

Roseman (3) and his group (10) have suggested that glycosyltransferase activities may exist at the surface of cell membranes and might therefore be involved in cell to cell recognition and adhesion. Ultrastructural and biochemical evidence have shown the existence of ectoglycosyltransferase activities in various cell types (11-13). In the central nervous system, these enzymes may be involved in the metabolism of membrane glycoconjugates which are known to have a central role in the regulation of membrane function (see for example 20). The present data indicate the existence, at the neuronal surface of UDP-galactose:galactosyltransferase, GDP-fucose: fucosyltransferase and CMP-NeuAc:sialyltransferase activities which transfer galactose, fucose and sialic acid units on endogenous membrane acceptors and possibly on acceptors present on the membrane of other cells.

Some caution should however be taken when asserting the existence of ecto-membrane enzyme activities because of possible sources of error when ectoglycosyltransferase activities are measured after incubation of intact neurons with labelled nucleotide sugars (for review, see 15, 21).

In the present study, the cellular uptake of free labelled sugars produced by the hydrolysis of nucleotide sugars by membrane-bound pyrophosphatases and phosphatases was examined and the maximal error was found to be 12.7 %, 1.4 % and 8.3 % of the glycosyltransferase activity for ectogalac-

tosyl-, ectofucosyl- and ectosialyltransferase respectively. These values were taken into consideration in the final calculation.

A possible alteration of the cell integrity was also excluded since under the present experimental conditions, the exclusion of trypan blue was observed in 99.5 % of the cells, while the presence in the incubation medium of enzymatic activities eventually released from the cells was negligeable (close to the blank values, Table 1,C). The possibility of a pinocytosis of exogenous acceptors and their further excretion into the medium was discarded using the resin bead-coupled acceptor. Finally, a possible adsorption at the cell surface of glycosyltransferases present in the calf serum used in the culture medium was also eliminated since among the three glycosyltransferase activities, those of fucosyl- and sialyltransferase are very low in the serum. Moreover the three ectoglycosyltransferase activities were present and actually increased when the cells were grown from the 2nd to the 7th day of culture in the synthetic medium described by Sato (22) in the absence of fetal calf serum (unpublished data).

Chick neurons in culture thus appear to possess ectogalactosyl-, ectofucosyl- and ectosialyltransferase activities capable to transfer glycosyl residues upon glycoproteic or glycolipidic substrates and/or upon exogenous acceptors via a transglycosylation. These results further suggest that the ectoglycosyltransferases present on a neuronal cell membrane may utilize the glycoconjugates present on the membrane of another cell as substrate. Thus, enzymatic interaction might occur between cells suggesting an additional role of ectoglycosyltransferases in cell-cell interaction ; these enzymes may also act in repair mechanism (such as it has been observed for DNA strands, 23) after local damage of plasma membranes.

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