NEW EVIDENCE FOR CELL SURFACE GALACTOSYLTRANSFERASE

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Received 12 January 1976

1. Introduction

Since Roseman [1] has proposed implications of ectoglycosyltransferase systems in intercellular adhesion and cell recognition, many works tend to demonstrate the occurrence of cell surface glycosyltransferase ([2,7] and for review, see ref. [8]). Except for ectosialyltransferase for which ultrastructural evidence has been obtained [9] the biochemical demonstration for ectoglycosyltransferase is not without certain failure. Recently, Keenan and Morre [10] reviewed the different reasons which can be misleading to conclusive demonstration for the occurrence of this enzymatic system on the outer surface of the plasma membrane. One way to characterize these enzymes is to isolate plasma membranes, however, this approach may be faulty by the contamination with intracellular membrane associated enzymes. Moreover it does not prove the external orientation of the active sites. These are the main reasons why most authors deal with intact cell systems. As it has been pointed out by Keenan and Morre [10], incubation of cells with nucleotide sugars with or without exogenous acceptors leads to the following causes of errors: (i) precursor hydrolysis and entry of the free carbohydrate into the cells, (ii) release or secretion of intracellular glycosyltransferases, (iii) exogenous acceptors glycosylation may be due to phagocytosis or pinocytosis and later excretion in the incubation medium.

Being aware of these facts, we have developed a new methodology to demonstrate clearly the occurrence of ectogalactosyltransferase. The use of acceptors coupled on Sepharose beads far larger in size than the cells allows us to eliminate the third remark. Measurement of the galactosyl transferase activity in the post incubation supernatant medium allows us to answer the second point. Finally, separation of cells from the insoluble acceptors after incubation enables us to discriminate between the glycosylation of the acceptor and the incorporation by the cell.

2. Materials and methods

2.1. *Preparation of cells*

To test the validity of our method we used cells for which ectogalactosyl transferase activities have been mentioned [5] i.e. lymphocytes. Spleen lymphocytes were prepared from three months old Sprague–Dawley rats according to Lamont et al. [5]. Erythrocytes lysis was achieved through a 15 min incubation in 0.154 M ammonium chloride in the presence of 10% fetal calf serum (Novogrodsky, personal communication).

2.2. *Preparation of the acceptor*

According to Spik et al. (unpublished results) ovomucoid is a good galactose acceptor as each of its glycan moieties possesses 5 GlcNAc residues in non-reducing terminal position [11]. Ovomucoid prepared according to Fredericq and Deutsch [12] was coupled to Sepharose 4B by the CNBr method described by March et al. [13]. Fifty miligrams of glycoprotein were fixed per ml of Sepharose beads.

2.3. *Galactosyl transferase assays*

The incubation mixture contained 0.1 M sodium

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cacodylate pH 7.4, 0.154 M NaCl, 10 mM $MnCl_2$, 10 μM UDP-[^{14}C]galactose (Amersham, brought to the specific activity of 100 mCi/mM with unlabeled UDP-Gal from Sigma). For a final volume of 50 μl, standard assays contained 6 X 10^6 cells and, either 20 μl of insolubilized acceptor or 300 μg of native ovomucoid. Incubation was achieved at 37°C, with a constant slow rotation of the tube to be sure of a good contact between cells and beads.

2.4. *Separation of cells from acceptors*

When native ovomucoid was used, cells were sedimented by low speed centrifugation. Acceptor containing supernatant was precipitated by the addition of 2 ml of cold 5% phosphotungstic acid in 2 M NaCl. The precipitate was collected on a glass fiber filter (Whatman GF/83) and extensively washed with the above phosphotungstic solution and then with cold absolute ethanol. Cells were washed in 0.154 M NaCl, then precipitated and collected as above.

When Sepharose-bound ovomucoid was used, the separation was achieved through adding 200 μl of 40% metrizamide (Nyegaard and Co.) and a further 5 min 5000 *g* centrifugation. In the final 32% metrizamide medium, the floating cells were pipetted, washed in 0.154 M NaCl, precipitated and collected as above. The pelleted beads were washed with 1.5 M NaCl to avoid any radioactive precursor adsorption and collected on glass fiber. They are extensively rinsed with 1.5 M NaCl and then with ethanol. Radioactivity of the collected materials was determined by counting in scintillation liquid.

2.5. *Analysis of nucleotide sugar degradation*

Several authors [14,15] have suggested that cell surface nucleotide pyrophosphatase degrading the sugar nucleotides would prevent the detection of ecto-glycosyltransferase activities. Consequently precursor degradation has been monitored during the incubation period by using paper chromatography developed for 18 h, in the following solvent: ethyl acetate–pyridine–glacial acetic acid–water (5:5:1:3) as recommended by Spik and Six (unpublished results). A strip along the chromatographic path is cut into 1 X 5 cm bands, radioactivity of which was determined by counting in toluol based scintillation medium.

3. Results and discussion

3.1. *Conditions for galactosyltransferase activity with native ovomucoid.*

Preliminary studies and determination of various parameters of the glycosylation process have been realized with native ovomucoid. Fig.1 reports a time-dependent incorporation of galactose both on exogenous ovomucoid and by cells, showing that ovomucoid can be used as an acceptor for the lymphocyte galactosyltransferase system. As shown in fig.2a, optimal galactose transfer on ovomucoid is obtained with a final concentration of 10^8 cells/ml, which is not the case for the galactose incorporated by the cells. These results may be accounted for by the fig.2b: the plateau observed for transfer activity is due to an extensive degradation of UDP-Gal when cell concentration exceeds 10^8 cells/ml. Moreover, similarity is observed between the increasing cell incorporated radioactivity and free galactose formation from UDP-Gal via Gal-1-P. These facts suggest that incorporation by the cells is due, to a certain extent, to galactose entry and its incorporation by the cells. The effect of ovomucoid concentration on the transfer reaction is shown in fig.3: while a plateau is observed from the concentration of 8 mg of ovomucoid per ml, the galactose incorporated by the cells remains

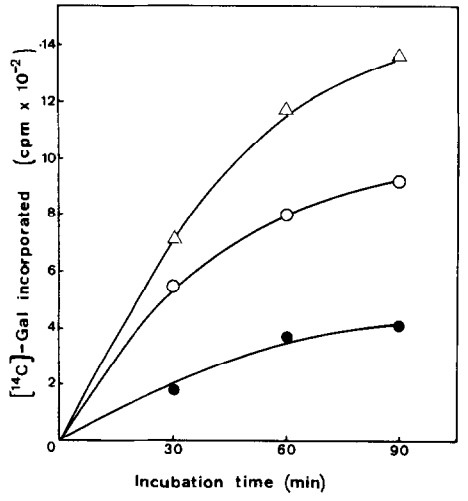

Fig.1. Kinetic study of the incorporation of radioactive galactose on native ovomucoid (●—●), by lymphocytes (○—○) and for total incorporation, cells plus acceptors (△—△). The final cell concentration was 6 X 10^7 cells/ml.

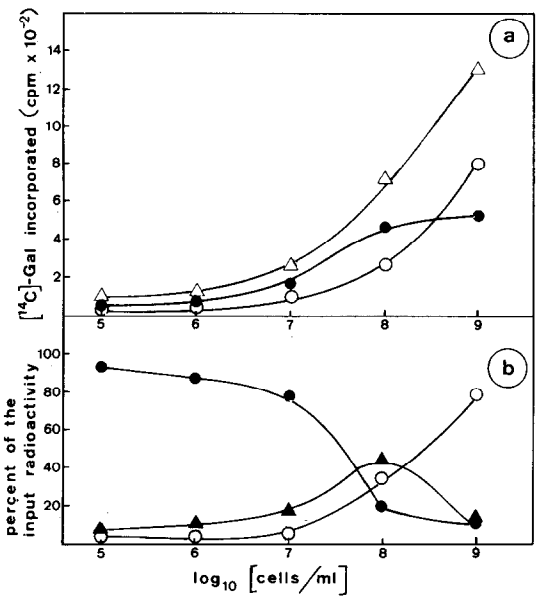

Fig.2(a) Effect of cell concentration on the incorporation of radioactive galactose on native ovomucoid (●—●) by the lymphocytes (○—○) and for total incorporation, cells plus acceptors (△—△). (b) Effect of cell concentration on the UDP-Gal degradation (●—●), free galactose appearance (○—○) and Gal-1-P evolution (▲—▲). Assays were made in the standard conditions for a 60 min incubation time.

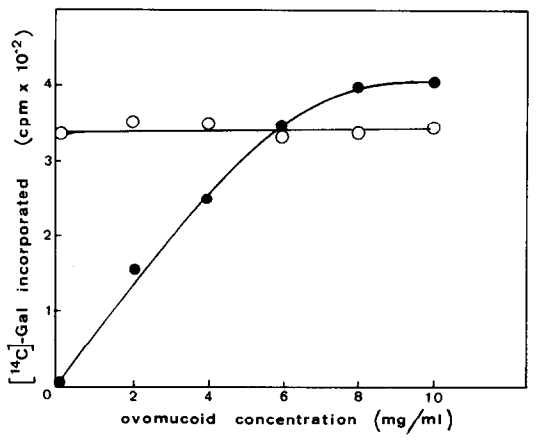

Fig.3. Effect of the native ovomucoid concentration on the incorporation of radioactive galactose on native ovomucoid (●—●), and by the lymphocytes (○—○).

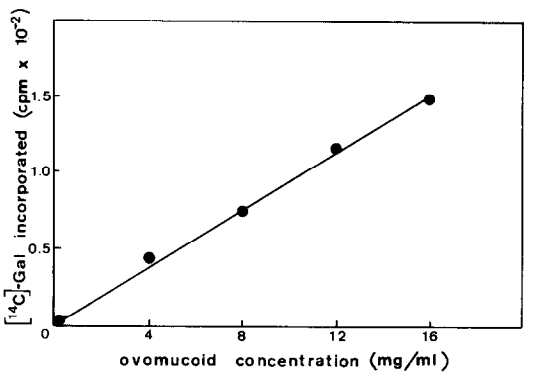

Fig.4. Radioactive galactose transfer reaction measured with increasing amounts of insolubilized ovomucoid. 15 μl of ovomucoid coupled beads represent an acceptor concentration of 4 mg/ml. The cell concentration was 10^8 cells/ml in a final volume of 75 μl. 200 cpm correspond to the transfer of 1 pM of galactose.

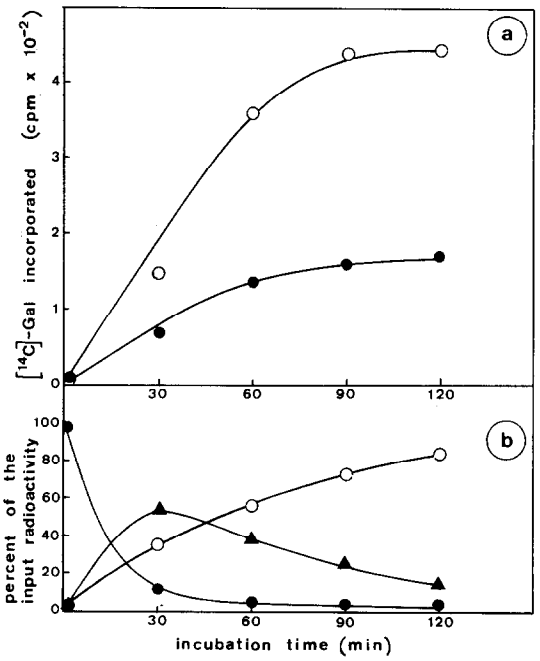

Fig.5(a) Kinetic studies of the incorporation of radioactive galactose on the insolubilized ovomucoid (●—●), by the lymphocytes (○—○). (b) Kinetic studies of the UDP-Gal degradation (●—●), of the free galactose appearance (○—○) and of the Gal-1-P evolution (▲—▲). The cell concentration was 10^8 cells/ml. 20 μl of ovomucoid coupled beads were used in a final volume of 50 μl.

constant and is not competed by the transfer reaction, which suggests again two separate phenomena.

The experiments described above are those commonly used by authors, yet, they do not allow any discrimination between intracellular and cell surface glycosyl transferases. Using these precedently determined parameters, we have measured the ability of intact lymphocytes to catalyse the transfer of galactose from UDP-Gal to nonphagocytosable ovomucoid-coupled beads.

3.2. *Ectogalactosyltransferase activity measured with exogenous insolubilized acceptors*

The transfer activity is proportional to the amount of ovomucoid-coupled beads added to the incubate with the cells. In this case, up to 16 mg/ml, no plateau is reached; this can be due to the fact that reaction does not depend on the acceptor concentration but rather on the number of true contacts between cells and beads. The transfer reaction is time-dependent as shown in fig.5. As previously observed, the transfer of galactose on immobilized ovomucoid depends on the UDP-Gal really present during the incubation (i.e. not yet degraded) and the incorporation by the cells follows the free galactose formation.

The zero-time incubation shows that there is no unspecific adsorption of radioactive material on the immobilized ovomucoid. These blank values are also shown in table 1a, where incubation of radioactive UDP-Gal is made either with non-coupled beads and cells or with coupled beads but no cells.

These results show that there is an effective glycosylation outside the cells. Moreover, as shown in table 1b, no detectable incorporation is observed on the insolubilized ovomucoid when incubation is made with the supernatant of the cell plus beads medium before or during incubation. On the contrary, after removing the supernatant, the whole galactosyltransferase activity is recovered within the cell plus beads pellet mixture. These results definitely exclude

Table 1

		Incubation time (min)	Radioactive galactose transferred on ovomucoid coupled beads (cpm)	Radioactive galactose incorporated by the cells (cpm)
(a)	Standard mixture:			
	Complete	60	320	430
	Complete	0	30	30
	Minus cells	60	35	–
	Minus coupled beads, plus uncoupled beads	60	35	410
(b)	Standard cell + beads mixture			
	No preincubation:			
	supernatant activity	60	62	–
	pellet activity	60	353	329
	30 min. preincubation:			
	supernatant activity	60	69	–
	pellet activity	60	330	347
(c)	Standard mixture:			
	no preincubation	60	310	472
	15 min. preincubation	60	362	470
	30 min. preincubation	60	364	504

(a) Evaluation of eventual unspecific adsorption of radioactive precursors on the Sepharose beads.
(b) Studies of an eventual release of intracellular galactosyltransferase by determination of the supernatant activity before (no preincubation) or during (30 min preincubation) the reactime time.
(c) Studies of an eventual release of intracellular galactosyltransferase by adding the radioactive precursor after various preincubation periods.

the possibility of the release of intracellular enzyme before the incubation (cell lysis) or during the incubation (secretion or cell breakage). In addition to this, when radioactive precursor is added after various preincubation time, the transfer activity is not increased in a noticeable proportion (table 1c) showing that no additional galactosyltransferase activity has appeared during the incubation.

4. Conclusions

The methodology described above enables us to prove unambiguously the occurrence of galactosyltransferase on the lymphocyte cell surface. This enzyme is able to transfer galactose from UDP-Gal to an exogenous acceptor. The proofs for the external position of the active site are as follows: (i) a non-phagocytosable exogenous acceptor can be glycosylated, (ii) the separation of cells from the acceptor after the incubation eliminates errors due to incorporation of free carbohydrate by cells, (iii) the glycosylation is not due to a release of intracellular enzymes during incubation. However, it is difficult to associate this ectogalactosyltransferase with membrane protein or lipid glycosylation because we have observed the presence of a very active membranous system degrading UDP-Gal. Nevertheless, our results point out the existence of sites on the cell surface able to recognize terminal GlcNAc residues of the glycan moiety of a glycoprotein.

Acknowledgements

This research was supported by grants from the C.N.R.S. (L.A. n° 217: Biologie Physico-Chimique et Moléculaire des Glucides Libres et Conjugués and A.T.P. Pharmacodynamie et Chimiothérapie) and by the Commissariat à l'Energie Atomique. We are grateful to Nyegaard and Co for their generous gift of metrizamide.

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