

ECTO GALACTOSYLTRANSFERASE STUDIES IN FIBROBLASTS AND CONCANAVALIN A-STIMULATED LYMPHOCYTES

LEONARD M. PATT, ROBERT O. ENDRES, DAVID O. LUCAS, and WILLIAM J. GRIMES. From the University of Arizona College of Medicine, Departments of Biochemistry and Microbiology, Tucson, Arizona 85724

Suspensions of intact animal cells are capable of transferring the sugar moiety of exogenous nucleotide sugars to endogenous glycoprotein and glycolipid acceptors (10, 12, 13). It has frequently been assumed that incorporation by suspended cells incubated with nucleotide sugars indicates the presence of an ectoglycosyltransferase. Ecto-enzymes are defined as membrane-bound enzymes whose active sites are accessible from the outside of the cell (3).

Several recent publications have challenged the suggestion that glycosyltransferases are ectoenzymes. Evans found a nucleotide pyrophosphatase on the plasma membrane of hepatocytes which could degrade exogenously added nucleotide-sugar substrates (5). Deppert et al. (4) have reported evidence that all the incorporation by baby hamster kidney (BHK) monolayers is due to hydrolysis and intracellular utilization of the labeled galactose released by degradation of exogenous UDP-galactose.

In this communication, we show that while suspensions of mouse spleen cells incorporate label from exogenous UDP-[¹⁴C]galactose, there are no ectogalactosyltransferases. The spleen cell preparations degrade the nucleotide sugar, releasing galactose which is utilized for complex carbohydrate synthesis within the cell. In contrast, BALB/c 3T3 and simian virus 40-transformed 3T3 cells in suspension possess an ectogalactosyltransferase capable of transferring the carbohydrate moiety of exogenous UDP-galactose to endogenous acceptor molecules.

MATERIALS AND METHODS

Cell Cultures

BALB/c 3T3 cells (clone A31) and simian virus 40-transformed 3T3 cells (SVT2) were the gift of Dr. G. Todaro, National Institutes of Health, Bethesda, Md. The procedures for fibroblast growth and suspension using 0.01 M EDTA in saline for studies of ectogalactosyltransferases have been described (10).

Cells were teased out of spleens from BALB/c or C3H mice into Hanks' balanced salts solution with 0.6%

dextran (HBSS + dex). Cells were dispersed by expressing through a 20-G needle. They were then washed twice with HBSS + dex and resuspended in RPMI 1640 medium supplemented with 5% fresh human serum, 2 mM glutamine, 40 U/ml penicillin, and 50 μg/ml streptomycin. Suspensions were diluted to 5 × 10⁶ mononuclear cells per milliliter and 2-ml aliquots were placed in 17 × 100-mm plastic culture tubes (Falcon Plastics, Oxnard, Calif.). Concanavalin A (Con A)¹ from Calbiochem (San Diego, Calif.) was added in 0.2-ml aliquots of a 100 μg/ml solution (final concentration 10 μg/ml). Incubation was carried out for 48 h at 37°C in a humidified atmosphere of 5% CO₂ in air. The 48-h point coincides with the maximum rate of [³H]thymidine incorporation. After Con A stimulation, cells were centrifuged, resuspended, and washed once with TBS.

Measurement of galactose incorporation into acid-precipitable material was performed essentially as described (10). The incubations with lymphocyte or fibroblast cell suspensions contained 80 pmol UDP-[¹⁴C]galactose, 80 pmol [³H]galactose, 0.5 μmol MnCl₂, and 1–3 × 10⁶ cells in a final volume of 0.06 ml of TBS. We have previously reported the optimal reaction conditions and kinetic constants for reactions of cell suspensions with nucleotide sugars (10).

For experiments requiring the addition of unlabeled galactose, galactose-1-phosphate, and UDP-galactose, solutions were prepared in TBS at concentrations 10–10,000 times that of the labeled compound. A volume of the unlabeled solution containing the required concentration of inhibitor was then added to the standard incubations as described above.

Chromatography

Aliquots of the supernatant solutions from the first trichloroacetic acid wash were subjected to descending paper chromatography on Whatman No. 1 paper. The solvent system used was 95% ethanol-1 M ammonium acetate, pH 3.6 (75:30). The chromatograms were developed for 17–20 h. Before spotting on the paper strips, the aliquots were made 20–50 mM in EDTA and neutralized with additions of 2.5 N NaOH.

The addition of EDTA is necessary to prevent forma-

¹ Abbreviations used in this paper: Con A, concanavalin A; 5'-AMP, adenosine-5'-monophosphoric acid; TBS, tris-buffered saline, 0.15 M NaCl and 0.02 M Tris-HCl, pH 7.4.

tion of a complex between the nucleotide sugar and the Mn^{2+} ions. This complex runs in the solvent used with an *R*_g of 0.9 and can cause high estimates of the extent of nucleotide sugar decomposition. Standards were detected with a periodate-permanganate spray (9).

Determination of Radioactivity

All samples were dissolved in 10 ml of a scintillation fluid composed of xylene, 2,900 ml; Triton X-114, 960 ml; and Liquifluor (New England Nuclear, Boston, Mass.), 125 ml. Vials also contained 1 ml 10% acetic acid. Counting efficiency was 67% for ^{14}C and 33% for 3H .

Chemicals

UDP- ^{14}C galactose (281 mCi/mmol) and 3H galactose (2.04 Ci/mmol) were purchased from New England Nuclear. The 3H galactose was diluted to a specific activity of 250 mCi/mmol with unlabeled galactose before use. UDP-galactose, galactose-1-phosphate, galactose, and 5'-adenosine monophosphate were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Suspensions of BALB/c fibroblasts and spleen lymphocytes incorporate radioactivity into acid-precipitable material when incubated with UDP- ^{14}C galactose. Adding Con A to spleen lymphocytes results in a fivefold stimulation of incorporation, with a maximum effect at 48 h. The increase in UDP- ^{14}C galactose utilization coincides with the peak of 3H thymidine incorporation. The results from these lymphocyte experiments confirm those from experiments on rat thymocytes in which Con A-stimulated blast transformation also resulted in increased galactose incorporation from exogenous UDP-galactose (8). We initiated studies to determine whether the incorporation into lymphocytes and fibroblasts was due to substrate degradation or ectogalactosyltransferases.

Table I shows the results of an experiment in which unlabeled UDP-galactose, galactose-1- PO_4 , and galactose were added to suspensions of fibroblasts or Con A-stimulated spleen cells incubated with equal concentrations of UDP- ^{14}C galactose and 3H galactose. The UDP- ^{14}C galactose was intended to measure ectoenzyme activity; the 3H galactose was included to measure intracellular activity. The addition of unlabeled UDP-galactose to suspended A_{91} cells decreased the incorporation of radioactivity from the UDP- ^{14}C galactose and had little effect on the incorporation from 3H galactose. The addition of unlabeled galactose had the opposite effect. The incorporation from

the UDP- ^{14}C galactose was lowered much less than that from the 3H galactose. In a separate experiment, the addition of unlabeled galactose-1-phosphate had no effect on the incorporation from either compound.

The results with suspensions of Con A-stimulated spleen cells were quite different. The incorporation of radioactivity from UDP- ^{14}C galactose and 3H galactose was inhibited to roughly the same extent by the addition of unlabeled UDP-galactose, galactose-1-phosphate, and galactose. The inhibition of incorporation from UDP- ^{14}C galactose by unlabeled galactose indicates that the nucleotide sugar does not serve as a direct glucose donor in the spleen cell suspensions. Also, the inhibition of 3H galactose incorporation by UDP-galactose and galactose-1-phosphate suggests that these compounds may be extensively degraded by the spleen cell preparations.

It has been reported that 5'-AMP is a potent inhibitor of nucleotide sugar hydrolysis (1, 2, 6). Table II shows the effect of the addition of a low concentration of 5'-AMP on the incorporation of radioactivity into acid-precipitable material and on the decomposition of UDP- ^{14}C galactose. The inhibition of UDP-galactose hydrolysis in the fibroblast suspensions resulted in an increase in incorporation from the nucleotide sugar, again suggesting that the UDP-galactose is the direct glucose donor. The spleen cell suspensions show the opposite effect. Inhibition of UDP- ^{14}C galactose decomposition completely prevents incorporation into acid-precipitable material. These results show that UDP- ^{14}C galactose must be broken down to the free sugar before it can be utilized by the spleen cells.

Additional information about the pathway for incorporation of galactose from exogenous UDP-galactose was obtained from analyses of the products formed in the reactions. Strong acid hydrolysis of the acid-precipitable material from spleen cells incubated with UDP- ^{14}C galactose yielded a mixture of labeled galactose and glucose, indicating that the labeled sugar was exposed to the action of intracellular enzymes before incorporation. Acid hydrolysis of the products from fibroblast incubations yields only labeled galactose.

DISCUSSION

Ectoglycosyltransferases have usually been studied by incubating intact cells with nucleotide sugars. Our results establish controls which can show

TABLE I
Effect of Addition of Unlabeled UDP-galactose, Galactose-1-Phosphate, and Galactose on Incorporation from UDP-[¹⁴C]Galactose and [³H]Galactose by Suspensions of A31 and Con A-Stimulated Spleen Cells

Cell type	Addition	Concentration <i>mM</i>	Total incorporation	
			¹⁴ C <i>cpm</i>	³ H <i>cpm</i>
Spleen cells	none	—	880 ± 70	800 ± 50
	UDP-Gal	0.1	80 ± 4	250 ± 10
	Gal-1-PO ₄	0.1	110 ± 2	120 ± 10
	Galactose	0.1	105 ± 20	90 ± 10
A31 cells	(A) none	—	1,640 ± 40	1,080 ± 20
	UDP-Gal	1.1	60 ± 10	760 ± 30
	Galactose	12.5	1,270 ± 40	120 ± 10
	(B) none	—	1,290 ± 40	1,340 ± 60
	Gal-1-PO ₄	1.1	1,220 ± 100	1,010 ± 70

Incubations were done as described in Materials and Methods. Reactions contained 1.6×10^6 suspended A31 cells or 4.7×10^6 Con A-stimulated spleen cells. Incorporation is \pm the standard deviation.

TABLE II
Effect of 5 mM 5'-AMP on the Hydrolysis of UDP-[¹⁴C]Galactose and Incorporation into Acid-Precipitable Material

Cell type	5'-AMP	Percent of radioactivity as			Total incorporation <i>cpm</i>
		UDP-Gal	Gal-1-PO ₄	Gal	
A31	—	32	63	6	1,970 ± 100
	+	93	3	4	2,730 ± 110
SVT2	—	29	65	6	880 ± 20
	+	89	9	2	1,120 ± 130
Spleen + Con A	—	30	13	34	880 ± 70
	+	96	4	—	20 ± 10

Incubations were set up as described in Materials and Methods. Reactions contained 0.7×10^6 A31 cells, 3×10^6 SVT2 cells, or 4.7×10^6 Con A-stimulated spleen cells. Reactions with 5'-AMP received 5 μ l of a solution of 0.075 M 5'-AMP. Chromatography was performed as described in Materials and Methods.

whether incorporation stems from ectoglycosyltransferases or uptake and incorporation of the released free sugar. First, it is important to show that excess unlabeled galactose in quantities sufficient to inhibit [³H]galactose incorporation has no effect on UDP-[¹⁴C]galactose incorporation. The unlabeled galactose concentration (10 mM) used was approximately five times the K_m of sugar transport reported for many cells (7, 11, 14). Second, adding 5'-AMP to reduce nucleotide sugar degradation should not inhibit incorporation. Finally, the incorporated sugar should not have been altered by cellular metabolism.

The results from studies of spleen lymphocytes and fibroblasts demonstrate how these controls differentiate between substrate degradation and ectogalactosyltransferases. Our results indicate that suspended fibroblasts but not spleen lymphocytes possess ectoglycosyltransferases.

SUMMARY

In this communication, we have demonstrated that hydrolysis of the nucleotide sugar can cause errors in the detection of an ectoglycosyltransferase. Spleen cell suspensions can incorporate radioactiv-

ity when incubated with labeled UDP-galactose, but all the activity is due to decomposition of the nucleotide sugar and uptake of the free sugar. The fibroblast cell lines can incorporate carbohydrate directly from UDP-galactose. Several criteria are presented which can be used to demonstrate that a nucleotide sugar is the direct carbohydrate donor.

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