ECTOGLYCOSYLTRANSFERASE ACTIVITY IN SUSPENSIONS AND MONOLAYERS OF CULTURED FIBROBLASTS

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SUMMARY. Fibroblasts suspended by a brief exposure to EDTA have the ability to transfer the carbohydrate moiety of exogenous nucleotide-sugars to endogenous acceptors. Monolayers of the same cells do not have this ability. Both suspensions and monolayers can transfer carbohydrate to exogenous glycose acceptors. The cells can glycosylate exogenous desialized, β-galactosidase treated fetuin utilizing either UDP-[^{14}C]-galactose as a direct donor or [^{3}H]-galactose as a precursor to a glycose donor.

Ectoenzymes are membrane bound enzymes whose active site is accessible from the exterior of the cell (1). Ectoglycosyltransferases have been reported to be present on the surface of many cell types (2-5). These enzymes can utilize exogenous nucleotide-sugars to synthesize cellular complex carbohydrates. Most ectoglycosyltransferases have been detected with cells suspended from a monolayer by EDTA or trypsin. These methods could alter the organization of the cell membrane. A crucial question is whether cells growing in a monolayer have ectoglycosyltransferase activities. Deppert et al (6) have suggested that monolayers of BHK cells lack ectoglycosyltransferases and Webb and Roth (5) observed that only mitotic cells in a monolayer will have detectable ectoglycosyltransferases. We have examined suspensions and monolayers of cultured fibroblasts for the ability to utilize exogenous nucleotide-sugars directly as glycose donors in carbohydrate synthesis using both endogenous and exogenous glycose acceptors. Both the suspensions and monolayers can be shown to have ectoglycosyltransferases.

METHODS

Cell Cultures

The A31 and SV40 transformed A31 cells used in those experiments were the gift of Dr. G. Todaro, N.I.H. The Baby Hamster Kidney fibroblasts, BHK,

were purchased from Grand Island Biological Co., Grand Island, New York. All cell lines were cultured routinely in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and streptomycin and penicillin at concentrations of 76 units/ml and 50 μg/ul respectively. All cell cultures were tested routinely for mycoplasma contamination by the method of Levine (7).

Measurement of Transferase Activity

Measurement of glycose incorporation into acid precipitable material was performed essentially as described (3). Incubation conditions are described in the Table legends. Cell suspensions were prepared by treating cell monolayers with 10^{-2} M EDTA for 10 minutes at 37°. For the measurement of glycosyltransferase activity in monolayers, cells were grown on 35 mm plastic petri dishes (Falcon Plastics, Oxnard, CA). The monolayers were washed twice with prewarmed TBS prior to enzyme assay. (Tris buffered saline, 0.02 M tris-HCl, pH 9.4, 0.15 m NaCl).

At the end of the incubation period, reactions with cell suspensions containing exogenous acceptors were terminated by the addition of 1 ml of cold TBS, vortexed, and centrifuged. The resulting supernatant solution was withdrawn and the cell pellet washed with an additional 1 ml of cold TBS. The pooled washes were added to an equal volume of cold 5% phosphotungstic acid in 0.5 N HCl and allowed to precipitate at 2° for several hours. The precipitate was centrifuged and washed three times with cold 1% phosphotungstic acid in 0.5 N HCl and once with cold 5% trichloroacetic acid. The washed precipitate was dissolved in 1 ml of 0.5 N NaOH for scintillation counting. Reactions with cell monolayers were terminated by the addition of 1 ml of cold TBS as above and the wash decanted. The monolayer was washed again with 1 ml of cold TBS. The pooled washes were precipitated and washed as above. The resulting incorporation was in the supernatant fraction.

Preparation of Exogenous Acceptors

Desialized fetuin was prepared by heating 500 mg of fetuin (Grand Island Biological Co., Grand Island, New York) in 40 ml of 0.05 N H_2SO_4 for 1 hour at 80°. Sialic acid released was measured by the thio-barbituric acid assay of Warren (8). The solution was cooled, neutralized, dialized against several changes of distilled water, and finally lyophilized.

Desialized, β-galactosidase treated fetuin was prepared using β-galactosidase immobilized on an Aqarose gel. 200 mg of Almond Emulsion β-glucosidase (Sigma Chemical Co., St. Louis, MO.) was dissolved in 25 ml of 0.1 m KPO_4 buffer, pH 7.2 and added to 1 gm of Affi-Gel 10 (N-hydroxysuccinimide ester of succinylated aminoalkyl Bio-Gel A, Bio Rad Laboratories, Richmond, CA.). The reaction was allowed to proceed for 24 hours at 2° and then terminated by the addition of 1 ml of 1 M ethanolamine. The gel was poured into a 2.5 cm diameter column and washed with 25 ml 0.1 M KPO_4 buffer, pH 7.2, 50 ml of 1 M NaCl in 0.1 M KPO_4 buffer, pH 7.2, and finally with 100 ml of 0.1 M sodium acetate buffer, pH 5.0. The washed gel was suspended in 40 ml of the acetate buffer.

β-Galactosidase treated, desialized fetuin was prepared by mixing 140 mg of desialized fetuin dissolved in 20 ml of the 0.1 M sodium acetate buffer, pH 5.0, with 20 ml of the β-galactosidase-Agarose suspension. The mixture was shaken at 37° for 72 hours. After incubation, the mixture was placed in a 2.5 cm diameter column and washed with 100 ml of the sodium acetate buffer. The eluate was dialized against distilled water and lyophilized. This procedure removes approximately 10% of the galactose residue from the fetuin.

Chemicals

UDP-[^{14}C]-galactose (281 mCi/mmole), UDP-[^{14}C]-glucose (227 mCi/mmole), GDP-[^{14}C]-mannose (276 mCi/mmole), CMP-[^{14}C]-N-acetylneuraminic acid (217

mCi/mmole), and [^{3}H]-D-galactose (2.04 Ci/mmole) were purchased from New England Nuclear, Boston, MA. The [^{3}H]-galactose was diluted to a specific activity of 250 mCi/mmole with unlabeled galactose. All other chemicals were purchased from Sigma Chemical Company, St. Louis, MO.

Protein Determination

Protein was determined by the method of Lowry (9) using Bovine Serum Albumin as standard.

Radioactivity Determinations

All samples were dissolved in a scintillation fluid composed of 2900 ml Xylene, 960 ml of Triton X-114 and 125 ml of liquifluor (New England Nuclear, Boston, MA.).

RESULTS

Table I shows that BHK fibroblasts suspended by EDTA treatment incorporate the carbohydrate moiety of UDP-[^{14}C]-galactose, CMP-[^{14}C]-sialic acid, UDP-[^{14}C]-glucose and GDP-[^{14}C]-mannose into acid precipitable material.

Table I. Ectoglycosyltransferase Activity in Monolayers and Suspensions of BHK Cells.

Substrate	Total Incorporation cpm/mg ± S.D.	
	Suspension	Monolayer
CMP-Sialic Acid	460 ± 73	17 ± 7
UDP-Galactose	692 ± 120	18 ± 17
UDP-Glucose	52 ± 18	8 ± 7
GDP-Mannose	3603 ± 264	22 ± 2

Table 1. Suspensions and monolayers of BHK cells were prepared as described under Methods. Reactions with suspended cells contained 80 pmoles of radioactive substrate in a final volume of 0.100 ml TBS. Monolayers received 320 pmoles of substrate in a final volume of 0.400 ml TBS. Reactions with CMP-[^{14}C]-Sialic Acid and UDP-[^{14}C]-galactose contained 5 mM $MnCl_2$, those with UDP-[^{14}C]-glucose and GDP-[^{14}C]-mannose contained 15 mM $MgCl_2$. All incubations contained 3 mM 5'-Adenosine monophosphate. After incubation at 37° for 1 hr., all reactions were assayed for acid precipitable radioactivity as described under Methods for analysis of monolayers. Incorporation is expressed as CPM per mg protein ± the standard deviation from samples taken in triplicate from one cell preparation.

Confirming the results of Deppert, et al., monolayers have little detectable activity compared with cell suspensions. In these experiments, cell monolayers and cell suspensions were assayed in the same manner and in the presence of identical nucleotide sugar concentrations. Several authors have suggested that cell surface nucleotide pyrophosphatase would prevent the detection of ectoglycosyltransferases (5, 6). The experiment shown in Table I was done in the presence of 5'-adenosine monophosphoric acid which prevents nucleotide-sugar degradation (Table II, ref. 10-12). We have shown that UDP-galactose is a direct galactose donor in these reactions. Adding a 100-fold excess of unlabeled UDP-galactose abolishes the incorporation while a 10,000-fold excess of free galactose has no effect. Monolayers of BALB/c A31 and SVT2 cells also lack detectable ectoglycosyltransferase activity.

There are two likely explanations for the difference in ectotransferase

Table II. Stability of UDP-Galactose Incubated With Suspensions and Monolayers of BHK Cells.

Cell Type	5'-AMP	% CPM As		
		UDP-Gal	Gal-1-PO_4	Gal
Monolayers	-	3.8	94.4	1.8
	+	91.7	7.4	0.9
Suspensions	-	58.3	36.5	5.2
	+	95.4	3.4	1.1

Table II. Incubations were prepared as described in the legend to Table I. At the end of the incubation period, the reactions were terminated by the addition of 1 ml of cold 5% trichloroacetic acid. Aliquots of the trichloroacetic acid wash were made 20-50 mM in EDTA, neutralized, and spotted with standards on Whatman No. 1 paper. Chromatograms were developed in a solvent composed of 95% Ethanol:1 M ammonium acetate, pH 3.6 (75:30) for 17-20 hours. Strips were cut into 1 cm strips for determination of radioactivity.

levels in suspensions and monolayers. Monolayers may require EDTA treatment to expose the transferases. Alternately, the monolayers may have ectoglycosyltransferases but lack the appropriate enzyme-acceptor configuration for detectable activity. We have explored these possibilities by the use of high molecular weight glycose acceptors. Two acceptors have been prepared from the glycoprotein fetuin; desialized fetuin (F^-), an acceptor for sialic acid residues, and desialized, β-galactosidase treated fetuin ($F^=$), an acceptor of galactose residues.

Table III shows the results obtained when suspensions and monolayers of BHK cells are incubated with CMP-[^{14}C]-sialic acid and desialized fetuin.

Table III. Ability of Monolayers and Suspensions of BHK Cells to Catalyze Sialic Acid Transfer to Desialize Fetuin.

Cells	Acceptor	Amount	Acid Precipitable CPM ± S.D. Cells	Supernatant
Suspension	F	1 mg	1509 ± 145	62 ± 26
	F^-	0.5 mg	1608 ± 89	128 ± 45
	F^-	1.0 mg	1659 ± 70	218 ± 24
Monolayer	F	1 mg	39 ± 2	32 ± 21
	F^-	1 mg	53 ± 3	161 ± 8
	F^-	2 mg	77 ± 28	337 ± 31

Table III. Incubations were set up with monolayers and suspensions as described under Methods. Reactions with suspensions contained 160 pmoles CMP-[^{14}C]-Sialic Acid, 5 mM $MnCl_2$, and the indicated amount of acceptor in a final volume of 0.100 ml TBS. Reactions with monolayers contained 800 pmoles CMP-[^{14}C]-Sialic Acid, 5 mM $MnCl_2$ and the indicated amount of acceptor in a final volume of 0.400 ml TBS. After incubation at 37° for 1 hour, reactions were assayed as described under Methods. F indicates native fetuin, F^-, desialized fetuin. Incorporation is expressed as CPM per mg protein ± the standard deviation from triplicate samples taken from the same preparation of cells.

The control incubations contained equal amounts of native fetuin. The exogenous acceptor remains in the supernatant fraction and does not become cell associated. Both suspensions and monolayers can glycosylate the desialized fetuin utilizing the CMP-[^{14}C]-sialic acid as a glycose donor. These results indicate that monolayers have ectoglycosyltransferases. Both the suspensions and monolayers glycosylate the acceptor to about the same extent on a per cell basis.

Similar results are obtained with desialized, β-galactosidase treated fetuin as an acceptor. Table IV shows the results obtained with monolayers of A31 and SVT2 cells. These experiments utilize UDP-[^{14}C]-galactose to measure ectoglycosyltransferases and [^{3}H]-galactose to measure intracellular complex polysaccharide synthesis. The monolayers glycosylate the acceptor molecule (supernatant and precipitable material) utilizing either extracellular UDP-galactose (^{14}C-CPM) or [^{3}H]-galactose which must be converted to UDP-[^{3}H]-galactose intracellularly.

DISCUSSION

We have shown that both monolayers and suspensions of fibroblasts have ectoglycosyltransferases which can utilize exogenous nucleotide-sugars. Detecting the activity in monolayers requires the use of exogenous glycose acceptors. It is known that EDTA can alter the permeability of bacterial cells (13), however this does not appear to be the case with the EDTA suspended cells. We have been unable to detect any uptake of nucleotide sugars into EDTA treated cells (3). Suspending the cells by EDTA treatment may alter the natural relationship between the transferases and endogenous acceptors on the cell surface.

Roth and White (5, 14) have suggested that suspended transformed cells can catalyze glycosylation without cell contacts. In their hypothesis, normal cells require cell to cell interaction for glycosylation using exogenous nucleotide-sugars. Our results are consistent with a model of cell to cell glycosylation, but monolayers of normal and transformed cells both have a

Table IV. Ability of Monolayers of A31 and SVT2 Cells to Catalyze Galactose Transfer to Desialized, β-Galactosidase Treated Fetuin.

Cell Type	Acceptor	Acid Precipitable Incorporation CPM ± S.D.			
		Monolayers		Supernatant	
		^{14}C	^{3}H	^{14}C	^{3}H
A31	F^-	32 ± 4	247 ± 67	43 ± 8	206 ± 2
	$F^=$	20 ± 3	204 ± 86	166 ± 20	430 ± 10
SVT2	F^-	16 ± 5	758 ± 100	12 ± 3	156 ± 10
	$F^=$	16 ± 3	1202 ± 120	155 ± 76	359 ± 43

Table IV. Monolayers of A31 and SVT2 cells were prepared as described under Methods. Incubations contained 320 pmoles of UDP-[^{14}C]-galactose and 320 pmoles of [^{3}H]-galactose, 5 mM $MnCl_2$, 5 mM 5'-AMP, the indicated amount of acceptor in a final volume of 0.400 ml TBS. Reactions were incubated at 37° for 1 hr. and assayed as described under Methods. F^- indicates desialized fetuin, $F^=$, indicates desialized, β-galactosidase treated fetuin. Incorporation is expressed as CPM per mg protein ± the standard deviation from triplicate samples taken from the same preparation of cells.

requirement for exogenous acceptor in ectoglycosyltransferase reactions.

The presence of nucleotide pyrophosphatases on cell surfaces (6, 15) argues against extracellular nucleotide sugars as normal substrates for ectoglycosyltransferases. We have observed that intracellular sugar can be transferred to the exogenous glycoprotein acceptor. These results confirm studies by Yogeeswaran et al. (16) using cells incubated with labeled galactose and glycolipids attached to glass beads. The most likely role for ectoglycosyltransferases is in the process of cell surface and extracellular complex polysaccharide synthesis using intracellular substrates. The observations can be interpreted as indicating that a significant part of cellular complex polysaccharide synthesis occurs at the cell surface.

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REFERENCES

1. DePierre, J. W. and Karnovsky, M. L. (1973) J. Cell Biol. 56:275-303.
2. Roth, S. (1973) Quart. Rev. Biol. 48:541-563.
3. Patt, L. M. and Grimes, W. J. (1974) J. Biol. Chem. 249:4157-4165.
4. Patt, L. M. and Grimes, W. J. (1975) Cancer Res. 35:438-441.
5. Webb, G. C. and Roth, S. (1974) J. Cell Biol. 63:796-805.
6. Deppert, W., Wechau, H. and Walter, G. (1974) Proc. Natl. Acad. Sci. U.S.A. 71:3068-3072.
7. Levine, C. M. (1972) Exptl. Cell Res. 74:99-109.
8. Warren, L. (1959) J. Biol. Chem. 234:1971-1975.
9. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193:265-]75.
10. Geren, L. M. and Ebner, K. E. (1974) Biochem. Biophys. Res. Comm. 59: 14-21.
11. Bischoff, E., Liersch, M., Keppler, D. and Decker, K. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351:729-736.
12. Bischoff, E., Wilkfning, J. and Decker, K. (1973) Hoppe-Seyler's Z. Physiol. Chem. 354:1112-1114.
13. Leive, L. (1968) J. Biol. Chem. 243:2373-2380.
14. Roth, S. and White, D. (1972) Proc. Natl. Acad. Sci. U.S.A. 69:485-489.
15. Evans, W. H. (1974) Nature 250:391-394.
16. Yogeeswaran, G., Laine, R. A., and Hakomori, S. (1974) Biochem. Biophys. Res. Comm. 59:591-599.