GLYCOSYL TRANSFER ACTIVITIES IN INTACT LIVER CELLS OF EMBRYONIC CHICK

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Summary Intact liver cells of chick embryos catalyze the transfer of radioactive monosaccharides from several labeled sugar nucleotides to endogenous acceptors. Cells of 11 day old embryos incorporate mannose faster than the other sugars tested. Incorporation of mannose into glycoprotein depends on the developmental stage of the embryos. At certain stages of development intermediary mannose containing compounds are probably utilized as substrates for the incorporation of mannose into glycoprotein.

Introduction In embryonic chick liver a progressive, age-dependent decrease in reaggregation competence of trypsin-dissociated cells occurs (1,2). We are interested in determining if the reason for this decrease is a variation in the biosynthesis of cell surface glycoproteins due to changes of glycosyl transfer activities at the cell surface during embryonic development. It is generally assumed that the cell surface plays an important role in cell recognition and intercellular adhesion. Complex carbohydrates have been shown to be involved in intercellular adhesion (3,4). Recent theories on the mechanism of intercellular adhesion propose that cell surfaces contain complex carbohydrates and glycosyltransferases which may act as "keys" and "locks" in the formation of specific cell contacts (5,6). In light of this hypothesis results have been interpreted which were obtained with cell surface glycosyltransferases of chick embryo neural retina cells (6), human platelets (7) and mouse fibroblasts (8). In this paper evidence is presented which indicates that intact viable cells, obtained by dissociation of embryonic chick livers with trypsin, catalyze the transfer of ^{14}C-sugar from exogenous ^{14}C-sugar nucleotides to endogenous acceptors and that in the case of mannose transfer

both transfer activity and probably the mechanism of transfer depend on the developmental stage of the embryos.

Materials and Methods

Materials The following materials were purchased from commercial sources: guanosine-diphosphate-^{14}C-D-mannose (160 mCi/mmole), uridine-diphosphate-^{14}C-D-galactose (254.5 mCi/mmole) and cytidine-monophosphate-^{14}C-sialic acid (229 mCi/mmole) from New England Nuclear Corp., Boston, Mass. Uridine-diphosphate-^{14}C-D-N-acetyl-glucosamine (275 mCi/mmole) and guanosine-diphosphate-^{14}C-L-fucose (175 mCi/mmole) from the Radiochemical Centre Amersham, Buckinghamshire, England. Trypsin, crystallized twice, soyabean trypsin inhibitor and DNA-ase (2800 U/mg), Worthington Biochemical Corp., Freehold, N.J.

Medium The medium for all incubations contained 0.137 M NaCl, 5.37 mM KCl and 1 mM EDTA, all buffered to pH 7.5 with 0.01 M Tris-HCl. For special additives see the text.

Preparation of cell suspensions Suspensions of viable liver cells were obtained by dissociation of freshly removed organs of White Leghorn chick embryos. The washed and minced livers were incubated at 37° C for 20 min in medium containing trypsin (0.5 %) and DNA-ase (56 U/ml) and then washed three times with trypsin-free medium and once with 0.05 % trypsin inhibitor in medium. The tissues were dissociated by repeated suction through a long blunt needle (100 x 1.2 mm) into a 5 ml syringe. The resulting suspensions were combined, diluted to approximately 10^7 cells/ml and filtered through cheese cloth. The filtrate was centrifuged for 5 min. at 400 x g and the pellet washed three times with medium. The cells were resuspended to an appropriate volume and used immediately.

Assay of glycosyl transfer activities Incubation mixtures consisted of 110 µl of liver cell suspensions (0.5 to 1.0 mg protein as estimated by the biuret method with bovine serum albumin as standard) in medium, the labeled sugar nucleotides, and all additives. Incubations were made at 37° C. Reactions were stopped by heating

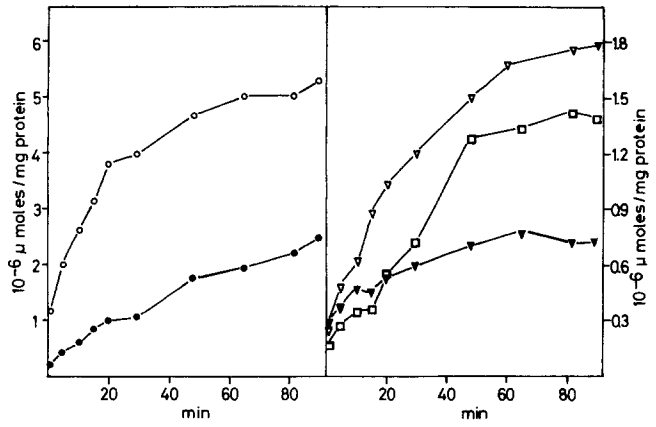

Fig. 1 Time course of incorporation of ^{14}C-sugars from ^{14}C-sugar nucleotides into endogenous acceptors of whole liver cells of 11 day old embryos. Reaction mixtures: cells corresponding to 0.83 mg protein, 1.5 µM radioactive sugar nucleotide, 5 mM $Mg^{++}$and 5 mM Mn^{++}. The enzyme assay is described in "methods". Substrates: O——O guanosine-diphosphate-mannose, ●——● uridine-diphosphate-galactose, ▽——▽ uridine-diphosphate-N-acetyl-glucosamine, □——□ cytidine-monophosphate-sialic acid, ▼——▼ guanosine-diphosphate-L-fucose. The ordinate shows sugar incorporated.

at 100° C. After 3 min. at 100° C the samples were chilled to -25° C and lyophilized. The lyophilized samples were extracted three times with a 10:10:3 mixture of chloroform-methanol-water. Residues were precipitated with 10 % trichloroacetic acid , washed twice with 5 % TCA and once with ethanol-ether (1:1), dissolved in 0.25 ml of Hyamin (Hewlett-Packard) plus 15 ml of 0.4 % Omnifluor (NEN-Chemicals) in toluene and counted in a Beckman model DPM 100 liquid scintillation counter. Cpm were corrected to dpm.

Results and Discussion

In preliminary experiments intact liver cells from 11 day old embryos catalyzed ^{14}C-glycosyl transfer from ^{14}C-labeled exogenous sugar nucleotides to endogenous acceptors using five different sugar nucleotides. With the conditions described in Fig. 1 mannosyl transfer showed the highest activity.

The transfer of mannose was dependent on divalent cations. Although Ca^{++}, Mg^{++} and Mn^{++} stimulated the reaction, Ca^{++} was less effective than Mg^{++} and Mn^{++} whereas the relative stimulating effects of Mg^{++} and Mn^{++} depended on the age of the em-

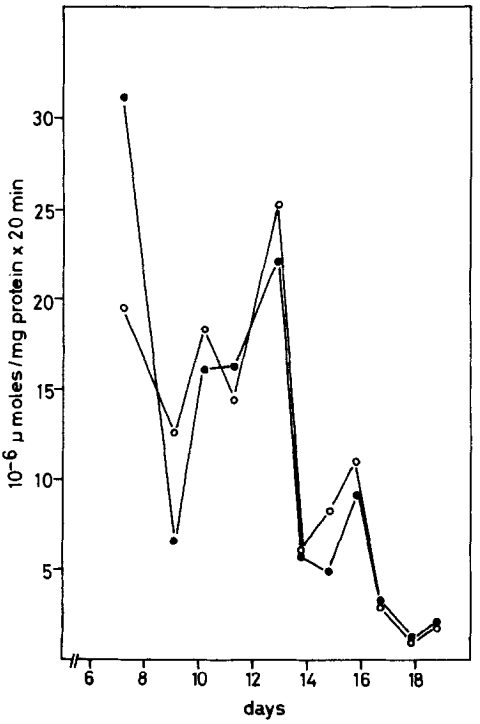

Fig. 2 Variation of mannosyl transfer activity in liver cells during development of chick embryos. Incubation mixtures were assayed as described. ●——● Mg^{++}-stimulated activities at optimal Mg^{++}-concentrations, O——O Mn^{++}-stimulated activities at optimal Mn^{++}-concentrations. Optimal cation concentrations for the individual embryonic stages were determinated in the same experiment.

bryos. In the presence of 1 mM EDTA and without added divalent cations guanosine-diphosphate-^{14}C-mannose (GDP-^{14}C-mannose) was rapidly degraded to ^{14}C-mannose-1-phosphate and ^{14}C-mannose and no label could be found in the endogenous acceptors. At optimal concentrations of Mg^{++} and Mn^{++}, 5 mM Cu^{++} inhibited 95 % of mannosyl transfer activity. Saturation of the mannose transferring system could be achieved only at high concentrations of GDP-^{14}C-mannose (8×10^{-4} M) and when Mg^{++} was used as sole divalent cation. Therefore standard experiments were carried out at a GDP-^{14}C-mannose concentration of 1.5 µM. Incorporation of radioactive label was proportional to the number of cells present in incubation mixtures. Enzyme activities were calculated per mg of protein, the protein content of the liver cells being variable during embryonic development.

Activities at different embryonic stages were compared with 10 different concentrations of Mg^{++} and Mn^{++} respectively

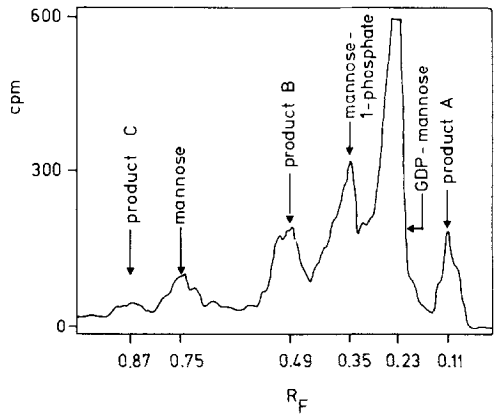

Fig. 3 Typical chromatogramm of the radioactive products formed by liver cells of 14 day old embryos using radioactive GDP-mannose as substrate. Chloroform-methanol-water-extracts (10:10:3) of lyophilized incubation mixtures were chromatographed on cellulose thin layer plates with n-propanol-water (7:3) followed by ethanol-1 M ammoniumacetate pH 7.0 (7:3) as solvents and then scanned.

in the range of 1 mM to 10 mM. Fig. 2 shows the incorporation of ^{14}C-mannose into endogenous acceptors as a function of embryonic age. The results reported in the Fig. 2 could be repeated qualitatively at many different concentrations of Mg^{++} and Mn^{++} within the same experiment and were reproducible in another independent experiment. These results raise the interesting question whether or not different mannosyltransferases and (or) different acceptors were active at different embryonic stages.

By analyses of the reaction products we found that at any given time of incubation 70-85 % of the radioactivity, not covalently bound to protein, was extractable from lyophilized incubation mixtures with chloroform-methanol-water (10:10:3). Reaction mixtures stopped by rapid freezing, were lyophilized and extracted three times with this solvent mixture. The extracts were chromatographed on cellulose thin layer plates (Merck, Darmstadt, West-Germany) with n-propanol-water (7:3) followed by ethanol-1 M ammonium acetate pH 7.0 (7:3) as solvents. Six radioactive fractions with the R_F-values shown in Fig. 3 could be detected.

The unidentified products A and B in Fig. 3 could not be found before the 10th to 11th day of incubation of the fertilized eggs. The highest concentrations occur in liver cells of

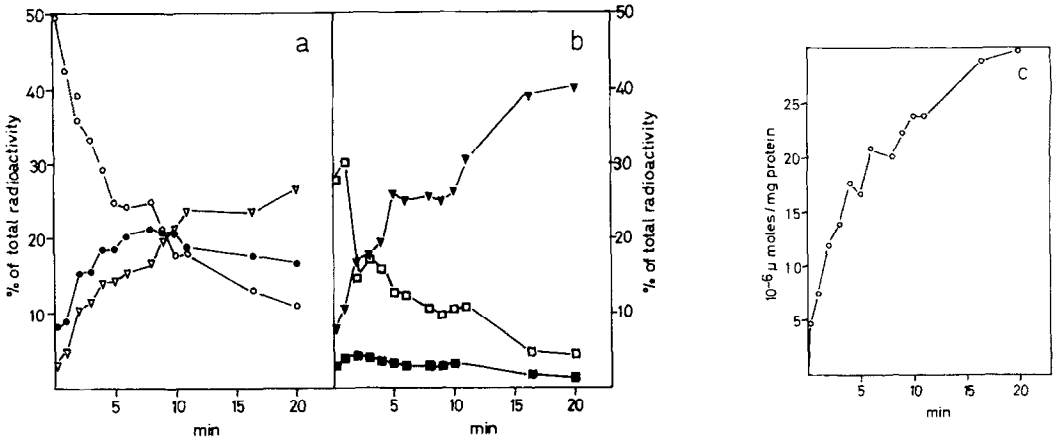

Fig. 4 Time course of degradation of GDP-mannose catalyzed by liver cells from 14 day old embryos. Chloroform-methanol-water-extracts of lyophilized incubation mixtures were chromatographed as described in the legend to Fig. 3. Radioactive fractions were scraped off and counted in toluene-Omnifluor. Recovery of radioactivity was 80 ± 2 %. Residues obtained after the extraction procedure were assayed for the incorporation of radioactive mannose as described in "methods".
a: O—O GDP-mannose, ●—● mannose-1-phosphate, ▽—▽ mannose.
b: □—□ product A, ▼—▼ product B, ■—■ product C.
c: O—O incorporation into glycoprotein. Reaction mixtures for each point contained cells corresponding to 0.97 mg protein and 4 µM radioactive GDP-mannose and 10 mM Mg^{++}.

13-14 day old embryos. After sixteen days of incubation of the embryos only small amounts of these products were present. The product C was detectable in small quantities at all embryonic stages from 7 to 19 days. The relative quantities of the products A, B and C also depended on cation concentrations in the incubation mixtures. Fig. 4 shows the formation of A, B and C, ^{14}C-mannose-1-phosphate, ^{14}C-mannose and ^{14}C-labeled glycoprotein as a function of time of incubation of the cells with ^{14}C-GDP-mannose. In no case were significant quantities of ^{14}C-mannose-6-phosphate detected. The radioactive label in glycoprotein was stable in 0.1 N HCl at 80° C. The cells for this experiment had been obtained from 14 day old embryos.

The low yield of product C has so far prevented its characterization. Product B is highly unstable. Its radioactive products after mild acid hydrolysis are mannose-1-phosphate and mannose. Product A was always bound tightly to the intact cells. It precipitates with the protein, when acid is added in the cold

to the incubation mixtures and it cannot be extracted with lipid solvents from aqueous incubation mixtures. However, a radioactive lipid was extractable with lipid solvents which migrated similarly to product C on TLC. Product A is extracted from lyophilized mixtures with chloroform-methanol-water.

Chloroform-methanol-water extracts of lyophilized incubation mixtures were evaporated, dissolved in 0.01 M ammonium acetate pH 7.0 and adsorbed to DEAE-cellulose columns (Whatman DE 32, acetate form). When eluting the columns with linear gradients from 0.01 to 0.3 M ammoniumacetate pH 7.0, product A migrates together with mannose. Compound A and two other compounds from the DEAE-cellulose-chromatography which are not GDP-mannose acted as substrates in the mannose incorporation reaction. Further experiments on the role of these substances as precursors for glycoprotein synthesis are in progress.

In control experiments no significant incorporation of radioactivity from free ^{3}H-mannose was observed. A hundredfold molar excess of unlabeled mannose resulted only in a 40 % decrease in mannosyltransfer activity in standard incubation mixtures with cells of 11 day old embryos.

The experiments reported here clearly show that intact embryonic liver cells are capable of transfering monosaccharides using exogenous sugar nucleotides as substrates. In the case of mannose transfer, incorporation of radioactivity is not produced by GDP-mannose resynthesized internally. Interpreting our results, it remains uncertain whether at any embryonic stage a direct single step transfer of mannose from exogenous GDP-mannose to glycoprotein occurs. We are assuming that, at least in 11-16 day old embryos, substrates distinct from GDP-mannose are used in the incorporation of mannose into glycoprotein. For other mannose incorporating systems derived from cells of higher organisms intermediates of mannose transfer have been identified (9,10)

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