Enzymatic transfer of galactosyl phosphate from UDP-galactose to UDP-*N*-acetylglucosamine

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The microsomal fraction of hen oviduct homogenate has been shown to contain an enzyme capable of catalyzing a transfer of galactosyl phosphate from UDP-galactose to UDP-*N*-acetylglucosamine. The product was isolated and identified as UDP-*N*-acetylglucosamine-6-phosphogalactose, the same compound as that found as a normal constituent in hen oviduct. The enzyme is analogous in reaction type to UDP-*N*-acetylglucosamine: glycoprotein *N*-acetylglucosamine-1-phosphotransferase (the enzyme responsible for introducing the recognition marker of newly synthesized lysosomal enzymes), which suggests that the galactosyl phosphotransferase is involved in galactose 1-phosphate transfer to *N*-acetylglucosamine residues of newly synthesized glycoproteins.

UDP-N-*acetylglucosamine* *Galactose-1-phosphotransferase* *UDP-galactose*
UDP-N-*acetylglucosamine-6-phosphogalactose*

1. INTRODUCTION

Previous studies [1–4] showed that the oviduct of a laying hen contains a novel oligosaccharide-carrying nucleotide (UDP-GlcNAc-P-Gal; see fig. 1 for the structure) in which galactose 1-phosphate is linked to the 6-hydroxyl of *N*-acetylglucosaminyl residue of UDP-*N*-acetylglucosamine. The discovery of galactose residue in phosphodiester linkage to *N*-acetylglucosamine residue suggested the existence of an enzyme capable of catalyzing a transfer of galactose 1-phosphate residue from UDP-galactose to *N*-acetylglucosamine residue, although it was a common notion that UDP-galactose is not a galactose-1-phosphate donor but a galactose donor.

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Abbreviations: UDP-GlcNAc-P-Gal, uridine diphosphate *N*-acetylglucosamine-6-phosphogalactose; P-GlcNAc-P-Gal, 1-phospho-*N*-acetylglucosamine-6-phosphogalactose; GlcNAc-P-Gal, *N*-acetylglucosamine-6-phosphogalactose

In [5–8] microsomes prepared from several cells and tissues contain an enzymatic activity that transfers *N*-acetylglucosamine 1-phosphate from UDP-*N*-acetylglucosamine to the 6-hydroxyl of mannose residues present in high mannose-type oligosaccharides of glycoproteins or of exogenous glycopeptides. This enzyme is believed to donate *N*-acetylglucosamine 1-phosphate to mannose residues of newly synthesized lysosomal enzymes. Demonstration of this new type of transferase prompted us to examine the possible occurrence of an enzyme transferring galactose 1-phosphate from UDP-galactose to UDP-*N*-acetylglucosamine to form UDP-GlcNAc-P-Gal.

2. MATERIALS AND METHODS

2.1. *Preparation of [β-^{32}P]UDP-galactose*

The synthesis of this compound involved the enzymatic production of galactose 1-[^{32}P]phosphate followed by enzymatic conversion to the sugar nucleotide and finally enzymatic removal of contaminating UDP-glucose. The initial reaction mixture contained 0.2 μmol of D-galactose, 1 mCi of

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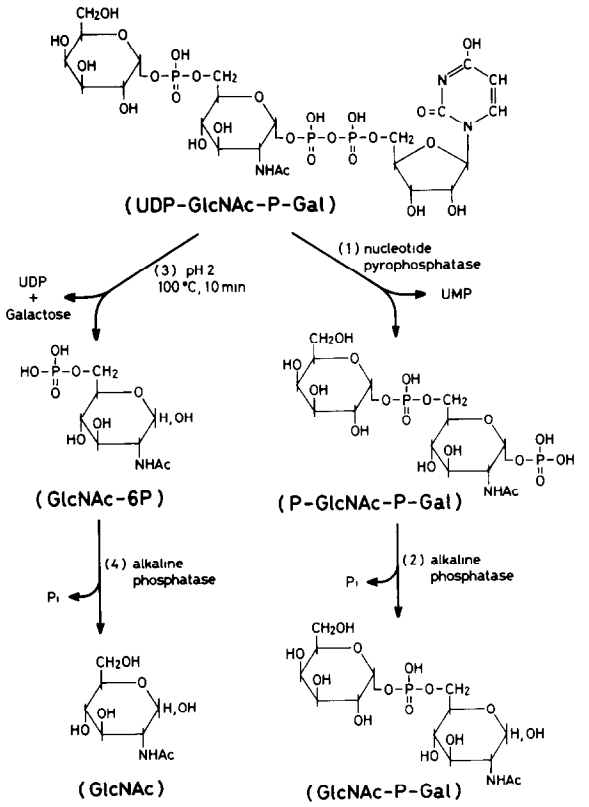

Fig. 1. Summary of reactions of UDP-GlcNAc-P-Gal.

[γ-^{32}P]ATP (5000 Ci/mmol), 5 μmol of Tris–HCl (pH 7.2), 1 μmol of $MgCl_2$, and 1 unit of galactokinase, in a total volume of 250 μl. The reaction mixture was incubated at 37°C for 1 h. To this mixture were added 0.1 μmol of UDP-glucose, 0.75 nmol of α-D-glucose 1,6-bisphosphate, 30 μmol of Tris–HCl (pH 8.5), 6 μmol of $MgCl_2$, 1 unit of uridyltransferase, 0.5 unit of phosphoglucomutase, and water to give a total volume of 300 μl. The reaction mixture was incubated at 37°C for 1 h. The pH of the mixture was then adjusted to 5.0 with acetic acid. After heating for 1 min in a boiling water bath, 0.3 μmol of NAD, 0.5 unit of UDP-glucose dehydrogenase, 30 μmol of Tris–HCl (pH 8.5), and water were added to a total volume of 370 μl, and the mixture was incubated at 37°C for 1 h. The pH of the reaction mixture was then adjusted to 5.0 with acetic acid. The sample was subjected to preparative paper chromatography in solvent A and then in solvent B (for desalting). The radioactive spot with the mobility of UDP-galactose was eluted with water. The overall yield of [β-^{32}P]UDP-galactose was about 90%. Digestion with nucleotide pyrophosphatase resulted in recovery of > 95% of the ^{32}P in the galactose 1-phosphate fragment, indicating that the radioactivity is associated almost exclusively with the β-phosphate. Before use, the specific radioactivity of the sample was adjusted to 192 mCi/mmol by diluting with unlabeled UDP-galactose.

2.2. *Measurement of UDP-GlcNAc-P-Gal synthesis*

Microsomes were prepared from the isthmus region of hen oviduct as in [9]. The final pellet of microsomes was suspended in 20 mM Tris–HCl (pH 7.2) at about 15 mg (as protein)/ml and used for enzyme assay.

For the measurement of galactosyl phosphotransferase activity, the standard incubation mixture contained 1.5×10^5 dpm (about 0.36 nmol) of UDP-[^{14}C]galactose or 1.6×10^6 dpm (about 3.8 nmol) of [β-^{32}P]UDP-galactose, 0.1 μmol of UDP-*N*-acetylglucosamine, 420 μg (final concentration = 0.6%) of Nonidet P-40, 0.14 μmol of 5′-AMP, 0.35 μmol of $MnCl_2$, 3.5 μmol of sodium acetate/acetic acid buffer (pH 5.4) and about 0.5 mg of microsome protein, in a total volume of 70 μl. Controls contained heat-killed microsomes. Following incubation at 37°C for the time indicated in individual experiments, the samples were boiled for 1 min, cooled, applied to Toyo no. 50 filter paper (48 cm long), and chromatographed in Solvent A for 48 h. The zone corresponding to the UDP-GlcNAc-P-Gal standard (for preparation see [4]) was cut out and placed in scintillation fluid for counting.

2.3. *Other methods*

Paper chromatography was performed on 48-cm strips of Toyo no. 50 paper by the descending technique in the following solvent systems: (A) *n*-butyric acid–0.5 M ammonia (5:3, v/v); (B) *n*-butanol–ethanol–water (13:8:4, by vol.); (C) 95% ethanol–1 M ammonium acetate (5:2, v/v). Paper electrophoresis was performed in a CCl_4-cooled apparatus on 60-cm strips of Toyo 51A paper in 0.05 M ammonium acetate/acetic acid buffer (pH 5.0) at 30 V/cm. The distribution

of radioactivity on chromatograms and electrophoretograms was determined by placing 1- or 1.5-cm segments of the paper strip in scintillation fluid for counting. Nucleotides and reducing sugars on paper were detected by viewing under a UV-lamp and by visualizing with the aniline hydrogen phthalate reagent [10], respectively. Protein was determined as in [11] using bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

In preliminary experiments, using either UDP-[^{14}C]galactose or [β-^{32}P]UDP-galactose as the nucleotide sugar donor, and UDP-*N*-acetylglucosamine as the acceptor, we found that the microsomes from hen oviduct catalyzed the incorporation of both the ^{14}C or ^{32}P label into a compound corresponding in chromatographic and electrophoretic mobilities to UDP-GlcNAc-P-Gal.

Using the standard assay conditions, the incorporation of ^{14}C from UDP-[^{14}C]galactose proceeded in time-dependent fashion for 60 min. The amount of substrate transferred was proportional to microsome concentration of 0.1–0.5 mg (as protein)/70 μl. The activity showed an optimum between pH 5.0 and 5.9 (acetate buffer). At pH 7.2 and 8.1 (Tris–HCl), the activities were 60 and 9%, respectively, of the activity at pH 5.4.

Table 1

Requirements for galactosylphosphotransferase activity

Incubation mixture	Product formed/h
	^{14}C, dpm
Complete	2000
– Microsomes + boiled microsomes	20
– UDP-*N*-acetylglucosamine	25
– UDP-*N*-acetylglucosamine + 0.1 μmol UDP-*N*-acetylgalactosamine	340
– $MnCl_2$	450
– $MnCl_2$ + 0.35 μmol $MgCl_2$	200
– Nonidet P-40	730
– Nonidet P-40 + 420 μg Triton X-100	1500
– Nonidet P-40 + 420 μg Tween 20	1200

The standard assay for [^{14}C]galactose-1-phosphate incorporation into added UDP-*N*-acetylglucosamine was performed as described in section 2 with modifications as indicated

The requirements for transferase activity are summarized in table 1. There is an absolute requirement for UDP-*N*-acetylglucosamine. Although UDP-*N*-acetylgalactosamine (for preparation see [12]) showed weak acceptor-activity, it is uncertain whether the activity is due to the nucleotide itself or to a small amount of UDP-*N*-acetylglucosamine which may have been formed from the added nucleotide by a microsomal 4-epimerase.

The results in figs. 1 and 2 indicate that the galactose and β-phosphate in UDP-galactose were transferred as a galactose 1-phosphate moiety to position 6 of the *N*-acetylglucosamine residue in UDP-*N*-acetylglucosamine. Thus, the products from both UDP-[^{14}C]galactose and [β-^{32}P]UDP-galactose were identical in chromatographic and electrophoretic properties with authentic UDP-GlcNAc-P-Gal; R_{UMP} = 0.37 in solvent A, and 0.83 in solvent C; paper electrophoretic mobility relative to 5′-UMP = 1.50. When an aliquot of the ^{14}C-labeled product, purified by consecutive paper chromatography and paper electrophoresis, was treated with nucleotide pyrophosphatase plus alkaline phosphatase (reaction 1 and 2 in fig. 1), and then subjected again to electrophoresis, about 90% of the original ^{14}C radioactivity was found in a fragment migrating as GlcNAc-P-Gal (fig. 2a). The same treatment of an aliquot of the ^{32}P-labeled product also caused greater than 90% of the ^{32}P to migrate as GlcNAc-P-Gal (fig. 2a). When a second aliquot of the ^{14}C-labeled material was treated with mild acid (fig. 1, reaction 3), and then chromatographed again in solvent B, the released ^{14}C migrated with authentic D-galactose (fig. 2b). The same treatment of a second aliquot of the ^{32}P-labeled material caused greater than 90% of the ^{32}P to migrate as *N*-acetylglucosamine 6-phosphate (fig. 2c). Alkaline phosphatase treatment (fig. 1, reaction 4) of a third aliquot of the ^{32}P-labeled material that had been treated with weak acid resulted in liberation of about 80% of the original ^{32}P label as inorganic phosphate (fig. 2d).

Microsomes prepared from various cells have been shown to contain UDP-*N*-acetylglucosamine:glycoprotein *N*-acetylglucosamine-1-phosphotransferase which is capable of introducing *N*-acetylglucosamine 1-phosphate to mannose

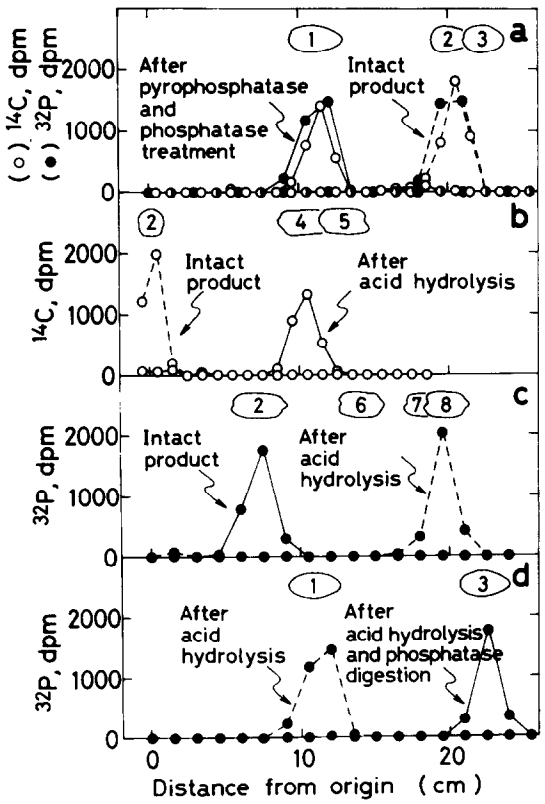

Fig. 2. Identification of the product of galactosyl-phosphotransferase: (a) paper electrophoretogram of the ^{14}C-labeled product (○) and ^{32}P-labeled product (●), before (-----) or after (——) treatment with nucleotide pyrophosphatase and alkaline phosphatase (fig. 1, reactions 1 and 2); (b) paper chromatogram in solvent B of the ^{14}C-labeled product, before (-----) or after (——) mild acid hydrolysis (fig. 1, reaction 3); (c) paper chromatogram in solvent A of the ^{32}P-labeled product, before (——) or after (-----) mild acid hydrolysis (fig. 1, reaction 3); (d) paper electrophoretogram of the acid hydrolysate of ^{32}P-labeled product, before (-----) or after (——) treatment with alkaline phosphatase (fig. 1, reaction 4). See [2] for details of the procedure. Standards: 1, GlcNAc-P-Gal; 2, UDP-GlcNAc-P-Gal; 3, inorganic phosphate; 4, galactose; 5, glucose; 6, UDP; 7, UMP; 8, GlcNAc-6-P.

residues of newly synthesized lysosomal enzymes [5–8]. In view of the similarity in reaction type, we assume that the *N*-acetylglucosamine linked to UDP is not the sole acceptor for galactosyl phosphate transfer but some *N*-acetylglucosaminyl end groups in glycoproteins may also be active acceptors. The activity of galactose-1-phosphotransferase in the assay described here is dependent upon exogenous acceptor (UDP-*N*-acetylglucosamine). This assay has allowed us to determine the distribution of the enzyme in microsomes prepared from various organs and tissues of a laying hen; relative specific activities were 1.0 (liver), 1.2 (ovary), 1.3 (uterus), 1.8 (magnum of oviduct), 2.2 (kidney), and 4.0 (isthmus of oviduct). The wide distribution of activity is consistent with the hypothesis that the synthesis of the hen oviduct nucleotide is not the only function of this enzyme.

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