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Chick Neural Retina *N*-Acetylgalactosaminyltransferase/Acceptor Complex: Catalysis Involves Transfer of *N*-Acetylgalactosamine Phosphate to Endogenous Acceptors†

Janne Balsamo, Rodney S. Pratt, and Jack Lilien*
Department of Zoology, University of Wisconsin—Madison, Madison, Wisconsin 53706
Received December 31, 1985; Revised Manuscript Received April 14, 1986

ABSTRACT: Homogenates of embryonic chick neural retina prepared in 1% Triton X-100 have the ability to transfer *N*-acetyl[^{32}P]galactosamine ([^{32}P]GalNAc) from β-^{32}P-labeled uridine diphosphate *N*-acetylgalactosamine ([β-^{32}P]UDP-GalNAc) to endogenous macromolecular acceptors. The phosphotransferase activity sediments as three distinct peaks upon centrifugation on sucrose gradients. These peaks are coincident with the transferase/acceptor complexes previously described [Balsamo, J., & Lilien, J. (1982) *J. Biol. Chem. 257*, 345–354]. The parameters of the ^{32}P transfer reaction closely parallel those observed with UDP-[^{3}H]GalNAc as substrate when the densest particles, H, are used as a source of transferase/acceptors. Treatment of ^{3}H- and ^{32}P-labeled products with α-*N*-acetylgalactosaminidase removes [^{3}H]GalNAc residues and exposes ^{32}P-labeled groups. These data suggest that the sugar–phosphate is transferred intact, resulting in a terminal phosphodiester linkage. The resistance of the macromolecular products to digestion by endoglycosidase F and its sensitivity to hydrolysis under mild alkaline conditions suggest that the α-linked sugar is transferred to an oligosaccharide chain attached to the protein core via an *O*-serine or threonine residue. Characterization of the ^{32}P- and ^{3}H-labeled H particle products by sodium dodecyl sulfate–polyacrylamide gel electrophoresis reveals a series of coincident high molecular weight polypeptides.

Intact embryonic chick neural retina cells catalyze the incorporation of *N*-acetyl[^{3}H]galactosamine ([^{3}H]GalNAc)[1] from UDP-[^{3}H]GalNAc into endogenous macromolecular acceptors (Balsamo & Lilien, 1980). Both biochemical (Balsamo & Lilien, 1982) and immunological (Balsamo et al., 1986) approaches have verified the presence of enzyme and its acceptor at the cell surface. The enzyme and its endogenous acceptors can be obtained as three distinct complexes, stable in 1% Triton, each of which migrates to a defined density upon equilibrium centrifugation (Balsamo & Lilien, 1982). The properties of these complexes, in particular the densest, are similar to the properties of the enzyme as it exists at the cell surface. Catalysis of the transferase reaction among intact cells results in release of the glycosylated products into the reaction medium (Balsamo & Lilien, 1980), leaving the enzyme still associated with the cell surface and able to glycosylate exogenously added acceptors (Balsamo & Lilien, 1982). In the particulate complexes, the reaction catalyzed by the GalNAc-transferase results in the release of both the enzyme and the glycosylated reaction products into the reaction medium. Like the cell-surface enzyme, the released soluble GalNAc-transferase is now able to glycosylate exogenously added acceptors (Balsamo et al., 1986). The isolated complex thus provides an opportunity to study the properties of the transferase and its acceptor under more defined conditions.

Attempts to identify the GalNAc-transferase glycosylated products have suggested to us that some of the transferred GalNAc residues are very labile, possibly attached to the acceptor molecule via a phosphodiester bond. To investigate this possibility, we have synthesized [β-^{32}P]UDP-GalNAc and used it as a substrate for the particulate form of the enzyme. In this report, we demonstrate that catalysis of the cell-surface GalNAc-transferase/acceptor complex involves the transfer of GalNAc phosphate to endogenous glycoprotein acceptors.

Materials and Methods

Materials. UDP-[^{3}H]GalNAc (10.7 Ci/mmol; 0.0058 mg/mL), [^{3}H]acetic anhydride (50 mCi/mmol), [^{32}P]orthophosphate (25 mCi/250 μL), and Enlightening were purchased from New England Nuclear.

Antipain, leupeptin, chymostatin and PMSF (phenylmethanesulfonyl fluoride) were obtained from Sigma. Antipain and leupeptin were kept at −20 °C at 5 mg/mL in H_2O.

† This work was supported by National Institutes of Health Grant GM29131.
* Correspondence should be addressed to this author.

[1] Abbreviations: GalNAc, *N*-acetylgalactosamine; GalNAc-transferase, *N*-acetylgalactosaminyltransferase; UDP-GalNAc, uridine diphosphate *N*-acetylgalactosamine; GalNAc-1-P, *N*-acetylgalactosamine 1-phosphate; UDP-Glc, uridine diphosphate glucose; Glc-1-P, glucose 1-phosphate; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine; ATP, adenosine 5′-triphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; Me_2SO, dimethyl sulfoxide; DTT, DL-dithiothreitol; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; PTA, phosphotungstic acid.

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Chymostatin was kept at −20 °C at 5 mg/mL in Me_2SO (dimethyl sulfoxide, Sigma). PMSF was kept at 4 °C as a 200 mM solution in 2-propanol.

The following materials were also purchased from Sigma: Hepes, Tris, glycine, sucrose, DTT (DL-dithiothreitol), β-mercaptoethanol, SDS (sodium dodecyl sulfate), Triton X-100, UDP-GalNAc, UDP-GlcNAc, UDP-Glc, Glc-1-P, GalNAc, ATP, and UDP.

Prestained molecular weight markers for PAGE were purchased from Bethesda Research labs. Acrylamide and bis(acrylamide) were obtained from Bio-Rad labs; X-ray film (XAR-5) from Eastman Kodak and intensifying screens used for ^{32}P gels were Cronex Lightning-Plus (Du Pont). Gel filtration material, Sephacryl S-300, and Sephadex G-50 are products from Pharmacia.

Enzymes. Alkaline phosphatase type I, from calf intestine, and β-*N*-acetylhexosaminidase were purchased from Sigma. The β-*N*-acetylhexosaminidase activity was found to have 0.2 unit of β-*N*-acetylgalactosaminidase activity for each unit of β-*N*-acetylglucosaminidase activity, when assayed with the appropriate substrates, at pH 4.0. Purified α-*N*-acetylgalactosaminidase from *Charonia lampas* was obtained from Miles-Yeda. The lyophilized enzyme was resuspended in 0.1 M citrate/phosphate buffer, pH 4.0, at a concentration of 2 units/mL and kept refrigerated for no more than 1 week. Endoglycosidase F was obtained from New England Nuclear. Uridyltransferase from calf liver (UDP-glucose: α-D-galactose-1-phosphate uridyltransferase) was purchased from Boehringher-Mannheim Biochemicals. Glyceraldehyde-phosphate dehydrogenase and 3-phosphoglycerate kinase used in the ATP synthesis were obtained from Sigma as a mixture of the semipurified enzymes.

Preparation of [β-^{32}P]UDP-GalNAc. This preparation was based on the following series of reactions:

$$\text{ADP} + {}^{32}\text{P} \rightarrow [\gamma\text{-}{}^{32}\text{P}]\text{ATP} \quad (1)$$

$$[\gamma\text{-}{}^{32}\text{P}]\text{ATP} + \text{GalNH}_2 \rightarrow \text{GalNH}_2\text{-1-}{}^{32}\text{P} + \text{ADP} \quad (2)$$

$$\text{GalNH}_2\text{-1-}{}^{32}\text{P} + \text{UDP-Glc} \rightleftarrows [\beta\text{-}{}^{32}\text{P}]\text{UDP-GalNH}_2 + \text{Glc-1-P} \quad (3)$$

$$[\beta\text{-}{}^{32}\text{P}]\text{UDP-GalNH}_2 + \text{acetic anhydride} \rightarrow [\beta\text{-}{}^{32}\text{P}]\text{UDP-GalNAc} \quad (4)$$

In eq 1, [γ-^{32}P]ATP was synthesized by ADP phosphorylation using enzymes of the glycolytic pathway as described by Schendel and Wells (1973). Labeled ATP was isolated by paper chromatography on Whatman 3MM paper using 95% ethanol/1 M ammonium acetate, pH 3.8 (5:2), for 16 h, at room temperature, eluted, and lyophilized. In step 2, the reaction was catalyzed by a crude preparation of galactosamine kinase from rat liver, prepared according to Cardini and Leloir (1955). The phosphorylated sugar was separated on paper chromatography using the system described in step one, eluted, and lyophilized. Step 3 is an exchange reaction catalyzed by Gal-1-P:UDP-Glc uridyltransferase. The reaction mixture contained 1 mg/mL $GalNH_2$-1-P, 1 mg/mL UDP-Glc, 10 mg/mL dithiothreitol, 50 μL of the $GalNH_2$-1-^{32}P obtained in step 2, and 1 μL of uridyltransferase in 0.07 M Tris, pH 8, in a total volume of 300 μL. Step 4, the acetylation of [β-^{32}P]UDP-$GalNH_2$, was accomplished by incubating the uridyltransferase reaction mix with acetic anhydride dissolved in methanol, in the presence of 1 M ethanolamine, pH 8.5. The resulting mixture was subjected to paper chromatography for 24 h at room temperature, and the ^{32}P-labeled spot comigrating with standard UDP-[^{3}H]GalNAc was cut out, eluted, lyophilized, redissolved in 50% ethanol/H_2O (v/v), and stored at −20 °C until used. A detailed description of this preparation will be published elsewhere. The double-labeled compound [β-^{32}P]UDP-[^{3}H]GalNAc was synthesized by using [^{3}H]acetic anhydride in the acetylation reaction.

Preparation of Particulate N-Acetylgalactosaminyltransferase/Acceptor Complexes and Their Glycosylated Products. Preparation of neural retina tissue homogenates, fractionation of the particulate transferase/acceptor complexes on sucrose gradients, and preparation of H particle enriched fractions were accomplished as described previously (Balsamo & Lilien, 1982; Balsamo et al., 1986). Glycosylated products were obtained from catalysis of the GalNAc-transferase in H particles. In a typical preparation, H particles from 60 retinas were incubated in 1 mL of HSTI (Hepes-buffered NaCl, pH 7.2, containing 1% Triton and 50 μg/L each of antipain, leupeptin, and chymostatin) containing ca. 20% sucrose, 2 mM Mn^{2+}, and 10 μL (1 μCi) of UDP-[^{3}H]GalNAc or [β-^{32}P]-UDP-GalNAc (10^5–10^6 cpm). Counts incorporated into macromolecules were determined by PTA/TCA (0.5:6%) precipitation, as described (Balsamo & Lilien, 1980). When necessary, glycosylated product was concentrated by precipitation with 5 volumes of 95% ethanol.

Column Chromatography of GalNAc-transferase Products. After catalysis with UDP-[^{3}H]GalNAc, the reaction mixture was mixed with an equal volume of column sample buffer (6% SDS, 20% glycerol, and 5 mM DTT in 0.1 M Tris, pH 8.3), and 500 μL of the resulting mixture was applied to a Sephacryl S-300 molecular sieve column (dimensions: 1.2-cm diameter × 45-cm height) equilibrated in 10 mM Tris, pH 8.3, 0.1% SDS, and 0.5 mM DTT. Fractions of approximately 1 mL were collected, and an aliquot was used for determining radioactivity by liquid scintillation counting. Peaks of radioactive material were pooled, concentrated on Centricon 10 (Amicon), and treated as described in the figure legends. The Sephadex G-50 column was equilibrated in 0.1 M sodium acetate/0.05 M NaCl, pH 5.0.

Analysis of the ^{32}P- and ^3H-Labeled Products by SDS–Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of SDS was performed in slabs, using the method of Laemmli (1970). The products of the glycosylation reaction were concentrated and redissolved in Tris-HCl buffer (62.5 mM, pH 6.8) containing 3% SDS, 5% β-mercaptoethanol, 10% glycerol, and bromophenol blue (50 μg/mL). Separating gels contained 7% or a 5–10% gradient of acrylamide/bis(acrylamide) (30:0.8). After electrophoresis, the gels of ^{3}H-labeled material were soaked in Enlightening for 20 min, dried overnight under vacuum without heat, and autoradiographed at −70 °C. The ^{32}P-labeled gels were soaked in 10% glycerol for 30 min, dried under vacuum without heat, and autoradiographed at −70 °C with intensifying screens.

Results

Particulate Forms of GalNAc-transferase Catalyze the Transfer of GalNAc-1-^{32}P from [β-^{32}P]UDP-GalNAc to Endogenous Acceptors. Equilibrium centrifugation of homogenates of embryonic chick neural retina reveals three distinct sedimentable *N*-acetylgalactosaminyltransferase-containing complexes (Balsamo & Lilien, 1982). The transferase and its endogenous acceptors band at the same density on recentrifugation in sucrose gradients and comigrate both on molecular sieve and on lectin affinity chromatography columns (unpublished results).

When a mixture of UDP-GalNAc preparations labeled with ^{32}P in the β-position of the nucleotide residue or ^3H in the GalNAc residue is used as substrate, both ^{32}P and ^3H are incorporated into macromolecular products. The distribution

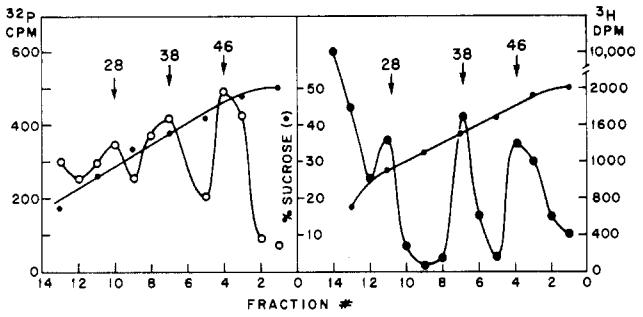

FIGURE 1: Incorporation of ^{32}P and ^{3}H from [β-^{32}P]UDP-GalNAc and UDP-[^{3}H]GalNAc into endogenous acceptors following fractionation of neural retina homogenates by density centrifugation. Triton X-100 extracts of neural retinas were prepared and fractionated on sucrose gradients as described under Materials and Methods. Each fraction was divided in half, made 2 mM in Mn^{2+}, and incubated for 1 h at 37 °C with UDP-GalNAc labeled in the nucleotide ^{32}P or sugar residue ^{3}H. The amount of radioactivity incorporated into macromolecules is plotted for each gradient fraction.

of this activity on a sucrose gradient is shown in Figure 1; ^{32}P-labeled product is seen to codistribute with ^{3}H-labeled product. The ratio of $^{32}P/^{3}H$ incorporated into PTA/TCA-precipitable material is highest for the densest peak, with little or no incorporation of ^{32}P in the fractions banding at the top of the gradient. Similar results were obtained when the double-labeled compound [β-^{32}P]UDP-[^{3}H]GalNAc was used as substrate (results not shown). Incubation of the fractions at the top of the gradient with UDP-[^{3}H]GalNAc has been shown to result in a population of very high molecular weight labeled components sensitive to digestion by hyaluronidase (Balsamo & Lilien, 1982).

To analyze and compare the parameters of the ^{32}P-labeled sugar transfer reaction to those of the ^{3}H-labeled sugar, we have used the densest complexes, termed H particles, as a source of transferase/acceptor. These particles are localized to the plasma membrane (Balsamo & Lilien, 1982) and can be obtained virtually free of contamination by the other forms (Balsamo et al., 1986).

Incorporation of ^{32}P and ^{3}H into endogenous acceptors in H particles is totally inhibited by 5 mM EDTA, while Mn^{2+} is stimulatory with an optimum at 2 mM (not shown). In the presence of 2 mM Mn^{2+}, addition of 1 mM Ca^{2+} to the reaction medium greatly reduces ^{3}H incorporation and totally inhibits incorporation of ^{32}P (not shown). The reaction shows little dependence on pH from 6 to 8, with an optimum at 7.2 (not shown). Incorporation increases with increasing concentrations of H particles, up to 200 μg/mL protein (not shown).

Incorporation for both ^{32}P and ^{3}H is linear up to 20 min (Figure 2). After that period, the amount of radioactivity due to ^{32}P incorporation declines slowly. Incorporation of ^{3}H consistently follows a bimodal curve; like ^{32}P, a plateau is reached at about 20 min, after which incorporation increases linearly up to 120 min (Figure 2). The consistency with which the incorporation of both substrates reaches plateau at approximately 20 min suggests that the initial phase of the reaction is similar for both substrates. The bimodal nature of the ^{3}H incorporation curve does suggest, however, that two distinct reactions are occurring within the same time period. This suggestion is further supported by analysis of the reaction products (see below). The apparent rates of incorporation for each isotope cannot be directly compared as the specific activity of the ^{32}P-labeled substrate could not be determined accurately due to extremely low yields.

Transfer of both ^{32}P and ^{3}H decreases when increasing concentrations of unlabeled UDP-GalNAc are included in the

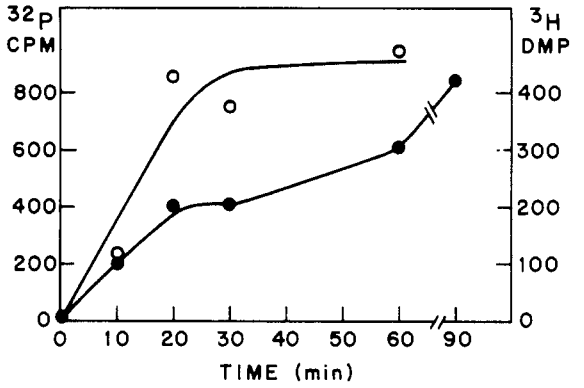

FIGURE 2: Time course of incorporation of ^{32}P and ^{3}H into macromolecular endogenous acceptors. H particles were incubated with ^{32}P (O) or ^{3}H (●) labeled UDP-GalNAc and 2 mM Mn^{2+} for the indicated times.

Table I: Incorporation of ^{32}P by H Particles in the Presence of Various Additions to the Reaction Mixture[a]

addition	cpm of ^{32}P incorporated[b]	% of control
none	1046	100
50 mM UDP-GalNAc	0	0
100 mM UDP-GlcNAc	1100	100
0.5 mM UDP-Glc	1160	110
1 mM Glc-1-P	1122	107
1 mM GalNAc	722	69
1 mM GalNAc-1-P	688	66
1 mM ATP	991	95
10 mM P_i	1306	125

[a] Equal aliquots of an H particle preparation were incubated with [β-^{32}P]UDP-GalNAc, 2 mM Mn^{2+}, and the indicated addition for 30 min at 37 °C. The amount incorporated into macromolecules was determined as described in the text. [b] cpm incorporated represents the average of duplicate samples. Background values were subtracted from total counts.

reaction mixture, reaching background levels at about 50 μM UDP-GalNAc. In contrast, concentrations of up to 100 μM UDP-GlcNAc have no effect on the level of ^{32}P or ^{3}H incorporation. Addition of 1 mM GalNAc inhibits incorporation of both isotopes by about 60% (Table I; Balsamo & Lilien, 1980). Also listed in Table I are the effects of addition to the reaction mixture of UDP-Glc, Glc-1-P, inorganic phosphate, and ATP on the incorporation of ^{32}P. Only ATP shows a slight inhibitory effect, at a concentration of 1 mM, causing a decrease of 5% in the incorporation of ^{32}P. Using GalNAc-1-^{32}P to replace [β-^{32}P]UDP-GalNAc resulted in no detectable incorporation of ^{32}P into endogenous macromolecular material (not shown).

Phosphosugar Linkage in Glycosylated Transferase Product Is Sensitive to Digestion by α-N-Acetylgalactosaminidase. Loss of ^{3}H counts due to digestion with α-*N*-acetylgalactosaminidase increases with enzyme concentration; typically, for an H particle preparation, a plateau is reached when about 50% of the originally incorporated counts are lost (Table II). Treatment of product with 0.1 unit/mL α-*N*-acetylgalactosaminidase exposes all terminal ^{32}P-labeled phosphate residues which then become susceptible to hydrolysis by alkaline phosphatase. Hydrolysis of the phosphate residues was followed by monitoring the loss of radioactivity from the ^{32}P-labeled transferase product (Table II). Treatment with alkaline phosphatase alone is ineffective in releasing either ^{32}P or ^{3}H counts from the labeled product (Table II). These results indicate that (1) ^{32}P is not incorporated into macromolecular products as a terminal residue and (2) the GalNAc-1-P is transferred as a unit from UDP-GalNAc, maintaining the α configuration of the sugar–phosphate linkage.

Table II: α- and β-*N*-Acetylhexosaminidase Digestion of Labeled Products

sample treatment[a]	^3H cpm	^{32}P cpm[b]
none (control)	388	796
α-*N*-acetylgalactosaminidase[c]	222	898
β-*N*-acetylhexosaminidase[c]	187	881
α-*N*-acetylgalactosaminidase[d] followed by alkaline phosphatase	ND[f]	13
β-*N*-acetylgalactosaminidase[d] followed by alkaline phosphatase	ND	737
alkaline phosphatase[e]	420	910

[a] Sample was prepared by incubating an H particle preparation with 2 mM Mn^{2+} and the indicated substrate for 30 min at 37 °C; aliquots of equal volume were treated as indicated. [b] cpm incorporated represents the results from a single experiment typical of many. [c] Hexosaminidase digestion was carried out at pH 4 with 0.1 unit/mL of α-*N*-acetylgalactosaminidase or 0.5 unit/mL of β-*N*-acetylhexosaminidase for 30 min at room temperature. [d] After hexosaminidase digestion, the pH of the solution was raised to 8 with 1 M Tris, and 10 units/mL of alkaline phosphatase were added, followed by 1-h incubation at 37 °C. [e] The sample was held at pH 4 for 30 min, at room temperature; the pH was then raised to 8.0 and incubation with alkaline phosphatase followed for 1 h at 37 °C. [f] ND, not determined.

Loss of ^3H counts is also observed following treatment of ^3H-labeled GalNAc-transferase product with β-*N*-acetylhexosaminidase at concentrations equivalent to that of α-*N*-acetylhexosaminidase (see Materials and Methods). Digestion of ^3H-labeled products is dose dependent, reaching a plateau at about 0.5 unit/mL, with 40–60% loss of ^3H counts (Table II). Treatment of transferase product with β-*N*-acetylhexosaminidase, however, does not render ^{32}P-labeled residues labile to alkaline phosphatase (Table II). These results suggest that there are two different GalNAc-transferase activities in H particles, one catalyzing the transfer of α-GalNAc-1-P and the other catalyzing the transfer of β-GalNAc residues.

To further analyze the GalNAc-transferase products containing terminal α- or β-linked GalNAc residues, preparations of H particles were incubated with UDP-[^3H]GalNAc and the reaction products chromatographed on Sephacryl S-300 under denaturing and reducing conditions. ^3H-Labeled material elutes as a high molecular weight, polydisperse peak and a low molecular weight, polydisperse peak comigrating with unreacted substrate. The pooled high molecular weight peak elutes at the same position upon rechromatography and is totally digested by Pronase (Figure 3A,B). After extensive dialysis against sodium acetate, pH 4.0, the pooled, concentrated, high molecular weight peak was subjected to digestion with α-*N*-acetylgalactosaminidase (0.01 unit/mL) or β-*N*-acetylhexosaminidase (0.1 unit/mL) for 1 h at 37 °C and rechromatographed on the same column. Treatment with α-*N*-acetylhexosaminidase resulted in about 80% of the label eluting at the column salt volumn (Figure 3d). In contrast, treatment with β-*N*-acetylhexosaminidase had no effect on the elution profile (Figure 3c). The β-*N*-acetylhexosaminidase labile material is contained within the polydisperse, low molecular weight peak from Sephacryl S-300 along with unreacted substrate. This material can be resolved from unreacted substrate on a Bio-Gel P-2 column (not shown). Enzyme catalysis in H particles thus results in two distinct types of product: a high molecular weight glycoprotein population containing α-linked terminal GalNAc residues and a low molecular weight population containing β-linked terminal GalNAc residues. It is not clear at the moment if both activities are due to a single enzyme.

GalNAc-P Residue Is Attached to an O-Linked Oligosaccharide Chain. The nature of the oligosaccharide/protein linkages in the labeled product was analyzed by treating the high molecular weight peak from the Sephacryl S-300 column with endoglycosidase F or mild alkaline hydrolysis. No change in the elution profile was observed after an overnight incubation with endoglycosidase F; however, after treatment with 0.1 N NaOH overnight, the radioactivity was eluted in the position of the salt volume markers (not shown).

To verify that the labeled sugar within the released oligosaccharide is attached via an α linkage, as in the phosphodiester-linked sugar, the labeled high molecular weight material obtained from Sephacryl S-300 was incubated in 0.1 N NaOH at 45 °C, for 30 min, and rechromatographed on Sephadex G-50 (Figure 4a). Under these conditions, hydrolysis is incomplete, and some labeled material elutes with the void volume following gel filtration on Sephadex G-50. The recovered oligosaccharide was treated with α-*N*-acetylhexosaminidase and rechromatographed on the same column. This resulted in radiolabel eluting in the position of [^3H]-

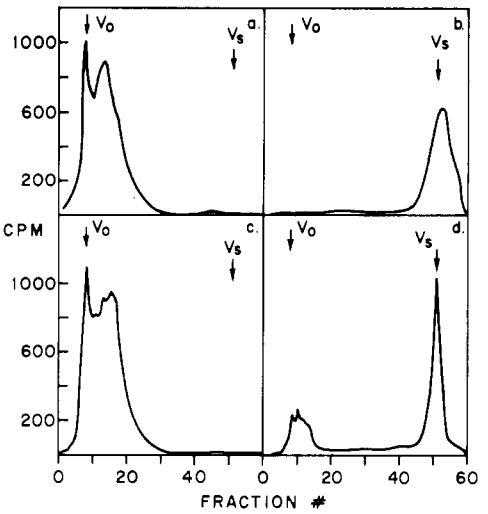

FIGURE 3: Column chromatography of ^3H-labeled products on Sephacryl S-300. H particle preparation incubated with UDP-[^3H]-GalNAc was mixed with an equal volume of column sample buffer (see Materials and Methods) and applied to the column. The eluted fractions containing labeled macromolecular material were pooled, made 1 mM in iodoacetamide, and concentrated as described under Materials and Methods. Equal aliquots were reapplied to the column after the following treatments: (a) sample incubated at 37 °C for 16 h; (b) sample incubated with 1 mg/mL Pronase at 37 °C for 16 h; (c) sample incubated with 0.1 unit/mL β-*N*-acetylhexosaminidase at pH 4.0 for 60 min at 37 °C; (d) sample incubated with 0.01 unit/mL α-*N*-acetylgalactosaminidase at pH 4.0 for 60 min at 37 °C.

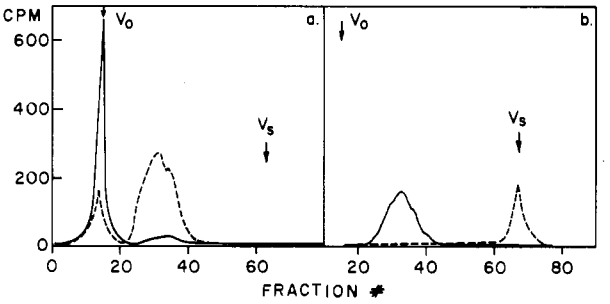

FIGURE 4: Elution profile of high molecular weight products on Sephadex G-50. (a) The high molecular weight product peak eluted from the Sephacryl column was concentrated and applied to a Sephadex G-50 column: (solid line) sample incubated for 30 min at 45 °C at neutral pH; (dashed line) sample incubated in 0.1 N NaOH/1 M $NaBH_4$ for 30 min at 45 °C. Before being reapplied to the column, the pH was adjusted to neutral. (b) Fractions 20–40 from the experiment shown in (a) were pooled and incubated at 37 °C for 30 min with (dashed line) or without (solid line) α-*N*-acetylhexosaminidase at pH 4.0.

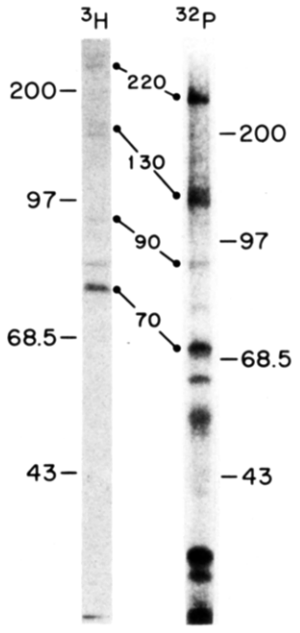

FIGURE 5: SDS–polyacrylamide gel electrophoresis of H particle products labeled with ^{3}H and ^{32}P. Labeled products were prepared by incubating H particles with UDP-[^{3}H]GalNAc or [β-^{32}P]UDP-GalNAc in the presence of 2 mM Mn^{2+} for 1 h at 37 °C. The ^{32}P and ^{3}H products were run on different gel systems. The molecular weights of the labeled bands were calculated on the basis of the migration of a set of prestained SDS–PAGE molecular weight standards. The gels were processed for autoradiography as described under Materials and Methods.

GalNAc (Figure 4b). In contrast, treatment of the G-50 voided peak or oligosaccharide peak with β-*N*-acetylhexosaminidase had no effect on the position of eluted radioactivity. Sugar transferred to macromolecular product is thus linked in an α configuration to an *O*-serine- or threonine-linked oligosaccharide.

^{32}P- and ^{3}H-Labeled Products Comigrate in SDS–Polyacrylamide Gel Electrophoresis. The results of analysis of macromolecular products generated by using ^{32}P- and ^{3}H-labeled substrate by SDS–PAGE are presented in Figure 5. Comparison of the autoradiographs shows that all the bands present in the ^{3}H-labeled preparation are also represented in the ^{32}P-labeled product. These areas of coincidence correspond to relative molecular masses of approximately 220 000, 130 000, 90 000, 80 000, and 70 000. As shown in Figure 5, the polypeptides often appear as doublets in the ^{3}H-labeled preparations and as somewhat broad, diffuse bands on the ^{32}P-labeled gel. Although this array of high molecular weight bands is very reproducible, the intensity of each band varies from experiment to experiment. The banding pattern in the low molecular weight region of the gel is much more variable. One important source of variability is the lability of the distal ester linkage. This results in a variable amount of release of the ^{3}H-labeled sugar. In preliminary experiments attempting to determine the effect of the gel buffers on hydrolysis of the terminal sugar, we found that as much as 50% of the ^{3}H precipitable counts were lost. Since the phosphate residue is retained on hydrolysis, product labeled with ^{32}P is unaffected.

Discussion

The neural retina *N*-acetylgalactosaminyltransferase described here and in previous publications (Balsamo & Lilien, 1980, 1982; Balsamo et al., 1986) is unique in several ways. First, it exists in discrete multimolecular complexes which undergo a series of ordered interconversions (Balsamo & Lilien, 1982). Second, its endogenous acceptors remain stably associated with the enzyme (Balsamo & Lilien, 1982; Balsamo et al., 1986). We now add to these characteristics the fact that catalysis involves addition of a terminal phosphodiester-linked GalNAc to an O-linked oligosaccharide chain.

The data presented in this paper show that the *N*-acetylgalactosaminyltransferase present in partially purified H particles can catalyze the transfer of terminal α- or β-linked GalNAc residues to endogenous acceptors. The α-linked sugar is attached to the carbohydrate chain via a phosphodiester bond. When [^{32}P]UDP-GalNAc is used as a substrate, digestion of the sugar by α-*N*-acetylhexosaminidase leaves exposed terminal phosphate groups on the acceptor; GalNAc-P is apparently transferred intact from UDP-GalNAc, maintaining the linkage present in the nucleotide sugar. Although the β-linked terminal GalNAc residues account for a significant percentage of the total sugar incorporated into acid-insoluble molecules, they do not chromatograph with the high molecular weight ^{3}H-labeled product after denaturation and reduction. It is possible that they are the result of incorporation of terminal GalNAc residues into small molecules, perhaps glycolipids, which become incorporated into Triton X-100 micelles. It is not clear, at present, whether both types of glycosylation can be accomplished by the same enzyme.

Sugar phosphotransferases have been identified in at least two other higher eukaryotic systems. In both cases, the role of the phosphodiester-bonded sugar appears to be in "targeting" the product to the appropriate cellular destination. Lysosomal enzymes are terminally glycosylated by an *N*-acetylglucosaminylphosphotransferase (Reitman & Kornfeld, 1981; Hasilik et al., 1981). The *N*-acetylglucosamine is subsequently removed, leaving exposed phosphomannose residues (Varki & Kornfeld, 1980, 1981; Waheed et al., 1981). The transferase recognizes high mannose containing oligosaccharide chains and shows selectivity for lysosomal enzymes. The phosphomannose residues are critical for the specific binding and uptake of these enzymes by intact cells (Fischer et al., 1980a,b). However, the normal processing and targeting of these enzymes presumably do not involve secretion and rebinding but interaction with internal phosphomannose receptors and translocation to the lysosome (Sly & Fischer, 1982; Gabel et al., 1982).

Phosphodiester-linked terminal sugars have also been implicated in the interaction of hexosaminidase (Jakoi et al., 1976, 1981) and acetylcholinesterase (Gaston et al., 1982) with ligatin, a 10 000-kilodalton polypeptide present at the cell surface in filamentous arrays. Marchase et al. (1981) have additionally identified a population of glycoproteins from chick neural retinas which bind to ligatin and are eluted with Glc-1-P. Koro and Marchase (1982) have reported the existence of a glucosephosphotransferase among embryonic chick neural retina cells. The endogenous acceptors for this enzyme are also high mannose type oligosaccharides and presumably identical with the previously identified ligatin binding glycoproteins.

Unlike these other systems, the neural retina *N*-acetylgalactosaminylphosphotransferase transfers the sugar to an oligosaccharide chain linked to the protein core via the β-hydroxyl group of serine and/or threonine. This conclusion is based on the lability of the labeled oligosaccharide chain to mild alkaline hydrolysis and on the removal of the terminal GalNAc from the free sugar chain by α-*N*-acetylhexosaminidase. The fact that treatment of the high molecular weight ^{3}H-labeled product with endoglycosidase F does not result in the release of ^{3}H-labeled oligosaccharides does not

rule out the presence of *N*-aspragine-linked oligosaccharide chains on the product molecules.

O-Serine/threonine-linked oligosaccharides are found in a variety of glycoproteins, mucins, and proteoglycans, and there is evidence that they play a role in protecting mucins against proteolysis (Allen, 1983), in platelet adhesion (Judson et al., 1982; Tsiyi et al., 1983), in vaccinia virus hemagglutination (Shida & Dales, 1981), and in binding of sperm to egg receptors in mice (Florman & Wasserman, 1985). This last system is of particular interest to us. In recent publications, Lopez et al. (1985) have demonstrated that a galactosyltransferase present on sperm is involved in sperm/egg binding, and Florman and Wasserman (1985) have identified a sperm binding component as an O-linked oligosaccharide chain present on the egg. It may well be that mouse sperm/egg interactions are the result of sperm galactosyltransferase interacting with O-linked egg oligosaccharides. In a previous publication (Balsamo & Lilien, 1982), we have pointed out that the presence of the GalNAc-transferase/acceptor complex at the cell surface is correlated with the ability of the cells to form Ca^{2+}-dependent adhesive bonds. It is our working hypothesis that this transferase is involved in the formation and/or modulation of Ca^{2+}-dependent adhesions.

Registry No. [β-^{32}P]UDP-GalNAc, 103904-68-3; UDP-acetylgalactosamine–glycoprotein acetylgalactosaminyltransferase, 9075-15-4.

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