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UDP-*N*-Acetylglucosamine:Glycoprotein *N*-Acetylglucosamine-1-phosphotransferase

PROPOSED ENZYME FOR THE PHOSPHORYLATION OF THE HIGH MANNOSE OLIGOSACCHARIDE UNITS OF LYSOSOMAL ENZYMES*

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The recognition marker for the targeting of lysosomal enzymes contains mannose 6-phosphate. The recent discovery of phosphate in diester linkage between *N*-acetylglucosamine (GlcNAc) and mannose in newly synthesized β-glucuronidase led to the proposal that the phosphate might be acquired via *N*-acetylglucosamine-phosphate transfer from UDP-GlcNAc (Tabas, I., and Kornfeld, S. (1980) *J. Biol. Chem.* 255, 6633-6639). We describe the synthesis of [β-^{32}P]UDP-[^{3}H]GlcNAc and the use of this compound to demonstrate a UDP-GlcNAc:glycoprotein *N*-acetylglucosamine-1-phosphotransferase. The basis of the enzyme assay is the incorporation of ^{32}P and ^{3}H into glycopeptides with a high affinity for Concanavalin A-Sepharose. This membrane-associated transferase is neither inhibited by tunicamycin nor stimulated by dolichol-phosphate, indicating that the reaction does not proceed via a dolichylpyrophosphoryl-*N*-acetylglucosamine intermediate. Characterization of the enzyme reaction products (derived from either endogenous or exogenous acceptors) demonstrated that α-linked *N*-acetylglucosamine 1-phosphate is transferred *en bloc* to the 6-hydroxyl of mannose in high mannose oligosaccharides of glycoproteins. We propose that the function of this enzyme is to donate *N*-acetylglucosamine 1-phosphate to mannose residues of newly synthesized lysosomal enzymes.

It is now established that 6-phosphomannosyl residues present on high mannose-type oligosaccharide units of acid hydrolases serve as an essential component of the recognition marker that mediates enzyme uptake by various cell types and targeting to lysosomes (1-12, reviewed in 13). We have reported that the biosynthetic intermediates of the acid hydrolase β-glucuronidase contain phosphate groups in diester linkage between mannose residues of the underlying oligosaccharide and outer α-linked *N*-acetylglucosamine residues (14). Since the phosphate is present in the mature enzyme as a phosphomonoester moiety (1-3, 5, 9, 11), it was proposed that the mechanism of phosphorylation of acid hydrolases involves the transfer of α-*N*-acetylglucosamine 1-phosphate to mannose residues of the high mannose-type oligosaccharide followed by the selective removal of the *N*-acetylglucosamine residue to expose a phosphomannosyl group (14). We recently demonstrated that rat liver smooth membrane preparations contain an α-*N*-acetylglucosaminyl phosphodiesterase which is capable of removing the "blocking" *N*-acetylglucosamine residues from phosphorylated high mannose-type oligosaccharides (15). We now present evidence for the existence of the postulated UDP-GlcNAc:glycoprotein *N*-acetylglucosamine-1-phosphotransferase.

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EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from the following sources: [6-^{3}H]glucosamine (19 Ci/mmol) and [γ-^{32}P]ATP (2000 Ci/mmol), New England Nuclear; α-minimum essential medium, Flow Laboratories, Rockville, MD; fetal bovine serum, penicillin, and streptomycin, GIBCO; Triton X-100 and 3a70 scintillation mixture, Research Products International Corp., Elk Grove Village, IL; Concanavalin A[1]-Sepharose, Pharmacia; Bio-Gel P-6 (200-400 mesh), Bio-Rad Laboratories; QAE-Sephadex (Q-25-120), thyroglobulin, dolicholphosphate, and other reagents, Sigma. DEAE-cellulose (DE-52, Whatman) was converted to the acetate form as described (16). Tunicamycin was a gift of Professor G. Tamura, University of Tokyo, Japan.

Enzymes—Homogeneous *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) was a gift from Dr. M. Schlesinger, Washington University. Pig liver α-*N*-acetylglucosaminidase (EC 3.2.1.50) was prepared as previously described (17). Pronase, grade B, was obtained from Calbiochem. Rat liver α-*N*-acetylglucosaminyl phosphodiesterase (15), purified 1800-fold, was obtained from Dr. A. Varki.[2] Phosphomannose isomerase (EC 5.3.1.8), phosphoglucose isomerase (EC 5.3.1.9), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and hexokinase (EC 2.7.1.1) (all from yeast), and rabbit muscle phosphoglucomutase (EC 2.7.5.1) were purchased from Boehringer-Mannheim. *Streptomyces griseus* endo-β-*N*-acetylglucosaminidase H was obtained from Miles. A crude *Staphylococcus aureus* extract with UDP-*N*-acetylglucosamine pyrophosphorylase (EC 2.7.2.23) activity was prepared as described by Strominger and Smith (18), stopping before the calcium phosphate step. Under the conditions in which it was used, the preparation did not exhibit UDP-*N*-acetylglucosamine 4-epimerase activity as determined by the absence of *N*-acetylgalactosamine on mild acid hydrolysis. The *S. aureus* (Cowan I) was provided by Dr. Steven Rose, Washington University, and was grown in brain-heart infusion media.

Paper Chromatography—Descending paper chromatography was performed using Whatman No. 3MM paper with the following solvent systems: Solvent A, ethanol/1 M ammonium acetate, pH 3.8 (7.5:3); Solvent B, ethanol/1 M ammonium acetate, pH 7.5 (7.5:3); Solvent C, the upper phase from ethyl acetate/pyridine/water (360:100:115); and Solvent D, ethyl acetate/pyridine/acetic acid/water (5:5:1:3). Reducing sugar standards were visualized by the alkaline silver nitrate technique (19). Free phosphate was detected as described by Burrows *et al.* (20). Nucleotides were visualized by UV illumination. Radioactivity was detected by cutting the paper into 1-cm strips, adding 0.4

[1] The abbreviations used are: Con A, Concanavalin A; Endo H, endo-β-*N*-acetylglucosaminidase H; GlcNAc, *N*-acetylglucosamine. All sugars are of the D configuration.

[2] A. Varki and S. Kornfeld, manuscript in preparation.

ml of water, 4 ml of 3a70, and counting in a scintillation spectrometer.

Preparation of [β-^{32}P]UDP-[^{3}H]GlcNAc—The synthesis of this compound involved the enzymatic production of [^{3}H]glucosamine 1-[^{32}P]phosphate followed by chemical acetylation (21) to form *N*-acetyl[^{3}H]glucosamine 1-[^{32}P]phosphate and finally enzymatic conversion to the nucleotide sugar (18). The initial reaction mixture contained the following components in a final volume of 0.3 ml: 2 μmol of [6-^{3}H]glucosamine (1 mCi), 1 μmol of [γ-^{32}P]ATP (2 mCi), 0.5 μM glucose 1,6-diphosphate, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 100 mM Tris-Cl, pH 8.0, 0.1 mg of hexokinase, and 1 mg of phosphoglucomutase. The hexokinase and phosphoglucomutase were dialyzed against 10 mM Tris-Cl, pH 8.0, 1 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 0.1 mM EDTA immediately prior to use. The reaction mixture was incubated for 5 h at 37 °C and then 0.8 ml of 130 mM $NaHCO_3$ was added followed by 8 μl of acetic anhydride in 0.2 ml of water. After 5 min at room temperature, the reaction mixture was heated for 3 min in a boiling water bath. The pH of the incubation mixture was then adjusted to 7.5 with 1 M acetic acid, UTP was added to a final concentration of 10 mM, and 50 μl of the UDP-*N*-acetylglucosamine pyrophosphorylase preparation (194 μg of protein, 0.13 unit) was added. The reaction mixture was incubated for 1 h at 37 °C and then heated again for 3 min in a boiling water bath. The sample was streaked over 8 cm on Whatman No. 3MM paper, chromatographed in solvent A for 10 h, and the material migrating with the UDP-GlcNAc standard was eluted and rechromatographed in solvent B for 10 h. The UV absorbing spot with the mobility of UDP-GlcNAc was eluted and stored at −20 °C in 1:1 (v/v) ethanol/H_2O for up to 1 month. The overall yield of [β-^{32}P]UDP-[^{3}H]GlcNAc ranged from 5–10%. The maximal theoretical yield is limited to 15–20% by the equilibrium constant of the phosphoglucomutase reaction (22).

Rechromatography of the final product in solvents A and B demonstrated co-migration with UDP-GlcNAc and >99% radiochemical purity for both isotopes. Following mild acid hydrolysis (0.05 M HCl, 100 °C, 10 min), greater than 99% of the ^{3}H migrated with *N*-acetylglucosamine in solvents A and C and 85% of the ^{32}P migrated with UDP while 15% migrated with P_i in solvent A.

Preparation of Thyroglobulin Glycopeptides—One g of bovine thyroglobulin was dissolved in 20 ml of 100 mM Tris buffer, pH 8.0, containing 2 mM $CaCl_2$. Pronase (10 mg) was added and the reaction mixture was incubated at 56 °C for 48 h. The digestion was terminated by boiling for 5 min and insoluble material was removed by centrifugation. The supernatant was desalted in aliquots on Sephadex G-25. The fractions containing glycopeptide material were detected with the phenolsulfuric acid assay (23). These fractions were pooled, concentrated, and applied to a Bio-Gel P-6 column (1.5 × 95 cm) using 0.1 M NH_4HCO_3 as eluting buffer. Two broad peaks of glycopeptide material eluted from the column. The second peak, which is enriched in high mannose-type glycopeptides, was pooled and used as exogenous acceptor in the enzyme assays.

Cells—The isolation and growth of the Chinese hamster ovary cell line 15B has been described previously (24). This line is deficient in UDP-GlcNAc:glycoprotein *N*-acetylglucosaminyltransferase I (25).

Preparations of Cell Homogenates—Suspension cultures of 15B cells were harvested by centrifugation and washed in Dulbecco's phosphate-buffered saline (26). Two volumes of Buffer A (50 mM Tris-Cl, pH 7.4, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 1% Triton X-100, 0.25 mM dithiothreitol) were added to the cell pellet and the cells were disrupted with 10 strokes of a Teflon pestle in a Potter-Evehjem homogenization apparatus. The protein concentration of the homogenate was determined by a modification of the method of Lowry *et al.* (27), including 0.5% sodium dodecyl sulfate.

Assay of N-Acetyl[^{3}H]glucosamine-[^{32}P]Phosphate Transfer to Endogenous and Exogenous Acceptors—The standard reaction mixture contained 1.25 nmol of [β-^{32}P]UDP-[^{3}H]GlcNAc (approximately 10^6 cpm of ^{32}P and 10^5–10^6 cpm of ^{3}H), 5 mM ATP, 0.75 mg of cell extract protein, and, when indicated, thyroglobulin glycopeptide (650 nmol of hexose) in a final volume of 0.08 ml of buffer A in a 1.5-ml polypropylene tube. Following incubation at 37 °C for 60 min, the samples were boiled for 5 min, cooled, and then 1.5 ml of chloroform/methanol (2:1) were added. After 15 min at room temperature the tubes were centrifuged at 12,000 × *g* for 2½ min and the supernatant fluid removed by aspiration. The chloroform/methanol extraction was repeated. The pellet was then air-dried, extracted three times with 1.0 ml of water, and suspended in 0.5 ml of buffer B (100 mM Tris-Cl, pH 8.0, 0.1 M glucose 6-phosphate, 20 mM $CaCl_2$) containing 1 mg of pronase. The samples were incubated for 1 h at 56 °C and then heated for 10 min at 100 °C to inactivate the pronase. The samples were diluted to 1.5 ml and applied to Con A-Sepharose columns (0.5 × 2.0 cm). The columns were washed with phosphate-buffered saline until the eluate contained no radioactivity (usually about 100 ml) and then elution was begun with 4 ml of 10 mM α-methylglucoside in buffer C (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.02% NaN_3). The final elution was with four 1-ml aliquots of 0.5 M α-methylmannoside in buffer C with 1-h intervals between aliquot additions. The radioactivity in the α-methylglucoside and the α-methylmannoside eluates was determined after the addition of 10 ml of 3a70.

When thyroglobulin glycopeptide was present as exogenous acceptor, the three water extracts were pooled and chromatographed on Con A-Sepharose as described for the endogenous acceptor.

RESULTS

Demonstration of N-Acetyl[^{3}H]glucosamine-[^{32}P]Phosphate Transfer to Endogenous Acceptors—Since cell extracts contain a number of different *N*-acetylglucosaminyltransferases capable of transferring *N*-acetylglucosamine from UDP-GlcNAc to both endogenous and exogenous acceptors, it was necessary to design an assay which would be selective for the postulated UDP-GlcNAc:mannose *N*-acetylglucosaminylphosphotransferase. In developing the assay, we attempted to gain selectivity at three different steps. First, we utilized [β-^{32}P]UDP-[^{3}H]GlcNAc as the nucleotide sugar donor so that the transfer of both *N*-acetylglucosamine and phosphate to the acceptor could be followed. Second, the product of the reaction was purified by Con A-Sepharose chromatography using conditions that select for high mannose-type oligosaccharides. In purifying product from reactions using endogenous glycoprotein acceptors, the boiled reaction mixtures were first extracted with chloroform/methanol (2:1), and then with water to remove remaining substrate and breakdown products. Pronase was next added to convert the endogenous glycoproteins to glycopeptides. The soluble glycopeptides were separated on Con A-Sepharose into species with no or low affinity *versus* those with high affinity (*e.g.* high mannose-type oligosaccharides). The latter are not eluted with 10 mM α-methylglucoside but are eluted with 0.5 M α-methylmannoside. Third, we performed these initial experiments with homogenates of 15B cells. This cell line lacks UDP-GlcNAc:glycoprotein *N*-acetylglucosaminyltransferase I, the major enzyme capable of transferring *N*-acetylglucosamine residues to high mannose-type oligosaccharides (25, 28). Therefore, by using this cell line we were able to virtually eliminate the transfer of *N*-acetylglucosamine to oligosaccharides that bind tightly to Con A-Sepharose, and therefore prevented their interference in the detection of oligosaccharides to which *N*-acetylglucosamine-phosphate had been transferred.

Using these assay conditions, extracts of 15B cells transferred *N*-acetyl[^{3}H]glucosamine and [^{32}P]phosphate to endogenous acceptors in a time-dependent fashion (Fig. 1*A*). The amount of substrate transferred was proportional to the amount of cell extract added (Fig. 1*B*). The finding that the ^{3}H and ^{32}P were transferred in a 1:1 molar ratio with the same time course and protein concentration dependence indicated that the two labels were being transferred as a single moiety, *e.g.* as *N*-acetylglucosamine 1-phosphate. Paper chromatography in solvent A demonstrated that no intact substrate remained at prolonged incubation times, accounting for the lack of further ^{32}P transfer.

The requirements for *N*-acetylglucosaminylphosphotransferase activity are summarized in Table I. There is an absolute requirement for divalent ions, with Mg^{2+} and Mn^{2+} being more effective together than when present individually. Triton X-100 and dithiothreitol are not required for enzymatic activity although both appear to potentiate activity to a small extent. The addition of 100-fold excess of unlabeled UDP-GlcNAc (1.6 mM) completely inhibited the transfer of labeled

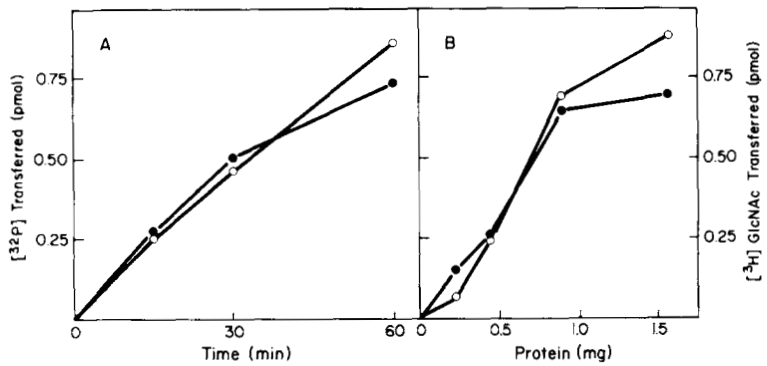

FIG. 1. **Time course and protein concentration dependence of *N*-acetyl[^{3}H]glucosamine-[^{32}P]phosphate transfer to endogenous acceptors.** Clone 15B cell homogenates were assayed as described under "Experimental Procedures." In *A* each reaction contained 0.75 mg of protein and was incubated for the indicated time. Incubations in *B* were for 60 min at the indicated protein concentration. Both ^{3}H (○——○) and ^{32}P (●——●) transfer were monitored.

TABLE I

Requirements for N-acetylglucosaminylphosphotransferase activity

The standard assay for *N*-acetyl[^{3}H]glucosamine-[^{32}P]phosphate incorporation into endogenous acceptors was performed as described under "Experimental Procedures" with modifications as indicated in the table.

Incubation mixture	Product formed
	^{32}P *cpm*
Complete	425
Boiled homogenate	20
− $MgCl_2$	240
− $MnCl_2$	209
− $MgCl_2$; − $MnCl_2$	10
− Dithiothreitol	320
− Triton X-100	358
+ 100-fold excess (1.6 mM) unlabeled UDP-GlcNAc	21

sugar phosphate while 5 mM *N*-acetylglucosamine 1-phosphate inhibited only 37%. Treatment of the [β-^{32}P]UDP-[^{3}H]GlcNAc with mild acid (0.01 N HCl, 100 °C, 30 min) prior to the assay totally abolished product formation. These data demonstrate that the donor is the [β-^{32}P]UDP-[^{3}H]GlcNAc and not a trace contaminant or breakdown product of the labeled substrate.

Dolichylpyrophosphoryl-N-acetylglucosamine Is Not an Intermediate in the N-Acetylglucosamine-Phosphate Transfer Reaction—Since UDP-GlcNAc serves as the *N*-acetylglucosamine phosphate donor in the synthesis of the lipid intermediate dolichylpyrophosphoryl-*N*-acetylglucosamine, we attempted to determine whether the *N*-acetylglucosamine-phosphate is transferred to the endogenous acceptor directly from UDP-GlcNAc or via this lipid intermediate. Assays were performed in the presence of dolichol-phosphate, which stimulates dolichylpyrophosphoryl-*N*-acetylglucosamine production, and in the presence of tunicamycin, an inhibitor of dolichylpyrophosphoryl-*N*-acetylglucosamine synthesis (29, 30). The effect on *N*-acetylglucosaminylphosphotransferase was then determined. As shown in Table II, the addition of dolichol-phosphate greatly stimulated lipid-linked sugar synthesis without affecting *N*-acetylglucosamine-phosphate transfer to glycoprotein. Likewise, tunicamycin virtually abolished incorporation of *N*-acetylglucosamine-phosphate into lipid-linked sugar without inhibiting transfer to the glycoprotein acceptors. These data demonstrate that dolichylpyrophosphoryl-*N*-acetylglucosamine is not the *N*-acetylglucosamine-phosphate donor in this system.

Lack of N-Acetylglucosamine-Phosphate Transfer to Lipid-linked Oligosaccharide—In another experiment we searched for the transfer of *N*-acetylglucosamine-phosphate to endogenous lipid-linked oligosaccharides. Eight standard reaction mixtures which had been extracted with chloroform/methanol (2:1) and water were subjected to extraction (3 × 1 ml) with chloroform/methanol/water (10:10:3). The pooled

TABLE II

Effects of tunicamycin and dolichol-phosphate on N-acetyl[^{3}H]-glucosamine-[^{32}P]phosphate transfer to lipid and to glycoprotein

Assays of *N*-acetylglucosamine-phosphate transfer to glycoprotein are described under "Experimental Procedures," with tunicamycin or dolichol-phosphate additions as noted. Quantitation of the lipid-linked sugar was then performed on the chloroform/methanol extracts of each incubation mixture. The extracts were chromatographed on Sephadex G-25-80 swollen in chloroform/methanol/water (20:10:1.5) to remove hydrophilic species. The unbound fraction was chromatographed on DEAE-cellulose exactly as described by Leloir *et al.* (31). The polyprenol pyrophosphate sugar region from each sample was pooled and an aliquot counted (see table). This material behaved as lipid pyrophosphate sugar on treatment of aliquots with mild acid (32) (0.1 M HCl, 50 °C, 60 min) or mild base (33) (0.1 M NaOH, 37 °C, 20 min) followed by phase partitioning (34). After acid hydrolysis, 97% of the ^{32}P remained in the nonaqueous phase while 90% of the ^{3}H partitioned to the aqueous phase. Following mild base hydrolysis, 96% of the ^{32}P and 92% of the ^{3}H remained in the nonaqueous phase.

Additions		^{32}P radioactivity in	
Tunicamycin (1 μg)	Dolichol-phosphate (10 μg)	Lipid-linked sugar	Glycopeptide
		cpm	
−	−	178	376
−	+	7619	362
+	−	0	418
+	+	26	322

extracted material, which should contain the lipid-linked oligosaccharides, was subjected to mild acid treatment to release the oligosaccharides (32) and then applied to a column of Con A-Sepharose. The column was washed in the standard fashion and the high mannose-type oligosaccharides were eluted with 0.5 M α-methylmannoside. This eluted material contained 48 cpm of ^{32}P compared to 3780 cpm of ^{32}P present in the same Con A-Sepharose fraction from the pronase digested glycoprotein material. These data indicate that the *N*-acetylglucosamine-phosphate is most likely being transferred directly to the endogenous glycoprotein acceptor rather than being transferred to a lipid-linked oligosaccharide intermediate which is then transferred to the protein. Further support for this conclusion is provided in the next section.

Thyroglobulin Glycopeptide Serves as an N-Acetylglucosamine-Phosphate Acceptor—In order to assay for transfer of *N*-acetylglucosamine-phosphate to an exogenous acceptor, we prepared a thyroglobulin glycopeptide fraction enriched in high mannose-type oligosaccharides and tested this preparation as an exogenous acceptor. The standard incubation conditions were utilized, but the product isolation was modified since the thyroglobulin glycopeptide is extracted by the water washes. Therefore, the water extracts, as well as the pronase-solubilized material, were applied separately to Con A-Sepharose columns which were eluted in the usual way. As shown in Fig. 2, the thyroglobulin glycopeptide material acted as an

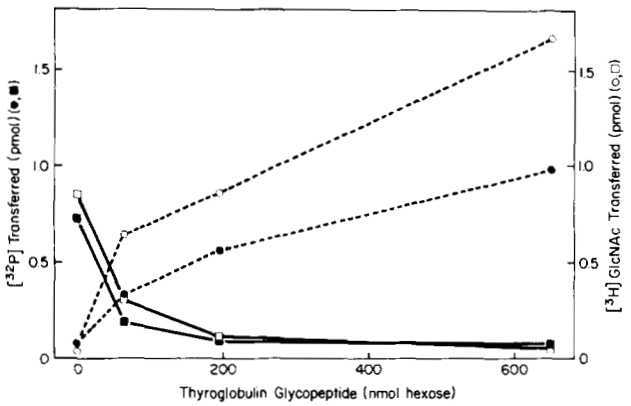

FIG. 2. **Effect of thyroglobulin glycopeptide on *N*-acetyl-[^{3}H]glucosamine-[^{32}P]phosphate incorporation into water-insoluble and -soluble acceptors.** Tritium (□, ○) and ^{32}P (■, ●) incorporation into water-soluble (○- - -○, ●- - -●) and pronase-solubilized (□——□, ■——■) acceptors were assayed as described under "Experimental Procedures."

TABLE III

Effect of mild acid and various enzyme treatments on the endogenous reaction product

Incubations were performed in the standard manner (see "Experimental Procedures"). After elution from the Con A-Sepharose, the glycopeptide was desalted on Sephadex G-25 (1.5 × 50 cm, developed with water) and aliquots were treated with mild acid (pH 2.0, 100 °C, 30 min), alkaline phosphatase (0.2 unit in 0.02 ml of 50 mM Tris-Cl, pH 8.0, for 14 h at 37 °C), α-*N*-acetylglucosaminidase (8 milliunits in 0.03 ml of 100 mM Na-citrate, pH 4.6, for 48 h at 37 °C), or α-*N*-acetylglucosaminyl phosphodiesterase (31 units[2], 7.8 μg in 0.03 ml of 50 mM Tris-Cl, pH 7.4, 10 mM *N*-acetylmannosamine, 0.5% Triton X-100 for the indicated time at 37 °C) as indicated. Specific inhibitors were included as noted. The samples were neutralized, diluted to 1 ml with buffer C, and chromatographed on Con A-Sepharose omitting the α-methylglucoside step (see "Experimental Procedures").

Treatment	Behavior on Con A-Sepharose			
	Unbound		Eluted with α-methyl-mannoside	
	^{32}P	^{3}H	^{32}P	^{3}H
	cpm			
Experiment 1				
1. None	12	1	277	128
2. Alkaline phosphatase	26	2	267	126
3. Mild acid	9	119	302	18
4. Mild acid, then alkaline phosphatase	222	123	24	13
Experiment 2				
5. α-*N*-Acetylglucosaminidase	7	201	313	44
6. α-*N*-Acetylglucosaminidase + 20 mM *N*-acetylmannosamine	10	37	327	186
Experiment 3				
7. α-*N*-Acetylglucosaminyl phosphodiesterase, 3½ h	10	161	379	167
8. α-*N*-Acetylglucosaminyl phosphodiesterase, 3½ h +10 mM GlcNAc 1-phosphate	11	52	319	218
9. α-*N*-Acetylglucosaminyl phosphodiesterase, 15 h	10	294	358	47

acceptor for both *N*-acetyl[^{3}H]glucosamine and [^{32}P]phosphate although somewhat more ^{3}H was transferred than ^{32}P. This excess ^{3}H transfer presumably reflects the activity of some *N*-acetylglucosaminyltransferase which is distinct from the *N*-acetylglucosaminylphosphotransferase and which is acting on an oligosaccharide acceptor that is present in the thyroglobulin preparation but not in the 15B cell extracts. The excess ^{3}H transfer was variable, and was usually less than the amount shown in Fig. 2. In addition, the exogenous glycopeptide inhibited the transfer of *N*-acetyl[^{3}H]glucosamine-[^{32}P]phosphate to the endogenous acceptors in a concentration-dependent fashion.

N-Acetylglucosaminylphosphotransferase Is a Membrane-associated Enzyme—The transferase was localized to the particulate fraction by the following experiment. Cells were homogenized in buffer A without Triton X-100 and centrifuged (145,000 × *g*, 45 min). The supernatant was adjusted to 1% Triton X-100 and the pellet was suspended in buffer A. Both fractions were assayed for *N*-acetylglucosaminylphosphotransferase activity. The particulate fraction contained 97–99% of the enzyme activity using either endogenous or exogenous acceptors (data not shown).

Characterization of the Phosphorylated Glycopeptide Product—A series of experiments were performed to establish that the phosphorylated glycopeptide product actually contained a high mannose-type oligosaccharide with phosphate in diester linkage between a mannose residue of the underlying oligosaccharide and an outer α-linked *N*-acetylglucosamine residue.

When an aliquot of the glycopeptide material derived from endogenous acceptor was treated with alkaline phosphatase and then reapplied to a Con A-Sepharose column, greater than 90% of the ^{32}P still bound to the column, demonstrating that the ^{32}P was not present in the form of a monoester bond (Table III). When a second aliquot of the material was treated with mild acid under conditions that release phosphodiester-linked moieties, 87% of the ^{3}H did not bind to the Con A-Sepharose, while 97% of the ^{32}P still bound. The released ^{3}H radioactivity migrated with authentic *N*-acetylglucosamine upon paper chromatography in solvent D. Alkaline phospha-

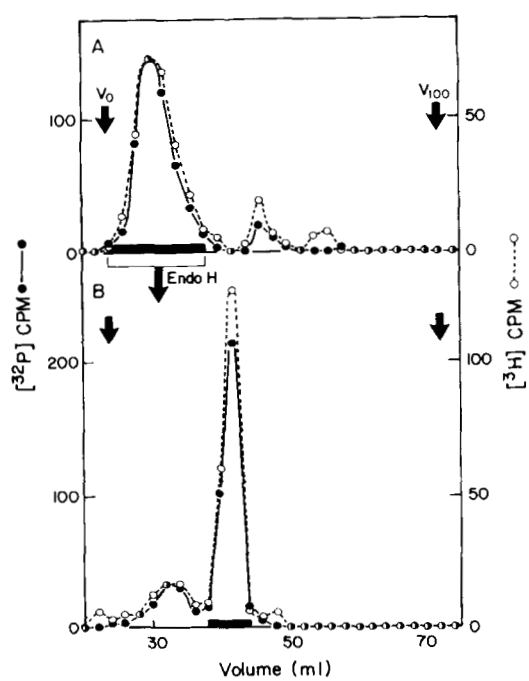

FIG. 3. **Bio-Gel P-6 gel filtration chromatography of endogenous reaction product, before and after treatment with endo-β-*N*-acetylglucosaminidase H.** The glycopeptide from eight standard incubations was pooled, desalted, and chromatographed on Bio-Gel P-6 (1.5 × 54 cm) with 0.1 M ammonium bicarbonate as the developing buffer (*A*). The material indicated by the *bar* in *panel A* was pooled, treated with Endo H (1 milliunit in 0.05 ml of 50 mM citrate-phosphate, pH 5.5, for 7 h at 37 °C), and rechromatographed on the Bio-Gel P-6 column. The void volume (V_0) was measured with bovine serum albumin and the included volume (V_{100}) was measured with mannose. The specific activity (counts per min per mol) of the ^{32}P relative to ^{3}H is 1.83 in *panel A* and 1.66 in *panel B*.

tase treatment of the material previously treated with mild acid resulted in cleavage of the ^{32}P from the Con A-Sepharose-binding glycopeptide. α-*N*-Acetylglucosaminidase treatment of an aliquot of the reaction product caused 82% of the ^{3}H to run through Con A-Sepharose. *N*-Acetylmannosamine, a specific inhibitor of this enzyme, inhibited the cleavage. An 1800-fold purified preparation of α-*N*-acetylglucosaminyl phosphodiesterase, substantially free of *p*-nitrophenol-α-*N*-acetylglucosaminidase activity,[2] released 86% of the ^{3}H, and was inhibited by *N*-acetylglucosamine 1-phosphate. Taken together, these data indicate that the endogenous glycopeptide product contains phosphate in diester linkage between a residue of the underlying oligosaccharide and an outer α-linked *N*-acetylglucosamine.

As shown in Fig. 3*A*, the endogenous glycopeptide product migrated as one major species on Bio-Gel P-6 gel filtration chromatography. Treatment of this glycopeptide with endo-β-*N*-acetylglucosaminidase H (35) converted most of the labeled material to a smaller species, providing further evidence that the glycopeptide contains a high mannose-type oligosaccharide unit (Fig. 3*B*). The material indicated by the *bar* in Fig. 3*B* was pooled, desalted, and then chromatographed on QAE-Sephadex (Table IV). Virtually all of the radioactivity bound to the column and was eluted with 20 mM NaCl. This behavior is indicative of oligosaccharides that contain one blocked (phosphodiester) phosphate (36). When the oligosaccharide was treated with mild acid and reapplied to the QAE-Sephadex column, the *N*-acetyl[^{3}H]glucosamine now passed through the column while the ^{32}P-labeled material required 70 mM NaCl for elution, characteristic of oligosaccharides with one phosphate in monoester linkage (36).

When the endogenous glycopeptide material was subjected to strong acid hydrolysis (1 N HCl, 100 °C, 4 h) followed by paper chromatography, 90% of the ^{32}P radioactivity comigrated with mannose 6-phosphate in both solvent B (Fig. 4*A*) and solvent D. In solvent D, mannose 6-phosphate can be readily distinguished from glucose 6-phosphate, glucosamine 6-phosphate and free phosphate. The remaining 10% of the ^{32}P migrated as free phosphate, which we attribute to overdegradation, since 18% of the internal [^{3}H]mannose 6-phosphate tracer was also hydrolyzed. To confirm that the hydrolyzed product was mannose 6-phosphate, it was treated with phosphomannose isomerase, phosphoglucose isomerase, and glucose-6-phosphate dehydrogenase. This treatment would result in the conversion of the material to 6-phosphogluconate only if the phosphorylated hydroxyl on the mannose were in position 6. As shown in Fig. 4*B*, most of the presumptive mannose 6-phosphate was converted to a product that comigrated with 6-phosphogluconate. Since mannose 6-phosphate was the only phosphorylated sugar detected after strong acid hydrolysis, we conclude that most if not all of the *N*-acetylglucosamine 1-phosphate donated by UDP-GlcNAc is transferred to the 6-hydroxyl of mannose residues of the acceptor oligosaccharide.

Similar experiments were performed to characterize the product formed in assays using exogenous thyroglobulin glycopeptide acceptor. The results were essentially identical.

TABLE IV

QAE-Sephadex chromatography of endo-β-N-acetylglucosaminidase H-cleaved oligosaccharides

Endo H-cleaved oligosaccharides were pooled as indicated by the bar in Fig. 3*B*, desalted on Sephadex G-25, and chromatographed on QAE-Sephadex (2 × 0.5 cm), in 2 mM Tris base. The column was eluted with 8-ml aliquots of 2 mM Tris base containing increasing concentrations of NaCl as indicated. Each fraction was concentrated and assayed for radioactivity (left side). The material that eluted with 20 mM NaCl was desalted, treated with mild acid (pH 2.0, 100 °C, 30 min), neutralized with Tris base, and rechromatographed on QAE-Sephadex (right side).

NaCl	Untreated oligosaccharide		20 mM NaCl fraction after mild acid treatment	
	^{32}P	^{3}H	^{32}P	^{3}H
mM	*cpm*			
0	7	13	3	526
20	1040	667	4	8
70	16	13	811	14
100	11	14	14	3
140	6	2	4	2
1000/0.1 N HCl	4	6	6	2

DISCUSSION

This study demonstrates the existence of an enzymatic activity that transfers *N*-acetylglucosamine 1-phosphate from UDP-GlcNAc to the 6-hydroxyl of mannose residues present in high mannose-type oligosaccharides of endogenous glycoproteins and of exogenous thyroglobulin glycopeptide. The use of [β-^{32}P]UDP-[^{3}H]GlcNAc as the nucleotide sugar donor in the assays greatly aided in the detection of this enzyme activity since products that contained both ^{3}H and ^{32}P could be readily identified and easily characterized. Another critical feature of the assay is the isolation of the desired product on Con A-Sepharose. Since *N*-acetyl[^{3}H]glucosamine is transferred to many different compounds in incubations utilizing cell homogenates, the Con A-Sepharose step allowed us to select for glycopeptides with high mannose-type units, the

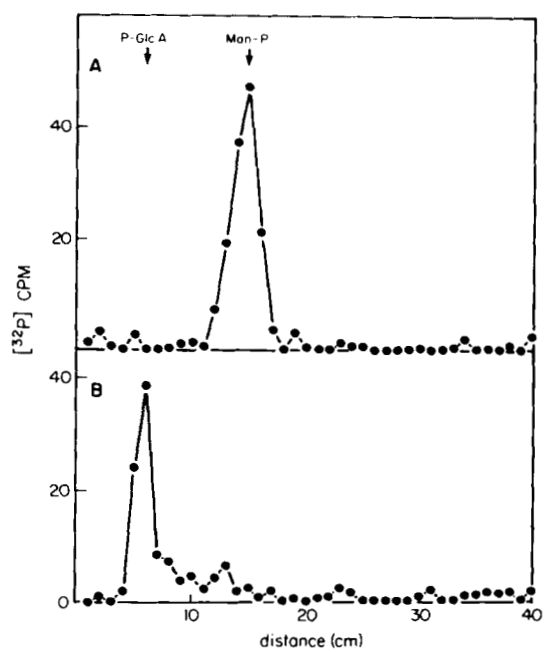

FIG. 4. **Mannose 6-phosphate is the ^{32}P-labeled compound obtained following acid hydrolysis of the *N*-acetylglucosaminylphosphotransferase reaction product.** Product of reactions using endogenous acceptor was desalted on Sephadex G-25, hydrolyzed (1 N HCl, 100 °C, 4 h), evaporated to dryness, and dissolved in 0.3 ml of 50 mM Tris buffer, pH 8.0. Phosphomannose isomerase (0.36 unit), phosphoglucose isomerase (3.5 units), glucose-6-phosphate dehydrogenase (0.35 unit), unlabeled mannose 6-phosphate (0.15 μmol), $NADP^{+}$ (1.5 μmol), and $MgCl_2$ (3.0 μmol) were added to an aliquot of the hydrolysate to give a final volume of 0.30 ml. The course of the reaction was monitored by following the increase in A_{340} and when completion was achieved (2 h), the incubation mixture was boiled. Internal standards, [^{3}H]mannose 6-phosphate (*Man-P*) and 6-[^{3}H]-phosphogluconate (*P-GlcA*) were added to both the control (*A*) and enzyme-treated (*B*) hydrolysates, and each was chromatographed in solvent B for 18 h.

acceptors of interest. However, the product of *N*-acetylglucosaminyltransferase I ($GlcNAcMan_5GlcNAc_2$) (25, 28) also binds tightly to Con A-Sepharose (28). Therefore clone 15B cells, lacking *N*-acetylglucosaminyltransferase I, were used as the *N*-acetylglucosaminylphosphotransferase source. The result was the 1:1 stoichiometry of *N*-acetylglucosamine:phosphate transfer. We have also demonstrated *N*-acetylglucosamine-1-phosphotransferase in human diploid fibroblasts, the murine macrophage cell line J774.2, and rat liver.[3] Each of these cell types contains *N*-acetylglucosaminyltransferase I, and in the *N*-acetylglucosaminylphosphotransferase assays the *N*-acetylglucosamine:phosphate transfer ratio is greater than 1.0.[3]

The discovery of *N*-acetylglucosamine residues in phosphodiester linkage to mannose residues of the oligosaccharide units of β-glucuronidase (14) and other lysosomal enzymes (37) suggested the existence of a UDP-GlcNAc:glycoprotein *N*-acetylglucosamine-1-phosphotransferase. Our demonstration of such an enzyme fulfills this prediction. The action of the transferase followed by that of the recently described α-*N*-acetylglucosaminyl phosphodiesterase (15) will produce the phosphomannosyl residues present in many lysosomal enzymes (1-3, 5, 9, 11). Indeed, in this study we have demonstrated *in vitro* that these enzymes can act sequentially on the same substrate.

Since acid hydrolases are the only glycoproteins known to contain mannose 6-phosphate, either with or without the outer *N*-acetylglucosamine, it seems likely that the physiologic substrates for the *N*-acetylglucosamine 1-phosphotransferase are newly synthesized acid hydrolases. The data presented in this paper only allow us to conclude that the endogenous acceptors are glycoproteins with high mannose-type units. The finding that thyroglobulin glycopeptides also act as acceptors demonstrates that in these *in vitro* assays the transferase does not have an absolute specificity for the oligosaccharide units of acid hydrolases. The role of this transferase in the phosphorylation of acid hydrolase oligosaccharides can be assessed by assaying fibroblasts from patients with mucolipidosis II (I-cell disease) and mucolipidosis III (pseudo-Hurler polydystrophy) for this enzyme activity. In both of these disorders there is a deficiency of many acid hydrolases and, in the case of mucolipidosis II, there is a failure to phosphorylate the oligosaccharide units of the acid hydrolases (9, 10). Consequently these enzymes are secreted by the cells rather than being targeted to lysosomes. In experiments to be reported elsewhere, we have tested fibroblasts from patients with these disorders. Assays using endogenous acceptors demonstrated that fibroblasts from patients with mucolipidosis II and III are severely deficient in *N*-acetylglucosaminylphosphotransferase.[4] The decreased enzyme activity in these patients strongly suggests that the *N*-acetylglucosaminylphosphotransferase activity measured by this assay is responsible for lysosomal enzyme phosphorylation.

The availability of an *in vitro* assay for *N*-acetylglucosamine-1-phosphotransferase which can be made dependent upon exogenous acceptors will allow further study of this reaction. Assuming that this enzyme is responsible for the phosphorylation of the oligosaccharide units of acid hydrolases, there must be some mechanism that allows the enzyme to distinguish acid hydrolases from other glycoproteins which appear to have identical oligosaccharide units. For example, the peptide fragments removed during proteolytic processing of lysosomal enzymes (38, 39) may serve this function. It should also be possible to determine the order of *N*-acetylglucosamine 1-phosphate transfer to different mannose residues of the oligosaccharide acceptor and to determine whether there is only one or whether there are several *N*-acetylglucosamine-1-phosphotransferases. This is of interest because 5 of the 9 mannose residues of the high mannose oligosaccharides may be phosphorylated (36). Finally, it will be important to define the precise subcellular location of this enzyme. The elucidation of these processes should aid our understanding of the mechanism whereby acid hydrolases are targeted to lysosomes.

[3] M. Reitman and S. Kornfeld, unpublished observations.

[4] Reitman, M. L., Varki, A., and Kornfeld, S. (1981) *J. Clin. Invest.* **67,** in press.

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