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-galactosidase 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-32P]UDP-[3H]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 32P and 3H 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 dolichol-pyrophosphoryl-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 1-phosphate 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.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from the following sources: [6-3H]GlcNAc (19 Ci/mmol) and [3H]ATP (2000 Ci/mmol), New England Nuclear; a minimum essential medium, Flow Laboratories, Rockville, MD; fetal bovine serum, penicillin, and streptomycin, GIBCO; Triton X-100 and 3α70 scintillation mixture, Research Products International Corp., Elk Grove Village, IL; Concanavalin A-Sepharose, Pharmacia; Bio-Gel P-6 (200-400 mesh), Bio-Rad Laboratories; QAE-Sephadex (Q-25-100), thyroglobulin, dolicholphosphate, and other reagents, Sigma. DEAE-cellulose (DE-32, Whatman) was converted to the acetate form as described (16). Tunicamycin was a gift from Professor G. Tamura, University of Tokyo, Japan.

Enzymes—Homogeneous Escherichia coli alkaline phosphatase (EC 3.1.3.1) was a gift from Dr. M. Schüssinger, 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-acetylglucosaminidase (EC 3.2.1.50), purified 1000-fold, was obtained from Dr. A. Varki.β Phosphomannosidase (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 a-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 (1:5.53); 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

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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 3% NaOCH, and counting in a scintillation spectrometer.

Preparation of $[^{32}P]$UDP-$[^{14}N]H$GlcNAc—The synthetic 32P UDP-N-acetylglucosamine-1-phosphate compound involved the enzymatic production of $[^{14}N]H$glucosamine-1-phosphate by chemical acetylation (21) to form N-acetyl-$[^{14}N]H$glucosamine-1-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): 0.1 mM EDTA immediately prior to use. The reaction mixture was incubated for 1 h at 37 °C and then eluted was with 4 ml of 10 mM $\alpha$-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 4 ml of 10 mM $\alpha$-methylglucoside and the 0.5 ml of 2-mercaptopethanol, 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 M sodium 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 μM, 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 out on Whatman No. 1 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$_2$O for up to 1 month. The concentration of $[^{32}P]$UDP-$[^{14}N]H$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 >98% radiochemical purity for both isotopes. Following mild acid hydrolysis (0.05 M HCl, 0.1 M NH$_4$HCO$_3$, 0.1 M sodium EDTA), the mixture was refluxed for 3 h. The products were eluted and stored at −20 °C in 1:1 (v/v) ethanol/H$_2$O for up to 1 month. The overall yield of $[^{32}P]$UDP-$[^{14}N]H$GlcNAc ranged from 4276 to 65%.

Preparation of N-Acetyl$[^{14}N]$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:glycoprotein N-acetylglucosaminylphosphotransferase. In developing the assay, we attempted to gain selectivity at three different steps. First, we utilized [$^{32}P$]UDP-$[^{14}N]H$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 mannosetype oligosaccharides. In purifying product from reactions using endogenous glycoprotein acceptors, the boiled reaction mixtures were first extracted with chloroform/methanol (2:1), 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 mannosetype oligosaccharides). The latter are not eluted with 10 mM $\alpha$-methylglucoside but are eluted with 0.5 M $\alpha$-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 responsible for transfer of N-acetylglucosaminylphosphate to high mannosetype 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$[^{14}N]$Hglucosamine and $[^{32}P]$phosphate to endogenous acceptors in a time-dependent fashion (Fig. 1A). The amount of substrate transferred was proportional to the amount of cell extract added (Fig. 1B). 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 $M^2$ 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 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 $\alpha$-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 $\alpha$-methylmannoside in buffer C with 1 h of enzyme incubation for each aliquot addition. The radioactivity in the $\alpha$-methylglucoside and the $\alpha$-methylmannoside eluates was determined after the addition of 10 ml of scintillation fluid.
Glycoprotein N-Acetylglucosaminyl Phosphotransferase

4277

FIG. 1. Time course and protein concentration dependence of N-acetyl[3H]glucosamine-32P phosphate transfer to glycoprotein. Clone 15B cell homogenates were assayed as described under "Experimental Procedures." Assays of N-acetylglucosamine-phosphate transfer to glycoprotein were performed as 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 effects were chromatographed on Sephades G-25-80 swollen in chloroform/methanol/water (20:10:1.5) to remove hydrophobic species. The unbound fraction was chromatographed on DEAE-cellulose exactly as described by Leloir et al., (31). The polypreol 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 (33) (0.1 M HCl, 50 °C, 60 min) or mild base (33) (1.0 M NaOH, 37 °C, 20 min) followed by phase partitioning (34). After acid hydrolysis, 97% of the 32P remained in the nonaqueous phase while 90% of the 3H partitioned to the aqueous phase. Following mild base hydrolysis, 96% of the 3P and 92% of the 3H remained in the nonaqueous phase.

| Additions | Lipid-linked sugar | Glycopeptide |
|-----------|--------------------|--------------|
| -         | -                  | 178 | 376 |
| -         | +                  | 7619 | 362 |
| -         | -                  | 0 | 418 |
| +         | +                  | 26 | 322 |

TABLE I
Requirements for N-acetylglucosaminylphosphotransferase activity

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

| Incubation mixture | Product formed |
|--------------------|----------------|
| Complete           | 425 32P cpm    |
| Boiled homogenate  | 20 32P cpm     |
| - MgCl2            | 240 32P cpm    |
| - MnCl2            | 200 32P cpm    |
| - Dithiothreitol   | 320 32P cpm    |
| - Triton X-100     | 358 32P cpm    |
| + 100-fold excess (1.6 mM) unlabeled UDP-GlcNAc | 21 32P cpm |

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 dolichyl-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-acetylglucosaminylphosphate 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 x 1 ml) with chloroform/methanol/water (10:10:3). The pooled 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 mannosetype oligosaccharides were eluted with 0.5 M α-methylmannoside. This eluted material contained 48 cpm of 32P compared to 3780 cpm of 32P 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 mannosetype 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...
acetylglucosaminyl phosphotransferase 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 an 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 repleted to a Con A-Sepharose column, greater than 90% of the 32P still bound to the column, demonstrating that the 32P 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 H did not bind to the Con A-Sepharose, while 97% of the 32P still bound. The released 3H radioactivity migrated with authentic N-acetylglucosamine upon paper chromatography in solvent D. Alkaline phospho-

Table III

| Treatment | Unbound | Eluted with α-methylmannoside |
|-----------|---------|-----------------------------|
| 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-Acetylgulosaminidase | 7 201 313 44 |
6. α-N-Acetylgulosaminidase + 20 | 10 37 327 186 |

Experiment 3

7. α-N-Acetylgulosaminyl phosphodiesterase, 3% h | 10 161 379 167 |
8. α-N-Acetylgulosaminyl phosphodiesterase, 3% h +100 mM GlcNAc | 11 52 319 218 |
9. α-N-Acetylgulosaminyl phosphodiesterase, 13 h | 10 294 358 47 |

FIG. 2. Effect of thyroglobulin glycopeptide on N-acetyl-[H]glucosamine-[32P]phosphate incorporation into water-insoluble and -soluble acceptors. Tritium (□ □) and 32P (■ ■) incorporation into water-soluble (O---O), •---• and pronase-solubilized (□ □ □) acceptors were assayed as described under "Experimental Procedures.”

FIG. 3. Bio-Gel P-6 gel filtration chromatography of endogenous reaction product, before and after treatment with endo-β-N-acetylgulosaminidase 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 (V0) was measured with bovine serum albumin and the included volume (V1/2) was measured with mannose. The specific activity (counts per min per mol) of the 32P relative to 3H 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 32P from the Con A-Sepharose-binding glycopeptide. α-N-Acetylgalactosaminidase treatment of an aliquot of the reaction product caused 82% of the 3H to run through Con A-Sepharose. N-Acetylmannosamine, a specific inhibitor of this enzyme, inhibited the cleavage. An 1800-fold purification of α-N-acetylgalactosaminylation phosphodiesterase, substantially free of p-nitrophenol-α-N-acetylgalactosaminidase activity, released 86% of the 3H, 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-acetylgalactosamine.

As shown in Fig. 3A, 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-acetylgalactosaminidase 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. 3B). The material indicated by the bar in Fig. 3B 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[3H]glucosamine now passed through the column while the 32P-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 32P radioactivity migrated with mannose 6-phosphate in both solvent B (Fig. 4A) 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 32P migrated as free phosphate, which we attribute to over-degradation, since 18% of the internal [3H]mannose 6-phosphate tracer was also hydrolyzed. To confirm that the hydrolyzed product was mannose 6-phosphate, it was treated with phosphomannose isomerase, phosphoglucone 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. 4B, most of the presumptive mannose 6-phosphate was converted to a product that co-migrated 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-acetylgalactosaminyl 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.

**DISCUSSION**

This study demonstrates the existence of an enzymatic activity that transfers N-acetylgalactosamine 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-32P]UDP-[3H]GlcNAc as the nucleotide sugar donor in the assays greatly aided in the detection of this enzyme activity since products that contained both 3H and 32P could be readily identified and easily characterized. Another critical feature of the assay is the isolation of the desired product on the Con A-Sepharose step. Since N-acetylgalactosamine 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

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**TABLE IV**

**QAE-Sephadex chromatography of endo-β-N-acetylgalactosaminidase H-cleaved oligosaccharides**

| NaCl (mM) | Unlabeled oligosaccharide | 20 mM NaCl fraction after mild acid treatment | 6P | 3H | 32P | 3H |
|-----------|---------------------------|---------------------------------------------|-----|----|-----|----|
| 0         | 7                         | 13                                          | 526 | 3  |
| 20        | 1040                      | 667                                         | 4   | 8  |
| 70        | 16                        | 13                                          | 811 | 14 |
| 100       | 11                        | 14                                          | 4   | 3  |
| 140       | 6                         | 2                                           | 4   | 2  |
| 1000/0.1 N HCl | 4                      | 6                                           | 6   | 2  |

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**FIG. 4. Mannose 6-phosphate is the 32P-labeled compound obtained following acid hydrolysis of the N-acetylgalactosaminylphosphotransferase 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), phosphoglucone isomerase (3.5 units), glucose-6-phosphate dehydrogenase (0.35 unit), unlabeled mannose 6-phosphate (0.15 μmol), NADP⁺ (1.5 μmol), and MgCl₂ (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 A₅₂₀ and when completion was achieved (2 h), the incubation mixture was boiled. Internal standards, [3H]mannose 6-phosphate (Man-P) and 6-[3H]phosphoglucone (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 (GlcaNACMan:GlcNAc-G) (25, 26) 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. Each of these cell types contains N-acetylglucosaminyltransferase I, and in the N-acetylglucosaminylphosphotransferase assay the N-acetylglucosamine-phosphate transfer ratio is greater than 1.0.3

The discovery of N-acetylglucosamine residues in lysosomal acceptors demonstrates that in these cases phosphomannosyl residues present in many lysosomal enzymes (15) will produce the phosphomannosyl residues present in many lysosomal en-

strongly suggests that the N-acetylglucosaminylphospho-
transferase does not have an absolute specificity for the oligosaccharide units of acid hydrolases. The role of this transferase in the phosphorylation of acid hydrolases (9, 10) 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.

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