Kidney N-Acetylglactosamine (GalNAc)-1-phosphate Kinase, a New Pathway of GalNAc Activation*

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A new enzyme that phosphorylates GalNAc at position 1 to form GalNAc-α-1-P was purified 1275-fold from the cytosolic fraction of pig kidney, and the properties of the enzyme were determined. The kinase is quite specific for GalNAc as the phosphate acceptor and is inactive with GlcNAc, ManNAc, glucose, galactose, mannose, GalN, and GlcN. This enzyme is clearly separated from galactokinase by chromatography on phenyl-Sepharose. The GalNAc kinase has a pH optimum between 8.5 and 9.0 and requires a divalent cation in the order Mg2+ > Mn2+ > Co2+, with optimum Mg2+ concentration at ~5 mM. The enzyme was most active with ATP as the phosphate donor, but slight activity was observed with ITP, acetyl-P, and phosphoenolpyruvate. Enzyme activity was highest in porcine and human kidney and porcine liver, but was low in most other tissues. Cultured HT-29 cells also had high activity for this kinase. The purified enzyme fraction was incubated with azido-[32P]ATP, exposed to UV light, and run on SDS gels. A 50-kDa protein was labeled, and this labeling showed saturation kinetics with increasing amounts of the probe and was inhibited by unlabeled ATP. Although the most purified GalNAc kinase preparation still had two bands that labeled with ATP, maximum labeling of the 50-kDa protein, but not the 66-kDa band, was coincident with maximum GalNAc kinase activity on a column of DEAE-Cibacron blue. On Sephacryl S-300, the native enzyme has a molecular mass of 48-51 kDa, indicating that the active kinase is a monomer. The product of the reaction was characterized as GalNAc-α-1-P by various chemical procedures.

GalNAc is an important sugar in complex carbohydrates since it is usually the sugar that links the carbohydrate chains to protein in mucins (1-3) and other O-linked oligosaccharides (4-6). In these glycoproteins, the linkage usually involves a GalNAc to serine or threonine attachment site. GalNAc is also linked to protein in mucins (1-3) and other complex carbohydrates. In this regard, it is important to note that we recently purified a UDP-HexNAc pyrophosphorylase from pig liver (and kidney) that catalyzes the formation either of UDP-GlcNAc from UDP and GlcNAc-1-P or of UDP-GalNAc from UDP and GalNAc-1-P (11). Although the measured K_m for GalNAc-1-P is somewhat high, this enzyme may still function as a UDP-GalNAc pyrophosphorylase in certain tissues or under particular conditions. Thus, some animal tissues appear to have the necessary enzymes to convert free GalNAc to its “activated” form, i.e. UDP-GalNAc, for polymerization without the intervention of UDP-GlcNAc.

EXPERIMENTAL PROCEDURES

Materials

[6-3H]GlcN (40 Ci/mmol), [1-3H]Galactose (10-25 Ci/mmol), [6-3H]GalNAc (20-30 Ci/mmol), [6-3H]GlcNAc (20-30 Ci/mmol), UDP-[6-3H]GlcNAc (25 Ci/mmol), and UDP-[6-3H]GalNAc (15 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. Unlabeled sugars, sugar phosphates, and deoxynucleoside diphosphate sugars were obtained from Sigma. Various absorbents were obtained from the following sources: DEAE-cellulose (DE52) from Whatman and Polybuffer Exchanger 94, Sephacryl S-300, red Sepharose, phenyl-Sepharose, and DEAE-Cibacron blue 3GA from Sigma. Polyethyleneimine-cellulose TLC plates were purchased from EM Science, cellulose TLC plates were from Eastman Kodak Co., and silica gel TLC plates were from Merck. The following materials were obtained from Bio-Rad: SDS, acrylamide, bisacrylamide, Coomassie Blue, protein assay reagent, hydroxylapatite, and Affi-Gel 50L. All other chemicals were from reliable commercial sources and were of the best grade available.

GalNAc Kinase Assay

GalNAc kinase activity was assayed in incubation mixtures of 100 μl containing the following components: 200 μCi [3H]GalNAc (30,000 cpm), 5 mM ATP, 5 mM MgCl2, 5 mM NaF, 100 mM Tris-HCl buffer, pH 8.5, and various amounts of the enzyme preparations to be assayed. Following an incubation for the appropriate time (usually 5 min), the reaction was stopped by heating at 100 °C for 1 min, and the incubation mixture was applied to a column of DE52. The column was washed with at least 5 column volumes of 10 mM (NH4)2CO3 to remove unbound material, and then GalNAc phosphate was eluted with 500 mM ammonium bicarbonate. Aliquots of the wash and eluent were removed and assayed for their radioactive content by scintillation counting.

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Kidney GalNAc-1-P Kinase

**Purification of GalNAc Kinase**

**Step 1: Preparation of Crude Extract**—Fresh pig kidneys from a local slaughterhouse were generally used in these studies, but kidney could be quick-frozen and used at a later time with equally good results. The kidneys were cut into small pieces and homogenized in Buffer A (10 mM Tris-HCl buffer, pH 7.8, containing 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin A) in a Waring blender for 3–4 min. The homogenate was centrifuged at 12,000 × g for 50 min, and the supernatant liquid was filtered through four layers of cheesecloth and centrifuged at 100,000 × g for 45 min. The supernatant liquid was used in subsequent purification steps.

**Step 2: Ammonium Sulfate Precipitation**—Solid ammonium sulfate was added to the crude extract to 75% saturation, and the mixture was left standing on ice for 15 min. The precipitate was discarded, and solid ammonium sulfate was slowly added with stirring to the supernatant liquid to reach 60% saturation. After standing on ice for 15 min, the supernatant was centrifuged to obtain the precipitate, which was dissolved in Buffer A containing 1 M ammonium sulfate.

**Step 3: Phenyl-Sepharose Column Chromatography**—The dissolved enzyme was applied to a 2.5 × 28-cm column of phenyl-Sepharose that had been equilibrated with Buffer A containing 1 M ammonium sulfate. The column was then washed with the equilibration buffer until the filtrate was almost colorless. The kinase was eluted from the column with a linear gradient of 1 to 0 M ammonium sulfate in Buffer A. Ten ml fractions were collected, and active fractions were pooled and dialyzed against Buffer A, with several changes of the dialysis fluid during the 12-h dialysis time.

**Step 4: DE52-Cellulose Chromatography**—The active fractions from Step 3 were applied to the DE52 column, which was washed with Buffer A containing 20 mM NaCl. The enzyme was eluted with 300 ml of a linear gradient of 20–200 mM NaCl in Buffer A. The active fractions were pooled, concentrated to 10 ml on an Amicon apparatus using a PM-10 membrane, and dialyzed against Buffer B (5 mM sodium phosphate buffer, pH 7.0, containing 1 mM β-mercaptoethanol and 1 mM EDTA).

**Step 5: Hydroxylapatite Column Chromatography**—The dialyzed enzyme from Step 4 was applied to a 2.5 × 5-cm column of hydroxylapatite that had been equilibrated with Buffer B. The column was washed with Buffer B, and the enzyme was then eluted with a 5–40 mM linear gradient of phosphate buffer. The enzyme activity emerged from the column at ~10–25 mM sodium phosphate buffer. Active fractions were pooled and concentrated by Amicon filtration, and Buffer B was exchanged for Buffer C (50 mM Heps buffer, pH 7.5, containing 1 mM β-mercaptoethanol, 1 mM EDTA, and 10% glycerol) by continually adding Buffer C to the concentrated enzyme solution and concentrating on the filter (three to seven times).

**Step 6: Red Sepharose Chromatography**—The concentrated enzyme fraction from the hydroxylapatite chromatography step was applied to a 1.5 × 8-cm column of red Sepharose 300 CL that had been equilibrated with Buffer C. The column was then washed with Buffer C, and the enzyme activity emerged in the wash.

**Step 7: Aminoethyl-Agarose Chromatography**—The active enzyme from the red Sepharose chromatography step was applied to an aminoethyl-Sepharose column (1.5 × 12 cm) that had been equilibrated with Buffer C. The column was washed with Buffer C, and the enzyme was eluted with 150 ml of a linear gradient of 0–140 mM NaCl in Buffer C. Fractions containing the active enzyme were pooled, and NaCl was removed by filtration on an Amicon apparatus.

**Step 8: DEAE-Cibacron Blue Chromatography**—The enzyme solution from Step 7 was applied to a 1.5 × 5-cm column of DEAE-Cibacron Blue 3G A, and the column was washed with 1.5 ml ATP in Buffer C. The enzyme was then eluted with a linear gradient of 1.5–8 mM ATP in Buffer C. Active fractions were pooled and concentrated several times in the presence of 20 mM Hepes buffer, pH 7.5, containing 1 mM ATP and 10% glycerol to remove Buffer C and β-mercaptoethanol.

**Step 9: Affi-Gel 501-Agarose Chromatography**—An Affi-Gel 501 or-ganomercurial-agarose column (0.9 × 7 cm) was equilibrated with 20 mM Hepes buffer, pH 7.5, containing 1 mM ATP and 10% glycerol. The enzyme was then applied to the column, which was then washed with the same buffer. The enzyme was then eluted with a 0–20 mM gradient of dithiothreitol. The enzyme emerged from this column at ~6–10 mM dithiothreitol. The enzyme was concentrated to a small volume and stored in 20 mM Hepes buffer, pH 7.0, containing 10% glycerol.

**Native and SDS Gel Electrophoresis**

Native PAGE was performed as described by Laemmli (12) with 8% gel and a discontinuous buffer system, but under non-denaturing conditions. Two samples were run in parallel: one lane was stained with Coomassie Blue to detect proteins, and the other lane was cut into 1-cm pieces for assay of enzyme activity. SDS-PAGE was done as described (13). Prior to electrophoresis, protein samples were mixed with the same buffer (62 mM Tris·HCl, pH 6.8, 5% β-mercaptoethanol, 2% SDS, 10% glycerol, and 0.002% bromphenol blue) and heated in a boiling water bath for 5 min.

**Product Characterization**

Large-scale incubations were prepared with radioactive GalNAc in order to isolate sufficient amounts of product for characterization. These large-scale incubations contained the same components as in the GalNAc kinase assay mixtures, but were scaled up by a factor of 10. After incubation, the reactions were stopped by heating, and the product was isolated by chromatography on a column of DE52.

The radioactive material was examined for purity and characterization by paper chromatography on Whatman No. 3MM paper in 70% ethanol and 1 M ammonium acetate, pH 7.5 (7:3), or in 70% ethanol and 1 M ammonium acetate, pH 3.5 (7:3). The product was also subjected to HPLC on a Dionex apparatus using a column designed to separate various sugar phosphates from each other. Sugar 1-phosphates were distinguished from sugar 6-phosphates by their susceptibility to acid hydrolysis in 0.05 N HCl at 100 °C. Under these conditions, sugar 1-phosphates are rapidly hydrolyzed, whereas sugar 6-phosphates are quite stable.

The phosphorylated sugar product was also characterized by NMR spectroscopy. 300-mHz proton NMR and 31P-decoupled (GARP) NMR on the sample of GalNAc-1P were performed on a Bruker ARX300 NMR apparatus. Data were acquired in D2O at pH 6.0.

**RESULTS**

Purification of GalNAc Kinase—The GalNAc kinase was purified from the cytosolic fraction of pig kidney using the purification scheme described under "Experimental Procedures" and outlined in Table I. One important step in this purification was the chromatography step using phenyl-Sepharose, which is shown in Fig. 1. It can be seen that the GalNAc kinase was completely resolved from galactokinase on this column, demonstrating that these activities are distinct from each other.

Using the purification procedure outlined in Table I, the GalNAc kinase was purified ~1275-fold from crude extracts, with a recovery of ~6%. Fig. 2A shows the protein profiles at various stages of the purification procedure. It can be seen that the most purified enzyme fraction (lane 7) showed one major and several minor protein bands on SDS-PAGE. However, the major band to label with an azido-[32P]ATP photoaffinity probe was the 50-kDa band, as seen in Fig. 2B. Labeling of this band

**Table I**

| Step            | Protein Activity | Specific Activity | Purification Yield |
|-----------------|------------------|-------------------|--------------------|
|                 | mg units         | units/mg          | total %            |
| 1. Crude extract | 7752             | 8740              | 1.12               | 1.00  100.0 |
| 2. Ammonium sulfate | 422            | 9600              | 2.27               | 2.02  109.8 |
| 3. Phenyl-Sepharose  | 806            | 5850              | 7.26               | 6.48  66.9 |
| 4. DE52-cellulose  | 150             | 4320              | 25.80              | 25.72 49.4 |
| 5. Hydroxylapatite | 12.1           | 1650              | 136.36             | 121.75  18.9 |
| 6. Red Sepharose  | 5.95            | 1680              | 282.35             | 252.10  19.2 |
| 7. Aminoethylagarose | 3.85        | 1370              | 357.14             | 318.87  15.7 |
| 8. DEAE-Cibacron blue | 0.30       | 910               | 700.00             | 625.00 10.4 |
| 9. Affi-Gel 501  | 0.35            | 500               | 1428.57            | 1275.5  5.7 |

**TABLE I**

| Step | Purification of pig kidney GalNAc kinase units |
|------|-----------------------------------------------|
|      | Units are expressed as nanomoles GalNAc-1-P formed per minute. |

The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; GARP, globally optimized alternating phase rectangular pulse.
Sepharose that had been equilibrated with 1 M ammonium sulfate in Buffer A. The column was washed with this buffer (containing the ammonium sulfate) and then eluted with a gradient of 1 to 0 M ammonium sulfate in Buffer A. Ten-ml fractions were collected and assayed for the GalNAc kinase, the enzyme preparation was chromatographed on a column of DEAE-Cibacron Blue, and fractions were assayed for GalNAc kinase activity. In all purification procedures, the 50-kDa band varied considerably in amount from one purified preparation to another and sometimes was present in almost undetectable amounts. It is also important to point out that gel filtration of the native enzyme on Sephacryl S-300 gave an activity peak in the region where proteins of 48–51 kDa elute. Thus, the native protein is probably a monomer of 50 kDa. The 50-kDa band has been eluted from SDS gels and appears to be a homogeneous protein.2 We are currently preparing enough of this protein for amino acid sequencing.

Sugar Specificity of GalNAc Kinase as Compared with Yeast Galactokinase—The specificity of the GalNAc kinase for the sugar substrate was determined as shown in Table I. A variety of sugars were tested, and in each case, the specific radioactivity of the labeled sugar was adjusted with unlabeled material so that all of the sugars were of the same specific activity in order to allow a true comparison of their ability to serve as substrates for the kinase. Thus, each sugar was used at a final concentration of 200 μM, containing 52,000 cpm of radioactivity. The amount of radioactivity that bound to DE52 and was eluted with (NH₄)HCO₃ was taken as a measure of phosphorylation by the enzyme. Table II clearly demonstrates that only GalNAc could readily serve as a substrate, and essentially no phosphorylation was observed with any other sugar. Galactose and perhaps galactosamine did show slight activity, but in either case, it was probably only 1% of the activity observed with GalNAc. These data indicate that this enzyme is very specific for GalNAc.

Galactokinase is an enzyme known to phosphorylate galactose at position 1. The sugar specificity of the yeast and pig kidney galactokinases indicated that these enzymes were very active with galactose as the substrate and had slight activity with glucose and galactosamine (data not shown). More important, no phosphorylation was observed when GalNAc was tested as the substrate in place of galactose. The specificity of the galactokinase and the GalNAc kinase and their separation on phenyl-Sepharose columns demonstrate that they are two different enzymes.

Specificity for Nucleoside Triphosphates as Phosphate Donor—A variety of nucleoside mono-, di-, and triphosphates were tested as phosphate donors for the GalNAc kinase, using 400 μM GalNAc as the sugar substrate, as shown in Table III. ATP was by far the best phosphate donor, but some activity was also observed with ITP, acetyl-P, and phosphoenolpyruvate. The activity observed with these other phosphate donors appears to

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2 Harvard Microchemistry Facility (22).
be real since no radioactivity bound to DE52 in the absence of the active enzyme. However, the activity with these other phosphate donors was too low to be able to characterize the product, and therefore, it is not known whether the product is also GalNAc-1-P. All the other nucleoside triphosphates and diphosphates tested were inactive in this reaction.

Requirement for Divalent Cation for Activity—The purified enzyme was essentially inactive in the absence of a divalent cation as seen in Fig. 4. The addition of Mg\(^{2+}\) greatly stimulated the enzyme, and maximum activity occurred at 6 mM Mg\(^{2+}\). Other divalent cations such as Mn\(^{2+}\) and Co\(^{2+}\) were also somewhat stimulatory, but much less so than Mg\(^{2+}\). Thus, optimum activity was observed in the presence of 2–8 mM Mn\(^{2+}\) or 10 mM Co\(^{2+}\), but this maximum activity was only about one-half of that seen with optimum concentrations of Mg\(^{2+}\). Various other cations were tested at a number of concentrations, including Mo\(^{2+}\), Zn\(^{2+}\), Ni\(^{2+}\), Cu\(^{2+}\), Ca\(^{2+}\), and Fe\(^{3+}\), and all of these were inactive in stimulating the kinase.

**TABLE II**

| Sugar\(^a\) | Enzyme amount | Radioactivity incorporated | Specific activity  |
|------------|---------------|---------------------------|-------------------|
| GalNAc     | µl            | cpm                       | nmol/mg protein   |
| 0          | 105           |                           |                   |
| 4          | 14,094        | 146.5                     |
| 8          | 18,448        |                           |
| GalN       | 227           | 1.0                       |
| 4          | 449           |                           |
| 8          | 468           |                           |
| Gal        | 183           | 1.64                      |
| 4          | 301           |                           |
| 8          | 442           |                           |
| GlcNAc     | 121           | 0.27                      |
| 4          | 135           |                           |
| 8          | 147           |                           |

\(^a\) Glucosamine, glucose, and N-acetylmannosamine were also tested and found to be inactive.

**TABLE III**

| Nucleotide added (at 5 mM) | Radioactivity in sugar-P | Specific activity (cpm nmol/mg) |
|---------------------------|--------------------------|-------------------------------|
| ATP                       | 10,195                   | 160.0                         |
| ADP                       | 260                      | 4.0                           |
| CTP                       | 370                      | 5.8                           |
| CDP                       | 490                      | 7.6                           |
| GTP                       | 300                      | 4.7                           |
| GDP                       | 10                       | 0.1                           |
| ITP                       | 900                      | 14.1                          |
| UTP                       | 180                      | 2.8                           |
| UDP                       | 30                       | 0.5                           |
| PEP\(^a\)                 | 1120                     | 17.6                          |
| Acetyl-P                  | 815                      | 12.8                          |
| No enzyme                 | 150                      |                               |

\(^a\) PEP, phosphoenolpyruvate.

**FIG. 3.** Demonstration that the 50-kDa protein band is the GalNAc kinase activity. A partially purified preparation of the GalNAc kinase (from Step 7) was applied to a DEAE-Cibacron Blue column. The column was washed with Buffer C, and the enzyme was eluted with a linear gradient of 0–200 mM NaCl (fractions 1–14). Various fractions were assayed for enzyme activity (●●●) and protein (●●●) (A), and aliquots of each fraction around the activity peak were incubated with azido-[\(^32\)P]ATP and then run on SDS gels (B). Maximum activity and maximum staining of the 50-kDa band coincided in fractions 10 and 11. C shows the protein staining in fractions 8–13 using Coomassie Blue.

**FIG. 4.** Effect of divalent cations on the GalNAc kinase. Various amounts of Mg\(^{2+}\), Mn\(^{2+}\), or Co\(^{2+}\) were added to incubations prepared as indicated under "Experimental Procedures." The amount of GalNAc phosphorylated in each incubation by the purified enzyme was determined by measuring the amount of radioactive GalNAc bound to DE52. Various other cations, such as Mo\(^{2+}\), Zn\(^{2+}\), Ni\(^{2+}\), Cu\(^{2+}\), Ca\(^{2+}\), and Fe\(^{3+}\), were tested at a number of concentrations and were found to be inactive.
Effect of pH and Substrate Concentration on Activity—The effect of the pH of the incubation mixture on enzyme activity was examined in two different buffers as shown in Fig. 5. Activity was somewhat better in Tris-HCl buffer, where optimum activity occurred at pH 8.9. In Tris maleate buffer, activity was somewhat lower, and the pH optimum was somewhat broader, occurring at pH 8.5–8.9.

The effect of substrate concentration on the formation of GalNAc-1-P is shown in Fig. 6. The formation of GalNAc-1-P is shown in Fig. 6. Fig. 6A demonstrates the dependence of the reaction rate on the concentration of GalNAc up to 250 μM, and the Kₘ for GalNAc was calculated to be 1.4 × 10⁻⁵ M (see inset). In this experiment, the ATP concentration was kept at 10 mM, which was well above saturation for this substrate. Fig. 6B shows the effect of ATP concentration on the formation of GalNAc-1-P at 2 mM GalNAc. This concentration of GalNAc is above saturation for this substrate. The reaction rate was proportional to the concentration of ATP up to 400 μM, and the Kₘ for ATP was calculated to be 6.3 × 10⁻⁵ M (see inset).

Tissue Distribution of GalNAc Kinase—To determine the distribution of this enzyme activity in porcine tissues, we prepared crude extracts from a variety of freshly collected pig tissues and assayed several different amounts of each extract for its ability to phosphorylate GalNAc. Table IV presents the results of these studies. Pig kidney showed the highest total activity and the highest specific activity, while pig liver was the next best tissue, with about one-half of the total activity. Some activity was also observed in the spleen and lung, but other tissues such as the pancreas, brain, aorta, and heart were considered to be devoid of this enzyme. Human kidney was also assayed and found to have good GalNAc kinase activity, as seen in Table IV, although the specific activity was considerably lower than that of pig kidney. GalNAc kinase activity was also present in rabbit kidney and liver, in both adult and neonatal tissues. Several cultured cell lines were examined for this kinase, and its activity was found to be quite high in HT-29 cells, but very low to nonexistent in Madin-Darby canine kidney cells. In fact, based on specific activity, the extracts from HT-29 cells had the highest GalNAc kinase activity of any tissue examined.

Effect of Various Inhibitors of Sulfhydryl Groups on GalNAc Activity—To determine whether sulfhydryl groups are necessary for catalytic activity, we examined the effects on the kinase activity of various compounds known to react with and to inactivate sulfhydryl groups. The data from these experiments are presented in Table V. It can be seen that p-chloromercuribenzoate was a very effective inhibitor of the kinase and caused >70% inhibition at a concentration of 0.02 mM and complete inhibition at 0.04 mM. N-Ethylimaleimide was also inhibitory, but required much higher concentrations (0.5 mM) for 90% inhibition, while iodoacetamide was only effective as an inhibitor at concentrations over 1 mM. The presence of dithio-
threitol or \(\beta\)-mercaptoethanol (at 0.5–8 mM) in the incubation mixtures was found to protect the kinase from inactivation by 0.1 mM \(p\)-chloromercuribenzoate or 1 mM \(N\)-ethylmaleimide.

**Product Characterization**—Since most sugar kinases phosphorylate the hydroxymethyl carbon at C-6 or C-5, it was important to determine the location of the phosphate group on the sugar and to be certain that the sugar product was still GalNAc. Thus, large-scale incubations were prepared, and the product was isolated as indicated above and purified by ion-exchange and thin-layer chromatography. A single symmetrical radioactive peak was obtained from the DE52 column, and this peak emerged in the same area as the GlcNAc-1-P standard. In addition, a single radioactive band was detected on TLC plates.

The radioactive product from the ion-exchange columns was suspended in 0.05 N HCl and heated at 100°C for various periods of time. An aliquot of the reaction mixture was removed at each of the times shown in Fig. 7 and passed through a column of DE52. The amount of radioactivity that emerged in the wash was taken as a measure of removal of the phosphate group. Fig. 7 shows that the GalNAc phosphate produced by the purified kinase was very susceptible to acid hydrolysis, and almost all of the phosphate was removed within 10 min of hydrolysis, as was also observed for the Glu-1-P and GalNAc-1-P standards. On the other hand, Glc-6-P and GlcNAc-6-P were quite stable to the hydrolysis conditions, and there was essentially no loss in their binding to the DE52 columns even after 50 min of heating. Thus, the phosphate group on the GalNAc phosphate must be located at position 1.

To be certain that the sugar was still GalNAc, the product of mild acid hydrolysis was identified by paper chromatography and by HPLC. The radiolabeled sugar was mixed with unlabeled GalNAc and subjected to Dionex chromatography. Fractions were collected, and their radioactive content was determined, whereas unlabeled sugars were determined by amperometric detection. The radioactivity emerged in the same position as authentic GalNAc (~17.08 min) and was clearly separated from GlcNAc (15.78 min). The radioactive sugar also migrated with authentic GalNAc on paper chromatograms in several solvents that separate these amino sugars from each other as well as from ManNAc (data not shown).

The anomeric configuration of the phosphate group was determined by high resolution NMR. GalNAc-1-P was subjected to \(D_2O\) NMR analysis. The anomeric proton signal at 5.3 ppm

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**TABLE V**

| Inhibitor conc (mM) | Activity in the presence of NEM* | Activity in the presence of IAA | Activity in the presence of pCMB |
|---------------------|---------------------------------|-----------------------------|---------------------------------|
| 0 (control)         | 8700                            | 8700                        | 8700                            |
| 0.02                | 2660                            | 2660                        | 2660                            |
| 0.04                | 250                             | 250                         | 250                             |
| 0.06                | 30                              | 30                          | 30                              |
| 0.1                 | 8260                            | 8260                        | 8260                            |
| 0.25                | 50                              | 50                          | 50                              |
| 0.5                 | 7660                            | 7660                        | 7660                            |
| 1                   | 5490                            | 5490                        | 5490                            |
| 2                   | 2170                            | 2170                        | 2170                            |
| 4                   | 658                             | 658                         | 658                             |

* NEM, \(N\)-ethylmaleimide; IAA, iodoacetamide; pCMB, \(p\)-chloromercuribenzoate.
exhibited a doublet of doublets (Fig 8A) due to splitting by the axial proton at carbon 2 and the $^{31}$P-labeled $\alpha$-anomeric phosphate. Using a GARP $^{31}$P decoupling protocol, the doublet of doublets collapsed to one doublet at 5.44 ppm, with a J $^{1,2}$ coupling constant of 4 Hz (Fig. 8B). These data are entirely consistent with an $\alpha$-anomeric configuration for the phosphate group (14, 15). In addition, the product of this reaction, i.e. GalNAc-1-P, is a substrate for a recently purified UDP-HexNAc pyrophosphorylase that condenses GlcNAc-$\alpha$-1-P and UTP to form UDP-GlcNAc and inorganic pyrophosphate or GalNAc-$\alpha$-1-P and UTP to form UDP-GalNAc and inorganic pyrophosphate (11). Since the sugar in both of these nucleoside diphosphate sugars is in the $\alpha$-configuration, this is strong additional evidence that the GalNAc-1-P is GalNAc-$\alpha$-1-P.

**DISCUSSION**

This study reports the identification and purification of a new phosphorylating enzyme in pig kidney and liver that transfers a phosphate group from ATP to GalNAc to form GalNAc-$\alpha$-1-P. A previous study by Maley et al. (16) demonstrated the formation of GalNAc-1-P from GalNAc and ATP by a crude extract from rat liver, but it was not clear from that study whether that activity was due to the enzyme galactokinase or to another kinase that could utilize GalNAc as a substrate.

More recently, a very interesting and useful Chinese hamster ovary cell mutant, referred to as IdlD, was isolated and shown to be missing the enzyme that converts UDP-GalNAc to UDP-Gal-NAc, i.e. the UDP-GalNAc 4-epimerase (17). This mutant, when grown in the absence of GalNAc or galactose, is defective in both O- and N-linked glycosylation because it cannot synthesize the necessary precursors, UDP-galactose and UDP-HexNAc. However, supplementation of the medium with free galactose and GalNAc allows the mutant cells to produce UDP-GalNAc, although the affinity of the enzyme is much lower for GalNAc than for GlcNAc derivatives. Based on the specificity of the GalNAc kinase for GalNAc but not galactose and the activity of the UDP-HexNAc pyrophosphorylase toward GalNAc-1-P, it seems likely that these two enzymes do represent a specific salvage mechanism to reutilize GalNAc that is produced in the kidney and liver by the turnover of O-linked glycoproteins.

Thus, there now appears to be two different mechanisms for producing UDP-GalNAc. The major pathway would be from Fru-6-P via GlcNAc-6-P and UDP-GlcNAc to form UDP-GalNAc by the UDP-galactose (GalNAc) 4-epimerase. The second pathway is the one described here with the GalNAc-1-P kinase and the UDP-HexNAc pyrophosphorylase. This second pathway would mostly depend on the production of GalNAc from the turnover of O-linked glycoproteins and glycolipids. We have already found that the kinase is present in HT-29 cells, but it will be interesting to determine the distribution and level of these two enzymes in other cultured cells.

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**REFERENCES**

1. Devine, P. L., and McKenzie, I. F. C. (1992) Bioessays 14, 619–625
2. Carraway, K. L., and Hull, S. R. (1991) Glycobiology 1, 131–138
3. Rose, M. C. (1992) Am. J. Physiol. Lung Cell. Mol. Physiol. 263, L413–L429
4. Sadler, J. E., Paulson, J. C., and Hill, R. L. (1979) J. Biol. Chem. 254, 2112–2119
5. Davies, C. G., Elhammer, A., Russell, D. W., Schneider, W. J., Kornfeld, S., Brown, M. S., and Goldstein, J. L. (1986) J. Biol. Chem. 261, 2828–2838
6. Fukuda, M. N., Sasaki, H., Lopez, L., and Fukuda, M. (1989) Blood 73, 84–89
7. Ilyas, A. S., Quarles, R. H., Dalakas, M. C., and Brady, R. O. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6697–6700
8. Hakomori, S. (1981) Annu. Rev. Biochem. 50, 733–764
9. Ruoslahti, E. (1988) Annu. Rev. Cell Biol. 4, 229–255
10. Davidson, E. (1966) in The Aminosugars (Balazs, E., and Jeanloz, R., eds), pp. 2–43, Academic Press, New York
11. Szumilo, T., Zeng, Y., Pastuszak, I., Drake, R., Szumilo, H., and Elbein, A. D. (1996) J. Biol. Chem. 271, 13147–13154
12. Laemmli, U. K. (1970) Nature 227, 680–685
13. Kaushal, G. P., Szumilo, T., Pastuszak, I., and Elbein, A. D. (1990) Biochemistry 29, 2168–2176
14. Rao, B. N., and Bush, C. A. (1988) Carbohydr. Res. 180, 111–123
15. Dua, V. K., Rao, B. N. N., Wu, S.-S., Dubé, V. E., and Bush, C. A. (1986) J. Biol. Chem. 261, 1599–1608
16. Maley, F., Tarentino, A. L., McGarrahan, J. F., and DelGiaco, R. (1988) Biochem. J. 261, 2828–2838
17. Kingsley, D. M., Kozarsky, K. F., Hobbs, L., and Krieger, M. (1986) Cell 44, 749–759
18. Matzuk, M. M., Krieger, M., Corless, C. L., and Baire, I. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6354–6358
19. Reddy, P., Caras, I., and Krieger, M. (1989) J. Biol. Chem. 264, 17329–17336
20. Strominger, J. L., and Smith, M. S. (1959) J. Biol. Chem. 234, 1822–1827
21. Pattabiraman, T. N., and Bachhawat, B. K. (1961) Biochim. Biophys. Acta 50, 129–134
22. Pastuszak, I., O’Donnell, J., and Elbein, A. D. (1996) J. Biol. Chem., in press