Communication

Identification of the GalNAc Kinase Amino Acid Sequence*

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A new kinase that forms GalNAc-1-P was purified from pig kidney cytosol and identified on gels by labeling with N3-[32P]ATP (Pastuszak, I., Drake, R., and Elbein, A. D. (1996) J. Biol. Chem. 271, in press). A 50-kDa labeled protein was eluted, digested with trypsin, and the sequences of four peptides representing 49 amino acids showed 90% identity to sequence described human galactokinase reported to be on chromosome 15. To resolve this dilemma, activities and substrate specificities of galactokinase and GalNAc kinase from human and pig kidney, as well as of galactokinase from the yeast clone transfected with the cDNA from presumptive human galactokinase, were compared. The purified galactokinases phosphorylated galactose, but not GalNAc, whereas GalNAc kinase also phosphorylated galactose when this sugar was present at millimolar concentrations. Extracts of gal 1- yeast clone, transfected with the cDNA from presumptive human galactokinase, were compared. The purified galactokinases phosphorylated galactose, but not GalNAc, whereas GalNAc kinase also phosphorylated galactose when this sugar was present at millimolar concentrations. The cDNA for this galactokinase was isolated from human HepG2 cells by expression cloning using a yeast clone transfected with the human expression library. This cDNA enabled this clone to grow on galactose. Transfection with this cDNA enabled this clone to grow on galactose.

In order to resolve the dilemma between these two sequences, we have reexamined the specificity of the human and pig kidney GalNAc kinase, as well as the galactokinase isolated from pig and human kidney. We have also examined these enzymatic activities in the parent yeast and in the yeast clone transfected with the human expression library. This report demonstrates that the previously reported sequence is that of GalNAc kinase and shows that this enzyme can phosphorylate galactose when this sugar is present at millimolar concentrations.

EXPERIMENTAL PROCEDURES

Materials—[1-3H]N-acetylglucosamine (GalNAc, 10–25 Ci/mmol) and [6-3H]galactose (20 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. Unlabeled sugars and various adsorbents used for purification of the enzymes were from Sigma. Coomassie Blue, protein assay reagent, sodium dodecyl sulfate, acrylamide, and hydroxyapatite were from Bio-Rad. All other chemicals were from reliable chemical sources and were of the best grade available.

Kinase Assays—GalNAc kinase activity was assayed in incubation mixtures of 100 μl containing the following components: 200 μM [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 examined. 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, contained in a Pasteur pipette (about 1.5 ml of resin). The column was washed with at least 5 column volumes of 10 mM (NH4)HCO3 to remove unbound material and then GalNAc-1-P was eluted with 500 mM (NH4)HCO3. Aliquots of the wash and eluate were removed and assayed for their radioactive content by scintillation counting. In some experi-

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the literature indicates that these two sugars are produced from the corresponding glucose derivatives, i.e. UDP-GlcNAc and UDP-glucuronic acid, by the action of specific UDP-sugar 4-epimerases (5). In fact, the UDP-gal 4-epimerase was purified to homogeneity, and the homogenous protein was shown to catalyze the epimerization of UDP-GlcNAc to UDP-GalNAc at the same rate as the epimerization of UDP-glucose to UDP-galactose (5). In mung bean seedlings, a UDP-galacturonic acid pyrophosphorylase was identified (6), suggesting the presence of a series of reactions for the activation of galacturonic acid that involves another kinase and the above-mentioned pyrophosphorylase.

Recent studies from our laboratory have identified and purified a GalNAc kinase from pig liver that phosphorylates GalNAc in the one position to form GalNAc-α-1-P (7). In addition, we previously purified a UDP-GlcNAc pyrophosphorylase from pig kidney and pig liver and found that this enzyme had strong activity with GalNAc-1-P and UDP for the synthesis of UDP-GalNAc (8). Thus, animal cells may also possess another mechanism for activation of GalNAc, and this pathway probably represents a salvage mechanism to rescue GalNAc arising from the degradation of complex carbohydrates. The purified GalNAc kinase was subjected to SDS-gel electrophoresis and a 50-kDa band that had strong GalNAc kinase activity, that was specifically labeled with N3-[32P]ATP, was subjected to peptide sequencing. Four peptides from this protein, containing a total of 49 amino acids, showed 90% homology to the sequence reported from the human galactokinase gene located on chromosome 15 (9). The cDNA for this galactokinase was isolated from human HepG2 cells by expression cloning using a yeast mutant that lacked galactokinase and could not grow on galactose. Transfection with this cDNA enabled this clone to grow on galactose (9).

Galactose, N-acetylgalactosamine, and galacturonic acid are all common components of complex carbohydrates in eucaryotic cells, and they all have the same configuration of hydroxyl groups at carbons 2 through 5, as well as similar pathways of biosynthesis (1). Thus, the major pathway for synthesis and activation of galactose is via the conversion of UDP-glucose to UDP-galactose by a 4-epimerase (2). However, another pathway for the formation of UDP-galactose also exists in some animal tissues such as liver and kidney. This pathway involves the phosphorylation of galactose in the one position by a specific galactokinase (3) and then transfer of galactose-1-P to UDP-glucose to give UDP-galactose and glucose-1-P (4). For synthesis of UDP-GalNAc and UDP-galacturonic acid,
The pig GalNAc kinase amino acid sequence is determined as follows:

| Source | Enzyme | Position of first amino acid | Sequence |
|--------|--------|-----------------------------|----------|
| Human  | GK     | 2                           | Ala-Thr-Glu-Ser-Pro-Ala-Thr-Arg-Arg-Val-Gln-Val-Ala-Ala-Glu-His-Pro-Arg |
| Pig    | GlcNAcK | 235                         | Ala-Ala-Thr-Arg-Arg-Val-His-Lys_Val-Gln_Val-Gln-Val-Ala-Glu-His-Pro-Arg |
| Human  | GK     | 342                         | Val-Leu-Gln-Phe-Lys-Lys |
| Pig    | GalNAcK | 441                         | Leu_Gln_Ile-Lys_Gln |

The pig GalNAcK sequence is determined as follows:

| Pig GalNAcK | Human GK 342 |
|-------------|--------------|
| Ala-Thr-Glu-Ser-Pro-Ala-Thr-Arg-Arg-Val-Gln-Val-Ala-Ala-Glu-His-Pro-Arg | Ala-Ala-Thr-Arg-Arg-Val-His-Lys_Val-Gln_Val-Gln-Val-Ala-Glu-His-Pro-Arg |
| Pig GlcNAcK | Human GK 2   |
| Ala-Ala-Thr-Arg-Arg-Val-His-Lys_Val-Gln_Val-Gln-Val-Ala-Glu-His-Pro-Arg | Ala-Ala-Thr-Arg-Arg-Val-His-Lys_Val-Gln_Val-Gln-Val-Ala-Glu-His-Pro-Arg |
| Pig GalNAcK | Human GK 235 |
| Ala-Ala-Thr-Arg-Arg-Val-His-Lys_Val-Gln_Val-Gln-Val-Ala-Glu-His-Pro-Arg | Ala-Ala-Thr-Arg-Arg-Val-His-Lys_Val-Gln_Val-Gln-Val-Ala-Glu-His-Pro-Arg |

The results and discussion section of the document discusses the enzymatic activity of pig (or human) kidney galactokinase. The partially purified galactokinase was incubated with various amounts of the radioactive substrates, galactose or GalNAc, plus ATP, and the amount of radioactivity binding to DE52 was determined as a measure of phosphorylation of the sugars. A, galactose concentration; ●, GlcNAc concentration.

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sequenced. As shown in Table I, these four peptides showed 90% homology to the amino acid sequence of human galactokinase deduced from the cDNA of a HepG2 expression library (9). This cDNA expression library was introduced into the yeast strain YM20, which contains a deletion of the gene encoding galactokinase (GAL1). Transformants were plated on media containing galactose as the carbon source and one transformant in 150,000 was identified. The cDNA involved in “curing” this mutant encodes for a 458-amino acid polypeptide of 50,386 daltons.

Since the sequence of the purified pig kidney GaINAc kinase was identical to that of the reported human galactokinase, we reexamined the specificity of our GaINAc kinase to determine whether it had any activity on galactose. Fig. 1 shows that the purified enzyme can phosphorylate galactose when this sugar is present at millimolar concentrations. A rough estimation of the \( K_m \) for galactose based on this experiment is about 4 mM. The data in Fig. 1 also demonstrate that this enzyme was much more active toward GaINAc and showed approximately the same activity with 50–100 \( \mu \)M GaINAc as with 10 \( \mu \)M galactose. Similar results were observed with the partially purified GaINAc kinase from human kidney.

We also purified the galactokinase from pig and human kidney and examined the specificity of this enzyme as shown in Fig. 2. The GaINAc kinase and the galactokinase were previously shown to be well separated from each other by chromatography on phenyl-Sepharose (7). Fig. 2 shows that the kidney galactokinase had good phosphorylating activity toward galactose with an approximate \( K_m \) of 0.5 mM, but it did not show any detectable activity toward GaINAc, even when this sugar was present at 10 mM concentrations. Thus the purified 50-kDa protein from pig kidney appears to be a true GaINAc kinase, but it does have the ability to phosphorylate galactose when this sugar is present in high concentrations. Since the transformants are selected based on their ability to grow on plates containing 2% galactose (about 10 mM), it seems likely that introduction of the GaINAc kinase into these cells would allow them to grow, albeit slowly, on galactose.

In order to determine if the yeast clone had been rescued by introduction of the GaINAc kinase gene, the wild type yeast and the transformant were grown on peptone-yeast extract medium containing either galactose or glucose as the sugar source. The cells were then ruptured, and the cytosolic fraction was assayed for the presence of galactokinase or GaINAc ki-
nase. Fig. 3 shows that the wild type S. cerevesiae, having the normal galactokinase gene, has strong phosphorylating activity for galactose when the cells were grown on galactose (panel A), but very low galactokinase activity when cells were grown on glucose (panel B). More importantly, the wild type cells had no ability to phosphorylate GalNAc, regardless of whether they were grown on glucose or galactose (panels A and B).

On the other hand, the yeast clone obtained by transformation with the HepG2 cDNA library showed quite different kinase activity as seen in Fig. 4. In this case, the cell free extracts had very low activity for phosphorylating galactose, regardless of whether cells were grown on glucose or galactose. However, these cells were able to phosphorylate GalNAc, and this activity was present regardless of whether cells were grown on glucose or galactose. In fact, the activity was significantly better when cells were grown on glucose, probably because these cells grow much better on glucose. The data with the various yeast clones are summarized in Table II, which shows the reasonably high GalNAc activity in the transformed yeast and the complete absence of this activity in wild type.

An earlier report had indicated that the human galactokinase resided on chromosome 17, but the subunit molecular weight was in conflict. One study reported a molecular mass of 38 kDa (13), while another reported it as 55 kDa (14). A second "galactokinase" was identified in the HepG2 expression library by complementing a yeast strain with a galactokinase deficiency that allowed this yeast to grow on galactose. This gene encoded a protein of 458 amino acids (50,386 kDa) and was localized to chromosome 15 (9). The data provided in this paper demonstrate that the sequence for this second gene is really that of the human (and pig) GalNAc kinase and that this enzyme is able to utilize galactose as a substrate and phosphorylate it to form galactose-1-P. Thus introduction of the gene for GalNAc kinase into the galactokinase negative yeast will apparently "cure" this organism and allow it to grow, although poorly, on galactose. Thus, the sequence of the GalNAc kinase and its location on chromosome 15 have now been established.

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