A 17-Amino Acid Insert Changes UDP-N-Acetylhexosamine Pyrophosphorylase Specificity from UDP-GalNAc to UDP-GlcNAc*

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We previously reported the purification of a UDP-N-acetylhexosamine (UDP-HexNAc) pyrophosphorylase from pig liver that catalyzed the synthesis of both UDP-GlcNAc and UDP-GalNAc from UTP and the corresponding HexNAc-1-P.1 Previous studies on the partial purification of UDP-GlcNAc pyrophosphorylase from Staphylococcus aureus (3) and animal tissues (4) indicated that those enzymes had very low activity with GalNAc-1-P as compared with GlcNAc-1-P. Recently, the human UDP-GlcNAc pyrophosphorylase gene was cloned from a testis cDNA library (5) and shown to be identical to AGX1, a protein of unknown function previously isolated from human sperm (6). However, the substrate specificity of the UDP-GlcNAc pyrophosphorylase was not determined in that study (5).

We sequenced three peptides from the pig liver 64 kDa pyrophosphorylase subunit and two from the 57 kDa subunit and found that they all had 100% identity to AGX1. An isoform of AGX1 called AGX2, also of unknown function and found in various tissues, was reported to be identical in sequence to AGX1, except that it had an additional 16-amino acid insert near the carboxyl terminus (6). In this report, we describe the expression of AGX1 and AGX2 in Escherichia coli, and we demonstrate that these two proteins are both UDP-HexNAc pyrophosphorylases that differ markedly from each other in specificity for HexNAc-1-P. Our results indicate that AGX2 differs from AGX1 by insertion of a 17-amino acid peptide and that this insertion changes the specificity of AGX1 from a UDP-GalNAc to a UDP-GlcNAc pyrophosphorylase.

MATERIALS AND METHODS

Plasmid Construction—AGX1 and AGX2 DNAs were amplified by polymerase chain reaction from a cDNA library made from breast cancer cells using primers: 5′GGCTTTAGTGACTATGTTCATTAATGACCTCAAA and 5′ TCTTATGCCGGCCGCAAATAATGTCTAGGTCCATGTTCA3. The amplified inserts were digested with NotI and SalI and ligated to pGEX-4T-2 expression vectors (glutathione S-transferase expression system from Amersham Pharmacia Biotech). The final constructs were transformed in E. coli JM109 for GST fusion protein expression. GST-AGX1 and GST-AGX2 colonies were confirmed by sequencing the coding region of the constructs.

Protein Purification and Expression—GST-AGX1 and GST-AGX2 colonies were grown in 500 ml of LB medium containing 0.1 mg/ml ampicillin at 37 °C until the optical density at 600 nm had reached a value of 1.5. At that point, GST fusion protein synthesis was induced by the addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside, and the cells were allowed to grow for an additional 6 h. The cells were isolated by centrifugation, suspended in phosphate-buffered saline, and ruptured by sonication. The cell debris was removed by centrifugation, and the supernatant fraction was retained for the assay of UDP-GlcNAc (GalNAc) pyrophosphorylase(s) and the isolation of AGX1 and AGX2. The fusion proteins were isolated on glutathione-Sepharose beads, and the AGX1 and AGX2 colonies were obtained by thrombin digestion of the purified GST fusion proteins. Purified AGX1 and AGX2 were used for enzyme assays and immunoblot analysis.

Western Blot Analysis—The purified recombinant AGX1 and AGX2 were subjected to 10% SDS-PAGE, along with purified UDP-GlcNAc/GalNAc pyrophosphorylase from human sperm. An isoform called AGX2 is identical in sequence to AGX1 except that it has an additional 16-amino acid insert near the carboxyl terminus. We expressed the AGX1 and AGX2 genes in Escherichia coli. The protein isolated from the AGX1 clone comigrated on SDS gels with the liver 57-kDa pyrophosphorylase subunit and was 2–3 times more active with GalNAc-1-P than with GlcNAc-1-P. On the other hand, the protein from the AGX2 clone migrated with the liver 64-kDa pyrophosphorylase subunit and had 8-fold better activity with GlcNAc-1-P than with GalNAc-1-P. These results indicate that insertion of the 17-amino acid peptide modifies the specificity of the pyrophosphorylase from synthesis of UDP-GalNAc to synthesis of UDP-GlcNAc.

Nucleotide diphosphate sugar pyrophosphorylases are important enzymes in the synthesis of complex carbohydrates as well as in other aspects of basic metabolism (1). These enzymes are thought to be the product of “housekeeping genes," and nothing is known about the regulation of these enzymes or their products. We recently purified the UDP-GlcNAc pyrophosphorylase from pig liver cytosol to apparent homogeneity (2) and found that the native enzyme had a molecular mass of about 120 kDa and was composed of two subunits of 64 and 57 kDa. Surprisingly, the native enzyme catalyzed the synthesis of both UDP-GlcNAc and UDP-GalNAc from UTP and the corresponding HexNAc-1-P.1 The abbreviations used are: HexNAc, N-acetylhexosamine; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.

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assayed in the forward (formation of UDP-HexNac) or in the reverse directions. In the forward direction, the incubation mixtures contained, in a final volume of 50 μl, 50 mM Tris buffer, pH 7.5, 2 mM MgCl₂, 2 mM [³H]UTP (55,000 cpm), 5 mM GlcNAc-1-P or GalNAc-1-P, and various amounts of the GST fusion proteins or the thrombin-released AGX1 and AGX2. After an incubation of 5 min at 37°C, the reaction was terminated by heating the tubes in a boiling water bath for 30 s. The amount of [³H]UDP-HexNAc formed was determined by applying reaction mixtures to DE52 and measuring the amount of radioactivity that eluted from the column in 70 mM (NH₄)HCO₃. The products of these reactions were characterized by paper and TLC chromatography as described previously (2).

RESULTS AND DISCUSSION

We previously reported the purification to apparent homogeneity of a pig liver UDP-HexNAc pyrophosphorylase that catalyzed the synthesis of both UDP-GlcNAc from GlcNAc-1-P and UTP or UDP-GalNAc from GalNAc-1-P and UTP (2). On SDS gels, the enzyme showed two protein bands of about 64 and 57 kDa in almost equal amounts. Three peptides from the 64- and two from the 57-kDa protein had complete identity to AGX1 (see Fig. 2), a protein of unknown function, located in the tail of human sperm (6). Because AGX1 is highly expressed in testis whereas AGX2 is not, we purified the UDP-GlcNAc pyrophosphorylase from pig testis and found that this enzyme was composed almost entirely of the 57-kDa protein. However, the exact relationship of AGX1 and AGX2 to the UDP-HexNAc pyrophosphorylase, or to each other, was still not clear.

To resolve this question, we amplified the AGX1 and AGX2 genes by polymerase chain reaction using a breast cancer cell cDNA library and expressed these genes in E. coli as GST fusion proteins. Fig. 1A shows that the recombinant AGX1 protein (lane 2), after release of the GST tag by thrombin, migrated in the same position as the 57-kDa liver pyrophosphorylase subunit on SDS gels (lane 1), whereas the recombinant AGX2 protein (lane 3) migrated with the 64-kDa liver subunit. Fig. 1B shows that both of these proteins, as well as the UDP-HexNAc pyrophosphorylase subunits, also reacted by Western blotting with an antibody prepared against an internal peptide from the AGX1 sequence. The Western blots further demonstrate the similarity between AGX1 and the 57-kDa subunit and AGX2 and the 64-kDa subunit. It should be noted that AGX2 shows several faster moving protein bands on these gels (Fig. 1A, lane 3; Fig. 1B, lane 2). These bands apparently result because AGX2 contains another thrombin cleavage site.

**TABLE I**

UDP-GlcNAc (GalNAc) pyrophosphorylase activity

The GST fusion proteins and purified AGX1 and AGX2 were prepared as described under "Materials and Methods." Assays were performed in the forward direction with 2 mM UTP (55,000 cpm) and 2–5 mM sugar-1-P. Each assay was done in triplicate.

| Protein assayed | GlcNAc-1-P | GalNAc-1-P |
|-----------------|------------|------------|
| nmol/min/mg     | nmol/min/mg|
| GST-AGX1        | 525        | 1342       |
| GST-AGX2        | 887        | 119        |
| GST             | 33         | 20         |
| AGX1            | 5919       | 14,902     |
| AGX2            | 5873       | 300        |

**TABLE II**

Kinetic constants for UDP-GlcNAc (GalNAc) pyrophosphorylase

The enzyme assays were performed in the forward direction as described under "Materials and Methods," except that the concentration of HexNAc-1-P was varied over a wide range to determine Kₘ values. The values in the table are the means of several experiments, and they were obtained by nonlinear regression analysis.

| Protein assayed | Substrate | Kₘ   | Vₘₐₓ/Kₘ |
|-----------------|-----------|------|---------|
|                 | GlcNAc-1-P|       |         |
|                 | GalNAc-1-P|       |         |
| GST-AGX1        | 0.24      | 105.2|
| GST-AGX2        | 0.38      | 165.1|
| GST-AGX1        | 0.32      | 262.8|
| GST-AGX2        | 1.3       | 10.9 |

**Fig. 1.** Migration of recombinant AGX1 and AGX2 as compared with pig liver UDP-HexNAc pyrophosphorylase subunits by SDS-PAGE. GST-AGX1 and GST-AGX2 were produced in E. coli as described and were released from GST by treatment with thrombin. Proteins were subjected to SDS-PAGE and identified by staining with Coomassie Blue (A: lane 1, UDP-GlcNAc pyrophosphorylase; lane 2, AGX1; lane 3, AGX2) and Western blotting with anti-AGX1 antibody (B: lane 1, UDP-HexNAc pyrophosphorylase; lane 2, AGX1; lane 3, AGX2).

**Fig. 2.** Amino acid sequence of AGX1 and AGX2. AGX1 and AGX2 cDNAs were amplified from breast cancer cell cDNA by polymerase chain reaction using appropriate primers. The amplified inserts were ligated to pGEX-4T-2 and expressed in E. coli. GST-AGX1 and GST-AGX2 colonies were confirmed by sequencing the coding region of the constructs. The coding sequences are converted to amino acid sequences in this figure. The sequences for AGX1 and AGX2 are identical except for the bracketed insert representing the 17-amino acid peptide insert (amino acids 454–468) in AGX2. The sequences underlined with a solid line were determined by us by sequencing peptides from the 57-kDa UDP-HexNAc pyrophosphorylase subunit, whereas those shown with a broken line were from the 64-kDa subunit.
A GlcNAc or UDP-GalNAc. In reaction mixtures were removed and assayed for the formation of UDP-recombinant fusion proteins. At the times indicated, aliquots of the Incubations were as described in the text and contained 7 mM were obtained as described and were assayed in the forward direction (6). We sequenced the except for a 16-amino acid insert near the carboxyl end of AGX2 indicated that these two proteins had identical sequences, ex-

phorylase was also a dimer of about 120 kDa (2). Our previous studies suggested that the pig liver UDP-HexNAc pyrophosphorylase in the AGX1 sequence.

The enzymatic activities of the GST fusion proteins of AGX1 and AGX2 were tested in the forward reaction with [3H]UTP and either GlcNAc-1-P or GalNAc-1-P (Table I). The activity of GST-AGX1 was 2–3 times higher with GalNAc-1-P than with GlcNAc-1-P (plus UTP), whereas GST-AGX2 activity was about 8 times higher with GlcNAc-1-P than with GalNAc-1-P. Table I also indicates that control cells, transformed with the same expression vector but without AGX1 or AGX2 inserts, had barely detectable activity, indicating that endogenous UDP-HexNAc pyrophosphorylase activity in E. coli cells is very low. Removal of GST by treatment of GST-AGX1 or GST-AGX2 with thrombin did not affect the substrate specificity of these two proteins, but it did appear to significantly enhance the enzymatic activity in both cases.

The kinetic constants of the two recombinant proteins were also determined as shown in Table II. The data indicate that the 17-amino acid insert causes a significant reduction in the $K_m$ for GalNAc-1-P as shown by the fact that AGX2 has a $K_m$ for this substrate of 1.3 mM, as compared with 0.38 mM for AGX1. As a result, the $V_{max}$ of AGX2 for synthesizing UDP-GalNAc is only about 12% (10.9 nmol/min) of that shown by AGX1 (165 nmol/min). In addition, there is a 2–3-fold increase in the rate of synthesis of UDP-GalNAc by AGX2 as compared with AGX1.

A time course study comparing the rates of synthesis of both sugar nucleotides by the two recombinant fusion proteins (GST-AGX1 and GST-AGX2) is presented in Fig. 3 (A and B). It can be seen in A that the rate of formation of UDP-GalNAc by AGX1 is about 2–3 times that of UDP-GlcNAc. AGX1 is equivalent to the 57-kDa UDP-HexNAc pyrophosphorylase subunit of pig liver and testis. This protein appears to function better as a UDP-GalNAc synthase. Fig. 3A also demonstrates that control cells containing the expression vector without the AGX1 or AGX2 inserts have very low enzymatic activity. On the other hand, Fig. 3B shows that AGX2, having the 17-amino acid insert, has much better activity as a UDP-GlcNAc synthase than as a UDP-GalNAc synthase. This protein is equivalent to the 64-kDa protein from pig liver and is present in very low amounts in testis. The products formed in these reactions were shown by paper and thin layer chromatography to be the expected UDP-HexNAcs using previously reported methods (2). These experiments suggest that insertion of the 17-amino acid peptide into the UDP-GalNAc pyrophosphorylase (AGX1) changes the substrate specificity to a UDP-GlcNAc pyrophosphorylase (AGX2). It was previously shown that AGX1 and AGX2 result from alternative splicing (6), suggesting that some animal tissues have the ability to utilize the same gene and convert a UDP-GalNAc to a UDP-GlcNAc synthesizing enzyme. These interconversions may represent a control mechanism to allow for the optimum synthesis of various types of complex carbohydrates. It will be important to determine the level of these two enzymatic activities in tissues that are synthesizing large amounts of O-linked oligosaccharides, as compared with those tissues that synthesize mostly N-linked oligosaccharides.

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**FIG. 3.** Enzymatic activities and substrate specificities of recombinant GST-AGX1 and GST-AGX2. The recombinant proteins were obtained as described and were assayed in the forward direction with GlcNAc-1-P or GalNAc-1-P and [3H]UTP as described in the text. Incubations were as described in the text and contained 7 μg of the recombinant fusion proteins. At the times indicated, aliquots of the reaction mixtures were removed and assayed for the formation of UDP-GlcNAc or UDP-GalNAc. In A, assays were done with AGX1, whereas in B, assays were done with AGX2.

near the 17-amino acid insert, giving rise to multiple protein bands. On native gels, both recombinant AGX1 and AGX2 (after thrombin cleavage) migrate like proteins of 100–120 kDa, suggesting that they are both homodimers. Our previous studies suggested that the pig liver UDP-HexNAc pyrophosphorylase activity in the 57- and 64-kDa subunits of the pig liver UDP-HexNAc inserts, had barely detectable activity, indicating that endogenous UDP-HexNAc pyrophosphorylase activity in E. coli cells is very low.