Lysosomal Enzyme Targeting

N-ACETYLGLOUCOSAMINYLPHOSPHOTRANSFERASE SELECTIVELY PHOSPHORYLATES NATIVE LYSOSONAL ENZYMES*

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A new assay for the N-acetylglocosaminylphosphotransferase, using α-methylmannoside as substrate, is presented. Using this assay, we partially purified the transferase and examined its substrate specificity. The transferase exhibited a very high affinity toward lysosomal enzymes (apparent K_m values of <20 μM) and was greater than 100-fold more efficient (V_max/K_m) when using lysosomal enzymes as acceptors as compared to nonlysosomal glycoproteins that contain high mannose oligosaccharide units. Heat denaturation of the lysosomal enzymes resulted in the loss of acceptor activity. The model compounds α-methylmannoside and Manα-GlcNAc were poor acceptors. We propose that this enzyme catalyzes the initial, determining step by which newly synthesized acid hydrolases are distinguished from other newly synthesized glycoproteins and thus are eventually targeted to lysosomes.

Lysosomal enzymes carry a recognition marker which mediates their translocation to lysosomes and their receptor-mediated cellular uptake (1). This recognition marker is a high mannose-type oligosaccharide containing one or two 6-phosphomannosyl groups (2–8). The biosynthesis of the phosphomannosyl residues occurs in two steps. First, N-acetylglucosamine 1-phosphate is transferred en bloc to an acceptor mannose by UDP-N-acetylgucosamine-lyosomal enzyme N-acetylglucosamine-1-phosphotransferase, resulting in a phosphogluco unit in diester linkage between the outer "blocking" N-acetylglucosamine and the inner mannose (9, 10). Next, an α-N-acetylgucosaminyl phosphodiesterase removes the N-acetylglucosamine, leaving the phosphate in monooester linkage to the underlying mannose residue (11–13).

Although the biochemical pathway for the generation of the phosphomannosyl residues is now established, little information is available pertaining to the specificity of the process for lysosomal enzymes. In this report, we examine the substrate specificity of partially purified rat liver N-acetylgucosaminylphosphotransferase. The transferase exhibits a striking preference for acid hydrolases over other potential glycoprotein acceptors. We propose that N-acetylgucosaminylphosphotransferase is responsible for the initial recognition of lysosomal enzymes as distinct from other newly synthesized glycoproteins.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from the following sources: Sprague-Dawley rats, Harlan Industries, DEAE-cellulose (DE52), Whatman; 3a70 scintillation mixture, Research Products International; Con A-Sepharose, Pharmacia; Lubrol PX, QAE-Sephadex (Q-25-120), α-methyl-b-mannoside, bovine serum albumin (A-5403), bovine thryoglobulin (T-1001), hen ovalbumin (A-5503), and bovine pancreatic ribonuclease B (R-8575), Sigma. [β-32P]UDP-GlcNAc was prepared as described (10). The ribonuclease B was purified by Con A-Sepharose chromatography (14). Human immunoglobulin M (Wa) and soybean agglutinin (16) were prepared by Walter Gregory of this laboratory. Partially purified lysosomal enzymes from porcine liver, α-N-acetylgucosaminidase, β-hexosaminidase A, and β-hexosaminidase B were supplied by Walter Gregory. Human placental β-hexosaminidase A (17), human placental β-hexosaminidase B (17), and human hepatic β-galactosidase (18) were provided by Dr. Arnold Miller, University of California at San Diego.

General Methods—Protein concentrations were determined by a modification of the method of Lowry et al. (19) or by absorbance at 280 nm. Descending paper chromatography was performed using Solvent D as previously described (10).

Assay of N-Acetylglucosaminylphosphotransferase Using a Man-Linked Mannoside as Acceptor—Each incubation contained 150 μM [γ-32P]ATP, 10 mM MnCl_, 10 mM MgCl_, 250 μM diethiolethiol, 100 μg of bovine serum albumin (preincubated 30 min at 56 °C), 0.3% (w/v) Lubrol PX, 50 mM Tris-HCl, pH 7.4, and enzyme in a final volume of 0.06 ml in a 1.5-ml polypropylene tube. Following incubation at 37 °C for 30 min, the reactions were stopped by placing in a boiling water bath for 5 min. Next, 1.3 ml of 2 M Tris base was added and the centrifuged at 12,000 × g for 5 min. The supernatant was applied to a column of QAE-Sephadex (0.5 × 30 cm) and washed with 2 ml of 2 M Tris base. The GlcNAc-[32P]-methylmannoside was selectively eluted with four 1-ml aliquots of 30 mM NaCl in 2 M Tris base. The four fractions were pooled and counted after the addition of 12 ml of scintillation mixture. Control incubations contained either no enzyme, 40 mM EDTA, or α-methylgalactosidase in place of the α-methylmannoside. All three controls gave similar background values of <0.01% of the radioactivity used. Evidence that this assay measures N-acetylgucosaminylphosphotransferase activity includes the fact that fibroblasts from patients with mucolipidosis II (I-cell disease) have <1% of the activity of normal controls (20). Further details of the validation of the assay and proof of the product will be presented in a subsequent publication. One unit of enzyme activity is defined as the transfer of 1 pmol of N-acetylgucosaminylphosphotransferase activity includes the fact that fibroblasts from patients with mucolipidosis II (I-cell disease) have <1% of the activity of normal controls (20).

Assay of N-Acetylglucosaminylphosphotransferase Using a Man-Linked Mannoside as Acceptor—Each incubation contained 150 μM [β-32P]UDP-GlcNAc (usually 300,000 cpm), 180 mM α-methylmannoside, 5 mM ATP, 10 mM MnCl_, 10 mM MgCl_, 250 μM diethiolethiol, 100 μg of bovine serum albumin (preincubated 30 min at 56 °C), 0.3% (w/v) Lubrol PX, 50 mM Tris-HCl, pH 7.4, and enzyme in a final volume of 0.06 ml in a 1.5-ml polypropylene tube. Following incubation at 37 °C for 30 min, the reactions were stopped by placement in a boiling water bath for 5 min. Next, 1.3 ml of 2 M Tris base was added and the centrifuged at 12,000 × g for 5 min. The supernatant was applied to a column of QAE-Sephadex (0.5 × 30 cm) and washed with 2 ml of 2 M Tris base. The GlcNAc-[32P]-methylmannoside was selectively eluted with four 1-ml aliquots of 30 mM NaCl in 2 M Tris base. The four fractions were pooled and counted after the addition of 12 ml of scintillation mixture. Control incubations contained either no enzyme, 40 mM EDTA, or α-methylgalactosidase in place of the α-methylmannoside. All three controls gave similar background values of <0.01% of the radioactivity used. Evidence that this assay measures N-acetylgucosaminylphosphotransferase activity includes the fact that fibroblasts from patients with mucolipidosis II (I-cell disease) have <1% of the activity of normal controls (20). Further details of the validation of the assay and proof of the product will be presented in a subsequent publication. One unit of enzyme activity is defined as the transfer of 1 pmol of N-acetylgucosaminylphosphotransferase activity includes the fact that fibroblasts from patients with mucolipidosis II (I-cell disease) have <1% of the activity of normal controls (20).

1 The abbreviation used is Con A, concanavalin A.
Lysosomal Enzyme Phosphorylation

**TABLE I**

| Acceptor glycoprotein | Acceptor site concentration (µM) | N-Acetylglucosaminylphosphotransferase activity (cpm transferred) | pmol transferred |
|-----------------------|---------------------------------|---------------------------------------------------------------|------------------|
| None                  |                                 |                                                               |                  |
| α-N-Acetylglucosaminidase | 0.2                           | 4.0                                                           | 168              |
|                       |                                 |                                                               |                  |
| Ovalbumin             | 0.4                             | 1.0                                                           | 279              |
|                       |                                 |                                                               |                  |
| Thryoglobulin         | 0.4                             | 0.3                                                           | 41               |
|                       |                                 |                                                               |                  |
| Immunoglobulin M      | 0.4                             | 0.2                                                           | 4.0              |
|                       |                                 |                                                               |                  |
| Ribonuclease B        | 0.4                             | 0.8                                                           | 67               |
|                       |                                 |                                                               |                  |
| Soybean agglutinin    | 0.4                             | 0.2                                                           | 4.0              |

*a* The transfer to endogenous acceptors (0.20 pmol) has been subtracted.

**Assay of N-Acetylglucosamine 1-Phosphate Transfer to Glycoprotein**

The incubation mixtures contained 150 µM [32P]P/UDP-GlcNAc (1-2 x 10^6 cpm), 5 mM ATP, 10 mM MgCl2, 250 µM dithiothreitol, 0.1 to 1.0% (w/v) Lubrol PX, 50 mM Na-acetylglucosamine, 50 mM Tris-HCl, pH 7.45, N-acetylglucosaminylphosphotransferase (usually 150 to 250 units), and acceptor protein in a volume of 0.05 ml. After incubation (37 °C, 30 min) the reactions were stopped by the addition of 0.1 ml of 1.55 (w/v) phosphotungstic acid/0.75 N HCl at 0 °C. After 5 min at 0 °C, the tubes were centrifuged (5 min, 12,000 x g, 4 °C) and the supernatant was discarded. The pellets were washed with 1 ml of 1% phosphotungstic acid/0.5 N HCl, centrifuged as above, and resuspended by sonication in 0.5 ml of 100 mM Na-acetylglucosamine/50 mM CaCl2/100 mM Tris-HCl, pH 8.0. One mg of pronase was added and the samples were incubated at 37 °C. After 1 h, the samples were heated at 100 °C for 10 min and then cooled. One ml of water was added and the samples were centrifuged (5 min, 12,000 x g, room temperature). The supernatant was applied to a pipette column (0.5 x 1.5 cm) of Con A-Sepharose and washed with 100-200 ml/column of 150 mM NaCl/10 mM Na-phosphate, pH 7.5, in 1 h. The Con A-Sepharose was then eluted from the column and counted in a scintillation spectrometer after the addition of 1 ml of water and 10 ml of 30% H2O2. Care was taken so that the capacity of the Con A-Sepharose (~60 nmol of high mannose oligosaccharide chain/ml of resin) was not exceeded. When the washed protein pellet was hydrolyzed (n HCl, 4 °C, 100 °C), desalted on Dowex 50, H+ form (0.5 x 4.5 cm), and subjected to paper chromatography, the amount of 32P co-migrating with mannose 6-phosphate was the same as that binding to Con A-Sepharose in the standard assay.

**Calculation of Acceptor Site Concentration**—The concentration of glycoprotein acceptor has been calculated based on the number of high mannose-type oligosaccharides/polyelectrolyte chain. The following acceptor site molecular weights were used: ribonuclease B, 15,000 (21, 22); soybean agglutinin, 30,000 (23, 24); ovalbumin, 45,000 (25); thryoglobulin, 118,000 (26); immunoglobulin M, 47,500 (27, 28); α-N-acetylglucosaminidase, 30,000 (arbitrary estimate); β-hexosaminidase A, 50,000 (29, 30) and β-hexosaminidase B, 54,000 (29, 30).

**Partial Purification of N-Acetylglucosaminylphosphotransferase**—Details of the enzyme preparation will be presented elsewhere. Briefly, smooth membranes were prepared from rat liver (13) and extracted with 10 mM mannose 6-phosphate/20 mM Na-acetate/0.1% NaCl at pH 5.0 and 100-200 ml/column of 150 mM NaCl/10 mM Na-phosphate, pH 7.5, in 1 h. The Con A-Sepharose was then eluted from the column and counted in a scintillation spectrometer after the addition of 1 ml of water and 10 ml of 30% H2O2. Care was taken so that the capacity of the Con A-Sepharose (~60 nmol of high mannose oligosaccharide chain/ml of resin) was not exceeded. When the washed protein pellet was hydrolyzed (n HCl, 4 °C, 100 °C), desalted on Dowex 50, H+ form (0.5 x 4.5 cm), and subjected to paper chromatography, the amount of 32P co-migrating with mannose 6-phosphate was the same as that binding to Con A-Sepharose in the standard assay.

**RESULTS**

**Partial Purification of N-Acetylglucosaminylphosphotransferase**—Our previous assay for N-acetylglucosaminylphosphotransferase activity depended on the transfer of N-acetylglucosamine 1-[32P]phosphate to endogenous glycoprotein acceptors (10). Therefore, in order to purify the enzyme it was necessary to develop an assay which is independent of endogenous acceptors. We found that the enzyme can transfer N-acetylglucosamine 1-[32P]phosphate to α-methylmannoside and this forms the basis for a sensitive, specific, and facile assay (see "Experimental Procedures"). Using this assay to follow activity, the transferase was solubilized from rat liver smooth membranes with 0.3% Lubrol PX and partially purified by DEAE-cellulose chromatography. The final preparations were 75-110-fold purified. The enzyme had a pH optimum of 7 to 8 with 50% activity at pH 6 and less than 1% activity at pH 4 to 5. The apparent K, for UDP-GlcNAc was 21 µM.

**Activity toward Glycoprotein Acceptors**—Using such partially purified preparations, we determined the ability of the enzyme to phosphorylate the oligosaccharide units of various exogenous glycoproteins. Table I shows the results of a typical experiment using a series of glycoprotein acceptors that have in common the presence of high mannose-type oligosaccharide units. It is evident that the lysosomal enzyme α-N-acetylglucosaminidase is the best acceptor, being active at low protein concentrations. In contrast, none of the five nonlysosomal glycoproteins are good acceptors. To facilitate comparison, the acceptor concentrations have been expressed in terms of the concentration of high mannose-type oligosaccharide units. The best nonlysosomal acceptor on a weight basis, ribonuclease B, has an unusually large oligosaccharide content. In other experiments, we found that human placental β-hexosaminidase A, human placental β-hexosaminidase B, human hepatic β-galactosidase, and partially purified porcine hepatic β-hexosaminidases A and B were all comparable in acceptor activity to the α-N-acetylglucosaminidase.

In order to quantitatively compare the acceptor activity of lysosomal enzymes with other possible acceptors, we measured the rate of N-acetylglucosamine 1-[32P]phosphate transfer as a function of acceptor concentration. Fig. 1 shows that α-N-acetylglucosaminidase is an excellent acceptor with an apparent K, of 8.9 µM. Ribonuclease B, on the other hand, is a much poorer acceptor with an apparent K, of 916 µM. The V, values for these two acceptors are similar. Studies with human placental β-hexosaminidases A and B showed that the apparent K, values for these substrates are in the low micromolar range (Table II). The acceptor activity of the other nonlysosomal glycoproteins was so low that it was not possible to determine their apparent K, and V, values. Table II also presents the kinetic parameters for two carbohydrate acceptors, α-methylmannoside and Man₆GlcNAc oligosaccharide. These molecules have very high apparent K, values, being 10⁵-10⁷-fold greater than those of lysosomal enzymes. Their V, values are 10-100-fold greater than those of the glycoprotein acceptors.

The relative catalytic efficiency (V, divided by apparent K,) of the transferase toward the various acceptors is also shown in Table II. The three lysosomal enzymes are phosphorylated at least 100-fold more efficiently than either ribonuclease B, Man₆GlcNAc oligosaccharide, or α-methylmannoside. Different preparations of the transferase exhibited vari
were used in the experiments.

Mand3cNAc oligosaccharide 32,000

Coproteins. Left, transfer to porcine hepatic α-N-acetylglucosaminidase by 260 units of N-acetylglucosaminylphosphotransferase. Right, transfer to bovine pancreatic ribonuclease B by 147 units of transferase. The insets show the Lineweaver-Burk plots of the same data. See "Experimental Procedures" for details. The transferase preparations used in this experiment were free of endogenous acceptors.

These results strongly suggest that acceptor protein conformation is recognized by N-acetylglucosaminylphosphotransferase.

The reason for this variation is not clear. However, since both ribonuclease B (with

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an exposed oligosaccharide unit (17, 32)) and free high mannosyl
oligosaccharides are poor acceptors, it is unlikely that accessibility of the carbohydrate chain to the transferase is the sole conformational requirement for recognition.

With the demonstration of its specificity, the N-acetylglu-
cosaminylphosphotransferase reaction becomes the earliest known point at which newly synthesized acid hydrolases are distinguished from other newly synthesized glycoproteins. While it is possible that lysosomal enzymes undergo some other specific modification prior to exposure to the transferase and that the observed specificity of the phosphorylation reaction is secondary to this other (heat-labile) modification, there is no reason to postulate this more complex pathway. Similarly, the specificity of the transferase makes it unnecessary to postulate the existence of a mechanism for the specific segregation of newly synthesized acid hydrolases which would precede the exposure to the N-acetylglucosaminylphosphotransferase. Based on the data presented, we propose that the N-acetylglucosaminylphosphotransferase is the initial and determining enzyme for the pathway which eventually results in the segregation of acid hydrolases into lysosomes.

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