Murine UDP-GlcNAc:Lysosomal Enzyme N-Acetylglucosamine-1-phosphotransferase Lacking the \( \gamma \)-Subunit Retains Substantial Activity toward Acid Hydrolases*

Received for publication, May 16, 2007, and in revised form, June 29, 2007. Published, JBC Papers in Press, July 25, 2007, DOI 10.1074/jbc.M704067200

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UDP-GlcNAc lysosomal enzyme \( N \)-acetylglucosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase) mediates the first step in the synthesis of the mannose 6-phosphate recognition marker on acid hydrolases. The transferase exists as an \( \alpha \beta_2 \gamma_2 \) hexameric complex with the \( \alpha - \) and \( \beta - \) subunits derived from a single precursor molecule. The catalytic function of the transferase is attributed to the \( \alpha - \) and \( \beta - \) subunits, whereas the \( \gamma - \) subunit is believed to be involved in the recognition of a conformation-dependent protein determinant common to acid hydrolases. Using knock-out mice with mutations in either the \( \alpha/\beta \) gene or the \( \gamma \) gene, we show that disruption of the \( \alpha/\beta \) gene completely abolishes phosphorylation of high mannose oligosaccharides on acid hydrolases whereas knock-out of the \( \gamma \) gene results in only a partial loss of phosphorylation. These findings demonstrate that the \( \alpha/\beta \)-subunits, in addition to their catalytic function, have some ability to recognize acid hydrolases as specific substrates. This process is enhanced by the \( \gamma - \) subunit.

In many cell types the targeting of newly synthesized acid hydrolases to lysosomes requires the synthesis of mannose 6-phosphate (Man-6-P) residues on the \( N \)-linked high mannose glycans of these enzymes (1). This post-translational modification is mediated by the sequential action of two enzymes, UDP-GlcNAc lysosomal enzyme \( N \)-acetylglucosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase) and phosphodiester glycosidase (“uncovering” enzyme). The first enzyme binds to a conformation-dependent protein determinant on the acid hydrolases and then transfers GlcNAc-1-P from UDP-GlcNAc to selected mannose residues on the oligosaccharide. This is followed by removal of the outer GlcNAc residue by uncovering enzyme to produce the Man-6-P monomer, which serves as a high affinity ligand for binding to Man-6-P receptors in the trans-Golgi network. The ligand-receptor complex is packaged into clathrin-coated transport vesicles for delivery to endosomes and lysosomes. Mutations in GlcNAc-1-phosphotrans-

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* This work was supported by National Institutes of Health Grant CA08759 (to S.K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: Man-6-P, mannose 6-phosphate; ES, mouse embryonic stem cells; GlcNAc, \( N \)-acetylglucosamine.

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subunits have some ability to recognize lysosomal enzyme subunits, but this function is enhanced by the $\gamma$-subunit. Our result provides an explanation for why mutations in the $\gamma$-subunit produce a milder phenotype than $\alpha/\beta$ mutations.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents were obtained from Sigma unless otherwise noted. Cathepsin D antiserum and the cation-independent mannose 6-phosphate receptor (CI-MPR)-affinity column were provided by Walter Gregory (Washington University School of Medicine in St. Louis, MO).

**Generation of Knock-out Mice and Fibroblasts**—Mouse embryonic stem (ES) cells carrying a mutation in the GlcNAc-1-phosphotransferase GNPTAB gene (GenBank accession number AK173132) were obtained from OmniBank, a library of gene-trapped ES cell clones identified by a corresponding OmniBank Sequence Tag (OST) (18, 19). The gene-trapping vectors were shown to insert in intron 1 of the GNPTAB gene, resulting in truncation of the $\alpha/\beta$ precursor after 39 amino acids of a total of 1256 residues. The ES cells with a mutation in the GNPTG gene (GenBank accession number AK078230) were obtained with a directed gene deletion approach that deleted exons 4–11 of the gene. This resulted in truncation of the $\gamma$-subunit after 59 amino acids (including 24 amino acids that comprise the signal sequence) of a total of 305 residues. These ES cells were used to generate mice heterozygous for the mutations using standard methods (20). Mating between the heterozygous mice gave rise to homozygous animals. The characterization of the mutant mice will be described elsewhere (21).

Skin fibroblasts were prepared from wild-type and mutant mice. The cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 100 $\mu$g/ml penicillin and 100 units/ml streptomycin.

**Cathepsin D Sorting Assay**—Mouse fibroblasts at 90% confluence in 6-well plates were labeled for 2 h with 1.07 ml cysteine/methionine-free Dulbecco's modified Eagle's medium containing 700 $\mu$Ci of TRAN $^{35}$S-LABEL (conc., 10.5 mCi/ml, MP Biomedicals, Inc. Irvine, CA) and chased for 1 h. Cells were harvested and extracted sequentially with 2 ml of CHCl$_3$:MeOH (2:1), 2 ml of EtOH:dH$_2$O (1:1) $\times$ 2, 2 ml of EtOH:dH$_2$O (1:1):H$_2$O (10:10:3) $\times$ 1.

The residual protein pellet was solubilized in 50 mM Tris, pH 6.8, 0.5% SDS by boiling and incubated overnight at 37 °C with 2 units of endoglycosidase H (New England Biolabs, Beverly, MA) following addition of citrate buffer, pH 5.5. Endo H-sensitive oligosaccharides were separated from resistant material using a Centricon10 filtration unit. The filtrate, containing the released high mannose oligosaccharides, was subjected to mild acid hydrolysis (pH 2.0, 100 °C for 30 min) to release any covering GlcNAc residues. The samples were diluted 10 times with 2 mM Tris buffer, pH 8.0 and applied to a 2 ml of QAE-Sephadex column equilibrated with the same buffer. The neutral high mannose oligosaccharides passed through the column, oligosaccharides with one uncovered phosphate bound to the column and were eluted with 70 mM NaCl in 2 mM Tris buffer, pH 8.0, and oligosaccharides with two uncovered phosphates were eluted with 140 mM NaCl in 2 mM Tris buffer, pH 8.0. The Centricon retentate, containing the Endo H-resistant complex-type oligosaccharides, was digested with Pronase, and the glycans were fractionated in Con A-Sepharose as previously described with a polyclonal anti-cathepsin D antibody and analyzed by SDS-PAGE as previously described (22).

**Lysosomal Enzyme Assays**—Acid hydrolase assays were performed as previously described with minor modifications (23, 24). $\beta$-Hexosaminidase and $\beta$-galactosidase were assayed with 5 mM 4-methylumbelliferyl(MU)-N-acetyl-$\beta$-D-glucosaminide (Sigma M-2133) and 5 mM 4-MU-$\beta$-D-galactopyranoside (Calbiochem 474424), respectively, in 50 mM citrate buffer containing 0.5% Triton X-100 (pH 4.5). $\beta$-Glucuronidase was assayed with 5 mM 4-MU-$\beta$-D-glucuronide (Calbiochem 474427) in 0.1 M sodium acetate buffer containing 0.5% Triton X-100 (pH 4.6).

$\beta$-Mannosidase was assayed with 5 mM 4-MU-$\beta$-D-mannopyranoside (Sigma M0905) in 50 mM sodium citrate buffer containing 0.5% Triton X-100 (pH 5.0). $\alpha$-Mannosidase was assayed with 5 mM 4-MU-$\alpha$-D-mannopyranoside (Sigma M3657) in 50 mM sodium citrate buffer containing 0.5% Triton X-100 (pH 4.0). Acid $\beta$-glucosidase was assayed with 5 mM 4-MU-$\beta$-D-glucopyranoside (Sigma M3633) in a buffer containing 0.1 M sodium citrate, 0.2 M Na$_2$HPO$_4$, 0.2% Triton X-100, and 0.25% sodium taurocholate (pH 5.2). The assay mixtures containing 90 $\mu$M of 5 mM substrate solution and 10 $\mu$L of sample were incubated for 1 to 5 h at 37 °C and then quenched with 900 $\mu$L of 0.4 M glycine-NaOH buffer, pH 10.8. The fluorescence was measured in a TURNER Model 450 Fluorometer (Barnstead Thermolyne Corporation, Dubuque, Iowa) using excitation and emission wave lengths of 360 and 450 nm, respectively.

**Man-6-P Receptor Affinity Chromatography**—Brain tissue was obtained from mice that had been perfused with phosphate-buffered saline buffer to remove contaminating blood and immediately frozen on dry ice. 100 mg of the frozen tissue was resuspended in 250 $\mu$L of buffer containing 154 mM NaCl, 1.0% Triton X-100, and 1 mM dithiothreitol (pH 4.5), homogenized in a 1.5-ml tube (catalog number 749510-1590, KONTES) using a Pellet Pestle Motor Homogenizer (KONTES), and sonicated for 5 s using Model 550 Sonic Dismembrator (Fisher Scientific). The homogenate was centrifuged at 29,000 g for 10 min and the supernatant fluid, which contained more than 90% of the acid hydrolase activity, was collected.

50 $\mu$L of the homogenate was diluted 10-fold with Buffer A (50 mM imidazole-HCl, pH 7.25, 150 mM NaCl, 0.05% Triton X-100) and loaded onto a 1-ml CI-MPR-Sepharose column (0.86 mg/ml). The column was washed with 7 ml of Buffer A, then with 7 ml of 5 mM glucose 6-phosphate in Buffer A, and eluted with 5 ml of 10 mM mannose 6-phosphate in Buffer A (25).

**Oligosaccharide Analysis**—Mouse fibroblasts at 90% confluency in 60-mm plates were labeled for 4 h in low-glucose (1 mM) medium with 150 $\mu$Ci/ml of [2-3H]mannose (MP Biomedicals, Inc. Irvine, CA) and chased for 1 h. Cells were harvested and extracted sequentially with 2 ml of CHCl$_3$:MeOH (2:1) $\times$ 2, 2 ml of EtOH:dH$_2$O (1:1):H$_2$O (10:10:3) $\times$ 1.

The residual protein pellet was solubilized in 50 mM Tris, pH 6.8, 0.5% SDS by boiling and incubated overnight at 37 °C with 2 units of endoglycosidase H (New England Biolabs, Beverly, MA) following addition of citrate buffer, pH 5.5. Endo H-sensitive oligosaccharides were separated from resistant material using a Centricon10 filtration unit. The filtrate, containing the released high mannose oligosaccharides, was subjected to mild acid hydrolysis (pH 2.0, 100 °C for 30 min) to release any covering GlcNAc residues. The samples were diluted 10 times with 2 mM Tris buffer, pH 8.0 and applied to a 2 ml of QAE-Sephadex column equilibrated with the same buffer. The neutral high mannose oligosaccharides passed through the column, oligosaccharides with one uncovered phosphate bound to the column and were eluted with 70 mM NaCl in 2 mM Tris buffer, pH 8.0, and oligosaccharides with two uncovered phosphates were eluted with 140 mM NaCl in 2 mM Tris buffer, pH 8.0. The Centricon retentate, containing the Endo H-resistant complex-type oligosaccharides, was digested with Pronase, and the glycans were fractionated in Con A-Sepharose as previously described.
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described (26). The radioactivity in all of the fractions was determined.

The percent phosphorylation was calculated as cpm recovered in Endo H-released oligosaccharides with one or two phosphates/total cpm in Endo H-released oligosaccharides (phosphorylated plus neutral) + 2 (Endo H-resistant complex-type oligosaccharides). The values of the complex oligosaccharides were multiplied by 2 to correct for the fact that they contain 3 mannosyl residues versus an average of 6 mannosyl residues per high mannose oligosaccharide (26).

RESULTS

Cathepsin D Targeting in Fibroblasts—In our initial experiment, we followed the sorting of newly synthesized cathepsin D, a typical lysosomal protease, in fibroblasts obtained from wild-type, α/β gene knock-out, and γ gene knock-out mice. The cells were incubated with [35S]Met/Cys for 2 h and then

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TABLE 1

Binding of acid hydrolases in brain tissue to CI-MPR-affinity column

| Enzyme          | Activity |
|-----------------|----------|
|                 | Wild type | γ-PT KO | α-β PT KO |
|                 | (n = 4)    | (n = 4)  | (n = 3)    |
| Average ± S.D.  | % bound to CI-MPR-column | % bound to CI-MPR-column | % bound to CI-MPR-column |
| μmol/h/mg      | %        | μmol/h/mg | %        | μmol/h/mg | %        |
| β-Hexosaminidase| 68.6 ± 14.2 | 40.7 | 126.2 ± 29.0 | 6.4 | 77.5 ± 20.8 | <2.0 |
| α-Mannosidase   | 11.9 ± 3.5 | 50.1 | 19.3 ± 3.9 | 40.0 | 21.0 ± 7.2 | <2.0 |
| β-Mannosidase   | 12.0 ± 3.8 | 50.8 | 11.7 ± 2.3 | 12.8 | 10.5 ± 2.8 | <2.0 |
| β-Galactosidase | 14.4 ± 7.3 | 82.1 | 15.5 ± 8.2 | 11.2 | 9.6 ± 0.8 | <2.0 |
| β-Glucuronidase | 18.4 ± 6.1 | 57.9 | 37.6 ± 8.3 | 41.6 | 23.4 ± 6.3 | <2.0 |
| Acid β-Glucosidase* | 12.9 ± 5.0 | <2.0 | 17.7 ± 7.7 | <2.0 | 10.3 ± 9.2 | <2.0 |

* Trafficking to the lysosome is independent of Man-6-P recognition marker.

Fig. 1. Cathepsin D sorting in fibroblasts. Wild type, α/β knock-out, and γ knock-out fibroblasts were metabolically labeled with [35S]methionine/cysteine as described under “Experimental Procedures.” Secreted and intracellular cathepsin D molecules were immunoprecipitated with a polyclonal anti-cathepsin D antibody, resolved by 10% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. M, medium; P, cell pellet. Quantitation of the autoradiograms showed that the wild-type, α/β knock-out, and γ knock-out cells secreted less than 1, 63, and 19% of the activity of the various acid hydrolases occurred with the γ gene knock-out samples, with the exception of the acid β-glucosidase. The highest binding was observed with α-mannosidase (40% versus 50.1% with wild type) and β-glucuronidase (41.6% versus 57.9% with wild type).
β-Mannosidase bound at 25% of the wild type level whereas β-hexosaminidase and β-galactosidase bound at 16 and 14% of their respective wild type levels. The lack of acid β-glucosidase binding indicates that the GlcNAc-1-phosphotransferase maintains its specificity for selected acid hydrolases in the absence of the γ-subunit.

Analysis of [2-3H] Mannose-labeled Oligosaccharides—To obtain a more quantitative measure of the effect of the α/β and γ gene knockouts on the phosphorylation of the acid hydrolase oligosaccharides, we determined the total synthesis of phosphorylated high mannose oligosaccharides in the various fibroblasts cell lines. Prior studies have established that GlcNAc-1-phosphotransferase accounts for all of the synthesis of this type of oligosaccharides in fibroblasts (9). The cells were incubated with [2-3H]mannose for 4 h followed by a 1-h chase. The lipid-linked oligosaccharides were then extracted, leaving a glycoprotein residue that contained the total cellular content of N-linked glycans, both high mannose and complex type. These glycoproteins were treated with endo H to release the high mannose glycans, including those derived from the acid hydrolases. The high mannose oligosaccharides were then separated into neutral, monophosphorylated, and diphasphyphorylated species by QAE-Sephadex chromatography. These fractions were quantified along with the endo H-resistant complex type species, allowing the determination of the total content of phosphorylated high mannose oligosaccharides in fibroblasts from the wild-type and the two knock-out lines. The results of this analysis are shown in Table 2. On average, 1.34% of the total N-linked glycans of wild-type fibroblasts contained Man-6-P residues, the bulk of which were present on oligosaccharides with two Man-6-Ps. The γ gene knock-out fibroblasts had 0.28% phosphorylated high mannose oligosaccharides (21% wild-type level) while the α/β gene knock-out fibroblasts had only 0.009% of these species (<1% of wild type level). In fact, the amount of radioactivity recovered in the phosphorylated oligosaccharide fractions of the α/β-subunits knock-out cells was so low that it may just reflect variation in background counting. Interestingly, the majority of the phosphorylated oligosaccharides recovered from the γ gene knock-out fibroblasts contained two Man-6-P residues, similar to the finding with the wild-type fibroblasts.

| Fibroblast type | Total cpm | Oligosaccharide species | % Oligosaccharide phosphorylation | Average phosphorylation |
|----------------|-----------|------------------------|----------------------------------|------------------------|
|                | Complex   | NHM*                   | 1-PM*                            | 2-PM*                  | % total | % total |
| Wt 402         | 298,183   | 44.2                   | 54.6                             | 0.41                   | 0.77    | 1.18    | 1.34    |
| Wt 402         | 377,718   | 44.6                   | 53.8                             | 0.11                   | 1.42    | 1.53    |
| Wt 405         | 42,734    | 43.4                   | 55.6                             | 0.24                   | 0.81    | 1.05    |
| γ-KO 208       | 180,220   | 48.3                   | 51.4                             | 0.10                   | 0.24    | 0.34    | 0.28    |
| γ-KO 403       | 553,298   | 50.3                   | 49.5                             | 0.05                   | 0.17    | 0.22    |
| γ-KO 403       | 270,484   | 49.8                   | 49.9                             | 0.058                  | 0.255   | 0.31    |
| γ-KO 953       | 353,268   | 47.6                   | 52.2                             | 0.016                  | 0.213   | 0.23    |
| α,β-KO 953     | 144,449   | 54.7                   | 45.3                             | 0.007                  | 0.009   | 0.016   | 0.01    |
| α,β-KO 953     | 232,497   | 50.9                   | 49.1                             | 0.002                  | 0.000   | 0.002   |

DISCUSSION

The findings presented in this study show that loss of the α/β gene of GlcNAc-1-phosphotransferase decreases phosphorylation of lysosomal acid hydrolases to undetectable levels. This would be expected in view of the data implicating the α/β-subunits as the catalytic components of phosphotransferase (4). On the other hand, knock-out of the γ gene resulted in only a partial loss of lysosomal enzyme phosphorylation. These results are consistent with the fact that the γ gene knock-out mice have a significantly milder phenotype than the α/β gene knock-out mice. While both types of knock-out mice have striking elevations of their serum acid hydrolases, the α/β gene knock-out mice are small and develop severe retinal degeneration that leads to blindness by 5 months of age whereas the γ gene knock-out mice are of normal size and do not exhibit retinal degeneration (21). The findings are also in line with the genetic analysis of patients with ML II and ML III showing that mutations of the γ gene result in the milder ML III phenotype whereas mutations of the α/β gene can lead to either ML II or ML III (8–17).

The major implication of these findings is that the α/β-subunits of GlcNAc-1-phosphotransferase have the ability to recognize lysosomal acid hydrolases as specific substrates in the absence of the γ-subunit, although not nearly as efficiently as the holoenzyme. An alternative interpretation is that the mannose phosphorylation observed in the absence of the γ-subunit is the result of nonspecific activity of the α/β-subunits toward high mannose oligosaccharides present on all classes of glycoproteins. We believe that this explanation is unlikely based on the fact that even though acid β-glucosidase contains four N-linked glycans (32) and would be expected to acquire Man-6-P residues if GlcNAc-1-phosphotransferase was acting in a nonspecific manner, it failed to bind to the CI-MPR-affinity column.

The finding that the α/β-subunits of GlcNAc-1-phosphotransferase are involved in substrate recognition necessitates a revision of the current view of how this enzyme functions. GlcNAc-1-phosphotransferase is known to bind a conformation-dependent protein determinant and then phosphorylate selected mannose residues on the N-linked glycans of the substrate (33–39). In the case of cathepsin D and arylsulfatase A, the protein determinant has been shown to extend over a broad
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surface of the molecule (26, 34, 40). The current model is that the γ-subunits mediate recognition of the protein determinant while the α/β-subunits catalyze mannos phosphorylation. This was based in part on the observation that some mutations in the γ-subunit impair recognition of the protein determinant without altering phosphorylation of the simple sugar α-methylmannoside (5–8). While these studies implicated the γ-subunit in protein recognition, they did not exclude a role for the α/β-subunits in the process. In fact, kinetic studies showed that the GlcNAc-1-phosphotransferase of several patients whose γ-subunit is truncated phosphorylated lysosomal acid hydrolases 13–88 times more efficiently than it did α-methylmannoside (6). While this was much poorer than observed with the wild-type enzyme (314–332 times better than α-methylmannoside), it indicated that the mutant enzymes retained some capacity to recognize acid hydrolases as specific substrates (6). Our current results confirm a role for the α/β-subunits in substrate recognition.

In view of the finding that the α/β-subunits recognize lysosomal acid hydrolases, albeit imperfectly, a number of models must be considered when defining the role of the γ-subunits. One possibility is that recognition of the common protein determinant is mediated exclusively by the α/β-subunits with the γ-subunit serving to either facilitate the proper folding of these subunits or functioning to maintain them in a conformation competent for substrate recognition and binding. Alternatively, both the α/β- and γ-subunits could be directly involved in substrate recognition, either via binding to the same protein elements of the substrates or by interaction with different components of the protein determinant.

Any model must explain the observation that in the absence of the γ-subunit, the phosphorylation of the five glycosidases examined was impaired to different extents. Thus two of the glycosidases (α-mannosidase and β-glucuronidase) were phosphorylated considerably better than the others (40 versus 6–13%). One potential explanation is that the protein recognition determinants of the various glycosidases interact with phosphotransferase with different affinities. In the case of the wild-type phosphotransferase, all of the interactions would be sufficient to give good phosphorylation. However, in the absence of the γ-subunit, the interaction with the glycosidases with the weaker signal would be most affected, resulting in the greatest drop in phosphorylation. If the γ-subunit and the α/β-subunits interact with different elements of the protein determinant, the extent of phosphorylation will be more or less affected depending on the nature of these elements on the different glycosidases.

In prior studies we have generated a GlcNAc-1 phosphotransferase protein recognition motif in the secretory protein peptinogen by substituting elements from the related aspartic protease cathepsin D into equivalent positions of peptinogen (22, 26, 35, 40). The minimum number of substitutions needed to generate a recognition motif was two lysines at the correct positions (22). However, the binding signal was substantially enhanced by the substitution of additional residues. It will be of considerable interest to determine whether these residues have the same effect on GlcNAc-1-phosphotransferase that is missing the γ-subunit or if only a subset of the residues is active in the absence of this subunit. Such studies of mutant GlcNAc-1-phosphotransferase should provide further insight into the mechanism whereby lysosomal acid hydrolases are recognized as specific substrates for phosphorylation.

Acknowledgments—We thank Drs. Xiucui Ma and Katherine Ponder for their help in obtaining the brain tissues and Drs. Rosalind Kornfeld, Balraj Doray, Intaek Lee, and Jennifer Martinez for critical reading of the manuscript.

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