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Enzymatic identification of mannose 6-phosphate on the recognition marker for receptor-mediated pinocytosis of β-glucuronidase by human fibroblasts

(adsorptive endocytosis/lysosomal enzymes/phosphoglycoproteins)

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ABSTRACT **Human β-glucuronidase (β-D-glucuronide glucuronosohydrolase, EC 3.2.1.31), like many other glycoprotein lysosomal hydrolases, is subject to receptor-mediated endocytosis by fibroblasts. Prior work demonstrated charge heterogeneity in β-glucuronidase and showed that high-uptake forms are more acidic than slowly internalized forms. Considerable indirect evidence implicated mannose 6-phosphate as an essential part of the recognition marker on high-uptake enzyme forms. Here we report the purification of β-glucuronidase from human spleen and demonstrate enzymatically that mannose 6-phosphate is released on acid hydrolysis of pure enzyme. Furthermore, the mannose 6-phosphate content of the enzyme varies directly with its susceptibility to pinocytosis by fibroblasts. Enzyme forms resolved by CM-Sephadex chromatography differed over an 18-fold range in uptake rate and in mannose 6-phosphate content. The most acidic forms had 4.4 mol of mannose 6-phosphate per mol of enzyme. The mannose 6-phosphate was released from the enzyme by treatment with endoglycosidase H with concomitant loss of susceptibility to adsorptive endocytosis. Thus, these studies provide direct evidence that mannose 6-phosphate is present on high-uptake enzyme forms, that it is present in the recognition marker for uptake, and that it is present on oligosaccharide that is released by endoglycosidase H.**

The uptake of lysosomal enzymes by human fibroblasts displays the selectivity and saturability expected for a receptor-mediated process (1). Hickman and Neufeld proposed that many lysosomal glycosidases share a common structural feature that is essential for their recognition by pinocytosis receptors on such cells (2). This hypothesis was based on the observation that fibroblasts from patients with a presumed single gene mutation (mucolipidosis II) secrete multiple hydrolases which are not specifically pinocytosed. Further work suggested that the common recognition marker contained carbohydrate (3).

Work from this laboratory implicated phosphohexose as an essential component of the recognition marker on human platelet β-glucuronidase (β-D-glucuronide glucuronosohydrolase, EC 3.2.1.31) (4). Phosphohexose was suspected on the basis of the potent competitive inhibition of enzyme uptake by Man6*P* and by yeast phosphomannans containing Man6*P*. Presence of the phosphate in phosphomonoester linkage was deduced from the observation that alkaline phosphatase treatment of high-uptake forms of β-glucuronidase converted them to forms that were very poorly pinocytosed. These findings were subsequently corroborated and extended to several other acid hydrolases from various tissue sources (5–8). The generality of these findings led us to propose that Man6*P*, or a structural analogue of Man6*P*, is an essential component of the postulated common recognition marker for uptake (5).

Until recently, however, the presence of phosphohexose on lysosomal enzymes has been supported only by indirect evidence. In this communication we provide direct evidence that β-glucuronidase purified from human spleen contains Man6*P* and that the susceptibility of this enzyme to adsorptive pinocytosis correlates directly with its Man6*P* content. In addition, we present evidence that the Man6*P* is present on oligosaccharide released from the enzyme by treatment with endoglycosidase H.

MATERIALS AND METHODS

Materials. Most reagents were purchased from Sigma, including glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase; EC 1.1.1.49) and yeast phosphomannose isomerase (D-mannose-6-phosphate ketolisomerase; EC 5.3.1.8). Yeast hexokinase (ATP: D-hexose 6-phosphotransferase; EC 2.7.1.1) and yeast phosphoglucoisomerase (D-glucose-6-phosphate ketolisomerase; EC 5.3.1.9) were products of Boehringer Mannheim. Endoglycosidase H was purchased from Miles. Cultured fibroblasts were established from skin biopsies obtained from patient J.E. with β-glucuronidase deficiency mucopolysaccharidosis (available as cell strain GM 151 from the Human Mutant Cell Repository, Camden, NJ). Human spleens were obtained from the Department of Pathology, Washington University School of Medicine (St. Louis, MO) and from the St. Louis City Morgue.

Enzyme Assays. β-Glucuronidase activity was determined fluorometrically as described (9). Purified enzyme was assayed for the following potential contaminating hydrolase activities: α-fucosidase, α-galactosidase, α-glucosidase, α-mannosidase, β-glucosidase, and β-hexosaminidase. These assays were carried out by using 4-methylumbelliferyl substrates (10).

Protein Determination. Protein was measured colorimetrically (11) with bovine serum albumin as the standard.

Sodium Dodecyl Sulfate ($NaDodSO_4$) Gel Electrophoresis. $NaDodSO_4$/polyacrylamide gels were prepared in 1.5-mm-thick slabs (10% gel) in Tris glycine buffer as described by Laemmli (12).

Pinocytosis Measurements. Pinocytosis of β-glucuronidase was measured by using subconfluent β-glucuronidase-deficient fibroblasts in 35-mm petri dishes. After exposure to enzyme for 2 hr, cells were washed and lysed and lysates were assayed for β-glucuronidase and protein as described (4). Results are ex-

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Abbreviations: Con A, concanavalin A; $NaDodSO_4$, sodium dodecyl sulfate.

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pressed as the percentage of added enzyme internalized per mg of protein per hr.

Man6*P* Assay. This assay is a modification of the Man6*P* assay described by Gawehn (13). After completion of the enzymatic reactions (see below), the reduced pyridine nucleotide that was generated was subsequently amplified by enzymatic cycling.

Appropriate aliquots of β-glucuronidase (1–4 μg) were centrifuge-evaporated and hydrolyzed in 0.1 ml of 1 M HCl for 4 hr at 100°C. Samples were immediately centrifuge-evaporated and redissolved in 50 μl of 50 mM Tris·HCl, pH 8.1. A 2-μl aliquot was added to an equal volume of reagent containing 20 μg of phosphomannose isomerase (PMI) per ml, 10 μg of phosphoglucoisomerase (PGI) per ml, 10 μg of glucose-6-phosphate dehydrogenase (G6PDH) per ml, 0.04% bovine serum albumin, 2 mM $MgCl_2$, 10 μM $NADP^+$, and 100 mM Tris·HCl, pH 8.1. Any Man6*P* present as monosaccharide would therefore be sequentially transformed via the following series of reactions: Man6*P* $\xrightarrow{PMI}$ Fru6*P* $\xrightarrow{PGI}$ Glc6*P* + $NADP^+$ $\xrightarrow{G6PDH}$ 6-*P*-gluconate + NADPH. This reaction was carried out under oil in an "oil well rack." (For details of the "oil well technique," including loading of samples, see ref. 14, p. 253.) The reactions were allowed to go to completion (40 min at 23°–25°C) and were terminated by addition of 1 μl of 0.4 M NaOH and heating at 80°C for 20 min. Four microliters were then withdrawn from the oil well and added to 0.05 ml of $NADP^+$–NADPH cycling reagent in a 10 × 75 mm tube. The enzymatic cycling reactions were terminated after 60 min at 38°C by heating at 100°C for 3 min, after which 6-*P*-gluconate was measured. The cycling and 6-*P*-gluconate reagents and the conditions for these reactions have been described elsewhere (14, 15). All assays were conducted in duplicate with both the complete reagent and with reagents from which either phosphomannose isomerase or both isomerases had been omitted, allowing separate calculation of Man6*P*, Fru6*P*, and Glc6*P*. Man6*P* standards (10 pmol) were carried through the entire procedure to correct for the loss of Man6*P* (approximately 20%) that occurs during hydrolysis of the β-glucuronidase. The calculation of the Man6*P* content of β-glucuronidase was based on an estimated molecular weight of 3×10^5 and a specific activity of pure enzyme of 4×10^6 units/mg (16). In addition, enzymatic determinations of the mannose content of β-glucuronidase were also performed on some samples. Because of the much larger amount of mannose present, 25-μl aliquots (0.5–2 μg of enzyme) could be assayed directly in the fluorometer in 1 ml of reagent containing 0.02% bovine serum albumin, 1 mM $MgCl_2$, 30 μM $NADP^+$, 100 μM ATP, and 50 mM Tris·HCl, pH 8.1. After an initial reading, enzymes were added as follows with readings after each addition (when the reaction, if any, was complete): glucose-6-phosphate dehydrogenase (5 μg), yeast hexokinase (5 μg), phosphoglucoisomerase (5 μg), and phosphomannose isomerase (10 μg).

Enzyme Preparations. Two protocols were used to obtain homogeneous spleen β-glucuronidase, both of which utilized the same initial steps: extraction procedure, heat inactivation, and ammonium sulfate fractionation. Approximately 400 g of tissue was cut into small pieces and homogenized with a Polytron homogenizer in 25 mM EDTA/25 mM tartrate/3 mM parahydroxymercuribenzoate at pH 6.3 (5 ml/g of tissue). Sodium deoxycholate was added to 0.2% final concentration and the homogenate was centrifuged at 13,800 × *g* for 20 min. The supernatant was filtered through cheesecloth, heated at 65°C for 60 min, and centrifuged at 13,800 × *g* for 20 min. This supernatant was brought to 55% saturation with solid ammonium sulfate, stirred at 4°C for 60 min, and centrifuged at 13,800 × *g* for 20 min. The pellet was dissolved in one-fifth the supernatant volume of 25 mM sodium acetate/0.025% sodium azide at pH 5.5 (buffer A). Subsequent steps in the two protocols differ as described below.

Method A. The resuspended ammonium sulfate pellet was extensively dialyzed vs. buffer A, centrifuged to remove a small precipitate, made 1 mM in $CaCl_2$, $MgCl_2$, and $MnCl_2$, and then adsorbed batchwise to concanavalin A-Sepharose (Con A-Sepharose) at room temperature (approximately 1 ml of resin per 30 ml of the resuspended ammonium sulfate pellet). The Con A-Sepharose was extensively washed and poured into a column and the enzyme was eluted at 37°C with buffer A made 1 M in α-methylmannoside. The eluate was concentrated and extensively dialyzed by using an Amicon model 52 apparatus with an XM 100 filter and buffer A containing 1 M α-methylmannoside (to remove any free Con A). The concentrated enzyme solution was dialyzed vs. buffer A and adsorbed to a column (11 × 1.6 cm) of CM-Sephadex equilibrated with that buffer. A linear 0–0.18 M NaCl gradient in buffer A was applied and a flow rate of 25 ml/hr was used. Pooled peak activity fractions were concentrated and applied to a column (95 × 2.6 cm) of Bio-Gel A-0.5 equilibrated with buffer A containing 150 mM NaCl. The column was eluted with the same buffer at a flow rate of 30 ml/hr and 2.5-ml fractions were collected. Peak activity fractions were pooled, reduced with dithiothreitol, and alkylated with iodoacetic acid as described (16). This treatment carboxymethylates cysteine-containing proteins, which are then separated by a second CM-Sephadex chromatography step from β-glucuronidase which contains no cysteine (16). This material was then extensively dialyzed, adsorbed to a new (3 × 0.9 cm) column of CM-Sephadex equilibrated with that buffer, and eluted under the same conditions used for the first CM-Sephadex column.

Method B. In this method the resuspended pellet from the $(NH_4)_2SO_4$ step was dialyzed vs. 0.01 M Tris·HCl/0.025% sodium azide at pH 7.5 (buffer B) containing 1 mM sodium phosphate and 150 mM NaCl and then batch adsorbed to antibody–Sepharose (16) overnight (after being made 0.5 M in NaCl). The resin was sequentially washed with 3 vol each of buffer B with NaCl added to 1 M and 0.5 M concentrations in the first two, respectively, of the three washes. The enzyme was batch eluted with one resin volume of 0.01 M acetic acid and dialyzed vs. buffer A. It was then chromatographed on CM-Sephadex as described above. Peak activity fractions were subsequently pooled, reduced and alkylated, dialyzed, and rechromatographed on CM-Sephadex as described for method A. No other purification steps intervened between the two ion exchange procedures. Unless otherwise indicated, all procedures for both protocols were performed at 4°C.

RESULTS

Purification of Human Spleen β-Glucuronidase. Table 1 presents the results of the two different purification methods whereby human spleen β-glucuronidase can be purified to homogeneity in relatively high yield. Both protocols have a common final purification step (CM-Sephadex chromatography) which shows a coincident profile of enzyme activity and protein (Fig. 1). Both methods yielded enzyme that was comparable in purity to homogeneous human placental β-glucuronidase as judged by specific activity and $NaDodSO_4$ gel electrophoresis patterns (ref. 16; Table 1) and that contained $<10^{-5}$ units of seven potential contaminant hydrolase activities per unit of β-glucuronidase.

Uptake Properties of Human Spleen β-Glucuronidase. Charge heterogeneity has been reported for human β-glucuronidase from several tissue sources (17). In all cases the high-uptake property has been associated with the more acidic forms

Table 1. Purification of human spleen β-glucuronidase

Purification step	Total activity, units	Specific activity, units/mg	Yield, %*	Fold purification
Homogenate	24,400,000	580†	100	1
Heat step	23,100,000	1,840	95	3.2
$(NH_4)_2SO_4$ fractionation	23,100,000	6,100	95	10.5
Method A				
Con A-Sepharose	9,600,000	190,000	74	327
Amicon concentration	10,300,000	ND‡	80	ND‡
CM-Sephadex 1	6,200,000	1,080,000	48	1860
Bio-Gel A-0.5	4,480,000	2,300,000	35	3970
CM-Sephadex 2§	2,010,000	3,510,000	16	6050
Method B				
Antibody-Sepharose	7,910,000	1,800,000	69	3100
CM-Sephadex 1	4,780,000	3,070,000	42	5290
CM-Sephadex 2§	3,150,000	3,900,000	28	6720

* After the $(NH_4)_2SO_4$ step, 53% of the enzyme was processed by method A; the remainder was processed by method B. Yields are adjusted accordingly.

† The specific activity in homogenates varied from 340 to 741 units/mg, affecting the fold purification but not the final specific activity.

‡ Not determined.

§ Prior to this step, the preparation was treated with dithiothreitol and iodoacetic acid.

of the enzyme (17). The chromatogram presented in Fig. 1 includes an analysis of the uptake properties of fractions of pure human spleen β-glucuronidase resolved on CM-Sephadex. Although enzyme from all fractions had significant uptake, enzyme eluting at lower salt concentrations—i.e., the more acidic enzyme—had approximately 20-fold the uptake of enzyme from later fractions. This result is consistent with the possibility that the high-uptake forms of the enzyme contain more Man6*P* than do the less acidic, low-uptake forms.

Correlation between Man6*P* Content and Uptake Potency. Fig. 2 shows the correlation between the Man6*P* content of the enzyme and its susceptibility to pinocytosis. The open circles in this figure indicate enzyme from fractions of the isolation presented in Fig. 1. The solid circles represent results from different spleen preparations that were also analyzed for uptake and Man6*P* content. It is clear from this figure that there is a direct correlation between Man6*P* content and uptake, whether the samples were derived from different fractions of a single preparation or from pooled fractions from completely different isolations. The sample with the highest uptake contained enzyme with an average of 4.4 mol of Man6*P* per mol of enzyme tetramer. Enzymatic determinations of the mannose content of β-glucuronidase failed to demonstrate a similar correlation with uptake, although enzyme from earlier fractions from the preparation shown in Fig. 1 (fractions 11–15) appeared to have a slightly greater mannose content (81 ± 5 mol of mannose per mol of β-glucuronidase) than enzyme present in fractions 16–24 (69 ± 1 mol of mannose per mol of enzyme). Negligible amounts of Fru6*P* or Glc6*P* were found in these enzyme samples.

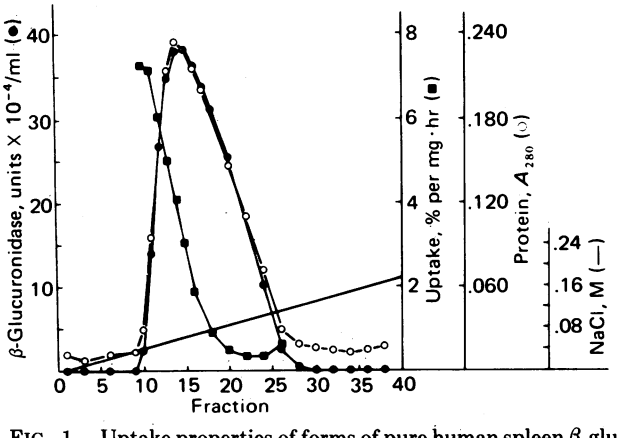

FIG. 1. Uptake properties of forms of pure human spleen β-glucuronidase resolved by CM-Sephadex chromatography. This profile represents the second ion exchange step from purification method B. Uptake was expressed as the percent of added enzyme internalized per mg per hr when incubation was for 2 hr at 37°C with 2000 units of β-glucuronidase.

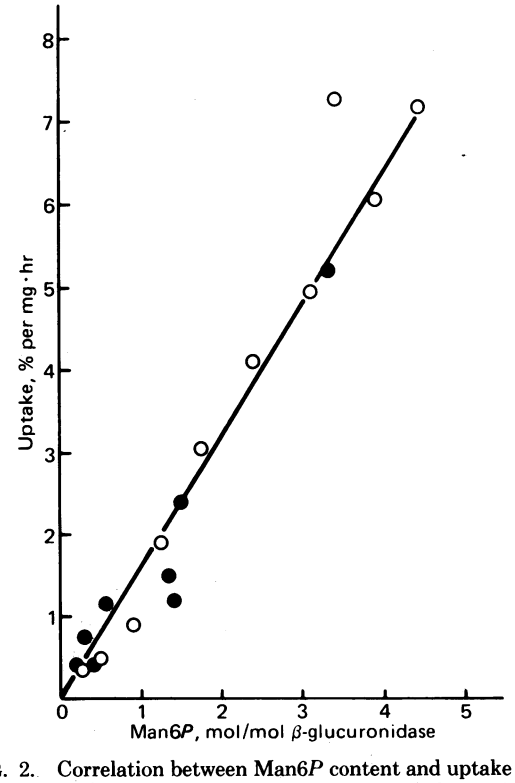

FIG. 2. Correlation between Man6*P* content and uptake of pure human spleen β-glucuronidase. ○, Results from analyses of enzyme from fractions across the column presented in Fig. 1; ●, results obtained from analyses of pooled fractions from several different enzyme purifications prepared by method A and from high-, moderate-, and low-uptake activity fractions of enzyme obtained by method B.

Table 2. Effect of endoglycosidase H on uptake properties and Man6*P* content of high-uptake β-glucuronidase

β-Glucuronidase	Uptake activity, % per mg/hr	Reduction of uptake activity, %	Man6*P*, mol/mol of enzyme		Man6*P* released, %
			Ethanol ppt	Supernatant	
Untreated	3.8	—	3.5	0.0	—
Endoglycosidase H-treated	0.1	97%	0.3	3.9	93%

Aliquots of pure spleen β-glucuronidase (10,000 units) were incubated with or without 9 milliunits of endoglycosidase H in a final volume of 0.115 ml containing 24 mM sodium acetate/0.012% sodium azide, pH 5.5, and 0.02% bovine serum albumin for 24 hr at 37°C. No β-glucuronidase catalytic activity was lost during this incubation. After the incubation, some samples were diluted into minimum essential medium containing 15% fetal calf serum and assayed for enzymatic activity and susceptibility to pinocytosis. Other samples were precipitated with ice-cold 80% ethanol and Man6*P* was measured on the precipitated β-glucuronidase and on the oligosaccharides present in the supernatant. Ninety percent of the initial β-glucuronidase catalytic activity was recovered in the ethanol precipitate; the supernatant contained no measurable enzymatic activity.

Effect of Endoglycosidase H Treatment on Human Spleen β-Glucuronidase. Endo-β-*N*-acetylglucosaminidase H, an endoglycosidase that hydrolyzes the core di-*N*-acetylchitobiose linkage in various high-mannose glycopeptides and glycoproteins, has been used as a probe in analyzing the carbohydrate structure of glycoproteins (18, 19). In an attempt to determine the type(s) of oligosaccharide chains containing Man6*P*, the β-glucuronidase was subjected to endoglycosidase H treatment and its uptake ability and Man6*P* content were measured. Table 2 presents data from a typical experiment. Endoglycosidase H treatment destroyed almost 100% of the uptake of β-glucuronidase without affecting its catalytic activity. Moreover, there was a similar quantitative loss of Man6*P* from the protein. That the loss of Man6*P* was not due to a contaminating phosphatase present in the endoglycosidase H preparations was established by the inability of the endoglycosidase to hydrolyze Man6*P* (data not shown) and by the quantitative recovery of the Man6*P* in oligosaccharides released from the protein (Table 2).

DISCUSSION

Our earlier studies showed that high-uptake forms of human β-glucuronidase represented only a small, relatively acidic fraction of enzyme from most organ sources (17). Human platelet enzyme was exceptionally rich in high-uptake enzyme (10). Limited availability of blood platelets led us to purify β-glucuronidase from human spleen, which is known to be a reservoir of platelets (20) and which had been reported to be a relatively rich source of high-uptake enzyme (21, 22). Purification of enzyme from this source allowed us to test the major prediction of our earlier studies, namely that Man6*P* would be present in the recognition marker for uptake of lysosomal enzymes by fibroblasts. This prediction was clearly confirmed, first by the finding that the Man6*P* content of the enzyme correlates directly with its susceptibility to pinocytosis, and second by the demonstration that endoglycosidase H treatment both releases the Man6*P* and destroys the uptake potency of the enzyme.

Are there additional structural features of the recognition marker that contribute to the high affinity of the enzymes for the pinocytosis receptor? The affinity of Man6*P* for the receptor, estimated from its potency as an inhibitor of enzyme pinocytosis (K_i, 5×10^{-5} M), was nearly 4 orders of magnitude lower than the affinity of the enzyme (K_{uptake}, 3×10^{-9} M). This difference in affinities (4, 6) indicates either that there are additional features of the recognition marker that are important for uptake or that acid hydrolases contain more than one 6-phosphomannosyl recognition marker.

We recently reported (22, 23) that Man6*P* in certain macromolecules was far more potent in inhibiting pinocytosis than free Man6*P*. In fact, one large phosphomonoester mannan fragment (containing 1000 Man6*P* groups) is 100,000 times as potent an inhibitor as Man6*P*, and its affinity approaches that of the high-uptake enzymes. This multivalent fragment is also pinocytosed by fibroblasts and is taken up by the same receptors that mediate pinocytosis of acid hydrolases (23). These studies led us to suggest that pinocytosis of mannan fragments (and, by analogy, of acid hydrolases) may depend on an interaction of a multivalent ligand (multiple 6-phosphomannose groups) with pinocytosis receptors on fibroblasts. Von Figura and Weber (24) made a similar suggestion based on other evidence. Higher uptake of a multivalent ligand might result from higher affinity binding to pinocytosis receptors or from stimulation of adsorptive endocytosis by crosslinking more than one receptor through the multivalent ligand (25). The finding of up to 4.4 Man6*P* groups per mol of tetrameric β-glucuronidase is compatible with the suggestion that this enzyme can be a multivalent ligand. However, even though β-glucuronidase and probably other acid hydrolases may be multivalent ligands and their pinocytosis may depend on an interaction with multiple pinocytosis receptors, there may be some yet undiscovered additional structural features of the recognition marker(s) that contribute to high-affinity binding.

Although bovine β-galactosidase uptake by human fibroblasts was initially attributed to a mannosyl recognition system (26), Sahagian *et al.* (8) recently reported evidence that this enzyme contains Man6*P* and that its pinocytosis involves phosphomannosyl recognition. However, unlike the findings with β-glucuronidase presented here, they failed to observe a correlation between the Man6*P* content of the β-galactosidase and its uptake properties. In other words, low-uptake enzyme fractions had as much Man6*P* per enzyme monomer as high-uptake enzyme fractions. These results suggest that Man6*P* on bovine β-galactosidase is essential to, but insufficient for, pinocytosis of this enzyme. If, as suggested above, a multivalent interaction facilitates enzyme pinocytosis, the findings with bovine β-galactosidase might be explained by different states of aggregation of this enzyme. Aggregation of Man6*P*-containing monomers could produce a multivalent ligand. Although the molecular weight of the purified bovine enzyme corresponds to that expected for a monomer (27), changes in the aggregation state of mammalian β-galactosidases have been amply documented (28–31). Of particular importance was the report that high molecular weight aggregates were enriched in fractions eluting at higher salt concentrations from DEAE-cellulose (28). Sahagian *et al.* (8) used DEAE chromatography to resolve low- and high-uptake fractions, with the high-uptake β-galactosidase eluting at high salt concentrations at which aggregation would be expected to be maximal. Thus, knowledge of the state of aggregation of the different uptake forms of β-galactosidase may help explain the lack of correlation of Man6*P* content and uptake observed with that enzyme, although other explanations are also possible.

The results of endoglycosidase H treatment on enzyme uptake and on release of Man6*P*-containing oligosaccharides from the enzyme argue that the Man6*P* in the recognition marker of spleen β-glucuronidase is not on a complex type of oligosaccharide chain. Similar results on the effects of endoglycosidase H on enzyme uptake have been seen with β-galactosidase from bovine testes (G. W. Jourdian, personal communication), α-L-iduronidase (R. Myerowitz and E. F. Neufeld, personal communication), and α-*N*-acetylglucosaminidase (32) from human urine and β-hexosaminidase from fibroblast secretions (unpublished data). Furthermore, von Figura and Klein recently showed release of acidic oligosaccharides from α-*N*-acetylglucosaminidase by endoglycosidase H treatment and conversion of the oligosaccharides to neutral species by phosphatase treatment (32). Thus, the Man6*P* in the common recognition marker for uptake of acid hydrolases by fibroblasts (5) appears to be present on endoglycosidase H-sensitive structures on all of these enzymes.

Until the indirect evidence for Man6*P* on human β-glucuronidase was reported, there was no precedent for Man6*P* on mammalian glycoproteins. However, there was precedent for this structure in yeast phosphomannans (33). There was also evidence for phosphomannosyl glycoprotein from rat brain, with the phosphate tentatively identified as being present on a secondary alcohol group of the mannose (34, 35). The direct evidence that Man6*P* is present in acid hydrolases and plays a role in directing them to lysosomes raises a number of new and interesting questions. What is the mode of biosynthesis of the 6-phosphomannosyl group on glycoproteins? How does the 6-phosphomannosyl group act to segregate acid hydrolases from secretory glycoproteins destined for export? Finally, what is the mechanism by which the cell chooses which glycoproteins should bear Man6*P*?

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