Steps in the phosphorylation of the high mannose oligosaccharides of lysosomal enzymes

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Abstract The phosphomannosyl recognition marker of acid hydrolases, which mediates their translocation to lysosomes, has been shown to be synthesized in two steps. First, N-acetylglucosamine 1-phosphate is transferred to an acceptor mannose by UDP-N-acetylglucosamine:lysosomal enzyme N-acetylglucosamine 1-phosphotransferase, resulting in a phosphate group in diester linkage between the outer N-acetylglucosamine and the inner mannose. Next, an a-N-acetylgalactosaminyl phosphotransferase removes the N-acetylglucosamine, leaving the phosphate in monoester linkage with the underlying mannoside residue. This exposed phosphomannosyl residue serves as the essential component of a recognition marker which leads to binding to high-affinity receptors and subsequent translocation to lysosomes. We propose that the first enzyme in this scheme, N-acetylglucosaminylphosphotransferase, catalyzes the initial step. A second enzyme, carrying α-linked N-acetylgalactosaminylphosphotransferase, catalyzes the second step, the synthesis of a phosphomannosyl residue in the oligosaccharide. This second enzyme is therefore responsible for the initial step in the synthesis of oligosaccharides. The absence of this enzyme activity, as in inclusion-cell disease, precludes the receptor-mediated targeting of newly synthesized oligosaccharides to lysosomes. Consequently, the enzymes are secreted into the extracellular milieu.

It is now well established that adsorptive pinocytosis of lysosomal enzymes is mediated by phosphomannosyl residues on these enzymes (Kaplan et al 1977, Sando & Neufeld 1977, Kaplan et al 1977. Ullrich et al 1978. Natowitz et al 1978. Distler et al 1979, von Figura & Klein 1979. Bach et al 1979, Fischer et al 1980a, b. Hasilik & Neufeld 1980a, b Gonzalez-Noriega et al 1980). These residues are the essential components of a recognition marker that is necessary for binding to high-affinity receptors on the cell surface and for subsequent translocation to lysosomes (Kleiman et al 1974). Initially, adsorptive pinocytosis was believed to be the major route by which newly synthesized acid hydrolases were delivered to lysosomes (Neufeld et al 1977). Recently, Sly and co-workers have demonstrated that most of the receptor is located intracellularly rather than on the cell surface (Fischer et al 1980a)

From this finding and other evidence, the investigators have proposed that the primary function of this receptor system is to mediate the intracellular transport of acid hydrolases from the Golgi to lysosomes (Sly & Stahl 1978, Sly et al 1981). According to this model, adsorptive pinocytosis serves as an alternative route for enzyme delivery to lysosomes.

Our work has been concerned with two basic aspects of this pathway. First, we have investigated the biochemical steps in the phosphorylation of the mannose residues of lysosomal enzymes and, second, we have attempted to determine the basis by which the cell selectively phosphorylates the mannose residues of lysosomal enzymes. Our initial goal was to elucidate the structure of the phosphorylated oligosaccharide units present on lysosomal enzymes because these structures might provide clues about the biosynthetic steps in phosphorylation. We labelled cells in tissue culture with [2-3H]mannose and then we isolated the lysosomal enzyme β-D-glucuronidase (EC 3.2.1.31) by immunoprecipitation. The [2-3H]mannose-labelled oligosaccharide units of the enzyme were then purified and structurally analyzed. This approach offered several advantages: it enabled us to determine the structure of the units by using extremely small amounts of material; and it allowed us to define the kinetics of phosphorylation in the intact cell. Previous workers had established that the initial glycosylation of asparagine-linked oligosaccharides occurs by the α-D-glucosyltransferase of a high mannose type oligosaccharide to the nascent polypeptide chain (Kiel et al 1976, Parodi & Lefoit 1979). With the [2-3H]mannose labelling, our studies proceeded from their site of synthesis in the rough endoplasmic reticulum to their final destination in the lysosomes.

When we analysed the phosphorylated oligosaccharide units of newly synthesized β-D-glucuronidase, we found that most of the phosphate groups were present in diester linkage between the sixth OH group of mannose residue in the underlying oligosaccharide and the first carbon of outer. α-linked N-acetylglucosamine residues (Tabas & Körhfeld 1980). A similar finding was also made by Hasilik et al 1980. Our studies of the structure of these unusual oligosaccharides (Varki & Körhfeld 1980b) showed that the phosphates are linked to five different mannose residues on the oligosaccharide, and that individual molecules may contain one or two phosphates, thus generating many isomers. A composite picture is shown in Fig. 1, where the asterisks identify the mannose residues that can be phosphorylated in the
LYSOSOMAL ENZYME PHOSPHORYLATION

In order to prove that the diesters are actually converted to monooesters in vivo (i.e., by 'uncovering'), we examined the kinetics of the phosphorylation pathway in the murine macrophage line P388D1 (Goldberg & Kornfeld 1981). Cells were incubated with [2-3H]mannose for 15-20 min and then chased in unlabelled media for various times up to 5 h. β-D-Glucuronidase was immunoprecipitated and its oligosaccharide units were examined (for extent of phosphorylation and uncovering) by chromatography of the oligosaccharides on quaternary aminomethyl-Sepharose using an elution system that separates neutral oligosaccharides from oligosaccharides with one or two phosphomonoesters or phosphodiesters. The results are shown in Fig. 3. At the end of the 20-min labelling period most of the β-D-glucuronidase oligosaccharides were neutral; the only detectable phosphorylated species was a small amount of oligosaccharide with one covered phosphate. During the first 40 min of the chase, the amount of oligosaccharide with one covered phosphate greatly increased and material having two covered phosphates became detectable, as did material with uncovered phosphates. The total extent of phosphorylation increased from 5% at the end of the pulse period to 20% after 40 min of chase and to 25% after 80 min of chase. As the chase proceeded, most of the phosphodiesters were converted to phosphomonoesters, as predicted by the scheme shown in Fig. 2. Since the total amount of labelled mannose in newly synthesized β-D-glucuronidase increased only slightly during the first 40 min of the chase period, the increase in the amount of phosphorylated species during this time must have resulted from phosphorylation of neutral high-mannose-type oligosaccharides transferred to the enzyme during the 20 min pulse. This finding demonstrates that phosphorylation is a post-translational event which takes place on protein-bound oligosaccharide and not on lipid-linked oligosaccharide precursors.

In our most recent experiments we have attempted to define the state of the oligosaccharides on the lysosomal enzymes when these molecules first bind to the high-affinity intracellular receptors. To approach this question we did a pulse-chase experiment similar to the one described above, but in this case we isolated a total cell-membrane fraction at each time point. This fraction, which contained the high-affinity receptor with its bound ligand, was treated with mannose 6-phosphate to release the bound lysosomal enzymes. The oligosaccharide units of the released glycoproteins were then analysed and compared to the phosphorylated oligosaccharides that remained in the soluble fraction of the cell. The results were striking. There was a tremendous enrichment in oligosaccharides with one and two phosphomonoesters in the material released from the receptor with mannose 6-phosphate. In contrast, most of the enzyme in the soluble fraction contained oligosaccharides with phosphodiester units. These data indicate that conversion of phosphodiesters to phosphomonoesters precedes binding to the intracellular receptors.
UDP-N-acetylglucosamine:lyosomal enzyme N-acetylglucosamine-1-phosphotransferase

As shown in Fig. 2, the proposed biosynthetic pathway requires two enzymes: an N-acetylglucosaminylphosphotransferase and an α-N-acetylglucosaminyl phosphoesterase. Assays for each of these enzymes have been developed and the enzyme activities have been detected in a number of tissues. UDP-N-acetylglucosamine:lyosomal enzyme N-acetylglucosamine-1-phosphotransferase activity has been detected in homogenates of Chinese hamster ovary cells, human diploid fibroblasts and rat liver (Reitman & Kornfeld 1981a, Hasilik et al. 1981). Using [β-32P]UDP-[3H]N-acetylglucosamine as donor, we showed that the enzyme transfers N-acetylglucosamine 1-phosphate to the sixth OH group of mannose residues in high mannose-type oligosaccharides of lysosomal enzymes. The enzyme is not inhibited by tunicamycin or stimulated by doxorubicin phosphate, indicating that the reaction does not proceed via a dolichol-pyrophosphoryl-N-acetylglucosamine intermediate. Fibroblasts from patients with inclusion-cell (I-cell) disease (mucolipidosis II) and pseudo-Hurler polydystrophy (mucolipidosis III) are severely deficient in this enzyme activity (Hasilik et al. 1981, Reitman et al. 1981, Varki et al. 1981). These autosomal recessive storage diseases are characterized by a general failure to target acid hydrolases to lysosomes in spite of normal rates of acid hydrolase synthesis (Fickman & Neufeld 1972) and by the failure to incorporate [32P]phosphate into newly synthesized acid hydrolases (Hasilik & Neufeld 1980, Bach et al. 1979). The absence of the N-acetylglucosaminylphosphotransferase explains the lack of phosphorylation of the newly synthesized acid hydrolases. This failure to generate the phosphomannosyl recognition signal precludes the receptor-mediated targeting of the newly synthesized acid hydrolases to lysosomes, and consequently the enzymes are secreted into the extracellular milieu.

One of the most intriguing questions about this system concerns the basis for the specificity of the selective phosphorylation of the oligosaccharide units of lysosomal enzymes. If we assume, as the data indicate, that lysosomal enzymes and non-lysosomal glycoproteins are each glycosylated with the identical oligosaccharide precursor, then we must explain why only the lysosomal enzymes are phosphorylated. The simplest explanation is that all
lysosomal enzymes share a common protein recognition site or marker and that this marker causes one of two possible effects. First, it could lead to the segregation of newly synthesized lysosomal enzymes into a specialized subcellular compartment which contains the N-acetylglucosaminylphosphotransferase. In this case the transferase would not have to recognize lysosomal enzymes specifically but, rather, it would phosphorylate the oligosaccharide units of any glycoprotein in the compartment. The other possibility is that the N-acetylglucosaminylphosphotransferase is able to distinguish between lysosomal and non-lysosomal enzymes on the basis of this common protein recognition marker. To evaluate this we partially purified the N-acetylglucosaminylphosphotransferase from rat liver and determined its ability to phosphorylate the oligosaccharide units of a series of lysosomal and non-lysosomal glycoproteins (Reitman & Kornfeld 1981b). Fig. 4 shows the results of a typical experiment using glycoprotein acceptors that have in common the presence of high mannose-type oligosaccharide units. It is evident that the lysosomal enzymes β-hexosaminidase A and B are the best acceptors, being active at very low protein concentrations. In contrast, none of the four non-lysosomal glycoproteins is a good acceptor. The data in Table 1 show some of the kinetic parameters of the enzyme towards various acceptors. The apparent $K_m$ values for the three lysosomal enzymes are in the low micromolar range whereas the $K_m$ for ribonuclease B is approximately 100-fold greater. The acceptor activity of the other non-lysosomal glycoproteins was so low that it was not possible to determine their apparent $K_m$ and $V_{max}$ values. Table 1 also presents the kinetic parameters for two carbohydrate acceptors, α-methylmannoside and Man$_{5}$N-acetylglucosamine oligosaccharide. These molecules have very high apparent $K_m$ values, being 10$^3$-10$^4$-fold greater than those of lysosomal enzymes. Their $V_{max}$ values are 10-100-fold greater than those of the glycoprotein acceptors. The relative catalytic efficiency ($V_{max}$ divided by apparent $K_m$) of the transferase towards the various acceptors is also shown in Table 1. The three lysosomal enzymes are phosphorylated at least 100-fold more efficiently than either ribonuclease B, Man$_{5}$N-acetylglucosamine oligosaccharide or α-methylmannoside. This preference of the N-acetylglucosaminylphosphotransferase for lysosomal enzymes in these in vitro assays demonstrates the remarkable specificity of a glycosyltransferase for the class of glycoproteins on which it acts in vivo.

![Graph showing activity of N-acetylglucosaminylphosphotransferase towards various glycoproteins containing high mannose-type oligosaccharides. The two lysosomal enzyme acceptors were human placental β-hexosaminidase A and B (after Reitman & Kornfeld 1981b).](image)

<table>
<thead>
<tr>
<th>acceptor</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (pmol/min/mg protein)</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human placental β-hexosaminidase A</td>
<td>0.020</td>
<td>251</td>
<td>163</td>
</tr>
<tr>
<td>Human placental β-hexosaminidase B</td>
<td>0.016</td>
<td>270</td>
<td>601</td>
</tr>
<tr>
<td>Purine hepatic α-N-acetylglucosaminidase</td>
<td>0.009</td>
<td>181</td>
<td>258</td>
</tr>
<tr>
<td>Bovine pancreatic ribonuclease B</td>
<td>0.9</td>
<td>116</td>
<td>1.6</td>
</tr>
<tr>
<td>Man$_{5}$N-acetylglucosamine oligosaccharide</td>
<td>32</td>
<td>3103</td>
<td>1.2</td>
</tr>
<tr>
<td>α-Methylmannoside</td>
<td>113</td>
<td>8940</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*pmol N-acetylglucosamine 1-phosphate transferred h$^{-1}$mg$^{-1}$ enzyme protein. $^b$Relative acceptor activity is defined as the $V_{max}$ divided by the apparent $K_m$, normalized to α-methylmannoside (after Reitman & Kornfeld 1981b).
N-acetylglucosaminylphosphotransferase. In other words, the N-acetylglucosaminylphosphotransferase appears to be specific for a particular protein conformation that is unique to lysosomal enzymes. The nature of this conformation requirement is obscure at present. Since both ribonuclease B (with an exposed oligosaccharide unit [Baynes & Wold 1976, Tarentino et al. 1974]) and free high mannose-type oligosaccharides are poor acceptors, it is unlikely that accessibility of the carbohydrate chain to the transferase is the sole conformational requirement for recognition.

The specificity of the N-acetylglucosaminylphosphotransferase towards lysosomal enzymes makes it unnecessary to postulate a mechanism for the specific segregation of newly synthesized acid hydrolases which would precede exposure to the N-acetylglucosaminylphosphotransferase. From these data we propose that the N-acetylglucosaminylphosphotransferase is the initial and determining enzyme for the pathway that eventually results in the segregation of acid hydrolases into lysosomes.

\[ \text{\textalpha-N-Acetylglucosaminylphosphodiesterase} \]

An \textalpha-N-acetylglucosaminylphosphodiesterase capable of removing the covering N-acetylglucosamine residues has been purified from rat liver (Varki & Kornfeld 1981) and human placenta (Waheed et al. 1981). The enzyme is distinct from a previously described lysosomal \textalpha-N-acetylglucosaminidase, which can also remove covering N-acetylglucosamine residues (Varki & Kornfeld 1980a, Waheed et al. 1981b), and it is active against N-acetylglucosamine residues that are \textalpha-linked to an underlying phosphate, although it is inactive against \textalpha-nitrophenyl \textalpha-N-acetylglucosaminide. Furthermore, the activity is inhibited by \textit{PO}_4\textsuperscript{2-} ions. Recently we have obtained evidence that the enzyme reaction may be proceeded by a phosphodiesterase mechanism that is true phosphodiesterase mechanism. We are pursuing this further to determine if a change in the nomenclature of the enzyme is appropriate.

Subcellular localization of N-acetylglucosaminylphosphotransferase and \textalpha-N-acetylglucosaminylphosphodiesterase

Both the N-acetylglucosaminylphosphotransferase and \textalpha-N-acetylglucosaminylphosphodiesterase have been localized to Golgi-enriched smooth membranes (Waheed et al. 1981b, Varki & Kornfeld 1981a). We have succeeded in separating both activities from the trans Golgi marker galactosyltransferase by subjecting mouse lymphoma and P388D1 macrophage membranes to centrifugation on a continuous sucrose density gradient. The precise location of these two enzyme activities is not known.

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