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Studies of the synthesis, structure and function of the phosphorylated oligosaccharides of lysosomal enzymes

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Abstract. Newly synthesized lysosomal enzymes were found to contain N-acetylglucosamine residues in phosphodiester linkage to the 6 position of the mannose residues on high-mannose type oligosaccharides. The formation of these structures was shown to be catalyzed by a specific N-acetylglucosaminylphosphotransferase enzyme, that utilises UDP-N-acetylglucosamine as a donor. The phosphorylation reaction can take place on any of four or five positions on the high-mannose oligosaccharide. Subsequently an α-N-acetylglucosaminylphosphodiesterase removes the outer blocking N-acetylglucosamine residues to generate the mature phosphomannsoyl recognition signal. This signal is responsible for the targetting of newly synthesized lysosomal enzymes to lysosomes. The human syndromes of I-cell disease (Mucolipidosis II) and pseudo-Hurler polydystrophy (Mucolipidosis III) were shown to be caused by deficiency of the first enzyme in the pathway, the UDP-N-acetylglucosamine: Glycoprotein N-acetylglucosaminylphosphotransferase.

Keywords. Lysosomal enzymes; I-cell disease; phosphorylated oligosaccharides; glycoproteins.

Previous studies have shown that a common phosphomannosyl recognition marker present on lysosomal enzymes appears to be important in their endocytotic uptake and their intracellular targetting (Kaplan *et al.*,1977; Sando and Neufeld, 1977; Kaplan *et al.*, 1978; Natowicz *et al.*, 1979; Distler *et al.*, 1979; von Figura and Klein, 1979; Bach *et al.*, 1979; Fischer *et al.*, 1980; Hasilik and Neufeld, 1980). In order to examine the structure of this recognition marker, we labelled mouse lymphoma cells for 3 h with [2-^{3}H]-mannose, and then immunoprecipitated the lysosomal enzyme β-glucuronidase. The high mannose oligosaccharides of the enzyme were released by endo-β-N-acetylglucosaminidase H and then fractionated into neutral and negatively charged species on QAE-Sephadex. The negatively charged oligosaccharides from newly synthesized β-glucuronidase were found to contain phosphate residues in diester linkage, with outer 'blocking' α-N-acetylglucosamine residues (Tabas and Kornfeld, 1980). Similar findings have been reported by Hasilik *et al.* (1980).

We next performed detailed structural studies on similar oligosaccharides isolated from whole-cell labelled material. These studies revealed a whole family of such phosphorylated oligosaccharides, all of which had a high-mannose type oligosaccharide core. Individual molecules contained one or two phosphate residues, either as phosphomonoesters, or as phosphodiesters, with outer 'blocking' α-N-

acetylglucosamine residues. The phosphate residues were present at five possible positions on the high mannose oligosaccharide, thus generating many different isomers. The phosphomonoester containing molecules had fewer mannose residues, suggesting that they were the "oldest", and had undergone oligosaccharide processing (Varki and Kornfeld, 1981).

These findings, along with other previous evidence suggesting that the mature phosphomannosyl recognition marker contained alkaline phosphatase-susceptible phosphomonoesters (Kaplan *et al.*, 1977; Sando and Neufeld, 1977; Kaplan *et al.*, 1978; Natowicz *et al.*, 1979; Distler *et al.*, 1979; von Figura and Klein, 1979), suggested that removal of the outer N-acetylglucosamine residues must occur *in vivo*. We therefore searched for and identified a rat liver α-N-acetylglucosaminylphosphodiesterase that catalyzes such a reaction. We have now purified this enzyme about 1800-fold from rat liver, using subcellular fractionation, differential detergent extraction, DEAE-cellulose and heparin-Sepharose chromatography, concanavalin A-Sepharose affinity chromatography, and gel filtration on Sephacryl S-300. The purified preparation is completely free of a previously described lysosomal α-N-acetylglycosaminidase (Weismann *et al.*, 1967). The enzyme is a smooth membrane bound glycoprotein that migrated as a single form in all the purification steps. It has a broad pH optimum, between 6.0–8.0, and is unaffected by divalent cations or reducing agents. It is capable of removing α-linked N-acetylglucosamine residues from all of the five positions on the high mannose oligosaccharides that we had previously found. It is also capable of removing α-N-acetylglucosamine residues from phosphodiester linkage in molecules such as [illegible] *p*-nitrophenyl-α-N-acetylglucosaminide (Varki and Kornfeld, 1980, 1981).

We have also identified the UDP-GlcNAc : glycoprotein N-acetylglucosamine-1-phosphotransferase that carries out the phosphorylation reaction. The basis of the assay was to follow incorporation of ^{3}H and ^{32}P from [β-^{32}P]-UDP-[6-^{3}H]GlcNAc into glycopeptides with high affinity for Concanavalin A-Sepharose. Characterization of the enzyme reaction products (derived from either endogenous or exogenous acceptors) showed that α-linked GlcNAc 1-phosphate is transferred *en bloc* to the 6 hydroxyl position of mannose in the high mannose oligosaccharides of the acceptor glycoproteins. This membrane-associated transferase was neither inhibited by tunicamycin, nor stimulated by dolichol phosphate, indicating that the reaction does not proceed *via* a dolichol pyrophosphoryl-N-acetylglucosamine intermediate (Reitman and Kornfeld, 1981).

We propose that the sequential action of the two enzymes described above results in the generation of the phosphomannosyl recognition marker of lysosomal enzymes that is involved in the targetting of these proteins to the lysosomes. In this scheme (see figure 1) the newly synthesized lysosomal enzyme is glycosylated by transfer of the lipid-linked oligosaccharide to the nascent polypeptide chain (structure 1). This oligosaccharide is then phosphorylated by the transfer of N-acetylglucosamine-1-phosphate from the donor, UDP-GlcNAc. Each oligosaccharide thus acquires one or two phosphate residues (the commonest of the many isomers thus generated is shown in structure 2). The outer blocking N-acetylglucosamine residues are then removed to generate structure 3, the mature phosphomannosyl recognition marker. This marker is then involved in the targetting of the lysosomal enzyme to its ultimate destination, in the lysosome.

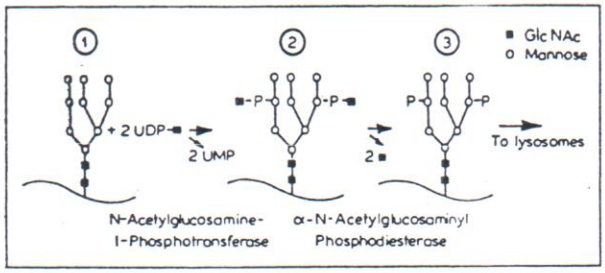

Figure 1. Mechanism for the phosphorylation of mannose residues of lysosomal enzyme oligosaccharides.

I-cell disease and pseudo-Hurler polydystrophy (Mucolipidoses II and III) are autosomal recessive lysosomal storage disorders. Both diseases are characterized by decreased intracellular activities of many lysosomal enzymes, and by markedly elevated levels of the same enzymes in the body fluids (McKusik *et al.*, 1978). Previous studies suggested that these abnormalities might arise from deficient phosphorylation of lysosomal enzymes (Bach *et al.*, 1979; Hasilik and Neufeld, 1980). We therefore studied the generation of the phosphomannosyl recognition marker in fibroblasts from patients with these diseases. The N-acetylglucosaminyl-phosphotransferase activity (using endogenous acceptors) in cultures of fibroblasts from six normal subjects ranged from 0.67 to 1.46 pmol N-acetylglucosamine 1-phosphate transferred/mg protein per h, whereas five pseudo-Hurler polydystrophy and five I-cell cultures transferred less than 0.02 pmol/mg protein per h. The activity in five other pseudo-Hurler cultures ranged from 0.02 to 0.27 pmol transferred/mg protein per h. The activity of the α-N-acetylglucosaminyl-phosphodiesterase was normal or elevated in all the I-cell and pseudo-Hurler cultures. Thus, the deficiency of the first enzyme in the pathway for the generation of the phosphomannosyl recognition marker can explain the biochemical abnormalities previously described in these diseases (Reitman *et al.*, 1981).

Note:

Many similar findings have been independently reported by K. von Figura, A. Hasilik, A. Waheed, and others (see references Hasilik *et al.*, 1980; Waheed *et al.*, 1981a, b; Hasilik *et al.*, 1981, and their accompanying papers).

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