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ENZYMATIC PHOSPHORYLATION OF LYSOSOMAL ENZYMES IN THE PRESENCE OF UDP-N-ACETYLGLUCOSAMINE. ABSENCE OF THE ACTIVITY IN I-CELL FIBROBLASTS.

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Summary: Recent finding of α-N-acetylglucosamine(1)phospho(6)mannose diesters in lysosomal enzymes suggested that formation of mannose 6-phosphate residues involves transfer of N-acetylglucosamine 1-phosphate to mannose. Using dephosphorylated ß-hexosaminidase as acceptor and [ß-^{32}P] UDP-N-acetylglucosamine as donor for the phosphate group, phosphorylation of ß-hexosaminidase by microsomes from rat liver, human placenta and human skin fibroblasts was achieved. The reaction was not affected by tunicamycin. Acid hydrolysis released mannose 6-[^{32}P] phosphate from the phosphorylated ß-hexosaminidase. Our results suggest that lysosomal enzymes are phosphorylated by transfer of N-acetylglucosamine 1-phosphate from UDP-N-acetylglucosamine. The transferase activity was deficient in fibroblasts from patients affected with I-cell disease. This deficiency is proposed to be the primary enzyme defect in I-cell disease.

Introduction: Lysosomal enzymes from various sources contain high mannose oligosaccharides with mannose 6-phosphate residues. In fibroblasts, these mannose 6-phosphate residues mediate the transfer of lysosomal enzymes into lysosomes as suggested by the multiple deficiency of lysosomal enzymes intracellularly and accumulation of these enzymes extracellularly in I-cell disease (mucolipidosis II) where non-phosphorylated lysosomal enzymes are formed (1-3).

Recently, it has been found that a considerable fraction of phosphate groups in lysosomal enzymes is present in a diester linkage within α-N-acetylglucosamine(1)phospho(6)mannose groups (4, 5). These diesters were proposed to be intermediates in the biosynthesis of mannose 6-phosphate termini in high mannose oligosaccharides in lysosomal enzymes. In the present communication we describe phosphorylation of ß-hexos-

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Abbreviations: GlcNAc, N-Acetylglucosamine

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aminidase by microsomes derived from rat liver, human placenta and human fibroblasts in the presence of [ß-^{32}P] UDP-N-acetylglucosamine and the deficiency of that reaction in fibroblasts from patients affected with I-cell disease.

Materials and Methods: Cell culture: Human skin fibroblasts were maintained at 37°C in 5% CO_2 in Eagle's Minimal Essential Medium supplemented with antibiotics, non-essential amino acids and 10% fetal calf serum (Boehringer Mannheim, Mannheim) as described (6). Fibroblasts from two patients affected with I-cell disease were kindly provided by Dr. J. Boué, Paris (cell line 1) and Dr. J. G. Leroy (cell line 2).

Preparation of ß-hexosaminidase (EC 3.2.1.52): Fibroblasts were incubated in a serum free medium that was supplemented with 10 mM NH_4Cl to increase the fraction of secreted ß-hexosaminidase (3, 7). ß-Hexosaminidase was purified by affinity chromatography according to ref. 7, except that 0.01% (w/v) bovine serum albumin (crystalline grade from Serva, Heidelberg) was added to the elution buffer. Purified ß-hexosaminidase, 21 units (equivalent to about 0.4 mg enzyme protein) was incubated with 3500 units of α-N-acetylglucosaminyl phosphodiesterase partially purified from human placenta microsomes (8) in 1.15 ml of 15 mM sodium acetate, pH 5.5, containing 0.5% (w/v) octylglucoside and 1 mM EDTA for 8 h at 37°C. ß-Hexosaminidase was rechromatographed on the affinity column and then incubated with 3 units/ml alkaline phosphatase (EC 3.1.3.1) from E. coli (Sigma Chem., St. Louis, 44 units/mg protein) for 14 h at 37°C as described (9). After rechromatography on the affinity column, ß-hexosaminidase was stored in 10 mM sodium phosphate, pH 6.0, containing 0.15 M NaCl. ß-Hexosaminidase recovered, 45% of the initial, was completly devoid of the uptake property assayed as described (9).

Preparation of [ß-^{32}P] UDP-N-acetylglucosamine: The procedure followed closely the method described by Wagner and Cynkin (10). Glucosamine was converted into [^{32}P] glucosamine 6-phosphate with hexokinase and [γ-^{32}P] ATP (New England Nuclear, Boston) with a specific radioactivity of 6.8 Ci/mmol. The [^{32}P] glucosamine 6-phosphate was chemically acetylated with acetic anhydride and enzymicly converted into [ß-^{32}P] UDP-N-acetylglucosamine with a crude yeast extract and UTP. After desalting on Bio-Gel P-4 (BioRad, Richmond) the [ß-^{32}P] UDP-N-acetylglucosamine was purified on AG 1-X8 (BioRad), formate. The final preparation was homogeneous as judged by paper electrophoresis in 1.9 M HCOOH, pH 1.7, for 40 min at 50 V/cm. The yield was 30% by radioactivity.

Preparation of microsomes: Microsomes from rat liver and human placenta were prepared as described (8). Trypsinized human skin fibroblasts were disrupted by N_2-cavitation (11). Nuclei and unbroken cells were removed by centrifugation at 600 x g for 10 min and the microsomal pellet was obtained by centrifugation at 105,000 x g for 90 min. The microsomal membranes were suspended in water and kept on ice. The activity in the liver preparation was stable for at least two weeks.

In vitro phosphorylation of ß-hexosaminidase: Unless otherwise stated the assay contained 0.25 units of ß-hexosaminidase, 20 mM sodium phosphate, pH 6.7, 5 mM $MgCl_2$, 1.5% (v/v) Triton X-100, 2 mM AMP, 25 µM [ß-^{32}P] UDP-N-acetylglucosamine (3.4-1.4 Ci/mmol) and 0.4-0.6 mg of microsomal protein in a final volume of 40 µl. After incubation for 6 h at 37°C 0.45 ml of ice cold 10 mM sodium phosphate, pH 7.0, containing 0.15 M NaCl was added and the mixture subjected to centrifugation at 50,000 x g for 1 h. ß-Hexosaminidase was immunoprecipitated and the radioactivity analyzed by gel electrophoresis in presence of sodium dodecyl sulfate and fluorography as described (7). Film exposure periods of 6 to 10 d were used.

Other methods: Activity of ß-hexosaminidase was determined by hydrolysis of p-nitrophenyl ß-N-acetylglucosaminide (9), that of α-N-acetylglucosaminyl phosphodiesterase by hydrolysis of oligosaccharide M I-2 (8). Protein was determined by the method of Lowry et al. (12) using bovine serum albumin as standard.

Results and Discussion: Phosphorylation of ß-hexosaminidase by rat liver and human placenta microsomes: Phosphorylation was studied using [ß-^{32}P] UDP-N-acetylglucosamine as donor for the N-acetylglucosamine 1-phosphate residue. With this donor it was expected that phosphorylation would be detectable even if it occured via [ß-^{32}P] N-acetylglucosamine(1)diphosphodolichol or if the product was cleaved by the microsomal α-N-acetylglucosaminyl phosphodiesterase. The precursor form of ß-hexosaminidase (7) was chosen as acceptor. Removal of N-acetylglucosamine and of phosphate residues by two subsequent enzymatic treatments of ß-hexosaminidase isolated from secretions of fibroblasts was thought to increase the number of sites suitable for phosphorylation.

When such a preparation of ß-hexosaminidase was incubated with rat liver microsomes in the presence of [ß-^{32}P] UDP-N-acetylglucosamine incorporation of [^{32}P] phosphate into α- and ß-chains of ß-hexosaminidase was observed (Fig. 1, lane 3, 8 and 9). The yield of incorporation was increased in the presence of AMP (Fig. 1, lane 2); presence of tunicamycin, 12 µM, had no effect (Fig. 1, lane 1). This excludes N-acetylglucosamine(1)diphosphodolichol as the donor of the phosphate group (13). AMP inhibits the rapid breakdown of UDP-N-acetylglucosamine to N-acetylglucosamine 1-phosphate and UMP, which occurs under the experimental conditions used (not shown). Phosphate incorporation was lower, when treatment of the accepting ß-hexosaminidase with α-N-acetylglucosaminyl phosphodiesterase prior to dephosphorylation was omitted (Fig. 1, lane 4). Under standard conditions phosphorylation of ß-hexosaminidase in the presence of human placenta microsomes was less efficient than in the presence of rat liver microsomes (Fig. 1, lane 10).

The amount of [^{32}P] phosphate incorporated into ß-hexosaminidase was very low (30-60 cpm per assay in the presence of AMP). The low incorporation can be attributed in part to the degradation of [ß-^{32}P] UDP-N-acetylglucosamine which is complete within 30 min of incubation even in the presence of 2 mM AMP. The radioactivity incorporated into ß-hexosaminidase was identified as [^{32}P] mannose 6-phosphate. The major part of the ^{32}P - labelled material released by acid hydrolysis of the immunoprecipitate comigrated in electrophoresis with mannose 6-phosphate (Fig. 2 A) and

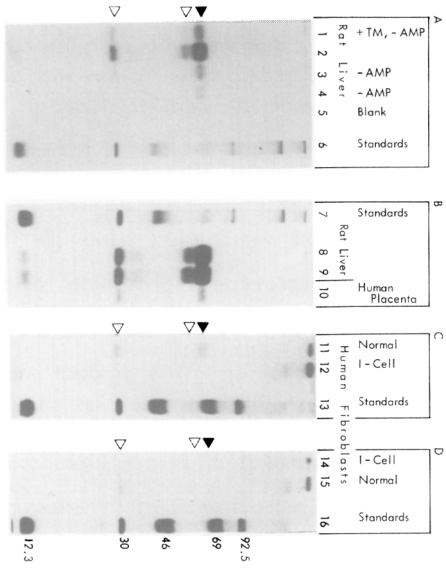

Fig. 1: Phosphorylation of ß-hexosaminidase in the presence of microsomes and [ß-^{32}P] UDP-N-acetylglucosamine. Panels A-D represent four different experiments. Panel A: Incubation with rat liver microsomes (lane 1-4) in the presence of 12 µM tunicamycin (TM) and omission of AMP as indicated above the photograph. In the blank (lane 5) microsomes were omitted. Panel B: Incubation with fresh rat liver microsomes (lane 8), rat liver microsomes stored for 12 days at 0°C (lane 9) and with human placenta microsomes (lane 10). Panels C and D: Incubation with microsomes from two normal fibroblast lines (lanes 11 and 15) and two I-cell lines (lane 12, cell line 1, lane 14, cell line 2). In these experiments the addition of microsomes, [ß-^{32}P] UDP-N-acetylglucosamine and AMP was repeated after 90 min, whereby the final volume was increased by 16 µl. The incubation was carried out for 16 h at 37°C.
The observed processing of ß-hexosaminidase precursors (63,000-67,000, filled arrows) to the previously characterized mature 54,000 α- and 29,000 ß-chains (open arrows) (7) occured during the incubation with microsomes, either before or after the phosphorylation. The molecular weights of human ß-hexosaminidase differ from those of rat liver ß-hexosaminidase, where the processed forms have molecular weights of 53,000 and 59,000 and the precursor forms a molecular weight of 69,000 (unpublished). The following [^{14}C] methylated standards (lanes 6, 7, 13, 16) obtained from New England Nuclear, were used: phosphorylase B, 92,500; bovine serum albumin, 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000 and cytochrome c, 12,300.

after enzymatic conversion with 6-phosphogluconate (Fig. 2 B). Less than 25% of the ^{32}P- labelled material released by acid hydrolysis behaved as inorganic phosphate.

Absence of the phosphorylating enzyme in membranes from I-cell fibroblasts: As shown in Fig. 1, panels C and D phosphorylation of ß-hexosaminidase occured with membranes from normal fibroblasts but not with those from fibroblasts of two unrelated

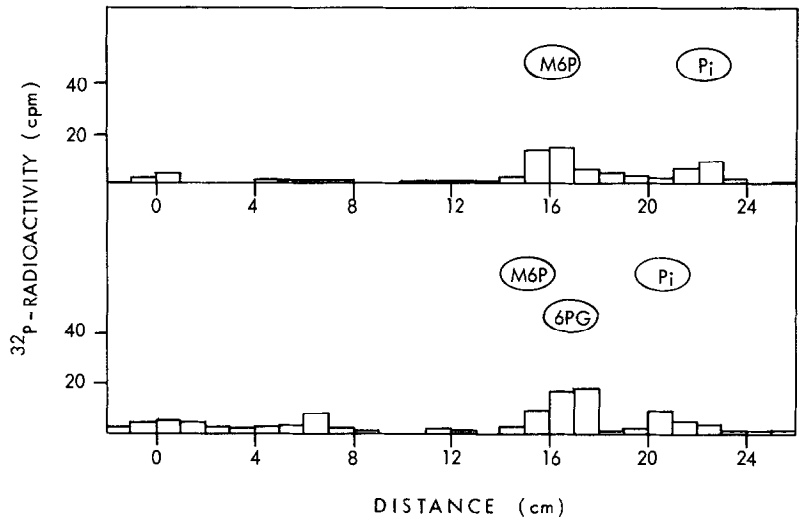

Fig. 2: Identification of mannose 6-phosphate in ß-hexosaminidase phosphorylated in vitro. Panel A: ß-Hexosaminidase immunoprecipitated from two standard reaction mixtures was subjected to acid hydrolysis (4 M trifluoroacetic acid, 4 h at 100°C). The hydrolysate was lyophilized and analyzed by high voltage electrophoresis at pH 8.0 (5). Panel B: The hydrolysate prepared as above from four standard reaction mixtures was incubated with phosphomannoisomerase (EC 5.3.1.8), phosphoglucoisomerase (EC 5.3.1.9), glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and NADP (3) and analyzed as above. As standards mannose 6-phosphate (M6P), 6-phosphogluconate (6PG) and inorganic phosphate were used.

patients affected with I-cell disease. The membrane preparations of normal and I-cell fibroblasts contained similar activities of the microsomal α-N-acetylglucosaminyl phosphodiesterase, 17 and 20 units/mg protein, respectively. A mixture of equal amounts of membranes from normal and I-cell fibroblasts catalyzed the phosphorylation (not shown). Therefore, the absence of the phosphorylation in I-cell fibroblasts is not due to the excess of an inhibitor.

Pathway for the biosynthesis of mannose 6-phosphate in lysosomal enzymes:

UDP-GlcNAc + Man··acceptor ⟶ UMP + αGlcNAc-1-Ⓟ-6-Man··acceptor (A)

αGlcNAc-1-Ⓟ-6-Man··acceptor + H_2O ⟶ GlcNAc + Ⓟ-6-Man··acceptor (B)

(acceptor stands for precursor forms of lysosomal enzymes)

It is proposed that high mannose oligosaccharides attached to lysosomal enzymes rather than the lipid linked oligosaccharide precursor serve as acceptors for the phosphate. This assumption is based on the present findings and the failure to achieve

phosphorylation of high mannose oligosaccharides isolated from lysosomal enzymes (unpublished results). Reaction A is similar to phosphorylation of mannans in yeast, where mannose 1-phosphate is transferred from GDP-mannose to C6 hydroxyl of mannose residues in nascent mannans (14). With [ß-^{32}P] UDP-N-acetylglucosamine as donor the transfer of N-acetylglucosamine together with phosphate cannot be demonstrated directly. Presence of N-acetylglucosamine 1-[^{32}P] phosphate groups in the phosphorylated ß-hexosaminidase would be indicated by the resistance of the ^{32}P-label to phosphatase. The low yield and the presence of microsomal α-N-acetylglucosaminyl phosphodiesterase precluded such an analysis in this study. In rat liver homogenate both the N-acetylglucosamine 1-phosphate transferase and the α-N-acetylglucosaminyl phosphodiesterase, which is supposed to uncover mannose 6-phosphate residues (reaction B), are present in a membrane fraction enriched in the Golgi marker galactosyltransferase (15, 16). Previous studies had established the deficiency of phosphorylated oligosaccharides in lysosomal enzymes from I-cell fibroblasts (2,3). The present findings indicate that the primary defect in I-cell disease is a deficiency in UDP-N-acetylglucosamine:lysosomal enzyme N-acetylglucosamine 1-phosphate transferase.

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