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Deficient phosphorylation of mannose residues of mannan in fibroblasts of patients with mucolipidoses II and III

Yoav BEN-YOSEPH, Lisa C. HAHN, C. L. DEFRANCO and Henry L. NADLER
Department of Pediatrics, Northwestern University Medical School, Division of Genetics, Children's Memorial Hospital, Chicago, IL 60614, U.S.A.

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Incorporation of ^{32}P from [γ-^{32}P]ATP into mannan could not be detected in homogenates of cultivated skin fibroblasts from patients with mucolipidosis II, and accounted for only up to 10% of normal control activity in cell lysates from patients with mucolipidosis III. Parents of patients with mucolipidosis II demonstrated 60–70% of normal control activity. On high-voltage electrophoresis, the hydrolysed mannan from reactions performed with normal cells, over the pH range 5.5–7.5, yielded a radioactive band migrating with the same mobility as mannose 6-phosphate, whereas no such product could be demonstrated in fibroblasts of patients with mucolipidosis II.

Mucolipidosis types II and III (I-cell disease and pseudo-Hurler polydystrophy respectively) are autosomal recessive lysosomal storage disorders, characterized by deficiency of multiple lysosomal hydrolases in fibroblasts and elevated activities of these enzymes in body fluids and in the medium in which the cells were cultured (McKusick *et al.*, 1978). Mucolipidosis III is less severe than mucolipidosis II, as evident from the milder manifestation, later onset and longer life span (Leroy *et al.*, 1971; Kelly *et al.*, 1975). Hickman & Neufeld (1972) proposed that lysosomal enzymes share a common 'recognition marker' that is essential for their receptor-mediated uptake by fibroblast, and that this recognition marker is absent from, or defective in, lysosomal hydrolases from mucolipidosis-II and mucolipidosis-III patients. The presence of carbohydrate within the common recognition site was suggested (Hickman *et al.*, 1974), and further studies implicated mannose 6-phosphate or some closely related phosphorylated sugar as the recognition marker required for incorporation of enzymes into lysosomes of fibroblasts (Kaplan *et al.*, 1977*a*,*b*; Sando & Neufeld, 1977; Neufeld *et al.*, 1977; Ullrich *et al.*, 1978; Von Figura & Klein, 1979; Distler *et al.*, 1979; Natowicz *et al.*, 1979). Skin fibroblast cultures from patients with mucolipidosis II have shown a marked disturbance of enzyme distribution between cells and medium, and the intracellular enzymes remained incompletely processed (Hasilik & Neufeld, 1980*a*). Incorporation of phosphate into precursor and processed chains of lysosomal enzymes was detected in normal fibroblast cultures, and mannose 6-phosphate residues were identified on the precursor chains (Hasilik & Neufeld, 1980*b*). However, in cultures from patients with mucolipidosis II, phosphorylation was essentially undetectable (Bach *et al.*, 1979; Hasilik & Neufeld, 1980*b*).

It should be noted that the defect in mucolipidosis II and mucolipidosis III is expressed in only few cell types (e.g., fibroblasts, certain kidney cells and Schwann cells), and even in fibroblasts, acid phosphatase and β-glucosidase activities are not decreased (Neufeld *et al.*, 1977). These lysosomal hydrolases showing no decrease in activity, from mucolipidosis-II fibroblasts, demonstrate decreased binding to lectins, similar to that found for the enzymes having decreased activity (Rousson *et al.*, 1979). This observation implies that the carbohydrate moiety of these apparently unaffected glycoprotein enzymes is altered. The defect does not affect the antigenic properties of mucolipidosis-II lysosomal hydrolases or the function of their catalytic site, as indicated by normal specific activity of the cross-reacting material of the intracellular enzymes showing an activity decrease (Ben-Yoseph *et al.*, 1977, 1978).

The present study describes the use of mannan as a model substrate for the phosphorylation reaction, and our findings indicate that this phosphorylation is severely defective in mucolipidosis II and mucolipidosis III.

Materials and methods

Cell cultures

Skin fibroblasts obtained by punch biopsy from six normal controls, two obligate heterozygotes for

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mucolipidosis II, four mucolipidosis-II patients and two mucolipidosis-III patients were cultured and harvested as previously described (Ben-Yoseph *et al.*, 1977). Fibroblast lysates were prepared in 5 vol. of water by five consecutive cycles of freeze–thawing. Whole homogenates were used for enzyme assays and boiled cell lysates served as blanks. Protein was determined by the dye-binding assay (Bradford, 1976), with bovine γ-globulin as a standard.

Hexokinase determination

Hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1) activity toward D-mannose was determined by incubating, at 25°C, 200μg of cell-lysate protein with 1μmol of D-mannose (Calbiochem), 2μmol of ATP (Sigma), 10μg of yeast phosphomannose isomerase (Sigma), 10μg of yeast phosphoglucose isomerase (Sigma), 10μg of *Leukonostoc mesenteroides* glucose 6-phosphate dehydrogenase (Sigma) and 20nmol of NADP (Sigma) in a final volume of 1 ml of 0.1 M-Tris/HCl buffer, pH 8.1 (Gawehn, 1974). The conversion of NADP into NADPH was monitored at 340nm in a Beckman double-beam spectrophotometer. All assays were performed in duplicate. Reaction mixtures with no cell lysate, with boiled cell lysate or with D-glucose (Calbiochem) substituting D-mannose, and those from which either phosphomannose isomerase or both isomerases had been omitted, served as blanks.

Phosphorylation of mannan

Incorporation of ^{32}P from [γ-^{32}P]ATP (sp. radioactivity 2600Ci/mmol; New England Nuclear Corp.) into mannan (from baker's yeast; Calbiochem) or alkaline phosphatase (from *Escherichia coli*; Sigma)-treated (Sando & Neufeld, 1977) mannan was determined in whole fibroblast lysates. The reaction mixture (0.5 ml) contained 100μg of fibroblast protein, 2mg of mannan, 1μmol of [γ-^{32}P]-ATP (1430d.p.m./nmol), 2μmol of $MgCl_2$, 1μmol of NaF and 75μmol of glycylglycine, pH 7.0. After 2h incubation at 37°C, 0.125ml of 5 M-KOH was added, the mixture was heated in boiling-water bath for 40min, the precipitate formed was spun down (20min, 12000*g*, 4°C) and mannan was precipitated from the supernatant in 66% (v/v) ethanol (Algranati *et al.*, 1966). The precipitation was allowed to proceed for 16h at 4°C, the precipitate was washed twice in 66% ethanol, redissolved in 0.2ml of water and further purified by paper electrophoresis (90min at 9V/cm) in 0.05 M-pyridine/acetate buffer, pH 6.5 (Algranati *et al.*, 1966). The mannan-containing area was cut out from the electrophoretogram and the radioactivity was measured in a Tri-Carb liquid scintillation spectrometer. Reaction mixture with no cell lysate, with boiled cell lysate or those to which mannan was added only after completion of the reaction, served as blanks.

Electrophoretic analysis

Acid hydrolysis of the ethanol-precipitated mannan was performed in 2.5 M-trifluoroacetic acid (Sigma) in sealed vials for 3h at 105°C (Hasilik & Neufeld, 1980*b*). Mannose 6-phosphate, *O*-phospho-L-serine or *O*-phospho-DL-threonine (Sigma) were added as carriers (0.2μmol/vial). Acid was removed by freeze-drying. High-voltage electrophoresis (Hasilik & Neufeld, 1980*b*) was performed on Whatman no. 3MM paper in a Savant flat-plate electrophoresis apparatus, for 2.5h at 65V/cm at pH 5.3 (pyridine/acetic acid/water, 5:2:493, by vol.). Radioactivity was quantified in 1 cm^2 squares. Standards of mannose 6-phosphate, phosphoserine, phosphothreonine, ATP and P_i were located with phosphate reagent (Burrows *et al.*, 1952).

Results

Table 1 shows hexokinase activities toward D-mannose and D-glucose in fibroblast extracts from three patients with mucolipidosis II and from three normal controls. No significant differences were found between the two groups. The activity toward D-glucose was higher than towards D-mannose in both cell lines. Exclusion of either phosphomannose isomerase or both isomerases from the reaction mixture did not affect the reaction with glucose, but diminished the production of NADPH in the reaction with mannose to about 5% of that obtained with the complete reaction mixture, in both mucolipidosis-II patients and normal patients.

The incorporation of ^{32}P from [γ-^{32}P]ATP into mannan in cell homogenates of cultured skin fibroblasts from six normal controls, from two obligate heterozygotes for mucolipidosis II and from four patients with mucolipidosis II and two patients with mucolipidosis III is summarized in Table 2. No

Table 1. *Phosphorylation of* D-*mannose and* D-*glucose in fibroblast extracts*

Activities are expressed as nmol of hexose 6-phosphate formed/min per mg of protein.

Cell line	Substrate ...	Hexokinase activity: D-Mannose	D-Glucose
Control	1	1.95	10.2
	2	2.24	11.6
	3	2.38	13.3
Mucolipidosis-II	1	2.17	11.0
	2	1.69	10.4
	3	2.08	12.1

phosphorylation of mannan could be detected in cell extracts from the mucopididosis-II patients, and less than 10% of normal phosphorylating activity was determined in fibroblast lysates from the mucolipidosis-III patients. The rate of phosphorylation of mannan in cell lysates from the parents of mucolipidosis-II patients (obligate heterozygotes for mucolipidosis II) was 60–70% of that found for the normal controls. In normal cells, activity could be detected over the pH range 5.5–7.5, but no phosphorylation could be determined below pH 4.5 in any of the cell lines.

Table 2. *Incorporation of ^{32}P from [γ-^{32}P]ATP into mannan in fibroblast extracts*

Activities are expressed as nmol of ^{32}P incorporated into 1 mg of mannan/h per mg of protein.

Cell line	No.	Mannan kinase activity
Normal controls	6	2.25–4.02
Mucolipidosis-II heterozygotes	2	1.89–2.28
Mucolipidosis-II patients	4	0
Mucolipidosis-III patients	2	0–0.22

Fig. 1 illustrates the electrophoretic pattern of mannan and acid-hydrolysed mannan from reactions performed with normal and mucolipidosis-II fibroblasts. Alkaline phosphatase-pretreated mannan was used in these experiments. Incorporation of ^{32}P into mannan was observed in normal cell preparations, whereas the corresponding radioactivity determined in mucolipidosis-II cells was as low as

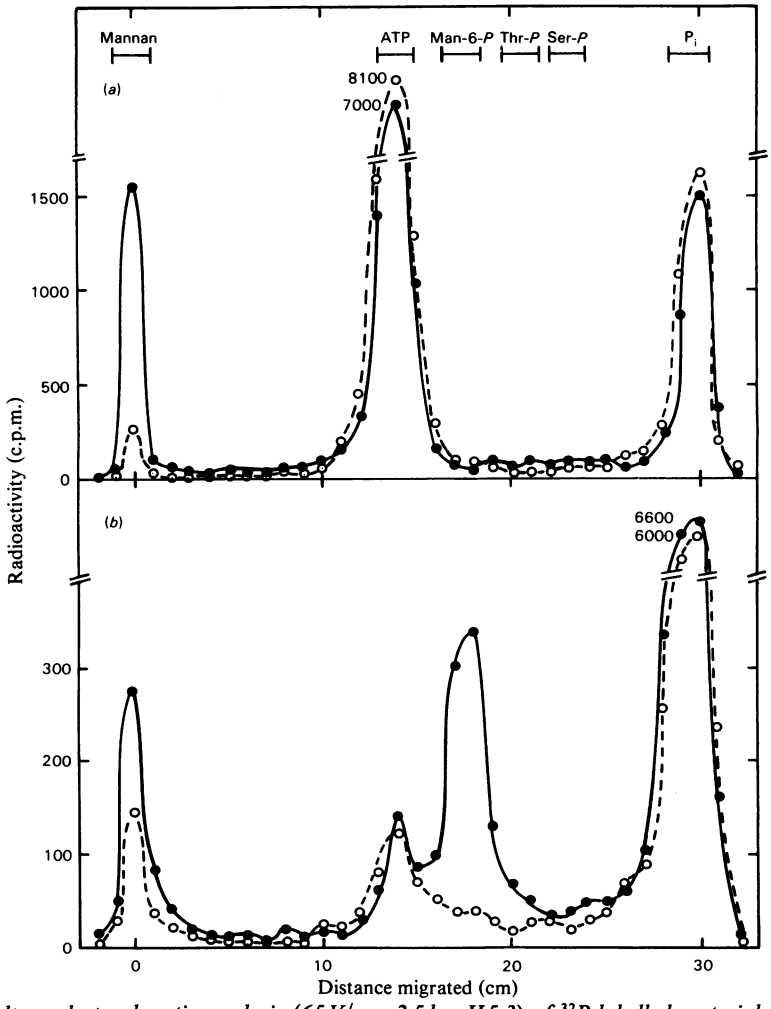

Fig. 1. *High-voltage-electrophoretic analysis (65 V/cm, 2.5 h, pH 5.3) of ^{32}P-labelled material in mannan (a) and acid hydrolysate (2.5 M-trifluoroacetic acid, 3 h, 105°C) of mannan (b) from incubations performed with normal (●) and mucolipidosis-II (○) fibroblast extracts*

Standards of ATP, mannose 6-phosphate (Man-6-*P*), phosphothreonine (Thr-*P*), phosphoserine (Ser-*P*) and inorganic phosphate (P_i) were located with phosphate reagent (Burrows *et al.*, 1952).

that of the reaction blanks. On the other hand, the pattern of radioactivity corresponding to ATP and P_i was similar in both cell lines. The hydrolysed mannan from incubations performed with normal cells yielded a radioactive band with the same mobility as mannose 6-phosphate, whereas no such product could be demonstrated with fibroblast lysates from patients with mucolipidosis II. No radioactivity corresponding to phosphothreonine or phosphoserine was observed, and that corresponding to ATP and free phosphate was similar in both cell lines.

Discussion

The present paper demonstrates a severe deficiency in phosphorylation of mannan in fibroblast extracts from patients with mucolipidosis II and mucolipidosis III. Although the content of protein in mannan is very low and accounts for only 0.4% (w/w), the yield of incorporation of ^{32}P into mannan, as determined with normal cells, is also low and could be explained by phosphorylation of amino acid residues. However, the high-voltage-electrophoretic pattern of acid-hydrolysed mannan, from incubations with normal fibroblasts, demonstrated a radioactive peak corresponding to mannose 6-phosphate, but no radioactivity that corresponds to either phosphoserine or phosphothreonine. Thus it appears that the product of this phosphorylating activity is a mannose 6-phosphate residue.

Since mannose 6-phosphate was identified as an essential component of the recognition marker of lysosomal enzymes, required for their transport into lysosomes and for their uptake by fibroblasts, and since lysosomal hydrolases synthesized by mucolipidosis-II fibroblasts are deficient in phosphorylated residues, it is reasonable to assume that the deficient phosphorylation of mannan in mucolipidosis II and mucolipidosis III reflects the aberrant phosphorylation of lysosomal enzymes in these disorders.

The absence of phosphorylation of mannan below pH 4.5 rules out the possibility that this is the activity of one of the lysosomal enzymes, many of which are known to have decreased activities in mucolipidosis-II and mucolipidosis-III fibroblasts. In view of our findings that phosphorylation of mannose residues on the polysaccharide mannan is deficient in mucolipidosis-II and mucolipidosis-III fibroblasts, whereas hexokinase activity toward free mannose appears to be normal, we suggest that the phosphorylation of mannose residues of lysosomal enzymes possibly takes place on either the glycoprotein itself or on the oligosaccharide chain before its transfer to the protein core. The deficiency of a mannosyl-glycoprotein phosphotransferase might be the primary defect in mucolipidosis types II and III.

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