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INTERGENIC COMPLEMENTATION AFTER FUSION OF FIBROBLASTS FROM DIFFERENT PATIENTS WITH β-GALACTOSIDASE DEFICIENCY

H.L. HOEKSEMA, O.P. VAN DIGGELEN and H. GALJAARD

Department of Cell Biology and Genetics, Erasmus University, Medical Faculty, P.O. Box 1738, Rotterdam (The Netherlands)

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Summary

Acid β-galactosidase from human liver consists, after gel filtration at pH 7.0, of a monomeric isoenzyme, β-galactosidase A and small amounts of a multimer, β-galactosidase B (Norden, A.G.W., Tennant, L. and O'Brien, J.S. (1974) J. Biol. Chem. 249, 7969—7976).

Our studies showed identical gel filtration patterns for β-galactosidase from human liver and cultured skin fibroblasts. Gel filtration in the buffer used for enzyme assays (pH 4.5) however, revealed primarily dimeric β-galactosidase, minor amounts of β-galactosidase A but no multimers. As the transitions between monomeric and dimeric β-galactosidase were reversible, dimeric β-galactosidase is apparently the enzymatically active isoenzyme.

The isoenzymes from patients with four different clinical variants of β-galactosidase deficiency showed altered aggregation patterns, although their molecular weights corresponded to the normal molecular weights.

It could be demonstrated that the restoration of β-galactosidase activity, which occurred after cell fusion of certain combinations of different β-galactosidase deficient fibroblasts, is not the result of intragenic complementation. Mixing experiments with cell-free extracts ruled out that the formation of dimeric β-galactosidase is required for complementation.

To explain the complementation a model is suggested involving two different genes: a structural gene responsible for the synthesis of the polypeptide chain and a second gene which is involved in a modification process.

Introduction

A deficiency of the acid forms of β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) is responsible for the autosomal recessive disease G_{M1}-gangliosidosis, where gangliosides and glycosaminoglycans are accumulated in

various tissues [2]. Several clinical forms of β-galactosidase deficiency have been described which differ in time of onset of the symptoms, the involvement of visceral organs and the presence of mental retardation [3—5].

Galjaard et al. [6] and Reuser et al. [7] showed that the gene mutation in the classical, infantile G_{M1}-gangliosidosis (type 1) and its juvenile form (type 2) differed from the mutations in three other clinical variants of β-galactosidase deficiency (type 3 [8] and adult type 4[9] and the patient described by Andria et al. [10]). This was shown by genetic complementation analysis in which cultured skin fibroblasts from different patients were fused yielding heterokaryons. The presence of the genetic information of both parental cells strains in these heterokaryons can give rise to restoration of β-galactosidase activity. So far, two complementation groups have been demonstrated.

An understanding of the mechanism of complementation requires knowledge about the molecular structure of β-galactosidase in control and patient's cell strains. In liver, two β-galactosidase isoenzymes have been demonstrated: a monomer, β-galactosidase A, and its multimeric aggregate β-galactosidase B [1,11].

In the present study we have used gel filtration techniques to analyse normal β-galactosidase, the residual activity in cultured skin fibroblasts of patients and the restored activity after genetic complementation. To explain this genetic complementation an intergenic model is proposed.

Materials and Methods

Cell and tissue materials. Skin fibroblasts were cultured in Ham's F10 medium, supplemented with 15% fetal calf serum and antibiotics as described earlier [12]. The cells were free of mycoplasma contamination judged by the method of Chen [13]. The β-galactosidase-deficient cell strains used were from patients with the infantile type 1 [6], the juvenile type 2 [14] or the adult type 4 [9] and from a patient recently described by Andria et al. [10]. Subcultures from early passages were harvested by trypsinization and cell homogenates were prepared by repeated freezing and thawing in the same buffer used for the gelfiltration. After centrifugation for 20 min at 1500 $\times g$, the supernatant fractions were used for biochemical analysis.

Cell fusion using 10^6 cells from each β-galactosidase-deficient strain was carried out as described [6].

Liver autopsy tissue was frozen 5—15 h after death and kept at −20°C until use. It was homogenized with a Waring Blendor in ice-cold buffer and centrifuged for 20 min at 1500 $\times g$. The supernatant fractions were separated by column chromatography.

Gelfiltration. Packing and elution of Sephacryl S200 superfine and Sepharose 6B (Pharmacia) columns (0.5 × 35 cm) were performed under gravity at 4°C.

Two different buffers were used: buffer I (10 mM sodium phosphate/10 mM NaCl, pH 7.0) and buffer II (0.1 M sodium acetate/0.1 M NaCl, pH 4.5), using the same buffer throughout the whole experimental procedure. Routinely 100 μl lysate, representing 10^6 cells, were applied to the columns and 100-μl fractions (Sephacryl S200) and 150-μl fractions (Sepharose 6B) were collected. The recovery of β-galactosidase activity after gelfiltration was about 50%. The

columns were calibrated by the method of Andrews [15] by using the Combithek calibration protein set from Boehringer (Mannheim). As markers were included: cytochrome *c* (M_r = 12 500), chymotrypsinogen *a* (25 000), albumin from hen egg (45 000), albumin from bovine serum (67 000), aldolase (158 000), catalase (240 000), ferritin (450 000). Dextran blue was used to estimate the void volume.

Interconversion of monomeric, dimeric and multimeric β-galactosidase. After gelfiltration on Sephacryl S200, the fractions containing the various isoenzymes were collected and concentrated. The buffer system was changed and the enzyme was incubated for 30 min at 37°C, followed by 60 min at 4°C. The enzyme activity was then measured and the remaining volume was applied to a Sephacryl S200 column, equilibrated with the other buffer system.

Enzyme assay. β-Galactosidase activity was assayed with 1 mM 4-methylumbelliferyl-β-D-galactopyranoside in buffer II by the method of Galjaard et al. [16].

Results

The most widely used buffer for gel filtration studies of acid β-galactosidase is a low ionic strength buffer at pH 7.0 (buffer I) [11,17]. However, enzyme activity is always measured at low pH, which might influence the aggregation forms of the enzyme present at pH 7.0.

The elution patterns of β-galactosidase in homogenates of normal human skin fibroblasts and normal human liver in buffer I are shown in Fig. 1A. Both patterns are similar and comprise a major peak, β-galactosidase A (M_r 65 000–75 000), and a minor peak, β-galactosidase B (M_r 600 000–800 000 on a

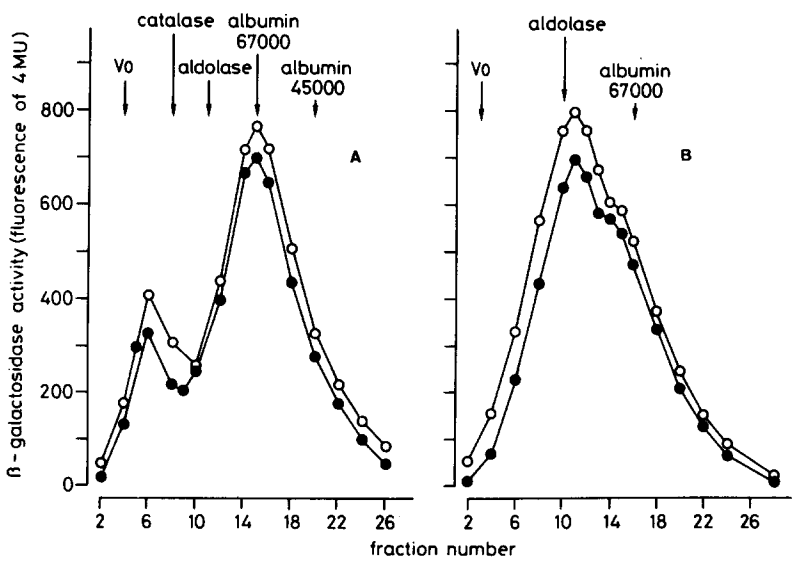

Fig. 1. Gelfiltration pattern of human liver and fibroblasts β-galactosidase, Sephacryl S200 elution patterns of homogenates from normal human liver (○———○) and cultured human skin fibroblasts (●———●). A, eluted in buffer I; B, eluted in buffer II.

Sepharose 6B column). In buffer II, β-galactosidase B has disappeared, and a peak with a molecular weight of 130 000—150 000 becomes prominent (Fig. 1B). The presence of minor quantities of β-galactosidase A is seen by the shoulder in the elution pattern.

To investigate whether the observed differences in the two buffer systems are based on reversible aggregation and dissociation phenomena, fractions isolated in one of the buffer systems were subjected to a second gel filtration in the other buffer (for details see Materials and Methods). The data showed that all of the changes in molecular weight are reversible except the multimeric isoenzyme cannot be generated from the M_r = 140 000 fractions. These findings indicate that we are dealing with the multimeric and dimeric aggregation forms of the monomeric β-galactosidase polypeptide. As transitions between monomer and dimer are reversible, most of the β-galactosidase is in the dimeric from during the enzyme assay at pH 4.5 in buffer II.

The isoenzymes in fibroblasts from patients with the infantile (type 1), juvenile (type 2) or adult (type 4) form of β-galactosidase deficiency had molecular weights indistinguishable from those of the normal isoenzymes. The aggregation patterns in buffer I differed from the normal enzyme (Table I). The aggregation forms in buffer II are similar, except for the infantile type 1.

The restored β-galactosidase activity after complementation between fibroblasts of the infantile or juvenile types and fibroblasts from either a patient with the adult type or the patient described by Andria et al. [10] was analysed by gel filtration. Fig. 2 shows the data for a fusion of infantile type 1 and adult type 4 cells. The heterokaryon homogenate shows after gel filtration in buffer I increased activity in both the fractions corresponding to the monomeric and multimeric β-galactosidase (Fig. 2). In buffer II, the dimeric and monomeric β-galactosidase were increased. Similar results were obtained in fusions between fibroblasts of the patient described by Andria et al. [10] and those of infantile type 1 or juvenile type 2 patients.

To investigate the possibility whether the increase in β-galactosidase activity after cell fusion is due to the formation of dimeric enzyme, studies with mixtures of cell free extracts were performed. The data presented in Table II show

TABLE I

AGGREGATION FORMS OF RESIDUAL β-GALACTOSIDASE ACTIVITY IN FIBROBLASTS FROM PATIENTS WITH G_{M1}-GANGLIOSIDOSIS

Monomer: β-galactosidase with molecular weight (M_r) 65 000—75 000; dimer: β-galactosidase with M_r = 130 000—150 000; multimer; β-galactosidase with M_r = 600 000—800 000.

	10 mM phosphate/10 mM NaCl buffer (pH 7.0)			0.1 M sodium acetate/0.1 M NaCl buffer (pH 4.5)		
	Monomer	Dimer	Multimer	Monomer	Dimer	Multimer
Control	++	—	+	+	++	—
Infantile Type 1	—	—	+	+	++	+
Juvenile Type 2	+	—	—	±	++	—
Type 'Andria'	+	—	—	+	++	—
Adult Type 4	+	—	—	+	++	—

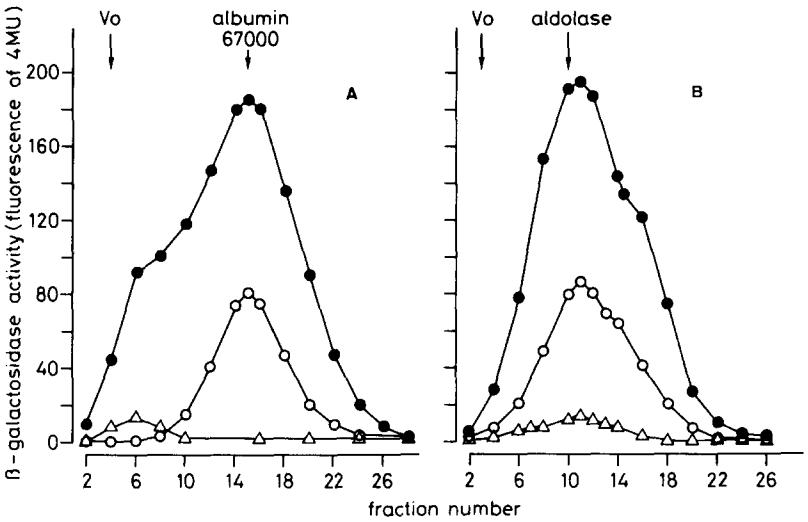

Fig. 2. Gelfiltration pattern of β-galactosidase after genetic complementation. Sephacryl S200 elution patterns of homogenates after fusion of: infantile (type 1) and adult (type 4) fibroblasts (●———●); type 1 and type 1 fibroblasts (△———△); and type 4 and type 4 fibroblasts (○———○). A, eluted in buffer I, pH 7.0; B, eluted in buffer II, pH 4.5.

that mixing of cell-free extracts of type 1 and type 4 fibroblasts, under conditions where monomeric β-galactosidase is prevailing (buffer I) and subsequently allowing dimers to be formed (buffer II) does not lead to increased β-galactosidase activity. Gel filtration of the mixture in buffer II showed that the β-galactosidase indeed exists predominantly in the dimeric form. Similar results were obtained when homogenates of type 2 were mixed with homogenates of type 4 fibroblasts or fibroblasts of the patient described by Andria et al. [10].

TABLE II

ATTEMPTED COMPLEMENTATION IN MIXED CELL HOMOGENATES OF TYPE 1 AND TYPE 4 CELLS

Equal amounts of homogenates of cells from the infantile and adult type were mixed in buffer I. The pH was adjusted to 4.5 with buffer II. After 30 min incubation at 37°C, followed by 60 min at 4°C the mixture was assayed for β-galactosidase activity. The protein content was determined according to Lowry et al. [27]. The aggregation state of the β-galactosidase was determined with a Sephacryl S200 column. 1 X 4: β-galactosidase activity after fusion of infantile (type 1) and adult (type 4) fibroblasts. The heterokaryons and cells used for the mixing experiments have been cultivated for the same period of time at the same cell density. This is essential for a meaningful comparison of the specific activities [28].

Cell strains	β-Galactosidase activity (nmol 4-methylumbelliferone/mg protein per h)
Infantile Type 1	3
Adult Type 4	44
Type 1 X Type 4	190
Type 1 + Type 4	23
Control	493

Discussion

Subunit structure of β-galactosidase

Human acid β-galactosidase in liver consists of a monomeric polypeptide, β-galactosidase A (M_r = 70 000) and a multimeric aggregate of the monomeric form, β-galactosidase B (M_r = 600 000—800 000) [1], under conditions of low ionic strength and neutral pH. Whether this multimeric form also contains additional subunits remaines uncertain.

The enzyme from human fribroblasts shows identical gel filtration patterns compared to that of human liver, indicating that the isoenzymes have the same subunit composition. At high ionic strength and acid pH (buffer II) dimeric β-galactosidase was the predominant form, whereas the multimeric form was absent.

Repeated gel filtration of isolated fractions in both buffers used indicated that dimeric β-galactosidase is the enzymatically active form, irrespective of whether monomers or multimers are isolated initially. In this light, characterization of separated β-galactosidase A and B [3,18] is meaningless.

A dimeric form of β-galactosidase has already been demonstrated in human liver in varying amounts depending on the experimental conditions [17,19] and in human small intestine [20].

The β-galactosidase isoenzymes from all four patients tested have molecular weights which are indistinguishable from those found in normal fibroblasts, suggesting that complete or nearly complete polypeptide chains are synthesized. However, β-galactosidase present in these patients shows aggregation patterns different from normal, indicating that we are dealing with structurally altered β-galactosidase. This is in agreement with immunological studies showing an increased amount of antigen per unit enzyme activity in adult type [21] and in infantile type β-galactosidase deficiency [22]. No difference in heat stability, K_m and pH optimum of β-galactosidase in normal, type 4 and type 1 × type 4 heterokaryons could be demonstrated [4].

Mechanism of complementation

For dimeric enzymes, Crick and Orgel [23] have postulated a model for intragenic complementation, based on interaction of defective subunits. The reversibility in the formation and dissociation of dimeric β-galactosidase in cell-free extracts made an experimental test of this model possible. As no complementation could be demonstrated, the Crick and Orgel model for intragenic complementation is not applicable.

An intragenic mechanism similar to α-complementation described for β-galactosidase deficient strains of *Escherichia coli* [24] is unlikely because of the presence of nearly normal amounts of immunological cross-reacting material in type 1 as well as in type 4 cells [21,22,25] and the identical heat sensitivity of restored β-galactosidase activity in heterokaryons and normal β-galactosidase [4,26].

The intergenic complementation model, proposed by O'Brien and Norden [21] in which they postulated that β-galactosidase B, the multimeric isoenzyme, contains a subunit not shared with β-galactosidase A can be ruled out. This model would predict that the increased β-galactosidase activity represents

exclusively multimeric enzyme; this is not observed.

We propose the following intergenic complementation model which is compatible with the experimental results. In one of the complementation groups, (e.g. in type 4 cells), a normal structural β-galactosidase gene is present, however, the lack of a second gene product results in defective β-galactosidase with abnormal aggregation properties. The β-galactosidase deficiency in the other complementation group, type 1 and type 2 G_{M1}-gangliosidosis, is thought to result from allelic mutations in the structural β-galactosidase gene. After fusion of cells from different complementation groups the modification factor or process can act on the defective β-galactosidase, resulting in a structurally normal β-galactosidase.

Enucleation studies, recently carried out in our laboratory, showed that the presence of the type 4 nucleus is required for complementation, whereas the cytoplasm of type 1 cells is sufficient [26]. These results are compatible with the proposed model for complementation.

Biochemical and gene localization studies are currently underway in our laboratory to establish this model.

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