Molecular defect in combined β-galactosidase and neuraminidase deficiency in man
(precursor β-galactosidase/leupeptin/corrective factor)


Department of Cell Biology and Genetics, Medical Faculty, Erasmus University, Rotterdam, the Netherlands

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ABSTRACT In normal human fibroblasts, an enzymically active 85,000-dalton precursor form of β-galactosidase is processed, via a number of intermediates, into a mature 64,000-dalton form. In addition there is an enzymically inactive 32,000-dalton component and its 54,000-dalton precursor. In fibroblasts from patients with a combined deficiency of β-galactosidase and neuraminidase these last two components are absent and hardly any mature β-galactosidase can be demonstrated. Nevertheless, in the mutant fibroblasts, precursor β-galactosidase is synthesized and processed normally. The excessive intralysosomal degradation that is responsible for the deficiency of mature β-galactosidase can be partially corrected by addition of the protease inhibitor leupeptin, which results in the accumulation of 85,000-dalton precursor β-galactosidase and of a partially processed 66,000-dalton form. When mutant cells were grown in the presence of a "corrective factor" purified from the medium of NH4Cl-stimulated cell cultures, both β-galactosidase and neuraminidase activities were restored to low control levels. The immunoprecipitation pattern was completely normal after addition of the corrective factor, and mature 64,000-dalton β-galactosidase accumulated in the mutant fibroblasts. We propose that the combined β-galactosidase/neuraminidase deficiency is caused by a defective 32,000-dalton glycoprotein which is normally required to protect β-galactosidase and neuraminidase against excessive intralysosomal degradation and to give these enzymes their full hydrolytic activity.

Acid β-galactosidase in human liver (1) and cultured fibroblasts (2) consists of a 70,000-dalton (Dal) monomeric form and an aggregate of the same polypeptide (600,000–500,000 Dal). Since the observation by Okada and O’Brien (3) that the autosomal recessive disease Gm1-gangliosidosis is due to a β-galactosidase deficiency, various patients with infantile, juvenile, and adult forms of this disease have been reported (4–7). Analyses of interspecies hybrids indicated that the β-galactosidase deficiency in the various forms of Gm1-gangliosidosis is based on a mutation of a structural gene on chromosome 3 coding for the enzyme polypeptide (8).

A number of patients have been described with a coexistent deficiency of β-galactosidase and neuraminidase (6, 7, 9, 10). Previous studies on fibroblasts from these types of patients had shown that the 10–15% residual β-galactosidase had normal kinetic properties (11) and that the aggregation of monomeric β-galactosidase was impaired (2). No evidence for a mutation of the structural locus was found (8).

Neuraminidase deficiency, however, also can occur without any abnormality of β-galactosidase, as in various types of patients with sialidosis (mucolipidosis I) (12–15). Complementation studies after somatic cell hybridization (10) indicated that three different gene mutations are responsible for the enzyme deficiencies in Gm1-gangliosidosis (β-galactosidase deficiency (β-gal−)), sialidosis (neuraminidase deficiency (neur−)), and combined β-galactosidase and neuraminidase deficiency (β-gal−/neur−).

The turnover time of β-galactosidase has been measured in different cell strains (16, 17). In normal fibroblasts and in Gm1-gangliosidosis cells, the enzyme has a half-life of approximately 10 days, whereas in β-gal−/neur− fibroblasts it is <24 hr. Subsequent studies revealed that this reduction is due to enhanced degradation of β-galactosidase; the enzyme is synthesized at a normal rate (18). It was also shown that both the β-galactosidase and neuraminidase activities in β-gal−/neur− fibroblasts could be restored by a "corrective factor" of a glycoprotein nature that is produced by normal fibroblasts and other mutant cells, including Gm1-gangliosidosis fibroblasts (19). Because the latter contain no measurable amount of β-galactosidase, a convenient source of this factor is the medium obtained when these mutant cells are cultured in the presence of ammonium chloride which diverts newly synthesized precursor forms of glycoproteins into the medium, thus enhancing their secretion (20–22).

In this paper we examine the molecular nature of the genetic defect responsible for β-gal−/neur−. Using immunoprecipitation studies on radiolabeled β-galactosidase and related components, we demonstrate the sequence of events involved in the processing of mature β-galactosidase from its precursor forms in control and mutant fibroblasts. We also show the molecular background of leupeptin inhibition of the excessive degradation of β-galactosidase in β-gal−/neur− cells and that of full correction by addition of the putative factor.

MATERIALS AND METHODS

Cell Culture. Human skin fibroblasts were maintained in Ham’s F10 medium (Flow Laboratories, McLean, VA) supplemented with 10% fetal calf serum and antibiotics. Fibroblasts from patients with the infantile or adult form of Gm1-gangliosidosis were kindly provided by H. Goldman (Montreal) and Y. Suzuki (Tokyo), respectively. Cells with isolated neur− were derived from a patient with classical mucolipidosis I (sialidosis) (from H. D. Bakker, Amsterdam). Two cell strains with β-gal−/neur− were obtained from patients described earlier (23, 24).

Preparation and Assay of NH4Cl-Induced Secretions. Normal culture medium above confluent cultures was replaced by serum-free medium supplemented with NH4Cl at a final concentration of 10 mM. Two days later the medium was collected.

Abbreviations: β-gal−, β-galactosidase deficiency or deficient; β-gal+/neur−, β-galactosidase and neuraminidase deficiency or deficient; β-gal−/neur−, neuraminidase deficiency or deficient; Dal, dalton.

* Permanent address: Dept. Biochemistry, Queen Elizabeth College, University of London.
and centrifuged (90 × g, 5 min) and enzymic activities were measured in 20-μl aliquots. The cells were harvested by trypsinization, rinsed in saline, and disrupted in distilled water. Enzymic analyses were carried out by using the appropriate 4-methylumbelliferyl substrates (Koch–Light) as described by Galjaard (25). Activities measured in 1 ml of culture medium are derived from approximately 10⁸ fibroblasts corresponding to 0.3 mg of cellular protein.

Enzyme Purification. Fibroblasts were grown to confluency in 200-cm² flasks and subcultured for 2 days in 40 ml of serum-free medium containing 10 mM NaHCl. The medium was collected and ultrafiltered to approximately 5 ml on an Amicon PM10 filter; then its pH was adjusted to 5.20 with 20 mM sodium acetate/0.1 M NaCl. The concentrate was applied to a 2.5-ml p-aminophenylthiogalactoside-CH-Sepharose affinity matrix (17). The column was washed with 20 mM sodium acetate, pH 5.2/1.0 M NaCl and eluted with 20 mM sodium acetate, pH 5.2/0.5 M NaCl containing 0.1 M γ-galactonolactone. The eluate was dialyzed against Dulbecco's phosphate-buffered saline and concentrated on an Amicon PM10 filter to a final volume of about 1 ml. The whole procedure was performed at 4°C.

Gel filtration studies of β-galactosidase were carried out on Sephacryl S-200 as described by Hoeksema et al. (2).

Uptake Studies. Cells were seeded at near confluency in 24-well plates (Costar) and cultured for 3–4 days. The purified β-galactosidase (2.5 × 10⁻⁸ unit) was added to the medium (1 unit hydrolyzes 1 μmol of 4-methylumbelliferyl-β-D-galactopyranoside per min). After 16-hr incubation in the presence of exogenous enzyme, the cells were cultured in fresh medium for 2 days prior to analysis. Where appropriate mannose-6-phosphate was used at a final concentration of 1 mM.

Immunoprecipitation of Labeled β-Galactosidase. This was carried out by using purified specific immunoglobulins immobilized to Sepharose-4B beads. The rabbit antiserum used for their production was raised against a highly purified human placental β-galactosidase prepared by affinity chromatography. It was virtually homogeneous and showed one 64,000-Dal band on polyacrylamide gel electrophoresis, corresponding to the known size of mature human β-galactosidase with traces of a smaller molecule (30,000 Dal). The amount of coupled IgG that precipitated 95% of the β-galactosidase in the cells was judged from a previously determined enzyme activity/IgG ratio.

Normal and mutant fibroblasts were grown in 75-cm² flasks for 2 weeks, to a cell density equivalent to 1.5 mg of protein per flask. At 3–4 days before labeling, Ham's F10 medium was replaced by Dulbecco's modification of Eagle's medium (Flow Laboratories, McLean, VA) supplemented with 10% fetal calf serum and antibiotics. Prior to labeling, the cultures were rinsed and incubated for 1 hr with 6 M of the medium free of leucine to induce the depletion of the intracellular leucine pool. The labeling medium (6 ml) was this same medium supplemented with 0.2 ml of dialyzed fetal calf serum and 0.2 ml of [4,5⁻¹³C]leucine (0.2 mCi; 135 Ci/μmol; Amersham Radiochemical Center; 1 Ci = 3.7 × 10¹⁰ becquerels). After incubation in the presence of the label, the cells were harvested and processed according to the method of Hasilik and Neufeld (20).

Polyacrylamide Gel Electrophoresis and Fluorography. Electrophoresis in the presence of NaDodSO₄ was performed on 12% slab gels according to Laemmli (26), except that the ratio of acrylamide to methylene-bisacrylamide was changed to 39:1. The gels were prepared for fluorography as described (27). [¹⁴C]methyl-labeled protein molecular weight standards (from Amersham Radiochemical Center) were: phosphorylase B, 92,000; bovine serum albumin, 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000.

Protease Inhibition. In these experiments leupeptin (Sigma) was included in the medium above normal and mutant fibroblasts at a final concentration of 0.02 mM. The incubation period varied between 2 and 5 days.

Correction Studies. The corrective factor from G₄⁺gαnia1-gangliosidosis fibroblasts was prepared as described (19). β-gal⁻/neur⁻ fibroblasts were incubated with concentrated corrective factor for 2 days in the presence of [³H]leucine and the radiolabeled products were then examined by NaDodSO₄ electrophoresis.

RESULTS

Because the β-galactosidase deficiency in β-gal⁻/neur⁻ cells is due to enhanced degradation (7, 17), the effect of leupeptin, a thiol protease inhibitor, was studied. Addition of 0.02 mM leupeptin to the culture medium for 5 days resulted in an increase in β-galactosidase activity from 40 and 47 nmol/hr per mg of protein in two different β-gal⁻/neur⁻ cell strains (from patients described in refs. 23 and 24) to 305 and 315 nmol/hr per mg. This is a restoration to low control levels (activity range in normal fibroblasts is 350–1,650). The effect of leupeptin on neuraminidase activity was less clear. Only in one cell strain (23) was there a significant increase and that only reached 10% of normal levels.

In medium from cultures of normal and different types of mutant fibroblasts, hardly any β-galactosidase activity could be detected (0.3–0.4 nmol/hr per ml). Addition of NH₄Cl increased the extracellular β-galactosidase activity to 10–14 nmol/hr per ml for both β-gal⁻/neur⁻ fibroblasts and control cells. Such increase did not occur for G₄⁺gαnia1-gangliosidosis fibroblasts. Neuraminidase activity could not be detected in the medium from any of the cells tested. The ability of β-gal⁻/neur⁻ cells to synthesize β-galactosidase precursor therefore is not impaired. This extracellular β-galactosidase was purified from both normal and β-gal⁻/neur⁻ culture media by affinity chromatography. Comparison of the two preparations showed them to be identical with regard to a number of criteria.

Gel filtration studies indicated an apparent size of 80,000–90,000 Dal, consistent with this being the precursor form of the enzyme. Both samples had a pH optimum of 3.9 and a K₅₀ of 1.4 mM, compared to values of 4.3 and 0.25 mM for the mature intracellular enzyme.

Both preparations were taken up from medium from G₄⁺gαnia1-gangliosidosis fibroblasts, and this uptake could be strongly inhibited by 1 mM mannose-6-phosphate (Table 1). A similar amount of β-galactosidase was ingested after a 16-hr incubation period when either preparation was used, and the levels in the recipient cells remained unchanged when cultured for a further 48 hr in fresh medium. However, when purified β-galactosidase precursor from control fibroblasts was administered to β-gal⁻/neur⁻ fibroblasts, no increase in enzyme activity could be detected after a 16-hr incubation. The excessive degradation of β-galactosidase in β-gal⁻/neur⁻ cells therefore is not due to obvious mutant properties of the β-galactosidase precursor.

In order to study possible molecular differences in the enzyme components, immunoprecipitation was carried out on radiolabeled normal and mutant cells and on media collected from the different cultures after NH₄Cl treatment. The constituent polypeptides were then examined by gel electrophoresis under reducing and denaturing conditions.

Control Fibroblasts. After incubation for 24 hr in the presence of [³H]leucine, control fibroblasts showed four radiolabeled immunoprecipitable components (Fig. 1). Two of these corresponded to the components of the placental preparations that showed a prominent 64,000-Dal form and traces of a 32,000-Dal form in Coomassie blue-stained gels. The 85,000-Dal precursor form that was also seen and the 64,000-Dal mature β-
galactosidase were enzymically active. The 51,000- and 32,000-Dal components were inactive, as can also be deduced from their presence in Gm1-gangliosidosis fibroblasts which have less than 1% enzyme activity (unpublished data). After NH4Cl stimulation the immunoprecipitable forms in the medium had apparent sizes of 88,000 and 54,000 Dal but the cells lacked the 64,000- and 32,000-Dal components. This suggests that the medium forms are precursors of the mature 64,000-Dal β-galactosidase and of the enzymically inactive 32,000-Dal component.

The sequence of events and the relationship among these different constituents were clarified in pulse-chase experiments. After a 3-hr pulse, almost all immunoprecipitable radioactivity was present in the 85,000-Dal form of β-galactosidase (Fig. 1). After a 5-hr chase this band disappeared and transient intermediates gave rise to the 64,000-Dal mature form that was fully established over a period of 3 days. The 32,000-Dal component, which was not seen after the 3-hr pulse, became evident after the shortest period of chase with a concomitant disappearance of the 54,000-Dal component. A 51,000-Dal component was visible during the whole period of the pulse-chase experiment.

**β-gal-*/neur-** fibroblasts. Corresponding immunoprecipitation experiments with radiolabeled β-gal-/neur- fibroblasts showed clear differences from control cells. Intracellularly, the mature 64,000-Dal β-galactosidase was barely visible and the 32,000-Dal component was absent (Fig. 2). As with the controls, 85,000- and 51,000-Dal bands were visible. After NH4Cl stimulation the intracellular pattern remained unchanged. Extracellularly, unlike the control, the 54,000-Dal band was absent and the only detectable constituent was 88,000 Dal.

In pulse-chase experiments on β-gal-/neur- cells the labeling pattern after 3 hr was similar to that in control cells. After

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**Table 1. Uptake of β-galactosidase precursor purified from control medium and β-gal-/neur- medium by human mutant fibroblasts**

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Incubation</th>
<th>No addition</th>
<th>With β-galactosidase from control medium</th>
<th>With β-galactosidase from β-gal-/neur- medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-gal*</td>
<td>16 hr</td>
<td>3.7</td>
<td>13.0</td>
<td>15.1</td>
</tr>
<tr>
<td></td>
<td>16 hr followed by 46-hr chase</td>
<td>3.3</td>
<td>12.6</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td>16 hr in presence of 1 mM Man-6-P</td>
<td>3.0</td>
<td>3.9</td>
<td>3.8</td>
</tr>
<tr>
<td>β-gal-/neur-</td>
<td>16 hr</td>
<td>40.5</td>
<td>42.0</td>
<td>39.5</td>
</tr>
</tbody>
</table>

*From Gm1-gangliosidosis.

†Each value is the mean of two or three separate experiments.

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**Fig. 1. NaDodSO4/polyacrylamide gel electrophoresis of β-galactosidase from normal fibroblasts and culture medium immunoprecipitated with antiserum after [3H]leucine labeling. Lanes: A, placental β-galactosidase (40 μg) stained with Coomassie blue; B, radiolabeled marker proteins; C, control fibroblasts labeled for 24 hr; D, control fibroblasts labeled for 24 hr in the presence of NH4Cl; E, medium from control cells labeled for 24 hr in the presence of NH4Cl. Lanes F–I show pulse labeling of β-galactosidase from control fibroblasts: F, 3-hr labeling; G, 3-hrs labeling followed by 5-hr chase in the presence of unlabeled leucine; H, 3-hr labeling and 20-hr chase; I, 3-hr labeling and 70-hr chase. Numbers show sizes in kDal.**

**Fig. 2. NaDodSO4/polyacrylamide gel electrophoresis of β-galactosidase from β-gal-/neur- fibroblasts and culture medium immunoprecipitated with antiserum after [3H]leucine labeling. Lanes: A, radiolabeled marker proteins; B, β-gal-/neur- cells labeled for 24 hr; C, β-gal-/neur- cells labeled for 24 hr in the presence of NH4Cl; D, medium from β-gal-/neur- cells labeled for 24 hr in the presence of NH4Cl. Lanes E–H show pulse labeling of β-galactosidase from β-gal-/neur- fibroblasts: E, 3-hr labeling; F, 3-hr labeling followed by 5-hr chase; G, 3-hr labeling and 20-hr chase; H, 3-hr labeling and 70-hr chase.**
a 5-hr chase, when 85,000-Dal precursor β-galactosidase was still present, a 66,000-Dal form appeared (Fig. 2). Subsequently, both these bands disappeared but, unlike with the controls, mature 64,000-Dal β-galactosidase did not accumulate in significant quantity. Another feature was the absence of the 32,000-Dal component in β-gal"/neur" cells during the whole period of chase, whereas the 51,000-Dal band was visible, as in controls.

Treatment with leupeptin led to an accumulation of both 85,000-Dal precursor β-galactosidase and of a partially processed 66,000-Dal form in β-gal"/neur" cells and in control fibroblasts (Fig. 3).

Addition of the corrective factor (19) had an effect quite different from that of leupeptin: a complete normalization of the β-galactosidase labeling pattern occurred (Fig. 3). There was no accumulation of precursor form but instead, as in control cells, this was rapidly converted to the mature 64,000-Dal β-galactosidase which seemed to be resistant to excessive intralysosomal degradation.

**DISCUSSION**

Previous studies (2, 7, 17) indicated that the 10–15% residual β-galactosidase activity in β-gal"/neur" fibroblasts has normal properties but that there is an excessive intralysosomal degradation of β-galactosidase, shortening its half-life to about 1/10th (17, 18). The present paper shows that, in normal human fibroblasts, mature 64,000-Dal β-galactosidase is produced via an 85,000-Dal precursor form. Similar observations have been made for β-galactosidase in mouse macrophages (28). We have shown that in β-gal"/neur" fibroblasts the 85,000-Dal form is synthesized normally and that all properties tested are similar to those of precursor β-galactosidase in control cells. The excessive degradation of β-galactosidase in β-gal"/neur" fibroblasts therefore cannot be due to an abnormality of the precursor form.

In immunoprecipitation studies the main mutant characteristics of β-gal"/neur" cells are a decreased amount of mature β-galactosidase and the absence of a nonenzymatic 32,000-Dal component. Pulse-chase experiments in the mutant cells demonstrated conversion of the 85,000-Dal precursor to a 66,000-Dal form. Both forms disappear within 20 hr without the appearance of the mature 64,000-Dal β-galactosidase, which became apparent in control cells.

Addition of leupeptin to both normal and β-gal"/neur" fibroblasts results in an increase of the 85,000-Dal precursor and of the 66,000-Dal form. This suggests that during normal processing the 85,000-Dal precursor may be degraded to some extent by intralysosomal proteases. The accumulation of the 66,000-Dal form possibly is due to leupeptin inhibition of the last maturation step to the 64,000-Dal form [like β-hexosaminidase (29)]. In experiments not reported here we found that the effect of the corrective factor was not inhibited by leupeptin. It is therefore unlikely that this factor is involved in this final maturation step. Instead, the corrective factor seems to protect β-galactosidase monomer against excessive degradation and, at the same time, enables its aggregation into the high molecular weight forms that were absent from β-gal"/neur" fibroblasts (2).

Our immunoprecipitation studies demonstrate complete lack of a 32,000-Dal component that is present in control cells (Figs. 1 and 2) and in other mutant cells (unpublished data). Similarly, in the culture medium of β-gal"/neur" cells a 54,000-Dal component is absent. Together with the results of pulse-chase experiments, this indicates that the latter larger form is a precursor of the 32,000-Dal component. The exact relationship between intracellular 51,000- and 54,000-Dal forms is not yet understood. Neither of them has β-galactosidase activity but apparently they are copurified with the precursor and mature forms of β-galactosidase.

We propose that the 32,000-Dal component is the genetically defective factor causing the combined β-galactosidase/neuraminidase deficiency and is identical to the corrective factor described by Hoogeveen et al. (19). It is able to convert the β-galactosidase labeling pattern completely to normal (Fig. 3). Unlike leupeptin, it allows complete processing of the precursor form to a 64,000-Dal mature β-galactosidase which can accumulate normally.

The 32,000-Dal glycoprotein lacking in “I-cell” disease (19) apparently plays a role in a final intralysosomal step leading to protection of β-galactosidase against excessive proteolytic degradation. It also stabilizes neuraminidase but, unlike the case with β-galactosidase, the presence of the 32,000-Dal glycoprotein seems to be essential to neuraminidase’s catalytic activity. After our report that leupeptin results in an increased activity of both β-galactosidase and neuraminidase in one cell strain (7), Suzuki et al. (30) did not find an effect of protease inhibition on neuraminidase in cells from their β-gal"/neur" patients. These observations and our analyses of the corrective factor in different types of β-gal"/neur" fibroblasts (unpublished data) point to molecular heterogeneity within this group of patients.

It may well be that the 32,000-Dal glycoprotein is normally required to unite β-galactosidase monomers and neuraminidase in a complex attached to the lysosomal membrane. Further studies on the nature of this mutation which affects the protection of lysosomal enzymes against the aggression of neighboring “colleague enzymes” are likely to give more insight in the normal cell biology of lysosomal enzymes.

**FIG. 3.** Effects of leupeptin and corrective factor on the NaDodSO4/polyacrylamide gel electrophoresis patterns of immunoprecipitated radiolabeled β-galactosidase from control fibroblasts and β-gal"/neur" cells. Lanes: A, radiolabeled marker proteins; B, control fibroblasts; C, control fibroblasts plus leupeptin; D, β-gal"/neur" cells; E, β-gal"/neur" cells plus leupeptin; F, β-gal"/neur" fibroblasts plus corrective factor. The cells were grown in the presence of leupeptin or factor for 2 days.
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