A RECOGNITION MARKER REQUIRED FOR UPTAKE OF A LYSOSOMAL ENZYME BY CULTURED FIBROBLASTS

Scot Hickman, Larry J. Shapiro, and Elizabeth F. Neufeld

National Institute of Arthritis, Metabolism, and Digestive Diseases
National Institutes of Health, Bethesda, Maryland 20014

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N-acetyl-β-hexosaminidase secreted by cultured skin fibroblasts from normal individuals, or from patients with mucopolysaccharidoses, is effectively captured by fibroblasts derived from patients with Sandhoff disease. On the other hand, N-acetyl-β-hexosaminidase from several other sources (including placenta, urine, and secretions of fibroblasts from patients with mucolipidosis II or III) is taken up to a much lesser extent. The uptake of the normal fibroblast hydrolase can be abolished by periodate oxidation under conditions that do not affect its catalytic activity, stability, or binding to Concanavalin A - Sepharose. These results suggest that uptake into fibroblasts requires a specific marker on the enzyme.

The uptake of certain lysosomal enzymes into cultured fibroblasts is so efficient as to suggest a selective recognition mechanism. When α-L-iduronidase is added to culture medium surrounding Hurler fibroblasts, nearly half the enzymatic activity can be recovered intracellularly 48 hours later (1,2). The α-L-iduronidase is presumably taken up by "adsorptive pinocytosis" (3), which implies a recognition marker on the enzyme, a receptor on the fibroblast membrane, and concentration of the enzyme against a gradient. This process contrasts with "fluid" or "bulk" pinocytosis, a non-selective uptake of medium and its solutes, requiring no specific interaction with the cell membrane (3).

The manner in which a particular hydrolase might be captured is not readily predicted. For instance, the N-acetyl-α-glucosaminidase purified from human urine is taken into Sanfilippo B fibroblasts adsorptively (4) whereas the same enzyme purified from human placenta is taken up non-adsorptively (5). Whatever the mechanism of uptake from culture medium, the enzymes are eventually sequestered into lysosomes, as shown histochemically for β-glucuronidase from human liver (6) and for horseradish peroxidase (7) (thought to enter by adsorptive and fluid pinocytosis, respectively).

We previously presented data suggesting that in the heritable disorder, mucolipidosis II (I-cell disease) the mutation affects the recognition marker on the hydrolase, thereby preventing adsorptive pinocytosis (2). Analogous observations have been made for a milder genetic disease, mucolipidosis III (pseudoHurler polydystrophy) (8).

We now present more direct evidence for a recognition marker that is required for the capture of a hydrolase by fibroblasts.

METHODS

The system chosen for study is the uptake of N-acetyl-β-hexosaminidase (2-acetamido-2-deoxy-β-hexoside acetamidodeoxyhexohydrolase, EC 3.2.1.52; designated below as "hexosaminidase") into fibroblasts derived from patients with the 0 variant of Tay-Sachs disease ("Sandhoff disease"), which are almost completely devoid of that enzymatic activity (9). Implicit in this experimental design is the assumption that although the mutation in Sandhoff disease prevents the formation of active hexosaminidase, it affects neither the uptake nor the catabolism of hexosaminidase that is supplied exogenously.

Methods for the maintenance of cell cultures and for the harvesting of serum-free secretions have been described previously (2). Hexosaminidase preparations were routinely dialysed against 0.01 M sodium phosphate, pH 6.0 containing 0.15 M NaCl (Buffer A) and stored in that buffer. A unit of hexosaminidase is defined as the activity catalysing the hydrolysis of one μmole of p-nitrophenyl N-acetyl-β-glucosaminide (Calbiochem) per hour at 37°, in a mixture containing enzyme, 0.005 M substrate, and buffer (0.02 M Na_2HPO_4 that had been brought to pH 4.4 with citric acid) in a total volume of 0.5 ml.

For measurement of uptake, 2 or more units of hexosaminidase in one ml of Buffer A were mixed with 5 ml of medium, filtered through a Millipore membrane into a 100 mm Falcon Petri plate containing ca. 10^6 fibroblasts (0.5 mg cell protein) from a Sandhoff disease patient. Internalized enzyme

activity was measured approximately 44 hours later as described (2).

Identification of the A and B isozymes of hexosaminidase was performed by stepwise elution from microcolumns of DEAE-cellulose (adapted from 10), or by polyacrylamide gel electrophoresis (stacking gel, pH 6.7, 2.5%; separation gel, pH 4.3, 7.5%; personal communication from Dr. John Tallman, NINDS, Bethesda) followed by elution of the enzyme from the sliced gel.

Fibroblast secretions in Buffer A were subjected to oxidation with 0.01 M sodium metaperiodate at 4°, in the dark, for specified periods of time. The reaction was terminated by addition of excess ethylene glycol, and soluble oxidation products as well as residual reagents were removed by dialysis against the above buffer.

Binding to concanavalin A was determined by filtration of the fibroblast secretions in Buffer A to which NaCl had been added to 1.0 M (final pH 5.3) over a microcolumn of Concanavalin A-Sepharose 4B (Pharmacia) prequilibrated with the same buffer, and determination of hexosaminidase activity in the effluent.

RESULTS AND DISCUSSION

Uptake of hexosaminidase - The enzyme activity present in secretions of fibroblasts derived from normal individuals is taken up by Sandhoff disease fibroblasts (2). Of the activity applied to the medium (2-3 units), about 10% was recovered within the cells 44 hours later in most experiments. However, there was considerable and poorly understood variability between cell lines, or between successive harvests from one cell line, so that uptake ranged from 3% to 25%. Uptake within that range was found for hexosaminidase activity extracted from normal fibroblasts by freeze-thawing, or from secretions of fibroblasts derived from at least one patient with each of the following lysosomal storage diseases: classical Tay-Sachs disease (a deficiency of the A isozyme of hexosaminidase), the Hurler, Hunter, Sanfilippo A and B

and Maroteaux-Lamy syndromes, and β-glucuronidase deficiency disease. There was no correlation between the uptake of hexosaminidase from fibroblast secretions and the proportion of A and B isozymes therein, since the B-isozyme secreted by Tay-Sachs fibroblasts, and the A-isozyme isolated from normal secretions by chromatography on DEAE-cellulose, were taken up equally well.

On the other hand, hexosaminidase elaborated by fibroblasts from two patients with mucolipidosis II and one patient with mucolipidosis III entered Sandhoff disease fibroblasts to a barely detectable extent (1% or less of the applied dose). Hexosaminidase from the following sources was likewise taken up poorly, if at all: human placental homogenate; highly purified preparations of human placental hexosaminidase A and B isozymes (a gift of Dr. John Tallman); unfractionated human urinary proteins; purified hexosaminidase A from human urine (a gift from Dr. Eugene Grebner, Albert Einstein Medical Center, Philadelphia); partially purified hexosaminidase from jack bean meal (11). Exclusion of hexosaminidase A and B isozymes purified from human liver has been reported (12,13). One may presume that Sandhoff disease fibroblasts also fail to take up the hexosaminidase of fetal calf serum, which is present in the culture medium to the extent of 1 unit per ml.

Since uptake was not altered in serum-free medium, the components of fetal calf serum are neither required for nor inhibitory to the adsorptive pinocytosis of hexosaminidase of fibroblast secretions.

Periodate oxidation - Fibroblast secretions treated with sodium meta-periodate for 8 hours under conditions specified in METHODS retained full hexosaminidase activity. However, uptake of hexosaminidase activity into Sandhoff disease fibroblasts decreased rapidly with increased oxidation time; from Fig. 1, the half-life of the potential for uptake can be estimated to be 20 minutes.

We considered the possibility that the apparent decrease in uptake was in fact a lowered intracellular recovery due to increased lability of the periodate-treated hexosaminidase. However, increased lability in

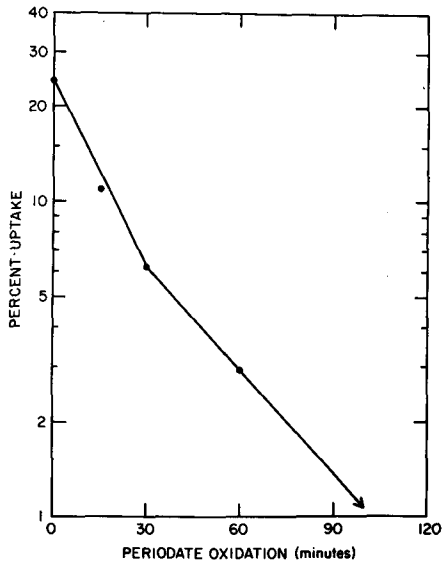

Fig. 1. Effect of periodate oxidation of fibroblast secretions on subsequent uptake of hexosaminidase. The uptake is expressed as percent of the applied dose (2.5 units) that was recovered within the Sandhoff fibroblasts after 40 hours. The preparation selected for this experiment contained hexosaminidase that was taken up exceptionally well.

the medium is ruled out since at least 75% of periodate-treated hexosaminidase applied to Sandhoff cultures was still present in the medium two days later. Accelerated intracellular catabolism also appears unlikely in light of the following experiment. Secretions were oxidized with periodate for 30 minutes, with the result that the apparent uptake in 48 hours was reduced from 8% in the untreated preparation to 2% in the treated one. In spite of this quantitative difference, the periodate-treated hexosaminidase appeared within the cells with the same linear kinetics, and persisted intracellularly with the same half-life (7 days) as the untreated enzyme.

These results imply that the periodate treatment diminishes the ability of hexosaminidase to enter cells, presumably because of the oxidation of some structural feature requied for adsorptive pinocytosis but not required for catalytic activity or for stability. Since periodate preferentially

oxidizes carbohydrate residues, and since hexosaminidase, as other lysosomal hydrolases, is a glycoprotein[1], we speculate that the uptake marker might be a carbohydrate side chain on the enzyme.

Hexosaminidase of fibroblast secretions was found to be quantitatively adsorbed to concanavalin A-Sepharose (but not to Sepharose itself), from which it could be eluted with 0.5 M α-methylmannoside. Binding to concanavalin A probably requires the presence of terminal and/or internal α-mannosyl residues (14). In an experiment analogous to that shown in Fig. 1, the binding of hexosaminidase to concanavalin A remained unaffected by exposure to periodate for two hours, and decreased slowly thereafter so that half the enzyme was still adsorbed after four additional hours of oxidation. As before, catalytic activity was not affected. The difference in the rates of destruction suggests that the concanavalin A binding site on the enzyme is structurally distinct from the uptake marker, and that both are distinct from the catalytic site.

The uptake of lysosomal hydrolases by human fibroblasts may be based on a principle similar to the uptake of plasma proteins by rat hepatocytes, which requires exposed β-galactosyl termini (15). It is unlikely, however, that the recognition markers for uptake into fibroblasts and hepatocytes are identical, for addition of 1.5 mg/ml of asialofetuin (which has the marker for uptake by hepatocytes) to the culture medium failed to inhibit the entry of hexosaminidase into Sandhoff disease fibroblasts.

The observation that catalytic activity and uptake into cells are distinct and separable properties of a lysosomal hydrolase is of importance to enzyme replacement therapy for lysosomal storage disorders. Administration of highly purified enzymes that fail to enter the affected cells must be considered a futile therapeutic endeavor. On the other hand, preparation of enzymes with the uptake marker (perhaps by chemical coupling of marker

[1] Hexosaminidase A and B purified from human placenta contain approximately 10% carbohydrate (Srivastava, S. K., Coligan, J., Yoshida, A., and Beutler, E., personal communication).

to protein) may permit targeting of the enzymes to the lysosomes of the relevant cells. The possibility of such an approach has been suggested by Rogers and Kornfeld (16), who showed that lysozyme and albumin were effectively taken up by rat liver if they were first modified by attachment of appropriate oligosaccharides.

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