

Subcellular Location of Two Enzymes Involved in the Synthesis of Phosphorylated Recognition Markers in Lysosomal Enzymes*

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Phosphorylated recognition markers in lysosomal enzymes appear to be synthesized by transfer of α -N-acetylglucosamine 1-phosphate groups to C6 hydroxyl of mannose residues in glycosylated enzyme precursors and a subsequent hydrolysis from the diester groups of the N-acetylglucosamine residues. The transfer and the diesterase activities were studied in subcellular fractions of rat liver. Both activities fractionated like the Golgi marker galactosyltransferase.

Lysosomal enzymes contain mannose 6-phosphate residues that act as recognition markers for receptor-mediated endocytosis of lysosomal enzymes (for review see Ref. 1). Recently, phosphorylated high mannose oligosaccharides have been isolated from lysosomal enzymes with phosphate groups resistant to alkaline phosphatase (2-4). Structural analysis of these oligosaccharides with blocked phosphate groups revealed that they contain phosphodiester with the phosphate linking C-1 of N-acetylglucosamine to C-6 of mannose. It has been suggested that such oligosaccharides are precursors of the phosphorylated recognition marker. Subsequently, a N-acetylglucosaminyl phosphodiesterase uncovering mannose 6-phosphate residues has been found in microsomal fractions in several cell types (5, 6). The enzyme has been partially purified from human placenta (6). Using rat liver homogenates, Varki and Kornfeld (5) have demonstrated that the enzyme is enriched in the Golgi fraction. We have been able to transfer *in vitro* N-acetylglucosaminyl 1-phosphate from UDP-N-acetylglucosamine to a lysosomal enzyme.¹ The transfer activity was found also in membranes from normal cultured human fibroblasts, but not in those from two mucopolidosis II (I-cell disease) fibroblast lines.¹ The finding of α -N-acetylglucosaminyl phosphodiesterase in membranes prepared from various cells (5, 6), the enrichment of the activity in the Golgi fractions (5), and finally the possibility of assaying also the phosphorylation of lysosomal enzymes prompted us to study in parallel the subcellular location of the two enzymes involved in the formation of the phosphorylated recognition marker in lysosomal enzymes.

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¹ A. Hasilik, A. Waheed, and K. von Figura (1981) *Biochem. Biophys. Res. Commun.* **98**, 761-767.

EXPERIMENTAL PROCEDURES

Subcellular Fractionation—Female Wistar rats were purchased from Hannoversche Versuchstieranstalt. After starving a rat overnight, the liver was perfused with 0.3 M sucrose under anesthesia. Golgi membrane and smooth and rough endoplasmic reticulum membranes were isolated by the procedure of Sandberg *et al.* (7). Sucrose gradient fractions thus obtained were diluted to 0.25 M sucrose by water and membranes of each fraction were pelleted by centrifugation at 105,000 $\times g$. The membrane pellet was suspended in 0.3 M sucrose. The fractions were characterized by determining the activity of several marker enzymes and the ratio of RNA to protein. Protein was determined as described by Lowry *et al.* (8) with bovine serum albumin as standard. RNA was assayed by the procedure of Fleck and Begg (9).

Enzyme assays—UDP-N-acetylglucosamine:lysosomal enzyme N-acetylglucosamine-1-phosphate transferase was assayed using as substrates [β -³²P]UDP-N-acetylglucosamine (2 Ci/mmol) and the precursor form of β -hexosaminidase, which was partially purified from secretions of cultured human fibroblasts and subjected to dephosphorylation. The preparation of the substrates is described elsewhere.¹ In the transfer reaction, fractions of rat liver membranes, 100 μ g of protein, were incubated with 25 μ M [β -³²P]UDP-N-acetylglucosamine and 0.25 unit of β -hexosaminidase in the presence of 20 mM sodium phosphate, 5 mM MgCl₂, 1.5% (v/v) Triton X-100, and 2 mM AMP, final pH 6.7, in a total volume of 40 μ l for 6 h at 37 °C. The reaction was terminated by adding 11 volumes of ice-cold 10 mM sodium phosphate-buffered saline, pH 7.0. The mixture was subjected to centrifugation and the supernatant to immunoprecipitation using goat antiserum against human placenta β -hexosaminidase (for details see Ref. 10). The immunoprecipitates were washed as before except for adding 5 mM UDP-N-acetylglucosamine into the wash buffers. The radioactively labeled products were analyzed by gel electrophoresis in the presence of sodium dodecyl sulfate and by fluorography with the impregnated gel backed by an intensifying screen (11).

Microsomal α -N-acetylglucosaminyl phosphodiesterase was assayed with radioactive oligosaccharide MI-2 as substrate. The preparation and characterization of the oligosaccharide and the assay conditions of the enzyme assay were those described (3). A typical assay mixture contained 600 cpm of MI-2, 10 μ l of enzyme in a final volume of 20 μ l of 20 mM sodium acetate, pH 5.5, and 1% Triton X-100. The reaction mixture was incubated for 2 h at 37 °C and then diluted with 4 ml of water. Substrate and products were separated by ion exchange chromatography on AG 1-X2 (3). One unit is defined as that amount of enzyme catalyzing the hydrolysis of 1% by radioactivity of oligosaccharide I-2 per h.

Glucose-6-phosphatase activity was determined in a reaction mixture containing 2 μ mol of sodium phosphate, pH 6.5, 0.8 μ mol of sodium tartrate, 0.04 μ mol of EDTA, 2 μ mol of [U-¹⁴C]glucose 6-phosphate (equivalent to 20,000 cpm), and enzyme protein in a final volume of 40 μ l. After incubation for 20-40 min at 37 °C, the mixture was diluted with 60 μ l of H₂O, applied to an AG 1-X2 column (0.5 \times 2 cm, Cl⁻ form), and equilibrated with H₂O. The product was eluted with 3 ml of H₂O and the unreacted substrate with 3 ml of 1 M NaCl. One unit is defined as that amount of enzyme catalyzing the hydrolysis of 1 μ mol of substrate per min.

UDP-galactosyltransferase was measured according to the procedure of Bauer *et al.*² using ovalbumin as acceptor. One unit is defined as the amount of enzyme transferring 1 μ mol of [³H]galactose per min onto ovalbumin.

Published procedures were used to determine the activities of β -hexosaminidase (12), β -glucuronidase (12), 5'-nucleotidase (13), and succinate dehydrogenase (14).

RESULTS AND DISCUSSION

Using the procedure of Sandberg *et al.* (7), membrane fractions highly enriched in Golgi apparatus, smooth endo-

² C. H. Bauer, B. F. Hassels, and W. G. Reutter (1976) *Biochem. J.* **154**, 141-147.

plasmic reticulum, or rough endoplasmic reticulum was obtained in a single centrifugation from the $10,000 \times g$ supernatant of liver homogenate. The biochemical and morphological characteristics of thus prepared membrane fractions is well documented (7). The distribution of protein, RNA, and marker enzymes as shown in Fig. 1 closely resembled the values reported (7). Galactosyltransferase was enriched 80-fold in the Golgi fraction, glucose-6-phosphatase, 2.7- and 6.6-fold in the smooth and the rough microsomal fractions, respectively, as compared to the postlysosomal supernatant (Fig. 1).

The two enzyme activities tentatively involved in the formation of the phosphorylated recognition markers in lysosomal enzymes also showed a characteristic distribution among the membranes separated. The activity phosphorylating β -hexosaminidase was detected in all three membrane fractions (Fig. 2). However, the labeling of the enzyme with the radioactive phosphate was much higher in the incubation with the Golgi than in those with the smooth and the rough endoplasmic reticulum fractions.

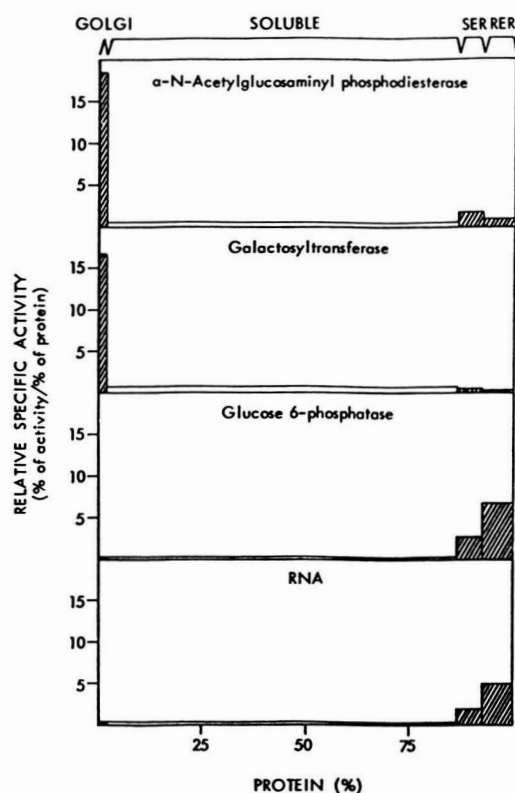


FIG. 1. Distribution profile of α -N-acetylglucosaminyl phosphodiesterase in Golgi apparatus and smooth and rough microsomes. Fractions enriched in Golgi apparatus, soluble material, and smooth and rough microsomes (from left to right) were obtained from a discontinuous sucrose gradient. The specific activities of α -N-acetylglucosaminyl phosphodiesterase, galactosyltransferase, and glucose-6-phosphatase in the homogenate were (units/mg of protein) 24, 26×10^{-6} , and 89×10^{-3} , respectively. After the discontinuous sucrose gradient, the recovery expressed in per cent of the homogenate was as follows: protein, 58%; RNA, 70%; α -N-acetylglucosaminyl phosphodiesterase, 82%; glucose-6-phosphatase, 69%; galactosyltransferase, 88%. The ratio of RNA to protein (mg/mg) for smooth and rough microsomes was 0.1 and 0.3, respectively. The values represent the mean of two experiments. Amounts of marker enzymes for other organelles found in the Golgi fraction were as follows (the values represent the yield in per cent and, in parentheses, the enrichment, both as compared to the homogenate): β -hexosaminidase, 0.4% (0.3); β -glucuronidase, 0.4% (0.2); succinate dehydrogenase, 1.0% (0.4); and 5'-nucleotidase, 14% (8.6).

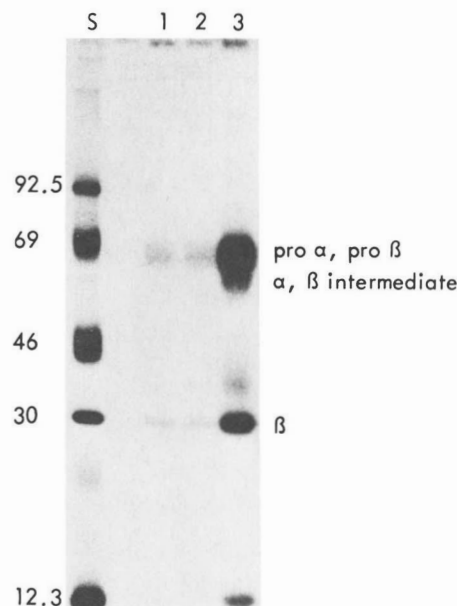


FIG. 2. Phosphorylation with [β - 32 P]UDP-N-acetylglucosamine of β -hexosaminidase in the presence of different membrane fractions from rat liver. Lane 1, rough endoplasmic membranes; lane 2, smooth membranes; lane 3, Golgi membranes. Lane S shows size markers used as previously (10) with the molecular weights in thousands indicated on the left margin. The labels to the right correspond to the various polypeptides of β -hexosaminidase: 67,000, pro- α -chain; 69,000, pro- β -chain; 54,000, α -chain; 52,000, β -chain intermediate; 29,000, β -chain (10). The incorporation of phosphate into the different polypeptides of β -hexosaminidase is discussed elsewhere.¹

The other enzyme studied, which uncovers mannose 6-phosphate residues in lysosomal enzymes,¹ was distributed in a similar manner. The activity of α -N-acetylglucosaminyl phosphodiesterase was enriched 18-fold in the Golgi fraction, 1.7-fold in the smooth microsomes, and 0.9-fold in the rough microsomes (Fig. 1). Although the specific activity appears low in smooth and rough microsomes, these fractions contain a significant amount of the total α -N-acetylglucosaminyl phosphodiesterase. The enzyme distributes among rough and smooth microsomes and Golgi apparatus in a ratio of 1:1.5:6. The distribution of α -N-acetylglucosaminyl phosphodiesterase is similar, although not identical, to that of galactosyltransferase. Nearly 60% of the enzyme present in the homogenate were recovered in the Golgi fraction.

These results show that two enzymes generating phosphorylated recognition markers in high mannose oligosaccharides in lysosomal enzymes are located in the same organelle fraction as galactosyltransferase, which is believed to participate in the synthesis of the complex type oligosaccharides (15). Unless the distribution of the enzymes and their substrates within the Golgi fraction is heterogeneous, certain signals in the substrate glycoproteins should exist for their recognition by specific carbohydrate processing enzymes. As the receptors for phosphorylated lysosomal enzymes have been found to be present in part within the cells (16), the sorting of lysosomal enzymes with the proper recognition markers from other biosynthetic products may occur intracellularly.

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