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Molecular size of *N*-acetylglucosaminylphosphotransferase and α-*N*-acetylglucosaminyl phosphodiesterase as determined *in situ* in Golgi membranes by radiation inactivation

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The radiation inactivation method was used to determine the molecular size of the two enzymes that participate in the synthesis of the phosphomannosyl recognition marker of lysosomal proteins. The determinations were carried out *in situ*, in Golgi membranes isolated from normal human placenta and cultured skin fibroblasts. A molecular size of 228 ± 29 kDa was found for placental *N*-acetylglucosaminylphosphotransferase, and 129 ± 11 kDa for placental α-*N*-acetylglucosaminyl phosphodiesterase. The values for the fibroblast enzymes were about 20% higher, 283 ± 27 kDa and 156 ± 14 kDa for the transferase and phosphodiesterase respectively. Triton X-100 had no effect on the molecular size of these enzymes.

INTRODUCTION

Two enzymes participate in the synthesis of the mannose 6-phosphate recognition marker of lysosomal proteins. The first enzyme, UDP-*N*-acetylglucosamine: lysosomal-protein precursor *N*-acetylglucosaminylphosphotransferase, transfers *N*-acetylglucosamine 1-phosphate from UDP-*N*-acetylglucosamine to mannose residues of high-mannose-type oligosaccharide chains of lysosomal-protein precursors (Reitman & Kornfeld, 1981; Hasilik *et al.*, 1981). The second enzyme, α-*N*-acetylglucosaminyl phosphodiesterase, cleaves the outer *N*-acetylglucosamine residues from these phosphodiester termini (Varki & Kornfeld, 1980; Waheed *et al.*, 1981). I-cell disease and pseudo-Hurler polydystrophy comprise a heterogeneous group of autosomal recessive disorders of lysosomal enzyme processing and targeting, caused by deficiency of *N*-acetylglucosaminylphosphotransferase activity (Hasilik *et al.*, 1981; Reitman *et al.*, 1981). In fibroblasts from patients with these disorders, the unphosphorylated high-mannose-type oligosaccharides are converted into complex or hybrid type, and the lysosomal-protein precursors are secreted from the cells rather than targeted into lysosomes. The results of kinetic studies by Varki *et al.* (1981) and complementation analyses by Honey *et al.* (1982), Shows *et al.* (1982) and Mueller *et al.* (1983) have suggested that the transferase is encoded by at least two genes, one presumably responsible for a catalytic subunit, and the other one for as lysosomal-protein-recognizing subunit. To examine the molecular size of the transferase as well as of the phosphodiesterase, we have used the radiation inactivation method (Kepner & Macey, 1968; Kempner & Schlegel, 1979), which determines *in situ* the smallest molecular size required to carry out the enzyme function. If the two subunits are required for the expression of transferase activity, then the size of the molecule obtained by radiation inactivation will correspond to the sum of the sizes of the subunits, i.e. destruction of any one of the subunits by a hit will cause the loss of activity of the whole oligomer.

MATERIALS AND METHODS

Preparation of Golgi membranes

Full-term placentae were collected at the time of delivery and kept frozen at −70 °C until used. Cultured skin fibroblasts from apparently normal individuals were obtained and maintained as previously described (Ben-Yoseph *et al.*, 1983). Tissue and cells were homogenized in 4 vol. of 0.25 M-sucrose in 10 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-iodoacetamide and 0.1 mM-leupeptin, by eight strokes in a Con-Torque homogenizer. Golgi membranes from placentae and fibroblasts were isolated by the procedure of Sandberg *et al.* (1980), with a sucrose density gradient. The Golgi-membrane fraction obtained from the gradient was diluted to 0.25 M-sucrose, and the membranes were pelleted by centrifugation at 105000 *g* for 90 min. The Golgi membranes were suspended by homogenization in water or in various concentrations (0.2–1.2%, v/v) of Triton X-100, and portions containing 1–2 mg of protein with or without Triton X-100 were freeze-dried in 1.5 ml Eppendorf micro test tubes. Protein was determined by the dye-binding method of Bradford (1976), with bovine γ-globulin as standard. In accordance with the treatment of the various preparations, protein standard solutions were made in water, or in various concentrations of Triton X-100, or were reconstituted in the presence of Triton X-100 following freeze-drying and irradiation.

Enzyme assays

Freeze-dried Golgi-membrane preparations with or without Triton X-100 were reconstituted in a final concentration of 1% (v/v) Triton X-100, and whole extracts of tissue and cells were adjusted to 1% (v/v)

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Triton X-100. The samples were sonicated by three pulses, 5 s each, with an Ultrasonics cell disruptor with a microtip at setting 7.

N-Acetylglucosaminylphosphotransferase activity with α-methyl mannoside acceptor and UDP-*N*-acetyl[1-^{14}C]glucosamine (ICN Radiochemicals, specific radioactivity 35 mCi/mmol) donor was assayed by a modification of previously described methods (Reitman & Kornfeld, 1981; Waheed *et al.*, 1982; Ben-Yoseph *et al.*, 1984). The reaction mixture contained 3 μmol of α-methyl mannoside, 3 nmol of UDP-*N*-acetyl[1-^{14}C]-glucosamine (205000 c.p.m.) and 10–50 μg of Golgi-membrane protein or 50–250 μg of whole-extract protein in a final volume of 30 μl of 35 mM-sodium phosphate buffer, pH 6.8, containing 1% (v/v) Triton X-100, 10 mM-$MgCl_2$, 5 mM-dimercaptopropanol, 10 mM-CDP-choline, 2 mM-ADP and 0.125 mM-leupeptin.

After incubation for 30 min at 37 °C, the reaction was terminated and the transfer product (α-methyl *N*-acetyl-[1-^{14}C]glucosaminyl-α1-phospho-6-mannoside) was separated from the breakdown product (*N*-acetyl[1-^{14}C]-glucosamine) on a QAE-Sephadex (Q-25-120; Sigma Chemical Co.) column (0.5 cm × 5.0 cm), and quantified, as previously described (Ben-Yoseph *et al.*, 1984). Enzyme specific activity was expressed as pmol of *N*-acetylglucosamine 1-phosphate transferred/h per mg of protein.

α-*N*-Acetylglucosaminyl phosphodiesterase activity was assayed as previously described (Ben-Yoseph *et al.*, 1984), with the isolated and desalted transfer product as substrate for this reaction. UDP-*N*-acetyl[U-^{14}C]glucosamine of higher specific radioactivity (310 mCi/mmol) (New England Nuclear) was used for preparation of the substrate. The reaction mixture contained 10 pmol of α-methyl *N*-acetyl[U-^{14}C]glucosaminyl-α1-phospho-6-mannoside (6000 c.p.m.) and 6–30 μg of Golgi-membrane protein or 30–150 μg of whole-extract protein in a final volume of 20 μl of 50 mM-Tris/HCl buffer, pH 7.4, containing 0.5% (v/v) Triton X-100, 10 mM-*N*-acetylmannosamine and 5 mM-EDTA. After incubation for 4 h at 37 °C, the reaction was terminated and the *N*-acetyl[U-^{14}C]glucosamine product was separated and quantified as previously described (Ben-Yoseph *et al.*, 1984). Enzyme specific activity was expressed as percentage of the initial substrate cleaved/h per mg of protein.

Radiation inactivation

Freeze-dried Golgi-membrane preparations, which were prepared in the absence or in the presence of Triton X-100 in 1.5 ml Eppendorf micro test tubes, were flushed with N_2, capped, and irradiated at room temperature (26±2 °C) in a ^{60}Co irradiator at a dose rate of about 2.8 Mrad/h (Gammacell model 220; Atomic Energy of Canada, Ottawa, Ont., Canada), as described by Beauregard & Potier (1982) and Beauregard *et al.* (1983). After exposure to radiation for various periods of time, samples were resuspended in a final concentration of 1% (v/v) Triton X-100 and assayed for enzyme activities. The freeze-dried samples were kept at −20 °C except for the time of the radiation inactivation experiments, and were shipped on solid CO_2. The non-irradiated samples retained more than 92% of their initial enzyme activities. The relative molecular mass of the functional unit was calculated according to the equation of Kepner & Macey (1968):

$$M_r = 6.4 \times 10^5 / D_{37}$$

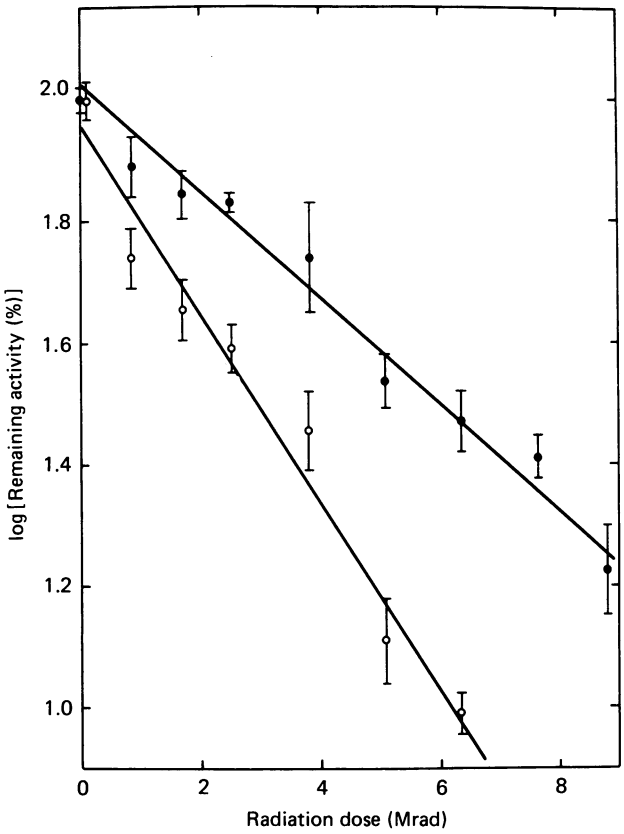

Fig. 1. Radiation inactivation of human placenta *N*-acetylglucosaminylphosphotransferase and α-*N*-acetylglucosaminyl phosphodiesterase

Freeze-dried Golgi-membrane preparations without detergent were irradiated and the residual activities of the transferase (○) and the phosphodiesterase (●) were determined as described in the Materials and methods section. The results represent the means±S.D. of the logarithm values of the percentage remaining activities for three experiments. Curve fitting was achieved by linear regression.

where D_{37} is the radiation dose in Mrad necessary to inactivate the enzyme to 37% of the initial activity. Enzyme preparations of known D_{37} and M_r values were used for calibration of the ^{60}Co source (Beauregard *et al.*, 1983). The empirical equation of Kepner & Macey (1968) appeared to be valid although it had been established under different experimental conditions (high dose rate and vacuum in their method as compared with low dose rate and N_2 in ours).

RESULTS AND DISCUSSION

The specific activities of both *N*-acetylglucosaminylphosphotransferase and α-*N*-acetylglucosaminyl phosphodiesterase in the Golgi-membrane preparations were 20–40 times higher than the respective activities in whole extracts of tissue and cells. Placental and fibroblast *N*-acetylglucosaminylphosphotransferase activities increased from 36.2 to 1594 pmol/h per mg and from 189 to 3817 pmol/h per mg respectively, and their α-*N*-acetylglucosaminyl phosphodiesterase activities increased from 3.70 to 163%/h per mg and from 12.6 to 277%/h per mg respectively. The yield for both enzymes in both

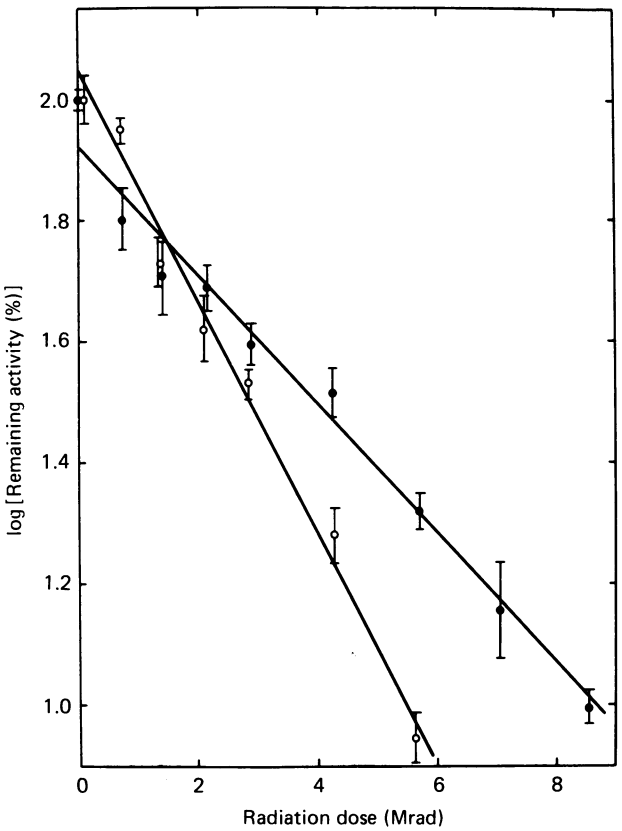

Fig. 2. Radiation inactivation of human fibroblast *N*-acetylglucosaminylphosphotransferase and α-*N*-acetylglucosaminyl phosphodiesterase

Freeze-dried Golgi-membrane preparations without detergent were irradiated and the residual activities of the transferase (○) and the phosphodiesterase (●) were determined as described in the Materials and methods section. The results represent the means ± S.D. of the logarithm values of the percentage remaining activities for three experiments. Curve fitting was achieved by linear regression.

sources was over 85%. The concurrent purification of these two enzymes indicates that they are located within the Golgi membranes, as previously shown by Pohlmann *et al.* (1982).

The radiation inactivation method (Kepner & Macey, 1968; Kempner & Schlegel, 1979) was chosen as means for molecular-mass determination, since on gel permeation of solubilized Golgi-membrane preparations (with Sepharose CL-4B) both activities were found to coincide in an aggregate form with an apparent molecular mass of 1.2–1.5 MDa. In such instances the radiation inactivation method is advantageous over other physicochemical methods, since it does not require a purified or even a soluble protein preparation. Thus membrane enzymes or other biologically active membrane proteins can be studied in their natural environment, in the absence of detergent.

After exposure to high-energy ionizing radiation for various periods of time, the residual activities of both enzymes were determined in the same irradiated specimens. The initial activities were determined in unexposed specimens, which were treated identically otherwise. The radiation inactivation of both enzymes in both sources yielded a linear relationship between the logarithm of the residual enzyme activity and the radiation dose. Figs. 1 and 2 show the decay curves of enzyme activities in irradiated Golgi membranes prepared in the absence of detergent from placenta and fibroblasts respectively. Radiation inactivation of Golgi membranes that were prepared in the presence of 0.2–1.2% (v/v) Triton X-100 yielded essentially identical decay curves.

The molecular-size values for fibroblast *N*-acetylglucosaminylphosphotransferase and α-*N*-acetylglucosaminyl phosphodiesterase were about 20% higher ($P < 0.05$, paired χ^2 test) than the respective values for the placenta enzymes, and in both sources the size of the transferase was about 1.8-fold larger ($P < 0.01$) than that of the phosphodiesterase (Table 1). Triton X-100 had no effect on the aggregation state of these enzymes or on their radiation-sensitivity (Beauregard & Potier, 1984). The higher molecular-size values obtained for the fibroblast enzymes may be due to an additional component not present in placenta or to different processing of the enzymes. The relatively large size of *N*-acetylglucosaminylphosphotransferase (228 ± 29 kDa for placenta and 283 ± 27 kDa for fibroblasts) indicates that this is probably a multimeric enzyme. This finding implies either that the integrity of several subunits within the enzyme molecule is required for performance of the catalytic activity or that close proximity between the subunits

Table 1. Molecular size of human *N*-acetylglucosaminylphosphotransferase and α-*N*-acetylglucosaminyl phosphodiesterase by radiation inactivation

Freeze-dried Golgi-membrane preparations without detergent were irradiated and the residual enzyme activities were determined as described in the Materials and methods section. The results represent the means ± S.D. of the D_{37} and M_r values for three experiments. D_{37} is the radiation dose that inactivates the enzyme to 37% of its initial activity. The relative molecular mass was calculated according to the equation of Kepner & Macey (1968):

$$M_r = 6.4 \times 10^5 / D_{37}$$

where D_{37} is given in Mrad.

Enzyme source	*N*-Acetylglucosaminyl-phosphotransferase		α-*N*-Acetylglucosaminyl phosphodiesterase	
	D_{37} (Mrad)	Molecular size (kDa)	D_{37} (Mrad)	Molecular size (kDa)
Placenta	2.84 ± 0.36	228 ± 29	5.00 ± 0.46	129 ± 11
Fibroblasts	2.27 ± 0.23	283 ± 27	4.13 ± 0.36	156 ± 14

allows for inter-subunit energy transfer. In either way, a hit at any subunit will bring about the inactivation of the whole oligomer. These results are in agreement with the results of kinetic (Varki *et al.*, 1981) and complementation (Honey *et al.*, 1982; Shows *et al.*, 1982; Mueller *et al.*, 1983) studies in cell preparations from patients with I-cell disease and pseudo-Hurler polydystrophy, which have suggested the presence of at least two distinct subunits within the *N*-acetylglucosaminylphosphotransferase molecule: a catalytic subunit and a lysosomal-protein-recognizing subunit. These values represent the size of each enzyme in the membrane and they may be used as guides for further purification or studies on these enzymes.

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