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STUDIES OF THE RESIDUAL GLYCOGEN BRANCHING ENZYME ACTIVITY PRESENT IN HUMAN SKIN FIBROBLASTS FROM PATIENTS WITH TYPE IV GLYCOGEN STORAGE DISEASE

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Human skin fibroblasts from patients with Type IV glycogen storage disease, in which there is a demonstrable deficiency of glycogen branching enzyme,were shown to be able to synthesize [^{14}C]glycogen containing [^{14}C]-glucose at branch points when sonicates containing endogenous glycogen synthase a were incubated with UDP[^{14}C]glucose. The branch point content of the glycogen synthesized by the Type IV cells was essentially the same as that formed by normal cells, but the total synthetic capacity of the Type IV cells was lower. A new assay for the branching enzyme using glycogen synthase as the indicator enzyme has been developed. Using this assay it has been shown that the residual branching enzyme of affected children and of their heterozygote parents is less easily inhibited by an IgG antibody raised in rabbits against the normal human liver enzyme than is the branching enzyme of normal fibroblasts.

Type IV glycogen storage disease is a rare, fatal congenital disease which has been shown to be due to a deficiency of the glycogen branching enzyme (EC 2.4.1.18) (1). Branching enzyme deficiency may have a generalized tissue distribution, since it has been shown to be demonstrable in liver, leukocytes, and cultured skin fibroblasts (1-4). The discovery that a branching enzyme deficiency is responsible for this disease was made possible by the development of an indirect assay which measures the stimulatory effect of the branching enzyme on the synthetic activity *in vitro* of glycogen phosphorylase when it is incubated with Glc-1-P in the absence of added polysaccharide acceptor (1). In this non-physiological assay, Type IV tissues have from 1 to 10% of the activity of normal tissues. By the use of this assay the detection of heterozygote carriers of the disease, and successful antenatal diagnosis of fetuses at risk has been possible. Although the reliability of the assay is thoroughly documented, it has seemed difficult to account adequately for the presence of an essentially normal content of glycogen with a lower than normal,

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but still substantial quantity of branch points in tissues which have so little detectable branching enzyme activity. Among other explanations, it has been suggested (a) that Type IV tissues may contain a second and less active branching enzyme which is under separate genetic control (1), or (b) that such tissues may contain a fetal branching enzyme which decreases in activity at birth, implying a partial, rather than a complete enzyme deficiency (5). We report now on studies of branching enzyme activity in sonicates of normal and Type IV human skin fibroblasts under physiologically relevant conditions. Structural analysis of the [^{14}C]glycogen synthesized from UDP[^{14}C]Glc *in vitro* by these cell lines has permitted a direct comparison to be made of their relative capacity for branch point synthesis. In addition, by the use of a coupled assay for the branching enzyme using glycogen synthase as the indicating enzyme, it has been possible to compare normal and mutant cells with respect to the cross-reactivity of their branching enzyme(s) with an inhibiting IgG antibody raised in rabbits against purified normal human liver branching enzyme.

MATERIALS AND METHODS

Cells and Cell Culture. Primary cultures of human skin fibroblasts were from skin samples removed at biopsy at various medical centers, or, in some cases, cultures were received from the Human Genetic Mutant Cell Repository, Camden, N.J. Fibroblasts were grown in monolayer culture and harvested as described by Brown *et al.* (6).

Synthesis of [^{14}C]Glycogen In vitro. Fibroblast sonicates in 0.9% NaCl were immediately made 50 mM in NaF in order to minimize the action of any phosphatase on endogenous glycogen synthase *b*, so that the activity of the indicator enzyme, glycogen synthase *a*, would not increase during the time of incubation. The quantity of [^{14}C]G_n synthesized from UDP[^{14}C]Glc, as described in Table I, was determined by the method of Thomas et al. (7). Rabbit muscle glycogen synthase (containing 70% *a*) was prepared by the method of Nimmo *et al.* (8). UDP[^{14}C]Glc (281 mCi/mmol) in ethanol/water (7:3) was from New England Nuclear. It was added to carrier UDPGlc (Sigma), and a solution (pH 6.8) of the sodium salt (60 dpm/nmol) was lyophilized to dryness in order to remove all ethanol, which is a potent inhibitor of the branching enzyme, and then redissolved in water.

Analysis of Polysaccharide Structure. The total quantity, extent of [^{14}C]Glc labeling, and the structure of each product were determined as described previously (6). Phosphorylase *a* (E.C.2.4.1.1) free of glycogen debranching enzyme was prepared according to Illingworth and Cori (9), and pure debranching enzyme (E.C.3.2.1.33) from rabbit muscle according to Taylor *et al.* (10).

Preparation of Antibody to Normal Human Liver Branching Enzyme. Branching enzyme was purified from human liver obtained at autopsy. Purification

was by ammonium sulfate fractionation, chromatography on DE-52 cellulose and on ω-aminohexyl agarose (Miles) and will be described elsewhere (11). A rabbit was immunized by subcutaneous injection of a mixture of the purified enzyme and Freund's complete adjuvant (Cappel). After one boosting with enzyme plus incomplete adjuvant, and a second boosting with enzyme alone, the globulin fraction of the serum was passed over a column of Protein A-Sepharose CL-4B (Sigma). The IgG fraction obtained in this way was used in the experiments described.

RESULTS AND DISCUSSION

The data of Tables I and II show that sonicates of human fibroblasts deprived of glucose contain glycogen synthase a which can act in vitro, to-

TABLE I

Synthesis in vitro of [^{14}C]Polysaccharide (G_n) from UDP[^{14}C]Glucose by Sonicates of Previously "Fasted" Human Skin Fibroblasts

The confluent fibroblast cultures were exposed to media containing no glucose for 24 h at 37° prior to their harvest by trypsinization. The cells then were disrupted by sonification and the sonicate was immediately made 50 mM in NaF. An aliquot portion of each sonicate (containing 1 to 2 mg protein) was incubated for 6 h at 37° with 7.4 mM UDP[^{14}C]Glc in 0.1 M glycylglycine buffer, pH 7.5, containing 25 mM NaF (total vol. = 0.42 ml). At 15 min intervals during the first hour and at hourly intervals thereafter 15 µl aliquots were removed and spotted on Whatman ET-31 filter paper which was then treated as described in the text for the determination of the quantity of [^{14}C]G_n which had been formed. After 6 h the remaining reaction mixture was heated in boiling water and the [^{14}C]polysaccharide product was isolated after dialysis and its structure analyzed (Table II).

Cell line	Specific activity[a] of branching enzyme	Synthase activity[b] $\frac{a}{a+b}$	Endogenous G_n in fasted cells	Initial rate of [^{14}C]G_n synthesis	Total [^{14}C]G_n formed in 6 h
	µmolP_i/min/mg	%	µmol$(Glc)_n$/mg	nmol [^{14}C]Glc /min/mg	nmol/mg
J.G. (control)	4.69	53.9	0.08	4.41	727
GM244 (Type II GSD)[c]	2.01	47.7	0.18	2.97	583
GM572 (Type IV GSD)	0.1 - 0.2	35.1	0.44	2.74	299

[a] Assayed by Method 1 of Gibson et al. (12) except that incubation was at 37°.

[b] Measured as the initial linear rate of [^{14}C]G_n synthesis in the presence of 100 µg/ml of added glycogen (synthase a) and with glycogen and added 7.1 mM Glc-6-P (synthase a + b). The rates measured under these conditions reflect the combined actions of any branching enzyme present, as well as synthase, since the latter enzyme can use the product of the action of the former as an acceptor.

[c] Type II GSD signifies that the cells were derived from a patient who lacked lysosomal α-glucosidase.

TABLE II

Composition and Structural Features of the Polysaccharides Synthesized in vitro from UDP[^{14}C]Glucose by Sonicates of Human Fibroblasts

The data in this Table refer to the polysaccharide products whose preparation is described in Table I.

Cell line	Content of [^{14}C]-α-1,6-linked Glc units[a]		Fraction of total [^{14}C]-α-1,6-linked Glc units in outer tier of product isolated[b]	Total [^{14}C]Glc branch points synthesized
	In product isolated	In [^{14}C]G_n synthesized[c]		
	%	%	%	nmol/mg
J.G. (Control)	6.49	7.23	55.6	52.5
GM244 (Type II GSD)	4.11	5.36	73.5	32.0
GM572 (Type IV GSD)	2.56	6.34	ca. 100	18.1

[a]Measured as the quantity of [^{14}C]Glc formed when the polysaccharide was degraded by the combined actions of glycogen phosphorylase (with inorganic phosphate) and the glycogen debranching enzyme.

[b]The expression "outer tier" refers to that portion of the polysaccharide which can be converted to α-Glc-1-P and then to Glc by the successive (not simultaneous) actions of glycogen phosphorylase (with inorganic phosphate) and the glycogen debranching enzyme.

[c]Calculated from the measured total content of [^{14}C]Glc in each isolated product.

gether with any endogenous glycogen branching enzyme which may be present, to form a branched polysaccharide from UDP[^{14}C]Glc. The newly formed α-1,6-linked glucose units at the branch points were [^{14}C]-labelled, and, most importantly, they were present to essentially the same extent in the product synthesized by the Type IV cell line (GM 572) as in those made by two other cell lines which were not deficient in branching enzyme (column 3, Table II). Although the Type IV fibroblasts had a very low residual branching activity, as judged from the phosphorylase assay (column 2, Table I), this activity was sufficient for these mutant cells to synthesize a polysaccharide with a normal degree of branching under conditions when the overall synthetic rate was limited by the activity of 35% of the total endogenous glycogen synthase.

However, the data of Table I (column 6) also show that the Type IV cell was relatively deficient in its total capacity for polysaccharide synthesis, since the quantity of $[^{14}C]G_n$ formed was only 63% of that which would have been expected from the synthase a activity of GM 572 compared to that of the control cell line, J.G., while that of GM 244, not deficient in branching enzyme, was 91% of that of the control. This difference is also shown clearly by the data in Table II (column 5) which compares the total number of branch points synthesized by these cell lines. It is not possible to obtain such information about the physiologically relevant capacity of Type IV cells to synthesize branched polysaccharide as compared to that of normal cells when only data from the phosphorylase assay for branching enzyme are available. This is due to the fact that in this assay the potential activity of the enzyme responsible for chain elongation is about 30-fold greater than the increment in its activity which the quantity of branching enzyme in normal cells is capable of producing, and 1000-fold greater than the increment produced by Type IV cells. The product formed in the phosphorylase assay has very long chains of α-1,4-linked glucose units with a very low total branch point content. The relative insolubility of this non-physiological product makes its structural analysis essentially impossible. The data presented here show that such considerations do not apply to polysaccharides formed by glycogen synthase and branching enzyme when they act in concert in relative concentrations approximately equal to those prevailing in the living fibroblast.

The data in Table III show that with cultures of skin fibroblasts derived from two other Type IV patients (A.L. and J.B.) results similar to those in Tables I and II were obtained when the cells were not deprived of glucose, with the consequence that more than 90% of their glycogen synthase was in the inactive b form (data not shown), and when, instead, purified rabbit muscle glycogen synthase a (shown to be free of branching enzyme) was added. In such experiments the lower potential activity of Type IV cells to synthesize $[^{14}C]$-glycogen with labeled branch points becomes more evident when the added synthase a activity is increased (lines 1 and 2, Table III). Under these con-

TABLE III

Synthesis *in vitro* of [^{14}C]Polysaccharide (G_n) from UDP[^{14}C]Glucose by Sonicates of Human Skin Fibroblasts in the Presence of Added Rabbit Muscle Glycogen Synthase *a*

The conditions of incubation and the composition of the reaction mixtures were similar to those described in Table I except that the fibroblasts were not deprived of glucose prior to harvest and purified rabbit muscle glycogen synthase *a* was added to the reaction mixture. Incubation was for 5 hours.

Cell line	Added synthase *a*	Total [^{14}C]G_n formed	Content of [^{14}C]-α-1,6-linked Glc units in [^{14}C]G_n products	Total [^{14}C]Glc branch points synthesized
	nmol/min/ml[a]	nmol/mg	%	nmol/mg
J.P. (Control)	25.8	9483	4.00	379
GM 572 (Type IV GSD)	25.8	1466	3.75	55
A.L. (Type IV GSD)	6.3	298	4.53	13.5
J.B. (Type IV GSD)	6.7	401	3.48	14.0

[a]Measured as the initial linear rate of [^{14}C]G_n synthesis in the absence of Glc-6-P and in the presence of the quantity of endogenous G_n present in a *boiled* aliquot of the fibroblast sonicate equal in volume to that used in the reaction mixture containing active (unboiled) enzyme.

ditions the GM 572 cells synthesized only 15% as much glycogen as the control cell line, but the degree of branching in the products was equal. Based on such experiments a new assay for the branching enzyme has been developed in which added glycogen synthase *a* is the indicating enzyme. Figure 1 shows representative results obtained by the use of this assay. Data for two control, two Type IV, and both heterozygote parents of one of the Type IV children are shown. The clearly intermediate activity observed for each of these parents shows that the assay is reliable. We have obtained similar results (not shown) for another such family, and 8 other Type IV cell and 18 other control cell lines have been assayed with concordant results.

The question of whether the branching enzyme activity in Type IV fibroblasts is due to a small amount of the normal enzyme or to a modified protein

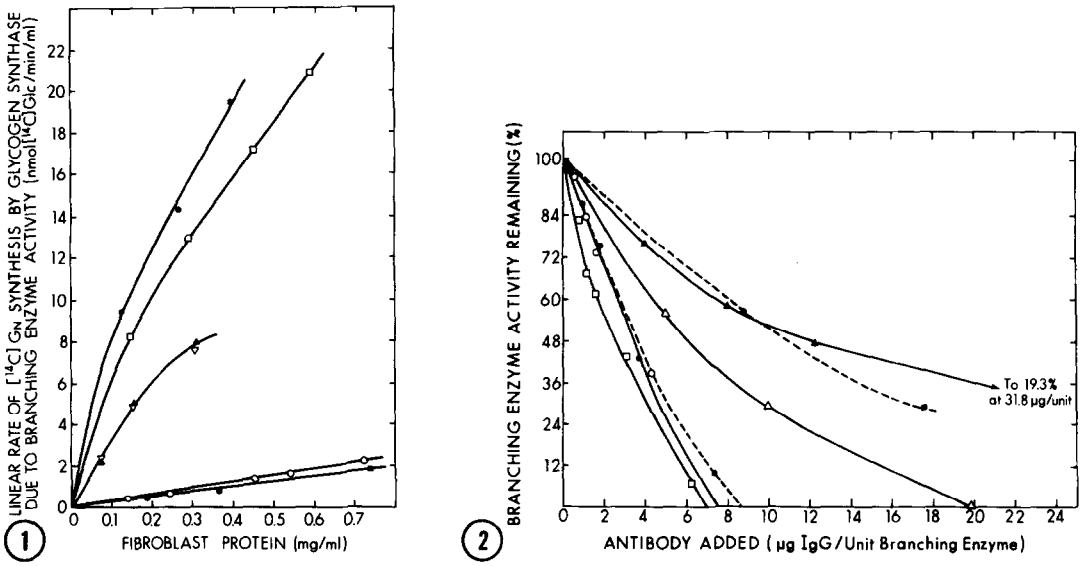

Figure 1. Assay of branching enzyme in fibroblast sonicates. Several aliquot portions of each sonicate (containing less than 60 µg protein) were incubated in duplicate (one without and one after prior heating in boiling water to destroy enzyme activity) for 80 min at 37° with 5.6 mM UDP[^{14}C]Glc in 16 mM glycylglycine buffer, pH 7.2, containing 8 mM dithiothreitol, 50 mM NaF, and 0.1 unit/ml of rabbit muscle glycogen synthase a (vol. = 0.08 ml). At four 20 min intervals, 15 µl aliquots were removed and spotted on Whatman ET-31 filter paper for determination of the quantity of [^{14}C]polysaccharide formed. The quantity found in each reaction mixture containing inactivated sonicate was subtracted from the amount found in the corresponding unheated sample. Branching enzyme activity was expressed as the net linear rate of polysaccharide (G_n) synthesis. Control cells: K.E., ●——●; J.C., □——□. Type IV cells: J.I., ○——○; M.W., ■——■. Father of M.W., ▽——▽. Mother of M.W., ▲——▲.

Figure 2. Inhibition of fibroblast branching enzyme by antibody to the normal human liver enzyme. Reaction mixtures were as described in Figure 1 except that a 10 µl aliquot of various dilutions of IgG in 0.15 M NaCl was included in each appropriate mixture. Control cells: K.E., □- - -□; J.C., ●- - -●; J.L., ○- - -○. Type IV cells: A.L., ▲- - -▲; A.T., ■- - -■. Father of Type IV: N.W., △- - -△

with reduced activity has been studied preliminarily by raising in rabbits an IgG antibody against purified branching enzyme from normal human liver, and then by comparing its action in inhibiting the branching enzyme activities in various kinds of fibroblasts. Results are shown in Figure 2 for two Type IV, one heterozygote, and three control cell lines. The differences in slope of these inhibition curves show that the antibody's effect is not due simply to inhibiting a residual amount of normal enzyme. The affected cells may contain an enzyme which cross-reacts with the antibody less completely than the enzyme in normal cells. However, in view of the result shown for one heterozygote

cell line (and for another for which data is not shown), it also seems likely that the fibroblasts of affected children as well as of their parents contain a substantial quantity of protein which is enzymatically inactive but cross-reactive with the antibody. These possibilities are now being investigated more completely as are other possible differences in the branching enzymes of normal and Type IV cells.

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