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Research Article

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Deficiency of the Pyruvate Dehydrogenase Component in Pyruvate Dehydrogenase Complex–deficient Human Fibroblasts

Immunological Identification

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Abstract

A previously reported deficiency of "total" pyruvate dehydrogenase complex activity is further characterized. Dihydrolipoyl transacetylase (E_2) and lipoamide dehydrogenase (E_3) activities in the patient's fibroblasts were normal. Pyruvate dehydrogenase activity (E_1) was 33% of that in fibroblasts from an age-matched control. The amounts of each of the components of pyruvate dehydrogenase complex were analyzed using an immunoblot technique and specific antibodies. Levels of components E_2 and E_3 were the same in fibroblasts from the patient and control, confirming the activity measurements. However, the levels of $E_1\alpha$ and $E_1\beta$ were reduced markedly in fibroblasts from the patient. Thus, impairment in the pyruvate dehydrogenase complex activity was due to a reduction in the amount of the E_1 component of the complex.

Introduction

Genetic defects involving the enzymes of pyruvate metabolism, pyruvate dehydrogenase complex (PDHC),[1] pyruvate carboxylase, lactate dehydrogenase, and *P*-enolpyruvate carboxykinase, are reported frequently (for review, see 1–4). The most common of these involves the PDHC (1–3). However, characterizations of the defects involving this complex have been limited to the measurements of the enzymatic activities of the complex and its components in extracts of skin fibroblasts, and in very rare cases, extracts of other tissues from affected patients. PDHC is composed of three catalytic components, pyruvate dehydrogenase (E_1) (EC 1.2.4.1), dihydrolipoyl transacetylase (E_2) (EC 2.3.1.12), and lipoamide dehydrogenase (E_3) (EC 1.6.4.3), and two regulatory enzymes, pyruvate dehydrogenase kinase (EC 2.7.1.99) and pyruvate dehydrogenase phosphatase (EC 3.1.3.43) (5). Phosphorylation of the α-chain of the E_1 component by its specific kinase inactivates E_1 (and hence the complex) while dephosphorylation by its phosphatase restores catalytic activity of the complex (5). The presence of an additional polypeptide (relative molecular mass [M_r], 50,000) in mammalian PDHC (6), has been established using immunological as well as structural analyses (7, 8).

Classification of PDHC deficiencies has been based on measurements of component activities (partial reactions). Defects in enzymatic activities have been reported for E_1, E_2, E_3, and pyruvate dehydrogenase phosphatase (for review see 1–3). By contrast, there are few reports of measurements of the mass of the various component enzymes. Using specific antisera we have raised against each of the three catalytic components of PDHC, we now document a defect in the E_1 component in extracts of fibroblasts from a PDHC-deficient patient.

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1. *Abbreviations used in this paper:* E_1, pyruvate dehydrogenase; E_2, dihydrolipoyl transacetylase; E_3, lipoamide dehydrogenase; PDHC, pyruvate dehydrogenase complex.

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Methods

Patient. A detailed case history of the patient (J.S.) was reported recently (9). Significant findings included lactic acidemia, intermittent ataxia, moderate psychomotor retardation, opthalmoplegia, and retinal pigment epithelial changes. A central hypoventilation syndrome also was documented. On postmortem histopathologic examination, the brain was shown to exhibit the specific central nervous system pathology of Leigh's disease (10). Deficiency of PDHC was established by measuring "total" PDHC activity in disrupted skin fibroblasts after pretreatment with dichloroacetate (11).

Assays for the complex and its catalytic components. Human skin fibroblasts were grown in Eagle's minimum essential medium, basically as described (11). Confluent monolayers were trypsinized and the released cells were washed twice with phosphate-buffered saline (11). To measure "total" PDHC and pyruvate dehydrogenase (E_1 component) activities, we suspended the cells in phosphate-buffered saline containing 5 mM dichloroacetate and incubated them at 37°C for 15 min with shaking. Activation was stopped (11), and the suspension was immediately frozen and stored at −76°C. Before enzyme assays, the suspension was further frozen and thawed two more times. Activity of PDHC was determined by measurement of $^{14}CO_2$ production from [1-^{14}C]pyruvate (11), in a total volume of 80 μl. The blank did not contain coenzyme A (CoA) and thiamin pyrophosphate. Three or four replicates were performed for each determination.

E_1 component was assayed by measuring the decarboxylation of [1-^{14}C]pyruvate to $^{14}CO_2$ in the presence of ferricyanide as an electron acceptor (12). The blank did not contain thiamin pyrophosphate. The activities of E_2 and E_3 components of the complex were measured in cells that were treated as described above except that the dichloroacetate-activation step was omitted. E_2 component activity was measured by following the transfer of the [1-^{14}C]acetyl moiety from [1-^{14}C]acetyl-CoA (12). E_3 component was assayed in the direction of lipoamide reduction (12). All assays were linear with respect to both time and the

amount of added protein. 1 mU of activity is defined as 1 nmol of substrate oxidized or product formed per min at 37°C. Protein was determined by the method of Lowry et al. (13), with bovine serum albumin as standard.

Immunological identification of E_1, E_2, and E_3 of PDHC. Human skin fibroblasts in culture were harvested by scraping with a rubber policeman. Cells were washed twice with ice-cold phosphate-buffered saline, and crude mitochondrial pellets were prepared using digitonin (14). Whole cells or crude mitochondrial pellets were stored at −76°C.

PDHC components were characterized by Western blot analysis. Frozen cells or mitochondrial pellets were resuspended in ice-cold 20 mM potassium phosphate, pH 7.0, 1% Triton X-100. The samples were sonicated twice for 10 s at continuous pulse with a microtip and were dissociated by boiling for 10 min in 17 mM dithiothreitol, 2% sodium dodecyl sulfate (SDS). The samples were separated by electrophoresis in discontinuous SDS polyacrylamide gels (6% polyacrylamide stacking, 9% polyacrylamide separation).

Proteins were transferred to a nylon-based membrane (Zeta-probe, Bio-Rad Laboratories, Richmond, CA) using a Bio-Rad transblot apparatus essentially as described in Bio-Rad Instruction Manual #85-0176-485, except that electroelution was conducted at room temperature at 75 mA, constant current, for 8 h. After transfer, the membrane was incubated with a specific antibody followed by ^{125}I-protein A. Immunoreactive proteins were visualized by exposing the membrane to X-Omat AR film (Eastman Kodak Co., Rochester, NY). In preliminary experiments using ^{125}I-labeled E_1 (15) from bovine kidney, both α- subunit and β-subunit were quantitatively transferred to the Zeta-probe membrane. Based on staining of the gel with Coomassie Brilliant Blue R-250 after electrotransfer, electrotransfers of E_2 and E_3 also were quantitative using these conditions.

Preliminary experiments showed that E_2 is highly susceptible to proteolytic degradation. Proteolysis was minimized by including protease inhibitors in the solutions during the preparation and solubilization of the mitochondrial fraction (results not shown). The following mixture of protease inhibitors was found to be effective in minimizing E_2 degradation: (final concentration) 2 mM EDTA, 2 mM EGTA, 0.2 mM phenylmethenesulfonyl fluoride, 0.5 mg/liter Leupeptin, and 0.7 mg/liter Pepstatin.

Rabbit antisera against individual components E_1 (16), E_2 (16), and E_3 (17) of PDHC were raised using highly purified bovine kidney E_1, bovine heart E_2, and a commercially available E_3 preparation from porcine heart (Sigma Chemical Co., St. Louis, MO). The anti-E_3 serum reacts with not only the E_3 component of PDHC, but also the E_3 components of α-ketoglutarate and branched-chain ketoacid dehydrogenase complexes. In contrast, the anti-E_2 serum reacts only with the E_2 component of PDHC (Carothers, D. J., and M. S. Patel, unpublished observations). For immunoblot analyses, antisera were diluted with 20 mM Trizma base, 500 mM NaCl, pH 7.5, 5% bovine serum albumin. Antisera for E_1, E_2, and E_3 were diluted 20-fold, 50-fold, and 50-fold, respectively.

Results

"Total" PDHC activity (dichloroacetate-activated) in extracts of fibroblasts from patient J.S. was considerably lower than that observed in the fibroblasts from three age-matched controls (F, P.H., and C.S.) and a younger control (B.M.) (Table I), confirming our earlier report (9). Treatment of the extract of fibroblasts from patient J.S. with added purified pyruvate dehydrogenase phosphatase caused a degree of activation of the PDHC equal to that caused by dichloroacetate, Mg^{++} and Ca^{++} (9), indicating that the phosphatase activity in the patient's fibroblasts was normal and suggesting that the low PDHC activity in these fibroblasts was due to a defect in one of the other components.

To determine which component of the PDHC was affected, the enzymatic activities of the E_1, E_2, and E_3 components of the complex were assayed. E_2 and E_3 activities of the patient's fibroblasts were not significantly different from those of an age-matched control (F) (Table I). E_1 activity in the patient's fibroblasts, however, was only 33% of that observed in the control F. The ferricyanide assay used to determine E_1 activity in crude cellular preparations measures only a small fraction of "total" PDHC activity. Therefore, identification of E_1 deficiency based solely on the ferricyanide assay may not be adequate. Immunological detection offers an alternate approach to characterize PDHC activity deficiency. Additionally, this approach should discriminate between a change in enzyme content and a change in the catalytic efficiency of the enzyme.

Mitochondrial extracts of equal quantities of fibroblasts from the control P.H. and patient J.S. had approximately similar amounts of immunoreactive protein at a position corresponding to E_3 (Fig. 1, panel *1*). Skin fibroblasts from patient J.S., therefore, contained normal quantities of E_3. A similar analysis established that the mutant fibroblast contained normal amounts of immunoreactive E_2 (Fig. 1, panel *2*). The relative amounts of $E_1\alpha$ and $E_1\beta$ were analyzed in mitochondrial pellets derived from equal quantities of fibroblasts from the two age-matched controls (P.H. and C.S.) and patient J.S. (Fig. 1, panel *3*). Fibroblasts from two controls had similar amounts of immunoreactive proteins at the positions corresponding to $E_1\alpha$ and $E_1\beta$ (panel *3*, lanes *B* and *D*). In contrast, very little E_1 α-subunit or β-subunit were detected in fibroblast extracts from patient J.S. (lane *C*). In a separate experiment (Fig. 2), amounts of E_1 subunits were analyzed in extracts of whole cell (*right*) and isolated mitochondria (*left*). The amounts of immunoreactive proteins at the positions corresponding to $E_1\alpha$ and $E_1\beta$ are drastically decreased

Table I. Measurement of Pyruvate Dehydrogenase Complex and Its Component Activities in Fibroblasts from Controls and Patient J.S.

Subject	Age	"Total" PDHC activity*	PDHC component activities* E_1	E_2	E_3
			nmol/min per mg protein	*nmol/min per mg protein*	*nmol/min per mg protein*
Patient J.S.	5 yr	0.32±0.17	0.06±0.002	2.3	42.1
Control F	7 yr	2.77±0.20	0.18±0.020	2.7	48.4
Control P.H.	7 yr	4.03±0.15	—	—	—
Control C.S.	7 yr	2.40	—	—	—
Control B.M.	1 mo	2.22±0.31	—	—	—

* Values are mean±SD of three or four determinations or when no SD is indicated, single observations.

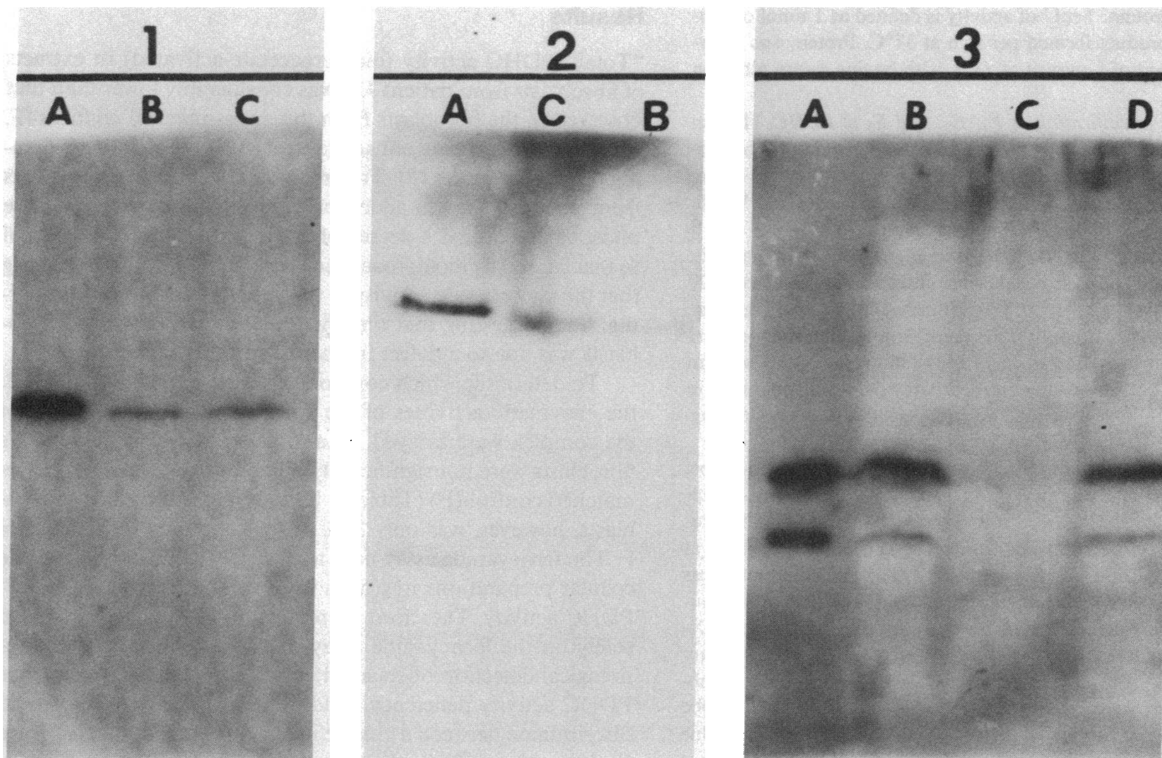

Figure 1. Immunoblot analysis of PDHC components in fibroblasts from age-matched controls and patient J.S. For each panel, crude mitochondrial pellets prepared from fibroblasts containing similar quantities of cellular protein were analyzed. (Panel *1*) E_3 immunoblot. The membrane was treated with an anti-E_3 serum; *A*, 100 ng of purified bovine heart E_3; *B*, 0.27 mg protein from control P.H.; *C*, 0.30 mg protein from patient J.S. (Panel *2*) E_2 immunoblot. The membrane was treated with an anti-E_2 serum; *A*, 100 ng of purified bovine heart E_2[2] (upper band, E_2; lower band, degradative product of E_2); *B*, 0.33 mg protein from control P.H.; *C*, 0.41 mg protein from patient J.S. (Panel *3*) E_1 immunoblot. The membrane was treated with an anti-E_1 serum; *A*, 150 ng of purified bovine kidney E_1 (upper band, $E_1\alpha$; lower band, $E_1\beta$); *B*, 0.53 mg protein from control P.H.; *C*, 0.59 mg protein from patient J.S.; *D*, 0.40 mg protein from control C.S.

in extracts from patient J.S. as compared with those from the control B.M. Further, there is no evidence for the accumulation of aberrant precursor-peptides of $E_1\alpha$ or $E_1\beta$ in whole cell extracts of patient's fibroblasts (Fig. 2, *right,* lane *B*). These reduced amounts of $E_1\alpha$ and $E_1\beta$ are consistent with residual "total" PDHC activity (<15% of control values; Table I) in the patient's fibroblasts.

Discussion

About 50 of the ~80 reported cases of PDHC deficiency in children have been attributed to defects in a particular component of the complex, based on measurements of component activities (partial reactions) of the complex. Of the three catalytic component assays used in the previous reports and in this report, the E_1 assay (the decarboxylation of [1-^{14}C]pyruvate in the presence of ferricyanide as an electron acceptor) is most problematic, as it measures <10% of "total" PDHC activity. Reduction in enzyme activity of a component and hence the complex activity could be due to a decrease in the catalytic efficiency of that component or a decrease in the mass of the component. These two possibilities can be discriminated with immunological analysis.

Among the reported cases of PDHC deficiency attributable to a reduction in the activity of a specific PDHC component, only in one case, an E_3 deficiency, has the defect been confirmed by demonstrating the absence of immunoreactive material in the patient's tissue (18). A preliminary report suggests an alteration of $E_1\alpha$ in a PDHC-deficient patient (No. 1373) based on SDS-polyacrylamide electrophoretic mobility of immunoprecipitated $E_1\alpha$ from ^{35}S-methionine–labeled fibroblasts (4). In this study, fibroblasts were labeled overnight with ^{35}S-methionine before immunoprecipitation of PDHC, and the comparison was based on the radioactivity present in the $E_1\alpha$ band, not on the amount of $E_1\alpha$ protein. The PDHC deficiency in our patient is due to a reduction in the amount of E_1 immunoreactive protein. Our analysis indicates that $E_1\alpha$ and $E_1\beta$ peptides of normal length are present at low levels in fibroblasts from patient J.S. (Fig. 2), which indicates that alterations, if any, in the primary structure of either α-peptide or β-peptide of E_1 are minor.

Possible mechanisms for decreased levels of the mature mitochondrial peptide include defects at transcriptional, posttranscriptional, translational, or posttranslational steps. Many mitochondrial proteins encoded by nuclear genes are synthesized as precursors with a NH_2-terminal leader peptide (19). In a poly(A)$^+$RNA-dependent cell-free system (wheat germ), both $E_1\alpha$ and $E_1\beta$ are synthesized as precursor polypeptides ~ 4,000-mo-

2. We have routinely observed that bovine heart E_2 migrates slightly slower than E_2 from human fibroblasts and rat liver (unpublished observations). The latter observation is consistent with that of DeMarcucci and Lindsay (7).

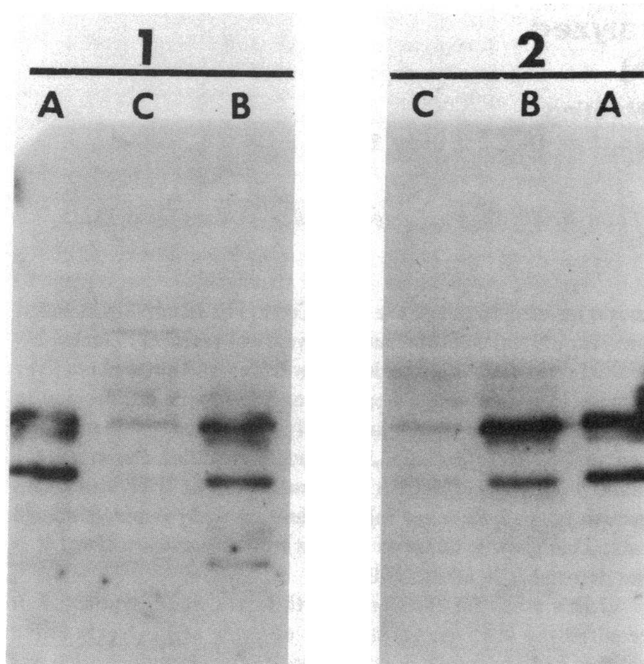

Figure 2. Immunoblot analysis of E_1 in fibroblasts from patient J.S. and control B.M. Whole cell extracts (*right*) and crude mitochondrial extracts (*left*) prepared from similar quantities of fibroblasts based on cellular protein content were analyzed in each case using anti-E_1 serum. *A,* 250 ng of purified bovine kidney E_1 (upper band, $E_1\alpha$; lower band, $E_1\beta$); *B,* 0.50 mg protein from control B.M.; *C,* 0.54 mg protein from patient J.S.

lecular weight heavier than their respective mature peptides (Raefsky, C., and M. S. Patel, unpublished observations). In the present study (Fig. 2, *right*), no precursor peptides of either $E_1\alpha$ or $E_1\beta$ were detected from whole cell extracts of the patient's fibroblasts. A point mutation should theoretically affect only one of the subunits. However, a defect in one of the two peptides of E_1 might inhibit transport of mutated precursor-peptide into the mitochondria or assembly of the heterotetramer ($\alpha_2\beta_2$), leading to rapid degradation of one or both subunits. Studies using specific cDNA probes and in vitro translation are needed to establish the nature of the defect of E_1 in this patient.

In summary, an impairment in the PDHC activity in this patient is attributable to a reduction in the amount of the E_1 component of the complex. The immunoblot technique employed in this study should prove useful in identifying defects in the PDHC components in fibroblasts from a large number of patients.

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