Pyruvate Dehydrogenase Complex Deficiency Caused by Ubiquitination and Proteasome-mediated Degradation of the E1β Subunit*

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Congenital deficiencies of the human pyruvate dehydrogenase (PDH) complex are considered to be due to loss of function mutations in one of the component enzymes. Here we describe a case of PDH deficiency associated with the PDH E1β subunit (PDHB) gene. The clinical phenotype of the patient was consistent with reported cases of PDH deficiency. Cultured skin fibroblasts demonstrated a 55% reduction in PDH activity and markedly decreased immunoreactivity for PDHβ protein, compared with healthy controls. Surprisingly, nucleotide sequence analyses of cDNAs corresponding to the patient PDH E1α (PDHA1) and PDHB genes revealed no pathological mutations. Moreover, the relative expression level of PDHB mRNA and the rates of transcription and translation of the PDHB gene were normal. However, PDH activity could be restored in cells from this patient following treatment with MG132, a specific proteasome inhibitor, and normal levels of E1β could be detected in MG132-treated cells. Similar results were obtained following treatment with Tyrosine 23 (Tyr23), a specific inhibitor of epidermal growth factor receptor-protein-tyrosine kinase (EGFR-PTK), which also restored E1β protein levels to those in healthy subjects or from patients with PDHA1 deficiency. The index patient's cells contained a high basal level of EGFR-PTK activity that correlated with the high level of ubiquitination of cellular proteins, although the total EGFR protein levels were similar to those in cells from E1α-deficient subjects and healthy subjects. These data indicate that PDH deficiency in our patient involves a post-translational modification in which EGFR-PTK-mediated tyrosine phosphorylation of the E1β protein leads to enhanced ubiquitination followed by proteasome-mediated degradation. They also provide a novel mechanism accounting for congenital deficiency of the PDH complex and perhaps other inborn errors of metabolism.

The nuclear encoded mitochondrial pyruvate dehydrogenase (PDH)2 complex plays major roles in regulating cellular fuel metabolism, acid-base equilibrium and energetics (1, 2). The complex consists of three catalytic components: pyruvate dehydrogenase (E1; EC 1.2.4.1), dihydrolipoamide transacylase (E2; EC 2.3.1.12), and dihydrolipoamide dehydrogenase (E3; EC 1.8.1.4), and an additional protein known as the E3-binding protein (E3BP). Rapid post-translational regulation of the complex is mediated in large part by reversible phosphorylation of the α subunit of E1 that is catalyzed by isoforms of PDH kinase and PDH phosphatase.

The E1 enzyme (EC 1.2.4.1) is a heterotetrameric (α2 β2) α-ketoacid decarboxylase that irreversibly oxidizes pyruvate to acetyl-CoA in the presence of thiamine pyrophosphate (TPP) and also catalyzes the subsequent reductive acetylation of the lipoyl moiety of E2. Most reported mutations of the PDH complex are located in the X-linked gene for the α subunit of the E1 component (MIM 312170). Only a small number of patients have been described with mutations in the E2, E3, E3BP, or PDH phosphatase genes (4–8). Several cases of PDH deficiency involving distinct mutations in the PDH E1β (PDHB) gene were reported recently (9, 10). Here we describe an unusual case of functional E1β deficiency which is not due to a pathological mutation in the E1β gene but to increased turnover of the E1β protein following activation of epidermal growth factor receptor-protein-tyrosine kinase (EGFR-PTK) by autophosphorylation at its tyrosine residues. This appears to be caused by enhanced ubiquitination of the E1β protein and its subsequent degradation by the ubiquitin-proteasome system.

EXPERIMENTAL PROCEDURES

This study was approved by the Institutional Review Board of Shands Hospital, University of Florida.

Case Report—The patient was a 5 and 10/12-year-old female who presented in infancy with delayed developmental milestones, microcephaly, agenesis of the corpus callosum, and bilateral hip displacement. The lactate concentration in whole blood was only modestly increased (1.1–2.3 mmol/liter), whereas the level in cerebrospinal fluid was high (5.3 mmol/liter). She was referred for metabolic testing at age five years, and enzymological studies conducted with her cultured skin

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‡ The abbreviations used are: PDH, pyruvate dehydrogenase; mAb, monoclonal antibody; EGFR, epidermal growth factor receptor; PTK, protein-tyrosine kinase; Tyr23, Tyrosinphant 23.
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fibroblasts disclosed PDH deficiency. She was the second child of healthy parents. The mother’s first child was also healthy and had a different paternal lineage than the patient. The remainder of the family history was unremarkable except for a maternal sibling who died in infancy from an unknown cause.

**Cell Culture, Treatment, and Transfection**—Primary cultures of fibroblasts were established from skin biopsies of the index patient (Patient 1), two patients with proven PDH deficiency due to a pathological mutation in the E1α subunit gene (Patient 2: c.904C > T and Patient 3: c.642G > T), and three healthy subjects. Cells were cultured as monolayers in T-flasks in complete Dulbecco’s modified Eagle’s medium (Sigma). The medium contained 25 mM glucose and was supplemented with 20% fetal bovine serum (Hyclone, Logan, UT), 1% penicillin/streptomycin solution (10,000 units of penicillin and 10 mg/ml of streptomycin) (Sigma), 1% insulin-transferrin solution (0.5 mg/ml), 0.2% fibroblast growth factor solution (1 µg/ml), and 1% l-glutamine solution (0.1 mM). Cultures were maintained at 37 °C in a humidified 5% CO2/95% air atmosphere. The human epidermoid carcinoma cell line A431 and the human lung small-cell carcinoma cell line H69 were obtained from the American Type Culture Collection. Monolayer cultures of A431 and suspension cultures of H69 were maintained in Iscove’s modified Dulbecco’s medium (Sigma) supplemented with 10% fetal bovine serum and antibiotics.

Cells were cultured in 6-well plates, with or without 20 µM of the proteasome inhibitor MG132 (Pepro Tech; Rocky Hill, NJ) for 24 h. Cells were treated with lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) and the lysates were immunoblotted with anti-E1α and anti-E1β monoclonal antibodies (mAbs). Stock solutions of Tyrphostin 23 (Tyr23, 500 mM; Sigma) were made in dimethyl sulfoxide (Me2SO) and stored at −20 °C. Cells were grown to 70–80% of confluence according to the manufacturer’s protocol. Briefly, 1.5 µl of Lipofectamine 2000, and 3 µg of DNA were each mixed with Opti-MEM (Invitrogen, Carlsbad, CA) and then following a 5-min incubation, the DNA was added to the Lipofectamine 2000 and further incubated at room temperature for 20 min. Then, the DNA-Lipofectamine 2000 complexes (1 ml per well) were added to the cells and incubated overnight. The next day, an additional 1 ml of normal medium was added to each well. Cells were harvested for Western blot analysis 48 h after transfection.

**PDH Enzyme Assay**—PDH specific activity was measured, as reported previously (11), by the rate of 14CO2 formation from 14C pyruvate as follows. Fibroblasts harvested by trypsinization from a confluent T-150 flask were washed in phosphate-buffered saline (PBS), then resuspended in PBS buffer containing serine protease inhibitors (leupeptin and phenylmethylsulfonyl fluoride). Cells were incubated for 15 min at 37 °C with dichloroacetate (DCA, final concentration of 3 mM) to activate the PDC, then reactions were halted by addition of a stop solution (25 mM NaF, 25 mM EDTA, 4 mM dithiothreitol, 40% ethanol) and cells were lysed by repeated freezing and thawing. Sixteen aliquots of each fibroblast sample were assayed in test tubes for PDC activity; four blanks (lysate and water) and four test samples (lysate and water containing thiamine pyrophosphate and co-enzyme A) were assayed at reaction times of 5 and 10 min. An aliquot of 14C-pyruvate of known radioactivity was added to the cell lysates, and the test tubes were immediately closed with a rubber stopper that was outfitted with a center well (Kimble-Kontes, Vineland, NJ) containing 1 cm2 chromatography paper soaked with 100 µl of hyamine hydroxide. Reactions were halted by adding stopping buffer. The 14CO2 evolved because of PDH-mediated catalysis and trapped in the paper was determined by liquid scintillation. PDH specific activity was expressed as nmol 14CO2 produced/min/mg protein. Excess cell lysate was used to determine total protein by the Lowry method (12).

**Western Immunoblot and Immunoprecipitation (WB/IP) Analysis**—Protein lysates from each cultured cell line were run on a 7.5% or 10% polyacrylamide-sodium dodecyl sulfate (SDS-PAGE) gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The blots were probed with mAbs (Invitrogen) to the α and β subunits of the human E1 enzyme, or with phosphorylated EGFR and EGFR antibodies (Santa Cruz Biotechnology) or anti-ubiquitin (P4D1, Santa Cruz Biotechnology, Inc.). The membrane was incubated with a horseradish peroxidase (HRP)-conjugated bovine anti-mouse IgG-HRP or donkey anti-goat IgG-HRP (Santa Cruz Biotechnology), and the secondary antibody was detected using a chemiluminescence luminol reagent (Santa Cruz Biotechnology). For IP, patient fibroblast cells, control fibroblast cells, A431 cells and H69 cells were grown in 10-cm culture dishes or T-75 flask (H69 suspension cells) to about 90% confluence (~106 of H69 cells). Because of the inherent instability of the E1β protein in the patient cells as well as in A431 cells, we divided the cells in two groups and either mock-treated them, or treated with MG132 (final concentration 20 µM) overnight before they were harvested. Cells were lysed in 1 ml of radioimmune precipitation assay buffer (Santa Cruz Biotechnology). The debris were removed by centrifugation at 10,000 × g for 10 min. Lysates were precleared by adding 1.0 µg of mouse IgG (Santa Cruz Biotechnology), together with 20 µg of agarose conjugate (protein A/G-agarose, Santa Cruz Biotechnology) at 4 °C for 30 min. 1 ml of supernatant was transferred to a microcentrifuge tube, and 0.4 µg of E1α and 10 µg of E1β mAb were added and incubated with the precleared lysates for 2 h at 4 °C prior to addition of protein-A/G for a further overnight incubation at 4 °C on a rocker platform. Sample beads were then washed four times with radioimmune precipitation assay buffer by centrifugation at 3,000 rpm for 30 s at 4 °C and the final immune complexes were resuspended in 40 µl of electrophoresis sample buffer (Santa Cruz Biotechnology). Samples were boiled for 2–3 min prior to adding 10 µl of complexes per well for Western analysis using tyrosine phosphor-specific antibody, PY99 (1:300), and secondary antibody, goat anti-mouse IgG-HRP (1:10,000, Santa Cruz Biotechnology), as described above.

**Sequence Analysis**—Skin fibroblast mRNA was extracted (RiboPure™ Kit, Ambion, Austin, TX), and cDNA was made with a Qiagen Omniscript RT Kit according to the manufactur-
er’s instructions. Overlapping segments of E1α and E1β cDNA were amplified with Pfx DNA polymerase (Invitrogen) by PCR. Products were assessed by running on 1% agarose gels, purified with a Qiagen QIAquick Purification kit (Qiagen, Valencia, CA) and sequenced using a Perkin Elmer/Applied Biosystems by the DNA Sequencing Core at the University of Florida.

Quantitative PCR—Quantitative PCR (qPCR) was conducted in a total volume of 20 μl on the DNA Engine Opticon (MJ Research, Waltham, MA), using a DyNamo Hot Start SYBR Green qPCR kit (MJ Research, Waltham, MA). cDNA syntheses were carried out using Oligo(dT) primers with the SuperScript III First-Strand Synthesis System (Invitrogen, La Jolla, CA). Patient and healthy control fibroblast cDNAs, each synthesized from 50 ng of total RNA, were used for each qPCR reaction. Thermal cycle parameters were set according to the manufacturer’s instructions. Human PDHB mRNA primers (forward, 5’-GGCCACAGTTTGGAGTAGGA-3’; reverse, 5’-GGAGCATCCAGGAATGTGA-3’; product size: 76 bp) was designed for qPCR. The human β-actin (forward, 5’-GGATCTGCTCAGCTGAAGTA-3’; reverse, 5’-AGGTGTCCAGATTTC-3’; product size 82 bp) was used as an internal control. One to three independent qPCR trials were conducted for each template source. In each trial, triplicate samples of template were analyzed. qPCR assays were used to measure human PDHB gene expression relative to human β-actin gene expression.

Plasmid Construction—Patient and control cells were cultured and total cellular RNAs were isolated by a RiboPure kit (Ambion), followed by reverse transcription using Omniscript RT kit (Qiagen), according to the manufacturers’ instructions. A cDNA segment over-covering the PDHB sequence was amplified using Pfx DNA polymerase (Invitrogen). The PCR products were cloned into pCR2.1-TOPO (Invitrogen) under control of a T7 promoter to obtain the plasmids pT7/ p-PDHb and pT7/c-PDHb.

In Vitro Transcription and Translation—1 μg each of pT7/ p-PDHb and pT7/c-PDHb was added to an aliquot of the Master Mix (TNT T3 System, Promega, Madison, WI), and was incubated in a 50-μl reaction volume for 60–90 min at 30 °C. The reaction mixture combines the RNA polymerase, nucleotides, salts, all 20 of the common amino acids required for protein synthesis and Recombinant RNasin® Ribonuclease Inhibitor with the reticulocyte lysate solution. Synthesized proteins were analyzed by SDS-PAGE with anti-E1α and anti-E1β mAbs.

RESULTS

The sequence of the cDNA corresponding to the PDHA1 gene was normal in this patient. Analysis of the PDHB cDNA revealed an A → G sense mutation at nucleotide 438 in exon 6 that results in a new codon that still codes for the same amino acid glycine (G146G; data not shown).

The activity of PDH in cultured fibroblasts from the patient was 45% of that measured in control cells (Table 1). Western immunoblot analysis revealed a marked reduction in the expression of the E1β subunit in patient cells, compared with that expressed by a normal control cell line (Fig. 1). In contrast, E1α protein expression in the patient’s cells was similar to that of the control. Transcription efficiency of the E1β gene as determined by qPCR was performed on RNA extracted from fibroblasts of our patient and a control subject. The transcript copy numbers relative to the reference gene β-actin in patient cells (0.023 ± 0.0005) was the same as that measured in control cells (0.024 ± 0.0004, data are means ± S.D. of triplicate determinations). This was further confirmed by an in vitro coupled transcription/translation reaction, which involved transcription of DNA into mRNA and its translation into polypeptides (Fig. 2).

Because no mutations could be identified in the patient’s E1β gene, we hypothesized that a post-translation event accounted for the observed loss of the E1β protein. The ubiquitin-proteasome is the major route for the degradation of cellular proteins and ubiquitin-like modifiers have emerged as regulators of various cellular activities (13–16). We therefore investigated the turnover rate of the E1β protein in fibroblasts from this subject, two patients with E1α deficiency and three normal controls by inhibiting proteasome activity using a specific inhibitor, MG132, a low-molecular-weight molecule that is a common peptide aldehyde inhibitor of the proteasome. Exposure of the patient’s cells to MG132 resulted in a marked decrease in the turnover rate of the E1β protein. However, since accumulation of ubiquitinated proteins were also detected in the control and E1α deficiency cell lines, it was not possible to conclude that
Proteasome-mediated degradation of E1β protein was inhibited by MG132 (Fig. 3A).

Growing evidence indicates that EGFR-PTK-mediated tyrosine phosphorylation is a prerequisite for ubiquitination of cellular proteins that are targeted for proteasome-mediated degradation (17–20). Tyr23 is one of a series of small molecular weight specific inhibitors of EGFR-PTK that can decrease ubiquitination of cellular proteins by binding to the substrate subsite of the EGFR-PTK domain (21). To investigate whether Tyr23 exposure affected protein ubiquitination, we treated fibroblast cells from this subject, two patients with E1α deficiency and three normal controls with Tyr23 prior to Western immunoblotting (Fig. 3B). Immunoreactivity of the E1β subunit in the index patient was clearly detectable with anti-E1β mAb after Tyr23 treatment. In contrast, Tyr23 did not change on E1β or E1α expression in either the E1α deficiency cell lines or the normal control cell lines. Therefore, we suspected that the protein degradation of the E1β subunit in our patient was due to post-translational modification that in turn was related to phosphorylation of its tyrosine residues by EGFR-PTK. We therefore determined the total EGFR protein levels and the EGFR-PTK activities in each cell line using anti-EGFR and anti-phosphotyrosine-EGFR antibodies. As shown in Fig. 4, total EGFR levels were similar among the six cell lines. However, the patient’s cells contained a high basal level of tyrosine-phosphorylated EGFR, indicative of high EGFR-PTK expression. In contrast, cells from patients with E1α deficiency or from control subjects had very low levels of tyrosine-phosphorylated EGFR, indicating that E1β degradation in our patient was dependent on EGFR-PTK activity.

To further confirm whether EGFR-PTK activity correlated directly with the extent of ubiquitination of cellular proteins, we utilized normal control fibroblasts, two cell lines that express high (A431) and low (H69) levels of EGFR-PTK activity, and compared the extent of ubiquitination of cellular proteins in these cells to that in the E1β-deficient cells in the absence and presence of MG132. Western blot analyses were performed using anti-Ub Ab (P4D1). The results showed that accumulation of ubiquitinated proteins in the patient’s cells was lower than that in A431 cells, but higher than that in H69 cells and normal control fibroblasts, and that treatment with MG132 markedly increased ubiquitination in A431 and in the E1β-deficient patient cells (Fig. 5).

In addition, A431 cells, H69 cells, E1α patient fibroblast cells and healthy control fibroblast cells were also transfected with a plasmid containing the human cytomegalovirus immediate-early promoter (CMVp)-driven cDNA for human EGFR-R (pEGFR) (22). Cell lysates were immunoblotted with anti-E1α and anti-E1β monoclonal antibodies.
To further investigate whether EGFR-PTK-mediated specific phosphorylation of tyrosine residue in E1β subunit, but not in E1α subunit, additional immunoprecipitate/Western blotting (IP/WB) experiments were performed with each cell type with or without pretreatment with MG132. E1α and E1β antibody-captured complexes were recovered with protein A/G-agarose beads and the immunoprecipitates were subjected to WB analysis using antibody specific for phosphorylated tyrosine residues. As shown in Fig. 7, the E1β subunit, but not the E1α subunit, of this patient's fibroblast cells and A431 cells, which contain high levels of EGFR, was clearly detected by IP/WB, following MG132 treatment (lanes 6 and 8). Since ~20-fold more protein was used in these experiments than in WB, the tyrosine-phosphorylated E1β proteins were also detectable in these two cell types even in the absence of MG132 treatment (lanes 2 and 4). These data corroborate our hypothesis that in cells that overexpress EGFR-PTK, the E1β subunit becomes phosphorylated at tyrosine residues, which leads to ubiquitination, and subsequent degradation by the proteasome. In contrast, the E1β subunit in healthy control fibroblast cells or H69 cells, which contain normal or low levels of EGFR-PTK, is not subject to this post-translational modification. The tyrosine residues in the E1α subunit in each cell type likewise escape phosphorylation by a hitherto unknown mechanism, thereby avoiding ubiquitination and subsequent proteasome-mediated degradation.

**DISCUSSION**

The patient described in this report had several features characteristic of PDH deficiency, including early age of clinical presentation, neurological dysfunction, elevated cerebrospinal lactate concentration relative to that in peripheral blood and agenesis of the corpus callosum (1, 2). The activity of PDH in cultured fibroblasts was moderately decreased and was associated with normal expression of the E1α subunit but with markedly decreased expression of the β subunit, based on Western immunoblotting (Fig. 1).

The patient's PDHB cDNA harbored a sense mutation that did not alter the amino acid sequence of the β subunit. Thus, we found no evidence for a pathological gene mutation to account for the enzyme deficiency in this subject. Furthermore, the level of E1β mRNA and the linked rate of transcription and translation of the PDHB gene were normal, indicating that these processes could not account for the marked decrease in subunit expression or enzyme activity.

Rapid intracellular degradation of newly synthesized proteins is facilitated ultimately by the ATP-dependent protease activity of the proteasome (23, 24). In most instances, proteasome activity on polypeptides that have been covalently linked to ubiquitin, which targets proteins for digestion. MG 132 is a low-molecular-weight molecule that is among the most widely used peptide aldehyde inhibitors of the proteasome. It readily enters cells in vitro and retards proteasomal destruction of ubiquitinated proteins by inhibiting proteolysis (25–28). Consequently, MG132 has been employed to identify conditions in which altered expression of cellular proteins occurs through changes in proteasomal function. We found that exposure of the patient's cells to MG132 resulted in a detectable

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ASTDYYKRG and 243, STDYYKRGD) are on the surface of E1α subunit in the predicted structure of the human PDH E1 enzyme (PDB accession no. 1ni4).
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EGFR-PTK activity in our patient. However, we have found that the phosphorylated EGFR level in her cells was detectable, in contrast to the absence of detectable phosphorylated EGFR in fibroblasts from the E1α patients and controls. In addition, the steady-state level of the E1β protein in our patient was markedly increased by Tyr23 treatment, whereas the levels of expression of the E1β and E1α subunits in cells from control subjects and from the E1α deficient patients were not appreciably affected. Furthermore, we showed that the nature of high EGFR-PTK activity in our subject was associated with an increased level of protein ubiquitination. Taken together, these data suggest that PDH deficiency in our patient is due to increased turnover of the E1β protein by activation of EGFR through the auto phosphorylation of its tyrosine residues that are targeted for ubiquitination. Although increased protein turnover has been invoked to explain some cases of E1α deficiency (35–37), to our knowledge, none has specifically implicated activation of EGFR as a cause of increased protein degradation. It is likely that in living cells, dynamic protein-protein interactions might prevent phosphorylation of surface-exposed tyrosine residues, which might account for the data obtained in the present study (Fig. 7). These data, nonetheless, provide corroborating evidence that PDH deficiency in our patient involves a post-translational modification in which EGFR-PTK-mediated tyrosine phosphorylation of the E1β protein leads to enhanced ubiquitination followed by proteasome-mediated degradation.

Based on these findings, we propose a model, shown schematically in Fig. 8, of post-translational modification the E1β protein. High EGFR-PTK activity leads to phosphorylation of tyrosine residues. In turn, these phosphorylated residues serve as docking sites, and the protein is readily ubiquitinated. The ubiquitinated protein is then recognized and degraded by the proteasome (Fig. 8A). MG132 inhibits the proteasome pathway, thus preventing protein degradation (Fig. 8B).
Similarly, inhibitors of EGFR-PTK, such as Tyr23, attach to the substrate subsite of the PTK domain and decrease the ubiquitination of the protein (Fig. 8C). Consequently, the protein escapes ubiquitination and, thus, proteosome-mediated degradation. This results in successful translocation to the mitochondrion, reconstitution of the PDH complex and restoration of its catalytic activity.

Two male children with mutations in the E1β gene have been described who had more severe clinical and laboratory findings than our patient (9). Both involved distinct missense mutations that cause marked reduction in E1β expression, modest decrease in E1α expression and no involvement of the active site of the PDHB gene. Similar findings were reported recently in four patients studied by Okajima et al. (10). The relative sparing of E1α expression in all cases contrasts with many reported examples of PDH deficiency due to deletions, missense or nonsense mutations in the E1α gene that result in loss of both immunoreactive E1α and E1β (1, 4, 5). This implies that the stability of the E1β subunit depends on the formation of an enzyme complex with an intact E1α subunit. However, as is apparent in our patient, loss of E1β immunoreactivity need not result in a change in E1α protein expression.

It is intriguing to speculate whether other causes of functional deficiency of the PDH complex or of other critical enzymes of fuel metabolism and energetics may be a consequence of a primary abnormality of the ubiquitin-proteasome system. Our patient represents the first reported case of PDH complex deficiency due to an unstable E1β protein, rather than to a pathological mutation in the E1β gene. The increased rate of E1β turnover we observed appears to be due to EGFR-PTK-mediated phosphorylation. In turn, this promotes ubiquitination and accelerated destruction of the protein by the proteasome.

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