

Loss of Kinase Activity in a Patient With Wolcott-Rallison Syndrome Caused By a Novel Mutation in the *EIF2AK3* Gene

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Wolcott-Rallison syndrome (WRS) is an autosomal recessive disorder characterized by neonatal or early infancy type 1 diabetes, epiphyseal dysplasia, and growth retardation. Mutations in the *EIF2AK3* gene, encoding the eukaryotic initiation factor 2 α -kinase 3 (EIF2AK3), have been found in WRS patients. Here we describe a girl who came to our attention at 2 months of age with severe hypertonic dehydration and diabetic ketoacidosis. A diagnosis of type 1 diabetes was made and insulin treatment initiated. Growth retardation and microcephaly were also present. Anti-islet cell autoantibodies were negative, and mitochondrial diabetes was excluded. Imaging revealed a hypoplastic pancreas and typical signs of spondylo-epiphyseal dysplasia. The diagnosis of WRS was therefore made at age 5 years. Sequencing analysis of her *EIF2AK3* gene revealed the presence of a homozygous T to C exchange in exon 13 leading to the missense serine 877 proline mutation. The mutated kinase, although it partly retains the ability of autophosphorylation, is unable to phosphorylate its natural substrate, eukaryotic initiation factor 2 α (eIF2 α). This is the first case in which the pathophysiological role of EIF2AK3 deficiency in WRS is confirmed at the molecular level. Our data demonstrate that EIF2AK3 kinase activity is essential for pancreas islet function and bone development in humans, and we suggest EIF2AK3 as a possible target for therapeutic intervention in diabetes. *Diabetes* 51:2301–2305, 2002

The Wolcott-Rallison syndrome (WRS) is named after the two authors who first described two brothers and a sister affected by infancy-onset diabetes, multiple epiphyseal dysplasia, and growth retardation (1). Other multisystemic manifestations, such as psychomotor retardation and hepatic dysfunction, become evident later in life.

Mutations in the *EIF2AK3* gene have been found in two families affected by WRS (2), although no pathophysiological basis for the disease was addressed. Eukaryotic

initiation factor 2 α -kinase 3 (EIF2AK3, also called PERK or PEK) regulates protein synthesis during stress by phosphorylating the α -subunit of the eukaryotic initiation factor 2 (eIF2). There are four protein kinases known to phosphorylate eIF2 α : GCN2, heme-regulated inhibitor (HRI), double-stranded RNA-activated protein kinase (PKR), and EIF2AK3/PERK. PERK, identified as the eIF2 α kinase enriched in pancreatic cells, is uniquely located to the endoplasmic reticulum (ER) (3) and is activated by the accumulation of unfolded proteins in the ER lumen (4). Activation of PERK results in phosphorylation of eIF2 α that acts as a dominant inhibitor of the guanine nucleotide exchange factor eIF2B and prevents the recycling of eIF2 between successive rounds of protein synthesis. Through halting translation initiation and protein synthesis, PERK may relieve ER stress by reducing the number of unfolded proteins in the ER (5,6) and prevent β -cell death.

The patient described herein is the third reported case of a mutation in the *EIF2AK3* gene as a cause of WRS, but it is the first demonstration that loss of PERK kinase activity leads to human disease and confirms that PERK is necessary for normal islet β -cell function and bone development in humans.

RESEARCH DESIGN AND METHODS

Case report. The girl came to our attention at 2 months of age, as the only child of nonconsanguineous parents. She presented with severe hypertonic dehydration and diabetic ketoacidosis with two prolonged convulsive episodes. A diagnosis of type 1 diabetes was made and insulin treatment initiated. There was no remission phase. The family history revealed that the mother suffers from a mild form of deafness and that a maternal uncle is affected by type 2 diabetes. After the age of 3 years, mild psychomotor development retardation was recognized. Physical examination showed growth retardation, microcephaly, limited shoulder mobility, and *genua valga* (Fig. 1A). The anti-islet cell autoantibodies (insulin autoantibody [IAA], insulinoma antigen 2 [IA2], and GAD) were negative. Mitochondrial diabetes was excluded. Average glycosylated hemoglobin since diagnosis was elevated (8.7%, normal value <6%). Abdominal ultrasonography showed a hypoplastic pancreas, and skeletal X-ray examination showed typical signs of spondylo-epiphyseal dysplasia (Fig. 1B). Based on these findings, the diagnosis of WRS was made. At age 2.7 years, the girl experienced an episode of atypical prolonged hypoglycemia despite greatly reduced insulin therapy during an acute infection with ataxia and neuromuscular hypotonia. At age 5 years, under similar circumstances, she showed high fever, prolonged hypoglycemia, and highly elevated liver enzymes (aspartate aminotransferase 4,753 units/l, alanine aminotransferase 2,429 units/l, and γ -glutamyl transferase 170 units/l). Hepatitis A, B, and C and Epstein-Barr virus (EBV) were excluded. Spontaneous normalization of clinical and laboratory abnormalities occurred within a few days. So far, no clinical or biochemical evidence of exocrine pancreas insufficiency is present.

Genomic DNA mutation analysis. Genomic DNA was extracted from peripheral blood leukocytes of the patient, her parents, and 50 normal control subjects using the Qiagen DNA blood and cell culture kit (Qiagen, Hilden,

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eIF2, eukaryotic initiation factor 2; EIF2AK3, eukaryotic initiation factor 2 α -kinase 3; ER, endoplasmic reticulum; IA2, insulinoma antibody 2; IAA, insulin autoantibody; WRS, Wolcott-Rallison syndrome.

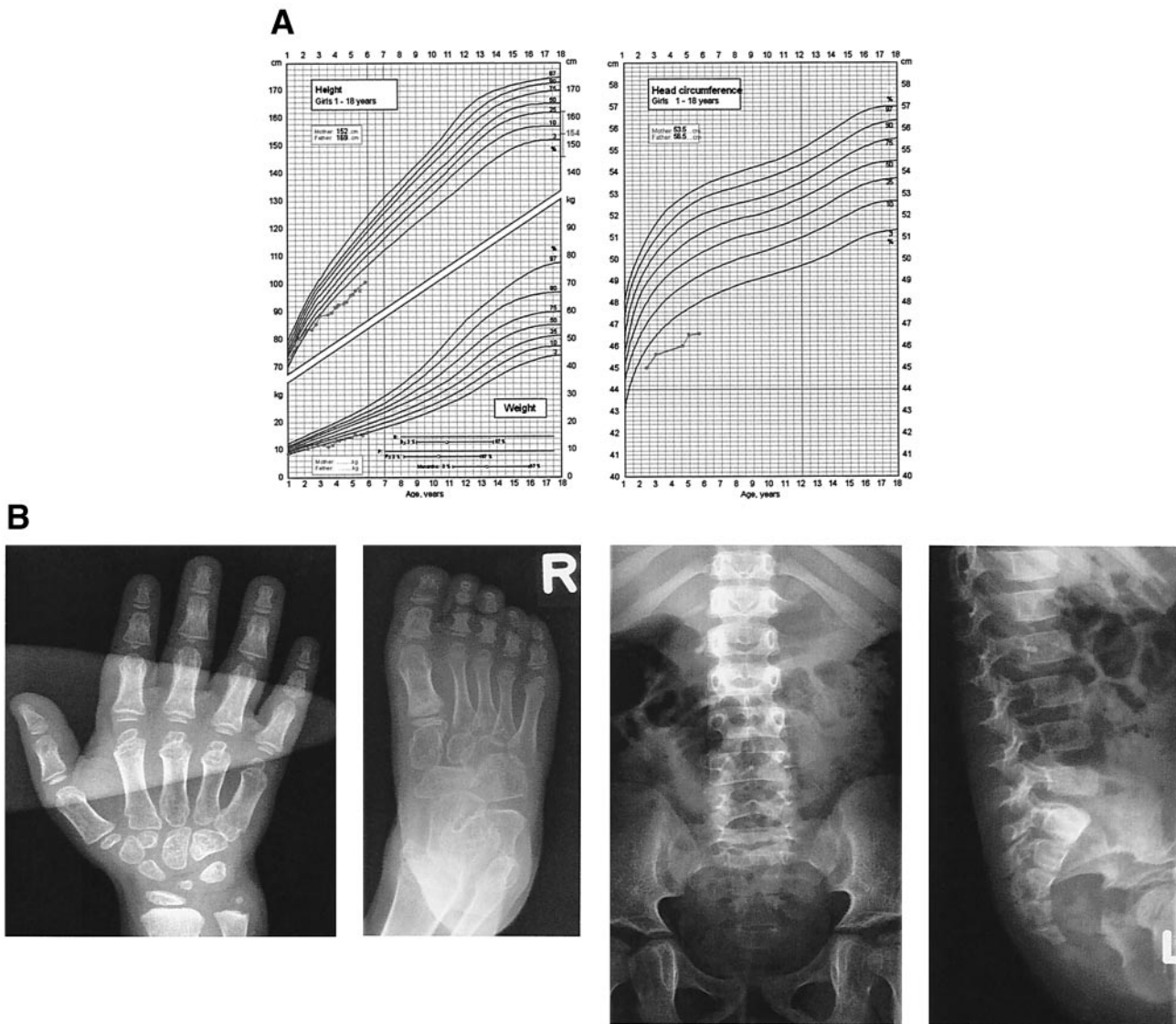


FIG. 1. A: Growth curve and head circumference chart showing growth retardation and microcephaly of our patient. **B:** X-ray imaging depicting the characteristic features of spondylo-epiphyseal dysplasia.

Germany) to perform PCR exonic amplification (Fig. 2A). Sequences of the primers used for amplification of *EIF2AK3* (accession no. 16162274) and *GNC2* (accession no. NT_010194) genes are available upon request (all primers were purchased from Mycosynth, Balgach, Switzerland). Direct cycle sequencing of the PCR products was performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit and analyzed by electrophoresis on the ABI Prism 310 Genetic Analyzer (Perkin Elmer Applied Biosystems, Rotkreuz, Switzerland). *HphI* restriction analysis was performed as suggested by the supplier (New England Biolabs, Allschwil, Switzerland).

Expression studies. To study the functional implications of the mutations found, we established an RT-PCR method using *EIF2AK3* mRNA ectopically expressed in peripheral blood leukocytes of normal subjects. Total RNA was extracted from 500 μ l whole blood using the RNeasy minikit (Qiagen). RT was performed on 100 ng total RNA using Superscript reverse transcriptase (Invitrogen/Life Technologies, Basel, Switzerland). PCR amplification of *EIF2AK3* cDNA was performed using the direct primer 5'-GCGGGAGAGGC AGGCGTCAGT-3' and the reverse primer 5'-TTGGCTCAAATTAGGT TATGC-3' under the following cycling conditions: 93°C for 1 min, 44°C for 2 min, and 68°C for 1 min \times 40 cycles. The primers contained a *Bam*HI and an *Eco*RI restriction sites, respectively, at their 5'-end, to facilitate subcloning. The wild-type cDNA was subjected to PCR-mediated site-directed mutagenesis. The wild-type and the mutant *EIF2AK3* cDNA were subcloned into a pCMV-Script vector (Stratagene, Basel, Switzerland) and used for functional studies. Confluent COS1 cells (American Type Culture Collection no. CRL 1650) were transiently transfected using 50 μ g lipofectamine and 10 μ g DNA on a 10-cm plate (Life Technologies). The transfection efficiency, assessed via

a β -galactosidase-containing vector (SV-40 β -gal; Promega, Wallisellen, Switzerland), ranged between 10 and 30%. Western blot analysis was performed using standard procedures. The first antibody, used at a titration of 1:200, was a polyclonal goat anti-human α PERK (Santa Cruz Biotechnology, Lucerne, Switzerland) and a rabbit polyclonal phosphorylation site-specific α EIF2 α (Biosource International, Lucerne, Switzerland). The second antibody was a rabbit anti-goat and a goat anti-rabbit IgG alkaline phosphatase conjugate (BioRad, Glattbrugg, Switzerland).

Immunoprecipitation kinase assay. Cellular extracts were obtained from transfected COS1 cells (48 h after transfection) as already described (6). The activity of wild-type and mutant EIF2AK3 (PERK) was measured in immune-complexed kinase assay using 1, 3, and 5 μ g of purified human eIF2 α as substrate (Biosource International) as previously described (4). As negative control, equivalent amounts of the nonphosphorylatable eIF2 α 51A peptide were used (Biosource International).

Routine diagnostic analyses. GAD, IAA, and IA2 were measured using commercially available kits (Schering, Geneva, Switzerland). Glycosylated hemoglobin levels were determined using the Bayer DCA 2000 method (Bayer, Leverkusen, Germany).

RESULTS

Direct sequencing of PCR fragments amplified from genomic DNA of the patient revealed the presence of a homozygous T to C exchange in exon 13 of the *EIF2AK3*

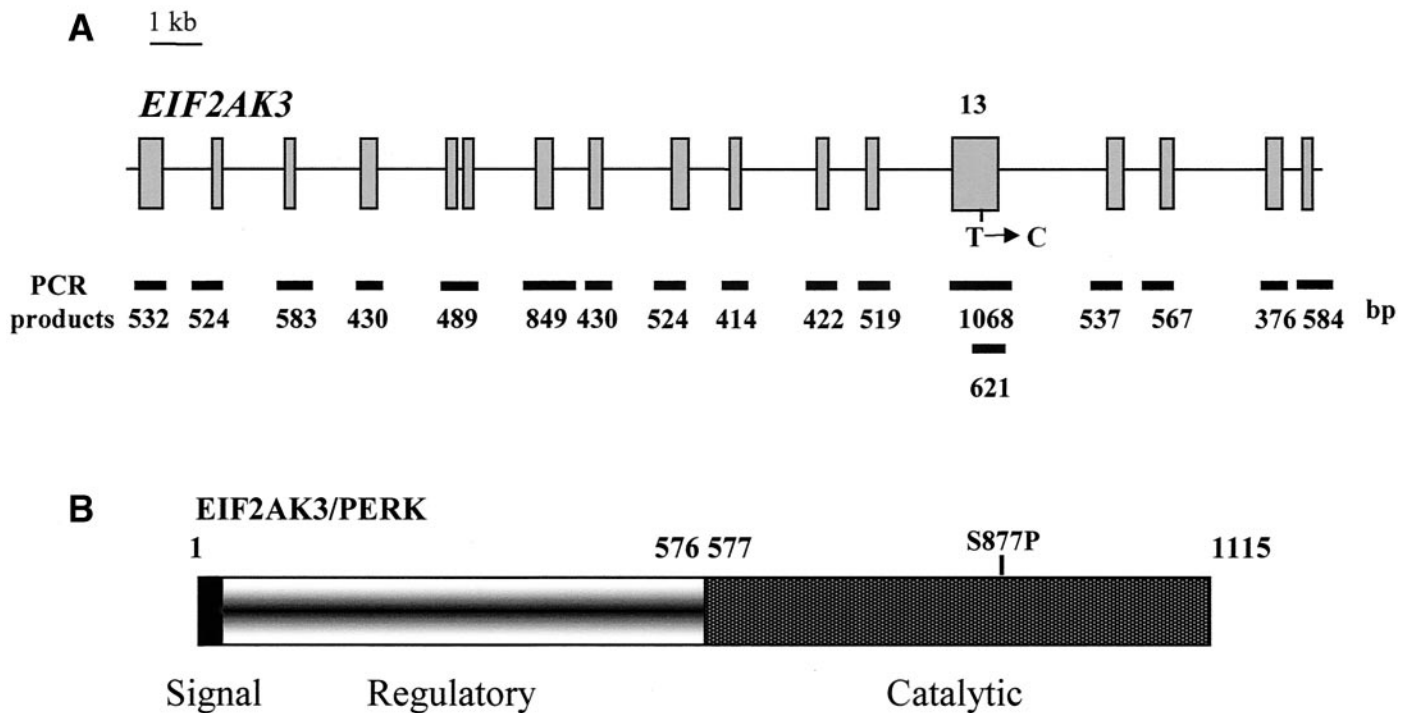


FIG. 2. Schematic representation of *EIF2AK3* gene (**A**) and protein (**B**). Gray boxes represent the coding exons. The sizes in base pairs of the PCR products are also indicated. The position of the mutation in exon 13 and the corresponding S877P missense mutation in the catalytic region of the *EIF2AK3*/PERK protein are sketched.

gene leading to the missense S877P mutation in the catalytic domain of the *EIF2AK3* protein (Figs. 2 and 3A). Serine 877 is conserved in human, mouse, and rat proteins. The rearrangement induces the loss of an *HphI* restriction site that is instead present in the heterozygous state in the parents of the patient (Fig. 3B) and on both alleles of the 50 normal control subjects (100 alleles; representative experiments, Fig. 3C). These data suggest that the T to C exchange is not a polymorphism. The mutation in the *EIF2AK3* gene of our patient was re-created by site-directed mutagenesis and used for functional studies. This rearrangement does not interfere with protein translation and stability, as demonstrated by the Western blot analysis (Fig. 4A and B). The P877 mutated protein is less efficient than the wild-type kinase in phosphorylating itself and is completely unable to phosphorylate *eIF2 α* (Fig. 4C), independently from the amount of substrate (Fig. 4D and E). This loss of kinase activity is likely the pathophysiological basis of the disease in our patient. The lack of an increase in the immunoprecipitable phosphorylated *eIF2 α* (Fig. 4C) between 3 and 5 μ g substrate indicates 3 μ g substrate as the saturation limit of our system.

Because of the occurrence of hypoglycemia in our patient, which is not present in *Perk*^{-/-} mice, we also analyzed the primary sequence of the *GCN2* gene. *GCN2* is thought to be responsible for *eIF2 α* phosphorylation in the liver in response to nutrient depletion, with consequent activation of gluconeogenic enzymes. No mutation was found in the *GCN2* gene of our patient (not shown).

DISCUSSION

The study of the molecular basis of Mendelian types of diabetes-related disorders, such as maturity-onset diabe-

tes of the young, severe insulin resistance, and WRS, has improved our knowledge of factors involved in the regulation of pancreatic β -cell function. WRS provides further intriguing insights into the relation between glucose homeostasis and protein translation control. In our patient, all the hallmarks of WRS were present, including infancy-onset type 1 diabetes, spondylo-epiphyseal dysplasia, hepatic dysfunction, and growth retardation. The diagnosis was confirmed at the molecular level by the presence of a homozygous mutation of a conserved residue (S877) located in the catalytic domain of the *EIF2AK3*/PERK protein. The single S877P amino acid exchange completely abolishes the ability of PERK of phosphorylating its physiological substrate *eIF2 α* . This is the first functional demonstration of a link between lack of *eIF2 α* phosphorylation and human inherited disease, although mutations in the *EIF2AK3* gene have been demonstrated to be linked to WRS (2). The recent generation of mice bearing targeted disruption of the *Perk* gene (7) gave new insights in the function of the enzyme and may allow extrapolation for human disease. For instance, in both mice and humans, type 1 diabetes is the main phenotypic feature, and it is caused by loss of β -cells (8). This β -cell depletion does not begin in utero but after birth in *Perk*^{-/-} mice and in patients with WRS (9 and present work), and it is largely caused by β -cell apoptosis. These observations support the model of the role of PERK in pancreatic β -cell function, in which *eIF2 α* phosphorylation causes a decrease in the number of unfolded proteins in the ER and promotion of β -cell survival. *eIF2 α* phosphorylation might also result in the transcriptional activation of genes that are important for cell survival. In the absence of a functional PERK, the accumulation of unfolded proteins in the ER and the

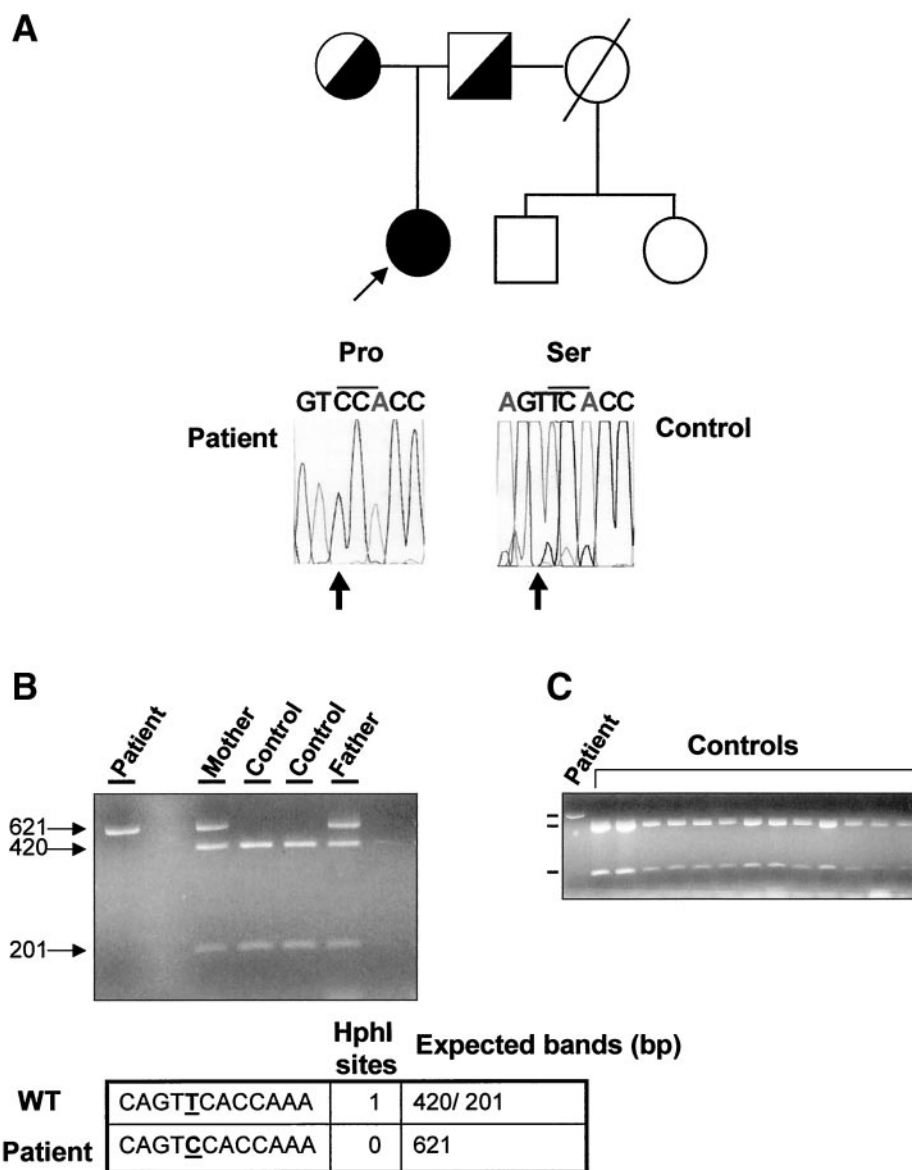


FIG. 3. A: DNA sequence chromatograms obtained by direct sequencing of PCR products showing the presence of the homozygous T to C substitution in exon 13, not present in normal individuals (control, representative example of 50, 100 alleles). **B:** Restriction endonuclease analysis of a 621-bp exon 13 PCR product. The rearrangement causes the loss of one *HphI* [(N)₈TCACC] restriction site and the generation of one undigested band in the patient instead of the expected normal two (420 and 201 bp) as summarized in the table. The parents, who are obligate heterozygotes, display the expected three bands of the carrier state. **C:** The exchange is not a common polymorphism because it is not present in 50 normal control subjects (representative experiments).

lack of survival factors eventually lead to β -cell apoptosis and diabetes.

However, the WRS and the *Perk*^{-/-} mice phenotypes display significant differences. For example, the liver involvement and the hypoglycemic crises suffered by our patient are absent in the *Perk*^{-/-} mice. Severe hypoglycemia caused by defective hepatic gluconeogenesis is rather a feature of the *Ser*⁵¹ mutant mouse (10), carrying an eIF2 α that cannot be phosphorylated. In the liver, eIF2 α phosphorylation seems to be mediated by GCN2 because deletion of the other eIF2 α kinases (including PERK) does not result in hypoglycemia in mice. We therefore searched for mutations in the *GCN2* gene of our patient and found none. It is then unlikely that our patient's hypoglycemic crises are caused by concomitant GCN2 defect, although we cannot exclude abnormalities in the expression or posttranslational modifications of GCN2. These observations suggest that, unlike in the mouse, PERK might be important for eIF2 α phosphorylation in the human liver. In particular, lack of activation of PERK in amino acid

depletion states and/or in stress would prevent the physiological activation of gluconeogenic enzymes, causing blunted stress-related hyperglycemic response and, in concomitance with insulin administration, prolonged but transient hypoglycemia, as our patient also experienced. Any broad conclusions, however, must await the description and characterization of more WRS patients.

Finally, the bone involvement indicates a pleiotropy in humans that might not be present in mice and, more importantly, demonstrates a crucial role of EIFAK3/PERK (and eIF2 α phosphorylation) in skeletal development in humans.

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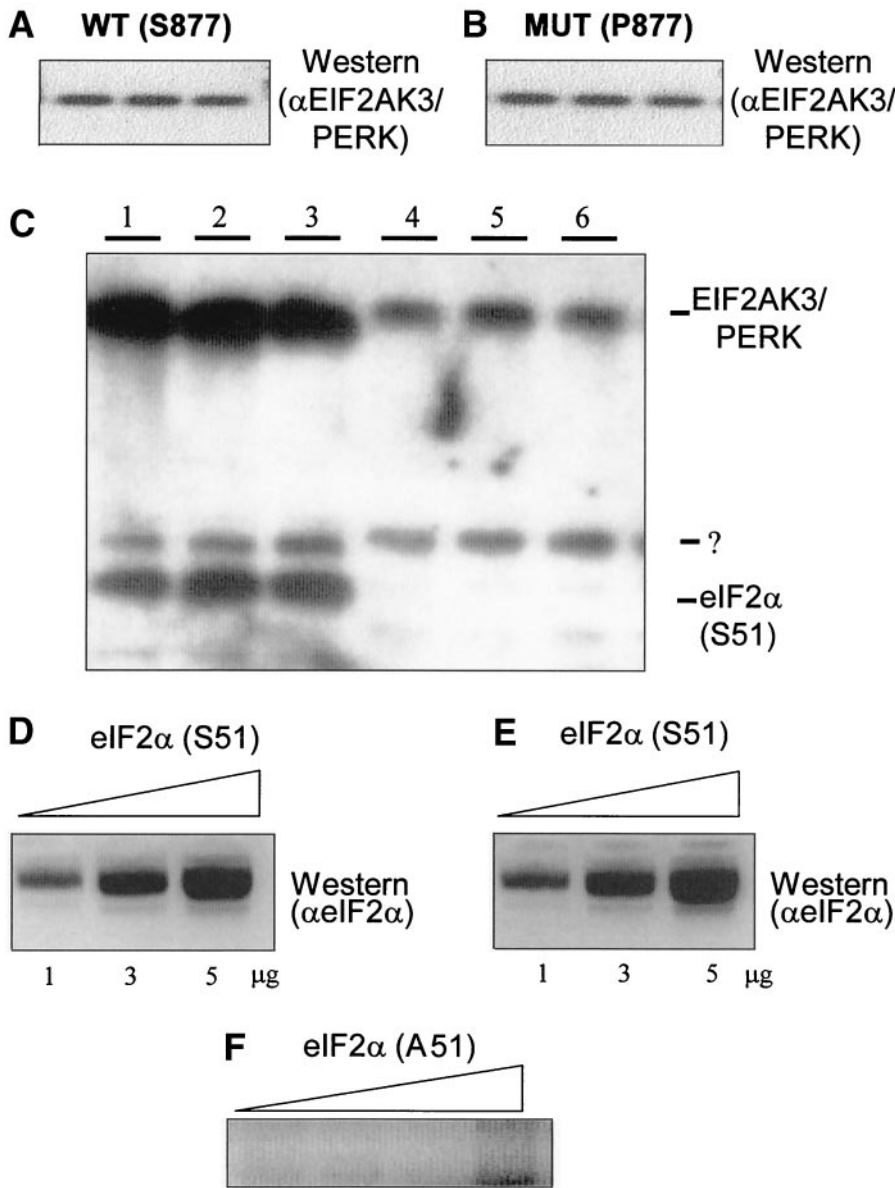


FIG. 4. Expression of wild-type and mutant EIF2AK3. *A* and *B*: Western blot analysis was performed on extracts of COS1 cells transiently transfected with a pCMV-Script vector containing the wild-type EIF2AK3 cDNA (*A*) or the mutated EIF2AK3 P877 (MUT) cDNA (*B*). *C*: The activity of the wild-type (WT) (S877, lanes 1–3) and mutant (MUT) EIF2AK3 (MUT P877, lanes 4–6) from COS1 cell lysate was assessed in immuno-complexed kinase assay using recombinant eIF2α as a substrate. The immunoprecipitation was carried out with 2 μg polyclonal anti-PERK antibody. After washing, the eIF2α kinase assay was carried out by addition of 1, 3, and 5 μg of purified human eIF2α (*D* and *E*, anti-eIF2α used at a 1:200 dilution) and 20 μCi of [γ - 32 P]ATP. The samples were then analyzed by SDS-polyacrylamide gel electrophoresis. As negative control for the phosphorylation reaction, the same increasing amount of the nonphosphorylatable S51A eIF2α was used (*F*). The amino acid substitution at residue 877 of the EIF2AK3 protein does not interfere with protein translation and stability but decreases PERK autophosphorylation and completely abolishes the ability of PERK to phosphorylate its natural substrate eIF2α.

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