Endocrine, *vol. 16, no. 1, 39–42, October 2001* 0969–711X/01/16:39–42/$11.00

Identification and Functional Analysis of Mutations in FAD-Binding Domain of Mitochondrial Glycerophosphate Dehydrogenase in Caucasian Patients with Type 2 Diabetes Mellitus

Mònica Gudayol,[1] Josep Vidal,[1] Elena F. Usac,[1] Albert Morales,[2]
Marta E. Fabregat,[1] José C. Fernández-Checa,[2] Anna Novials,[3] and Ramon Gomis*,[2]

[1]Endocrinology and Diabetes Unit; [2]Liver Unit, IDIBAPS, Hospital Clínic de Barcelona, Spain; and [3]Department of Diabetes, Sardà-Farriol Foundation, Barcelona, Spain

Ca^{2+}-responsive mitochondrial FAD-linked glycerophosphate dehydrogenase (mGPDH) is a key component of the pancreatic β-cell glucose-sensing device. The purpose of this study was to examine the association of mutations in the cDNA coding for the FAD-binding domain of mGPDH and to explore the functional consequences of these mutations in vitro. To investigate this association in type 2 diabetes mellitus, we studied a cohort of 168 patients with type 2 diabetes and 179 glucose-tolerant control subjects of Spanish Caucasian origin by single-stranded conformational polymorphism analysis. In vitro site-directed mutagenesis was performed in the mGPDH cDNA sequence to reproduce those mutations that produce amino acid changes in a patient with type 2 diabetes. We detected mutations in the mGPDH FAD-binding domain in a single patient, resulting in a Gly to Arg amino acid change at positions 77, 78, and 81 and a Thr to Pro at position 90. In vitro expression of the mutated constructs in *Xenopus* oocytes resulted in a significantly lower enzymatic activity than in cells expressing the wild-type form of the enzyme. Our results indicate that although mutations in the mGPDH gene do not appear to have a major role in type 2 diabetes mellitus, the reduction in mGPDH enzymatic activity associated with the newly described mGPDH mutations suggests that they may contribute to the disease in some patients.

Key Words: Mitochondrial FAD-linked glycerophosphate dehydrogenase; genetics; insulin secretion; amino acid variants; polymerase chain reaction single-stranded conformational polymorphism.

Introduction

The generation of metabolic signals derived from glucose metabolism in pancreatic β-cells has a pivotal role in glucose-induced insulin release *(1)*. Transference of cytosolic NADH generated by the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase into the mitochondria through NADH shuttles is currently considered essential for coupling glycolysis and activation of the mitochondrial energy metabolism that finally triggers insulin secretion *(2)*. Thus, the Ca^{2+}-responsive mitochondrial FAD-linked glycerophosphate dehydrogenase (mGPDH), the limiting enzyme of the glycerol-phosphate shuttle, is considered a key component of the glucose-sensing device *(3)*. A deficiency in mGPDH enzymatic activity has been related to the impairment of glucose-stimulated insulin release both in several animal models of noninsulin-dependent diabetes mellitus and in islets from a short series of humans with type 2 diabetes mellitus *(3–5)*. Although most studies in animals have suggested that the low enzymatic activity of mGPDH is an acquired defect *(6,7)*, the origin of this in humans remains an open question. Linkage studies have failed to find an association between mGPDH gene mutations and type 2 diabetes, which suggests that mutations in this gene are not a major cause of type 2 diabetes *(8,9)*. However, some studies based on single-stranded conformational polymorphism (SSCP) analysis of the mGPDH gene found in some patients that mutations affecting the mGPDH Ca^{2+}-binding domain, which codes exons 15 and 16 and a conserved region of exon 6, may be linked to the disease *(10, 11)*. The gene has 17 exons with a coding region length of 2667 bp (GB#U36310). The critical activity regions are as follows: FAD binding (exons 3 to 4); glycerol phosphate binding (exons 10 to 11), and EF-hand calcium binding (exons 15 to 16) *(12)*.

The aims of the present study were to examine the association of mutations in the cDNA coding for the FAD-binding domain of mGPDH and to explore the functional consequences of these mutations in vitro.

Results

An abnormal mobility pattern in the FAD-binding domain SSCP analysis was observed in the sample of one patient with diabetes (Fig. 1), a 65-yr-old man (body mass index [BMI]

Received July 13, 2001; Revised October 1, 2001; Accepted October 2, 2001.
Author to whom all correspondence and reprint requests should be addressed: Dr. Ramon Gomis, Endocrinology and Diabetes Unit, Hospital Clínic, Villaroel 170, 08036 Barcelona, Spain. E-mail: gomis@medicina.ub.es

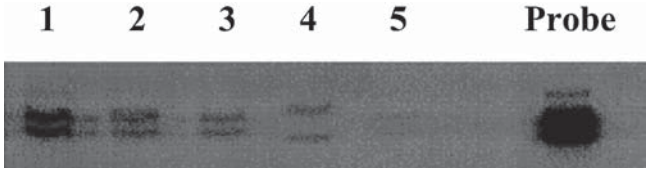

Fig. 1. SCCP analysis of mGPDH FAD-binding domain of human cDNA from two patients without diabetes (lanes 1 and 2) and three patients with type 2 diabetes (lanes 3–5). Lanes 1–3 and 5 display the same mobility pattern as the probe. Automated sequencing of the PCR product corresponding to the lane with altered mobility pattern (lane 4) yielded four nonconservative base pair mutations.

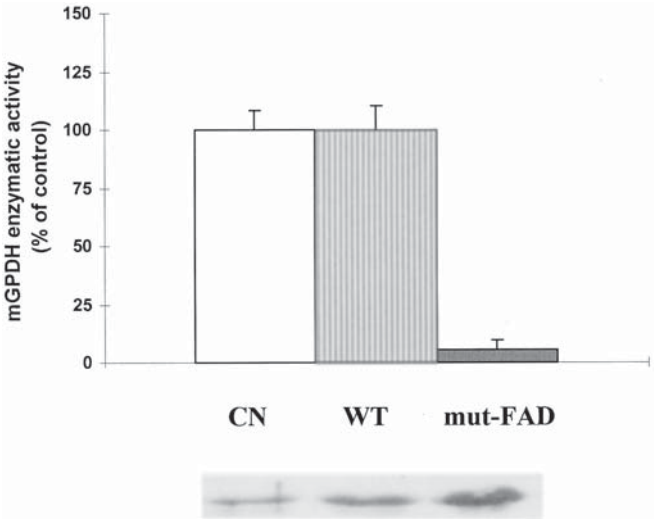

Fig. 2. mGPDH enzymatic activity in microinjected *Xenopus* oocytes with wild-type (WT) form or FAD-binding domain mutated construct (mut-FAD) of mGPDH. *Xenopus* oocytes microinjected with water were used as controls (CN). Data were adjusted to mGPDH protein content determined by densitometry of Western blot (a representative Western blot is shown). The enzymatic activity of mGPDH in CN was normalized to 100 (equivalent to 190 atmol/[min · cell] for *Xenopus* oocytes). Data represent the mean ± SD of three to five individual experiments.

= 26.2 kg/m$^{2)}$ in whom type 2 diabetes was diagnosed at the age of 53 and who had been treated for the previous 7 yr with hypoglycemic sulfonylurea. Automated sequencing of the polymerase chain reaction (PCR) product yielded four nonconservative base pair mutations. G→A substitutions at positions 352, 355, and 364 caused Gly to Arg amino acid change at positions 77, 78, and 81. A→C at position 390 yielded a Thr^{90}→Pro change. These amino acid changes were found in a homozygous form. In this patient, insulin secretion was assessed by an iv glucose tolerance test. The sum of immunoreactive insulin levels at min 1 and 3 was used to estimate first-phase insulin release. Results were compared with those obtained in 10 previously reported healthy control subjects *(13)*. First-phase insulin release was lower (10.6 mU/L) than in the control group (70.8 mU/L). Homeostasis Modelling Assessment analysis revealed insulin sensitivity (%S) in the normal range in the proband (%S of 44.9% vs healthy control population %S of 55 ± 21% mean ± SD, range 14.2–90.2, n = 44) *(14)*.

Xenopus oocytes microinjected with the FAD-binding domain–mutated mRNA displayed on average mGPDH enzymatic activity of 5.8 ± 4.3% (n = 3) of the activity in those microinjected with the wild-type form (100 ± 11.7%) (p < 0.0l) (Fig. 2). mGPDH enzymatic activity *(15)* was adjusted to the mGPDH protein content determined by densitometry of a Western blot *(16)*.

Discussion

In the present study, we failed to find a positive association between type 2 diabetes mellitus and mutations in the FAD-binding domain of mGPDH cDNA in Spanish Caucasian patients. Of the 347 patients studied, we identified only one patient with type 2 diabetes with mutations in the FAD-binding domain, leading to four base pair substitutions. The patient was the diabetic proband of a previously reported family in which mutations in the Ca^{2+}-binding domain of mGPDH cDNA were identified *(10)*.

A genetically determined impaired β-cell function has been considered an essential component of the pathogenesis of type 2 diabetes mellitus *(17)*. The genetic basis of such a defect is currently unknown, but various evidence suggests that it may be multifactorial and heterogeneous in populations *(18)*. Linkage analysis in Mexican-American *(8)* and Caucasian *(9)* patients has failed to find an association between mGPDH and type 2 diabetes, excluding mGPDH as a major gene involved in type 2 diabetes mellitus in these populations. However, SSCP analysis has not excluded a possible minor role for mGPDH in some Japanese *(19)* or Caucasian American *(11)* patients. Accordingly, using the same approach, we identified a patient with type 2 diabetes harboring mutations at the FAD-binding site. The Gly to Arg substitutions at positions 77, 78, and 81 represent a replacement of conserved amino acids in humans *(20)*, rats *(21)*, mice *(21)*, *Sarachomices cerevisiae (22)*, and *Bacillus subtilis (21)*. Thr at position 90 is also conserved in these species, except in *B. subtilis (21)*. All the mutations observed affect the most conserved part of the expected FAD-binding site *(12)*.

Enzymatic mGPDH activity measurements in *Xenopus* oocytes support the view that there is a possible effect of mGPDH mutations on impairment of β-cell function. When compared with the wild-type form of the enzyme, the in vitro expression of the mGPDH mutant form reproducing the mutations in the FAD-binding domain led to a significant reduction in enzymatic activity (5.8 ± 4.3%). This reduction in enzymatic activity is comparable to those reported both in human islets of patients with type 2 diabetes *(5)* and

Table 1
Characteristics of Study Subjects[a]

	Control subjects (n = 179)	Patients with type 2 diabetes (n = 168)
Age (yr)	61.2 ± 12.7	61.3 ± 12.2
Sex (male/female)	72/107	101/67
BMI (kg/m^2)	26.0 ± 4.9	28.3 ± 5.2*
Fasting plasma glucose (mmol/L)	5.06 ± 0.31	9.72 ± 3.86**
Duration of diabetes (yr)		10.9 ± 8.1

[a]Data are presented as mean ± SD. *$p < 0.001$; **$p < 0.01$.

animal models of the disease *(3,4)* and are linked to a reduction in glucose-induced insulin secretion.

Eto et al. *(2)* recently reported metabolic studies on mice with a targeted disruption of mGPDH gene. Even though mice homozygous for the mutated mGPDH gene lacked mGPDH protein, they were glucose tolerant both when fasting and after an ip glucose tolerance test. These results imply that mutations in the mGPDH gene are unlikely to play a major role in the pathogenesis of type 2 diabetes mellitus. However, we do not rule out the possible influence of mutations in the mGPDH gene in a multifactorial or polygenic model of the disease. In that respect, it has been proposed that the interaction between experimental *(2)* or dietary *(23)* and genetic β-cell defects is a mechanism leading to impaired β-cell function in animal models of type 2 diabetes mellitus.

In summary, mutations in the FAD-binding domain of the mGPDH gene are not a common cause of type 2 diabetes mellitus in Spanish Caucasians. However, in vitro expression studies of the mGPDH mutant forms reproducing the observed mutations suggest that a contributory effect of mutations in the mGPDH gene to the pathogenesis of type 2 diabetes mellitus should not be completely ruled out.

Materials and Methods

Subjects

A cohort of 168 unrelated type 2 diabetic and 179 sex- and age-matched glucose-tolerant individuals of Spanish Caucasian origin participated in the study (Table 1). The study was approved by the Hospital Clínic ethical committee. Informed consent was obtained from all participants.

Reverse Transcriptase-PCR and PCR-SSCP Analysis

RNA was obtained from peripheral leukocytes using a Quick prep total RNA extraction reagent kit (Pharmacia Biotech, Uppsala, Sweden). RNA was treated with RNase-free DNAase I (10 U for 30 µg of total RNA) in order to avoid any contamination by genomic DNA. cDNA synthesis was performed in a reaction mixture containing 100 U/µg of RNA of M-MLV Reverse Transcriptase (Gibco-BRL, Life Technologies, Gaithersburg, MD), 1 m*M* dNTPs, random primers (500 ng), and 20 U of RNasin Inhibitor enzyme (Promega, Madison, WI). The cDNA obtained from total RNA was then amplified in a 10 µL radioactive PCR. The radioactive PCR reaction for the FAD-binding domain was performed using [α-^{32}P]-dCTP as a tracer and a specific set of primers: forward primer, 5'-TCC AGA GAA GCT CAG CTA CT; reverse primer, 3'-GTC TTC GTG ATT TAA CTA GG. The standard conditions were 1 µL of the cDNA, 1 µ*M* of each primer, 70 µ*M* of dNTPs, 1.5 m*M* $MgCl_2$, 0.2 µL of [α-^{32}P]-dCTP (3000 Ci/mmol, 10 mCi/mol, 10 mCi/mL), and 0.2 U of Taq DNA polymerase (Gibco-BRL). The cycling protocol consisted of 30 cycles of denaturation (94ºC, 30 s), annealing (58ºC, 30 s), and extension (72ºC, 50 s).

SSCP analysis of the FAD-binding domain was conducted on 1 µL of each PCR product, which was mixed with 95% formamide, 10 m*M* EDTA, 0.05% bromophenol blue, and 0.05% xylen cyanol. Samples were heat denatured at 94ºC for 5 min and then electrophoresed under two specific conditions: samples for the FAD-binding domain study were run at room temperature at 15-W constant power for 4 h, 30 min. cDNA sequencing was performed on the PCR products displaying an abnormal migration pattern in SSCP analysis with an ABI-PRISM 377 automatic sequencer and dye terminator kit (Applied Biosystems, Perkin-Elmer, Branchburg, NJ).

Construction of mGPDH Mutants

Standard molecular cloning techniques were used to subclone human mGPDH cDNA from Bluescript, kindly provided by Dr. J. Ferrer (Boston, MA), to pcDNA3 mammalian expression vector. Site-directed mutagenesis was performed in the mGPDH cDNA sequence (GB#U36310) to reproduce those mutations that produce amino acid changes in the patient with type 2 diabetes. The construct, called mutFAD, had four amino acid changes in the FAD-binding domain: three G→A substitutions at positions 352, 355, and 364 causing Gly to Arg amino acid changes at positions 77, 78, and 81, and A→C substitution at position 390 yielding a Thr^{90}→Pro. The mutations were confirmed by cDNA sequencing, using an ABI-PRISM 377 automatic sequencer and dye terminator kit (Applied Biosystems, Perkin-Elmer).

Cell Culture and In Vitro Protein Expression

The mutated form plus the wild-type mGPDH cDNA were studied by in vitro expression systems. Capped mRNA to microinject *Xenopus* oocytes was synthesized by the T7 cap-site method (Boehringer Mannheim GmbH, Germany). The procedures for preparation and injection of *Xenopus* oocytes were performed in line with previously described protocols *(24)*.

Wild-Type and Mutated mGPDH Activity

The assay of FAD-glycerol phosphate dehydrogenase was based on the generation of ^{3}HOH from L-(2-^{3}H) glycerol 3-phosphate (5 µCi/mL) during a 30-min incubation at 37ºC in 90 µL of HEPES buffer (20 m*M*, pH 7.2) containing 1 m*M* glycerol 3-phosphate, 1 m*M* EGTA, and 0.1 m*M* FAD with the material derived from five oocytes per sample. Cells were previously washed twice with HEPES buffer containing 1 m*M* EGTA for 5 min at 780 *g*. The supernatant was removed, and the remaining material was mixed with the same buffer, sonicated, and centrifuged for 1 to 2 min at 500 *g*. The supernatant was then used for the enzymatic activity assay. The reaction was halted by adding 90 µL of citrate/NaOH buffer (0.4 *M*, pH 4.9) containing KCN (5 m*M*), rotenone (10 µ*M*), and antimycin A (10 µ*M*). ^{3}HOH was recovered as described elsewhere *(15)*. Enzymatic activity was adjusted to mGPDH protein content determined by densitometric analysis of Western blots *(16)*.

Western Blot Analysis

Western blot was performed to analyze the effect of these mutations on correct folding and protein maturation and to evaluate and quantify protein expression. Groups of five oocytes were homogenized in a Tris-HCl buffer (50 m*M*, pH 8.0) containing 150 m*M* NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Igepal, 0.5% sodium deoxycholate, 0.02% sodium azide, 1 µg/mL of aprotinin, and 100 µg/mL of phenylmethylsulfonyl fluoride. Proteins were resolved by electrophoresis on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose filters (Bio-Rad, Hercules, CA). The filters were blocked in 7% nonfat dry milk/0.2% Igepal in Tris-buffered saline (pH 7.4) for 60 min at room temperature. Membranes were incubated with a polyclonal rabbit antiserum against mGPDH recombinant protein prepared in our laboratory *(16)*. Bound antibodies were detected by peroxidase-conjugated antirabbit IgG (Sigma, St. Louis, MO) and an electrochemiluminescence system (Amersham Life Science, Bucks, UK).

Statistical Analyses

Patients with diabetes and control subjects were compared for the prevalence of mutations by the χ^2 test. Differences between groups in mGPDH enzymatic activities in the in vitro expression studies were assessed using the Mann-Whitney U test.

Acknowledgments

This study was supported by grants from the Associació Catalana de Diabetis, the Fondo de Investigación Sanitaria 96/0164; and the Comisión Interministerial de Ciencia y Tecnología, Ministerio de Educación y Cultura (SAF97/0023).

References

1. Malaisse, W. J., Sener, A., Herchuelz, A., and Hutton, J. C. (1979). *Metabolism* **28,** 373–386.
2. Eto, K., Tsubamoto, Y., Terauchi, Y., et al. (1999). *Science* **283,** 981–985.
3. Giroix, M.-H., Rasschaert, J., Bailbe, D., Leclercq-Meyer, V., Sener, A., Portha, B., and Malaisse, W. J. (1991). *Diabetes* **40,** 227–232.
4. Östenson, C. G., Abdel-Halim, S. M., Rasschaert, J., Malaisse-Lagae, F., Meuris, S., Sener, A., Efendic, S., and Malaisse, W. J. (1993). *Diabetologia* **36,** 722–276.
5. Fernández-Alvarez, J., Conget, I., Rasschaert, J., Sener, A., Gomis, R., and Malaisse, W. J. (1994). *Diabetologia* **37,** 177–181.
6. Giroix, M.-H., Malaisse-Lagae, F., Portha, B., Sener, A., and Malaisse, W. J. (1997). *Biochem. Mol. Med.* **61,** 72–81.
7. MacDonald, M. J., Efendic, S., and Östenson, C.-G. (1996). *Diabetes* **45,** 886–890.
8. Hanis, C. L., Boerwinkle, E., Chakraborty, R., et al. (1993). *Nat. Genet.* **13,** 161–166.
9. Warren-Perry, M. G., Stoffel, M., Saker, P. J., Zhang, Y., Brown, L., MacDonald, M. J., and Turner, R. C. (1996). *Diabetes* **45,** 639–641.
10. Novials, A., Vidal, J., Franco, C., Ribera, F., Sener, A., Malaisse, W. J., and Gomis, R. (1997). *Biochem. Biophys. Res. Commun*. **231,** 570–572.
11. MacDonald, M. J., Brown, L. J., Hasan, N. M., Stoffel, M., and Dills, D. G. (1997). *Diabetes* **46,** 1660–1661.
12. MacDonald, M. J. and Brown, L. J. (1996). *Arch. Biochem. Biophys.* **326,** 79–84.
13. Rodriguez-Villar, C., Conget, I., González-Clemente, J. M., et al. (1996). *Metabolism* **45,** 873–875.
14. Costa, A., Rios, M., Casamitjana, R., Gomis, R., and Conget, I. (1998). *Diabetes Res. Clin. Pract.* **41,** 191–196.
15. Rasschaert, J. and Malaisse, W. J. (1991). *Biochem. J.* **278,** 335–340.
16. Novials, A., Fabregat, M. E., Benito, C., Fernández-Alvarez, J., Gallart, T., Malaisse, W. J., and Gomis, R. (1996). *Biochem. Mol. Med.* **57,** 187–191.
17. Polonsky, K. S., Sturis, J., and Bell, G. I. (1996). *N. Engl. J. Med*. **334,** 777–783.
18. McCarthy, M. I., Froguel, P., and Hitman, G. A. (1994). *Diabetologia* **14,** 628–638.
19. Takeuchi, Y., Matsutani, A., and Oka, Y. (1997). *Diabetologia* **40,** 339–343.
20. Lehn, D. A., Brown, L. J., Simonson, G. D., Moran, S. M., and MacDonald, M. J. (1994). *Gene* **150,** 417–418.
21. Brown, L. J., MacDonald, M. J., Lehn, D. A., and Moran, S. M. (1994). *J. Biol. Chem.* **269,** 14,363–14,366.
22. Muller, S. and Seitz, H. (1994). *Proc. Natl. Acad. Sci. USA* **91,** 10,581–10,585.
23. Nesher, R., Gross, D. J., Donath, M. Y., Cerasi, E., and Kaiser, N. (1999). *Diabetes* **48,** 731–737.
24. Garcia-Ruiz, C., Morales, A., Colell, A., Rodos, J., Yi, J. R., Kaplowitz, N., and Fernández-Checa, J. C. (1996). *J. Biol. Chem*. **270,** 15,946–15,949.