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Site-Directed Mutations of the FAD-Linked Glycerophosphate Dehydrogenase Gene Impairs the Mitochondrial Anchoring of the Enzyme in Transfected COS-7 Cells

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COS-7 cells were transfected with the green fluorescent protein (GFP) of *Aequorea victoria*, human mitochondrial FAD-linked glycerophosphate dehydrogenase (mGDH), a $mGDH_{wt}$-EGFP construct, or two mutant mGDH-proteins fused with EGFP. The site of mutation was selected to affect cationic amino acids in the peptide signal sequence currently believed to play a key role in the subcellular distribution of mitochondrial proteins. All proteins were suitably expressed in the COS-7 cells. However, an increase in mGDH enzymatic activity above the control value in nontransfected COS-7 cell homogenates was only observed in cells transfected with mGDH, indicating that the catalytic activity of mGDH was masked in fused proteins. Confocal microscopy documented that, in the cells transfected with the $mGDH_{wt}$-EGFP construct, the fusion protein was located exclusively in mitochondria, this contrasting with the nuclear labelling of cells expressing the green fluorescent protein alone. The mitochondrial anchoring of the mutated mGDH fused protein was altered, this alteration being most obvious in the $mGDH_{313233}$-EGFP mutant. These findings raise the idea that a conformation change of the mGDH protein, as resulting from either an inherited or acquired alteration of its amino acid sequence, may affect its subcellular distribution and, hence, modify its immunogenic potential.

Many nuclear-encoded polypeptides destined for import into mitochondria are synthesized as precursors, bearing a transient amino-terminal presequence that is removed upon transport of the polypeptide to its correct location within the organelle (1). Experiments employing gene fusion have demonstrated that the information required for finding mitochondria and the correct submitochondrial compartment can be contained within the presequence (2). Presequences can thus act independently of the attached protein as signals for intracellular protein sorting. The known mitochondrial presequences are not highly conserved but some analogies have been noted. Mitochondrial targeting signals lack extended uninterrupted stretches of hydrophobic residues, are relatively poor in acidic residues and are rich in basic and hydroxylated residues. The most interesting feature of these sequences is their ability to form positively charged amphiphilic α-helices. They are capable of adopting α-helical structure in apolar solvents, detergents or acidic phospholipids, such as cardiolipin, which are prominent in mitochondrial membranes.

The mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase is a nuclear-encoded protein and is located at the outer surface of the inner mitochondrial membrane. This enzyme plays a key role in the process of glucose-induced insulin release from pancreatic islet β-cells (3, 4). The activation of the enzyme by cytosolic Ca^{2+} indeed accounts for the preferential stimulation of the aerobic modality of glycolysis in glucose-stimulated β-cells (3, 4). An impaired activity of the islet enzyme was previously reported in several animal models of non-insulin-dependent diabetes mellitus and in a few type 2 diabetic patients examined for such a purpose (5, 6). A mutation of the mGDH gene was also documented in a family of patients with non-insulin-dependent diabetes (7). Moreover, antibodies against mGDH are detected in a large number of patients with type 1, but not type 2, diabetes (8, 9). The protein sequence of this enzyme has 42

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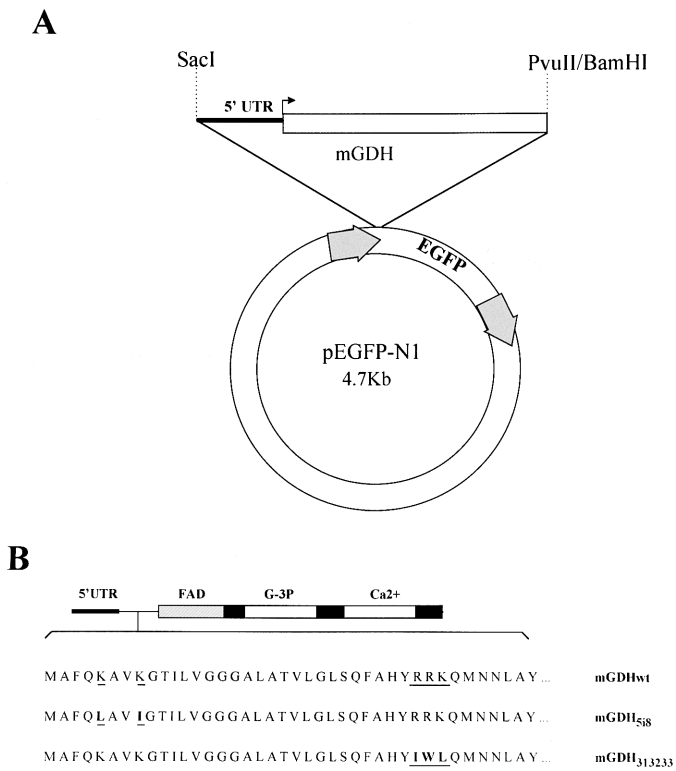

FIG. 1. Construction of the wild type and mutated mGDH cDNA fused with EGFP containing vector.

amino acids at the N-terminal end that are compatible in size and positive charge with known mitochondrial leader sequences. Garrib and McMurray (10) detected a Mr5000 difference between precursor and mature mitochondrial glycerol phosphate dehydrogenase. Clay and Ragan (11), however, could not find a mGDH precursor by *in vitro* translation experiments.

These findings led us to investigate, in the present study, whether site-directed mutations at the mitochondria signal coding region of the mGDH gene may affect the mitochondrial anchoring of the enzyme in an eukaryotic cell line. These experiments were motivated by the consideration that an altered conformation of the enzyme could conceivably affect its subcellular distribution and, by doing so, increase its potential as an antigenic determinant.

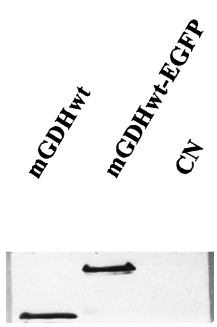

FIG. 2. Western blot analysis of $mGDH_{wt}$ and $mGDH_{wt}$-EGFP. Proteins from non-transfected COS-7 cells (CN) and cells transfected with either $mGDH_{wt}$ or $mGDH_{wt}$-EGFP were resolved by electrophoresis and transferred onto nitro-cellulose filters. After incubation with a polyclonal rabbit antiserum against mGDH recombinant protein bound antibodies were detected with peroxidase-conjugated anti-rabbit IgG.

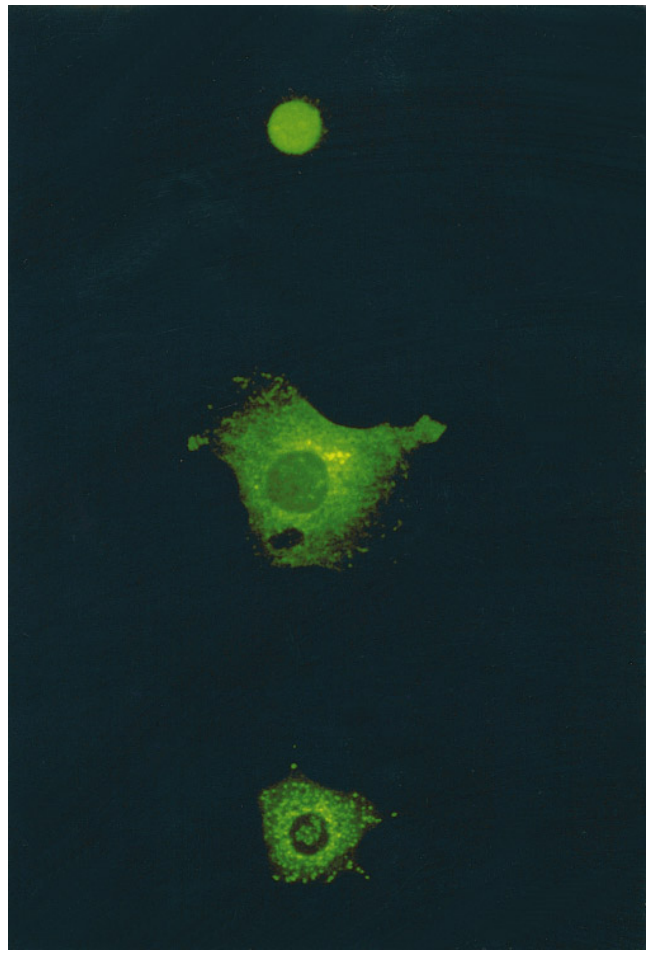

FIG. 3. Localization of GFP alone (top), $mGDH_{313233}$-EGFP (middle), and $mGDH_{5i8}$-EGFP (bottom) in transfected COS-7 cells. Note the nuclear localization of GFP, the cytoplasmic localization of $mGDH_{313233}$-EGFP, and the mixed mitochondrial-cytoplasmic pattern of $mGDH_{5i8}$-EGFP.

MATERIALS AND METHODS

Plasmid construction. Standard molecular cloning techniques were used throughout. Five amino acid changes were introduced in the amino-terminal import signal sequence of human mGDH cDNA (GeneBank No. U36310) using the QuikChange site-directed mutagenesis Kit (Stratagene, Cambridge, UK). As shown in Fig. 1B, amino acids 5 and 8 (K and K) were replaced by L and I ($mGDH_{5i8}$) for one construct, and amino acids 31, 32 and 33 (R, R and K) were replaced by I, W and L for the other construct ($mGDH_{313233}$). The oligonucleotides used were CCCTTTCACTGC-C**AG**TTGAAATGC (sense), GCATTTCAA**CT**GGCAGTGAAAGGG

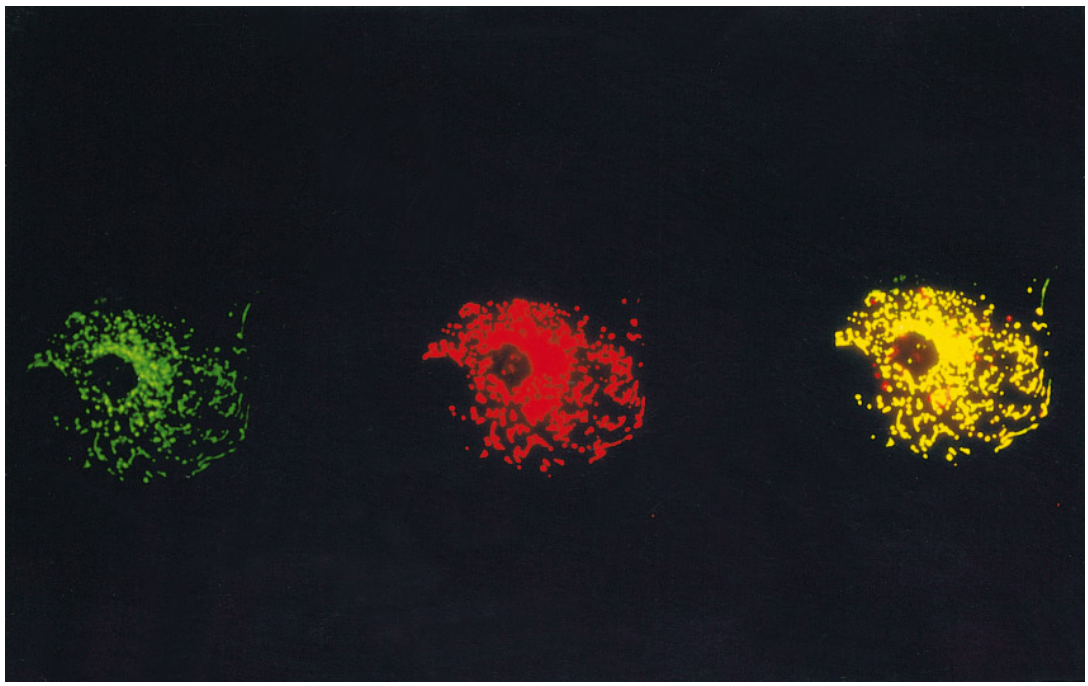

FIG. 4. Localization of $mGDH_{wt}$-EGFP in transfected COS-7 cells (left). The localization of an antimitochondrial antibody (middle) and its colocalization with $mGDH_{wt}$-EGFP (right) are also documented.

(antisense), GAATCGTCCCT**A**TCACTGCC (sense), GGCAGT-GA**T**AGGGACGATTC (antisense), CATTTGGATCC**ATA**TGTA-ATGAGC (sense), GCTCATTACA**T**A**T**GGATCCAAATG (anti-sense), GTTCATTTGG**A**TCCTTCTG (sense), CAGAAGGA**T**-CCAAATGAAC (antisense) respectively. DNA was sequenced using the ABI-PRISM DNA sequencing Kit and the ABI-PRISM 377 automatic DNA sequencer (Perkin Elmer Applied Biosystems, Branchburg, NJ, USA). Full-length coding region of wild type and mutated human mGDH ($mGDH_{wt}$, $mGDH_{5i8}$ and $mGDH_{313233}$) together with 121 bases of the 5′ untranslated region were excised from the expression plasmid pCDNA3 with PvuII, blunt-ended with the klenow fragment of E. coli DNA polymerase I, followed by digestion with SacI. They were ligated (Fig. 1A) into pEGFP-N1 (Clontech, Palo Alto, California, USA) which had been previously digested with BamH I, klenow-filled and digested with SacI. This ensured the in-frame fusion of mGDH at the N-terminus of the GFP coding sequence. The final plasmids ($mGDH_{wt}$-pEGFP-N1, $mGDH_{5i8}$-pEGFP-N1 and $mGDH_{313233}$-pEGFP-N1) were purified by ionic exchange chromatography and finally dissolved in 10 mM Tris-HCl (Quiagen, Hilden, Germany).

Cell culture and transfection. COS-7 cells were grown on tissue culture testplate in Dulbecco's Modified Eagle's medium (Gibco-BRL, Life Technologies Inc., Gaithersburg, MD, USA) supplemented with 10 % fetal calf serum (Gibco-BRL), 2 mM L-glutamine (Gibco-BRL), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco-BRL). Liposome-mediated transfections were performed at 70-80 % of cell confluence using 12 μl of lipofectamine (Gibco-BRL) and 1 μg of plasmid cDNA per 35 mm diameter dish. After 48 h of transfection, cells were seeded on 8 well glass chamber slide (Nunc, Roskilde, Denmark) and returned to culture overnight.

Immunofluorescence. Cells were washed with PBS and fixed in 4 % paraformaldehyde for 30 min at room temperature, washed twice with PBS and mounted for confocal analysis. Colocalization was performed with a monoclonal antibody to mitochondria (Biogenesis, Poole, UK) using as a second antibody a goat anti-mouse Cy3 (Amersham, Buckinghamshire, UK). Fluorescence images were observed with a Leica TCS NT confocal scanning laser microscope adapted to an immunofluorescence microscope. Green fluorescence from GFP fusion proteins was excited at 488 nm with the laser and optical sections were obtained.

Wild type and mutated mGDH 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 37°C in 30 μl Hepes buffer (20 mM, pH 7.2) containing 1 mM glycerol 3-phosphate, 1 mM EGTA, and 0.1 mM FAD with the material derived from 2.5×10^4 transfected cells per sample. Cells were previously washed twice with Hepes buffer containing 1 mM EGTA and centrifuged for 5 min at 780 g. The supernatant was removed and the remaining material was mixed with the same buffer, sonicated and centrifuged for 1-2 min at 500 g. The supernatant was used for the enzyme assay. The reaction was halted by adding 30 μl of a citrate/NaOH buffer (0.4 M, pH 4.9) containing 5 mM KCN, 10 μM rotenone, 10 μM antimycin A, the ^{3}HOH being then recovered as described elsewhere (12).

Western blot analysis. For Western blot analysis, groups of 4×10^4 transfected cells were homogenized in a Tris-HCl buffer (50 mM, pH 8.0) containing 150 mM NaCl, 0.1 % SDS, 1 % Igepal, 0.5 % sodium deoxycholate, 0.02 % sodium azide, 1 μg/ml aprotinin and 100 μg/ml phenylmethylsulfonyl fluoride. Proteins were resolved by electrophoresis on a 10 % SDS-polyacrilamide gel and transferred onto nitro-cellulose filters (BioRad, St. Louis, MO, USA). The filters were blocked in 7 % non-fat dry milk/0.2 % Igepal in Tris-buffered saline (pH 7.4) for 60 min at 20°C. Membranes were incubated with a polyclonal rabbit antiserum against mGDH recombinant protein prepared in our laboratory (13). Detection of bound antibodies was performed using peroxidase-conjugated anti-rabbit IgG (Sigma, St Louis, MO, USA) and the ECL chemiluminescence system (Amersham Life Science, Buckinghamshire, UK).

Presentation of results. All results are presented as means ± SEM together with the number of individual determinations (n). The statistical significance of differences between mean values was assessed by use of Student's *t*-test.

RESULTS

Western blot analysis indicated that both mGDH itself and the mGDH-protein fused with EGFP were expressed in the COS-7 transfected cells, whilst little immunoreactive material was detected in control COS-7 cells (Fig. 2). The two mGDH mutated fusion proteins were also adequately expressed in the COS-7 cells (Fig. 3).

After fixation, COS-7 cells expressing the green fluorescent protein alone (GFP) showed an uniform labelling of the nucleus as has been observed in other cell types (14) (Fig. 3A). This distribution changed when cells were transfected with the $mGDH_{wt}$-EGFP construct. The fusion protein was found exclusively in the mitochondria and it colocalized with an monoclonal antibody to a human 65Kd mitochondrial protein (Fig. 4). In contrast, the expression of $mGDH_{313233}$-EGFP in COS-7 cells showed a different distribution of the protein. The fluorescent label was mostly localized in the cytoplasm (Fig. 3B). The $mGDH_{5i8}$-EGFP protein was located in part in mitochondria, but some of it was also localized in the cytoplasm (Fig. 3C). The cytoplasmic expression of mutated fusion proteins disappeared when cells were previously permeabilized with triton X-100 (0.05 %, v/v; data not shown).

The activity of mGDH averaged 431 ± 36 amol/min per cell ($n = 7$) in control COS-7 cell homogenates. It was increased to 200.5 ± 18.4 % ($n = 3$; $P < 0.05$) of the paired control value in cells transfected by $mGDH_{wt}$. In cells transfected by $mGDH_{wt}$-EGFP, however, the enzymatic activity averaged 99.6 ± 18.6 % ($n = 3$) of the same paired control value. Likewise, in cells transfected with either $mGDH_{5i8}$-EGFP or $mGDH_{313233}$-EGFP, the enzymatic activity did not exceed 89.2 ± 12.7 % ($n = 4$) of the paired control value.

DISCUSSION

We used the intrinsic fluorescence of the *Aequorea victoria* green fluorescent protein (GFP) to study the subcellular distribution of the human mitochondrial glycerol phosphate dehydrogenase.

Despite suitable expression of the corresponding proteins in COS-7 cells, as documented by Western blot analysis, only the cells transfected with $mGDH_{wt}$ itself displayed increased enzymatic activity. This indicates that the catalytic activity of mGDH is masked in the fused proteins.

In COS-7 cells transfected with the wild type mGDH fused protein, all the protein was located in the mitochondria in sharp contrast to the nuclear pattern found in COS-7 cells expressing the green fluorescent protein (EGFP) alone. The mitochondrial anchoring of the mutated mGDH fused protein was altered, this alteration being most obvious in the $mGDH_{313233}$-EGFP mutant. In this respect, it should be stressed that the site of mutation was selected to affect cationic amino acids in the peptide signal sequence currently believed to play a key role in the subcellular distribution of mitochondrial proteins. It is known that an amphipathic structure at the amino terminus of the cytoplasmic protein must contain a minimum number of basic residues to initiate productive interaction with the energized mitochondrial membrane and subsequent transfer across the bilayer (15). This raises the intriguing possibility that some proteins with weak import signals might partition between mitochondria and other compartments of the cell in response to a given cellular perturbation.

We have shown in this study the significance of the N-terminal sequence for targeting the mGDH protein to the mitochondria. The present findings raise the idea that a conformation change of the mGDH protein, as possibly resulting from either an inherited or acquired alteration of its amino acid sequence, may affect its subcellular distribution and, hence, modify its immunogenic potential in the autoimmune process implied in the pathogenesis of type 1 diabetes mellitus. An otherwise sequestered organellar protein could indeed be redirected and gain access to the cell surface or compartments involved in antigen presentation.

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