Interaction of Glutaric Aciduria Type 1-Related glutaryl-CoA Dehydrogenase with Mitochondrial Matrix Proteins

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Abstract

Glutaric aciduria type 1 (GA1) is an inherited neurometabolic disorder caused by mutations in the GCDH gene encoding glutaryl-CoA dehydrogenase (GCDH), which forms homo- and heteromeric complexes in the mitochondrial matrix. GA1 patients are prone to the development of encephalopathic crises which lead to an irreversible disabling dystonic movement disorder. The clinical and biochemical manifestations of GA1 vary considerably and lack correlations to the genotype. Using an affinity chromatography approach we report here for the first time on the identification of mitochondrial proteins interacting directly with GCDH. Among others, dihydrolipoamide S-succinyltransferase (DLST) involved in the formation of glutaryl-CoA, and the β-subunit of the electron transfer flavoprotein (ETFB) serving as electron acceptor, were identified as GCDH binding partners. We have adapted the yellow fluorescent protein-based fragment complementation assay and visualized the oligomerization of GCDH as well as its direct interaction with DLST and ETFB in mitochondria of living cells. These data suggest that GCDH is a constituent of multimeric mitochondrial dehydrogenase complexes, and the characterization of their interrelated functions may provide new insights into the regulation of lysine oxidation and the pathophysiology of GA1.

Introduction

The inherited neurodegenerative disorder glutaric aciduria type 1 (GA1, OMIM 231670) is caused by mutations in the gene for the mitochondrial matrix enzyme glutaryl-CoA dehydrogenase (GCDH, E.C. 1.3.99.7). GCDH belongs to the acyl-CoA dehydrogenase family of mitochondrial flavoproteins and catalyzes the oxidative decarboxylation of glutaryl-CoA in the degradative pathway of the amino acids lysine, hydroxylysine and tryptophan [1,2]. The heterodimeric electron transfer flavoprotein (ETF) transfers electrons from GCDH to the respiratory chain [3,4]. Mutations in the GCDH gene lead to formation and accumulation of the dicarboxylates glutaric acid (GA) and 3-hydroxyglutaric acid (3OHA) in tissues and body fluids. Affected patients are at risk to develop encephalopathic crises triggered by catabolic situations such as infectious diseases, fever, vomiting or diarrhea. During crises a further increase of GA and 3OHA concentrations were observed, accompanied by the selective destruction of striatal neurons with a subsequent development of an irreversible dystonic/dyskinetic movement disorder [4,5]. Newborn screening programs allow the early identification of GA1 patients and the initiation of lysine and tryptophan restricted diet therapy prior to the development of encephalopathic crises [6]. Considerable variation in severity of the clinical and biochemical phenotype is observed showing no correlation to the genotype of the patients [7,8]. More than 150 different mutations in the GCDH gene with predominance in specific populations have been described, which lead to a wide spectrum of clinical symptoms in GA1 patients ranging from an asymptomatic course to severe disabling dystonia [8–10].

The GCDH is synthesized as a precursor protein of 438 amino acids. After import into mitochondria the 44 N-terminal amino acid mitochondrial targeting sequence is cleaved off [9], and the assembly of four GCDH monomers containing a non-covalently bound flavin adenine dinucleotide (FAD) results in the enzymatically active tetrameric protein complex [11]. In addition to homotetramerization, cross-link experiments revealed that GCDH forms heteromeric higher molecular mass protein complexes with so far unidentified interaction partners [12].

In this report we used GCDH affinity chromatography, co-precipitation and protein complementation assays to identify and verify dihydrolipoamide S-succinyltransferase (DLST) and the electron transfer flavoprotein subunit beta (ETFB) as GCDH interacting proteins.

Materials and Methods

Antibodies

Rabbit anti-human GCDH antibody was kindly provided by Dr. S. I. Goodman (University of Colorado Health Sciences Center, Denver). The polyclonal mouse anti-human DLST and rabbit anti-human ETFA antibodies were purchased from Sigma (Munich, Germany), rabbit anti-human ETFB from Abcam (Cambridge, UK), and rabbit anti-LC3 from Abgent (San Diego, USA). The monoclonal mouse anti-GFP antibody was obtained from Roche (Mannheim, Germany) and rabbit anti-MnSOD from...
DNA constructs

The human GCDH-Myc in the pcDNA6.2/V5/GW/TOPO vector has been described previously [12]. The LC3-GFP in the pEGFP-N1 (Clontech, Saint-Germain-en-Laye, France) vector was kindly provided by Dr. G. Gigliotti (this institute). The human DLST and DLST cDNAs (GenBankTM accession numbers NM_000126.3 and NM_001933.4, respectively) were isolated from total cDNA by PCR using Phusion (Aarhus University, Denmark) and isolated from the pCRII-ETFA (Invitrogen). HMGCL cDNA was kindly provided by Dr. S. Gersting, LMU Munich, Germany) and cloned into the pcDNA3.1D/V5-His-TOPO vector (Merck, Darmstadt, Germany) as 3' His6 sequence into the pcDNA3.1D/V5-His-TOPO vector using the corresponding Directional TOPO Expression Kit (Invitrogen). The human EFA and ETFB cDNA (GenBankTM accession numbers NM_000126.3 and NM_001933.4, respectively) were kindly provided by Dr. P. Brox (Aarhus University, Denmark) and isolated from the pCRII-ETFA and -ETF vectors [13] by PCR using Phusion polymerase (Thermo Scientific, St. Leon-Rot, Germany) and subcloned with a 3' His6 sequence into the pcDNA3.1D/V5-His-TOPO vector using the corresponding Directional TOPO Expression Kit (Invitrogen). HMIGCL cDNA was kindly provided by Dr. S. Gersting, LMU Munich, Germany) and cloned into the pcDNA3.1D/V5-His-TOPO as described above. For expression in bacteria, the mature GCDH, EFA and ETFB cDNA were additionally cloned into the pET28a(+) vector (Merck, Darmstadt, Germany) as 3' or 5' His6- linkers, using NcoI and HindIII (GCDH) or NdeI and HindIII (ETF, ETFB), respectively. For the protein complementation assay, DNA sequences of YFP fragments 1 (YFP1; amino acids 1–150) and 2 (YFP2; amino acids 159–239) with an introduced 5' linker (GGGGS3) were amplified by PCR using the vectors pcDNA3-MCFD2-cYFP1 and pcDNA3-MCFD2-cYFP2 (kindly provided by Dr. H.P. Haeri, University of Basel, Switzerland [14]) as template. Afterwards, the YFP1 and YFP2 cDNA was subcloned 3' of DLST, HMIGCL and GCDH into pcDNA3.1 by using the restriction enzymes EcoRV and XhoI. For the subcloning into pcDNA3.1-ETFA and -ETF vector, EcoRV and XhoI were used. All expression vectors were sequenced (Seqlab, Goettingen, Germany) and subcloned with a 3' His6-fusion constructs, using NcoI and HindIII of DLST, HMGCL and GCDH, ETF, ETFB, respectively.

Cell culture and transfection

Baby hamster kidney 21 (BHK) cells and HeLa cells were cultured in Dulbecco's modified eagle medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (FCS; PAA Laboratories, Colbe, Germany) and penicillin/streptomycin (Invitrogen). Cells grown on 10-cm plates were transfected with the indicated cDNAs using jetPEI SM Transfection Reagent (Peglab Biotechnology, Erlangen, Germany) according to the manufacturer's instructions. The cells were used 24 h after transfection.

Isolation of mitochondrial matrix proteins

Heavy mitochondrial fractions were prepared from pig liver (Ellegaard Gottingen Minipigs ApS, Dalnose, Denmark) using the basic protocol 1, previously described for rat liver [13], except centrifugation of the samples at 9,500 g. Purified mitochondria were resuspended in 0.25 M sucrose (40 mg/ml), followed by sequential fractionation into outer membranes, inner membranes and matrix proteins as described for rat liver [16].

Preparation of GCDH affinity matrix

GCDH cDNA encoding the mature enzyme was subcloned into the E. coli expression vector pET28a (+) (Merck, Darmstadt, Germany) as with the C-terminal His6-tag. E. coli BL21(DE3) (Merck) were transformed using standard procedures and were grown in 400 ml Luria-Bertani (LB) medium supplemented with kanamycin (50 µg/ml) at 37°C in a shaking incubator until OD600 of 0.6 was reached. Cells were than induced for 4 h with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG, Roth, Karlsruhe, Germany). Cells were resuspended in lysis buffer (50 mM NaH2PO4, pH 7.8 containing 500 mM NaCl, 10 mM imidazole, 2 mg/ml lysozyme, 20 μg/ml DNase, 1% Triton X-100 and protease inhibitor cocktail), sonicated and centrifuged for 15 min at 3,000 g at 4°C. To the supernatant 0.2 ml Ni-NTA agarose (Invitrogen) was added, incubated by rotating for 4 h at 4°C, washed and bound proteins were eluted sequentially with 50 mM NaH2PO4, pH 7.8 containing 500 mM NaCl and 150 mM or 250 mM imidazole. Purified GCDH (2 mg) was coupled to 2 ml bed volume of Affi-Gel 10 (Bio-Rad, Munich, Germany) according to the manufacturer's instructions.

Identification of GCDH-binding proteins

Mitochondrial matrix protein extracts (1 mg) were applied to the GCDH-affinity matrix and incubated for 12 h at 4°C on a rotating wheel. Unbound material was discarded, and the column was washed with 10 vol. buffer A (50 mM Hepes, pH 7.5 containing 5 mM KCl and 120 mM NaCl). Bound proteins were eluted with 0.5 ml buffer A containing 1.5 M NaCl and protease inhibitor cocktail. The eluate was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described previously [17].

GCDH co-precipitation experiments

HeLa cells grown on 10 cm plates were transfected with DLST-His6 cDNA alone or co-transfected with cDNA of GCDH-myc or LC3-GFP. Twenty-four hours after transfection cells were lysed in buffer B (10 mM Tris/HCl pH 7.5 containing 150 mM NaCl, 0.5 mM EDTA, 0.5% NP40, 1 mM PMSE and protease inhibitor cocktail), centrifuged for 10 min at 14,000 g and 4°C and the supernatants were incubated with 0.1 ml Ni-NTA agarose for 4 h at 4°C on a rotating wheel to bind DLST-His, protein complexes. Unspecific bound proteins were removed by a series of washes (3×0.5 ml buffer B, without NP40) and DLST-protein complexes were recovered by solubilization of beads in SDS-sample buffer and by SDS-PAGE followed by western blotting.

Pull-down precipitation experiments

An overnight starter culture of pET28-ETFA or ETFB was established in LB-medium supplemented with kanamycin (50 µg/ml). Two ml of the culture was added to 200 ml of LB media supplemented with 50 µg/ml kanamycin. The culture was grown with shaking at 37°C to OD600 = 0.6. The expression of recombinant ETFA/ETFB was induced by addition of 0.5 mM IPTG for additional 4 h shaking at 37°C. Cells were pelleted at 4,000 g for 15 min. The pellet was resuspended in 10 ml cold buffer C (50 mM NaH2PO4 pH 7.8, containing 1% Triton X-100, 300 mM NaCl, 10 mM imidazole, 1 mg/ml lysozyme, 0.1 mg/ml DNase and protease inhibitor cocktail) and disrupted by ultrasonic treatment (6 times 20 sec). The resulting lysate was centrifuged at 10,000 g for 5 min at 4°C. The protein concentration of the supernatant containing the soluble ETFA or ETFB was determined by the Bio-Rad protein assay. 1.5 ml Ni-NTA agarose columns were equilibrated with buffer C, loaded with 4 mg/ml...
protein of the supernatant containing ETFA/ETFB and incubated by gently shaking for 4 h at 4 °C. Afterwards the resin was divided into two columns of 0.75 ml each and incubated for additional 2 h with 0.3 mg of BHK cell extracts overexpressing GCDH or LC3, respectively. Unbound proteins were removed by a series of washes using buffer C (without Triton X-100, lysozyme and DNase) and protein complexes were recovered by solubilization of beads in SDS-sample buffer and by SDS-PAGE followed by western blotting.

Other methods

Protein interactions were determined with the Bio-Rad protein assay (Munich, Germany). Western blotting was performed as previously described [12] using anti-GCDH (1:5,000), anti-ETFA (1:1,000), anti-ETFB (1:1,000), anti-DLST (1:500), anti-GFP (1:1,000) and anti-LC3 (1:200) antibodies. For YFP fluorescence microscopy transfected BHK cells were grown on glass coverslips for 6 h and transfected with 2.5 μg pcDNA3.1 vectors (Invitrogen) containing the YFP1 or YFP2 fusion constructs. After 24 h the cells were fixed with 4% paraformaldehyde in 10 mM phosphate buffer saline, pH 7.4 (PBS). After washing and DAPI staining (Roht, Karlsruhe, Germany; 1:1,000) the cells were embedded in Mowiol (Merck). Fluorescence was detected and images were obtained using a Leica DMIRE2 digital scanning confocal microscope with TCS NT software (Leica Microsystems Scientific Instruments Group, Wetzlar, Germany).

Results

GCDH affinity chromatography

To identify proteins interacting with GCDH, we employed an affinity chromatography approach. Human GCDH fused to a His6-tag was expressed in E. coli and purified to homogeneity by Ni-chelate affinity chromatography (Fig. 1A). The purified GCDH has a molecular mass of 43 kDa in accordance with that predicted from the cDNA sequence. The identity of the purified polypeptide was confirmed by western blotting (Fig. 1B). The GCDH was immobilized covalently to beads and incubated with mitochondrial matrix protein extracts isolated from porcine liver (Fig. S1). Mass spectrometric analysis of polypeptides eluted with high salt buffer and digested with trypsin resulted in the identification of five mitochondrial matrix proteins, two inner mitochondrial membrane proteins and three peroxisomal proteins (Table 1; Table S2). Two mitochondrial matrix proteins, dihydrolipoamide S-succinyltransferase (DLST) and electron transfer flavoprotein subunit beta (ETFB) have been studied in more detail in this study because both proteins are directly involved in the degradation pathway of lysine. DLST as well as GCDH and ETFB in living cells, we adapted the YFP-based protein complementation assay (PCA) approach. To visualize and verify the interaction between GCDH and DLST, we carried out His6-pulldown experiments. Purified ETFB-His6, immobilized on Ni-NTA agarose was incubated with cell extracts overexpressing GCDH-Myc. GCDH was recovered in the elution fraction from ETFB-agarose matrix (Fig. 4). No interaction was observed between ETFB-His6 and the cytosolic protein LC3-GFP. The expression of the 28 kDa ETFB-His6 protein used for precipitation was verified by anti-DLST immunoblotting.

In order to confirm the interaction between GCDH and ETFB, we employed a YFP-based pulldown experiment. Purified ETFB-His6, immobilized on Ni-NTA agarose was incubated with cell extracts overexpressing GCDH-Myc. GCDH was recovered in the elution fraction after GCDH affinity chromatography (Fig. S2). The affinity elution fraction also contained the 15 kDa ETFB (Fig. 2B). Neither DLST nor ETFB bound to the control chromatography matrix.

GCDH binds directly to DLST and ETFB

To confirm the results of GCDH affinity chromatography, the interaction between GCDH and DLST was examined by co-precipitation experiments. When HeLa cells were transfected with cDNA encoding the DLST-His6 protein followed by Ni-NTA-agarose precipitation, about 2% of endogenous GCDH could be recovered from the beads (Fig. 3, DLST). After co-expression of DLST-His6 with GCDH-Myc (DLST+GCDH) a strong increase of GCDH in the eluted (E) fraction was observed. Non-bound GCDH was found in the supernatant (S). Co-expression of DLST with a cytosolic protein LC3-GFP (DLST+LC3) was used as a negative control. As expected, LC3-GFP did not bind to DLST-His6. Immunoreactive 40 kDa LC3-GFP polypeptides were only detectable in the input (I) and in the supernatant fraction (Fig. 3). The expression of the 49 kDa DLST-His6 protein used for precipitation was verified by anti-DLST immunoblotting.

Since ETFB is known to form stable complexes with ETFA [21], we performed ETFA-His6 pulldown experiments. Very low amounts of GCDH-immunoreactive material were detected in the elution fraction, suggesting a weak binding of GCDH to ETFA (Fig. S3).

YFP-based protein complementation assay (PCA)

To visualize and verify the interaction between GCDH and DLST as well as GCDH and ETFB in living cells, we adapted the protein fragment complementation assay (PCA) to the mitochondrial compartment. The assay relies on yellow fluorescent protein (YFP) fragments, YFP1 and YFP2, fused to two interacting proteins. Interaction of the fusion proteins brings the two YFP-fragments into close proximity allowing their folding into the active YFP structure [14,22]. In this study GCDH, DLST, ETFB and ETFA were C-terminally either tagged with YFP1 (amino acids 1–158) or YFP2 (amino acids from 159 to 239) (Fig. 5A and 6A). As negative controls, we included the multiple coagulation factor deficiency protein 2 (MCFD2), localized in the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) [23], and the mitochondrial matrix enzyme 3-hydroxy-3-methylglutaryl-CoA lyase (HMGLC) [24]. Individual expression of different YFP1- and YFP2-fusion proteins in BHK cells was confirmed by western blotting. Anti-GFP antibodies recognize YFP2-fusion proteins but not YFP1 constructs (Fig. 5B and 6B). To test the application of YFP-PCA for mitochondrial matrix proteins, we first studied the homooligomerization of GCDH. Strong YFP fluorescence was observed when GCDH-YFP1 and GCDH-YFP2 were co-expressed, indicating fragment complementation upon GCDH oligomerization (Fig. 5C). No YFP fluorescence was detected in BHK cells expressing GCDH-YFP1 or GCDH-YFP2 alone, demonstrating that YFP fragments per se have no intrinsic
fluorescence (Fig. 5C). Co-expression of GCDH-YFP1 with DLST-YFP2 and GCDH-YFP2 with DLST-YFP1, respectively, resulted in a strong fluorescence signal (Fig. 5C).

Additionally, co-expression of GCDH-YFP1 and ETFB-YFP2 or GCDH-YFP2 and ETFB-YFP1 in BHK cells revealed a strong YFP fluorescence (Fig. 6C). No intrinsic YFP fluorescence was

Table 1. Mitochondrial proteins binding to GCDH.

| Protein                          | Gene   | Localization | Function                                                                 |
|----------------------------------|--------|--------------|--------------------------------------------------------------------------|
| aldehyde dehydrogenase 2         | ALDH2  | M            | oxidation of aldehydes to generate carboxylic acids                      |
| dihydrolipoamide-succinyltransferase | DLST   | M            | component of the 2-oxoglutarate dehydrogenase complex which catalyzes the conversion of 2-oxoglutarate to succinyl-CoA in the tricarboxylic acid cycle and the conversion of \( \alpha \)-ketoadipate to glutaryl-CoA in the degradation pathway of lysine |
| electron transfer flavoprotein subunit beta | ETFB | M            | ETF subunit acting as electron acceptor for several acyl-CoA dehydrogenases and transfers the electrons to the main mitochondrial respiratory chain via ETF-ubiquinone oxidoreductase |
| glutamate dehydrogenase 1        | GLUD1  | M            | key enzyme in the nitrogen and glutamate/\( \alpha \)-ketoglutarate metabolism |
| thioredoxin-dependent peroxide reductase | PRDX3 | M            | member of the peroxiredoxin family of antioxidant enzymes involved in cellular redox regulation |
| ATP synthase subunit alpha       | ATP5A1 | IM           | part of the \( F_0 \) domain of ATP synthase, functions as a proton channel |
| ATP synthase subunit beta        | ATP5B  | IM           | part of the \( F_0 \) domain of ATP synthase, functions as a proton channel |

GCDH-His6 was immobilized on beads and incubated with isolated mitochondrial matrix proteins from pig liver. The identity of specifically co-purifying proteins was determined by LC-MS/MS.

M: mitochondrial matrix; IM: inner mitochondrial membrane.

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detected in BHK cells expressing ETFB-YFP1 or ETFB-YFP2 alone.

The GCDH-DLST and GCDH-ETFB fluorescence patterns are typical for mitochondria. Overlay of PCA-positive signals generated by co-expression of GCDH- with either DLST- or ETFB-YFP constructs with the counterstaining of the endogenous mitochondrial matrix protein manganese-dependent superoxide dismutase (MnSOD; Fig. S4) confirmed the localization of the interactions between GCDH and either DLST or ETFB in the mitochondrial matrix. In contrast to the strong signal intensity detecting the interaction between GCDH and ETFB distributed over the cell, the co-expression of GCDH-YFP1 and ETF1-YFP2 showed a dramatically restricted number of stained mitochondria (Fig. S5). As expected, the co-expression of ETFA-YFP2 with ETFB-YFP1 revealed a strong PCA signal, confirming that the constructs are functional.

No YFP fluorescence has been observed when GCDH-YFP1 was co-expressed with the ERGIC marker protein MCFD2 fused to YFP2, or with the YFP2-fused mitochondrial matrix protein HMGCL, verifying the specific interaction between GCDH and DLST or ETFB (Fig. S4). The data demonstrate the selectivity and specificity of GCDH-DLST and GCDH-ETFB interactions in the mitochondrial compartment.

Both co-precipitation and YFP-PCA verified the direct interactions between GCDH and DLST as well as GCDH and ETF, in particular ETFB.
Discussion

In the present study we have identified protein-protein interactions between GCDH, a mitochondrial matrix enzyme involved in the degradation of lysine and tryptophan, and several other mitochondrial metabolic proteins using immobilized GCDH affinity chromatography coupled with mass spectrometric proteome analysis. Among these proteins, dihydrolipoamide S-succinyltransferase (DLST) and electron transfer flavoprotein subunit beta (ETFB) were examined in more detail.

DLST constitutes the oligomeric E2-core subunit of the large mitochondrial matrix 2-oxoglutarate dehydrogenase complex (OGDC) containing additionally multiple copies of 2-oxoglutarate dehydrogenase (E1) and dihydrolipoamide dehydrogenase (E3). OGDC catalyzes the rate-limiting step of oxidative decarboxylation of both α-ketoglutarate and α-ketoadipate to succinyl- and glutaryl-CoA, respectively, in the tricarboxylic acid (TCA) cycle [25,26]. In addition to OGDC, pyruvate dehydrogenase (PDC) and branched-chain α-ketoacid dehydrogenase complex (BCKDC) belong to the family of α-ketoacid dehydrogenase multienzyme complexes containing homologous DLST-E2 subunits and may explain the 55 and 69 kDa DLST immunoreactive bands detected in the elution fraction. The enzymatic activity of OGDC is regulated by feedback inhibition of the glutaryl-CoA reaction product which has been shown to inhibit the DLST subunit of OGDC in vitro [18]. The interaction between GCDH and DLST suggests that both consecutive enzymes function in a multienzyme complex to allow sufficiently short distance for efficient oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA.

The other GCDH binding partner, ETFB, forms with ETFA an FAD-containing heterodimer that serves as electron acceptor for at least nine mitochondrial matrix flavoprotein dehydrogenases of fatty acid oxidation and amino acid catabolism. Subsequently the electrons are passed through the membrane-bound ETF ubiquinone oxidoreductase to the respiratory chain [21]. Defects in ETF cause multiple acyl-CoA dehydrogenase (MAD) deficiency, also called glutaric aciduria type 2 (GA2) [27].

In addition, three other mitochondrial matrix proteins have been identified as potential GCDH-binding partners. First, the aldehyde dehydrogenase 2 (ALDH2) is an allosteric tetrameric enzyme that catalyzes the oxidation of ethanol-derived acetaldehyde to acetate [28]. Second, peroxiredoxin 3 (PRDX3) is a peroxidase exclusively localized in the mitochondrial matrix and has protective effects to mitochondrial oxidative stress [29]. Of note, PRDX3 has been reported to be closely linked to the DLST expression in murine adipocytes [30]. Third, glutamate dehydrogenase (GLUD1) is a homohexameric enzyme that catalyzes the reversible oxidative deamination of glutamate to α-ketoglutarate and plays a central role in the nitrogen and glutamate metabolism as well as the cellular energy homeostasis. In mammals, GLUD1 is
highly regulated by allosteric effectors [31]. Dominant mutations in GLUD1 that cause a loss of allosteric inhibition lead to unusual hyperinsulinism/hyperammonemia syndrome [32]. Interestingly, ammonium accumulation and occasionally hypoglycemia have been reported in a rat 3D brain cell model of GA1 and in GA1 children, respectively [33,34], supporting a potential functional interaction between Glud1 and GCDH. Furthermore, in the brain Glud1 is predominantly expressed in astrocytes, and the loss of Glud1 reduces the oxidative catabolism of glutarate to α-ketoglutarate [35], which secondarily impairs the anaplerotic transfer of TCA cycle intermediates to neurons [36]. Since the accumulation of GA and 3OHGA associated with GCDH-deficiency also inhibits the astrocytic efflux and neuronal uptake of TCA cycle intermediates [37], it is tempting to speculate that patients with GCDH mutations interfering with the binding or allosteric control of Glud1 activity are more severely affected than others. However, the potential interactions of GCDH with ALDH2, PRDX3, and Glud1 detected by affinity chromatography have to be confirmed by detailed experimental studies as done for ETFB and DLST. Furthermore, studies are needed to examine the biological significance of the interaction of the various mitochondrial matrix and inner mitochondrial membrane proteins with GCDH, which might be important for modulation of GCDH activity or the coordinated allosteric control of other multimeric dehydrogenase complexes.

At present the amino acid residues on GCDH, involved in the direct binding to DLST and ETFB are unknown. These residues are predicted to be located at the surface of GCDH. About 20 mutations found in GA1 patients affect amino acid residues on the GCDH surface [8–10] associated with residual GCDH activities of 0–30% of controls and a wide spectrum of clinical symptoms in the respective GA1 patients. Thus, the p.Met263Val mutation, exhibiting 30% residual enzyme activity in patient fibroblasts [38], is located at the surface of GCDH, and failed to form heterologous GCDH-protein complexes upon chemical cross-linkage [12]. Co-crystalization of ETF with another mitochondrial dehydrogenase, medium-chain acyl-CoA dehydrogenase (MCAD), revealed that ETFB interacts with nine residues in the N-terminal domain of MCAD, Glu22, Phe 23, Thr26, Glu34, Gly60, Thr64, Leu73, Leu75 and Ile83, which mediate hydrogen bonds to ETFB or form a hydrophobic pocket [39]. Because MCAD and GCDH share a sequence homology of 28%, it appeared likely that also amino acid residues in the N-terminal domain of GCDH are involved in ETFB binding. A direct comparison, however, of the amino acids of the GCDH protein that correspond to the ETFB-binding residues in the MCAD protein, revealed a low homology with only 2 out of 9 identical amino acids between GCDH and MCAD, Thr26 and Gly60 (Fig. S6). Further mutational analyses on GCDH are needed to identify the residues involved in ETFB binding.

Taken together, in this study five mitochondrial matrix proteins have been identified to be able to bind to GCDH. Among these, the physical interaction between DLST, constituting an oligomeric core subunit of the multienzyme α-ketoglutarate dehydrogenase complex, and ETFB, serving as electron acceptor for several mitochondrial dehydrogenases, and GCDH have been verified.
with different experimental approaches. The identification of the first GCDH interacting proteins provides new insights into the functional linkage between multienzyme complexes required for efficient metabolism of glutaryl-CoA, and its role in the pathogenesis of glutaric aciduria type 1.

Supporting Information

Figure S1 Isolation of mitochondrial matrix proteins. Crude mitochondrial extracts were fractionated into outer membrane, inner membrane and matrix proteins. Ten μg of the fraction with mitochondrial matrix proteins was separated by SDS-PAGE (10% acrylamide) and proteins were visualized by Coomassie Blue staining. The positions of molecular mass marker proteins (in kDa) are indicated. (JPG)

Figure S2 Co-precipitation of DLD with GCDH. Extracts from HeLa cells overexpressing DLD-His6-V5 together with GCDH-YFP2 (DLD+GCDH) or together with LC3-GFP (DLD+LC3) were incubated with Ni-NTA agarose for 4 h. Aliquots of the cell extract (input, I: 10% of total), the unbound protein supernatant after precipitation of Ni-NTA beads (S, 10%), and the eluted fraction (E, 100%) representing bound proteins, were analyzed by anti-GFP western blotting detecting GCDH-YFP2 and LC3-GFP. Extracts of HeLa cells overexpressing DLD-His6-V5 and LC3-GFP (DLD+LC3) were used as negative control. The expression of DLD was analyzed by anti-V5 western blotting. The positions of the 55 and 40 kDa molecular mass marker proteins are indicated. Arrow: DLD-immunoreactive 52 kDa band. (JPG)

Figure S3 Binding of purified ETFA to GCDH. ETFA-His6 expressed and purified from E. coli was immobilized on Ni-NTA agarose and incubated with extracts from BHK cells overexpressing GCDH-Myc (ETFA+GCDH) for 2 h. Cell extracts overexpressing LC3-GFP were used as negative control (ETFA+LC3). Aliquots of cell extract (input, I: 10% of total), the unbound protein supernatant after precipitation of ETFA-Ni-NTA beads (S: 10%), last wash (W: 25%), and the eluted fraction (E: 100%), containing the bound proteins were separated by SDS-PAGE (10% acrylamide) and analyzed by anti-GCDH and anti-LC3 immunoblotting. The expression of ETFA used for the pull-down experiments was analyzed by anti-ETFA western blotting. The image shows representative blots of n = 5 independent experiments. (JPG)

Figure S4 YFP fragment complementation assay and mitochondrial counterstaining. A Schematic composition of C-terminal YFP1 (dark blue) and YFP2 (red) fusion proteins of GCDH, ETFB, DLST, and HMGCL used in this study. The 10-amino acid linker (GGGGS)2 is indicated in green. The calculated molecular masses of the fusion proteins are shown in brackets. The mitochondrial matrix protein HMGCL was used as negative control. B Expression analysis in HeLa cells of all fusion proteins visualized by western blotting, using anti-GCDH and anti-GFP antibodies. *endogenous GCDH protein. C Fluorescence microscopy of the indicated co-expressed fusion proteins. Strong YFP fluorescence was observed in cells co-expressing GCDH-YFP1 with either GCDH-YFP2, ETFB-YFP2, or DLST-YFP2. No YFP fluorescence signal was observed when GCDH-YFP1 was co-expressed with HMGCL-YFP2. Nuclei were visualized using DAPI (blue). Mitochondria were counterstained with anti-MnSOD antibody. Merged signals indicate co-localization of PCA signal with MnSOD-positive mitochondria. Scale bars = 40 μm (merge) or 10 μm (zoom). (JPG)

Figure S5 Interaction of GCDH with ETFA in vivo. A Fluorescence microscopy of fixed BHK cells co-expressing GCDH-YFP1 and ETFA-YFP2 fusion proteins showed a YFP fluorescence signal only in few mitochondria. B In contrast, co-expression of ETFB-YFP1 with ETFA-YFP2 revealed a strong fluorescence signal with a typical mitochondrial expression pattern. Nuclei were visualized using DAPI (blue). Scale bars = 40 μm. (JPG)

Figure S6 Comparison of the ETFB-binding site of MCAD with GCDH. Sequence alignment of mature GCDH and MCAD proteins. Identical amino acids are presented in blue. MCAD amino acid residues that have been reported to interact with ETFB [39] and to form hydrogen bonds (yellow), a hydrophobic pocket (green), or both (orange), are indicated. (JPG)

Table S1 Sequences of primers used in this study. (DOC)

Table S2 LC-MS/MS analyses of the GCDH affinity chromatography elution fraction identifying mitochondrial and non-mitochondrial proteins. (DOC)

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Author Contributions

Conceived and designed the experiments: JS TB KU CM. Performed the experiments: JS HS. Analyzed the data: JS HS KU TB CM. Contributed reagents/materials/analysis tools: JS HS. Wrote the paper: JS TB CM.

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