Mutations in GDAP1 lead to recessively or dominantly inherited peripheral neuropathies (Charcot–Marie–Tooth disease, CMT), indicating that GDAP1 is essential for the viability of cells in the peripheral nervous system. GDAP1 contains domains characteristic of glutathione-S-transferases (GSTs), is located in the outer mitochondrial membrane and induces fragmentation of mitochondria. We found GDAP1 upregulated in neuronal HT22 cells selected for resistance against oxidative stress. GDAP1 over-expression protected against oxidative stress caused by depletion of the intracellular antioxidant glutathione (GHS) and against effectors of GHS depletion that affect the mitochondrial membrane integrity like truncated BH3-interacting domain death agonist and 12/15-lipoxygenase. Gdap1 knockdown, in contrast, increased the susceptibility of motor neuron-like NSC34 cells against GHS depletion. Over-expression of wild-type GDAP1, but not of GDAP1 with recessively inherited mutations that cause disease and reduce fission activity, increased the total cellular GHS content and the mitochondrial membrane potential up to a level where it apparently limits mitochondrial respiration, leading to reduced mitochondrial Ca$^{2+}$ uptake and superoxide production. Fibroblasts from autosomal-recessive CMT4A patients had reduced GDAP1 levels, reduced GHS concentration and a reduced mitochondrial membrane potential. Thus, our results suggest that the potential GST GDAP1 is implicated in the control of the cellular GHS content and mitochondrial activity, suggesting an involvement of oxidative stress in the pathogenesis of CMT4A.

INTRODUCTION

Charcot–Marie–Tooth (CMT) disease is the most frequent inherited peripheral neuropathy in humans and affects 1 in 2500 people. Mutations of the gene GDAP1 (ganglioside-induced differentiation associated protein 1) cause CMT4A (1,2), the most common recessive subtype, and CMT2K, a dominant form of CMT (3). To date, more than 40 disease-causing GDAP1 mutations have been described, including missense mutations, mutations leading to truncated proteins and alterations that affect splice sites in the GDAP1 transcript (4). CMT4A is clinically characterized by an early onset in the first decade of life, severe distal muscle weakness and frequent vocal cord paresis, leading to a substantial degree of disability.
GDAP1 was first identified as a transcript upregulated after ganglioside-induced cholinergic differentiation of the mouse neuroblastoma cell line Neuro2a (5) and is expressed in neurons of the peripheral nervous system and in Schwann cells (6). Others found a predominant expression in neurons (7). GDAP1 is located in the outer mitochondrial membrane and its over-expression in COS-7 cells induces fragmentation of mitochondria without inducing apoptosis or interfering with mitochondrial fusion (6). Recessively inherited CMT-associated forms of GDAP1 have reduced fission activity, whereas dominantly inherited forms interfere with mitochondrial fusion (6). Over-expression of GDAP1 with dominantly inherited mutations increases the production of reactive oxygen species (ROS), leads to uneven mitochondrial transmembrane potentials and enhances the susceptibility to apoptotic stimuli (8).

As a clue to another function distinct of mitochondrial shape, it is of interest that GDAP1 and related proteins contain domains characteristic of glutathione-S-transferases (GSTs), a family of enzymes that detoxify a large variety of compounds by catalyzing their conjugation with glutathione (GSH) (9). However, prokaryotic expression of truncated GDAP1 lacking its transmembrane region showed no GSH-dependent activity and did not bind to GSH immobilized on agarose (10), whereas insect cell-expressed recombinant GDAP1 appears to be a catalytically active GST-enzyme (11).

We found that GDAP1 plays a protective role in neuronal cell death associated with GSH depletion, which can be excellently studied in neuronal HT22 cells (reviewed in 12). In these cells, high concentrations of extracellular glutamate induce a programmed cell death called oxidative glutamate toxicity by inhibiting the gradient-driven glutamate-cystine antiporter system xC−, which subsequently depletes cells of the reduced form of cystine, cysteine. Cysteine is required for the synthesis of GSH. GSH depletion renders the cells incapable to neutralize ROS, which are constantly produced in living cells, mostly in the mitochondria and also during various enzymatic reactions, and this eventually causes cell death by oxidative stress. In contrast to other paradigms of oxidative stress-induced cell death such as hydrogen peroxide, ROS are not applied externally but are generated endogenously within the cell. Cell death proceeds in a well-defined manner and includes activation of the enzyme 12/15-lipoxygenase (12/15-LOX) (13), translocation of the pro-apoptotic BCL-2 family member BID (BH3-interacting domain death agonist) to mitochondria and loss of mitochondrial transmembrane integrity (14).

Here, we show that GDAP1 is upregulated in HT22 cells selected for resistance against glutamate; over-expression protects against oxidative glutamate toxicity and against over-expression of truncated BID and 12/15-LOX. Gdap1 knockdown, in contrast, increases the susceptibility of HT22 cells and motor neuron-like NSC34 cells against GSH depletion. Using neuronal cell lines and fibroblasts from CMT4A patients, we demonstrate that GDAP1 is implicated in the control of the cellular GSH content and mitochondrial activity. Together these studies describe a novel function of GDAP1 in the defense against oxidative stress.

**RESULTS**

GDAP1 is upregulated in glutamate-resistant HT22R cells and by GHS depletion

Extracellular glutamate kills wild-type HT22S cells in a concentration-dependent manner. No HT22S cell survives 10 mM glutamate, whereas >50% of HT22R cells are still alive at concentrations of 40 mM (Fig. 1A). HT22R cells were selected by repeated and prolonged exposure to glutamate and are also resistant against other stressors like direct oxidative stress induced by hydrogen peroxide or induction of endoplasmic reticulum stress with the N-glycosylation inhibitor tunicamycin (15). The cell protection of HT22R cells correlates with a higher basal concentration of total cellular GSH, which nevertheless declines in response to glutamate (Fig. 1B). HT22R cells have been described before and were shown to differ from HT22S by the upregulation of various proteins involved in the defense against oxidative stress, suggesting transcriptional changes involved in the antioxidant response (15–18). We therefore conducted a gene array analysis to identify new antioxidative proteins by the expression pattern of their transcripts. This identified the system X− subunit xCT, which is also functionally upregulated in these cells (16), to be expressed stronger in HT22R cells, proving the relevance of this screen. We also found transcripts belonging to the GDAP1 family to be upregulated and quantitated their expression in HT22S and HT22R cells by quantitative real-time PCR. This showed a similar upregulation of Gdap1 and xCT of ~15-fold and a 5-fold upregulation of the GDAP1 homologue Gdap1I1 (Fig. 1C). We further focused on GDAP1, because of its more prominent regulation and its role in human disease despite its apparently lower expression level in HT22R cells (not shown). The upregulation of Gdap1 in cells under continuous oxidative stress and the fact that GDAP1 contains domains characteristic of GSTs in its cytosolic N-terminus suggested that Gdap1 might be regulated by the redox state of the cell and indeed, GHS depletion by buthionine sulfoximine (BSO), which inhibits γ-glutamylcysteine synthetase, the key enzyme in GSH synthesis, resulted in a prominent 7-fold upregulation of Gdap1, whereas N-acetylcysteine, which increases the cellular GSH content, had the opposite effect (Supplementary Material, Fig. S1). We concluded that Gdap1 is upregulated in HT22R cells and in response to GHS depletion and aimed to test the hypothesis that this upregulation is involved in resistance against oxidative stress.

GDAP1 protects from oxidative glutamate toxicity

To test whether GDAP1 plays such a role, we asked whether it protects against oxidative glutamate toxicity. Over-expression of GDAP1, but not empty vector, in glutamate-sensitive HT22 cells protected against high concentrations of glutamate, but not as strong as xCT, which we used as positive control in these experiments (Fig. 1D). To evaluate the contribution of endogenous GDAP1 to the glutamate-resistant phenotype of HT22R cells, we used two small hairpin (sh)-RNA constructs targeting different regions of Gdap1. Both constructs, but not the control shRNA, were effective in knocking down endogenous Gdap1 mRNA (Fig. 1E) and, when transfected into HT22R cells, significantly reduced viability of these cells in...
GDAP1 is upregulated in glutamate-resistant HT22R cells and protects from oxidative glutamate toxicity. (A) HT22R but not the parental cell line HT22S is resistant against glutamate. 5000 cells/well were seeded in 96-well plates and treated with the indicated concentrations of glutamate for 24 h. Survival was measured by the MTT assay and normalized. (B) Cell protection of HT22R cells is mediated by an increased concentration of total cellular GSH, which was measured enzymatically and normalized to protein content. Cells were treated with the indicated concentrations of glutamate for 6 h. (C) Expression of the induced transcripts in HT22R cells compared with HT22S cells quantitated by real-time RT–PCR and normalized to the expression of housekeeping genes Gapdh and β-actin. Regulation was calculated using the ΔΔCT method. (D) GDAP1 and xCT over-expression protects transiently transfected HT22S cells. Cells were transfected, replated after 24 h, and exposed to 10 mM glutamate again 24 h later. Survival was measured by the MTT assay. (E and F) Endogenous GDAP1 contributes to the glutamate resistance of HT22R cells. HT22R cells were transiently transfected with two shRNA constructs directed against Gdap1 or control and (E) Gdap1 mRNA abundance quantitated by quantitative real-time RT–PCR and normalization to the expression of housekeeping genes Gapdh and β-actin, respectively. (F) Survival of cells in 96-well plates quantitated again 24 h later by MTT assays. In all experiments, each data point corresponds to the mean ± SEM of three experiments done in triplicate. The asterisk in all panels indicates P < 0.05 as determined by one-way ANOVA and Dunnett’s multiple comparison test.

GDAP1 increases GSH content and protects against loss of the mitochondrial membrane integrity

As oxidative glutamate toxicity involves GSH depletion and glutamate resistance was associated with increased GSH levels, we investigated whether GDAP1 over-expression alters the GSH content of HT22S cells. HT22S cells over-expressing GDAP1 contained ~10% more GSH prior to the application of glutamate. Following treatment with 2.5 mM glutamate for 6 h, GDAP1-over-expressing cells contained 270% more GSH than cells expressing empty vector (Fig. 2A). However, no differences in cellular GSH were obvious at higher glutamate concentrations, where GDAP1 was still protective (Fig. 2B). We concluded that GDAP1 might have a second, GSH-independent protective activity. This prompted us to investigate whether GDAP1 also protects against the steps in the cell death pathway downstream of GSH depletion, which take place at the mitochondria. Activation of neuronal 12/15-LOX (13) directly targets mitochondria and leads to increased permeability of the mitochondrial membrane and breakdown of the membrane potential (19). We therefore asked whether GDAP1 protects against 12/15-LOX over-expression, which by itself had a small but reproducible detrimental effect of ~10% on cell viability that was completely inhibited by co-expression of GDAP1 (Fig. 2C). GDAP1 over-expression also protected significantly against a combined assault of 12/15-LOX over-expression and 1 mM glutamate treatment (Fig. 2D). The next step and a key feature of oxidative glutamate toxicity and neuronal cell death, in general, is the translocation of the pro-apoptotic BCL-2 family member BID to mitochondria, which results in a perinuclear accumulation of BID-loaded mitochondria and loss of mitochondrial membrane integrity. BID siRNA and a small molecule BID inhibitor both protect HT22 cells against glutamate, suggesting an important role for BID in GSH-depletion-mediated cell death (14). We therefore investigated the effect of GDAP1 on cell death mediated by over-expression of truncated, active BID (tBID) in the presence and absence of glutamate. A large fraction of unchallenged tBID-transfected cells died, whereas a significant amount of GDAP1-over-expressing cells survived (~60 versus ~74%; Fig. 2E). This effect was enhanced when we applied 1 mM glutamate to the transfected cells (~91% compared with ~69%; Fig. 2F). We conclude that GDAP1 protects against three different key steps involved in endogenous oxidative stress caused by GSH depletion: GSH depletion itself, activation of 12/15-LOX and disruption of the mitochondrial membrane potential by truncated BID.

GDAP1 increases the mitochondrial membrane potential

It was previously shown that oxidative glutamate toxicity causes disruption of the mitochondrial membrane potential...
(ΔΨm) as measured with JC-1, a lipophilic, cationic dye that can selectively enter mitochondria and reversibly changes color from green to red as the membrane potential increases (14,19). Others, however, found the opposite using the same method and the same cells (20). We therefore decided to first clarify the role of membrane potential loss in oxidative glutamate toxicity and its relation to ROS production. We measured ΔΨm with TMRE (tetramethylrhodamine ethyl ester), another cell-permeable, cationic, red-orange fluorescent dye that is readily sequestered by active mitochondria, and ROS production using DCFDA in parallel every 2 h after addition of 5 mM glutamate to HT22 cells. This showed that the expected increase in ROS induced by GSH depletion is associated with a parallel loss of ΔΨm (Fig. 3A and B). Moreover, we found that HT22R cells have an increased ΔΨm as shown with TMRE and JC-1 (Fig. 3C), whereas their ROS production is reduced compared with HT22S cells (Fig. 3D). Thus, there is an inverse relationship of ROS and ΔΨm during oxidative glutamate toxicity, and an increased ΔΨm associated with lower ROS might be part of the protective phenotype of HT22R cells.

Toxicity mediated by 12/15-LOX (19) and tBID (14) involves a decrease in ΔΨm. We therefore asked whether GDAP1 confers part of its function by affecting ΔΨm. In HT22 cells transiently transfected with GDAP1, we noted a significant increase in ΔΨm measured with JC-1 and TMRE (Fig. 3E, exemplary FACS profiles are shown in Supplementary Material, Fig. S2). This correlated with a decreased ROS content (Fig. 3F) similar to the results obtained with HT22R cells (Fig. 3D). We conclude that GDAP1 protects from GSH depletion at least in part by increasing the mitochondrial membrane potential.

Disease-causing mutations attenuate or even abolish GDAP1’s effects on cell protection and mitochondrial fragmentation.

To evaluate how these observations relate to the pathogenesis of CMT4A, we next tested various disease-causing autosomal-recessive GDAP1 point mutations (M116R, R120Q, R310Q) for their ability to protect against oxidative glutamate toxicity. Only wild-type and, to a lesser degree, the M116R mutant were able to protect against 10 mM glutamate, whereas the R120Q and R310Q mutants had no effect (Fig. 4A). Our results were therefore consistent with the effect of these mutants on mitochondrial fragmentation in transiently transfected COS7 cells, where M116R was almost indistinguishable from wild-type GDAP1, and R120Q and R310Q resembled empty vector (6). To rule out cell-type-specific activities of GDAP1 on the mitochondrial fragmentation, we repeated these experiments using wild-type GDAP1 and the least functioning R310Q mutant in HT22 cells. We transiently over-expressed these constructs together with a mitochondrially targeted EGFP and observed that GDAP1 also leads to fragmented mitochondria in HT22 cells and that the R310Q mutant has no effect (Fig. 4B). The effect of GDAP1 in these cells was also quantitated, which showed that GDAP1...
over-expression leads to an increase in fragmented mitochondria and a decrease in tubular mitochondria in HT22S cells (Supplementary Material, Fig. S3). To further evaluate the effect of the disease-causing point mutation R310Q on the presumed cytoprotective functions of GDAP1, we then measured total cellular GSH content and the mitochondrial membrane potential in HT22 cells transiently transfected with GDAP1, R310Q GDAP1 or empty vector as control. In these experiments, the R310Q mutant did not alter the GSH content (Fig. 4C) and only had a small effect on \( \Delta \Psi m \) (Fig. 4D) in line with its inability to protect against oxidative glutamate toxicity. We conclude that GDAP1 with disease-associated mutations lacks the cytoprotective function of wild-type GDAP1 and its effects on mitochondrial shape, suggesting that the two functions are connected.

GDAP1, but not R310Q GDAP1, attenuates mitochondrial activity, \( Ca^{2+} \) uptake and superoxide production

To rule out that GDAP1-mediated changes are only caused by short-term massive over-expression, we next generated HT22 cells stably transfected with GDAP1, R310Q GDAP1 or empty vector as control. These cells expressed similar amounts of GDAP1 as shown by immunoblotting using β-actin as loading control (Fig. 5A). It is also evident that GDAP1 is not detectable with this antiserum in untransfected HT22 cells. Stable over-expression of GDAP1 also increased the total cellular GSH concentration (Fig. 5B) and the mitochondrial membrane potential (Fig. 5C), whereas the R310Q mutant had no effect. To clarify whether these effects of GDAP1 might be caused by an increase in mitochondrial...
mass, we then compared the abundance of the mitochondrial housekeeping protein HSP70 with the abundance of β-actin by immunoblotting of mitochondrial fractions obtained by differential centrifugation. It is evident that GDAP1 overexpression has no positive effect on the mitochondrial mass (Fig. 5D).

To obtain further insight into how GDAP1 affects mitochondrial function, we evaluated the activity of the mitochondrial respiratory chain in these cells using three independent methods. We first used polarometric measurements of the oxygen consumption of intact mitochondria, which showed that GDAP1 caused a decreased respiratory chain activity in the presence of ADP (State 3) and ATP (State 4 respiration), whereas the R310Q GDAP1 mutant did not exhibit any significant changes (Fig. 5E). This was in line with a reduced mitochondrial superoxide production measured with flow cytometry using MitoSox staining and (G) reduced mitochondrial Ca²⁺ concentration measured by flow cytometry using Rhod-AM staining only in GDAP1-expressing cells. (H) No change in the cytosolic Ca²⁺ concentration measured by single-cell Ca²⁺ imaging (n > 1000 cells each from three independent experiments) using cells stained with the ratiometric dye Fura2. Each data point in (B), (C) and (E–G) corresponds to the mean ± SEM of three experiments done in triplicate. Asterisk indicates P < 0.05 as determined by one-way ANOVA and Dunnett’s multiple comparison test.

**Figure 5.** GDAP1, but not R310Q GDAP1, attenuates mitochondrial respiratory chain function. (A) Immunoblot of lysates from HT22S cells stably transfected with empty vector, GDAP1 or R310Q GDAP1 probed with antiserum against GDAP1. Equal amounts of protein were applied per lane. Note unchanged bands at the bottom. β-Actin as loading control demonstrates equal expression levels of GDAP1. (B) Increased GSH content measured enzymatically and normalized to protein content, and (C) increased mitochondrial membrane potential measured by flow cytometry using JC-1 staining in HT22S cells stably expressing GDAP1, but not R310Q GDAP1. (D) Immunoblot of extracts from purified mitochondria from cells shown in (E) were probed with an antiserum against the mitochondrial housekeeping protein HSP70 and β-actin and shows similar amounts of mitochondrial proteins in HT22S cells stably expressing vector, GDAP1 or R310Q GDAP1. (E) Reduced mitochondrial respiratory activity of intact mitochondria isolated from HT22S cells stably expressing GDAP1 but not empty vector or R310Q GDAP1 measured by polarometry in the presence of ADP or ATP. (F) Reduced mitochondrial superoxide production measured by flow cytometry using MitoSox staining and (G) reduced mitochondrial Ca²⁺ concentration measured by flow cytometry using Rhod-AM staining only in GDAP1-expressing cells. (H) No change in the cytosolic Ca²⁺ concentration measured by single-cell Ca²⁺ imaging (n > 1000 cells each from three independent experiments) using cells stained with the ratiometric dye Fura2. Each data point in (B), (C) and (E–G) corresponds to the mean ± SEM of three experiments done in triplicate. Asterisk indicates P < 0.05 as determined by one-way ANOVA and Dunnett’s multiple comparison test.

**GDAP1 is mainly expressed in neuronal cells of the peripheral and central nervous system**

We then evaluated the expression levels of GDAP1 in the peripheral and central nervous system in order to choose an adequate model system to test our findings in physiologically relevant cells. Expression of GDAP1 in Schwann cells and neurons of the peripheral nervous system has been demonstrated previously by immunohistochemistry (6), whereas others mainly found an expression in neurons using northern blotting (21). We used quantitative real-time PCR to evaluate mRNA expression levels in cultured cells from the peripheral and central nervous system from rats and humans. We cultured primary astrocytes from embryonic rats characterized by staining against GFAP (glial fibrillary acidic protein) and pure β-tubulin III-positive cortical neurons—pure S100-positive human Schwann cells and motor neurons derived from human embryonic stem cells directed to the motor neuron lineage and sorted by flow cytometry based on EGFP fluorescence driven from the motor neuron-specific promoter Hb9,
which we described previously (22,23), and co-cultures of dorsal root ganglion cells and Schwann cells derived from embryonic rats (Fig. 6A). Gdap1 mRNA was ≏300-fold more abundant in rat neuronal than in glial cultures and 10-fold more abundant in human motor neurons than in pure Schwann cells (Fig. 6B). In co-culture of Schwann cells and dorsal root ganglia, the number of neurons remains constant but the number of Schwann cells increases over time. Gdap1 perfectly matched the decline of the neuronal marker β-tubulin III caused by the increasing number of Schwann cells in the culture (Fig. 6C). We conclude that in cultured rat and human cells, the Gdap1/GDAP1 mRNA is mainly found in neurons of the central and peripheral nervous system.

Over-expression of the R310Q mutant and Gdap1 knockdown enhances the susceptibility of NSC34 motor neurons to GSH depletion

Based on the preferential neuronal expression pattern observed above, we sought to reproduce our findings in peripheral neuronal cells. NSC34 cells are a murine neuroblastoma × motor neuron cell line that exhibits motor neuron characteristics (24). NSC34 cells were not susceptible to glutamate toxicity, but succumbed to excessive amounts of BSO, which inhibits γ-glutamylcysteine synthetase, the key enzyme in GSH synthesis. In NSC34 motor neurons, GDAP1 over-expression was only slightly and not significantly protective against GSH depletion by BSO, but the R310Q mutant was clearly and significantly detrimental (Fig. 6D). Also, knockdown of Gdap1 using the same shRNA constructs directed against Gdap1 or control shRNA (E). Cells were replated after 24 h and treated with the indicated concentrations of BSO for 24 h. Survival was measured by the CTB assay. Each data point corresponds to the mean ± SEM of three experiments done in triplicate. The asterisk indicates P < 0.05 as determined by one-way ANOVA and Dunnett’s multiple comparison test.

GSH content and mitochondrial function in CMT4A-derived fibroblasts

To test whether our findings are linked to human disease, we then extended our studies to fibroblasts obtained from two patients suffering from CMT4A. Fibroblasts designated CMT#1 were obtained from a 25-year-old still ambulant male patient from a non-consanguineous family with a compound heterozygosity (L239F/R273G) of mutations in the C-terminal GST domain of GDAP1 (Fig. 7A). This patient
and his clinical characteristics were described in full detail elsewhere (25). The second fibroblast cell line, designated CMT#2, was generated from a 40-year-old wheelchair-bound male. His parents were cousins from the maternal and the paternal side, because both grandparents married siblings (see pedigree in Fig. 7B). GDAP1 sequencing in this patient disclosed a homozygous mutation of the intron 4 splice donor site (c.579+1G→A, Fig. 7B), which results in skipping of exon 4 as previously assumed (26); amplification of GDAP1 mRNA from CMT#2 and control fibroblasts resulted in a shortened transcript that lacks exon 4, leading to frame-shift and a truncated protein lacking the C-terminal GSTs and the transmembrane domain of GDAP1 (Fig. 7C).

We first investigated the expression of GDAP1 at the mRNA level using quantitative real-time PCR in control fibroblasts and in human motor neurons derived from human embryonic stem cells. This demonstrated that human fibroblasts express only 2.6% of GDAP1 of motor neurons (Fig. 7D).

Interestingly, expression of mutated GDAP1 in both patient-derived fibroblast cell lines was dramatically reduced to 1.6% in CMT#1 and 13.2% in CMT#2 when compared with the expression levels in six different fibroblast cell lines obtained from adult healthy donors, which were passaged in parallel (Fig. 7E). It was not possible to visualize GDAP1 at the protein level in these fibroblasts probably due to the very minute amounts present. We observed no changes in mitochondrial morphology in these cells, where already 90% of all mitochondria had a tubular morphology (Fig. 7F).

CMT4A is an autosomal-recessive disease and therefore most likely caused by loss-of-function of GDAP1; we therefore hypothesized a reduction in the protective functions of GDAP1 in such cells. It was not possible to conduct cell death studies in these cell lines, as their growth characteristics were too divergent. We were, however, able to quantify GSH levels, which demonstrated a significant reduction in both patient cell lines. GSH levels were reduced to ~57% in CMT#1 and ~81% in CMT#2 when compared with control fibroblast cell lines (Fig. 7G). The same applied to the mitochondrial membrane potential measured by flow cytometry using TMRE staining, which was significantly reduced in CMT fibroblasts. Shown are the median and box and whisker plots of three experiments done in triplicate. The whiskers correspond to the minimum and maximum of values obtained in these experiments. The asterisk indicates P < 0.05 as determined by one-way ANOVA and Dunnett’s multiple comparison test.
~82% of control levels in CMT#2 (Fig. 7H). All these results were statistically significant ($P < 0.05$, ANOVA). We conclude that non-functional GDAP1 leads to downregulation of GDAP1 mRNA and a reduction in GSH content and mitochondrial membrane potential in human fibroblasts in line with the findings obtained by over-expression in neuronal cell lines.

**DISCUSSION**

Our results demonstrate a novel role for GDAP1 in the regulation of GSH metabolism and of cell death caused by GSH depletion. First, GDAP1 is upregulated in hippocampal HT22 cells selected for resistance against oxidative glutamate toxicity, a form of programmed cell death induced by GSH depletion, and knockdown of GDAP1 sensitizes these cells to glutamate. Second, knockdown of GDAP1 also increases cell death of motor neuron-like NSC34 cells to inhibition of GSH synthesis by the γ-glutamylcysteine inhibitor BSO. Third, over-expression of GDAP1 induces glutamate-resistance and increases GSH levels in glutamate-sensitive HT22 cells. Finally, GSH is downregulated in fibroblasts derived from CMT4A patients with mutations in both GPAP1 alleles.

In addition to these effects on the cellular GSH content, we also observed GDAP1-mediated changes of the mitochondrial membrane potential $\Delta \Psi m$, which is increased in HT22 cells transiently and stably transfected with GDAP1 and reduced in fibroblasts from CMT4A patients. GDAP1 over-expression also protects against downstream effectors of GHS depletion that affect the mitochondrial membrane integrity like truncated BID and 12/15-lipoxygenase. This is astonishing, as with GDAP1, the increased $\Delta \Psi m$ is associated with a decreased ROS production although the mitochondria are the predominant source of ROS that finally triggers cell death in oxidative glutamate toxicity (27). This is, however, in part reconciled by our results showing increased $\Delta \Psi m$ together with a decreased mitochondrial activity in GDAP1-over-expressing cells, which might be explained by an inhibitory effect of GDAP1 on the later stages of oxidative phosphorylation, e.g. at the ATP synthase level and subsequent alternative routes of energy production like enhanced flux of glucose through both the glycolytic pathway and the hexose monophosphate shunt, which have been shown before to be cytoprotective in oxidative glutamate toxicity (28).

HT22R cells also show an increased $\Delta \Psi m$ and decreased ROS production, and it is probable that these changes contribute to the resistant phenotype, because glutamate treatment induces opposing effects with increasing ROS and decreasing $\Delta \Psi m$. At the first glance, it seems plausible that hyperpolarization of mitochondria might prevent the mitochondrial depolarization that is associated with cell death in oxidative glutamate toxicity (Fig. 3A and data shown in 14,19). However, in general, an increased production of ROS is associated with a $\Delta \Psi m > 140$ mV, whereas mild depolarization, e.g. by uncoupling proteins, decreases ROS production (reviewed in 29). We performed no absolute quantification of the mitochondrial membrane potential, but the negative correlation of increased $\Delta \Psi m$ and decreased ROS production induced by GDAP1 indicates that the GDAP1-induced changes are $< 180$ mV, when the $\Delta \Psi m$-mediated excessive mitochondrial ROS production becomes detrimental to cells. Additionally, the decreased mitochondrial ROS levels could explain the higher or more preserved GHS levels in GDAP1-expressing cells. In this context, it is of interest that mutations of the mitofusin MFN2, which has an opposite effect on the mitochondrial morphology than GDAP1, cause CMT2A, a clinically almost undistinguishable disease (30). Similar to GDAP1, over-expression of MFN2 increases $\Delta \Psi m$ and knockdown decreases $\Delta \Psi m$ (31). Other studies also indicate a role of MFN2 in cell death protection against DNA damage and oxidative stress (32) similar to the effects of GDAP1. It is tempting to speculate that both proteins might have another, and possibly similar, function in addition to the regulation of the mitochondrial shape.

Changes in mitochondrial form induced by GDAP1 were previously shown in COS-7 cells (6,7), and we demonstrate that GDAP1 also induces mitochondrial fragmentation in neuronal HT22 cells. Disease-causing mutations of GDAP1 lack the effect on mitochondrial fragmentation and the novel functions described here, suggesting that these are connected. GDAP1 shares structural similarities with GSTs (10,33), an enzyme family that is able to catalyze the conjugation of reduced GSH to non-polar compounds that contain an electrophilic carbon, nitrogen or sulfur atom. It is conceivable that GDAP1 might act as a GSH sensor, which transmits information about the cytosolic GSH content through its GST-like domains to the mitochondria, leading to changes in mitochondrial form and function. Most mutations that cause CMT4A are found in the C-terminal GST-like domain of GDAP1 (4). In this context, it appears of interest that inhibition of GST function by ethacrynic acid increases mitochondrial fusion (34). However, ethacrynic acid has a wide variety of cellular functions, including GSH depletion and alkylation, and a number of experiments suggest that its effect on mitochondrial fusion is mediated via alkylation of cysteine residues (34,35). It is unlikely that GDAP1 displays classical GST activity, because GSTs, in general, consume and do not increase GSH (36). Also, others observed no GSH-dependent activity and no GSH binding typical for GSTs of bacterially expressed GDAP1 lacking its transmembrane region (10). Thus, the GDAP1-mediated increase in GSH is most probably independent of a direct GST activity.

In summary, we show that GDAP1 increases cellular GSH in neuronal cells, and decreases ROS production potentially due to stabilization of the mitochondrial membrane potential and respiratory chain activity, thereby protecting against oxidative stress. GSH is the most abundant small-molecule antioxidant in the nervous system, and decreased GSH levels are observed during neurodegeneration in specific areas of the nervous system in Parkinson’s disease (37) and in an animal model of motor neuron degeneration (38). Oxidative stress has also been implicated in the pathogenesis of various neuropathies, including diabetic neuropathy (39,40), neuropathy in familial amyloidosis (41) and alcoholic neuropathy (42). It is therefore likely that GSH metabolism plays a role in the pathogenesis of CMT4A.

CMT4A is an autosomal-recessive neuropathy and is therefore most probably caused by loss-of-function of GDAP1. We hypothesize that the lack of the new functions of GDAP1 described herein, elevating cellular GSH and decreasing...
ROS production in association with an increased mitochondrial membrane potential and decreased respiratory chain activity, is involved in the degeneration of the peripheral nervous system in this disease. This would implicate that CMT4A is caused or at least aggravated by oxidative stress and might be amenable to therapies targeting this. We hope that our results shed light on the pathogenesis of CMT4A and possibly open up new therapeutic opportunities.

MATERIALS AND METHODS

Cell culture

Glutamate-sensitive wild-type HT22 cells (HT22S) were cultured in DMEM high glucose (PAA) supplemented with 5% fetal calf serum (FCS, Thermo Fisher Scientific), 100 IU/ml penicillin and 100 µg/ml streptomycin. Glutamate-resistant HT22 cells (HT22R) were additionally maintained in 10 mM glutamate and stably transfected HT22 cells in 2 mg/ml geneticin. NSC-34 cells were grown in DMEM high glucose medium containing 10% FCS. Human fibroblast cell lines were generated from disinfected skin biopsies. The biopsies were transferred into a Petri dish and dissected carefully. The skin fragments were cultured undisturbed in MEM media (Invitrogen), sublimated with 20% FCS (Invitrogen), 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich), 1% l-glutamine (Sigma-Aldrich) at 37°C with 95% air and 5% CO2 for 1 week. Growing fibroblasts were enzymatically detached by Trypsin/EDTA (Sigma-Aldrich), transferred into new flasks and maintained in DMEM/F-12 (Invitrogen) containing 10% FCS, 2 mM l-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. All cells were grown at 37°C with 95% air and 5% CO2. Purified primary cultures from astrocytes, neurons and Schwann cells were prepared from embryonic E15 Wistar rats as described (22). DRG neurons were prepared as described earlier (43). Entire ganglia were plated on collagen-coated 35 mm plastic dishes (Greiner Bio-One) and kept in neurobasal medium for 2 days. Myelination was initiated by switching from neurobasal medium to Eagle’s medium after 24 h, containing MEM medium with Earle’s salts and l-glutamine, 10% FCS (PAA), 100 IU/ml penicillin and 100 µg/ml streptomycin, 100 ng/ml nerve growth factor (Sigma-Aldrich) and 50 µg/ml ascorbic acid (Sigma-Aldrich).

Plasmids and transfections

GDAp1 and R310Q in pcDNA3.1 were a kind gift of A. Niemann (Zürich, Switzerland). 12/15-LOX in pCMV-Sport was obtained from RZPD (Berlin, Germany). A pIRES-EGFP vector expressing truncated BID and a mitochondria-targeted EGFP-expression vector were gifts from C. Culmsee (Marburg, Germany). For vector-based expression of shRNA, two Gdap1-specific shRNAs were designed using Invitrogen’s RNAi Designer and cloned into the pdDNA6.2-GW/EmGFP-miR vector expression according to the manufacturer’s instructions (Invitrogen). The antisense target sequences were 5’-AACCTATGCACTGATAGAAA-3’ (sh#1) and 5’-CCA TATGGAAGACCGTTC3’ (sh#2). Transfections were performed using Attractene, according to the recommendations of the manufacturer (Qiagen), and cells were harvested for further analysis 48 h after the start of transfection.

Microscopy

To visualize the mitochondria, a vector encoding mitochondria-targeted GFP was transfected into cells prior to plating cells onto cover slips. For staining F-actin with rhodamine phalloidin, cells were fixed with 3.7% paraformaldehyde for 15 min at room temperature and permeabilized with blocking buffer (0.2% bovine serum albumin, 0.2% FCS, 0.01% Triton) for another 10 min. After washing with phosphate-buffered saline (PBS), cells were incubated in 100 nM rhodamine phalloidin for 30 min at room temperature and washed again several times with PBS. Cell nuclei were stained with 0.5 µg/ml DAPI. Images were taken using a BX51 fluorescence microscope (Olympus). Alternatively, we labeled the mitochondria with MitoTracker Red CMXRos (Invitrogen) according to the manufacturer’s instruction. In brief, 20,000 cells on cover slips (Fisherbrand) were stained with 0.4 µM MitoTracker Red in serum-free medium for 15–20 min under growth condition. After removing the staining solution, cells were cultivated for 15–20 min in fresh growth medium without the dye. Cells were fixed in 4% PFA/PBS (15 min, room temperature), permeabilized with 0.2% Triton X-100/ PBS (15 min, room temperature) and their nuclei stained with Hoechst (1:1000, 7 min). The cover slips were mounted on slides using Fluoro-Gel (Electron Microscopy Sciences, Hatfield, PA, USA). Cells were examined by a blinded observer using a fluorescence microscope (Olympus BX60) and the morphology of mitochondria in 500 cells (100 cells per cover slip) rated as described (6).

To visualize myelin-containing cultures, DRG cultures were stained with Sudan Black. Cultures were treated with media supplemented with ascorbic acid, washed with PBS and fixed for 1 h with 4% PFA, followed by 0.1% osmium for 1 h. After sequential ethanol treatment (25, 50, 70, 75% each for 5 min), 0.5% Sudan Black solution (Sigma Aldrich), dissolved in 70% ethanol, was added for 1 h. Descending ethanol treatment (70, 50, 25% each for 1 min) was followed by optical microscopy.

Cell viability

5000 HT22 cells/well were seeded into 96-well plates. Glutamate was added 24 h later and cell viability assessed 24 h after the start of the respective treatment using MTT assay or Cell Titer Blue (Promega) and a GENios Pro microplate reader (Tecan) as described (16).

Microarray analysis

Total RNAs from cells were prepared using the RNAeasy mini-prep kit according to the manufacturer’s (Qiagen) protocol. RNA was quantified with a NanoDrop ND-1000 spectrophotometer and quality assessed by a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Double-stranded cDNA synthesis and in vitro transcription were carried out according to Affymetrix protocols. In brief, 3 µg of total RNA from cultured cells were reverse-transcribed with SuperScriptII and
T7-Oligo(dT) Promoter Primer for 1 h at 42°C, followed by 2 h incubation at 16°C for second-strand cDNA synthesis. After sample clean-up, the cDNA served as a template for subsequent in vitro transcription. The IVT reaction was carried out in the presence of T7 RNA polymerase and a biotinylated nucleotide analog/ribonucleotide mix for complementary (cRNA) amplification and biotin labeling for 16 h at 37°C. The quality of cRNA was assessed by a 2100 Bioanalyzer. Biotinylated cRNA was cleaned up and 15 µg of it were fragmented by metal/heat-induced fragmentation. The fragmented cRNA was hybridized against GeneChip Mouse Genome 430A 2.0 in a Affymetrix GeneChip hybridization oven for 16 h at 45°C and 60 r.p.m. Each microarray was washed and stained with streptavidin-phycocerythrin using Affymetrix GeneChip Fluidics Station 450 and then scanned with Affymetrix Scanner 3000. Raw data were analyzed with Affymetrix MAS5 algorithm, and probe sets with a detection P-value of P < 0.01 were used for further analysis.

**Quantitative RT–PCR**

Total RNA was extracted using the Illustra RNAspin Mini Kit (GE Healthcare) following the manufacturer’s instructions and reverse-transcribed with oligo(dT) primers using the SuperScript Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was performed with a 7500 real-time PCR system (Applied Biosystems), TaqMan Gene Expression Master Mix (Roche) and 5′-FAM-labelled probes of the Universal Probe Library (Roche). TaqMan primers were designed using the Universal Probe Library Design Tool (Roche): mouse/rat Gdap1 forward, 5′-AAGAAGAAATGAGGAACTCCAGA-3′, and reverse, 5′-ACATCTGGCATGTGAAGAGGATT-3′; human GDAP1 forward, 5′-TGCAAGAAGAAGAACC-3′, and reverse, 5′-GCTGCACTCAACTCTC-3′; mouse Gdap11 forward, 5′-CTGAGCCTACTTTCTAAACA-3′, and reverse, 5′-TTTTCGATGTAAGCTCATTAT-3′; rat β-Tubulin-III forward, 5′-CA GAGCCATTCTGGTGAGC-3′, and reverse, 5′-GCCAGCAACCTCTGAGC-3′. Expression levels were normalized by expression of the housekeeping genes hypoxanthine phosphoribosyltransferase (Hprt) and β-actin, with the following primer sequences: mouse Hprt forward, 5′-GATTTAACACTCA-3′, and reverse 5′-GGTTGCAAAGCTT GCTGGTGAA-3′; rat Hprt forward, 5′-GACCGTTTCTCTCATGTGTCG-3′, and reverse 5′-ACCTGGTTCATCATCAC TAATCAC-3′; human HPRF7 forward, 5′-TGGACCTTGGATT ATTGTGCTACC-3′, and reverse, 5′-CGAGCAAGACGT TCAGTCTCACC-3′; rat β-actin forward, 5′-CTAAGGCGCTAC CGTGTAAAG-3′, and reverse, 5′-TCAATGCTTGGGTGT TTGAA-3′; human β-actin forward, 5′-ATGGCAGAACGG GGTTGCTC-3′, and reverse, 5′-GGATGGCCAGACCTCTCA-3′. Moreover, a human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Primer-Probe-Assay (Applied Biosystems) was used as a second endogenous control.

**Measurement of total cellular GSH**

Forty-eight hours after transfection, cells were washed twice with ice-cold PBS, resuspended in 200 µl of PBS and transferred to a microcentrifuge tube containing 100 µl of 10% SSA. Samples were vortexed and incubated on ice for 10 min and centrifuged at 16000g for 10 min. Supernatants were transferred into a second microcentrifuge tube containing 24 µl of triethanolamine/H2O2 1:1. GSH was then measured by monitoring NADPH consumption by GSH reductase in an assay buffer (100 mM Na2PO4, 1 mM EDTA, pH 7.5) containing 0.6 mM DTNB (5,5′-dithiobis-(2-nitro- benzoic acid)) and 0.8 mM NADPH at 390 nm using GENios Pro microplate reader (Tecan). Chemicals were obtained from Sigma-Aldrich.

**Detection of ROS**

Intracellular ROS levels were assayed by FACS analysis with the fluorescent dye 2,7′-dichlorodihydrofluorescein diacetate (H2DCFDA). Cells were washed with PBS, detached with trypsin, resuspended with PBS and centrifuged at 1000 r.p.m. for 5 min. Pellets were resuspended in 1 ml of PBS containing 10 µM H2DCFDA. After 20 min incubation at 37°C, cell suspensions were washed twice by centrifugation and resuspension in PBS. Fluorescence intensity at 519 nm was measured with a FACSCalibur flow cytometer (Beckton Dickinson) and quantified using the CellQuest3.3 software (BD Biosciences).

**Mitochondrial membrane potential determination**

The mitochondrial membrane potential was analyzed using JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide, Molecular Probes) and TMRE (Sigma). Forty-eight hours after transfection, cells were washed, resuspended in PBS and treated with 2 µM JC-1 or 10 µM TMRE, respectively. Pre-incubation with 200 µM CCCP (carbonyl cyanide 3-chlorophenylhydrazone, Sigma), an ionophore and uncoupler of oxidative phosphorylation in mitochondria, served as control for the breakdown of the mitochondrial membrane potential. Fluorescence was analyzed using a FACS Calibur flow cytometer and quantified using the CellQuest3.3 software (BD Biosciences). The JC-1 ratio was calculated as the ratio of the fluorescence at 590 nm to that at 530 nm. TMRE fluorescence was monitored at 590 nm.

**Subcellular fractionation**

Mitochondrial and cytosolic fractions were obtained by differential centrifugation. Stably transfected HT22 cell lines were grown in 175 cm² flasks. Cells were washed with PBS, harvested by trypsinization and centrifuged at 1000g for 6 min. Cell pellets were resuspended in 200 µl of mitochondria isolation buffer (200 mM mannitol, 100 mM sucrose, 10 mM HEPES, 1 mM EGTA, 5 mM MgCl2, pH 7.4) containing a protease inhibitor (Complete Mini Protease Inhibitor Cocktail Tablets, Roche) and cells were disrupted by 50 strokes of a Dounce homogenizer. The homogenates were centrifuged at 1000g for 10 min in order to remove nuclei and unbroken cells, and the post-nuclear supernatants were centrifuged at 16,000g for 30 min to obtain mitochondria-enriched pellets. All steps were performed at 4°C and fractions were stored at −80°C.
Immunoblotting

For total cellular protein, cells were lysed with RIPA lysis buffer (Thermo Fisher Scientific) supplemented with one tablet per 10 ml of Complete Mini Protease Inhibitor Cocktail Tablets (Roche). Lysates were centrifuged at 16 000g for 30 min at 4°C and supernatants were stored at −80°C. Protein quantification was performed using the BC Assay Protein Quantification Kit (Interchim). A polyclonal rabbit anti-GDAP1 antibody against the peptide MARRQDEARAGVPL (amino acids 1–14) was a gift of A. Niemann (Zürich, Switzerland). Monoclonal mouse anti-mitochondrial heat shock protein 70 was obtained from Affinity BioReagents. Equal protein loading was examined with a mouse monoclonal anti-actin antibody (MAB1501, Millipore). Following SDS–PAGE, proteins were transferred onto a nitrocellulose membrane using the iBlot Dry Blotting System (Invitrogen) and the blot probed with primary antibodies at 4°C overnight. After washing with TBS containing 0.05% Tween, membranes were exposed to IRDye 800CW goat anti-mouse, IRDye 680 Goat Anti-Rabbit (LiCor) or HRP goat anti-rabbit (Morphosys) IgG secondary antibodies. IRDye-conjugates were detected with the Odyssey Infrared Imaging System (LiCor), HRP conjugates were detected with ECL substrates (Thermo Scientific) on chemiluminescence films (Amersham).

Mitochondrial activity measurements

Respiration of intact mitochondria was measured polarographically at 25°C in a volume of 500 μl of a buffer consisting of 20 mM HEPES, pH 7.0, 125 mM KCl, 2 mM K2HPO4, 1 mM MgCl2, 10 μM EGTA. The measurements were conducted after subsequent additions of 10 mM pyruvate + 2 mM malate, 30 μM ADP and 3 μM CCCP using an Oxygraph system (Helmut Saur Laborbedarf, Reutlingen, Germany). The activity of mitochondria is given as turnover number—consumed O2 (μM)/(time (min) × total amount of protein (mg))—determined in the presence of ADP and ATP. The latter stage is reached when the exogenously added ADP has been phosphorylated by respiring mitochondria and becomes visible by a decrease of mitochondrial activity during the polarographic experiment.

Mitochondrial Ca2+ content and superoxide production

Mitochondrial matrix Ca2+ content and superoxide production were determined by flow cytometric analysis with Rhod2-AM (ABD Bioquest) or MitoSOX red mitochondrial superoxide indicator (Invitrogen). Cells were seeded in six-well plates on the day before the experiment. Staining was done in HBSS with 2 μM Rhod2-AM for 30 min or 5 μM MitoSOX for 10 min at 37°C. After two wash steps, mean red fluorescence was analyzed on a FACScalibur flow cytometer (BD Biosciences).

Single-cell Ca2+ imaging

For Ca2+ imaging, cells were seeded in 96-well plates on the day before the measurement in a density of 5000 cells per well. Loading with Fura2-AM (Invitrogen) was done in HBSS (Gibco) at a concentration of 2 μM for 30 min at 37°C. After washing twice, cells were subjected to measurement in HBSS and images were taken at 340 and 380 nm excitation on a BD Pathway 855 High Content Imaging System. For cytosolic baseline Ca2+ analysis, five data points with a delay of 5 s were recorded and the ratio calculated.

Statistical analysis

Data were summarized as mean ± SEM and the statistical significance assessed using two-tailed Student’s t-tests or analysis of variance (ANOVA) with post hoc Tukey’s or Dunnett’s multiple comparison test as indicated.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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