GDAP1, the protein causing Charcot–Marie–Tooth disease type 4A, is expressed in neurons and is associated with mitochondria

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Mutations in GDAP1, the ganglioside-induced differentiation-associated protein 1 gene, cause Charcot–Marie–Tooth (CMT) type 4A, a severe autosomal recessive form of neuropathy associated with either demyelinating or axonal phenotypes. Here, we demonstrate that GDAP1 has far greater expression in neurons than in myelinating Schwann cells. We investigated cell localization of GDAP1 in a human neuroblastoma cell line by means of transient overexpression and co-localization with organelle markers in COS-7 cells and by western blot analysis of subcell fractions with anti-GDAP1 polyclonal antibodies. We observed that GDAP1 is localized in mitochondria. We also show that C-terminal transmembrane domains are necessary for the correct localization in mitochondria; however, missense mutations do not change the mitochondrial pattern of the wild-type protein. Our findings suggest that CMT4A disease is in fact a mitochondrial neuropathy mainly involving axons and represents a disease belonging to the new category of mitochondrial disorders caused by mutations in nuclear genes. We postulate that GDAP1 may be related to the maintenance of the mitochondrial network.

INTRODUCTION

Charcot–Marie–Tooth disease (CMT) refers to inherited peripheral neuropathies named for three investigators who described them in the late 1800s (1,2). CMT neuropathies affect approximately one in 2500 people, and are among the most common inherited neurological disorders (3,4). The majority of patients have autosomal dominantly inherited neuropathies that are separable into demyelinating (CMT1) or axonal (CMT2) forms of CMT (5). CMT1 patients have slow nerve conduction velocities (NCV) and features of demyelination on nerve biopsies, whereas CMT2 patients have normal or near normal NCV and pathological evidence of axonal damage without demyelination (5,6). Interestingly, patients with CMT1 have evidence of axonal loss in addition to demyelination (6). Moreover, this axonal loss may correlate more with disability than with demyelination in CMT1 patients (7), suggesting that communication between Schwann cells and axons plays an important role in the pathogenesis of CMT1. The molecular basis for these axo–glial interactions remains unknown.

Some patients develop autosomal recessively inherited forms of CMT (CMT4), which are particularly severe and disable the patient in infancy. Both demyelinating and axonal forms of CMT4 exist. In some cases, the nerve damage is so severe that it has been difficult to determine whether the primary pathology was in myelin or in axon. This is the situation with CMT4A, the most common recessively inherited neuropathy. CMT4A is a severe disorder caused by mutations in the ganglioside-induced differentiation-associated protein 1 (GDAP1) gene (8). Mutations in GDAP1 have been reported to cause both demyelinating
and axonal (10) forms of CMT. mRNA studies using RT–PCR have demonstrated ubiquitous expression of GDAP1. It is not known whether the primary site of pathology in CMT4A is in myelin or in axon, although it has been speculated that GDAP1 may play a role in glial–axonal signalling.

GDAP1 is a 358 amino acid protein whose function is unknown. Phylogenetic and structural analyses suggest that GDAP1 belongs to a new subfamily of glutathione S-transferases (GSTs) (11). GDAP1 has two typical GST domains at the N-terminal region: domain I that corresponds to the glutathione binding site and domain II that corresponds to sites associated with presumed cytotoxic and xenobiotic activities. The C-terminus contains two predicted transmembrane domains not found in other GSTs (11). In most patients, nonsense or frameshift mutations of GDAP1 cause autosomal recessive neuropathies associated with severe phenotypes, suggesting that CMT4A phenotypes are due to loss of normal GDAP1 function. However, missense mutations of GDAP1 have also recently been described in two families with autosomal dominant neuropathies and in one sporadic patient (12). Thus, some cases may cause neuropathy by a toxic gain of function.

In order to better understand the function of GDAP1, both in normal and during neuropathy, we investigated both its tissue distribution and its intracellular localization. We found that GDAP1 is predominantly expressed in axons, not in myelin. By overexpressing the protein and utilizing specific polyclonal antibodies to GDAP1 raised in our laboratory, we also demonstrated that GDAP1 is localized within the mitochondrial membranes of the cell. Taken together, these results suggest that CMT4A is primarily an axonal neuropathy, which, like CMT2A (13), is caused by abnormalities in mitochondrial function.

RESULTS
GDAP1 is mainly expressed in neuronal cells
Because neuropathies due to GDAP1 mutations have been described causing both axonal and demyelinating phenotypes, we investigated cell expression of GDAP1 by analyzing mRNA in several rat neuronal and non-neuronal tissues. Consistent with previous reports (9,10), RT–PCR experiments demonstrated GDAP1 mRNA expression in brain, spinal cord, dorsal root ganglia (DRG), peripheral nerve and skeletal muscle, although not from cultured Schwann cells or liver (Fig. 1A and B). However, real-time PCR, which permits quantification of mRNA, demonstrated that GDAP1 mRNA levels were exponentially higher in brain, spinal cord and DRG than they were in sciatic nerve (Fig. 1C). Moreover, by northern blot analysis, we could not detect GDAP1 in sciatic nerve or muscle from adult rat (Fig. 1D). These results demonstrated that GDAP1 has far greater expression in neurons than in myelinating Schwann cells.

In order to detect endogenous GDAP1 protein, we next generated two polyclonal antibodies; one against amino acid residues 9–25 in the N-terminus, RGSPPLRAEGKADAENVK (GDAP1-N), and a second generated against the C-terminal peptide KRLGSMILAFRPFPNYF corresponding to amino acid residues 342–358 (GDAP1-C). Both antibodies specifically recognized human GDAP1 protein expressed in bacteria. We used GDAP1-N antibody to detect endogenous GDAP1 on western blots of SH-SY5Y human neuroblastoma cells (Fig. 2).
We observed two bands of 40 and ~88 kDa, respectively. Following incubation with 1 μM retinoic acid (RA), which induces neuroblastoma cells to differentiate into neurons (14), we observed a new band of 54 kDa. The 40 kDa band corresponds to the expected size of the GDAP1 protein. In both culture conditions, the 88 kDa band almost disappeared after treatment with 50 mM 1,4-dithio-DL-threitol (DTT), suggesting that it may represent a dimeric form of the protein. The extra 54 kDa band may correspond to an isoform of the protein expressed by an alternative splicing of the gene (http://www.ncbi.nlm.nih.gov/mapview/) induced by RA.

To confirm tissue expression of GDAP1, we performed immunocytochemistry on sagittal sections of mice. The maximal gene expression levels of mouse GDAP1 is observed at early postnatal ages as well as during adulthood (8). Therefore, studies were performed in newborn mice, an age at which PNS myelin gene expression is well underway (15). Using the GDAP1-C antibody for immunocytochemistry, we observed expression in brain, spinal cord, muscles and intestinal villi (data not shown).

**GDAP1 is located in mitochondria**

A major clue for unravelling the function of a new protein is to identify its subcellular localization. To investigate the localization of GDAP1 in the cell, we generated pEGFP::GDAP1 and pEGFP-C1::GDAP1 cDNA constructs and we transiently transfected them into monkey kidney COS-7 cells. Overexpressed GDAP1 was localized in the membrane of medium-sized cytoplasmic vesicular structures (Fig. 3A). To confirm this finding, we performed immunocytochemistry studies with GDAP1-C antibody. We detected endogenous GDAP1 in SH-SY5Y cells (Fig. 3B) and also in COS-7 cells overexpressing GDAP1 (Fig. 3C). Previous analysis in silico using the PSORT II programme suggested that GDAP1 might be located in cytoplasm (26%) and cytoplasmic vesicles (21.7%). Thus, we postulated that GDAP1 might be a vesicular protein involved in cell trafficking or axon transport. To address this point, we first investigated potential changes in the central vacuolar system by adding unlabelled brefeldin-A (BFA) and BODIPY-labelled BFA to cell cultures. BFA blocks protein export from the endoplasmic reticulum to the Golgi complex and causes dismantling of the Golgi complex. Although the expression pattern of GDAP1 constructs did not change in these studies, we observed that BFA was localized within GDAP1-positive vesicles (Fig. 3D–I). We therefore proceeded to systematically analyze membrane trafficking of GDAP1 by co-localization experiments using chimeric constructs with specific antibodies or markers for different organelles. Surprisingly, we found that GDAP1 did not co-localize with antibodies labelling the endoplasmic reticulum, cis-Golgi vesicles, trans-Golgi network, late endosomes, lysosomes or clathrin-coated vesicles. We also excluded localization in peroxisomes or lipid bodies. Alternatively, GDAP1 did co-localize with the mitochondrial marker MitoTracker, suggesting that GDAP1 is localized into mitochondria (Fig. 4A–C).

To obtain independent support for a mitochondrial localization of endogenous GDAP1, we prepared subcellular fractionation using differential centrifugation. Using western blotting we confirmed the presence of GDAP1 in the mitochondrial fraction obtained from SH-SY5Y cells (Fig. 5).

**The intracellular localization of GDAP1 is altered by truncation of transmembrane domains but not by disease-causing missense mutations**

To test the effect of nonsense and frameshift GDAP1 mutations on cell expression and to determine the relevance
of C-terminal amino acids in protein targeting, we transfected COS-7 cells with constructs expressing either known disease-causing missense mutations (R120Q, R120W, T157P, R161H and R282C) (http://www.molgen.ua.ac.be/CMTmutations/) (9,12,16–19) or a truncated form of GDAP1 lacking transmembrane domains (pEGFP-N1::GDAP1Δ320–358 and pEGFP-C1::GDAP1Δ320–358). The truncated chimeric protein showed diffuse cytoplasmic and nuclear localization (Fig. 4D). Alternatively, all the missense mutation constructs showed a mitochondrial pattern similar to that observed in the wild-type protein (Fig. 4E–I).

GDAP1 does not express GST activity

Because GDAP1 protein has sequence similarity with GSTs and may characterize a novel class of GST-related proteins, we investigated the GST conjugating activity of GDAP1 against 1-chloro-2,4-dinitrobenzene (CDNB), an electrophilic substrate used to study the kinetic properties of GSTs. The full-length protein contains two transmembrane domains and is not soluble; therefore, we expressed a truncated GDAP1 (GDAP1Δ320–358), containing the putative GST domains, in the BL21 (DE3) strain of Escherichia coli. We did not observe any GST activity of GDAP1 either at physiological or at acidic pH conditions (Fig. 6).

DISCUSSION

The discovery of mutations in GDAP1 as the cause of two forms of autosomal recessive CMT, demyelinating CMT4A (MIM 214400) and axonal CMT with vocal cord paresis (MIM 607706) (http://www.ncbi.nlm.nih.gov/Omim/), raised the question about the localization of the primary lesion in peripheral nerve and the disease mechanisms involved. As a first step towards understanding the pathogenesis of the neuropathy, we searched for cells that express the highest levels of GDAP1. Analysis of gene expression by real-time RT–PCR and northern blot showed robust expression of GDAP1 in neurons, but not in Schwann cells. Expression in neurons was also confirmed by western blot analysis of neuroblastoma cells. Taken together, these results suggest that CMT4A, originally reported as a demyelinating disorder (20), is in fact likely to be primarily an axonal neuropathy. Consistent with this concept, to the best of our knowledge, all published reports of sural nerve biopsies from CMT4A patients have demonstrated a profound loss of myelinated axons, consistent with axonal degeneration (18,19,21). In some reports, features of demyelination (thinly myelinated axons, segmental demyelination and frequent onion bulbs), in addition to marked axonal loss, have been identified (18,19,22). Perhaps in exceptional cases, mutant GDAP1 can cause features of demyelination as well as axonal loss by disrupting communication between the degenerating axon and the ensheathing Schwann cells.

GDAP1 clearly appears to be localized in mitochondria based on immunocytochemical studies and subcellular fractionation in neuronal cells from the SH-SY5Y cell line and overexpressed COS-7 cells transiently transfected with GDAP1 constructs. Mitochondrial disorders such as MELAS (MIM 540000), MERRF (MIM 545000), Leigh syndrome (MIM 256000), Kearns–Sayre syndrome (MIM 530000) and NARP syndrome (MIM 551500) are known to cause axonal neuropathy (reviewed in 23,24). Recently, mutations in the nuclear-encoded mitochondrial GTPase mitofusin 2 (MFN2) gene have been shown to cause the most common, axonal form of CMT, CMT2A (MIM 118210) (13). Thus, CMT4A...
appears to be the most recent of a group of inherited neuropathies caused by mitochondrial abnormalities.

How mitochondrial abnormalities cause axonal neuropathy is not known. Mitochondria are essential for normal cellular function because they are the site of aerobic energy mobilization and biosynthesis of metabolites including amino acids, fatty acids and pyrimidines. When this central function of mitochondria is disrupted, disease occurs. For example, MELAS, MERRF, Leigh syndrome, Kearns–Sayre syndrome and NARP syndrome are all linked to defects in mitochondrial respiratory function. Potentially related mechanisms have recently been postulated in other neurodegenerative disorders: mutations of \textit{PINK1} gene in Parkinson’s disease (PARK6; MIM 605909) may be related to both stress-induced mitochondrial dysfunction and apoptosis (25), and the human deafness dystonia syndrome (MIM 304700) associated with mutations in the \textit{DDP1} gene is likely to be caused by a defective mitochondrial protein-import system (26). Moreover, in peripheral nerves, defects in mitochondrial fusion or axonal transport may also cause disability, presumably by depriving the distal axon of a needed source of energy. Mitochondria undergo a dynamic equilibrium between fusion, in which they line up in chains, and fission, in which these chains dissociate. Following fusion, mitochondria are coupled to a kinesin, KIF1B, and transported along axons by fast anterograde transport. In nerves, mitochondria are enriched in the nerve terminal (27,28). In light of this, it is possible that defects in transport of mitochondria to nerve terminals may lead to defects in energy-dependent processes in this region (23). Indeed, the fact that disability in most cases of CMT are predominantly distal can be explained at least in part by the concept that longer axons have greater need for axonal transport and that the consequences of disrupted axonal transport are most pronounced distally in the axon. MFN2, the protein responsible for CMT2A (13), is necessary for both mitochondrial fusion and transport along microtubules or actin (13). Human MFN2 overexpression affects the morphology of mitochondria filaments in muscle cells, disrupting the mitochondrial network. Interestingly, the cytoplasmic medium-sized vesicles we observed when overexpressing GDAP1 by transient transfection in COS-7 cells were really spheroidal mitochondria that may represent the collapse of the mitochondrial network (29). Whether GDAP1 causes neuropathy by disrupting this process remains to be determined, but will be important for understanding mitochondrial function and neuropathy.

We have also demonstrated that transmembrane domains are necessary for the correct localization of GDAP1 in the mitochondrial membranes and are necessary for GDAP1 to perform its normal function in the neuron. These results, along with identification of mutations in patients that truncate the protein, suggest that loss of function is the most frequent mechanism for the disease, as would be expected in an autosomal recessive disorder. However, recently several patients with presumably autosomal dominant GDAP1 mutations, such as R120W and T157P, have been described (12). Because these mutations lead to amino acid substitutions that appear properly expressed in the mitochondria membrane, it is likely that at least some mutations may also cause neuropathy by a gain of function mechanism although the nature of this mechanism(s) also remains to be determined.

Finally, phylogenetic and structural analyses suggest that GDAP1 belongs to a novel family of GST proteins. However, a measure of GST enzymatic activity using the model substrate CDNB did not confirm GST activity for GDAP1. We tested GST activity utilizing an incomplete protein with no transmembrane domain and it remains possible that GST activity for GDAP1 requires a proper localization of the protein into the mitochondria. There are two proteins belonging to the class Kappa family of GSTs, one
in rat and one in mouse, that have been isolated from the mitochondrial matrix or associated with mitochondrial fractions (30). Conceivably, GDAP1 could be a third member of this group and CMT4A might be caused by disruption of its specific biological role within this organelle.

MATERIALS AND METHODS

Real-time PCR and northern blotting

PCR products of 278 bp were generated using 1 μg of rat liver, spinal cord, DRG, sciatic nerve, muscle, brain and cultured Schwann cell mRNA as templates. Total RNA was extracted using Trizol Reagent (Gibco-BRL, UK) and was reverse transcribed by oligo-dT primers and RNase H- reverse transcriptase Superscript II (Invitrogen, UK) using oligonucleotides 5'-GAACATGATGTAAGTCTGCC-3' and 5'-TCAGGATGAAATTGCAGCC-3'. Quantitative real-time PCR was carried out with SYBR Green Master Mix (Applied Biosystems, UK) at the DNA Engine Opticon CFD-0200 (MJResearch Inc., MA, USA). Real-time PCR oligonucleotides were 5'-AGCACATGAGGCCCTTTGTT-3' and 5'-CAGGAGTAACCTGGGTGTT-3'. PCR was performed in 25 μl total volume including 12.5 μl SYBR Green Master Mix, 1 μl primers of 5 pmol/μl and 1 μl cDNA of 100 ng/μl. Cycling conditions included preincubation 2 min at 52°C and 10 min at 94°C, and 40 cycles of 15 s at 94°C and 1 min at 62°C.

Northern blot analysis was performed with a 1.077 bp cDNA probe amplified from a GDAP1 cDNA clone as template. PCR products were gel purified [32P]-labelled with Primer II Kit (Stratagene) and hybridized to rat adult multiple tissues: liver, spinal cord, DRG, sciatic nerve, muscle and brain. Hybridization and blotting were performed according to Alwine et al. (31). Control hybridization was performed employing GAPDH cDNA as a probe.

Plasmids and site-directed mutagenesis

The full-length human cDNA of GDAP1 gene was cloned into the pGEM-T vector (Promega, WI, USA). The cDNA was amplified by PCR using primers containing restriction enzyme sites for EcoRI and BamHI, and then fused in-frame into the expression vectors for mammalian cells pEGFP-N1 and pEGFP-C1 (Clontech, CA, USA). To obtain a soluble form of the protein, human cDNA of GDAP1 was modified by PCR at its 3' end to remove the two putative transmembrane domains corresponding to amino acids 320–358 (primers 5'-ATGGGCTGAGGACGAGAAGAG-3' and 5'-A ACTTTTGGGGGCTTTTCT-3'). It was then cloned into the expression vectors pCR T7/Nt-TOPO TA and pCR T7/Ct-TOPO TA (Invitrogen); the incomplete GDAP1 protein was in-frame with a His6 tag. Overexpression of the fusion protein was performed in the E. coli BL21 (DE3) strain and the protein was purified according to the manufacturer’s instructions. Human GDAP1 missense mutations (R120Q, R120W, T157P, R161H and R282C) were generated by PCR with specific primers containing the nucleotide changes. GDAP1 mutations (missense and nonsense) were cloned into pEGFP-C1 and pEGFP-N1 expression vectors as described previously.

Cell culture, transfection, immunofluorescence and co-localization experiments

COS-7 and SH-SY5Y cells were grown in DMEM containing 10% (v/v) fetal bovine serum (FBS) supplemented with 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen). To carry out the protein overexpression and localization, COS-7 cells were cultured in six-well plates on glass coverslips and then transfected by calcium phosphate precipitation. For co-localization studies, cells were fixed and then probed with primary antibodies diluted in blocking solution. Primary antibodies were then detected using goat anti-mouse or goat anti-rabbit immunoglobulins coupled to Alexa Fluor 488 or Texas Red (Molecular Probes, The Netherlands) and the samples were mounted in Fluoromount-G (Southern Biotech, AL, USA). Cells were examined using a Leica DM RXA2 microscope and Leica TCS SP Confocal System.

We used antibodies and probes for co-localization of GDAP1 in subcellular organelles in both COS-7 and SH-SY5Y cells. The following antibodies were used: mouse monoclonal antibody B-COP for cis-Golgi compartment (Sigma-Aldrich, MO, USA), mouse antibody TGN38 for trans-Golgi network and mouse monoclonal anti-Clathrin heavy chain for clathrin-coated vesicles (BD Biosciences, CA, USA), rabbit polyclonal anti-Calnexin for endoplasmic reticulum (Calbiochem, CA, USA), polyclonal rabbit anti-Rab-7 for late endosomes (Santa Cruz Biotechnologies, CA, USA), polyclonal rabbit antibody anti-PMP70 for peroxisomes (Molecular Probes) and mouse monoclonal antibody anti-adipophilin for lipid bodies (Progen, Germany), Brefeldin A-BODIPY, Nile Red (lipid bodies), Lysotracker (lysosomes), MitoTracker Red CMXRos and MitoTracker Orange CMTMRos (mitochondria) were from Molecular Probes.

Polyclonal antibody production

Two polyclonal antibodies were produced against two synthetic peptides of GDAP1 (http://immunax.dfc.new.harvard.edu/Tools/antigenic.html) conjugated with Keyhole limpet haemocyanin (Calbiochem) prior to injection in rabbits. The N-terminal peptide is RGSPPLRAEGKADAEVK (amino acids 9–25) and C-terminal peptide is KRLGSMILAFPRPNYF (amino acids 342–358). For each peptide, two rabbits were immunized at multiple subcutaneous sites (32). Antibodies were purified using the immuno affinity support Affi-Gel 10 (Bio-Rad Laboratories, UK) according to instruction manual. Endogenous GDAP1 protein was detected by immunofluorescence in SH-SY5Y cells with the primary antibodies against GDAP1.
Western blot analysis and cell fractionation

SH-SY5Y cells and SH-SY5Y cells induced by 1 μM RA during 24 h were scrapped from two 150 mm dishes and washed twice with PBS. Proteins were extracted and analyzed by western blotting using the GDAP1-N antibody. In order to analyze dimeric forms, samples were loaded with additional 50 mM DTT. For cell fractionation, SH-SY5Y cells were resuspended and homogenized in cold hypotonic buffer A (10 mM Tris–HCl, pH 7.4; 0.33 M sucrose; 1 mM EDTA; 0.5 mM DTT; 1 mM PMSF; 40 μg/ml leupeptin; 40 μg/ml apro tinin; 1 mM Na2VO4; 1 mM NaF). The lysate was centrifuged at 800g for 10 min. The supernatant was removed and retained. The pellet was rehomogenized and spun as before. Supernatant was removed and both supernatants were then centrifuged at 16 100g for 10 min to obtain the cytoplasmic (supernatant) and mitochondrial (pellet) fractions. The cytoplasmic fraction was then concentrated using centrifron YM-30 devices (Millipore, UK) as per the manufacturer’s instructions. Equal proportions of these fractions (supernatant and pellet), each representing the same number of cells, were analyzed by western blotting using GDAP1-N antibody. To confirm relative purity and relative quantity of fractions, the membranes were stripped and reprobed with the following primary antibodies: mouse monoclonal Porin (Molecular Probes) and rabbit polyclonal Actin (Sigma-Aldrich).

Immunohistochemistry

Sections of 4 μm of ethanol 70% fixed wild-type P0 mice embedded in paraffin were collected in Polyline microscope slides (BD Biosciences). The sections were dewaxed, rehydrated and then the endogenous peroxidase activity was blocked, following which they were fixed in paraformaldehyde (4%). Immunoperoxidase staining was then carried out with GDAP1-C primary antibody and detected with horse anti-rabbit biotinilated secondary antibody. The signal amplification and developing were performed using Vectastain Elite ABC Kit and DAB substrate kit (Vector Laboratories, CA, USA). The sections were counterstained with Harris Haematoxylin solution (Sigma-Aldrich), dehydrated, mounted with DPX (Scharlab, Spain) and then analyzed in Leica MS FLIII sterosmicroscope.

GST activity assays

Enzymatic detection was performed using a GST Detection Module kit (Amersham Biosciences, UK). All GST enzyme activity assays were conducted at 37°C using protein, expressed and purified in E. coli. In all instances, the non-enzymatic reaction was measured and subtracted from the overall reaction rate. Activity towards CDNB was measured at 340 nm with an UltraSpec 3000 UV/Visible spectrophotometer (Pharmacia Biotech, UK) at different pH values (5.0, 6.5 and 7.4) in 0.1 M sodium phosphate/1 mM EDTA buffer. As a positive and negative control, GST liver rat and serum albumin (Sigma-Aldrich), respectively, were used. Background activity was measured in the purified protein extract of the E. coli BI21 (DE3) strain.

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