

Report

A Mutation in the Gene for the Neurotransmitter Receptor–Clustering Protein Gephyrin Causes a Novel Form of Molybdenum Cofactor Deficiency

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Gephyrin was originally identified as a membrane-associated protein that is essential for the postsynaptic localization of receptors for the neurotransmitters glycine and GABA_A. A sequence comparison revealed homologies between gephyrin and proteins necessary for the biosynthesis of the universal molybdenum cofactor (MoCo). Because gephyrin expression can rescue a MoCo-deficient mutation in bacteria, plants, and a murine cell line, it became clear that gephyrin also plays a role in MoCo biosynthesis. Human MoCo deficiency is a fatal disease resulting in severe neurological damage and death in early childhood. Most patients harbor *MOCS1* mutations, which prohibit formation of a precursor, or carry *MOCS2* mutations, which abrogate precursor conversion to molybdopterin. The present report describes the identification of a gephyrin gene (*GEPH*) deletion in a patient with symptoms typical of MoCo deficiency. Biochemical studies of the patient's fibroblasts demonstrate that gephyrin catalyzes the insertion of molybdenum into molybdopterin and suggest that this novel form of MoCo deficiency might be curable by molybdate supplementation.

In mammals, molybdenum cofactor (MoCo) is essential for the activity of sulfite oxidase, xanthine dehydrogenase, and aldehyde oxidase (Johnson and Wadman 1995). Mutations affecting the MoCo biosynthetic pathway result in the simultaneous loss of all molybdoenzyme activities. The cerebral atrophy associated with MoCo deficiency (MIM 252150) is also observed in isolated sulfite oxidase deficiency (MIM 272300) and can be attributed exclusively to the loss of this enzyme. Both the isolated and the combined form are inherited as autosomal recessive traits, without any symptoms in heterozygous carriers, and they come to clinical attention because of untreatable neonatal seizures, with opisthotonos and facial dysmorphism. The combined form is found more often, with ~100 cases known worldwide.

We had examined 42 of these patients and had found mutations in *MOCS1* (MIM 603707) (Reiss et al. 1998b) or *MOCS2* (MIM 603708) (Stallmeyer 1999a) in 40 cases (Reiss et al. 1998a, 1999; Reiss 2000).

We studied the last of three affected infants born to a Danish mother and father who were cousins. All three died in the neonatal period (day 12, 29, and 3, respectively), with symptoms identical to MoCo deficiency. Three other pregnancies of the mother resulted in two healthy sibs and one spontaneous abortion. There was no family history of genetic disease, and all three affected infants had a normal karyotype. The first infant was a boy, and the other two were girls. All showed hypotonia combined with hyperreflexia, as well as tonic-clonic convulsions. Fibroblasts of the third infant, the patient, were used to verify a MoCo deficiency by biochemical and in vitro complementation assays and to isolate DNA for genetic analysis. The study was approved by the ethics committee of the Medical Faculty of Göttingen. Complete sequencing of all *MOCS1* and *MOCS2* exons, plus adjacent splice-junction sequences, did not reveal a disease-causing mutation in the patient. The human *MOCS3* cDNA and genomic sequence, encoding the

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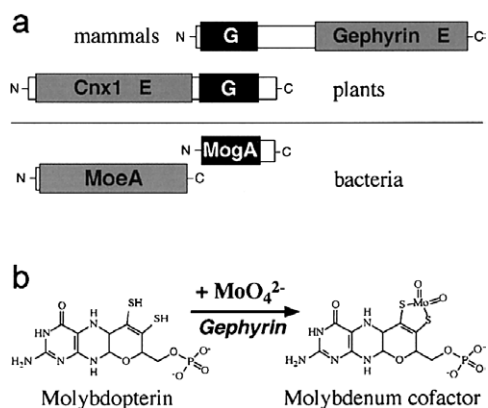


Figure 1 Gephyrin and MoCo biosynthesis. *a*, Schematic representation of gephyrin and homologous proteins. The domain symbols G and E correspond to the respective bacterial operons. *b*, Illustration of the last step in MoCo biosynthesis, the gephyrin-catalyzed insertion of molybdenum into molybdopterin.

molybdopterin synthase sulfurylase (Appleyard et al. 1998), were also found to be devoid of mutations in the patient’s DNA (data not shown).

We analyzed the *GEPH* gene (MIM 603930) for the receptor-clustering neuroprotein gephyrin (Kirsch et al 1973; Essrich et al. 1998; Kneussel and Betz 2000) as a candidate gene for the MoCo deficiency in this family. Gephyrin consists of two distinct protein domains separated by a linker sequence (fig. 1*a*). The N-terminal domain of gephyrin is homologous to the bacterial protein MogA, and the C-terminal domain is homologous to bacterial MoeA, both proteins being involved in the biosynthesis of MoCo (Stewart 1988). This architecture

is shared by the *Drosophila* protein Cinnamon (Kamdar et al. 1994) and, with a reversed domain order, the plant protein Cnx1 (Stallmeyer et al. 1995). The bacterial *moaB* gene is also homologous to the 5’ region of the *GEPH* cDNA, as well as to the bacterial *mogA* gene. However, no bacterial mutant and no function is known for *moaB*. The *moeA* gene product is believed to activate molybdenum before its incorporation into molybdopterin (Hasona et al. 1998; Kuper et al. 2000). Bacterial *mogA* mutants can produce active MoCo in media supplemented with high concentrations of molybdate (Stewart and MacGregor 1982). This “molybdenum rescue” is also observed for the corresponding plant mutants (Stallmeyer et al. 1995) and the MoCo-deficient murine cell line L929 (Falciani et al. 1994).

Geph cDNA derived from rats could restore molybdenum-repairable plant mutants and bacterial *mogA* mutants, as well as L929 cells (Stallmeyer et al. 1999*b*). Likewise, *Geph* knockout mice showed the expected absence of synaptic glycine-receptor clustering and developed symptoms identical to those of MoCo deficiency (Feng et al. 1998). Therefore, gephyrin combines an evolutionarily novel function (neuroreceptor clustering) with a conserved old function (MoCo biosynthesis), the latter being essential for activation (E domain) and insertion (G domain) of molybdenum into molybdopterin, thereby forming the biologically active cofactor (fig. 1*b*).

The rat *Geph* cDNA sequence (Prior et al. 1992) was used to screen the nonredundant GenBank database for human cDNA sequences with the homology search program Advanced BLAST. A 4,193-bp cDNA clone derived from brain tissue contains the complete coding sequence for human gephyrin, with the start codon at position

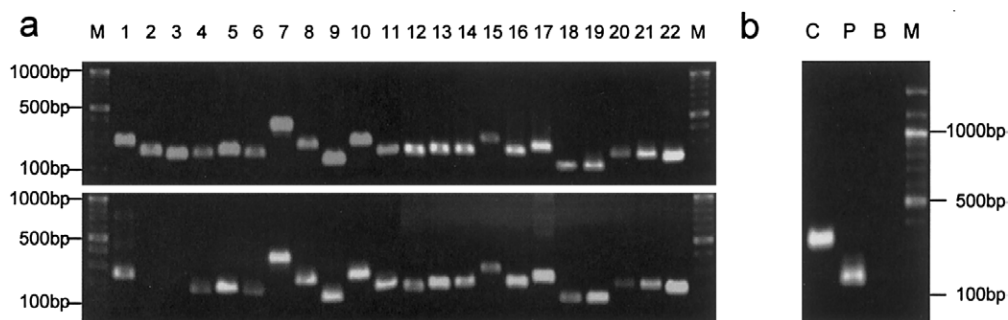


Figure 2 Detection of a gephyrin gene deletion. *a*, PCR amplification of individual exons (1–22) from genomic DNA of a control (*top*) and the patient (*bottom*). DNA was isolated from fibroblasts with standard phenol/chloroform extraction methods. PCR (40 cycles at 94°C for 45 s, at 50°C–60°C for 45 s, and at 72°C for 2 min after an initial incubation of 15 min at 94°C) was done with HotStar*Taq* PCR master mix (Qiagen) and exon-flanking primers (table 1). *b*, Nested PCR cDNA amplification from exon 1 to exon 4 after reverse transcription. Total RNA was isolated from fibroblasts with an RNeasy minikit combined with QIAshredder columns (Qiagen). First-strand synthesis was done with the Omniscript RT kit (Qiagen), including RNasin (Promega) and a sequence-specific primer in a 20- μ l volume. In a first PCR of 25 cycles, 10 μ l of this reaction was used, with the conditions specified above, and 1 μ l of first-round PCR product was the template for a second-round PCR with 40 cycles and two nested primers. B = blank (no template); C = healthy control; M = size marker (100-bp ladder) (New England Biolabs); P = patient.

Table 1
Oligonucleotides Used in Study of Patient with Gephyrin Gene Deletion

Method ^a	Primer	Sequence
EA:		
E1	G1F	5'-CCTAGCTGTCGCGCTCTCCT-3'
	G1R	5'-TCCCAGGCCGCAGAGAAGG-3'
E2	G2F	5'-GGTCAGCAATAGCTTAAATG-3'
	G2R	5'-CTCTTTTGAGAAAAGGAACAC-3'
E3	G3F	5'-GCATTCTGATGGTAATGGCA-3'
	G3R	5'-ACCCCTACCAAGATGCTAA-3'
E4	G4F	5'-GGGATGTTTTGAGCAAGCAG-3'
	G4R	5'-CCATGATTAGTTTAACTCTTG-3'
E5	G5F	5'-GTGGGTTTACTAGTCTGAC-3'
	G5R	5'-ACAGTTCACCTAGCAAATGG-3'
E6	G6F	5'-CTTAATGTATTTAAACCGGGC-3'
	G6R	5'-TTGGCTCCCTAACTTTCAC-3'
E7	G7F	5'-CAGTTTGATTGCCACCATCT
	G7R	5'-TTACCTGTGGGTCCTTTAGG-3'
E8	G8F	5'-AAGGGGGTCTTGATTCTACA-3'
	G8R	5'-CCCAGATTACTATAGAAGAGC-3'
E9	G9F	5'-ACCTCAGGAGCTTGCCATT-3'
	G9R	5'-AAGCTCTAGTTCAGCAGCCC-3'
E10	G10F	5'-GTCATTGCCACTTTTTAATCA-3'
	G10R	5'-CAGGAAAACCTGTCATTAATG-3'
E11	G11F	5'-CAAGCACTCATGCCCATCTT-3'
	G11R	5'-CAGTGCCTGATTATGTTAAG-3'
E12	G12F	5'-CTTGTCCATGCTGTAGGTC-3'
	G12R	5'-TCCACTAAACTGATAGGAGA-3'
E13	G13F	5'-CTTTTCCTTTGCAGCAGCAA-3'
	G13R	5'-ACTGCCATAGGAACAACAGC-3'
E14	G14F	5'-TATCCTGGGCTATCTGATG-3'
	G14R	5'-GCCAGGGTTTCTGAGTAAA-3'
E15	G15F	5'-CACTAAAGTTTCCCTCTGAG-3'
	G15R	5'-CAACACAGAACATATGTCAG-3'
E16	G16F	5'-TATGCAACATTAACCTAATAG-3'
	G16R	5'-ATGAGTATCCAAAACTCG-3'
E17	G17F	5'-GCCTATTAGTGAATAAGGCG-3'
	G17R	5'-AGATGCCTACCAGACCACAG-3'
E18	G18F	5'-TCATTTAAAGTGTTGAAAGTC-3'
	G18R	5'-CCCATATATGAGATAACAAGA-3'
E19	G19F	5'-AAAACACTGGAGTACTTAATG-3'
	G19R	5'-CCAGAAAAGGAAAGGAAAC-3'
E20	G20F	5'-TAGACAGACATAATTATTTGGC-3'
	G20R	5'-TTAGGAAATCATATCCCTAAC-3'
E21	G21F	5'-GTCCACTGTATTCTTTGCAC-3'
	G21R	5'-CAGGATAGGTGCTAGGAA-3'
E22	G22F	5'-AGGGCCCAACTGTATACGCC-3'
	G22R	5'-GCTTCTCCTGCTGGTGACC-3'
RT-PCR:		
RT	RT1	5'-CAGGGCCATCCCTGGTGCTT-3'
PCR1	G1F	5'-CCTAGCTGTCGCGCTCTCCT-3'
	RT1	5'-CAGGGCCATCCCTGGTGCTT-3'
PCR2	RT2	5'-TTCTCCCGGCTCCTGTCAGT-3'
	RT3	5'-TCGTGGTGCAAATCCTGTTC-3'

^a A = amplification; E = exon.

1122 and the stop codon at position 3330. The nucleotide and amino acid sequence homologies are 93% and 100%, respectively. The human cDNA sequence was used to identify the genomic sequences in the HTGS (high throughput genomic sequences) library. A homol-

ogy search revealed that the gene is located on chromosome 14 and consists of 22 exons covering a genomic region of ≥ 375 kbp. These genomic sequences were used as primer targets to amplify all exons individually, plus each of their adjacent splice-signal sequences (table 1). Amplification of patient and control DNA revealed a deletion encompassing exons 2 and 3 in the patient (fig. 2a). This deletion was verified by reverse transcriptase

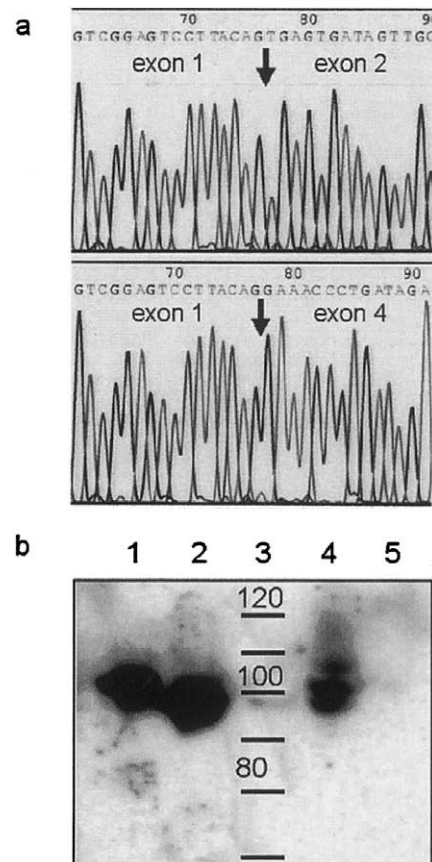


Figure 3 a, Sequence analysis of the RT-PCR product of control (top) and patient (bottom). Sequencing was done commercially (SeqLab) after purification of the amplification products with the QIA quick PCR purification kit (Qiagen) with the PCR primers and dideoxy fluorescent dye terminators (ABI). The transition from exon 1 to exon 2 or 4, respectively, is indicated by the arrow. b, Western blot analysis of gephyrin expression in crude protein extracts using a polyclonal anti-gephyrin antibody. Crude protein extracts were prepared from fibroblasts, separated by SDS-PAGE using a 7.5% polyacrylamide gel, and blotted onto a polyvinylidene fluoride membrane. Positive controls were recombinant rat *Geph* P1 and P2 splice forms expressed in *Escherichia coli* (G.S., unpublished data). A primary polyclonal antibody generated against recombinant rat gephyrin P1 protein was used (diluted in serum at 1:2000), followed by chemiluminescent detection (secondary antibody, Promega; ECL system, Amersham/Pharmacia). Lane 1, recombinant rat gephyrin P2 (20 ng); lane 2, recombinant rat gephyrin P1 (20 ng); lane 3, size marker (labeled in kDa); lane 4, positive control (18 μ g); lane 5, patient with gephyrin gene deletion (20 μ g).

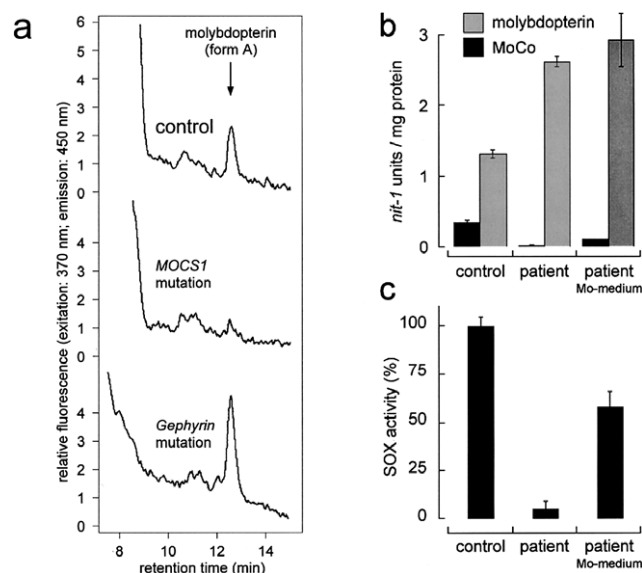


Figure 4 Biochemical characterization of fibroblasts with the gephyrin gene deletion. *a*, HPLC detection of the molybdopterin derivative “form A” in crude fibroblast extracts of a healthy control subject (*top*), a patient with a *MOCS1* mutation as negative control (*middle*), and the patient with the gephyrin gene deletion (*bottom*). Molybdopterin was detected by conversion to its stable oxidation product form A-dephospho. Oxidation (1 mg protein per sample) with I_2/KI , QAE-sephadex chromatography and HPLC analysis on a C18 reversed-phase column were performed as described elsewhere (Schwarz et al. 1997). *b*, nit-1 reconstitution assay for the detection of active MoCo (–Mo) and total molybdopterin, including both the metal-free and metal-loaded pterin (+Mo) of a control (*left*) and of the patient without (*middle*) and with (*right*) molybdate supplementation of the medium. *c*, Sulfite oxidase activity of control fibroblasts (*left*), the patient’s fibroblasts grown on normal medium (*middle*) and the patient’s fibroblasts grown on medium supplemented with molybdate (10 mM) (*right*). *Neurospora crassa* nit-1 extract was prepared as described elsewhere (Nason et al. 1971). The assay was performed in a 90- μ l (+Mo) or a 180- μ l (–Mo) volume containing 30 μ l or 60 μ l, respectively, of nit-1 extract freshly filtered on gel (Nick columns; Amersham/Pharmacia) in the presence of 2 mM reduced glutathione. Protein extracts from fibroblasts were prepared in 50 mM sodium phosphate, 200 mM NaCl, 5 mM EDTA (pH 7.2) and were added in various amounts (20–120 μ l) to the nit-1 extract, according to the linear range of the assay. Total molybdopterin content was determined by performing the reconstitution in the presence of 10 mM sodium molybdate. MoCo was detected in the absence of external molybdate. Complementation was carried out anaerobically overnight at 4°C. After addition of 20 mM NADPH for 10 min, reconstituted NADPH-nitrate reductase activity was determined. One nit-1 unit is defined as the A540 of 1.0/30 min nitrate reductase reaction time. Sulfite oxidase activity was determined as described elsewhere (Johnson et al. 1991).

PCR, in which exclusively truncated transcripts were detected (fig. 2*b*), followed by sequencing of the amplification products (fig. 3*a*). On the mRNA level, this deletion results in a frameshift after only 21 codons of the normal coding sequence. Therefore, neither of the two gephyrin domains (fig. 1) is expressed. Western blot analysis confirmed the complete absence of gephyrin protein

in crude extracts of the patient’s fibroblasts, whereas the expression of at least two splice variants (Prior et al. 1992) can be seen in control cells (fig. 3*b*).

Because of the presumed function of gephyrin in MoCo formation (fig. 1*b*), loss of this protein should result in the accumulation of molybdenum-free molybdopterin. We demonstrated the presence of the fluorescent oxidation product of molybdopterin (“form A”) (Johnson and Wadman 1995) by high-performance liquid chromatography analysis (HPLC) (fig. 4*a*). Biologically active molybdopterin and MoCo can be quantified by the in vitro nit-1 reconstitution assay, which is based on the transfer of MoCo to the nitrate reductase apoprotein of the *Neurospora crassa* nit-1 mutant (Nason et al. 1971). Depending on presence or absence of molybdate in the assay, it can be used for the detection of total molybdopterin (+Mo), including all forms of the pterin and MoCo (–Mo). HPLC analysis of the patient’s cells shows an increased level of molybdopterin (fig. 4*b*), compared with control cells. Active MoCo could be detected only in control cells and in those patient cells that were supplemented with 1 mM molybdate (fig. 4*b*). This molybdate repair of the cofactor could also be demonstrated by the regained sulfite oxidase activity (fig. 4*c*).

After the primary sequence of the receptor-associated gephyrin revealed homologies to MoCo biosynthetic enzymes, it appeared counterintuitive that a eukaryotic protein might have two such different functions as (i) a structural role in receptor clustering and (ii) a biosynthetic activity in MoCo formation. It has been suggested that two bacterial biosynthetic genes (*mogA* and *moeA*) have been fused during evolution to form a multidomain protein with a novel function (Kamdar et al. 1994; Stallmeyer et al. 1995; Feng et al. 1998). This has happened under persistence of the biosynthetic activities, whose abrogation, as shown here, results in a lethal malfunction. Moreover, the fusion of the same prokaryotic genes, in reversed orientation, in higher plants (Stallmeyer et al. 1995) (fig. 1*a*) demonstrates that there is a strong selective pressure for this convergent development in eukaryotes.

The gravity of the disease in the family described here is very similar to the serious phenotype seen in the *Geph* knockout mice (Feng et al. 1998). Such mice could be used to test whether MoCo-deficient patients with *GEPH* mutations could be treated successfully by administering high concentrations of molybdate. In attempts reported elsewhere, administration of an oral (Duran et al. 1978; Munnich et al. 1983; Endres et al. 1988) or an intravenous (Bamforth et al. 1990) molybdate supplementation to MoCo-deficient patients did not lead to any clinical or biochemical improvement. These patients, however, have not been examined genetically, and, on the basis of the observed mutation frequencies, it seems likely that they carried mutations in the “non-

repairable” genes *MOCS1* or *MOCS2* (Reiss 2000). The dysfunction of gephyrin as a receptor-clustering molecule in the postsynaptic membrane cannot be restored by these means. Hyperekplexia (MIM 149400), as a consequence of isolated receptor dysfunction, is a relatively mild neurological disorder, which can be ameliorated by pharmaceutical agents (Andrew and Owen 1997). These drugs could be combined with a molybdate therapy in gephyrin-deficient patients to treat the synaptic receptor dysfunction, as well as the biosynthetic deficiency. As in the other forms of MoCo deficiency, the most successful approaches may include prenatal treatment to prevent neurological damage.

Acknowledgments

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for rat gephyrin cDNA [accession number X66366] and for human sequences *MOCS3* cDNA [accession number AF102544], *MOCS3* genomic [accession number HS914P20], gephyrin cDNA [accession numbers AB037806 and AB272663], and gephyrin genomic [accession numbers AC021012.2, AL139295.2, AL117667.2, AL049835.3, AL159179.2, AL135978.2, and AL133241.2])

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for MoCo deficiency [MIM 252150], sulfite oxidase deficiency [MIM 272300], *MOCS1* [MIM 603707], *MOCS2* [MIM 603708], *GEPH* [MIM 603930], and hyperekplexia [MIM 149400])

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