Splice-specific Functions of Gephyrin in Molybdenum Cofactor Biosynthesis

Received for publication, February 6, 2008, and in revised form, April 1, 2008. Published, JBC Papers in Press, April 14, 2008, DOI 10.1074/jbc.M800985200

Birthe Smolinsky†, Sabrina A. Eichler§, Sabine Buchmeier†, Jochen C. Meier†, and Guenter Schwarz†,‡

From the †Institute of Biochemistry and Center for Molecular Medicine, University of Cologne, 50674 Cologne, Germany, the ‡Max-Delbrueck-Center for Molecular Medicine, 13125 Berlin-Buch, Germany, and the §Institute of Zoology, Technical University Braunschweig, 38106 Braunschweig, Germany

Gephyrin is a multifunctional protein involved in the clustering of inhibitory neuroreceptors. In addition, gephyrin catalyzes the last step in molybdenum cofactor (Moco) biosynthesis essential for the activities of Mo-dependent enzymes such as sulfite oxidase and xanthine oxidoreductase. Functional complexity and diversity of gephyrin is believed to be regulated by alternative splicing in a tissue-specific manner. Here, we investigated eight gephyrin variants with combinations of seven alternatively spliced exons located in the N-terminal G domain, the central domain, and the C-terminal E domain. Their activity in Moco synthesis was analyzed in vivo by reconstitution of gephyrin-deficient L929 cells, which were found to be defective in the G domain of gephyrin. Individual domain functions were assayed in addition and confirmed that variants containing either an additional C5 cassette or missing the C6 cassette are inactive in Moco synthesis. In contrast, different alterations within the central domain retained the Moco synthetic activity of gephyrin. The recombinant gephyrin G domain containing the C5 cassette forms dimers in solution, binds molybdopterin, but is unable to catalyze molybdopterin (MPT) adenylylation. Determination of Moco and MPT content in different tissues showed that besides liver and kidney, brain was capable of synthesizing Moco most efficiently. Subsequent analysis of cultured neurons and glia cells demonstrated glial Moco synthesis due to the expression of gephyrin containing the cassettes C2 and C6 with and without C3.

*This work was supported in part by the DFG (Schw759/2-2 and FOR471 (to G. S.) and ME2075/3-1 (to J. C. M.)), Helmholtz Association (VN-NG-246, to J. C. M.), and the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2 and Table S1.

‡Both authors contributed equally to this report.

§To whom correspondence should be addressed: Institut für Biochemie, Universität zu Köln, Otto-Fischer-Str. 12–14, 50674 Köln, Germany. Tel.: 49-221-470-6432; Fax: 49-221-470-6731; E-mail: gschwarz@uni-koeln.de.

The abbreviations used are: GlyR, glycine receptor; GephE, gephyrin E domain; GephG, gephyrin G domain; Mo, molybdenum; Moco, molybdenum cofactor; MPT, molybdopterin; IPTG, isopropyl-1-thio-β-D-galactopyranoside; EGFP, enhanced green fluorescent protein; GABA_A, γ-aminobutyric acid, Type A.

G. Schwarz, unpublished results.
Splice-specific Functions of Gephyrin

The nomenclature of these exons shows some irregularities because of different and independent identification. It is remarkable that except for two cases, all resulting mRNAs express full-length gephyrins without early termination. Gephyrin isoforms with the C2 and C6 cassette or an additional C4 exon in the C domain are the most common and first described variants (17) also referred to as P1 and P2 variants. A tissue-specific distribution of some splice variants (21, 22) raised the question if the different functions of gephyrin in neurons as well as in basic metabolism may be undertaken by or restricted to different isoforms.

Here, we studied the Moco biosynthetic activity of eight gephyrin splice variants isolated from rat spinal cord (20) (Fig. 1C), by functional reconstitution of L929 cells, a gephyrin-deficient line for which we found a defect in the G domain of gephyrin (GephG). Individual domain functions were investigated in Escherichia coli mogA and moeA mutants. Both experiments demonstrated that C2 and C6 cassettes are essential for the gephyrin enzymatic function; cassettes in the C domain had no significant affect on the activity while the insertion of C5 resulted in a loss of GephG activity. Finally, we demonstrated that high levels of Moco are synthesized in brain, which was traced back to glial cells expressing metabolically active gephyrin variants.

EXPERIMENTAL PROCEDURES

Gephyrin Domain Constructs and Splice Variants—Sequences encoding gephyrin variant Geph2,6 or domain fragments Geph-E, Geph-GC, and Geph-GCE3/4 were derived from pcDNA3 constructs (5) and PCR, cloned into the EcoRI/Sall or EcoRI/XhoI sites of pEGFP-C2 (Clontech). pEGFP-Geph2,4,6EE was described elsewhere (12). GFP-tagged gephyrin splice variants Geph2,6, Geph2,3,6, Geph2,4,6, Geph2,5,6, Geph2,4,6/H11032, Geph2,4,5,6, Geph1,2,4/H11032,5,6, and Geph2 were described previously (Meier et al., Ref. 20). For the expression in E. coli, splice variants were PCR-cloned into the AgeI and SacI sites of pQE80-Geph2,6 (12) or KpnI site of pQE30 (Qiagen).

In Vivo Localization—L929 cells were grown in RPMI medium (PAA Laboratories) containing 10% fetal calf serum (Perbio) at 37 °C and 10% CO2. Transfection of GFP-tagged gephyrin constructs (pEGFP-N1-Geph2,6, -Geph2,3,6, -Geph2,4,6, -Geph2,5,6, -Geph2,4,6/H11032, -Geph2,4,5,6, -Geph1,2,4/H11032,5,6, -Geph2) was performed with a nucleofector device (NucleofectorTM II, Amaxa Biosystems) according to the manufacturer’s protocol. After transfection, cells were seeded at 1 × 10^6 cells per nine 6-cm² dishes and fixed 24 h later with 4% paraformaldehyde. GFP fluorescence was visualized by confocal laser-scanning microscopy (Leica DM TCS SP2).

Determination of MPT and Moco Content in L929 Cells—Gephyrin domain constructs (pEGFP-C2-Geph2,6, -Geph2,4,6-E, -Geph-GCE3/4, -Geph-E, -Geph-GC, and pcDNA3-Geph2,6), gephyrin splice variants (pEGFPN1-Geph2,6, -Geph2,3,6, -Geph2,4,6, -Geph2,5,6, -Geph2,4,6/H11032, -Geph2,4,5,6, -Geph1,2,4,5,6, -Geph2), and empty pEGFP-C2

(19–21), mouse (22), and men (23) (Fig. 1B). The nomenclature of these exons shows some irregularities because of different and independent identification. It is remarkable that except for two cases, all resulting mRNAs express full-length gephyrins without early termination. Gephyrin isoforms with the C2 and C6 cassette or an additional C4 exon in the C domain are the most common and first described variants (17) also referred to as P1 and P2 variants. A tissue-specific distribution of some splice variants (21, 22) raised the question if the different functions of gephyrin in neurons as well as in basic metabolism may be undertaken by or restricted to different isoforms.

Here, we studied the Moco biosynthetic activity of eight gephyrin splice variants isolated from rat spinal cord (20) (Fig. 1C), by functional reconstitution of L929 cells, a gephyrin-deficient line for which we found a defect in the G domain of gephyrin (GephG). Individual domain functions were investigated in Escherichia coli mogA and moeA mutants. Both experiments demonstrated that C2 and C6 cassettes are essential for the gephyrin enzymatic function; cassettes in the C domain had no significant affect on the activity while the insertion of C5 resulted in a loss of GephG activity. Finally, we demonstrated that high levels of Moco are synthesized in brain, which was traced back to glial cells expressing metabolically active gephyrin variants.
Splice-specific Functions of Gephyrin

vector were transiently expressed in L929 cells, grown as described above. Cells were seeded at a density of 5.4 × 10^5 cells on 85-mm plates, transfected with EFFECTENE (Qiagen), harvested after 48 h, washed twice with phosphate-buffered saline, and sonicated in 100–200 μl of nit-1 buffer (50 mM sodium phosphate, 200 mM NaCl, 5 mM EDTA). For the determination of Moco and MPT, 3–30 μl of crude extract were incubated with 20 μl of Neurospora crassa nit-1 extract supplemented with 2 mM reduced glutathione. Moco and MPT were determined by reconstitution (overnight) of nit-1 nitrate reductase in the absence or presence of 5 mM sodium molybdate, respectively, as described (24).

Determination of Moco Content in E. coli mogA and moeA Mutant Cells—Gephyrin constructs pQE30-Geph2,6, pQE30-Geph2,4,6, pQE80-Geph2,3,6, -Geph2,5,6, -Geph2,4,6, and Geph-2 as well as empty pQE80 vector were expressed in the E. coli mogA mutant RK5206 (25) and moeA mutant SE1588 (26), respectively. 50-ml cultures were inoculated to an initial A600 of 0.06 (SE1588) and 0.14 (RK5206), induced with 10, 50, and 200 μM IPTG, grown overnight anaerobically at 25 °C. harvested, washed with nit-1 buffer, solubilized in 1 ml of nit-1 buffer, and homogenized by sonication on ice. The expression level of each sample was analyzed by Western blot. Aliquots of equally expressed proteins were used for the determination of Moco by nit-1 reconstitution using 5–15 μl of crude protein extract and 25 μl of desalted nit-1 extract. After 2 h of anaerobic reconstitution, nitrate reductase was determined (24).

Quantification of Expression Levels—Expression levels of the different gephyrin domain constructs and splice variants in L929 and E. coli cells were analyzed by Western blots using either mouse monoclonal anti-gephyrin antibodies mAb3B11 (1:25 dilutions) or polyclonal rabbit anti-gephyrin antibodies pAb-GephG (1:2000, “Puszta-Serum”) as primary and anti-mouse or anti-rabbit alkaline phosphatase-coupled secondary antibodies (Sigma). Densitometric quantification of detected bands was performed with GeneTools software from SynGene, and the resulting differences were used to normalize the determined activities.

MPT Binding and Adenylylation Experiments—Gephyrin G domain as well as G domain containing the C5 cassette were expressed from pQE30-GephG (10) or pQE30-GephG5 (PCR cloned as pQEP4 (Qiagen)) into the E. coli strain BL21. Proteins were expressed and purified using a Ni-nitrolotriacetic acid column (Qiagen) as described (10) followed by size exclusion chromatography with a Superdex 200 XK16/60 column (GE Healthcare). MPT synthesis was performed in 100 mM Tris/HCl, pH 8.0 with 500 pmol of cyclic pyranopterin monophosphate (28), 100 pmol of Moco in a total volume of 140 μl per reacting unit (time point) under anaerobic conditions. Adenylylation was induced with 0.5 mM ATP and 10 mM MgCl_2, final concentration. The reaction was stopped at different time points by acidic iodine oxidation of MPT and MPT-AMP. Based on the formation of the fluorescent products FormA and FormA-AMP, the content of MPT and MPT-AMP was quantified by HPLC analysis as described (17).

Determination of MPT and Moco Content in Murine Organs—Organs (brain, heart, kidney, liver, lung) of 6-month-old mice were pestled in liquid nitrogen, solubilized in 100 mM Tris/HCl, pH 8.0, sonicated, and centrifuged for 20 min at 4 °C. 100 and 300 μg of crude extract were used to determine total MPT and Moco content by HPLC analysis as described above. The amount of Moco in all samples was further determined through the nit-1 reconstitution assay as described before.

Generation of Monoclonal Antibodies—Purified Geph-EE (5) was used to immunize mice following a standard immunization protocol. After hybridization and cloning, antibody producing hybridoma cells were screened for their binding to holo-gephyrin as well as the individual domains by ELISA. Supernatants were grown either according to standard protocols or in serum-free medium in the presence of 500 mg/liter albumin and 10 mg/liter transferrin. The resulting mAb3B11 was used as culture supernatant.

Cortex Cell Culture—Cortical cultures were prepared from E19 Wistar rats. All animals were sacrificed according to the permit (LaGeSo, 0122/07) given by the Office for Health Protection and Technical Safety of the regional government of Berlin and obeyed the rules laid down in the European Community Council Directive.

For glia-free cortical neuron cultures, cells were plated at an initial density of 75,000 cells/cm² and maintained for 2 weeks in Neurobasal medium, supplemented with 2% B27 (30), 0.25 mM glutamine, 25 μM β-mercaptoethanol, and 0.05% penicillin/streptomycin (Invitrogen). To inhibit glia cell proliferation, 5 μM 1-β-d-arabinofuranosylcytosine was added 2 days after plating.

For neuron-free glia cultures were plated at an initial density of 40,000 cells/cm² and maintained for 2 weeks in glycine-free Earle’s minimal essential medium (MEM), supplemented with 0.25 mM glutamine, 10 mM HEPES, 30 mM glucose, 0.23 mM Na⁺-pyruvate, 0.05% penicillin/streptomycin, and 10% heat-inactivated horse serum (Invitrogen). Harvested cell pellets were washed with phosphate-buffered saline buffer and sonicated in nit-1 buffer. Crude extracts were anaerobically concentrated with a 3-kDa concentrator (Amicon) to a protein concentration of approximately 10 mg/ml (with exception of the neuron-sample) and used for the nit-1 reconstitution assay.

Transcript Analysis of Gephyrin Splice Variants—Gephyrin splicing was analyzed in neurons and glia cells as previously described (31, 32). Briefly, primers were designed to bind to the vicinity of either C2 and C5 (gephyrin ±C5: GAGTCTCTCACAGTGAGTATA & TCAAGTTCATCATGCACCTCC, yielding 537/498 bp; respectively), C3 and C4 (gephyrin C3/C4: GGAGTGCACTGAACTTGA & TTGCTGCTGACCTGGACTG, yielding −/−: 356 bp, +/−: 398 bp, +/+: 464 bp, respectively), or C6 (gephyrin +/−C6: GCATCAGAAAAAGATGGCTATG & GGTCATCTTCAGGATTTAGTAG, yielding 441/321 bp, respectively). The presence of these cassettes was determined on the basis of size comparison with PCR products of identified gephyrin clones (20). PCR products of the fragments containing one of the C4 cassettes were further characterized by DNA sequencing.

RESULTS

Mapping the Gephyrin Defect in L929 Cells—L929 cells are gephyrin-deficient mouse fibroblasts (13). First, EGF-tagged full-length gephyrin (Geph2,6) as well as domain constructs
encoding GephG with the central domain (Geph-GC) and subdomains 3 and 4 of the GephE (Geph-GCE3/4), GephE (Geph-E) and a chimeric construct containing a second E domain fused to the C terminus of gephyrin (Geph-EE) (5) were transfected into L929 cells, and Moco and MPT content were determined by the nit-1 reconstitution assay (24).

While total MPT levels in L929 cells were comparable in all samples, including non-transfected cells (supplemental Fig. S1A), clear differences were seen for the levels of reconstituted Moco (Fig. 2A). Geph2,6 exhibited maximal activity, and no activity was found for GephE. Constructs with no or modified E domains showed a somewhat lower activity than full-length gephyrin, pointing to a functional advantage of the domain fusion. In conclusion, L929 cells express functional GephE, as isolated GephG is able to restore Moco biosynthesis.

Expression of transfected gephyrin variants was confirmed by Western blot using a newly generated monoclonal anti-gephyrin antibody (mAb3B11, for details see “Experimental Procedures”) and a polyclonal anti-gephyrin antibody (pAb-GepG) that recognizes GephG (Fig. 2B) (27). As mAb3B11 only gave signals for constructs containing the entire E domain, we conclude that it recognizes an epitope located within GephE. It is important to note that no signal for endogenous gephyrin was detected with mAb3B11, although L929 complementation confirmed GephE activity. Successful expression of Geph-GC was demonstrated with pAb-GepG antibodies. Only Geph-GCE3/4 was hard to detect, probably due to a rapid degradation as reported recently (12).

These experiments were performed with EGFP-tagged proteins, similar to the following experiments with gephyrin splice variants in order to monitor transfection efficiency visually. To exclude any effect derived from the EGFP tag, we investigated the functionality of Geph2,6 as a representative control. Geph2,6 was expressed as non-tagged (pcDNA3-Geph2,6) or N- or C-terminally labeled EGFP fusion protein (pEGFP-Geph2,6). All constructs resulted in similar Moco synthetic activities in L929 cells (supplemental Fig. S2).

Moco Activity of Gephyrin Splice Variants in L929 Cells—To determine Moco synthetic activity of gephyrin splice variants, they were first expressed in L929 fibroblasts. Upon expression of gephyrin splice variants in non-neuronal cells, different types of cytoplasmic aggregates were reported previously (20) that are believed to form imperfect scaffolds due to the lack of neuronal gephyrin-associated proteins such as collybistin (33, 34). Consistent with previous findings in HEK293 and COS7 cells (20) Geph2,6, Geph2,3,6, Geph2,4,6, Geph2,5,6, and Geph2 formed round aggregates in L929 cells, the so-called "gephyrin blobs" (35) (Fig. 3A). While variants containing the C5 cassette form very elongated spike-like structures as observed before (20), in contrast to the well-known gephyrin "blobs" (35). Expression of Geph1,2,4,5,6 was not detectable. B, reconstitution of Moco biosynthesis in L929 cells transfected with the above mentioned constructs. The Moco content was determined as described in Fig. 2A; n.d., not detectable. C, Western blots of crude protein extracts used for Moco analysis in B. Gephyrin was detected with mAb3B11 (mAb) and pAb-GepG (pAb) antibodies. For each sample, 50 μg of protein were loaded onto 7.5% SDS-polyacrylamide gels.

FIGURE 2. Reconstitution of Moco biosynthesis in L929 cells by gephyrin domain constructs. A, reconstitution of L929 cells transfected with pEGFP-Geph2,6, Geph1,2,4,6-EE, GephGC3/4, GephG, Geph-E, and control vector (pEGFP-C2). The Moco content in crude extracts is derived from multiple measurements with different protein amounts according to the linear range of reconstitution. Units are defined as reconstituted enzyme activity (AA₄₅₀ over 20 min of reaction time). B, Western blots of crude protein extracts used for Moco analysis in A. Gephyrin was detected with mAb3B11 (mAb) and pAb-GepG (pAb) antibodies. For each sample, 50 μg of total protein were loaded onto 10% SDS-polyacrylamide gels.

FIGURE 3. Expression of gephyrin splice variants in L929 cells. A–C, L929 cells were transfected with pEGFP-Geph2,6, Geph2,3,6, Geph2,4,6, Geph2,5,6, Geph2,4,6-EE, GephGCE3/4, Geph2,4,5,6, Geph1,2,4,5,6, Geph2, and as control empty vector (pEGFP). A, GFP fluorescence of transfected cells observed after 24 h of incubation. Note that variants containing the C5 cassette form very elongated spike-like structures as observed before (20), in contrast to the well-known gephyrin "blobs" (35). Expression of Geph1,2,4,5,6 was not detectable. B, reconstitution of Moco biosynthesis in L929 cells transfected with the above mentioned constructs. The Moco content was determined as described in Fig. 2A; n.d., not detectable. C, Western blots of crude protein extracts used for Moco analysis in B. Gephyrin was detected with mAb3B11 (mAb) and pAb-GepG (pAb) antibodies. For each sample, 50 μg of protein were loaded onto 7.5% SDS-polyacrylamide gels.
Splice-specific Functions of Gephyrin

the following functional data are derived from gephyrin variants that form the previously described intracellular aggregates (except Geph1,2,4',5,6) in non-neuronal cells (20, 35).

Consistent with the results from the domain complementation, the overall MPT content in cells expressing the different splice variants was comparable (supplemental Fig. S1B) and independent of reconstituted Moco levels (Fig. 3B). As seen for the domain reconstitutions, we found Moco synthetic activity for all gephyrin variants that contained G domains without splice modification. Variants with an additional C5 cassette (Geph2,5,6; Geph2,4,5,6; Geph1,2,4',5,6) were completely impaired in Moco synthesis (Fig. 3B), suggesting a loss in GephG function. Although L929 cells were shown to be GephG-deficient, alteration at the E domain seems to impact the overall Moco synthetic activity of gephyrin, as deletion of the C6 cassette resulted in a significantly lower activity of Geph2. Interestingly, variants Geph2,6, Geph2,3,6, Geph2,4,6, and Geph2,4',6 were in a similar range of activity, indicating that the insertion of cassettes C3, C4, and C4' into the C domain did not alter the overall function of gephyrin in Moco biosynthesis.

Equal expression of all eight gephyrin variants was confirmed by Western blot (Fig. 3C). Band intensities were densitometrically determined and used to normalize Moco levels to that of Geph2,6, resulting in even more similar activities (Fig. 3B, white bars). Remarkably, for Geph2, no signal was detected with mAb3B11, while pAb—GepE antibodies showed a smaller but equally intense band corresponding to a 4-kDa reduction in molecular weight compared with Geph2,6, due to the missing C6 cassette in GephE. This finding suggests that mAb3B11 specifically recognizes residues within C6, which form the majority of subdomain 2 (see below). Therefore, mAb3B11 can be used as a splice-specific gephyrin antibody that binds only variants with the C6 cassette. Finally, the absence of any signal in Geph1,2,4',5,6 confirmed the very weak expression of this variant (Fig. 3C).

Domain-specific Functions of Gephyrin Isoforms—Given the fact that L929 cells are defective in GephG, we could not investigate the functional properties of GephE in more detail. Therefore, individual domain functions of gephyrin variants were analyzed in E. coli by reconstituting Moco synthesis in the GephG—corresponding mogA mutant and GephE—corresponding mogA mutant, because bacteria express two separate proteins homologous to GephG and GephE, respectively. mogA mutants are unable to adenylylate MPT (16), while mogE mutants accumulate adenylylated MPT as they are defective in molybdenum insertion (18). Seven gephyrin splice variants were re-cloned into bacterial expression vectors (pQE30/pQE80) and recombinantly expressed in both E. coli mutants. As gephyrin isoforms varied in their expression levels, different IPTG amounts were used for induction of protein synthesis. Crude extracts with comparable amounts of expressed gephyrin isoforms were identified by Western blots (Fig. 4, C and F), and Moco content was determined by nit-1 reconstitution (Fig. 4, A and D). Furthermore, the resulting Moco levels were normalized to the levels of Geph2,6 expression (Fig. 4, B and E).

Similar to L929 reconstitutions, all non-modified GephG-containing gephyrin variants were able to restore Moco synthesis in mogA mutants, confirming the catalytic activity of their G domains (Fig. 4A). After normalizing the activities to the expression levels of each variant to the levels of Geph2,6 expression (Fig. 4, B and E), two forms, Geph2,3,6 and Geph2,4',6 were twice as active as the other positive gephyrin forms. Variants containing C5 were again inactive and indistinguishable from controls. Note that Moco levels were significantly higher than in L929 cells because of anaerobic growth conditions and stronger expression of gephyrins (expression was induced with 10–200 μM IPTG). Therefore, it cannot be excluded that MPT synthesis presents a limiting factor that might explain the increased normalized activity of variants with lower expression
level (Geph2, 3, 6 and Geph2, 4, 6). Furthermore, Geph2 shows almost the same activity as Geph2, 6, while it was much less active in L929 cells. An explanation might be the low abundance of the remaining E domain, whose activity is lowered because of a dominant negative effect of the truncated E domain in Geph2. Such an effect is not seen in E. coli, because MoeA is strongly expressed.

In contrast to the L929 complementation, Geph2 also showed complete loss of activity upon expression in moeA mutants, because large parts of GephE are deleted in this splice form (Fig. 4D). All other variants contained high levels of Moco, which correlated with their expression levels (Fig. 4E), resulting in very similar activities (Fig. 4E). The overall Moco content in gephyrin-reconstituted moeA cells was 2–5 times higher than in mgoA mutants, which goes back to the synthesis of eukaryotic Moco by gephyrin, while in MgoA-deficient cells MoeA competes for MPT-AMP to drive the synthesis of the bacterial bis-MPT guanine dinucleotide cofactor (14) that is not detected in the nit-1 assay.

Biochemical Properties of C5-containing GephG—While the loss of activity in Geph2 was not surprising because of deletions of large parts of the active site within GephE (7), the loss of function in C5 cassette-containing variants was not so obvious. As only GephG function is altered in Geph2, 5, 6 and Geph2, 4, 5, 6, we generated GephG with the C5 cassette. Biochemical characterization of GephG containing the C5 cassette (GephG5). A, size exclusion chromatogram (Superdex 200 16/60) of 14 mg of purified GephG5. B, catalytic MPT synthesis in the presence or absence of 200 pmol of GephG or GephG5 using 500 pmol of cyclic pyranopterin monophosphate. C, adenyllylation of in vitro synthesized MPT (panel B) by the addition of 0.5 mM ATP and 10 mM MgCl2. MPT and MPT-AMP were determined by HPLC FormA analysis described under “Experimental Procedures” and elsewhere (18).

Splice-specific Functions of Gephyrin

Distribution of MPT and Moco in Different Murine Organs—Tissue/organ distribution of gephyrin splice variants has been reported previously using either mRNA expression (19, 20, 22, 23) or proteomic analysis of GlyR-associated proteins (21), summarized in supplemental Table S1. Both studies confirmed that brain and liver are the organs with major gephyrin expression where most of the alternatively spliced cassettes were found. In addition, transcripts containing C2 and C6 cassettes were also identified in heart, kidney, and lung, but with lower abundance. Next, we investigated Moco and total MPT levels in these five tissues with proven gephyrin expression. Highest levels of MPT and Moco (measured together as the FormA oxidation product) were found in liver followed by kidney with the lowest readings in brain (3% of liver, Fig. 6A). However, the Moco content was found to be much higher in brain (22% of liver, Fig. 6B). This finding shows a more efficient conversion of MPT into Moco by gephyrins in neuronal tissue as illustrated by the Moco content with respect to total MPT, which is 7.3-fold higher in brain than in liver (Fig. 6C). When comparing these data with gephyrin expression in the respective organs by Western blot using the mAb3B11 antibody, the strongest signals were found in brain followed by liver and kidney. The presence of at least three gephyrin bands in the brain extract points to several splice forms presumably generated by the splicing of cassettes located in the C domain.

Moco Synthesis in Glia Cells—To identify the cell type that contributes to Moco synthesis in brain, we prepared primary cultures of rat cortex neurons and glia cells that were subjected to Moco and gephyrin expression analyses (Fig. 7). Western blots using mAb3B11 showed that cortical neurons mainly express a gephyrin variant running around 95 kDa, while in glia cells an additional band, ~3–5 kDa larger than the lower one, was detectable (Fig. 7A). Transcript analysis by PCR indicated that cortical neurons mainly express gephyrin variants containing C2 and C6 with minor contribution of C4 and C5. The latter might be reflected by the weak signals seen above the major
gephyrin band in Western blots (Fig. 7A). Sequence analysis of the C4-assigned PCR bands showed that three of four C4 cassettes identified in humans (23) are also present in a similar ratio in rat cortical neurons (C4a, C4c, and C4d, data not shown). In contrast, glia cells express mainly two gephyrin variants containing C2, C3, and C6 or only C2 and C6. Therefore, the larger gephyrin band seen in the glia Western blot can be assigned to C3-containing variants, which are similar in size to those variants detected in liver and kidney (Fig. 6D).

For Moco analysis, cells were harvested, extracted, and concentrated prior to use. Specific Moco activity was detected in glia cells, which was 30% higher than in total brain extract, indicating that glia cells mainly, if not exclusively, contribute to Moco synthesis in the brain (Fig. 7C). In contrast, no Moco activity was detectable in cortex neurons.

In summary, variants containing C2, C6 with and without C3 were found to be capable of synthesizing Moco in vivo. Moco synthesis in the brain is based on gephyrins expressed in glial cells, while gephyrins in neurons, biochemically able to synthesize Moco, do not seem to participate in Moco synthesis.

**DISCUSSION**

Gephyrin is a multifunctional protein that participates in at least two different processes: clustering of inhibitory neurotransmitters and biosynthesis of Moco. Alternative splicing and tissue-specific expression of gephyrin might contribute to the functional diversity including receptor binding (31), clustering (36), phosphorylation (37), binding to other proteins involved in gephyrin-mediated synapse formation, and/or controlling the gephyrin metabolic function in Moco synthesis. The latter was investigated in this study using eight splice variants that have been identified in rat spinal cord with modifications in both domains (G and E) essential for Moco synthesis as well as the C domain, which is believed to be crucial for brain-specific functions because it contains binding sites for a number of interacting proteins (8). Six of these variants represent the most abundant gephyrin forms identified in rat, mouse, and human and are found in different organs including brain and liver (supplemental Table S1).

First, we used gephyrin-deficient L929 cells (13) to study Moco synthesis of full-length gephyrin variants. Whereas Western blots with G and E domain-specific antibodies could not detect any gephyrin in L929 cells, we showed that only the G domain function is lost in those cells. Therefore, either very low levels of full-length gephyrin or an N-terminal-truncated form is expressed. The latter might be caused by mutations that result in the use of an alternate start site that has been proposed also for gephyrin variants lacking the C2 cassette (19). With respect to the functionality of gephyrin and its splice variants, it is important to note that all variants showed the same type of
aggregation seen in other non-neuronal cells (20, 36) (Fig. 3A). Variants that form typical gephyrin “blobs” (Geph2,6, Geph2,3,6, Geph2,4,6, Geph2,4) were able to synthesize Moco with Geph2 as the only exception. Therefore, one can argue that these intracellular aggregates contain metabolically active gephyrin.

An important finding is that the C5 cassette inactivates GephG. C5 is inserted into α-helix 4 (10), which holds a loop in place that hosts a conserved Gly-Gly-Thr-Gly motif (Fig. 8A), in close proximity to the pyrophosphate bond in MPT-AMP (16). For the homologous plant Cnx1 G domain, Thr-542, located in the aforementioned loop, is vital for both MPT binding and catalysis (16, 38). Furthermore, this loop also hosts an invariant aspartate residue, which is functionally important (39). Our in vitro studies on GephG containing C5 demonstrated that the overall folding of this domain is not altered, because GephG5 is able to bind MPT. However, catalysis of MPT-AMP synthesis, is completely destroyed by C5, which explains the loss of function in Geph2,5,6 and Geph2,4,5,6.

In addition, a significant change in the oligomerization shifting from trimeric GephG to dimeric GephG5 was observed. Previous gel filtration studies on C5-containing GephG were interpreted as a monomeric G domain because of an elution at 27 (36) and 21 kDa (11). Here, the observed elution at 38 kDa is closer to the theoretical mass of a GephG5 dimer (41 kDa). Therefore we favor a dimer, which could also explain the observed spike-like structures upon expression in non-neuronal cells (20) due to a uni-directional oligomerization of gephyrin dimers.

Recently, variants with the C5 cassette were mainly found in liver and to a somewhat lower extent in brain (21) (Fig. 7B). Previous findings have also shown that neuronal expression of C5-containing gephyrins might have a function in regulating inhibitory synapse composition (31). The presence of C5 in liver, as the main Moco-synthesizing organ, is somewhat surprising because C5-containing gephyrins were now found to be inactive in Moco synthesis. As C5 gephyrins show an altered oligomerization, they might help to disrupt gephyrin aggregates in order to maintain high levels of soluble gephyrin in liver. The fact that in brain Moco synthesis is most efficient suggests a very effective molybdenum insertion catalyzed by the gephyrin variants expressed in brain. The 7-fold higher Moco per MPT content in brain is partially mirrored by an increased expression of gephyrin (2-fold, Fig. 6D).

Two other cassettes in GephG, C1, and C2, also influence gephyrin stability/solubility and function in Moco synthesis (Fig. 8A). The C2 cassette was present in all examined splice variants, and previous data revealed that C2 is widely distributed in neuronal and non-neuronal tissue (21–23). A loss of C2 leads to an alternate start codon in the C-terminal region of GephG, resulting in N-terminal truncation by 115 residues (19) that would entirely destroy GephG function. Geph1,2,4,5,6 harbors an additional C1 exon and showed very weak expression and/or rapid degradation with no detectable activity in Moco synthesis. The low abundance of Geph1,2,4,5,6 might be related to structural changes at the N terminus of GephG because the individual insertions of C4 and C5 did not change protein expression/stability.

The only variant affecting GephE was Geph2, a form that was generated in vitro. The missing C6 cassette forms large parts of subdomain 2 (Fig. 8B), which forms the upper half of the GephE active site harboring residues important for Moco biosynthesis as shown for the homologous Cnx1 protein (40) as well as MoeA (41). The fact that Geph2 is expressed at similar levels as other variants (including blob formation) points to an overall maintenance of the structural integrity of GephE. It remains to be elucidated whether the binding of certain gephyrin ligands is dependent on the presence of C6 and might serve as an entry point to regulate gephyrin-clustering.

Recent progress in the understanding of the Moco biosynthetic function of the gephyrin-homologous Cnx1 protein (17, 18) has uncovered the functional basis of domain fusion in Cnx1 as well as gephyrin (16–18). Product substrate channeling was shown to be the main reason to fuse both domains within one protein. MPT-AMP, synthesized by the G domain, has to be efficiently transferred to the adjacent E domain. The main difference between Cnx1 and gephyrin is the inverted order of the domains, which might be one cause for the elongated C domain connecting the G and E domain in gephyrin. Consequently, this domain has gained unique features such as binding sites for proteins that control gephyrin-clustering and neuronal transport, including the GABA<sub>A</sub> receptor-associated protein GABARAP (42), dynein light chain proteins (43), or

![FIGURE 8. Structure of GephG trimer (A) and GephE dimer (B) with highlighted splice cassettes.](image-url)
microtubules (22). Therefore it is not surprising that the C domain undergoes intensive alternative splicing.

1,929 cells present a tool to investigate the function of full-length gephyrins, and therefore our results further allow conclusions regarding the role of C domain-related splice variations. Geph2,6, Geph2,3,6, Geph2,4,6, and Geph2,4’6 showed similar activities in Moco synthesis, suggesting that splice cassettes in the C domain do not alter the function of gephyrin. Although previous studies reported a strong expression of Geph2,3,6 in non-neuronal tissue (23, 44) such as liver (21) (Fig. 6), the organ with highest Moco synthesis required for sulfite oxidase catalytic activity (45), we could not detect increased Moco synthesis upon expression of Geph2,3,6.

As seen in Fig. 6D, the strongest expression of gephyrin is seen in brain with several splice forms detected by the E domain-specific mAb3G11 antibody. Consistent with our biochemical data, these splice variants should have Moco synthetic activity because high Moco levels were found with respect to the overall MPT content. Finally, we investigated if neurons, glia cells or both contributed to the observed Moco synthesis in brain. Our results clearly show that glia cells express two gephyrin variants (Geph2,6 and Geph2,3,6) at almost equal levels and that these variants are the main contributors to Moco synthesis in brain. To date, only residual activity of sulfite oxidase has been detected in brain.4 Consequently, future studies need to investigate the expression and activity of Moco-dependent enzymes in brain tissue. Although, neuronal gephyrins were found to be able to synthesize Moco, no Moco synthesis has been detected in neurons. Therefore, we conclude that synaptic gephyrin does not participate in Moco synthesis suggesting that both the scaffolding and metabolic functions exist independent of each other. The latter is also supported by the finding that Moco-deficient MOC51 knockout mice show wild-type-like gephyrin and GlyR clustering (46).

In summary, we have demonstrated that (i) the insertion of C5 into GephG abolishes Moco synthetic function of gephyrin, (ii) C6 is crucial for GephE function, and (iii) modification within the linker such as insertions of C3, C4, or C4’ do neither affect individual domain functions nor the activity of the entire protein. These findings are in contrast to the GlyR binding properties of gephyrin splice variants (21, 36) where none of the known splice cassettes are located in the GlyR-binding site of GephE (7) and therefore do not affect GlyR binding. (iv) Finally, we showed efficient Moco synthesis in the brain, which we traced back to glia cells, while cultured cortex neurons did not contribute to Moco synthesis. Therefore, we suggest that synaptic gephyrin is not involved in Moco metabolism.

Acknowledgments—We thank Franziska Koenig and Tanja Otte (TU Braunschweig, Germany) for the cloning of pcDNA3- and pEGF-gephyrin domain constructs, Carola Bernert (MDC Berlin, Germany) for excellent technical assistance with neuronal and glial cell cultures and gephyrin splice analysis, Tania Messerschmidt (TU Braunschweig) for technical assistance, Dr. Katrin Fischer (University of Cologne, Germany) for rendering figures, Prof. Jens Bruening (Institute of Genetics, University Cologne, Germany) for providing murine organs, Prof. Brunhilde Wirth (Institute of Genetics, University Cologne, Germany) for help with the transfection, and Brigitte M. Jockusch (TU Braunschweig) for helpful discussions.

REFERENCES

1. Pfeiffer, F., Graham, D., and Betz, H. (1982) J. Biol. Chem. 257, 9389–9393
2. Feng, G., Tintrap, U., Kirsch, J., Nichol, M. C., Kuhse, J., Betz, H., and Sanes, J. R. (1998) Science 282, 1321–1324
3. Meyer, G., Kirsch, J., Betz, H., and Langosch, D. (1995) Neuron 15, 563–572
4. Tretter, V., Jacob, T. C., Mukherjee, J., Fritschy, J. M., Pangalos, M. N., and Moss, S. J. (2008) J. Neurosci. 28, 1356–1365
5. Schrader, N., Kim, E. Y., Winking, J., Paulakut, J., Schindelin, H., and Schwarz, G. (2004) J. Biol. Chem. 279, 18733–18741
6. Sola, M., Bavro, V. N., Timmins, I., Franz, T., Richar-Blum, S., Schoehn, G. Ruigrok, R. W., Paarmann, I., Saiyed, T., O’Sullivan, G. A., Schmitt, B., Betz, H., and Weissenhorn, W. (2004) EMBO J. 23, 2510–2519
7. Kim, E., Schrader, N., Smolinsky, B., Bedet, C., Vannier, C., Schwarz, G., and Schindelin, H. (2006) EMBO J. 25, 1385–1395
8. Kneussel, M., and Betz, H. (2000) Trends Neurosci. 23, 429–435
9. Sola, M., Kneussel, M., Heck, I. S., Betz, H., and Weissenhorn, W. (2001) J. Biol. Chem. 276, 25294–25301
10. Schwarz, G., Schrader, N., Mendel, R. R., Hecht, H. J., and Schindelin, H. (2001) J. Mol. Biol. 312, 405–418
11. Saiyed, T., Paarmann, I., Schmitt, B., Haeger, S., Sola, M., Schmalzing, G., Weissenhorn, W., and Betz, H. (2007) J. Biol. Chem. 282, 5625–5632
12. Lardi-Studler, B., Smolinsky, B., Petitjean, C. M., Koenig, F., Sidler, C., Meier, J. C., Fritschy, J. M., and Schwarz, G. (2007) J. Cell. Sci. 120, 1371–1382
13. Stammeyer, B., Schwarz, G., Schulze, J., Nerlich, A., Reiss, J., Kirsch, J., and Mendel, R. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1333–1338
14. Schwarz, G. (2005) Cell. Mol. Life Sci. 62, 2792–2810
15. Reiss, J., and Johnson, L. L. (2003) Hum. Mutat. 21, 569–576
16. Kuper, J., Llamas, A., Hecht, H. J., Mendel, R. R., and Schwarz, G. (2004) Nature 430, 806–806
17. Llamas, A., Mendel, R. R., and Schwarz, G. (2004) J. Biol. Chem. 279, 55241–55246
18. Llamas, A., Otte, T., Multhaupt, G., Mendel, R. R., and Schwarz, G. (2006) J. Biol. Chem. 281, 18343–18350
19. Prior, P., Schmitt, B., Grenningloh, G., Pribilla, I., Multhaupt, G., Beyreuther, K., Maulet, Y., Werner, P., Langosch, D., Kirsch, J., and Betz, H. (1992) Neurosci 8, 1161–1170
20. Meier, J., De Chadde, M., Triller, A., and Vannier, C. (2000) Mol. Cell. Neurosci. 16, 566–577
21. Paarmann, I., Schmitt, B., Meyer, B., Karas, M., and Betz, H. (2006) J. Biol. Chem. 281, 34918–34925
22. Ramming, M., Kins, S., Werner, N., Hermann, A., Betz, H., and Kirsch, J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10266–10271
23. Rees, M. I., Harvey, K., Ward, H., White, J. H., Evans, L. I., Duguid, I. C., Hsu, C. C., Coleman, S. L., Miller, J., Baer, K., Waldvogel, H. J., Gibbon, F., Smart, T. G., Owen, M. J., Harvey, R. J., and Snell, R. G. (2003) J. Biol. Chem. 278, 24688–24696
24. Reiss, J., Gross-Hardt, S., Christensen, E., Schmidt, P., Mendel, R. R., and Schwarz, G. (2001) Am. J. Hum. Genet. 68, 208–213
25. Kuper, J., Palmer, T., Mendel, R. R., and Schwarz, G. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6475–6480
26. Hasona, A., Ray, R. M., and Shanmugam, K. T. (1998) J. Bacteriol. 180, 1466–1472
27. Giese, T., Schwarz, G., Navrotzki, R., Berhörster, K., Rothkegel, M., Schlüter, K., Schröder, N., Schindelin, H., Mendel, R. R., Kirsch, J., and Jockusch, B. M. (2003) J. Neurosci. 23, 8330–8339
28. Santamaria-Araujo, J. A., Fischer, B., Otte, T., Nitz, M., Mendel, R. R., Wray, V., and Schwarz, G. (2004) J. Biol. Chem. 279, 15994–15999
29. Gützkow, G., Fischer, B., Mendel, R. R., and Schwarz, G. (2001) J. Biol. Chem. 276, 36268–36274
30. Brewer, G. J., and Cotman, C. W. (1989) Brain Res. 494, 65–74
31. Meier, J., and Grantyn, R. (2004) J. Neurosci. 24, 1398–1405
32. Meier, J., Juttner, R., Kirischuk, S., and Grantyn, R. (2002) Mol Cell. Neurosci. 21, 324–340
33. Kins, S., Betz, H., and Kirsch, J. (2000) Nat. Neurosci. 3, 22–29
34. Harvey, K., Duguid, I. C., Aldred, M. J., Beatty, S. E., Ward, H., Keep, N. H.,
Lingenfelter, S. E., Pearce, B. R., Lundgren, J., Owen, M. J., Smart, T. G., Luscher, B., Rees, M. I., and Harvey, R. J. (2004) *J. Neurosci.* 24, 5816–5826
35. Kirsch, J., Kuhse, J., and Betz, H. (1995) *Mol. Cell Neurosci.* 6, 450–461
36. Bedet, C., Bruusgaard, J. C., Vergo, S., Groth-Pedersen, L., Eimer, S., Triller, A., and Vannier, C. (2006) *J. Biol. Chem.* 281, 30046–30056
37. Zita, M. M., Marchionni, I., Bottos, E., Righi, M., Del Sal, G., Cherubini, E., and Zacchi, P. (2007) *EMBO J.* 26, 1761–1771
38. Kuper, J., Winking, J., Hecht, H. J., Mendel, R. R., and Schwarz, G. (2003) *Arch. Biochem. Biophys.* 411, 36–46
39. Liu, M. T., Wuebbens, M. M., Rajagopalan, K. V., and Schindelin, H. (2000) *J. Biol. Chem.* 275, 1814–1822
40. Schwarz, G., Schulze, J., Bittner, F., Eilers, T., Kuper, J., Bollmann, G., Nerlich, A., Brinkmann, H., and Mendel, R. R. (2000) *Plant Cell* 12, 2455–2472
41. Nichols, J. D., Xiang, S., Schindelin, H., and Rajagopalan, K. V. (2007) *Biochemistry* 46, 78–86
42. Kneussel, M., Haverkamp, S., Fuhrmann, J. C., Wang, H., Wassle, H., Olsen, R. W., and Betz, H. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 8594–8599
43. Fuhrmann, J. C., Kins, S., Roostaing, P., El Far, O., Kirsch, J., Sheng, M., Triller, A., Betz, H., and Kneussel, M. (2002) *J. Neurosci.* 22, 5393–5402
44. David-Watine, B. (2001) *Gene* 271, 239–245
45. Kisker, C., Schindelin, H., Pacheco, A., Webbi, W. A., Garrett, R. M., Rajagopalan, K. V., Enemark, J. H., and Rees, D. C. (1997) *Cell* 91, 973–983
46. Lee, H.-J., Adham, I. M., Schwarz, G., Kneussel, M., Sass, J.-O., Engel, W., and Reiss, J. (2002) *Hum. Mol. Gen.* 11, 3309–3317