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# Identification of Multiple Gephyrin Variants in Different Organs of the Adult Rat

Achim Hermann, Matthias Kneussel, and Heinrich Betz[1]
*Department of Neurochemistry, Max-Planck-Institute for Brain Research, Deutschordenstrasse 46, D-60528 Frankfurt/Main, Germany*

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**The neurotransmitter receptor anchoring protein gephyrin is encoded by a highly mosaic gene whose primary transcript is subject to extensive alternative splicing. Gephyrin mRNAs are widely expressed in various mammalian tissues, and gephyrin has been implicated in neuron-specific and general metabolic functions. Using a novel affinity isolation procedure, we report the identification of different gephyrin variants in various organs of the adult rat. In particular, polypeptides of 52, 56, 60, and 91 kDa were detected in addition to the previously characterized 93-kDa protein. Our results suggest tissue-specific functional differences between gephyrin variants.** 

***Key Words:*** **gephyrin; glycine receptor; $GABA_A$ receptor; receptor clustering; inhibitory synapse; molybdenum cofactor.**

Gephyrin, a polypeptide of 93 kDa, was originally identified as a membrane-associated protein copurifying with the mammalian inhibitory glycine receptor (GlyR) (1–3). During the past decade, essential functions of gephyrin have been revealed in both the CNS and various peripheral organs (for review, see 4, 5). Biochemical and cotransfection studies have shown that gephyrin interacts with polymerized tubulin (6), the GlyR $\beta$ subunit (7, 8) and, to a lesser extent, the $GABA_A$ receptor ($GABA_AR$) subunit $\beta$3 (9). Gephyrin was therefore proposed to anchor and immobilize inhibitory neurotransmitter receptors at the subsynaptic cytoskeleton (10). Consistent with this view, gephyrin is highly enriched at the cytoplasmic face of inhibitory postsynaptic membrane differentiations and colocalizes with both GlyRs and $GABA_AR$s in spinal cord, retina and various brain regions (11–15).

Abbreviations used: GFP, green fluorescent protein; PBS, phosphate buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

[1] To whom correspondences should be addressed. Fax: ++49-69-96769-441. E-mail: neurochemie@mpih-frankfurt.mpg.de.

A key role of gephyrin in the synaptic anchoring of inhibitory receptors was established by antisense oligonucleotide depletion (16, 17) and gene knockout in mice (18, 19); both paradigms prevented the synaptic localization of GlyRs and $GABA_AR$s. Gephyrin in addition interacts with other proteins localized in dendrites, such as the GDP/GTP exchange factor collybistin (20, 21), the actin monomer-binding protein profilin (22), the $GABA_AR$-associated protein GABARAP (23) and the translational regulator RAFT-1 (24). Gephyrin has therefore been proposed to recruit both membrane receptors and subsynaptic structural and signaling proteins to inhibitory synapses (5, 25).

Gephyrin has also been shown to have a general function in intermediary metabolism (18, 26). Gephyrin-deficient mice display a loss of molybdenum cofactor (MoCo)-dependent enzyme activities in liver and intestine (18), and transfection of gephyrin subdomains in MoCo biosynthesis mutants of *E. coli* and plants rescues their MoCo deficiency (26). These data are consistent with gephyrin catalyzing crucial steps in MoCo biosynthesis, a notion supported by significant sequence similarities between gephyrin (3) and MoCo biosynthetic proteins from bacteria (27), invertebrates (28) and plants (29). Interestingly, individuals suffering from hereditary MoCo deficiency show neurological abnormalities indicative of impaired inhibitory neurotransmission (for review, see 5).

The molecular basis for the distinct functions of gephyrin is only poorly understood. The murine gephyrin gene displays a highly mosaic organization, with 8 of its 29 exons being subject to alternative splicing (30). In this study, we have used a novel affinity-isolation protocol to enrich and immunologically detect distinct gephyrin variants in different organs of the adult rat. Our data reveal an unexpected heterogeneity of gephyrin proteins and extend previous Northern and *in situ* hybridization data that have demonstrated multiple forms of alternatively spliced gephyrin mRNAs in distinct mammalian tissues (3, 30). In particular, shorter



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(52–60 kDa) gephyrin variants were found in all organs, examined.

## MATERIALS AND METHODS

*Subloning of gephyrin P1 cDNAs.* The gephyrin cDNA clone P1, encoding amino acids 1–736 (3), was subcloned as a *Hin*dIII–*Xho*I fragment generated by PCR into the pCDNA 3 expression vector (Invitrogen, Groningen, The Netherlands). For immunodetection, VSV or FLAG epitope tags were inserted 3′-adjacent to the ATG start codon or 5′-adjacent to the stop codon, respectively. A green fluorescent protein (GFP)–gephyrin fusion construct was generated by shuttling a *Sma*I–*Bgl*II fragment of the gephyrin–P1 cDNA into the *Eco*47III–*Bgl*II sites of the pEGFP-N3 vector (Clontech, Heidelberg, Germany).

*Preparation of tissue and cell homogenates.* Adult rats were anaesthetized with halothane and sacrificed by neck dislocation. Freshly dissected organs were homogenized in TBS-lysis buffer (150 mM NaCl, 50 mM KCl, 14 mM $Na_2HPO_4$, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM ATP, 25 mM Tris–HCl, pH 7.5, Complete proteinase inhibitors/EDTA-free (Roche Molecular Biochemicals, Mannheim) containing 2% (v/v) Triton) using a Polytron potter (Kinematica, Littau, Switzerland). Human embryonic kidney (HEK) 293 cells were transfected with the respective plasmids encoding gephyrin, essentially as described (9), and homogenized in TBS-lysis buffer. After centrifugation at 15,000*g* for 30 min, the resulting supernatants were stored at −70°C until use or directly used for pull-down experiments.

*Affinity isolation.* The plasmid GST-49 harboring nucleotides encoding amino acids 378-426 of the GlyR $\beta$ subunit as a glutathione-*S*-transferase (GST) fusion protein has been described previously (7). GST-49 was transformed for isopropyl $\beta$-D-thiogalactopyranoside (IPTG)-induced protein production into *Escherichia coli* XL1-blue. Resulting fusion protein was purified using glutathione-Sepharose beads.

Glutathione–Sepharose beads charged with 5 $\mu$g of GST-49 fusion protein, or GST alone, were incubated with tissue or cell homogenate, respectively. After 12 h at 4°C, the beads were washed three times with TBS-buffer and eluted by adding 20 $\mu$l of protein sample buffer (25 mM Tris–HCl, 192 $\mu$M glycine, 0.1% (w/v) SDS). The eluted proteins were separated by 10% SDS–PAGE and analyzed by Western blotting.

*Antibodies and Western blotting.* After 10% SDS–PAGE, proteins were blotted onto nitrocellulose. Membranes were blocked with 3% (w/v) low fat milk in phosphate-buffered saline (PBS) for 20 min. Incubation with the primary antibody was performed for 90 min in 3% (w/v) low-fat milk/PBS at a dilution of 1:500 (anti-Gephyrin, Transduction Laboratories, Lexington, KY; anti-VSV, Roche Molecular Biochemicals, Mannheim, Germany; anti-FLAG, Sigma, St. Louis, MO; or anti-GFP, Clontech, Heidelberg, Germany). After three washes with PBS, HRP-conjugated secondary antibody was applied in 3% low-fat milk/PBS for 45 min. After three additional washes, bound antibodies were detected using a chemiluminescent substrate (Pierce, Rockford, IL).

## RESULTS AND DISCUSSION

Originally, gephyrin was considered to be a brain-specific protein. Consistent with gephyrins enrichment at inhibitory synapses (11, 31, 12–15), the previously available monoclonal antibodies (32) failed to detect gephyrin in non-neuronal tissues (33). Furthermore, the size of the gephyrin polypeptide has consistently been reported to be 93 kDa (1, 2, 34). First evidence for a presence of gephyrin in peripheral tissues came from Northern blot studies, which revealed gephyrin transcripts not only in brain, but also in kidney, lung and liver (3). Moreover, the gephyrin pre-RNA was found to be subject to extensive alternative splicing in its 5′ region (3). The failure to detect the corresponding gephyrin variants immunologically might thus be attributed to a location of the respective epitopes in highly variable regions (30). Since the nucleotide sequence encoding the C-terminal region of gephyrin is conserved in the known gephyrin mRNAs (30), the recent availability of a monoclonal antibody against the invariant C-terminal region (Transduction Laboratories) prompted us to search for gephyrin variants in different organs of the adult rat.

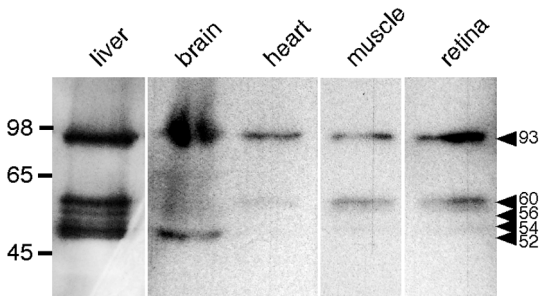


**FIG. 1.** Western blotting with a monoclonal antibody against the C-terminal region of gephyrin detects polypeptides of different molecular weight in homogenates prepared from liver, brain, heart, muscle, and retina. The samples derived from liver, heart, muscle, and retina contain 93- and 60-kDa bands, whereas the brain sample contains a 93- and 52-kDa band. In addition, three distinct bands of 56, 54, and 52kDa are detectable in liver. Apparent molecular weights of proteins are given in kDa; left, positions of marker proteins.

Western blot analysis with this C-terminus-specific gephyrin antibody of homogenates from rat liver, brain, heart, muscle and retina revealed a 93 kDa band in all these tissues examined as well as different weaker bands of lower molecular weight (Fig. 1). Specifically, the following immunoreactive polypeptides were detected: 52, 54, 56, and 60 kDa in liver, 52 kDa in brain, 60 kDa in heart, 60 kDa in muscle, and 60 kDa in the retina (Fig. 1). Thus, shorter variants of gephyrin seem to exist in all tissues, examined.

To reveal further diversity of gephyrin and to enhance the sensitivity of its detection, we employed a pull-down assay with the previously described GST-fusion construct GST-49 (7). GST-49 encodes 49 amino acids of the GlyR $\beta$ subunit large intracellular loop, which include the high-affinity gephyrin binding site of this protein, as a C-terminal fusion to GST (7, 8). To examine whether this fragment was suitable for affinity isolation of gephyrin, we tested heterologously expressed gephyrin (variant P1, see (3)) for its capacity to bind GST-49 (Fig. 2). Recombinant gephyrin, which was detectable in the input lane, was neither found in the supernatant after washing nor did it bind to GST

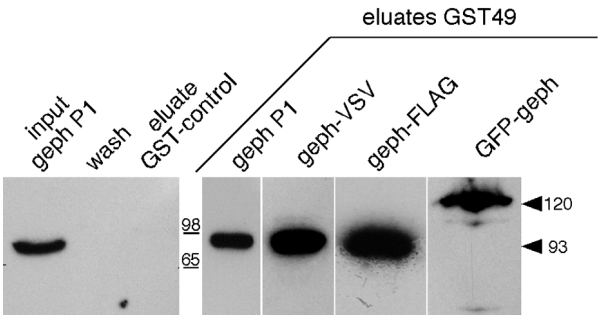


**FIG. 2.** GST pull-down assay for the enrichment of recombinant gephyrin variants. Glutathione–Sepharose beads charged with GST-49, or GST alone, were incubated with homogenates of HEK 293 cells which had been transfected with either gephyrin (geph) P1, gephyrin-VSV, gephyrin-FLAG or GFP-gephyrin, respectively. Gephyrin P1, which was revealed by anti-gephyrin antibody in the input fraction, but not the washes, was not retained by GST alone. In contrast, gephyrin P1, gephyrin-VSV, gephyrin-FLAG, and GFP-gephyrin all efficiently bound to GST-49. Apparent molecular weights are given in kDa.

alone (eluate GST control). In contrast, untagged gephyrin P1 as well as VSV- or FLAG-tagged gephyrin and GFP-gephyrin all bound the Sepharose-coupled GST-49 fusion-proteins as revealed by Western blotting with monoclonal antibodies against either the gephyrin C-terminus or the respective epitope tags and GFP (Fig. 2).

Taking advantage of this sensitive pull-down assay and the C-terminus-specific antibody, we examined the presence of gephyrin variants in different peripheral tissues (Fig. 3). This led to the detection of two additional gephyrin variants in brain tissue other than the known 93 kDa gephyrin, with apparent molecular weights of 60 and 52 kDa. Moreover, we found immunoreactive bands of the following molecular weights in peripheral tissues: 52, 60, and 93 kDa in heart, 52 and 75 kDa in kidney, 52, 56, 60, and 93 kDa in muscle, 52, 56, 60, 91, and 93 kDa in lung, 52, 54, 56, 60, and 93 kDa in liver, a barely detectable 52 kDa and a prominent 93 kDa band in testis, 52 and 93 kDa in retina and 54, 60, 91, and 93 kDa in spleen (Fig. 3). The shorter polypeptides are unlikely to represent proteolysis products of the full-length 93 kDa gephyrin because of the following reasons: (i) incubation of the tissue homogenates at room temperature for up to 20 min did not alter the pattern of bands detected; and (ii), the same lower molecular weight bands were also seen when the pull-down experiments were performed in the simultaneous presence of a commercial protease inhibitor mix (Protease inhibitor cocktail Complete, containing antipain–HCl, bestatine, chymostatin, leupeptin, pepstatin, phosphoramidone, Pefabloc SC, aprotinin), 1 mM phenylmethylsulfonyl fluoride and 5 mM EDTA and EGTA, each. We therefore conclude that all immunoreactive bands represent variants of the gephyrin protein.

Our data demonstrate for the first time that gephyrin polypeptides other than the previously described 93 kDa protein indeed exist in both neuronal and non-neuronal tissues. This is consistent with the gephyrin mRNAs to be highly heterogeneous, due to extensive alternative splicing of the mRNA (3, 30). Insertion and/or removal of individual exonic sequences might directly change the molecular weight of the protein and/or create translational stop codons or frame shifts, which result in reading frames of variable lengths. In addition, in mouse a gephyrin cDNA has been isolated that due to the presence of an alternative stop codon directs the synthesis of a polypeptide of about 65 kDa (35). Protein sequencing of individual bands will have to clarify the precise nature of the polypeptides identified here by immunoblotting. Gephyrin is known to interact with different proteins including the GlyR (2, 3), tubulin (6), profilin (22), RAFT-1 (24), collybistin (20), and GABARAP (23), and has been proposed to assemble into larger scaffolds via respective interaction domains (30, 5). Furthermore, gephyrin is involved in the biosynthesis of a coenzyme, the molybdenum cofactor MoCo in liver and intestinal tissue (18). It

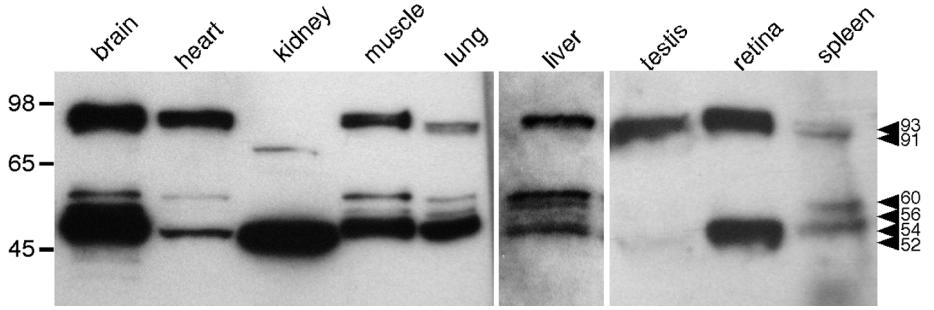


**FIG. 3.** Affinity isolation of gephyrin variants from different organs of the adult rat. GST-49 pull-down assays were performed with homogenates prepared from different organs. After elution, the bound proteins were analyzed by Western blotting using a C-terminal monoclonal antibody. All organs examined, with the exception of kidney, contained the 93-kDa variant of gephyrin. In addition, extracts from all tissues except testis contained either one or multiple immunoreactive polypeptides of lower molecular weight. Apparent molecular weights of proteins are indicated in kDa; left, positions of marker proteins.

would therefore not be surprising if the various functions of the gephyrin protein, encoded by a single gene, would be mediated by a set of distinct polypeptides that originate from a complex regulation of tissue-specific mRNA processing. For example, the presence of at least four individual gephyrin variants in liver might reflect the high MoCo-dependent enzymatic activity in this organ (36, 37). Future gene replacement studies should help to disclose the specific functions of the different gephyrin variants in various mammalian tissues.

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