MASS SPECTROMETRIC ANALYSIS OF GLYCINE RECEPTOR-ASSOCIATED GEPHYRIN SPLICE VARIANTS

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Running title: Glycine receptor-associated gephyrin

Gephyrin is an ubiquitously expressed protein, which in the nervous system, is essential for synaptic anchoring of glycine receptors (GlyRs) and major GABA<sub>A</sub> receptor subtypes. The binding of gephyrin to the GlyR depends on an amphipathic motif within the large intracellular loop of the GlyR<sub>β</sub> subunit. The mouse gephyrin gene consists of 30 exons. Ten of these exons, encoding cassettes of 5 - 40 amino acids, are subject to alternative splicing (C1 to C7, C4′ to C6′). Since one of the cassettes, C5′, has recently been reported to exclude GlyRs from GABAergic synapses, we investigated which cassettes are found in gephyrin associated with the GlyR. Gephyrin variants were purified from rat spinal cord, brain and liver by binding to the GST-tagged GlyR<sub>β</sub> loop, or copurified with native GlyR from spinal cord by affinity chromatography, and analyzed by mass spectrometry. In addition to C2 and C6′, already known to be prominent, C4 was found to be abundant in gephyrin from all tissues examined. The non-neuronal cassette C3 was easily detected in liver, but not in GlyR-associated gephyrin from spinal cord. C5 was present in brain and spinal cord polypeptides, while C5′ was coisolated mainly from liver. Notably C5′-containing gephyrin bound to the GlyR<sub>β</sub> loop, inconsistent with its proposed selectivity for GABA<sub>A</sub> receptors. Our data show that GlyR-associated gephyrin, lacking C3, but enriched in C4 without C5, differs from other neuronal and non-neuronal gephyrin isoforms.

Glycine and γ-aminobutyrate (GABA)<sup>1</sup> are the major inhibitory neurotransmitters in the mammalian CNS. Both amino acids activate postsynaptic ligand-gated chloride channel proteins, the glycine receptor (GlyR) and GABA<sub>A</sub> receptor proteins. These receptors are clustered in the postsynaptic membrane by the receptor anchoring protein gephyrin (1), which links both GlyRs and selected GABA<sub>A</sub> receptors to the cytoskeleton (2-4). Apart from its role in clustering GlyRs and GABA<sub>A</sub> receptors, gephyrin is involved in the synthesis of the molybdenum cofactor (5). This explains gephyrin’s ubiquitous expression also in non-neuronal tissues (1,6,7).

The mouse gephyrin gene consists of approximately 30 exons (6). Alternative splicing has been reported so far for 10 of these exons, which encode cassettes of 5 - 40 amino acids, thereby allowing for the generation of a large number of possible gephyrin splice variants (1,6,8,9). The gephyrin protein is composed of a N-terminal G-domain that is homologous to the E. coli MoeA protein, a central linker region, and a C-terminal E-domain with homology to the E. coli MoeA protein. Gephyrin shares with these bacterial proteins common enzymatic functions in the biosynthesis of the molybdenum cofactor (5), an essential coenzyme of oxidoreductases (10). In all three domains, alternative splicing can generate diversity. The G-domain of gephyrin forms a trimer, whereas the E-domain can form dimers (11,12). Both N-terminal trimerization and C-terminal dimerization have been proposed to be essential for postsynaptic receptor clustering (12,13).

The interaction between gephyrin and the GlyR<sub>β</sub> subunit is well characterized and stable enough to allow copurification of gephyrin with the GlyR from rat spinal cord (14,15). A peptide containing 49 amino acids of the cytoplasmic loop between the third and fourth transmembrane domain of GlyRβ is sufficient to obtain robust gephyrin binding (16) and can be used for affinity purification of gephyrin splice variants from different organs (7). Within gephyrin, the GlyR<sub>β</sub> binding site is located within the E-domain (12,17). Cassette 5′, which is located in the G-domain, has been reported to interfere with binding to the
GlyR and proposed to prevent GlyR accumulation at gephyrin scaffolds of GABAergic synapses (9,18). This indicates that only specific cassettes are present in GlyR-associated gephyrin. Therefore, we examined which gephyrin splice variants are bound to the GlyR.

In the present study, we copurified gephyrin with the native GlyR from rat spinal cord and, for comparison, from rat spinal cord, brain and liver via GST pulldown using the GlyRβ loop sequence. The presence of the different cassettes was investigated by mass spectrometry. So far, proteomic approaches have been seldom used to identify splice variants in higher eukaryotes, due to limitations in sequence coverage and sensitivity (19-21). Here, affinity purification combined with mass spectrometry was found to reliably disclose differences in cassette composition of tissue-specific and receptor-bound gephyrin isoforms. Cassettes C2 and C6 were found in all preparations examined. In addition, GlyR-associated gephyrin was revealed to significantly differ from the GlyRβ loop-isolated proteins in showing a strict absence of the C3-cassette and a preserved presence of the C4-cassette.

**EXPERIMENTAL PROCEDURES**

**Protein expression and purification** - pGEX4T1 and pGEX-5x1 were obtained from Amersham Biosciences (Freiburg, Germany), GlyRβ(378-426)-pGEX-5x1 (16) and gephyrin-P1-pRSET (12) have been described previously. E-domain-pGEX-4T1 was generated by cloning the E-domain of GEA5-pRSET (12) into pGEX-4T1. The proteins GST, GlyRβ(378-426)-GST, E-domain-GST, and His-gephyrin-P1 were produced in *E. coli* BL21 (DE3) (Merck Biosciences, Schwalbach, Germany). Expression was induced by addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside at 25°C overnight. Clear lysates were prepared according to the manufacturer’s protocol (Amersham Biosciences, Freiburg, Germany). His-tagged gephyrin-P1 was purified according to standard protocols (Qiagen, Hilden, Germany). The GST pulldown procedure was performed at 4°C as follows: 30 µl bed volume of glutathione-Sepharose beads (Amersham Biosciences, Freiburg, Germany) were incubated with 0.2 - 0.5 ml of clear bacterial lysate for 2 h. After washing with phosphate-buffered saline, the beads were incubated with 7.5 ml of a Triton X-100 extract prepared from rat brain, liver, or spinal cord for 2 h, and washed four times with phosphate-buffered saline and 0.5 % (w/v) Triton X-100. The beads were eluted three times with 30 µl each of 10 mM reduced glutathione in 50 mM Tris-Cl, pH 8.0, for 10 min at 25°C under vigorous mixing. The eluates were combined.

**Preparation of tissue extracts** - Triton X-100 extracts were prepared by adding 5 volumes of cold phosphate-buffered saline containing protease inhibitor cocktail (complete, Roche Diagnostics, Mannheim, Germany) and 100 µM phenylmethylsulfonyl fluoride to the tissue followed by homogenization for 3 min at 1000 rpm on ice using a Potter-homogenizer (Braun Melsungen AG, Melsungen, Germany). The homogenate then was centrifuged at 2000 x g for 10 min at 4°C. To the supernatant, Triton X-100 was added to a final concentration of 1 % (w/v), and dithiothreitol to 1 mM. After 75 min under mild mixing at 4°C, samples were centrifuged at 27,000 x g for 30 min at 4°C. The supernatant was used directly in the GST pulldown.

GlyR-associated gephyrin was copurified with the glycine receptor from spinal cord using an aminostrychnine column as described (14,15).

**Sample preparation for mass spectrometry** - To the samples, reducing SDS-PAGE loading buffer was added, followed by incubation for 5 min at 48°C for the GlyR-associated gephyrin or at 95°C for gephyrin purified via GST pulldown. The samples were resolved on one-dimensional 8 to 10% polyacrylamide gels. To visualize proteins, gels were silver-stained with a Silver Stain plus kit (Bio-Rad, Munich, Germany). Gephyrin bands were identified by size (see Fig. 1), cut out, and stored at -80°C. The protein bands were destained with 100 µl 15 mM potassium ferricyanide III and 50 mM sodium thiosulfate, followed by two washes with 400 µl H2O for 15 min and two washes with 400 µl 50% (v/v) acetonitrile/25 mM NH4HCO3, pH 8.0, for 15 min. Subsequent preparation of the samples for mass spectrometry was essentially performed as described (22). After trypsin digestion, the samples were spun in a table-top microcentrifuge. The aqueous solution was collected, and the gel pieces were extracted once for 45 min with 20 mM NH4HCO3. After brief centrifugation, the supernatant was collected, followed by extraction of the gel pieces for 30 min with 70 % (v/v)
acetonitrile/5% (v/v) formic acid. After centrifugation, the resulting supernatant was combined with the two previous supernatants from the same sample. The combined extracts were dried in a Speed Vac and stored at -20°C until mass spectrometric analysis.

**Mass spectrometry** - All MS experiments were performed on the Voyager-DE™ STR (Applied Biosystems, MDS SCIEX). Additionally, MS/MS spectra were acquired with the 4800 MALDI TOF/TOF™ Analyzer (Applied Biosystems, MDS SCIEX) to verify the MS data. Prior to analysis the pellets were dissolved in 5 µl 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid under mixing for 3 h. The sample (0.4 µl) was mixed with 0.4 µl matrix (5 mg/ml α-cyano-4-hydroxycinnamic acid; Bruker Daltonik, Bremen, Germany) in 50% (v/v) acetonitrile/0.5% (v/v) trifluoroacetic acid directly on a stainless-steel target (Applied Biosystems, MDS SCIEX) and dried in ambient air. The crystals were washed briefly with 1 µl ice-cold 5% (v/v) formic acid. The acquisition range for the MS analysis was set from 600 to 5000 Da, and the low mass gate to 500 Da. The spectra were first externally calibrated using a peptide mass mixture (Peptide Calibration Standard II, Bruker Daltonik, Bremen, Germany). Trypsin autolysis peaks and prominent gephyrin peaks were then used for internal calibration. Between 400 and 1000 single scans were accumulated for each mass spectrum. Representative spectra are shown in Fig. 2 and Supplemental Fig. 1. Selected peaks characterizing the gephyrin splice variants were fragmented to get sequence information (see Supplemental Fig. 3 and Supplemental Table 1).

**Analysis of mass spectrometric data** - All MS spectra were smoothed, noise-filtered and deisotoped using the Data Explorer™ software (Applied Biosystems, MDS SCIEX). Deisotoped peaks were automatically labeled by the software, and the peaks were used for database search. Gephyrin was identified using Mascot™ (23) and MS-Fit (Expasy) via web interface. Gephyrin splice variants not present in the NCBI and Swissprot databases were generated in silico. The presence and absence of the cassettes was determined using the web-based FindMod (24). For all cassettes, Aldente (Expasy) was used to identify false positive results due to keratin contamination. Additionally, the cassettes were checked for trypsin (25) and other common contaminations (26). FindMod was also used to check for GlyR contamination in GlyR-associated gephyrin, and for GlyRβ(378-426)-GST in case of the other gephyrin protein bands. MS/MS spectra were processed similarly and searched against the NCBI database. Mass accuracy was set to 50 ppm for the precursor and 0.25 Da for the fragments. Unidentified spectra of splice variants were interpreted manually.

**Determination of gephyrin cassette boundaries at the amino acid level** - The genomic rat and mouse sequences containing the gephyrin gene were obtained from the genome databases (NCBI). Using the published gephyrin cDNA sequences and the intron-exon boundaries described (6), the exact sequence of all cassettes, except for C1, could be verified. Since the genomic rat sequence is apparently incomplete, the mouse sequence was used when no published rat sequence was available such as for C6 and C7.

**Statistical analysis** - For all bands examined, 10 independent experiments were performed. Statistical significance of differences between gephyrin protein bands for a given cassette was calculated using Fisher’s exact test (27).

**RESULTS**

GlyR-associated gephyrin (SC GlyR) was copurified with the GlyR from spinal cord extracts on an aminostrychnine column as described (14,15). Additionally, gephyrin able to interact with the GlyR was isolated from detergent extracts of spinal cord, brain, and liver by a GST pulldown approach with the gephyrin binding motif of GlyRβ (16). This identified in both brain and spinal cord two major gephyrin bands of different apparent molecular weights in one-dimensional SDS polyacrylamide gels, which we named brain 93 kDa and brain 90 kDa, and SC 93 kDa and SC 90 kDa, respectively (see Fig. 1). Such a separation of gephyrin bands was not seen in liver (see Fig. 1). To reveal whether these gephyrin polypeptides contained different alternatively spliced exons, or cassettes, the individual bands were cut out and subjected to mass spectrometry. For GlyR-associated gephyrin, the observed upper and lower bands were indistinguishable by mass spectrometry. A representative spectrum of a brain 90 kDa band is shown in Fig. 2 in its raw, deisotopized and simplified form.

Mass spectrometry data for the different gephyrin bands were obtained at comparable quality. For the calculation of average sequence
coverage, a reference sequence with the following features was used: rat gephyrin containing cassettes C2 and C6’ (Genbank Acc. Nr. CAA47009), methionine at position 1 replaced by an acetyl group, and alanine at position 242. C2 and C6’ were found in all samples analyzed. Gephyrin without N-terminal modification was found in only 3%, but with N-terminal acetylation in 95% of the experiments. The N-terminal acetylation was confirmed by MS/MS (see Supplemental Fig. 3F and Supplemental Table 1). At position 242 of the published rat gephyrin sequence (1), an arginine residue is present, whereas in all other mammals an alanine residue is conserved. However, in none of the experiments with the rat strain used (Wistar rats) was an arginine 242 found but, rather, an alanine in one third of the experiments. Re-examination of our original clones (1) revealed a sequencing error at position 242 in the published sequence. Deviations of peptide masses within one experiment were determined using Mascot™ (23) and MS-Fit (Expasy).

The average sequence coverage of the peptide mass fingerprints varied from 44 to 49% (see Fig. 3), a value characteristic of in-gel digests with trypsin. The average deviation was approximately 20 ppm (see Fig. 3). This indicates that mass spectrometry results were obtained for all gephyrin protein bands with similar reliability. Under the same conditions, the presence and absence of all cassettes was correctly identified in recombinant gephyrin-P1 and the recombinant E-domain (see Supplemental Fig. 2A and B), whereas no false positive results were obtained from actin and myosin heavy chain 9 copurified as non-specific proteins with gephyrin from liver (data not shown).

The amino acid sequences of different peptides were confirmed by MS/MS (see Supplemental Table 1 and Supplemental Fig. 3). Only a limited number of theoretically possible peaks identified splice variants; presumably this reflects difficulties with peptide extraction from the gel, unused cleavage sites etc. Cassettes C4 and C5 were defined by the presence or absence of a single tryptic fragment. C3 could only be detected with a peak at 1056 Da. Cassettes C2 and C6’ were identified easily because multiple peaks were found in the MS spectra; their identities were verified in MS/MS experiments. The Supplemental Fig. 3 shows a selection of the acquired MS/MS spectra. All MS/MS spectra resulted in significant database identifications except for the peak at 1056 Da found for C3; this is consistent with fragmentations in the low mass region frequently creating only manually interpretable spectra (Supplemental Table 1). The presence of the fragments at the hyphenation points of peptide 1056 Da (residues P and D) identified it unambiguously in this special chase (Supplemental Fig. 3B). The borderline MS/MS database identification of the tryptic fragment of cassette C4 (1786.8 Da) must be correct, because the MS/MS spectrum contains a satisfactory y-ion series (Supplemental Fig. 3C). Additionally, N-terminal acetylation of gephyrin was proven in a MS/MS experiment (Supplemental Fig. 3F).

No significant differences were observed for the cassettes located in the E- and G-domain. In all experiments, the presence of the cassettes C2 and C6’ was observed (see Fig. 4), indicating that these cassettes are highly abundant. Additionally, they clearly do not interfere with GlyRβ binding since they were also present in gephyrin obtained from affinity-purified GlyR preparations (SC GlyR). The GlyR binding site of gephyrin resides in the E-domain (12).

In contrast to C2 and C6, cassettes C1, C6 and C7 were hardly or not at all detected (see Table 1). For C1, this might reflect the fact that this is a very rare cassette. In the case of C6, there exists an alternative explanation. Since it is located in the E-domain, it might interfere with GlyR binding. The most prominent feature of C7 is that its presence introduces a translational stop codon after amino acid position 463 in rat gephyrin containing C2 and C6’ (6). As a consequence, a major portion of the E-domain containing the GlyR binding site is deleted, and the calculated molecular weight of a gephyrin polypeptide containing C7 is approximately 30 kDa smaller as compared to the gephyrin bands investigated in this study. Hence, it should not be contained in the 90-93 kDa bands analyzed here.

The presence of C5’ was detected only occasionally. Though C5’ was observed more often in liver as compared to the other bands (see Fig. 4), this difference is not statistically significant (p < 0.25 for SC GlyR). Since C5’ has been reported to interfere with GlyRβ binding (9), its presence in affinity-purified GlyR preparations and GlyRβ(378-426)-GST
pulldowns was a surprising finding. However, binding of C5'-containing gephyrin to the GlyRβ loop used in this study has also been seen recently with the purified recombinant proteins. For precise localization of the cassettes, see Fig. 6.

Within the central linker region, differences for most of the cassettes were observed. In contrast to the cassettes C1, C2, C6, and C6' located within the E- or G-domain, most of the cassettes found in the central linker region were differentially distributed between the bands defined above. C3 was abundantly represented in the liver, brain 93 kDa and SC 93 kDa bands, but not detected in brain 90 kDa, SC 90 kDa and GlyR-associated gephyrin (see Fig. 5). This difference turned out to be highly significant (p < 0.005). Obviously, C3 does not directly interfere with GlyRβ binding. C3 is largely repressed in neurons by NOVA proteins, neuronal regulators of pre-mRNA splicing (28,29) indicating that GlyR-associated gephyrin, brain 90 kDa and SC 90 kDa are indeed derived from neurons, but brain 93 kDa and SC 93 kDa might originate from non-neuronal, e.g., glial, cells. C3 consists of 36 amino acids, corresponding to a molecular weight of 4 kDa. Therefore, the observed difference in the molecular weight of SC 93 kDa versus SC 90 kDa, and brain 93 kDa versus brain 90 kDa, can be easily explained by the presence of the C3 cassette in the bands of higher molecular weight.

C4 was readily disclosed in all bands, being most prominent in GlyR-associated gephyrin and brain 93 kDa, but significantly less abundant in the SC 90 kDa, brain 90 kDa and liver isolates (see Tab. 1). This was the most obvious difference observed between GlyR-associated gephyrin and the SC 90 kDa band (see Fig. 5), suggesting a special importance of the C4 cassette for the GlyR-associated variant (SC GlyR). C4 obviously seems to not impair binding to GlyRβ and might in addition provide some functionally relevant features, such as phosphorylation and protein binding sites. Among the splice variants containing C4, those without C5 were detected most frequently. C4 without C5 was found in significantly higher amounts in GlyR-associated gephyrin as compared to the SC 90 kDa and brain 90 kDa polypeptides (see Fig. 5).

The results for C3 and C4 show that the GlyR-associated gephyrin clearly differs from the other bands isolated. Therefore, the ability to bind to GlyRβ is an essential prerequisite, but not sufficient, for being associated with the GlyR in spinal cord. SC 90 kDa and brain 90 kDa, likely of neuronal origin, probably not only contain GlyR-associated gephyrin, but also gephyrin with different neuronal functions, such as clustering of GABA<sub>2</sub> receptors.

Similar to C4, for cassette C5 clear differences were observed between the bands examined. C5 was often seen in brain 93 kDa, less often in spinal cord bands and brain 90 kDa, but not detected in liver (see Fig. 5). Like C4, C5 also might provide phosphorylation and protein binding sites important for the proper function of splice variants.

In all bands, in which both C4 and C5 were frequently represented, C4 was often directly followed by C5 (see Fig. 5); this was particularly true in case of the brain 93 kDa band. The brain 93 kDa band contained significantly more C4 combined with C5 as compared to the brain 90 kDa and liver bands (p < 0.01). Since C4 with and without C5 occasionally were detected in the same sample, this indicates that the gephyrin bands isolated were not entirely homogeneous.

C4' was less often detected as compared to C3, C4 and C5, and found mainly in the bands from brain, but also in GlyR-associated gephyrin (see Fig. 4). However, the total number of experiments was too small to obtain statistically significant data for C4'.

Peptides excluding certain cassettes were observed frequently. For C5' and C6 in most, and for C7 in about 25%, of the experiments, peptides were found which indicate their absence (see Tab. 1). The identification of peptides indicative of both the presence (see above) and absence of C5' within one protein band again underlines that, within the bands examined, splice variants are not homogeneously represented. The observation of peptides excluding C4', C4 and C5 in 5% of the experiments (see Tab. 1) indicates that it was at least technically possible to detect them.

**DISCUSSION**

In order to identify GlyR-associated gephyrin splice variants, we copurified gephyrin with the GlyR on an aminostyrchnine column. For comparison, gephyrin polypeptides were isolated from spinal cord, brain and liver by a GST pulldown approach using the GlyRβ loop
sequence containing the gephyrin binding motif. The presence and absence of alternatively spliced exons, encoding cassettes, was determined by mass spectrometry. Our data constitute the first detailed analysis of gephyrin splice variants associated with the native GlyR.

Previous studies on the expression of gephyrin cassettes addressed only the transcript level, whereas we analyzed gephyrin cassettes in protein bands using mass spectrometry. Contamination of the gel bands by non-receptor binding gephyrin variants was minimized by the affinity purification procedures used. About 50% of the protein sequence was not detected by our approach. Hence, peptides characterizing splice variants may have gone undetected due to the difficulty to obtain full sequence coverage. Additionally, the amino acid sequences of the gephyrin splice variants are very similar. Therefore, the number of fragments defining the presence or absence of a cassette is low (see Supplemental Figs. 1 and 3, and Table 1). Despite this limitation, the high reproducibility of the MS experiments, the mass accuracy and the high specificity of trypsin digestion allow an unambiguous peak assignment of peptide fragments. All MS/MS experiments performed in this study also show that false assignments were not found. We therefore conclude that one or two tryptic fragments are sufficient for a reliable identification of splice variants in purified protein samples.

Gephyrin is N-terminally acetylated. The two cotranslational processes N-terminal methionine cleavage and N-terminal acetylation are two major types of protein modifications (30,31). The initial methionine is cleaved by methionine aminopeptidase in mammalia if it is followed by glycine, alanine, serine, cysteine, threonine, and valine (32). Principle substrates of N\(^n\)-acyetyltransferases in higher eukaryotic cells are proteins that have N-terminal glycine, alanine, serine, or threonine residues (32). Since gephyrin contains an alanine at position two, this makes it an excellent substrate for both the methionine aminopeptidase and the N\(^n\)-acyetyltransferases. Hence, our finding that gephyrin is N-terminally acetylated, is in complete agreement with the previously described rules for both N-terminal methionine cleavage and N-terminal acetylation.

Comparison with previous studies on expression and GlyR\(^\beta\) binding. The cassettes C2 and C6\(^\prime\), found in all experiments in this study, have already been described as being prominent in spinal cord, brain and liver and are known not to interfere with GlyR\(^\beta\) binding (6,9,33,34). Though being located in the E-domain, C6\(^\prime\) is not essential for the GlyR\(^\beta\) binding ability of this domain (9).

C1, C6 and C7, in contrast to C2 and C6\(^\prime\), are rather rare cassettes (6,9,33) which are not present in human gephyrin (34). However, C6 transcripts are prominent in rat liver (6). Here C6 was detected by mass spectrometry only in one out of 60 experiments in our study. A possible explanation for this discrepancy may be that C6 is located in the E-domain and thus might directly affect GlyR\(^\beta\) binding. C7 introduces a stop codon after position 463 in gephyrin containing only C2 and C6\(^\prime\). Therefore, the GlyR binding site as defined previously (12) is not properly generated, and hence any respective gephyrin variant would not have been purified under our conditions. Additionally, in our study we selected for gephyrin polypeptides of 90-93 kDa, much longer than those predicted to contain C7.

At least in neuronal tissue, C4\(^\prime\) containing gephyrin transcripts are not as rare as C1, C6, and C7 (9,34). In contrast, non-neuronal expression of C4\(^\prime\) has not been observed (34). Gephyrin containing C2, C6\(^\prime\), and C4\(^\prime\) is able to bind to GlyR\(^\beta\) (9). Our finding that C4\(^\prime\) is exclusively detected in neuronal tissue derived gephyrin, but not in liver, is in complete agreement with these earlier observations.

C5\(^\prime\), as C1, C6 and C7 not detected in human (34), has been found by PCR in rat brain and spinal cord (9,18), whereas its expression in non-neuronal tissues has not been investigated. Interestingly, C5\(^\prime\) though located in the G-domain has been reported to interfere with GlyR\(^\beta\) binding (9) which involves the E-domain (12,17). Our data clearly indicate that C5\(^\prime\) is also present in liver (see Fig. 6) and in affinity purified GlyR preparations. Thus, this cassette does not directly interfere with GlyR\(^\beta\) binding. The differences in GlyR\(^\beta\) binding observed between (9) and our study might reflect the fact that we used a much shorter GlyR\(^\beta\) loop in our GST pulldown experiments. Since our GlyR\(^\beta\) construct also binds recombinant C5\(^\prime\)-containing gephyrin\(^\prime\), artifacts produced by the mass spectrometry approach can be excluded. In spinal cord, C5\(^\prime\) has been proposed to be preferentially associated with GABA\(_\lambda\) receptors (18). However, the low detection rate of C5\(^\prime\) in
both spinal cord and brain is difficult to reconcile with a general role of this gephyrin cassette in specific GABA<sub>A</sub> receptor clustering (see Fig. 4).

For C3 expression in neuronal tissues, the results of previous studies are somewhat contradictory. Though C3 was identified by cloning (1,9) and by RT-PCR (34) in spinal cord and brain, it was hardly detected by in-situ hybridization on brain sections (33) and not at all by Northern blotting (6). C3 insertion into gephyrin transcripts has been reported to be repressed by NOVA proteins, neuronal regulators of pre-mRNA splicing (28,29). This is consistent with the prominent expression of C3 in liver (1,6,34), which was also observed in our study. Here, we were able to isolate gephyrin polypeptides clearly distinguishable by size due to the presence of C3 from both brain and spinal cord by the GST pulldown approach, but did not observe C3 in GlyR-associated gephyrin. Therefore, our data are consistent with the hypothesis that the C3 containing polypeptides are from non-neuronal, most probably glial, origin.

The previously reported presence of C4 encoding RNA in spinal cord (9,34), brain (33,34) and liver (6) is similar to our findings. Like C3, C4 has been shown to not affect GlyR<sub>B</sub> binding (9,16).

C5, more recently identified than C4, has not yet been studied in detail. Its expression has been observed in rat exclusively in muscle (6), whereas in human C5 is clearly present in brain (34). In both studies, C5 containing transcripts were not found in liver, similar to the study presented here (see Fig. 6). The detection of C5 in human brain might reflect a higher sensitivity of the RT-PCR approach (34) compared to Northern blotting (6). Our data indicate the presence of C5 in rat brain, similar to human brain, and also in spinal cord, where its expression has not been studied so far. Additionally, our data suggests that C5 does not interfere with GlyR<sub>B</sub> binding. The only clear discrepancy between our data and previous studies relates to the detection of splice variants not containing C4, C4' and C5. At the RNA level, in spinal cord and brain more than 50% of the transcripts do not contain C4, C4' and C5 sequences (1,34). Additionally, gephyrin containing only C2 and C6' binds to GlyR<sub>B</sub> (9,12,16), whereas we could detect it only in 5% of the experiments. The reason for this is unclear and might reflect restrictions of the mass spectrometry approach. A common posttranslational modification, such as the N-terminal acetylation observed in 95% of the experiments in this study, was not seen. Also, in one control experiment using recombinant gephyrin containing only C2 and C6' (P1 according to (1)), no C4', C4, C5 bridging peptides were found, although overall sequence coverage was 70% (data not shown). This might indicate that these particular peptides are not easily detected by mass spectrometry. A summary of our results is given in Fig. 6. It shows that GlyR-associated gephyrin has a unique cassette composition in the linker region, which distinguishes it from the other bands isolated. The difference between GlyR-associated gephyrin and the other bands isolated is very likely to result from binding of non-synaptic gephyrin to the GST-GlyR<sub>B</sub> loop.

Potential functions of C3, C4 and C5.

The E- and G-domains of gephyrin share two functional roles. First, the E- and the G-domains are both involved in molybdenum cofactor synthesis (5). Second, both domains contribute to N- and C-terminal oligomerization processes (11,12) that are thought to be crucial for gephyrin scaffold formation beneath the developing postsynaptic membrane (12,13). The intact domains are needed to fulfill these two functional roles. Therefore, insertion of cassettes in the E- and G-domain clearly might interfere with their essential functions. Hence, the central linker region appears to be better suited for modulation of gephyrin function by differential insertion of alternatively spliced cassettes.

Inclusion of exon 10 encoding for C3 into the mature gephyrin mRNA is largely repressed in neurons by NOVA proteins, neuronal regulators of pre-mRNA splicing (28,29). This indicates that C3 might either interfere with the neuronal functions of gephyrin or be essential for its non-neuronal functions. Using ELM prediction (35), none of the established protein motifs could be identified in the C3 amino acid sequence. However, interestingly the G-domain is thought to adenylate molybdopterin, whereas the E-domain is believed to cleave the molybdopterin-adenosine monophosphate complex and to transfer a molybdobium atom to molybdopterin (36), thereby completing molybdobium cofactor biosynthesis. In general, the advantage of bifunctional enzymes catalyzing consecutive reactions is that dissociation of reaction
intermediates into solution is prevented. Furthermore, substrate channeling and interdomain communication are often found in bifunctional enzymes (37). C3, located in the linker region, might affect gephyrin’s activity in molybdenum cofactor synthesis by bringing the molybdenum cofactor precursor binding sites of the E- and the G-domain closer together, or by coordinating their different activities.

Gephyrin binds with high affinity to tubulin (38). The tubulin binding site has been proposed to be encoded by exon 15 (6). Interestingly, Pfam (39) predicts that additionally the last amino acid encoded by exon 11, a lysine, is essential for tubulin binding. C4 and C5, in contrast to C4’, do not contain a lysine codon at the last position. Therefore, the presence of C4 or C5 might directly interfere with tubulin binding.

Both the C4 and C5 peptide sequences contain a potential phosphorylation site, and an additional phosphorylation site is formed if both cassettes are present, as predicted by NetPhos (40). The C4 phosphorylation site is part of a potential 14-3-3 binding site (ELM prediction (35)). The only other potential 14-3-3 binding site of gephyrin is encoded by exon 11, close to exon 13 representing C4. The presence of two 14-3-3 sites in close proximity might help to stabilize an interaction of 14-3-3 proteins, versatile multifunctional adaptors (41), with gephyrin.

Translation of C5 in silico predicts a potential sumoylation site (ELM prediction (35)). The functional significance of such a modification in GlyR-associated gephyrin is unclear, since sumoylation has been reported to occur mainly in the nucleus (42). However, recently sumoylation modulating the activity of a potassium channel at the plasma membrane has been reported (43).

In summary, our work shows that GlyR-associated gephyrin clearly differs from other neuronal and non-neuronal gephyrin. Alternative splicing in the central linker region provides the basis for this difference and is likely to affect gephyrin’s interaction with cytoskeletal proteins as well as its activity in molybdenum cofactor biosynthesis.

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FOOTNOTES

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The on-line version of this article contains supplemental data.

The abbreviations used are: GlyR, glycine receptor; GABA, γ-aminobutyrate; GST, glutathione S-transferase.

T. Saiyed, personal communication

TABLE

Table 1: Cassette detection and exclusion rates (%)

The cassette detection rates (%) were obtained by the number of independent experiments in which peptides containing at least a part of the cassette was detected, divided by the total number of experiments. Exclusion rates were calculated on the detection of peptides encoded by both the exon before and after the one encoding for the cassette, thereby excluding the latter. All data are based on ten independent experiments. ΔCx indicates the detection rate of peptides excluding cassette x.

| cassette | liver 93 kDa | brain 90 kDa | brain 93 kDa | SC 93 kDa | SC 90 kDa | SC GlyR |
|----------|--------------|--------------|--------------|-----------|-----------|---------|
| C1       | 10           | 0            | 0            | 0         | 0         | 0       |
| C4       | 50           | 100          | 40           | 80        | 50        | 100     |
| C6       | 0            | 0            | 0            | 0         | 10        | 0       |
| C7       | 0            | 0            | 0            | 0         | 0         | 0       |
| ΔC5'     | 100          | 100          | 100          | 100       | 100       | 100     |
| ΔC4’C4C5 | 0            | 0            | 0            | 10        | 10        | 10      |
| ΔC6      | 80           | 100          | 100          | 90        | 60        | 100     |
| ΔC7      | 30           | 40           | 20           | 30        | 10        | 20      |

FIGURE LEGENDS

Fig. 1. Purification of gephyrin bands. Gephyrin bands were purified by GST pulldown with GST-tagged GlyRβ-loop from spinal cord (A), brain (B) and liver (C). GlyR-associated gephyrin bands were copurified with the GlyR by affinity chromatography using an aminostrochnine column (D). The purified proteins were separated by SDS-PAGE followed by silver staining. Using the GST pulldown approach, two gephyrin bands were isolated from both spinal cord (A) and brain (B). Upper and lower bands from SC GlyR were indistinguishable by mass spectrometry.
Fig. 2. Representative mass spectrum of a brain 90 kDa gephyrin band. After purification and SDS-PAGE, the gephyrin band was cut out from the polyacrylamide gel and digested with trypsin. The resulting peptides were subjected to mass spectrometry. Gephyrin peaks were identified using Mascot™ and MS-Fit. A: Non-deisotopized spectrum. Prominent peaks are highlighted with numbers corresponding to the molecular weights of the protonated peptides. B: Spectrum resulting from deisotoping of the spectrum shown in A. All peaks specific for gephyrin are marked with dashed lines. C: Simplified spectrum in which only the peaks indicating the presence or absence of cassettes are shown. Peaks corresponding to identified cassettes are indicated.

Fig. 3. Quality analysis of the mass spectrometry data. Average sequence coverage at the amino acid level was calculated with gephyrin (C2, C6´) as reference; for the different protein bands, it varied from 44 to 49 %, without significant differences. The deviation, i.e., the accuracy with which the peptide masses were determined, was obtained by analysis of the mass spectrometry data with Mascot™ and MS-Fit. Deviation values varied from 20 to 22 ppm, and thus were similar for all protein bands analyzed. Error bars indicate the standard deviation.

Fig. 4. Abundance of cassettes C2, C4´, C5´ and C6´. The amino acid sequences of gephyrin splice variants were generated in silico. Using FindMod in combination with the deduced amino acid sequences, the presence of the different cassettes was determined. C2 and C6´ peptide sequences were both found in all experiments. In contrast, C4´ and C5´ were only detected occasionally, C4´ primarily in brain and C5´ primarily in liver. No highly significant band-specific differences were observed for C2, C4´, C5´ and C6´.

Fig. 5. Summary of the results for cassettes C3, C4 and C5. The presence of these cassettes was determined as described in Fig. 4. C3, not present in SC GlyR, was exclusively found in the higher molecular weight bands from brain and spinal cord (brain 93 kDa and SC 93 kDa) and in liver. C4 without C5 (C4ΔC5) was more often found in SC GlyR as compared to SC90 kDa. C4 with C5 (C4C5) was detected in all spinal cord bands and in brain 93 kDa, but not in liver or brain 90 kDa. The significance of the differences in detection rates is indicated by asteriks, with SC GlyR serving as a reference. The different gephyrin bands isolated clearly differed in their content of C3, C4 and C5.

Fig. 6. Summary of the results obtained for all gephyrin bands analyzed. In the top row, a gephyrin domain map is shown. The row below represents a gephyrin exon map. Cassettes which can be spliced out are indicated in grey, constitutive exons in white. Arrows indicate start and stop codons. The structures of the gephyrin bands isolated are schematically indicated in the rows below. Black: constitutive or more than 66 %, light grey: 33 to 66%, white: 15 to 32% detection rate. Excluded cassettes or cassettes below 15 % detection rate are not represented.
Figure 1

A

Spinal cord

SC 93 kDa - 98 kDa
SC 90 kDa

B

Brain

Brain 93 kDa - 98 kDa
Brain 90 kDa

C

Liver

Liver - 98 kDa

D

Spinal cord

SC GlyR - 98 kDa
Figure 3
Figure 4

![Bar chart showing detection rates for different samples and molecular weights.](http://www.jbc.org/)

- **Detection rate (%)**
- **Y-axis**: 0 to 100
- **X-axis**: SC GlyR, SC 90 kDa, SC 93 kDa, Brain 90 kDa, Brain 93 kDa, Liver
- **Legend**:
  - C2
  - C4’
  - C5’
  - C6’
Figure 6
Mass spectrometric analysis of glycine receptor-associated gephyrin splice variants
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