THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 46, pp. 34918–34925, November 17, 2006

Mass Spectrometric Analysis of Glycine Receptor-associated Gephyrin Splice Variants*[S]

Received for publication, August 14, 2006, and in revised form, September 20, 2006 Published, JBC Papers in Press, September 25, 2006, DOI 10.1074/jbc.M607764200

Ingo Paarmann[‡], Bertram Schmitt[‡], Björn Meyer[§], Michael Karas[§], and Heinrich Betz[‡1]

From the [‡]Department of Neurochemistry, Max-Planck-Institute for Brain Research, Deutschordenstrasse 46, D-60528 Frankfurt, Germany and [§]Institute of Pharmaceutical Chemistry, Johann Wolfgang Goethe University, D-60438 Frankfurt am Main, Germany

Gephyrin is an ubiquitously expressed protein that, in the nervous system, is essential for synaptic anchoring of glycine receptors (GlyRs) and major $GABA_A$ receptor subtypes. The binding of gephyrin to the GlyR depends on an amphipathic motif within the large intracellular loop of the GlyRβ 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–C7, C4′–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 glutathione *S*-transferase-tagged GlyRβ 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 nonneuronal 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, whereas C5′ was coisolated mainly from liver. Notably C5′-containing gephyrin bound to the GlyRβ loop, inconsistent with its proposed selectivity for $GABA_A$ receptors. Our data show that GlyR-associated gephyrin, lacking C3, but enriched in C4 without C5, differs from other neuronal and nonneuronal gephyrin isoforms.

Glycine and γ-aminobutyrate (GABA)[2] are the major inhibitory neurotransmitters in the mammalian central nervous system. Both amino acids activate postsynaptic ligand-gated chloride channel proteins, the glycine receptor (GlyR), and $GABA_A$ receptor proteins. These receptors are clustered in the postsynaptic membrane by the receptor-anchoring protein gephyrin (1), which links both GlyRs and selected $GABA_A$ receptors to the cytoskeleton (2–4). Apart from its role in clustering GlyRs and $GABA_A$ receptors, gephyrin is involved in the synthesis of the molybdenum cofactor (5). This explains the ubiquitous expression of gephyrin also in nonneuronal tissues (1, 6, 7).

The mouse gephyrin gene consists of ~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* MogA 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 GlyRβ 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 GlyRβ 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 pull-down 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

* This work was supported by Deutsche Forschungsgemeinschaft (SFB628) and 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.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and supplemental Figs. 1–3.

[1] To whom correspondence should be addressed: Dept. of Neurochemistry, Max-Planck-Institute for Brain Research, Deutschordenstrasse 46, D-60528 Frankfurt, Germany. Tel.: 49-69-96769-260; Fax: 49-69-96769-441; E-mail: neurochemie@mpih-frankfurt.mpg.de.

[2] The abbreviations used are: GABA, γ-aminobutyrate; GlyR, glycine receptor; GST, glutathione *S*-transferase; MS, mass spectrometry.

proteins in showing a strict absence of the C3 cassette and a conserved presence of the C4 cassette.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—pGEX-4T-1 and pGEX-5X-1 were obtained from Amersham Biosciences, and GlyRβ-(378–426)-pGEX-5X-1 (16) and gephyrin-P1-pRSET (12) have been described previously. E-domain-pGEX-4T-1 was generated by cloning the E-domain of GE45-pRSET (12) into pGEX-4T-1. The proteins GST, GlyRβ-(378–426)-GST, E-domain-GST, and His-gephyrin-P1 were produced in *E. coli* BL21 (DE3) (Merck). Expression was induced by the 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). His-tagged gephyrin-P1 was purified according to standard protocols (Qiagen, Hilden, Germany). The GST pull-down procedure was performed at 4 °C as follows: 30-μl bed volume of glutathione-Sepharose beads (Amersham Biosciences) was 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 mixture (complete; Roche Applied Science) 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 $\times$ *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 was added to 1 mM. After 75 min under mild mixing at 4 °C, samples were centrifuged at 27,000 $\times$ *g* for 30 min at 4 °C. The supernatant was used directly in the GST pull-down.

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 pull-down. The samples were resolved on one-dimensional 8–10% polyacrylamide gels. To visualize proteins, gels were silver-stained with a Silver Stain Plus kit (Bio-Rad). Gephyrin bands were identified by size (see Fig. 1), cut out, and stored at −80 °C. The protein bands were destained with 100 μl of 15 mM potassium ferricyanide III and 50 mM sodium thiosulfate, followed by two washes with 400 μl of H_2O for 15 min and two washes with 400 μl of 50% (v/v) acetonitrile, 25 mM NH_4HCO_3, 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 NH_4HCO_3. 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 SpeedVac 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 of 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 of 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 of 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 was set 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 data base search. Gephyrin was identified using Mascot™ (23) and MS-Fit (Expasy) via World Wide Web interface. Gephyrin splice variants not present in the NCBI and Swiss-Prot data bases were generated *in silico*. The presence and absence of the cassettes was determined using the World Wide 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 data base. 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 data bases (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.

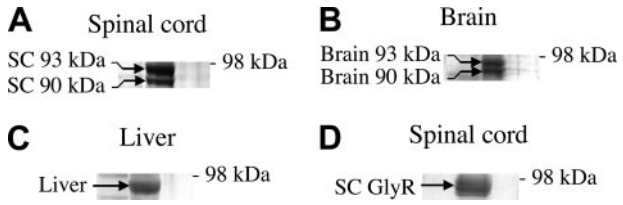

FIGURE 1. **Purification of gephyrin bands.** Gephyrin bands were purified by GST pull-down with GST-tagged GlyRβ loop from spinal cord (*A*), brain (*B*), and liver (*C*). GlyR-associated gephyrin bands were copurified with the GlyR by affinity chromatography using an aminostrychnine column (*D*). The purified proteins were separated by SDS-PAGE followed by silver staining. Using the GST pull-down 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.

Statistical Analysis—For all bands examined, 10 independent experiments were performed. The 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 pull-down 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^{TM} accession number 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 gephyrin with N-terminal acetylation was found in 95% of the experiments. The N-terminal acetylation was confirmed by MS/MS (see supplemental Fig. 3*F* 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 was found in one-third of the experiments. Reexamination 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^{TM} (23) and MS-Fit (Expasy).

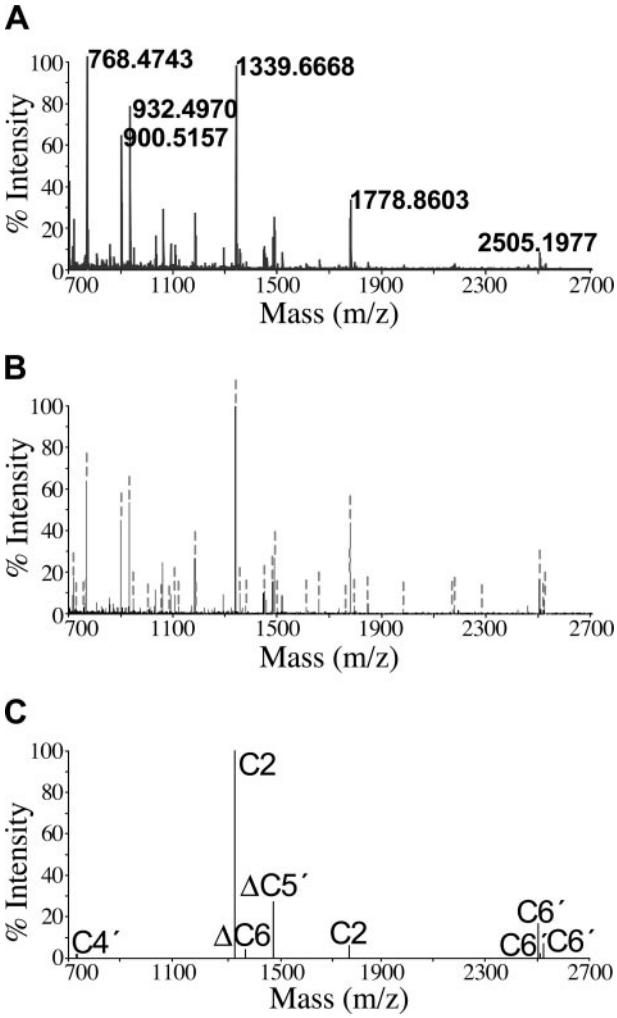

FIGURE 2. **Representative mass spectrum of a brain 90 kDa gephyrin band.** After purification and SDS-PAGE, the gephyrin band was cut out from the polyacrylamide gel and digested with trypsin. The resulting peptides were subjected to mass spectrometry. Gephyrin peaks were identified using Mascot^{TM} and MS-Fit. *A*, nondeisotopized 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.

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 ~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. 2, *A* and *B*), whereas no false positive results were obtained from actin and myosin heavy chain 9 copurified as nonspecific 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.

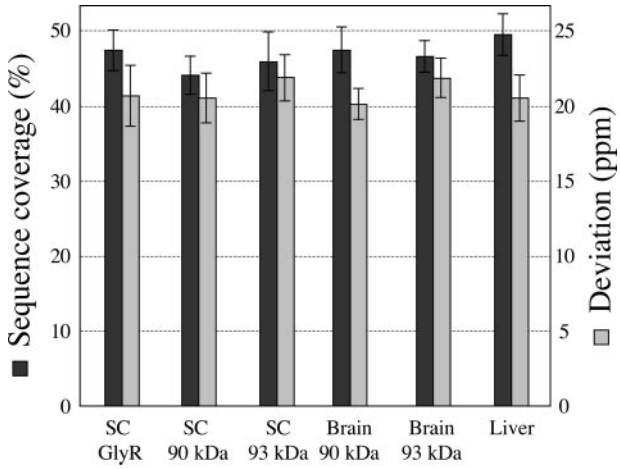

FIGURE 3. **Quality analysis of the mass spectrometry data.** Average sequence coverage at the amino acid level was calculated with gephyrin (C2, C6′) as reference; for the different protein bands, it varied from 44 to 49%, without significant differences. The deviation (*i.e.* the accuracy with which 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 S.D.

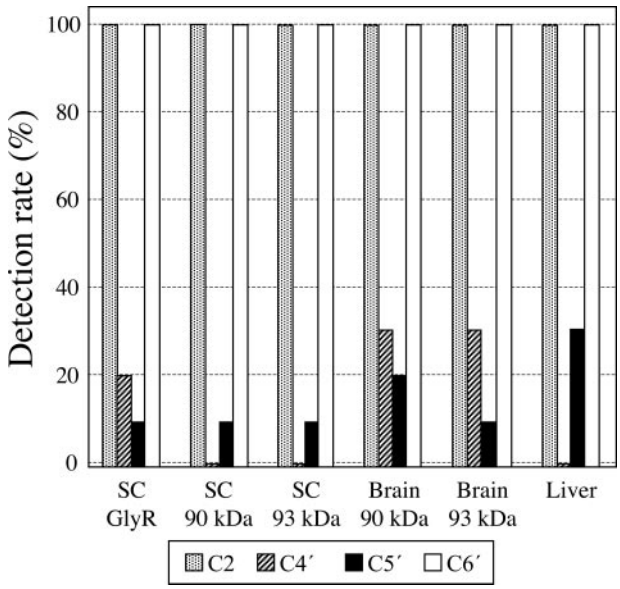

FIGURE 4. **Abundance of cassettes C2, C4′, C5′and C6′.** The amino acid sequences of gephyrin splice variants were generated *in silico*. Using FindMod in combination with the deduced amino acid sequences, the presence of the different cassettes was determined. C2 and C6′ peptide sequences were both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

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. Supplemental Fig. 3 shows a selection of the acquired MS/MS spectra. All MS/MS spectra resulted in significant data base 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. 3*B*). The borderline MS/MS data base 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. 3*C*). Additionally, N-terminal acetylation of gephyrin was proven in an MS/MS experiment (supplemental Fig. 3*F*).

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 mass of a gephyrin polypeptide containing C7 is ~30 kDa smaller as compared with the gephyrin bands investigated in this study. Hence, it should not be contained in the 90–93 kDa bands analyzed here.

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 were detected, divided by the total number of experiments. Exclusion rates were calculated on the detection of peptides encoded by both the exon before and the one after the one encoding for the cassette, thereby excluding the latter. All data are based on 10 independent experiments. ΔCx indicates the detection rate of peptides excluding cassette *x*.

Cassette	Liver	Brain 93 kDa	Brain 90 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

The presence of C5′ was detected only occasionally. Although C5′ was observed more often in liver as compared with 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 pull-downs 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.[3] For precise localization of the cassettes, see Fig. 6.

[3] T. Saiyed, personal communication.

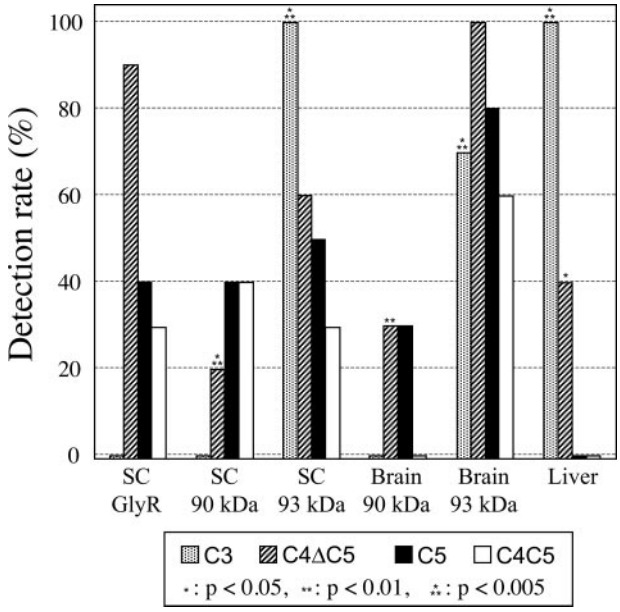

FIGURE 5. **Summary of the results for cassettes C3, C4, and C5.** The presence of these cassettes was determined as described in Fig. 4. C3, not present in SC GlyR, was exclusively found in the higher molecular weight bands from brain and spinal cord (brain 93 kDa and SC 93 kDa) and in liver. C4 without C5 (*C4ΔC5*) was more often found in SC GlyR as compared with SC 90 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 *asterisks*, with SC GlyR serving as a reference. The different gephyrin bands isolated clearly differed in their content of C3, C4, and C5.

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 nonneuronal (*e.g.* glial) cells. C3 consists of 36 amino acids, corresponding to a molecular mass of 4 kDa. Therefore, the observed difference in the molecular mass of SC 93 kDa *versus* SC 90 kDa and of brain 93 kDa *versus* brain 90 kDa can be easily explained by the presence of the C3 cassette in the bands of higher molecular mass.

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 Table 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 not to 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 with 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, probably of neuronal origin, may contain not only GlyR-associated gephyrin but also gephyrin with different neuronal functions, such as clustering of $GABA_A$ receptors.

Similar to C4, for cassette C5, clear differences were observed between the bands examined. C5 was often seen in brain 93 kDa and 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 the case of the brain 93 kDa band. The brain 93 kDa band contained significantly more C4 combined with C5 as compared with the brain 90 kDa and liver bands ($p < 0.01$). Since C4 both with and without C5 occasionally was detected in the same sample, this indicates that the gephyrin bands isolated were not entirely homogeneous.

C4′ was less often detected as compared with C3, C4, and C5 and found mainly in the bands from the 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 that indicated their absence (see Table 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 Table 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 aminostrychnine column. For comparison, gephyrin polypeptides were isolated from spinal cord, brain, and liver by a GST pull-down 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

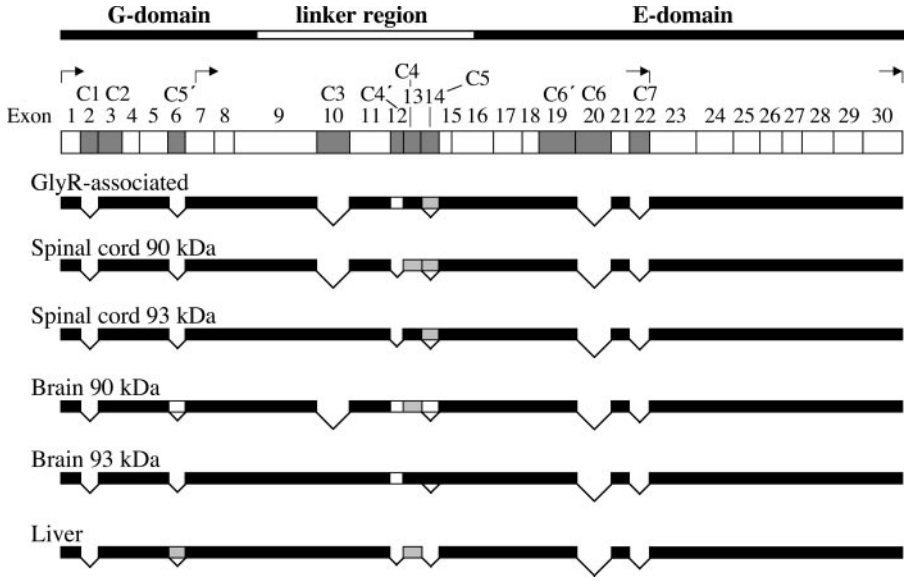

FIGURE 6. **Summary of the results obtained for all gephyrin bands analyzed.** In the *top row*, a gephyrin domain map is shown. The *row below* represents a gephyrin exon map. Cassettes that can be spliced out are indicated in *gray*, and constitutive exons are shown in *white*. The *arrows* indicate 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 gray*, 33–66%; *white*, 15–32% detection rate. Excluded cassettes or cassettes below a 15% detection rate are not represented.

may have gone undetected due to the difficulty of obtaining 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 of 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^{α}-acetyltransferases in higher eukaryotic cells are proteins that have N-terminal glycine, alanine, serine, or threonine residues (32). Since gephyrin contains an alanine at position 2, this makes it an excellent substrate for both the methionine aminopeptidase and the N^{α}-acetyltransferases. 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 GlyRβ Binding—The cassettes C2 and C6′, 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 GlyRβ binding (6, 9, 33, 34). Although it is located in the E-domain, C6′ is not essential for the GlyRβ binding ability of this domain (9).

C1, C6, and C7, in contrast to C2 and C6′, 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 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 GlyRβ binding. C7 introduces a stop codon after position 463 in gephyrin containing only C2 and C6′. 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′-containing gephyrin transcripts are not as rare as transcripts containing C1, C6, and C7 (9, 34). In contrast, nonneuronal expression of C4′ has not been observed (34). Gephyrin containing C2, C6′, and C4′ is able to bind to GlyRβ (9). Our finding that C4′ is exclusively detected in neuronal tissue-derived gephyrin, but not in liver, is in complete agreement with these earlier observations.

C5′, like C1, C6, and C7 not detected in humans (34), has been found by PCR in rat brain and spinal cord (9, 18), whereas its expression in nonneuronal tissues has not been investigated. Interestingly, C5′, although located in the G-domain, has been reported to interfere with GlyRβ binding (9), which involves the E-domain (12, 17). Our data clearly indicate that C5′ is also present in liver (see Fig. 6) and in affinity-purified GlyR preparations. Thus, this cassette does not directly interfere with GlyRβ binding. The differences in GlyRβ binding observed between (9) and our study might reflect the fact that we used a much shorter GlyRβ loop in our GST pull-down experiments. Since our GlyRβ construct also binds recombinant C5′-containing gephyrin,[3] artifacts produced by the mass spectrometry approach can be excluded. In spinal cord, C5′ has been proposed to be preferentially associated with $GABA_A$ receptors (18). However, the low detection rate of C5′ in both spinal cord and brain is difficult to reconcile with a general role of this gephyrin cassette in specific $GABA_A$ receptor clustering (see Fig. 4).

For C3 expression in neuronal tissues, the results of previous studies are somewhat contradictory. Although C3 was identified by cloning (1, 9) and by reverse transcription-PCR (34) in the 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 regula-

tors 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 pull-down approach but did not observe C3 in GlyR-associated gephyrin. Therefore, our data are consistent with the hypothesis that the C3-containing polypeptides are of nonneuronal, 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 GlyRβ binding (9, 16).

C5, more recently identified than C4, has not yet been studied in detail. Its expression has been observed in rats exclusively in muscle (6), whereas in humans C5 is clearly present in the 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 the human brain might reflect a higher sensitivity of the reverse transcription-PCR approach (34) compared with Northern blotting (6). Our data indicate the presence of C5 in rat brains, similar to human brains, and also in spinal cord, where its expression has not been studied so far. Additionally, our data suggest that C5 does not interfere with GlyRβ 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 the 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 GlyRβ (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 Ref. 1), no C4′, C4, and 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 nonsynaptic gephyrin to the GST-GlyRβ 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 nonneuronal 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 molybdenum atom to molybdopterin (36), thereby completing molybdenum 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 the activity of gephyrin 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, iPfam (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 nonneuronal gephyrin. Alternative splicing in the central linker region provides the basis for this difference and is likely to affect the interaction of gephyrin with cytoskeletal proteins as well as its activity in molybdenum cofactor biosynthesis.

REFERENCES

1. Prior, P., Schmitt, B., Grenningloh, G., Pribilla, I., Multhaup, G., Beyreuther, K., Maulet, Y., Werner, P., Langosch, D., Kirsch, J., and Betz, H. (1992) *Neuron* **8,** 1161–1170
2. Kirsch, J., and Betz, H. (1995) *J. Neurosci.* **15,** 4148–4156
3. Kirsch, J., Meyer, G., and Betz, H. (1996) *Mol. Cell Neurosci.* **8,** 93–98
4. Kneussel, M., Brandstatter, J. H., Laube, B., Stahl, S., Muller, U., and Betz, H. (1999) *J. Neurosci.* **19,** 9289–9297

5. Stallmeyer, 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
6. Ramming, M., Kins, S., Werner, N., Hermann, A., Betz, H., and Kirsch, J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 10266–10271
7. Hermann, A., Kneussel, M., and Betz, H. (2001) *Biochem. Biophys. Res. Commun.* **282,** 67–70
8. Heck, S., Enz, R., Richter-Landsberg, C., and Blohm, D. H. (1997) *Brain Res. Dev. Brain Res.* **98,** 211–220
9. Meier, J., De Chaldee, M., Triller, A., and Vannier, C. (2000) *Mol. Cell. Neurosci.* **16,** 566–577
10. Johnson, J. L., and Wadman, S. K. (1989) Molybdenum cofactor deficiency. In: Stanbury, J. B., and Wyngaarden, J. B. (eds). *Inherited Basis of Metabolic Disease*, McGraw-Hill, New York
11. Sola, M., Kneussel, M., Heck, I. S., Betz, H., and Weissenhorn, W. (2001) *J. Biol. Chem.* **276,** 25294–25301
12. Sola, M., Bavro, V. N., Timmins, J., Franz, T., Ricard-Blum, S., Schoehn, G., Ruigrok, R. W. H., Paarmann, I., Saiyed, T., O'Sullivan, G. A., Schmitt, B., Betz, H., and Weissenhorn, W. (2004) *EMBO Journal* **23,** 2510–2519
13. Kneussel, M., and Betz, H. (2000) *Trends Neurosci.* **23,** 429–435
14. Pfeiffer, F., Graham, D., and Betz, H. (1982) *J. Biol. Chem.* **257,** 9389–9393
15. Schmitt, B., Knaus, P., Becker, C. M., and Betz, H. (1987) *Biochemistry* **26,** 805–811
16. Meyer, G., Kirsch, J., Betz, H., and Langosch, D. (1995) *Neuron* **15,** 563–572
17. Schrader, N., Kim, E. Y., Winking, J., Paulukat, J., Schindelin, H., and Schwarz, G. (2004) *J. Biol. Chem.* **279,** 18733–18741
18. Meier, J., and Grantyn, R. (2004) *J. Neurosci.* **24,** 1398–1405
19. Godovac-Zimmermann, J., Kleiner, O., Brown, L. R., and Drukier, A. K. (2005) *Proteomics* **5,** 699–709
20. Roth, M. J., Forbes, A. J., Boyne, M. T., 2nd, Kim, Y. B., Robinson, D. E., and Kelleher, N. L. (2005) *Mol. Cell. Proteomics.* **4,** 1002–1008
21. Thiede, B., Hohenwarter, W., Krah, A., Mattow, J., Schmid, M., Schmidt, F., and Jungblut, P. R. (2005) *Methods* **35,** 237–247
22. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anal. Chem.* **68,** 850–858
23. Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999) *Electrophoresis* **20,** 3551–3567
24. Wilkins, M. R., Gasteiger, E., Gooley, A. A., Herbert, B. R., Molloy, M. P., Binz, P. A., Ou, K., Sanchez, J. C., Bairoch, A., Williams, K. L., and Hochstrasser, D. F. (1999) *J. Mol. Biol.* **289,** 645–657
25. Harris, W. A., Janecki, D. J., and Reilly, J. P. (2002) *Rapid Commun. Mass Spectrom.* **16,** 1714–1722
26. Ding, Q., Xiao, L., Xiong, S., Jia, Y., Que, H., Guo, Y., and Liu, S. (2003) *Proteomics* **3,** 1313–1317
27. Sachs, L. (1984) *Angewandte Statistik*, 6. Edition Ed., Springer Verlag, Berlin/Heidelberg/New York/Tokyo
28. Ule, J., Jensen, K. B., Ruggiu, M., Mele, A., Ule, A., and Darnell, R. B. (2003) *Science* **302,** 1212–1215
29. Ule, J., Ule, A., Spencer, J., Williams, A., Hu, J. S., Cline, M., Wang, H., Clark, T., Fraser, C., Ruggiu, M., Zeeberg, B. R., Kane, D., Weinstein, J. N., Blume, J., and Darnell, R. B. (2005) *Nat. Genet.* **37,** 844–852
30. Bradshaw, R. A., Brickey, W. W., and Walker, K. W. (1998) *Trends Biochem. Sci.* **23,** 263–267
31. Polevoda, B., and Sherman, F. (2000) *J. Biol. Chem.* **275,** 36479–36482
32. Boissel, J. P., Kasper, T. J., and Bunn, H. F. (1988) *J. Biol. Chem.* **263,** 8443–8449
33. Kirsch, J., Malosio, M. L., Wolters, I., and Betz, H. (1993) *Eur. J. Neurosci.* **5,** 1109–1117
34. Rees, M. I., Harvey, K., Ward, H., White, J. H., Evans, L., Duguid, I. C., Hsu, C. C. H., 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
35. Puntervoll, P., Linding, R., Gemund, C., Chabanis-Davidson, S., Mattingsdal, M., Cameron, S., Martin, D. M., Ausiello, G., Brannetti, B., Costantini, A., Ferre, F., Maselli, V., Via, A., Cesareni, G., Diella, F., Superti-Furga, G., Wyrwicz, L., Ramu, C., McGuigan, C., Gudavalli, R., Letunic, I., Bork, P., Rychlewski, L., Kuster, B., Helmer-Citterich, M., Hunter, W. N., Aasland, R., and Gibson, T. J. (2003) *Nucleic Acids Res.* **31,** 3625–3630
36. Kuper, J., Llamas, A., Hecht, H. J., Mendel, R. R., and Schwarz, G. (2004) *Nature* **430,** 803–806
37. Nagradova, N. (2003) *IUBMB Life* **55,** 459–466
38. Kirsch, J., Langosch, D., Prior, P., Littauer, U. Z., Schmitt, B., and Betz, H. (1991) *J. Biol. Chem.* **266,** 22242–22245
39. Finn, R. D., Marshall, M., and Bateman, A. (2005) *Bioinformatics* **21,** 410–412
40. Blom, N., Gammeltoft, S., and Brunak, S. (1999) *J. Mol. Biol.* **294,** 1351–1362
41. van Heusden, G. P. (2005) *IUBMB Life* **57,** 623–629
42. Gill, G. (2004) *Genes Dev.* **18,** 2046–2059
43. Rajan, S., Plant, L. D., Rabin, M. L., Butler, M. H., and Goldstein, S. A. (2005) *Cell* **121,** 37–47