Molecular Basis of Gephyrin Clustering at Inhibitory Synapses

ROLE OF G- AND E-DOMAIN INTERACTIONS*

Gephyrin is a bifunctional modular protein that, in neurons, clusters glycine receptors and γ-aminobutyric acid, type A receptors in the postsynaptic membrane of inhibitory synapses. By x-ray crystallography and cross-linking, the N-terminal G-domain of gephyrin has been shown to form trimers and the C-terminal E-domain dimers, respectively. Gephyrin therefore has been proposed to form a hexagonal submembranous lattice onto which inhibitory receptors are anchored. Here, crystal structure-based substitutions at oligomerization interfaces revealed that both G-domain trimerization and E-domain dimerization are essential for the formation of higher order gephyrin oligomers and postsynaptic gephyrin clusters. Insertion of the alternatively spliced C5′ cassette into the G-domain inhibited clustering by interfering with trimerization, and mutation of the glycine receptor β-subunit binding region prevented the localization of the clusters at synaptic sites. Together our findings show that domain interactions mediate gephyrin scaffold formation.

The precise localization and a high density of neurotransmitter receptors at postsynaptic sites is a prerequisite for proper synaptic transmission. During the development of inhibitory synapses, the peripheral membrane protein gephyrin accumulates beneath the postsynaptic plasma membrane and plays a key role in recruiting inhibitory receptors under the contacting nerve terminals (1, 2). Both attenuation of gephyrin expression and, hence, the clustering of inhibitory receptors at postsynaptic membrane specializations (1, 15) by reducing their lateral mobility (17, 18).

In this study, we investigated whether G-domain trimerization and E-domain dimerization are essential for gephyrin scaffold formation. Using structure-deduced mutations that disrupt oligomerization interfaces, we found that both G- and E-domain interactions are required for gephyrin scaffolding. In addition, we report that the postsynaptic localization of the gephyrin scaffold depends on the GlyRβ binding region of the E-domain. Intact E- and G-domains are also a prerequisite for the formation of gephyrin hexamers, which we propose to represent novel intermediates of the scaffold assembly reaction. Together, our data indicate that oligomerization via the G- and E-domains is essential for gephyrin scaffold formation and, hence, the clustering of inhibitory receptors at developing synapses.

EXPERIMENTAL PROCEDURES

Generation of Gephyrin Constructs—The region encoding the G-domain of gephyrin (amino acids 1–181) was amplified by PCR using Geph-pRSET (15) as a template and subcloned into pBluescript II SK (+) (pBSK) (Stratagene) using XmaI/XhoI to generate G-pBSK. The full-length coding region of wild-type P1-gephyrin (gephyrin containing the cassettes 2 and

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4 The abbreviations used are: GlyR, glycine receptor; GlyRβ, glycine receptor β-subunit; BN-PAGE, blue-native PAGE; DIV, days in vitro; GFP, green fluorescent protein; GABA_A, γ-aminobutyrate acid, type A; GABA_R, GABA_A receptor; HEK, human embryonic kidney; VIAAT, vesicular inhibitory amino acid transporter.

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Domain Interactions in Gephyrin Scaffold Formation

6') was excised from Geph-pBSK (11) using Xmal/NsiI restriction sites and cloned between the Xmal and blunted Apal sites of the pNKS 2 vector (19) to generate Geph-pNKS 2. Codons for an AHIIHHHHH sequence tag were inserted directly behind the initiator ATG by using the QuikChange mutagenesis kit (Stratagene) to yield His-Geph-pNKS 2. The additional alanine codon serves to maintain the Kozak initiation sequence of Geph-pNKS 2. Using PCR-based mutagenesis, the mutations F90R, L113R, L128R, and L168R were introduced into G-pBSK using NdeI/XhoI sites to generate His-Geph2xR-pBSK. The isolated G-domain was excised from Geph-pBSK (11) using a PCR-based strategy and NotI/PstI sites to generate the full-length cDNA fragment allowing cloning into pEGFP-C2 (Clontech) via SacI/KpnI sites to generate Gephmut-pBSK (11). Using PCR-based mutagenesis, the mutations G483R, R523E, and A532R were introduced into G-pBSK (11) using EcoRI/NcoI sites and into Geph-pEGFP-C2 (20) using PstI restriction sites to generate the constructs GephRER-pPSET, GephRER-PSET, and GephRER-PPEGF-C2, respectively. Transfer of the mutant E-domain from GephRER-pPSET into Gephmut-pBSK via EcoRI/HindIII sites generated the double mutant Gephmut-pBSK, and Geph RER-pEGFP-C2, respectively.

The construct Gephmut-pEGFP-C2 (15) has been described previously. All constructs were verified by DNA sequencing.

Expression and Purification of Recombinant Proteins—N-terminal His$_6$-tagged wild-type and mutant domain proteins were expressed using the pQE-30/31 (Qiagen) expression system in E. coli BL21 DE3 (Novagen), whereas G$_{4xR}$ was expressed in E. coli C41 DE3 (21). Recombinant proteins were purified as described (15) and directly used for gel filtration chromatography.

Size Exclusion Chromatography—The recombinant wild-type and mutant G- and E-domain proteins were used. The purified proteins were subjected to chromatography on a Superdex 200 column (2.4 ml) in His$_6$ elution buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 250 mM imidazole, 20 mM β-mercaptoethanol) using a SMART separation unit (Amersham Biosciences). All samples including standard marker proteins (Bio-Rad) were analyzed under identical conditions (6 °C, flow rate 40 μl/min, 50-μl fractions).

BN-PAGE of [$^{35}$S]Methionine-labeled Full-length Gephyrin Purified from Xenopus laevis Oocytes—Collagenase-defolliculated oocytes were injected with capped cRNAs and metabolically labeled by overnight incubation at 19 °C in frog Ringer’s solution (90 mM NaCl, 1 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, and 10 mM HEPES, pH 7.4) supplemented with ~40 MBq/ml L-$[^{35}$S]methionine (>40 TBq/mmol, Amersham Biosciences, ~0.1 MBq/oocyte). Oocytes were lysed in homogenization buffer consisting of 1% (w/v) digitonin (Merck Biosciences) in 0.1 M phosphate buffer, pH 8.0, 10 mM iodoacetamide, and protease inhibitors (10 μM antipain, 5 μM pepstatin A, 50 μM leupeptin, 100 μM Pefabloc SC). Full-length wild-type and mutant gephyrin proteins were purified as His-tagged proteins under non-denaturing conditions from the centrifugation-cleared digitonin extracts using nickel-nitriilotriacetic acid-agarose (Qiagen) essentially as described previously (22). Pilot experiments revealed that the migration of gephyrin in the BN-PAGE gel was not affected by the inclusion of digitonin in the washing and elution buffers. Accordingly, digitonin was only used for the initial homogenization of the oocytes and excluded from all further purification steps. Proteins were eluted from the beads by two subsequent incubations with 250 mM imidazole/HCl, pH 7.4, each for 15 min at ambient temperature. Within 1–2 h of purification, proteins were separated on BN-PAGE gels (4–16% acrylamide) (23) as described (22). Gels were fixed, dried, and exposed to a PhosphorImager screen, which was scanned with a Storm 820 PhosphorImager (Amersham Biosciences) and analyzed using the ImageQuant software.

Transfection of HEK 293T Cells and Hippocampal Neurons—HEK 293T cells were cultured on glass coverslips and transfected with cDNAs encoding gephyrin constructs using the calcium phosphate co-precipitation method as detailed previously (24). After 24 h of transfection, cells were fixed and processed for immunocytochemistry. Primary hippocampal neurons were prepared from 18 day-old rat embryos and newborn gephyrin knock-out mice and cultured as described (20). Neurons were transfected at days in vitro (DIV) 12 or 13 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol and fixed at DIV 18.

Immunofluorescence Staining—HEK 293T cells and hippocampal neurons were fixed with 4% paraformaldehyde for 10–12 min. Fixation and immunostaining were performed essentially as described (20). Cells were blocked with 1% (w/v) bovine serum albumin in phosphate-buffered saline for 1 h and incubated with primary antibody for 90 min. GFP was visualized by autofluorescence. For the detection of VIAAT, a pri-
Design of Gephyrin Constructs with Impaired Oligomerization Properties—The crystal structure of the G-domain of gephyrin (13) shows that 4 hydrophobic amino acid residues (Phe-90, Leu-113, Leu-128, and Leu-168) are located at the trimer interface (Fig. 1B). We used site-directed mutagenesis to replace these G-domain residues by 4 arginines, which due to hydrophilicity and charge were anticipated to abolish the interactions required for trimerization (Fig. 1A). Similarly, based on the crystallographic data available for the E-domain dimer (15), 3 amino acids (Gly-483, Arg-523, and Ala-532) predicted to be located at the dimer interface (Fig. 1C) were substituted with arginines or glutamic acid (Fig. 1A). The murine gephyrin gene comprises 30 exons. Of these, 10 exons or “cassettes”, named C1 to C7 and C4’, to C6’, have been found to be subject to alternative splicing, thus giving rise to a potentially large diversity of gephyrin isoforms (11, 25–27). One of these cassettes, C5’ (13 amino acids), encoded by exon 6, has been proposed to interfere with gephyrin binding to the GlyR and thereby to generate a GABAAR-specific postsynaptic gephyrin scaffold (26, 28). To examine the role of C5’ in gephyrin interaction, we also generated constructs containing this cassette for oligomerization studies (Fig. 1A).

The different domain constructs were named G_4xR (harboring substitutions F90R, L113R, L128R, and L168R), E_RER (G483R, R523E, and A532R), and G_C5’ (containing cassette C5’) and the corresponding full-length constructs Geph_4xR, Geph_RER, and Geph_C5’, respectively. In addition, we used Geph_mut containing an E-domain mutation (see “Experimental Procedures”), which abolishes GlyRβ binding but does not affect C-terminal dimerization (15).

Gel Filtration Chromatography of Gephyrin Domain Constructs—After bacterial expression and affinity purification, recombinant wild-type and mutant gephyrin domain proteins were subjected to gel filtration chromatography on a Superdex 200 column. The wild-type G-domain eluted at a position corresponding to a size of 58 ± 8 kDa (n = 5) (Fig. 1D). Because the calculated molecular mass of the recombinant G-domain is ~22 kDa, this result is consistent with the previously reported trimer formation of the G-domain (13). In contrast, recombinant G_4xR eluted in a major peak at 21 ± 6 kDa, which corre-
Domain Interactions in Gephyrin Scaffold Formation

responds to the molecular mass of the monomeric G-domain (Fig. 1D). Thus, the 4 arginine substitutions within the G-domain interface disrupted the trimerization of this N-terminal region of gephyrin. Recombinant G_{CS}, eluted in a major peak corresponding to 22 ± 4 kDa and a minor peak of 44 ± 5 kDa (Fig. 1D). Apparently, insertion of the C5′ cassette impairs G-domain trimerization.

The isolated gephyrin E-domain has been shown to form dimers in solution (15). In agreement with these earlier data, recombinant wild-type E-domain protein, with a calculated mass of 48 kDa, eluted from the column at a volume corresponding to 102 ± 13 kDa (Fig. 1E). In contrast, for the E_{mut} mutant protein carrying 3 charged amino acid substitutions at its dimer interface a major peak was observed at a position corresponding to 59 ± 4 kDa, i.e. a molecular mass corresponding to the E-domain monomer (Fig. 1E). Additionally, a minor peak at the position of the dimer was detectable. Thus, the mutations introduced at the predicted dimer interface of the E-domain largely disrupted dimer formation. For E_{mut}, an E-domain construct impaired in GlyR binding (Fig. 1A), a dimeric structure has been established previously (15).

Oligomerization Properties of Full-length Gephyrin Constructs—The gel filtration data shown above indicate that charge substitutions at G- and E-domain interfaces impair oligomerization of the individual gephyrin subdomains. To assess the effect of these assembly mutations on full-length gephyrin, we used BN-PAGE, which permits gel electrophoresis under non-denaturing conditions and, thus, determination of the oligomeric structure of proteins (22, 29). Recombinant full-length gephyrin purified by metal affinity chromatography from [35S]methionine-labeled X. laevis oocytes migrated upon BN-PAGE as a major band with an apparent mass of ~640 kDa (Fig. 2, lane 1) as assessed by comparison with soluble mass markers. In addition, higher order complexes accumulated at the interface between stacking and separating gels. We then treated the natively purified gephyrin with urea and SDS to dissociate the protein oligomer into lower order intermediates by weakening non-covalent subunit interactions (30). The ~640-kDa gephyrin band and the high molecular mass complexes seen at the top of the separating gel proved to be very sensitive to SDS and were converted almost completely to monomeric gephyrin migrating at ~110 kDa in SDS concentrations ≥0.01% (lane 7). By careful titration with low concentrations of SDS, intermediate oligomeric forms of gephyrin could be generated (lanes 3–6), which were judged to constitute dimers and trimers according to their apparent masses. Some trimers were also produced when full-length gephyrin was treated with 1 M urea (lane 2). By referring to the migration of monomers and trimers at ~110 and ~330 kDa, respectively, the ~640-kDa band was concluded to represent a gephyrin hexamer.

The hexameric structure of full-length gephyrin can be readily reconciled with the existence of the two independent oligomerization interfaces that together define the overall assembly state. Accordingly, trimers formed through G-domain interactions dimerize through E-domain interactions into a hexameric complex. In support of this view, the G-domain mutants Geph_{4xR} (lane 8) and Geph_{CS} (lane 12) migrated as dimers upon BN-PAGE. The somewhat slower mobility of Geph_{4xR} and Geph_{CS}, dimers as compared with that of the major dimers produced by partially denaturing SDS treatment of wild-type gephyrin (lane 6) may reflect conformational differences. Indeed, more slowly migrating dimers were also formed as a minor byproduct of SDS-induced dissociation of wild-type gephyrin (lanes 5–7).

For the E-domain mutant Geph_{mut}, containing the intact trimerization interface but lacking residues crucial for dimerization, both the major trimer band and a band of slightly reduced mobility were found (lane 9). SDS increased the intensity of the more slowly migrating band at the expense of the faster major band (results not shown), suggesting that partial unfolding of the native structure increases the effective radius of the Geph_{mut} trimer, which then becomes trapped in larger pores. Accordingly, we postulate that the double band is due to the presence of two trimer conformers. The existence of two independent assembly interfaces on gephyrin is further confirmed by mutant Geph_{4xR,mut} (lane 11), which combines substitutions of crucial side chains at both assembly interfaces and migrated as a monomer (lane 10). As expected, Geph_{mut} (lane 11) migrated to the position of wild-type gephyrin. This is consistent with this E-domain substitution not affecting dimerization (15). Together these data extend our gel filtration analysis of the isolated subdomains and indicate that wild-type gephyrin forms hexamers in cells by a combination of G- and E-domain interactions.

Heterologous Expression of Full-length Gephyrin Constructs in HEK 293T Cells—The results described above indicate that the G- and E-domains of gephyrin are important for oligomerization in vivo. To investigate whether the disruption of domain oligomerization affects the properties and subcellular distribution of gephyrin in mammalian cells, we expressed N-terminal GFP-tagged wild-type and mutant gephyrin constructs in HEK
a diffuse distribution in HEK 293T cells (Fig. 3D). However, the fraction of cells containing gephyrin aggregates (22.8 ± 8.8%) was larger compared with that of GFP-Geph_{4xR} and GFP-Geph_{C5}-transfected cells (Fig. 3F). The GFP-Geph_{mut} construct, which is oligomerization competent in vitro, generated large aggregates to an extent comparable with that found with wild-type GFP-gephyrin (89.4 ± 3.0%) (Fig. 3, E and F). Together, these data show that both the G- and E-domains of gephyrin are required for efficient aggregate formation in HEK 293T cells. Thus, aggregate formation in non-neuronal cells directly reflects the ability of gephyrin subdomains to oligomerize.

**Gephyrin Clustering in Cultured Hippocampal Neurons**—To determine whether oligomerization is required for the formation of gephyrin clusters at synaptic sites, we also expressed the GFP-gephyrin constructs in hippocampal neurons and analyzed the subcellular localization of GFP fusion proteins. In cultured hippocampal neurons transfected on DIV 12–13 and analyzed at DIV 18, wild-type GFP-gephyrin was localized in small clusters that were visible as punctate staining along the dendrites (Fig. 3G) in the majority of the transfected neurons examined (83.3 ± 11.5%, Fig. 3L). Most of these GFP-gephyrin clusters (~60%) colocalized with VIAAT, a marker of inhibitory nerve terminals (Fig. 4, A and E) as reported previously (20). This is consistent with proper synaptic clustering of the gephyrin fusion protein. In contrast, GFP-Geph_{4xR} and GFP-Geph_{C5}-produced a diffuse distribution of GFP fluorescence throughout the soma and dendritic shafts (Fig. 3, H and I, respectively). In both cases, a small fraction of the GFP-positive neurons showed an abnormal patchy or punctate fluorescence (20.0 ± 10.0% and 13.3 ± 4.7% for GFP-Geph_{4xR} and GFP-Geph_{C5}, respectively; Fig. 3L). However, these punctae did not significantly colocalize with the presynaptic marker VIAAT (Fig. 4E). We conclude that G-domain trimerization is important for clustering and synaptic targeting of gephyrin.

The E-domain dimerization mutant GFP-Geph_{ER} also displayed a diffuse distribution throughout the soma and dendritic regions in a vast majority of the transfected neurons (Fig. 3J). However, a slightly higher fraction of the transfected neurons (26.7 ± 9.4%) showed punctate fluorescence as compared with GFP-Geph_{4xR} and GFP-Geph_{C5}-produced (Fig. 3L). Notably, a punctate distribution similar to that of GFP-Gaph was detected with GFP-Geph_{mut} (Fig. 3K) in most of the transfected neurons (80.0 ± 10.0%, Fig. 3L). Double immunostaining with VIAAT revealed that clusters formed by both mutants, GFP-Geph_{mut} and GFP-Geph_{ER}, were rarely apposed to presynaptic terminals (Fig. 4, C and E).

To exclude the possibility that the residual colocalization with VIAAT seen with the GFP-gephyrin mutant proteins (Fig. 4E, black bars) is due to an interaction of the mutants with endogenous gephyrin, we repeated all transfection experiments with hippocampal neurons from gephyrin knock-out mice (see Fig. 4, B, D, and E). Again, GFP-gephyrin colocalized to a much higher extent (47.4 ± 11.1%) with VIAAT than all mutants, which exhibited colocalization values (Fig. 4E, white bars) indistinguishable from those obtained in wild-type neurons. Thus, the overlap of VIAAT immunoreactivity with the residual clusters formed by the mutant proteins is not dependent on

293T cells. In these cells, wild-type gephyrin has been shown to accumulate in intracellular aggregates, or “blobs” (8, 24). Here, transfection of GFP-tagged full-length gephyrin cDNA also resulted in the formation of large fluorescent aggregates (Fig. 3A) in most of the transfected cells (92.6 ± 3.1%, Fig. 3F). In contrast, transfection of GFP-Geph_{ER}, the mutant with impaired G-domain trimerization ability, produced a diffuse distribution of GFP fluorescence (Fig. 3B). Only 10.8 ± 5.3% of the transfected cells showed large aggregates (Fig. 3F). Identical findings as with GFP-Geph_{ER} were obtained with GFP-Geph_{C5}-transfected HEK 293T cells. Again, a diffuse distribution of the recombinant protein was seen (Fig. 3C) and large aggregates were found only in a small fraction (7.4 ± 2.9%) of the transfected cells (Fig. 3F). GFP-Geph_{ER} similarly displayed
Domain Interactions in Gephyrin Scaffold Formation

FIGURE 4. Synaptic targeting of mutant gephyrin proteins in transfected hippocampal neurons. GFP-tagged gephyrins (green) were expressed in hippocampal neurons and analyzed for colocalization with VIAAT (red), a marker for inhibitory terminals. Representative high resolution confocal images are displayed for wild-type gephyrin and Gephmut (A–D). A, wild-type gephyrin accumulated at inhibitory synapses formed by rat hippocampal neurons as revealed by colocalization (yellow punctae) or close apposition of green and red fluorences. In rat neurons transfected with Gephmut (C), apposition or colocalization of gephyrin with VIAAT was not observed. B, wild-type gephyrin also accumulated at inhibitory synapses of hippocampal neurons of gephyrin knock-out mice (Geph−/−), whereas for Gephmut again no colocalization with VIAAT was observed (D). E, the percentage of gephyrin punctae colocalizing with VIAAT is presented as means ± S.D. from four to six individual experiments in which 90–200 punctae were analyzed for each of the different constructs (**, statistically different from wild-type by p < 0.001). Arrowheads indicate colocalization and arrows a lack of colocalization. Scale bars, 5 μm.

endogenous gephyrin but may reflect an incomplete impairment of oligomer formation (see also Fig. 2) or some contribution of the fused GFP moiety known to be able to form dimers (31). Together, our results indicate that both G- and E-domain interactions are necessary but not sufficient for the clustering of gephyrin at inhibitory postsynaptic sites.

DISCUSSION

In this study, we show that oligomerization of the G- and E-domains of gephyrin is essential for its clustering at synaptic sites. In addition, the E-domain region interacting with GlyRβ is required for proper synaptic targeting. We also demonstrate that upon recombinant expression in Xenopus oocytes gephyrin forms hexamers that are likely to represent precursors of scaffold formation. Together our data provide strong experimental evidence for the concept that, at synapses, gephyrin forms a hexagonal submembranous lattice that recruits inhibitory neurotransmitter receptors under glycinergic and GABAergic nerve terminals.

G-domain Trimerization and E-domain Dimerization Are Required for Postsynaptic Gephyrin Clustering—Because the crystal structures of both the G-domain trimer and the E-domain dimer of gephyrin had been solved previously (13–15), we used a structure-based mutagenesis strategy to impair oligomerization of the N- and C-terminal regions of gephyrin. To abolish trimerization of the G-domain, 4 hydrophobic residues at the trimer interface were replaced by arginines. Similarly, charges were introduced at the E-domain dimer interface to interfere with dimerization. Gel filtration chromatography revealed that oligomerization of the individual recombinant G- and E-domains was largely prevented by these substitutions. Similarly, when the corresponding full-length gephyrin constructs were expressed in Xenopus oocytes, BN-PAGE of the affinity-purified Geph4xR and GephER mutant proteins revealed only dimers and trimers, respectively, but not the hexamer (and higher order complexes) characteristic of wild-type gephyrin. We therefore conclude that the mutations introduced were highly effective in perturbing gephyrin oligomerization.

Upon expression in hippocampal neurons, the GFP-fused Geph4xR and GephER mutant proteins were not clustered at synaptic sites but diffusely distributed throughout the somatic and dendritic cytoplasm. Thus, disruption of both N-terminal trimerization and C-terminal dimerization interferes with the formation of dendritic gephyrin clusters. This indicates that oligomerization is crucial for gephyrin scaffolding at developing postsynaptic sites. Based on the crystal structures of the G- and E-domains, gephyrin has been proposed to form a hexagonal submembranous lattice that recruits inhibitory neurotransmitter receptors to postsynaptic membrane specializations (1, 15). The results presented here are in full agreement with this model.

Although we cannot entirely exclude that the oligomerization defects of Geph4xR and GephER might reflect changes in the tertiary structure of gephyrin, different lines of evidence argue against indirect effects of the substitutions introduced. First, in all our experiments the results for the GephC5 construct containing the C5’ cassette in the G-domain were indistinguishable from those obtained for the trimerization-deficient mutant Geph3xR. GephC5 also migrated only as a dimer, and hexamers were never observed in our oocyte expression experiments. Second, all gephyrin mutants used here bound the established gephyrin interaction partner dynein light chain 1 (20) and, with the exception of Gephmut, GlyRβ. Thus, characteristic properties of gephyrin were retained upon substitution.

During the preparation of this report, Bedet et al. (32) also reported that C5’ interferes with N-terminal trimerization, a finding that is confirmed by our results. The same report in addition shows that in spinal cord neurons impairment of gephyrin trimerization leads to an enhanced internalization of GlyRs and their loss from synaptic sites. Additionally, gel filtration chromatography of gephyrin domain constructs indicated that the linker region inhibits dimerization of the E-domain, but not trimerization of the G-domain (32). These results complement and extend the data obtained here with hippocampal neurons.

Oligomerization has been shown to be important also for other synaptic scaffolding proteins. Rapsyn is essential for the formation of synaptic nicotinic acetylcholine receptor clusters at developing neuromuscular junctions (33). Recently, it has been shown that Rapsyn oligomerizes through its tetra-
coppeptide repeat domains (34). Similarly, PSD-95 (postsynaptic density protein of 95 kDa), which scaffolds N-methyl-D-aspartate receptors at excitatory synapses (35), can form multimers. For multimerization, the first 13 amino acids of PSD-95 and palmitoylation of 2 cysteine residues within this 13-amino acid motif are essential (36). Shank, another scaffolding protein in the postsynaptic density that seems not to be directly involved in the clustering of ion channel receptors, has recently been reported to require oligomerization for synaptic targeting (37).

The Gephyrin Hexamer, an Intermediate of Postsynaptic Scaffold Assembly?—Our BN-PAGE experiments identify a new hexameric assembly state of native gephyrin. Previously, purified full-length trimeric gephyrin generated in bacteria had been shown to reversibly assemble into a proteinaceous network upon ammonium acetate treatment (15). Electrospray ionization mass spectrometry revealed peaks corresponding to dimers, tetramers, and hexamers in these ammonium acetate-treated gephyrin samples. Here, gephyrin expressed in Xenopus oocytes was found to run as a hexamer after affinity purification. In addition, some higher order complexes accumulated at the interface between stacking and separating gels. The formation of both hexamers and higher order complexes was strikingly reduced in the oligomerization mutants Geph4xR and GephC5. These data are consistent with the hexameric state identified here representing a defined higher order conformer of gephyrin that forms in vivo and may represent a natural intermediate in postsynaptic scaffold formation. Its precise structure is presently unknown. One possibility is that this hexamer is a dimer of trimers, in which one E-domain of the first trimer interacts with one E-domain of a second trimer (see Fig. 2). Such a model would leave the remaining E-domains free for interactions with additional gephyrin molecules and hence explain the tendency of gephyrin to also form higher order complexes. Alternatively, the hexameric state might be stabilized further by additional interactions between the other E-domains, e.g. result in hexamers in which the three C-terminal regions of each gephyrin trimer are bound to the corresponding E-domains of the other trimer. An essential prerequisite for such a “condensed” structure would be a highly flexible linker region between the G- and E-domains. Indeed, secondary structure prediction algorithms suggest that the linker region of gephyrin is largely unstructured.

The GlyRβ Binding Region of the E-domain Is Essential for Synaptic Localization—In contrast to the mutants displaying impaired domain oligomerization, GFP-Gephmut substituted in the region of the E-domain that mediates high affinity GlyRβ binding (15, 16) readily formed clusters in transfected hippocampal neurons. However, these clusters did not colocalize with VIAAT. This indicates that GFP-Gephmut is not targeted to developing postsynaptic sites. Apparently, interaction with cytoplasmic receptor domains is required for proper localization of gephyrin scaffolds under contacting inhibitory nerve terminals. A similar conclusion has also been reached from experiments in which colocalization of recombinant GlyR subunits with neuronal gephyrin clusters was found to require insertion of a functional gephyrin binding motif derived from the GlyRβ cytoplasmic loop domain (38). It should be noted, however, that in hippocampal neurons gephyrin is primarily found at GABAergic synapses (39, 40). The mechanism by which GABAergic synapses interact with postsynaptic gephyrin is presently unclear. Our findings imply that the region substituted in GFP-Gephmut is important for both GlyR and GABAAR interaction and that gephyrin-receptor interactions are essential for proper postsynaptic targeting of gephyrin.

Formation of Intracellular Gephyrin Aggregates Depends on Domain Interactions—Upon expression in HEK293 cells, gephyrin forms large intracellular aggregates or blobs (24). This phenomenon has been widely utilized to study the interactions of gephyrin with other proteins by heterologous expression (8, 15, 20, 41). Recently, gephyrin aggregates have been shown to accumulate at microtubule-organizing centers, due to dynein light chain 1-dependent transport (42). However, the ability to bind dynein light chain 1 clearly is not a prerequisite for aggregate formation in heterologous cells (20). Here, all gephyrin mutants with impaired domain oligomerization, e.g. GFP-Geph4xR, GFP-GephC5R, and GFP-GephC5, displayed a diffuse cytoplasmic distribution and strongly reduced blob formation in HEK293 cells. This result is consistent with oligomerization being required for aggregate formation. Similarly, the inability of the mutants to efficiently form aggregates in HEK293 cells correlated with reduced cluster formation in neurons. Apparently, aggregate formation directly reflects the ability of gephyrin to assemble into higher order oligomeric complexes.

Conclusion—The data presented in this report establish an important role of both G-domain trimerization and E-domain dimerization in different aspects of gephyrin function in neurons. In addition, these domain interactions appear to be crucial for molybdenum cofactor biosynthesis in non-neuronal cells, because they are conserved in the bacterial G- and E-domain precursors, the MoeA and MoeB proteins (43, 44). For MoeA, dimerization is known to be essential for catalytic activity (44). The striking extent of structural and sequence conservation seen between bacterial and plant molybdenum cofactor-synthesizing proteins and gephyrin (13–15,27) thus probably reflects common quaternary structure requirements in scaffold formation and enzymatic function.

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Domain Interactions in Gephyrin Scaffold Formation

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