Gephyrin is required for the formation of clusters of the glycine receptor (GlyR) in the neuronal postsynaptic membrane. It can make trimers and dimers through its N- and C-terminal G and E domains, respectively. Gephyrin oligomerization could thus create a submembrane lattice providing GlyR-binding sites. We investigated the relationships between the stability of cell surface GlyR and the ability of gephyrin splice variants to form oligomers. Using truncated and full-length gephrins we found that the 13-amino acid sequence (cassette 5) prevents G domain trimerization. Moreover, E domain dimerization is inhibited by the gephyrin central L domain. All of the gephyrin variants bind GlyR β subunit cytoplasmic loop with high affinity regardless of their cassette composition. Coexpression experiments in COS-7 cells demonstrated that GlyR bound to gephyrin harboring cassette 5 cannot be stabilized at the cell surface. This gephyrin variant was found to deplete synapses from both GlyR and gephyrin in transfected neurons. These data suggest that the relative expression level of cellular variants influence the overall oligomerization pattern of gephyrin and thus the turnover of synaptic GlyR.

Fast neurotransmission at synapses depends on the enrichment of ionotropic receptors in the postsynaptic membrane. Scaffolding proteins present in the postsynaptic densities participate in the local and selective accumulation of most excitatory and inhibitory receptors in front of the corresponding transmitter release sites (1, 2). Synaptic localization of clusters of inhibitory γ-aminobutyric acid, type A and glycine receptors relies on gephyrin, initially discovered as a GlyR-associated extrinsic membrane protein (3, 4). The pivotal role of gephyrin has been largely demonstrated by antisense experiments (5, 6) and raises the questions of the function of the cassettes (22, 25). The effect of gephyrin variability on receptor recognition. This had led us to suggest that the presence of a cassette in the gephyrin molecule to form trimers and dimers via distinct homophilic interactions, which rely on the ability of the molecule to form trimers and dimers via distinct domains, are thought to account for the clustering of gephyrin-interacting GlyR in the postsynaptic membrane. Because of the variable primary structure of gephyrin, we investigated here the effect of the cassettes within splice variants on
its dual function as scaffold protein for GlyR and in polymerization. We have designed novel molecular tools to analyze in vitro the GlyR binding properties of gephyrin and its oligomerization capability. We show that the cassette composition of gephyrin has no effect on a high affinity interaction with the β subunit, provided that its gephyrin-binding site is presented within a closed M3-M4 cytoplasmic loop. In contrast, we found that 1) trimerization of gephyrin can be impaired by the insertion of a defined cassette (cassette 5) in the G domain and 2) gephyrin trimerization correlates with the ability to stabilize GlyR at the cell surface. In cultured neurons, the incorporation of a trimerization-defective gephyrin molecule into the postsynaptic gephyrin polymer interfered with the presence of GlyR at synapses. Altogether, these results indicate that the relative cellular expression levels of gephyrin variants influence the overall oligomerization pattern of gephyrin, thereby controlling GlyR number at synapses.

**EXPERIMENTAL PROCEDURES**

Constructs and expression plasmids, protein expression, and purification are described in the supplemental materials (27, 29, 30).

**In Vitro Binding Assays**—Tagged gephyrin fragments and PH3 chimeras in PBSM (5 mM β-mercaptoethanol in phosphate-buffered saline, pH 7.4) buffer were used immediately after elution from Ni-NTA-agarose. Two binding protocols were used. Solid phase binding assays (first protocol) were performed in ELISA plates. 96-Well microtiter plates (PVC plates; Costar) were coated with 50 μl of well of 60 nM PH or GST chimera solution in 0.1 M Na2CO3/NaHCO3, pH 9.6. Nonspecific binding sites were blocked with BPBSM buffer (4 mg/ml bovine serum albumin, 10% (w/v) sucrose, 0.25% (w/v) gelatin, 0.25% Tween 20, 5% (w/v) milk in PBSM). Then, unless otherwise stated, gephyrin fragments were incubated for 16 h at 4 °C using 50 μl/well of solutions at concentrations specified in the figure legends. After washing, bound gephyrin was detected with either anti-Myc or anti-T7 (Novagen) tag-specific antibodies and horseradish peroxidase-conjugated rabbit anti-mouse antibody. For quantitation, ABTS (Sigma-Aldrich) was used as substrate, and development was monitored in an ELISA reader (Bio-Tek). For solution assays (second protocol) of gephyrin promoter binding capacity, the tested fragments at the final concentration indicated in the figure legend were incubated in a final volume of 15 μl for 16 h at 4 °C in PBSMG buffer (PBSM containing 20% (w/v) glycerol). Then 50 μl of 12-fold dilutions of reaction mixes were incubated at 25 °C in Ni-NTA HisSorb strips (Qiagen) for capture. After washes with the same buffer, bound gephyrin species were detected separately with either anti-Myc, anti-T7, or anti-His6 (BD Biosciences Clontech) antibodies and horseradish peroxidase-conjugated antibody as above. Calibration of tag reactivity (see Fig. 4) was performed in normal Ni-NTA HisSorb strips (Qiagen), except for T7mycGL (2) and T7mycLE, which were adsorbed to strips pretreated with 20 mM EDTA in 0.1 M Na2CO3/NaHCO3, pH 9.6. 

**Cell Culture and Transfection**—African green monkey kidney (COS-7) cells were plated on glass coverslips and grown in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal calf serum (Invitrogen) at 37 °C and 7.5% CO2. For transfection, the experiments were performed on subconfluent cultures (60% confluency) using the FuGENE 6 (Roche Applied Science) method. Usually, 2 μg of plasmid DNA were added to 35-mm dishes. Transient protein expression was allowed to proceed for up to 16 h at 37 °C. Spinal cord neurons were prepared from embryonic Sprague-Dawley rats as described previously (28). Routinely, the cells were plated at a density of 5.104 cells/cm² onto glass coverslips (Assistent, Winigor, Germany) and grown in neurobasal medium supplemented with B27, 2 mM glutamine, and antibiotics (Invitrogen, France) at 36 °C in a 5% CO2 atmosphere. They were transfected 8–10 days after plating using the Lipofectamine2000™ method (Invitrogen) according to the manufacturer’s protocol. The cells were usually transfected with 2 μg of plasmid DNA in 20-mm wells.

**Fluorescence Microscopy**—Immunofluorescence labeling was performed essentially as described (31) on cells that were fixed in 4% (w/v) paraformaldehyde. The primary antibodies were used at the following concentrations: mouse anti-GlyR α1 subunit: 1 μg/ml (clone mAb2b; Synaptic System GmbH), mouse anti-c-Myc monoclonal antibody: 0.5 μg/ml (clone 9E10; Roche Applied Science), rabbit anti-synapsin I antibody: 1 μg/ml (Chemicon), rat anti-HA monoclonal antibody: 1 μg/ml (clone 3F10; Roche Applied Science), rabbit anti-green fluorescent protein antibody: 1 μg/ml (BD Biosciences Clontech). The secondary antibodies were: goat anti-mouse or anti-rabbit antibodies conjugated with fluorescein isothiocyanate, tetramethylrhodamine isothiocyanate, Cy3 or Cy5 (1:250–1:400; Jackson Immunoresearch Laboratories), or Alexa 488 (Molecular Probes). Extracytoplasmic Myc tags were detected as described (31). Observations were made using the 63×/1.32 and 100×/1.3 objectives of a Leica DMR fluorescence microscope. Fluorescence pictures were usually acquired with a Leica DMR/HCS microscope (63× immersion objective) (Nussloch, Germany) equipped with a CCD camera (Coolscan; Princeton Instruments). Acquisition was done in black and white in 12-bit mode using appropriate filters, and quantification was done with Metamorph software (Princeton Instruments). GlyR and gephyrin clusters were quantified on digitized images on neurons from two independent cultures. Integrated synaptic GlyR-associated fluorescence was measured on cells chosen randomly on the basis of Venus:Ge fluorescence. For each transfected neuron the GlyR-associated fluorescence intensity colocalized with synapsin I staining was measured on neurites using the gephyrin fluorescence as a mask, after subtraction of the background signal. All of the measurements were performed using Metamorph software. Statistical significance was determined by means of Student’s t test using StatView Software (Abacus Concepts, Berkeley, CA). Fluorescence of clusters was determined on pictures acquired under a 100× objective lens. When required, pictures were pseudocolored with Photoshop (Adobe Systems, San Jose, CA).

The abbreviations used are: PH, pleckstrin homology; ABTS, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid; GlyR, glycine receptor; Ni-NTA, nickel-nitrilotriacetic acid; ELISA, enzyme-linked immunosorbent assay; GST, glutathione S-transferase.
**Gephyrin Oligomers and Postsynaptic GlyR Stability**

**Endocytosis Test**—The cells were incubated at 2 °C with 1 μg/m anti-c-Myc antibody for 30 min (prelabeling step) and then extensively washed (time 0 min of chase). They were returned to their initial culture medium pre-equilibrated at 37 °C to allow endocytosis for 40 min. Low pH stripping was then performed (time, 40 min of chase) by incubating cells at 2 °C in 0.2 M acetic acid, 0.5 M NaCl, pH 2.2, for 30 min. At any step, the cells were either processed for immunofluorescence staining or lysed for two-site ELISA. For two-site ELISA, washed and pelleted cells were solubilized in 50 mM Tris/HCl, 100 mM NaCl, 0.2% (w/v) Triton X-100, pH 8.0, for 30 min on ice. The cleared detergent extract was supplemented with 0.2% (w/v) gelatin, 0.05% (v/v) Tween 20 and incubated for 18 h at 4 °C with rabbit anti-mouse antibody coated in 96-well microtiter plates (10 μg/ml, 50 μl/well). 9E10 IgG was detected with horseradish peroxidase-conjugated rabbit anti-mouse antibody and ABTS reaction as described above.

**Analytical Size Exclusion Chromatography**—Sephacryl S-200 (Amersham Biosciences) was packed in a 4.1-ml column (4.3 mm/27.5 cm) and equilibrated in PBSMG buffer at a flow rate of 24 μl/min. 30-μl samples of solutions (9–14 μl) of freshly eluted gephyrin fragments in PBSMG, GL (2), GL (2,4,5), G(2), G(2,5), E, and LE, were loaded onto the column within 5–10 min following elution from Ni-NTA-agarose. Fractions were collected in 96-well microplates, and proteins were adsorbed to nitrocellulose membrane in a 96-well dot-blot apparatus. The His6 tag was detected as described for binding assays, using 3,3’-diaminobenzidine as horseradish peroxidase substrate. Dot density was determined after scanning of wet membrane using National Institutes of Health 1.52 software. The molecular mass standards used for the calibration of the column were proteins (68 kDa), ovalbumin (43 kDa), and RNase A (13.7 kDa).

**RESULTS**

**Interaction of Gephyrin with GlyR Subunit M3-M4 Loops**—The influence of the various domains and cassettes of gephyrin on its functional properties was analyzed with in vitro assays using recombinant proteins obtained after synthesis in E. coli and His6-mediated purification (Fig. 1).

First, in an attempt to mimic their native conformation, we designed recombinant versions of GlyR cytoplasmic M3-M4 loops in which the N and C termini were brought close together. For this, the subunit loop sequences were substituted for the small loop connecting the anti-parallel β-strands 6 and 6’ in the cytohesin PH domain, which contributes to hydrogen bonding with 5-phosphate in inositol-(1,3,4,5)tetrakis-triphosphate (33). The structure and stability of the PH domain are not altered by the insertion of the loop. These chimeras can be considered as displaying the loop in a restricted conformation, and the 18-amino acid gephyrin-binding sequence, βgb, should be properly displayed within the β loop (9). Second, a series of tagged, truncated forms of gephyrin in addition to full-length versions were constructed (Fig. 1) and used for binding experiments. The insertion of a tobacco etch virus protease (TEV) cleavage site in some constructs offered the possibility of investigating gephyrin protomer-protomer interactions in capture assays utilizing the His6 tag. The appropriate immunoreactivity of the sequence tags, as well as the efficiency of TEV digestion, was verified (supplemental Fig. S1).

To characterize a possible differential binding of gephyrin-derived molecules (Fig. 1) to the cytoplasmic loop of the GlyR β subunit, the interaction of fragment LE was first compared with that of E, taken here as the reference binding entity. In the solid phase assay (first protocol, see “Experimental Procedures”) using immobilized PH-βLoop, maximal binding was reached after 3–4 h of incubation for both ligands and for all of the concentrations tested. In addition to the same kinetics, a saturable association of E and LE molecules to PH-βLoop was also described by superimposable curves (Fig. 2A). The same KD value in the nanomolar range was estimated for binding to PH-βLoop. In contrast, very little binding to GST-βLoop could be detected over this concentration range. A similar and high affinity interaction was also observed with the various LE, variants (Fig. 2B), indicating that none of the cassette sequences (C3, C4, and C4’) present in the L domain affect the recognition of PH-βLoop. Moreover, the association of full-length (GLE) molecules, 4

---

*S. Eimer, C. Bedet, and C. Vannier, unpublished results.*

**FIGURE 1. Structure and sequence tags of expressed recombinant proteins.** For truncated (LE, E, G, and GL) and GL forms of gephyrin, names (right) are given according to the domain structure of the full-length molecule (top, clone P1, Ref. 23; or Ge2,6 or Ge clone 80, Ref. 24) and to the various splice cassettes found in the four variants selected for this study: Ge(2), Ge(2,4,5), Ge(2,4’), and Ge(2,3). For the sake of clarity, cassette 6, which is present in invariant domain E, is neglected in the nomenclature. The fragment harboring the domains G, L, and the N-terminal third of E is a construction intermediate, incapable of either GlyR binding or dimerization. It is referred to as GL in this work. Tag combinations (right) fused to the N terminus of the various constructs are indicated. The numbers on the diagrams refer to amino acid residues of clone P1. For the PH-based fusion proteins, the numbers refer to the amino acid residue preceding the inserted M34 loop.
when tested with identical ligand to PH-βLoop concentration ratios (Fig. 2C), revealed not only similar respective binding curves but affinities close, if not identical, to those of the corresponding LE fragment (Fig. 2, compare B and C). Therefore, PH-βLoop binding to the E domain was not influenced by either G domain, implying that cassette C5 of Ge(2,4,5) does not alter the high affinity interaction between gephyrin and the β loop. The GL construct, which contains neither the GlyR β subunit-binding site nor the E subdomains involved in protomer interface within the E dimer (18), could be used as a control. The curves of Fig. 2D confirmed that G domains had no binding ability whatever the variants tested. In this assay, no binding to the α1 loop or to the α2 loop occurred (Fig. 2, B and C). These data indicate that the formation of a high affinity complex with the GlyR β subunit cytoplasmic M3-M4 loop is not influenced by variations in the primary structure of gephyrin outside its E domain.

In a previous work (24), we showed that a differential β loop binding of gephyrin variants could be measured using GST pull-down assays. In an attempt to better characterize the effect of the structure surrounding the βγβ gephyrin-binding sequence of β loop, we probed the association of Ge(2,4,5) and Ge(2) with PH-βLoop, GST-βLoop, and PH-α1βγβLoop using the solid phase assay. As observed with E and LE, the strength of the Ge/GST-βLoop complex was strikingly low as compared with that of Ge/PH-βLoop (supplemental Fig. S2A). However, in contrast to the interaction between gephyrins and PH-βLoop, the distinct ability of the two gephyrin variants to bind GST-βLoop (as exhibited in GST pull-down assays), was reproduced here, because Ge(2,4,5) was captured more efficiently than Ge(2,4,5) by immobilized GST-βLoop (supplemental Fig. S2A, inset). This difference was not observed when βγβ was inserted in the PH-α1βγβLoop chimera, which binds gephyrin as strongly as PH-βLoop (supplemental Fig. S2B). This suggests that structural elements promoting gephyrin recognition are maintained in the PH-M3-M4 loop proteins and lost in GST-βLoop. PH domain-based carriers of the βγβ sequence thus provide high affinity binding sites for both gephyrin variants. The present results (Fig. 2C) demonstrate that gephyrins Ge(2,4,5) and Ge(2) bind with similar affinities to PH-βLoop. Furthermore, we show that high affinity binding
to βγβ is dependent on structural elements that are only maintained in a closed M3-M4 loop conformation.

Size Exclusion Chromatography of Gephyrin Fragments—We then investigated whether cassette composition could affect the quaternary structure of the molecule. Based on reports from others (19), the more stable oligomeric state of purified full-length gephyrin (GLE) is a trimer behaving as a 300-kDa assembly, in agreement with the crystal structure of the G domain (16, 17) but suggesting that the E domain does not possess a conformation suitable for dimerization. Here, only the truncated gephyrin versions were used, potentially able to form either trimers or dimers. Polypeptides were analyzed immediately after purification to minimize self-aggregation. Size exclusion chromatography of fragments corresponding to Ge(2,4,5) and Ge(2) and detected by dot-blotting is illustrated (Fig. 3A). The elution profiles obtained for G(2) and GL(2) were consistent with those of homogeneous trimers with peaks at 81 and 178 kDa, respectively (Fig. 3B). This was not the case for G(2,5) and GL(2,4,5), mainly eluted at volumes nearly corresponding to the size of the monomers (peaks at 27 and 60 kDa, as compared with 25.9 and 55.8 kDa, respectively). This demonstrated a lack of trimer formation by the G domain of Ge(2,4,5). A striking difference was also observed in the behavior of the E and LE fragments. E, which is a dimer at concentrations in the micro-molar range (20), was indeed recovered in a single peak (123 kDa), consistent with the size of the dimerized polypeptide (50.2 kDa/monomer). In contrast, LE was recovered as two distinct entities: predominantly a monomeric form (68 kDa, predicted: 65.8) and a dimer (145 kDa), clearly eluting ahead of the E dimer. This result suggested that the L domain does not favor the formation of an LE dimer. These results indicate that trimer formation by the G domain of gephyrin can be prevented by the insertion of cassette C5 and support the notion that E domain dimerization is impeded by the L linker domain.

Gephyrin Oligomer Formation via the G and E Domains—Interactions between either G or E domains of gephyrin were analyzed using a solution assay (second protocol, see “Experimental Procedures”). This assay was designed to measure the potential exchange of protomers between oligomeric forms of gephyrin at equilibrium. Briefly, His-tagged (capture molecules) and Myc-tagged (ligand molecules) fragments of gephyrin (Fig. 1A) were coincubated (at concentrations in the micro-molar range), prior to binding to Ni-NTA-coated wells of ELISA plates. The epitope tags associated with the wells were assayed, and the recovery of the Myc tag was taken as the indication of capture and ligand molecule association. His-tagged G or GL fragments from Ge(2,4,5) and Ge(2) (capture molecules) were mixed with Myc-tagged GL (2) (ligand molecule) at varying concentration ratios (Fig. 4, A and B). In these experiments, when T7mycGL (2) was incubated with either HisT7G(2) or HisT7GL (2), the Myc tag was recovered as complexed with the His tag according to a saturable process (Fig. 4A). The progressive decrease in the reactivity of the His tag, despite its obligatory capture, likely results from steric hindrance in antibody binding (compare anti-His6 reactivity toward trimeric HisT7GL (2) and monomeric HisT7LE80 in Fig. 4D). Therefore, the partial loss of measurable His tag with increasing T7mycGL (2) concentrations is consistent with the appearance of His(T7GL (2))T7mycGL(2) and His(T7GL(2))T7mycGL(2) trimers during incubation. Taken as an internal standard in the test, the amount of captured T7 tag remains constant, as expected if protomers can be exchanged between trimers. When HisT7G(2,5) was used as the capture molecule, no binding of the Myc tag could be detected (Fig. 4B). This confirmed that trimer formation cannot occur between G(2,5) domains and implies that G(2,5) and G(2) cannot form heterotrimers.

A similar experiment was performed using mixes of MycLE with either HisT7E or HisT7LE. It revealed that whereas little capture of mycLE by HisT7E could be obtained, none occurred with HisT7LE (Fig. 4C). These results indicated that LE exists mainly as a monomer. These data therefore support the notion that, whatever its cassette composition, the L domain acts as an inhibitor of the E-E interaction in the solution assay. These experiments corroborated the monomeric state of the LE molecule observed upon size exclusion chromatography.

GlyR-Gephyrin Association and Cell Surface Stability—Fluorescence microscopy analysis of full-length (GLE) Ge(2,4,5) and Ge(2) in COS-7 cells (supplemental Fig. S3) confirmed that
gephyrin, independently of its primary structure, can strongly interact with the βgb sequence. Ge(2,4,5) and Ge(2) have distinct oligomerization abilities. Therefore, the possibility arises that the gephyrin/receptor coclusters (supplemental Fig. S3, C2 and D2) were not similar with regard to the extent of GlyR bridging. To examine whether the stability of cell surface GlyR was influenced by the nature of gephyrin, transfected COS-7 cells were subjected to an endocytosis test. A–C, time, 0 min of chase. After the anti-Myc antibody prelabeling step at 2 °C, the α1βgb subunit displays only diffuse cell surface staining. D–F, time, 40 min of chase. After the 40-min incubation at 37 °C, the cells expressing Ge(2,4,5) exhibit higher punctate labeling of α1βgb subunit (arrowheads) than cells expressing Ge(2). Higher magnifications of fields selected in E and F are shown below the rows. Note the presence of both gephyrin variants at the plasma membrane (arrows). The cells shown are representative of the whole population of transfected cells in each condition. G, quantification of internalized anti-Myc antibody by two-site ELISA. Examples of assays comparing endocytosis of α1 subunits harboring or not βgb either in the presence or absence of Ge(2) (G1) or in the presence of Ge(2,4,5) or Ge(2) (G2). The results are expressed as percentages of the anti-Myc antibody determined at the end of the prelabeling step. Bar, 20 μm.

**FIGURE 5. Gephyrin variants and cell surface stabilization of GlyR.** The cells were transfected either with pC-Myc::α1βgb (A and D) or with both pC-Myc::α1βgb and the indicated pC-monomeric red fluorescent protein::Gei (B, C, E, and F). Following 14 h of expression they were subjected to an endocytosis test. A–C, time, 0 min of chase. After the anti-Myc antibody prelabeling step at 2 °C, the α1βgb subunit displays only diffuse cell surface staining. D–F, time, 40 min of chase. After the 40-min incubation at 37 °C, the cells expressing Ge(2,4,5) exhibit higher punctate labeling of α1βgb subunit (arrowheads) than cells expressing Ge(2). Higher magnifications of fields selected in E and F are shown below the rows. Note the presence of both gephyrin variants at the plasma membrane (arrows). The cells shown are representative of the whole population of transfected cells in each condition. G, quantification of internalized anti-Myc antibody by two-site ELISA. Examples of assays comparing endocytosis of α1 subunits harboring or not βgb either in the presence or absence of Ge(2) (G1) or in the presence of Ge(2,4,5) or Ge(2) (G2). The results are expressed as percentages of the anti-Myc antibody determined at the end of the prelabeling step. Bar, 20 μm.

gephyrin, independently of its primary structure, can strongly interact with the βgb sequence.

Ge(2,4,5) and Ge(2) have distinct oligomerization abilities. Therefore, the possibility arises that the gephyrin/receptor coclusters (supplemental Fig. S3, C2 and D2) were not similar with regard to the extent of GlyR bridging. To examine whether the stability of cell surface GlyR was influenced by the nature of gephyrin, transfected COS-7 cells were subjected to an endocytosis test of surface GlyR molecules. The respective typical diffuse or punctate distribution pattern of α1βgb after such labeling is illustrated (Fig. 5, A–C). Coclusters of cell surface GlyR and either Ge(2) or Ge(2,4,5) were formed. After a 40-min chase period at 37 °C, the acid wash-resistant labeling was only found in endocytic vesicles or cisternae (Fig. 5, D–F, and supplemental Fig. S4). At this stage, however, labeled GlyR was still present at the surface of cells not exposed to an acid wash (not shown). The intracellular label (Fig. 5, E and F, arrowheads) was abundant in cells coexpressing Ge(2,4,5), and their fluorescence pattern was reminiscent of that found in cells expressing only α1βgb (Fig. 5D). In contrast, very little labeling of intracellular vesicles was observed in cells coexpressing Ge(2) (supplemental Fig. S4, compare E1 and F1). Whether Ge(2,4,5) or Ge(2) was expressed, clusters of gephyrin could be observed beneath the plasma membrane (Fig. 5, E2 and F2, arrows). Colocalization of Ge(2,4,5) and internalized GlyR was also observed (Fig. 5, F3, crossed arrows).

The extent of α1 or α1βgb subunit internalization was measured by ELISA of the receptor-bound anti-Myc antibody. (Fig. 5G). The results of two sets of duplicate representative experiments are shown, demonstrating that GlyR–Ge(2) interaction through βgb largely contributed to maintain the receptor at the cell surface because α1βgb expressed alone was internalized as rapidly as the gephyrin-
non interacting α1 in the presence of Ge(2) (Fig. 5G1). Internalization of α1βgb in the presence of Ge(2,4,5) occurred as rapidly as that of α1 (Fig. 5G2). Altogether, our data demonstrate that the interaction of cell surface GlyR with Ge(2,4,5), in contrast to that involving Ge(2), was not sufficient to prevent internalization. Consequently, the cell surface stability
of GlyR, in addition to binding to gephyrin, is also determined by the oligomeric state of gephyrin itself.

**Gephyrin Can Displace GlyR from Synaptic Loci**—The reduced cell surface stability of GlyR when associated with small clusters of Ge(2,4,5) in COS-7 cells suggested that the latter could interfere with Ge(2) to maintain the receptor under a clustered form in neurons. This hypothesis was scored by counting clusters of both Venus-tagged gephyrin and endogenous synaptic GlyR (supplemental Fig. S6). This redistribution was accompanied by a disappearance of GlyR clusters from the postsynaptic surface (Fig. 6G) but showed no significant change. These results favor the notion that Ge(2,4,5), which can bind both Ge(2) (and endogenous gephyrin) and GlyR, was acting as a dominant-negative variant in the formation of gephyrin/GlyR coclusters. This could only result from the titration by Ge(2,4,5) of its two binding partners, which acquired the diffuse distribution expected for small complexes. Altogether these data show that Ge(2,4,5), which exhibits a limited oligomerization potential, can compete with Ge(2) for the binding to GlyR, leading to a loss of synaptic receptor upon expression.

**DISCUSSION**

In this work, we demonstrate that: 1) the ability of gephyrin to stabilize GlyR at the cell surface is correlated with its trimerization and 2) gephyrin unable of trimerization displaces both gephyrin and GlyR from postsynaptic loci, when expressed in cultured neurons. These results suggest that modifications of the relative cellular expression level of gephyrin variants influence the turnover and number of GlyR at synapses.

**Gephyrin Interacts with GlyR Independently of Its Structure**—Gephyrin is likely the main regulator of receptor organization. The identification of the gephyrin E domain as bearing the GlyR-binding site has been largely substantiated recently (19, 20, 25). In this study we designed a new tool to probe GlyR-gephyrin interaction. A soluble pleckstrin homology domain was used as a carrier for isolated GlyR large cytoplasmic loops to maintain their native tertiary structure. We present evidence that a high affinity interaction occurs between the large gephyrin E domain and the cytoplasmic loop of the GlyR β subunit when presented as the PH-βLoop chimera. ELISA-based assays revealed PH-βLoop binding characteristics consistent with surface plasmon resonance experiments reported by Sola et al. (19), indicating dissociation constants in the nanomolar range. Our data now establish that β subunit association with the E domain is not modulated by cassettes encountered in the gephyrin L domain studied here. Notably, the E domain displayed either alone or in truncated or full-length molecules had unchanged binding capacity and affinity for PH-βLoop.
Gephyrin Oligomers and Postsynaptic GlyR Stability

This indicates that the E domain association with β subunit is autonomous.

We have found no detectable effect of cassette C5, when present in the G domain of full-length gephyrin, on the PH-β-loop binding. Clearly, the measurement of the strength of the interaction between gephyrin and its binding site on β subunit, βgb, depends on the experimental design (see Ref. 24). Schrader et al. (20) also showed that affinities almost 2 orders of magnitude higher could nevertheless be detected by isothermal titration calorimetry, corroborating the surface plasmon resonance data of Sola et al. (19) performed with the same GST-β-loop chimera. Our data favor the notion that the βgb sequence is not displayed properly when the entire β subunit M3-M4 loop is fused by its N terminus to a leader protein. In contrast, it becomes a strong binding site in vitro and in vivo (Fig. 2 and supplemental Fig. S2), when the fusion protein mimics the topology of the β subunit loop. We propose that βgb is optimally recognized by gephyrin in a folded, constrained structure provided by proteins such as the native β subunit, the α1βγβ subunit, and PH-β-loop or PH-α1βγβ loop, but not GST-β-loop. The possibility of measuring strong interactions with chimera of the 49-residue sequence is plausibly due to the absence of additional and misfolded polypeptide impeding recognition.

Trimerization and Dimerization Are Controlled by Gephyrin Structure According to Distinct Mechanisms—Intrinsic structural elements govern the oligomerization of gephyrin. On the one hand, G domain trimerization can be prevented in a splice variant harboring cassette C5, and on the other hand, dimer formation by the E domain is impeded by the central L domain. The basic molecular form of full-length gephyrin is a highly stable trimer as recently determined by dynamic light scattering and chemical cross-linking (19, 20). Such quaternary structure agrees well with the strength of the interaction between protomers in purified G domain trimers and suggests that the E domain, which otherwise forms dimers when isolated, is in a metastable conformation within the full-length polypeptide (19, 20). These quaternary structures of gephyrin are in both cases consistent with their behavior of soluble proteins when expressed in transfected cells.

We now provide evidence of a lack of trimer formation by Ge(2,4,5) resulting from the presence of cassette C5. This cassette, which has been found following cloning of several rat gephyrin variants, was also identified in the human gene and attributed to exon 5 (24, 34). Actually, a mere alteration of the trimer interface might alter the formation of gephyrin oligomers (16). We propose that the Ge(2,4,5) monomer results from the disruption of the highly conserved α-helix 4 upon insertion of the 13 residues of splice cassette C5 between residues Glu99 and Ala99 (16, 17, 23). Interestingly, we found that monomeric full-length Ge(2,4,5) still binds GlyR β subunit, in agreement with evidence that dimerization of the E domain is not required for GlyR binding (20).

The inhibition of E domain dimerization in full-length gephyrin results either from an inappropriate conformation or from the masking of the interface, which is defined between protomers in the crystal structure (19). Altogether, available data and our findings support the notion that the two types of oligomerization events within holo-gephyrin are not simultaneous. Regulation of dimerization is therefore likely to be a mechanism whereby formation of GlyR clusters is achieved.
Furthermore, the control of dimerization would prevent inappropriate lattice formation outside synapses.

Control Receptor Cell Surface and Synaptic Stability by Gephyrin Oligomerization—We found in a previous study (32) that gephyrin contributes to the accumulation of GlyR in the plasma membrane when interaction is permitted. Such a situation may in part rely on co-clustering events similar to those already reported for Kv1.4 and Kv4.2 channels, which undergo reduced internalization when complexed with postsynaptic differentiation-95 (35, 36). The distinct effects of Ge(2,4,5) and Ge(2) on endocytosis of bound GlyR support two conclusions: 1) a GlyR-interacting gephyrin variant unable to trimerize is not sufficient to stabilize GlyR at the cell surface and 2) formation of complexes between Ge(2) and gephyrin variants with altered oligomerization can lower the half-life of this cell surface receptor. Therefore, in addition to receptor-gephyrin interaction, the stability of GlyR in the plasma membrane also depends on the extent of gephyrin oligomerization. As a matter of fact, gephyrin variant Ge(2,4,5), which can transiently populate postsynaptic loci in neurons, is responsible for the removal of GlyR from these sites. The parallel loss of postsynaptic cassette 5-lacking gephyrin provides evidence that Ge(2,4,5) acts as a dominant-negative molecule titrating both receptor and gephyrin within synapses. The disappearance of GlyR from synapses could therefore result from the formation of small GlyR-Ge(2,4,5) complexes able to undergo diffusion in the plasma membrane and subsequent internalization (37).

Postsynaptic Scaffold and Regulation of GlyR Clustering—Our findings now allow us to propose a model for the formation and plasticity of the inhibitory postsynaptic lattice (Fig. 7). The general organization (18, 22) of the hexagonal gephyrin lattice remains. However, variants of gephyrin allow the construction of a dynamic lattice, which influences GlyR clustering. It is assumed that E domains within the lattice can be engaged in dimers for a majority of gephyrin variants because of the low frequency of splicing in this region (however, see Ref. 12). This is not necessarily the case because dimerization could be modulated by specific partner proteins or phosphorylation. Based on our data, a disruption of the hexagonal oligomerization pattern results from a failure of the trimerization process. In the presence of a mixture of the two types of gephyrin, Ge(2,4,5) and Ge(2), this mechanism would contribute to a regulated steady state size of a nonhexagonal, branched polymer (Fig. 7). Moreover, changes in the relative expression level of these variants would result in disassembly or assembly of the scaffold (Fig. 7).

The gephyrin lattice assembly/disassembly substantiated here has consequences for the local density as well as for the dynamics of its partners and is consistent with the diffusion-retention mechanism underlying clustering of GlyR (31, 37, 38). In particular, gephyrin may shuttle between the lattice and the cytosolic phase in a GlyR-bound state. Our data and model thus challenge the hypothesis that simple incorporation of gephyrins harboring C5 in a lattice might selectively reduce the density of GlyR at synaptic sites but not that of γ-aminobutyric acid receptor (39). Instead, the association of GlyR with C5-containing gephyrin present in the lattice is possible, but impairment of dimerization would drive redistribution of such a complexed receptor toward the extrasynaptic space. The cassette-dependent oligomerization of gephyrin has mechanistic implications for both the control of GlyR clustering at synapses and dynamic events in the postsynaptic differentiation. As a conclusion, we provided evidence that the functional heterogeneity of gephyrin with regard to lattice formation creates an additional, nonexclusive mechanism regulating the presence, density, and dynamics of the inhibitory postsynaptic differentiation components.

Acknowledgments—We thank Drs. J. L. Bessereau, B. Dargent, and B. Gasnier for discussion and critical reading of the manuscript. We thank R. Y. Tsien for the gift of the monomeric red fluorescent protein cDNA.

REFERENCES
1. Craig, A. M., and Boudin, H. (2001) Nat. Neurosci. 4, 569–578
2. Nussel, Z. (2000) Curr. Opin. Neurobiol. 10, 337–341
3. Pfeiffer, F., Graham, D., and Betz, H. (1982) J. Biol. Chem. 257, 9389–9393
4. Schmitt, B., Knaus, P., Becker, C. M., and Betz, H. (1987) Biochemistry 26, 805–811
5. Kirsch, J., Wolters, L. Triller, A., and Betz, H. (1993) Nature 366, 745–748
6. Essrich, C., Lorez, M., Benson, J. A., Fritschy, J. M., and Luscher, B. (1998) Nat. Neurosci. 1, 563–571
7. Feng, G., Tintrup, H., Kirsch, J., Nichol, M. C., Kuhse, J., Betz, H., and Sanes, J. R. (1998) Science 282, 1321–1324
8. Kneussel, M., Brandstatter, J. H., Laube, B., Stahl, S., Muller, U., and Betz, H. (1999a) J. Neurosci. 19, 9289–9297
9. Meyer, G., Kirsch, J., Betz, H., and Langosch, D. (1995) Neuron 15, 563–572
10. Kneussel, M., Hermann, A., Kirsch, J., and Betz, H. (1999) J. Neurochem. 3, 1323–1326
11. Kirsch, J., Langosch, D., Prior, P., Littauer, U. Z., Schmitt, B., and Betz, H. (1991) J. Biol. Chem. 266, 22242–22245
12. Ramming, M., Kins, S., Werner, N., Hermann, A., Betz, H., and Kirsch, J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10266–10271
13. Kamdar, K. P., Shelton, M. E., and Finnerty, V. (1994) Genetics 137, 791–801
14. Stallmeyer, B., Nerlich, A., Schiemann, J., Brinkmann, H., and Mendel, R. R. (1995) Plant J. 8, 751–762
15. Liu, M. T., Wuebbens, M. M., Rajagopalan, K. V., and Schindelin, H. (2000) J. Biol. Chem. 275, 1814–1822
16. Schwarz, G., Schrader, N., Mendel, R. R., Hecht, H. V., and Schindelin, H. (2001) J. Biol. Chem. 312, 405–418
17. Solà, M., Kneussel, M., Heck, I. S., Betz, H., and Weissenhorn, W. (2001) J. Biol. Chem. 276, 25294–25301
18. Xiang, S., Nichols, J., Rajagopalan, K. V., and Schindelin, H. (2001) Structure (Camb) 9, 299–310
19. Solà, M., Barro, V. N., Timmins, I., Franz, T., Richar-Blum, S., Schoenh, G., Ruigrok, R. W., Paarmann, I., Saiyed, T., O’Sullivan, G. A., Schmitt, B., Betz, H., and Weissenhorn, W. (2004) EMBO J. 23, 2510–2519
20. Schwäder, N., Kim, E. Y., Winkling, J., Paulukat, J., Schindelin, H., and Schwarz, G. (2004) J. Biol. Chem. 279, 18733–18741
21. Kim, E. Y., Schrader, N., Smolinsky, B., Bedet, C., Vannier, C., Schwarz, G., and Hermann Schindelin, H. (2006) EMBO J. 25, 1385–1395
22. Kneussel, M., and Betz, H. (2000) Trends Neurosci. 23, 429–435
23. 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
24. Meier, J., De Chaldee, M., Triller, A., and Vannier, C. (2000) Mol. Cell. Neurosci. 16, 566–577
25. Rees, M. I., Harvey, K., Ward, H., White, J. H., Evans, L., Duguid, I. C., Hsu,
Gephyrin Oligomers and Postsynaptic GlyR Stability

C. C., Coleman, S. L., Miller, J., Baer, K., Waldvogel, H. J., Gibbon, F., Smart, T. G., Owen, M. J., Harvey, R. J., and Snell, R. G. (2003) J. Biol. Chem. 278, 24688–24696

26. Vannier, C., and Triller, A. (2004) in Excitatory-Inhibitory Balance, (Hensch, T. K., and Fagiolini, M., eds) pp. 59–74, Kluwer Academic/Plenum Publishers, New York

27. Sheffield, P., Garrard, S., and Derewenda, Z. (1999) Protein Expression Purif. 15, 34–39

28. Meier, J., Meunier-Durmort, C., Forest, C., Triller, A., and Vannier, C. (2000) J. Cell Sci. 113, 2783–2795

29. Nagai, T., Ibata, K., Park, E. S., Kubota, M., Mikoshiba, K., and Miyawaki, A. (2002) Nat. Biotechnol. 20, 87–90

30. Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Baird, G. S., Zacharias, D. A., and Tsien, R. Y. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7877–7882

31. Rosenberg, M., Meier, J., Triller, A., and Vannier, C. (2001) J. Neurosci. 21, 5036–5044

32. Hanus, C., Vannier, C., and Triller, A. (2004) J. Neurosci. 24, 1119–1128

33. Ferguson, K. M., Kavran, J. M., Sankaran, V. G., Fournier, E., Isakoff, S. J., Skolnik, E. Y., and Lemmon, M. A. (2000) Mol. Cell 6, 373–384

34. David-Watine, B. (2001) Gene (Amst.) 271, 239–245

35. Jugloff, D. G., Khanna, R., Schlichter, L. C., and Jones, O. T. (2000) J. Biol. Chem. 275, 1357–1364

36. Wong, W., Newell, E. W., Jugloff, D. G., Jones, O. T., and Schlichter, L. C. (2002) J. Biol. Chem. 277, 20423–20430

37. Choquet, D., and Triller, A. (2003) Nat. Rev. Neurosci. 4, 251–265

38. Meier, J., Vannier, C., Serge, A., Triller, A., and Choquet, D. (2001) Nat. Neurosci. 4, 253–260

39. Meier, J., and Grantyn, R. (2004) J. Neurosci. 24, 1398–1405