Neuron, Vol. 15, 563–572, September, 1995, Copyright © 1995 by Cell Press

# Identification of a Gephyrin Binding Motif on the Glycine Receptor β Subunit

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## Summary

**The tubulin-binding protein gephyrin copurifies with the inhibitory glycine receptor (GlyR) and is essential for its postsynaptic localization. Here we have analyzed the interaction between the GlyR and recombinant gephyrin and identified a gephyrin binding site in the cytoplasmic loop between the third and fourth transmembrane segments of the β subunit. GlyR α subunits and $GABA_A$ receptor proteins failed to bind recombinant gephyrin. However, insertion of an 18 residue segment of the GlyR β subunit into the $GABA_A$ receptor β1 subunit conferred gephyrin binding both in an overlay assay and in transfected mammalian cells. These results indicate that β subunit expression is essential for the formation of a postsynaptic GlyR matrix.**

## Introduction

Fast neurotransmission is mediated by ligand-gated ion channel proteins that are highly concentrated at postsynaptic sites of central neurons. The dense packing of these neurotransmitter receptors is considered to constitute an important determinant of synaptic efficacy (Korn et al., 1987; Busch and Sakmann, 1990). The cytoplasmic face of postsynaptic specializations containing the inhibitory glycine receptor (GlyR) is decorated with the tubulin-binding protein gephyrin (Triller et al., 1985; Altschuler et al., 1986; Kirsch et al., 1991; Prior et al., 1992). Originally, gephyrin was identified as a peripheral membrane protein that copurifies with the GlyR and forms large complexes (Pfeiffer et al., 1982; Schmitt et al., 1987). Molecular cloning disclosed different gephyrin isoforms that result from alternative splicing of four distinct oligonucleotide cassettes (Prior et al., 1992). In situ hybridization and immunocytochemical studies revealed a widespread expression of gephyrin in embryonic and adult rat brain, which suggests a more general role of this protein in synapse formation and maintenance (Kirsch and Betz, 1993; Kirsch et al., 1993a). Studies on cultured spinal neurons have shown that the formation of membrane-associated gephyrin clusters precedes the postsynaptic localization of the GlyR in cultured spinal neurons. Furthermore, depletion of gephyrin by treatment with antisense oligonucleotides was found to prevent the accumulation of GlyRs under contacting nerve terminals (Kirsch et al., 1993b). Also, gephyrin–GlyR clusters are rapidly disrupted by colchicine, a microtubule-dissociating agent (Kirsch and Betz, 1995). These data are consistent with a model in which gephyrin serves as an anchor molecule that links the GlyR to the submembranous cytoskeleton (Kirsch et al., 1991; Kirsch and Betz, 1995).

The GlyR shares a number of structural features with other ligand-gated ion channel proteins, in particular the γ-aminobutyric acid type A receptor ($GABA_AR$). Both inhibitory receptors are pentameric integral membrane proteins composed of at least two different types of subunits (Pfeiffer et al., 1982; Sigel et al., 1983). These subunits display considerable sequence homology and a common predicted transmembrane topology, with an extracellular N-terminus followed by four membrane-spanning regions (M1–M4), which are separated by short and long cytoplasmic loops (reviewed in Betz, 1992). For the GlyR, presently four α (α1–α4) and a single β subunit are known, whereas a much larger diversity exists for $GABA_AR$ subunits (reviewed in Wisden and Seeburg, 1992). The GlyR α subunits harbor the binding sites for glycinergic agonists and the competitive antagonist strychnine and oligomerize into functional glycine-gated chloride channels upon heterologous expression (Schmieden et al., 1989; Sontheimer et al., 1989; Kuhse et al., 1990b). The β subunit does not form GlyRs on its own, but its incorporation into recombinant receptors changes several functional characteristics of the ion channel, such as binding of noncompetitive antagonists and the selection of elementary chloride conductance states; these effects have been attributed to its channel-forming M2 segment and established a modulatory role of the β subunit in GlyR channel function (Pribilla et al., 1992; Bormann et al., 1993; Rundström et al., 1994).

In this study, we analyzed the interaction between gephyrin and the GlyR in overlay assays using affinity-purified native GlyR and recombinant subunit fragments, and in human embryonic kidney 293 cells cotransfected with GlyR, or $GABA_AR$, and gephyrin cDNAs. Our results reveal a direct interaction of gephyrin with the GlyR β subunit and identify a binding region within its large cytoplasmic loop between transmembrane segments M3 and M4. These data extend the functional roles of the β subunit from channel modulation to the postsynaptic localization of the GlyR.

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## Results

To reconstitute the gephyrin–GlyR complex, we examined the binding of recombinant gephyrin to the native GlyR, to electrophoretically separated GlyR polypeptides, and to bacterially expressed fusion proteins. In addition, we analyzed by immunocytochemistry whether the subcellular distribution of the GlyR and $GABA_AR$ in 293 cells was altered upon coexpression of recombinant gephyrin.

### Recombinant Gephyrin Isoforms Bind to the GlyR but Not to the $GABA_AR$

Gephyrin copurifies with the GlyR α and β subunits upon solubilization of spinal cord membranes with the nonionic detergent Triton X-100 and is highly enriched in aminostrychnine–agarose affinity column eluates (Pfeiffer et al., 1982). However, ionic detergents like cholate or deoxycholate dissociate the vast majority of gephyrin from the receptor (Graham et al., 1985), resulting in the purification of a gephyrin-depleted GlyR complex. Taking advantage of this sensitivity of the GlyR–gephyrin interaction to ionic detergents, we depleted the GlyR of endogenous gephyrin by solubilization and washing with buffers containing cholate after the receptor was bound to the affinity column. Subsequently, we exchanged this detergent for Triton X-100 and tested the ability of soluble recombinant gephyrin isoforms expressed in 293 cells (Prior et al., 1992) to bind immobilized GlyR. The major gephyrin isoforms differ by the presence of cassette 2 only (P1), cassettes 2 plus 4 (P2), or cassettes 2 plus 3 (P3), respectively (Prior et al., 1992). All three recombinant gephyrin polypeptides bound to GlyR immobilized on aminostrychnine–agarose, as revealed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and silver staining of the respective glycine eluates of the affinity column (Figure 1A). The identity of the eluted gephyrin bands was confirmed by immunoblotting (Figure 1B) using the gephyrin-specific monoclonal antibody (MAb) 5a (Pfeiffer et al., 1984), and the amount of coeluted GlyR-protein (Figure 1C) was revealed using the GlyR α subunit-specific MAb 4a (Pfeiffer et al., 1984). By several criteria, the eluted gephyrin did not represent residual endogenous gephyrin. First, the variants P1 and P2 differed from endogenous gephyrin by their lower molecular weight (90 kDa; Figure 1A; also see Prior et al., 1992). Second, no gephyrin band was detected in a control experiment in which a cytosolic extract of untransfected 293 cells was applied to the column (Figure 1A). Finally, the use of P1 protein metabolically labeled with [$^{35}$S]methionine demonstrated unequivocally that recombinant gephyrin was indeed bound to the immobilized GlyR (Figure 2).

The specificity of this protein–protein interaction was examined by analyzing the binding of gephyrin to the $GABA_AR$, which is structurally related to the GlyR but does not copurify with gephyrin (Stephenson, 1988). To this end, GlyR and $GABA_AR$ were solubilized and immobilized on the appropiate affinity columns under conditions (0.5% [w/v] deoxycholate) identical to those of the original $GABA_AR$ purification protocol (Sigel and Barnard, 1984).

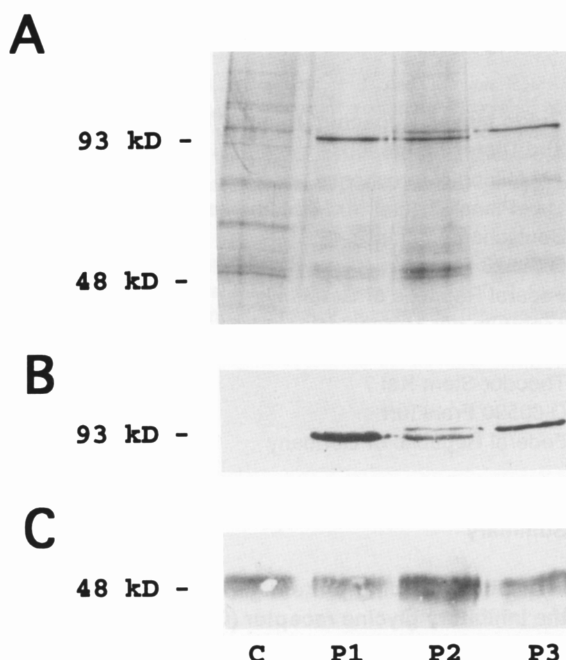



Figure 1. Binding of Recombinant Gephyrin Isoforms to Immobilized GlyR

GlyR, depleted of endogenous gephyrin by cholate solubilization, was bound to aminostrychnine–agarose and incubated with the cytosolic fractions of 293 cells that were either untransfected (lane C) or transfected with the gephyrin P1, P2, or P3 cDNAs. The glycine eluates of the columns were then analyzed by SDS–PAGE followed by silver staining (A) or by immunoblotting with gephyrin-specific MAb 5a (B) or GlyR α subunit-specific MAb 4a (C). The faint 93 kDa band above the immunoreactive band of P2 seen in (B) represents endogenous gephyrin, since, owing to low expression levels of the P2 construct, more column eluate was applied to SDS–PAGE.

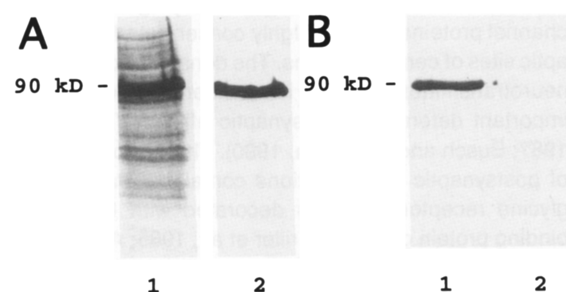



Figure 2. Recombinant Gephyrin Binds to GlyR Columns but Not to $GABA_AR$ Columns

GlyR and $GABA_AR$ were solubilized by deoxycholate from rat spinal cord or brain membranes, respectively. Receptors were immobilized on the appropiate affinity columns and incubated with the cytosolic fraction of [$^{35}$S]methionine-labeled 293 cells transfected with the gephyrin P1 cDNA.

(A) The cytosolic fraction contained P1 gephyrin of 90 kDa as a major protein, as shown by autoradiography (lane 1) and immunoblotting with MAb 5a (lane 2) of the electrophoretically separated cytosol.

(B) Receptor-bound gephyrin was detected after SDS–PAGE and autoradiography in the eluate of the GlyR column (lane 1) but not of the $GABA_AR$ column (lane 2).

Then, [$^{35}$S]methionine-labeled recombinant gephyrin P1 was passed over the columns, and its binding to the immobilized receptors was examined. Gephyrin did not bind to the $GABA_AR$ bound to a benzodiazepine–agarose affinity resin, whereas the aminostrychnine–agarose-immobilized GlyR retained the radiolabeled protein, as monitored by SDS–PAGE and autoradiography of the respective column eluates (Figure 2). Control experiments showed that comparable amounts of receptor proteins were detected by protein staining and immunoblotting in the electrophoretically separated glycine and clorazepate eluates (data not shown).

### Gephyrin Binds to the GlyR β Subunit

To identify the GlyR subunit that mediates gephyrin binding, we used an overlay procedure. GlyR purified from cholate-solubilized membranes was separated by SDS–PAGE and transferred to nitrocellulose. After renaturation by incubation with a series of guanidine–HCl solutions of decreasing concentrations, the nitrocellulose filter was incubated with $^{35}$S-labeled recombinant gephyrin P1. Autoradiography visualized binding of radioactive material to a single band on the nitrocellulose, which was identified as the GlyR β subunit by molecular weight and immunostaining (Figure 3, lanes 2 and 4) with a β subunit–specific polyclonal antiserum (Pribilla et al., 1992). Binding was not detected when using a cytosolic extract from [$^{35}$S]methionine-labeled untransfected cells (data not shown). In addition, gephyrin immunoreactivity was revealed within the radioactive band by staining with MAb 5a, thus confirming the identity of the bound material as gephyrin (Figure 3, lane 3). Traces of endogenous gephyrin in the affinity-purified GlyR preparation were immunostained by MAb 5a at 93 kDa, and the GlyR α subunit was identified by MAb 4a at 48 kDa (Figure 3, lanes 3 and 5). Staining with an anti-tubulin antibody showed that the cholate-solubilized GlyR sample did not contain detectable amounts of tubulin, which was previously found to copurify with Triton X-100–solubilized GlyR and to comigrate with the 58 kDa GlyR β subunit upon SDS–PAGE (Kirsch et al., 1991). Furthermore, after SDS–PAGE, purified tubulin from rat brain failed to bind detectably to recombinant gephyrin in our overlay assay (data not shown), although native tubulin binds to immobilized gephyrin under tubulin-polymerizing conditions (Kirsch et al., 1991). These data exclude the fact that gephyrin binding to the 58 kDa band was due to endogenous tubulin rather than the GlyR β subunit.

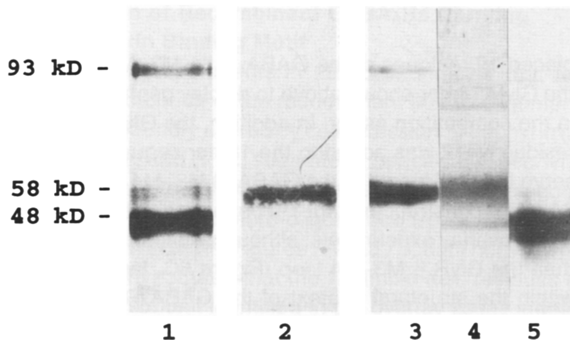



Figure 3. Gephyrin Binds to the GlyR β Subunit

Affinity-purified GlyR was separated by SDS–PAGE and either silver stained (lane 1) or transferred to nitrocellulose (lanes 2–5). The filter was probed with the cytosolic fraction of [$^{35}$S]methionine-labeled 293 cells transfected with the gephyrin P1 cDNA. Recombinant gephyrin bound to a single band at 58 kDa as detected by autoradiography (lane 2) and gephyrin-specific MAb 5a staining (lane 3). The 58 kDa band was also stained by a β subunit–specific polyclonal antiserum (lane 4); the 48 kDa α subunit band was stained by MAb 4a (lane 5).

### The Gephyrin Binding Domain Lies within the Large Cytoplasmic Loop of the GlyR β Subunit

The gephyrin binding region on the β subunit was mapped by overlay experiments in which GlyR polypeptides were replaced by subunit fragments. A large loop between transmembrane regions M3 and M4 provides the major cytoplasmic domain of the receptor subunits (Figure 4A). Therefore, we fused M3–M4 loop sequences to the C-terminus of glutathione-S-transferase (GST) and subjected the bacterially expressed fusion proteins to the gephyrin overlay procedure. A fusion protein containing the entire M3–M4 loop of the GlyR β subunit (GST-123) bound the $^{35}$S-labeled gephyrin probe, whereas a corresponding loop of the neonatal GlyR (Becker et al., 1988) α2 subunit (GST-81) did not (Figure 4C, lanes 1 and 2). This demonstrates that gephyrin specifically interacts with an intracellular domain of the β subunit. To delineate further those portions of the β loop region mediating gephyrin binding, we examined a series of shorter fragments (Figure 4A). Out of these, a GST fusion construct containing a 49 amino acid stretch starting at position 378 of the β subunit (GST-49) produced a binding signal similar to that seen with the entire M3–M4 loop sequence (Figure 4C, lane 3). Further deletion of this 49 amino acid fragment at both termini generated fusion proteins exhibiting a strongly reduced binding (GST-34 and GST-33; Figure 4C, lanes 5 and 6) and finally abolished all binding detectable by the overlay assay (GST-22 and GST-18; Figure 4C, lanes 4 and 7). In an alternative approach, we examined the competition of gephyrin binding to nitrocellulose-immobilized fusion protein by including the soluble purified fusion proteins in the gephyrin-containing overlay solution. By testing a concentration series, we found that the soluble GST-49 construct was able to compete for >90% of the gephyrin binding when present at about only 10% of the total amount of its immobilized counterpart (Figure 5B). This suggests that only a low proportion of the immobilized polypeptide refolds properly, or that immobilization reduces the protein's binding affinity. Interestingly, inclusion of GST-18, but not of GST alone, in the overlay solution also inhibited gephyrin binding to the immobilized GST-123 (Figure 5A) and GST-49 (Figure 5B) polypeptides. However, efficient competition of gephyrin binding to immobilized GST-49 by the inclusion of soluble competitor protein required about 100-fold more GST-18 than GST-49 (Figure 5B). This indicates that GST-18 also binds to gephyrin, albeit with an affinity below that detectable by the direct overlay assay.

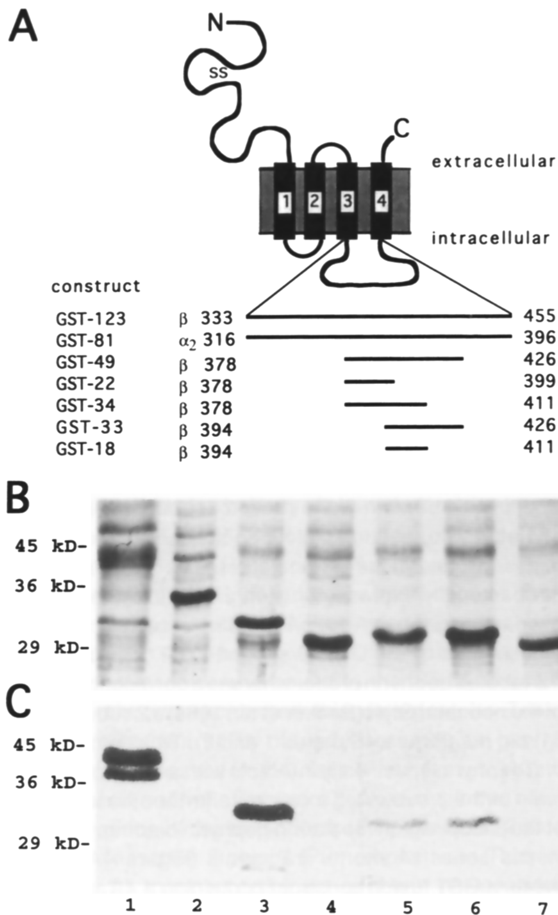



Figure 4. Mapping of the Gephyrin Binding Site on the GlyR β Subunit

(A) Schematic diagram of the GlyR β and α2 subunit fragments contained in the GST fusion proteins. The constructs are named according to fragment lengths; the residue numbers of the N- and C-termini of the individual fragments are indicated.

(B and C) The GST fusion proteins listed in (A) were expressed in Escherichia coli. Bacterial lysates containing ~9 μg of fusion protein were separated by SDS-PAGE and stained by Coomassie blue to control for comparable expression levels (B) or transferred to a nitrocellulose filter. The filter was probed with the cytosolic fraction of [35S]methionine-labeled 293 cells transfected with the gephyrin P1 cDNA. Bound recombinant gephyrin was then detected by autoradiography (C). GST-123 (lane 1), GST-49 (lane 3), GST-34 (lane 5), and GST-33 (lane 6) bound detectable amounts of the protein, whereas GST-81 (lane 2), GST-22 (lane 4), and GST-18 (lane 7) gave no signal. Partial degradation of the larger GST fusion proteins may account for the additional radioactive bands seen in lanes 1 and 3.

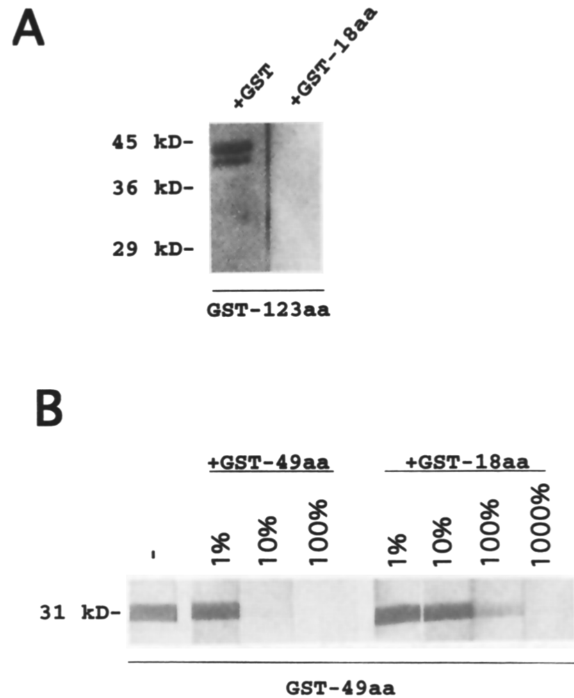



Figure 5. Competition of Gephyrin Binding to Immobilized β Subunit Fusion Proteins by Different Soluble Fragments

(A) Bacterial lysate containing the GST-123 fusion protein (~9 μg/lane) was electrophoretically separated and transferred to nitrocellulose. The filter was probed for the binding of [35S]methionine-labeled gephyrin in the presence of ~100 μg/ml purified GST or GST-18. Note that gephyrin binding to GST-123 was not observed when GST-18 was present in the overlay solution. Partial proteolysis may account for the additional band seen at 36 kDa in lane 1.

(B) Filter-bound purified GST-49 fusion protein (~20 μg/lane) was probed with 35S-labeled gephyrin in the absence (−) or presence of purified GST-49 or GST-18 fusion proteins added to the overlay solution. The amount of soluble competitor protein is expressed as a percentage relative to the amount of filter-bound protein. Note that an ~100-fold higher concentration of GST-18 as compared with GST-49 was required for efficient competition of gephyrin binding.

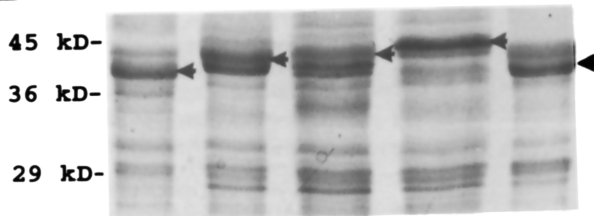
Primary structure alignments reveal that, of the known $GABA_AR$ subunits, the β1–β3 polypeptides (Ymer et al., 1989) share the highest homology with the GlyR β 49mer within their M3–M4 loop regions (Figure 6A). Therefore, GST fusion proteins containing the large cytoplasmic loops of the $GABA_AR$ β subunit isoforms 1, 2, and 3 were generated (Figure 6B) and subjected to the overlay procedure (Figure 6C, lanes 2–4). None of the constructs detectably bound gephyrin, which corroborates our negative results with the $GABA_AR$ affinity column. Next, we replaced 19 residues of the $GABA_AR$ β1 M3–M4 loop with the GlyR 18mer shown above to display gephyrin binding in the competition assay. In addition, the GlyR β subunit residue N412 was added to the 18mer sequence to conserve the total length of the $GABA_AR$ M3–M4 loop (Figure 6A). This construct (mutant 19) bound recombinant gephyrin in overlay experiments, although with lower efficiency than the GlyR β M3–M4 loop (Figure 6C, lane 5). Thus, within the structural context of the $GABA_AR$ β1 M3–M4 loop sequence, the GlyR 18mer conveyed detectable gephyrin binding. This suggests that these 18 residues harbor major determinants of gephyrin binding.

To delineate the gephyrin binding site further, we mutated all residues within this segment that are nonconservatively exchanged between the $GABA_AR$ β1 and the GlyR β subunit to generate the $GABA_AR$ β1 sequence 391-KATDYSIDSSIPRDFELS or, alternatively, introduced all hydrophobic residues of the GlyR 18mer into the equivalent position of the $GABA_AR$ β1 subunit, resulting in a 391-

**A**

| | | |
|---|---|---|
| GlyR β | 378 | V G E T R C K K V C T* S K S D L R S N D F S I V G S - L P R D F E L S - N Y* D C Y G K P I E V N N G L |
| GABA$_A$R β1 | 375 | R N E T S G S E V L T G V S D P K A T M Y S Y D S A S I Q Y R K P L S - S R G E F G R - G L D R H G V |
| GABA$_A$R β2 | 374 | K N E M A T S E A V M G L G D P R S T M L A Y D A S S I Q Y R K A G L - P R H S F G R M A L E R H V A |
| GABA$_A$R β3 | 375 | H N E M - - N E V A G S V G D T R N S A I S F D N S G I Q Y R K Q S M - P K E G H G R Y M G D R S I P |
| Mutant 19 | 375 | R N E T S G S E V L T G V S D P [R S N D F S I V G S - L P R D F E L S N] S R G E F G R - G L D R H G V |

**B**




**C**

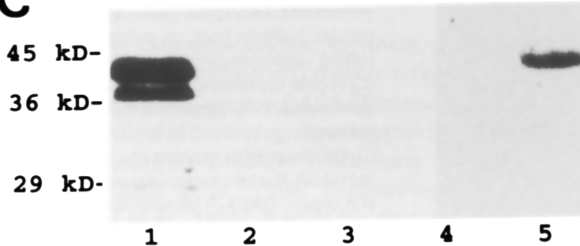



Figure 6. Gephyrin Binding to a GABA$_A$R β1 Cytoplasmic Loop Sequence Containing the Central 18 Amino Acids of the Gephyrin Binding Segment of the GlyR β Subunit

(A) Sequence comparison of the 49 amino acid gephyrin binding motif of the GlyR β subunit with corresponding regions of GABA$_A$R β subunits. Residues conserved between GlyR and GABA$_A$R β subunits are shaded, and the central 18 amino acid competing fragment is underlined. The sequence of mutant 19, in which the homologous 18 amino acid region of the GABA$_A$R β1 subunit loop is replaced by the corresponding residues (boxed) of the GlyR β subunit, is also shown. The consensus sites on the GlyR β subunit for phosphorylation by protein kinase C or tyrosine kinases are marked by asterisks.

(B and C) Bacterial lysates containing ~6 μg of GST fusion proteins (arrowheads), including the whole M3–M4 loops of the GlyR β subunit (lane 1), of the GABA$_A$R β1 (lane 2), β2 (lane 3), and β3 (lane 4) subunits, and of mutant 19 (lane 5), were separated by SDS–PAGE and stained by Coomassie blue to control for comparable expression levels (B) or transferred to nitrocellulose. The filter was probed with the cytosolic fraction of $^{35}$S-labeled 293 cells transfected with the gephyrin P1 cDNA, and bound recombinant gephyrin was detected by autoradiography (C). Note that both the GlyR β subunit and mutant 19 sequences bind gephyrin.

KATMFSIVSALQYRFPLS motif (underlined residues indicate positions where the GABA$_A$R β1 sequence was substituted by the corresponding amino acid of the GlyR β subunit; see Figure 6A). None of these constructs, however, displayed detectable gephyrin binding (data not shown). We conclude, therefore, that the GlyR β 18mer sequence is close to a minimal gephyrin binding motif.

### Gephyrin Expression Alters the Subcellular Distribution of Recombinant GABA$_A$Rs Carrying the Gephyrin Binding Motif

Transfection experiments in 293 cells indicate that the cellular distribution of the recombinant hetero-oligomeric GlyR is profoundly altered by coexpression of gephyrin (J. K., J. Kuhse, and H. B., submitted). Specifically, gephyrin forms large intracellular aggregates upon heterologous expression that trap hetero-oligomeric α1/β, but not homo-oligomeric α1, GlyRs (Figures 7A and 7B; data not shown). To test the hypothesis that the 18 amino acid residues of the GlyR β subunit identified in the overlay assay indeed represent a gephyrin binding motif that is functional in a cellular context, we investigated the subcellular distribution of the GABA$_A$R β1 mutant 19 harboring the GlyR β 18mer in 293 cells expressing recombinant gephyrin.

When hetero-oligomeric GABA$_A$R was generated by cotransfection of GABA$_A$R α1 and β1 subunits, most of the receptor antigen revealed by a GABA$_A$R α1 subunit–specific polyclonal antibody was found in the plasma membrane (Figure 7C). This distribution was not changed upon coexpression of gephyrin (Figure 7D), a situation which resembles that seen in cells expressing homo-oligomeric α1 GlyR together with gephyrin (J. K., J. Kuhse, and H. B., submitted). A different localization was observed when mutant 19 was used instead of the wild-type GABA$_A$R β1 subunit. In the absence of gephyrin, the α1/mutant 19 hetero-oligomer was again found at the cell surface (Figure 7E). In cells cotransfected with the gephyrin P1 cDNA, however, α1/mutant 19 receptors colocalized with intracellular gephyrin (Figure 7F). A similar intracellular accumulation of receptor protein upon coexpression of gephyrin was seen when GABA$_A$R α2 or α3 subunits were assembled with mutant 19 (data not shown). This rerouting of hetero-oligomeric GABA$_A$Rs containing mutant 19 closely resembles the sorting behavior of hetero-oligomeric α1/β GlyRs coexpressed with gephyrin. We therefore conclude that the GlyR β 18mer sequence is sufficient to target receptors to gephyrin-rich subcellular domains.

## Discussion

The data presented in this paper constitute the first direct demonstration that gephyrin binds to the large cytoplasmic loop of the GlyR β subunit. Our results indicate an important role of this subunit in the formation of the glycinergic postsynaptic membrane.

### Implications for Receptor Localization

The synaptic localization of neurotransmitter receptors and their aggregation to high receptor densities are prerequisites for efficient neurotransmission. The cyto-

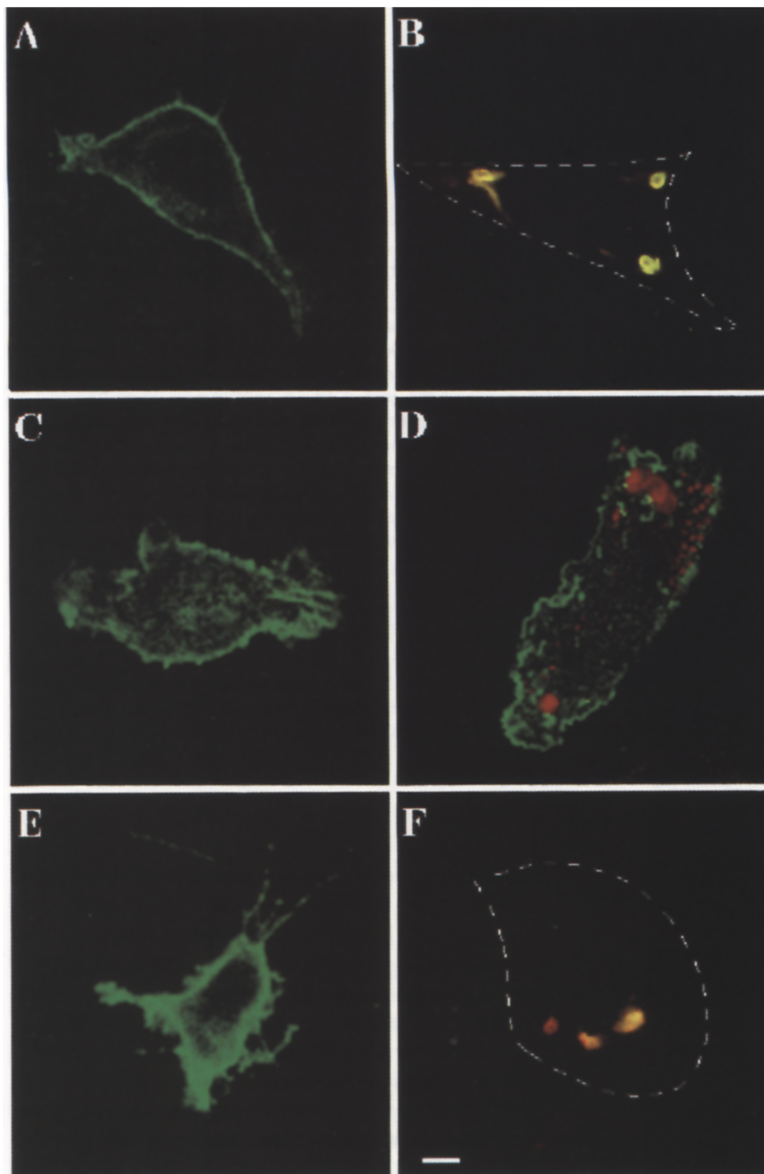



Figure 7. Coexpression of Gephyrin Alters the Subcellular Distribution of GlyR and Mutant 19-Containing $GABA_AR$ Hetero-Oligomers in 293 Cells

HEK 293 cells were cotransfected with GlyR α1 and β subunit cDNAs (A and B), $GABA_AR$ α1 and β1 subunit cDNAs (C and D), or $GABA_AR$ α1 and mutant 19 cDNAs (E and F) in the absence (A, C, and E) and presence (B, D, and F) of the gephyrin P1 cDNA and then immunolabeled as described in Experimental Procedures. The subcellular localization of the resulting hetero-oligomeric receptors was visualized using MAb 4a for GlyR proteins or an α1 subunit-specific polyclonal antibody for $GABA_AR$ proteins (green) and using MAb 5a or a monospecific polyclonal antiserum for gephyrin (red), respectively.

(A) α1/β GlyR (green) is localized at the plasma membrane of transfected 293 cells.

(B) Upon cotransfection with gephyrin, GlyR immunoreactivity (green) colocalizes with intracellular gephyrin (red), as indicated by the resulting yellow labeling.

(C) In cells expressing α1/β1 $GABA_AR$, specific immunoreactivity (green) is found at the cell surface.

(D) Double immunostaining of a cell coexpressing $GABA_AR$ α1/β1 hetero-oligomers and gephyrin. Again, $GABA_AR$ immunoreactivity (green) is mainly localized at the plasma membrane; no label is found over intracellular gephyrin aggregates (red).

(E) In cells expressing recombinant α1/mutant 19 $GABA_AR$, immunoreactivity is found primarily at the plasma membrane (green).

(F) Upon coexpression of the α1/mutant 19 $GABA_AR$ (green) and gephyrin (red), the $GABA_AR$ colocalizes with intracellular gephyrin aggregates, as indicated by the yellow labeling.

In (B) and (F), cell contours are indicated by stippled lines. Bar, 10 μm.

plasmic receptor-associated proteins rapsyn (or 43 kDa protein) and gephyrin are known to be crucial for the clustering of the nicotinic acetylcholine receptor at the neuromuscular junction or of the GlyR at CNS inhibitory synapses, respectively (reviewed in Froehner, 1993; Kuhse et al., 1995; Kirsch and Kröger, 1995). However, no sequence homology is found between these peripheral membrane proteins, suggesting that their role at the synapse may be different. While in muscle cultures rapsyn and acetylcholine receptors are diffusely distributed until the extracellular matrix protein agrin initiates their aggregation (Wallace, 1989), gephyrin accumulates subsynaptically in cultivated spinal neurons prior to incorporation of the GlyR into the postsynaptic membrane (Kirsch et al., 1993b). Antisense inhibition of gephyrin synthesis indicates that a preformed gephyrin matrix is essential for subsequent GlyR aggregation (Kirsch et al., 1993b). Thus, at central synapses, binding of the GlyR to gephyrin constitutes a crucial step in the formation of postsynaptic receptor clusters.

Binding of gephyrin to the GlyR had been inferred from the consistent copurification of both proteins from different vertebrate species (Pfeiffer et al., 1982; Graham et al., 1985). Here we have reconstituted this interaction by heterologous expression of gephyrin and GlyR polypeptides in 293 cells and demonstrate in overlay assays that gephyrin binds to the β subunit, but not the α subunits, of the GlyR. Previous work from our laboratory has shown that the β subunit modulates the pharmacological and conductance properties of the GlyR ion channel upon incorporation into recombinant receptors (Pribilla et al., 1992; Bormann et al., 1993; Rundström et al., 1994). This channel-modulatory function of the β subunit, which is determined by particular residues within and around the channel-forming M2 segment (Bormann et al., 1993), is extended here by a role of its cytoplasmic domain, i.e.,

anchoring of the GlyR to subsynaptic gephyrin. Based on the exclusive binding of gephyrin to the β subunit, we propose the β subunit as a GlyR component indispensable for the formation of postsynaptic receptor clusters. This is consistent with the α/β hetero-oligomeric structure previously established for the adult-type GlyR (Pfeiffer et al., 1982; Langosch et al., 1988) and more recently proposed for a subpopulation of embryonic GlyRs in spinal cord cultures (Walstrom and Hess, 1994).

### Gephyrin and $GABA_AR$s?

The in vitro results obtained here failed to demonstrate an interaction of gephyrin with affinity-purified $GABA_AR$ and its recombinant β subunits. However, our results cannot exclude binding of gephyrin to a minor $GABA_AR$ population, a low affinity binding not detectable under our assay conditions, or binding of $GABA_AR$s to yet unidentified gephyrin isoforms.

Immunocytochemical studies, in which gephyrin and GABA, the GABA-synthesizing marker enzyme glutamic acid decarboxylase, and/or $GABA_AR$ immunoreactivities were colocalized at central synapses, provide indirect evidence for gephyrin–$GABA_AR$ interactions (Triller et al., 1987; Bohlhalter et al., 1994; Sassoè-Pognetto et al., 1995; Cabot et al., 1995). Part of the data from synaptic profiles of rat spinal cord (Triller et al., 1987; Bohlhalter et al., 1994) may originate from synapses that use both glycine and GABA as cotransmitters (Todd et al., 1995), and consequently should have a postsynaptic receptor matrix containing both GlyRs and $GABA_AR$s. However, in the inner plexiform layer of the retina and in preganglionic sympathetic neurons of the intermediolateral column of spinal cord, $GABA_AR$-containing synapses staining for gephyrin are clearly devoid of GlyR α subunit immunoreactivity or glycinergic input (Sassoè-Pognetto et al., 1995; Cabot et al., 1995). Thus, gephyrin appears to be important for the localization of at least some $GABA_AR$ subtypes. Whether this interaction reflects a direct binding of gephyrin to particular $GABA_AR$ subunits or the presence of the GlyR β polypeptide in some $GABA_AR$ hetero-oligomers is presently unsolved. However, in view of the close phylogenetic distance between the GlyR and the $GABA_AR$ subunits, an incorporation of the GlyR β polypeptide into a subset of $GABA_AR$s appears conceivable. Indeed, the widespread expression of GlyR β subunit transcripts in rat brain exceeds the spatial distribution of all known GlyR α subunit mRNAs (Malosio et al., 1991) and matches the broad expression pattern of the gephyrin gene (Kirsch et al., 1993a; Kirsch and Betz, 1993).

### Primary Structure of the Gephyrin Binding Site

An overlay assay and GST fusion proteins allowed us to assign the gephyrin binding region of the GlyR β subunit to the large cytoplasmic loop located between transmembrane segments M3 and M4. A series of deletion constructs of this M3–M4 loop defined a 49 amino acid stretch as sufficient for binding gephyrin upon filter immobilization. Interestingly, the N-terminus of this binding segment is defined by the 5′-terminal region of exon 9 of the β subunit gene (Mühlhardt et al., 1994). A homologous sequence is lacking in the equivalent exons of the GlyR α subunit genes (Matzenbach et al., 1994), which results in their considerably shorter M3–M4 loops. Indeed, gephyrin did not bind to a GST fusion protein encompassing the M3–M4 loop of the neonatal α2 subunit (Kuhse et al., 1990a), a result that complements our failure to demonstrate gephyrin binding to the purified GlyR α1 subunit contained within adult-type GlyR. M3–M4 loops of similar length and limited sequence homology with the GlyR β subunit are found in $GABA_AR$ β1–β3 subunits. Corresponding GST fusion proteins, however, did not display gephyrin binding, which again emphasizes the specificity of the interaction seen with the GlyR β subunit.

Three lines of evidence indicate that the major binding determinants are contained within a central 18 amino acid segment of the gephyrin binding 49mer: a soluble purified fusion protein containing this 18 amino acid sequence inhibited gephyrin binding to both the entire GlyR β M3–M4 loop region and the 49 amino acid construct, albeit with a decreased efficacy as compared with the full-length 49mer sequence; mutational insertion of these 18 amino acid residues into the homologous position in the $GABA_AR$ β1 subunit created a gephyrin binding site detectable by our filter binding assay; and this same mutation led to a colocalization of the $GABA_AR$ with gephyrin upon coexpression in 293 cells. It is likely, therefore, that, within the structural context of the M3–M4 loop of the $GABA_AR$ β subunit, this GlyR β subunit 18mer adopts a conformation that allows gephyrin binding. On the other hand, functional complementation of this region by adjacent conserved residues of the $GABA_AR$ β subunit cannot be excluded. Notably, in the GlyR β subunit this central 18 amino acid segment of the gephyrin binding region is flanked by potential phosphorylation sites for both protein kinase C and tyrosine kinases (Grenningloh et al., 1990a) (see Figure 6A). Tyrosine phosphorylation of nicotinic acetylcholine receptor subunits parallels the aggregation of this receptor at the neuromuscular junction (Wallace et al., 1991). By analogy, usage of the GlyR β subunit phosphorylation sites may allow regulation of GlyR–gephyrin interactions in vivo, and thus determine the number of postsynaptic receptors docked to a subsynaptic gephyrin matrix. In conclusion, gephyrin–receptor interactions may be dynamic in vivo and thereby contribute to the developmental and functional plasticity of glycinergic synapses in the mammalian CNS.

## Experimental Procedures

### Heterologous Expression and Metabolic Labeling

The human embryonic kidney cell line 293 (ATCC #CRC 1573) was transfected with the gephyrin P1–P3 cDNAs, and cytosolic cell fractions were prepared as described previously (Prior et al., 1992). For metabolic labeling, cells were washed 24 hr after transfection in methionine-deficient medium, and incubation in the presence of [$^{35}$S]methionine (100–200 µCi per 5 ml; Amersham) was continued for another 12 hr. For the column binding assay, the cytosolic fraction was dialyzed against 25 mM $KP_i$ (pH 7.4), 100 mM KCl, 5 mM EDTA, 5 mM β-mercaptoethanol and a cocktail of protease inhibitors (Pfeiffer et al., 1982) before adding a stock solution of Triton X-100 to a final concentration of 0.1% (v/v).

**Column Binding Assay**

Rat spinal cord membranes were solubilized using 1% (w/v) sodium cholate, and the centrifuged detergent extract was loaded onto an aminostrychnine–agarose column as described (Graham et al., 1985). The column was washed with 10 bed volumes of solubilization buffer including 0.18% (w/v) phosphatidylcholine followed by 10 volumes of buffer A (25 mM $KP_i$ [pH 7.4], 1 M KCl, 1% (v/v) Triton X-100, 0.18% (w/v) phosphatidylcholine, and protease inhibitors as above). The column was then reloaded by circulating 10–15 bed volumes of dialyzed cytosolic fraction prepared from 293 cells transfected with the gephyrin P1 cDNA through the resin for 12 hr. After washing with 20 bed volumes of buffer A, GlyR–gephyrin complexes were then isolated by recycling 4 bed volumes of 100 mM glycine in buffer A through the column (Pfeiffer et al., 1982).

$GABA_AR$ was solubilized from rat brain membranes using 0.5% (w/v) deoxycholate and isolated on a benzodiazepine Ro7/1986-1–agarose column (Sigel and Barnard, 1984; Stephenson, 1990). After receptor binding, the column was washed with 20 bed volumes of buffer B (10 mM $KP_i$ [pH 7.4], 200 mM KCl, 10% (w/v) sucrose, 0.2% (v/v) Triton X-100, 0.18% (w/v) phosphatidylcholine, and protease inhibitors as above) and probed with recombinant gephyrin by recirculating 100 μl of a cytosolic fraction from [$^{35}$S]methionine-labeled 293 cells transfected with the gephyrin cDNAs in 3 bed volumes of buffer B through the resin for 12 hr. After washing with 20 volumes of buffer B, $GABA_AR$ was cycle-eluted with 4 volumes of 10 mM clorazepate in buffer B. All chromatographic steps were carried out at 4°C.

Eluted proteins were precipitated by trichloracetic acid and subjected to 10% SDS–PAGE (Laemmli, 1970). Polypeptides were visualized by silver staining (Merril et al., 1982) or transferred onto nitrocellulose for immunoblotting as described (Schmitt et al., 1987).

**Fusion Protein Construction and Expression**

Fragments of the GlyR β (Grenningloh et al., 1990a) and α2 (Grenningloh et al., 199b) subunit cDNAs and of the $GABA_AR$ β1, β2, and β3 (Ymer et al., 1989) subunit cDNAs were isolated by PCR, performing 25 cycles of 1 min at 92°C, 45 s at 50°C–55°C, and 45 s at 74°C, respectively. Sense primers contained a BamHI restriction site 5′-CGCGCGGATCCTG-3′ upstream, and antisense primers contained an EcoRI restriction site 5′-CGCGAATTC-3′ downstream of the complementary bases of the respective rat cDNA sequences encompassing the following positions (Grenningloh et al., 1990a; Kuhse et al., 1990a): GST-123 (GlyR β sense 1286–1303/antisense 1637–1654), GST-81 (GlyR α2 1546–1563/1771–1788), GST-49 (GlyR β 1421–1438/1550–1567), GST-22 (GlyR β 1421–1438/1469–1486), GST-34 (GlyR β 1421–1438/1505–1522), GST-33 (GlyR β 1469–1486/1550–1567), and GST-18 (GlyR β 1469–1486/1505–1522). For all three $GABA_AR$ β subunits, degenerated sense (5′-CGCGCGGATCCTGAT(C/T)TTCTT-(C/T)GG(A/G)(A/C)(A/G)AGG(A/C/T)CC(C/T)CA-3′) and antisense (5′-CCGGAATTC(A/G)TTCAC(A/G)TC(A/G)GT(C/T)A(A/G)(A/G)TC(A/G)GGGAT-3′) primers complementary to the sequence positions 1059–1082 and 1408–1430, respectively, of the $GABA_AR$ β1 subunit cDNA were used (Ymer et al., 1989). Fragments were cloned into the BamHI and EcoRI sites of the pGEX-5X-1 vector (Pharmacia), and all constructs were confirmed by automated dideoxysequencing (Applied Biosystems). The $GABA_AR$ constructs showed sequence divergence in the region corresponding to the degenerated primers, however, without creating a stop codon. The recombinant DNAs were electrotransformed into JM109 cells (Stratagene). Protein production was induced by 1 mM isopropyl-β-D-thiogalactopyranoside for 1–3 hr at 37°C. Cells were washed with PBS and lysed in SDS-loading buffer, or the GST fusion proteins were purified on glutathione Sepharose-4B according to the manufacturer's instructions (Pharmacia). Protein amounts were determined after SDS–PAGE and Coomassie blue staining by densitometry in a Hirschmann Elscript 400 densitometer using ovalbumin (Sigma) as a standard.

Mutant 19 was constructed by oligonucleotide-directed mutagenesis of single-stranded DNA derived from the original $GABA_AR$ β1 construct (Bio-Rad T7 MutaGene kit). The mutagenic oligonucleotide (antisense) was 5′-GAAGCCCTCACGGCTGTTCGATAGTTCGAAGT-CCCGGGGGAGGGAGCCGACGATTGAGAAATCATTGGACCTGG-GGTCACTTACACC-3′. The mutagenized fragment was amplified and subcloned into pGEX-5X-1 as described above.

**Overlay Assay**

Purified GlyR (Pfeiffer et al., 1982), bacterial lysates, or purified GST fusion proteins were separated by SDS–PAGE on 10% or 15% gels and transferred to nitrocellulose filters (Kyhse-Andersen, 1984). After incubation for 10 min in buffer C (10 mM HEPES–KOH [pH 7.5], 60 mM KCl, 1 mM EDTA, 1 mM β-mercaptoethanol), the filters were incubated in 6 M guanidine–HCl in buffer C followed by six buffer changes in which the guanidine–HCl concentration was sequentially reduced to 50% for 10 min each. Afterwards, the filters were blocked by 5% (w/v) followed by 1% (w/v) nonfat milk powder in buffer C for 1 hr each. Filters were probed with 200–800 μl of [$^{35}$S]methionine-labeled cytosol of 293 cells, which had been transfected with the gephyrin P1 cDNA, in 2–6 ml of buffer C for 12 hr and then washed three times with buffer C. All incubations were performed at 4°C. Filters were exposed to Kodak X-Omat AR film for 12–36 hr. For competition experiments, purified GST or GST fusion proteins (in 50 mM Tris–HCl [pH 8.0], 10 mM glutathione) were added to the radioactive cytosol fractions in concentrations specified in the respective figure legends.

**Coexpression Experiments and Confocal Microscopy**

For subcellular distribution studies, 293 cells were cotransfected with cDNAs encoding GlyR or $GABA_AR$ α and β subunits and/or gephyrin. Immunolabeling and subsequent analysis with a confocal microscope (Molecular Dynamics) was performed as described (Kirsch and Betz, 1995). MAbs 4a and 5a, used for the immunodetection of GlyR subunits or gephyrin, respectively, have been characterized previously (Pfeiffer et al., 1984; Triller et al., 1985; Kirsch and Betz, 1993). In double-labeling experiments, gephyrin was immunolabeled by a monospecific polyclonal antibody generated against a C-terminal fusion protein encompassing residues I447–D652 (J. K., J. Kuhse, and H. B., submitted).

An antiserum directed against a $GABA_AR$ α1 subunit-specific specific epitope (Fritschy et al., 1992) was a gift from Dr. J.-M. Fritschy, University of Zürich.

**Acknowledgments**

We thank A. Herbold and J. Lorentz for expert technical assistance with mutagenesis and tissue culture, Dr. A. Stephenson for providing the Ro7/1986-1–agarose, Dr. J. M. Fritsch for the $GABA_AR$ α1 subunit-specific antibody, and Drs. P. Seeburg and H. Lüddens for $GABA_AR$ β subunit cDNAs. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 169 and Schwerpunkt "Funktionelle Domänen") and the Fonds der Chemischen Industrie. J. K. and D. L. are supported by Heisenberg Fellowships of the Deutsche Forschungsgemeinschaft.

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Received February 13, 1995; revised June 27, 1995.

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