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CLONING OF THE cDNA FOR A BRAIN GLYCINE-, GLUTAMATE- AND THIENYLCYCLOHEXYLPIPERIDINE-BINDING PROTEIN

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Summary. Polyclonal antibodies (Ab's) were raised against a 43-kDa component of a protein complex that has ligand recognition sites similar to those of brain *N*-methyl-D-aspartate (NMDA) receptors. The Ab's were used to immunopurify from brain synaptic membranes a 60-kDa glycine (Gly), glutamate (Glu) and thienylcyclohexylpiperidine (TCP)-binding protein and to screen a rat hippocampal cDNA expression library. A 1.85-kb clone, pGlyBP, coding for a protein of 470 amino acids (52.7 kDa) was identified. Northern blot analyses performed on poly(A$^+$) RNA from brain revealed hybridization of the labeled cDNA probes to transcripts of 1.9 kb. *E. coli* transformed with the pGyBP expressed a protein that was recognized by the anti-43 kDa Ab's and had recognition sites for Gly, Glu and TCP. The cloned protein has 2 glycosylation sites, 3 hydrophobic domains, 4 cysteine-rich motifs (**C**-X_2-**C**-X_{16-20}-**C**-X_{5-11}), and 2 regions homologous to the NR1 receptor protein.

A group of proteins purified from brain synaptic menbranes have ligand binding sites for agonists, antagonists, and ion channel modulators of neuronal NMDA receptors (1). The role of this complex of proteins in neuronal function is still not completely defined. Because of the importance of glutamate (Glu) receptors in normal neuronal development and synaptic plasticity as well as in pathological states in the nervous system (e.g. 2-6) warrants the pursuit of more complete knowledge about the structure and function of the proteins associated with the isolated complex. Two proteins in the complex were previously isolated, defined in terms of predominant ligand-binding sites associated with each protein, and shown to be membrane glycoproteins with distinct immunochemical identities (7-10). These are the Glu-binding (7,8) and the carboxypiperazinylphosphonic acid (CPP)-binding protein (9,10). The cDNA for the Glu binding protein has a sequence that is not homologous to any other proteins (11), including previously cloned Glu receptors, Glu-metabolizing enzymes, or Glu transporters. Present efforts were focused on cloning the cDNA for another protein in the previously isolated complex, a protein identified on the basis of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as a 40-43 kDa component of the complex (1).

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METHODS

Antiserum to the ~43 kDa Protein of the Complex Isolated from Brain Synaptic Membranes—Synaptic membrane proteins were solubilized and chromatographed on an L-Glu-derivatized ReactiGel matrix as described previously (1). The fraction eluted from the column by the introduction of 30 mM KCl was enriched in the ~43 kDa protein and was used for the immunization of rabbits following electroelution of individual proteins from SDS-PAGE. Antibody titers were estimated by ELISA and the specificity of the antibodies was determined by immunoblots (8,10).

Immunopurification of Synaptic Membrane Proteins Recognized by the Anti-43 kDa Ab's—Immune IgGs were purified and coupled to ReactiGel (Pierce Chemical) (8). Synaptic membranes were solubilized in 10 mM potassium phosphate buffer, pH 7.4, containing 1% Triton X-100, 0.5% *n*-octylglucoside, 10% glycerol, 10 mM ε-aminocaproic acid, 0.1 mM EGTA, 0.1 mM benzamide, and 10 mg/ml of a phosphatidylethanolamine:cholesterol mixture (4:1). Solubilized proteins were applied to the IgG-ReactiGel columns (0.7 x 3 cm), the columns were washed extensively with solubilization buffer in the absence of any detergents but in the presence of 200 mM KCl, and the proteins bound to the matrix were specifically eluted with 1.0 M propionic acid, pH 3.0, and collected in tubes containing 0.5 ml of 0.5 M Tris-HCl buffer, pH 10.

Measurement of [^{3}H]Gly, [^{3}H]TCP, and L-[^{3}H]Glu Binding to the Isolated Proteins—The binding of [^{3}H]Gly, [^{3}H]TCP and L-[^{3}H]Glu was determined by the polyethyleneglycol–γ-globulin precipitation procedure (1,7,9). The final concentrations of [^{3}H]Gly and L-[^{3}H]Glu in each assay were 100 nM (unless otherwise stated) and those of [^{3}H]TCP were 50 nM. The final concentration of the displacing ligands used to define non-specific ligand binding was 100 μM and that of agents used to activate the [^{3}H]TCP binding entities was 50 μM, unless indicated otherwise. Specific [^{3}H]Gly binding was defined as the difference between the binding of Gly to the respective protein preparation in the absence of 100 μM Gly or D-serine minus that in the presence of the displacing ligands. When [^{3}H]Gly was used as the ligand, the samples were pretreated with 100 μM strychnine in order to prevent glycine binding to strychnine-sensitive sites (1). L-Glu (100 μM) was used to define the non-specific binding of L-[^{3}H]Glu, and 100 μM dizocilpine (MK-801) to define the non-specific binding of [^{3}H]TCP (1). All data were analyzed as described previously (1,7,9).

Screening of Hippocampal cDNA Libraries with the Anti-43 kDa Ab's—A hippocampal cDNA expression library harbored in the phage λZap (provided by Dr. J. Boulter, Salk Institute) and plated on *E. coli* XL-1 Blue strain was screened using the anti-43 kDa Ab's according to procedures described previously (11). A 1:500 dilution of the anti-43 kDa antiserum (pre-reacted with proteins from *E. coli* transformed with non-recombinant λZap) was used in screening the library. Phages from positive plaques were replated and rescreened with the same antiserum until a homogeneous population of immunopositive recombinant phages was obtained (4 re-screenings). Recombinant phage DNA was isolated and the phage with the largest insert (~1.8 kb) was subcloned into pBS (Stratagene) by manual excision. The phagemid harboring this cDNA insert, pGlyBP, was used to transform *E. coli* (DH5α strain, Clontech).

cDNA Sequencing, Northern Blot Hybridizations, and Protein Expression in Bacteria—The cDNA insert of the phagemid pGlyBP was sequenced on both strands (11). The Seqaid software package was used to analyze the sequence data and the BLAST program to search the NCBI gene bank for possible homologies. Isolation of total RNA from various tissues and brain regions and the Northern blot hybridization reactions were carried out as described previously (11). Poly(A$^+$)-RNA from each population of total RNA was prepared using an oligo-dT-cellulose column (12). A PstI fragment of the pGlyBP insert (0.8 kb) in pBS vector was purified, labeled with [^{32}P]-dATP by the random primer labeling method (total activity of 5.2 x 10^9 dpm in 15 ml hybridization buffer), and hybridized to the RNA transfer blots.

E. coli were transformed with either pGlyBP or pBS vector only. A 40 ml culture of transformed *E. coli* was grown in LB medium that contained ampicillin and induced with 2.5 mM IPTG for 1.5–2 h at 37 °C. *E. coli* cells were harvested by centrifugation, homogenized on ice and subjected to 30 s periods of sonication in a bath sonicator, and the proteins extracted in buffer that contained either 1% (v/v) Triton X-100 and 0.1% (v/v) Tween-20 or 2% (v/v) Triton X-114 in 10 mM potassium phosphate, pH 7.4. The suspension was transferred to microfuge tubes (1.5 ml) and centrifuged at 28,300 x *g* for 45 min at 4 °C and the supernatant was aspirated and its volume doubled to 3–4 ml by the addition of deionized, distilled H_2O. This extract was passed through BioBeads SM-2 (BioRad) to remove excess detergent associated with the proteins (13) or applied to a PharmaLink matrix (Pierce) derivatized with 5,7-dichlorokynurenic acid (5,7-DCK) (14).

RESULTS AND DISCUSSION

Reaction of the anti-43 kDa Ab's with a 60 kDa Protein and Its Immunopurification from Synaptic Membranes— Antiserum obtained following immunization of rabbits with the ~43 kDa protein reacted selectively in ELISA assays with as little as 16 ng of the proteins in the partially purified complex and dilutions of antiserum greater than 1:1000 produced positive reactions with 1 µg or less of the proteins in the complex (15). The antiserum strogly labeled a synaptic membrane protein that had an estimated size of 60 kDa (Fig. *1A*). Prolonged storage of synaptic membranes at 4 °C led to an increased number of protein bands of molecular size smaller than 60 kDa that were labeled by the antiserum on immunoblots. This indicated that the protein of 43 kDa against which the antiserum was raised was probably a degradation product of the 60 kDa protein. Immunopurification of solubilized synaptic membrane proteins on ReactiGel matrices derivatized with the IgG fraction of the antiserum led to the isolation of a 60 kDa protein (Fig. *1B*). The eluted protein was recognized by the anti-43 kDa anti-serum (Fig. *1B*) and was also labeled by concanavalin A, an indication that it represented a glycosylated protein (Fig. *1B*).

The immunopurified 60 kDa protein exhibited two types of ligand binding activities, strychnine-insensitive but D-serine-sensitive [^{3}H]Gly binding, and L-Glu- and Gly-activated MK-801-sensitive [^{3}H]TCP binding activity. The D-serine-sensitive [^{3}H]Gly (100 nM) binding to the purified protein was equal to 32.46 ± 6.92 (S.E.M., n = 3) pmol/mg protein. MK-801-sensitive [^{3}H]TCP (50 nM) binding

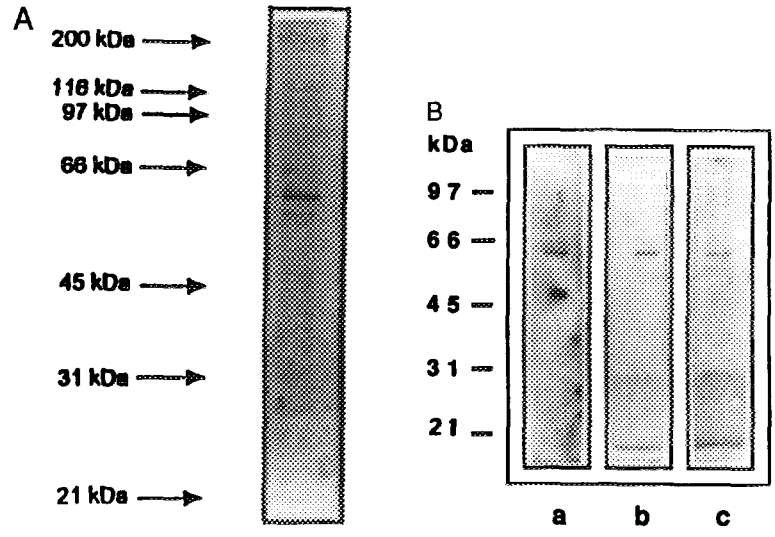

FIG. 1. **Immune labeling by the anti-43-kDa antiserum of a 60-kDa protein in synaptic membranes and the purification of a 60-kDa protein by immunoaffinity chromatography.** *A*, immune labeling of synaptic membrane proteins (12 µg) with the antiserum to the 43 kDa. The molecular sizes of standard proteins are shown on the left. *B*, purity, immunochemical reactivity, and reactivity with concanavalin A of the protein fraction isolated through immunoaffinity chromatography on a ReactiGel matrix derivatized with the anti-43-kDa antibodies (2 µg loaded per lane). Following SDS-PAGE and electrotransfer onto a nitrocellulose filter, each of the lanes on the filter was cut and reacted either with Aurodye (*lane a*), or with antiserum to the 43-kDa protein (*lane b*), or with concanavalin A-biotin (*lane c*).

to the immunopurified protein was not detectable unless the protein was pre-incubated with L-Glu and Gly. In the presence of 500 μM L-Glu and 100 μM Gly, [^{3}H]TCP binding was equal to 35.17 ± 6.8 (S.E.M., n = 3) pmol/mg protein. Preliminary studies performed with the immunopurified 60 kDa protein indicated that this protein lost much of its ligand binding activity during exposure to propionic acid. Nevertheless, results of the immunochemical studies indicated that the 43 kDa component of the previously identified complex was a proteolytic fragment of a 60 kDa synaptic membrane glycoprotein that had Gly, L-Glu, and TCP recognition sites.

Cloning of the cDNA for a Protein Recognized by the Anti-43 kDa Ab's— From 600,000 phage plaques of a hippocampal cDNA library screened, 11 were identified as expressing a protein that was immunoreactive with the antiserum. The sequence of the clone with the longest cDNA insert (1,851 base pairs) is shown in Fig. *2*. This clone had one major open reading frame which coded for a protein of 470 amino acids and a molecular size of 52.7 kDa (Fig. *2*). The protein has no significant homology to any Glu or Gly receptors, enzymes, or transport proteins and has only a 20% identity to the previously cloned Glu-binding protein. Despite the low level of homology between this protein and the NMDA receptor protein NR1 (11% homology), there were two regions of relatively high homology (40%) between these two proteins (Fig. *3A*). These two regions in NR1, and especially the five residues indicated in Fig. *3A*, have been identified as being important in maintaining Gly binding and activation of the receptor-ion channels formed by NR1 homomeric expression (16). Four identical residues are present in the homologous regions of GlyBP, an indication that these regions may also contribute to the formation of a Gly binding site in GlyBP.

The major hydrophobic domains in the cloned pGlyBP were confined to the NH_2-terminal of the protein (Fig. *3B*). One of these was within the first 23 amino acids of the protein, a region that has some of the characteristics of a signal peptide. The structure of the protein within the second and third hydrophobic regions was predicted by the Chou-Fasman calculations to be primarily that of an α-helical (residues 41–61) and a β sheet (residues 96–106) conformation. Two canonical *N*-glycosylation sites were identified in the sequence beyond the third hydrophobic domain (Fig. *2*), indicating that this region may be in the extracellular domain of cell membranes. Four repeats of the structure **C**-X_2-**C**-X_{16-20}-**C**-X_{5-11} were identified starting with residue 308 and terminating at 434 Fig. *2*). The C-X_2-C initial sequence of each repeat was followed by a domain of 16 to 20 amino acids that contained several negatively charged residues, another cysteine, and a 5 to 11 amino acid domain with one or more positively charged residues. The first C-X_2-C sequence was preceded by a proline-rich region (residues 285–299), possibly representing a hinge or folded region of the protein. The predicted structure of the protein around the C-X_2-C sequence of each repeat as well as in the regions between the other cysteine residues was that of multiple turns. The structure of the repeats in the GlyBP was similar, but not identical, to those of the Zn (or Fe)-binding motifs of LIM and RING finger proteins (17,18,19,20), the Zn and Cd-binding motifs of variable surface proteins of *Giardia lamblia* (21), or the Fe-binding domains of ferredoxin (22).

cDNA probes of the pGlyBP clone hybridized with a 1.9 kb poly(A$^+$)-RNA from brain (Fig. *3C*). Heart and liver mRNA exhibited low but detectable levels of hybridization with the pGlyBP probe (Fig.

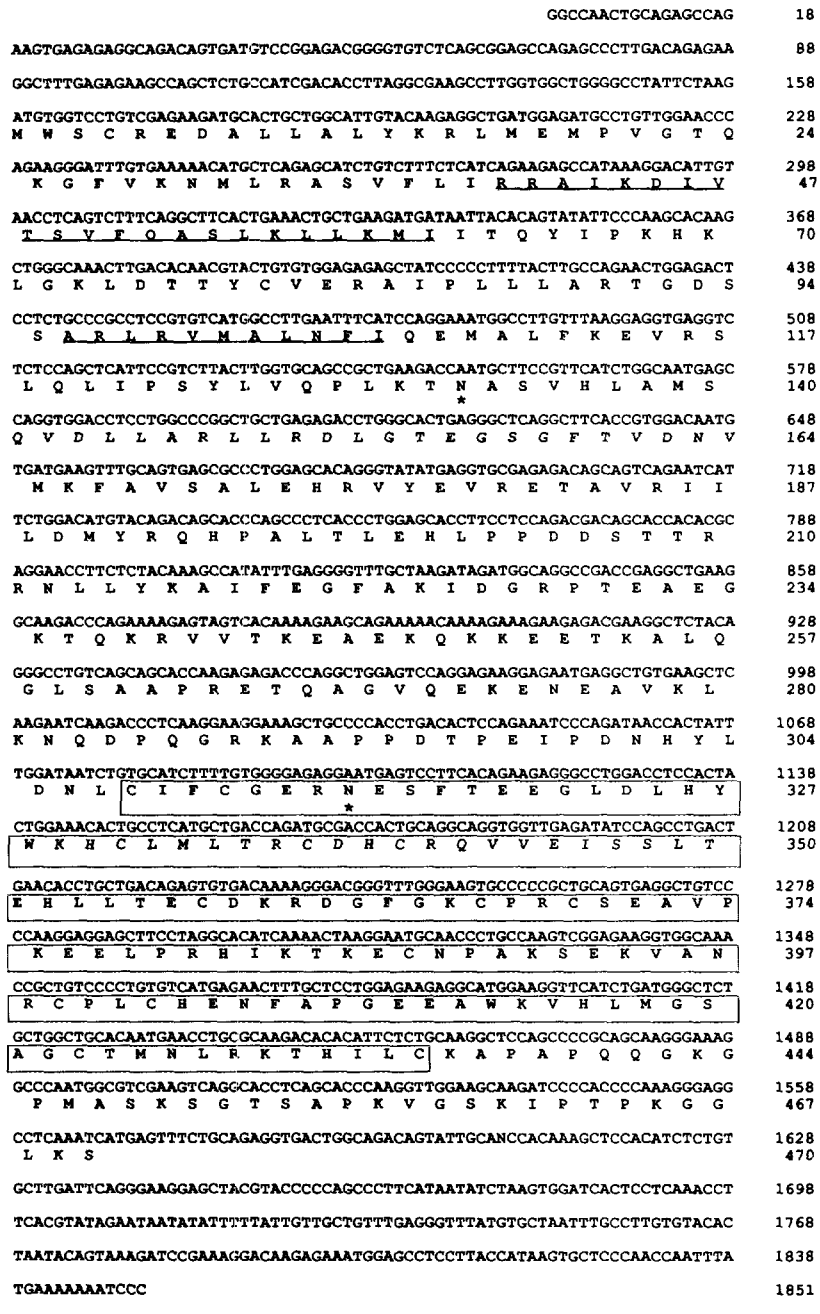

FIG. 2. **Nucleotide sequence of pGlyBP and deduced amino acid sequence of the protein.** The hydrophobic domains of this protein are underlined and the consensus *N*-glycosylation sites are marked by an asterisk. The four cysteine-rich repeats near the C-terminal of the protein are boxed.

3C), but the size of the mRNA's labeled differed from that of brain mRNA. The relative strength of hybridization of the pGlyBP probe with samples of total RNA from the cerebral cortex, hippocampus, cerebellum, and brainstem indicated fairly widespread expression of the cognate mRNA in rat brain (Fig. *3D*).

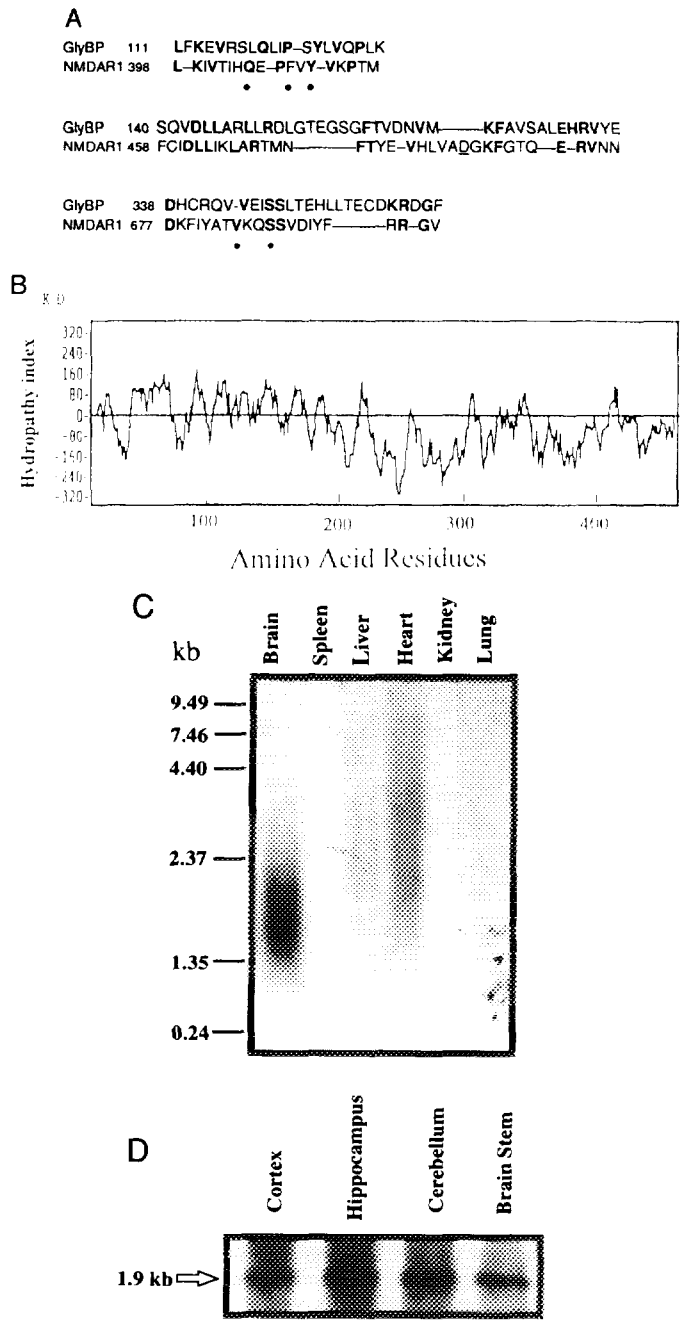

FIG. 3. **Homologous regions between GlyBP and NR1, hydropathy analysis of GlyBP, and Northern blot analysis of mRNA in brain and other tissues.** *A*, comparison of the sequence of GlyBP with that of NR1. The key amino acids for glycine activation of NR1 are indicated by a dot. Residues that are identical between the two sequences are shown in bold letters. *B*, the Seqaid DNA/protein analysis program and a window of 15 residues were used for the analysis of the hydrophobicity of the GlyBP according to procedures described previously (7). *C*, Northern blot analysis of 3-μg aliquots of poly(A$^+$)-RNA extracted from various tissues. A [^{32}P]-labeled, 0.8-kb PstI restriction fragment of the pGlyBP cDNA was used as the probe. *D*, Northern blot analysis of 10-μg aliquots of total RNA extracted from different regions of rat brain.

Ligand-binding Characteristics of the Cloned and Expressed Protein— In extracts of proteins from *E. coli* transformed with pGlyBP, a doublet of 58 and 61 kDa proteins was strongly labeled by the anti-43 kDa Ab's (Fig. *4A*). Proteins extracted from *E. coli* transformed with pGlyBP and induced with IPTG had binding sites for both [^{3}H]Gly and [^{3}H]TCP (Table I). The interaction of glycine with these proteins was not sensitive to strychnine but was partially inhibited by other ligands that bind to the glycine sites of NMDA receptors, *i.e.* **R**-(+)-HA-966 and 5,7-DCK (Table I). The protein extracts had low [^{3}H]TCP-binding activity when such binding was measured in the absence of either L-Glu or Gly,

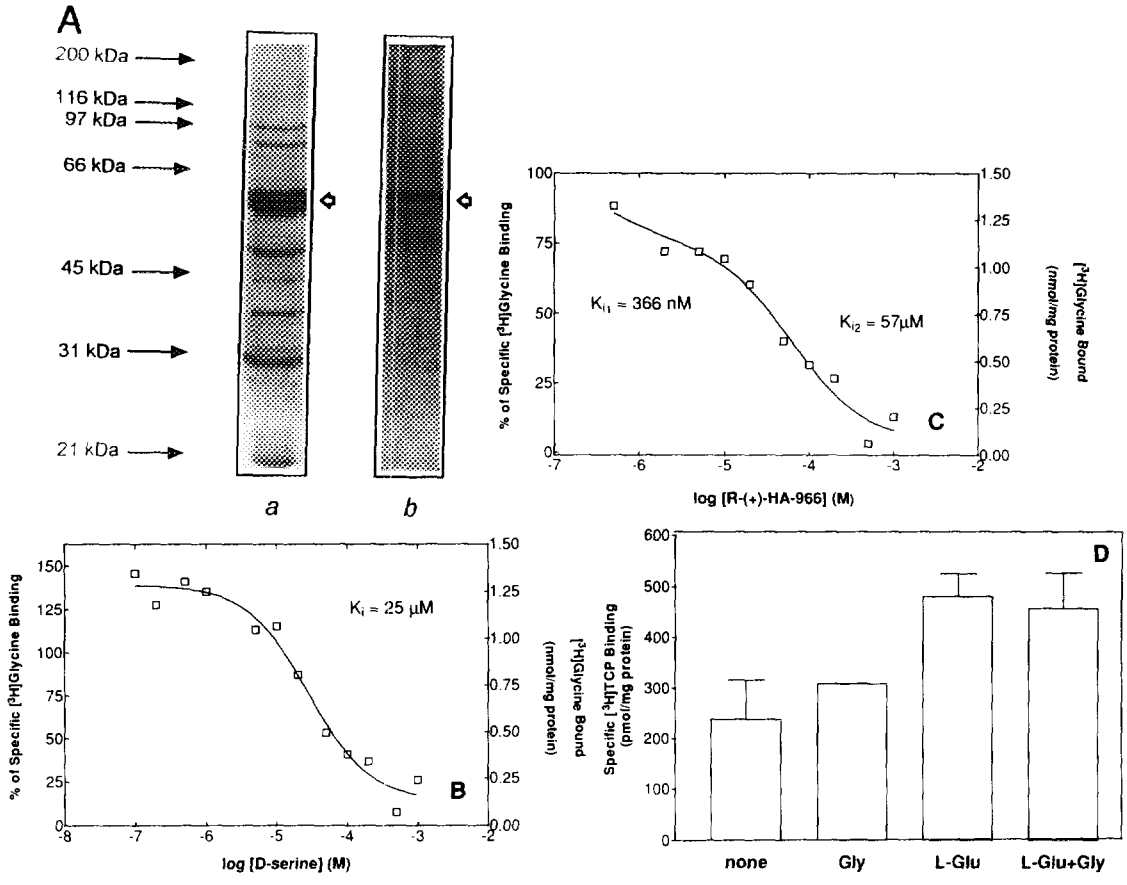

FIG. 4. **Immune labeling with the anti-43-kDa antiserum of proteins expressed in *E. coli* transformed with the pGlyBP plasmid and characterization of glycine and TCP binding to the proteins expressed in *E. coli*.** *A*, immune labeling by the anti-43-kDa antiserum of the proteins in the soluble extract (*lane a*) and the proteins purified by 5,7-DCK chromatography, i.e., the fraction eluted by the introduction of 1 mM D-serine/NMDA. This fraction was extensively dialyzed against H_2O at 4 °C, precipitated with 10% (w/v) TCA, and a 6-µg aliquot was loaded onto the electrophoresis gel. *B and C*, displacement by D-serine and **R**-(+)-HA-966 of [^{3}H]Gly (100 nM) bound to the partially purified proteins. The same protein fraction as described in *A* was extensively dialyzed and used in these binding studies. Each point is the mean of triplicate determinations and the curve shown was fitted to the data (1,8). For **R**-(+)-HA-966, optimal fitting was achieved under the assumption that HA-966 interacted competitively with two sites. *D*, activation by L-Glu, Gly, or both of the MK-801-sensitive [^{3}H]TCP binding to the same protein fraction described above. Activation of TCP binding was achieved by preincubating the proteins for 30 min with 50 µM Gly, or 50 µM L-Glu, or 50 µM of both. Each value is the mean (± S.D.) from 2–5 preparations of proteins.

Table I [^{3}H]Gly and [^{3}H]TCP binding to solubilized proteins extracted from *E. coli* transformed with the pGlyBP plasmid and induced with IPTG

Radioligand	Competing Ligand (100 µM)	Activating Ligand (50 µM)	Ligand-binding Activity (pmol/mg protein)	n
[^{3}H]Glycine	none		4.3 ± 1.2	3
	HA-966		1.9 ± 0.8	3
	5,7-DCK		2.3 ± 1.7	3
[^{3}H]TCP		none	0.5	2
		L-Glu	17.6	2
		Gly	10.5	2
		L-Glu + Gly	12.5	2

Strychnine-insensitive [^{3}H]Gly (100 nM) and MK-801-sensitive [^{3}H]TCP (50 nM) binding to the proteins were measured as described in "Methods". The values for [^{3}H]Gly binding are the mean ± SEM for the determinations shown (n).

but [^{3}H]TCP binding was markedly enhanced when either Glu or Gly was present (Table I). There was no evidence of glycine or TCP-binding entities in protein extracts obtained from *E. coli* transformed with pBS vector without the 1.85 kb insert.

The synaptic membrane 60 kDa protein has been highly enriched by chromatographic separation of solubilized membrane proteins on 5,7-DCK-derivatized matrices (14). When the bacterial proteins extracted with Triton X-114 were chromatographed on a 5,7-DCK PharmaLink matrix, an ~60 kDa protein was highly enriched in the fraction eluted by the introduction of a solution containing 1 mM D-serine-1 mM NMDA and this protein was strongly labeled by the anti-43 kDa Ab's (Fig. *4A*). Proteins isolated by 5,7-DCK chromatography exhibited high [^{3}H]Gly binding capacity (B_{max} = 62 nmol/mg protein) and two K_D values for glycine binding to these proteins (K_{D1} = 647 nM and K_{D2} = 20 µM) that were similar to those of the isolated brain protein (K_{D1} = 200 - 400 nM and K_{D2} = 9 -34 µM) (14). Low concentrations of the glycine agonist D-serine caused activation and high concentrations inhibited Gly binding to the expressed and partially purified proteins (Fig. *4B*). The antagonist of Gly sites of NMDA receptors, **R**-(+)-HA-966, inhibited Gly binding to the same proteins by interacting with high (K_i = 366 nM) and low (K_i = 57 µM) affinity recognition sites (Fig. *4C*). The same proteins also contained MK-801-sensitive [^{3}H]TCP-binding sites that were activated by the introduction of 50 µM of either Gly or L-Glu or both (Fig. *4D*). These observations indicate that the cloned GlyBP is a new form of a Gly-, L-Glu-, and TCP-binding protein, i.e. a protein that has some of the ligand binding sites associated with NMDA receptors as well as the complex that contains the Glu-binding and CPP-binding proteins (Kumar et al., 1994). The role of this newly identified Glu, Gly and TCP-binding protein in neuronal function is currently being investigated.

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REFERENCES

1. Kumar, K. N., Babcock, K. K., Johnson, P. S., Chen, X., Eggeman, K. T., and Michaelis, E. K. (1994) *J. Biol. Chem.* **269,** 27384–27393
2. Balazs, R., Jorgensen, O. S., and Hack, N. (1987) *Neurosci.* **27,** 437–451
3. Mattson, M. P. (1988) *Brain Res. Rev.* **13,** 179–212
4. Cotman, C. W., and Monaghan, D. T. (1987) in *Psychopharmacology: The Third Generation of Progress* (Metzler, H. R., Coyle, J. T., Bunney, W. E., Kopin, I. J., and Davis, K. L., eds.) pp. 197–210, Raven Press, New York.
5. Meldrum, B. (1985) *Clin. Sci.* **68,** 113–122
6. Choi, D. W. (1992) *Science* **258,** 241–243
7. Chen, J.-W., Cunningham, M. D., Galton, N. and Michaelis, E. K. (1988) *J. Biol. Chem.* **263,** 417–427
8. Eaton, M. J., Chen, J.-W., Kumar, K .N., Cong, Y. and Michaelis, E. K. (1990) *J. Biol. Chem.* **265,** 16195–16204
9. Cunningham, M. D., and Michaelis, E. K. (1990) *J. Biol. Chem.* **265,** 7768–7778
10. Eggeman, K. T., Pal, R., Walsh, J., Kumar, K. N., and Michaelis, E. K. (1993) *Neurosci. Lett.* **158,** 173–176
11. Kumar, K. N., Tilakaratne, N., Johnson, P. S., Allen, A. E., and Michaelis, E. K. (1991) *Nature* **354,** 70–73
12. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13. Ly, A. M., and Michaelis, E. K. (1991) *Biochemistry* **30,** 4307–4316
14. Babcock, K.A., Eggeman, K.T., Chen, X., and Michaelis, E.K. (1994) *Neuroscience Abs.* **20**, 463
15. Eggeman, K.T. (1992) Ph.D. Dissertation, University of Kansas
16. Kuryatov, A., Laube, B., Betz, H., and Kuhse, J. (1994) *Neuron* **12**, 1291-1300
17. Schirmer, T., Keller, T. A., Wang, Y-F., and Rosenbusch, J. P. (1995) *Science* **267**, 512–514
18. Hofnung, M. (1995) *Science* **267**, 473–474
19. Li, M., Reichert, J., Freyd, G., Horvitz, H. R., and Walsh, C. (1991) *Proc. Nat. Acad. Sci. (USA)* **88,** 9210–9213
20. Michelsen, J. W., Schmeichel, K. L., Beckerle, M. C., and Winge, D. R. (1993) *Proc. Nat. Acad. Sci. (USA)* **90,** 4404–4408
21. Nash, T.E. and Mowatt, M.R. (1993) *Proc. Nat. Acad. Sci. (USA)* **90,** 5489-5493
22. Lovering, R., Hanson, I. M., Borden, K. L. B., Martin, S., O'Reilly, N. J., Evan, G. I., Rahman, D., Papin, D. J. C., Trowsdale, J., and Freemont, P. S. (1993) *Proc. Nat. Acad. Sci. (USA)* **90,** 2112–2116