

Subunit Selectivity and Epitope Characterization of mAbs Directed against the GABA_A/Benzodiazepine Receptor

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Abstract. mAbs bd 17, bd 24, and bd 28 raised against bovine cerebral γ -aminobutyric acid (GABA_A)/benzodiazepine receptors were analyzed for their ability to detect each of 12 GABA_A receptor subunits expressed in cultured mammalian cells. Results showed that mAb bd 17 recognizes epitopes on both β_2 and β_3 subunits while mAb bd 24 is selective for the α_1 subunit of human and bovine, but not of rat origin.

The latter antibody reacts with the rat α_1 subunit carrying an engineered Leu at position four, documenting the first epitope mapping of a GABA_A receptor subunit-specific mAb. In contrast to mAbs bd 17 and bd 24, mAb bd 28 reacts with all GABA_A receptor subunits tested but not with a glycine receptor subunit, suggesting the presence of shared epitopes on subunits of GABA-gated chloride channels.

γ -AMINO BUTYRIC ACID (GABA)¹ is the major inhibitory neurotransmitter in the mammalian central nervous system and gates a chloride-conducting channel intrinsic to the GABA_A receptor. GABA-gated channel activity leading to neuronal hyperpolarization can be modulated by a variety of compounds, e.g., benzodiazepines and barbiturates, some of which are in clinical use (for review, see Olsen and Venter, 1986). The GABA_A receptor complex has been purified from several species (Sigel et al., 1983; Schoch et al., 1984; Sweetnam et al., 1987; Barnard et al., 1987; Bureau and Olsen, 1988) by affinity chromatography using the immobilized benzodiazepine Ro7-1986/1. This receptor is resolved by gel electrophoresis into two major bands with M_r of 50 (termed α) and 55 kD (termed β), as well as several minor bands, up to an M_r of \sim 59 kD (Fuchs et al., 1988).

The molecular cloning of cDNAs encoding GABA_A receptor subunits showed that these subunits share sequence similarities with subunits of both the glycine receptor (Grenningloh et al., 1987) and the nicotinic acetylcholine receptor (Schofield et al., 1987), indicating that GABA_A receptors may well consist of a pentameric arrangement of subunits (for review, see Unwin, 1989). Although these cloning studies have revealed the existence of a large family of GABA_A receptor subunits, suggesting extensive receptor heterogeneity, the exact subunit composition and stoichiometry of a natural GABA_A receptor has yet to be determined. In particular, several variants of both the α and β subunit classes are sequence characterized (Levitan et al., 1988; Ymer et al., 1989 *a, b*; Pritchett and Seeburg, 1990), and the existence of members of novel subunit classes is docu-

mented (Pritchett et al., 1989*b*; Shivers et al., 1989). As a consequence similarities in predicted size and the unknown extent of glycosylation have hampered the direct assignment of gel-resolved receptor polypeptides to cloned subunits.

Photoaffinity labeling of tritiated receptor ligands as well as mAbs have been used extensively to characterize the GABA_A receptor constituents. Similarly, these tools have been employed in radio- and immunohistochemistry to characterize the distribution of this important receptor in the mammalian central nervous system (Möhler et al., 1980; Häring et al., 1985; Schoch et al., 1985; Stephenson et al., 1986; Richards et al., 1987; Sieghart et al., 1987; de Blas et al., 1988; Houser et al., 1988; Somogyi et al., 1989). However, the molecular heterogeneity of this receptor raises the issue of subunit specificity of these antibodies.

Three mAbs, bd 17, bd 24, and bd 28 developed in one of our laboratories (Häring et al., 1985) have been employed successfully in the characterization and localization of GABA_A receptors (Schoch et al., 1984, 1985; Häring et al., 1985; Richards et al., 1987; Houser et al., 1988; Fuchs et al., 1988). Antibody bd 17 binds to a 55-kD polypeptide and has been designated as β -subunit specific (Häring et al., 1985). Antibody bd 24 specifically reacts with a 50-kD polypeptide thought to represent a particular α -subunit. Tritiated flunitrazepam photolabels this polypeptide and additionally proteins of M_r 53 and 59 kD. These larger proteins as well as the 50-kD polypeptide reacted with mAb bd 28, which suggested that this antibody is α subunit-specific (Häring et al., 1985; Fuchs et al., 1988). Using an array of cultured cells transiently expressing 12 GABA_A receptor subunits and a glycine receptor subunit, we show that mAbs bd 17 and bd 24 are highly subunit specific, while mAb bd 28 reacts with all GABA_A receptor subunits tested. We also determined the epitope recognized by mAb bd 24.

1. Abbreviation used in this paper: GABA, γ -aminobutyric acid.

Materials and Methods

Transfection of Cultured Cells

Transformed human embryonic kidney cells 293 (ATCC CRL 1573) (Gorman et al., 1989) were grown at 37°C on coverglass coated with fibronectin (25 µg/µl in PBS) in MEM (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS containing 100 U of penicillin (Gibco Laboratories) and 100 µg of streptomycin (Gibco Laboratories) per ml in a 5% CO₂/95% air incubator. Exponentially growing cells were trypsinized and seeded at 2 × 10⁵ per coverglass in 2 ml of growth medium. The transfection was performed by using the calcium phosphate precipitation technique (Chen and Okayama, 1987). The cloned cDNAs of human GABA_A receptor subunits, inserted singly into the eukaryotic expression vector CIS2 (Pritchett et al., 1988; Gorman et al., 1989) or CDM8 (Ymer et al., 1989 a, b), were used for transfection. The cells were incubated in the presence (2 µg/ml) of one or several supercoiled expression plasmids for 12–16 h at 37°C under 3% CO₂/97% air. The medium was removed, and the cells were rinsed twice with culture medium, refed, and incubated in the same medium for 24 h at 37°C under 5% CO₂/95% air before beginning the immunological studies.

Immunocytochemistry

Medium was removed by aspiration and cells were washed twice with 10 mM PBS, pH 7.4. Fixation was performed in 3% PBS-buffered paraformaldehyde for 5 min. Cells were rinsed twice with PBS and incubated with 50 mM glycine in PBS. In some cases, cells were permeabilized by the addition of 0.3% Triton X-100. After an additional 5 min, 1% blocking serum (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) was applied for 30 min. The mAbs were added to cells for 2–3 h in a 1:5 dilution using supernatants of cultured hybridoma cells. Coverglasses with cells were washed gently with PBS and the second biotinylated antiserum (Vectastain 1:200) was added for 60 min. After washing with PBS the avidin-biotinylated HRP complex (1:100) was applied for 1.5 h. Cells were rinsed with PBS and the reaction was started by incubation in PBS containing 0.05% diaminobenzidine/0.03% H₂O₂ typically for 5–15 min. To stop this reaction, cells were again rinsed with PBS. Coverglasses were dipped for a few seconds into distilled water and mounted on glass microscope slides with Moviol (Hoechst AG, FRG).

Insertional Mutagenesis

The single-stranded M13 mp 18 DNA, containing the cloned rat α_1 GABA_A receptor subunit cDNA, served as a template for insertional oligonucleotide-mediated DNA synthesis (Adelman et al., 1983) in the presence of *E. coli* polymerase I, Klenow fragment (Boehringer Mannheim Biochemicals, Indianapolis, IN), and dATP, dTTP, dGTP, and dCTP α S (Taylor et al., 1985). The oligonucleotide (5'-TATGGACAGCCCTCC77CAAGATGAACCTT-3') was complementary to the sequence encoding the amino acid residues YGQPSLQDEL, and specified an additional leucine codon at position four of the rat α_1 polypeptide sequence. The mutant detection system developed by Eckstein and collaborators (Taylor et al., 1985) was employed to minimize screening efforts, using conditions as specified in the protocol accompanying the Amersham Corp. in vitro mutagenesis kit (Arlington Heights, IL). Resulting M13 plaques were analyzed by DNA sequencing (Sanger et al., 1977). For eukaryotic expression, the cDNA fragments containing the entire coding regions for the wild type and mutated rat α_1 subunits were excised with endonucleases Hind III and Pst I from the respective M13 DNAs after double-strand DNA synthesis primed with the lac 17-mer primer (Messing et al., 1981). These DNA fragments were gel purified and cloned into linearized CDM8 vector DNA (Invitrogen, San Diego, CA).

Results

Single GABA_A receptor subunits as well as subunit combinations can be transiently expressed in cultured mammalian cells and the receptors which form in these cells can be readily characterized by pharmacological and electrophysiological means (Pritchett et al., 1988, 1989a,b; Shivers et al., 1989). We have used this system to investigate the specificity of mAbs bd 17, bd 24, and bd 28 in their detection of 12 GABA_A

receptor subunits. Human embryonic kidney cells grown on coverslips were transfected with expression vectors containing cloned cDNAs that encode these subunits. After subunit expression, fixed, and either permeabilized or nonpermeabilized cells, were incubated with the antibodies which, in turn, were visualized by the avidin-biotinylated HRP method. Neither untransfected nor mock-transfected 293 cells showed immunoreactivity with any of the three antibodies (Fig. 1 a).

Displaying high specificity, mAb bd 24 reacted only with bovine and human α_1 subunits (Fig. 1 b), but did not detect the α_1 subunit of rat origin. This antibody did not react with any other α subunit variant nor any of the other members of the known GABA_A receptor subunit classes. Thus, it appears that mAb bd 24 is specific for the α_1 subunit of selected species.

The mAb bd 17 displayed intensive staining of cells expressing the rat β_3 subunit (Fig. 1 c), a reproducibly weaker reaction with the rat β_2 subunit and no staining with rat or human β_1 subunits. No other GABA_A receptor subunit was recognized by this mAb (Fig. 2). These results confirm that bd 17 is β -subunit specific as proposed previously (Häring et al., 1985; Fuchs et al., 1988). However, mAb bd 17 is selective for β_2 and β_3 subunits and fails to react with the β_1 subunit.

In marked contrast to the specificity of mAbs bd 17 and bd 24, mAb bd 28 recognized all six α subunits, although with different intensities (Fig. 2), and reacted additionally with β , γ (Fig. 1 d), and δ subunits. This nonselectivity of mAb bd 28 suggested that it might also bind to subunits of the glycine receptor, as subunits of GABA_A and glycine receptors are highly homologous (Barnard et al., 1987; Grenningloh et al., 1987; Schofield et al., 1987). However, no immunocytochemical reaction was observed upon expression of the 48-kD glycine receptor subunit (Grenningloh et al., 1987), although this subunit was detected by mAb 4 specific for the glycine receptor (Pfeiffer et al., 1984; Becker et al., 1988). Hence, GABA_A receptor subunits may share epitopes not present on the glycine receptor.

In nonpermeabilized cells the antibodies have access only to epitopes located at the outer surface of the plasma membrane. Under these conditions, mAbs bd 24 and bd 17 did, while bd 28 did not, immunoreact with all GABA_A receptor subunits detected in permeabilized cells. This indicates extracellularly located epitopes for mAbs bd 24 and bd 17, while the epitope recognized by mAb bd 28 is either intracellularly located or hidden in a folded structure within the extracellular domain. We favor the latter possibility since the extracellular domain of the δ subunit contained in a bacterial fusion protein reacted in an ELISA with mAb bd 28 when pretreated with Triton X-100 (not shown). However, in sections of rat brain, mAb bd 28 did not yield an immunoreaction even after treatment with detergent (J. G. Richards, personal communication).

Sequence comparisons among the GABA_A receptor subunits failed to reveal likely epitopes for bd 17 and bd 28, indicating that features other than primary structure may determine the reactivity of these antibodies. However, comparison of the α_1 subunit sequences from three species was more informative as it revealed very few amino acid substitutions. In particular, an extra leucine residue occurs in position four of the predicted mature extracellular domain of the

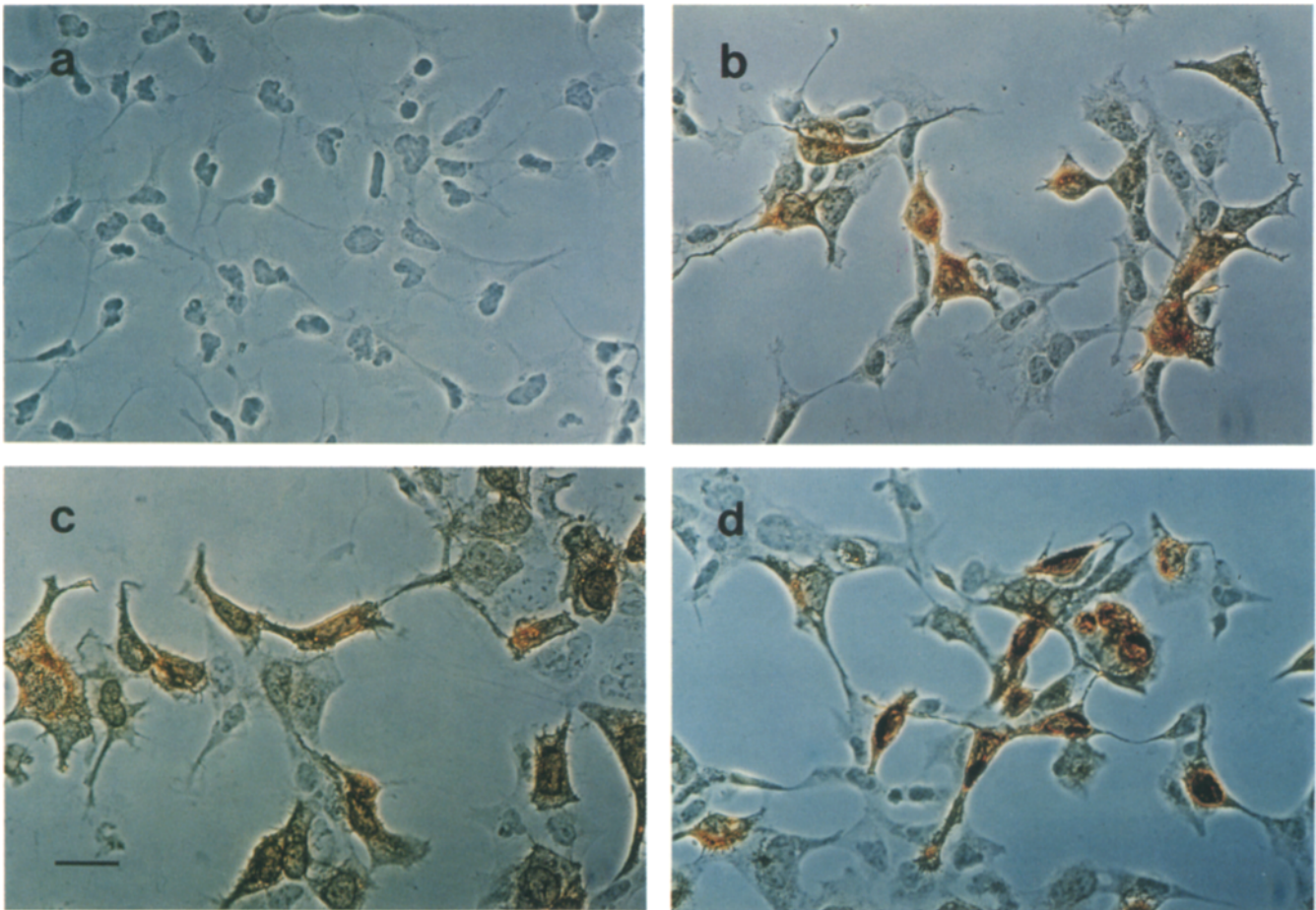


Figure 1. Immunocytochemistry using different mAbs was performed on 293 cells transiently transfected with GABA_A receptor subunits and photographed using phase-contrast optics. Brown staining stems from a DAB precipitate as a result of the peroxidase-coupled reaction. (a) Mock-transfected 293 cells do not show any immunoreactivity with the mAb bd 24 (which is shown as representative of all three mAbs); (b) human GABA_A receptor subunit α_1 is recognized by mAb bd 24; (c) mAb bd 17 reacting with the rat β_3 subunit; and (d) mAb bd 28 reacting with the human γ_2 GABA_A receptor subunit. Bar, 30 μ m.

bovine and human versus the rat α_1 subunits. To test whether this difference correlates with the species selectivity of mAb bd 24, we inserted the leucine residue by site-directed mutagenesis into the appropriate position of the cloned rat α_1 subunit cDNA. Upon expression, the mutated rat α_1 subunit indeed reacted with mAb bd 24 (Fig. 3). As predicted, both the normal and mutated rat α_1 subunits were detected by mAb bd 28 in a control experiment. Further support that mAb bd 24 specifically recognizes this epitope is provided by the positive reaction of the antibody to an immobilized 14-mer peptide covering amino acid residues 1–14 of the bovine GABA_A receptor α_1 subunit (not shown).

Discussion

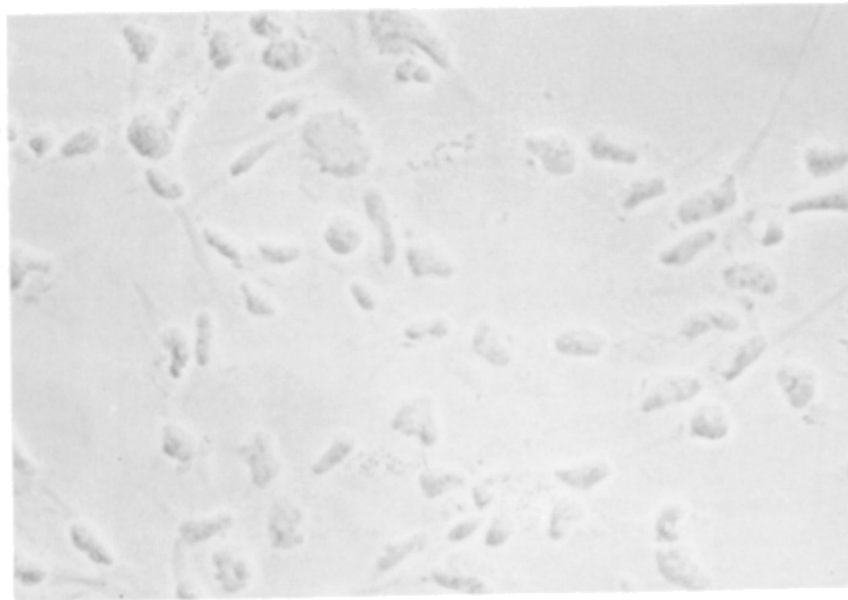
We have tested the specificity of subunit recognition by the three most widely used mAbs for GABA_A receptor characterization and immunocytochemistry. Two of these antibodies displayed a high specificity towards particular subunits. Thus, bd 17 is clearly selective for subunits of the β class, detecting β_2 and β_3 , though it does not show any reactivity towards the β_1 subunit (Figs. 1 and 2). The latter subunit is the least expressed among the β subunits in the CNS as judged by Northern analysis (Ymer et al., 1989 b). Sequence

comparisons among the three β subunits did not give any clues as to which epitope mediates β_2 and β_3 subunit recognition by mAb bd 17. In brain sections, bd 17 recognizes GABA_A receptors with the pattern of epitope distribution

	bd 24	bd 17	bd 28
α_1	Black	White	Black
α_2	White	White	Black
α_3	White	White	Black
α_4	White	White	Black
α_5	White	White	Black
α_6	White	White	Black
β_1	White	White	White
β_2	White	Black	Black
β_3	White	Black	Black
γ_1	White	White	Black
γ_2	White	White	Black
δ	White	White	Black
Gly48k	White	White	White

Figure 2. Differential reactivity of mAbs bd 17, bd 24, and bd 28 towards 12 GABA_A receptor subunits and a glycine receptor subunit. The different shadings of each field represent the immunoreactivities of mAbs with specific GABA_A receptor subunits. The following GABA_A receptor subunits from the following species were tested: α_1 , rat, bovine, human (r, b, h); α_2 , h; α_3 , h; α_4 , b (Ymer et al., 1989a); α_5 , r (Khrestchatsky et al., 1989; Pritchett and Seeburg, 1990); α_6 , b; β_1 , r and h; β_2 , r; β_3 , r; γ_1 , h; γ_2 , h; δ , r; Gly 48K, r and h.

wt α_1 unstained by bd24



$\alpha_1 + L$ stained by bd24

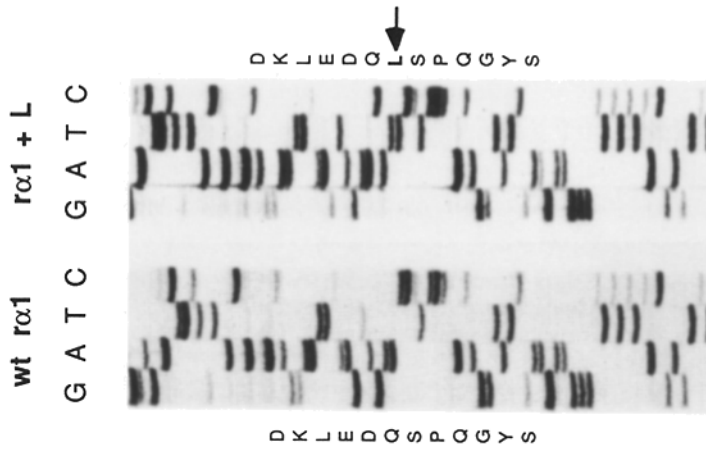
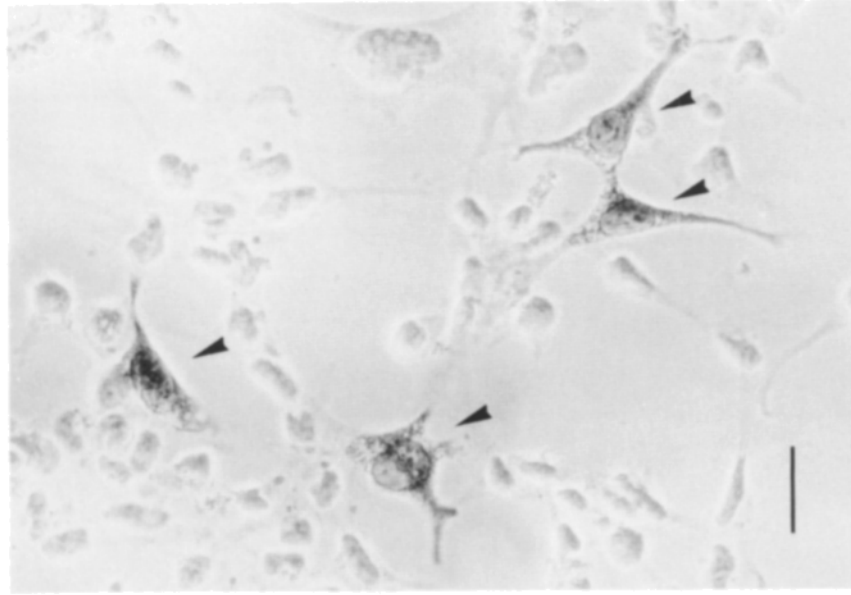


Figure 3. Site-directed mutagenesis to characterize the epitope recognized by mAb bd 24. The sequencing gel on the left shows the rat GABA_A receptor α_1 subunit (wt α_1) with the nucleotide sequence shown from 107–186 bp (premature form). The corresponding amino acid sequence is shown on the side. The right sequencing gel shows the mutated rat GABA_A receptor α_1 subunit ($\alpha_1 + L$) with the inserted leucine at amino acid position two (mature form). The reaction of mAb bd 24 with the normal and mutated α_1 subunits is shown next to the respective sequences. Immunopositive cells are indicated by arrowheads. Bar, 30 μ m.

often corresponding to the distribution of radioligand binding sites (Schoch et al., 1985; Richards et al., 1987). Thus, in the absence of data from high-resolution immunocytochemistry, the β_2 and/or β_3 subunits seem frequently components of natural GABA_A receptors.

Antibody bd 24 presented the highest selectivity because it reacted solely with the α_1 subunit of human and bovine origin but failed to react with rat α_1 (Figs. 1 and 2). This species specificity has been noticed previously (Häring et al., 1985). However, the selectivity for α_1 excluding all other α subunits, documented here, had not been previously appreciated. Currently, six α subunits are characterized (Schofield et al., 1987, 1989; Ymer et al., 1989a; Khrestchatsky et al., 1989; Pritchett and Seeburg, 1990) that display ~70% sequence homology in their extracellular domain (>80%). Hence the selectivity of mAb bd 24 makes this antibody a particularly useful tool to study α_1 subunit-containing GABA_A receptors. The latter receptors are highly expressed in many parts of the mammalian central nervous system and are constituents of the pharmacologically important BZ I type of GABA_A/benzodiazepine receptors (Pritchett et al., 1989a). In sections of brain from several species, the epitope distribution for mAb bd 24 is very similar to that of mAb bd 17 (Schoch et al., 1985; Richards et al., 1988; Somogyi et al., 1989). Thus, the natural GABA_A/benzodiazepine receptors frequently contain α_1 subunits in some combination with β_2 and/or β_3 subunits.

In stark contrast to mAbs bd 17 and bd 24, mAb bd 28 is indiscriminate in its binding to all GABA_A receptor subunits tested (Figs. 1 and 2). Interestingly, it reacts with subunits of all classes including the β and δ classes having low homology to α subunits. Although mAb bd 28 reacts with all subunits, the extent of this reaction is not as uniform as α_4 , β_1 , and β_3 subunits are less intensively stained. Such lower reactivity could be due to a reduced expression efficiency of these subunits. While we cannot investigate this possibility due to the lack of subtype-specific antibodies, we would like to stress that DNA constructs for expression of the β_1 and α_4 subunits injected into *Xenopus* oocyte nuclei lead to large chloride currents upon superfusion with GABA (Ymer et al., 1989a). The lack of selectivity of mAb bd 28 is unexpected considering earlier findings in which bd 28 reacted mainly with α subunit proteins in Western blots (Häring et al., 1985; Fuchs et al., 1988). The epitope recognized by this antibody may not be preserved to the same extent in all subunits under Western blot conditions.

As for mAb bd 17, a sequence search among GABA_A receptor subunits for a common epitope to account for mAb bd 28 recognition was unsuccessful. Our finding that mAb bd 28 does not react with the 48-kD subunit of the glycine receptor although subunits of both receptors share ~35% sequence similarity points to an epitope unique to GABA_A receptor subunits (Barnard et al., 1987; Grenningloh et al., 1987; Schofield et al., 1987). This extent of sequence similarity is also found between members of different classes of GABA_A receptor subunits. A successful epitope characterization was achieved in the case of mAb bd 24 where a leucine residue present in the human and bovine α_1 subunits, but absent in the rat homologue, was identified as an essential part of the specific epitope recognized by this antibody (Fig. 3).

The present characterization should greatly aid in the in-

terpretation of immunocytochemical results obtained with the use of these mAbs. Our present studies underscore the importance of determining the specificities of antibodies directed against subunits of ligand-gated ion channels. As these subunits are members of the large gene family with significant similarity, the reactivity towards several subunits of antibodies once thought to be subunit specific becomes a likely possibility. Hence, an immunomethod that utilizes cells expressing an array of related subunits is a convenient and necessary tool to characterize such antibodies. Furthermore, modifications of this method should also permit an investigation into intracellular processing assembly and transport (Hurtley and Helenius, 1989) as well as receptor insertion into the cell surface of subunits expressed singly or in combination with others.

The authors would like to thank our colleagues Dolan B. Pritchett, Sanie Ymer, Pia Werner, and Martin Köhler for the expression vector constructs for various GABA_A receptor subunit cDNAs; Heike Wieland for the bacterial fusion protein of the extracellular domain of the δ subunit; and Cord-Michael Becker and Heinrich Betz for use of an mAb specific for the 48-kD subunit of the glycine receptor. We gratefully acknowledge the excellent secretarial help of Jutta Rami. M. Ewert is the recipient of a doctoral fellowship by Fonds der Chemischen Industrie. This research was supported by Deutsche Forschungsgemeinschaft, SFB grant 317, to P. H. Seeburg.

Received for publication 1 December 1989 and in revised form 26 January 1990.

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