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Alpha subunit variants of the human glycine receptor: primary structures, functional expression and chromosomal localization of the corresponding genes

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Two cDNAs encoding variants (α_1 and α_2) of the strychnine binding subunit of the inhibitory glycine receptor (GlyR) were isolated from a human fetal brain cDNA library. The predicted amino acid sequences exhibit ~99% and ~76% identity to the previously characterized rat 48 kd polypeptide. Heterologous expression of the human α_1 and α_2 subunits in *Xenopus* oocytes resulted in the formation of glycine-gated strychnine-sensitive chloride channels, indicating that both polypeptides can form functional GlyRs. Using a panel of rodent–human hybrid cell lines, the gene encoding α_2 was mapped to the short arm (Xp21.2–p22.1) of the human X chromosome. In contrast, the α_1 subunit gene is autosomally located. These data indicate molecular heterogeneity of the human GlyR at the level of α subunit genes.

Key words: synaptic receptor/glycine/strychnine/receptor heterogeneity/oocyte expression/X chromosome

Introduction

Inhibitory neurotransmission in the central nervous system (CNS) is primarily mediated by the amino acids glycine and γ-aminobutyric acid (GABA), which both inhibit neuronal firing by increasing the chloride permeability of the neuronal membrane (Barker and McBurney, 1979). Although similar in molecular weight (Pfeiffer *et al.*, 1982; Sigel and Barnard, 1984) and conductance properties (Hamill *et al.*, 1983; Bormann *et al.*, 1987), the postsynaptic receptors for these amino acids exhibit a distinct pharmacology. The convulsive plant alkaloid strychnine inhibits glycine responses, whereas bicuculline antagonizes, and benzodiazepines potentiate, $GABA_A$ receptor activation (for review see Betz and Becker, 1988). By cDNA cloning, subunits of glycine and $GABA_A$ receptors were found to share significant sequence similarity and a conserved transmembrane topology with subunits of the nicotinic acetylcholine receptor, an agonist-gated cation channel (Grenningloh *et al.*, 1987a; Schofield *et al.*, 1987). In particular, a disulfide-bonded extracellular domain and four hydrophobic membrane-spanning segments are highly conserved between these channel proteins (Grenningloh *et al.*, 1987b). A common evolutionary origin has therefore been proposed for these ligand-gated ion channels of excitable membranes.

In vertebrates, ligand-gated ion channels are composed of different homologous subunits (Pfeiffer *et al.*, 1984; Changeux *et al.*, 1987; Grenningloh *et al.*, 1987a; Schofield *et al.*, 1987). In the case of neuronal nicotinic acetylcholine and $GABA_A$ receptors, considerable heterogeneity has been shown to exist for individual subunit polypeptides. For both receptors, various α and β subunit isoforms have been identified in the vertebrate CNS (Deneris *et al.*, 1988; Levitan *et al.*, 1988; Nef *et al.*, 1988; Ymer *et al.*, 1989). Biochemical and immunological data (Becker *et al.*, 1988) indicate developmental heterogeneity of the ligand binding (α) subunit of the inhibitory glycine receptor (GlyR). Here we report isolation of two cDNAs encoding variants of this GlyR subunit from human brain. We show that these cDNAs can direct the expression of functional glycine receptors in heterologous cells, and that the corresponding genes are located on different human chromosomes.

Results

Isolation of human cDNA clones

Radiolabelled DNA of clone GR-2, which encodes most of the strychnine binding 48 kd (α) subunit of the GlyR from adult rat spinal cord (Grenningloh *et al.*, 1987a), was used as a probe to screen a λgt10 cDNA library prepared from human fetal brain. Three hybridizing overlapping cDNAs (huB1, 1700 bp; huB7, 1475 bp; huB20, 627 bp) were isolated which covered an open reading frame (ORF) of 1356 bp (Figure 1A). After a hydrophobic putative signal peptide, the subsequent deduced amino acid sequence of 426 residues (calculated molecular weight 48 900) exhibited extensive homology (76% identical amino acids) to the rat protein (Figure 2). Conserved characteristics include four putative membrane-spanning segments as well as four cystein residues and a possible *N*-glycosylation site in the extracellular domain. Most sequence differences occur in the presumptive cytoplasmic region between the third and fourth transmembrane segment, as previously seen for subunits of other ligated-gated ion channels (Deneris *et al.*, 1988; Levitan *et al.*, 1988). In addition, the N-termini of the mature proteins exhibit considerable sequence variation.

Screening of the same library with a 96-fold degenerate ^{32}P-labelled 23mer oligonucleotide covering possible coding sequences for a contiguous stretch of eight amino acids that are conserved between rat glycine and $GABA_A$ receptor subunits (Grenningloh *et al.*, 1987b) resulted in identification of another cDNA (clone hGly, 1715 bp). The protein

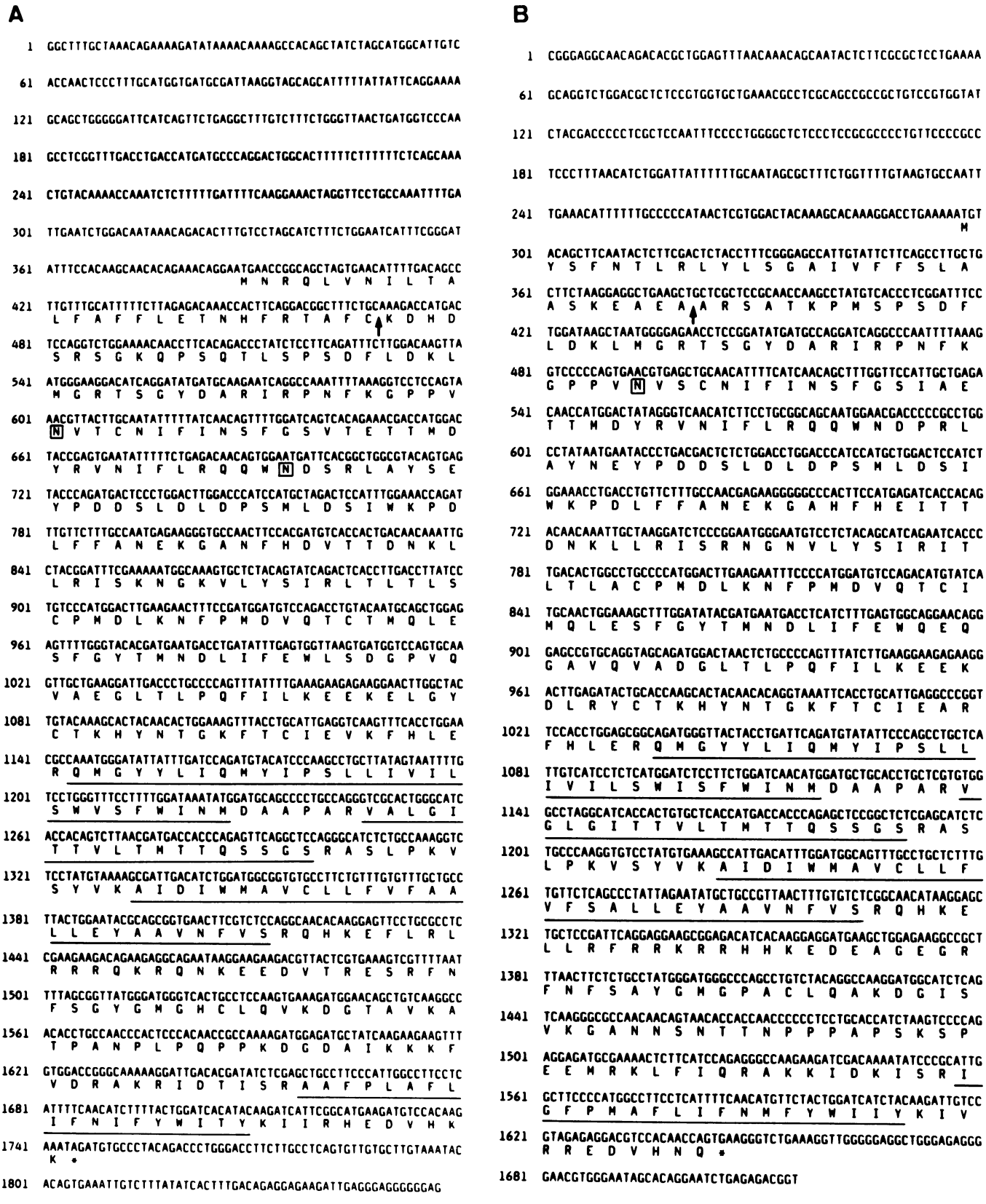

Fig. 1. Nucleotide and deduced amino acid sequences of human α_2 (**A**) and α_1 (**B**) subunit cDNAs. Nucleotide numbers are indicated at the left, and putative signal peptide cleavage sites are marked by arrows. The putative membrane-spanning segments M1 to M4 are underlined; possible extracellular *N*-glycosylation sites are boxed. Asterisks denote 3′-terminal stop codons.

sequence (421 residues, calculated molecular weight 48 412) predicted from its ORF of 1347 bp (Figure 1B) displayed very high homology (99%) to the previously deduced rat protein. Only six amino acid substitutions are found within 421 residues between the putative mature rat and human polypeptides (Figure 2). This low degree of interspecies

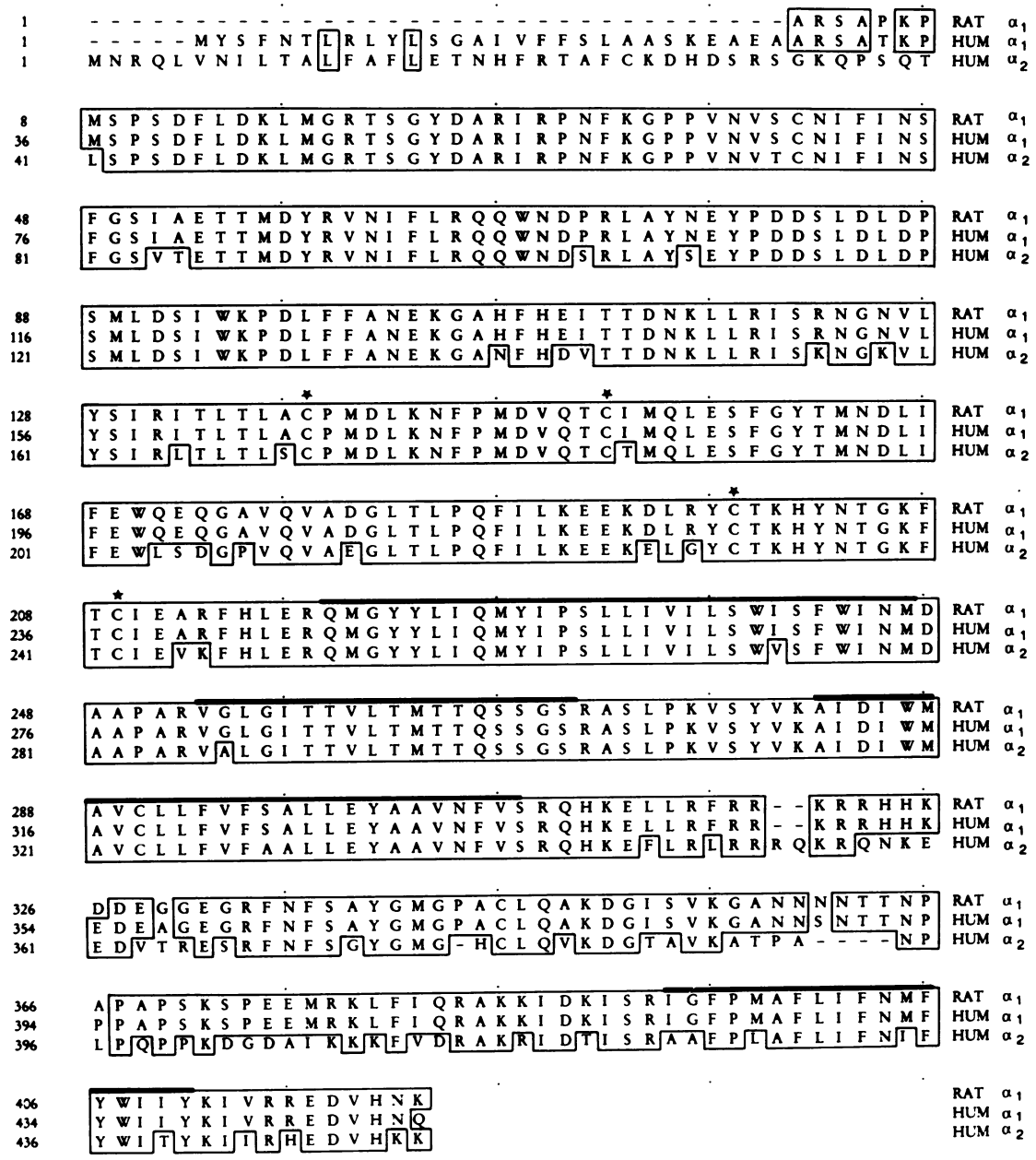

Fig. 2. Alignment of the human α_1 and α_2 polypeptide sequences with the mature rat α_1 subunit sequence. Numbering starts with the N-terminal amino acid of the mature rat, and the signal peptide sequences of the human proteins. Identical residues are indicated by solid boxes. Gaps were introduced to maximize homology. Conserved cysteines are indicated by asterisks, and the membrane-spanning segments M1 to M4 by solid lines.

variation indicates that hGly encodes the human equivalent of the 48 kd subunit from adult rat spinal cord (Grenningloh, 1987a), whereas huB corresponds to a novel variant of this receptor polypeptide. Hence, we refer to the hGly and rat 48 kd polypeptides as α_1, and to the huB protein as α_2, subunits of the mammalian GlyR.

Functional expression

Expression of the rat GlyR α_1 subunit in *Xenopus* oocytes or in mammalian cells generates functional glycine-gated chloride channels which display most of the pharmacology typical of the native receptor protein in spinal cord (Schmieden *et al.*, 1989; Sontheimer *et al.*, 1989). To establish that the human cDNAs isolated here correspond to functional ligand binding receptor subunits, they were engineered for expression by subcloning into the eukaryotic expression vector pCIS2 (Gorman *et al.*, 1989) and into the plasmid pSPT19 which permits *in vitro* transcription of cloned fragments.

Transfection of the human embryonic kidney cell line 293 with a human α_1-pCIS2 construct created glycine-activated, strychnine-sensitive chloride channels which, in whole cell patch clamp experiments, were indistinguishable from those formed upon rat α_1 subunit cDNA expression (Sontheimer *et al.*, 1989). Very similar results were obtained upon injection of synthetic human α_1 (hGly) RNA into *Xenopus* oocytes (Table I). Expression of human α_2 (huB) RNA in the oocyte system also generated similar chloride currents upon bath application of 50−500 μM glycine (Figure 3). At higher agonist concentrations, significant desensitization of the response was found. Again, activation of the expressed channels was blocked by 10−100 nM strychnine. No

Table I. Pharmacology of GlyR channels generated upon expression of α_1 and α_2 subunit RNAs in *Xenopus* oocytes

RNA injected	EC_{50} (mM)		K_i (nM) strychnine
	Glycine	Taurine	
Human α_1	0.29 ± 0.02	3 ± 2	16 ± 2
Human α_2	0.31 ± 0.01	6 ± 3	18 ± 3
Rat α_1[a]	0.30–0.40	≥5	9–15

[a]Data for rat from Schmieden *et al.* (1989) are shown for comparison. Agonist concentrations eliciting a half-maximal response (EC_{50}) were determined at a holding potential of −70 mV as described (Schmieden *et al.*, 1989). Because of differences in cooperativity of channel activation by different agonists (Schmieden *et al.*, 1989), no attempt has been made to calculate absolute K_d values for agonists. K_i values for strychnine (Hill coefficient $n \sim 1.0$) were calculated from data obtained at a glycine concentration eliciting a half-maximal response (300 μM). All data represent the mean ± SD of between two and six independent determinations.

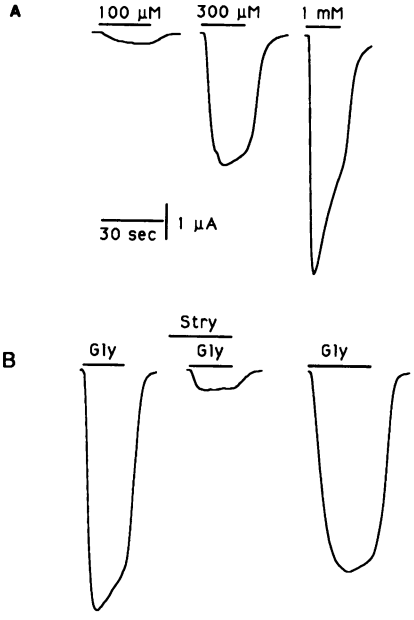

Fig. 3. Functional expression of the human α_2 subunit. RNA prepared by *in vitro* transcription of linearized pSPT19 hub B DNA was injected into *Xenopus* oocytes, and, after two days of incubation, the latter subjected to voltage clamp recording at a holding potential of −60 mV. (**A**) Response to different concentrations of glycine. (**B**) Inhibition of the response to glycine (300 μM) by 100 nM strychnine. Periods of agonist and antagonist application are indicated by bars. Downward deflections denote inward current that reversed to outward current near −25 mV (not shown).

significant differences were detected between human α_1 and α_2 subunit channels in apparent glycine and strychnine affinities (Table I). Taurine, another glycinergic agonist (Betz and Becker, 1988; Schmieden *et al.*, 1989), also gated both α_1 and α_2 subunit GlyRs; however, millimolar concentrations were required (Table I). Preliminary data indicate significant differences in relative taurine responses (Schmieden *et al.*, 1989) between α_1 and α_2 subunit GlyRs. Their characteristics will be presented in detail elsewhere (J.Kuhse, V.Schmieden and H.Betz, in preparation).

Chromosomal localization of human α subunit genes

We have shown previously that a gene homologous to the α_1 subunit of rat GlyR exhibits restriction fragment length polymorphism (RFLP) in humans (Siddique *et al.*, 1989).

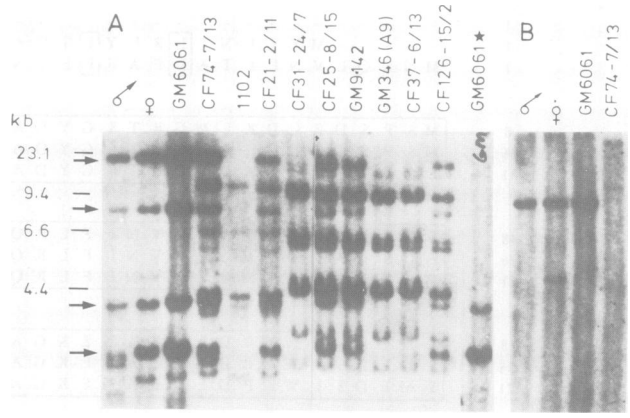

Fig. 4. Southern blot analysis of human genomic DNA and DNA preparations from human–hamster and human–mouse hybrid cell lines. (**A**) Hybridization with α_2 subunit cDNA probes encompassing either the 5′ non-translated and N-terminal coding regions (*Eco*RI–*Apy*I fragment of huB20 corresponding to nucleotides 310–483 in Figure 1A) or the cytoplasmic domain (*; *Hin*pI–*Eco*RV fragment of huB7 representing nucleotides 1434–1649) of the predicted protein sequence. (**B**) Hybridization with an α_1 subunit specific cDNA probe (nucleotides 1293–1540 in Figure 1B). DNA (20 μg) digested with *Eco*RI was extracted from the following sources: ♂, ♀, normal human lymphoblasts; GM 6061, human lymphoid cell line; CF 74-7/13, human X chromosome in a hamster cell background; 1102, hamster parental cell line for CF74-7 and CF21-2; CF21-2/11, Xp/20p translocation [short arm of the human X chromosome (Xp) in a hamster background]; CF31-24/7 and CF31-1, Xq/20p translocation, reciprocal of CF21-2 [long arm of the human X chromosome (Xq) in a mouse (A9) background]; CF25-8/15, X/13 translocation (xp22.1 to Xqter in A9 background); GM 9142, X/21 translocation (Xp21.2 to Xpter in A9 background); GM346 (A9), mouse parental cell line A9; CF37-6/13, X/11 translocation (Xp21.2 to xqter in A9 background); CF126-15/2, deletion in Xp22.3 (A9 background). Arrows in (A) indicate the position of hybridizing X chromosomal bands in human–rodent hybrid cells.

To determine whether this locus can be assigned to one of the α subunit variants described here, Southern blots of human chromosomal DNA were probed with different radiolabelled DNA fragments corresponding to homologous and unique sequences of α subunit cDNAs.

Probes encompassing the coding regions of human or rat α subunit cDNAs hybridized to several fragments in *Eco*RI digested human genomic DNA (data not shown). When using a human α_2 cDNA probe, five of these bands were also found in DNA preparations from human–hamster and human–mouse hybrid cell lines containing the human X chromosome, and four in hybrids containing specific fragments thereof (Xp, Xp 22.1 to Xqter, Xp 21.2 to Xpter; see Figure 4A). The same probe revealed the previously described RFLP in *Hinc*II digested human DNA (Siddique *et al.*, 1989; and data not shown). A DNA fragment encoding the cytoplasmic region of the α_2 subunit hybridized to two of these X-chromosomal bands (Figure 4A). In contrast, a probe selective for the corresponding region of the α_1 subunit revealed a band of ~9 kb in human genomic DNA and a human cell line, but not in hamster hybrid cells containing the human X chromosome (Figure 4B). From these hybridization data, the following conclusions can be drawn. (i) The α_2 subunit gene is located on the X chromosome. (ii) The gene encoding the α_1 subunit resides on an autosome. (iii) As α_2 subunit sequences are present in DNA from cell lines that contain X and Xp, but not Xq, Xp22.1 to Xqter and Xp21.2 to Xpter, but not Xp21.2 to

Xqter, the α_2 subunit gene must be located between Xp21.2 and Xp22.1.

Discussion

Here, two human GlyR α subunit cDNAs were isolated which exhibit a high degree of conservation with the rat α_1 subunit sequence. Amino acid sequence identity of 99% is seen between the mature human and rat α_1 polypeptides, suggesting that they both represent the ligand binding subunits of the biochemically characterized GlyR complex present in adult mammalian spinal cord (Pfeiffer *et al.*, 1982; Betz and Becker, 1988). Of five interspecies amino acid exchanges, four correspond to isofunctional replacements in the putative cytoplasmic domain. The human α_2 subunit has diverged considerably further (amino acid identity of 76% with both the human and rat α_1 proteins). Major differences concern the cytoplasmic region between transmembrane segments M3 and M4, which generally is highly variable among receptor subunit isotypes (Deneris *et al.*, 1988; Levitan *et al.*, 1988; Nef *et al.*, 1988), and the most N-terminal sequence. Here, the first eight residues following the putative signal peptide are entirely different from those of the α_1 protein.

The biological significance of the α_2 subunit is presently unclear. It may correspond to a developmentally or regionally regulated isoform of the GlyR. Indeed, an α subunit variant with an apparent molecular weight of 49 000 is prevalent in neonatal rodent spinal cord and cultured embryonic neurons, and differs from its adult counterpart of 48 kd in immunological and antagonist binding properties (Becker *et al.*, 1988; Hoch *et al.*, 1989). Also, differences in size have been observed between fractionated spinal cord and brain RNA samples enriched for expression of functional GlyR in *Xenopus* oocytes (Akagi and Miledi, 1988). Recent cDNA sequencing data indicate the existence of corresponding α_2 and novel α_3 subunit variants in the rat central nervous system (J.Kuhse, V.Schmieden and H.Betz, in preparation).

Both human α subunits form functional glycine-gated channels upon expression in heterologous cell systems. The previously noted ability to assemble into conducting channel structures (Schmieden *et al.*, 1989; Sontheimer *et al.*, 1989) thus apparently is inherent to GlyR α subunits. This may reflect the phylogenetically ancient origin of this inhibitory receptor, i.e. its similarity to a hypothesized ancestral homo-oligomeric channel protein (Grenningloh *et al.*, 1987a; Betz and Becker, 1988). Furthermore, it supports the interpretation that the α_1 and α_2 subunits described here are indeed part of distinct functional GlyR proteins.

Our Southern blot data indicate that the previously identified human GlyR gene (Siddique *et al.*, 1989) is located on the short arm (Xp21.2−p22.1) of the human X chromosome and encodes the α_2 subunit. In contrast, the α_1 subunit gene is autosomally located. The X chromosomal location of the α_2 subunit gene is of considerable interest. Our assignment places it in proximity to the Duchenne/Becker muscular dystrophy locus (Xp21.21) and other genes implicated in neurological disorders (Martin, 1989). In accord with the hybrid cell mapping data obtained in this study, a highly significant linkage between the α_2 subunit gene and the hypophosphatemic rickets locus has recently been detected (M.J.Econs, M.A.Pericak-Vance, H.Betz, R.J.Bartlett, M.C.Speer and M.K.Drezner, submitted for publication). The latter gene resides at Xp22.1−p22.2 (Thakker *et al.*, 1987). Because of the high synteny conservation of the X chromosome in mammals (Nadeau, 1989), the α_2 subunit gene in mouse should be close to *mdx*, the rodent homolog of the Duchenne/Becker locus.

Further genomic mapping of GlyR subunit genes may help to unravel the molecular basis of inherited spasticity and related motor disorders. Deficient glycinergic transmission has been implicated in the pathogenesis of acquired and genetic myoclonus, and low GlyR levels can explain the phenotype of homozygous Poll Hereford myoclonic cattle (Gundlach *et al.*, 1988) and *spastic* mouse mutants (White and Heller, 1982; Becker *et al.*, 1986). In the latter case, the disease locus has been mapped to mouse chromosome 3 (Eicher and Lane, 1980); its relation to GlyR structural genes is, however, unsolved (Becker *et al.*, 1986). Similar mutations in human GlyR genes might account for hereditary diseases of a related phenotype in man (Andermann *et al.*, 1980; Harding, 1981).

Materials and methods

Isolation of cDNAs

GlyR α_1 and α_2 subunit cDNAs were isolated from a λgt10 cDNA library of human fetal brain (Schofield *et al.*, 1989). Clones huB1, huB7 and huB20 were obtained by screening 10^6 p.f.u. of the library with a nick-translated GR-2 cDNA probe of the 48 kd α subunit of the rat GlyR (Grenningloh *et al.*, 1987a). Hybridization was in 4 × SET at 60°C, and washings at the same temperature in 2 × SET, 1 × SET and 0.2 × SET (Grenningloh *et al.*, 1987a). Clone hGly was obtained by screening of the same library with the radiolabelled oligonucleotide 5′ AC(A,C)AC(A,T)GT(G,T) CT(A,C,G)AC(A,C)ATGAC(A,C)AC 3′ (Ymer *et al.*, 1989). After purification and subcloning into pSPT18 or M13 vectors, DNA sequences were determined by the chain termination method (Sanger *et al.*, 1977).

Functional expression

Expression of the α_1 subunit encoded by clone hGly was performed after subcloning the insert into pCIS2 and pSPT19 as described (Pritchett *et al.*, 1988; Schmieden *et al.*, 1989; Sontheimer *et al.*, 1989). For expression of the α_2 subunit, a full-length construct encompassing 387 bp of the 5′ untranslated sequence, the coding region and a few bases of 3′ untranslated sequence was assembled from corresponding fragments of clones huB1 and huB7 and subcloned in pSTP19. Transfection of mammalian cells, RNA synthesis using SP6 polymerase, injection of *Xenopus* oocytes and voltage clamp recordings were done as detailed previously (Schmieden *et al.*, 1989; Sontheimer *et al.*, 1989).

Chromosomal mapping

Southern blot hybridizations to endonuclease-digested DNA extracted from human lymphoblasts, human−hamster and human−mouse hybrid cell lines, or rodent cells were performed using radiolabeled α subunit sequences as probes. Hybridization conditions and labelling procedures were as described by Siddique *et al.* (1988).

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