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Identification of amino acid residues important for assembly of $GABA_A$ receptor α1 and γ2 subunits

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Abstract

Comparative models of $GABA_A$ receptors composed of α1β3γ2 subunits were generated using the acetylcholine-binding protein (AChBP) as a template and were used for predicting putative engineered cross-link sites between the α1 and the γ2 subunit. The respective amino acid residues were substituted by cysteines and disulfide bond formation between subunits was investigated on co-transfection into human embryonic kidney (HEK) cells. Although disulfide bond formation between subunits could not be observed, results indicated that mutations studied influenced assembly of $GABA_A$ receptors. Whereas residue α1A108 was important for the formation of assembly intermediates with β3 and γ2 subunits consistent with its proposed location at the α1(+) side of $GABA_A$ receptors, residues γ2T125 and γ2P127 were important for assembly with β3 subunits. Mutation of each of these residues also caused an impaired expression of receptors at the cell surface. In contrast, mutated residues α1F99C, α1S106C or γ2T126C only impaired the formation of receptors at the cell surface when co-expressed with subunits in which their predicted interaction partner was also mutated. These data are consistent with the prediction that the mutated residue pairs are located close to each other.

Keywords: assembly, comparative models, cysteine substitution, $GABA_A$ receptor, structure.

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GABA is the quantitatively most important inhibitory neurotransmitter in the CNS. Most actions of GABA are generated via $GABA_A$ receptors. These receptors are chloride ion channels that can be opened by GABA (Macdonald and Olsen 1994) and are the targets of action of a variety of pharmacologically and clinically important drugs, such as benzodiazepines, barbiturates, steroids, anaesthetics and convulsants. These drugs modulate GABA-induced chloride ion flux by interacting with separate and distinct allosteric binding sites (Sieghart 1995).

$GABA_A$ receptors are composed of five subunits (Nayeem *et al*. 1994; Tretter *et al*. 1997) that can belong to different subunit classes. Six α, three β, three γ, one δ, one ε, one π, one θ, and three ρ subunits have been cloned from the mammalian nervous system (Barnard *et al*. 1998; Sieghart and Sperk 2002; Simon *et al*. 2004). Depending on their subunit composition, receptors exhibit distinct pharmacological and electrophysiological properties (Sieghart 1995).

Recombinant receptor studies (Im *et al*. 1995; Chang *et al*. 1996; Tretter *et al*. 1997; Farrar *et al*. 1999) as well as studies investigating the subunit composition of $GABA_A$ receptors in the brain (Jechlinger *et al*. 1998; Poltl *et al*. 2003) indicated that the vast majority of $GABA_A$ receptors found are composed of two α, two β and one γ subunit. Biochemical studies (Tretter *et al*. 1997; Baumann *et al*. 2001, 2002), as well as modelling of the $GABA_A$ receptor extracellular domain (Ernst *et al*. 2003) according to the structure of the acetylcholine-binding protein (AChBP) (Brejc *et al*. 2001), indicated the absolute arrangement of the subunits in $GABA_A$ receptors (Fig. 1a).

The assembly of hetero-oligomeric receptors is a complex multistep process that generally occurs in the endoplasmic reticulum (Connolly *et al*. 1996). To achieve the correct order of subunits around the pore, each subunit must be able to discriminate between different subunits and to interact

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Abbreviations used: AChBP, acetylcholine-binding protein; DTT, dithiothreitol; HEK, human embryonic kidney; IP low buffer, low salt buffer for immunoprecipitation; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulfate.

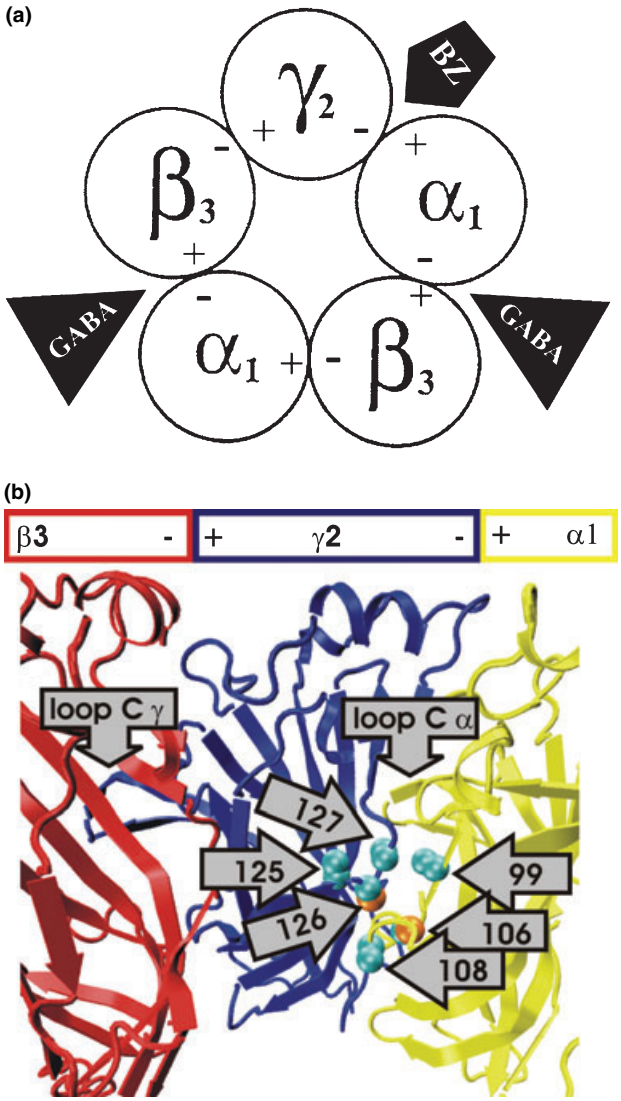

Fig. 1 $GABA_A$ receptor structure. (a) Schematic drawing of the stoichiometry and absolute subunit arrangement of the recombinant $\alpha1\beta3\gamma2$ $GABA_A$ receptor. The principal (+) and complementary (–) sides of a subunit are indicated. BZ or GABA indicate the sites of interaction of benzodiazepines or GABA with $GABA_A$ receptors, respectively. (b) Structure of one of the generated models of the $GABA_A$ receptor extracellular domain. View from inside the chloride channel to the surrounding β3, γ2 and α1 subunits, indicating the amino acid residues investigated in this study. Cα and Cβ atoms of the respective residues are rendered as van der Waals spheres. The Cβ atoms of residues α1S106 and γ2T126 are highlighted in orange to emphasize that, in the respective model, these side chains are in positions suitable for forming disulfide bonds when replaced by cysteines.

with its neighbours via specific high-affinity contact sites. Several amino acid sequences have been identified that seem to be important for assembly of α, β, and γ subunits of $GABA_A$ receptors (Taylor *et al.* 1999; Klausberger *et al.* 2000; Taylor *et al.* 2000; Klausberger *et al.* 2001; Sarto *et al.* 2002a,b; Bollan *et al.* 2003a,b; Ehya *et al.* 2003).

In the present study, we used the comparative models developed by our group (Ernst *et al.* 2003) for predicting amino acid residues on the α1 and the γ2 subunit, that might form direct contacts with each other. These residues were substituted by cysteines and a possible disulfide bond formation between subunits was investigated on co-transfection into human embryonic kidney (HEK) cells. Although disulfide bond formation between subunits could not be observed, results indicated that mutations studied influenced assembly of $GABA_A$ receptors.

Materials and methods

Antibodies

The antibodies anti-peptide α1(1–9), anti-peptide β3(1–13), anti-peptide γ2(1–33), anti-peptide γ2(319–366) and anti-peptide β3(345–408) were generated and affinity purified as described previously (Tretter *et al.* 1997; Jechlinger *et al.* 1998; Klausberger *et al.* 2000).

Generation of cDNA constructs

For the generation of recombinant receptors, α1, β3 and γ2 subunits of $GABA_A$ receptors from rat brain were cloned and subcloned into pCDM8 expression vectors (Invitrogen, San Diego, CA, USA) as described previously (Tretter *et al.* 1997). Mutated subunits were constructed by PCR amplification using the wild-type subunit as a template. For this, PCR primers were used to construct point mutations within the subunits by the 'gene splicing by overlap extension' technique (Horton *et al.* 1993). The PCR primers contained *Xho*I and *Nsi*I or *Xho*I and *Xba*I restriction sites, which were used to clone the α1 or γ2 fragments into pCI vectors, respectively (Promega, Madision, WI, USA). The mutated subunits were confirmed by sequencing.

Culture and transfection of HEK293 cells

Transformed HEK293 cells (CRL 1573; American Type Culture Collection, Rockville, MD, USA) were cultured as described in Tretter *et al.* (1997). Cells (3×10^6) were transfected with 20 μg of cDNA for a single subunit transfection using the calcium phosphate precipitation method (Chen and Okayama 1988). For co-transfection with two different subunits, for each subunit 10 μg of cDNA was used. When cells were co-transfected with three different subunits, 7 μg of cDNA was used per subunit. A total of ~20 μg of cDNA per transfection and a cDNA ratio of 1 : 1 : 1 seemed to be optimal for the expression of $GABA_A$ receptors under the conditions used as judged by receptor-binding studies in cells transfected with α1, β3, and γ2 subunits. Changing the subunit ratio by doubling the amount of cDNA for a single subunit at the cost of that for other subunits did not significantly change the number of [^{3}H]Ro15-1788 binding sites detected.

The cells were then harvested 48 h after transfection. At this time point the number of [^{3}H]Ro15-1788 binding sites formed per milligram of protein was at its maximum for cells transfected with α1, β3, and γ2 subunits. Results obtained, however, did not change when cells were harvested 34–48 h after transfection. In addition, western blot analysis revealed that expression levels of wild-type and mutated subunits were comparable at all harvesting times.

Purification and immunoprecipitation of wild-type and mutated subunits

The culture medium was removed from transfected HEK cells, and cells from four culture dishes were extracted with 1 mL of a C12E10 extraction buffer [1% polyoxyethylene-10-lauryl ether (Sigma, St Louis, MO, USA), 0.18% phosphatidylcholine (Sigma), 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, containing one 'Mini Complete Protease Inhibitor Cocktail' tablet per 10 mL extraction buffer (Roche Diagnostics GmbH, Mannheim, Germany)] for 8–12 h at 4°C. The extract was centrifuged for 40 min at 150 000 *g* at 4°C, and the clear superantant was incubated overnight at 4°C under gentle shaking with 20 μg antibodies. After addition of immunoprecipitin (Life Technologies, Gaithersburg, MD, USA; for preparation see Tretter *et al*. 1997) and 0.5% non-fat dry milk powder and shaking for an additional 2 h at 4°C, the precipitate was washed three times with a low-salt buffer for immunoprecipitation (IP low buffer) (50 mM Tris-HCl, 0.5% Triton X-100, 150 mM NaCl, and 1 mM EDTA, pH 8.0). The precipitated proteins were dissolved in sample buffer [108 mM Tris-sulfate, pH 8.2, 10 mM EDTA, 25% (w/v) glycerol, 2% sodium dodecyl sulfate (SDS), with or without 3% dithiothreitol (DTT)]. SDS–polyacrylamide gel electrophoresis (PAGE) and western blot analysis with digoxygenized antibodies were performed as described in Tretter *et al*. (1997). Secondary antibodies (alkaline phosphatase-coupled anti-digoxygenin $F(ab')_2$ fragments purchased from Boehringer Mannheim, Mannheim, Germany) were visualized by the reaction of alkaline phosphatase with CDP-Star or CSPD reagent (Tropix, Applied Biosystems, Bedford, MA, USA).

The chemiluminescent signal was quantified by densitometry after exposing the immunoblots to the Fluor-S MultImager (Bio-Rad Laboratories, Hercules, CA, USA) and evaluated using the Quantity One Quantitation Software (Bio-Rad Laboratories) and GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). The linear range of the detection system was established by measuring the antibody generated signal to a range of antigen concentrations. Under the experimental conditions used, the immunoreactivities were within the linear range, and this permitted a direct comparison of the amount of antigen per gel lane between samples. Data were generated from several different gels per subunit and expressed as mean ± standard error.

All mutated constructs used in this study could be expressed to a comparable extent after single transfection into HEK cells. After co-transfection of different constructs, however, the stability of mutated subunits that formed non-productive assembly intermediates was reduced, presumably by proteolytic degradation (Paulson and Claudio 1990; Paulson *et al*. 1991; Klausberger *et al*. 2000). In all control experiments, the extent of expression of mutated constructs was therefore determined in singly transfected HEK cells.

Immunoprecipitation of receptors expressed at the cell surface

The culture medium was removed from HEK cells transfected with cDNA (21 μg per 3×10^6 cells) of $GABA_A$ receptor subunits (cDNA ratio 1 : 1 : 1), and the cells were washed once with phosphate-buffered saline (PBS; in mM: 2.7 KCl, 1.5 KH_2PO_4, 140 NaCl, and 4.3 Na_2HPO_4, pH 7.3). Cells were then detached from the culture dishes by incubating with 2.5 mL of 5 mM EDTA in PBS for 5 min at 20°C. The resulting cell suspension was diluted in 6.5 mL of cold Dulbecco's modified Eagle's medium and centrifuged for 5 min at 1000 *g*.

For western blot analysis, the cell pellet from two dishes was incubated with 35 μg of α1(1–9) antibodies in 3 mL of the same medium for 30 min at 37°C. Cells were again pelleted, and free antibodies were removed by washing twice with 10 mL of PBS buffer. Then, receptors were extracted with IP low buffer containing 1% Triton X-100 for 1 h under gentle shaking. Cell debris was removed by centrifugation (30 min; 150 000 *g*; 4°C). After addition of immunoprecipitin and 0.5% non-fat dry milk powder and shaking for 2 h at 4°C, the precipitate was centrifuged for 10 min at 10 000 *g* at 4°C and dissolved in sample buffer (with or without DTT) and subjected to SDS–PAGE and western blot analysis using digoxygenized antibodies.

To verify that only receptors at the cell surface were labelled by the antibodies, parallel samples were incubated with antibodies directed against the intracellular loop of $GABA_A$ receptor subunits (experiments not shown). These antibodies could not precipitate any $GABA_A$ receptor subunits under the conditions used. A possible redistribution of the antibodies during the extraction procedure has been excluded previously (Klausberger *et al*. 2000).

SDS–PAGE, western blot, and chemiluminescence detection

SDS–PAGE was performed according to Neville and Glossmann (Neville and Glossmann 1974) using gels containing 10% polyacrylamide in a discontinuous system. Proteins separated on the gels were tank blotted onto pre-wetted polyvinylidene fluoride (PVDF) membranes. After blocking with 1.5% non-fat dry milk powder in PBS and 0.1% Tween20 for 1 h at room temperature, the membranes were incubated overnight with digoxygenized α1(1–9), β3(1–13) or γ2(319–366) antibodies (1 μg/mL) at 4°C. The membranes were extensively washed and incubated with secondary antibodies (anti-digoxygenin-AP, $F(ab)_2$ fragments, Roche Diganostics GmbH) for 1 h at room temperature. PVDF membranes were again washed extensively as described above, were then equilibrated in assay buffer (0.1 M diethanolamine and 1 mM $MgCl_2$, pH 10.0) for 10 min, and secondary antibodies were visualized by the reaction of alkaline phosphatase with CSPD or CDP Star (Applied Biosystems, Bedford, MA, USA). The chemiluminescent signal was quantified by densitometry after exposing the immunoblots to the Fluor-S MultImager (Bio-Rad Laboratories) and evaluated using the Quantity One Quantitation Software (Bio-Rad Laboratories). Under the experimental conditions used, the immunoreactivities were within the linear range, and this permitted a direct comparison of the amount of antigen per gel lane between samples.

Data were generated from several different gels and expressed as mean ± standard error. Student's unpaired *t*-test was used for comparing groups, and significance was set at $p < 0.05$.

Modelling

All comparative models of the $GABA_A$ receptor extracellular domain (Sali and Blundell 1993) were based on the AChBP structure 1I9B (Brejc *et al*. 2001). Based on seven alignments for the structurally variable regions, a total of 35 models were computed with Modeller version 6. After validation, a total of seven well-scoring models (one per alignment) of the $GABA_A$ receptor extracellular domain was used to predict the segments that participate in interface formation. Predictions were then made with

the program WHAT IF (Vriend 1990) on possible engineered disulfide bridges at the interface. The high uncertainty that is associated with amino acid side chain positions at most interface forming regions of cys-loop receptors was taken into account by using multiple different models.

Results

Prediction of amino acid residues on α1 and γ2 subunits that might directly interact with each other

The interfaces of the extracellular domain of the cys-loop receptors contain regions that are structurally not homologous to the AChBP. To account for these structurally variable regions, predictions on possible subunit contacts can be made only on a statistical basis using multiple different models. Based on the seven which scored best out of a total of 35 models that represented seven different alignments, a pool of putative direct contact sites was predicted. Results indicated, however, that most contact-forming regions possess a geometry that is not suitable for disulfide bond formation. If a contact forming region displayed a suitable geometry, at most one potential disulfide bridge was predicted in this region in a given model. At the α1/γ2 interface, the seven models predicted a total of 11 distinct putative cross-link sites. Four of these (each one in a different model) were predicted in the same contact region that was selected for further investigations because it yielded the largest number of individual predictions. In three of seven models, either residues α1F99 and γ2P127, α1S106 and γ2T126 or α1A108 and γ2T125 were predicted to form part of the α1(+)/γ2(–) interface and to be in an orientation suitable for disulfide formation if replaced by cysteines. (Fig. 1b). In one model, another amino acid residue pair on the α1 and γ2 subunit in this contact region was predicted to form disulfides after replacement by cysteines. Results on these mutants will be reported elsewhere (manuscript in preparation).

Generation and expression of mutated subunits

To investigate which of the three amino acid residue pairs might form contacts, residues α1F99, α1S106, α1A108 of the α1 subunit and γ2T125, γ2T126, γ2P127 of the γ2 subunit were substituted by cysteines individually (Fig. 2).

To study whether the mutated subunits were adequately expressed, wild-type α1 or mutated α1F99C, α1S106C or α1A108C subunits were transfected individually into HEK cells. The proteins were extracted, immunoprecipitated using α1(1–9) antibodies and subjected to SDS–PAGE and western blot analysis using digoxygenized α1(1–9) antibodies. As shown in Fig. 3(a), in agreement with previous results, the α1 subunit migrated as a major protein band with apparent molecular mass of 51 kDa (Zezula *et al*. 1991). Bands with lower apparent molecular mass presumably were degradation products. Comparison of western blots indicated that wild-type and mutated subunits were expressed to a similar extent,

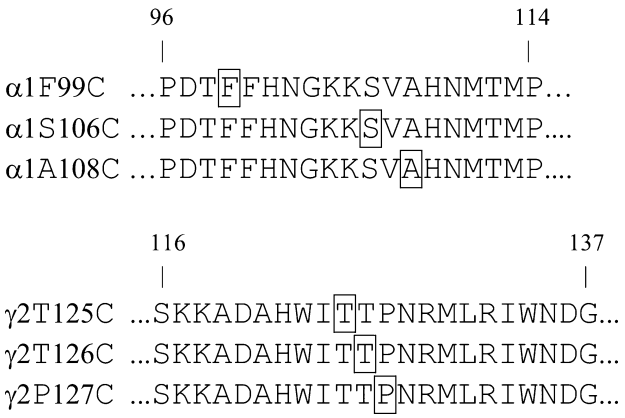

Fig. 2 Schematic drawing of parts of the N-terminal sequences of α1 and γ2 subunits. The name of the mutated subunit is given by the respective subunit (α1 or γ2), the wild-type residue in the amino acid single-letter code, followed by its position in the mature amino acid sequence and, finally, by the single-letter code for cysteine to which the wild-type residue has been mutated. The positions of the first and last residue of the sequences shown are indicated by the numbers above the amino acid single-letter code. The amino acid residues that have been substituted by cysteines are boxed.

indicating that there was no major structural rearrangement in the mutated subunits that would have caused enhanced degradation (Paulson and Claudio 1990; Paulson *et al*. 1991; Klausberger *et al*. 2000). In addition, these results indicated that the mutated as well as the wild-type α1 subunits could be precipitated and detected by α1(1–9) antibodies.

Similar experiments were performed with wild-type γ2 or mutated γ2T125C, γ2T126C or γ2P127C subunits. After individual transfection into HEK cells, the proteins were extracted, immunoprecipitated using γ2(319–366) antibodies and subjected to SDS–PAGE and western blot analysis. As shown in Fig. 3(b), wild-type and mutated γ2 subunits were expressed to a similar extent and, in agreement with previous results, could be detected on western blots, displaying a diffuse band migrating at 45–49 kDa (Ehya *et al*. 2003).

The expression of β3 subunits was not investigated separately as, in all cases, only wild-type β3 subunits were used for co-transfection experiments.

Investigation of cell surface receptors containing combinations of mutated subunits

To investigate a possible cross-link of amino acid residues located at different subunits, the mutated subunits α1F99C and γ2P127C, α1S106C and γ2T126C, and α1A108C and γ2T125C were co-transfected into HEK cells together with wild-type β3 subunits.

Receptors present at the cell surface were labelled using α1(1–9) antibodies. Receptors were then extracted under conditions in which the receptor–antibody complex was not drastically impaired and under which no redistribution of the

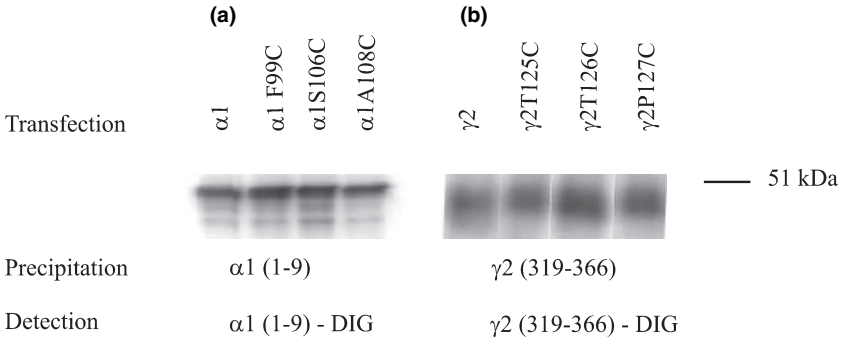

Fig. 3 Comparable expression of wild-type and mutated α1 or γ2 subunits in HEK cells. In control experiments, wild-type and mutated α1 or γ2 subunits were transfected into HEK cells, extracted and immunoprecipitated using (a) α1(1–9) or (b) γ2(319–366) antibodies, respectively. The precipitates were subjected to SDS–PAGE and the constructs were detected using digoxygenized α1(1–9) (a) or γ2(319–366) (b) antibodies. The α1 protein exhibited an apparent molecular mass of 51 kDa, whereas the γ2 protein migrated as a smear in the range of 45–49 kDa.

antibody could be observed (Klausberger *et al*. 2000). Receptors were immunoprecipitated by addition of immunoprecipitin, dissolved in SDS loading buffer without dithiothreitol (DTT) to allow the detection of subunit dimers formed by disulfide bonds, and subjected to SDS–PAGE and western blot analysis using digoxygenized α1(1–9) antibodies. Under these conditions, in another study (manuscript in preparation) we were able to identify $GABA_A$ receptor subunit dimers cross-linked by disulfide bonds, indicating the feasibility of this approach. Figure 4(a) shows that cells transfected with wild-type α1, β3 and γ2 subunits under these conditions exhibited a major protein band at about 51 kDa that could be labelled by α1(1–9) antibodies and, thus, represented α1 subunits.

Similar studies performed in cells transfected with α1F99C, β3 and γ2P127C or α1S106C, β3 and γ2T126C subunits indicated that the extent of cell surface expression of the mutated α1F99C and α1S106C subunits was significantly reduced (to 48.3 ± 3.9 and 56.7 ± 4.6%, respectively), as compared with wild-type α1 subunits (Fig. 4a). Cell surface expression of the mutated subunit α1A108C, however, was dramatically reduced (to 23.4 ± 4.8%) when cells were transfected with subunit combinations α1A108C, β3 and γ2T125C. In no case was a protein band with an apparent molecular mass of around 100 kDa identified that contained α1 subunits, indicating that no cross-link was formed between mutated subunit pairs. The protein smears at around 110–180 kDa could also be detected in cells transfected with wild-type α1, β3 and γ2 subunits. They presumably resulted from unspecific protein aggregates caused by oxidation of endogenous cysteines in these gels, that were run in the absence of DTT to allow the detection of possible cross-linked proteins. The protein bands below the major band of 51 kDa presumably represented degradation products of α1 subunits.

To demonstrate that completely assembled receptors were precipitated by the α1(1–9) antibodies, the same western blot was then stripped and re-probed using digoxygenized β3(1–13) or digoxygenized γ2(319–366) antibodies. Figure 4(b) shows that the β3(1–13) antibody, in agreement with previous results, could identify a major protein band of about 54 kDa and a minor one of 51 kDa in cells transfected with wild-type α1, β3 and γ2 subunits, both of which seem to be differentially glycosylated β3 subunits (Buchstaller *et al*. 1991). Western blot analysis of cells transfected with α1F99C, β3 and γ2P127C, α1S106C, β3 and γ2T126C, or α1A108C, β3 and γ2T125C subunits revealed that, in all three cases, there was a reduction of cell surface expression of the β3 subunits (to 51.6 ± 6.0, 58.3 ± 5.8 or 19.8 ± 4.7%, respectively) (Fig. 4b) to an extent similar to that observed for the respective mutated α1 subunit (Fig. 4a). This finding is consistent with the assumption that all receptors at the cell surface contained α and β subunits under the conditions used.

Re-probing this western blot using digoxygenized γ2(319–366) antibodies indicated a diffuse protein band in the range of 45–49 kDa in cells transfected with wild-type α1, β3, and γ2 subunits that represented the γ2 subunit (Ehya *et al*. 2003) (Fig. 4c). Whereas expression of the γ2T126C subunit at the cell surface was significantly reduced (to 64.3 ± 5.0%), surface expression of γ2T125C and γ2P127C subunits was dramatically reduced (to 38.1 ± 2.3 and 35.3 ± 4.9%, respectively).

The same results were obtained when the order of incubation with digoxygenized primary antibodies for detection of subunits was changed.

Again, in no case, a protein band with an apparent molecular mass of around 100 kDa containing γ2 subunits could be identified that was present in mutated, but not in wild-type, receptors, confirming the absence of a cross-link between the mutated γ2 and α1 subunits.

Mutated α1 subunits exhibit an impaired assembly with mutated γ2 subunits

In other experiments, it was investigated whether the mutated α1 and γ2 subunits could form assembly intermediates. For

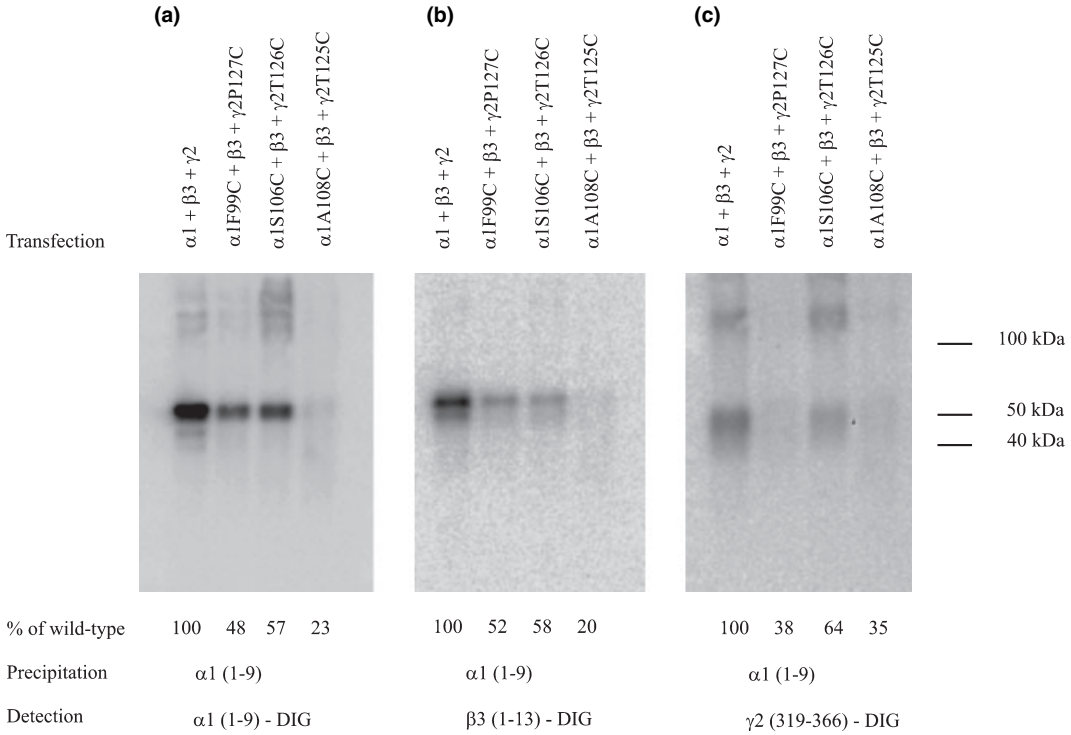

Fig. 4 Cell surface expression of $GABA_A$ receptors containing mutated α1 and γ2 subunits. HEK cells were co-transfected with α1, β3 and γ2, or α1F99C, β3 and γ2P127C, or α1S106C, β3 and γ2T126C, or α1A108C, β3 and γ2T125C subunits. $GABA_A$ receptors expressed on the surface were immunolabelled by an incubation of intact cells with α1(1–9) antibodies. Receptors were then extracted, precipitated by immunoprecipitin, and subjected to SDS–PAGE and western blot analysis using digoxygenized α1(1–9) antibodies (a). The blots were then stripped and reanalysed with digoxygenized β3(1–13) antibodies (b). The blots were then again stripped and reanalysed with digoxygenized γ2(319–366) antibodies (c). Comparable results were obtained when the order of detection of subunits was changed. These experiments were performed four times with comparable results. α1, β3 and γ2 subunits migrated as protein bands of 51, 54 and 45–49 kDa, respectively. The average staining of subunits detected with the respective antibodies in the α1 precipitates of receptors present at the cell surfaces are given as a percentage of wild-type staining and represents the mean values of four different experiments. The respective variability (± SD values) of data is given in the text.

that, mutated subunits α1F99C, α1S106C or α1A108C were co-transfected into HEK cells with γ2P127C, γ2T126C or γ2T125C, respectively. In control experiments, wild-type α1 subunits were co-transfected with γ2 subunits. Proteins were then extracted from HEK cells and assembly intermediates were immunoprecipitated with γ2(319–366) antibodies. The precipitates were subjected to SDS–PAGE and western blot analysis using digoxygenized α1(1–9) antibodies.

Results indicated (Fig. 5) that the subunits α1F99C or α1S106C could be co-precipitated with γ2 antibodies to a similar extent as wild-type α1 subunits (96.7 ± 6.2 or 97.2 ± 2.6%, respectively). In contrast, co-precipitation of α1A108C with γ2T125C was reduced to 35.1 ± 4.6%, indicating that assembly of these two mutated subunits was impaired. The extent of reduction in the formation of α1A108Cγ2T125C intermediates was comparable with that observed for α1A108Cβ3γ2T125C receptors at the cell surface (Fig. 4c). Again, in no case could a protein band with an apparent molecular mass of around 100 kDa be identified that was present in mutated, but not in wild-type intermediates, confirming the absence of a cross-link between the mutated γ2 and α1 subunits.

Investigation of cell surface receptors containing the mutated α1 and wild-type β3 and γ2 subunits

In order to investigate whether the reduction in cell surface expression of $GABA_A$ receptors was caused already by a mutation in a single subunit, or whether mutations in both α1 and γ2 subunits had to be present for this effect to occur, subunits α1F99C, α1S106C or α1A108C were transfected into HEK cells, together with wild-type β3 and γ2 subunits. Cell surface receptors were labelled using α1(1–9) antibodies, were then extracted, immunoprecipitated and subjected to SDS–PAGE and western blot analysis using digoxygenized α1(1–9) antibodies (Fig. 6a). The blot was then stripped and re-probed with digoxygenized β3(1–13) antibodies (Fig. 6b). Finally, the western blot was again stripped and re-probed with digoxygenized

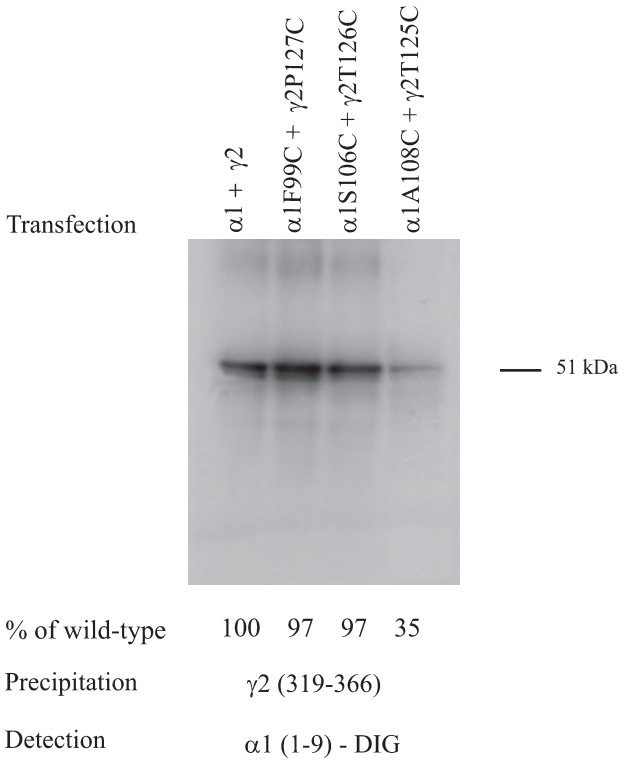

Fig. 5 Co-immunoprecipitation of assembly intermediates containing mutated α1 and γ2 subunits. HEK cells expressing the α1 and γ2, or α1F99C and γ2P127C, or α1S106C and γ2T126C, or α1A108C and γ2T125C subunits were extracted, and proteins were precipitated with γ2(319–366) antibodies and subjected to western blot analysis using digoxygenized α1(1–9) antibodies. This experiment was performed five times with comparable results. The average staining of subunits detected with digoxygenized α1(1–9) antibodies in the γ2 precipitates of assembly intermediates is given as a percentage of wild-type staining and represents the mean values of five different experiments. The respective variability (± SD values) of data is given in the text.

γ2(319–366) antibodies. As shown in Fig. 6(a–c), receptors containing α1, β3, and γ2, α1F99C, β3, and γ2, α1S106C, β3, and γ2, or α1A108C, β3, and γ2 subunits could be precipitated by α1(1–9) antibodies from the cell surface. Whereas the amount of α1 subunits in α1-precipitated α1F99Cβ3γ2 receptors was reproducibly larger (122.1 ± 3.0%) than that of wild-type receptors, presumably as a result of a more efficient assembly of the mutant α1F99C with the wild-type β3 and γ2 subunits, the amount of α1 subunits in α1S106Cβ3γ2 receptors was comparable (99.3 ± 1.1%). In contrast, the amount of α1A108Cβ3γ2 receptors was significantly reduced (to 50.8 ± 3.9%) at the cell surface (Fig. 6a).

Similar results were obtained when the amounts of β3 subunits in α1-precipitated α1F99Cβ3γ2 (109.6 ± 6.1%), α1S106Cβ3γ2 (84.9 ± 6.1%) or α1A108Cβ3γ2 (55.2 ± 7.2%) receptors were compared with that in α1β3γ2 receptors (Fig. 6b). Because of the variability of the results, however, the increase of β3 subunits in α1F99Cβ3γ2 receptors was not significant. Digoxygenized γ2(319–366) antibodies, however, detected 104.4 ± 3.8, 78.6 ± 11.8 or 48.6 ± 3.5% of γ2 subunits at the cell surface in α1F99Cβ3γ2, α1S106Cβ3γ2 or α1A108Cβ3γ2 receptors, respectively. This indicated that, out of the three mutated α1 subunits, only α1A108C displayed a significantly impaired assembly with other subunits.

The α1A108C subunit exhibits an impaired assembly with wild-type β3 as well as γ2 subunits

To further investigate the assembly behaviour of α1A108C, this subunit was co-transfected into HEK cells, together with either wild-type β3 or wild-type γ2 subunits. In control experiments, α1F99C or α1S106C subunits were also co-transfected with β3 or γ2 subunits. The protein complexes were then extracted and immunoprecipitated with β3(1–13) or γ2(319–366) antibodies, respectively. The precipitates were subjected to SDS–PAGE and western blot analysis using digoxygenized α1(1–9) antibodies.

Results indicated that the subunit α1A108C, in contrast to α1F99C (111.7 ± 4.6%) or α1S106C (97.7 ± 2.5%), could not be co-precipitated efficiently (only 28.2 ± 9.0%) with β3 subunits (Fig. 7a), as compared with wild-type α1 and β3 subunits, indicating that assembly of the mutated subunit α1A108C with the wild-type β3 subunit was impaired.

Interestingly, α1A108C, in contrast to α1F99C (80.4 ± 3.1%) and α1S106C (90.7 ± 2.9%) could also not efficiently be co-precipitated with γ2 subunits (30.9 ± 8.8%) after co-transfection of these subunits into HEK cells (Fig. 7b), indicating that the assembly of α1A108C with the wild-type γ2 subunit was also impaired.

Investigation of cell surface receptors containing the mutated γ2 and wild-type α1 and β3 subunits

In other experiments, the influence of the mutations γ2P127C, γ2T126C or γ2T125C on the expression of $GABA_A$ receptors at the cell surface was investigated. For that, subunits γ2P127C, γ2T126C or γ2T125C were co-transfected into HEK cells with wild-type α1 and β3 subunits. Cell surface receptors were again labelled using α1(1–9) antibodies and, after extraction, were precipitated by addition of immunoprecipitin and subjected to SDS–PAGE and western blot analysis using digoxygenized α1(1–9) antibodies (Fig. 8a). The blots were then stripped and re-probed with digoxygenized β3(1–13) antibodies (Fig. 8b). Subsequently, they were again stripped and re-probed with digoxygenized γ2(319–366) antibodies (Fig. 8c).

As shown in Fig. 8, α1(1–9) antibodies were able to co-precipitate α1 and β3 subunits from the cell surface of all receptors investigated. The amounts of α1 subunits detected were similar to that of α1β3γ2 receptors in the precipitated α1β3γ2P127C and α1β3γ2T126C receptors (102.6 ± 7.3

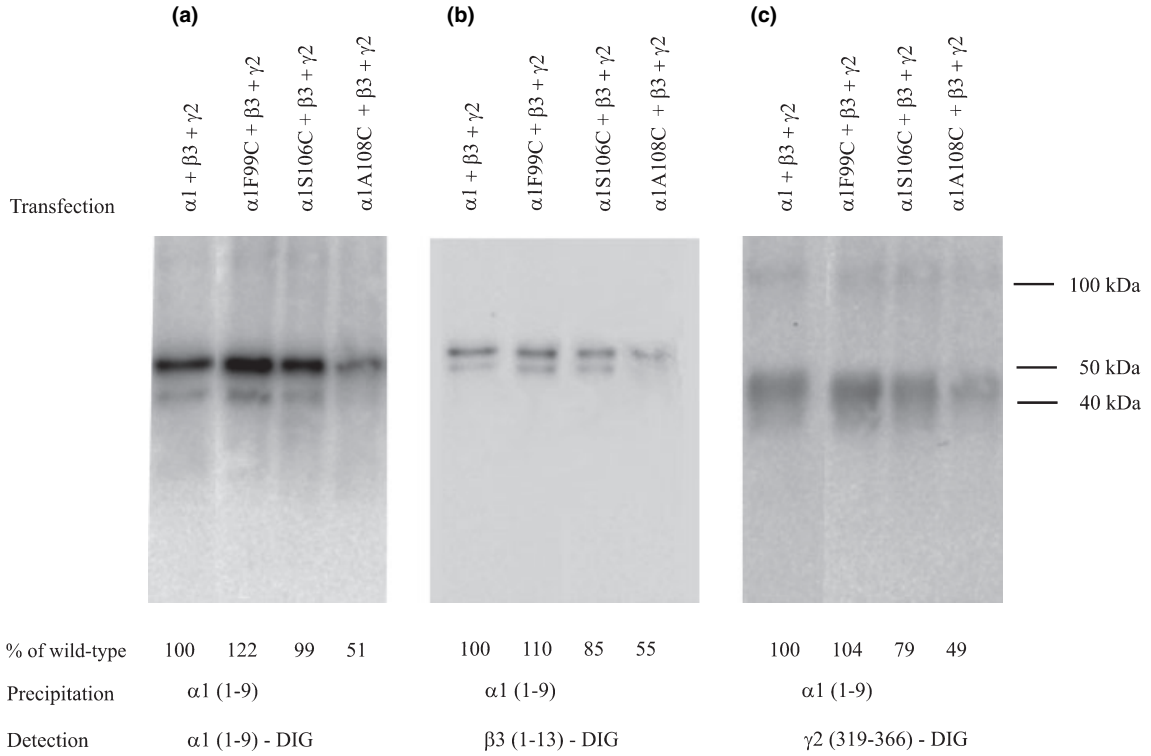

Fig. 6 Cell surface expression of $GABA_A$ receptors containing mutated α1 and wild-type β3 and γ2 subunits. HEK cells were co-transfected with α1, β3 and γ2, or α1F99C, β3 and γ2, or α1S106C, β3 and γ2, or α1A108C, β3 and γ2 subunits. $GABA_A$ receptors expressed on the surface were immunolabelled by an incubation of intact cells with α1(1–9) antibodies and were then extracted, precipitated by immunoprecipitin and subjected to SDS–PAGE and western blot analysis using digoxygenized α1(1–9) antibodies (a). The blots were then stripped and reanalysed with digoxygenized β3(1–13) antibodies (b). The blots were then again stripped and reanalysed with digoxygenized γ2(319–366) antibodies (c). Comparable results were obtained when the order of detection of subunits was changed. These experiments were performed three times with comparable results. The average staining of subunits present at the cell surface and detected with the respective antibodies in the α1 precipitated receptors is given as percentage of wild-type staining and represents the mean values of three different experiments. The respective variability (± SD values) of data is given in the text.

and 90.9 ± 6.3%, respectively), but were significantly reduced (to 47.8 ± 6.6%) in α1β3γ2T125C receptors. Similar results were obtained for β3 subunits. Thus, digoxygenized β3(1–13) antibodies detected 106.7 ± 4.5, 88.0 ± 1.9 or 50.8 ± 3.0% of β3 subunits in α1β3γ2P127C, α1β3γ2T126C or α1β3γ2T125C receptors, respectively. γ2 subunits in α1 precipitated receptors, however, amounted to 24.2 ± 6.1, 83.3 ± 3.8 or 40.7 ± 5.3% in α1β3γ2P127C, α1β3γ2T126C or α1β3γ2T125C receptors, respectively, when compared with wild-type receptors. These results suggested that, out of the three mutated γ2 subunits, the subunits γ2P127C and γ2T125C displayed an impaired subunit assembly.

Subunits γ2T125C or γ2P127C exhibit an impaired assembly with β3 subunits

To investigate which contacts of the γ2P127C and γ2T125C subunits were impaired, wild-type or mutated γ2 subunits were transfected into HEK cells, together with either wild-type α1 or wild-type β3 subunits. The protein complexes were extracted, and immunoprecipitated with α1(1–9) or β3(1–13) antibodies, respectively, and the precipitates were subjected to SDS–PAGE and western blot analysis using digoxygenized γ2(319–366) antibodies.

Western blot analysis of intracellular assembly intermediates showed that the mutated γ2P127C (86.3 ± 2.6%), γ2T126C (102.8 ± 3.8%) and γ2T125C (91.2 ± 1.4%) subunits could be co-precipitated with α1 subunits to about the same extent as the wild-type γ2 subunit (Fig. 9a). This indicated that the assembly of these mutated γ2 subunits with wild-type α1 subunits was not dramatically impaired (Fig. 9a). Whereas γ2T126C could be co-precipitated with β3 subunits to a similar extent (93.2 ± 13.3%) as wild-type γ2, co-precipitation of the mutated γ2P127C or γ2T125C subunits with β3 subunits was dramatically reduced (to 19.6 ± 6.8 or 44.3 ± 3.1%,

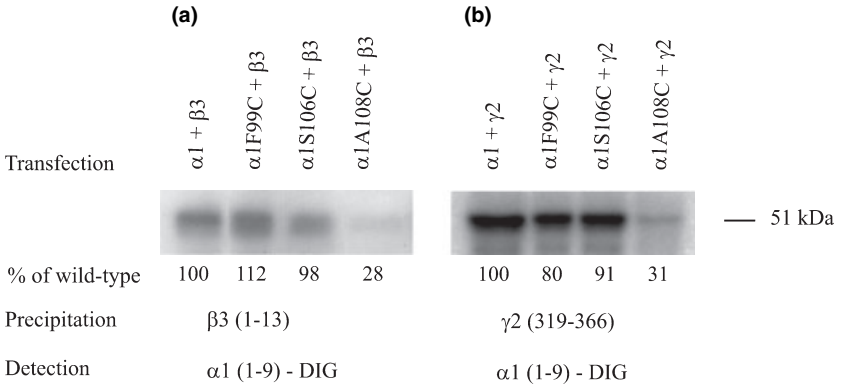

Fig. 7 Co-immunoprecipitation of assembly intermediates containing mutated α1 and wild-type β3 or γ2 subunits. (a) HEK cells expressing the α1 and β3, α1F99C and β3, α1S106C and β3, or α1A108C and β3 subunits were extracted, and proteins were precipitated with β3(1–13) antibodies and subjected to western blot analysis using digoxygenized α1(1–9) antibodies. (b) HEK cells expressing the α1 and γ2, α1F99C and γ2, α1S106C and γ2, or α1A108C and γ2 subunits were extracted, and proteins were precipitated with γ2(319–366) antibodies and subjected to western blot analysis using digoxygenized α1(1–9) antibodies. These experiments were performed four times with comparable results. The average staining of subunits detected with the respective antibodies in the precipitates of assembly intermediates are given as a percentage of wild-type staining and represents the mean values of four different experiments. The respective variability (± SD values) of data is given in the text.

respectively) (Fig. 9b). This indicated that the mutated γ2P127C and γ2T125C subunits displayed an impaired assembly with β3 subunits.

Discussion

The amino acid residue α1A108 is important for assembly of α1 subunits

Using comparative models of the extracellular domain of the GABA$_A$ receptor, we predicted that the segments α1F99 to α1A108, and γ2P127 to γ2T125 form a contact region between α1 and γ2 subunits. Moreover, residues α1F99 and γ2P127, α1S106 and γ2T126, or α1A108 and γ2T125 were identified as putative engineered cross-link sites. All these residues were therefore substituted by cysteines and it was investigated whether the introduced cysteines on co-transfection of the respective mutated α1 and γ2 subunit pairs with wild-type β3 subunits could form disulfide bonds detectable on western blots. Results indicated that receptors composed of β3 and mutated α1 and γ2 subunits were formed at the cell surface, but in no case was a cross-link of mutated α1 and γ2 subunits detectable. The lack of disulfide bond formation between the mutated α1 and γ2 subunits is not entirely surprising because the amino acid sequence γ2T125 to γ2P127 is located in a region that is not conserved in the superfamily. Predictions of amino acid positions might, thus, contain fairly large errors, and even slight changes in these positions could prevent the formation of disulfide bonds. Although this was known at the beginning of this work, this region was selected because it yielded the largest number of individual predictions for putative cross-links in different GABA$_A$ receptor models.

Although no spontaneous disulfide bond formation could be observed between the mutated α1 and γ2 subunits (Fig. 4), the amounts of mutated receptors at the cell surface were significantly smaller than those of receptors composed of wild-type α1, β3 and γ2 subunits, suggesting that the respective amino acid residues might be important for assembly. Alternatively, their mutation might interfere with transport of receptors to the cell surface. To clarify possible causes for the reduced expression of mutated receptors at the cell surface, mutated α1 and γ2 subunits were co-expressed in HEK cells and their ability to form assembly intermediates was investigated. Whereas assembly of α1F99C and γ2P127C, or α1S106C and γ2T126C subunits seemed not to be disturbed as compared with wild-type α1 and γ2 subunits, there was a significant reduction in assembly of α1A108C and γ2T125C subunits (Fig. 5).

Subsequent experiments indicated that, in several cases, cell surface expression of receptors was already disturbed when a single mutated subunit was co-expressed with two wild-type subunits. Thus, receptors composed of α1A108C, β3 and γ2 subunits were reduced at the cell surface (Fig. 6), although not as strongly as those composed of α1A108C, β3 and γ2T125C subunits (Fig. 4), indicating that the mutated α1A108C subunit alone is sufficient to cause an impaired expression of receptors at the cell surface. The observation of a reduced formation of assembly intermediates between α1A108C and β3 as well as between α1A108C and γ2 subunits (Figs 7a and b) indicates that the reduced cell surface expression of α1A108Cβ3γ2 (Fig. 6) and α1A108Cβ3γ2T125C (Fig. 4) receptors was a result of an assembly and not a trafficking problem. If the amino acid residue α1A108 is located at the

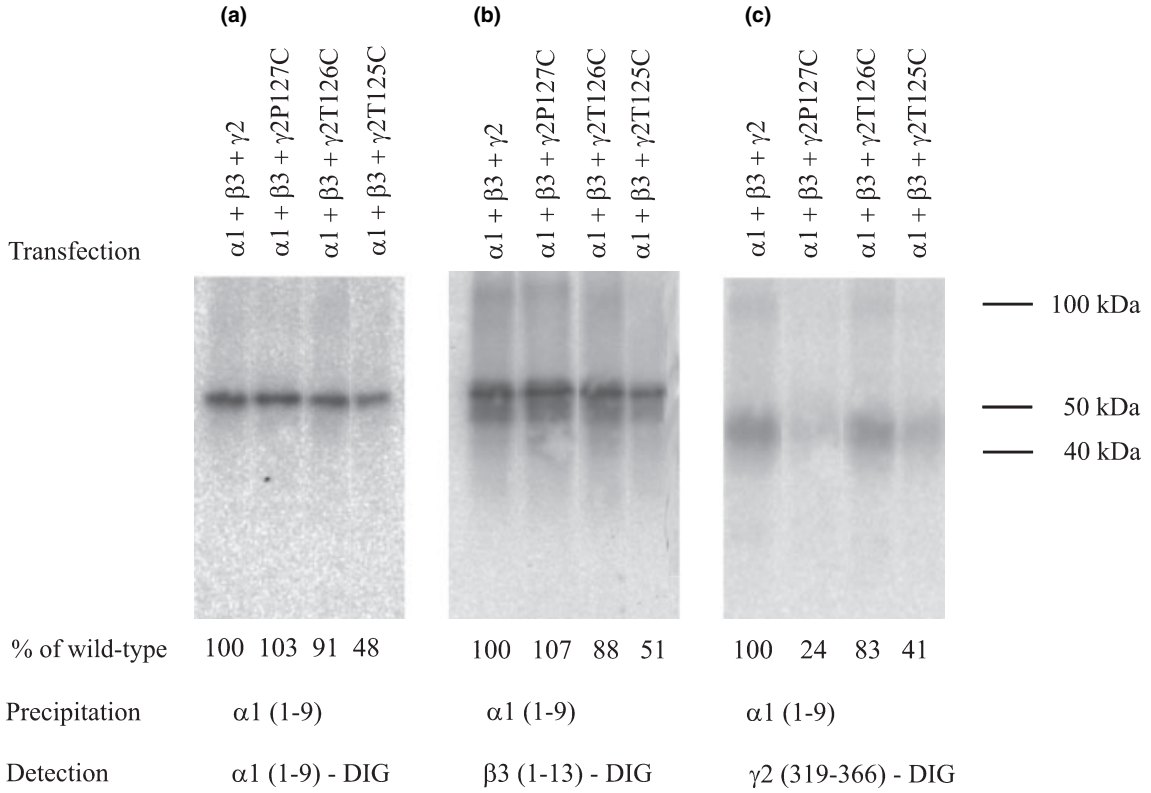

Fig. 8 Cell surface expression of $GABA_A$ receptors containing mutated γ2 and wild-type α1 and β3 subunits. HEK cells were co-transfected with α1, β3 and γ2, or α1, β3 and γ2P127C, or α1, β3 and γ2T126C, or α1, β3 and γ2T125C subunits. $GABA_A$ receptors expressed on the surface were immunolabelled by an incubation of intact cells with α1(1–9) antibodies, then extracted, precipitated by immunoprecipitin, and subjected to SDS–PAGE and western blot analysis using digoxygenized α1(1–9) antibodies (a). The blots were then stripped and reanalysed with digoxygenized β3(1–13) antibodies (b). The blots were then again stripped and reanalysed with digoxygenized γ2(319–366) antibodies (c). Comparable results were obtained when the order of detection of subunits was changed. These experiments were performed three times with comparable results. The average staining of subunits present at the cell surface and detected with the respective antibodies in the α1 precipitated receptors is given as a percentage of wild-type staining and represents the mean values of three different experiments. The respective variability (± SD values) of data is given in the text.

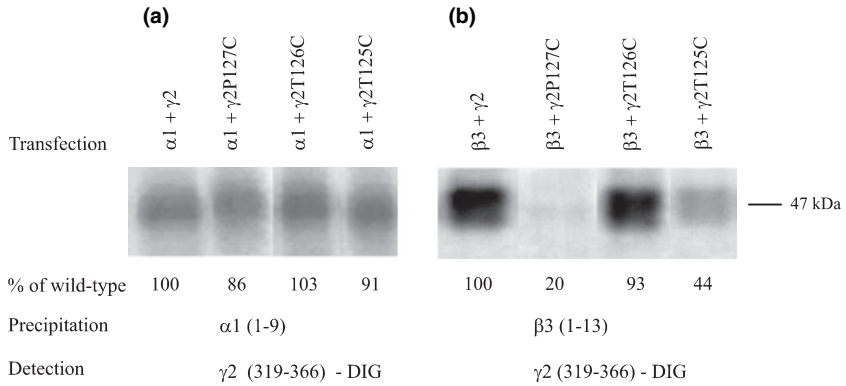

Fig. 9 Co-immunoprecipitation of mutated γ2 with wild-type α1 or β3 subunits. (a) HEK cells expressing the α1 and γ2, or α1 and mutated γ2 subunits were extracted, and proteins were precipitated with α1(1–9) antibodies and subjected to western blot analysis using digoxygenized γ2(319–366) antibodies. (b) HEK cells expressing the β3 and γ2, or β3 and mutated γ2 subunits were extracted, and proteins were precipitated with β3(1–13) antibodies and subjected to western blot analysis using digoxygenized γ2(319–366) antibodies. These experiments were performed three times with comparable results. The average staining of subunits detected with the respective antibodies in the γ2 precipitates of assembly intermediates is given as percentage of wild-type staining and represents the mean values of three different experiments. The respective variability (± SD values) of data is given in the text.

α1(+) side, as suggested by the structural models of $GABA_A$ receptors (Fig. 1b), a replacement of this amino acid by cysteine could have influenced intersubunit contacts with β3 and γ2 subunits (Fig. 1a).

The amino acid residues γ2T125 and γ2P127 are important for assembly of γ2 subunits

After co-transfection of cells with wild-type α1 and β3, and either one of the mutated γ2T125C or γ2P127C subunits, it was demonstrated that the mutated subunits were significantly reduced at the cell surface as compared with wild-type γ2 subunits present in α1β3γ2 transfected cells (Fig. 8c).

On co-expression with wild-type α1 subunits, mutated γ2T125C or γ2P127C subunits could still be co-precipitated by α1(1–9) antibodies (Fig. 9a), suggesting that residues γ2T125 and γ2P127 are not important for assembly with α1 subunits. In contrast, only minor amounts of γ2T125C or γ2P127C subunits could be co-precipitated with wild-type β3 subunits in appropriately transfected HEK cells (Fig. 9b), suggesting that residues γ2T125 and γ2P127 are important for assembly with β3 subunits. The reduced formation of α1β3γ2T125C and α1β3γ2P127C receptors at the cell surface, thus, could be attributed to an assembly problem caused by the mutated subunits. Interestingly, in the ε subunit of the nicotinic acetylcholine receptor, residue εS106, that is homologous to residue γ2P127, has also been demonstrated to be important for assembly (Gu *et al*. 1991).

As the only direct contact of β3 and γ2 subunits in α1β3γ2 receptors is at the β3(–)/γ2(+) interface (Fig. 1a) (Baumann *et al*. 2002; Ernst *et al*. 2003), these data indicated that mutations γ2T125C or γ2P127C impair assembly at the (+) side of the γ2 subunits. This could have been caused by residues γ2T125 or γ2P127 being located at the γ2(+) side, which is in contrast to the comparative models predicting their location at the α1(+)/γ2(–) interface (Fig. 1b). Alternatively, residues γ2T125 and γ2P127 are located at the γ2(–) side as predicted by the homology models, but their mutation could have caused conformational changes in the protein that influenced assembly via the γ2(+) side. Both alternatives seem possible, but the present results and currently available techniques cannot distinguish between these possibilities.

Residues γ2T125 and γ2P127 appear to contribute differentially to the assembly

By comparing the staining intensity of subunits in cells transfected with α1, β3 and γ2 or α1, β3 and γ2P127C subunits (Fig. 8), it is evident that, in the second case, the majority of receptors at the cell surface is composed of α1 and β3 subunits, and that only about 24% of the receptors seem to contain γ2P127C subunits. This is consistent with the finding that the formation of assembly intermediates between β3 and γ2P127C subunits was reduced to a similar extent (to about 20%) as compared with wild-type subunits (Fig. 9b). The extremely inefficient assembly of β3 with γ2P127C subunits might then have caused a preferential formation of α1β3 receptors at the cell surface.

Although the formation of assembly intermediates between β3 and γ2T125C subunits was also reduced (to about 44%) compared with that of wild-type β3 and γ2 subunits (Fig. 9b), it was more efficient than that of β3 and γ2P127C subunits. Furthermore, formation of pentameric receptors composed of α1, β3 and γ2P127C subunits probably was also more efficient than that of pentameric receptors composed of α1 and β3 subunits, as indicated by a predominant formation of α1β3γ2T125C receptors (Fig. 8) and by no or a minimal additional formation of α1β3 receptors.

Mutations in two different subunits can further reduce formation of receptors at the cell surface

When cells were transfected with α1S106C together with β3 (Fig. 7a), γ2 (Fig. 7b) or γ2T126C subunits (Fig. 5), no impaired formation of assembly intermediates was observed, consistent with the observation of a largely unimpaired expression of α1S106Cβ3γ2 receptors at the cell surface (Fig. 6). Similarly, when cells were transfected with γ2T126C together with α1 (Fig. 9a), β3 (Fig. 9b) or α1S106C subunits (Fig. 5), no impaired formation of assembly intermediates was observed. This, again, was consistent with the observation of a more or less unimpaired expression of α1β3γ2T126C receptors at the cell surface (Fig. 8). Interestingly, however, when both mutated subunits were co-expressed with wild-type β3 subunits (Figs 4a–c) a significantly impaired expression of cell surface receptors could be demonstrated, possibly supporting the conclusion that residues α1S106 and γ2T126 are located close to each other (Fig. 1b). The mutation of both of these residues, in contrast to single mutations, might then have impaired pentamer formation. Alternatively, a combination of these mutations within the same receptor might have impaired transport of receptors to the cell surface.

In other experiments, it was demonstrated that the formation of α1A108Cβ3γ2 receptors at the cell surface was significantly ($p < 0.01$) less impaired (to around 50% of wild-type receptors; Fig. 6) than expected from Fig. 7, where α1A108C formed assembly intermediates with β3 or γ2 subunits ($p < 0.01$) that represented only around 30% of those formed from the respective wild-type subunits. This seems to indicate that the additional assembly with the third kind of subunits stabilizes assembly intermediates and slightly enhances the formation of receptors at the cell surface. In experiments, however, where not only the mutated α1A108C, but also the mutated γ2T125C subunit, was co-expressed with wild-type β3 subunits, cell surface expression of the resulting receptors was further reduced to 20–35% of wild-type receptors (Fig. 4). The stronger reduction in the formation of α1A108Cβ3γ2T125C receptors

as compared with α1A108Cβ3γ2 (Fig. 6) or α1β3γ2T125C (Fig. 8) receptors can thus be explained by additive effects of the two mutated subunits.

Finally, the mutation α1F99C does not seem to significantly modulate assembly with β3 and γ2 subunits (Fig. 7) or with γ2P127C subunits (Fig. 5), and does not seem to significantly influence formation of cell surface receptors (Fig. 6). When mutated subunits α1F99C were combined with γ2P127C and wild-type β3 subunits, however, a drastic reduction in the formation of α1F99Cβ3 receptors (that are formed in addition to α1F99Cβ3γ2P127C receptors) was observed at the cell surface (Fig. 4) when compared with α1β3 receptors formed after transfection with α1, β3 and γ2P127C subunits (Fig. 8). This indicates a synergistic inhibition of the formation of receptors at the cell surface when two mutated subunits were present in the same receptor, and again supports the conclusion that the two mutated residues are located closely together.

Thus, effects observed at the level of assembly intermediates do not necessarily translate directly to the formation of pentameric receptors at the cell surface. It is possible that a mutation does not show an effect at the level of assembly intermediates, whereas expression of receptors at the cell surface is disturbed. It is also possible that the formation of assembly intermediates is strongly disturbed as a result of a point mutation of subunits, whereas formation of receptors at the cell surface is less disturbed because of beneficial effects of other assembly partners, or of conformational changes accompanying pentamer formation. Finally, an additive effect of point mutations in different subunits can also occur, supporting the conclusion that mutation of a single amino acid residue can not only change the function (Sigel 2002; Chang *et al*. 2003; Miko *et al*. 2004), but also the assembly of $GABA_A$ receptors (Jin *et al*. 2004; Sancar and Czajkowski 2004).

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