Solution Structure of Human GABA<sub>A</sub> Receptor-associated Protein GABARAP

IMPLICATIONS FOR BIOLOGICAL FUNCTION AND ITS REGULATION

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Control of neurotransmitter receptor expression and delivery to the postsynaptic membrane is of critical importance for neural signal transduction at synapses. The γ-aminobutyric acid, type A (GABA<sub>A</sub>) receptor-associated protein GABARAP was reported to have an important role for movement and sorting of GABA<sub>A</sub> receptor molecules to the postsynaptic membrane. GABARAP not only binds to GABA<sub>A</sub> receptor γ2-subunit but also to tubulin, gephyrin, and ULK1. We present for the first time the high resolution structure of human GABARAP determined by nuclear magnetic resonance in aqueous solution. One part of the molecule, despite being well ordered and rigid on a MHz time scale, exists in at least two different conformations that interchange with each other on a time scale slower than 25 Hz. An important feature of the solution structure is the observation that α- and carboxyl-terminal ends of the protein directly interact with each other, which is not seen in recently reported crystal structures. The possible biological relevance of these observations for the regulation of GABARAP interactions and functions is discussed.

Rapid signaling at synapses between neurons are mediated by small molecules called neurotransmitters. Among neurotransmitters, the most prominent are acetylcholine and glutamate for excitatory synapses and glycine and γ-aminobutyric acid (GABA) for inhibitory synapses, respectively. Receptors for these neurotransmitters are important targets for drugs used to treat mental disorders or to modulate sleep and mood. The principal GABA-gated ion channel is the GABA<sub>A</sub> receptor. Drugs that bind to GABA<sub>A</sub> receptors and modulate their activity, such as the benzodiazepines, offer both medical and economic potential.

Control of neurotransmitter receptor expression at the postsynaptic membrane is of critical importance for functional neurotransmission. Sorting, targeting, clustering, and degradation of neurotransmitter receptors as dynamic processes play a key role in the construction and functional maintenance of synapses.

Recently a novel protein was identified as a binding partner for the γ2-subunit of GABA<sub>A</sub> receptor, termed GABARAP (GABA<sub>A</sub> receptor-associated protein) (1). GABARAP was also reported to bind tubulin (1), gephyrin (2), and ULK1 (3). It is closely related to light chain-3 (LC-3) of microtubule-associated proteins 1A and 1B (MAP-1A and -1B) and to the “late acting intra-Golgi transport factor,” termed GATE-16, of which crystal structures are known (4). In contrast to GABARAP, however, GATE-16 does not interact with gephyrin and GABA<sub>A</sub> receptor γ2-subunit (2). GABARAP is postulated to have an important role for early steps in movement and sorting of GABA<sub>A</sub> receptors (5) and for GABA<sub>A</sub> receptor clustering at the postsynaptic membrane. Binding affinity of GABA to GABA<sub>A</sub> receptors as well as kinetics of inactivation and desensitization of the receptors are dependent on the clustering state of the GABA receptor, which was reported to be strongly modulated by GABARAP (6). Modulation of GABARAP binding to its interaction partners provides a new avenue for pharmacological intervention of receptor activity and neurotransmitter action at the synapse. We and others therefore started a detailed structural investigation of GABARAP. The first available crystal structure of GABARAP (7) turned out to be very similar to the crystal structures of GATE-16 (4), leading to the interesting since sequence identity between both proteins is 57%.

GATE-16 and the GABARAP crystal structures resemble ubiquitin folds with two additional amino-terminal helices. A difference in GATE-16 in the putatively flexible carboxyl-terminal residues and smaller differences in helix 2 and loop regions were found. An additional two different crystal structures of GABARAP are reported but not deposited in the Protein Data Bank (8) rendering them impossible to be studied in detail and compared with other structures. Coyle et al. (8) obtained the structures from two different crystal forms. One structure is reported to resemble closely that of GATE-16. The other crystal form was obtained under high salt conditions in which helix 1 is flipped by −180°, pointing away from the rest of the molecule and contacting the neighboring molecule in the crystal in a head to tail fashion. Whether this polymerized state of GABARAP might be of physiological relevance is not clear.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—GABARAP was expressed and purified as a glutathione S-transferase fusion protein. Thrombin (Merck) cleavage was performed yielding full-length GABARAP with additional glycine and serine residues at its amino terminus. Details of

Overhauser effect; NOESY, NOE spectroscopy; HSQC, heteronuclear single quantum correlation; r.m.s.d., root mean square deviation; E1, ubiquitin-activating enzyme.

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cloning, protein expression, and purification of GABARAP have been described elsewhere (9).

NMR Spectroscopy—NMR samples contained 0.8 mM protein in 25 mM sodium phosphate, 100 mM NaCl, 100 mM KCl, pH 6.9, in 95% H2O, 5% D2O with 100 µM phenylmethylsulfonyl fluoride, 0.02% (by weight) sodium azide, and 50 µM EDTA. NMR spectra were recorded at 298 K on a Varian Unity INOVA spectrometer equipped with a triple-axis pulse-field gradient 1H/15N/13C probe at proton frequencies of 600 and 750 MHz. The resonance assignment of GABARAP was described previously (9). Structural constraints were derived from 15N-edited NOESY-HSQC (100-ms mixing time) (10), aliphatic 13C-edited NOESY-HSQC (80-ms) experiments (11) in the described buffer, and aliphatic 13C-edited NOESY-HSQC (120-ms) experiments with protein in the buffer after replacement of H2O by D2O. Uniformly 13C-15N-labeled protein was used for these experiments. 15N-Labeled protein was used for the 1H-15N heteronuclear NOE experiments (12).

Data Evaluation and Structure Calculation—Based on the almost complete assignment of 1H, 13C, and 15N resonances of GABARAP, a total of 4577 NOE distance constraints (including 1375 long range NOEs) could be derived from three-dimensional 15N-edited NOESY spectra in an iterative procedure (Table I). NOE analysis and assignment were performed using NMRView (13) and ARIA (14). Interproton distances were used directly to calibrate experimental peaks and to extract distance constraints. Lower and upper bounds for distance constraints were derived from the target distances empirically by estimation of the error as 12.5% of the target distance squared. Distances involving ambiguous constraints, methyl groups, aromatic ring protons, and the nonstereo-specifically assigned methylene protons were treated as sum of separate contributions to the target function, known as “sum averaging” (15).

Final structures were calculated using the simulated annealing protocol with the program CNS version 1.0 (16) using standard parameters with the following modifications. For conformational space sampling 20 ps with a time step of 10 fs were simulated using torsion angle dynamics at a temperature of 50,000 K followed by 30 ps of slow cooling to 0 K with a time step of 15 fs. In an additional Cartesian slow cooling stage, the temperature was decreased in 20 ps from 2000 to 0 K with a time step of 5 fs. After simulated annealing the structures were subjected to 2000 steps of energy minimization.

A total of 15 structures that did not show any distance constraint violation of more than 0.0175 nm was used for further analysis. Geometry of the structures, structural parameters, and secondary structure elements were analyzed and visualized using the programs MOLMOL (17), PROCHECK (18), and WHATIF (19). The coordinates have been deposited in the Protein Data Bank with accession code 1KOT.

RESULTS AND DISCUSSION

Solution Structure and Comparison with Crystal Structures of GABARAP and GATE-16—Earlier we reported almost complete assignments for backbone and side chain 1H, 13C, and 15N resonances of human GABARAP (9). Simultaneously backbone resonance assignments were reported by others (20). Reinspec-

![Fig. 1. Solution structure of human GABARAP after simulated annealing and refinement calculations. A, shown is the superposition of the backbone of all 15 obtained structures. B, ribbon presentation of the averaged GABARAP structure. Secondary structure elements are labeled according to their sequential arrangement. Amino- (N) and carboxyl (C)-terminal ends are indicated. C, backbone worm presentation of GABARAP. Residues that contain amide groups with split or broadened resonance peaks are colored in red. Residues Val6 and Asp102 are also colored in red because their amide resonances were undetectable. This indicates that the respective residues are involved in conformational exchange on a slow to intermediate time scale. Prominent residues are labeled with amino acid type and sequence position. All figures were prepared using MOLMOL (17).](Image 0x-23 to 25x808)


\(\beta\)-sheet are aligned antiparallel to the inner strands, which are parallel to each other, and helices \(\alpha_3\) and \(\alpha_4\) are located on one side of the sheet. In addition to the ubiquitin fold, GABARAP contains two additional helices, \(\alpha_1\) and \(\alpha_2\), that are located on the opposite side of the \(\beta\)-sheet relative to \(\alpha_3\) and \(\alpha_4\).

Average local displacement values relative to the mean structure are a measure for the precision of the derived family of structures. Large values indicate either local flexibility of the protein or lack of experimental data for this region. Average local displacement values of the GABARAP solution structure indicate the regions Asp45–Lys47, Leu70–Glu74, and Glu101–Leu105 of the protein to be less defined (Fig. 2A). The first two regions also have slightly decreased heteronuclear NOE values (Fig. 2B) indicating increased dynamic behavior. \(^1H\)–\(^{15}N\) heteronuclear amide NOE values are a measure for the dynamics of the local environment within the time scale of the absolute NMR frequencies (60–750 MHz in the present study).

Overall the solution structure is very similar to that of the GABARAP crystal structure (7). Notable differences between the solution structure and all deposited crystal structures of GABARAP and GATE-16 map to residues 2–14, 37–46, 66–75, and 113–117. The first region largely overlaps with residues that appear in the NMR spectra as broadened and split resonances. Regions 37–46 and 66–75 coincide with those shown to have slightly increased mobility as inferred from heteronuclear NOE data (Fig. 2B).

The root mean square deviation (r.m.s.d.) value for the backbone coordinates of residues 1–112 is 0.134 nm between GABARAP solution and crystal structure (7) as well as 0.139 and 0.151 nm for GABARAP solution structure and GATE-16 crystal structures (4). The coordinates for the very carboxyl-terminal residues, however, differ remarkably.

Some Regions of GABARAP Are Involved in Conformational Exchange—Backbone amide resonances of a number of residues showed up in the NMR spectra as broadened and split lines, indicating conformational exchange on an intermediate and slow time scale. Due to the lack of concentration dependence, the observed phenomenon could not be assigned to a monomer-dimer equilibrium. The respective residues map to completely different sequence regions (Val1–Lys20 without Glu12 and Ser16, His99–Leu105 without Phe103, and Lys47) that are all close to each other in space (Fig. 1C). The first region encompasses helix \(\alpha_1\) and a large portion of helix \(\alpha_2\), and the second region belongs to the loop between helix \(\alpha_4\) and strand \(\beta_4\). The carboxyl-terminal Leu105 of the second region exhibits a split backbone amide resonance, but the corresponding proton is involved in stable \(\beta\)-sheet-like secondary structure hydrogen bonding with the Pro25 backbone carbonyl oxygen as suggested by inspection of the structure and the stability of the Leu105 amide proton in hydrogen exchange experiments (data not shown). Lys45 is located next to the amino terminus of strand \(\beta_2\), and its side chain protrudes to the amino terminus of helix \(\alpha_1\), thus obviously opposing the dipole of helix \(\alpha_1\). Phe103, Glu12, and Ser16 appear not to be affected by line broadening or splitting. Also the amino-terminal residues Met1, Lys2, and Phe3 are not visibly affected by conformational exchange phenomena. To estimate the time scale for a potential exchange between the different conformations corresponding to the different observed sets of resonances, the frequency distance for several pairs of split amide resonances was measured. Some of them (Arg15 and Lys20) yielded values of 25 and 27 Hz, respectively. That leads to the conclusion that, under the conditions used in the present study, any exchange between the conformers is slower than 25 Hz.

Interestingly all residues involved in this conformational exchange are spatially close to Pro10. This residue is discussed as the hinge for the interchange between two different conformations yielded for GABARAP under different crystallization conditions (8). Comparing both conformations relative to each other, helix \(\alpha_1\) is flipped by \(\sim180^\circ\) at residue Pro10. The potential of conformational changes around Pro10 is principally in accordance with the dynamics observations obtained for the GABARAP solution structure. However, the proposed head to tail polymerization could not be observed under the conditions used in the present study.

The Carboxyl-terminal Part of GABARAP Is Well Defined and in Direct Contact with the Amino-terminal Residues—
GABARAP and GATE-16 crystal structures indicate that the carboxyl-terminal part of the respective molecule is not an integral part of the protein scaffold. In the publicly accessible crystal structures of GABARAP and the two GATE-16 conformers the carboxyl termini differ significantly among themselves and compared with the solution structure. This may be due to different favorable crystal contacts of the carboxyl-terminal end observed in the crystal structures (4, 7). Inspection of the solution structure of GABARAP reveals that the hydroxyl oxygen of the Tyr\(^{115}\) phenolic ring is hydrogen-bonded to the backbone amide nitrogen of Lys\(^2\) (Fig. 3). Furthermore Tyr\(^{115}\) is involved in a network of hydrophobic interactions. The methyl groups of Met\(^1\), Ala\(^{36}\), Ala\(^{108}\), and Leu\(^{117}\), as well as the side chain of Pro\(^{97}\), form a hydrophobic pocket for Tyr\(^{115}\) as evidenced by a large number of direct NOE observations between these residues. In addition to Tyr\(^{115}\) and Leu\(^{117}\), most of the carboxyl-terminal residues are involved in numerous direct NOE-observable contacts to residues of the amino terminus and the loop connecting \(\beta\)-strands \(\beta1\) and \(\beta2\). Thus, clearly the carboxyl-terminal residues of GABARAP are an integral part of the globular and compact structure of GABARAP.

Throughout the GABARAP and GATE-16 family of proteins, Phe\(^{115}\) appears to be highly conserved (4). GABARAP itself and a Caenorhabditis elegans ortholog of GATE-16 are the only remarkable exceptions, containing a tyrosine at this position. Stabilizing the closed conformation of the carboxyl terminus in the GABARAP solution structure using a hydrogen bond in addition to hydrophobic interactions might therefore be a unique feature of GABARAP that is, however, not observed in the reported crystal structure. \(^{1}H,^{15}N\) NOE data confirm that Tyr\(^{115}\) does not exhibit a decreased heteronuclear NOE (Fig. 2B) as would be expected for a residue not rigidly connected to the globular fold of the protein.

The carboxyl terminus of GABARAP may play a decisive role in the biological function of the protein. GABARAP as well as GATE-16, MAP-LC-3, and human Apg12p were shown to be substrates for human Apg7p, a novel E1 enzyme essential for the Apg12p-conjugating system (21), a system involved in autophagy. Autophagy is a process that involves the bulk degradation of cytoplasmic components by the lysosomal/vascular system, which is conserved from yeast to mammalian cells. For the yeast system, it was shown that Apg12p is covalently attached to Apg5p via the carboxyl-terminal glycine residue of Apg12p, which is very similar to the ubiquitin system (22). For mammalian cells, however, a speculation that needs to be addressed by future investigations.

This kind of triggered conformational change may also be used for rational manipulation of GABARAP function. Selective stabilization of the conformation with a tightly attached carboxyl terminus may inhibit binding to interaction partners.

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**REFERENCES**