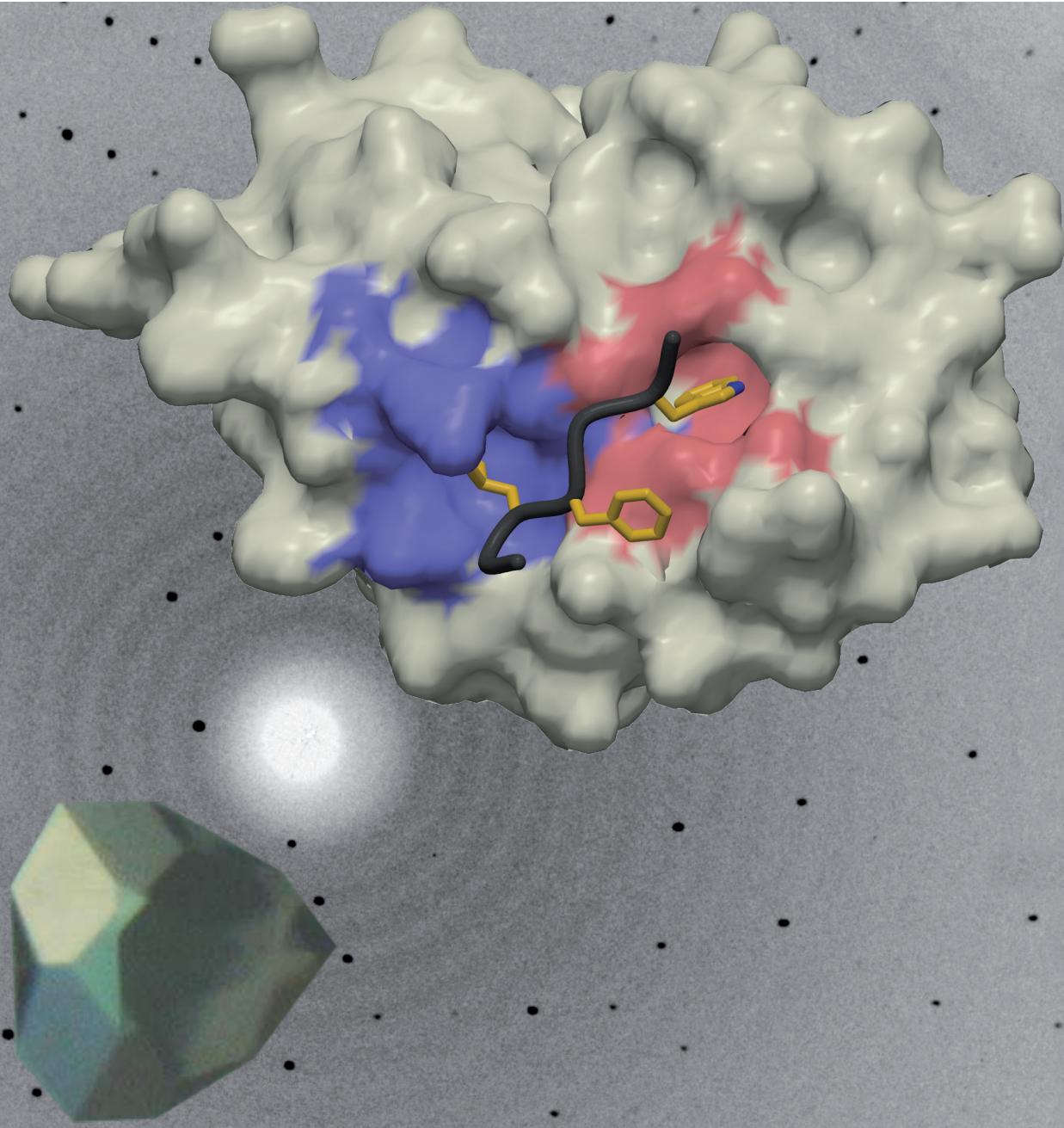


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REVIEW

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PAPER

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Structural characterization of GABARAP–ligand interactions†

Yvonne Thielmann,^a Oliver H. Weiergräber,^{*b} Jeannine Mohrlüder^{ac} and Dieter Willbold^{*ac}

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The GABA_A receptor-associated protein (GABARAP) plays an important role in intracellular trafficking of several proteins. It undergoes a C-terminal lipidation process that enables anchoring in the cytosolic leaflet of cellular membranes. While the three-dimensional structure of GABARAP itself has been determined, structural investigation of complexes with its interaction partners has just commenced. Studies with indole derivatives revealed that GABARAP features two hydrophobic binding sites (hp1 and hp2). These also play an essential role in complex formation with the native ligand calreticulin. Furthermore, a model of hexameric *N*-ethylmaleimide-sensitive factor (NSF) suggests that binding of GABARAP to this molecular machine may involve a similar site. Since hp1 and hp2 are highly conserved throughout the GABARAP family, the relevance of the structural data presented here is likely to extend to GABARAP homologues.

1. Introduction

Originally, GABARAP was identified as a protein interacting with the cytoplasmic loop between TM3 and TM4 of the GABA_A receptor γ 2 subunit. Colocalization with the GABA_A receptor in cultured cortical neurons and the presence of a tubulin binding site qualified this protein as a potential adaptor linking the GABA_A receptor to the cytoskeleton.¹

After some initial controversy, the punctate localization pattern of GABARAP is now believed to correspond to vesicular structures, rather than synapses.²

GABARAP belongs to a protein family which is highly conserved from yeast to mammals.³ The single member in *Saccharomyces cerevisiae* is Atg8, an essential regulator of autophagy, which involves non-selective sequestration of cytoplasmic material for vacuolar degradation. Homologues in mammals include GEC1 (glandular epithelial cell protein 1), which was characterized as an early estrogen-regulated protein, GATE-16 (Golgi-associated ATPase enhancer of 16 kDa), identified as an essential component of intra-Golgi transport, and LC3 (light chain 3), which is a subunit of microtubule-associated protein complexes. Proteins of the GABARAP family are involved in transport processes and display the following sequence identities with GABARAP: GEC1 86%, GATE-16 57%, Atg8 54% and LC3 28%. They are all members of the ubiquitin superfamily, which is characterized by a so-called β -grasp fold. The latter consists of a central

^a Institut für Strukturbioologie und Biophysik 3 (Strukturbiochemie), Forschungszentrum Jülich, 52425, Jülich, Germany.
E-mail: d.willbold@fz-juelich.de; Fax: +49 2461 612023;
Tel: +49 2461 612100

^b Institut für Strukturbioologie und Biophysik 2 (Molekulare Biophysik), Forschungszentrum Jülich, 52425, Jülich, Germany.
E-mail: o.h.weiergraebert@fz-juelich.de; Fax: +49 2461 611448;
Tel: +49 2461 612028

^c Institut für Physikalische Biologie und BMFZ,
Heinrich-Heine-Universität, 40225, Düsseldorf, Germany
† Electronic supplementary information (ESI) available: Movies of GABARAP–ligand interactions. See DOI: 10.1039/b900425d



Yvonne Thielmann

Yvonne Thielmann is a scientist in the Institute of Structural Biology and Biophysics at the Research Center in Jülich. She received a diploma in biotechnology from the University of Applied Sciences in Giessen and a diploma in biology from the University of Düsseldorf. Her current work is focused on structural characterization of GABARAP–ligand interactions. Recently, she received her PhD at the University of Düsseldorf.



Oliver H. Weiergräber

Dr Oliver H. Weiergräber is a scientist in the Institute of Structural Biology and Biophysics at the Research Centre Jülich, Germany. His current research interests comprise investigation of protein–protein and protein–ligand interactions using biophysical techniques, in particular X-ray crystallography. After receiving his degree in medicine at the RWTH Aachen, he performed postdoctoral studies at the University of Düsseldorf, dealing with the impact of cell volume on signal transduction processes.

four-stranded mixed β -sheet with two α -helices located on its concave side. A hallmark of the GABARAP family is an N-terminal extension containing two additional α -helices that are attached to the convex face of the β -sheet.^{4–7}

Ubiquitin is processed by a series of enzymes and finally conjugated to various target proteins, thus either labelling them for degradation or changing their localization. A similar mechanism was found for the GABARAP family, the first step being limited proteolysis by Atg4, which results in exposure of the penultimate glycine residue. Via ATP hydrolysis, the protein is coupled to an E1 enzyme (Atg7), transferred to an E2 enzyme (Atg3) and finally bound covalently to phosphatidylethanolamine or phosphatidylserine. This conjugation is reversible, and GABARAP can be cleaved from the membrane by Atg4.^{8,9}

The number of known GABARAP interaction partners is rising steadily. Their diversity currently complicates a clear-cut definition of GABARAP's cellular function. Nonetheless, current evidence points to a major role in subcellular transport of various cytosolic and membrane-associated proteins.

Structural information on GABARAP interactions with artificial as well as physiological ligands has only recently become available. The purpose of this review is to provide a brief account of the work in this area, which was mainly conducted in our laboratories during the past five years. These investigations include binding studies with indole and indole derivatives,¹⁰ an artificial peptide from a phage display screen,¹¹ as well as newly identified physiological interaction partners of GABARAP such as clathrin¹² and calreticulin.¹³ The latter were found in a database search with a position specific scoring matrix (PSSM) and confirmed by colocalization and pulldown experiments. The binding mode of these ligands was studied extensively by nuclear magnetic resonance (NMR) spectroscopy; in addition, the X-ray structures of two GABARAP-peptide complexes could be determined.^{11,14} Finally, comparative modeling was applied to obtain

conceptual information on the association of GABARAP and GATE-16 with the large hexameric ATPase NSF.¹⁵

Together, these investigations have provided novel insight into structural foundations and dynamic properties of protein–protein interactions of GABARAP and its homologues, which ultimately govern the biological functions of this exciting class of adaptor molecules.

2. Studies with artificial ligands

2.1 Indole derivatives

In a phage display screen of a randomized dodecapeptide library, 94% of sequences selected for GABARAP binding activity contained at least one tryptophan residue. This result is remarkable in view of the low overall prevalence (2.2%) for tryptophan in the library. Since the side chain of tryptophan contains a rigid, aromatic indole moiety, free indole and several derivatives [indole acetic acid (IAA), L-tryptophan (Trp) and N-acetyl tryptophanamide (NATA)] were chosen as probes to characterize the binding properties of GABARAP by two-dimensional NMR spectroscopy. Heteronuclear single quantum coherence (HSQC) titration experiments with ¹⁵N-GABARAP revealed similar chemical shift perturbation patterns for all four compounds. Major changes are seen in β -strands β 2 and β 1 as well as the α 2- β 1 loop, with minor changes in helices α 2 and α 3 and the β 3- α 4 and α 4- β 4 loops. In experiments with IAA, the trajectories of individual peaks showed qualitative differences. Some resonances shifted strongly at low concentrations whereas others moved predominantly at higher concentrations. This observation could be rationalized by determination of the dissociation constants for residues involved in the binding event: two apolar grooves on the surface of GABARAP display different affinities for IAA. These patches were termed hydrophobic pocket 1 (hp1, $K_D \approx 6$ mM) and hp2 ($K_D > 38$ mM). For indole ($K_D \approx 12$ mM) and Trp ($K_D > 30$ mM), dissociation constants were obtained for hp1 only, whereas the low



Jeannine Mohrlüder

Dr Jeannine Mohrlüder is a scientist at the Institute of Structural Biology and Biophysics at the Research Centre Jülich in Germany. Her current work focuses on the identification and characterization of protein–protein interactions of the human GABA type A receptor associated protein GABARAP. She majored in Biology at the Johannes Gutenberg University in Mainz, Germany and the University of Montpellier II in France, and carried out her diploma thesis

at the Institute of Biochemistry of the University of Cologne in Germany. She did her PhD at the Heinrich Heine University of Düsseldorf, Germany.



Dieter Willbold

Dr Dieter Willbold is a full professor for physical biology at the Heinrich-Heine-University of Düsseldorf and director of the Institute of Structural Biology and Biophysics at the Research Centre Jülich. His main research interest is the structural basis for affinity and specificity of protein–ligand interactions, focusing on viral proteins and proteins that are relevant for function and dysfunction of neurons. He majored in Biochemistry in Tübingen, Bayreuth and Boulder, CO and received a PhD in Biophysical Chemistry at the University of Bayreuth before performing postdoctoral studies there and at Tel-Aviv University, and heading a junior research group in Jena, Germany. In 2001, he accepted a professorship in Düsseldorf.

solubility of NATA precluded the reliable determination of a dissociation constant. For hp1 the indole derivatives can be ranked according to their affinities (IAA > indole > Trp), suggesting an electrostatic contribution to the binding energy from the carboxyl group of IAA and basic side chains K46 or K48 of GABARAP. Intriguingly, in the phage display-selected peptides the prominent tryptophan residue was frequently flanked by glutamic or aspartic acid.

While tryptophan is the least frequent among all proteinogenic amino acids, it shows the highest propensity for residing in a protein–protein interface. If the binding site for indole and indole derivatives is biologically relevant, GABARAP interaction partners should display a surface-exposed conserved tryptophan. Indeed, such an invariant tryptophan can be found in the binding motifs of the GABA_A receptor $\gamma 2$ subunit, calreticulin and clathrin heavy chain. It is noteworthy that mutation of the tryptophan to alanine in the respective calreticulin fragment (CRT^{178–188}, see below) decreased the affinity of the peptide by at least three orders of magnitude.

These results indicate that GABARAP displays a specific binding site for indole and indole derivatives, which seems to play an important role for protein–protein interactions. Whether this hydrophobic surface is a physiological docking site for low molecular weight compounds with similarity to indole remains to be determined¹⁰.

2.2 K1 peptide

Phage display screening with GABARAP led to identification of a peptide (DATYTWEHLAWP, hereafter termed “K1”) which was found numerous times in independent experiments. The interaction of the K1 peptide with GABARAP was studied using surface plasmon resonance (SPR) and NMR spectroscopy. SPR experiments yielded kinetic rate constants of $k_{\text{on}} = 2.23 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and $k_{\text{off}} = 7.88 \times 10^{-2} \text{ s}^{-1}$, resulting in a dissociation constant of 350 nM. These observations were confirmed by NMR experiments. Titration with the K1 peptide led to gradual disappearance of native GABARAP resonances in ¹H¹⁵N-HSQC spectra, with concomitant emergence of new signals corresponding to the GABARAP-K1 complex. This behaviour indicates medium to slow exchange rates on the chemical shift timescale, which is often found with low micromolar or submicromolar dissociation constants. The three-dimensional structure of the GABARAP-K1 complex was determined by X-ray crystallography.¹¹ The K1 ligand makes close contact with GABARAP in its entire length, burying approximately 620 Å² of the solvent-accessible surface. Based on its structural properties, the peptide can be divided into three parts: the N- and C-terminal segments (D¹ATY⁵ and A¹⁰WP¹², respectively) take on an extended conformation and are connected by a short central 3₁₀ helix (W⁶EHL⁹). Complex formation is dominated by hydrophobic interactions mediated by the 3₁₀ helix introducing W⁶ and L⁹ into hp2 and the C-terminal segment inserting W¹¹ into hp1 of GABARAP. It is important to note that ligand binding to GABARAP induces significant conformational changes, as illustrated in a movie which is available as ESI.† For additional details refer to the discussion of the GABARAP-CRT^{178–188} structure (section 3.2).

3. Investigation of physiological ligands

3.1 Clathrin heavy chain

In a pulldown experiment, the heavy chain of clathrin was identified as a GABARAP binding partner. Simultaneously, it was found in a database search with a PSSM established from phage display-derived peptide sequences, suggesting a probable GABARAP interaction site. Binding of this clathrin peptide (aa 510–522) to GABARAP was investigated by NMR experiments. HSQC spectra of native GABARAP show line broadening and reduction of peak intensities after addition of clathrin(510–522). These changes are clearly indicative of a direct interaction. Intriguingly, amino acids that are affected by ligand binding closely match those found in experiments with indole and indole derivatives as well as the K1 peptide. Both hydrophobic pockets of GABARAP appear to be involved in complex formation with clathrin(510–522), which features one conserved tryptophan.

Notably, native clathrin heavy chain can be displaced from binding to recombinant GABARAP by a second physiological ligand, calreticulin¹² (see below).

The biological significance of the clathrin–GABARAP interaction might relate to the GABA_A receptor: endocytosis of the GABA_A receptor is crucial for control of receptor numbers at the postsynaptic membrane of neurons. This process depends on the formation of coated pits and is mediated by clathrin. The interaction with clathrin heavy chain may therefore represent an important aspect of GABARAP function in GABA_A receptor trafficking.

3.2 Calreticulin

Calreticulin is another GABARAP binding partner identified by a database search with the PSSM mentioned above. HSQC spectra of GABARAP in the absence and presence of calreticulin showed clear indications of complex formation. In this case, determination of interacting residues by NMR spectroscopy was not possible due to the large size of the complex. SPR experiments with calreticulin and GABARAP yielded a dissociation constant of 64 nM. This is the strongest interaction reported thus far for either one of these proteins.

Calreticulin is known for its function as Ca²⁺-dependent chaperone and Ca²⁺ storage protein in the endoplasmic reticulum (ER). The structure of calreticulin has not yet been determined in its entirety. It consists of N- and C-terminal segments predicted to contribute to a globular domain, and an intermediate part, the so-called P domain (proline rich domain), which forms an arm-like structure. Although it is mostly located in the ER lumen, its retrotranslocation back into the cytosol has been demonstrated, thus allowing for interaction with GABARAP. Indeed, colocalization studies revealed similar patterns for both proteins. Additionally, it was shown that recombinant GABARAP can bind calreticulin from cell lysates, and this interaction can be prevented by addition of an artificial peptide.¹³

In a second study, different calreticulin fragments were investigated for their interaction with GABARAP. These included the complete P domain (aa 177–288), a short segment thereof (CRT^{178–188}), and a mutant of this peptide

(W¹⁸³A-CRT^{178–188}). All fragments contain the putative GABARAP binding motif identified in the database search. SPR measurements with these calreticulin constructs indicated an increase of affinity with peptide length (CRT^{178–188} 11 μM, P domain 930 nM, full-length calreticulin 64 nM). These data suggest that the peptide CRT^{178–188} contains the primary binding motif, but the P domain and the globular domain of calreticulin account for additional contacts. Furthermore, the mutation of tryptophan to alanine in W¹⁸³A-CRT^{178–188} shifts the dissociation constant from the micromolar into the millimolar range. These complexes were further characterized by NMR experiments with HSQC titrations. The HSQC spectrum of GABARAP showed only slight changes in the presence of W¹⁸³A-CRT^{178–188}. In contrast, addition of CRT^{178–188} had an obvious effect on all correlation signals, and the same was true for the P domain. Again, the NMR experiments essentially confirmed the SPR data and clearly indicated involvement of both hp1 and hp2 in calreticulin binding to GABARAP.

The three-dimensional structure of the GABARAP-CRT^{178–188} complex could be determined by X-ray crystallography. The CRT^{178–188} ligand attains an elongated conformation in close contact to GABARAP, burying 490 Å² of solvent-accessible surface. The central portion of the peptide establishes main chain hydrogen bonds to strand β2 and can therefore be viewed as an intermolecular extension of the central β-sheet. Side chain hydrogen bonds stabilize both termini of the peptide. Overall, the interaction is dominated by hydrophobic contacts of W¹⁸³, F¹⁸⁵ and L¹⁸⁶ to GABARAP. W¹⁸³ anchors the peptide in hp1, with its indole moiety forming contacts to residues in helix α2, strands β1 and β2 and the α4-β4 loop. The side chain of F¹⁸⁵ is reaching out across strand β2, interacting with apolar groups from the α2-β1 loop. The C-terminal part of CRT^{178–188} is held in position by hydrophobic contacts of L¹⁸⁶ to strand β2, helix α3 and the β2-α3 loop (hp2).

Formation of this complex induces a significant conformational change in the GABARAP molecule (Fig. 1); a movie

visualizing these effects is included as ESI.† The indole ring of W¹⁸³ (inserted in hp1) causes a slight displacement of helix α2, accompanied by changes in the side chain conformations of K⁴⁸ and F¹⁰⁴. In contrast, L¹⁸⁶ leads to a large movement of helix α3, resulting in hp2 taking an “open” conformation. This spatial rearrangement appears to be chiefly mediated by the displacement of L⁶³. It is interesting to note that GABARAP-bound CRT^{178–188} differs from the K1 peptide not only in its secondary structure but also in directionality: while the K1 ligand attaches to hp1 with its C-terminal side chains, a reversed orientation is found in the CRT^{178–188} complex.

A structure model of full-length calreticulin reveals that its proposed GABARAP binding site is located at the N-terminal junction between the globular domain and the P domain. The precise physiological significance of this interaction is currently unknown, but may be related to intracellular trafficking of membrane proteins, such as the N-cadherin/β-catenin complex or α₃β₁ integrin dimers.¹⁴

3.3 NSF

Fusion of vesicles with their target membranes *in vivo* is a reversible process. The minimal machinery required to maintain this circuit includes NSF, the adaptor SNAP (soluble NSF attachment protein) and the membrane-anchored SNAP receptor (SNARE) complexes. NSF is a member of the AAA (ATPases associated with various cellular activities) class within the superfamily of Walker-type ATPases. These proteins typically form ring-like oligomers, the physiological assembly of NSF being a symmetric hexamer. Each protomer is composed of three domains, an N-terminal substrate binding domain (N) and two ATPase domains (D1 and D2). While crystal structures have been determined for the N and D2 domains, a structure of the full-length protein including the D1 domain is still lacking. Therefore, a homology model of the entire NSF complex was generated, using the related ATPase p97/VCP as a template.¹⁵

Assuming an antiparallel orientation of the ATPase domains of NSF,¹⁶ the model reveals three hydrophobic side

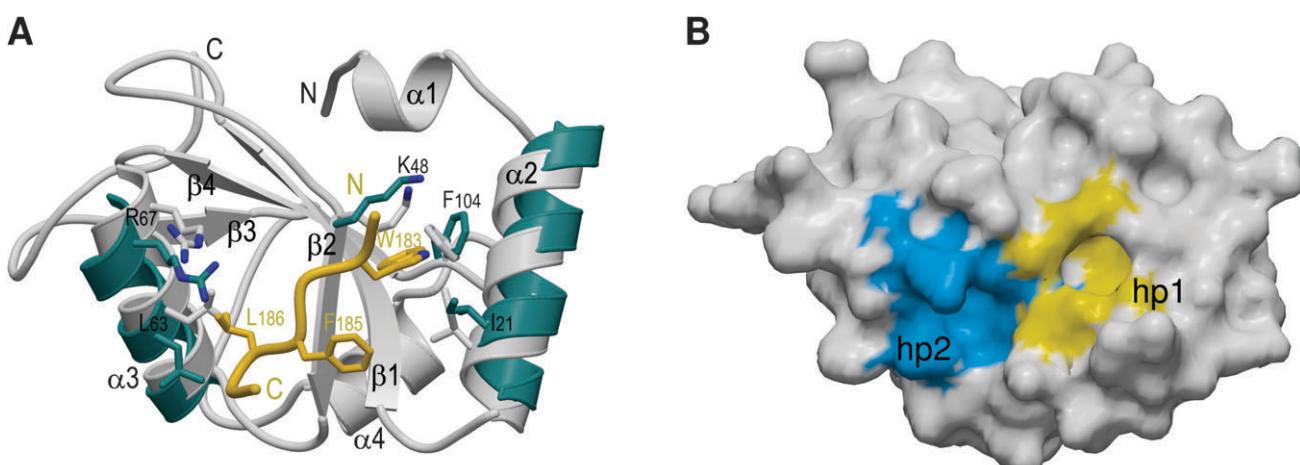


Fig. 1 Complex of GABARAP with a calreticulin fragment. (A) The CRT^{178–188} peptide (yellow) as well as GABARAP helices α2 and α3 (green), which are displaced upon ligand binding, are superposed on the structure of native GABARAP (1KOT, grey). Selected side chains involved in the interaction are indicated. (B) Surface representation of the GABARAP molecule from the complex structure (same orientation as in (A), but not to scale), with the hydrophobic pockets harboring W¹⁸³ and L¹⁸⁶ of calreticulin colored yellow (hp1) and blue (hp2), respectively.

chains exposed at the beginning of the D2 domain; this site appeared as a promising target for docking experiments with GABARAP. According to the resulting model, the NSF-GABARAP interface is composed of an apolar core, which is flanked by polar contacts, mainly salt bridges. Binding of GABARAP to NSF involves interaction with both AAA domains, in addition to the hydrophobic side chains residing in the D1–D2 domain linker. In the GABARAP molecule, contacts are chiefly mediated by hp1 residues. The GABARAP surface buried in the complex is 950 Å², which amounts to 14% of GABARAP's total surface area.

An interesting aspect of the complex model is the proximity of GABARAP to the ATP binding site in the D1 domain, suggesting a regulatory impact on ATP binding and/or hydrolysis. Indeed, the GABARAP homologue GATE-16 has been reported to increase the ATPase activity of NSF 3.5 fold. Due to its C-terminal lipidation, binding of GABARAP (or GATE-16) to NSF in this configuration could serve to anchor the complex to membranes. This hypothesis is supported by the observation that suppression of GABARAP lipidation results in an altered localization pattern of NSF in the cell¹⁷.

4. Conclusion

The work summarized in this review has focused on the characterization of GABARAP interactions with artificial as well as physiological ligands, applying a wide repertoire of methods from molecular and cellular biology, biochemistry and structural biology. Together, these investigations have provided insight into structural foundations and dynamic properties of protein–protein interactions of GABARAP and its homologues. Despite this progress, several important issues remain to be addressed, such as the precise molecular mechanism of GABARAP in GABA_A receptor regulation, intracellular vesicular transport, fusion processes and autophagy. The authors are confident that future research will continue to unveil exciting new aspects about this novel class of adaptor molecules.

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