Identification of Clathrin Heavy Chain as a Direct Interaction Partner for the γ-Aminobutyric Acid Type A Receptor Associated Protein†

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ABSTRACT: γ-Aminobutyric acid type A receptors ($GABA_A$ receptors) are the major sites of GABA-mediated fast synaptic inhibition in the central nervous system. Variation of the cell surface receptor count is postulated to be of importance in modulating inhibitory synaptic transmission. The $GABA_A$ receptor associated protein (GABARAP) is a ubiquitin-like modifier, implicated in $GABA_A$ receptor clustering, trafficking, and turnover. GABARAP pull-down experiments with brain lysate identified clathrin heavy chain to be GABARAP-associated. Phage display screening of a randomized peptide library for GABARAP ligands yielded a sequence motif which characterizes the peptide binding specificity of GABARAP. Sequence database searches with this motif revealed clathrin heavy chain as a protein containing the identified sequence motif within its residues 510−522, supporting the result of the pull-down experiments. Calreticulin, which was identified recently as a GABARAP ligand, contains a very similar sequence motif. We demonstrate that calreticulin indeed competes with clathrin heavy chain for GABARAP binding. Finally, employing nuclear magnetic resonance spectroscopy, we mapped the GABARAP residues responsible for binding to clathrin. The hereby mapped GABARAP regions overlap very well with the homologue residues in yeast Atg8 that were recently shown to be important for autophagy. Together with the knowledge that GABARAP and clathrin are known to be involved in $GABA_A$ receptor trafficking within the cell, this strongly suggests a clear physiological relevance of the direct interaction of GABARAP with clathrin heavy chain.

The ratio between neuronal excitation and inhibition is of critical importance for information processing in the brain. Disruption of this balance is hypothesized to be responsible for epilepsy and anxiety disorders (*1*−*4*). Neurons therefore require distinct mechanisms to regulate intracellular vesicular protein transport, sorting, targeting, clustering, and endocytosis of neurotransmitter receptors. $GABA_A$ receptors[1] are the major mediators of fast synaptic inhibition in the CNS and are the principal GABA-gated ion channels. They are important targets for drugs used to treat mental disorders or to modulate sleep and mood (*5*, *6*). Internalization of the $GABA_A$ receptor is mediated by clathrin-coated vesicles (*7*). The γ2-subunit of $GABA_A$ receptors was shown to bind the $GABA_A$ receptor associated protein (GABARAP) (*31*), a 14 kDa polypeptide implicated in trafficking $GABA_A$ receptors to the plasma membrane (*8*−*11*).

Atg8 is a GABARAP orthologue protein in yeast, essential for autophagy (*12*). Human GABARAP homologue proteins are members of the MAP1-LC3 family. Besides GABARAP, this family includes the GABARAP-homologous proteins GABARAPL1 (GEC1; sequence identity 87%), GABARAPL2 (GATE-16; 57%), and GABARAPL3 (82%) and the MAP-LC3 proteins MAP-LC3A (30%), MAP-LC3B (31%), and MAP-LC3C (38%). All members of this family are evolutionally conserved in eukaryotic cells from yeast to mammals, indicating a critical function of these proteins in mammalian cells. The high sequence identity between the GABARAP-like proteins and the missing phenotype of a GABARAP knockout mouse (*13*) suggest that the GABARAP-like proteins can functionally substitute each other.

Like other proteins of the MAP1-LC3 family, GABARAP exhibits a ubiquitin-like fold with two additional N-terminal α-helices (*14*−*19*), distinguishing the GABARAP-like protein family within the superfamily of ubiquitin-like proteins.

Besides structural similarity to ubiquitin, GABARAP is a ubiquitin-like modifier (UBL) and is involved in ubiquitin-like protein modification machinery (*20*, *21*). After processing of GABARAP to a glycine-exposed form by the cysteine-protease hAtg4 (*22*), GABARAP is activated by the E1 ubiquitin-activating enzyme hAtg7 (*23*) and subsequently transferred to the E2 ubiquitin-conjugating enzyme hAtg3 (*24*). Finally, GABARAP forms a protein−phospholipid

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[1] Abbreviations: $GABA_A$ receptor, γ-aminobutyric acid type A receptor; GABARAP, $GABA_A$ receptor associated protein; CNS, central nervous system; ER, endoplasmic reticulum; HSQC, heteronuclear single-quantum coherence, PSSM, position-specific scoring matrix; Ubq, ubiquitin; UBL, ubiquitin-like modifier; Ins(1,4,5)P3, inositol 1,4,5-triphosphate; SPR, surface plasmon resonance; NMR, nuclear magnetic resonance.

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conjugate with phosphatidylethanolamine or phosphatidylserine (*25*). No E3 enzyme has yet been identified carrying this modification. The lipid moiety can be removed by hAtg4 again, releasing the C-terminal glycine-exposed GABARAP for a new conjugation cycle (*26*). The protein−lipid conjugation is a unique feature of the MAP1-LC3 protein family. Other UBLs, such as SUMO, ubiquitin, and NEDD8, are well-known for conjugating exclusively proteins.

Close homology of GABARAP to proteins implicated in autophagy (*27*) and vesicular intra-Golgi transport processes (*28*), the interaction of GABARAP with NSF (*29*), tubulin (*30*), and $GABA_A$ receptor (*31*), and GABARAP's subcellular localization at intracellular membranes (*29*) suggest its role in intracellular trafficking of $GABA_A$ receptors. A functional effect of GABARAP on the trafficking of $GABA_A$ receptors has been shown in neurons (*32*). Promotion of $GABA_A$ receptor clustering as well as modulation of channel kinetics and conductance of $GABA_A$ receptors by GABARAP was recently demonstrated (*33*, *34*).

In addition to the aforementioned proteins, a wide range of other proteins such as ULK1 (*35*), transferrin receptor (*36*), PRIP-1 (*37*), GRIP (*38*), gephyrin (*39*), and DEAD box polypeptide 47 (DDX47) (*40*) were reported to interact with GABARAP.

In the present study, we intended to identify novel GABARAP interaction partners by simple pull-down experiments to potentially detect highly abundant proteins that interact with GABARAP. In addition, phage display screening of a randomized peptide library was carried out to identify artificial and physiological ligands of GABARAP.

MATERIALS AND METHODS

Pull-Down Assay. The target protein (GABARAP) was coupled to *N*-hydroxysuccinimide-activated Sepharose (NHS-activated Sepharose 4 Fast Flow, GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Brains from adult Sprague Dawley rats were homogenized in buffer A containing 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 1 mM β-mercaptoethanol, protease inhibitor (Complete Mini, Roche Diagnostics, Germany), 0.1 mg/mL DNAse, and 0.5% Triton. Insoluble material was removed by centrifugation at 13000*g* for 15 min. Extracts were then exposed to Sepharose-coupled target protein and incubated for 1.5 h at room temperature. After the Sepharose was washed with buffer A, bound proteins were eluted with buffer B containing 50 mM glycine, pH 2.3, and subsequently neutralized with a final concentration of 100 mM Tris, pH 8.8. Eluted proteins were precipitated using chloroform/methanol and subjected to SDS−PAGE. Identification of GABARAP−Sepharose-bound proteins was done by trypsin digestion and mass spectrometric analysis.

Immunoblotting. SDS−PAGE-separated proteins were analyzed by Western blot analysis. The antibody used was anti-clathrin heavy chain (C43820, BD Biosciences, Pharmingen). Blots were visualized using chemoluminescence (SuperSignal West Pico chemiluminescent substrate, Pierce, Rockford, IL) and documented using a chemiluminescence detection system (ChemiDoc, Bio-Rad, Hercules, CA).

Phage Display Screening. The phage display procedure was repeated exactly as described previously (*41*).

Motif Extraction and Database Search. Our approach for motif extraction and database search followed closely the approach outlined in ref *42*. Phage display selected peptide sequences were aligned with ClustalX using standard parameters (*43*, *44*). The alignment was based on the previously reported sequences obtained from randomly chosen true positive clones with sequence similarity to a peptide with the sequence SHKSDWIFLPNAA (named "N1"), which turned out to bind most tightly to GABARAP (*41*), and all the peptide sequences obtained in the above-described phage display screening that showed significant similarity to N1 as well. Although some sequences were found multiple times, and some were even identified from both screenings, each sequence was used only once in the resulting alignment to avoid inadequate weighting of sequences from both screenings. On the basis of the alignment, a position-specific scoring matrix (PSSM) was constructed (*45*) using the BLOCKS multiple-alignment processor tool (http://blocks.fhcrc.org/blocks/process_blocks.html). The PSSM was used in a BLAST search against the Swiss-Prot database using the motif alignment and search tool (MAST) (*46*).

Peptides and Proteins. The peptide clathrin(510−522) was purchased as a reversed-phase high-performance liquid chromatography-purified product (BMFZ Analytische Zentrallabor, Düsseldorf, Germany). Clathrin(510−522) was C-terminally amidated and N-terminally acetylated ($COCH_3$-TPDWIFLLRNVMR-NH_2). Cloning, expression, and purification of GABARAP (Swiss-Prot accession number O95166) (*47*) and calreticulin (Swiss-Prot accession number P27797) (*41*) has been described previously.

Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR spectra were recorded at 25 °C on a Varian Unity INOVA spectrometer equipped with a Varian Gen 2 HCN cryogenic probe at a proton frequency of 600 MHz. The sample contained 30 μM uniformly ^{15}N labeled GABARAP in 25 mM sodium phosphate, pH 7.0, 100 mM KCl, 100 mM NaCl, and 5% (v/v) deuterium oxide. Addition of clathrin(510−522) solution resulted in a sample containing GABARAP and 30 μM clathrin(510−522) peptide. $^1H-^{15}N$ heteronuclear single-quantum coherence (HSQC) spectra were collected with 96 complex points in the ^{15}N time domain, with 256 scans per point in t_1 and with a 1.2 s recycle delay. Data were processed with NMRPipe (*48*) and analyzed with CARA (*49*).

RESULTS

Pull-Down of the Endogenous Clathrin Heavy Chain. To identify physiological GABARAP binding proteins, we established a pull-down assay using recombinant GABARAP immobilized on NHS-activated Sepharose (GABARAP−Sepharose). The protein moieties from rat brain extract bound to Sepharose-coupled GABARAP were analyzed by SDS−PAGE. Upon staining with Coomassie, a clearly visible band was detected with an apparent molecular mass of about 180 kDa (Figure 1A). Untreated Sepharose, as a control, did not yield enrichment of this protein. The 180 kDa band was identified to be clathrin heavy chain by trypsin digestion and mass spectrometric analysis. This result was confirmed by a positive Western blot analysis for clathrin heavy chain immunoreactivity (Figure 1B). The drastically increased sensitivity of the Western blot analysis as compared to the

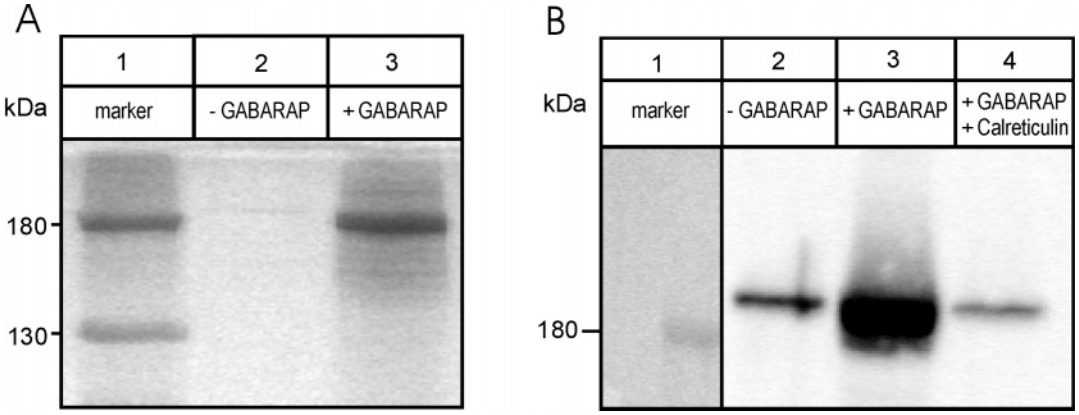

FIGURE 1: Endogenous clathrin heavy chain protein associates with immobilized GABARAP. (A) Untreated Sepharose as a control (lane 2) and GABARAP-coupled Sepharose (lane 3) were exposed to rat brain lysate. After extensive washing, bound material was resolved by SDS−PAGE (15%) and analyzed by Coomassie staining. Untreated Sepharose did not bind (lane 2), but GABARAP-coupled Sepharose did bind (lane 3) a ~180 kDa protein, which was identified by tryptic digestion and mass spectrometry analysis to be clathrin heavy chain. Lane 1: prestained protein marker (broad range, NEB, Beverly, MA). (B) Untreated Sepharose (lane 2) and Sepharose-coupled GABARAP in the absence (lane 3) and in the presence (lane 4) of 70 μM recombinant calreticulin protein were exposed to rat brain lysate. After extensive washing, bound material was resolved by SDS−PAGE (8%) and analyzed by immunoblotting with an anti-clathrin heavy chain antibody. Control Sepharose exhibits only weak immunoreactivity for clathrin heavy chain (lane 2), whereas Sepharose-coupled GABARAP shows a strong signal (lane 3). In the presence of 70 μM calreticulin, only a very weak immunoreactivity can be observed (lane 4). Lane 1: prestained protein marker (broad range, NEB, Beverly, MA).

Coomassie-stained gel band revealed that a small amount of clathrin bound unspecifically to untreated Sepharose. Sepharose-coupled GABARAP, however, bound a drastically increased amount of clathrin heavy chain.

Calreticulin was recently shown to be a ligand of GABARAP as well. To investigate whether calreticulin influences clathrin binding of GABARAP, we carried out the pull-down assay of GABARAP-coupled Sepharose with rat brain extract in the presence of recombinantly expressed calreticulin. In the presence of 70 μM calreticulin, clathrin binding to GABARAP was almost completely abolished.

In Vitro Selection of GABARAP Peptide Ligands. Calreticulin was recently identified as a GABARAP ligand by screening a randomized phage displayed peptide library for GABARAP ligands (*41*). A large variety of somehow similar peptide sequences from the GABARAP ligand screen were used to extract a sequence motif and search the sequence databases. The considerable sequence variability for GABARAP binding peptides suggests that GABARAP might be able to bind a variety of proteins containing nonidentical recognition peptide sequences.

To increase the basis of amino acid sequences from GABARAP binding peptides, we carried out an additional screening of a randomized phage displayed dodecapeptide library using recombinant GST−GABARAP fusion protein as a target. After four selection cycles, single clones were randomly chosen and assayed for GABARAP binding activity employing anti-phage ELISAs to eliminate false positive clones. Amino acid sequences of phage displayed peptides were deduced by DNA sequence analysis of true positive clones. A single dominating peptide sequence was not observed; however, the sequences exhibited significant similarity to each other and to the ones previously identified (*41*) (Figure 2A).

Motif Extraction and Database Search. Because the phage display screening did not result in a single dominating peptide sequence, we concluded that the considerable sequence diversity of the selected peptide sequences together could give a better description of GABARAP's peptide binding specificity than any single peptide. In the multiple-sequence alignment of the phage display selected peptides, a highly conserved tryptophan residue was observed. Besides the conserved tryptophan, further sequence properties could be derived, yielding a virtual sequence motif. Defining the tryptophan residue as sequence position 1 (Trp+1) within the potential sequence motif, aliphatic residues at positions +2 and +4 and an aromatic residue at position +3 seem to contribute to GABARAP binding. Position −1 contained a predominant hydrophilic and charged aspartic acid residue (Figure 2A).

This set of phage display selected peptides was used to create a representative consensus by deriving a sequence PSSM from the sequence alignment, which is depicted as a sequence logo in Figure 2B. The PSSM represents the amino acid tolerance and expected frequency at each position within a consensus block of similar sequences, in contrast to the limited information available from each individual peptide (*50*). We used the PSSM information to screen the sequence databases for stretches of amino acid sequences that fit best to the GABARAP binding consensus motif. We carried out a BLAST search of the obtained PSSM against the Swiss-Prot protein database and obtained residues 512−516 of clathrin heavy chain as a sequence fragment with significant similarity to the phage display derived consensus motif (Figure 2A). Clathrin heavy chain turned out to be among the top 10 human proteins of the resulting MAST hit list (*46*).

Clathrin Heavy Chain (510−522)−GABARAP Interaction. Further indication for a direct interaction of recombinant GABARARAP and clathrin heavy chain in solution was obtained by NMR spectroscopy. $^{1}H-^{15}N$ HSQC spectra of ^{15}N stable isotope labeled GABARAP were recorded in the course of a titration with unlabeled clathrin heavy chain peptide (510−522) corresponding to the phage display derived sequence motif. The NMR spectrum of GABARAP without clathrin(510−522) exhibited the known and expected

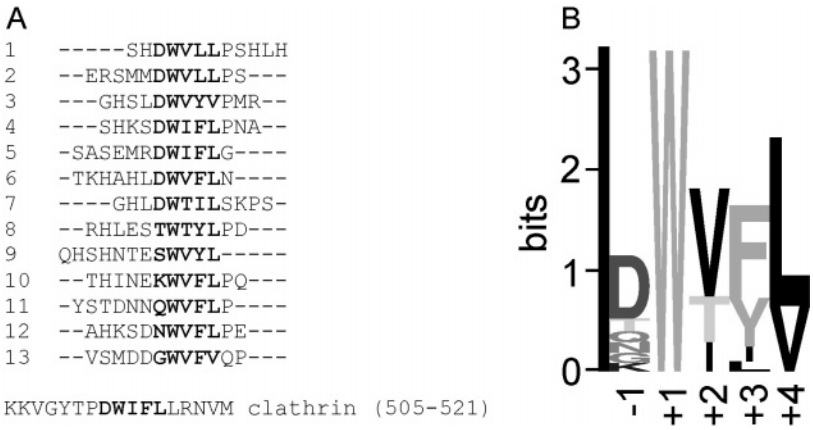

FIGURE 2: (A) Multiple-sequence alignment of phage display selected peptide sequences, exhibiting GABARAP binding properties, and clathrin heavy chain (504−521). The sequence fragment of clathrin heavy chain (504−521) is shown in the bottom line. Phage display derived sequence fragments that are depicted in bold were used to construct a PSSM (*50*). The two peptide sequences shown on top of the alignment have been identified in a display screening described previously (*41*), while peptide sequences 6−13 have been identified in the above-mentioned phage display screening. Peptide sequences 3−5 could be observed in both approaches. (B) A sequence logo computed from the PSSM represents a graphical illustration of aligned sequences where at each position the size of each residue is proportional to its frequency in that position and the total height of all the residues in the position is proportional to the conservation of this position.

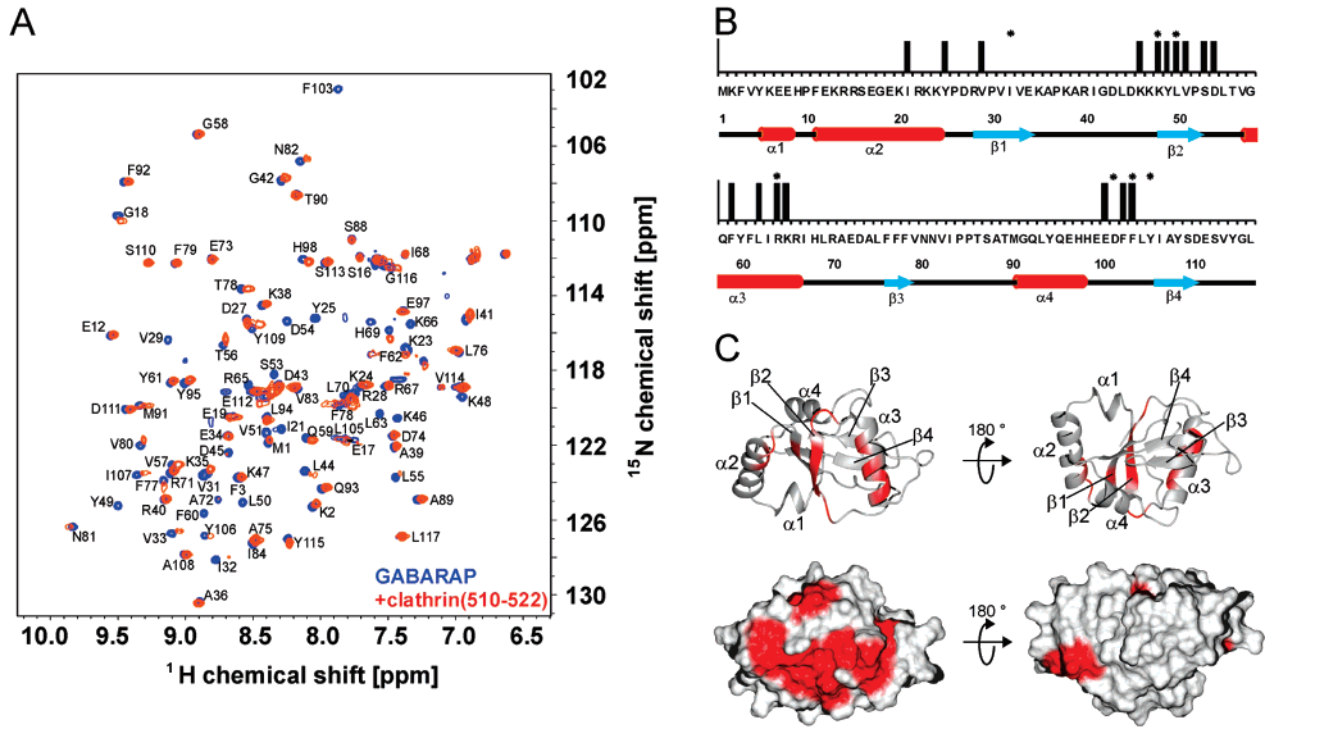

FIGURE 3: GABARAP interacts with clathrin(510−522). (A) Superimposed ^{15}N HSQC spectra of GABARAP in the absence and presence of clathrin(510−522). The sample contained 30 μM uniformly ^{15}N stable isotope labeled GABARAP in 25 mM sodium phosphate, pH 7.0, 100 mM KCl, 100 mM NaCl, and 5% deuterium oxide (blue). Addition of clathrin(510−522) resulted in a sample containing GABARAP and 30 μM clathrin(510−522) (red). In the presence of clathrin(510−522), the affected GABARAP resonances showed signal broadening and decreased intensities, indicating intermediate exchange on the NMR chemical shift time scale. The assignment of the resonances was done according to ref *15*. (B) Sequence topology representation of GABARAP. Secondary structure elements are shown below the sequence, and residues affected upon clathrin(510−522) peptide binding are marked with bars above the respective sequence position. Strands and helices are labeled in alphabetical order from the N-terminum to the C-terminus. Asterisks mark residues that were shown to be important for autophagy in the yeast orthologue of GABARAP, Atg8, at the respective homologue sequence position (*56*). (C) Ribbon and surface illustration of GABARAP residues affected upon clathrin heavy chain (510−522) binding: upper line, ribbon structure with the labeled secondary structure elements of GABARAP (PDP code 1kot); lower line, surface representation of GABARAP. GABARAP residues affected upon clathrin heavy chain (510−522) binding are highlighted in red (I21, Y25, V29, K46, K48, Y49, L50, V51, S53, D54, F60, L63, R65, K66, E101, F103, F104).

resonances typical for natively folded GABARAP (*47*, *51*, *52*). Adding clathrin(510−522) to GABARAP resulted in line broadening and a decrease of the signal intensity of many GABARAP resonances, a clear indication of direct binding (Figure 3A). To rule out any effects due to paramagnetic ions, precipitation, and pH shift, GABARAP and RP-HPLC-purified ligand peptide were solved in exactly the same buffer. The 17 residues of GABARAP affected by clathrin-(510−522) are spread over the complete GABARAP sequence (Figure 3B), but are located in several adjacent

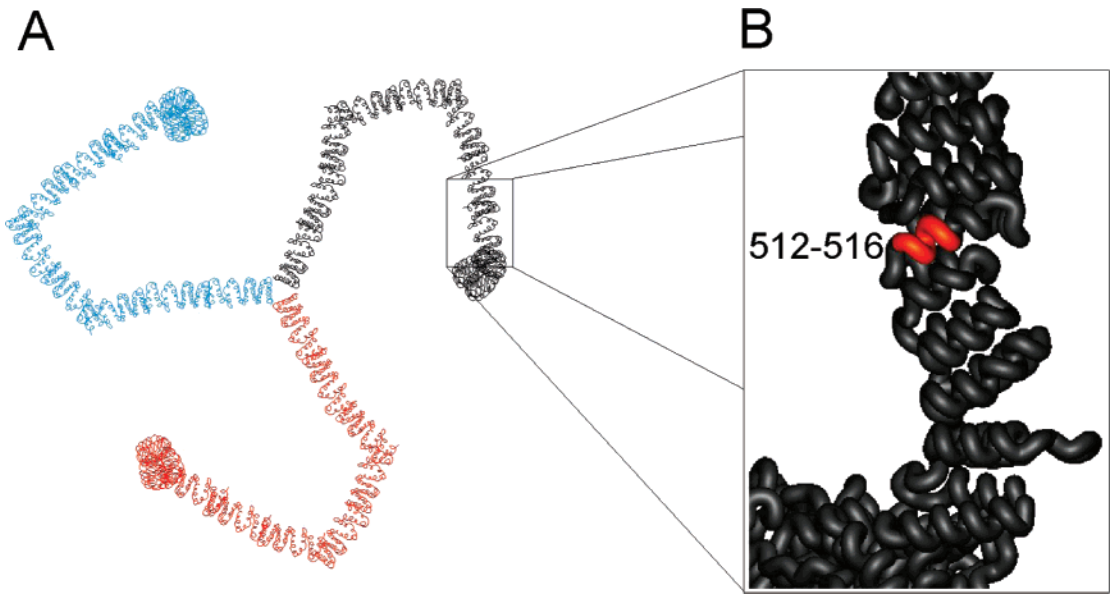

FIGURE 4: (A) Schematic representation of three clathrin heavy chain molecules (blue, red, and black), showing the structural composition of the clathrin heavy chain triskelion. The boxed area comprises the presumed GABARAP binding motif. (B) PDB structure of clathrin heavy chain (PDB code 1XI5). Enlarged view of the flexible linker region including the putative GABARAP binding site (amino acid residues 512−516), which is highlighted in red.

regions of the GABARAP surface (Figure 3C), most notably in the secondary structure elements of α2, α3, β1, and β2.

DISCUSSION

The Clathrin Heavy Chain Binds to GABARAP. Performance of a pull-down assay of rat brain homogenate with recombinant GABARAP protein coupled to NHS-activated Sepharose revealed a 180 kDa protein to bind GABARAP. This protein could be detected by simple Coomassie staining, indicating it to be a highly abundant cellular protein. Mass spectroscopic analysis revealed the protein to be clathrin heavy chain, which was subsequently verified by Western blot analysis.

Calreticulin Competes with Clathrin for Binding to GABARAP. Calreticulin, a recently identified GABARAP ligand was able to competitively inhibit GABARAP interaction with clathrin heavy chain (Figure 1). Calreticulin is a multifunctional lectin-like 46 kDa protein best known as a luminal Ca^{2+}-dependent chaperone of the endoplasmic reticulum (ER) (*53*).

Because calreticulin is reported to be present in the cytoplasm (*54*) and the reported dissociation constant for calreticulin and natively folded GABARAP is in the nanomolar range (*41*), calreticulin might have an important regulatory role for the GABARAP interaction with clathrin. This, however, needs substantial further investigation.

The GABARAP Binding Site Maps to Residues 512−516 of the Clathrin Heavy Chain. The GABARAP binding site within calreticulin was shown to contain residues 182−186 (DWDFL in the one-letter amino acid code) (*41*). This is a sequence motif which is very similar to the sequence DWIFL within the clathrin heavy chain (residues 512−516). A synthetic peptide comprising residues 510−522 bound directly with recombinant GABARAP protein in vitro (Figure 3A). Therefore, we propose residues 512−516 of clathrin heavy chain as the primary binding site for GABARAP. These residues are part of a surface-exposed α-helix, thus suggesting that at least a part of them is fully accessible for ligand interaction (Figure 4). In addition, this region of clathrin heavy chain was described as the "flexible linker region" on the basis of protease cleavage assays (*55*). This observation may even support a potential conformational change of the sequence region responsible for GABARAP interaction upon GABARAP binding. Interestingly, the flexible linker region of clathrin heavy chain is not known to be implicated in any other interaction events reported so far.

Mapping the Clathrin Binding Site on GABARAP. Using a chemical shift perturbation NMR experiment, numerous residues of GABARAP were identified to be involved in binding of clathrin heavy chain peptide comprising residues 510−522. Upon addition of the clathrin peptide, some GABARAP resonances were not affected at all, and others showed changes in their chemical shifts, line broadening, or reduction of their signal intensity (Figure 3A), indicating intermediate exchange on the NMR chemical shift time scale for at least some resonances. The amide proton resonance of Ile32, for example, almost disappeared after a shift of about 60 Hz (Figure 3A), indicating the off rate to be close to 100 Hz. Assuming the association rate to be diffusion controlled ($<10^8$ Hz M^{-1}) leads to a rough estimation of the dissociation constant (k_D) for GABARAP and clathrin heavy chain (510−522) in the low micromolar range. The GABARAP residues involved in binding are spread over the complete GABARAP sequence (Figure 3B), but map very close to each other on the surface of the protein (Figure 3C). The interaction of GABARAP with different peptides was investigated by NMR and is discussed in the literature, revealing a common peptide binding region on the GABARAP surface for all peptide−GABARAP interactions reported so far (*17*).

Recently, the role of several residues in the yeast orthologue of GABARAP, Atg8, for autophagy was investigated by mutational analysis (*56*). In this study, all residues identified to significantly contribute to autophagy are located in sequence regions of Atg8 that almost exactly overlap with the homologue GABARAP residues that were shown in the

present study to be involved in binding of clathrin heavy chain (Figure 3B).

Implications of an Interaction between GABARAP and Clathrin. We report for the first time a direct interaction between GABARAP and clathrin. Both clathrin heavy chain and GABARAP are reported to interact with phosphoinositide binding proteins. AP180 (*57*), OCRL (*58*), and EpsinR (*59*) represent appropriate examples of clathrin ligands, while GABARAP is reported to associate with the phospholipase C-related inactive protein 1 (PRIP-1) (*37*, *60*), which interacts with the β- and γ2-subunits of the $GABA_A$ receptor (*37*, *61*, *62*).

Probably the most obvious functional connection of GABARAP and clathrin heavy chain is their implication in $GABA_A$ receptor trafficking within the cell. Clathrin heavy chain protein is essential for clathrin-coated pit formation and thus for the ability of neurons to regulate the number of postsynaptic receptors. $GABA_A$ receptors are constitutively internalized by clathrin-coated vesicles (*63*, *7*).

GABARAP belongs to the UBL family, whose members are well-known as sorting signals for trafficking events. Lipids are reported as targets for GABARAP modification. This is in accordance with the subcellular distribution of GABARAP, which indeed localizes to membrane structures, possibly serving as a transport tag to membrane vesicles.

In this study we successfully screened for novel GABARAP binding partners by performing a simple pull-down assay, demonstrating that GABARAP associates with clathrin heavy chain protein and provided evidence for a direct interaction between both proteins. The involvement of GABARAP and clathrin heavy chain in $GABA_A$ receptor trafficking gives a strong hint for the relevance of their interaction: The precise function of this interaction, however, remains to be investigated.

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