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Association of GRIP1 with a $GABA_A$ receptor associated protein suggests a role for GRIP1 at inhibitory synapses

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Abstract

$GABA_A$ receptors mediate the majority of fast synaptic inhibition in the mammalian central nervous system. $GABA_A$ receptors associate with a number of cytosolic proteins important for regulating their function including the $GABA_A$ receptor γ2 subunit associated protein GABARAP. Here we show GABARAP associates with the synaptic PDZ domain containing protein GRIP1. GRIP1 has been localized to inhibitory synapses however the role of this protein with respect to neuronal inhibition remains unclear. Using in vitro protein interaction assays we show that GABARAP interacts directly with PDZ domains 4–6 of GRIP1. Furthermore, using coimmunoprecipitation assays we show that GABARAP interacts with GRIP1 in vivo. Finally, we show that GRIP1 colocalizes with γ2 subunit containing $GABA_A$ receptors in cultured hippocampal neurons. Our findings provide evidence that GRIP1 can associate with proteins important for regulating $GABA_A$ receptor function and suggest that GRIP1 may play a role at inhibitory synapses.

Keywords: Inhibitory; Synapse; GABA; Receptor; GABARAP; GRIP

1. Introduction

$GABA_A$ receptors represent the major sites of fast synaptic inhibition in the brain and are also drug targets for a variety of clinically important drugs including benzodiazepines, barbiturates and general anesthetics. $GABA_A$ receptors are hetero-pentameric chloride selective, ligand-gated ion channels, which can be constructed from seven subunit classes: α1–6, β1–3, γ1–3, δ, ε, π and θ [1]. It is widely believed that most benzodiazepine sensitive $GABA_A$ receptor subtypes in the brain are constructed from α, β and γ2 subunits [1]. It has become increasingly clear that receptors and ion channels in the central nervous system are not isolated entities but in fact form numerous interactions with other proteins important for regulating their function. A significant effort has been made to identify proteins that interact directly with the large intracellular domain (located between transmembrane domains III and IV) of the $GABA_A$ receptor subunits. This has revealed a number of receptor-associated proteins implicated in the regulation of the phosphorylation, clustering and membrane trafficking of these ion channels [1–4]. The $GABA_A$ receptor γ2 subunit is of central importance in conferring $GABA_A$ receptor benzodiazepine sensitivity and in controlling the synaptic targeting of these receptors [5,6]. The $GABA_A$ receptor associated protein GABARAP was identified in a yeast two-hybrid (Y2H) screen for proteins that associate with the intracellular domain of the $GABA_A$ receptor γ2 subunit [7]. GABARAP is a microtubule binding protein that belongs to a growing family of proteins implicated in intracellular transport processes [7–10]. From work in cell lines GABARAP has been proposed to modify $GABA_A$ receptor channel clustering and kinetics [11,12]. In addition GABARAP has also been suggested to function in the intracellular transport of $GABA_A$ receptors [9,10]. However, at present, the exact mechanisms by which GABARAP may regulate $GABA_A$ receptor function have remained elusive.

In addition to proteins that have been found to associate directly with $GABA_A$ receptor intracellular domains, a limited number of proteins have been found to specifically

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localize to inhibitory postsynaptic domains [13,14]. These include gephyrin, members of the dystrophin glycoprotein complex (dystrophin, syntrophin and beta-dystroglycan) and the glutamate receptor interacting protein 1 (GRIP1) [13–16]. Gephyrin colocalizes with $GABA_A$ receptor clusters in many brain regions and has been demonstrated to be an important determinant for the clustering of $GABA_A$ receptors containing the γ2 subunit [17–19]. However, gephyrin independent clustering of some $GABA_A$ receptor subtypes has also been demonstrated [20]. GRIP1 is a seven PDZ (for −PSD95, Discs Large, Z01) domain-containing protein that interacts with and plays an essential role in the clustering and trafficking of AMPA type glutamate receptors containing the Glutamate receptor 2 (GluR2) subunit [21]. GRIP1 is one of the few proteins present at both inhibitory and excitatory synapses although the role GRIP1 may play at inhibitory synapses is unknown [13].

In this study we demonstrate that GABARAP, a $GABA_A$ receptor associated protein, associates with the synaptic PDZ domain-containing protein GRIP1. We provide evidence that GRIP1 can associate with proteins important for $GABA_A$ receptor function and discuss the possible role of GRIP1 at inhibitory synapses.

2. Methods

2.1. Yeast two-hybrid screen

A bait encoding GABARAP (amino acids 1–117) was amplified by PCR and cloned into the yeast expression vector pPC97. This bait was used to screen a random-primed cDNA library from rat hippocampus subcloned into the *Sal*I/*Not*I sites of the pPC86 vector as described previously [21,22]. The plasmids were transformed into the yeast strain Y190 and transformants were selected on −Leu/His/Trp media containing 25 mM 3AT and assayed for β-galactosidase activity.

2.2. Antibodies

The 9E10 antibody was isolated from hybridoma cell lines via chromatography on protein A-Sepharose. Mouse anti GRIP1 was from BD Biosciences and used for immunoblotting at 1:250–1:500, rabbit anti-GRIP has been described previously [21]. Guinea-pig anti-$GABA_A$ receptor γ2 subunit and rabbit anti-GABARAP antibodies have been described previously [7,10,23].

2.3. Cell culture

COS cells were maintained in DMEM with 10% fetal bovine serum as previously described and transfected by electroporation using 10 μg of expression construct [10]. Cultures of hippocampal neurons were prepared as described previously [10,24]. Briefly, hippocampi were dissected from embryonic day 18 embryos followed by incubation for 15 min in 0.25% Trypsin followed by washes in HBSS. Hippocampi were then triturated with fire polished pipettes of decreasing bore size and then cells were immediately plated on 13 mm coverslips coated with 100 μg/ml poly (L) lysine and maintained in Neurobasal-A plus B27 supplement (GIBCO).

2.4. Immunofluorescence

For immunolocalization studies hippocampal neurons (21 days in vitro) were fixed in 4% paraformaldehyde and processed as described previously [10,24]. Guinea pig anti-$GABA_A$ receptor γ2 subunit antibody and rabbit anti-GRIP1 were used as previously described [10,15]. FITC and texas red-conjugated secondary antibodies were from Jackson and used at 1:500. Coverslips were examined using a confocal microscope (MRC1000, Bio-Rad) as previously described [10,24].

2.5. Affinity purification assays

pGEX, pGEX GABARAP, pGEX SGT and pGEX GRIP1 PDZ 4–6 were used to generate GST and GST fusion proteins of GABARAP, SGT and GRIP1 PDZ 4–6 as described previously [10,24,21,25] by expression in *E. coli* and purification with glutathione agarose. ^{35}S labeled GRIP1 PDZ 4–6 was prepared using TNT Quick Coupled Transcription/Translation System (Promega) following the manufacturer's instructions. GST fusion protein pull down assays from COS cell or brain lysates were carried as previously described [10,24] in HEPES pull down buffer (20 mM HEPES, 150 mM NaCl, 0.5 mM EDTA, 1% Triton, 1 mM PMSF and antipain, pepstatin and leupeptin at 10 μg/ml). Briefly, 50 μg of GST fusion protein, immobilized to glutathione agarose beads was incubated with 1 ml of lysate solubilized in HEPES pull down buffer, for 2 h at 4 °C followed by several washes with HEPES pull down buffer. Following washes remaining material attached to the beads was eluted with SDS-loading buffer and analyzed by SDS-PAGE and immunoblotting.

2.6. Immunoprecipitation

For immunoprecipitation, brain lysates (10 mg) were prepared in Tris immunoprecipitation buffer (50 mM Tris, 150 mM NaCl, 1% Triton, 5 mM EDTA, 1 mM PMSF and antipain, pepstatin and leupeptin at 10 μg/ml) as described previously [10] and incubated with either 10 μg non-specific rabbit IgG or GABARAP specific antibody [10], followed by precipitation with protein A sepharose. Immunoprecipitations were analyzed by SDS-PAGE and immunoblotting with mouse anti-GRIP1 at 1:500. Antibodies were detected with HRP-conjugated anti-mouse secondary antibodies at 1:5000 (Jackson) and visualized by enhanced chemiluminescence.

3. Results

3.1. In vitro association of GRIP1 with the $GABA_A$ receptor associated protein GABARAP

The $GABA_A$ receptor associated protein GABARAP associates with the γ2 subunit of $GABA_A$ receptors. To identify proteins that may interact with GABARAP and that therefore may be important for GABARAP's function we carried out a yeast two-hybrid screen (Y2H) of a rat hippocampal library using full length GABARAP as bait. From this screen we identified three clones encoding amino acids identical to amino acids 413–794 of the first glutamate receptor interacting protein 1 (GRIP1) family member identified [21]. GRIP1 contains 7 PDZ domains [15,21] and the clone we identified includes PDZ domains 4 (amino acids 471–558), 5 (amino acids 572–655) and 6 (amino acids 672–753) of this protein (Fig. 1A) in addition to 41 amino acids of sequence downstream of PDZ domain 6 but lacking the remaining C-terminal sequence of full length GRIP1.

To confirm our observations outside yeast we further examined the interaction of GRIP1 with GABARAP using GST fusion protein affinity chromatography (GST pull downs). In vitro translated ^{35}S labeled GRIP1 PDZ domains 4–6 (GRIP1 PDZ 4–6) was found to associate with GABARAP expressed as a GST fusion protein (GST-GAB ARAP) but not with GST alone (Fig. 1B). As a positive control we used a GST-fusion protein of GRIP1 PDZ 4–6 (GST-GRIP1 PDZ 4–6) as this fragment of GRIP1 has been shown to self-associate [15,16] and should therefore also associate with ^{35}S GRIP1 PDZ 4–6. In agreement with this GST-GRIP1 PDZ 4–6, also bound efficiently to ^{35}S GRIP1 PDZ 4–6. As an additional negative control we also tested the ability of another cytosolic protein, GST-fusion protein of SGT (small glutamine rich tetracopeptide repeat containing protein, GST-SGT, [25]) to associate with ^{35}S GRIP1 PDZ 4–6 and this showed no interaction (Fig. 1B). This result provides further support for an interaction between GABARAP and GRIP1 and provides evidence that GRIP1 may associate with proteins involved in the regulation of $GABA_A$ receptor function.

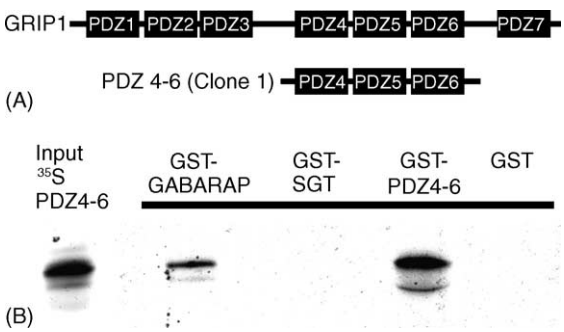

Fig. 1. (A) Schematic diagram showing full length rat GRIP1 containing seven PDZ domains and the GABARAP associated clone containing PDZ domains 4–6 of GRIP1. (B) Association of GRIP1 PDZ domains 4–6 with GST GABARAP in vitro. ^{35}S labeled GRIP1 PDZ 4–6 (10%) input is shown in left of panel. Both GST-GARABAP and the positive control, GST GRIP1 PDZ domains 4–6 (GST-PDZ 4–6), interact with ^{35}S labeled GRIP1 PDZ domains 4–6. In contrast GST and GST-SGT do not interact with ^{35}S labeled GRIP1 PDZ 4–6.

3.2. Association of GRIP1 with GABARAP in transfected cells

We further tested the specificity of the association of GABARAP with GRIP1 by probing cell lysates expressing a myc tagged version of GRIP1 PDZ 4–6 which represents the clone obtained from the GABARAP Y2H screen (myc-C1). GST-GABARAP and GST were incubated with detergent solubilized COS cell extracts expressing myc-C1 (Dong et al., 1997) and GST-GABARAP associated proteins were resolved by SDS-PAGE, followed by western blotting with anti-myc antibody. GST-GABARAP was found to interact with myc tagged GRIP1 PDZ 4–6 (myc-C1) whereas GST alone did not (Fig. 2). No anti-myc immunoreactivity could be detected when GST-GABARAP was incubated with cell extracts from untransfected COS cells.

3.3. Association of GABARAP with neuronal GRIP1

We also analyzed if neuronal GRIP1 interacts with GABARAP. GST-GABARAP immobilized on glutathione agarose beads was exposed to detergent solubilized brain extracts, followed by extensive washing. Bound proteins were resolved by SDS-PAGE, and probed with a monoclonal antibody that recognizes GRIP1. Neuronal GRIP1 was found to associate with GST-GABARAP (Fig. 3A) consistent with our approaches using in vitro translated GRIP1 PDZ 4–6 and myc tagged GRIP1 PDZ 4–6 expressed in COS cells. Neuronal GRIP1 did not associate with GST alone. This result confirms that GABARAP can associate with full length neuronal GRIP1.

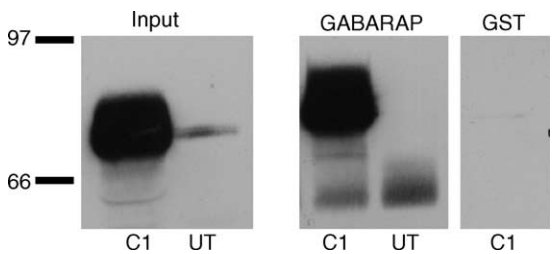

Fig. 2. Association of GRIP1 from COS cell lysates with GABARAP in vitro. GST-GAB ARAP and GST were exposed to COS cell lysates transiently transfected with myc-tagged GRIP1 clone containing PDZ domains 4–6 (C1). After extensive washing bound material was resolved by SDS-PAGE and analyzed by immunoblotting with an anti-myc antibody. Binding of C1 to GST-GAB ARAP could be detected but not to GST alone. No myc immunoreactive bands could be detected in untransfected COS cells (UT).

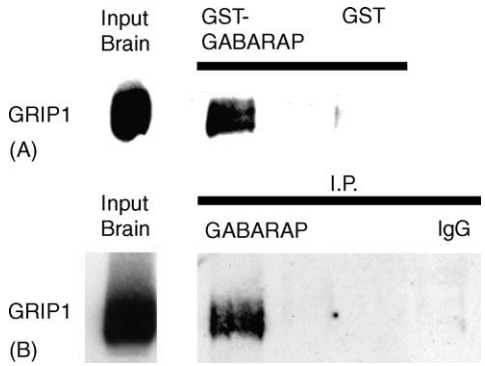

Fig. 3. Association of GABARAP with neuronal GRIP1. (A) GST-GABARAP and GST were exposed to neuronal lysates and after extensive washing bound material was resolved by SDS-PAGE and analyzed by immunoblotting with an anti-myc antibody. Binding of neuronal full length GRIP1 to GST-GAB ARAP but not GST was detected. (B) GABARAP coimmunoprecipitates with GRIP1 from neuronal extracts. GABARAP was immunoprecipitated from neuronal lysates using rabbit anti-GABARAP antibodies and bound material was resolved by SDS-PAGE followed western blotting with a monoclonal antibody against GRIP1. GRIP1 coimmunoprecipitates with GABARAP but not with control non-immune IgG.

Whether GABARAP and GRIP1 complexes occur in neurons was further examined using immunoprecipitation. Detergent solubilized brain extracts were subjected to immunoprecipitation with a rabbit antibody that recognizes GABARAP [8,10] and immunoblotted with a monoclonal antibody that recognizes GRIP1. GRIP1 could be readily detected coimmunoprecipitating with anti-GABARAP antibodies but not with control IgG (Fig. 3B).

3.4. Co-localization of GRIP1 with $GABA_A$ receptors in cultured hippocampal neurons

The localization of GRIP1 to inhibitory synapses has previously been shown using an antibody to the presynaptic inhibitory synaptic marker glutamic acid decarboxylase (GAD) [15,16]. To further confirm the localization of GRIP1 to inhibitory synapses and to further test if GRIP1 colocalizes with the $GABA_A$ receptor we carried immunofluorescence studies on cultured neurons. Twenty-one days old cultured hippocampal neurons were double labeled with antibodies to GRIP1 and to the γ2 subunit of the $GABA_A$ receptor (Fig. 4). GRIP1 (red) was distributed in the soma and in small puncta on dendrites (Fig. 4A). Staining for the $GABA_A$ receptor (green) could be detected as puncta in the soma and dendrites (Fig. 4B). Importantly, as can be seen in the merged image, many of the $GABA_A$ receptor puncta contained immunoreactivity for GRIP1 (Fig. 4C) supporting the idea that GRIP1 is present at inhibitory synapses.

4. Discussion

For efficient inhibitory synaptic transmission, it is critical that $GABA_A$ receptors are correctly targeted to the appropriate synaptic sites. However, the mechanism by which these receptors are selectively targeted to inhibitory synapses remains to be fully elucidated. It is highly probable that the selective targeting, membrane transport and clustering of $GABA_A$ receptors in addition to their functional modulation, will be dependent on protein-protein interactions made between the receptor and associated proteins. As a result, there has been a general interest in identifying $GABA_A$ receptor associated proteins using both biochemical and molecular methodologies. Using the yeast two-hybrid system a small molecular weight (17 kDa) microtubule binding protein termed GABARAP was isolated which binds specifically to $GABA_A$ receptor γ2 subunits [7]. GABARAP has been demonstrated to cluster $GABA_A$ receptors and modify

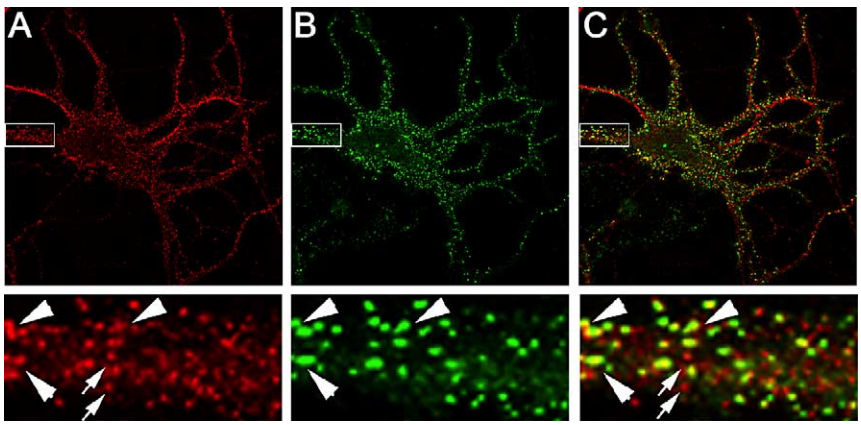

Fig. 4. (A–C) Colocalisation of GRIP1 with $GABA_A$ receptors containing the γ2 subunit in cultured hippocampal neurons. Three weeks old cultured hippocampal neurons were fixed and permeabilized and then probed with (A) rabbit anti-GRIP1 and (B) a guinea pig anti-$GABA_A$ receptor γ2 subunit antibody followed by detection with anti-mouse Texas red-conjugated and anti-guinea pig FITC-conjugated secondary antibodies. The merged image showing colocalisation of the γ2 subunit and GRIP1 can be seen in (C). An enlargement of the same dendrite is shown in each panel. Arrow heads show colocalising puncta. In addition a significant amount of GRIP1 that does not colocalise with the $GABA_A$ receptor γ2 subunit (presumably intracellular GRIP1 and GRIP1 at excitatory synapses) can also be detected (arrows in bottom panel of A and C).

their kinetics and it has also been suggested that GABARAP may participate in the intracellular trafficking of these ion channels [7,10].

In an attempt to identify GABARAP associated proteins we carried out a yeast two-hybrid screen. From this screen we identified an interaction with PDZ domains 4–6 of GRIP1. Using GST fusion protein affinity chromatrography, we were able to show that GABARAP can bind a region containing PDZ domains 4–6 of GRIP1, confirming the interaction outside of yeast. Using similar methodologies we were also able to demonstrate that GST-GABARAP can associate with full length GRIP1 from neuronal lysates. We further confirmed the association between GABARAP and GRIP1 using co-immunoprecipitation from neuronal lysates. In addition, we also confirmed the localization of GRIP1 to inhibitory synapses by demonstrating the colocalisation of GRIP1 with the $GABA_A$ receptor γ2 subunit.

GRIP1 and a recently identified novel four PDZ domain containing isoform of GRIP1 (GRIP1c 4–7) [34], are members of one of the few protein families that appear to be expressed at both excitatory and inhibitory synapses [14-16,21,34]. Although this present study, and the recent identification of GRIP1c 4–7 [34], provides further evidence for a role for GRIP family proteins at inhibitory synapses, the role GRIP1 may be playing remains unclear. GRIP1 was originally identified as a protein interacting with the GluR2 subunit of AMPA type glutamate receptors and substantial evidence supports a key role for GRIP1 in regulating AMPA receptor trafficking and clustering [21]. However, in addition to gephyrin and components of the dystrophin glycoprotein complex GRIP1 is one of the few proteins found enriched at inhibitory synapses [14]. Since it is original identification, GRIP1 has been found to associate with an increasing number of both synaptic and non-synaptic proteins. These include the ephrins and their receptors [26,27], the proteoglycan NG2 [28], GRASPs (for GRIP associated proteins) [29], DNA polymerase beta [30] and the putative extracellular matrix (ECM) protein Fraser syndrome protein (Frasl) [31]. This suggests that GRIP1 may be important for regulating the function of other proteins in addition to glutamate receptors. In agreement with this, GRIP1 has recently been proposed to be involved in several morphogenetic processes during early embryonic development through a role in the trafficking of cell surface-ECM molecules [31,32]. This suggests that GRIP1 may have a more general role in the sorting, transportation and organization of proteins in several contexts [15,29]. In agreement with the notion that GRIP may be an important trafficking molecule, GRIP1 has been demonstrated to associate directly with the motor protein, kinesin [33] and to enable kinesin to transport dendritic proteins such as the GluR2 subunit of the AMPA receptor. This suggests that GRIP1 could potentially play a role in the transport of membrane cargo to inhibitory synapses.

One inconsistency is that GRIP1 and two other GABARAP binding partners, the $GABA_A$ receptor γ2 subunit and gephyrin, may be more highly enriched at inhibitory synapses than GABARAP itself, which appears to be detectable at only low levels at these sites [9,10]. However, like GABARAP, GRIP1 has been localized to a number of intracellular membrane compartments. From electron micrograph studies, both GRIP1 and GABARAP have been found in the Golgi compartment [10,15], suggesting that these two proteins may interact at the level of the Golgi. This has been previously suggested for the interaction of GABARAP with gephyrin [9]. It is possible that GABARAP acts as a transient tag or signal for proteins to be transported to and/or from the inhibitory postsynaptic domain via specific transport vesicles.

Further work will be needed to determine what role GRIP1 may be playing at inhibitory synapses. It will also be of interest to determine if GRIP1 can be detected associated with $GABA_A$ receptor complexes themselves and if so, whether this is via an interaction with GABARAP. This is a pertinent question since, although GABARAP has been found to interact with the $GABA_A$ receptor γ2 subunit and gephyrin, it has thus far not been possible to demonstrate a complex between gephyrin and $GABA_A$ receptors themselves. GRIP1 may also potentially act to anchor/target other GRIP binding proteins to inhibitory synapses. It will be of interest to determine if any of the identified GRIP1 associated proteins can also be detected at inhibitory synaptic sites. The identification of an interaction between GABARAP and GRIP1 in combination with GRIP1's localization at inhibitory synapses suggests that GRIP family members may be involved in inhibitory synaptogenesis and/or the regulation of $GABA_A$ receptor function and suggests that further studies of the potential role of GRIP family members at inhibitory synapses are warranted.

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