Biochemical and Biophysical Research Communications 390 (2009) 82–86

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Metabotropic glutamate receptor 4 interacts with microtubule-associated protein 1B

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ARTICLE INFO

*Article history:*
Received 9 September 2009
Available online 23 September 2009

*Keywords:*
Metabotropic glutamate receptor 4
Microtubule-associated protein 1B
Protein interaction

ABSTRACT

The metabotropic glutamate receptor 4 (mGluR4) is a G-protein-coupled receptor that mediates inhibition of neurotransmitter release. Here, we used a proteomic approach to identify novel interaction partners of mGluR4 and report that the cytoplasmic C-terminal tail of mGluR4 interacts with microtubule-associated protein 1B (MAP1B). Binding of MAP1B to mGluR4 is inhibited by $Ca^{2+}$/calmodulin, and MAP1B and mGluR4 colocalize at excitatory synapses in cultured hippocampal neurons. Thus, MAP1B might be implicated in the synaptic trafficking and/or regulation of mGluR4.



## Introduction

Metabotropic glutamate receptors (mGluRs) constitute a family of eight G-protein-coupled receptors (GPCRs) that are involved in the modulation of synaptic transmission [1]. mGluRs of the predominantly presynaptically localized sub-group III comprising mGluR4, mGluR6, mGluR7 and mGluR8 inhibit adenylylcyclase via $G\alpha_i$ and are selectively activated by L-AP4. Their major function is the feedback regulation of glutamate release.

Different studies have shown that the intracellular C-termini of mGluRs are important binding sites for interacting proteins (reviewed in [2]). The latter may regulate processes as diverse as down-stream signal transduction, receptor trafficking and anchoring to the cytoskeleton. For example, $Ca^{2+}$/calmodulin (CaM) has been shown to compete with G-protein $\beta\gamma$-subunit binding to mGluR7 and other group III mGluRs [3] and is thought to facilitate mGluR-mediated inhibition of presynaptic $Ca^{2+}$ channels upon activity-induced $Ca^{2+}$ influx [4,5]. Yeast two-hybrid screens have revealed interactions of mGluR8 with PIAS1 [6], and of mGluR7 with the protein interacting with C-kinase 1 (PICK1) [2]. The latter interaction has been found to be important for receptor stability [7], down-stream signalling [8] and anti-epileptogenic activity [9].

mGluR4 belongs to the least characterized group III mGluRs although it constitutes a potential target for novel therapeutics, in particular in Parkinson's disease [10]. mGluR4 activation provides neuroprotection in models of excitotoxicity [11] and dopaminergic neuron degeneration [12]. Mice deficient in mGluR4 show resistance to GABA-A receptor antagonist induced absence seizures [13], and increased mGluR4 levels have been found in human temporal lobe epilepsy [14]. Here, we describe a proteomic approach for identifying mGluR4 binding proteins and show that microtubule-associated protein 1B (MAP1B) is a novel interaction partner of mGluR4.

## Materials and methods

*Animals and antibodies.* Adult Wistar rats and wild-type (C57/BL6) and mGluR4 −/− mice [15] backcrossed to the C57/BL6 background were deeply anaesthesized and killed by decapitation in accordance with national regulations. Brains were dissected, frozen in liquid nitrogen and stored at −80 °C. Primary antibodies used were a rabbit polyclonal antibody raised against amino acids 893–912 of rat mGluR4 (Upstate), a mouse monoclonal antibody AA6 against MAP1B (Sigma), and a guinea pig polyclonal antibody against the vesicular glutamate transporter 1 (vGluT1; Chemicon). Secondary antibodies were Alexa goat anti-rabbit-488, anti-guinea-pig-546, anti-mouse-488, and anti-rabbit-546 (all from Molecular Probes).

*GST pull-down assays.* The glutathione-*S*-transferase (GST) fusion protein expression constructs and the protocols for protein expression and purification have been described previously [3,6,16]. Rat brains were homogenized in ice-cold TBS (50 mM Tris buffer, pH 7.4, 125 mM NaCl) containing 100 μM phenylmethylsulfonyl fluoride and complete protease inhibitor cocktail (Roche). After centrifugation at 3500*g* for 10 min at 4 °C, the supernatant (S1) was adjusted to 1% (v/v) Triton X-100 and re-centrifuged at 15,000*g* for 30 min to result in supernatant S2.

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doi:10.1016/j.bbrc.2009.09.070

Glutathione-Sepharose beads (GE Healthcare Europe) were incubated with GST fusion protein for 1 h at 4 °C, washed, and re-incubated overnight with S2 fractions at 4 °C. Where indicated, calmodulin and $Ca^{2+}$ or EGTA were added to the S2 fraction before adding the immobilized GST fusion protein. After washing with TBS, bound proteins were eluted with SDS-sample buffer and analyzed by SDS–PAGE followed by silver staining or Western blotting. Loading of equal amounts of GST fusion proteins was confirmed by Ponceau S staining of the blot membranes.

*Protein identification by mass spectrometry.* Silver-stained protein bands were excised from SDS–polyacrylamide gels, processed for mass spectrometry (MS) and identified by MALDI-TOF-MS and tandem MALDI-TOF/TOF-MS as described [17].

*Co-immunoprecipitation.* Freshly dissected C57/BL6 mouse brains were homogenized in TBS containing 100 μM phenylmethylsulfonyl fluoride and complete protease inhibitor cocktail (Roche) followed by centrifugation at 2000*g* for 10 min. The supernatant was re-centrifuged at 100,000*g* for 1 h and the resulting pellet solubilized for 4 h in 1% (v/v) Triton X-100 in TBS. After centrifugation for 30 min at 14,000*g*, the resulting detergent extract was precleared by incubation with protein G-Sepharose (GE Healthcare, Europe). After incubation with antibodies against MAP1B (1:1000) for 2 h, antigen–antibody complexes were immobilized on protein G-Sepharose overnight. All steps were performed at 4 °C. After washing the beads with 0.05% (v/v) Tween 20 in 50 mM Tris/HCl, pH 7.4, 150 mM NaCl (TBS-T), bound proteins were eluted with 2× SDS-sample buffer and analyzed by Western blotting with mGluR4 antibody.

*SDS–PAGE and Western blotting.* Protein samples were separated on 6–8% SDS–polyacrylamide gels (PROTEAN II xi or Mini-PROTEAN III system, Bio-Rad) and silver-stained using the Silver Stain Plus Kit (Bio-Rad). Western blotting with anti-MAP1B (1:500) and anti-mGluR4 (1:1000) was performed as described [3].

*Primary cultures and immunofluorescence staining of rat hippocampal neurons.* Hippocampal neurons were cultured as previously described [18]. Neurons were fixed with 100% methanol at −20 °C. Cells were permeabilized with 0.2% (v/v) Triton X-100 for 20 min and then blocked with 5% (w/v) goat serum in TBS for 1 h. Incubation with primary antibodies was at 4 °C in blocking buffer for at least 24 h, and with secondary antibodies for 45 min, respectively. Antibody dilutions were 1:200 (anti-MAP1B), 1:100 (anti-mGluR4), 1:200 (anti-vGluT1), and 1:1000 (secondary antibodies). Images were captured with a Leica TCS-SP confocal laser scanning microscope equipped with a 63× objective.

## Results and discussion

An immobilized GST fusion protein comprising the intracellular C-terminus of mGluR4 (GST-mGluR4-C) was used to isolate interacting proteins from rat brain detergent extracts. Bound proteins were separated by SDS–PAGE and visualized by silver staining (Fig. 1). Protein bands bound only to GST-mGluR4-C but not GST were analyzed by mass spectrometry. This protocol identified ten proteins, most of which were initially found through MALDI-TOF-MS and later confirmed by MALDI-TOF/TOF-MS (Table 1). Two bands at 350 and 270 kDa corresponded to microtubule-associated proteins 1A and 1B (MAP1A and MAP1B). Other proteins linked to the cytoskeleton included spectrin-β2, non-muscle myosin and the cytoskeleton-associated protein 5. Bands of 200 kDa and 100 kDa represented clathrin and the adaptor complex protein AP-2, respectively, e.g. proteins involved in endocytosis. The stable tubule-only polypeptide (STOP) protein was found only once in MALDI-TOF-MS, but is included here because it also was detected by immunoblotting after GST-mGluR4-C pull-down (data not shown).

As in seven independent experiments MAP1B was the most frequently found mGluR4-C binding protein, we confirmed its interaction with mGluR4 by both Western blot analysis and co-immunoprecipitation. In the protein fraction isolated with GST-mGluR4-C but not GST, MAP1B immunoreactivity was enriched as compared to the supernatant (Fig. 2A). Hence, the mGluR4-C sequence specifically binds MAP1B. Furthermore, sequential incubation of brain detergent extracts prepared from wild-type and mGluR4 −/− mice with anti-MAP1B and protein G-agarose produced a 200 kDa (the molecular weight of the mGluR4 dimer; see [4]) mGluR4 immunoreactive band only with wild-type but not mGluR4 −/− samples (Fig. 2B). Thus, endogenous mGluR4 binds endogenous MAP1B in mouse brain extracts.

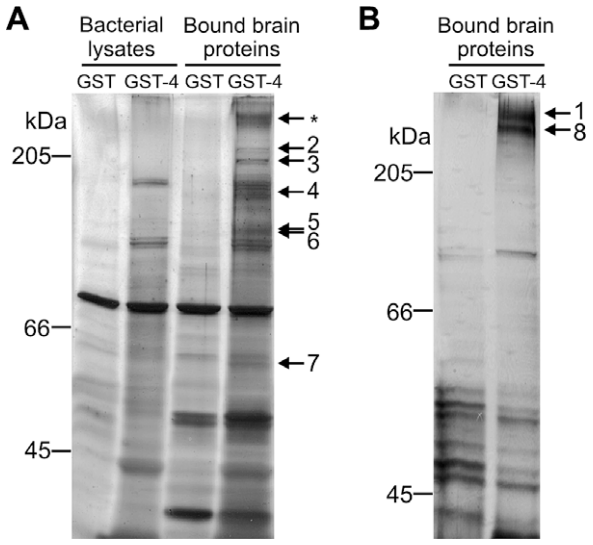


**Fig. 1.** Identification of proteins binding to GST-mGluR4-C. (A) Rat brain proteins bound to GST-mGluR4-C (GST-4) were separated by 8% SDS–PAGE followed by silver staining. For comparison, rat brain proteins bound to GST alone and the bacterial lysates containing overexpressed GST-mGluR4-C or GST were also analyzed. Arrows indicate bands specifically isolated with GST-mGluR4-C that were analyzed by MS (see Table 1). The asterisk shows a double band further resolved in (B). Positions of molecular-weight markers (in kDa) are indicated. (B) As (A), right lanes, but separation by 6% SDS–PAGE for better resolution of high molecular-weight proteins.

Different proteins have been shown to interact with the C-tails of all group III mGluR family members [3,6]. We therefore examined whether MAP1B binds selectively to mGluR4 or also to other closely related mGluRs. GST pull-down experiments with the intracellular C-terminal domains of mGluRs 6, 7a, 7b, 8a, and 8b showed that all these group III mGluRs bind MAP1B (Fig. 3A). In contrast, no interaction was found with the group II mGluRs 2 and 3 (Fig. 3B).

To localize the MAP1B binding site within the C-terminal domain of group III mGluRs, GST fusion proteins comprising the following peptidic sequences were used in binding assays with detergent extracts from rat brain: the very C-terminal regions of mGluR7a (C27, 27 amino acids) and mGluR8a (C44, 44 amino acids); and the complementary membrane-proximal fragments (N38, 38 amino acids of mGluR7a; and N24, 24 amino acids of mGluR8a) (Fig. 3C). Only the latter gave strong positive binding signals. Comparison of their sequences indicates that the MAP1B binding sites of mGluR7 and mGluR8 reside within a highly conserved 24 amino acid stretch located at the N-terminal regions of the group III mGluR-C tails.

CaM is known to interact with group III mGluRs in a $Ca^{2+}$-dependent manner [3,4]. The interaction site of $Ca^{2+}$/CaM lies within the same C-terminal sequence shown above to bind MAP1B. We therefore investigated whether the interaction of MAP1B with GST-mGluR4-C is affected by $Ca^{2+}$/CaM. In GST pull-down experiments with brain extracts, exogenously added CaM almost completely