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The actin-binding protein Filamin-A interacts with the metabotropic glutamate receptor type 7

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Abstract A yeast two-hybrid screen identified Filamin-A as a binding partner of the metabotropic glutamate receptor type 7b (mGluR7b) splice variant. In addition, Filamin-A interacted with mGluR4a, mGluR5a, mGluR5b, mGluR7a and mGluR8a. Domain mapping revealed that alternative splicing of mGluR4, mGluR7 and mGluR8 C-termini regulated the interaction. A conserved tyrosine within mGluR C-termini was identified to mediate the binding to Filamin-A. Protein interactions were verified in biochemical assays using recombinant and native proteins. Finally, co-expression of Filamin-A and mGluR7 splice variants was shown in brain regions. These findings suggest that Filamin-A may physically link metabotropic glutamate receptors to the actin cytoskeleton.

Key words: mGluR; Filamin-A; Protein–protein interaction; Yeast two-hybrid; Expression pattern

1. Introduction

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system acting on ion channel-associated (ionotropic) and G-protein-coupled (metabotropic) glutamate receptors. Metabotropic glutamate receptors (mGluRs) influence a variety of intracellular second messenger systems, modulating neuronal excitability, development, synaptic plasticity and transmitter release [1]. To date eight different mGluRs are known, which are subdivided into three groups based on sequence homology, pharmacological properties and second messenger systems [2]. Group III mGluRs (mGluR4, mGluR6, mGluR7, mGluR8) were primarily found at presynaptic active zones where they may function as autoreceptors [3–8]. Remarkably, mGluR7 in the hippocampus was localized at different densities at synaptic terminals of the same axon depending on the nature of the postsynaptic neuron [6,7]. Similarly, in retinal axon terminal systems of bipolar cell ribbon synapses the expression of mGluR7 was restricted to one side of the active zone [8].

Synaptic neurotransmission requires a highly ordered arrangement of neurotransmitter receptors at subcellular specializations. Mechanisms mediating the synaptic localization of mGluR7 are unknown. The intracellular C-terminus is important for targeting of mGluR7 [9,10] and recently, proteins interacting with this domain were identified: calmodulin, PICK1 (protein interacting with C-kinase) and PKC (protein kinase C) [11–16]. Here, a yeast two-hybrid screen showed interaction of Filamin-A, a member of the α-actinin/spectrin/dystrophin family of actin-binding proteins, with the mGluR7b C-terminus. Filamin-A induces polymerization of actin filaments by crosslinking them into orthogonal arrays or parallel fibers, contributing to the formation of the actin meshwork adjacent to the surface cell membrane [17]. Recently, Filamin-A was found to bind dopamine receptors and voltage-gated potassium channels, thus physically connecting neurotransmitter receptors and ion channels to the actin cytoskeleton [18,19].

2. Materials and methods

2.1. Yeast two-hybrid techniques

The mGluR7b C-terminus of rat (amino acids 851–922) was subcloned in pBTM116 for expression as a LexA fusion protein. Yeast L40 (Invitrogen) cells were sequentially transformed with the bait and 1 mg of bovine retinal cDNA library in pVP16 [20]. Colonies were grown for 5 days at 30°C on selection plates containing 3 mM 3-amino-1,2,4-triazole, then transferred to plates containing 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside. Library plasmids of positive clones were isolated and sequenced. To map interacting domains, amplified DNA fragments were subcloned in appropriate yeast vectors. MGluR7 point mutations were introduced by PCR: sense 5′-CG**GGATCC**ACCCTGAACTCAATGTC-3′, antisense Y916S 5′-AA**CTGCAG**CTATACTGTTGGTGGGATAGTACTCCAAGTTACGGACTTTTGTAC-3′, antisense Y916F 5′-AA**CTGCAG**CTATACTGTTGGTGGGATAGTAAACCAAGTTACGGACTTTTGTAC-3′ (restriction sites are bold, mutated codons underlined). Yeast transformations and quantification of protein–protein interactions were performed according to the 'Yeast Protocolls Handbook' (Clontech). A shaker β1-domain (aa 1–227, rat sequence) was used for controls. (Plasmids and cDNA were gifts of Drs. Jeremy Nathans and Min Li, Johns Hopkins University, Baltimore, MD, USA.)

2.2. Glutathione-S-transferase (GST) pull-down assays

MGluR C-termini (rat sequences) were ligated to the coding sequence of GST of pET-41 (Novagen), expressed in *Escherichia coli* BL21(DE3)pLysS and immobilized on glutathione Sepharose. Calmodulin (rat) and Filamin-A (bovine) were T7-tagged by cloning in pET-21 (Novagen). Membrane protein extracts of human embryonic kidney cells (HEK-293, ATCC CRL1573) were prepared as described [21]. Binding assays were performed according to the manufacturer's protocol (Novagen). For calmodulin, solutions were supplemented with 1 mM $CaCl_2$ or 5 mM EGTA. Bound proteins were eluted by boiling in SDS sample buffer, separated by SDS–PAGE, Coomassie-stained or detected by Western blotting and chemoluminescence

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Abbreviations: GST, glutathione-*S*-transferase; HEK-293 cells, human embryonic kidney cells; mGluR, metabotropic glutamate receptor; PICK1, protein interacting with C-kinase; PKC, protein kinase C

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(ECL; Amersham) using anti-T7 (1:10 000; Novagen) and anti-Filamin-A (1:200; Dianova) as primary antibodies.

2.3. Immunocytochemistry

Staining of HEK-293 cells was performed as described [22], using a Filamin-A-specific immunserum (1:300; Dianova) and Cy3-conjugated goat anti-mouse antibodies (1:500, Dianova). Immunofluorescence was accomplished with an epifluorescence microscope (Axioskop, Zeiss) and a cooled coupling device (CCD camera C4742-95, Hamamatsu).

2.4. RT-PCR

RNA purification, cDNA synthesis and PCR were performed as reported [23]. Total RNA was extracted from adult rat brain regions or HEK-293 cells and reverse transcribed. For PCR, oligonucleotides specific for Filamin-A (sense 5′-CCATCACTGACAACAAAGATG-3′, nt 5456–5478; antisense 5′-CTCAATGGCCAGAGACAAGC-3′, nt 5709–5690), mGluR7a (sense 5′-CACCCTGAACTCAATGTC-CAG-3′, nt 2551–2571; antisense 5′-TTAGATAACCAGGTTATTA-TAACTG-3′, nt 2748–2724), mGluR7b (sense 5′-CACCCTGAACT-CAATGTCCAG-3′, nt 2551–2571; antisense 5′-CTATACTGT-TGGTGGGATAGTG-3′, nt 2769–2748) and β-actin (sense 5′-TGA-GACCTTCAACACCCCAG-3′, nt 372–391; antisense 5′-GTAGAC-GACCTTCCACCTGT-3′, nt 1065–1046) were used. For HEK-293 cDNA, Filamin-A-specific PCR primers were: sense 5′-CTGTG-GCTTCTCCGTCTG-3′, nt 6972–6989; antisense 5′-AACTCG-GATCTTGAAGGGGC-3′, nt 7262–7243). Southern blot oligonucleotides for Filamin-A (5′-CTGTTACTGTGCGTTACTCAC-3′, nt 5482–5502), mGluR7a, mGluR7b (5′-GTTTGGGTCTACATTTT-CACAG-3′, nt 2655–2676) and β-actin (5′-CGGTCAGGTCATCAC-TATC-3′, nt 732–750) were tailed with DIG-ddUTP, hybridized and detected following the protocol of the manufacturer (Roche).

3. Results

3.1. Identification of Filamin-A as a binding partner of mGluR7b

A yeast two-hybrid screen for the mGluR7b C-terminus generated $\sim 3 \times 10^6$ primary transformants, of which 100 candidates were further analyzed. Clone 44 reproducibly interacted with mGluR7b, but not with control plasmids. Sequencing identified an open reading frame of the actin-binding protein Filamin-A (aa 2178–2647), followed by the 3′-untranslated region. The coding region varied little between bovine (GenBank accession number AY072716), human [24] and mouse sequences (GenBank accession number BC004061), most changes being conservative. Serine-2446 forms a putative consensus sequence for casein kinase-2 in bovine and mouse, but is absent in human. The bovine 3′-untranslated region differed by 48 and 61% from human and mouse genes, respectively.

3.2. Filamin-A interacts with splice variants of mGluR family members

To investigate whether Filamin-A interacts with other mGluR family members, their C-termini were individually tested in binding assays (Fig. 1). Protein interactions were monitored by the ability of transformed yeast cells to grow on selective media containing 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside, supplemented with either no or 3 mM 3-amino-1,2,4-triazole, indicating weak or strong binding affinities. Robust interaction with Filamin-A was observed for mGluR5a, 5b, 7b and 8a, while mGluR4a and 7a showed lower affinities (Fig. 1A). The mGluR1a C-terminus self-activated yeast reporter genes, as already reported [25]. MGluR6 was the only group III member not binding to Filamin-A. This is consistent with its unique expression in retinal bipolar cell dendrites, suggesting a specific mechanism mediating this localization [26]. Relative strengths of protein–protein interactions were calculated as arbitrary β-galactosidase units. Strongest binding was observed for mGluR5a (490 ± 102) and mGluR5b (531 ± 25), values were lower for mGluR7b (200 ± 50) and mGluR8a (155 ± 156) whereas mGluR4a (8.4 ± 1.4) and mGluR7a (22.5 ± 7.7) showed weakest affinities (Fig. 1A). For a control, the selective binding of group I mGluRs to Homer 1a was tested. As reported [27], mGluR5 (group I), but not mGluR4, mGluR7 and mGluR8 (group III), did bind to Homer 1a.

Since mGluR/Filamin-A interactions might depend on alternative splicing, domains representing identical or different regions within C-terminal tails were tested individually (Fig. 1B). The Filamin-A/mGluR5 interaction appeared independent of the splice variant, since an identical region (aa 827–875) revealed a binding strength of 473 ± 26, similar to the

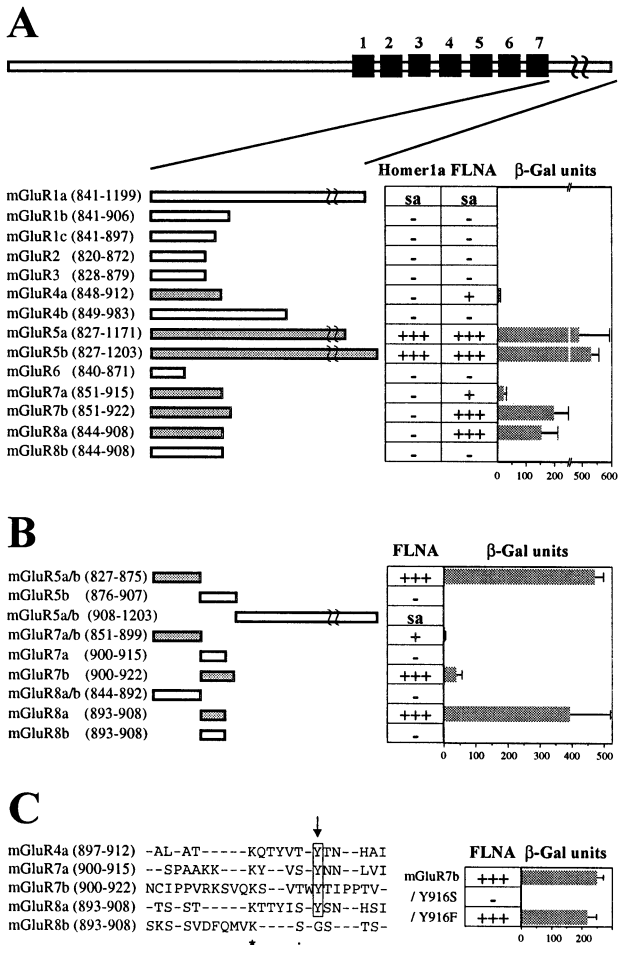

Fig. 1. Filamin-A binds to mGluR C-termini: MGluR C-termini (A), proximal and distal regions (B) or mutated domains (C) were individually tested in binary two-hybrid experiments for their ability to interact with Filamin-A (aa 2178–2647). The seven transmembrane regions of mGluR7 are visualized as black boxes, C-termini are drawn to scale and corresponding amino acid numbers are given in brackets. The arrow in (C) indicates the conserved tyrosine mutated in mGluR7b. Diagrams show activation of yeast reporter genes on selective media without (+) or with 3 mM 3-amino-1,2,4-triazole (+++); sa, self-activating. Affinities were quantified as arbitrary β-galactosidase units. Values represent the mean of three yeast colonies and are expressed as ± S.E.M.

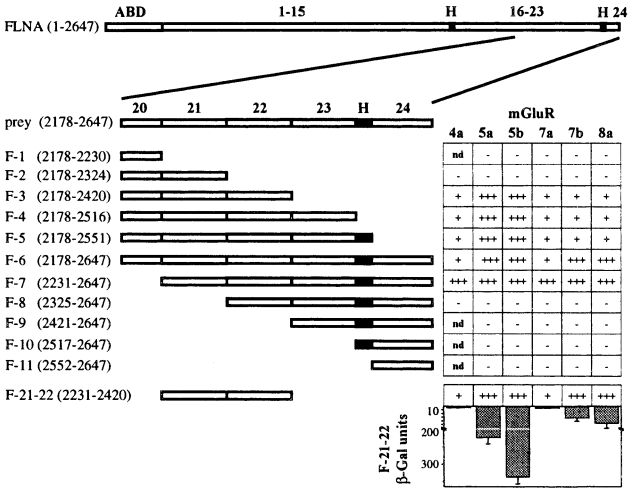

Fig. 2. Domain mapping of Filamin-A: Deletion constructs of Filamin-A (aa 2178–2647) were tested for binding to mGluR C-termini as described in Fig. 1. Filamin-A repeats are indicated by numbers; ABD, actin-binding domain; H, hinge; nd, not determined.

complete C-termini. This Filamin-A-binding site differs from the domain interacting with Homer (aa 1122–1129) [27], suggesting that Filamin-A and Homer do not compete for mGluR5 binding. Interaction of mGluR4, mGluR7 and mGluR8 with Filamin-A depended on the splice variant, with affinities of 40.4 ± 17.2 and 396 ± 125 for the distal splice variant-specific domains of mGluR7b and mGluR8a (Fig. 1B). The variant-specific domain of mGluR7a did not bind Filamin-A and the proximal part interacted extremely weak (0.26 ± 0.1), indicating that both regions contained binding sites needed in combination for the Filamin-A/mGluR7a association.

The specific interaction of Filamin-A with a subset of mGluRs allowed the identification of amino acids important for the binding. Alignment of interacting and non-interacting distal regions of mGluR C-termini identified a tyrosine (Y916 of mGluR7b), which is conserved in Filamin-A-binding C-termini, but is absent in the non-interacting mGluR8b (Fig. 1C). To test if size or hydroxyl group of Y916 are important determinants for Filamin-A binding, Y916 was mutated into serine (Y916S) or phenylalanine (Y916F). While Y916S abolished the mGluR7b/Filamin-A interaction, Y916F did not significantly alter the binding strength (252 ± 19.1) compared to wild-type mGluR7b (220 ± 28.8; Fig. 1C). Consistent with this finding, the tyrosine is conserved in all group III mGluRs binding to Filamin-A, but is absent in the non-interacting mGluRs [13].

3.3. Mapping domains of Filamin-A binding to mGluRs

Filamin-A consists of a rod-like structure composed of 24 homologous repeats, separated by two 'hinge' regions [24]. According to this modular structure, successive N- or C-terminal deletions were introduced to identify regions binding to mGluRs (Fig. 2). Truncating repeat 21 or 22 abolished interaction, indicating that sequences in these two domains were important for Filamin-A/mGluR association. Supporting this hypothesis, a construct containing repeats 21 and 22 (F21–22) did bind to all mGluR C-termini tested. Binding (mGluR4a: 0.82 ± 0.33; mGluR5a: 223 ± 18.2; mGluR5b: 336 ± 20.2; mGluR7a: 1.1 ± 0.3; mGluR7b: 31.7 ± 8.8; mGluR8a: 45.8 ± 13.7) was weaker compared to repeats 20–24, thus F21–22 might represent a minimal binding motive, while in the full-length protein additional domains could play some role in the formation of the complex. Interestingly, the dimerization domain (repeat 24) of Filamin-A [24], as well as the actin-binding domain, were not involved in mGluR binding, allowing dimerization and cytoskeleton association of Filamin-A to happen independent of mGluR binding.

3.4. Recombinant and native Filamin-A bind to mGluR7 C-termini

To confirm the Filamin-A/mGluR interactions, pull-down assays were performed using GST-mGluR7a and GST-mGluR7b fusion proteins immobilized on glutathione Sepharose incubated with *E. coli* protein extracts. Reliability of the pull-down assay was tested using the calcium-dependent binding of calmodulin to mGluR7. As reported [11], interaction was observed in the presence of calcium but not in the presence of the calcium chelator EGTA (Fig. 3A). When mGluR C-termini were incubated with Filamin-A (aa 2178–2647), binding was observed for mGluR7 splice variants but not for GST (Fig. 3B), consistent with the yeast-binding studies.

To test whether full-length Filamin-A was able to bind mGluR7, HEK-293 cells were identified to express endogenous concentrations of Filamin-A detectable by RT-PCR (Fig. 3C) and immunostaining (Fig. 3D). Incubation of mGluR C-termini with HEK-293 protein extracts demonstrated binding of mGluR7 to native Filamin-A (Fig. 3E).

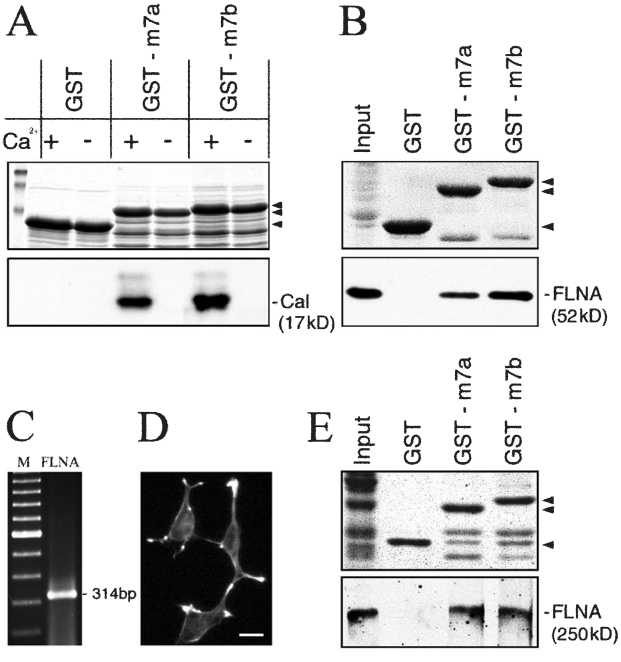

Fig. 3. Recombinant and native Filamin-A binds to mGluR7: GST fusion proteins immobilized on glutathione Sepharose were incubated with T7-tagged proteins (A, B) or with membrane-bound fractions of HEK-293 cells (E). Coomassie-stained gels show equal input of fusion proteins (arrowheads in A, B, E). Interacting proteins identified in Western blots are indicated as Cal (17 kDa) – calmodulin; FLNA (52 kDa) – recombinant Filamin-A; FLNA (250 kDa) – native Filamin-A. Filamin-A expression in HEK-293 cells was detected by RT-PCR (C) and immunostaining (D, white bar is 10 μm).

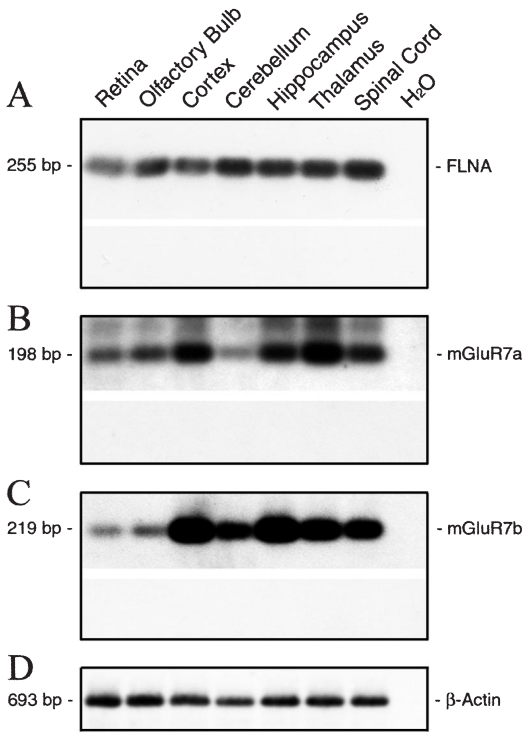

Fig. 4. Expression of Filamin-A, mGluR7a and mGluR7b in rat brain: Southern blots performed with PCR products representing Filamin-A (A), mGluR7a (B), mGluR7b (C) and β-actin (D), amplified after reverse transcription of total RNA isolated from the indicated tissues (upper panels) and control reactions without reverse transcriptase (lower panels). Calculated sizes of the PCR products are indicated on the left.

3.5. Localization of Filamin-A, mGluR7a and mGluR7b in the brain

Since physiological interaction of Filamin-A with mGluR7 requires the presence of binding partners in the same tissue, their expression was analyzed in various brain tissues. Filamin-A was present in retina and hippocampus, where asymmetric synaptic localization of mGluR7 has been reported [6–8], as well as in other brain regions, with slightly higher concentrations in olfactory bulb, cerebellum and spinal cord (Fig. 4A). Highest expression for mGluR7a was visible in cortex and thalamus (Fig. 4B), whereas transcripts for mGluR7b were enriched in cortex and hippocampus (Fig. 4C). Approximately equal amounts of cDNA from each tissue were used, as demonstrated by the similar intensity of β-actin PCR products (Fig. 4D).

4. Discussion

This study identified Filamin-A as a new binding partner of mGluR C-termini. Filamin-A has been co-localized with acetylcholine receptor clusters in cultured chick myotubes and has been proposed to play a role in agrin-mediated acetylcholine receptor clustering at neuromuscular junctions [28]. Recent studies demonstrated binding of Filamin-A to dopamine receptors D2 and D3 as well as to the voltage-gated potassium channel Kv4.2 [18,19]. Based on these findings, a scaffolding function for Filamin-A in formation and maintenance of macromolecular complexes at synapses has been proposed. According to a recent model, induction of filopodial-like precursors under synaptic axonal boutons results in the formation of dendritic spines [29], and indeed, Filamin-A is highly concentrated at cellular filopodial roots [30]. Considering the data of this study, mGluR7 might represent a new member of Filamin-A containing synaptic complexes.

Proteins binding to mGluR7 include PKCα, calmodulin and PICK1 [11–16]. Calmodulin and a G-protein βγ-subunit compete for binding to the mGluR7 C-terminus [11–13]. This binding site was located in the proximal part of the C-terminus, distinct from the Filamin-A-binding region. Thus, mGluRs could be linked to the cytoskeleton via Filamin-A independent of G-protein binding. In contrast, the binding motive for PICK1 was localized to the C-terminal 15 amino acids of mGluR7 [16], including the conserved tyrosine residue identified in this study. PICK1 interacts with PKCα and phosphorylation of mGluR7 has been demonstrated [13,31]. Therefore, the exclusive association of Filamin-A or PICK1/PKCα with mGluR7 could regulate phosphorylation of the mGluR7 C-terminus.

Within cells, Filamin-A exists in two different pools depending on its phosphorylation status, one being associated with the plasma membrane, the other crosslinking the actin cytoskeleton [19]. Since kinase activity can be influenced by synaptic signaling via second messenger systems, mGluR7 could regulate Filamin-A concentrations at synapses, thereby controlling the synaptic presence of cytoskeletal associated proteins.

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