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Homer Binds a Novel Proline-Rich Motif and Links Group 1 Metabotropic Glutamate Receptors with IP3 Receptors

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Summary

Group 1 metabotropic glutamate receptors (mGluRs) activate PI turnover and thereby trigger intracellular calcium release. Previously, we demonstrated that mGluRs form natural complexes with members of a family of Homer-related synaptic proteins. Here, we present evidence that Homer proteins form a physical tether linking mGluRs with the inositol trisphosphate receptors (IP3R). A novel proline-rich "Homer ligand" (PPXXFr) is identified in group 1 mGluRs and IP3R, and these receptors coimmunoprecipitate as a complex with Homer from brain. Expression of the IEG form of Homer, which lacks the ability to cross-link, modulates mGluR-induced intracellular calcium release. These studies identify a novel mechanism in calcium signaling and provide evidence that an IEG, whose expression is driven by synaptic activity, can directly modify a specific synaptic function.

Introduction

Group 1 metabotropic glutamate receptors (mGluRs) represent a family of seven membrane-spanning proteins that couple to G proteins and activate phospholipase C (Nakanishi, 1994). Members of the family include mGluR1 and mGluR5 (Masu et al., 1991; Abe et al., 1992; Aramori and Nakanishi, 1992). Activation of these receptors results in the hydrolysis of membrane phosphatidylinositol bisphosphate to diacylglycerol, which activates protein kinase C, and inositol trisphosphate (IP3), which activates the IP3 receptor (IP3R) to release intracellular calcium (Aramori and Nakanishi, 1992; Joly et al., 1995; Kawabata et al., 1998). Metabotropic signaling has been implicated in several forms of activity-dependent synaptic plasticity (Linden et al., 1991; Zheng and Gallagher, 1992; Bolshakov and Siegelbaum, 1994; Bortolotto et al., 1994; O'Connor et al., 1994), but its role in some of these processes remains controversial (Chinestra et al., 1994; Manzoni et al., 1994; Hsia et al., 1995). Consistent with a role in activity-dependent plasticity, mice with null mutations of either mGluR1 (Aiba et al., 1994; Conquet et al., 1994; Bordi, 1996) or mGluR5 (Lu et al., 1997) display reductions of hippocampal LTP, abnormalities of motor coordination and associative learning, and of fear conditioning. Metabotropic receptor signaling is also implicated in neurodegenerative diseases (Nicoletti et al., 1996; Conn and Pin, 1997), cortical development (Kaczmarek et al., 1997), and addiction (Wolf, 1998).

Group 1 mGluRs possess a long C-terminal intracellular tail that influences sensitivity to agonists (Flor et al., 1996) and may be important for subcellular localization of the receptor (Grandes et al., 1994). The C terminus is also the site of binding by the immediate early gene (IEG), Homer 1a (Brakeman et al., 1997). Homer 1a is the founding member of a new gene family that includes products of three distinct mammalian genes, as well as a single *Drosophila* gene (Xiao et al., 1998 [this issue of *Neuron*]). All share a highly conserved ~120 aa N-terminal EVH domain that defines the Homer family and all bind group 1 mGluRs. In contrast to Homer 1a, new members additionally encode a C-terminal coiled-coil (CC) domain and form multivalent complexes that bind group 1 mGluRs. Homer 1a competes with constitutively expressed CC-Homers to modify the association of group 1 mGluRs with CC-Homer complexes. Since Homer proteins are strikingly enriched at the postsynaptic density (PSD), these observations suggest a role for the Homer family in regulating synaptic metabotropic receptor function.

In the present study, we examine the molecular specificity of the interaction between Homer and group 1 mGluRs and identify a novel proline-rich "Homer ligand" motif. Interestingly, a search of sequence databases identified the Homer ligand motif in the IP3R, as well as other proteins involved in calcium signaling. Consistent with the multimeric binding property of CC-Homers, we demonstrate that mGluR1α and the IP3R coimmunoprecipitate from cerebellum with CC-Homers. We further demonstrate that this association is important for the signaling property of mGluRs in neurons. These studies provide molecular insight into the synaptic function of an IEG and define a novel mechanism in calcium signaling.

Results

Identification of a Homer Ligand Site in Group 1 mGluRs

In our original analysis of the interaction between Homer and group 1 mGluRs, we showed that C-terminal deletions of mGluR5 reduced its binding to Homer (Brakeman et al., 1997). In subsequent analyses, we found that substantial binding remained even with >20 amino acid deletions. Accordingly, we sought to identify a putative second (or alternative) site of interaction with Homer. We began by examining successively larger C-terminal deletions of a 241 aa C-terminal fragment of mGluR5 that binds Homer. Deletion of the C-terminal 31, 37, 40, or 41 aa did not affect binding (Figure 1). However, deletion of the C-terminal 50 aa destroyed binding to Homer, suggesting that amino acids in the region −50 to −41 are important for mGluR5 interaction with Homer. This sequence was noted to be identically conserved

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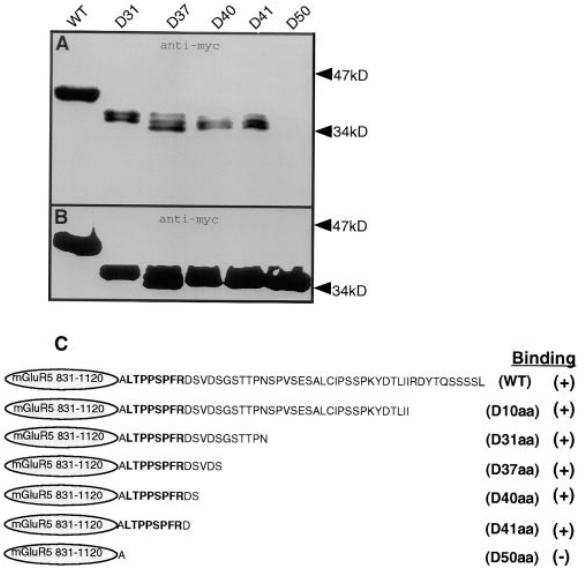


Figure 1. Deletion Analysis Defines a Region in the C Terminus of mGluR5 Essential for Binding Homer

(A and B) In vitro binding of mGluR5 C-terminal deletion mutants to GST-Homer 1a. myc-tagged mGluR5 mutants (wild type and deletions of the C-terminal 31, 37, 40, 41, and 50 aa) were expressed in HEK293 cells, and bound proteins were assayed by myc Ab immunoblot. (B) Expression of offered mGluR5 deletion mutants.

(C) Schematic of mGluR5 deletion mutants. Candidate Homer ligand domain is in bold.

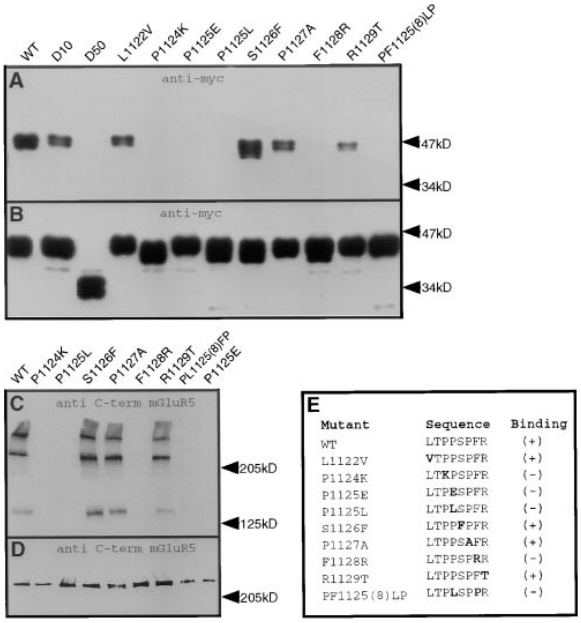


Figure 2. Point Mutagenesis Defines Homer Ligand Motif in mGluR5

(A and B) In vitro binding of mGluR5 C-terminal point mutants to GST-Homer 1a. N-myc-tagged mGluR5 mutants were expressed in HEK293 cells, and bound proteins were assayed by immunoblot with myc-Ab. Wild-type (WT) and Del 10 (deletion of the last 10 amino acids) provide positive controls; Del 50 (last 50 amino acid deletion) provides a negative control. Point mutations are referenced to full-length mGluR5. (B) Expression of offered mGluR5 point mutants.

(C and D) In vitro binding of full-length mGluR5 point mutants to GST-Homer 1a. Western blots use anti-mGluR5. Note presence of monomer 150 kDa, dimer 300 kDa, and tetramer 600 kDa forms of mGluR5. The dimer is the predominant form in offered extracts (D).

(E) Schematic of mGluR5 point mutants and binding activity. The mutated amino acids are in bold.

in mGluR1α, consistent with its representing a site of interaction with both group 1 mGluRs.

We next generated point mutations of the putative Homer interaction domain, again using the C-terminal 241 aa mGluR5 fragment (Figure 2). Binding assays included the 10 and 50 aa C-terminal deletion mutants of the same construct for direct comparison. Mutations of the first or second proline (proline 1124 or 1125 in full-length mGluR5) destroyed binding (Figure 2A). Effective mutations included both charge changing (P1124K and P1125E), as well as the more conservative substitution of leucine for proline at position 1125. Side by side comparisons of binding properties indicated that specific point mutations are as effective in disrupting binding as deletion mutations. These observations provided evidence that the proline-rich region is an important domain for interaction with Homer.

Dependence of binding on prolines at positions 1124 and 1125 suggested a role for these amino acids in either establishing the appropriate secondary structure of the ligand or in mediating a specific contact with Homer. We noted similarity between the Homer "ligand" sequence in group 1 mGluRs (ALTPPSPFRD) and the SH3 ligand (consensus for the class II SH3 ligand is XXXPPLPXR for binding both Src-SH3 and PI3K-SH3 domains [Feng et al., 1994; Yu et al., 1994]). Detailed mutagenesis and structural information is available for the SH3 ligand–Src family interaction (Yu et al., 1994), and we designed several of the mGluR5 mutants based on precedent from these studies. For example, the SH3 ligand forms a polyproline type II helix with a left-handed secondary structure. Because the period of the helix is $\sim$3.0, the amino acid backbone forms a trigonal prism (Yu et al., 1994). Sites of contact, between the SH3 ligand and Src, involve prolines and charged amino acid side chains on one face of the prism. Consequently, the amino acids that are critical for interaction with Src proteins show a predictable third position dependence in the primary sequence. Based on the observed dependence on prolines 1124 and 1125 of mGluR5 for binding, we predicted that amino acids at either position 1127 or 1128 might also be important for interaction. Mutation of proline 1127 of mGluR5 did not disrupt binding. By contrast, the F1128R mutation in the Homer ligand destroyed binding (Figure 2A). These results are interesting to contrast with studies of the SH3 ligand–SH3 interaction, since equivalent mutations in the SH3 ligand show reciprocal effects on binding. To evaluate the concern that the disruptive effect on binding of the F1128R mutation might be due to an indirect effect on the secondary structure, we tested a double point mutant in which the total proline content was conserved (P1125L and F1128P). This mutation also destroyed binding. These observations suggest a specific role for P1125 and F1128.

Two additional point mutations further differentiated the putative Homer ligand from the SH3 ligand. Neither

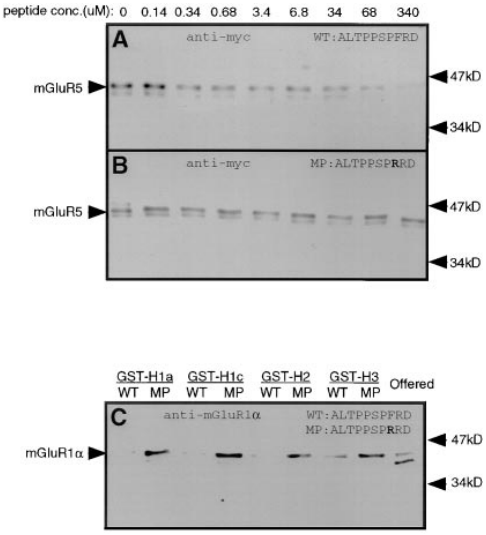


Figure 3. Homer Ligand Peptide Selectively Blocks Binding to Group 1 mGluRs

(A and B) In vitro binding of myc-tagged mGluR5 C terminus to GST-Homer 1a in the presence of indicated concentrations of wild-type (A) and mutant (B) peptides.

(C) In vitro binding of myc-tagged mGluR1α C terminus to full-length GST-Homer fusion proteins in the presence of wild-type and mutant peptide (68 μM).

S1126F nor R1129T destroyed binding to Homer, while amino acids at equivalent positions in the SH3 ligand are critical for binding Src (Yu et al., 1994). In repeated assays, we note that the R1129T appears to reduce, but not destroy, binding. Based on these observations, we propose a core Homer ligand consensus motif as PPXXFr.

We then examined the role of this motif in binding of the full-length mGluR5 to Homer. This analysis considered the possibility that there may be multiple sites of interaction, since the long intracellular C terminus of group 1 mGluRs includes several proline-rich regions (Tanabe et al., 1992). The full-length mutant mGluR5 exhibited identical Homer binding properties as did the equivalent C-terminal mGluR5 fragment mutants (Figure 2C). We conclude that the Homer ligand is the primary site of binding interaction with mGluR5.

Homer Ligand Peptide Selectively Blocks Binding to Group 1 mGluRs

To further assess the role of the Homer ligand sequence in the interaction between mGluR5 and Homer, we examined the ability of synthetic 10 aa peptides to block binding (Figure 3). SH3 ligand peptides as short as 9 aa bind with micromolar affinities to the SH3 domains of Src and PI3K (Chen et al., 1993; Yu et al., 1994) and have been useful to assess the physiological role of Src in synaptic physiology (Lu et al., 1998). As a control, we tested a second peptide in which the F was replaced with R. Peptides were mixed with extracts of HEK293 cells expressing the C-terminal mGluR5 fragment and then added to GST-Homer 1a on beads. Wild-type peptide was effective in blocking binding of mGluR5 (Figure 3A), while the mutant peptide did not inhibit binding (Figure 3B). The wild-type peptide blocked greater than half of the binding at 68 μM. In this assay, the dose-response curve showed a partial block at lower concentrations consistent with a higher affinity binding site.

Wild-type, but not mutant, peptide also blocked binding of a C-terminal fragment of mGluR1α to Homer 1a (Figure 3C). Peptides were additionally tested in binding assays with different Homer family members. The Homer family presently includes six proteins derived from three distinct genes (Xiao et al., 1998). Consistent with the high degree of conservation in their N-terminal domains, products of all three genes bind group 1 mGluRs. The wild-type Homer ligand peptide selectively blocked binding of each of the three Homer genes products to mGluR1α (Figure 3C).

Homer Ligand Site Is Present in IP3R and Dynamin III

We searched the GenBank database for other proteins that might contain the Homer ligand motif. We anticipated that Homer must interact with proteins in addition to the group 1 mGluRs, since various Homer family proteins are present at high levels in tissues that do not express mGluRs, including heart and muscle (Xiao et al., 1998). In our analysis, we focused on those proteins in which the putative motif is cytosolically exposed, since Homer is a soluble protein. Based on these criteria, we identified a list of candidate Homer interacting proteins (Figure 4A). The human type 1 IP3 receptor encodes a Homer ligand–like sequence (PPKKFRD) at aa 48–55 that is exposed in the cytosol and is identically conserved in *Drosophila*, rat, mouse, and human sequences. Further, mammals possess three distinct IP3R genes (type 1–3) (Ross et al., 1992), and the Homer ligand sequence is conserved in all forms. The dynamin homolog, dynamin III (Shpetner and Vallee, 1989; Nakata et al., 1993), also encodes a proline-rich sequence in its C terminus that conforms to the Homer ligand consensus sequence. Dynamin III is expressed in brain and testis (Cook et al., 1996) and is anticipated to play a role similar to dynamin 1 in vesicle formation (Oh et al., 1998; Sweitzer and Hinshaw, 1998); however, its specific function remains unknown. A putative Homer ligand is also present in the alpha 1D adrenergic receptor (accession number, 1168243), the ryanodine receptors (accession number, 134132), cytochrome P450 (accession number, 117260), and protein-tyrosine phosphatase 2E (accession number, 2493262). It is intriguing that several of these proteins are involved in calcium signaling.

We tested whether the IP3R and dynamin III might bind Homer. In our initial efforts, we presented brain extracts to GST-Homer 1a and blotted for the IP3R. This assay detects robust binding of group 1 mGluRs to Homer, but a similar assay for IP3R binding showed high nonspecific binding to beads. Accordingly, we prepared GST fusions of the relevant regions of the IP3R and dynamin III. Binding assays were performed with extracts of cerebellum and blotted for Homer 1 and Homer 2. Both Homer 1 and Homer 2 bound to the IP3R and dynamin (Figures 4B and 4C). The nature of the lower mw bands seen on these blots has not been determined. Deletion of the Homer ligand from the IPR3 fusion protein destroyed its binding to Homer (data not shown).

A	cDNA	sequence
	Rat-mGluR1αR	TPPSPFRD(1145-1152)
	Rat-mGluR5R	TPPSPFRD(1123-1130)
	Human IP3R	NPPKKFRD (48-55)
	Rat-DynaminIII	APPVPFRP (798-805)
	Human-Ryanodine	RPPHHFSA(1771-1778)
	Human-P450	LPPDLFRL (250-257)
	Human-a-adrener	KPPSAFRE (489-496)
	Rat-PTNF	RPPVVFRW (249-256)

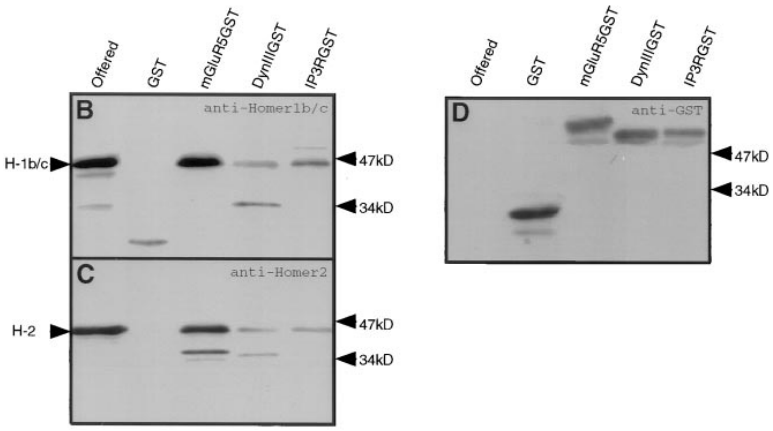


Figure 4. IP3R and Dynamin III Bind to Homer In Vitro

(A) Sequence alignment of genes that contain Homer binding motif. Numbers indicate the amino acid positions in the full-length proteins. Accession numbers are provided in text. (B and C) In vitro binding of Homer 1b/c and Homer 2a/b from detergent extracts of rat cerebellum to GST fusion proteins. Homer proteins bind GST-IP3R (N-terminal 260 amino acid of human type I IP3R), GST-Dynamin III (C-terminal 236 amino acid of rat dynamin III), and GST-mGluR5 (rat C-terminal 241 amino acid). (B) was probed with rabbit anti-Homer 1b/c and (C) with anti-Homer 2a/b antibody. (D) Equal loading of GST proteins was confirmed by GST-immunoblot.

These studies support the notion that Homer may bind to proteins other than the group 1 mGluRs.

Homer Forms a Complex with mGluR1α and IP3R

Previously, we demonstrated that Homer coimmunoprecipitates mGluR5 from hippocampus (Brakeman et al., 1997) and mGluR1α from the cerebellum (Xiao et al., 1998). Because Homer family proteins and the IP3R are both enriched in the cerebellum, we focused our efforts on examining the physiological binding partners of Homer in this tissue. The IP3R was specifically coprecipitated with antibodies for Homer 1, Homer 2, and Homer 3 (Figure 5). In support of the notion that Homer and the IP3R are natural partners, we note that both Homer and the IP3R are highly enriched in Purkinje cells of the cerebellum and that both are present in dendrites and spines (Satoh et al., 1990; Xiao et al., 1998).

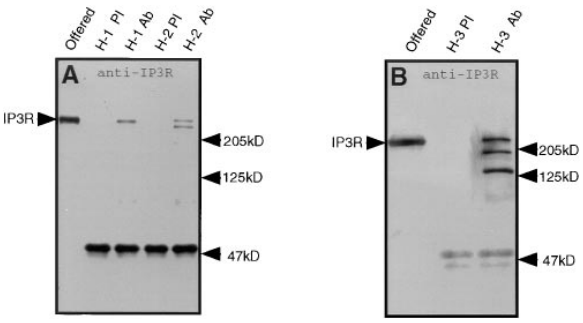


Figure 5. IP3R Coprecipitates with Homer Proteins from Rat Cerebellum

(A and B) Detergent extracts of cerebellum were immunoprecipitated with antibodies for Homer 1, 2, and 3. Control lanes use preimmune serums. One hundered microliters of extract was used for each IP, and 10 μl was loaded in the offered lane. Multiple IP3R bands in Homer 2 and Homer 3 precipitates are presumed proteolysis products. Bands at ~50 kDa are reduced heavy chains of the precipitating antibodies.

All members of the Homer family, with the single exception of the original IEG form, encode a ~100 aa CC domain and form homo- and heterodimers with other Homer family members (Xiao et al., 1998). This capacity for multimerization appears to be physiologically relevant, since Homer 1b/c Ab coimmunoprecipitates Homer 3. Thus, while Homer possesses a single binding site for the Homer ligand site, CC-containing forms of Homer (CC-Homer) can multimerize and thereby function to cross-link proteins. Accordingly, we examined the possibility that CC-Homers might cross-link group 1 mGluRs and IP3R.

Immunoprecipitation of the IP3R, using a goat primary antibody, provided a clean co-IP of mGluR1α (Figure 6A). Immunoprecipitates were also reprobed with antibodies for each of the CC-Homer proteins. Homer 3 is most clearly part of the immunoprecipitated complex (Figure 6B). Homer 1b/c was also present but less easily detected due to a similar-sized, cross-reacting band from the heavy chain of the goat IgG (data not shown). Homer 2 was not detected in the complex.

Transient Expression of the IEG Form of Homer Modulates Glutamate-Induced Intracellular Calcium Release

One prediction of the current data is that Homer may cross-link group 1 mGluRs and IP3R into a functional signaling complex. Group 1 mGluRs generate IP3, and their physical proximity to the IP3R would serve to optimize the functionality of the intracellular calcium response. To examine this hypothesis, we introduced the

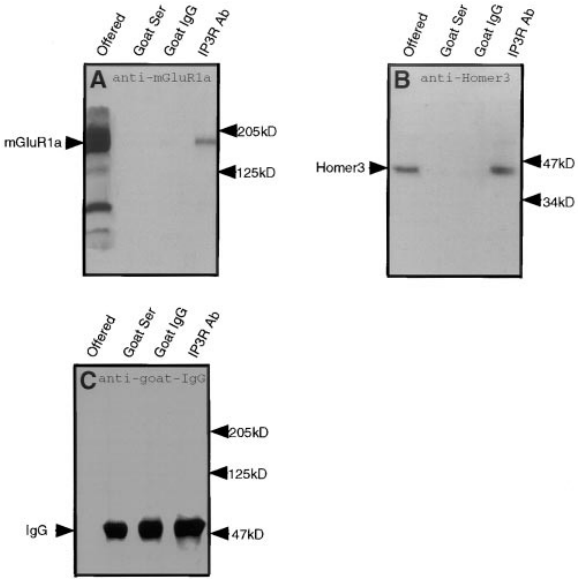


Figure 6. mGluR1α and IP3R Coprecipitate from Cerebellum, and the Complex Includes Homer

(A) Detergent lysates of cerebellum were immunoprecipitated with IP3R Ab (goat) and blotted for mGluR1α (mouse monoclonal). One hundred microliters of extract was used for each IP, and 20 μl was loaded in the offered lane. Negative controls included goat IgG fraction or goat serum.

(B) Reprobe of (A) with Homer 3 antibody. Homer 3 co-IPs with IP3R Ab.

(C) Reprobe of (A) with anti-goat antibody to confirm equal offering of IgGs.

IEG form of Homer 1 into Purkinje cells in primary cerebellar cultures and examined the effect on metabotropic glutamate-induced calcium transients. The Homer 1 IEG construct was an N-terminal 186 aa fragment of Homer 1b. This sequence does not encode a CC domain and is identical to Homer 1a with the exception of the C-terminal 11 aa. We used this deletion construct because it yielded better expression than Homer 1a in heterologous cells. Ca^{2+} mobilization, in the absence of influx, was measured by ratio imaging fura-2 in Purkinje cells bathed in Ca^{2+}-free external saline and stimulated with a micropressure pulse of quisqualate, an mGluR agonist (Linden, 1996). The resultant Ca^{2+} transient is triggered by an mGluR/IPR3 pathway, since it is completely blocked by either an mGluR antagonist ([+]-MCPG, 500 μM in the bath) or a novel specific IP3R–associated ion channel blocker, xestospongin C (xestospongin C, 1 μM in the internal saline; [Narasimhan et al., 1998]). Two to three days prior to recording, Purkinje cells were transfected using the biolistic method with plasmids that separately expressed Homer 1 and green fluorescent protein (GFP). Expression of GFP was used to label transfected Purkinje cells, which were identified based on their distinctive morphology (Figure 7A). In these experiments, we compared effects of the IEG form of Homer 1 with that of a CC-containing form of the same gene (Homer 1b).

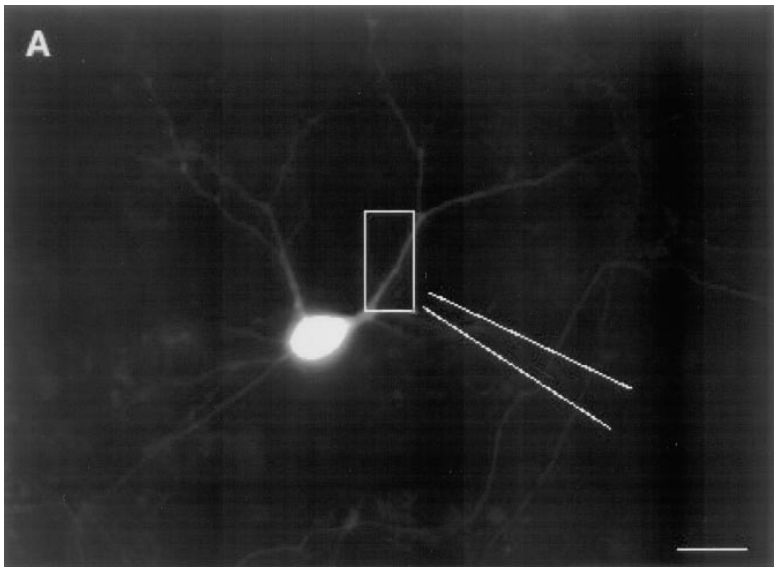
The main finding is that Purkinje cells transfected with an IEG form of Homer 1 showed mGluR-evoked Ca^{2+} responses with a decreased amplitude (170 ± 9 nM, mean ± SEM, n = 30 cells) and an increased latency (10.5 ± 1.8 s), as compared with cells transfected with Homer 1b (244 ± 17 nM, 4.2 ± 0.9 s, n = 23) or an empty vector control (239 ± 19 nM, 4.5 ± 1.1 s, n = 15) (Figure 7B). The decay phase of the Ca^{2+} response appeared somewhat slower in neurons transfected with the IEG form. While the total Ca^{2+} flux appeared similar in all three cases, this measurement could not be made definitively because the tail of the Ca^{2+} response was truncated due to the constraint of the image buffer capacity.

Discussion

The present study identifies a novel mechanism that appears to regulate the coupling between membrane mGluRs and ER-associated intracellular IP3R. Homer proteins bind a proline-rich motif that is present in both group 1 mGluRs and IP3R, and these receptors coimmunoprecipitate in a complex that includes Homer. Disruption of the complex, as a consequence of expression of the IEG form of Homer, results in an alteration of mGluR-induced Ca^{2+} release. Since Homer 1a is naturally upregulated in response to specific forms of synaptic activity that are associated with long-term plasticity (Brakeman et al., 1997), we hypothesize that Homer proteins function to regulate coupling between membrane group 1 mGluRs and intracellular IP3Rs.

Recent studies support the notion that proteins involved in signaling cascades may be physically coupled. For example, components of the MAP kinase pathway form a complex of cytosolic kinases with their specific substrates (Davis, 1995). Similarly, proteins such as AKAP function as scaffolds for specific kinases and their substrates (Lester and Scott, 1997). Recently, a multi-PDZ containing protein was identified in *Drosophila*, termed InaD, that couples the membrane light-activated ion channel with its effector enzymes (Tsunoda et al., 1997). The coupling activity of these proteins is essential to normal function of the signaling cascade (Lester and Scott, 1997; Tsunoda et al., 1997).

The present study suggests that Homer functions to couple membrane group 1 mGluRs with intracellular IP3R. Since the IP3R is associated with endoplasmic reticulum (ER), this model would predict that ER involved in group 1 mGluR Ca^{2+} signaling should be in close physical proximity to the plasma membrane. Moreover, the ER–plasma membrane apposition should occur precisely at sites with high levels of membrane group 1 mGluRs. In both the hippocampus and cerebellum, group 1 mGluRs are enriched at discrete sites surrounding the postsynaptic density (Baude et al., 1993; Nusser et al., 1994; Lujan et al., 1997). Spacek and Harris have recently published three-dimensional reconstructions of the ER pool in spines of hippocampal neurons (1997). Smooth ER is visualized to extend from the spine apparatus to the outer edge of the PSD. A similar distribution of SER in spines is reported in cerebellar Purkinje cells (Harris and Stevens, 1988). Moreover, the plasma membrane and the membrane of the SER are in such close apposition at these sites that the membranes have been hypothesized to fuse (Jones and Harris, 1995). Such SER/membrane appositions do not occur at other regions



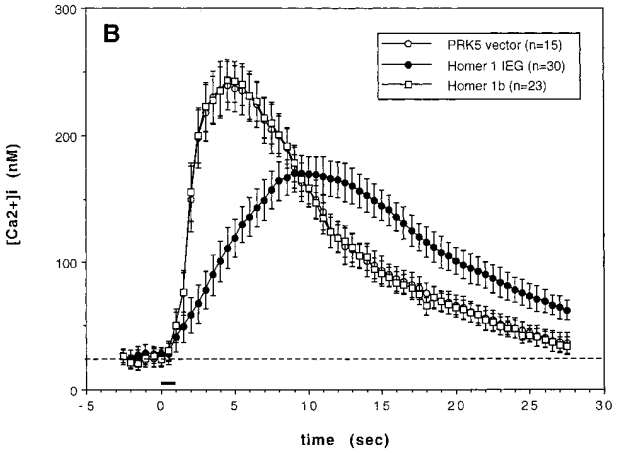


Figure 7. mGluR-Evoked Ca^{2+} Mobilization Is Attenuated in Purkinje Cells Transfected with Homer 1 IEG, but Not Homer 1b

Primary cultures of rat cerebellum were transfected with separate plasmids for GFP, in combination with Homer 1 IEG, Homer 1b, or the empty vector.

(A) A GFP-positive Purkinje cell is shown together with the orientation of the iontophoresis pipette and the analysis box containing the pixels used to derive the Ca^{2+} measurements. After locating transfected Purkinje cells using a GFP filter set, the set was changed to another for fura-2 ratio imaging. Scale bar, 25 μm.

(B) Population measures of mGluR-evoked Ca^{2+} mobilization. Following a brief baseline, quisqualate, an mGluR agonist, was ejected using a micropressure pulse (6 lb/in^2, 1 s, indicated by horizontal bar at t = 0 s). The peak amplitude of the mGluR-evoked Ca^{2+} transient is reduced, and the latency to peak amplitude is prolonged in Purkinje cells transfected with Homer 1 IEG, as compared with either full-length Homer 1b or an empty vector control.

in the spine, and the distribution of these appositions closely matches that of group 1 mGluRs in these neurons.

ImmunoEM studies of the IP3R and Homer are also informative. The IP3R localizes to the SER of Purkinje neurons and is present in dendrites and extends into the cytoplasm of the spine as morphologically identified cysternae (Satoh et al., 1990; Takei et al., 1992). In the accompanying paper, we demonstrate that Homer 1b/c and 3 localize to the PSD and are enriched in the region of the lateral PSD (Xiao et al., 1998). Thus, available biochemical and ultrastructural information supports the notion that Homer may form a physical tether linking membrane mGluRs and ER-associated IP3R.

Homer proteins are not exclusively restricted to the lateral region of the PSD (Xiao et al., 1998). Both Homer 1 and 3 are also present in the central region of the PSD. The group 1 mGluRs are present at the PSD and cytoplasm of the spine (Petralia et al., 1997), albeit at a lower concentration than at the lateral margin, and Homer at these central PSD sites may be coupled to these receptors. Alternatively, Homer may bind other proteins at this site, perhaps cross-linking IP3Rs.

It can be anticipated that additional functions for the Homer family will be identified. The Homer ligand motif in dynamin III suggests that Homer may play a role in ER vesicular mechanics. The putative Homer ligand present in the alpha 1D adrenergic and the ryanodine receptors suggests a broader potential role for Homer in calcium signaling.

The Homer motif possesses similarities to the SH3 ligand motif, including similarity of linear sequence, dependence on specific prolines, and binding activity of relatively short synthetic peptides. NMR and crystal structure determinations of the SH3 ligand interaction with the SH3 binding domain indicate that the ligand forms a class II proline helix (Feng et al., 1994; Yu et al., 1994). It is interesting to note that despite the sequence similarity between the SH3 and Homer ligands, the amino acids that are most critical for binding to their respective receptors are distinct. In particular, the phenylalanine at 1128 of mGluR5 is at a relatively silent position for the SH3 ligand, but it is critical for binding Homer. In contrast, the proline at equivalent position 1127 would be critical for SH3 motif binding, but it is relatively silent in the Homer motif. Another lesson from

the SH3 experience, which may be relevant to Homer, is that SH3 binds ligands with both parallel and antiparallel orientations (Feng et al., 1994). If this is true for Homer, we may anticipate a second motif, perhaps with the critical phenylalanine preceding the relevant prolines. Finally, it is possible that the first proline of the Homer ligand is important for establishing the secondary structure, rather than being a site of contact. If so, this would expand the possible targets for Homer. We note that the recently described human InaDL (Philipp and Flockerzi, 1997) and the TRP receptors (Xu et al., 1997) possess Homer ligand–like motifs. As with studies of SH3 interactions, the Homer ligand peptide should provide a valuable tool to assess the functions of Homer.

The N-terminal region of Homer, which is required for binding to group 1 mGluRs, was originally thought to possess homology to PDZ proteins (Brakeman et al., 1997). Others, however, pointed out the closer sequence homology of Homer with members of a recently described EVH family (Enabled/VASP homology) (Gertler et al., 1996; Kato et al., 1997; Ponting and Phillips, 1997). Proteins that encode the EVH1 domain include *Drosophila enabled* (termed Mena in mouse) (Gertler et al., 1996), yeast Bee1p (Li, 1997), vasodilator-stimulated phosphoprotein (VASP) (Haffner et al., 1995), and the Wiscott-Aldrich syndrome protein (WASP) (Symons et al., 1996). The putative EVH domain in Homer is highly conserved in the Homer-related family member (Xiao et al., 1998). There is precedent that EVH proteins may interact with proline-rich domains. The EVH proteins VASP and Mena bind a polyproline sequence, E/DFPPPPXD/E (Niebuhr et al., 1997). These proteins are believed to act as modulators of actin polymerization, and the identified ligand is present in the cytoskeletal proteins zyxin and vinculin. Interestingly, the ligand was first identified in the ActA protein of *Listeria monocytogenes* (Niebuhr et al., 1997) whose binding by cellular proteins is implicated in the intracellular motility and virulence of the bacteria. This ActA ligand is distinct from the Homer ligand, but its proline-rich character supports the notion that EVH-related proteins bind unique SH3 ligand–like sequences.

Of the neural genes that we have studied, the IEG form of Homer is one of the most responsive. Homer mRNA is induced within minutes of specific stimuli that are linked to long-term plasticity, and its induction occurs in relevant neural circuits (Brakeman et al., 1997). Thus, our studies provide mechanistic insight into how the IEG response can modify glutamate signaling at synapses. At present, it is not known whether the IEG form of Homer accumulates specifically at activated synapses or whether it is distributed cell wide. Once present at synapses, it could potentially alter not only basal synaptic function, but also the probability of subsequent induction of LTP or LTD (metaplasticity).

Another IEG, termed RGS2 (regulator of G-protein signaling), also appears to target metabotropic signaling (Ingi et al., 1998). The RGS family of proteins acts to accelerate the rate of GTPase activity of specific G proteins (Arshavsky and Pugh, 1998; Berman and Gilman, 1998). Of this family, RGS2 is selectively induced by synaptic activity in brain (Ingi et al., 1998). While many of the RGS proteins activate Gi, RGS2 and RGS4 selectively accelerate the GTPase activity of Gq. RGS4 has recently been shown to block metabotropic glutamate signaling (Saugstad et al., 1998). Thus, metabotropic signaling appears to be negatively regulated by two independent IEG-mediated mechanisms. An appreciation of the contribution of these mechanisms to synaptic physiology should provide important insights into the regulation of intracellular calcium responses and long-term neuronal plasticity.

Experimental Procedures

In Vitro Binding

GST fusion constructs were prepared by polymerase chain reaction with specific primers that included SalI and NotI sequences and subcloned into pGEX4T-2 vector (Pharmacia Biotech, Uppsala, Sweden). Constructs were confirmed by sequencing. GST-fusion proteins were expressed in BL21 bacterial strains. Bacteria were harvested and lysed in PBS, 1% Triton X100, 2 mM phenylmethylsulfonyl fluoride (PMSF) and pelleted at 13,000 rpm (Sorvall SS-34) at 4°C for 5 min. Proteins were purified by incubating 1 ml bed volume glutathione-sepharose (GST) beads (Sigma USA) with bacterial supernatant at 4°C for 10 min and washing twice with PBS and PBS plus 1% Triton X-100. Protein was eluted with 10 mM glutathione and dialyzed against PBS at 4°C. Protein concentrations were measured by BCA (Pierce, Illinois).

Mutant Construction, Expression, and In Vitro Binding

The C-terminal 723 nucleotide (241 aa) of rat mGluR5 was amplified by polymerase chain reaction and cloned into the mammalian expression vector pRK5 (Genentech). Deletion mutants of mGluR5 (del 10, 31, 37, 40, 41, and 50) were also made by PCR. Point mutants were made using the QuikChange TM Site-Directed Mutagenesis Kit (Stragene). The sequences of the primers used to generate each mutant will be supplied on request. Full-length mGluR5 mutants were made by subcloning Eco47III/NotI fragments of the C-terminal mutants into the wild type full length.

Proteins were expressed in HEK293 cells. Cells were transfected with 10 μg per 10 cm dish. After 8 hr, the medium was changed, and cells were washed twice with sterilized PBS + 1 mM EDTA. Ten milliliters of fresh DMEM (10 % FBS + 1% P-S) was added and the cells were grown ~48 hr before harvesting. Cells were lysed by adding 1 ml of PBS with 1% Triton X-100 plus PMSF and protease inhibitor cocktail.

In vitro binding assays were performed by adding HEK293 cell lysates or cerebellum lysate to GST fusion protein on agarose beads and incubated at 4°C for 2 hr. Beads were washed twice with PBS and once with PBS + 1% Triton X-100, and bound proteins were eluted in 4% SDS loading buffer. Proteins were assayed by SDS-PAGE and immunoblot. Identical assays were used to assess the effect of Homer ligand peptides (final concentration at 0–340 μM) on binding.

Immunoprecipitation Assays

Rats were sacrificed by decapitation, and the cerebella were dissected immediately. Cerebella were sonicated in TE buffer (50 mM Tris, 1 mM EDTA [pH 7.4]) containing 1% CHAPS and protease inhibitor cocktail (~100 mg wet weight/ml). The homogenate was centrifuged at 90,000 rpm, 20 min, 4°C in a TLA 100.3 rotor. One hundred microliters of the cerebellar extract was used for each immunoprecipitation assay with the following antibodies: 3 μl of crude Homer 1, Homer 2, or Homer 3 antibodies (Xiao et al., 1998); 20 μg of affinity purified IP3R antibody (gift from Alan Sharp). Antibodies and extract were incubated for 30 min at 4°C, then 60 μl of 1:1 protein A or protein G (for goat Ab) sepharose slurry was added. The antibody/extract/beads were incubated for an additional 90 min at 4°C. After washing three times for 10 min each in TE-CHAPS buffer, the proteins were eluted from the beads with 30 μl of 4% SDS loading buffer and analyzed by SDS-PAGE and immunoblot.

Calcium Imaging

Embryonic mouse cerebellar cultures were prepared and maintained according to the method of Schilling et al. (1991). At 4–5 DIV, cultures

were transfected with plasmids coding for E-GFP (Clontech) and either full-length Homer 1b or an IEG form of Homer 1. The IEG form of Homer 1 was a 186 aa N-terminal fragment of Homer 1b. Plasmids were purified by cesium banding. Three combinations of the plasmids were transfected. Group I (control), 20 μg of E-GFP and 40 μg of pRK5 vector; group II, 20 μg of E-GFP and 40 μg of pRK5 Homer 1 IEG; group III, 20 μg of E-GFP and 40 μg of pRK5 Homer 1b. Plasmid DNA was mixed with gold particles (0.6 micron) and coated onto plastic tubing. DNA was then ballistically transfected into cells according to the manufacturer's protocol (Helios Gene Gun System, BIO-RAD). After transfection, cultures were returned to the incubator and maintained for an additional 2 days for a total of 7–8 DIV at the time of use for imaging experiments.

Patch electrodes were attached to the somata of GFP-expressing Purkinje cells, and a holding potential of −60 mV was applied. Micropressure electrodes (1 μm tip diameter) were filled with quisqualate (100 μM in external saline) and were positioned ~20 μm away from large-caliber dendrites. Test pulses were delivered using positive pressure (6 lb/in^2, 1 s). Cells were bathed in a solution that contained (in mM) NaCl (140), KCl (5), EGTA (0.2), $MgCl_2$ (0.8), HEPES (10), glucose (10), tetrodotoxin (0.005), and picrotoxin (0.1), adjusted to pH 7.35 with NaOH, which flowed at a rate of 0.5 ml/min. The recording electrode contained CsCl (135), HEPES (10), fura-2 K_5 salt (0.2), and Na_2-ATP (4), adjusted to pH 7.35 with CsOH. Patch electrodes yielded a resistance of 3–5 MΩ when measured with the internal and external salines described above.

Fura-2 ratio imaging of intracellular free Ca^{2+} was accomplished by measuring the background corrected fluorescence ratio at 340 and 380 nm excitation using a cooled CCD camera system, as previously described (Linden et al., 1995). Exposure times were 200 ms per single wavelength image. Experiments were conducted at room temperature. Enhanced GFP is weakly excited by illumination in the 380–400 nm spectrum. Based upon the band-pass characteristics of our 340HT15 and 380HT10 excitation filters and the absorption spectrum of enhanced GFP (Clontech), we estimate that <1% of the signal at 340 nm excitation and <5% of the signal at 380 nm excitation are contributed by GFP, even in those cells where the fura/GFP loading ratio is smallest. This could lead to a small (<5%) systematic underestimation of Ca^{2+} concentration that should distribute randomly across experimental groups.

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