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Co-expression of metabotropic glutamate receptor type 1α with Homer-1a/Vesl-1S increases the cell surface expression of the receptor

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Homer-1a is a 30 kDa protein that forms part of a family of conserved Homer-related proteins that interact with the C-termini of the metabotropic glutamate receptors mGluR1α and mGluR5a. Analysis of HEK-293 cells by PCR showed that they contained mRNA coding for members of the Homer family with the predominant form being Homer-1b, which is consistent with the immunochemical analysis of these cells. Homer-1a could not be detected by immunochemical analysis. To examine the function of Homer-1a, HEK-293 cells were transfected with cDNA encoding mGluR1α or Homer-1a or co-transfected with both cDNAs. When cells were co-transfected with the cDNAs for both proteins, immunofluorescent staining and biotinylation of cell surface molecules revealed a significant increase in the amount of receptor present at the cell surface in contrast to cells transfected with mGluR1α cDNA alone. This finding was consistent with a concomitant increase in the production of inositol phosphates after treatment of the doubly transfected cells with agonist. Intracellular immunostaining for both proteins revealed that they were co-localized and underwent a redistribution into a large vesicular compartment when they were co-expressed.

Key words: cell surface expression, Homer/Vesl proteins, metabotropic, mGluR1α, receptor targeting.

INTRODUCTION

Metabotropic glutamate receptors (mGluRs) constitute a family of large G-protein-coupled receptors which show little sequence similarity with the superfamily of smaller G-protein-linked receptors [1]. Eight members of the mGluR family have been identified and categorized into three subgroups on the basis of their sequence similarity, agonist selectivity and signal transduction pathway. The receptors mGluR1 and mGluR5 and their splice variants make up the group 1 mGluRs [2,3]. These share the strongest sequence similarity (62 % identical at the amino acid level) and are coupled to phospholipase C in cells transfected with DNA encoding the receptor. Quisqualic acid is their most potent agonist.

Immunocytochemical studies have shown that the different groups of the mGluRs show differential targeting in neurons, with group 1 mGluRs being predominantly post-synaptic, whereas group 2 (mGluR2 and mGluR3) and group 3 (mGluR4, mGluR6, mGluR7 and mGluR8) receptors are generally pre-synaptic [4–7]. The precise synaptic location of mGluR1α appears to be highly ordered, with the protein present in an annulus that surrounds the post-synaptic density [4,8,9]. One possible explanation for this specific localization of mGluR1α is that the protein is directed by interactions of its cytoplasmic C-terminus with specific targeting proteins. Precisely this type of targeting mechanism appears to operate for the synaptic localization of the ionotropic glutamate receptors and a number of different proteins, containing PDZ protein-binding domains, which interact with specific C-terminal sequences of these receptors [10–14]. A novel PDZ-domain-containing protein, which binds specifically to the C-terminal residues of mGluR1α and mGluR5a, has recently been described [15,16]. This protein termed Homer was isolated as the product of a synaptic plasticity-regulated gene from rat hippocampus [15,16] but as yet its function remains unclear. Very recently, new proteins related to Homer have been described, namely Homer-1b, Homer-1c/Vesl-1L, Homer-2a/Vesl-2Δ11, Homer-2b/Vesl-2 and Homer-3 [17,18]. Homer-1b is a C-terminal splice variant of the original Homer protein (re-named Homer-1a/Vesl-1S and referred to as Homer-1a), with the two proteins sharing the same N-terminal 175 amino acids. Unlike Homer-1b/c, Homer-2a/b and Homer-3, which are constitutively expressed, Homer-1a/Vesl-1S is only expressed during brain development or in response to cortical activity [15], which might indicate a special role for it in mGluR regulation and targeting.

To examine the functional role of Homer-1a we have examined the consequences of its co-expression with mGluR1α in transiently transfected HEK-293 cells. The results indicate a role for Homer-1a in both the stabilization and the cell-surface targeting of the receptor, which in turn stabilizes the expressed levels of Homer-1a.

EXPERIMENTAL

Materials

myo-[^{3}H]Inositol (85 Ci/mmol) was purchased from Amersham. EZ-Link Sulfo-NHS-LC-Biotin and the Super Signal™ chemi-

Abbreviations and nomenclature used: DMEM, Dulbecco's modified Eagle's medium; EVH, ENA/VASP homology; GST, glutathione S-transferase; HRP, horseradish peroxidase; IP, inositol phosphate; mGluR, metabotropic glutamate receptor. Homer is used as a generic name for the related proteins Homer-1, Homer-2 and Homer-3, which are products of different genes. The splice variants of these genes are denoted by using a suffix, e.g. Homer-1a or Homer-1b. In cases where the splice variants are not distinguished, the suffix is modified, e.g. Homer-1b/c or Homer-2a/b. In cases where all of splice variants are included, the suffix is omitted, e.g. Homer-1 or Homer-2. Vesl is a widely used name for the same family of proteins. We have indicated in the text instances in which both nomenclature systems have been applied to the same splice variant of the same protein, e.g. Homer-1a/Vesl-1S or Homer-2b/Vesl-2.

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luminescence immunoblotting detection system were from Pierce (Rockford, IL, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), BSA, monoclonal anti-β-tubulin clone TUB 2.1, streptavidin–agarose and protein A–Sepharose were obtained from Sigma. Inositol- and glutamate-free DMEM were from ICN. Anti-FLAG™ M2 monoclonal antibody was purchased from Eastman Kodak Company (Rochester, NY, U.S.A.). Fluorescein isothiocyanate-conjugated AffiniPure donkey anti-mouse IgG and Texas Red™ dye-conjugated AffiniPure donkey anti-rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.). Horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG and HRP-conjugated swine anti-mouse IgG were from Dako (High Wycombe, Bucks., U.K.). All other products were of the best grade available.

Mammalian cell culture and transfection

Human embryonic kidney cells (HEK-293) were grown in DMEM supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin/streptomycin at 100 units/ml and 10 % (v/v) foetal calf serum at 37 °C in an atmosphere of air/CO_2 (19:1). Cells were passaged when they were 80–90 % confluent. For the transient expression of proteins the following procedures were followed. HEK-293 cells growing in 75 cm^2 dishes were transiently transfected (by calcium phosphate precipitation) with 10 μg of DNA encoding the indicated proteins [19]. To keep the amount of DNA used in the transfections constant, the appropriate amounts of pcDNA3 vector (Invitrogen, San Diego, CA, U.S.A.), containing the *LacZ* reporter, were added to the transfections. The cells were harvested at either 24 or 48 h after transfection.

Cloning of the rat Homer-1a protein and generation of fusion proteins

Rat brain poly(A)$^+$ RNA was isolated from 0.1 g of cortex using a Pharmacia Quick Prep kit following the manufacturer's protocol. cDNA was obtained using a random hexamer primer and SuperScript II M-MLV reverse transcriptase (Gibco BRL) according to the manufacturer's protocol. The first-strand reaction mixture was used for PCR amplification without further purification. Rat Homer-1a cDNA was amplified using proofreading *Pfu* DNA polymerase and primers HF (5′-TTGGAATTCATGGGGGAACAACCTATCTTC-3′) and HR (5′-ATCGAATTCATTTAATCATGATTGCTGAATTG-3′). Amplification conditions were as follows: 1 cycle of 1 min at 96 °C, 1 min at 60 °C, 10 min at 72 °C; 35 cycles of 1 min at 96 °C, 1 min at 65 °C and 3 min at 72 °C; 10 min at 72 °C. The amplified cDNA was cloned into the *Eco*RI site of the pcDNA3 vector. The sequences of the cDNAs and their orientation in the vector were confirmed by DNA sequencing.

To produce the glutathione S-transferase (GST)–Homer-1a fusion protein the cDNA coding for the full-length Homer-1a was subcloned into the *Eco*RI site of the *Escherichia coli* expression vector pGEX-4T-1 (Pharmacia Biotech). The recombinant fusion protein GST–Homer-1a was purified on glutathione–agarose (Sigma).

Homer-1a was isolated from the GST fusion protein by thrombin treatment of the fusion protein as described by the manufacturer (Pharmacia Biotech).

Antibody production

Two antisera were used in this study. The first one designated VHr4 was raised against the synthetic peptide IDPNTKKNWPASK, based on residues 16–28 in the N-terminal sequence present in Homer [15]. This polyclonal anti-serum was affinity purified as previously described [20]. All the initial experiments were performed with this antibody and confirmed using the second antibody described below.

The second antibody, VHr20, was raised against the recombinant GST–Homer-1a fusion protein containing the full sequence of Homer-1a. The immunization of rabbits and affinity purification of the antisera were performed as described previously [21,22].

The generation and characterization of antibodies against mGluR1 (F1-Ab) and mGluR1α (F2-Ab) used in this study have been described before [21,23].

Preparation of P2 membranes and cytosolic fraction from brain and HEK-293 cells

P2 membranes and cytosolic fraction were prepared as described earlier [24]. Briefly, five adult rats were decapitated, the whole brain dissected, cleaned of white matter and homogenized in 0.32 M sucrose (10 volumes). The homogenate was centrifuged twice at 1500 ***g*** for 10 min each. The combined supernatants (S1) were then centrifuged at 9500 ***g*** for 20 min. The resulting supernatant (S2) and pellet (P2 membranes) were carefully separated. S2 was then centrifuged at 80000 ***g*** for 90 min to pellet insoluble materials. The resulting supernatant was considered as a cytosolic fraction. The HEK-293 cell fractions S1, P2 membranes and cytosolic fraction were produced from 10^8 cells using this same protocol.

Protein was determined using the assay based on bicinchoninic acid [25] obtained from Pierce.

Gel electrophoresis, immunoblotting and immunoprecipitation

SDS/PAGE was performed using 6 % or 10 % (w/v) polyacrylamide gels [26] and immunoblotting was performed using specific affinity-purified VHr4 or VHr20 antibody (2–4 μg/ml) or affinity-purified F2-Ab antibody (2–4 μg/ml) as previously described [23].

For immunoprecipitation, supernatant S1 from whole rat brain was solubilized in ice-cold lysis buffer [PBS, pH 7.4, containing 1 % (v/v) Nonidet P-40, 0.5 % (w/v) sodium deoxycholic acid and 0.1 % (w/v) SDS] for 1 h on ice. The solubilized preparation was then centrifuged at 80000 ***g*** for 90 min. The supernatant (1 mg of protein/ml) was processed for immunoprecipitation using affinity-purified anti-mGluR1 antibodies (F1-Ab, 2 μg/ml), anti-Homer affinity-purified VHr4 antibody (5μg/ml), anti-Homer affinity-purified VHr20 antibody (5μg/ml) or a control rabbit IgG (5μg/ml) as described previously [23]. Immune complexes were dissociated by heating to 100 °C for 5 min and resolved by SDS/PAGE in 7 % (w/v) gels. The gels were run and immunoblotted as described above.

Expression of the Homer mRNAs in HEK-293 cells

Poly(A)$^+$ RNA was isolated from 10^6 HEK-293 cells using a Pharmacia Quick Prep kit and the protocol provided. cDNA was obtained using a random hexamer primer and SuperScript II M-MLV Reverse Transcriptase (Gibco BRL), following the manufacturer's protocol. A pair of partially degenerate primers for the PCR amplification of the Homer protein cDNAs was designed to anneal to the conservative 5′ fragments of the coding sequences of all human Homers (corresponding to $\sim$ 100 amino acids of the N-termini of Homer-1, -2 and -3) and thus to quantitatively amplify all corresponding cDNA frag-

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ments in one reaction. The primer sequences were PanHF [5′-G(AG)GA(AG)CA(AG)CC(AGCT)AT(ACT)TT-3′] and PanHR [5′-TC(CT)TG(AG)AA(CT)TT(CT)TC(AGCT)GC-(AG)AA-3′], and were based on the Homer/Vesl sequences available from the EMBL Nucleotide Sequence Database. An excess of each of the forward and the reverse primers (200 pmol each), 5 units of *Taq* polymerase and 1 μl of the first-strand reaction mixture (taken without purification) were used for each amplification. Amplification conditions were as follows: 1 cycle of 5 min at 96 °C, 30 s at 45 °C, 10 min at 72 °C; 30 cycles of 1 min at 96 °C, 20 s at 45 °C, 1 min at 72 °C; 10 min at 72 °C. The amplified cDNA fragments were analysed by electrophoresis in 2 % (w/v) agarose gels containing 0.5 μg/ml ethidium bromide. PCR products were of the expected length (310 bp). Molecular size markers were purchased from Gibco BRL.

To identify and quantify the amplified cDNAs corresponding to each of the Homer-1, -2 and -3 proteins (including all splicing forms for each), the 310 bp PCR product (obtained as above) was digested with the *Rsa*I restriction endonuclease (6 h at 37 °C) and analysed on 4 % (w/v) agarose gels. All of the observed cDNA fragments were of the predicted lengths: digestion of Homer-1 cDNA (310 bp) yielded 70 bp, 103 bp and 137 bp fragments; the Homer-2 fragment (310 bp) remained undigested; the Homer-3 cDNA (310 bp) yielded 129 bp and 181 bp fragments. Subsequently, these fragments were subcloned and sequenced. The relative abundance of each of the Homers in HEK-293 cells was determined by measuring the fluorescence of the corresponding cDNA fragments using the GelDoc 1000 gel imaging system and MultiAnalist software from Bio-Rad. Since ethidium bromide fluorescence is proportional to the amount of DNA, the amount of fluorescence measured was normalized in relation to the length of the cDNA fragments to calculate the molar ratios. The results obtained in this manner do not discriminate between alternative splicing forms of the Homer-1 and Homer-2 proteins, which, except for Homer-1a, differ marginally by deletion or insertion of a 12 amino acid fragment in Homer-1b and -1c or an 11 amino acid fragment in Homer-2a and -2b. Therefore the expression of the Homer-1a form compared with the Homer-1b and -1c forms was studied using the antisera raised against the Homer-1 proteins described above.

FLAG-tagging of the mGluR1α

The FLAG epitope (DYLKDDDDK) was introduced into the N-terminus of the mGluR1α between amino acids 57 and 58 using a PCR-based mutagenesis approach. Two fragments of the cDNA coding for mGluR1α [27] were amplified with primers containing the FLAG-epitope sequence. The first amplification was with primers GRF1 (5′-TCTTCGGTACCATGGTCCGG-CTCCTCTTGA-3′) and GRR1 (5′-TATCGTCATCGTCTTT-GTAATCAGGCTGGTGATGGAC-3′), and the second was with primers GRF2 (5′-ACAAAGACGATGACGATAAACC-AGCCGAGAAGGTA-3′) and GRR2 (5′-AGCCAGGGCCG-ATCACTCCAGCAATA-3′). Amplification conditions were as follows: 1 cycle of 1 min at 95 °C, 1 min at 50 °C, 3 min at 72 °C; 20 cycles of 1 min at 95 °C, 1 min at 60 °C and 3 min at 72 °C; 10 min at 72 °C. The amplified overlapping products were used as templates in a third amplification with primers GRF1 and GRR2.

A *Kpn*I–*Kpn*I fragment of the recovered cDNA, containing the sequence encoding the FLAG epitope, was used to substitute for a corresponding fragment of the rat mGluR1α cDNA in the pcDNA3 vector. The sequence of the resultant FLAG-encoded mGluR1α cDNA was confirmed by DNA sequencing.

Biotinylation of cell surface proteins

HEK-293 cells transiently transfected with mGluR1α and/or Homer-1a were washed three times in borate buffer (10 mM H_3BO_3/150 mM NaCl, pH 8.8) and then incubated with Sulfo-NHS-LC-Biotin (50 μg/ml borate buffer) for 5 min at room temperature. After incubation, cells were washed three times in borate buffer and again incubated with Sulfo-NHS-LC-Biotin (50 μg/ml borate buffer) for 10 min at room temperature. At the end of this incubation 100 mM NH_4Cl was added for 5 min to quench the remaining biotin. Cells were washed in PBS and solubilized in ice-cold lysis buffer as described above for immunoprecipitation. The solubilized preparation was then centrifuged at 80000 ***g*** for 90 min. The supernatant was incubated with 80 μl streptavidin–agarose beads for 3 h with constant rotation at 0–4 °C. The beads were washed as described before. Immunocomplexes were dissociated by adding 60 μl of SDS/PAGE sample buffer and heated to 100 °C for 5 min, and then resolved by SDS/PAGE on 6 % gels. The gels were run and immunoblotted as described above.

Inositol phosphate (IP) accumulation

HEK-293 cells transiently transfected with mGluR1α and/or Homer-1a were grown overnight in inositol- and glutamate-free medium [DMEM supplemented with 7.5 % (v/v) foetal calf serum and dialysed against inositol- and glutamate-free DMEM] containing *myo*-[^{3}H]inositol (5 μCi/ml). Cells were collected and washed exhaustively in prewarmed (37 °C) Hepes/Krebs solution (20 mM Hepes, pH 7.4, containing 145 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4 and 10 mM glucose), incubated for 1 h in Hepes/Krebs containing 2 mM pyruvate and glutamic–pyruvic transaminase at 2 units/ml. Cells were washed and incubated (10^6 cells/0.2 ml) in Hepes/Krebs containing 10 mM LiCl for 10 min. The IP production was initiated by the addition of 0.1ml of pre-warmed Hepes/Krebs containing various concentrations of quisqualic acid. After 20 min, the assay was ended by perchloric acid protein precipitation [5 % (v/v) for 30 min on ice]. The samples were neutralized with a solution containing 4 M KOH, 1 M Tris and 60 mM EDTA. The mixtures were centrifuged for 20 min at 13000 ***g***, and the aqueous phase was loaded into a Poly-Prep chromatography column (Bio-Rad) containing 1 g of an anion exchange resin (DOWEX 200-400 mesh, formate form). The total IP pool was eluted according to the method of Berridge et al. [28] and the radioactivity was measured. Results are expressed as specific IP production and correspond to the amount of IP produced in the quisqualic-acid-stimulated cells over the mock-stimulated cells. The EC_{50} of agonist-induced IP accumulation was determined by non-linear regression as described previously [29].

Immunofluorescence

For immunofluorescence staining, HEK-293 cells growing on glass coverslips were transiently transfected as described before. Cells were rinsed in PBS, fixed in 4 % (w/v) paraformaldehyde in PBS for 15 min, and washed in PBS containing 20 mM glycine (buffer A) to quench the aldehyde groups. Where indicated, cells were permeabilized with buffer A containing 0.2 % (v/v) Triton X-100 for 5 min. After a 30 min incubation in buffer A containing 1 % BSA (buffer B), cells were incubated with a mixture of anti-FLAG monoclonal antibody (M2-Ab, 10 μg/ml) and anti-Homer polyclonal antibody (VHr20, 2–4 μg/ml) in buffer B for 1 h at room temperature. They were then washed and stained with fluorescein isothiocyanate-conjugated donkey

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anti-rabbit IgG antibody (1:50) and Texas Red-conjugated donkey anti-mouse IgG antibody (1:50). The coverslips were rinsed for 30 min in buffer B and mounted with Vectashield immunofluorescence medium (Vector Laboratories, Burlingame, CA, U.S.A.). Confocal microscopic observations were made with a Leica TCS 4D (Leica Lasertechnik GmbH, Heidelberg, Germany) confocal scanning laser microscope adapted to an inverted Leitz DMIRBE microscope.

RESULTS

Characterization of anti-Homer antibodies

Two antibodies raised against Homer have been used in this study, VHr4 and VHr20. On immunoblots both antibodies reacted with the recombinant GST–Homer fusion protein expressed and purified from *E. coli* (Figure 1A, lane 1). To demonstrate the specificity of the antibodies, we cleaved Homer from the fusion protein by thrombin treatment of GST–Homer and immunoblotted the product using affinity-purified VHr4 (Figure 1A, lane 2). A major immunoreactive band with an apparent molecular mass of 30 kDa was detected by this antibody. The fusion-protein antibody VHr20 yielded identical results (not shown).

In the soluble fraction of rat brain the antibody combined with two immunoreactive proteins, a major protein with an apparent molecular mass of 48 kDa similar to that detected in P2 membranes (p48), and a minor immunoreactive protein with an apparent molecular mass of 63 kDa, designated p63 (Figure 1B). In a similar analysis of Homer species in rat brain Xiao et al. [18] also identified a 63 kDa protein that reacted with their anti-Homer-2 antiserum. Immunoblotting of membrane and cytosolic preparations from HEK-293 cells revealed that these cells also contained two proteins, both with cell-fraction profiles and molecular masses similar to those detected in rat brain by VHr4 or VHr20 (Figure 1B). No immunostaining was observed with either VHr4 or VHr20 when the antibody was preincubated with 1 μM of the peptide antigen or 100 μg of the GST–Homer-1a fusion protein respectively (results not shown). Since Homers interact strongly with mGluRs our antisera should immunoprecipitate the receptor from solubilized rat brain and the results from such experiments are shown in Figure 1(C). The specific antibody against mGluR1 (F1-Ab) immunoprecipitated an immunoreactive protein of 150 kDa corresponding to mGluR1α (Figure 1C, lane 2) and an immunoreactive band with identical gel migration was also seen in the immunoprecipitate formed using affinity-purified VHr4 (Figure 1C, lane 3) or VHr20 (Figure 1C, lane 4) antibodies. In contrast the immunoprecipitates performed with a control rabbit IgG (Figure 1C, lane 5) failed to yield mGluR1α immunoreactive material. Because of the reaction of the IgG heavy chains with the secondary antibody, it was not possible to probe the mGluR1α immunoprecipitates for p48 or p63. The molecular masses of Homer-1b/c, Homer-2a/b and Homer 3 range from 43–45 kDa based on their predicted amino acid composition [17,18]. On SDS/PAGE these proteins have been reported to migrate with molecular masses of approx. 47 kDa [17,18], similar to that reported in the present study. Together these results suggest that the antisera are reacting with Homer proteins, and that HEK-293 cells contain some members of the Homer gene family.

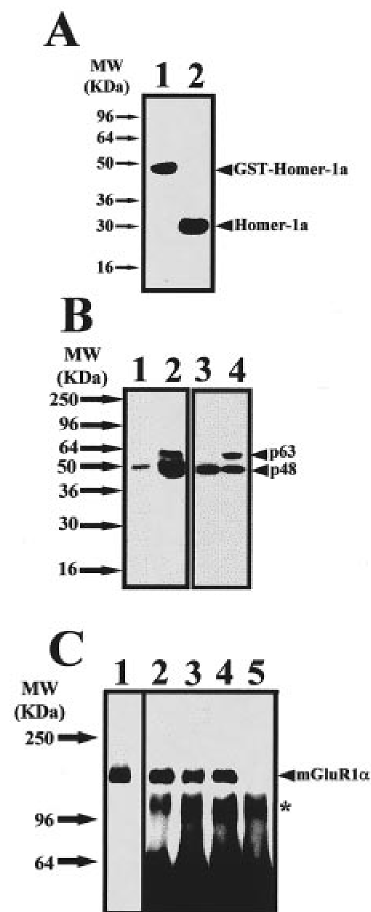

Figure 1 Characterization of anti-Homer antiserum

(**A**) Purified GST–Homer-1a fusion protein (5 μg, lane 1) and the same amount of fusion protein digested with thrombin (see the Experimental section) (lane 2) were analysed on SDS/PAGE (10% gel) and immunoblotted using anti-Homer affinity-purified VHr4 antibody (2–4 μg/ml). Immunoreactive bands were detected with swine anti-rabbit (1:5000) secondary antibody conjugated to HRP followed by chemiluminescence detection. Similar results were obtained in three different experiments or using anti-Homer affinity-purified VHr20 antibody. In this and all subsequent Figures the positions of the molecular mass markers (kDa) are indicated by arrows on the left. The positions of GST–Homer-1a and Homer-1a are indicated by arrowheads on the right. (**B**) P2 membranes (lanes 1 and 3) and cytosol fraction (lanes 2 and 4) from whole rat brain (lanes 1 and 2) or HEK-293 cells (lanes 3 and 4) were separated by SDS/PAGE and immunoblotted with anti-Homer affinity-purified VHr4 antibody (2–4 μg/ml). Immunoreactive bands were detected as described above. The arrowheads on the right indicate the positions of p63 and p48. (**C**) Co-immunoprecipitation of mGluR1α with anti-Homer antibodies. Supernatant S1 from total rat brain was solubilized in ice-cold lysis buffer as described in the Experimental section and immunoprecipitated using specific N-terminal anti-mGluR1 affinity-purified F1 antibody (lane 2), anti-Homer affinity-purified VHr4 antibody (lane 3), anti-Homer affinity-purified VHr20 antibody (lane 4) and an irrelevant rabbit IgG (lane 5). Immunocomplexes were isolated as described in the Experimental section and immunoblotted using anti-mGluR1α affinity-purified F2 antibody (2–4 μg/ml). The arrowhead on the right indicates the position of mGluR1α and the IgG used in the immunoprecipitation gives the chemiluminescence signal indicated by the asterisk (*).

Identification and quantification of Homer mRNAs in the HEK-293 cells

To further characterize these endogenously expressed Homers we employed reverse transcriptase PCR amplification to confirm the expression of Homer protein mRNAs in HEK-293 cells. First, a pool of 310 bp cDNA fragments containing all human Homer sequences expressed in HEK-293 cells was amplified using degenerate pan-Homer primers (see the Experimental section). Secondly, to identify and characterize the expression of each of the three known Homer protein mRNAs, the amplified 310 bp cDNA pool was subjected to restriction digestion using enzymes that gave a diagnostic pattern of cleavage fragments for

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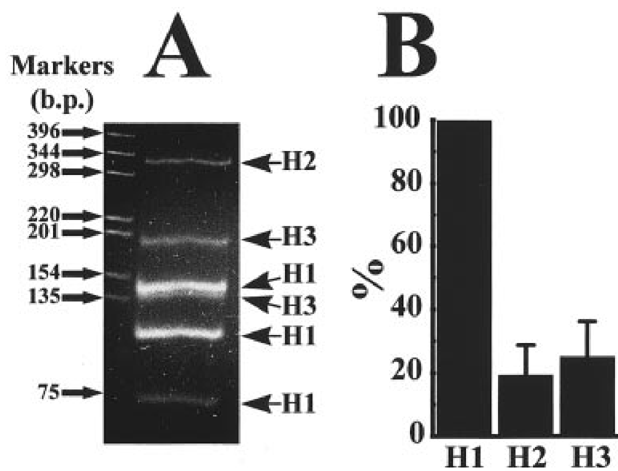

Figure 2 Analysis of the Homer mRNAs expression in HEK-293 cells

(**A**) Reverse transcriptase PCR amplification (with primers PanHF and PanHR) and *Rsa*I restriction digestion of an amplified product which contains 310 bp fragments of all Homer protein mRNAs expressed in HEK-293 cells. H1 denotes 70, 103 and 137 bp fragments of Homer-1 cDNA; H2, a 310 bp fragment of Homer-2 cDNA; H3, 129 and 181 bp fragments of Homer-3 cDNA. Molecular size (bp) markers are shown by arrows on the left. (**B**) Expression levels of Homer-2a/b and Homer-3 are shown as a fraction of the expression level of Homer-1 mRNA, which is taken as 100%. The values are means ± S.E.M. from seven independent determinations.

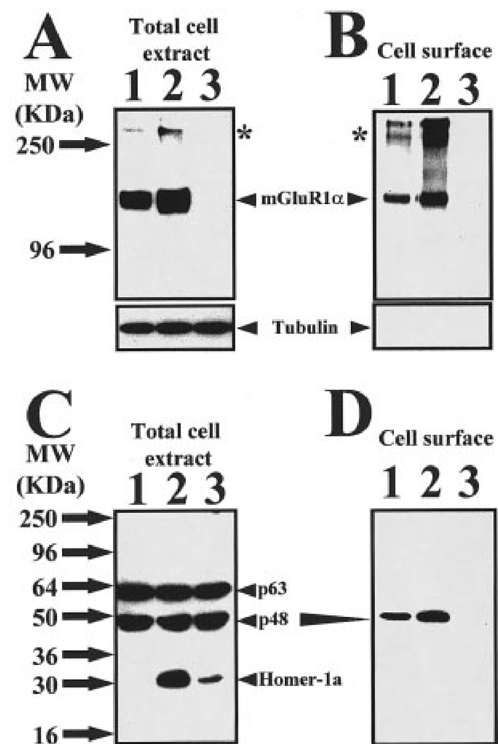

Figure 3 Co-expression of mGluR1α and Homer-1a in HEK-293 cells

HEK-293 cells were transiently transfected with 5 μg cDNA encoding mGluR1α and 5 μg cDNA encoding the *LacZ* reporter (lane 1), 5 μg cDNA encoding mGluR1α and 5 μg cDNA encoding Homer-1a (lane 2), and 5 μg cDNA encoding Homer-1a and 5 μg cDNA encoding the *LacZ* reporter (lane 3) as described in the Experimental section. After 48 h crude cell extracts (40 μg of protein) were analysed by SDS/PAGE and immunoblotted using anti-mGluR1α affinity-purified F2 antibody (**A**), anti-Homer affinity-purified VHr20 antibody (**C**) or a monoclonal anti-β-tubulin (1:200) (**A**, lower panel). Similarly, transiently transfected HEK-293 cells were biotinylated on the cell surface as described in the Experimental section. Biotin-labelled proteins were isolated with streptavidin–agarose beads, analysed by SDS/PAGE and immunoblotted with anti-mGluR1α affinity-purified F2 antibody (**B**), anti-Homer affinity-purified VHr20 antibody (**D**) or a monoclonal anti-β-tubulin (1:200) (**B**, lower panel). The arrowheads on the right indicate the positions of mGluR1α, Homer-1a, p48, p63, and tubulin. The position of the mGluR1α dimer is indicated with an asterisk (*).

the different DNA fragments (Figure 2A). Because all of the amplified fragments were of the same length and were amplified in one reaction with the same pair of primers, analysis of the relative abundance of each of the Homer-1, -2 and -3 sequence fragments allowed us to quantify the levels of the corresponding mRNAs in HEK-293 cells (Figure 2B). The relative level of Homer-1 mRNA expression (including all splicing forms) exceeded that of Homer-2 by 6-fold and that of Homer-3 by 4-fold (Figure 2B). Our method, based on the amplification of the 5′ coding regions of Homer genes, does not allow us to distinguish between the expression of the short (1a) and the long (1b, 1c) forms of Homer-1 protein. Since Homer-1a mRNA codes for a small 30 kDa protein [15] and this was not present at detectable levels in our immunoblots of HEK-293 cells (Figure 1B, lanes 3 and 4), we consider that it was either not expressed or quickly down-regulated. However, we can detect the expression of Homer long forms, which are of a sufficiently similar molecular mass that they would not be resolved under our polyacrylamide gel conditions. The p63 protein immunoreactive with the peptide- and fusion-protein-derived sera seen in both rat brain and HEK-293 cells may represent either a protein cross-reacting with the sera or a hitherto undescribed, alternatively spliced, form of a member of the Homer family. A similarly sized protein reacting with anti-Homer sera has been reported in rat brain and other tissue [18]. These results show that the endogenous expression of Homer mRNAs, and by implication proteins, in HEK-293 cells is limited to the long forms of Homers (1b, 1c, 2a, 2b and 3), among which the Homer-1b/c forms are predominant.

mGluR1α and Homer-1a are co-stabilized when co-expressed in HEK-293 cells

To study the interaction between mGluR1α and Homer-1a, HEK-293 cells were transiently transfected with cDNA encoding mGluR1α or Homer-1a or both cDNAs together. Cells transfected with mGluR1α and immunoblotted with F2-Ab showed an immunoreactive protein of molecular mass 150 kDa, corresponding to mGluR1α (Figure 3A, lane 1), together with a higher molecular mass band ($\sim$ 300 kDa) which may represent a dimeric form of the receptor [21]. Extracts from control untransfected HEK-293 cells did not show any immunoreactivity for mGluR1α (Figure 3A, lane 3), but did yield immunoreactive bands for p48 and p63 as noted previously (Figure 3C, lane 1).

When cells were transfected with Homer-1a alone and immunoblotted with VHr20, an immunoreactive protein with an apparent molecular mass of 30 kDa was detected (Figure 3C, lane 3) in agreement with previous results [15]. This protein could not be detected in untransfected HEK-293 cells (Figure 3C, lane 1). Surprisingly, when cells were simultaneously transfected with cDNAs encoding mGluR1α and Homer-1a, the immunoreactivity for Homer-1a was dramatically increased in contrast to the transfections with Homer-1a alone (Figure 3C, lane 2). Similarly, the expression of mGluR1α also appeared to be increased in those cells transfected with cDNAs for both proteins (Figure 3B, lane 2). It should be noted that the levels of p48 and p63 did not change with the transfection of the cells with cDNAs for either mGluR1α or Homer-1a (Figure 3C, lanes 1–3).

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Table 1 Quantification of mGluR1α and Homer-1a in transiently transfected HEK-293 cells

Experiments were performed as described in the legend to Figure 3, using HEK-293 cells transiently transfected with cDNAs for mGluR1α, mGluR1α plus Homer-1a and Homer-1a alone. The intensities of the immunoreactive bands on X-ray film corresponding to each protein (mGluR1α, p63, p48 and Homer-1a) were measured by densitometric scanning. All the values are normalized using tubulin as a control protein. The results are presented as the mean ± S.E.M. of three different experiments. $^*P < 0.01$ when compared with HEK-293 cells transfected with mGluR1α cDNA alone. $^\dagger P < 0.01$ when compared with HEK-293 cells transfected with Homer-1a cDNA alone. (–), Not detectable.

	Immunodetected protein (relative amount)					
	Total cell extract				Cell surface	
Transfection	mGluR1α	p63	p48	Homer-1a	mGluR1α	p48
mGluR1α	1.27 ± 0.01	1.21 ± 0.02	1.12 ± 0.02	–	1.08 ± 0.02	0.46 ± 0.03
mGluR1α + Homer-1a	2.40 ± 0.02*	1.21 ± 0.03	1.15 ± 0.03	0.94 ± 0.01†	3.32 ± 0.01*	0.83 ± 0.02*
Homer-1a	–	1.19 ± 0.02	1.14 ± 0.04	0.26 ± 0.01	–	–

Table 2 Quisqualic acid induced IP accumulation in HEK-293 cells transiently transfected with cDNAs for mGluR1α and Homer-1a

HEK-293 cells transiently transfected with cDNAs for mGluR1α or mGluR1α plus Homer-1a were labelled overnight with *myo*-[^{3}H]inositol, stimulated with various concentrations of quisqualic acid for 20 min and the production of total IPs was then determined. The basal levels of [^{3}H]IP accumulation for unstimulated cells were 2334 ± 428 d.p.m./10^5 cells and 2445 ± 603 d.p.m./10^5 cells in cells transfected with cDNAs for mGluR1α and mGluR1α plus Homer-1a respectively. E_{max} is the maximal level of cellular IP production at the highest dose of agonist added. The percentage increase in the E_{max} relative to that of the cells transfected with mGluR1α cDNA alone is given in parentheses. Results are presented as the mean ± S.E.M. for four independent experiments. $^*P < 0.01$ when compared with HEK-293 cells transfected with mGluR1α cDNA alone.

Transfection	EC_{50} (μM)	E_{max} (d.p.m./10^5 cells)
mGluR1α	1.2 ± 0.3	13 767 ± 428 (100%)
mGluR1α + Homer-1a	0.8 ± 0.3	18 338 ± 484 (140%)*

Densitometric scanning of the immunoreactive bands on the film was used to quantify these changes in protein expression (Table 1). The band intensities used for mGluR1α included both the monomeric and dimeric forms. The levels of each protein were normalized to the amount of tubulin in each lane as a measure of the amount of cell lysate present in each sample (Figure 3A, lower panel). The results, summarized in Table 1, showed that the total amount of mGluR1α in HEK-293 cells co-transfected with cDNAs for Homer-1a and mGluR1α increased 2-fold when compared with the cells transfected with mGluR1α cDNA alone. More strikingly, the amount of Homer-1a in the doubly transfected cells increased more than 3 times when compared with cells transfected with Homer-1a cDNA alone. The amounts of p48 and p63 were unchanged by the expression of either Homer-1a or mGluR1α. The results suggest that Homer-1a is being stabilized by the presence of the receptor and that Homer-1a increases the amount of cell surface mGluR1α.

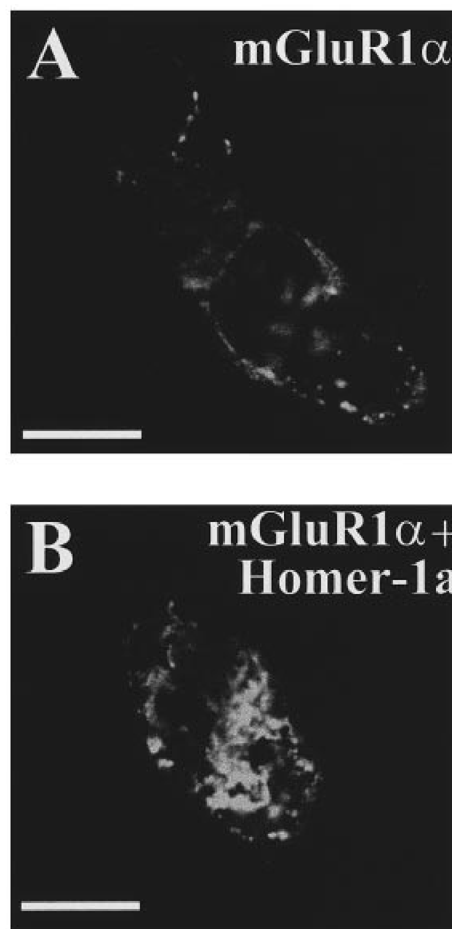

Figure 4 Cell surface distribution of mGluR1α in HEK-293 cells

HEK-293 cells were transiently transfected (see the legend to Figure 5) with cDNAs for mGluR1α (**A**) or mGluR1α and Homer-1a (**B**). After 48 h the cells were washed, fixed and processed for immunostaining as indicated in the Experimental section. Staining was performed with anti-FLAG monoclonal antibody (M2-Ab, 10 μg/ml), detected with a fluorescein-conjugated donkey anti-mouse IgG antibody (1:50) and observed by confocal microscopy. Each image shows a single horizontal section of representative cells. The scale bar represents 10 μm.

Cell surface expression of mGluR1α in co-transfected HEK-293 cells

To study the effects of Homer-1a on the mGluR1α surface expression we isolated the membrane receptor by cell-surface-protein biotinylation, using a membrane-impermeant biotin ester, and streptavidin–agarose affinity precipitation of the membrane proteins. The results show that the amount of receptor present in the cell surface was increased when mGluR1α and Homer-1a were co-expressed compared with when mGluR1α was expressed alone (Figure 3B, lanes 1 and 2). Quantification of this increase

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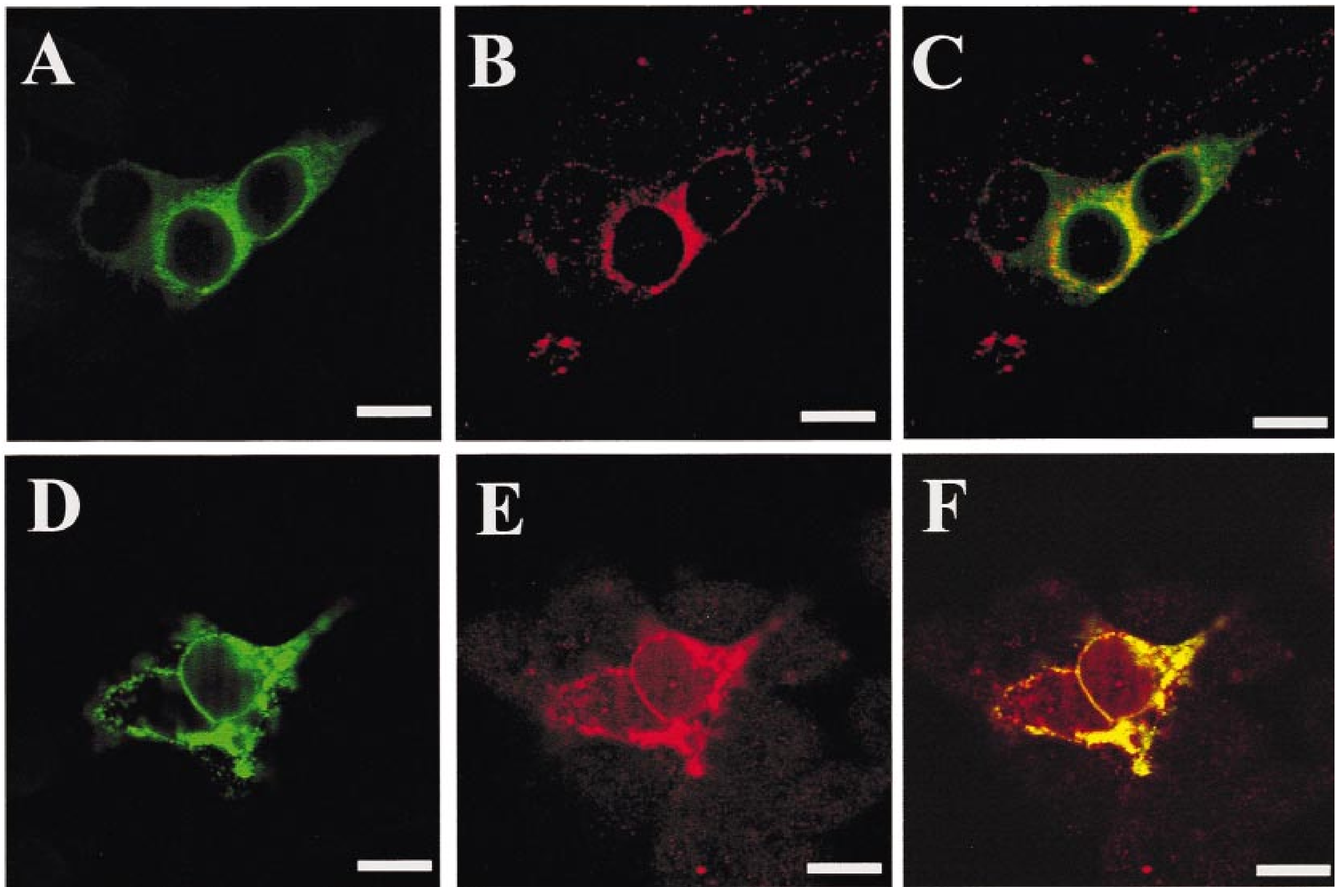

Figure 5 Co-localization of mGluR1α and Homer-1a in HEK-293 cells

HEK-293 cells were transiently transfected with cDNA for mGluR1α (**A**, **B**, **C**) or cDNAs for mGluR1α and Homer-1a (**D**, **E**, **F**). After 48 h the cells were washed, fixed, permeabilized and processed for immunostaining as described in the Experimental section. Immunostaining was performed with anti-FLAG monoclonal antibody (M2-Ab, 10 μg/ml) and anti-Homer affinity-purified VHr20 antibody. The bound primary antibody was detected using either Texas Red-conjugated donkey anti-rabbit IgG antibody (1 : 50) or fluorescein-conjugated donkey anti-mouse antibody (1 : 50). Cells were analysed by double immunofluorescence using a confocal microscope to detect mGluR1α (green images) and Homer (red images). Superimposition of images reveals mGluR1α/Homer co-localization in yellow. The images show a single horizontal section of representative cells. The scale bar represents 10 μm.

in membrane mGluR1α indicated that the amount of surface receptor had risen by up to 3-fold in the co-transfected cells (Table 1). Interestingly, when the streptavidin isolates were reacted with the Homer-specific serum VHr20, it became apparent that p48 could only be observed in the isolates from the cells that were transfected with mGluR1α cDNA (Figure 3D). Co-expression of Homer-1a and mGluR1α resulted in an increase of both proteins in the streptavidin isolates (Figure 3D). The increase in the amount of p48 in the streptavidin isolates in the Homer-1a-expressing cells was similar to that seen for the mGluR1α (Table 1). Since no tubulin could be detected in the streptavidin isolates, the biotin ester had not penetrated the cell membrane (Figure B, lower panel). As Homer-1a, tubulin, p48 and p63 were not biotin labelled in the cells transfected with Homer-1a alone, the appearance of p48 in the streptavidin isolates from mGluR1α-transfected cells must reflect its association with the receptor in the plasma membrane. Since neither Homer-1a nor p63 could be detected in the streptavidin isolates, even in the presence of transfected mGluR1α, these results strongly suggest that p48 is the dominant member of this protein family associated with the receptor at the plasma membrane.

The functional consequences of the co-expression of Homer-1a with mGluR1α on second messenger coupling of the receptor were examined. HEK-293 cells transiently transfected with cDNAs for mGluR1α, mGluR1α plus Homer-1a, or Homer-1a alone were treated with quisqualic acid and the accumulation of IP measured. As shown in Table 2 the quisqualic-acid-induced increase in IP production in HEK-293 cells co-transfected with cDNAs for mGluR1α plus Homer-1a was 1.4-fold higher than in the cells transfected with mGluR1α cDNA. No significant change in the EC_{50} was observed (Table 2). As expected, no increment in IP production was observed when HEK-293 cells were transfected with Homer-1a cDNA alone and stimulated with quisqualic acid (results not shown). The potentiation of IP production in the co-transfected cells is consistent with the increase in surface receptor as determined biochemically (see above).

Cell surface distribution of mGluR1α in co-transfected HEK-293 cells

The distribution of mGluR1α on the surface of transfected HEK-293 cells was determined by immunofluorescence staining using a monoclonal anti-FLAG antibody directed against a FLAG sequence introduced at the N-terminus of mGluR1α. The receptor distribution in non-permeabilized cells, fixed with paraformaldehyde, was analysed by confocal microscopy. In

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HEK-293 cells transiently transfected with mGluR1α cDNA the cell surface receptor appeared as bright punctate spots of fluorescence suggestive of receptor clustering in the plasma membrane (Figure 4A). This distribution of mGluR1α has been noted previously in a different cell line [30]. When the cells were co-transfected with mGluR1α and Homer-1a cDNAs, a significant increment in the level of cell surface fluorescence was detected (Figure 4B). This increment in the cell surface fluorescence correlates well with the results described above (Table 1). In addition, the surface distribution of the receptor changed, showing an increase in the size of the immunoreactive clusters at the cell surface. No cell surface staining for mGluR1α was detected in HEK-293 cells transiently transfected with Homer-1a cDNA alone. The transfection frequency of the differently transfected cultures was similar throughout these experiments.

Subcellular co-localization of mGluR1α and Homer-1a in HEK-293 cells

Homer-1a and mGluR1α were expressed alone or together in HEK-293 cells and the intracellular distribution of the proteins was examined following fixation and permeabilization of the cells with Triton X-100. The proteins p48 and p63 were constitutively present in HEK-293 cells and gave a low level of background immunofluorescence in all cells when the antibody VHr20 was used to detect them. This endogenous fluorescence resulted in a weak grainy or reticular pattern (Figure 5B). Expression of mGluR1α in HEK-293 cells resulted in a diffuse cytoplasmic stain (Figure 5A), which showed a partial but not complete co-localization with the endogenous p48 and p63. Occasionally, punctate staining of the endogenous Homer-related immunoreactive proteins, which appeared to co-localize with the mGluR1α could be seen and could represent the membrane clusters of mGluR1α associated with p48. When Homer-1a was expressed by itself in HEK-293 cells, no apparent change in distribution and fluorescence intensity of the VHr20 immunoreactive proteins was observed (results not shown) probably reflecting the low levels of Homer-1a expression in the absence of mGluR1α as noted above.

This pattern is in marked contrast to the result obtained when Homer-1a was co-expressed with mGluR1α (Figure 5E). In this case the immunofluorescence appeared in large intracellular accumulations which are precisely co-localized with those immunoreactive for mGluR1α (Figures 5D and 5F). Since our antibody reacts with all the members of the Homer gene family in HEK-293 cells, these intracellular accumulations could contain p48 and Homer-1a together with mGluR1α. However, in the absence of Homer-1a the intracellular pattern of immunostaining of HEK-293 cells transfected with mGluR1α cDNA does not look like that of the cells transfected with cDNAs for both Homer-1a and the receptor. In addition, the presence of Homer-1a and mGluR1α in HEK-293 cells did not alter the amount of either p63 or p48 in the cells as shown above. Therefore we would suggest that the dominant contribution to the vesicular immunofluorescence detected by the VHr20 serum is due to Homer-1a. Consequently, co-expression of Homer-1a and mGluR1α caused a dramatic change in the intracellular distribution of the two proteins, from a diffuse cytoplasmic/reticular pattern to one of large vesicular inclusions.

DISCUSSION

Recently, a family of Homer-related proteins has been identified. This family is comprised of several related proteins, two of which are C-terminally extended forms of Homer-1a, termed Homer-1b and Homer-1c, and two closely related but distinct proteins, Homer-2a/b and Homer-3 [17,18]. All the Homer-related proteins bind to the C-terminus of mGluR1α and have strong regions of similarity in their N-terminal amino acid sequences with which our antisera would react. Homer-1b/c and Homer-2a/b, but not Homer-1a, have been shown to form both homo- and heteromeric complexes with each other, via C-terminal protein–protein interaction domains [17]. They are also of comparable molecular mass and migrate at virtually the same position on SDS/PAGE, having apparent molecular masses of about 47 kDa [17,18].

The results of the present study show that HEK-293 cells, which have been widely used for the expression of different receptors, contain mRNAs for Homer-1, Homer-2 and Homer-3. Since the serum against Homer used in the present study could potentially react with all members of this protein family, and the long forms of Homer are of comparable molecular mass, our immunochemical data do not allow us to identify which of the long forms of the Homer protein are present in HEK-293 cells. However, we could not detect the presence of Homer-1a in HEK-293 cells, and based on the relative abundance of the mRNAs for Homer-1,-2 and -3, the 48 kDa immunoreactive band seen in our immunoblots must contain a substantial amount of Homer-1b/c. The 63 kDa immunoreactive band seen in our immunoblots of HEK-293 cells could either be an as yet undescribed splice variant/member of this gene family or a protein cross-reacting with our serum. It is interesting to note that a similar molecular mass band has been identified in rat brain and other tissues in studies using other Homer antisera [18].

Homer-1a itself acts to increase the surface expression of mGluR1α when it is co-expressed with the receptor in HEK-293 cells. This results in an increase in agonist-mediated IP accumulation and an increased amount of aggregation of the receptor in the plasma membrane. The only other study on the functional effects of expressing Homer-1a in cells was performed on cultured Purkinje cells, where transfection with Homer-1a cDNA resulted in a slower but more prolonged calcium response in the proximal dendrites in response to pressure-applied quisqualic acid. Whilst the peak calcium response in the transfected cells was reduced, the overall response was identical to that in the control cells or those transfected with Homer-1b cDNA [18]. Unfortunately, neither IP production nor the amount of receptor on the cell surface was determined in that study, which makes comparison of those results with the results reported in the present study difficult. The co-expression of Homer-1a and mGluR1α in HEK-293 cells caused a significant increase in the level of Homer-1a in the cells as compared to HEK-293 cells expressing Homer-1a alone, suggesting that the receptor was stabilizing Homer-1a. In the course of these experiments we have found that, in the absence of mGluR1α, it can be difficult to detect Homer-1a following transfection of its cDNA into HEK cells. This finding is echoed by the low levels of Homer-1a found in transfected COS cells by Kato et al. [17]. It is also consistent with their observation that despite high levels of Homer-1a mRNA in rat cortex there was little immunoreactive protein present. Together these results strongly suggest that Homer-1a is rapidly turned over and that it is stabilized by the presence of an interacting receptor.

The fact that the surface fluorescence of mGluR1α is patchy in the HEK-293 cells transfected with the receptor alone may well be a consequence of their containing Homer-1b/c, Homer-2a/b or Homer 3, since these members of the Homer family have been shown to form both homo- and heteromeric complexes, via C-terminal protein interaction domains [17]. The resulting dimeric molecules could then cross-link mGluR1α molecules via their

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single N-terminal EVH1-like domain (ENA/VASP homology domain 1), which interact with a proline-rich region in the C-terminal region of the receptor. It should be noted that the EVH1-like domain of these Homer proteins interacts with a proline-rich motif (PPXXF) which is present in proteins other than the mGluR, such as mGluR1α, mGluR5, dynamin and the inositol trisphosphate receptor. Thus, Homer-1b/c, Homer-2a/b and Homer-3 have the potential not only to link mGluR1α and mGluR5 receptors together but also to cross-link these to other molecules and therefore potentially give rise to large multimeric signalling complexes [18]. The increase in the size of the cell surface patches of mGluR1α in cells co-transfected with Homer-1a cDNA could therefore be explained by there being more plasma membrane receptor in these cells to complex with the endogenous Homer-related proteins or other signalling molecules.

Since Homer-1a, which has the EVH1-like domain, does not contain the C-terminal interaction domain, it cannot form dimers and therefore cannot directly cross-link mGluR1α to other molecules. The fact that the intracellular distributions of both Homer-1a and mGluR1α change from a diffuse, and sometimes reticular, to a distinct vesicular, intracellular pattern when they are co-expressed, suggests that Homer-1a may have a role as a regulator of the intracellular trafficking of the receptor. The facts that both proteins are co-localized in the cells expressing them and that their co-expression results in a significant increase in the cell surface expression of the receptor would be consistent with this suggestion. Thus we would suggest that one role for Homer-1a is to promote the cell surface targeting of mGluR1α. This could be achieved by either facilitating the transport of the receptor from the endoplasmic reticulum to the plasma membrane or by preventing its removal once it is inserted into the membrane. The alterations in the intracellular immunostaining of Homer-1a and the receptor in cells transfected for both proteins would be more consistent with the former mechanism, as would be our finding that Homer-1a was not associated with membrane mGluR1α. Once it reaches the plasma membrane the receptor can form complexes with the other Homer-related proteins to form the patches of immunoreactive protein visible in the plasma membrane of the transfected cells. This hypothesis is consistent with our observation that p48 was found at the cell surface only in cells transfected with mGluR1α and that there was a parallel increase in the amounts of membrane mGluR1α and p48 in cells expressing both Homer-1a and mGluR1α. The fact that Homer-1a was not found associated with mGluR1α in the streptavidin isolates from HEK-293 cells transfected with the cDNAs of both proteins suggests that it disassociates from the receptor having delivered it to the plasma membrane. If disassociation was followed by the degradation of the Homer-1a then the stabilization of the Homer-1a by the receptor could be explained. In addition to facilitating the membrane targeting of the receptor, Homer-1a could also regulate the formation and maintenance of the interaction of signalling complexes described above by competing with the other members of the Homer family for the proline-rich motif which binds to their EVH1-like domains. Either function for Homer-1a would be consistent with the observed rapid increase in its synthesis following neuronal stimulation and its rapid turnover. Either function would also allow it to play a role in synaptic remodelling.

It is unclear why HEK-293 cells, which do not express endogenous mGluR1α, express several forms of the Homer-related proteins. Since HEK-293 cells are derived from embryonic kidney cells, the proteins may be up-regulated during development. Using overlay techniques on rat brain Kato et al. showed that some members of the Homer family can interact with proteins other than mGluRs [17] and therefore these proteins may have different functions in cells other than neurons. Indeed, there is immunocytochemical evidence for the expression of the other Homer-family members in tissues other than brain [18]. It will be of some interest to characterize the functions of the other members of this gene family.

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