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Homer-1c/Vesl-1L Modulates the Cell Surface Targeting of Metabotropic Glutamate Receptor Type 1α: Evidence for an Anchoring Function

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Homer-1c/Vesl-1L is a 48-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 mGluR5. In order to examine the function of Homer-1c, HEK-293 cells have been transfected with mGluR1α, Homer-1c, and both proteins together. When cells were transfected with both proteins, biotinylation of cell surface molecules revealed a significant increase in the amount of receptor and Homer-1c associated with the cell surface compared with cells transfected with mGluR1α alone. This finding was paralleled by a concomitant increase in the production of inositol after treatment of the doubly transfected cells with agonist. Cell surface immunostaining of mGluR1α showed that Homer-1c can induce clustering of the receptor in the plasma membrane of HEK-293 cells and suggested that the surface receptor was associated with Homer-1c in the plasma membrane. The presence of Homer-1c reduced the rate of loss from the cell surface of mGluR1α from 5 to 1%/min and increased the extent of dendritic trafficking of the receptor in rat primary cultured neurons. Our results suggest that Homer-1c increases the cell surface expression of the metabotropic glutamate receptor type 1α by increasing its retention in the plasma membrane.

INTRODUCTION

Postsynaptic membranes in the nervous system are organized structures highly enriched in neurotransmitter receptors. Increasing evidence suggests that the clustering and immobilization of neurotransmitter receptors at synapses in the central nervous system are regulated by the direct interaction of these receptors with cytoplasmic proteins and that these interactions may be crucial for efficient signal transduction (see O'Brien *et al*., 1998).

Glutamate is the major excitatory neurotransmitter in the central nervous system and can exert its function through two different types of receptors, ionotropic and metabotropic glutamate receptors. Metabotropic glutamate receptors (mGluRs) constitute a family of large G-protein-coupled receptors which show little sequence homology with the superfamily of smaller G-protein-linked receptors (Tanabe *et al*., 1992). Eight members of the mGluR family have been identified and categorized into three subgroups on the basis of their sequence homology, agonist selectivity, and signal transduction pathway. The receptors mGluR1 and mGluR5 and their splice variants make up the group 1 mGluRs (Knopfel *et al*., 1995; Pin and Duvoisin, 1995). These share the strongest sequence homology (62% identify at the amino acid level) and are coupled to phospholipase C in transfected cells with quisqualic acid as their most potent agonist.

Immunocytochemical studies have shown that the different groups of the mGluRs show differential targeting in neurons with the group I mGluRs being predominantly postsynaptic while group 2 (mGluR2 and mGluR3) and group 3 (mGluR4, mGluR6, mGluR7, and mGluR8) receptors are generally presynaptic (Baude *et al*., 1993; Ohishi *et al*., 1995; Shigemoto *et al*., 1996, 1997). The precise synaptic location of mGluR1α is highly ordered, with the protein present in an annulus which surrounds the postsynaptic density (Baude *et al*., 1993; Lujan *et al*., 1996, 1997). One possible explanation for this specific localization of mGluR1α is that it is directed by interactions of the 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 domains,

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which interact with specific C-terminal sequences of these receptors, have been described (Dong *et al*., 1997; Hunt *et al*., 1996; Kim *et al*., 1996; Muller *et al*., 1996; Niethammer *et al*., 1996). Recently, a similar EVH domain-containing protein which binds specifically to the C-terminal residues of mGluR1α and mGluR5a has been described (Brakeman *et al*., 1997). This protein, termed Homer, was isolated as a synaptic plasticity-regulated gene from rat hippocampus (Brakeman *et al*., 1997; Kato *et al*., 1997) but as yet its function remains unclear. Additional proteins related to Homer have also been described, namely Homer-1b, Homer-1c/Vesl-1L, Homer-2a/Vesl-2Δ11, Homer 2b/Vesl-2, and Homer-3 (Kato *et al*., 1998; Xiao *et al*., 1998). The first is a C-terminal splice variant of the original Homer protein (renamed Homer-1a/Vesl-1S) but which shares the same N-terminal 175 amino acids. Unlike Homer-1b/c, Homer-2a/b, and Homer-3, which are expressed constitutively, Homer-1a/Vesl-1S is expressed only during brain development or in response to cortical activity (Brakeman *et al*., 1997; Kato *et al*., 1997), which might indicate a special role for it in mGluR regulation and targeting. Recently, Homer-1a/Vesl-1S has been shown to increase the cell surface expression of mGluR1α in transfected HEK 293 cells, but was not found at the cell surface associated with the receptor (Ciruela *et al*., 1999a).

Homer-1c contains a coiled-coil domain which is missing in Homer-1a, which can result in the homo- and heterodimerization of this molecule (Kato *et al*., 1998; Xiao *et al*., 1998). In order to examine the functional role of Homer-1c/Vesl-1L in the targeting and localization of mGluR1α we have examined the consequence of its coexpression with mGluR1α in transiently transfected HEK-293 cells and in primary cultures of rat cortical neurons. Our results show that Homer-1c also increases the cell surface expression of mGluR1α, by increasing its retention in the plasma membrane, and is associated with the receptor at the cell surface.

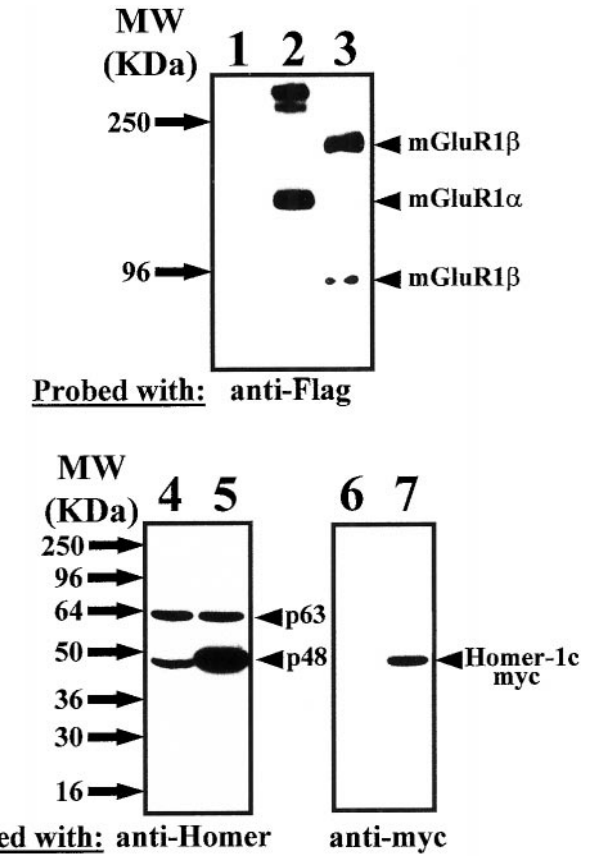


FIG. 1. Immunoblot showing expression of mGluR1α, mGluR1β, and Homer-1c. HEK-293 cells (lane 1, 4, and 6) were transiently transfected with 10 μg cDNA encoding mGluR1α-flag (lane 2), mGluR1β-flag (lane 3), Homer-1c (lane 5), and Homer-1c-myc (lane 7) as described under Experimental Methods. After 48 h crude cell extracts (40 μg of protein) were analyzed by SDS–PAGE and immunoblotted using anti-Flag monoclonal antibody (Clone M2; 10 μg/ml), anti-Homer affinity-purified VHr20 antibody (2–4 μg/ml), or anti-myc monoclonal antibody (Clone 9E10; 1/500). Immunoreactive bands were detected with swine anti-rabbit or swine anti-mouse (1:5000) secondary antibody conjugated to horseradish peroxidase followed by chemiluminescence detection. In this and all subsequent figures the position of the molecular mass markers in kilodaltons is indicated on the left. The positions of mGluR1α-flag, mGluR1β-flag, p48, p63, and Homer-1c-myc are indicated with arrows on the right.

RESULTS

Expression of mGluR1- Proteins in Transiently Transfected HEK-293 Cells

HEK-293 cells were transiently transfected with flag-tagged mGluR1α or mGluR1β and immunoblotted with an anti-Flag antibody. Cells transfected with mGluR1α-flag showed an immunoreactive protein with an apparent molecular weight of 150 kDa corresponding to mGluR1α-flag receptor (Fig. 1, lane 2). Additional immunoreactive bands with a higher molecular weight (~300 kDa) which may represent a dimeric form of the receptor (Ciruela and McIlhinney, 1997) were also observed. When cells were transfected with flag epitope-tagged mGluR1β, the flag antibody yielded two immunoreactive bands, with apparent molecular weights of 94 and 190 kDa (Fig. 1, lane 3), which correspond to mGluR1β receptor monomer and dimer, respectively, in good agreement with previous results on the native receptor (Ciruela and McIlhinney, 1997). Control untransfected HEK-293 cells did not show any immunoreactivity with the anti-flag antibody (Fig. 1, lane 1).

As described recently (Ciruela *et al*., 1999a) HEK-293 cells as well as other commonly used cell lines seem to constitutively express Homer-related proteins (Soloviev *et al*., 1999). In agreement with these observations, untransfected HEK-293 cells immunoblotted with anti-Homer antibody gave two specific immunoreactive proteins with apparent molecular weights of 48 (p48) and 63 kDa (p63) (Fig. 1, lane 4). The p48 represents the endogenously expressed Homers of which Homer-1b/1c are the most abundant (Ciruela *et al*., 1999a). The immunoreactive band at 63 kDa corresponds in size to those previously described by Xiao *et al.* (1998) and by Ciruela *et al.* (1999a) and may represent a Homer-related protein or a protein cross-reacting with the Homer antiserum. Expression of Homer-1a in HEK 293 cells was not detected here (Fig. 1) nor previously (Ciruela *et al*., 1999a). When the cells were transiently transfected with Homer-1c and immunoblotted using an anti-Homer serum an increment of 3.5 fold in the immunoreactivity of p48 compared with the untransfected cells was obtained (compare lane 4 with lane 5 in Fig. 1). Finally, when HEK-293 cells were transiently transfected with myc-tagged Homer-1c and immunoblotted with an anti-myc antibody, an immunoreactive protein with an apparent molecular weight of 48–50 kDa was observed (Fig. 1, lane 7). In contrast, control untransfected HEK-293 cells did not show any immunoreactivity with the anti-myc antibody (Fig. 1, lane 6). All of the epitope-tagged proteins therefore are expressed in HEK-293 cells. These results confirm that HEK 293 cells express endogenous Homer proteins and show that the transfection of the cells with Homer-1c cDNA leads to a significant increase in the amount of Homer-1c protein. Moreover, the recombinantly expressed Homer-1c can be distinguished from the endogenous protein using the myc-tagged version of the protein.

Cell Surface Expression of mGluR1α in Cotransfected HEK-293 Cells

In order to study the effects of Homer-1c on the mGluR1α cell surface expression we isolated the plasma membrane receptor by cell surface protein biotinylation, using a membrane-impermeant biotin ester, followed by streptavidin–agarose affinity precipitation of the membrane proteins. The results show that the amount of receptor present in the cell surface is increased when mGluR1α and Homer-1c are coexpressed compared to the amount found when mGlur1α is expressed alone (Fig. 2B, lanes 1 and 2). Quantitation of the increase of membrane mGluR1α indicated that the levels of surface receptor had risen by up to threefold in the Homer-1c

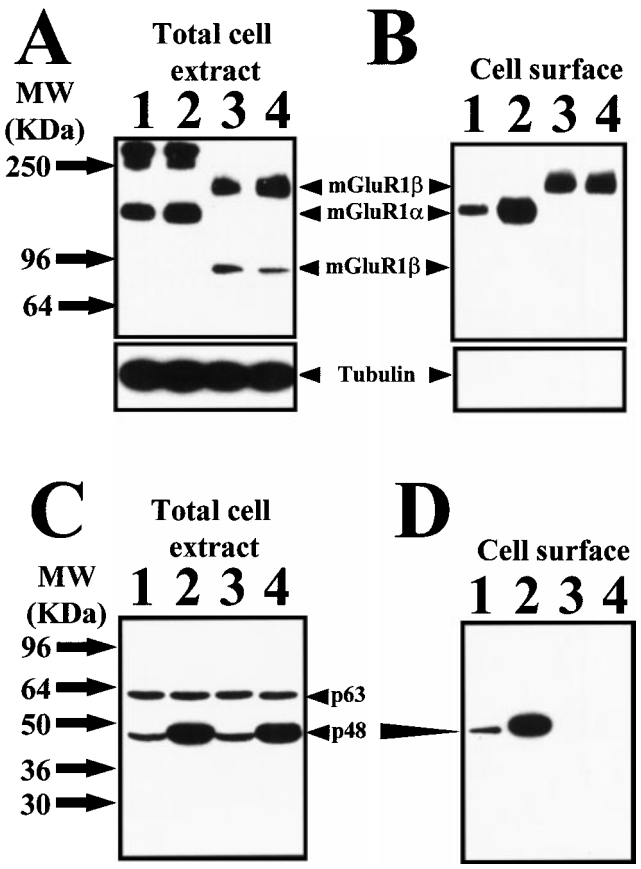


FIG. 2. Coexpression of mGluR1α, mGluR1β, and Homer-1c in HEK-293 cells. HEK-293 cells were transiently transfected with 5 μg cDNA encoding mGluR1α-flag and 5 μg cDNA encoding LacZ reporter (lane 1), 5 μg cDNA encoding mGluR1α-flag and 5 μg cDNA encoding Homer-1c (lane 2), 5 μg cDNA encoding mGluR1β-flag and 5 μg cDNA encoding LacZ reporter (lane 3), and 5 μg cDNA encoding mGluR1β-flag and 5 μg cDNA encoding Homer-1c (lane 4), as described under Experimental Methods. After 48 h crude cell extracts (40 μg of protein) were analyzed by SDS–PAGE and immunoblotted using anti-flag M2 antibody (A), anti-Homer affinity-purified VHr20 antibody (C), or a monoclonal anti-β-tubulin (1:200) (A, bottom). Similarly, transiently transfected HEK-293 cells were cell-surface biotinylated as described under Experimental Methods. Biotin-labeled proteins were isolated with streptavidin–agarose beads, analyzed by SDS–PAGE, and immunoblotted using anti-flag M2 antibody (B), anti-Homer affinity-purified VHr20 antibody (D), or a monoclonal anti-β-tubulin (1:200) (B, bottom). Immunoreactive bands were visualized as described before. The arrowheads indicate the positions of mGluR1α-flag, mGluR1β-flag, p48, p63, and tubulin.

cotransfected cells. Under the same conditions, no change in the cell surface expression of the mGluR1β was observed when it was coexpressed with Homer-1c (Fig. 2B, lanes 3 and 4), suggesting a high degree of specificity in the interaction. Interestingly, when the streptavidin isolates were reacted with the Homer-specific serum

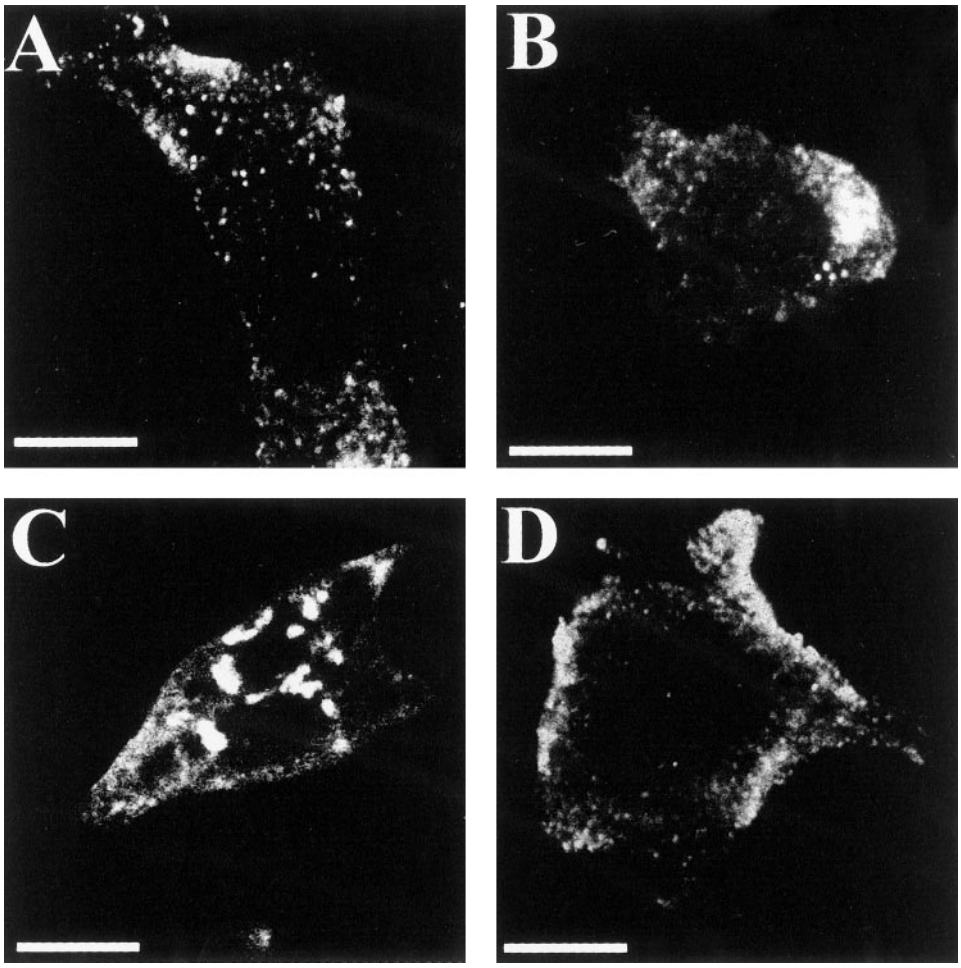


FIG. 3. Cell surface distribution of mGluR1α and mGluR1β in HEK-293 cells. HEK-293 cells were transiently transfected with mGluR1α-flag (A), mGluR1α-flag plus Homer-1c (C), mGluR1β-flag (B), or mGluR1β-flag plus Homer-1c (D). After 48 h cells were washed, fixed, and processed for immunostaining as indicated under Experimental Methods. Staining was performed with anti-Flag monoclonal antibody (Clone M2; 10 μg/ml), detected with a Texas red-conjugated donkey anti-mouse IgG antibody (1/50), and observed by confocal microscopy. The scale bar represents 10 μm.

TABLE 1

Quisqualic Acid-Induced IP Accumulation in HEK-293 Cells Transiently Transfected with mGluR1α and Homer-1c

Transfection	EC_{50} (μM)	E_{max} (dpm/10^5 cells)
mGluR1α	1.1 ± 0.2	13,600 ± 410 (100%)
mGluR1α + Homer-1c	0.9 ± 0.3	20,390 ± 480 (150%)*

Note. HEK-293 cells transiently transfected with mGluR1α and mGluR1α plus Homer-1c were labeled overnight with *myo*-[^{3}H]inositol and stimulated with quisqualic acid for 20 min and the production of total IP was then determined. The basal levels of [^{3}H]IP (unstimulated cells) were 2124 ± 420 and 2400 ± 540 dpm/10^5 cells in cells transfected with mGluR1α and mGluR1α plus Homer-1c, respectively. Results are means of four independent experiments ± SEM.

*$P < 0.01$ compared with HEK-293 transfected with mGluR1α alone.

VHr20 it became apparent that p48 could be observed only in the streptavidin isolates from the cells that were cotransfected with mGluR1α (Fig. 2D). Furthermore, coexpression of Homer-1c and mGluR1α resulted in an increase of both proteins in the streptavidin isolates (Fig. 2D). The increase in the amount of p48 in the streptavidin isolates in the Homer-1c-expressing cells was comparable to that seen for the mGluR1α. Since no tubulin could be detected in the streptavidin isolates the biotin ester had not penetrated the cell membrane (see bottom of Fig. 2B). As Homer-1c, tubulin, p48, and p63 were not biotin labeled in the cells transfected with mGluR1β, the appearance of p48 in the streptavidin isolates from mGluR1α-transfected cells must indicate its association with the receptor in the plasma membrane. Since p63

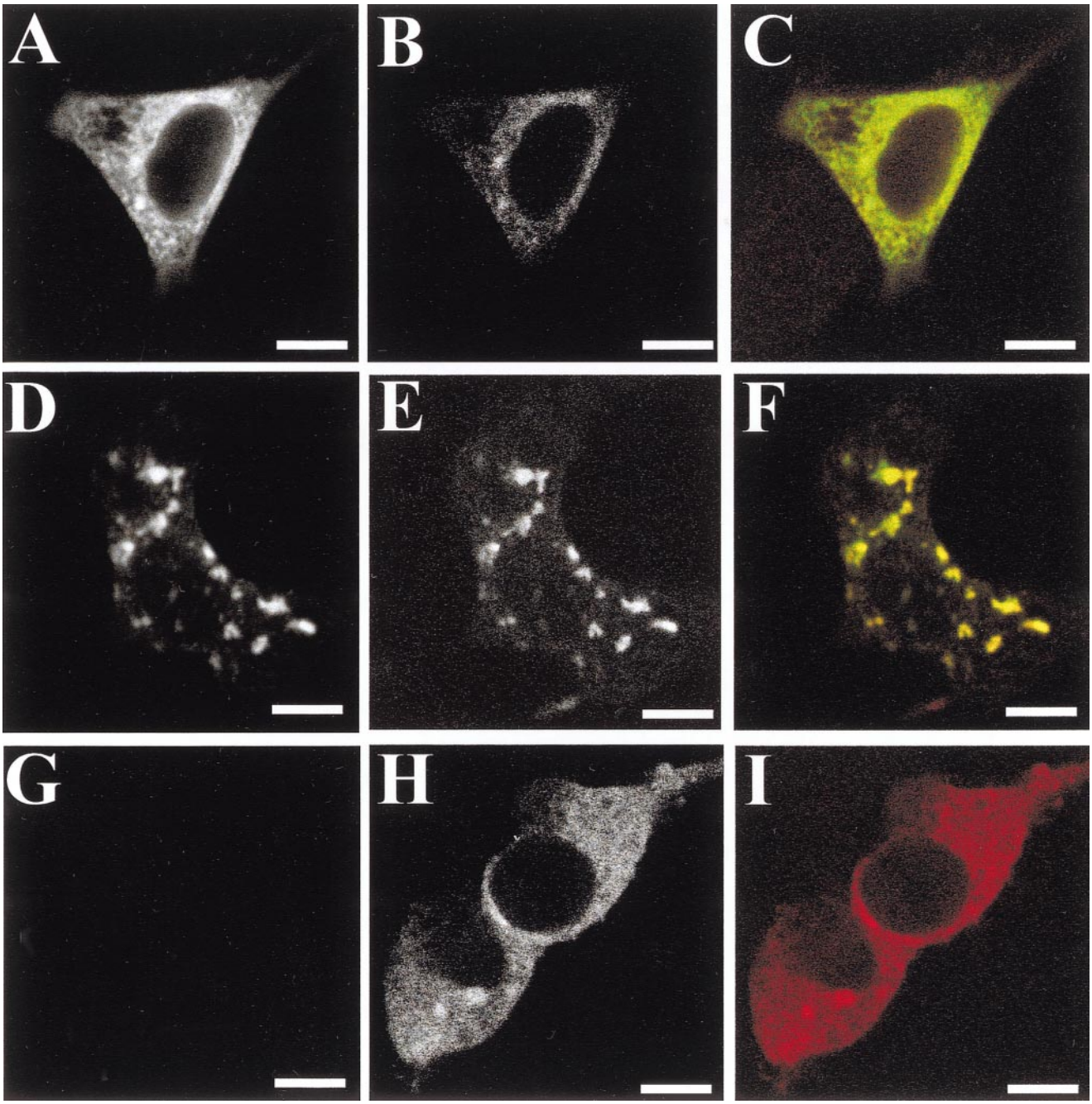
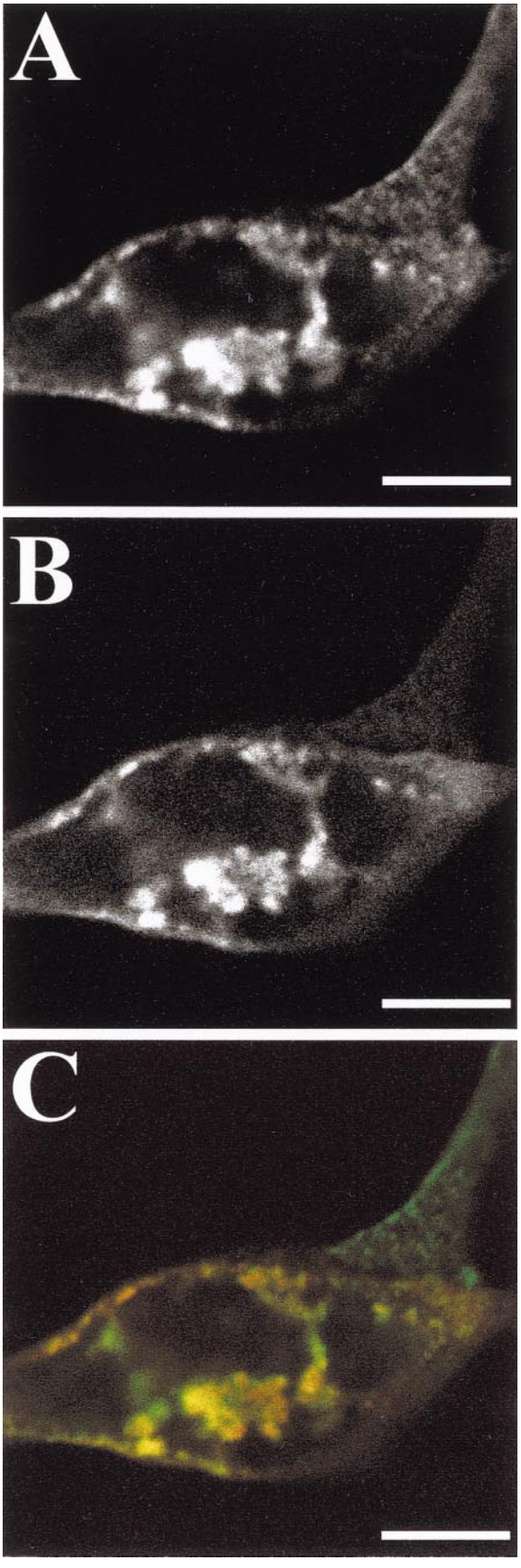


FIG. 4. Colocalization of mGluR1α and Homer-1c in HEK-293 cells. HEK-293 cells were transiently transfected with mGluR1α-flag (A, B, and C), mGluR1α-flag plus Homer-1c (D, E, and F), or Homer-1c alone (G, H, and I). After 48 h the cells were washed, fixed, permeabilized, and processed for immunostaining as indicated under Experimental Methods. Immunostaining was performed with anti-Flag monoclonal antibody (Clone M2; 10 μg/ml) and anti-Homer affinity-purified VHr20 antibody (5 μg/ml). The bound primary antibody was detected using either fluorescein-conjugated donkey anti-mouse IgG antibody (1/50) or Texas red-conjugated donkey anti-rabbit (1/50). Cells were analyzed by double immunofluorescence with confocal microscopy to detect mGluR1α (A and D) and Homer (B, E, and H). Superimposition of images reveals mGluR1α/Homer colocalization in yellow. The scale bar represents 10 μm.



could not be detected in the streptavidin isolates, even in the presence of transfected mGluR1α, these results strongly suggest that p48 is the only member of the Homer family associated with the receptor at the plasma membrane. Precisely, similar results were obtained when myc-tagged Homer-1c was used in these experiments, and in this case myc-tagged Homer-1c was also found only in the streptavidin isolates in cells cotransfected for Homer-1c and mGluR1α (data not shown). Therefore, Homer-1c, but not p63 or Homer-1a (Ciruela *et al.*, 1999a), is found at the plasma membrane associated with mGluR1α.

The functional consequences of the coexpression of Homer-1c with mGluR1α on the second-messenger coupling of the receptor were also examined. HEK-293 cells transiently transfected with mGluR1α, mGluR1α plus Homer-1c, or Homer-1c alone were treated with quisqualic acid and the accumulation of inositol phosphates (IP) was measured. As shown in Table 1, the quisqualic acid-induced increase in IP production in mGluR1α and Homer-1c cotransfected HEK-293 cells was 1.5-fold higher than in the cells transfected with mGluR1α alone and no significant change in the EC_{50} was observed. As expected, no increase in IP production was observed when HEK-293 cells transfected with Homer-1c alone were stimulated with quisqualic acid (data not shown). The potentiation of the production of IP in the cotransfected cells correlates well with the increase in surface receptor as determined biochemically above.

Cell Surface Distribution of mGluR1α in Cotransfected HEK-293 Cells

The distribution of mGluR1α on the surface of transfected HEK-293 cell was determined by immunofluorescence staining using a monoclonal anti-Flag antibody

FIG. 5. Cell surface colocalization of mGluR1α and Homer-1c in HEK-293 cells. HEK-293 cells were transiently transfected with mGluR1α-flag plus Homer-1c. After 48 h the cells were washed, fixed, and immunostained with anti-Flag monoclonal antibody (Clone M2; 10 μg/ml). After being washed, cells were postfixed with 4% paraformaldehyde in PBS for 5 min and permeabilized with 0.2% Triton X-100 for 5 min. Cells were then immunostained with anti-Homer affinity-purified VHr20 antibody (5 μg/ml). The bound primary antibody was detected using either fluorescein-conjugated donkey anti-mouse IgG antibody (1/50) or Texas red-conjugated donkey anti-rabbit (1/50). Cells were analyzed by double immunofluorescence with confocal microscopy to detect mGluR1α (A) and Homer (B). Superimposition of images reveals mGluR1α/Homer colocalization in yellow (C). The scale bar represents 10 μm.

directed against a flag sequence introduced at the amino terminus of mGluR1α. The receptor distribution in nonpermeabilized cells, fixed with paraformaldehyde, was analyzed by confocal microscopy. In HEK-293 cells transiently transfected with mGluR1α, the cell surface receptor appeared as bright punctate spots of fluorescence, indicating receptor clustering in the plasma membrane (Fig. 3A). This distribution of mGluR1α has also been noted previously in a different cell line (Pickering *et al*., 1993) and may reflect the interaction of the receptor with the endogenous Homer proteins. When the cells were cotransfected with mGluR1α and Homer-1c, a significant increase in the level of cell surface fluorescence was detected (Fig. 3C), together with a dramatic redistribution of the receptor into large receptor clusters in the plasma membrane. In contrast, no differences were observed in the cell surface distribution of mGluR1β when coexpressed with Homer-1c (compare Figs. 3B and 3D). No cell surface staining for mGluR1α was detected in HEK-293 cells transiently transfected with Homer-1c alone (data not shown), and the transfection frequencies of the differently transfected cultures were similar throughout these experiments.

Subcellular Colocalization of mGluR1α and Homer-1c in HEK-293 Cells

Homer-1c and mGluR1α were expressed alone and 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 constitutively expressed Homer-related proteins in HEK-293 cells give a low level of background immunofluorescence in all cells when the antibody VHr20 is used to detect them. This endogenous fluorescence gives a diffuse grainy or reticular pattern (Fig. 4B). Expression of mGluR1α in HEK-293 cells results in a similar pattern of cytoplasmic immunoreaction (Fig. 4A), which shows colocalization with the endogenous Homer-related proteins. Occasionally, weak punctate staining of the endogenous Homer-related immunoreactive proteins could be seen, which appeared to colocalize with the mGlur1α and could represent the membrane clusters of mGlur1α associated with the endogenous p48. When Homer-1c was expressed alone in HEK 293 cells the fluorescence intensity of the VHr20-immunoreactive proteins increased considerably, but remained cytosolic (Fig. 4H), as was the fluorescence of the endogenous Homer proteins in absence of the mGluR1α.

This was in marked contrast to the result obtained when Homer-1c was coexpressed with mGluR1α (Fig. 4E). Now the immunofluorescence appeared as large immunoreactive aggregates which are precisely colocalized with those immunoreactive for mGluR1α (Figs. 4D and 4F). These could be equivalent to the cell surface patches noted above. Since our antibody reacts with all the members of the Homer gene family, these aggregates could contain mGluR1α together with Homer-1c as well as the endogenous p48 or p63. However, similar results were obtained when cells were cotransfected using Homer-1c-myc and mGluR1α and stained with the anti-myc serum. This suggests that the major contribution to the aggregate immunofluorescence is due to the recombinantly expressed Homer-1c. Moreover, in the absence of recombinantly expressed Homer-1c the intracellular pattern of immunostaining of HEK 293 cells transfected with mGluR1α does not appear like that of the cotransfected cells. It is therefore the coexpression of Homer-1c and mGluR1α which causes the dramatic change in the intracellular distribution of both proteins from a diffuse cytoplasmic/reticular pattern to one of large aggregates. When HEK-293 were cotransfected with mGluR1β and Homer-1c the intracellular distribution of the former was not affected by the presence of Homer-1c, suggesting again a very specific interaction between mGluR1α and Homer-1c (data not shown).

Since the cell surface biotinylation data suggested an association of Homer-1c with mGluR1α in the plasma membrane, we have attempted to confirm this using immunofluorescence. HEK-293 cells doubly transfected with both Flag-tagged mGluR1α and Homer-1c were fixed with paraformaldehyde and reacted with the anti-Flag antibody. The cells were then washed, permeabilized, and incubated with the anti-Homer antibody. The results showed clearly that the patches of receptor in the cell surface corresponded precisely with the intracellular accumulations of Homer-1c (Fig. 5), suggesting that the two proteins were indeed closely associated and at the cell surface.

Stabilization of Cell Surface mGluR1α Receptors Coexpressed with Homer1-c in HEK-293 Cells

The increase in cell surface mGluR1α in the cotransfected HEK 293 cells described above could reflect either an increase in the rate of intracellular transport or an increased retention of the protein at the cell surface. In order to determine if the latter was the case we have used an ELISA assay to determine amount of cell surface mGluR1α and to measure the amount of the receptor over time in the presence and absence of Homer-1c. Thus cell surface Flag-tagged mGluR1α was reacted

with the anti-Flag antibody using conditions that inhibit endocytosis (4°C) (Pelchen-Matthews *et al*., 1989). The labeled cells are then warmed to temperatures permissive for endocytosis (37°C) and the amount of receptor uptake can be measured by monitoring the amount of cell surface antibody remaining at different time points determined by chilling the cells and incubating them with peroxidase-coupled secondary antibody. The primary antibody was used at a saturating concentration in order to ensure that the amount of receptor could be quantified. The initial color reaction at time zero for the Homer-1c/mGluR1α-transfected cells increased threefold over that of the mGluR1α-containing cells (see legend to Fig. 6), confirming that the surface expression of the receptor was increased by the cotransfection of Homer-1c and showing that the assay does give an estimate of the amount of surface receptor. As shown in Fig. 6 in cells expressing mGluR1α alone the receptor surface receptor was lost at a faster rate (5 ± 1.5%/min) than when it was coexpressed with Homer-1c (1 ± 0.25%/min), suggesting that the receptor was indeed being stabilized at the cell surface by the presence of Homer-1c. It should be noted that without formally demonstrating that the antibody bound to the receptor is internalized these data cannot be used to infer the endocytosis rate of the receptor, since the loss of the antibody could be due to some other cause, such as proteolysis or shedding of the receptor. However, the reduction in the rate of loss of the receptor to a plateau value is consistent with receptor endocytosis and recycling, rather than receptor proteolysis, and the rates of surface receptor loss are consistent with those described for other G-protein-coupled receptors (Koenig and Edwardson, 1997; Marsh and Pelchen-Matthews, 1996).

Subcellular Distribution of mGluR1α and Homer-1c in Neurons

To analyze the intracellular distribution of mGluR1α and Homer-1c in neurons, we transiently expressed both proteins in primary cultures of neurons from rat cortex. Consistently, when the neurons were transfected with mGluR1α alone, the receptor showed somatic staining together with a punctate distribution through the proximal dendrites (Fig. 7), with the latter fully colocalized with MAP-2 (data not shown). However, when mGluR1α was coexpressed together with Homer-1c, the receptor showed an extensive punctate distribution throughout the proximal and distal dendrites. Homer-1c was found to fully colocalize with these accumulations of receptor (Fig. 7). In two independent transfections of different cultures of 74 doubly transfected neurons 63 showed extensive dendritic staining with the remainder having the somatic/proximal dendrite pattern of staining. When Homer-1c was expressed alone, a grainy distribution through the cell soma and proximal dendrites was found (data not shown), similar to that found in transiently transfected HEK-293 cells (Fig. 6H). Thus the presence of Homer-1c appears to cause an increase in the dendritic trafficking of the mGluR1α. Owing to the cortical cultures being heterogeneous in cell type the numbers of neurons showing endogenous staining for mGluR1α was variable, and where present they were weakly reactive. Consequently when Homer alone was transfected into the cultures we were unable to ascertain the effect of this on the endogenous receptors.

In order to characterize the punctate distribution of mGluR1α induced by Homer-1c, doubly cotransfected neurons were immunostained simultaneously for mGluR1α and the synaptic marker synaptophysin. As shown in Fig. 8, some of the mGluR1α aggregates in distal dendrites colocalized with synaptophysin, suggesting that some of these aggregates correspond to synapses. Interestingly, Homer-1c aggregates previously known to colocalize with mGluR1α (Fig. 7) also show some synaptic localization (Figs. 8D, 8E, and 8F).

DISCUSSION

The results presented here show that Homer-1c itself increases the cell surface expression of mGluR1α when it is coexpressed with the receptor in HEK-293 cells. Coexpression of Homer-1c and mGluR1α in the HEK-293 cells had no effect on the EC_{50} of the inositol phosphate generation in cotransfected cells treated with quisqualate, but did increase the E_{max} of the response, consistent with the increase in the amount of cell surface receptor. So far, the only other study on the functional effects of expressing Homer-related proteins in cells has been performed by transfection of Homer-1b into cultured Purkinje cells (Xiao *et al*., 1998). This resulted in a faster calcium response in the proximal dendrites in response to pressure-applied quisqualate, which was interpreted by the authors as being due to a more efficient coupling of the receptor to IP3 receptors via its increased crosslinking to these via Homer-1b. The fact that we find the related Homer-1c at the cell surface in association with mGluR1α would be consistent with such a hypothesis.

The increase in the cell surface expression of mGluR1α in the cotransfected cells is accompanied by a dramatic redistribution of the surface receptor, into large patches,

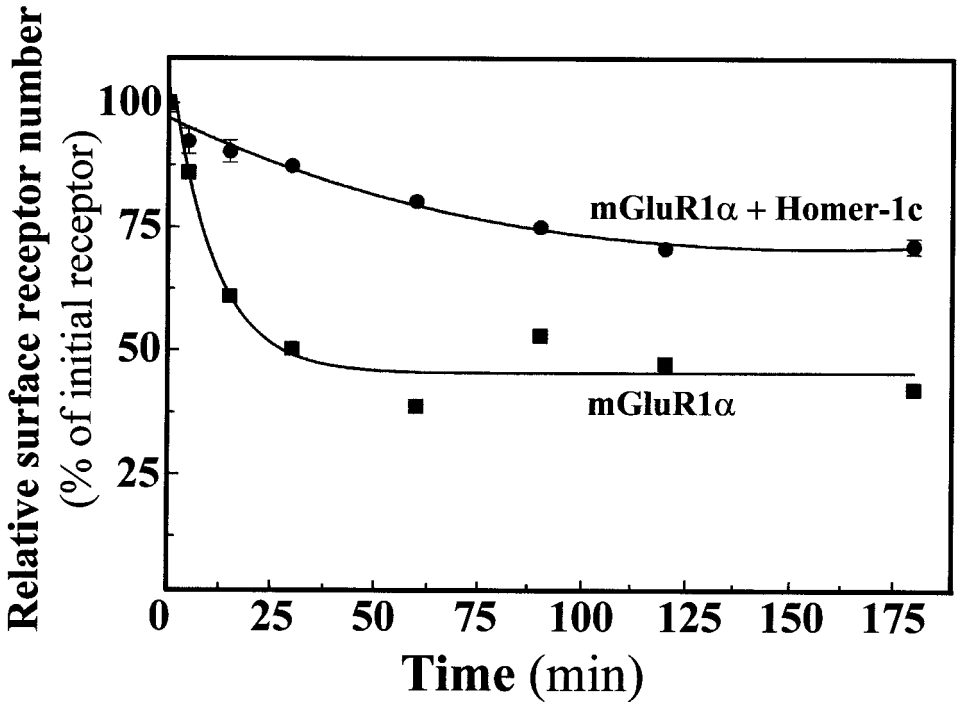


FIG. 6. Cell surface expression of mGluR1α in HEK-293 cells. HEK-293 cells were transiently transfected with mGluR1α-flag (■) or mGluR1α-flag plus Homer-1c (●), and the cell surface permanency of the receptor was determined by pulse–chase experiments as described under Experimental Methods. Values are expressed as percentages of the initial amount of receptor ($Abs_{620\ nm}$) at time zero. These were 0.323 ± 0.01 for the cells transfected with mGluR1α only and 0.969 ± 0.02 for cells doubly transfected with mGluR1α.and Homer-1c. Cells transiently transfected with Homer-1c alone did not show a significant increase in $Abs_{620\ nm}$ compared with untransfected cells, both of these control cells gave an Abs_{620nm} of less than 5% of the doubly transfected cells. Similar results were obtained in three different experiments. The results are the means ± SEM of values measured in quintuplicate.

and both the biotin labeling results and the immunofluorescence data suggest that these contain Homer-1c. This is consistent with a previous report which showed that Homer-1a increased the surface expression of mGluR1α and that this was accompanied by an increase in the association of the endogenous 45-kDa Homer proteins with the plasma membrane (Ciruela *et al.*, 1999a).

Since we do not see increased amounts of the receptor in the doubly transfected cells it seems unlikely that the increased cell surface expression of mGluR1α is due to a reduction in the degradation of the receptor. Raised levels of cell surface receptor could therefore be the result of either the facilitation of transport of the protein to the cell surface or its increased retention in the membrane. While we cannot exclude the former, the effect of cotransfection of Homer-1c, to reduce the rate of removal of the receptor from the cell surface, suggests that this may be one mechanism contributing to the increase in cell surface mGluR1α in the doubly transfected cells. The decreased rate of loss of the receptor could be the result of Homer-1c masking or disrupting any endocytosis sequence(s) present in the cytoplasmic domain of mGluR1α. Alternatively, Homer-1c may interact with other cellular components and thereby anchor mGluR1α in the plasma membrane. In this context it should be noted that Homer-1c, as a member of the Homer/Vesl-related proteins family, contains an EVH1-like domain (ENA/VASP homology domain 1) at its N-terminus (Kato *et al.*, 1998). In addition, Homer-1c has a C-terminal coiled-coil domain which allows both homo- and hetero-oligomerization of it with itself or other members of the protein family (i.e., Homer-2a/2b and Homer-3) (Kato *et al.*, 1998). EVH1 domains have also been reported to directly interact with zyxin, a component of the actin cytoskeleton (Beckerle, 1997), and Shank, a NMDA receptor scaffold protein present at the PSD-95 complex (Tu *et al.*, 1999). The EVH1 domain of these Homer proteins also interacts with a proline-rich motif (PPXXF) which is present in proteins other than the metabotropic glutamate receptor, such as dynamin and the IP3 receptor. Thus by forming either homo-

or hetero-oligomers Homer-1b/c, 2a/b, and 3 have the potential to link mGluR1α and mGluR5 receptors to themselves, to the cell cytoskeleton, and to other intracellular molecules. This could give rise to large multimeric signaling complexes (Xiao *et al*., 1998), the formation of which could stabilize the receptor in the plasma membrane. Homer-1a does not have the coiled-coil domain and can therefore not perform this crosslinking role. Interestingly, in HEK-293 cells, unlike the endogenous Homer proteins or recombinantly expressed Homer-1c, Homer-1a could not be detected in streptavidin isolates from cells transfected with mGluR1α and Homer-1a (Ciruela *et al*., 1999a), suggesting that this protein is not associated with the plasma membrane receptor.

The results of the transfection of Homer-1c and Flag-tagged mGluR1α in cerebral neurons were surprising in that the transfection of the receptor alone did not result in extensive dendritic staining of the neurons. Our results are consistent with those obtained using cultured cerebellar granule cells transfected with mGluR5, another group 1 metabotropic glutamate receptor, which was also retained in the cell body (Ango *et al*., 1999). This is in contrast with the transfection of mGluR1α into cultured hippocampal neurons in which extensive dendritic trafficking of the receptor was observed (Stowell and Craig, 1999). The apparent discrepancies in these results could be explained by the different cells used in the transfections or by the fact that the viral expression system used by Stowell and Craig (1999) may have resulted in overexpression of the receptor in these cells. The retention of mGluR1α in the cell soma of the singly transfected neurons could be due to their having a limiting concentration of endogenous Homer proteins or their expression of other variants of the protein. Alternatively, it may be the ratio of the different forms of the endogenously expressed neuronal Homer proteins which prevents either the dendritic trafficking or the retention of mGluR1α in the neurons expressing mGluR1α alone. However, coexpression of Homer-1c with flag-tagged mGluR1α caused extensive trafficking of both proteins into the distal dendrites. This could be due to a facilitation of receptor transport by Homer-1c or the receptor being retained in the dendrites due to the overexpression of the protein.

The correct targeting of neuronal proteins must involve their sorting into the appropriate axonal or dendritic compartment and subsequent delivery to specific synapses. Studies using polarized epithelial cells have shown that their initial sorting of membrane proteins into their basolateral or apical domains takes place in the *trans*-Golgi network and involves sequences contained in the proteins themselves (Matter and Mellman, 1994). Similar mechanisms may also operate in neurons (Bradke and Dotti, 1998). However, for correct positioning on the plasma membrane not only correct sorting, but also anchoring or retention, of the protein in the plasma membrane at specific locations is needed (Craven and Brendt, 1998; Kim, 1997; Rongo *et al*., 1998). This function also appears to be determined by specific sequences in the cytoplasmic domains of many proteins, including the ionotropic glutamate receptors which interact with PDZ domain-containing proteins at the synapse (Ehlers *et al*., 1996; Sheng, 1996). The data presented here suggest that Homer-1c may also act in this capacity for the group 1 metabotropic glutamate receptors since it is found associated with the receptor at the cell surface and in synapses.

EXPERIMENTAL METHODS

Mammalian Cell Culture and Transfection

HEK-293 cells were grown in DMEM (Sigma) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 10% (v/v) fetal calf serum (FCS) at 37°C and in an atmosphere of 5% CO_2. 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 25-cm^3 dishes were transiently transfected with 10μg of DNA encoding the indicated proteins by calcium phosphate precipitation (Jordan *et al*., 1996). In order to keep the ratio of DNA in cotransfections, pcDNA containing LacZ reporter was used to equilibrate the amount of total DNA. The cells were harvested at either 24 or 48 h after transfection.

Neuron Cultures and Transfection

Cortical hemispheres from E17 rat embryos were dissected out in Hanks' buffered saline solution (Sigma) supplemented with gentamicin (50 μg/ml) (Sigma) (HBSS-G), transferred to a new petri-dish containing HBSS-G, where the meninges were carefully removed, and washed twice with HBSS-G. Cortical tissue was treated with trypsin (0.5 g/L)–EDTA (2 g/L) solution (Sigma) for 10 min at 37°C. Trypsinization was stopped by addition of complete medium (DMEM containing Glutamax-I, 10% FCS, 50 μg/ml gentamicin) and tissue was gently triturated (10 passages) with a flame-sterilized glass Pasteur pipette. Cells were plated at 4–5 $\times$ 10^4 cells/cm^2 in DMEM containing Glutamax-I, 10% heat-inactivated FCS, and 50 μg/ml gentamicin, on

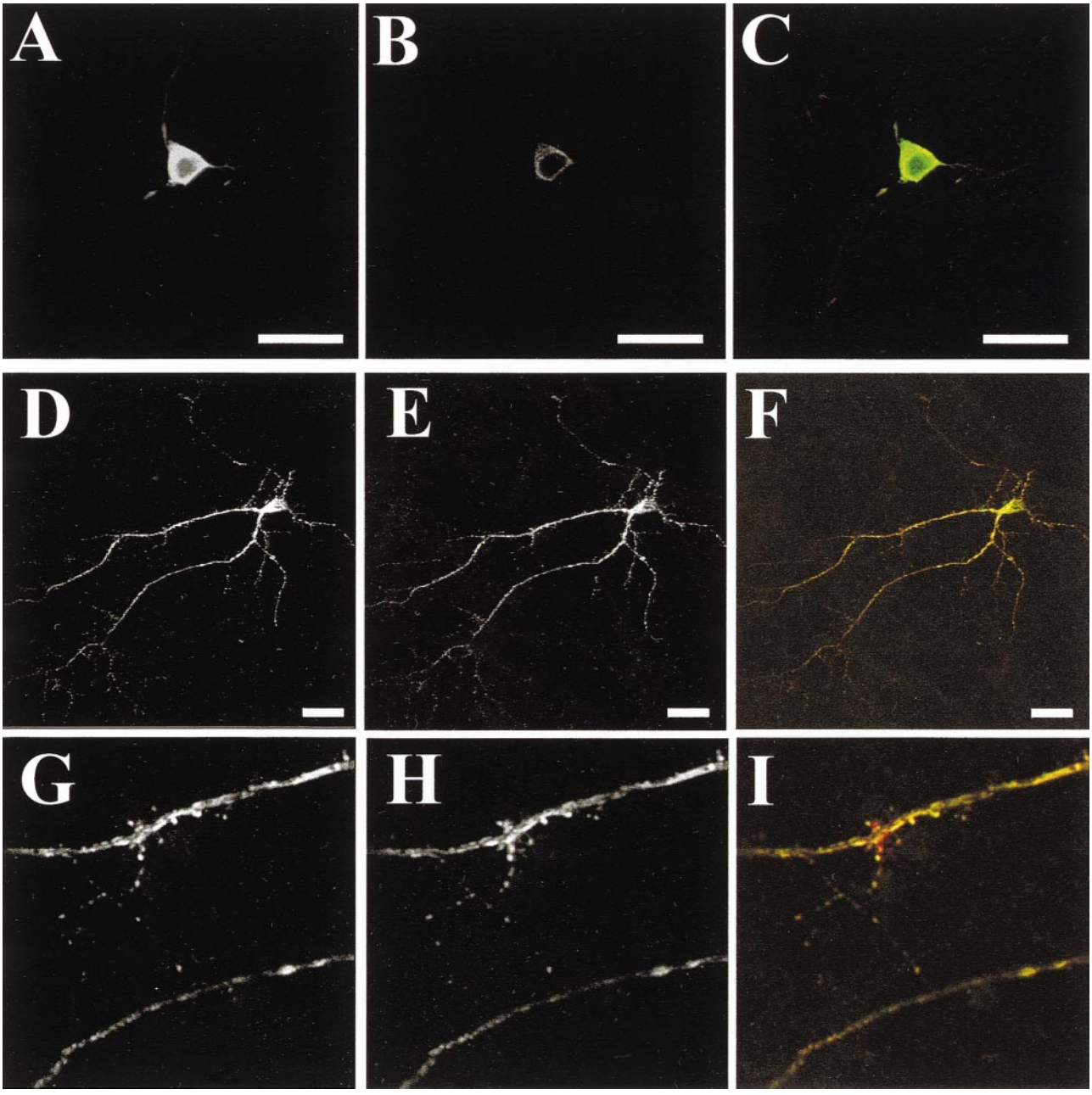


FIG. 7. Colocalization of mGluR1α and Homer-1c in neurons. Cultured cortical neurons were transiently transfected with mGluR1α-flag (A, B, and C) or mGluR1α-flag plus Homer-1c (D, E, and F) as described under Experimental Methods. After 48 h the cells were washed, fixed, permeabilized, and processed for immunostaining as described before. Immunostaining was performed with anti-Flag monoclonal antibody (Clone M2; 10 μg/ml) and anti-Homer affinity-purified VHr20 antibody (5 μg/ml). The bound primary antibody was detected using either fluorescein-conjugated donkey anti-mouse IgG antibody (1/50) or Texas red-conjugated donkey anti-rabbit (1/50). Cells were analyzed by double immunofluorescence with confocal microscopy to detect mGluR1α (A, D, and G) and Homer (B, E, and H). (G, H, and I) Four times magnification of D, E, and F, respectively. The scale bar represents 10 μm.

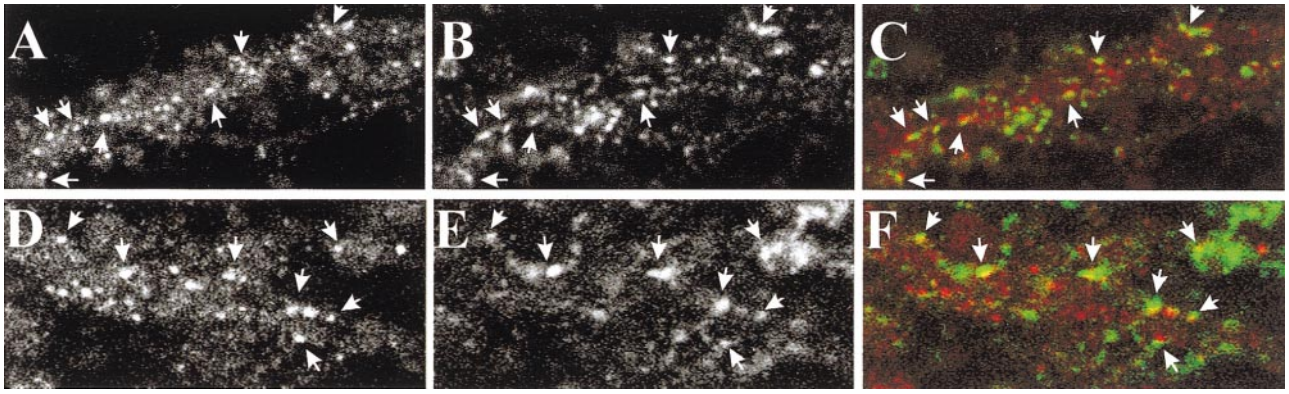


FIG. 8. Colocalization of mGluR1α and Homer-1c with synaptophysin in neurons. Cultured cortical neurons were transiently transfected with mGluR1α plus Homer-1c as described under Experimental Methods. After 48 h the cells were washed, fixed, permeabilized, and processed for immunostaining as described before. Immunostaining was performed with a mixture of affinity-purified anti-mGluR1α polyclonal antibody (F2-Ab; 5 μg/ml) and anti-synaptophysin monoclonal antibody (1/20) (A, B, and C) or anti-Homer affinity-purified VHr20 antibody (5 μg/ml) and anti-synaptophysin monoclonal antibody (1/20) (D, E, and F). The bound primary antibody was detected using either fluorescein-conjugated donkey anti-mouse IgG antibody (1/50) or Texas red-conjugated donkey anti-rabbit (1/50). A high-magnification image of a distal dendrite from a single transfected neuron is shown. Cells were analyzed by double immunofluorescence with confocal microscopy to detect mGluR1α (A), Homer (D), or synaptophysin (B and E). White arrows in C and F denote the colocalization between mGluR1α and Homer with synaptophysin, respectively.

poly-D-lysine-coated glass coverslips The medium was replaced after 1 h with DMEM containing 10% heat-inactivated FCS and 50 μg/ml gentamicin. The next day, the cells were washed with DMEM, and the medium was replaced with serum-free B27-supplemented Neurobasal medium (Gibco) containing 50 μg/ml gentamicin (BNG medium) (Brewer *et al.*, 1993). The cells were kept at 37°C in an atmosphere of 5% CO_2 for 4 weeks without frequent medium changes. On day 1 *in vitro* (1 DIV) the medium was replaced with fresh BNG medium containing 5 μM cytosine-β-D-arabinofuranose (Sigma) to inhibit glial growth and at 7 DIV the concentration of cytosine-β-D-arabinofuranose was diluted twofold.

Neurons were transfected using the cationic lipid transfection reagent Effectene (Qiagen) and the DNA encoding for the indicated proteins, using the protocol described by the manufacturer. Effectene–DNA complexes were added to the neurons (after 7 DIV) in fresh BNG medium and incubated for 18 h and the transfection was terminated by replacing the medium. Transient gene expression was continued for 24–48 h at 37°C.

Antibodies

The primary antibodies used for immunolabeling were anti-β-tubulin monoclonal antibody (Clone TUB 2.1; Sigma), affinity-purified anti-Flag monoclonal antibody (Clone M2; Eastman Kodak Company), affinity-purified anti-myc monoclonal antibody (Clone 9E10; Sigma), anti-synaptophysin monoclonal antibody (Boehringer Mannheim Biochemical), affinity-purified anti-Homer polyclonal antibody VHr20 (Ciruela *et al.*, 1999a), affinity-purified anti-mGluR1 polyclonal antibody F1-Ab (pan-mGluR1) (Ciruela and McIlhinney, 1997), and affinity-purified anti-mGluR1α polyclonal antibody F2-Ab (Ciruela *et al.*, 1999b). The secondary antibodies used were horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG and HRP-conjugated swine anti-mouse IgG (Dako); HRP-conjugated goat anti-mouse IgG (Promega); fluorescein-conjugated affinity-purified donkey anti-mouse IgG and Texas red dye-conjugated affinity-purified donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc).

Flag-Tagging of the mGluR1α and mGluR1β

The Flag epitope (DYKDDDDK) was introduced into the N-terminal of the mGluR1 between amino acids 57 and 58 using a PCR-based mutagenesis approach. Two fragments of the cDNA coding for mGluR1 (Masu *et al.*, 1991) were amplified with primers containing the Flag-epitope sequence. The first amplification was with primers GRF1 (5′-TCTTCGGTACCATGGTCCGGCTC-CTCTTGA-3′) and GRR1 (5′-TATCGTCATCGTCTTTG-TAATCAGGCTGGTGATGGAC-3′), and the second was with primers GRF2 (5′-ACAAAGACGATGACGATAA-ACCAGCCGAGAAGGTA-3′) and GRR2 (5′-AGCCAG-GGCCGATCACTCCAGCAATA-3′). Amplification con-

ditions were 1 cycle of 1 min at 95°C, 1 min at 50°C, and 3 min at 72°C and 20 cycles of 1 min at 95°C, 1 min at 65°C, and 3 min at 72°C followed by 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 obtained mGluR1 cDNA fragment, containing the Flag epitope, was used to substitute a corresponding fragment of the rat mGluR1α and mGluR1β cDNA in pcDNA3 vector (Invitrogen). The sequences of the Flag-tagged mGluR1α and mGluR1β cDNA were confirmed by DNA sequencing.

Cloning and Myc-Tagging of the Homer-1c/Vesl-1L Protein

Rat brain poly(A)$^+$ RNA was isolated from 0.1 g of cortex and cDNA was obtained using random hexamer primer as described previously (Soloviev *et al.*, 1999b). Homer-1c cDNA was amplified using proofreading *Pfu* DNA polymerase and primers RHF (5′-TTGGAATTC**ATG**GGGGAACAACCTATCTTC-3′) and RHR (5′-TACAGAGCCCAAACAGTCCTAC-3′), based on the sequences of the Vesl-1L/Homer-1c mRNAs (Kato *et al.*, 1998; Xiao *et al.*, 1998). The forward primer RHF included the *Eco*RI restriction endonuclease site (underlined) and encoded the first methionine of the Homer-1c protein (shown in bold). Amplification conditions were 1 cycle of 1 min at 96°C, 1 min at 60°C, and 10 min at 72°C and 35 cycles of 1 min at 96°C, 1 min at 65°C, and 3 min at 72°C followed by 10 min at 72°C. The amplified cDNA was cloned into the *Eco*RI–*Eco*RV sites of the mammalian expression vector pcDNA3 (Invitrogen). A cDNA coding for the *myc* epitope was obtained by annealing two synthetic oligonucleotides, MycF (5′-GATCATGGAACAAAAATTAATATCAGAAGAAGATTTAC-3′) and MycR (5′-AATTGTAAATCTTCTTCTGATATTAATTTTTGTTCCAT-3′). The obtained double-stranded DNA fragment (coding for MEQKLISEEDLQF) was ligated into the *Bam*HI and *Eco*RI sites of the Homer-1c expression plasmid in frame with and immediately preceding the first methionine of the Homer-1c protein.

Gel Electrophoresis and Immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) was performed using 6 or 10% polyacrylamide gels (Laemmli, 1970); proteins were immunoblotted to PVDF membranes (Immobilon-P; Millipore) using a semidry transfer system and developed with the enhanced chemiluminescence detection kit (Pierce), as described previously (Ciruela and McIlhinney, 1997).

Biotinylation of Cell Surface Proteins

HEK-293 cells transiently transfected with mGluR1α-Flag, mGluR1β-Flag, and/or Homer-1c were washed three times in borate buffer (10 mM H_3BO_3, pH 8.8; 150 mM NaCl) and then incubated with 50μg/ml sulfo-NHS-LC-biotin (Pierce) in borate buffer for 5 min at room temperature. After incubation, cells were washed three times in borate buffer and again incubated with 50μg/ml sulfo-NHS-LC-biotin in the same buffer for 10 min at room temperature, and then 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 (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 80,000*g* for 90 min. The supernatant was incubated with 80μl streptavidin–agarose beads (Sigma) for 3 h with constant rotation at 0–4°C. The beads were washed as described previously (Ciruela *et al.*, 1997). 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 Accumulation

HEK-293 cells transiently transfected with mGluR1α-Flag and/or Homer-1c were grown overnight in inositol and glutamate-free DMEM (ICN) supplemented with 7.5% dialyzed fetal bovine serum, containing *myo*-[^{3}H]inositol (5 μCi/ml) (Amersham). Cells were collected and washed exhaustively in prewarmed (37°C) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)-buffered Krebs solution (20 mM Hepes, pH 7.4; 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 2 units/ml glutamic-pyruvic transaminase. Cells were washed and incubated in Hepes/Krebs containing 10 mM LiCl (106 cells/0.2 ml) for 10 min. The IP production was initiated by the addition of 0.1 ml of prewarmed Hepes/Krebs containing various concentrations of quisqualic acid. After 20 min, the assay was ended by perchloric acid protein precipitation (5% perchloric acid; 30 min on ice). The samples were neutralized with neutralizing buffer (4 M KOH, 1 M Tris, 60 mM EDTA) and centrifuged for 20 min at 13,000*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.* (1983) 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 inositol phosphate accumulation was determined by nonlinear regression as described previously (Ciruela *et al*., 1997).

Cell Surface ELISA for Membrane mGluR1α

HEK-293 cells transiently transfected with mGluR1α-Flag and/or Homer-1c were assayed for surface expression of the Flag-epitope tag. Cells were chilled down and incubated at 4°C for 30 min in MPBS buffer (phosphate-buffered saline, pH 7.4, containing 4% nonfat milk powder and 1% normal goat serum). Then the cells were treated with 10μg/ml anti-Flag M2 antibody in MPBS buffer for 1 h at 4°C. After being washed with MPBS buffer, cells were incubated in normal DMEM growth medium at 37°C and in an atmosphere of 5% CO_2. At the indicated times the cells were quickly chilled, washed with PBS, and fixed with 4% paraformaldehyde in PBS for 15 min. Next, they were washed in PBS containing 20 mM glycine to quench the aldehyde groups. After further washing in MPBS buffer the cells were incubated with HRP-conjugated goat anti-mouse IgG (1:6000; 60 min, room temperature) and then washed three times in MPBS and twice in PBS. The reaction product was generated by addition of 0.5 ml K-Blue substrate (Neogen Corp., Kentucky) and quantitated after 10–15 min by measuring the absorbance at 620 nm. Data are presented as means ± SEM values from determinations performed in quintuplicate. The endocytic ratio of the receptor was calculated by nonlinear regression as described previously (Ciruela *et al*., 1997).

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% 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% Triton X-100 for 5 min. After a 30-min incubation in buffer A containing 1% BSA (buffer B), cells were incubated first with a mixture of anti-Flag monoclonal antibody (M2-Ab; 10μg/ml) and anti-Homer polyclonal antibody (VHr20; 2–4μg/m) in buffer B for 1 h at room temperature, washed, and stained with fluorescein-conjugated donkey anti-mouse IgG antibody (1/50) and Texas red-conjugated donkey anti-rabbit IgG antibody (1/50). The coverslips were rinsed for 30 min in buffer B and mounted with Vectashield immunofluorescence medium (Vector Laboratories, UK). Confocal microscope observations were made with a Leica TCS 4D (Leica Lasertechnik GmbH, Heidelberg, Germany) confocal scanning laser microscope adapted to an inverted Leitz DMIRBE microscope.

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REFERENCES

Ango, F., Albani-Torregrossa, S., Joly, C., Robbe, D., Michel, J. M., Pin, J. P., Bockaert, J., and Fagni, L. (1999). A simple method to transfer plasmid DNA into neuronal primary cultures: Functional expression of the mGluR5 receptor in cerebellar granule cells. *Neuropharmacology* **38:** 793–803.

Baude, A., Nusser, Z., Roberts, J. D. B., Mulvihill, E., McIlhinney, R. A. J., and Somogyi, P. (1993). The metabotropic glutamate receptor (mGluR1α) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. *Neuron* **11:** 771–787.

Beckerle, M. C. (1997). Zyxin: Zinc fingers at sites of cell adhesion. *BioEssays* **19:** 947–957.

Berridge, M. J., Dawson, R. M. C., Downes, C. P., Helson, J. P., and Irvine, R. R. (1983). Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J.* **212:** 473–492.

Bradke, F., and Dotti, C. G. (1998). Membrane traffic in polarized neurons. *Biochim. Biophys. Acta* **1404:** 245–258.

Brakeman, P. R., Lanahan, A. A., O'Brien, R., Roche, K., Barnes, C. A., Huganir, R. L., and Worley, P. F. (1997). Homer: A protein that selectively binds metabotropic glutamate receptors. *Nature* **386:** 284–288.

Brewer, G. J., Torricelli, J. R., Evege, E. K., and Price, P. J. (1993). Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J. Neurosci. Res.* **35:** 567–576.

Ciruela, F., and McIlhinney, R. A. J. (1997). Differential internalisation of mGluR1 splice variants in response to agonist and phorbol esters in permanently transfected BHK cells. *FEBS Lett.* **418:** 83–86.

Ciruela, F., Saura, C., Canela, E. I., Mallol, J., Lluis, C., and Franco, R. (1997). Ligand-induced phosphorylation, clustering and desensitisation of A1 adenosine receptors. *Mol. Pharmacol.* **52:** 788–797.

Ciruela, F., Soloviev, M. M., and McIlhinney, R. A. J. (1999a). Coexpression ofmGluR1α and Homer-1a/Vesl-1S increases the cell surface expression of the receptor. *Biochem. J.* **341:** 795–803.

Ciruela, F., Robbins, M. J., Willis, A. C., and McIlhinney, R. A. J. (1999b). Interactions of the C terminus of metabotropic glutamate

receptor type 1α with rat brain proteins: Evidence for a direct interaction with tubulin. *J. Neurochem.* **72:** 346–354.

Craven, S. E., and Brendt, D. S. (1998). PDZ proteins organize synaptic signaling pathways. *Cell* **93:** 495–498.

Dong, H. L., O'Brien, R. J., Fung, E. T., Lanahan, A. A., Worley, P. F., and Huganir, R. L. (1997). GRIP: A synaptic PDZ domain-containing protein that interacts with AMPA receptors. *Nature* **386:** 279–284.

Ehlers, M. D., Mammen, A. L., Lau, L. F., and Huganir, R. L. (1996). Synaptic targeting of glutamate receptors. *Curr. Opin. Cell. Biol.* **8:** 484–489.

Hunt, C. A., Schenker, L. J., and Kennedy, M. B. (1996). PSD-95 is associated with the postsynaptic density and not with the presynaptic membrane at forebrain synapses. *J. Neurosci.* **16:** 1380–1388.

Jordan, M., Schallhorn, A., and Wurm, F. M. (1996). Transfecting mammalian cells: Optimization of critical parameters affecting calcium phosphate precipitate formation. *Nucleic Acids Res.* **24:** 596–601.

Kato, A., Ozawa, F., Saitho, Y., Hirai, K., and Inokuchi, K. (1997). vesl, a gene encoding a VASP/Ena family related protein, is upregulated during seizure, long-term potentiation and synaptogenesis. *FEBS Lett.* **412:** 183–189.

Kato, A., Ozawa, F., Saitoh, Y., Fukazawa, Y., Sugiyama, H., and Inokuchi, K. (1998). Novel members of the Vesl/Homer family of PDZ proteins that bind metabotropic glutamate receptors. *J. Biol. Chem.* **273:** 23969–23975.

Kim, E., Cho, K. O., Rothschild, A., and Sheng, M. (1996). Heteromultimerization and NMDA receptor-clustering activity of chapsyn-110, a member of the PSD-95 family of proteins. *Neuron* **17:** 103–113.

Kim, S. K. (1997). Polarized signalling: Basolateral receptor localization in epithelial cells by PDZ-containing proteins. *Curr. Opin. Cell. Biol.* **9:** 853–859.

Knopfel, T., Kuhn, R., and Allgeier, H. (1995). Metabotropic glutamate receptors: Novel targets for drug development. *J. Med. Chem.* **38:** 1417–1426.

Koenig, J. A., and Edwardson, J. M. (1997). Endocytosis and recycling of G protein-coupled receptors. *Trends Pharmacol. Sci.* **18:** 276–286.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature (London)* **227:** 680–685.

Lujan, R., Nusser, Z., Roberts, J. D. B., Shigemoto, R., and Somogyi, P. (1996). Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the hippocampus. *Eur. J. Neurosci.* **8:** 1488–1500.

Lujan, R., Roberts, J. D. B., Shigemoto, R., Ohishi, H., and Somogyi, P. (1997). Differential plasma membrane distribution of metabotropic glutamate receptors mGluR1a, mGluR2 and mGluR5 relative to neurotransmitter sites. *J. Chem. Neuroanat.* **13:** 219–241.

Marsh, M., and Pelchen-Matthews, A., Eds. (1996). *Endocytic and Exocytic Regulation of CD4 Expression and Function.* Springer-Verlag, Berlin/Heidelberg.

Masu, M., Tanabe, Y., Tsuchida, K., Shigemoto, R., and Nakanishi, S. (1991). Sequence and expression of a metabotropic glutamate receptor. *Nature* **349:** 760–765.

Matter, K., and Mellman, I. (1994). Mechanisms of cell polarity: Sorting and transport in epithelial cells. *Curr. Opin. Cell. Biol.* **6:** 545–554.

Muller, B. M., Kistner, U., Kindler, S., Chung, W. J., Kuhlendahl, S., Fenster, S. D., Lau, L. F., Veh, R. W., Huganir, R. L., Gundelfinger, E. D., and Garner, C. C. (1996). SAP102, a novel postsynaptic protein that interacts with NMDA receptor complexes in vivo. *Neuron* **17:** 255–265.

Niethammer, M., Kim, E., and Sheng, M. (1996). Interaction between the C terminus of the NMDA receptor subunits and multiple members of the PSD-95 family of membrane associated guanylate kinases. *J. Neurosci.* **16:** 2157–2163.

O'Brien, R. J., Lau, L. F., and Huganir, R. L. (1998). Molecular mechanisms of glutamate receptor clustering at excitatory synapses. *Curr. Opin. Neurobiol.* **8:** 364–369.

Ohishi, H., Nomura, S., Ding, Y.-Q., Shigemoto, R., Wada, E., Kinoshita, A., Li, J.-L., Neki, A., Nakanishi, S., and Mizumo, N. (1995). Presynaptic localization of a metabotropic glutamate receptor, mGluR7, in the primary afferent neurons and immunohistochemical study in the rat. *Neurosci. Lett.* **202:** 85–88.

Pelchen-Matthews, A., Armes, J. E., and Marsh, M. (1989). Internalization and recycling of CD4 transfected into HeLa and NIH-3T3 cells. *EMBO J.* **8:** 3641–3649.

Pickering, D. S., Thomsen, C., Suzdak, P. D., Fletcher, E. J., Robitaille, R., Salter, M. W., Macdonald, J. F., Huang, X. P., and Hampson, D. R. (1993). A comparison of 2 alternatively spliced forms of a metabotropic glutamate receptor coupled to phosphoinositide turnover. *J. Neurochem.* **61:** 85–92.

Pin, J.-P., and Duvoisin, R. (1995). The metabotropic glutamate receptors: Structure and functions. *Neuropharmacology* **34:** 1–26.

Rongo, C., Whitfield, C. W., Rodal, A., Kim, S. K., and Kaplan, J. M. (1998). Lin-10 is a shared component of the polarized protein localization pathways in neurones and epithelia. *Cell* **94:** 751–759.

Sheng, M. (1996). PDZs and receptor/channel clustering: Rounding up the latest suspects. *Neuron* **17:** 575–578.

Shigemoto, R., Kinoshita, A., Wada, E., Nomura, S., Ohishi, H., Takada, M., Flor, P. J., Neki, A., Abe, T., Nakanishi, S., and Mizuno, N. (1997). Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. *J. Neurosci.* **17:** 7503–7522.

Shigemoto, R., Kulik, A., Roberts, J. D. B., Ohishi, H., Nusser, Z., Kaneko, T., and Somogyi, P. (1996). Target-cell-specific concentration of a metabotropic glutamate receptor in the presynaptic active zone. *Nature* **381:** 523–525.

Soloviev, M. M., Ciruela, F., Chan, W. Y., and McIlhinney, R. A. J. (1999a). The molecular characterization of a family of Homer proteins which are expressed constitutively in mammalian brain and cell lines. *Biochem. Soc. Trans.* **27:** A113.

Soloviev, M. M., Ciruela, F., Chan, W. Y., and McIlhinney, R. A. J. (1999b). Identification cloning and analysis of expression of a new alternatively spliced form of the metabotropic glutamate receptor mACaR, mRNA. *Biochem. Biophys. Acta* **1446:** 161–166.

Stowell, J. N., and Craig, A. M. (1999). Axon/dendrite targeting of metabotropic glutamate receptors by their cytoplasmic carboxy-terminal domains. *Neuron* **22:** 525–536.

Tanabe, Y., Masu, M., Ishii, T., Shigemoto, S., and Nakanishi, S. (1992). A family of metabotropic glutamate receptors. *Neuron* **8:** 169–179.

Tu, J. C., Xiao, B., Naisbitt, S., Yuan, J. P., Petralia, R. S., Brakeman, P., Doan, A., Aakalu, V. K., Lanahan, A. A., Sheng, M., and Worley, P. F. (1999). Coupling mGluR/Homer and PSD-95 complexes by the shank family of postsynaptic density proteins. *Neuron* **23:** 583–592.

Xiao, B., Tu, J. C., Petralia, R. S., Yuan, J. P., Doan, A., Breder, C. D., Ruggiero, A., Lanahan, A. A., Wenthjold, R. J., and Worley, P. F. (1998). Homer regulates the association of group 1 metabotropic glutamate receptors with multivalent complexes of Homer-related synaptic proteins. *Neuron* **21:** 707–716.

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