# Cell surface expression of the metabotropic glutamate receptor type 1α is regulated by the C-terminal tail

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**Abstract The cell surface expression of metabotropic glutamate receptor type 1 splice variants has been studied using cell surface biotinylation. Co-expression of the last 86 residues of the C-terminal tail of mGluR1α (F2-protein) with mGluR1α caused a significant reduction of the amount of the cell surface receptor when compared to that in cells transfected with mGlur1α alone, and this was accompanied by a reduction in the production of inositol following agonist stimulation of the cells. In contrast, cell surface expression of mGluR1β was unaltered by co-expression with the F2-protein. These results suggest that the C-terminal tail of mGluR1α regulates cell surface expression of the receptor.**


*Key words:* Cell surface expression; Metabotropic; Glutamate; mGluR1α; Receptor targeting

## 1. Introduction

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 [1]. 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 I mGluRs [2,3]. These share the strongest sequence homology (62% identical at the amino acid level) and are coupled to phospholipase C in transfected cells with quisqualic acid (Quis) as their most potent agonist.

Immunocytochemical studies have shown that the different groups of the mGluRs show differential targeting in neurones with the group I mGluRs being predominantly post-synaptic whilst the group 2 receptors (mGluR2 and mGluR3) and group 3 (mGluR4, mGluR6, mGluR7 and mGluR8) are generally pre-synaptic [4–7]. The precise synaptic location of mGluR1α is highly ordered, with the protein present in an annulus which surrounds the post-synaptic density [4,8,9]. One possible explanation for this specific localization of mGluR1α is that this 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, which interact with specific C-terminal sequences of these receptors have been described [10–14]. A novel PDZ domain containing protein which binds specifically to the C-terminal residues of mGluR1α and mGluR5, has recently been described [15]. This protein, termed Homer, was isolated as 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-2/Vesl-2 and Homer-3 [17,18]. The former is a C-terminal splice variant of the original Homer protein (re-named Homer-1a/Vesl-1S) but which shares the same N-terminal 175 amino acids. Unlike Homer-1b/Vesl-1L, Homer-2/Vesl-2 and Homer-3 which are constitutively expressed, Homer-1b, Homer-1c/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.

In order to examine the functional role of the C-terminal tail in the targeting of mGluR1 we have examined the consequence of its co-expression with mGluR1α and mGluR1β in transiently transfected HEK-293 cells. The results indicate a role for this domain in the cell surface targeting of mGluR1α.

## 2. Materials and methods

### 2.1. Mammalian cell culture and transfections

HEK-293 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 80–90% confluency was obtained. For the transient expression of proteins the following procedures were followed. HEK-293 cells growing in 75 $cm^3$ dishes were transiently transfected with 10 μg of DNA encoding for the indicated proteins by calcium phosphate precipitation [19]. In order to keep the ratio of DNA in co-transfections, 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.

### 2.2. Antibodies

The primary antibodies used for immunolabelling were: anti-β-tubulin monoclonal antibody (clone TUB 2.1, Sigma), affinity purified anti-mGluR1 polyclonal antibody F1-Ab (pan-mGluR1) [20] and affinity purified anti-mGluR1α polyclonal antibody F2-Ab [21]. The secondary antibodies used were: horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG and HRP-conjugated swine anti-mouse IgG (Dako).

### 2.3. Construction of F2-protein

A histidine tagged fusion protein containing the last 86 amino acids of mGluR1α carboxy-terminal tail (Fig. 1), residues 1114–1199 [21], was produced by cloning a *Pst*I-*Pst*I fragment of pmGR1 [22] into the bacterial expression vector pET-28c(+) (Novagen) and then the same fragment was subcloned into the pcDNA3 mammalian expression vector (Invitrogen) to produce a protein termed F2-protein.

### 2.4. Gel electrophoresis and immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 6% or 12% polyacrylamide gels [23], proteins were immunoblotted to PVDF membranes (Immobilon-P, Millipore) using a semi-dry transfer system and developed with the

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enhanced chemiluminescence detection kit (Pierce), as previously described [21].

*2.5. Biotinylation of cell surface proteins*

HEK-293 cells transiently transfected with mGluR1α, mGluR1β and/or F2-protein were washed three times in borate buffer (10 mM $H_3BO_3$, pH 8.8; 150 mM NaCl) and then incubated with 50 μg/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 50 μg/ml Sulfo-NHS-LC-Biotin in borate 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 $80\,000\times g$ for 90 min. The supernatant was incubated with 80 μl streptavidin-agarose beads (Sigma) for 3 h with constant rotation at 0–4°C. The beads were washed as described previously [21]. Immune complexes were dissociated by adding 60 μl of SDS-PAGE sample buffer and heated to 100°C for 5 min and then resolved by SDS-polyacrylamide gel electrophoresis on 6% gels. The gels were run and immunoblotted as described above.

*2.6. Inositol phosphate (IP) accumulation*

HEK-293 cells transiently transfected with mGluR1α, mGluR1β and/or F2-protein 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-piperazine ethanesulfonic 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 U/ml of glutamic-pyruvic transaminase (GPT). Cells were washed and incubated in HEPES/Krebs containing 10 mM LiCl ($10^6$ cells/0.2 ml) for 10 min. The IP production was initiated by the addition of 0.1 ml of pre-warmed HEPES/Krebs containing 100 μM quisqualic acid. After 20 min the assay was ended by protein precipitation (5% perchloric acid, 30 min on ice). Samples were neutralized with neutralizing buffer (4 M KOH, 1 M Tris, 60 mM EDTA) were centrifuged for 20 min at $13\,000\times g$, 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. [24] and the radioactivity was measured.

*2.7. Statistics*

Data are presented as mean ± S.E.M. of quintuplicate values from five separate experiments. One-way analysis of variance was used to determine significance among groups, after which the modified *t*-test with the Bonferroni correction was used for comparison. A value of $P < 0.01$ was considered to be statistically significant.

## 3. Results

HEK-293 cells transiently transfected with mGluR1α and immunoblotted with F1-Ab antibody, yielded an immunoreactive protein of 150 kDa of molecular weight (MW) corresponding to mGluR1α receptor (Fig. 2, lane 2), together with higher MW bands one of which (≈300 kDa) may represent a dimeric form of the receptor [20]. When cells were transfected with mGluR1β, the same antibody yielded two immunoreactive bands, with apparent MW of 94 kDa and 190 kDa (Fig. 2, lane 3), which correspond to mGluR1β receptor monomer and dimer respectively, which is in good agreement with results reported previously [20]. Finally, transient expression of F2-protein (Fig. 1) in HEK-293 cells and immunoblotting with F2-Ab antibody gave rise to an immunoreactive band

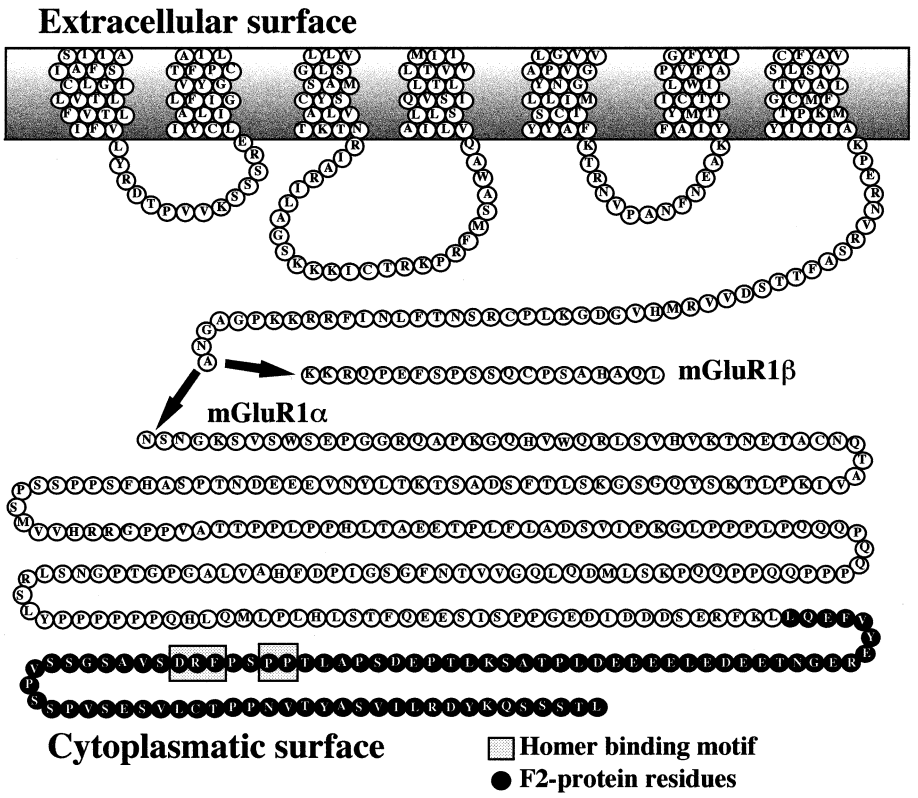


Fig. 1. Schematic representation of the rat mGluR1α and mGluR1β C-termini. The proposed membrane spanning topography of the mGluR1 splice variants is also shown. The amino acid sequence used to generate the F2-protein is denoted by the filled circles. In the dotted square is shown the proline rich motif (PPXXFr) which interacts with Homer proteins. The figure is adapted from that shown in Pin and Duvoisin [2].

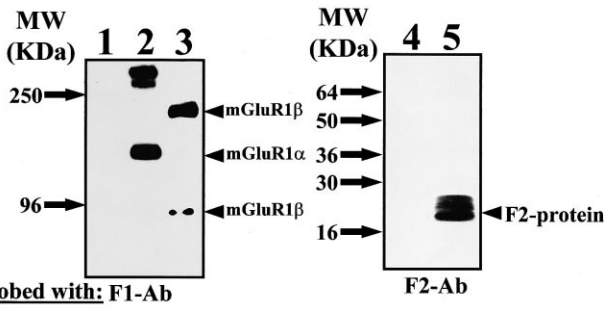


Fig. 2. Immunoblot of mGluR1α, mGluR1β and F2-protein in transiently transfected HEK 293 cells. HEK-293 cells (lanes 1 and 4) were transiently transfected with either 10 μg cDNA encoding mGluR1α (lane 2), mGluR1β (lane 3) or F2-protein (lane 5) as described in Section 2. After 48 h crude cell extracts (40 μg of protein) were analyzed by SDS-PAGE and immunoblotted using anti-mGluR1 affinity purified F1 antibody (2–4 μg/ml) (lanes 1, 2 and 3) or anti-mGluR1α affinity purified F2 antibody (2–4 μg/ml) (lanes 4 and 5). Immunoreactive bands were detected with swine anti-rabbit (1:5000) secondary antibody conjugated to horseradish peroxidase followed by chemiluminiscence detection. In this and all subsequent figures the position of the molecular mass markers in kDas is indicated on the left. The position of mGluR1α, mGluR1β and F2 protein are indicated with arrows on the right.

with an apparent MW of 18–20 kDa (Fig. 2, lane 5), close to that predicted from the F2-protein amino acid sequence. The appearance of this band suggested that it may have undergone some proteolysis despite the incorporation of protease inhibitors in the cell lysates. Control untransfected HEK-293 cells did not show any immunoreactivity for either F1-Ab or F2-Ab (Fig. 2, lanes 1 and 4).

In order to study the effects of F2-protein expression on the surface expression of mGluR1α and mGluR1β we isolated the membrane receptors 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 mGluR1α receptor present in the cell surface is decreased significantly when mGluR1α is co-expressed with F2-protein compared to the singly expressed mGlur1α (Fig. 3). However, it is important to note that under the same conditions the cell surface expression of mGluR1β was unaffected by co-expression of the F2-protein (Fig. 3). Quantitation of the immunoblots indicated that the levels of surface receptor had decreased by up to 5-fold in the F2-protein co-transfected cells. Since neither tubulin nor F2-protein could be detected in the streptavidin isolates the biotin ester had not penetrated the cell membrane (see insets to Fig. 3).

The functional consequences of the co-expression of F2-protein with mGluR1α on second messenger coupling of the receptor were also examined. HEK-293 cells transiently transfected with mGluR1α, mGluR1β and/or F2-protein, were treated with quisqualic acid and the accumulation of IPs measured. As shown in Fig. 4, the quisqualic acid induced increase of IP production in the HEK-293 cells co-transfected with mGluR1α and F2-protein was lower (five times less) than in the cells transfected with mGluR1α alone. No significant effect of F2-protein on agonist induced IP production was observed in cells co-transfected with mGluR1β. As expected HEK-293 cells transfected with F2-protein alone showed no effect on IP production (Fig. 4). This reduction of IP generation in the F2-protein and mGluR1α co-transfected cells is consistent with the decrement in cell surface mGluR1α in the same cells as shown above.

## 4. Discussion

The results presented here show that the last 86 residues of mGluR1α may play a role in regulating the plasma membrane targeting of this form of the receptor, since co-expression of a protein containing them with mGluR1α reduces the amount of receptor on the cell surface. Co-expression of the F2-protein with the mGluR1β splice variant of mGluR1 had no effect on its surface expression presumably due to the changes in the C-terminal amino acid sequence resulting from the alternative splicing of the mRNA. Together these results also suggest that the splice variants mGluR1α and mGluR1β contain motifs within their C-terminal tail that determine either their differential delivery to, and/or their differential anchoring in the plasma membrane. Such a suggestion is consistent with both, our earlier demonstration of the different rates of internalization of mGluR1α and mGluR1β in transfected cells exposed to agonist [20], and the more general finding that protein sorting to specific locations within cells is often determined by motifs located at the intracellular cytoplasmic tail of the protein [25]. It should be noted that C-terminal domain of mGluR1α cannot be essential for surface expression of the receptor, since extensive deletion mutants of this domain are

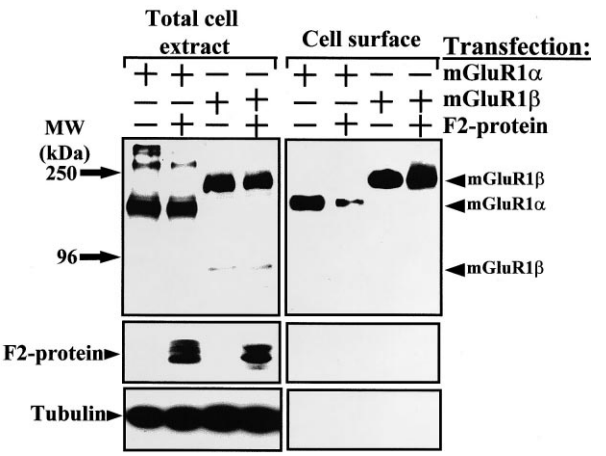


Fig. 3. Effects of co-expression of mGluR1α and mGluR1β with F2-protein on receptor surface expression in transiently transfected HEK-293 cells. HEK-293 cells were transiently transfected with either, 5 μg cDNA encoding mGluR1α and 5 μg cDNA encoding LacZ reporter (lane 1), 5 μg cDNA encoding mGluR1α and 5 μg cDNA encoding F2-protein (lane 2), 5 μg cDNA encoding mGluR1β and 5 μg cDNA encoding LacZ reporter (lane 3), or 5 μg cDNA encoding mGluR1β and 5 μg cDNA encoding F2-protein (lane 4), as described in Section 2. After 48 h crude cell extracts (40 μg of protein) was analyzed by SDS-PAGE and immunoblotted using anti-mGluR1 affinity purified F1 antibody (2–4 μg/ml) (left upper panel), anti-mGluR1α affinity purified F2 antibody (2–4 μg/ml) (left middle panel) or a monoclonal anti-β-tubulin (1:200) (left lower panel). Similarly transiently transfected HEK-293 cells were cell surface biotinylated as described in Section 2. Biotin labeled proteins were isolated with streptavidin-agarose beds, analyzed by SDS-PAGE and immunoblotted using the same antibodies as described before. Immunoreactive bands were detected with swine anti-rabbit (1:5000) secondary antibody conjugated to horseradish peroxidase followed by chemiluminiscence detection. The position of mGluR1α, mGluR1β, F2 protein and Tubulin are indicated with arrows.

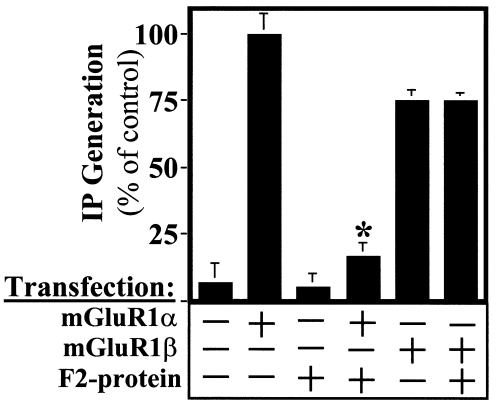


Fig. 4. Quisqualic acid induced IP accumulation in HEK-293 transiently transfected with mGluR1α, mGluR1β and F2-protein. HEK-293 were transiently transfected transfected with 5μg of the indicated cDNA as described in Fig. 3. After 24 h of expression cells were labeled overnight with *myo*-[$^3$H]inositol, stimulated with 100 μM quisqualic acid for 20 min and the production of total IP was then determined as described in Section 2. Results are mean of five independent experiments ± S.E.M. and expressed as a relative percentage of the maximal response in the single mGluR1α transfected cells. $*P < 0.01$ when compared with HEK-293 transfected with mGluR1α alone.

expressed at the cell surface and are functional [26]. However this does not preclude the C-terminus having a role in modulating plasma membrane levels of the receptor.

Once located in the appropriate cell compartment neurotransmitter receptors also need to be located at precise positions in the synapse. The discovery of a family of PDZ containing proteins which interact with the C-terminal-domains of specific ionotropic glutamate receptors has led to the suggestion that the location of these receptors at precise synaptic locations is mediated by these proteins [27]. Recently a similar family of proteins, termed Homers, has been described which bind to a proline rich region within the last 86 amino acids of the C-terminal tail of mGluR1α and mGluR5 [18]. Certain members of the Homer family can form homo- and heteromeric complexes, via C-terminal coil-coil protein interaction domains [17]. The resulting dimeric molecules would be capable of crosslinking mGluR1α molecules via their single N-terminal EVH (enabled/VASP homology) domains. These interact with a proline rich motif (PPXXFr) contained in the C-terminal region of mGluR1α or mGluR5 and in the F2-protein used in this study. This EVH domain can also interact with a the proline rich motif which is present in proteins other than the metabotropic glutamate receptor, such as dynamin and the IP3 receptor [18]. Thus the Homer related proteins have the potential not only to link mGluR1α and mGluR5 receptors but also to cross-link these to other molecules. They could therefore give rise to multimeric protein complexes important for the targeting to, or stabilizing the association of these receptors with, the plasma membrane [18]. We have preliminary results indicating that HEK-293 cells constitutively express members of the Homer family of proteins similar to those found in rat brain (F. Ciruela, M.M. Soloviev and R.A.J. McIlhinney, unpublished observation) and in similar ratios. Consequently the disruption of the plasma membrane expression of mGluR1α by the F2-fusion protein could be due to its competing for the endogenously expressed Homer proteins in HEK 293 cells and thereby preventing their trafficking to the cell surface. Alternatively the retention of the receptor at the cell surface, also potentially mediated by the Homer proteins, could similarly be disrupted. Either or both of these mechanisms may explain the results presented here, which emphasize the importance of the C-terminal domain of mGlur1α in regulating the level of cell surface expression of this receptor.

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