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Molecular Determinants of Metabotropic Glutamate Receptor 1B Trafficking

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The metabotropic glutamate receptor mGluR1 undergoes alternative splicing to generate isoforms differing in C-terminal sequence. The mechanism by which these isoforms give different functional responses to agonists *in vitro* is so far unclear. Using the native mGluR1 and CD2-mGluR1 chimeric molecules, as well as their C-terminal truncations and mutants, we identified an endoplasmic reticulum (ER) retention signal Arg-Arg-Lys-Lys within the C-terminal sequence of mGluR1b. Its presence results in a much reduced cell surface expression of the receptor and chimeric molecules in cell lines and their restricted trafficking in neurones. This motif is also present in the C-terminus of mGluR1a, but its effect is overcome by a region of the mGluR1a-specific C-terminal sequence (amino acids 975–1098). Our results indicate that these splice variants of mGluR1 utilize different targeting pathways and suggest that this may be a general phenomenon in the metabotropic glutamate receptor gene family.

INTRODUCTION

Glutamate is the major excitatory neurotransmitter in the central nervous system and can exert its function through two different types of receptors namely ionotropic and metabotropic glutamate receptors (mGluRs). The latter constitute a family of large G-protein coupled receptors (GPCRs) that show little sequence homology with the subfamily 1 and 2, which comprise the smaller GPCRs. They do, however, share sequence similarities with both the extracellular Ca^{2+}-sensing receptors and the recently cloned $GABA_B$ receptors (Couve *et al.*, 2000; Blein *et al.*, 2000; Conn and Pin, 1997; Nakanishi and Masu, 1994; Pin and Duvoisin, 1995; 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 (Conn and Pin, 1997; Knopfel *et al.*, 1995; Nakanishi and Masu, 1994; Pin and Duvoisin, 1995; Tanabe *et al.*, 1992). These share the strongest sequence homology, couple to phospholipase C in transfected cells, and quisqualic acid is their most potent agonist. Both mGluR1 and mGluR5 have large intracellular C-terminal tails that undergo alternative splicing to generate different forms of the receptors. The two splice variants of mGluR5 both have similarly sized C-termini, whereas the different splice variants of mGluR1 have severely truncated C-terminal tails (Conn and Pin, 1997; Nakanishi and Masu, 1994; Pin and Duvoisin, 1995). To date four C-terminal splice variants of mGluR1 receptor have been described, namely mGluR1a, mGluR1b, mGluR1c, mGluR1d receptors (Pin *et al.*, 1992; Tanabe *et al.*, 1992).

The functional significance of these different splice variants has not yet been fully explored but studies have shown subtle differences in the responses of the different forms of these receptors when expressed in mammalian cells. Thus mGluR1a shows a higher affinity for agonists than mGluR1b or other short forms of mGluR1 (Flor *et al.*, 1996; Pickering *et al.*, 1993; Prezeau *et al.*, 1994). Different agonist induced internalization rates between mGluR1a and mGluR1b have also been reported (Ciruela and McIlhinney, 1997). The long forms of both mGluR1 and mGluR5 also show a constitutive agonist independent activity when expressed in HEK293 cells, whereas the short forms of mGluR1 do not (Joly *et al.*, 1995; Prezeau *et al.*, 1996). Recently a

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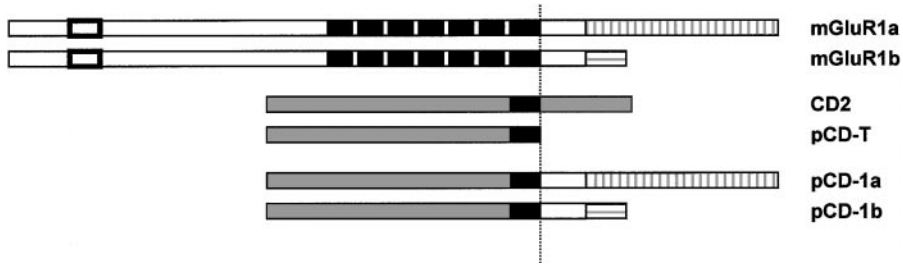

FIG. 1. Schematic representation of the pCD chimeras. The black boxes represent the transmembrane domains and the bold open boxes represent the site at which the FLAG epitope was introduced. The N-terminus and C-terminal domain of CD2 are shown as grey, the C-terminal tail of mGluR1a as vertical crosshatching, and that of mGluR1b as an open box with a horizontal line. The constructs pCD-1a and pCD-1b contain the full sequence of pCD-T followed by the full C-terminal sequence of mGluR1a or mGluR1b, respectively.

region of charged residues in the C-terminal tail of the short forms of mGluR1 has been suggested to negatively regulate the coupling of the mGluR1 splice variants to phospholipase C (Mary *et al.*, 1998). The short C-terminal splice variants of mGluR1 also seem to induce slower and more prolonged Ca^{2+} responses when expressed in both Xenopus oocytes and transfected cells than does mGluR1a (Joly *et al.*, 1995; Pin *et al.*, 1992; Simoncini *et al.*, 1993). In addition while the long form of mGluR1 exhibits coupling to adenylyl cyclase transfected cell lines the short forms do not (Joly *et al.*, 1995; Lin *et al.*, 1997; Pickering *et al.*, 1993).

Immunocytochemical studies have shown that the long forms of the group 1 mGluRs are dominantly post-synaptic and that they can be organized in a precise perisynaptic ring around the postsynaptic density (Baude *et al.*, 1993; Lujan *et al.*, 1996, 1997). While the C-terminal splice variants of mGluR1 are also postsynaptic, they may have, in some brain areas, a slightly different postsynaptic organization and extend further from the edge of the postsynaptic density than mGluR1a (Mateos *et al.*, 1998, 2000).

In order to examine if the C-terminal domains of mGluRs are sufficient to regulate their neuronal targeting, chimaeric molecules were made of mGluR1, using the rat T lymphocyte surface antigen CD2, which is a well characterized single transmembrane protein (He *et al.*, 1988; Moingeon *et al.*, 1989). Chimaeric proteins have been produced where the full cytoplasmic C-terminal tail of mGluR1a and 1b have replaced that of CD2. Using these molecules and their mutants, we have studied the trafficking of mGluR1s in both transiently transfected cell lines and primary cortical neuronal cultures.

RESULTS

Trafficking of mGluRs and CD2 Constructs in Rat Cortical Neurones in Primary Culture

In order to examine the molecular determinants important for the trafficking of the mGluR1b, we created the chimera pCD-1b in which the C-terminal domain of mGluR1b was transferred to CD2 (Fig. 1). Experiments using primary cultured neurones transfected with mGluR1b showed that the mGluR1b was predominantly found in the cell body with occasional punctate reactivity found in the proximal dendrites in permeabilized cells (Figs. 2a–2c). A similar pattern of staining was found when the pCD-1b chimera was transfected into the neurones (Figs. 2d–2f). In contrast transfection of pCD-T alone into the neurons resulted in an extensive expression of the molecule in the axon, cell soma, and dendrites (Figs. 2g–2i). Therefore splicing the C-terminal tail of mGluR1b to CD2 results in the restriction of the chimeric pCD-1b to the cell body and proximal dendrites and results in the pCD-1b having an intracellular cell distribution similar to that of the native mGluR1b receptor.

The C-Terminal Tail of mGluR1b Regulates Its Surface Expression

Expression of mGluR1a in HEK293 cells resulted in robust cell surface expression of the receptor and the intracellular immunoreactivity for the protein showed a reticular and perinuclear pattern of fluorescence (Fig. 3a). However, expression of mGluR1b in HEK293 cells resulted in a significantly reduced level of surface immunofluorescence which was characterized by the cultures having few relatively bright cells together with many more weakly stained cells with a granular or

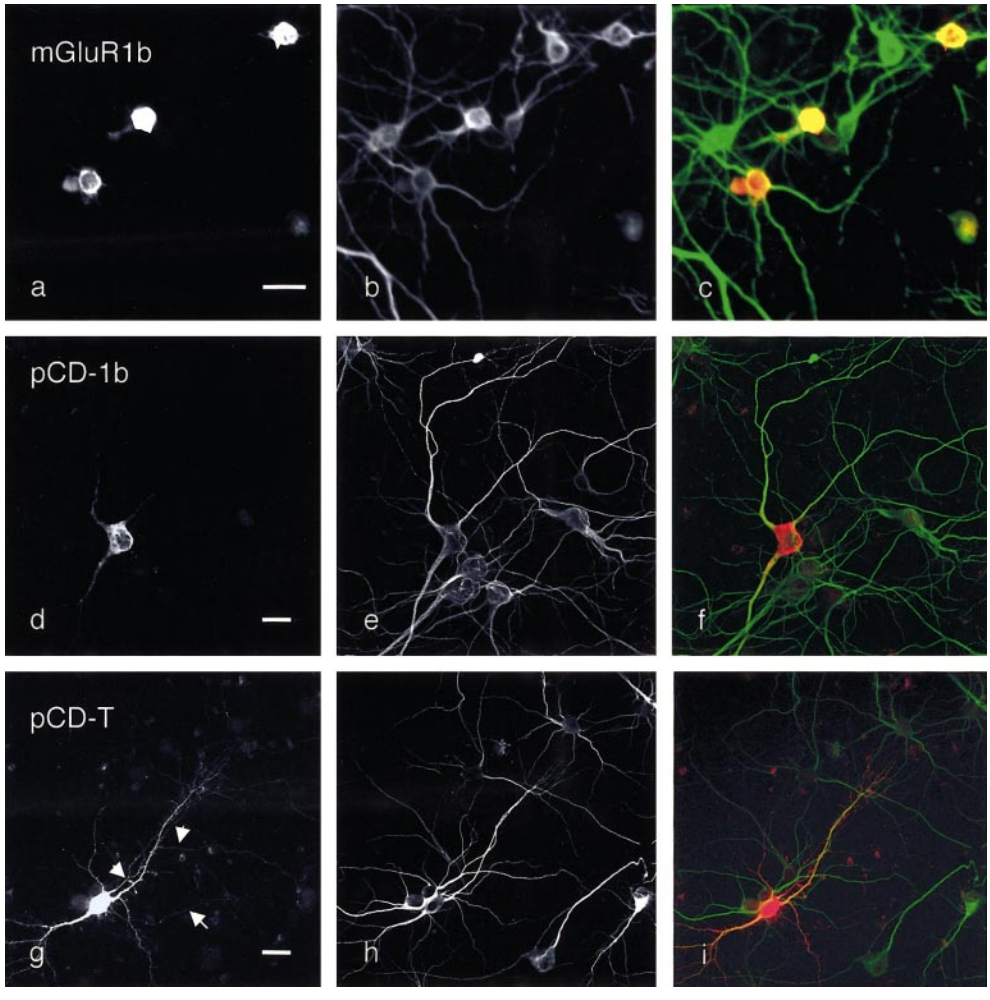

FIG. 2. mGluR1b and pCD-1b are retained in the soma of neurones. Immunofluorescent staining of primary cultures of 10-12 DIV rat cortical neurones 40 h after transfection with mGluR1b (a–c), pCD-1b (d–f), and pCD-T (g–i). The neurones were cultured and transfected with the different constructs as described under Materials and Methods. The cells were fixed and permeabilized with 0.2% Triton X-100 before being reacted with a mGluR1b specific antibody (a) and a rabbit anti-CD2 (d and g). At the same time the cells were also reacted with a mouse monoclonal antibody against MAP2 (b, e, h). These primary antibodies were detected using a Texas red-conjugated donkey anti-rabbit and an FITC-conjugated donkey anti-mouse antibody. The composite images are shown in c, f, and i, respectively. The scale bar is 20 μm and the arrows in (g) indicate a MAP2 negative process, which is reactive for pCD-T and is an axon.

punctate pattern of surface immunofluorescence (Fig. 3a). The intracellular staining for mGluR1b was also distinctive with a vesicular pattern of intracellular accumulations seen in the cells (Fig. 3a).

The surface immunofluorescence for pCD-T was extremely bright and the intracellular immunoreactivity for the protein showed a reticular and intense perinuclear distribution (Fig. 3b). The cell surface and intracellular immunofluorescent staining for pCD-1a is similar to that of pCD-T. Strikingly, introducing the C-terminus of mGluR1b into pCD-T resulted in a reduced surface expression of the protein, which like mGluR1b now showed a more heterogeneous intensity of surface staining in the cultures (Fig. 3b). In two quantitative cell surface ELISA experiments performed in COS-7 cells, the surface expression of pCD-1b was less than 10% of that of pCD-T. In permeabilized cells, the Elisa reaction gives equivalent signals for pCD-1b and pCD-T, indicating that the two proteins were synthesized to the same level. The effect of the introduction of the mGluR1b C-terminal sequence into pCD-T was even more dramatic on the intracellular distribution of the protein, causing it to become accumulated in intracellular vesicles (Fig. 3b).

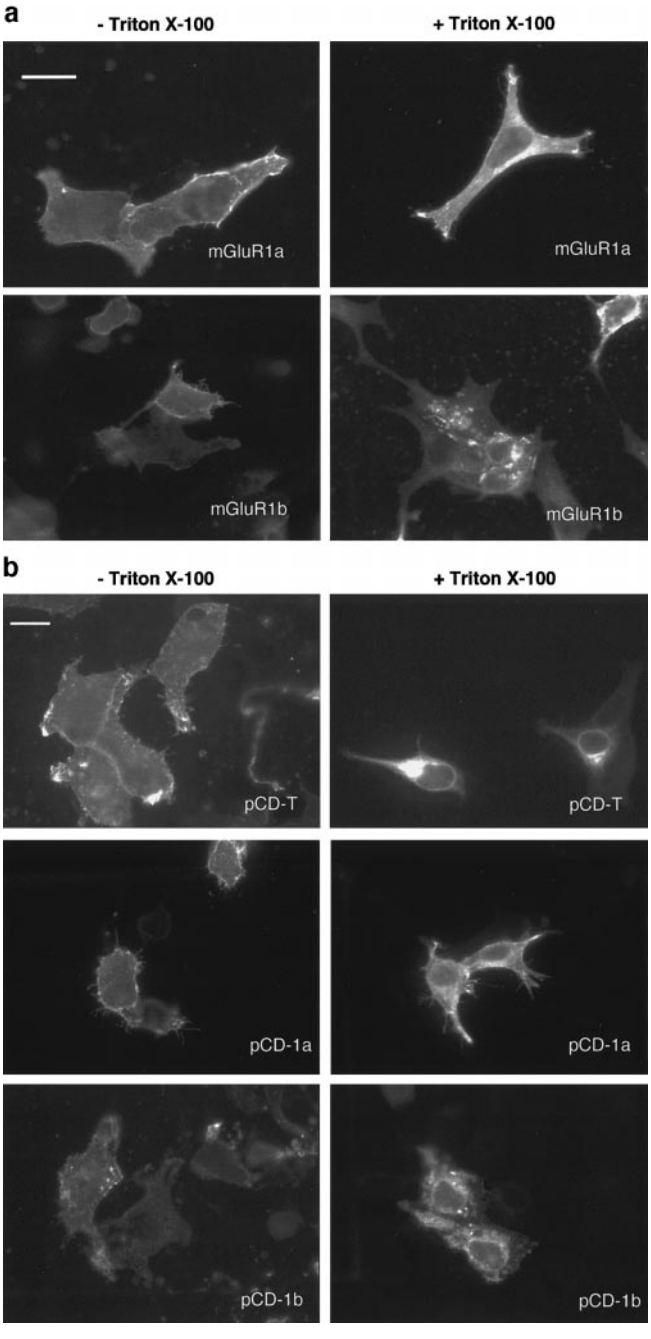

FIG. 3. mGluR1b and pCD-1b accumulate in an intracellular compartment. Immunofluorescence of HEK293 cells transfected with mGluR1a (a), mGluR1b (a), pCD-T (b), pCD-1a (b), and pCD-1b(b). Cells were either fixed with paraformaldehyde (−Triton X-100) or fixed and permeabilized with Triton X-100 (+Triton X-100), and reacted with a pan mGluR1 antibody F1 (a) or with OX34 (b) 48 h after transfection. The scale bar is 10 μm.

These data suggest that the C-terminal tail of mGluR1b is solely responsible for the alteration in the amount of cell surface pCD-1b and for the change in its intracellular distribution compared to that of pCD-T. The differences in the levels of cell surface expression between pCD-T and pCD-1b is not due to the two pCD constructs having different expression levels as, when the cells are permeabilized, both proteins give similar levels of immunofluorescence. In addition in immunoblotting experiments the expression levels of the different constructs were consistently equivalent for equal loadings of cell proteins.

mGluR1b and pCD-1b Are Retained in the Endoplasmic Reticulum

Because both mGluR1b and pCD-1b appear to be accumulating in intracellular vesicles, we investigated in which cell compartment they might be. For these experiments pCD-1b was transfected into COS-7 cells, as these allowed a better resolution of the intracellular compartments than the HEK293 cells. The results show clearly that the intracellular accumulations of pCD-1b co-stain for the endoplasmic reticulum marker calnexin, but not for the Golgi marker β-COP (Fig. 4). Thus the C-terminal tail of mGluR1b results in the accumulation of pCD-1b and by inference mGluR1b in the endoplasmic reticulum.

If the receptor was being retained in the endoplasmic reticulum then the majority of mGluR1b should be endoglycosidase H sensitive, since the receptor would not be passing through the Golgi apparatus. To test this hypothesis mGluR1b was expressed in HEK293 cells and the membranes treated with either endoglycosidase H (EndoH) or Protein N-glycosidase F (pNGase F). The latter should remove all the carbohydrate from the proteins. The results showed clearly that mGluR1b was indeed EndoH sensitive (Fig. 5a). That the EndoH sensitivity of mGluR1b was due to the C-terminal tail of the receptor is evidenced by the fact that introducing this into pCD-T caused this too to become predominantly EndoH sensitive (Fig. 5b). Interestingly rat brain cerebellar mGluR1b is fully EndoH resistant (Fig. 5c), suggesting that in neurons the receptor is not being retained in the endoplasmic reticulum. It should be noted that both CD2 and the truncated pCD-T form are heavily glycosylated at multiple sites and generally run as broad and heterogeneous bands. The effect of the introduced mGluR1b tail is therefore all the more striking. Since a limited amount of both mGluR1b and pCD-1b can be found on the cell surface we determined if the surface expressed material was EndoH resistant. In order to do this cells expressing pCD-1b were cell surface biotinylated and the streptavidin isolated protein subjected to glycosidase treatment. The results

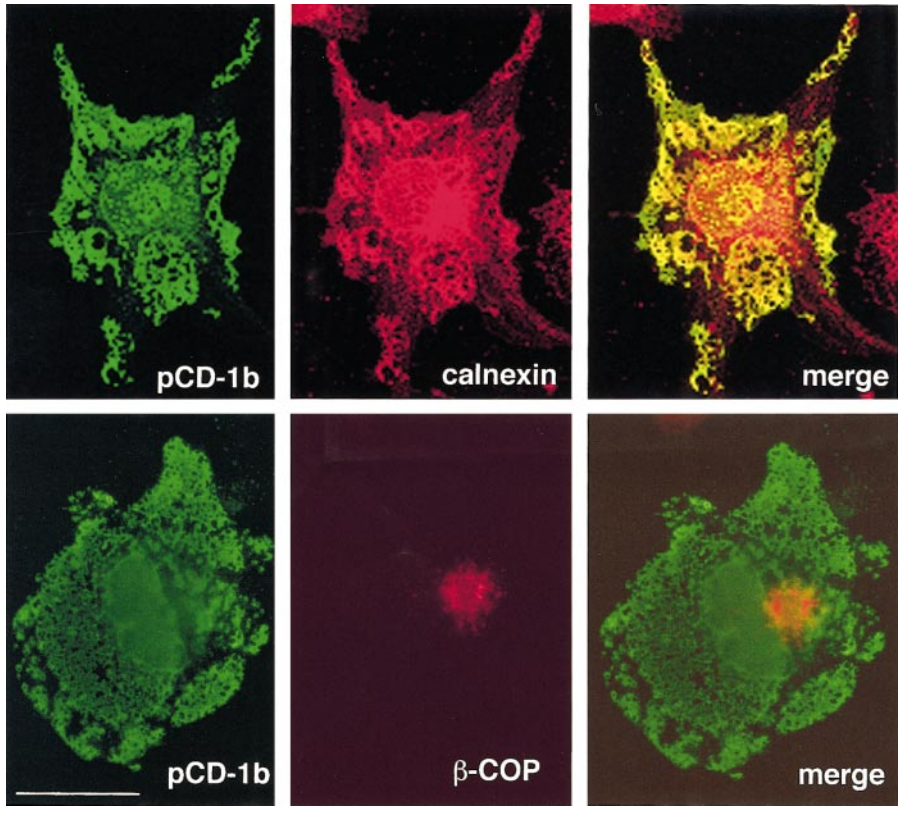

FIG. 4. Immunofluorescent identification of the cell compartment in which the pCD-1b accumulates. COS-7 cells were transfected with pCD-1b and 48 h after the transfected cells were fixed and permeabilized. They were then reacted with either rabbit anti-calnexin (ER specific) or rabbit anti β-COP (Golgi specific) together with OX34 to identify the pCD-1b. The bound primary antibodies were detected using goat Alexa Fluor 488-conjugated goat anti-rabbit and goat Alexa Fluor 543-conjugated anti-mouse antibodies.

showed that the cell surface pCD-1b was dominantly EndoH sensitive (Fig. 5d). Since tubulin could not be found in the streptavidin isolates, these were not contaminated with intracellular proteins (Fig. 5d) and therefore the result is not due to contamination of the streptavidin isolates with intracellular pCD-1b.

Identification of the Retention Signal

Scanning alanine mutagenesis, together with truncation mutants, of the C-terminal tail of mGluR1b were used to investigate which residues are important for the accumulation of the receptor in the endoplasmic reticulum. The mutated receptor was expressed in HEK293 cells and the effects of the mutation on the EndoH sensitivity of the receptor analyzed. The results showed that mutation of residues within the sequence after the splice site of the receptor or truncation of the receptor at this point had little effect on the EndoH sensitivity of the receptor (Fig. 6a). Mutation of the group of arginines and lysines to alanines caused the mutant protein to become fully EndoH resistant (Fig. 7a; mGluR1b-MM18). Since truncation of the receptor before the charged residues RRKK also results in full EndoH resistance (Fig. 7a; mGluR1-M9), whereas truncation after these residues results in EndoH sensitivity (Fig. 7a; compare mGluR1-M7 and mGluR1-M9), we conclude that these residues are those most important for the accumulation of the receptor in the endoplasmic reticulum.

mGluR1a Contains a Domain Which Overrides the Retention Signal

Since the RRKK motif is present in the C-termini of both mGluR1a and mGluR1b, but mGluR1a is well expressed at the cell surface and is predominantly EndoH resistant (Fig. 7c), mGluR1a must contain a sequence that prevents the endoplasmic retention signal from functioning. In order to determine where this lies

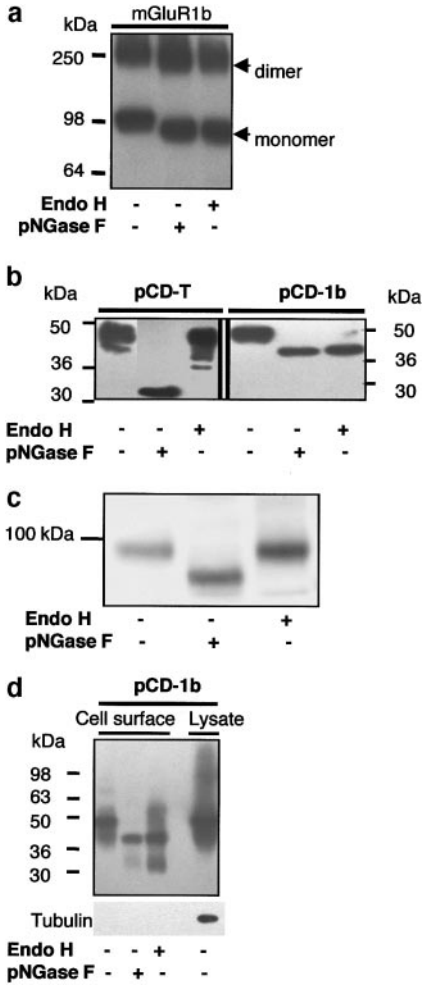

FIG. 5. Both mGluR1b and the pCD-1b chimeras are EndoH sensitive as is surface pCD-1b. Membranes prepared from cells transfected with mGluR1b (a), pCD-T and pCD-1b (b), or rat cerebellar membranes (c) were subjected to endoglycosidase treatment as shown and the membranes analyzed by SDS gel electrophoresis and immunoblotting with (a) anti-Flag, (b, d) rabbit anti-CD2, and (c) mGluR1b specific antibodies. The dimeric upper band of mGluR1b also shows a shift following endoglycosidase treatment with either pNGaseF or EndoH. However, because this band runs as a fairly broad band the differential migration of this band following enzyme treatment was not always so readily seen. Therefore we have in other figures shown only the lower monomeric form of the receptor. In (d), cells transfected with pCD-1b were cell surface biotinylated and the streptavidin isolates treated with the endoglycosidases as shown. The sensitivity of the cell surface protein to EndoH is clear. The lower molecular weight bands seen in the digested tracks may represent some degradation of the proteins during glycosidase treatment. The upper bands are probably aggregates of the proteins.

we examined the Endoglycosidase sensitivity of a series of truncation mutants of mGluR1a and their pCD equivalents (see Fig. 6). None of the truncations before that in mGluR1a-M5 affected the endoglycosidase sensitivity of the proteins which were as EndoH resistant as mGluR1a (data not shown). However, deletion of the region between mGluR1a-M5 mGluR1a-M6 resulted in the protein becoming largely Endo-H-sensitive (Fig. 7b). Therefore the region between residues 975–1098 of the C-terminal tail of mGluR1a is responsible for the blockade of the ER retention signal.

Mutation of the RRKK Motif Increases Cell Surface Expression and Neuronal Trafficking

Removal or mutation of the RRKK motif increases the cell surface expression of mGluR1b, as shown for constructs mGluR1-M9 and mGluR1b-MM18, respectively, when compared to mGluR1b (Fig. 8). Mutation of the RRKK motif also alters the neuronal trafficking of both mGluR1b and pCD-1b as shown in Fig. 9 for pCD-MM18 (a) and mGluR1b-MM18 (b). These mutants show enhanced dendritic trafficking, which is dramatically increased when compared to pCD-1b or mGluR1b in the permeabilized cells (+Triton X-100). In addition it increases significantly the surface staining for the pCD-MM18 mutant with the surface expressed protein widely distributed over the cell soma and neurites (−Triton X-100).

DISCUSSION

The results presented here show that mGluR1b contains an endoplasmic reticulum retention signal in the cytoplasmic domain and that this contains four positively charged amino acids that are essential for the trafficking signal. Unlike other ER retention signals such as KDEL and KKXX, which are C-terminal (Teasdale and Jackson, 1996), the sequence described here is found at some distance from the C-terminus. The accumulation of mGluR1b in the ER was surprising but could explain the different intracellular distributions of mGluR1a and mGluR1b noted by others (Pickering *et al.*, 1993). It is interesting to note that charged residue motifs contained within the C-terminal region, or in intracellular loops, of other proteins have been found to regulate the surface expression of both K_{ATP} channels (RKR) (Zerangue *et al.*, 1999) and the $GABA_BR1$ receptor subunit (RXR(R)) (Margeta-Mitrovic *et al.*, 2000). These motifs also cause the ER accumulation of these proteins.

Our results do show however that recombinant mGluR1b is less well represented at the surface of transfected cells than mGluR1a and that the majority of the

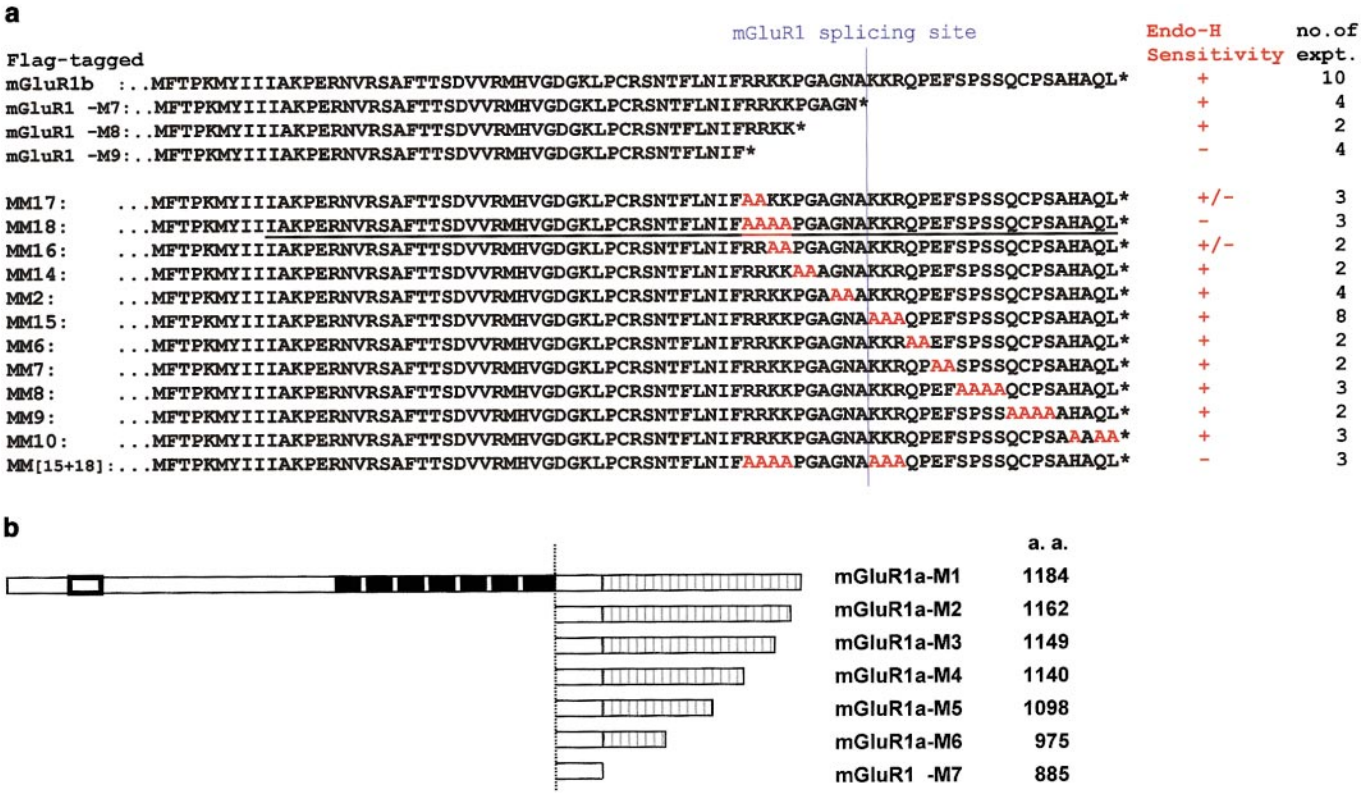

FIG. 6. Schematic representation of the mGluR1 truncations and mutants used to determine the retention signal. The mutated residues produced by scanning alanine mutagenesis are shown in red in (a). Here only the C-terminal sequence following the seventh transmembrane domain of the receptor is shown and the underlined sequence shows that used to produce the chimera pCD-MM18. Alongside the mutations and truncations the number of endoglycosidase experiments performed with each is shown together with the result obtained. (b) The same coding is used as described in the legend of Fig. 1 for the structural determinants of the constructs. The truncations of mGluR1a were introduced at residues 1184, 1162, 1149, 1140, 1098, 975, 885, 880, and 876. The position of the truncations mGluR1-M7, -M8, and -M9 are shown in more detail in (a).

membrane protein containing its C-terminal tail does not have mature carbohydrate groups, since it is Endo-H-sensitive. However, the surface expressed receptor is functional since we and others have demonstrated functional responses for mGluR1b when expressed in a variety of cell types (Ciruela *et al.*, 2000; Flor *et al.*, 1996; Pickering *et al.*, 1993; Prezeau *et al.*, 1994, 1996). The fact that the plasma membrane pCD-1b is predominantly EndoH sensitive suggests that it has not passed through the Golgi apparatus, and in this context, it is interesting to note that there was no detectable pCD-1b in the Golgi apparatus in the immunofluorescence experiments shown in Fig. 4. This suggests that both the pCD-1b and mGluR1b are not being properly routed to the cell surface in HEK293 cells and may not therefore be properly folded. It should be noted that all of the short forms of mGluR1 contain the RRKK motif, and it has been suggested that these residues may negatively regulate their coupling to phospholipase C (Mary *et al.*, 1998; Prezeau *et al.*, 1996). Our data could provide an alternative explanation for this finding since it shows that the short forms of mGluR1, which all have the RRKK sequence, could be expressed at the cell surface but at a lower level than mGluR1a and are not properly glycosylated. These combined effects could also contribute to some of the subtle differences in the kinetics of response of the short forms of mGluR1, and their altered affinity for some ligands, when compared to mGluR1a.

The fact that rat brain mGluR1b is fully glycosylated and is present at synapses suggests that neurones do have a means to overcome the retention signal present in mGluR1b. This could be due to the presence of interacting proteins in the neurons, which prevent the RRKK sequence from acting as an ER retention signal. Alternatively since mGluRs appear to be expressed as disulphide bonded dimers, mGluR1b might het-

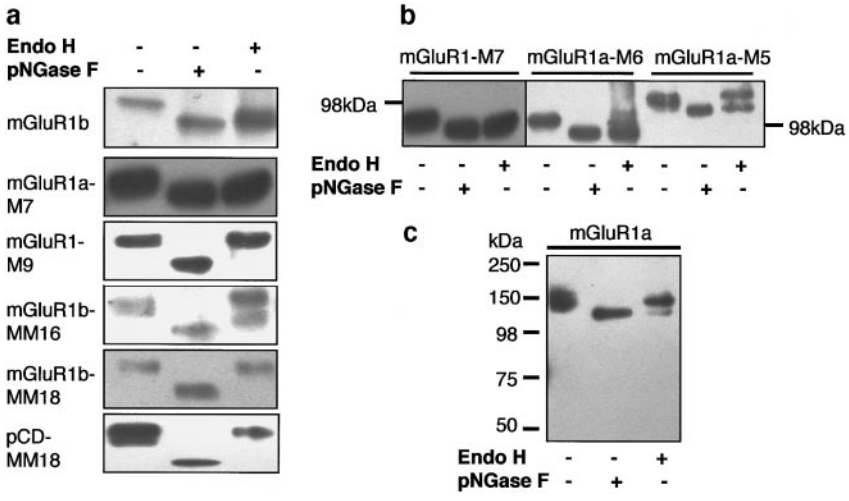

FIG. 7. Identification of the ER retention signal in the C-terminal tail of mGluR1b and the region of mGluR1a which prevents ER retention of the receptor. (a) Membranes from HEK293 cells expressing the mutants or truncations of mGluR1b were treated with endoglycosidases as described under Materials and Methods. The data are summarized in the legend of Fig. 6a. Shown here are representative digests from the indicated mutations (a) to show the effect of mutation of the dominant residues. Thus truncation of mGluR1b before the RRKK sequence results in full endoglycosidase H sensitivity (mGluR1-M9) as does mutation of all the RRKK residues to alanine (mGluR1b-MM18). However, mutation of only two of the residues results in partial EndoH sensitivity as shown here for mGluR1b-MM16. (b, c) Membranes from cells expressing the truncations of mGluR1a were subjected to endoglycosidase treatment and the digests analyzed on SDS–polyacrylamide gels. The results showed that mGluR1a-M5 (b) and mGluR1a (c) were predominantly EndoH resistant. The small amount of EndoH-sensitive material in these digests probably represents the degree of contamination of the membranes with ER. Truncation before mGluR1a-M5 caused no change in EndoH sensitivity. However, removal of the residues between 1098 and 975 resulted in almost complete EndoH sensitivity (mGluR1a-M6) comparable to that seen with the fully EndoH sensitive mGluR1-M7 truncation.

erodimerize with mGluR1a and this could overcome the retention signal. This seems unlikely since we have shown that these subunits do not heterodimerize when HEK293 cells are cotransfected with both receptors (Robbins *et al.*, 1999).

Immunocytochemical localization of mGluR1b in the rat hypothalamus and cerebellum has indicated a more restricted localization of this receptor subtype to the cell soma and proximal dendrites than mGluR1a (Grandes *et al.*, 1994; Mateos *et al.*, 1998). Therefore the restriction of mGluR1b and the pCD-1b construct to the cell soma and proximal dendrites of the neurons in culture described here, could reflect a real compartmentalization of the receptor to these regions of the cell. The neuronal somatic retention of mGluR1b shown here is strikingly similar to that of mGluR1a in neuronal cultures in the

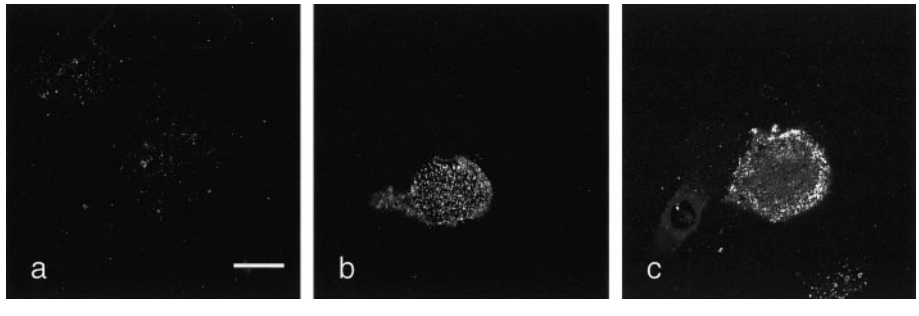

FIG. 8. Mutation of the ER retention signal increased cell surface expression. Cos-7 cells were transiently transfected with Flag-tagged constructs of mGluR1b(a), mGluR1-M9 (b), or mGluR1b-MM18 (c), and the cells were fixed for immunofluorescence after 24 h. The cells were reacted with anti-FLAG antibody without permeabilization. The weak cell surface staining of Flag-tagged mGluR1b is greatly enhanced when the RRKK motif is removed (b) or mutated (c), which shows that the cell surface expression is increased. The scale bar is 20 μm.

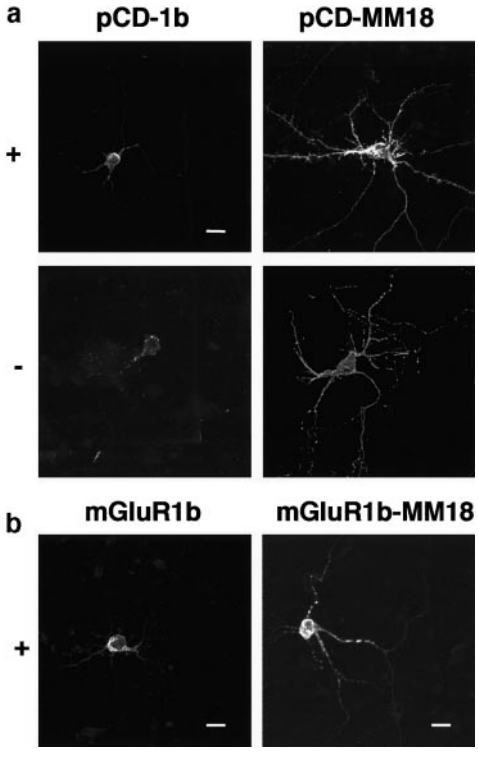

FIG. 9. Mutation of the ER retention signal restores dendritic trafficking and cell surface expression. Rat cortical primary cultures 12 DIV were transfected with pCD-MM18 (a) or mGluR1b-MM18 (b) and the cells prepared for immunofluorescence after 40 h. The cells were reacted with OX34 (a) or anti-Flag (b) antibody either without (−) or with (+) treatment with Triton X-100 as shown. The results show that the retention of pCD-1b in the cell soma is dramatically reduced following the mutation of the RRKK residues to alanine. In addition the weak cell surface expression of the pCD-1b found on the cell soma is greatly enhanced and the protein is now found extensively at the cell surface in dendrites (−). Similarly in the permeabilized (+) cells shown in (b) the mutation of the RRKK motif results in the full receptor being trafficked further in the dendrites. The scale bar is 20 μm.

absence of cotransfected Homer-1c (Ciruela *et al.*, 2000). In this context it is interesting to note that we and others have shown that coexpression of Homer proteins (Brakeman *et al.*, 1997; Kato *et al.*, 1998; Tu *et al.*, 1998; Xiao *et al.*, 1998) with the long forms of group 1 mGluRs (mGluR1a and mGluR5), results in their improved dendritic targeting in primary cultures of rat neurones (Ango *et al.*, 2000; Ciruela *et al.*, 2000), which suggests that the availability of these proteins is limiting in the cultured neurones. The restricted trafficking of mGluR1b in the primary neuronal cultures could therefore also be explained by a limiting concentration of proteins, currently unidentified, which specifically interact with it.

The results presented here also suggest that the presence of a functional receptor at the cell surface should not be taken as sufficient evidence that the protein has been properly processed during synthesis. The effects of truncating the C-terminal domain of mGluR1a, resulting in the unmasking of the ER retention signal, have clear implications for the use of truncation mutants in trafficking studies of these and other receptors, since the truncations may unmask other targeting signals within C-terminal domains.

In conclusion we have identified a novel ER retention signal, composed of four positively charged residues, in the C-terminal tail of mGluR1b, which is also present, but silent, in mGluR1a. The motif responsible for the ER retention is a chain of four positively charged amino acids RRKK, which are present in mGluR1a also, but are masked by the residues 975–1098. Removal of these residues results in the restoration of normal trafficking of the protein as evidenced by its resistance to EndoH treatment, increased surface expression and increased dendritic trafficking. Together these data suggest that the different mGluR1 splice variants may undergo differential trafficking in neurones and that the trafficking of other metabotropic glutamate receptors and their splice variants may be subject to differential regulation.

EXPERIMENTAL METHODS

Production of mGluR1 Chimeras, Truncations, and Mutants

Full-length mGluR1a and mGluR1b rat cDNAs were in the mammalian expression vector pcDNA3 and contained sequences encoding N-terminal FLAG epitope (DYKDDDDK) (Ciruela *et al.*, 2000). The lymphocyte antigen CD2 cDNA (EMBL Accession No. X05111) was a gift from Professor Niel Barclay (MRC Immunology Group, Oxford). The coding sequence of the CD2 was used to produce a number of constructs in pcDNA3 vector for expression in HEK293 cells and in cultured rat neurones. These included: a full-length CD2, a pCD-T in which the CD2 was truncated after its single transmembrane domain (at position 238 of the amino acid sequence X05111), and pCD-mGluR1 chimeras that contained fragments of the mGluR1a and 1b receptors (EMBL Accession No. Y18810; Fig. 1).

Translation termination ("stop") codons were introduced into the 3′ ends of the coding sequences of the mGluR1a cDNAs at and upstream of the site of alternative splicing between the mGluR1a and mGluR1b subunits, using a PCR-based mutagenesis approach.

We have also mutated individual, and groups, of amino acids to Ala, in a region containing the last 30 amino acids of the C-terminus of mGluR1b, which was responsible for its lack of surface expression and ER retention. Each mutant clone was produced by inserting synthetic DNA cassettes into the mGluR1b cDNA in pcDNA3 vector. The list and sequence of the mutants is shown on the Fig. 6a. The mutated C-terminal tails of the mGluR1b-MM15, -MM18, and -MM[15 + 18] mutants were also introduced into pCD-T. All of the truncations and mutated proteins were cloned into pCDNA3 and were confirmed by DNA sequencing. The structures and sequences of the different mutations and truncations are illustrated in Fig. 6.

Cell Culture and Transfection

HEK293 or COS-7 cells were grown in DMEM supplemented with 1 mM sodium pyruvate, 2 mM l-glutamine, 100 U/ml penicillin/streptomycin (all from Sigma Chemical Co.), and 10% (v/v) fetal calf serum (FCS) (Hyclone) at 37°C and in an atmosphere of 5% CO_2. Cells were passaged and transfected as described previously (Ciruela *et al.,* 2000; Jordan *et al.,* 1996). The cells were harvested 24 h after transfection.

Primary rat cortical cultures were prepared from E16 rat embryos and transfected using Effectene (Qiagen) as reported previously (Ciruela *et al.,* 2000). Transient gene expression was continued for 40 h at 37°C.

Immunofluorescence

Cells were processed for as described in Ciruela *et al.* (2000). In some experiments the secondary antibodies used were (1/5000) affinity purified Alexa Fluor 488-conjugated goat anti-rabbit or Alexa Fluor 543-conjugated goat anti-mouse antibodies (Molecular Probes, Oregon). Immunofluorescence was recorded on either a confocal microscope or a using a Leitz Dialux 20 epifluorescence microscope and OptiLab software. Confocal microscope observations were made with a Zeiss LSM510 NLO confocal scanning microscope mounted on an Axiovert 100M inverted microscope. All photomicrographs of immunofluorescence showing comparative staining were taken with fixed exposure times or if confocal, using identical pin hole and laser power settings.

The primary antibodies used for immunolabeling were, affinity purified anti-Flag monoclonal antibody (10 μg/ml; Clone M2, Sigma Chemical Co.), affinity purified anti-mGluR1 polyclonal antibody F1-Ab (pan-mGluR1) (Ciruela and McIlhinney, 1997), affinity purified anti-mGluR1a polyclonal antibody F2-Ab (Ciruela *et al.,* 1999b), a rabbit antibody R23 (1/1000), and a monoclonal antibody OX34 against rat CD2 N-terminal domain (1/20; Dr. Niel Barclay, Oxford UK). Immunoblotting for mGluR1b was performed using an antibody specific for the C-terminus of mGluR1b (M2; (Robbins *et al.,* 1999)). Antibodies against the Golgi marker β-COP and the ER marker calnexin were obtained from Stratagene and Cambridge Bioscience respectively.

Gel Electrophoresis and Immunoblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 6 or 10% polyacrylamide gels, proteins were immunoblotted to PVDF membranes (Immobilon-P, Millipore) using a semidry transfer system and developed with the enhanced chemilluminiscence detection kit (Pierce), as described previously (Ciruela *et al.,* 1999).

Cell Surface Elisa

COS-7 cells were transfected with pCD-T or pCD-1b and incubated for 24 h. They were then dissociated by trypsinisation and plated onto 12-well plates for another 24 h growth. The cells were then fixed with 4% paraformaldehyde, with or without permeabilization by 0.2% Triton X-100. The cells were reacted with OX34 antibody and the assay developed as described previously (Ciruela *et al.,* 2000).

Membrane Preparations

Membranes from cells transfected with the different constructs were prepared using hypotonic lysis and shearing as previously described (McIlhinney and Molnar, 1996). Rat brain synaptosomal membranes were prepared from the cerebella of 6-week old male Sprague–Dawley rats using sucrose gradients (Molnar *et al.,* 1994). Membranes were either used fresh or rapidly frozen in liquid nitrogen and stored at −70°C.

Cell surface biotinylation was performed as described previously (Ciruela *et al.,* 2000).

Endoglysosidase Digestion

Membranes from cells, or isolates from biotinylated cells, transfected with the different constructs, or cerebellar membranes, were heated at 100°C in 100 μl of 1% SDS for 5 min. The membranes were cooled and 50 μl of 10% *N*-octylglucoside solution added. The membranes were adjusted to a final volume of 500 μl using either 50

mM sodium acetate buffer, pH 5.5 (for Endoglycosidase H; EndoH), or 50 mM phosphate buffer, pH 7.2 (for pNGaseF), containing a protease inhibitor cocktail (Boehringer). Samples (150 μl) of the heated membranes were incubated with 1.5 μl of the different endoglycosidases for 16 h at 37°C. Control samples were incubated with no enzyme using the pH 7.2 buffer. After incubation the samples were treated with SDS-sample buffer and subjected to analysis as described above.

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