Homer 1b Regulates the Trafficking of Group I Metabotropic Glutamate Receptors

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The molecular basis for glutamate receptor trafficking to the plasma membrane is not understood. In the present study, we demonstrate that Homer 1b (H1b), a constitutively expressed splice form of the immediate early gene product Homer (now termed Homer 1a) regulates the trafficking and surface expression of group I metabotropic glutamate receptors. H1b inhibits surface expression of the metabotropic glutamate receptor mGluR5 in heterologous cells, causing mGluR5 to be retained in the endoplasmic reticulum (ER). In contrast, mGluR5 alone or mGluR5 coexpressed with Homer 1a successfully travels through the secretory pathway to the plasma membrane. In addition, point mutations that disrupt mGluR5 binding to H1b eliminate ER retention of mGluR5, demonstrating that H1b affects metabotropic receptor localization via a direct protein-protein interaction. Electron microscopic analysis reveals that the group I metabotropic receptor mGluR1a is significantly enriched in the ER of Purkinje cells, suggesting that a similar mechanism may exist in vivo. Because H1b is found in dendritic spines of neurons, local retention of metabotropic receptors within dendritic ER provides a potential mechanism for regulating synapse-specific expression of group I metabotropic glutamate receptors.

The responsiveness of a neuron to neurotransmitter released from a presynaptic cell is determined by the type and amount of receptor expressed on the postsynaptic membrane. Glutamate is the most prevalent excitatory neurotransmitter in the mammalian central nervous system, and it has multiple receptor subtypes that have unique distributions throughout the brain. The complex and distinct synaptic localization of different glutamate receptors in central nervous system neurons (1, 2) requires highly selective intracellular targeting mechanisms. The distributions of individual receptors within neurons are not uniform, including differences related to specific synaptic populations (3–5) as well as pre- and postsynaptic locations (1, 2, 6, 7). Since the initial observation that PSD-95 interacts with N-methyl-D-aspartate receptors (8), it has been proposed that PDZ domain-containing proteins such as PSD-95 play a role in clustering and/or anchoring of glutamate receptors at synapses (9–12). The synaptic expression of glutamate receptors in central nervous system neurons (1, 2, 6, 7) has been proposed with the immediate early gene form H1a, which binds metabotropic receptor but lacks the C-terminal coiled-coil domain. Although these experiments were performed in heterologous cells, we also observe enrichment of the group I metabotropic receptor mGluR1a in the ER of Purkinje cells. This suggests H1b-regulated ER retention may occur in vivo, possibly revealing a novel mechanism for synapse-specific receptor expression.

EXPERIMENTAL PROCEDURES
cDNA Constructs—mGluR1a, mGluR2, and mGluR5 cDNAs were generously provided by S. Nakanishi, and each was subcloned into the mammalian expression vector pBK5. H1a and H1b were also subcloned into pBK5 and included an N-terminal Myc epitope.

Cell Culture and Transfections—HeLa cells were grown on 10-cm dishes for biochemical analyses and on glass coverslips in 6-well tissue culture dishes for immunofluorescence microscopy. HeLa cells were transfected with mGluR5, mGluR1a, or mGluR2 cDNAs with empty vector, H1a, or H1b cDNAs (10 µg total/10-cm dish or 5 µg total/well of 6-well dish) using the calcium phosphate coprecipitation method (15). Transfected cells were analyzed 36 h after transfection.

Antibodies—Antibodies against mGluR5 (13), H1b (14), mGluR2/3 (7), mGluR1a (16), and Myc (17) (9E10) have been characterized previously. The BiP mouse monoclonal antibodies were obtained from StressGen Biotechnologies Corp. The N-mGluR5 antibodies were generated by immunizing rabbits with the synthetic peptide RLEGFAQEN-

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SKYNKTC (mGluR5 amino acids 365–380).

**Surface Labeling**—Transfected HeLa cells grown on coverslips were washed in PBS, incubated with primary antibodies (6 μg/ml affinity-purified N-mGluR5 antibodies in PBS containing 3% NGS) on ice for 1.5 h, washed in PBS, and fixed in 4% paraformaldehyde in PBS for 20 min. The coverslips were then washed in PBS, incubated in secondary antibodies (Cy3 anti-rabbit diluted 1:500 in PBS containing 1% bovine serum albumin and 1% saponin) at room temperature for 30 min, and then washed in PBS and mounted onto slides using Vectashield mounting media. 

**Immunocytochemistry**—Transfected HeLa cells grown on coverslips were washed in PBS, fixed in 4% paraformaldehyde in PBS for 20 min, washed in PBS, and permeabilized in 0.25% Triton X-100 in PBS for 5 min. The coverslips were washed in PBS and incubated with primary antibodies (mGluR5 antisera, 1:1000; Myc, 1:1000; mGluR1α antibodies, 1 μg/ml; mGluR2/3 affinity-purified antibodies, 1 μg/ml; or BiP ascites, 1:500 in PBS containing 3% NGS) for 1–2 h at room temperature, washed in PBS, and incubated with secondary antibodies (Cy3 anti-rabbit or Cy3 anti-mouse diluted 1:500 in PBS containing 3% NGS for single label or fluorescein isothiocyanate anti-mouse and rhodamine anti-rabbit diluted 1:500 in PBS containing 3% NGS for double label) for 30 min at room temperature, washed in PBS, and then mounted with Vectashield mounting media.

**Glycoshidase Treatment**—Transfected HeLa cells grown on 10-cm dishes were washed in TBS (2 mM EDTA, 0.1 mM EGTA 4-(2-aminoethylbenzenesulfonyl fluoride), 1 μg/ml leupeptin, 5 mM iodoacetamide), collected in lysis buffer without detergent and sonicated, and total membranes were collected. The membranes were resuspended in 50 μl of lysis buffer with 1% SDS and heated at 100 °C for 5 min. Then 5 volumes of cold 1% octyl glucoside was added, and the samples were divided three ways. The samples were incubated alone, with endoglycosidase H (Endo H), or with N-glycosidase F (6 units/ml) for 4–5 h at 37 °C, then frozen until further analysis. Samples were thawed, sample buffer was added, and the samples were incubated at 100 °C for 5 min before loading on the gel. The proteins were resolved by SDS-polyacrylamide gel electrophoresis on 4–20% (Fig. 4A) or 4–12% (Fig. 3) gradient gels. The proteins were transferred to polyvinylidene difluoride membranes, immunoblotted with mGluR5 antisera diluted 1:1000, and visualized with enhanced chemiluminescence.

**Generation of Point Mutations**—Point mutations within the Homer binding site of mGluR5 were generated using the QuikChange method (Stratagene) and are described in Tu et al. (22).

**EM Analysis**—The postembedding immunogold method has been described previously (18–20) and is modified from the method of Matsubara et al. (21). Male Sprague-Dawley rats were perfused with 4% paraformaldehyde plus 0.5% glutaraldehyde in 0.1 M phosphate buffer. Parasagittal sections of the rostral cerebellum (folia III–V) were cryo-protected in 30% glycerol, frozen in liquid propane in a Leica EM CPC, immersed in 1.5% uranyl acetate in methanol at −90 °C in a Leica AFS freeze-substitution instrument, infiltrated with Lowicryl HM 20 resin at −45 °C, and polymerized with UV light. Thin sections were incubated in 0.1% sodium borohydride plus 50 mM glycine in Tris-buffered saline (pH 7.4) for 5 min, then washed in PBS and incubated in secondary antibodies (1:500 in PBS containing 3% NGS) on ice for 1–2 h at room temperature, washed in PBS, and incubated with secondary antibodies (Cy3 anti-rabbit or Cy3 anti-mouse diluted 1:500 in PBS containing 3% NGS for single label or fluorescein isothiocyanate anti-mouse and rhodamine anti-rabbit diluted 1:500 in PBS containing 3% NGS for double label) for 30 min at room temperature, washed in PBS, and then mounted with Vectashield mounting media.

![FIG. 1. H1b inhibits surface expression of mGluR5.](image)

HeLa cells were transfected with mGluR5 and empty vector (top panel), mGluR5 and H1a (middle panel), or mGluR5 and H1b (bottom panel). In each condition, the cells were labeled live with N-mGluR5 antibodies on ice and fixed, and surface-expressed mGluR5 was visualized with Cy3-conjugated secondary antibodies.

In an attempt to understand the mechanism by which H1b regulates mGluR5 surface expression, we characterized the intracellular distribution of mGluR5 using immunofluorescence of permeabilized cells. mGluR5 expressed alone or with H1a was distributed diffusely throughout the cell. In contrast, mGluR5 expressed with H1b had a dramatically different subcellular localization. mGluR5 was present in perinuclear organelles as well as extensive reticular staining present throughout the cell that strongly resembled that of the ER (Fig. 2A). To confirm that the effect of H1b was not restricted to mGluR5, we expressed H1b with another group I metabotropic glutamate receptor, mGluR1α, and analyzed the intracellular distribution of the glutamate receptor. Like mGluR5, mGluR1α appeared to be localized to ER when coexpressed with H1b (data not shown).

To conclusively identify the intracellular compartment containing mGluR5, we double-labeled mGluR5/H1b cotransfected cells with antibodies recognizing mGluR5 and the ER-resident protein BiP. Staining with BiP antibodies revealed the extensive ER present in both transfected and untransfected cells. The mGluR5 antibodies only recognized transfected cells and revealed colocalization with the BiP-positive reticulum and the perinuclear organelles, confirming that mGluR5 was present within the ER and ER-derived structures (Fig. 2B). It should be noted that the perinuclear organelles were not present within nontransfected cells and therefore appear to be ER-derived structures unique to cells overexpressing mGluR5 and H1b. These observations suggest that H1b, but not H1a, causes cotransfection with H1b (Figs. 2A and 4).
mGluR5 to be retained in the ER.

We also performed double-labeling immunofluorescent microscopy of mGluR5 and H1b when these two proteins were coexpressed in HeLa cells. mGluR5 and H1b displayed considerable colocalization (Fig. 3) with bright immunoreactivity in the ER and perinuclear organelles. Although complete colocalization was common, we also often observed colocalization of the two proteins with excess plasma membrane localization of H1b.

As an additional assay for ER retention, we examined the status of the carbohydrates present on mGluR5 in cells expressing H1a or H1b. If H1b caused mGluR5 to be retained within the ER, then mGluR5 should contain immature, high mannose carbohydrates that are sensitive to digestion with the enzyme Endo H. Alternatively, if mGluR5 had successfully traveled through the ER and cis-Golgi, it would possess mature, complex carbohydrates that would be Endo H-resistant. Mature carbohydrates would be anticipated if mGluR5 was on the cell surface or if it was sequestered in a post-Golgi intracellular compartment such as endosomes. We prepared membranes from mock transfected cells, cells expressing mGluR5 alone, or cells coexpressing mGluR5 and H1a or H1b. These membranes were treated with no enzyme, Endo H, or N-glycosidase F, an enzyme that removes all Asn-linked carbohydrates. Immunoblotting with mGluR5 antibodies revealed that mGluR5 is Endo H-resistant when expressed alone or with H1a. However, when expressed with H1b, mGluR5 is Endo H-sensitive (Fig. 4), consistent with the hypothesis that expression of H1b leads to the retention of group I metabotropic glutamate receptors in the ER.

We were surprised that coexpression of mGluR5 with H1b caused complete ER retention of mGluR5 as detected by surface staining (Fig. 1) and carbohydrate analysis (Fig. 4). To determine whether H1b was present at saturating concentrations when coexpressed with mGluR5, we varied the amount of H1b expressed with mGluR5 to determine whether there existed a dose-response effect of H1b on mGluR5 trafficking to the cell surface (Fig. 5). We transfected cells with constant amounts of mGluR5 (5 μg) and increasing amounts of H1b (0, 0.5, 1.5, and 5 μg) to evaluate the effect H1b had on mGluR5 localization. We found that low levels of H1b expression did not result in the ER retention of mGluR5, whereas higher concentrations did result in ER retention. All other experiments we conducted used equal amounts of mGluR5 and H1b cDNA and therefore reflect the effect H1b has on mGluR5 under saturating conditions.

We next wanted to confirm that overexpression of H1b did not cause the nonspecific retention of proteins in the ER. The subcellular localization of the group II metabotropic glutamate receptor mGluR2 was the same whether expressed alone or with H1b (data not shown). In addition, we used a series of mGluR5 constructs containing point mutations within the Homer binding site and found that mutations that disrupt mGluR5/Homer interactions in vitro also prevent ER retention of mGluR5 coexpressed with H1b (Fig. 6). mGluR5 S1126F, which does not bind to Homer in vitro (22), was not retained in the ER when coexpressed with H1b. In contrast, mGluR5 S1128F, which does bind Homer in vitro, was ER-retained when coexpressed with H1b (Fig. 6A). Other point mutations in adjacent residues were analyzed, and the results were consistent with in vitro binding studies (summarized in Fig. 6B) (22), demonstrating that mGluR5 is retained within the ER by H1b only when its Homer binding site is intact.

Although our results clearly demonstrate that H1b retains group I metabotropic receptors in the ER of heterologous cells, its role in neurons may be more complicated. Therefore, we used immunoelectron microscopy to determine the intracellular distribution of mGluR1α, the group I metabotropic receptor...
expressed within Purkinje cells, which express high levels of H1b (14). In agreement with previous reports, mGluR1 is expressed at perisynaptic sites (20, 23) (Fig. 7, inset). Interestingly, we also find many examples in the cell body and dendrite of Purkinje cells where mGluR1 is concentrated in the ER (Fig. 7), indicating that a major pool of metabotropic glutamate receptors is retained in the ER. The retention in neurons is most apparent in ER of the cell body, although mGluR1 is also found associated with ER of the dendrite. A similar distribution for other metabotropic receptors or the ionotropic glutamate receptors has not been reported, although most receptors have a significant pool associated with a yet undefined membrane population. Thus it appears that group I metabotropic glutamate receptors, at least in some cell types, are enriched in the ER of neurons.

In the present study, we investigated the role of H1b in the trafficking and organization of group I metabotropic receptors. Our results show that H1b inhibits the surface expression of mGluR5 when the two proteins are coexpressed in heterologous cells. Immunofluorescent microscopy and carbohydrate analysis of mGluR5 definitively reveal that the impaired surface expression is due to retention of mGluR5 in the ER. We also demonstrate that this ER retention requires a direct interaction between H1b and mGluR5. It is important to note that the ER retention of mGluR5 is specific for H1b because H1a has no effect on mGluR5 localization even though it possesses the same mGluR5 binding site. This finding implicates the C-terminal coiled-coil domain of H1b in ER retention. The coiled-coil domain mediates homomultimerization of H1b and produces a multivalent complex that could cross-link mGluRs to other proteins such as the IP3 receptor (22), which is located in the ER. Although other receptor-binding proteins have been shown to alter receptor distributions in heterologous cells (24–29), to our knowledge, this is the first demonstration of the ER retention of an integral membrane protein upon coexpression with a cytosolic binding protein. This novel finding supports the idea that proteins of the Homer family are involved in the transport and localization of group I metabotropic receptors in neurons.

Activity-regulated mechanisms have been implicated in the
surface expression of mGluRs previously (30). Using synaptosomes prepared from the visual cortex, it was found that functional group I metabotropic glutamate receptors were developmentally regulated in normal animals but not in dark-reared animals. The activity-dependent increase in the expression of functional phosphatidylinositol-linked metabotropic glutamate receptors almost precisely parallels the expression of H1a observed in light-exposed versus dark-reared animals (13). These data are consistent with a model in which activity-regulated increases in H1a expression disrupt binding of H1b to mGluRs, leading to increased trafficking of metabotropic receptors to the plasma membrane and additional functional surface receptors.

In addition to the regulation of receptor transport, H1b could also form a functional link between the presynaptic mGluRs and inositol 1,4,5-trisphosphate receptors in the dendritic ER (22). Recent findings demonstrate that H1b binds to inositol 1,4,5-trisphosphate receptors. Furthermore, it was demonstrated that H1a injected into Purkinje cells causes a reduction in calcium released from intracellular stores, consistent with the long Homer isoforms coupling metabotropic receptors to inositol 1,4,5-trisphosphate receptors (22). This is a provocative idea because the ER is the functional target of phosphatidylinositol 1,4,5-trisphosphate receptors (22). Recent findings demonstrate that H1b binds to inositol 1,4,5-trisphosphate receptors in the dendritic ER and inositol 1,4,5-trisphosphate receptors in the perisynaptic mGluRs (22). In both cases, H1a, the immediate early gene product of Homer 1b, is anticipated to act locally to compete with the long Homer isoforms coupling metabotropic receptors to the ER leading to increased trafficking of metabotropic receptors to the postsynaptic membrane and additional functional surface receptors.

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