Involvement of the Proximal C Terminus of the AMPA Receptor Subunit GluR1 in Dendritic Sorting

Francesca Ruberti and Carlos G. Dotti

Cell Biology and Biophysics Programme, European Molecular Biology Laboratory, 69112 Heidelberg, Germany

Studies on dendritic sorting of transmembrane proteins in hippocampal neurons in culture have shown that these cells use similar mechanisms as epithelial cells to sort transmembrane proteins to the basolateral membrane domain. However, information is still scarce with regard to which amino acid sequences are required for dendritic sorting in neurons. The glutamate receptor 1 (GluR1) subunit of the AMPA receptor is present on the dendritic compartment of hippocampal neurons in culture. To identify the GluR1 sorting signal responsible for dendritic targeting, we have expressed the wild-type GluR1, a deletion mutant in the C-terminal cytoplasmic tail, and chimeric GluR1 proteins in hippocampal neurons using a calcium phosphate transfection method. The recombinant full-length GluR1 is polarized to the dendritic domain. Truncated GluR1 with a

deletion of the C-terminal cytoplasmic tail is still delivered to the somatodendritic domain. However a chimeric protein made of the luminal and transmembrane domain of the influenza virus hemagglutinin (HA) fused to the GluR1 C-terminal cytoplasmic tail (HaemR1) is detected in the somatodendritic domain. This finding indicates that the GluR1 C-terminal cytoplasmic tail contains a dendritic sorting signal, which redirects the axonal or axonal-dendritic protein HA to the dendritic compartment exclusively. Deletion analysis of HaemR1 shows that the proximal segment of the GluR1 C-terminal cytoplasmic tail contains a novel dendritic sorting signal.

Key words: dendrites; sorting; hippocampal neurons; GluR1; axonal; hemagglutinin

In neurons, as in any other polarized cell, proper function is primarily dependent on the correct segregation of membrane proteins to different cellular domains. How neurons sort proteins to specialized domains and how this differential protein distribution is maintained are only beginning to be understood. Studies performed in cultured hippocampal neurons during the last 10 years suggested that neurons can make use of different intracellular pathways to accomplish polarized segregation of membrane proteins (Dotti and Simons, 1990; Winckler and Mellman, 1999). For example, the axonal protein Ng-CAM (the chicken homologue of L1) is delivered directly to the axonal surface immediately after synthesis in the cell body, and the transferrin receptor, the polymeric Ig receptor (pIgR), the low-density lipoprotein receptor (LDLR) and the metabotropic glutamate receptor 2 (mGluR2) are instead delivered directly to the dendritic domain (de Hoop et al., 1995; West et al., 1997a; Jareb and Banker, 1998; Stowell and Craig, 1999). These results suggested the existence of a direct pathway from the cell body to either axons or dendrites, in analogy to what happens in epithelial cells, where apical and basolateral proteins are sorted at the level of the Golgi apparatus. However, other constitutively axonal proteins, such as synaptophysin, synaptotagmin, and mGluR7, when overexpressed, result in the filling of both axon and dendrites (West et al., 1997b; Stowell and Craig, 1999; Kaether et al., 2000b). Yet another pathway seems to be used by amyloid precursor protein (APP)

and a mutant form of the pIgR ($\Delta 655$ –668 pIgR mutant). When overexpressed, these proteins appear initially in the axonal domain and at later times also in the dendrites (de Hoop et al., 1995; Simons et al., 1995). The latter two types of distribution suggest the existence of a post-Golgi sorting organelle responsible for polarized distribution (in the dendritic surface or endosomes for synaptophysin and synaptotagmin and in the axonal surface or endosomes for APP).

The work on the dendritic sorting in hippocampal neurons highlighted the role of specific amino acid motifs in the cytoplasmic tail of polarized proteins. For example, the transferrin receptor, the LDLR, and the glycine transporter require tyrosine or dileucine motifs located in the cytoplasmic side close to the transmembrane domain for proper dendritic delivery (West et al., 1997a; Jareb and Banker, 1998; Poyatos et al., 2000). Different from these motifs, the neuronal mGluR2 uses yet a different sorting signal for its dendritic delivery (Stowell and Craig, 1999).

To advance our understanding in the field of neuronal membrane asymmetry, we have now studied the sorting signals required for the dendritic distribution of the AMPA GluR1. The AMPA receptors are ionotropic glutamate receptors, which are

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Correspondence should be addressed to Carlos G. Dotti, Cell Biology and Biophysics Programme, European Molecular Biology Laboratory, 69112 Heidelberg, Germany. E-mail: Dotti@embl-heidelberg.de.

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involved in fast excitatory synaptic transmission in the brain (Hollmann and Heinemann, 1994). Functional AMPA receptors are multimeric complexes formed by different combinations of the homologous subunits GluR1–4, which share 60–70% identity at the level of the primary sequence (Mano and Teichberg, 1998). Numerous studies have shown that in neurons AMPA receptors are highly concentrated at excitatory synapses on dendritic spines and shafts.

In the present study we show that overexpressed wild-type (wt) GluR1 and a truncated mutant with a deletion in the C-terminal cytoplasmic tail are delivered to the somatodendritic domain in hippocampal neurons in culture. However, the addition of the GluR1 C-terminal cytoplasmic tail redirects the sorting of a axonal or axonal–dendritic protein to the dendritic domain only. Deletion mutants of the chimeric protein allowed us to demonstrate that the proximal segment of the GluR1 C-terminal cytoplasmic tail contains a novel dendritic sorting signal.

MATERIALS AND METHODS

Plasmids. Human GluR1 flip was obtained from American Type Culture Collection (Manassas, VA); mGluR2 cDNA was kindly provided from S. Nakanishi (Kyoto University, Kyoto, Japan); and influenza hemagglutinin (HA) [A/Japan/305 (H2)] and hemagglutinin tail minus cDNAs were kindly provided from P. Scheiffele (European Molecular Biology Laboratory). All the cDNAs were subcloned in pcDNA3 mammalian expression vector (Invitrogen, San Diego, CA). For GluR1 constructs, amino acid numbers refer to published sequence excluding the signal peptide. Influenza hemagglutinin is listed with the amino acid number including the signal sequence.

A two-step PCR strategy was used to insert an HA epitope (YPYD-VPDYA) in human GluR1 (hGluR1). In the first step, two distinct PCR reactions, using as template an HA/mGluR2 construct (available in our laboratory), and hGluR1, respectively, were performed. One PCR reaction produced a fragment containing a 5' *EcoRI* restriction site, the 5' untranslated region, the signal sequence of mGluR2, and the HA tag; the other reaction, using as template hGluR1, produced a PCR fragment containing a 5' HA tag and the hGluR1 coding sequence from the first amino acid past the signal sequence (Sun et al., 1992) to the unique *ApaI* restriction site. In the second step, the two PCR products, which contain a 30 bp sequence overlap, were mixed together and amplified using external primers. The PCR product was cut with *EcoRI* and *ApaI* and subcloned in hGluR1 pCDNA3. The deletion mutant HA-hGluR1(d816–888) was produced by PCR starting from HA-hGluR1. The two-step PCR strategy has been also used to produce the chimeric construct HaemR1 (HA, 1–535; hGluR1flip, 809–888) and its deletion mutants HaemR1 proximal (HA, 1–535; hGluR1flip, 809–849) and HaemR1 distal (HA, 1–535; hGluR1flip, 809–815, 848–888).

All of the mutagenized cDNAs were sequenced by the EMBL sequences service to ensure that unwanted mutations had been introduced during PCR.

Neuron culture and immunocytochemistry. Primary hippocampal neurons were prepared from embryonic day 18 rat brains as described by Goslin and Banker (1991). For immunocytochemistry experiments cells grown on glass coverslips were fixed with 4% paraformaldehyde in PBS for 15 min. Paraformaldehyde was quenched with 50 mM ammonium chloride in PBS for 10 min. Then the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min. To prevent unspecific staining, the cells were incubated with blocking solution (2% BSA, 2% FCS, and 0.2% fish skin gelatin) in PBS for 30 min. For double labeling the cells were incubated with the primary antibodies diluted in 10% blocking solution in PBS for 1 hr at room temperature or overnight at 4°C. Cells were washed three times with PBS (5 min each) and then incubated with appropriate fluorochrome-conjugated secondary antibodies diluted in 10% blocking solution in PBS for 20–30 min at room temperature. After three washes with PBS the coverslips were mounted with Mowiol (Merck, Darmstadt, Germany) containing 100 mg/ml 1,4-diazabicyclo[2.2.2]octane (Sigma, St. Louis, MO) as an anti-fading agent.

The following primary antibodies have been used: mouse monoclonal antibody anti-HA (12CA5; Roche Molecular Biochemicals, Mannheim, Germany), polyclonal rabbit antibody 514 to MAP2 (a gift from C. Sanchez, Centro de Biología Molecular, Madrid, Spain), polyclonal

rabbit antibody anti-hemagglutinin (kindly provided by Peter Scheiffele, European Molecular Biology Laboratory), and mouse monoclonal antibody to MAP-2 (anti-MAP-2; Roche).

Calcium phosphate transfection method. Neurons 7–11 d *in vitro* (DIV) bearing coverslips were transferred from the home dish and flipped cell side-up to 35 cm dishes with 2 ml of N2 glia-conditioned medium. The DNA for transfection was prepared by EndoFree plasmid maxi kit (Qiagen, Hilden, Germany). Two to 5 μ g of DNA were mixed with 6 μ l of CaCl_2 solution (2.5 M) to a final volume of 60 μ l and then mixed with 60 μ l of 2 $\times$ BBS (280 mM NaCl, 1.5 mM Na_2HPO_4 , and 50 mM *N,N*-bis[2-hydroxyethyl]-2-amino-ethanesulfonic acid pH 7.1) and immediately (<2 min) added to the neurons. The dishes were incubated at 37°C and 2.5% CO_2 until a fine precipitate was formed. Cells were washed with HBS (135 mM NaCl, 20 mM HEPES, 4 mM KCl, 1 mM Na_2HPO_4 , 2 mM CaCl_2 , 1 mM MgCl_2 , and 10 mM glucose) and cultured in the original medium at 37°C and 5% CO_2 for 24–48 hr (Kaether et al., 2000a).

Quantitation of hemagglutinin distribution. Eleven DIV transfected neurons were analyzed by immunofluorescence by using antibodies against the dendritic marker MAP2 and against hemagglutinin. Phase-contrast, MAP2, and hemagglutinin images were captured for each transfected cell with a charged-coupled device camera (4910; Cohu, San Diego) connected to a Macintosh computer (Power Macintosh 7300/166; Apple, Cupertino, CA) equipped with an image recorder (LG3; Scion, Frederick, MD). Unprocessed images were stored by using NIH Image. For the images with hemagglutinin labeling, axonal and dendritic domains were identified by comparison with the corresponding MAP2 and phase-contrast images. Therefore, the area of axonal and dendritic segments was delineated, and the hemagglutinin signal within the area was quantitated (mean pixel intensity/area). Axonal and dendritic area were normalized to the same size, and the percentage of labeling intensity in dendrites and axons was calculated.

RESULTS

The wild-type human GluR1 and a C-terminal deletion mutant are sorted to the dendrites of cultured hippocampal neurons

The sorting of human GluR1 was analyzed in 9 DIV hippocampal neurons in culture. An HA epitope tag was introduced at the N terminus of human GluR1 to distinguish recombinant from endogenous proteins. A calcium phosphate precipitation expression system was used to study the polarization of newly synthesized recombinant proteins. The dendrites of the cells were identified by labeling with an antibody against MAP2, a cytoskeletal dendritic protein (Bernhardt and Matus, 1984). Indirect immunofluorescence microscopy after 24–48 hr of transfection with the wt HA-hGluR1(1–888) revealed an almost dendritic distribution (Fig. 1*A–D*). Indeed, MAP2 and HA exactly colocalized in all transfected cells. The distribution of the overexpressed human form of the receptor is consistent with the dendritic distribution of the endogenous receptor in these cells (results not shown; but see Craig et al., 1993). Furthermore, this result proves the validity of the method used to further characterize the sorting signals responsible for dendritic sorting of this subunit. Given that most dendritic membrane proteins studied until now contain sorting information within the cytoplasmic domain, we investigated whether this is also the case for the GluR1 subunit. To test the involvement of the C-terminal cytoplasmic tail, we expressed a deletion mutant truncated seven amino acids after the fourth transmembrane domain, HA-hGluR1(d816–888), into hippocampal neurons and analyzed the distribution by immunofluorescence microscopy. Surprisingly, the distribution of the overexpressed mutant protein HA-hGluR1(d816–888) was similar to that of the wild-type protein and appeared exclusively in the dendritic domain (Fig. 1*E,F*). Although the simplest interpretation of these results would be that dendritic delivery does not require the C terminus, it could also be that the deletion construct is sorted via interaction with endogenous subunits during pro-

Figure 1. Dendritic distribution of expressed hGluR1 constructs in 9 DIV hippocampal neurons. *A, E*, Scheme of wt HA-hGluR1 and HA-hGluR1(d816-888). The amino acid sequences of the C-terminal cytoplasmic tail of wild-type and mutant human HA-GluR1 are shown in single-letter code. *B-D, F-H*, Nine DIV neurons were transfected with wt HA-hGluR1 (*B-D*) or HA-hGluR1(d816-888) (*F-H*) and fixed 48 hr later. The neurons were analyzed by immunofluorescence using an antibody against the HA tag of the recombinant hGluR proteins (*B, F*) and antibodies against a dendritic marker, MAP2 (*C, G*). As shown in *B*, the anti-HA antibody gave specific labeling of the transfected cell on the left and only background labeling of the nontransfected cell on the right. *D, H*, Phase-contrast images indicating the cell body, dendrites, and putative axons (arrows) of cells of the same fields (*D, H*). Note that the expressed full-length HA-hGluR1 and HA-hGluR1(d816-888) gave the same distribution pattern of the cytoskeletal protein MAP2. All of the axons in the micrographs are negative for MAP2 and HA. Scale bar, 10 μm.

cessing along the secretory pathway. We tried to titrate out the endogenous subunits increasing the level of expression of HA-hGluR1(d816-888), but in this case, in most of the transfected cells the mutant protein was retained in the endoplasmic reticulum, and in the remaining cells it was still delivered to the somatodendritic domain, suggesting that only correctly folded, oligomerized protein can leave the secretory pathway.

The C terminus of GluR1 is sufficient to direct an axonal protein to the dendritic domain of cultured hippocampal neurons

To circumvent the problem of oligomers, we constructed a chimeric protein composed of the C terminus of GluR1 and the ectodomain and transmembrane domain of influenza virus glycoprotein HA. We called this chimera HaemR1. In hippocampal

Figure 2. Distribution of HA tail minus protein and HaemR1 chimera in 11 DIV hippocampal neurons. *A, E*, Scheme of HA tail minus and HaemR1 chimera. *B, C, F, G*, Double immunofluorescence performed 10–12 hr after transfection using polyclonal rabbit antibodies against the viral protein hemagglutinin (*B, F*) and monoclonal antibody against a dendritic marker, MAP-2 (*C, G*). *D, H*, Phase-contrast images. Putative axons of transfected cells are indicated (arrows). The hemagglutinin tail minus protein is distributed to both the axonal and the dendritic compartments, whereas the HaemR1 chimera is targeted to the dendritic compartment and excluded from the axon. Scale bar, 10 μm.

neurons HA is directed to the axon but in some neurons is also dendritic (Jareb and Banker, 1998; Ledesma et al., 1998). Hemagglutinin tail minus and HaemR1 were transiently transfected into 10–11 DIV hippocampal neurons by the CaPO₄ method and fixed 10–12 hr later. Most of the neurons expressing the tail-less HA showed clear axonal labeling and dendritic-positive patches (Fig. 2*A–D*). Different from the tail-less HA, HaemR1 was exclusively found on the dendritic compartment (Fig. 2*E, F*). These data imply that the GluR1 cytoplasmic tail contains a *cis*-acting dendritic sorting signal(s), sufficient to direct a heterologous protein from primarily axonal and dendritic distribution to the dendritic compartment exclusively.

The proximal segment of the GluR1 C terminus contains a somatodendritic sorting signal

To define with more precision the region of the GluR1 C-terminal cytoplasmic tail involved in dendritic sorting, we made two different deletion mutants in the GluR1 cytoplasmic tail of the HaemR1 construct: HaemR1 proximal and HaemR1 distal. HaemR1 proximal contains 39 amino acids of GluR1 C-terminal cytoplasmic tail proximal to the transmembrane region; HaemR1 distal is complementary to the previous one and has the last 41 amino acids of the GluR1 C-terminal cytoplasmic tail.

HaemR1 proximal and distal were separately transfected in

Figure 3. HaemR1 proximal but not HaemR1 distal is sorted to the somatodendritic domain. *A, E*, Cytoplasmic domain amino acid sequences of the chimeric proteins HaemR1 proximal and distal consisting of the ecto and transmembrane domains of HA and GluR1 C-terminal cytoplasmic domain sequences are shown in *single-letter code*. The first seven amino acids of the GluR1 cytoplasmic tail, common to the two constructs, are indicated in *gray*. *B, C, F, G*, Double immunofluorescence performed 12 hr after transfection using polyclonal rabbit antibodies against the viral protein hemagglutinin (*B, F*) and a monoclonal antibody against a dendritic marker, MAP-2 (*C, G*). *D, H*, Phase-contrast images of cell body, dendrites, and putative axons (*arrows*) of HaemR1 proximal- and HaemR1 distal-positive cells. As shown in these representative micrographs, HaemR1 proximal contains sorting information sufficient for dendritic targeting; instead, HaemR1 distal is distributed to the dendritic and axonal domains. Bar, 10 μ m.

10–11 DIV hippocampal neurons and the distribution of the mutant protein analyzed by immunofluorescence microscopy. The results of such experiments are shown in Figure 3, and the quantitation of protein distribution is shown in Figure 4. The HaemR1 proximal protein colocalized perfectly with MAP2 and is therefore efficiently delivered to the somatodendritic domain. In contrast, the HaemR1 distal chimeric protein filled both MAP2-positive and MAP2-negative processes, thus behaving like numerous overexpressed axonal proteins do (West et al., 1997b; Jareb and Banker, 1998; Kaether et al., 2000b). The application of a quantitative method on 24 transfected cells (from two different experiments) for the different chimeric constructs revealed that >90% of HaemR1 and HaemR1 proximal protein was in the dendritic compartment, whereas for HaemR1 distal 40% of the protein was axonal and 60% was dendritic (Fig. 4).

DISCUSSION

In this work we show that the GluR1 subunit contains dendritic sorting information in the proximal region of the C terminus.

Figure 4. Percentage of dendritic and axonal distribution mediated by the GluR1 C-terminal tail and its proximal and distal domains. Indicated is the percentage of the intensity of axonal (*gray bars*) and dendritic (*black bars*) labeling for HaemR1 chimera, HaemR1 proximal and HaemR1 distal (mean $\pm$ SD; $n = 24$ cells obtained from the transfection of neurons from two different cultures). The intensity of the labeling for HaemR1 and HaemR1 proximal was much higher in the dendritic compartment than in the axon, whereas the intensity of labeling for HaemR1 distal in the dendrite was comparable with that in the axon.

This is based on the fact that this region was able to direct the hemagglutinin ectodomain–transmembrane construct from axonal–dendritic to exclusively dendritic. Although the results with this chimera were clear, it is necessary to discuss why the absence of this region in the GluR1 protein itself did not affect proper dendritic sorting. It is possible that the lack of effect was attributable to interaction with the endogenous protein. Indeed, the assembly of multimeric channels may occur in the endoplasmic reticulum, as has been demonstrated for the acetylcholine receptor (Gelman et al., 1995). Alternatively, other signals in the native protein may play a role in dendritic sorting, and these might become activated in the absence of the proximal C terminus. If this is true, it would mean that the GluR1 contains redundant dendritic sorting information, similar to what has been observed in basolaterally sorted proteins (Matter et al., 1992).

A second aspect of our findings is that the sequence in the proximal segment of the GluR1 C-terminal cytoplasmic tail involved in dendritic delivery does not contain previously described (Tyr or Leu-Leu) dendritic sorting motifs (Keller and Simons, 1997; Jareb and Banker, 1998; Poyatos et al., 2000). Although the proximal segment contains a Leu-Ile motif, deletion of this region did not affect dendritic sorting (results not shown). Because the sequence here described is also unrelated to that found in another postsynaptic receptor, a metabotropic glutamate receptor subunit (Stowell et al., 1999), we can conclude that the sorting of membrane proteins to dendrites is controlled quite specifically, almost in a protein–protein fashion, which might reflect transport in different vesicles. For the cell such diversity of signals could represent a mechanism to guarantee the precise delivery to different membrane subdomains, i.e., different dendritic spines, different parts of individual dendritic spines, and the dendritic shaft. This also implies that the sorting machinery, i.e., cytoplasmic factors interacting with the sorting signal, also would be protein-specific.

A third conclusion from our results is that the well described

PSD-95/Dlg/ZO-1 (PDZ) motif present in the final region of the C terminus (Kim and Huganir, 1999) is not involved in sorting. Indeed, we show that deletion of this segment (distal mutant) does not alter proper dendritic distribution. This would point to a role of the PDZ domain for proper retention of the protein, for example, via interaction with the synaptic-associated protein SAP97 (Leonard et al., 1998), but not for its delivery from the cell body to dendrites.

Despite the clustered synaptic localization of endogenous GluR1 in cultured hippocampal neurons as reported by Craig et al. (1993), we were unable to observe such clustering of the recombinant HA-hGluR1(1–888). However, overexpression in hippocampal neurons of another synaptic protein, mGluR2, reproduces dendritic sorting but not synaptic clustering, suggesting that this process must involve late steps in targeting, which could be more susceptible to saturation respect to dendritic sorting (Stowell and Craig, 1999).

The molecular mechanism by which the proximal region of the C terminus here identified controls dendritic delivery remains to be resolved. One possible mechanism would be via interaction with the membrane cytoskeletal protein 4N1, which has been recently shown to bind to a membrane proximal region of the GluR1 cytoplasmic tail (Shen et al., 1999; Walensky et al., 1999). However, this remains to be demonstrated. Our results should facilitate the isolation of the cytosolic receptor(s) responsible for GluR1 sorting. Indeed, the proximal region of the C terminus here reported to have dendritic sorting information can be used to identify interacting components, either in the form of glutathione S-transferase fusion peptides or as bait for the yeast two-hybrid screen.

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