A novel anterograde trafficking signal present in the N terminal extracellular domain of ionotropic glutamate receptors

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Running title: GluR1 surface expression requires its extreme N terminus

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Trafficking of AMPA receptors to and from the postsynaptic membrane plays an important role in regulating transmission at excitatory synapses. AMPA receptor subunits contain a large extracellular N terminal domain that is important for receptor assembly (1). To further investigate the determinants of receptor assembly and surface expression, we have epitope-tagged the N terminal domain of the AMPA receptor subunit, GluR1, and expressed it in HEK 293 cells and hippocampal neurons. Full length GluR1 was readily detected on the cell surface in both cell types. However, surface expression was profoundly decreased by deletion or replacement of 9 amino acids in the extreme N terminus. Immunoprecipitation experiments demonstrated that the mutant GluR1, in which this sequence was deleted, still interacts with GluR2, suggesting that mutant GluR1 is capable of at least partial assembly into heteromeric structures. The mutant forms of GluR1 co-localize with an endoplasmic reticulum (ER) marker suggesting that they are retained in this structure. These results suggest a specific function of a short sequence present in the N terminal domain in controlling anterograde trafficking of ionotropic glutamate receptors.

Ionotropic glutamate receptors are routinely subdivided into three classes termed α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, N-methyl-D-aspartate (NMDA) receptors and kainate receptors. These are found at virtually all excitatory synaptic connections in the mammalian brain and mediate the majority of excitatory synaptic transmission (2,3). They are thought to be assembled as heteromers of most likely four (4-6) or perhaps five (7,8) individual subunits which share similar membrane topology (9,10). Starting from the amino terminal this includes an extracellular, N terminal domain (NTD) of ~400 amino acids and an S1 domain which precedes the first transmembrane domain and is necessary for ligand binding. A reentrant loop follows this transmembrane domain and forms the pore for the channel. This is followed by a second transmembrane domain and a large extracellular loop that contains the S2 region of the ligand-binding domain. Finally the protein transverses the membrane a third time and ends intracellularly with a 50 to 100 amino acid C terminal domain.

A major means by which glutamatergic signaling is modulated in vivo is by membrane trafficking processes controlling the surface expression of receptors (11). Protein interactions with the C-terminal cytoplasmic domains of glutamate receptor subunits are thought to play important roles in regulating both anterograde trafficking to, and endocytic removal from, the neuronal plasma.
membrane (11). Extracellular surfaces of ionotropic glutamate receptors are well known to mediate ligand binding and to contribute to inter-subunit interactions involved in the assembly of functional receptors (1). There is accumulating evidence that extracellular surfaces of glutamate receptors may also play additional roles in controlling the membrane trafficking of receptors. The neural activity-regulated pentraxin Narp is a secreted protein that interacts with extracellular domains of GluR1, GluR2 and GluR3 subunits and promotes synaptic clustering of receptors in certain neurons (12). Studies of other classes of membrane protein, such as voltage-gated potassium channels, indicate that subunit oligomerization can indirectly regulate membrane trafficking by controlling the exposure of anterograde or retrograde trafficking signals on the cytoplasmic surface (13,14). Interestingly, a large deletion in the NTD of GluR2 that strongly inhibits subunit assembly did not disrupt surface expression of receptor subunits in non-neural cells (which do not express Narp) (15). These observations suggest the possibility that there may exist additional mechanisms controlling the membrane trafficking of ionotropic glutamate receptors, which are not dependent on Narp protein interactions or on subunit oligomerization itself.

We have addressed this question by examining the surface targeting of epitope-tagged mutant versions of GluR1 and GluR2 expressed in heterologous non-neural cells and in cultured hippocampal neurons in which these subunits are endogenously expressed. Our results indicate that the extreme N-terminus of both GluR1 and GluR2 contains a sequence which is required for anterograde membrane trafficking of glutamate receptor subunits to the plasma membrane and, when mutated, results in ER retention of subunits without detectably affecting subunit oligomerization. A core motif (IQI) is highly conserved across species and is required for ER export and surface expression of receptor subunits both in non-neural cells and in hippocampal neurons. Thus we propose that ionotropic glutamate receptors contain a Narp-independent anterograde trafficking signal in the extreme N-terminal extracellular domain.

Experimental Procedures

Recombinant DNA—Standard molecular biology protocols were used to construct flag tagged GluR1 proteins (16,17). Serial deletions in the extreme N terminus of GluR1 were performed by PCR based mutagenesis and all mutant constructs were sequenced to confirm there were no PCR errors. Alanine mutation of GluR1 was accomplished by incorporating the mutation in the PCR
primers. All constructs were placed under the control of a CMV promoter. CD4-GFP-KKYL was kindly provided by Lily Jan (UCSF) and HA-NR1 by S. Okabe (Japan).

Cell culture and transfections--Hippocampal cell cultures were prepared as described previously (18). Briefly, hippocampi were taken from P0 rat pups and the dentate gyri were removed. Tissue dissociation was facilitated by papain treatment and followed by tituration with glass pipettes. Cells were plated on poly D-lysine coated cover slips and grown in neurobasal medium supplemented with B27. Media was changed by half on the day after plating and in part each week thereafter. Glial growth was inhibited by FUDR after one week in culture. Human embryonic kidney (HEK 293) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100-units/ml penicillin/streptomycin. Cells [both HEK 293 cells and primary hippocampal neurons (8-10 DIV)] were transfected using a calcium phosphate method (18) and were examined two days after transfection. All constructs were examined in at least three independent transfections.

Immunofluorescence methods--Flag monoclonal antibody M1(Sigma) was used at 1µg/ml for either live feeding or permeabilized staining. For live surface labeling, the flag antibody was diluted in conditioned medium and then incubated with cells for 15 min at 37°C. The excess antibody was washed out and the cells fixed by 4% paraformaldehyde (in PBS) for 15 min. The cells were then incubated with non-permeabilizing blocking buffer (NP buffer: 2% BSA in PBS) for at least 40 min before being stained with Alexa 568 conjugated anti-mouse secondary antibody (Molecular Probes). For permeabilized staining, the cells were fixed with 4% paraformaldehyde for 15 min, and then blocked in permeabilizing buffer (P buffer: 0.1% triton-X 100 in NP buffer) for at least 40 min. The primary antibody (anti-flag antibody) was then diluted in P buffer and incubated with the cells for 1 hour before they were stained with secondary antibody. Most experiments in this study involved first performing the live surface feeding procedure followed by the permeabilized staining procedure to label the intracellular protein using a different fluorophore. The antibody to the ER marker calnexin was from Stressgen and the antibody to the Golgi marker COP II was a gift from Dr. Suzanne R. Pfeffer (Stanford University). Coverslips were mounted on slides with fluoromount G (Electron Microscopy Sciences, PA) and cells were imaged using a 63X oil immersion objective on a Zeiss Axioskop2. Images were digitized using a cooled CCD camera (Hamamatsu) and were analyzed using Metamorph Software (Universal Imaging). For individual experiments, images for all
conditions were analyzed using identical acquisition parameters. Surface expression of constructs was visually obvious (>10 times background) and therefore was not quantified in most experiments.

To make a quantitative estimate of the relative surface expression of the Flag-(I7AQ8AI9A)-GluR1 construct compared to wild type Flag-GluR1, a three step amplification procedure was used (16) with two modifications: the secondary antibody was a biotinylated anti-mouse antibody (Jackson Immuno) which was amplified using streptavidin linked Alexa 564 (Jackson Immuno). The surface expression of Flag-(I7AQ8AI9A)-GluR1 was estimated by calculating the total surface immunofluorescence (above background) per unit cell area for cells expressing Flag-(I7AQ8AI9A)-GluR1 and directly comparing this value to that of cells expressing Flag-GluR1 from the same culture preparation and using identical acquisition parameters. The levels of expression of the two constructs were comparable as measured by the total immunofluorescence obtained using our standard two step procedure (primary antibody followed by fluorescent secondary antibody).

Western blotting and immunoprecipitation (IP)--HEK 293 cells were co-transfected with two glutamate receptor subunits and cells were harvested after two days of expression. The cells were resuspended in IP buffer [50mM Tris, pH 7.4, 150mM NaCl, 1% triton X100 and 1x of complete protease inhibitor cocktail (Boehringer Mannheim 1697498)]. After using a 25G needle to break the cell membranes, the total extracts were centrifuged at 14,000 rpm for 15 minutes. The supernatants were spun again at the same centrifugal force. The resulting supernatant was then subject to immunoprecipitation using flag monoclonal antibody (2 µg) which was added to 0.5-ml aliquots incubated on ice for 1 h. Protein G Sepharose (50 µl) was used to precipitate antibodies. Protein G pellets were washed three times with buffer containing 100 mM NaCl and 1% Triton X-100. Immunoprecipitated proteins were denatured with loading buffer and resolved by SDS-PAGE (7.5%) in duplicate. One membrane was probed with anti-flag antibody and the other was probed with an anti-GluR2/3 antibody (Chemicon) or an antibody against the C terminus of GluR1 (Chemicon).

RESULTS

For previous experiments designed to study the mechanisms which control the surface expression of AMPA receptors, we epitope tagged GluR1, an ubiquitous subunit of endogenous AMPA receptors, on the very N-terminus of its extracellular domain using a Flag epitope tag sequence (Fig. 1) (16). To maintain the exact length of this extracellular domain, the N-terminal first nine amino acids of the mature GluR1 (ANFPNNIQI) were replaced by the Flag tag. Two assays
suggested that this Flag-tagged GluR1 construct produced functional AMPA receptors. When expressed in HEK 293 cells, application of agonist generated characteristic AMPA receptor-mediated currents and when expressed in hippocampal neurons, we observed surface clusters of Flag-GluR1 at synapses (16). However, despite the robust overexpression of Flag-GluR1 the amount of recombinant protein on the cell surface was relatively low when compared to endogenous AMPA receptors as evidenced by the relatively small currents that were generated in HEK 293 cells and the fact that a three-step amplification procedure was required to visualize the surface Flag-GluR1 receptors (16). These observations raised the possibility that the 9 deleted N-terminal amino acids may play an important role in the normal surface expression of GluR1.

To test this possibility we compared the surface expression of this original construct (now termed Flag-ND9-GluR1) with the full length GluR1 tagged at the N-terminus with Flag (now termed Flag-GluR1) (Fig. 1). In both HEK 293 cells and hippocampal neurons, Flag-GluR1 exhibited robust, easily detectable surface staining using a standard surface staining procedure (see Methods) while Flag-ND9-GluR1 yielded very low or undetectable surface expression (Fig. 2). This was not due to significant differences in the level of expression of the two constructs since permeabilized staining revealed large amounts of recombinant GluR1 were present (Fig. 2). Thus while flag-ND9-GluR1 is capable of surface expression (16), it is severely defective compared with full length GluR1. Similar results were obtained when the AMPA receptor subunit GluR2 was examined in that Flag-ND9-GluR2 showed minimal or no surface expression when expressed in either HEK 293 cells or hippocampal neurons (data not shown).

To exclude the possibility that the Flag tag itself affects surface expression we also examined the consequences of deleting these 9, N-terminal amino acids in GluR1 which was not tagged with Flag. When expressed in HEK 293 cells wild type GluR1 exhibited clear surface expression (detected by an antibody to an extracellular domain of GluR1 (19)) while ND9-GluR1 was not detectable on the cell surface (Fig. 3). Again, permeabilized staining revealed that both proteins were robustly expressed.

Two general mechanisms can account for the defect in surface expression of ND9-GluR1. One is that it cannot be transported normally through the biosynthetic pathway and rarely reaches the cell surface. The second possibility is that the mutation greatly enhances the endocytosis of GluR1 relative to its delivery to the cell surface and thus very low levels of surface receptors are detected. To address this latter possibility we co-expressed Flag-ND9-GluR1 with a dominant negative form
of dynamin (mDynamin) which has been shown to block the endocytosis of endogenous AMPA receptors (19). Co-transfection with mDynamin still did not yield detectable surface expression of Flag-ND9-GluR1 even though an increase in the surface expression of Flag-GluR1 was observed when expressed with mDynamin (data not shown). An alternate way of blocking endocytosis is incubation of cells with a high concentration of sucrose, a manipulation that like the mDynamin blocked the endocytosis of endogenous AMPA receptors (19). However, incubation of HEK 293 cultures with 0.35 M sucrose for 30 minutes before the labeling procedure still did not result in detectable surface expression of Flag-ND9-GluR1 (data not shown).

These results suggest that some failure in its transport through the biosynthetic pathway accounts for the lack of surface expression of Flag-ND9-GluR1. To begin to address in which subcellular organelle Flag-ND9-GluR1 is retained we compared its intracellular localization with that of well-established protein markers of the Golgi and ER. Co-labeling with an antibody to the ER marker calnexin showed near perfect co-localization (Fig. 4B), while co-labeling with an antibody to the Golgi marker COP II gave two distinct immunofluorescence patterns (Fig. 4A). We also co-expressed Flag-ND9-GluR1 with an ER marker protein CD4-gfp-KKYL (18,20) and these two recombinant proteins exhibited strong co-localization (Fig. 4C) providing further evidence that Flag-ND9-GluR1 appears to be trapped in the ER.

Since transmembrane receptor proteins usually assemble in the ER before they are transported to the Golgi and the plasma membrane, it is possible that the trapping of Flag-ND9-GluR1 in the ER occurred because it cannot assemble with other AMPA receptor subunits. To address this possibility we performed co-immunoprecipitation experiments (15,21) with co-expressed GluR2 which is known to assemble with GluR1 in the formation of endogenous AMPA receptors (22). Flag-GluR1 and GluR2 were co-expressed in HEK 293 cells and detergent (1% Triton X 100) soluble fractions were used for immunoprecipitation with anti-Flag antibody. Figure 5 shows that GluR2 co-immunoprecipitated with Flag-GluR1, confirming that Flag-GluR1 and GluR2 interact with each other. We then performed the same assay using Flag-ND9-GluR1 and again found that immunoprecipitation of the Flag-tagged protein co-immunoprecipitated a substantial amount of GluR2 (Fig. 5). In control experiments using cells expressing Flag-GluR1 only, immunoprecipitation with the anti-Flag antibody did not yield a band on SDS-PAGE after probing with the anti-GluR2/3 antibody (data not shown), indicating (as specified by the manufacturer) that this antibody does not cross react with GluR1. To further test the specificity of this assay, we co-expressed Flag-GluR1 or
Flag-ND9-GluR1 with HA-NR1 (23), an epitope tagged NMDA receptor subunit. As expected, Flag-GluR1 and Flag-ND9-GluR1 co-immunoprecipitated a minimal amount of HA-NR1 (data not shown). Because Flag-ND9-GluR1 still binds and presumably assembles with GluR2, we wondered whether GluR2 would rescue the surface expression of Flag-ND9-GluR1. To test this we co-expressed Flag-ND9-GluR1 and GluR2 in both HEK 293 cell and neurons, but in neither cell type did GluR2 rescue the surface expression of Flag-ND9-GluR1 (data not shown).

We next performed a more detailed mutational analysis to determine which of the nine amino acids deleted in Flag-ND9-GluR1 are necessary for GluR1 surface expression. Surprisingly, the deletion of amino acids 2 to 6 [Flag-ND5-GluR1: Δ(NFPNN)] (Fig. 1) did not impair the surface expression of GluR1 in either HEK 293 cells or hippocampal neurons (Fig.6). In contrast, the deletion of the three membrane proximal amino acids [Flag-Δ(IQI)-GluR1] (Fig. 1) completely disrupted the surface expression of GluR1 in both cell types (Fig. 6) as did alanine scanning mutagenesis of these three amino acids [Flag-(I7AQ8AI9A)-GluR1] (Fig. 1,6). Thus the IQI motif at amino acids 7 to 9 appear to be necessary for the export of GluR1 out of the ER, and hence surface expression.

In a final set of experiments we attempted to estimate the relative percentage of the mutant constructs that can reach the plasma membrane of hippocampal neurons. To accomplish this we used the Flag-(I7AQ8AI9A)-GluR1 construct and performed a three step amplification procedure to label surface receptors (see Experimental Procedures). Using this approach, we were able to detect surface expression of Flag-(I7AQ8AI9A)-GluR1. However, the total surface immunoreactivity was only ~6% of that of Flag-GluR1. In independent experiments, we estimated that ~50-70% of the total Flag-GluR1 reaches the cell surface suggesting that only ~3-4% of the mutant Flag-(I7AQ8AI9A)-GluR1 reaches the plasma membrane.

DISCUSSION

The main new finding from this work is that the extreme N termini of GluR1 and GluR2 are critical for their surface expression in both HEK 293 cells and hippocampal neurons. The specific amino acid motif IQI at positions 7 to 9, which is conserved in both GluR1 and GluR2, is particularly important in that it appears to be necessary for the export of the receptor out of the ER although it is not required for binding to other subunits. Previous work suggests that the NTD’s of AMPA receptor subunits are necessary for dimer formation (15,24,25) while further dimerization of
two dimers to form a functional receptor is thought to be mediated by the interactions of the S2 and/or transmembrane domains (1,24). The higher affinity association between NTD’s and S2 domains within a subfamily determines the favorable association among intra-subfamily subunits (1,15,24). Our co-immunoprecipitation experiments (Fig. 5) indicate that amino acids 1 to 9 of the very NTD of GluR1 are not required for dimer formation. Furthermore, the fact that folding generally occurs before assembly (26,27) makes it unlikely that the mutant GluR1’s are misfolded. This conclusion is further supported by the observation that ND9-GluR1 and wild type GluR1 show a similar degree of detergent solubility (data not shown).

The very NTD of GluR1 could be required for export from the ER and surface expression in two general ways. First, it could be important for secondary dimerization if this process required both compatible S2 domains and full-length integral NTDs. The fact that the S1,S2 domain itself can form a dimer in crystal (28) makes this possibility unlikely. Second, the mutant GluR1 constructs could be fully capable of assembly into a functional receptor but not exported out of ER either because of the unmasking of an ER retention signal (13,14,18,29-31) or because they are missing an ER forward trafficking signal. The unmasking of an ER retention signal seems unlikely because there were no clear ER retention signal-like motifs present in either GluR1 or GluR2 (29). A forward trafficking signal, on the other hand, has also been described in the intracellular domain of certain potassium channels (20) and provides a potential novel mode of regulation of receptor number on the plasma membrane.

The proposal of an ER forward trafficking signal in the extracellular domain of GluR1 is provocative since the machinery regulating export from the ER is thought to be mainly concentrated in the cytosolic fraction, not in the lumen of the ER. However, in principle, transport receptors could recognize luminal domains of membrane receptors as hypothesized for the selective transport of class I MHC molecules out of the ER (32). Narp, which clusters AMPA receptors via interactions with extracellular domains (12,33), is one protein that might interact with AMPA receptors in the ER lumen and facilitate their export. Another intriguing candidate for this function is stargazin, which is necessary for the trafficking of AMPA receptor to the plasma membrane (34). Interestingly, non-neural cells (i.e. HEK 293 cells, COS cells) do not express Narp (33) or stargazin (34,35) indicating that different AMPA receptor-interacting targeting proteins must exist in these cells.

In summary, we have presented evidence that the extreme N terminus of GluR1, specifically the IQI motif at amino acids 7 to 9, is necessary for its normal trafficking out of the ER. Identical (in
GluR2) or similar (VQI in GluR4) NTD motifs exist in other AMPA receptor subunits suggesting that the same trafficking mechanisms likely apply to these subunits. It will be interesting in future studies to determine the mechanism by which this trafficking signal functions. As the mutations that disrupt ER export of glutamate receptor subunits did not detectably inhibit the formation of subunit oligomers, we favor the hypothesis that the IQI motif engages in a distinct protein interaction of the receptor ectodomain that is required for ER export. On the other hand, we cannot rule out more subtle effects of the IQI sequence on heteromer formation itself, which might not be detectable by co-immunoprecipitation analysis of assembly and might secondarily affect the exposure of trafficking signals present in the cytoplasmic domain. In either case, the present studies define a distinct sequence in the ectodomain of ionotropic glutamate receptors which functions as a novel anterograde membrane trafficking signal.

ACKNOWLEDGEMENTS

This work was supported by grants from N.I.H. H.X. was supported by an NRSA from N.I.M.H. We thank Tina Lizama for her expert assistance in preparing the neuronal cultures used in this study, and Jerron Fisher for expert technical help in molecular biology. We also thank Steven Braithwaite (Agy Inc, South San Francisco), Bill Ju, and Sheela Singla for helpful discussions.

REFERENCES

1. Madden, D. R. (2002) *Nat. Rev. Neurosci.* 3(2), 91-101.
2. Sheng, M., and Sala, C. (2001) *Annu. Rev. Neurosci.* 24, 1-29
3. Wisden, W., and Seeburg, P. H. (1993) *Curr. Opin. Neurobiol.* 3(3), 291-8.
4. Rosenmund, C., Stern-Bach, Y., and Stevens, C. F. (1998) *Science* 280(5369), 1596-9.
5. Mano, I., and Teichberg, V. I. (1998) *Neuroreport* 9(2), 327-31.
6. Laube, B., Kuhse, J., and Betz, H. (1998) *J. Neurosci.* 18(8), 2954-61.
7. Ferrer-Montiel, A. V., and Montal, M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93(7), 2741-4.
8. Premkumar, L. S., and Auerbach, A. (1997) *J. Gen. Physiol.* 110(5), 485-502.
9. Bennett, J. A., and Dingledine, R. (1995) *Neuron* 14(2), 373-84.
10. Hollmann, M., Maron, C., and Heinemann, S. (1994) *Neuron* 13(6), 1331-43.
11. Malinow, R., and Malenka, R. C. (2002) *Annu. Rev. Neurosci.* 25, 103-26
12. O'Brien, R. J., Xu, D., Petralia, R. S., Steward, O., Huganir, R. L., and Worley, P. (1999) *Neuron* **23**(2), 309-23.
13. Margeta-Mitrovic, M., Jan, Y. N., and Jan, L. Y. (2000) *Neuron* **27**(1), 97-106.
14. Zerangue, N., Schwappach, B., Jan, Y. N., and Jan, L. Y. (1999) *Neuron* **22**(3), 537-48.
15. Leuschner, W. D., and Hoch, W. (1999) *J. Biol. Chem.* **274**(24), 16907-16.
16. Lissin, D. V., Gomperts, S. N., Carroll, R. C., Christine, C. W., Kalman, D., Kitamura, M., Hardy, S., Nicoll, R. A., Malenka, R. C., and von Zastrow, M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**(12), 7097-102.
17. Xia, H., Winokur, S. T., Kuo, W. L., Altherr, M. R., and Bredt, D. S. (1997) *J. Cell. Biol.* **139**(2), 507-15.
18. Xia, H., Hornby, Z. D., and Malenka, R. C. (2001) *Neuropharmacology* **41**(6), 714-23.
19. Carroll, R. C., Beattie, E. C., Xia, H., Luscher, C., Altschuler, Y., Nicoll, R. A., Malenka, R. C., and von Zastrow, M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**(24), 14112-7.
20. Ma, D., Zerangue, N., Lin, Y. F., Collins, A., Yu, M., Jan, Y. N., and Jan, L. Y. (2001) *Science* **291**(5502), 316-9.
21. Wang, Z. Z., Hardy, S. F., and Hall, Z. W. (1996) *J. Biol. Chem.* **271**(44), 27575-84.
22. Wenthold, R. J., Petralia, R. S., Blahos, J., II, and Niedzielski, A. S. (1996) *J. Neurosci.* **16**(6), 1982-9.
23. Okabe, S., Miwa, A., and Okado, H. (1999) *J. Neurosci.* **19**(18), 7781-92.
24. Ayalon, G., and Stern-Bach, Y. (2001) *Neuron* **31**(1), 103-13.
25. Kuusinen, A., Abele, R., Madden, D. R., and Keinanen, K. (1999) *J. Biol. Chem.* **274**(41), 28937-43.
26. Green, W. N., and Claudio, T. (1993) *Cell* **74**(1), 57-69.
27. Netzer, W. J., and Hartl, F. U. (1998) *Trends Biochem. Sci.* **23**(2), 68-73.
28. Armstrong, N., and Gouaux, E. (2000) *Neuron* **28**(1), 165-81.
29. Ma, D., and Jan, L. Y. (2002) *Curr. Opin. Neurobiol.* **12**(3), 287-92.
30. Scott, D. B., Blanpied, T. A., Swanson, G. T., Zhang, C., and Ehlers, M. D. (2001) *J. Neurosci.* **21**(9), 3063-72.
31. Standley, S., Roche, K. W., McCallum, J., Sans, N., and Wenthold, R. J. (2000) *Neuron* **28**(3), 887-98.
32. Spiliotis, E. T., Manley, H., Osorio, M., Zuniga, M. C., and Edidin, M. (2000) *Immunity* **13**(6), 841-51.

33. Tsui, C. C., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Barnes, C., and Worley, P. F. (1996) *J. Neurosci.* **16**(8), 2463-78.

34. Chen, L., Chetkovich, D. M., Petralia, R. S., Sweeney, N. T., Kawasaki, Y., Wenthold, R. J., Bredt, D. S., and Nicoll, R. A. (2000) *Nature* **408**(6815), 936-43.

35. Letts, V. A., Felix, R., Biddlecome, G. H., Arikath, J., Mahaffey, C. L., Valenzuela, A., Bartlett, F. S., 2nd, Mori, Y., Campbell, K. P., and Frankel, W. N. (1998) *Nat. Genet.* **19**(4), 340-7.
FIGURE LEGENDS

Fig. 1. Schematic of the GluR1 constructs and their expression in HEK 293 cells. A, Topology of GluR1 is shown on the right with the first 13 amino acids of the mature GluR1 shown on the left. Residues deleted or mutated in this study are in bold type. Underlined amino acids are the flag motif. B, HEK 293 cells were transfected with the respective constructs and two days after transfection, cells were lysed and the triton X100 soluble fractions were run on SDS page and probed with flag antibody.

Fig. 2. Flag-ND9-GluR1 is defective in surface expression in both HEK 293 cells and neurons. Flag-GluR1 and Flag-ND9-GluR1 were transfected into either HEK 293 cells (top two panels) or primary hippocampal neurons (bottom two panels). Cells were subject to surface staining with flag antibody, after which cells were permeabilized for subsequent intracellular staining with a different (green) fluorophore.

Fig. 3. ND9-GluR1 is defective in surface expression in HEK 293 cells. HEK 293 cells were transfected with GluR1 or ND9-GluR1 and subject to surface staining with an antibody against a motif in the extracellular domain of GluR1 (19). Cells were subsequently permeabilized for labeling of intracellular GluR1 by the same antibody.

Fig. 4. Flag-ND9-GluR1 is retained in the ER in neurons. A, Flag-ND9-GluR1 does not colocalize with the Golgi marker COP II. B, Flag-ND9-GluR1 colocalizes with the ER marker calnexin. C, Flag-ND9-GluR1 colocalizes with the ER marker CD4-GFP-KKYL.

Fig. 5. Flag-ND9-GluR1 interacts with GluR2 as efficiently as wild type GluR1. HEK 293 cells were co-transfected with GluR2 and either Flag-GluR1 or Flag-ND9-GluR1. Immunoprecipitation of either construct with Flag antibody effectively co-precipitated approximately equal amounts of GluR2.

Fig. 6. IQI is necessary for surface expression of GluR1. Both deletion and alanine scan mutagenesis of IQI in the N terminus of GluR1 result in inhibition of surface expression, while
deletion of the other 5 amino acids has no effect on the surface expression of GluR1. Flag-ND5-GluR1, Flag-Δ(IQI)-GluR1 and Flag-(I7AQ8A9A)-GluR1 were transfected into neurons and HEK 293 cells. Cells were subject to surface staining with flag antibody, after which cells were permeabilized for subsequent intracellular flag motif staining with a different (green) fluorophore.
Wild type GluR1

Intracellular

Surface

ND2-GluR1
Δ(ANFPNNIQI)

Merge
Transfection

| GluR2/Flag-GluR1 | GluR2/Flag-ND9-GluR1 |
|------------------|---------------------|

Immunoprecipitation with anti-Flag antibody

Probed with:

- Anti-Flag antibody
- Anti-GluR2/3 antibody

[Image of immunoprecipitation gels]
Flag-ND5-GluR1

Flag-Δ(IQ1)-GluR1

Flag-(I7AQ8AI9A)-GluR1

HEK 293

Intracellular  Surface  Merge

Neuron

Intracellular  Surface  Merge
A novel anterograde trafficking signal present in the N terminal extracellular domain
of ionotropic glutamate receptors
Houhui Xia, Mark Von Zastrow and Robert C. Malenka

J. Biol. Chem. published online October 3, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M207122200

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