The Stargazin C Terminus Encodes an Intrinsic and Transferable Membrane Sorting Signal

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Activity-dependent plasticity of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors is regulated by their auxiliary subunit, stargazin. Association with stargazin enhances α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor surface expression and modifies the receptor’s biophysical properties. Fusing the cytoplasmic C terminus of stargazin to the C-terminal domains of either GluR1 or the gonadotropin-releasing hormone receptor permits efficient trafficking from the endoplasmic reticulum and sorting to the basolateral membrane without altering other properties of either receptor.

α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors are ionotropic glutamate receptors that mediate rapid excitatory neurotransmission in the central nervous system and undergo activity-dependent alterations in cellular localization and biophysical properties, contributing to cellular mechanisms of learning and memory (1–3). Transmembrane AMPA receptor regulatory proteins such as stargazin (STG, γ-2) physically associate with AMPA receptors (4). Upon association, stargazin enhances surface expression of AMPA receptors that otherwise appear to be trapped in the endoplasmic reticulum (ER) (5) and slows the kinetics of receptor deactivation and desensitization (6–9). The C-terminal domain of stargazin is necessary for its trafficking effects, presumably permitting efficient trafficking through the ER and cis-Golgi compartments (6, 8). However, it is not known whether stargazin masks ER retention signals on AMPA receptors or acts independently through an ER export signal (10, 11).

We previously performed site-directed mutagenesis on two ER retention sites, which had no effect on stargazin-mediated trafficking (12). We subsequently mutated residues EEEFE within the cytoplasmic loop to QQFEE or EEFQQ and found normal modulation and trafficking by stargazin, suggesting that this simple model of stargazin masking ER retention sites of the AMPA receptor was not likely.

MATERIALS AND METHODS

Generation of Constructs—The cytomegalovirus expression plasmids (pRK) for GluR1 were provided by Dr. Peter Seeburg (Max Planck Institute for Medical Research, Heidelberg, Germany). R1i46-YFP was generated using overlapping PCR to make an in-frame fusion protein with YFP, using pEYFP (Clontech) as a template. Stargazin cloned from Rattus norvegicus in the pcDNA3 vector was a generous gift from Dr. David Bredt (University of California, San Francisco). STG-YFP was created by inserting YFP in-frame at the BglII site in stargazin but truncating after amino acid 269. R1i46cSTG-YFP was made by inserting the restriction site MluI (ACCGGT) between amino acids GGG and SGE of the C-terminal domain using a QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The final sequence at the fusion was GGGTR(YFP). R1i46cSTG-YFP was created by fusing the stargazin cytoplasmic tail, amino acids 203–323, beginning DRHK and ending TTPV, followed by YFP. The final fusion had MluI sites between the C terminus of R1i46 and the 5′ end of the stargazin cytoplasmic tail and the 3′ end of the stargazin cytoplasmic tail and YFP. The cSTG-CFP fusion protein was created using the pECFP-N1 vector and inserting amino acids 203–323 of the stargazin cytoplasmic tail preceded by ATG at the Xhol and HindIII restriction sites. GnRHR-GFP (13) was created by inserting amino acids 203–323 of stargazin between GnRHR and pEGFP at the BamHI site.

Transient Transfections for Electrophysiology—Human embryonic kidney (HEK) 293 fibroblasts (CRL 1573; American Type Culture Collection) were cultured as described previously (14). Cells were transiently transfected using Lipofectamine 2000 (Invitrogen) with AMPA receptor cDNA (0.25–1 μg/35-mm dish). After transfection, 10–20 μM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo(f)quinoxaline-7-sulfonamide was added to the medium to prevent cell toxicity.

Transient Transfections for Confocal Microscopy—Collagen- or poly-d-lysine-coated 14-mm glass bottom culture dishes (MatTek Corp., Ashland, MA) were incubated with ECL attachment matrix (Upstate Cell Signaling Solutions, Lake Placid, NY) for 1 h at 37 °C and then washed with complete MEM before plating cells. Cells were transfected using Lipofectamine 2000 when 60–90% confluent and incubated under identical conditions as cells used for standard electrophysiology. For each transfection 70 μl of MEM was incubated with 3 μl of Lipofectamine, and in another tube 30 μl of MEM was incubated with 0.1–3 μg of total cDNA and thoroughly mixed. Contents of the tubes were combined, and after 20–30 min the solution was added to the cells. 6–24 h later, the solution was exchanged with fresh complete MEM with 10–20 μM 2,3-dioxo-6-nitro-1,2,3,4-tetrahy-
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drobenzo(f)quinoxaline-7-sulfonamide. All cells were imaged at room temperature 2–3 days after transfection. Immediately before imaging, the solution was exchanged with complete MEM containing no phenol red.

Outside-out Patch Recordings—Currents were recorded 2–3 days after transfection as described previously (14). Extracellular solutions contained the following: 20 mM sucrose, 145 mM NaCl, 5.4 mM KCl, 5 mM HEPES, 1 mM MgCl₂, 1.8 mM CaCl₂·H₂O, and 0.01 mg/ml phenol red, pH 7.3. Outside-out membrane patches were voltage-clamped at ~60 mV using an Axopatch 200B amplifier (Molecular Devices, Union City, CA). Synapse (Version 3.6d; Synergy Research, Silver Spring, MD) controlled piezoelectric movement, data acquisition, and trace analysis. Responses were filtered at 5 kHz, digitized at 10–500 μs/pulse, and stored on a Power Macintosh computer (Apple, Cupertino, CA) using an ITC-16 interface (InstruTech, Port Washington, NY). Micropipettes (2–5 megohms; TW150F; World Precision Instruments, Sarasota, FL) contained the following: 135 mM CsCl, 10 mM CsF, 10 mM HEPES, 5 mM Cs₂-1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid, 1 mM MgCl₂, and 0.5 mM CaCl₂, pH 7.2. Patches were perfused at 0.2 ml/min with solutions emitted from a two-barrel flow pipe made with θ tubing (BT150–10; Sutter Instruments, Novato, CA). One barrel contained vehicle (control) composed of the following: 145 mM NaCl, 5.4 mM KCl, 5 mM HEPES, 1 mM MgCl₂, 1.8 mM CaCl₂·H₂O, and 0.01 mg/ml phenol red, pH 7.3. The other barrel had this solution plus 1-glutamate (10 mM). After going into the voltage clamp, an outside-out patch was pulled, lifted up to the flow pipe, positioned near the interface between the glutamate-free and glutamate-containing solutions, and jumped rapidly from the vehicle control into glutamate. Rapid solution exchanges of 500 ms were driven by a piezoelectric device (Burleigh Instruments, Fishers, NY).

Confocal Imaging—Transfected cells were imaged using an LSM 510 META laser scanning confocal microscope (Zeiss, Thornwood, NY) with a Plan Achromat LSM 510 META laser scanning confocal microscope (Zeiss, Thornwood, NY) with a Plan Achromat 63×/1.4 oil. Confocal imaging revealed that R1i46-CFP could have a marked increase in surface expression. We also determined that modulation of AMPA receptor kinetics. We also determined that the C terminus of stargazin (amino acids 203–323) in-frame with the C terminus of GluR1 and continued in-frame with YFP. This construct (R1i46cSTG-YFP) (Fig. 1a) was expressed heterologously, and confocal microscopy was used to assess the trafficking pattern. R1i46cSTG-YFP was effectively trafficked from the ER to the plasma membrane as determined by identifying cells in which the surface expression was much greater than the cytosolic expression. In two separate experiments, 69% (43/62 cells) and 55% (55/100 cells) of cells expressing R1i46cSTG-YFP had predominant surface expression. This was unlike GluR1 in the absence of stargazin (≤1% cells with surface expression for R1i46cSTG-YFP and more comparable with GluR1 co-expressed with stargazin (5–35% surface expression for R1i46cSTG-YFP + STG) (12) (Fig. 1b). In contrast, a soluble cSTG-CFP fusion protein that contained amino acids 203–323 of the stargazin cytoplasmic tail did not traffic AMPA receptors to the plasma membrane (data not shown). We confirmed that R1i46cSTG-YFP ion channel density was greater than wild-type channel density by measuring current amplitude in outside-out patches of cells with visually enhanced surface expression (708 ± 130 pA) versus R1i46-YFP (206 ± 77 pA) (Fig. 1c). This value was similar to what we found previously for R1i36-YFP and R1i81-YFP (12). Without selecting for R1i46cSTG-YFP enhanced surface expression, we did not see a significant current amplitude increase (337 ± 220 pA). These results are similar to what is found upon co-transfecting stargazin and AMPA receptors (12).

To demonstrate that the C terminus of stargazin modulated only the trafficking and not other aspects of AMPA receptor function, we assessed the activity of the fusion proteins. The desensitization kinetics (3.0 ± 0.4 ms) (Fig. 1c) were typical of wild-type GluR1 (3.7 ± 0.4 ms) and different from stargazin-modulated wild-type GluR1 (8.5 ± 0.8 ms) (Ref. 12; see also Refs. 6, 8, and 9). This provides strong evidence that trafficking by stargazin of AMPA receptors occurs independently of its modulation of AMPA receptor kinetics. We also determined that R1i46-CFP could have a marked increase in surface expression when co-expressed with R1i46cSTG-YFP, presumably by hetero-oligomerization (Fig. 1d).

To test whether the reduced ER retention was related to a GluR1-specific interaction, we fused the same C-terminal domain of stargazin to the GnRHR (GnRHRcSTG-GFP). Although a member of the G-protein-coupled receptor superfamily, the GnRHR is inefficiently trafficked to the plasma membrane and, like AMPA receptors, is largely retained in the ER in both homologous and heterologous cells (16, 17). The C-terminal fusion of stargazin dramatically enhanced GnRHR transport to the cell surface (Fig. 1e), with nearly every cell demonstrating robust surface expression. This is significant as it reveals trafficking information that is intrinsic to stargazin and transferable to an unrelated plasma membrane receptor.

The enhanced trafficking of the GnRHR by GnRHRcSTG-GFP did not impair protein function. We tested ligand-induced
phosphorylation of ERK, a well established intracellular target of GnRH signaling (Fig. 1f). Also, specific binding of GnRH agonist was not affected by the C-terminal stargazin fusion, and consistent with the imaging analysis, total cell-surface binding was significantly greater for cells expressing GnRHRcSTG-GFP compared with GFP alone or wild-type GnRHR-GFP (Fig. 1g).

The results from Fig. 1 indicate that the C terminus of stargazin encodes a membrane sorting signal that can operate independently of the rest of the stargazin protein. It is likely that stargazin-mediated trafficking of AMPA receptors therefore occurs by virtue of this signal, such that the AMPA receptors are pulled to the surface through their specific interaction with stargazin in a region that is not the stargazin cytoplasmic tail. Furthermore, this ability is not receptor-dependent, as the C-terminal domain acts on the GnRHR, a protein with which it does not associate.

Because stargazin has been shown to direct AMPA receptors not only to the plasma membrane, but more specifically to the synapse (7, 18, 19), we tested whether the C-terminal domain was involved with protein sorting. Stargazin was heterologously expressed in Madin-Darby canine kidney cells, a model system for neuronal trans-Golgi network sorting, where basolateral sorting in Madin-Darby canine kidney cells may reflect somatodendritic sorting in neurons (20). STG-YFP expression was restricted to the basolateral membrane (Fig. 2). GnRHRs did not demonstrate membrane sorting. However, when the C terminus of stargazin was fused to the GnRHR, it was trafficked specifically to the basolateral membrane, suggesting that stargazin encodes a sorting signal that either directs sorting vesicles to the basolateral membrane or permits enhanced endocytosis from the apical membrane. A similar effect was seen for R1i46-YFP, although again, the ability of stargazin to direct AMPA receptor sorting was not as robust as its effect on the GnRHR.

Several protein motifs have been identified as positive ER export signals: LL, DXE, RXR, VXXSL, and hydrophobic motifs, including FXN.X2F.X, FXXXF.XXXF, and FCYENE (21). Amino acids 270–323 of the stargazin C terminus may be truncated without affecting its ability to traffic: within the remaining C-terminal

![Image](https://example.com/image.png)
domain (amino acids 203–269) there are several possible dibasic motifs, the first of which is proximal to stargazin transmembrane domain 4 (amino acids 204–206), an obvious target as an ER export site. We mutated this motif in the GnRHR fusion and found that neutralization of the charges did not reverse the effects of the C-terminal domain of stargazin on GnRHR trafficking (data not shown).

We assumed previously that stargazin-mediated surface expression of AMPA receptors in HEK cells was variable because of differences in the amount of stargazin per cell. We found that with increasing stargazin per constant AMPA receptor cDNA concentration, we could get a surface expression enhancement in ∼50% of cells, but this value approached an asymptotic line (refer to Fig. 1c of Ref. 12). Surprisingly, despite having homomeric AMPA receptor chimeras in this study, we still observe ∼40% of the cell population that does not demonstrate an enhancement of surface expression or current amplitude. These results suggest that there is likely intrinsic cell variability in protein expression. The differences in the way these proteins may interact with AMPA receptors and keep them from the surface membrane are not present in GnRHRs. Sorting the two populations of cell types for microarray analysis to determine gene candidates that hinder or allow AMPA receptor trafficking to surface membranes may be instructive.

In summary, we have shown that the C terminus of stargazin directs effective trafficking of itself from the ER, as well as of AMPA receptors complexed with it, or of other receptors to which its C terminus is directly fused. Unexpectedly, we also found that the C terminus of stargazin encodes a sorting signal, directing trafficking through the trans−Golgi network to specific membrane compartments. It remains to be shown whether the sorting is regulated in an activity-dependent manner.

REFERENCES

1. Sheng, M., and Kim, M. J. (2002) Science 298, 776–780
2. Nicoll, R. A. (2003) Philos. Trans. R. Soc. Lond. B Biol. Sci. 358, 721–726
3. Malenka, R. C. (2003) Annu. N. Y. Acad. Sci. 1003, 1–11
4. Vandenberghe, W., Nicoll, R. A., and Bredt, D. S. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 485–490
5. Hall, R. A., Hansen, A., Andersen, P. H., and Soderling, T. R. (1997) J. Neurochem. 69, 625–630
6. Tomita, S., Adesnik, H., Sekiguchi, M., Zhang, W., Wada, K., Howe, J. R., Nicoll, R. A., and Bredt, D. S. (2005) Nature 435, 1052–1058
7. 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, 936–943
8. Turetsky, D., Garringer, E., and Patneau, D. K. (2005) J. Neurosci. 25, 7438–7448
9. Priel, A., Kolleker, A., Ayalon, G., Gillor, M., Osten, P., and Stern-Bach, Y. (2005) J. Neurosci. 25, 2682–2686
10. Vandenberghe, W., Nicoll, R. A., and Bredt, D. S. (2005) J. Neurosci. 25, 1095–1102
11. Ziff, E. B. (2007) Neuron 53, 627–633
12. Bedoukian, M. A., Weeks, A. M., and Partin, K. M. (2006) J. Biol. Chem. 281, 23908–23921
13. Horvat, R. D., Roess, D. A., Nelson, S. E., Barisas, B. G., and Clay, C. M. (2001) Mol. Endocrinol. 15, 695–703
14. Leever, D. L., Clark, S. Z., Weeks, A. M., and Partin, K. M. (2003) Mol. Pharmacol. 64, 5–10
15. Tucker, T. A., Varga, K., Behok, Z., Zsembery, A., McCarty, N. A., Collawn, J. F., Schwiebert, E. M., and Schwiebert, L. M. (2003) Am. J. Physiol. 284, C791–C804
16. Navratal, A. M., Bliss, S. P., Berghorn, K. A., Haughian, J. M., Farmerie, T. A., Graham, J. K., Clay, C. M., and Roberson, M. S. (2003) J. Biol. Chem. 278, 31593–31602
17. Knollman, P. E., Janovick, J. A., Brothers, S. P., and Conn, P. M. (2005) J. Biol. Chem. 280, 24506–24514
18. Bats, C., Groc, L., and Choquet, D. (2007) Neuron 53, 719–734
19. Deng, F., Price, M. G., Davis, C. F., Mori, M., and Burgess, D. L. (2006) J. Neurosci. 26, 7875–7884
20. Horton, A. C., and Ehlers, M. D. (2003) Neuron 40, 277–295
21. Ma, D., and Jan, L. Y. (2002) Curr. Opin. Neurobiol. 12, 287–292
22. Scannevin, R. H., Murakoshi, H., Rhodes, K. J., and Trimmer, J. S. (1996) J. Cell Biol. 135, 1619–1632