Phosphorylation of Stargazin by Protein Kinase A Regulates Its Interaction with PSD-95*

Received for publication, January 17, 2002
Published, JBC Papers in Press, January 22, 2002, DOI 10.1074/jbc.M200528200

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Stargazin is the first transmembrane protein known to associate with AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionate) glutamate receptors (AMPARs) and regulate their synaptic targeting by two distinct mechanisms, specifically via delivery of AMPARs to the surface membrane and synaptic targeting of these receptors by binding to PSD-95/SAP-90 and related PDZ proteins. However, it is not known whether and how this stargazin-mediated synaptic targeting of AMPARs is regulated. Stargazin interacts with the PDZ domains of PSD-95 through the C-terminal PDZ-binding motif. The stargazin C terminus contains a consensus sequence for phosphorylation by cAMP-dependent protein kinase A (PKA). Phosphorylation site-specific stargazin antibodies reveal that the stargazin C terminus is phosphorylated at the Thr-321 residue in heterologous cells and in vivo. Stargazin phosphorylation is enhanced by the catalytic subunit of PKA. Mutations mimicking stargazin phosphorylation (T321E and T321D) lead to elimination of PKA, protein kinase A; PSD, postsynaptic density; EGFP, enhanced green fluorescent protein.

This paper is available on line at http://www.jbc.org

* This work was supported by the Korean Ministry of Science and Technology, the Korea Science and Engineering Foundation, and the Korea Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: AMPAR, AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionate) glutamate receptor; aa, amino acid(s); PKA, protein kinase A; PSD, postsynaptic density; EGFP, enhanced green fluorescent protein.
EXPERIMENTAL PROCEDURES

Antibodies—Anti-fusion protein stargazin antibody (Stg-Cyto 1222, guinea pig polyclonal) was generated using an H6 fusion protein containing two copies of the C-terminal cytoplasmic region of stargazin (aa 203–323) as immunogen. Anti-peptide stargazin antibody (Stg-C-term 1217, rabbit polyclonal) was generated using a synthetic peptide mimicking the last 10 aa residues of stargazin (CQTANRRTTPV). The underlined cysteine residue was added for coupling to keyhole limpet hemocyanin or the SulfoLink column (Pierce). Affinity purification of antibodies was performed using the SulfoLink column coupled with the peptides. To generate phosphorylation site-specific stargazin antibodies (Stg-pT321 1218 rabbit and 1229 guinea pig polyclonal), the same synthetic stargazin C-terminal peptide with phosphorylated T321 was employed as immunogen. For affinity purification of the Stg-pT321 antibody, antisera were passed through the SulfoLink column coupled with the phosphorylated peptide followed by a column coupled with unphosphorylated C-terminal peptide as described previously (20).

Rabbit polyclonal EGFP (1167) and PSD-95 (SM55) antibodies were generated using H6-EGFP (aa 1–240) and H6-PSD-95 (aa 77–299) (21) as immunogens. The PSD-95 (HM319) antibody is described in the literature (21).

Yeast Two-hybrid Analysis—The yeast two-hybrid assay was performed as described earlier (21). The L40 yeast strain harboring reporter genes HIS3 and LacZ, under control of the upstream LexA DNA-binding domain was used in the assay. For pBHA (a bait containing the LexA DNA-binding domain) constructs, the last 121 aa residues (aa 203–323) of stargazin were amplified by PCR and subcloned in-frame into pBHA. Mutant pBHA stargazin constructs (RRDPV, RRTEPV, and RRRTTPA) were generated using the QuikChange site-directed mutagenesis kit (Stratagene). For pGAD10 constructs, the following PDZ domains were subcloned in-frame into pGAD10 (a prey vector, CLONTECH): PDZ2 (aa 224–311), PDZ2 (aa 318–404), PDZ3 (aa 465–545), and PDZ2–2 (aa 224–404) of SAP97; PDZ 4–6 (aa 463–761) of GRIP1. pGAD10 constructs containing the PDZ domains of PSD-95 are described (21). Stargazin-PDZ interactions were measured by semiquantitative yeast two-hybrid assays using his3 and lacZ as reporter genes.

COS Cell Transfection, Coinmunoprecipitation, and Clustering Assays—The entire open reading frame and 75 bp of the 5′ untranslated region of stargazin were amplified by RT-PCR using mouse brain total RNA, digested with HindIII and EcoRI, and subcloned into GW1 (British Biotechnology). For EGFP tagging of stargazin (EGFP-stargazin), a fragment containing EGFP (aa 1–240) was amplified by PCR and subcloned in-frame into the BglII site at the C-terminal cytoplasmic region of stargazin, thus generating a construct containing EGFP between aa 203–323 of the protein. Mutant stargazin expression constructs of stargazin, thus generating a construct containing EGFP between aa 203–323) of GRIP1. pGAD10 constructs containing the PDZ domains of PSD-95 are described (21). Stargazin-PDZ interactions were measured by semiquantitative yeast two-hybrid assays using his3 and lacZ as reporter genes.

RESULTS

Stargazin Is Phosphorylated at T321—To determine whether the T321 residue of stargazin is the phosphorylation site, we generated phosphorylation site-specific (termed Stg-pT321) antibodies (1218 rabbit and 1229 guinea pig) using the last 10 residues of stargazin with phosphorylated T321 as immunogen. Stg-pT321 antibodies were tested against stargazin expressed in heterologous cells (Fig. 1). Immunoblotting of COS cell lysates transfected with EGFP-tagged stargazin (EGFP-stargazin) with Stg-pT321 antibodies revealed a single protein band of about 66 kDa (38 kDa stargazin + 28 kDa EGFP) (Fig. 1A). In contrast, mutant stargazin (T321A) lacking the hydroxyl group for phosphorylation was not detected by Stg-pT321 antibodies (Fig. 1A). Preincubation of Stg-pT321 antibodies with excess free phosphorylated (but not unphosphorylated) peptide abolished recognition of EGFP-stargazin (wild-type) (Fig. 1A). Moreover, pretreatment of the membrane with phosphatase abolished recognition of the EGFP-stargazin (wild-type) by Stg-pT321 antibodies (Fig. 1B). These results indicate that stargazin is phosphorylated at T321 in heterologous cells.

Fig. 1. Stargazin C terminus is phosphorylated at T321. A, T321 phosphorylation of stargazin. COS cells singly transfected with EGFP-tagged stargazin (EGFP-Stg), wild-type (WT) or mutant (T321A), were immunoblotted with Stg-pT321 (1218) antibodies. Stg-pT321 antibodies recognize wild-type stargazin (lane 1) but not the T321A mutant (lane 2). Immunoblotting with EGFP (1167) antibodies indicates loading of equal amounts of stargazin. B, specificity of the Stg-pT321 antibody. Recognition of EGFP-stargazin (wild-type) expressed in COS cells by Stg-pT321 antibodies (lane 1) was eliminated by preincubating antibodies with excess amounts of phosphorylated peptide (lanes 2) but not unphosphorylated peptide (lane 3). Recognition was also eliminated by preincubating the nitrocellulose membrane with λ-phosphatase (lane 4). P-pep, phosphorylated peptide; U-pep, unphosphorylated peptide.
PKA Regulation of Stargazin-PSD-95 Interaction

The Stargazin C Terminus Is Phosphorylated by PKA—To determine whether the stargazin C terminus is a substrate of PKA, we examined phosphorylation levels of this region of stargazin in COS cells doubly transfected with stargazin and the PKA catalytic subunit (wild-type and an inactive mutant) (Fig. 3). When COS cells singly transfected with stargazin were immunoblotted with Stg-pT321 antibodies, a relatively small but significant amount of phosphorylated stargazin was detected, suggesting that stargazin is basally phosphorylated in COS cells (Fig. 3). Stargazin phosphorylation was markedly increased upon coexpression with the PKA catalytic subunit but not with an inactive catalytic subunit (25) (Fig. 3). These results indicate that PKA phosphorylates the C terminus of stargazin at T321.

Mutations Mimicking Phosphorylation of the Stargazin C Terminus at T321 Disrupt Interactions with PSD-95 and SAP97 in the Yeast Two-hybrid Assay—To investigate whether phosphorylation of T321 of stargazin affects its binding to PSD-95, we generated two mutations mimicking the PKA-phosphorylated state of the protein (RRTDPV and RRTEPV; mutations are underlined). When these stargazin mutations were tested for their interactions with the PDZ domains of PSD-95 and SAP97 in the yeast two-hybrid assay, neither mutant bound PSD-95 or SAP97 (Fig. 4). In control experiments, the wild-type stargazin C terminus specifically interacted with the PDZ domains of PSD-95 and SAP97 but not with unrelated PDZ domains of GRIP1, an AMPAR-interacting multi-PDZ protein (26) (Fig. 4). Additional mutant stargazin (RRTTPA) lost the ability to bind both PSD-95 and SAP97, indicating that the C terminus of stargazin specifically interacts with the PDZ domains of the PSD-95 family members. These findings demonstrate that the stargazin mutations mimicking T321 phosphorylation disrupt interactions with members of the PSD-95 family.

Mutations Mimicking T321 Phosphorylation Disrupt Biochemical Association and Coclustering Between Stargazin and PSD-95 in Heterologous Cells—As an independent test of the effects of stargazin mutations on the stargazin-PSD-95 interaction, we performed coimmunoprecipitation experiments on COS cell lysates doubly transfected with EGFP-stargazin (wild-type and mutants) and PSD-95 (Fig. 5). PSD-95 antibodies pulled down, in addition to their cognate antigen PSD-95, wild-type stargazin (RRTTPV) but none of the mutant stargazins tested (RRTDPV, RRTEPV, and RRTTPA) (Fig. 5). In control immunoprecipitation, singly transfected stargazin (wild-type) was not brought down by PSD-95 antibodies (Fig. 5). These results indicate that stargazin mutations mimicking T321 phosphorylation disrupt the biochemical association between stargazin and PSD-95 in heterologous cells.

Coexpression of stargazin and PSD-95 in heterologous cells results in the formation of clusters where the two proteins colocalize (14). We examined the effects of stargazin mutations on the coclustering between stargazin and PSD-95 (Fig. 6). Wild-type EGFP-stargazin when coexpressed with PSD-95 in

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**FIG. 2.** Phosphorylation of stargazin in the brain. Subcellular fractions of rat brain (S2 and P2) were immunoblotted with Stg-pT321 (1229) antibodies and other stargazin antibodies raised against the C-terminal peptide (the last 10 residues with unphosphorylated T321) (Stg-C-term 1217) and the entire C-terminal cytoplasmic region (aa 203–322; Stg-Cyto 1220). All stargazin antibodies, including Stg-pT321, recognized bands of about 38 kDa corresponding to the reported size of stargazin (4) indicating in vivo phosphorylation. PSD-95 and synaptophysin (SynPhys) were visualized for comparison. P2, crude synaptoosomes; S2, supernatant after the removal of P2.

**FIG. 3.** The stargazin C terminus is phosphorylated by PKA. COS cells transfected with stargazin alone (EGFP-Stg), stargazin + PKA catalytic subunit, or stargazin + inactive PKA catalytic subunit were immunoblotted with Stg-pT321 (1218) and EGFP (1167) antibodies. Stargazin phosphorylation was markedly increased by coexpression with the PKA catalytic subunit (lane 2) but not an inactive subunit (lane 3). EGFP-Stg, EGFP-stargazin wild-type; PKA Cat WT, the wild-type PKA catalytic subunit; PKA Cat Mut, inactive PKA catalytic subunit. **Trans**, transfection; **IB**, immunoblot.

**FIG. 4.** Mutations mimicking stargazin phosphorylation at T321 eliminate the interaction between stargazin and PSD-95 in the yeast two-hybrid assay. Wild-type and mutant stargazin C termini were tested against the PDZ domains of PSD-95 and SAP97 in the yeast two-hybrid assay. Stargazin mutants mimicking phosphorylation of T321 (RRTDPV, RRTEPV) lost their ability to interact with the PDZ domains of PSD-95 and SAP97. Another stargazin mutant, RRTTPA, failed to bind PDZ domains, thus confirming that stargazin interacts with PDZ domains. Mutations are indicated by underlines. HIS3 activity: +++, >60%; +++, 30–60%; +, 10–30%; −, no significant growth. β-gal activity: +++, <45 min; +, 45–90 min; +, 90–240 min; −, no significant β-gal activity.

**FIG. 5.** Mutations mimicking T321 phosphorylation eliminate coimmunoprecipitation between stargazin and PSD-95 in heterologous cells. COS cell lysates doubly transfected with EGFP-stargazin (wild-type or mutants) + PSD-95 or singly with wild-type stargazin were immunoprecipitated (IP) with PSD-95 (HM319) antibodies and immunoblotted (IB) with EGFP (1167) and PSD-95 (SM55) antibodies. PSD-95 selectively coimmunoprecipitated with wild-type stargazin (lane 7) but none of the mutant stargazins. PSD-95 antibodies did not bring down singly transfected stargazin. WT, wild-type ending with RRTTPV; T321D, RRTDPV, T321E, RRTEPV, V323A, RRTTPA. **Input**, 10% lysates were used for each coimmunoprecipitation.
PKA Regulation of Stargazin-PSD-95 Interaction

**Fig. 6.** Mutations mimicking T321 phosphorylation disrupt co-clustering between stargazin and PSD-95 in heterologous cells. COS cells were doubly transfected with EGFP-stargazin (wild-type or mutants) and PSD-95 and visualized by double-label immunofluorescence staining with EGFP and PSD-95 antibodies. A, wild-type stargazin and PSD-95 formed clusters upon coexpression. B–D, in contrast, stargazin mutants did not form typical clusters with PSD-95 and displayed diffuse distribution of both proteins throughout the cells.

COS cells formed clusters in which both proteins colocalized (Fig. 6A), consistent with previous results (14). However, none of the stargazin mutants (RRTDPV, RRTEPV and RRTTPA) formed typical clusters on coexpression, and both proteins were diffusely distributed throughout the cells (Fig. 6, B–D). These data suggest that T321 phosphorylation of stargazin regulates the interaction between stargazin and PSD-95, in agreement with the yeast two-hybrid results.

**Phosphorylated Stargazin Selectively Loses Its Interaction with PSD-95**—To investigate whether phosphorylation of stargazin reduces its interaction with PSD-95, we performed coimmunoprecipitation experiments with COS cells triply transfected with stargazin (wild-type), PSD-95, and the PKA catalytic subunit (Fig. 7). Immunoprecipitation of COS cell lysates with PSD-95 antibodies did not bring down any detectable amount of phosphorylated stargazin, as visualized by Stg-pT321 antibodies (Fig. 7, top panel). Conversely, stargazin interacts with all known members of the PSD-95 family (Fig. 4) (14), which display diverse distribution patterns in the brain (16). These results indicate that phosphorylated stargazin is less tightly associated with the PSD.

We propose three possible roles of stargazin phosphorylation. First, stargazin phosphorylation may regulate the synaptic targeting of AMPARs by modulating interactions between the stargazin C terminus and the PDZ domains of PSD-95 and related PDZ proteins. Consistently, stargazinΔC rescues extrasynaptic but not synaptic AMPAR currents in cerebellar granule cells of stargazer mice (14). In cultured hippocampal neurons, stargazinΔC is diffusely distributed and down-regulates synaptic AMPAR currents (14).

Second, phosphorylation may regulate the stability of stargazin on the synaptic surface membrane. Phosphorylated stargazin may lose its ability to interact with synaptic PDZ anchors and consequently diffuse away laterally from the synaptic surface or become internalized. Consistently, PSD-95 markedly suppresses internalization of its binding partners including Kv1.4 potassium channel (27) and β-adrenergic receptor (28). Stargazin phosphorylation may not be the only factor that determines its synaptic stability, if stargazin delivered to synaptic sites still remains associated with AMPARs, which are known to interact with their own anchors such as GRIP/ABP (26, 29, 30). However, a mutant GluR2 subunit of AMPARs lacking GRIP binding loses its stability at the synaptic surface an altered enrichment in the PSD, we performed immunoblot analysis on PSD fractions of rat brain (Fig. 8). Stg-pT321 antibodies revealed that phosphorylated stargazin is minimally enriched in PSD fractions. In particular, phosphorylated stargazin was not detectable in the PSD III fraction, a core of the PSD extracted with Triton X-100 and sarcosyl detergents. In contrast, Stg-Cyto and Stg-C-term antibodies showed a significant enrichment of total stargazin in all PSD fractions. These results indicate that phosphorylated stargazin is less tightly associated with the PSD.

**DISCUSSION**

Our results demonstrate that phosphorylation of stargazin at T321 by PKA inhibits its interaction with PSD-95. The C-terminal sequence of stargazin (NRRTTPV) is identical or similar to those in other closely related γ-subunits of voltage-dependent calcium channels (NRRTTPV in γ-3 and γ-4 and NRRTTPV in γ-8) that are expressed in the brain (7, 8, 10), suggesting similar interactions with PSD-95 and phosphorylation by PKA. Conversely, stargazin interacts with all known members of the PSD-95 family (Fig. 4) (14), which display diverse distribution patterns in the brain (16). These results suggest that the PKA-dependent phosphorylation of stargazin at T321 regulates interactions between various members of the stargazin/PSD-95 family. To our knowledge, this is the second report thus far showing that phosphorylation of the C-terminal PDZ-binding motif regulates PDZ interactions.

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PKA Regulation of Stargazin-PSD-95 Interaction

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