The scaffolding protein CASK mediates the interaction between rabphilin3a and β-neurexins

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Abstract CASK, a member of the membrane-associated guanylate kinase (MAGUK) superfamily, binds to the carboxyl-terminus of β-neurexins on the intracellular side of the presynaptic membrane. The guanylate kinase-like (GUK) domains of MAGUKs lack kinase activities, but might be important for mediating specific protein–protein interaction. By a yeast two-hybrid approach, we identified an interaction between the GUK domain of CASK and the C2B domain of rabphilin3a, a presynaptic protein involved in synaptic vesicle exocytosis. The interaction was confirmed by in vitro GST pull-down and co-immunoprecipitation assays. It was proposed that presynaptic vesicles might be guided to the vicinity of points of exocytosis defined by β-neurexins via the interaction between rabphilin3a–CASK–β-neurexins.

Key words: CASK; β-Neurexin; Rabphilin3a; Synaptic vesicle exocytosis; Yeast two-hybrid system

1. Introduction

The membrane-associated guanylate kinases (MAGUKs) are a family of proteins that act as molecular scaffolds for signaling pathway components at the plasma membrane of animal cells. MAGUK proteins share a multidomain organization including one or three postsynaptic density (PSD)-95/SAP90 (PDZ) (Psd-Dlg-Zo1) domains, an Src homology 3 (SH3) domain, and a guanylate kinase-like (GUK) domain [1,2]. CASK, an important member of the MAGUK superfamily, was originally identified as a binding partner of β-neurexins located at the intracellular side of the presynaptic membrane [3]. CASK is composed of an N-terminal Ca^{2+}/calmodulin-dependent kinase-like domain, followed by a PDZ domain, an SH3 domain, and a C-terminal domain homologous to guanylate kinase [3]. In neuronal systems, CASK is allocated to the intracellular side of presynaptic membrane by binding to the cytoplasmatic carboxyl-terminus of β-neurexins. At synapse, CASK forms an asymmetric intercellular junction with the postsynaptic PSD-95–neuroligin complex, via the interaction between β-neurexins and neuroligins [4,5]. The N-terminal Ca^{2+}/calmodulin-dependent kinase-like domain of CASK interacts with Mint-1 [4] independent of the association of Ca^{2+}/calmodulin with CASK; and Mint-1 in turn binds to the trafficking protein Munc-18 [6] which is essential for exocytosis [7]. The Ca^{2+}/calmodulin-dependent kinase-like domain of CASK also binds to Veli-1, which may interact with receptor tyrosine kinases and other components via its PDZ domain. Thus, CASK, Mint-1 and Veli-1 form a specific salt-resistant complex functioning as a protein scaffold in the presynapses of neuronal cell terminals [4].

CASK is ubiquitously expressed, though highly enriched in the brain [3,8]. In the basolateral membranes of epithelial cells, the PDZ domain of CASK interacts with the cytoplasmatic carboxyl-terminus of syndecan-2 [9]. A region between the SH3 and GUK domains interacts with protein 4.1, which associates with the actin cytoskeleton via spectrin and is thus connected to the cytoskeletal system of the cell [8,9].

The GUK domains of MAGUKs lack enzymatic activity and may be involved in protein–protein interactions [4]. For example, the GUK domain of PSD-95 binds to guanylate kinase domain-associated protein (GKAP) in postsynaptic structures [10]. It was recently reported that the GUK domain of CASK could bind to Tbr-1, a transcription regulator, directly enter the nucleus, and thereby regulate gene expression. Thus, in contrast to the classical pathway, CASK and Tbr-1 may mediate a novel signal transduction pathway [11,12]. Here we report that in addition to Tbr-1, rabphilin3a, a presynaptic protein involved in synaptic vesicle exocytosis, was identified as the binding partner of the GUK domain of CASK using yeast two-hybrid screening. Glutathione *S*-transferase (GST) pull-down and co-immunoprecipitation assays confirmed the interaction. Possible roles of the rabphilin3a–CASK–β-neurexins interaction in the exocytosis of presynaptic vesicles are discussed.

2. Materials and methods

2.1. Plasmid construction and protein expression

PLexA/GUK was constructed by inserting the GUK domain of human CASK (GenBank GI: 2661105; SwissProt GI: 6166125), encoding amino acids 701–897, into pLexA (Clontech). The same GUK domain of CASK was expressed using pGEX-2T (Amersham Pharmacia Biotech) and pCDNA3.1(−)/myc-hisA (Invitrogen) in *Escherichia coli* strain BL21(DE3) and COS-7 cells, respectively. Human rabphilin3a gene, without N-terminal 415 amino acids, fused to a myc tag (EQKLISEEDL) was cloned into pT7his (kindly provided

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Abbreviations: MAGUK, membrane-associated guanylate kinase; GUK, guanylate kinase-like; PSD, postsynaptic density; PDZ, PSD-95/SAP90 discs large ZO-1 homologous; SH3, Src homology 3; GKAP, guanylate kinase domain-associated protein; NMDAR, *N*-methyl-D-aspartate receptor

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by Dr. C. Fang). Human full-length rabphilin3a (GenBank GI: 4589613) and human rabphilin3a without C-terminal sequence encoding the last 121 amino acids were inserted into pCDNA3 (Invitrogen) fused with a FLAG epitope (DYKDDDDK) tag to generate pCDNA3/rabphilin3a–FLAG and pCDNA3/rabphilin3a–Δ-C-FLAG plasmids, respectively. All constructions were confirmed by sequencing. For protein expression in *E. coli* strain BL21(DE3), pGEX-2T- or pT7his-based plasmids were induced using 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) at 30°C for 3 h and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.2. Yeast two-hybrid screening

A cDNA fragment encoding the human CASK/GUK domain (amino acids 701–897) was used as bait to screen a human adult brain cDNA library in pB42AD vector (Clontech). Approximately 2×10^6 yeast transformants were screened following the manufacturer's instructions.

2.3. GST pull-down assay

The recombinant proteins of the bacterial pellet from a 1.5 ml culture were solubilized in 250 μl buffer 1 (50 mM Tris–Cl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 50 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin and 40 μg/ml phenylmethylsulfonyl fluoride), and sonicated. 50 μl of each of the extract was mixed with 20 μl of glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) and 100 μl buffer 2 added (50 mM Tris–Cl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 50 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin and 40 μg/ml phenylmethylsulfonyl fluoride). The reaction was incubated for 1 h at room temperature with an end-over-end shaking. The beads were separated by a brief centrifugation, washed five times with buffer 2, and suspended in 25 μl sample-loading buffer (50 mM Tris–Cl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol). The bound proteins were analyzed by Western blot using an anti-myc antibody (Calbiochem).

2.4. Cell culture, transient transfection, co-immunoprecipitation and Western blot analysis

COS-7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Transient co-transfection of COS-7 cells with pCDNA3/rabphilin3a–FLAG and pCDNA3.1(−)/myc-hisA/GUK plasmids was performed for 3.5 h with the Lipofectamine plus reagent (Gibco) following the manufacturer's instructions. pCDNA3/rabphilin3a–Δ-C-FLAG plasmid was used as control. Cells were harvested 48 h after transfection and lysed in 20 mM Tris–Cl pH 8.0, 150 mM NaCl, 0.5% NP-40, 1 mM Na_2VO_4, 1 mM NaF, 1 mM PMSF, 10 μg/mg aprotinin, 10 μg/ml pepstatin and 10 μg/ml leupeptin. Nuclear and cellular debris was removed by centrifugation at $10\,000\times g$ for 15 min at 4°C. Cell extracts were incubated with 2 μg/ml of mouse anti-myc antibody (Calbiochem) overnight at 4°C. Protein A-agarose beads (Amersham Pharmacia Biotech) were then added and incubated for 2 h at room temperature. The immunoprecipitated samples were washed extensively with lysis buffer and then boiled in SDS-loading buffer. The supernatant was subjected to 12% SDS–PAGE. The membrane was immunoblotted with chicken anti-FLAG antibody and horseradish peroxidase-conjugated goat anti-chicken IgY (Aves Labs). Bond antibodies were detected using the ECL system (Amersham Pharmacia Biotech).

SDS–PAGE was performed in 12% polyacrylamide gels. Reduction was achieved by boiling the samples for 5 min in sample-loading buffer (see above). The gel was stained with Coomassie brilliant blue R250. For Western blot analysis, the proteins were transferred to nitrocellulose membrane (Schleicher and Schuell) at 0.65 mA/cm^2 for 2 h in transfer buffer (48 mM Tris, 39 mM glycine, 0.037% SDS, 20% methanol). The membrane was blocked for 1 h in PBST (0.1% Tween-20) containing 1% BSA and 4% milk, and incubated with 2 μg/ml mouse anti-myc antibody (Calbiochem) or 10 μg/ml chicken anti-FLAG antibody (Aves Labs) in PBST for 1 h and washed 3×10 min with PBST. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham Pharmacia Biotech) or horseradish peroxidase-conjugated goat anti-chicken IgY (Aves Labs) for 1 h and washed as described before. Detection made use of the enhanced chemiluminescence reaction (ECL, Amersham Pharmacia Biotech), followed by exposition to X-ray film.

3. Results

To identify proteins binding to the GUK domain of CASK, we screened a human adult brain cDNA library in a yeast two-hybrid assay using the C-terminal GUK domain of CASK as a bait. Screening of 2×10^6 yeast transformants was carried out in selective medium and plasmid DNA of the stronger interactions, which were able to grow in selective media and displayed a strong β-galactosidase signal, were isolated. Two kinds of clones encoding Tbr-1 and rabphilin3a were obtained. The C-terminal fragment of Tbr-1 protein (residues 398–681) was identified in the screening, consistent with a recent report that the amino acids 342–681 of Tbr-1 are necessary and sufficient for binding to the GUK domain of CASK [11].

The rabphilin3a cDNA encoded the C-terminal 573–694 amino acid residues of the protein. Rabphilin3a was originally identified as an upstream effector of rab3a [13]. It contains an N-terminal rab3a binding domain with a conserved Zn^{2+} finger motif, a central domain with phosphorylation sites recognizable by protein kinase A and Ca^{2+}/calmodulin-dependent protein kinase II, and two C-terminal C2 domains (C2A and C2B) which mediate protein–lipid or protein–protein interactions [13–15]. It binds to rab3a in a GTP-dependent manner,

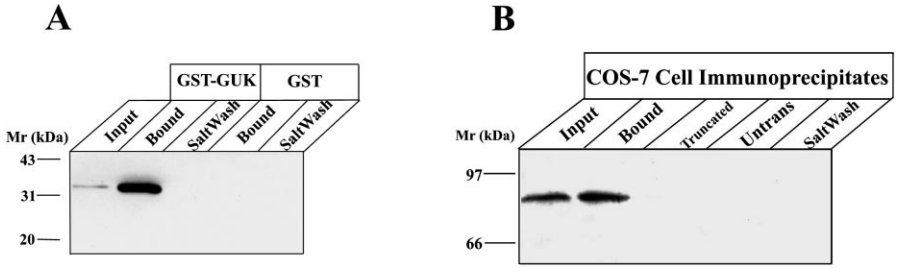

Fig. 1. Confirmation of yeast two-hybrid screening. A: GST pull-down assay of CASK and rabphilin3a. Bacterially expressed GST-GUK fusion protein was bound to truncated rabphilin3a recombinant proteins in vitro. GST protein was used as control. Anti-myc antibody was used to test the presence of rabphilin3a pulled down. Input: Bacterial expressed truncated rabphilin3a protein with a myc tag. Bound: Bound proteins eluted from the glutathione-Sepharose beads. Salt wash: Wash buffer used to remove unbound proteins. B: Co-immunoprecipitation of CASK-GUK-Myc with rabphilin3a–FLAG. Cell lysates from COS-7 cells co-transfected with pCDNA3.1(−)/myc-hisA/GUK and pCDNA3/rabphilin3a–FLAG or pCDNA3/rabphilin3a–Δ-C-FLAG were immunoprecipitates using anti-FLAG antibody. Input: Cell extracts from the COS-7 cells expressing recombinant rabphilin3a protein. Bound: Immunoprecipitates from COS-7 cells co-transfected with pCDNA3.1(−)/myc-hisA/GUK and pCDNA3/rabphilin3a–FLAG. Truncated: Immunoprecipitates from COS-7 cells co-transfected with pCDNA3.1(−)/myc-hisA/GUK and pCDNA3/rabphilin3a–Δ-C-FLAG. Salt wash: Wash buffer used to remove unbound proteins.

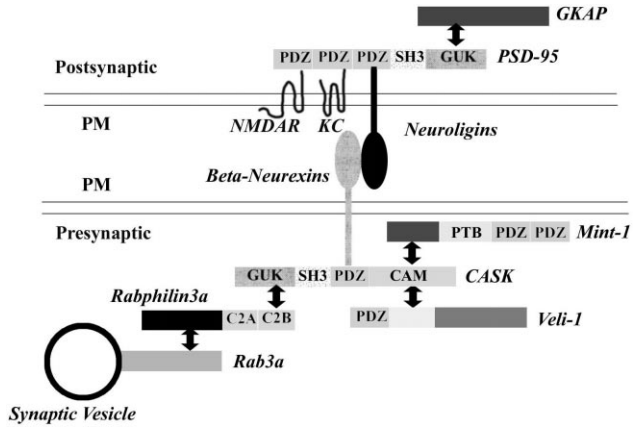

Fig. 2. Illustration of a possible mechanism in which synaptic vesicles were recruited to β-neurexins via interaction between CASK–rabphilin3a–rab3a at presynapse. PSD-95 binds to neuroligins, NMDAR and K^+ channel via its PDZ domains and binds to GKAP, a postsynaptic protein, via its GUK domain at postsynapse. β-Neurexin/neuroligin complexes mediate the formation of asymmetric junctions at the synapse. CASK binds to β-neurexins via its PDZ domain, and forms a tight tripartite complex with Mint-1 and Veli-1 at the presynapse. CASK also binds rabphilin3a via its GUK domain. Rabphilin3a attached to synaptic vesicles by binding to rab3a, which is localized to synaptic vesicles. Thus, the described protein–protein interactions result in alignment of synaptic vesicles and postsynaptic receptors near synaptic axis mediated by β-neurexin/neuroligin association.

and regulates synaptic vesicle exocytosis [16,17]. The cDNA clone we obtained encoded only the C2B domain. Since both CASK and rabphilin3a were reported to be predominantly localized at the presynaptic active zone [18], we focussed our attention on the interaction between CASK and rabphilin3a.

A GST pull-down assay was employed to independently confirm the rabphilin3a–CASK interaction identified in the yeast two-hybrid screening. The GUK domain (residues 701–897) of CASK fusing to the C-terminal of a bacterial GST, and the C2 domains of rabphilin3a protein (residues 416–694) fused myc epitope in its C-terminal were expressed in *E. coli* strain BL21(DE3). Fusion proteins were 48 and 34 kDa in molecular weight, respectively. To test the specificity of the binding, GST protein was used as a control. The mixtures of GST-GUK with rabphilin3a-C2, and GST with rabphilin3a-C2 recombinant proteins were incubated with glutathione-Sepharose 4B beads. Proteins bound to the GST fusion proteins were eluted and analyzed by Western blot using anti-myc antibody. The Western blot results indicated that the C2 domains of rabphilin3a protein bound to the GST-GUK fusion protein. No interaction with C2 domains of rabphilin3a was detectable for GST alone, confirming the specificity of the GST-GUK–rabphilin3a-C2 interaction (Fig. 1A).

The interaction between rabphilin3a and CASK was also confirmed by using the immunoprecipitation approach. FLAG-tagged rabphilin3a and myc-tagged GUK domain of CASK were expressed by transient transfection of pCDNA3.1(−)/myc-hisA/GUK and pCDNA3/rabphilin3a–FLAG plasmids in COS-7 cells. Truncated rabphilin3a protein without C-terminal 121 amino acids was used as a control. Binding of the expressed proteins was determined by co-immunoprecipitation. Anti-myc antibody and protein A-agarose were used to precipitate the truncated CASK protein. The rabphilin3a–FLAG protein was detected in the precipitate using an anti-FLAG antibody whereas the rabphilin3a without C-terminal 121 amino acids was not (Fig. 1B). The co-immunoprecipitation experiment was therefore consistent with the result of the in vitro interaction between CASK and rabphilin3a.

4. Discussion

The MAGUK superfamily constitutes a class of scaffolding proteins that recruit signaling molecules into localized multimolecular complexes. All MAGUK proteins contain PDZ and SH3 domains, which mediate specific protein–protein interactions and form scaffolds for protein networks at cell membranes [1]. The GUK domains of MAGUK proteins lack enzymatic activity, and might be responsible for protein–protein interactions as suggested by the fact that GKAP binds the GUK domain of PSD-95 [10]. Interaction between the GUK domain of CASK and Tbr-1 has been previously reported [11]. Here we report that in addition to Tbr-1, rabphilin3a, a presynaptic protein could bind to the same region of the CASK protein. The C2B domain of rabphilin3a mediates the interaction.

Rabphilin3a was the first identified rab3a effector [13] binding to rab3a through its N-terminus [19]. Rab3a is a member of a large family of low molecular weight GTPases that is localized to synaptic vesicles, and is thought to mediate directional vesicular trafficking and regulate synaptic vesicle exocytosis. Rab3a cycles between an active, GTP-bound form and an inactive, GDP-bound form, which is dependent upon the accumulation of rabphilin3a which stabilizes the GTP-bound form of rab3a on the vesicle [11,20].

The specific interaction between CASK and raphilin3a raised an interesting possibility that the scaffolding proteins might be involved in synaptic vesicle exocytosis at presynaptic terminal. CASK, Mint-1, and Veli-1 form a tight, salt-resistant scaffolding protein complex at the presynaptic terminal, and serve as a nucleus for the recruitment of channels and receptors to β-neurexin/neuroligin junction at synapses [4]. A previous report suggests that Mint-1 binds to the cytosolic carboxyl-terminus of the α_{1B} subunit of the N-type Ca^{2+} channel, and that the SH3 domain of CASK might bind to the proline-rich region of the same α_{1B} subunit [21]. In addition, the N-terminus of Mint-1 could bind to Munc-18 [22], a neuronal protein essential for synaptic vesicle exocytosis [23,24], and thus mediate the recruitment of Munc-18 to neurexins [25]. Our observation suggests that CASK could bind to rabphilin3a, and therefore might target trafficking of the presynaptic vesicles to β-neurexins via the interaction between rabphilin3a and rab3a.

CASK–β-neurexin–neuroligin–PSD-95 protein complexes form an asymmetric intercellular junction at synapses [4,5]. At the postsynapse, the PDZ domains of PSD-95 bind directly to the cytosolic carboxyl-terminus of the *N*-methyl-D-aspartate receptors (NMDARs) which bind to the neurotransmitter glutamate [5]. Our data suggest that at presynapse, CASK might recruit rabphilin3a, and thus guide the presynaptic vesicles to the sites at the plasma membrane that were defined by β-neurexins. Thus, the efficiency of neurotransmission might be greatly enhanced due to the high local concentration of neurotransmitters around the synaptic cleft area where NMDARs are located (Fig. 2).

In agreement with this model, neuroligin-induced synaptic

vesicle clusters were observed at the presynaptic terminal, and the synaptic scaffolding protein CASK was co-localized with presynaptic vesicle clusters in the pontine axon [26]. In the tips of an extending axon of a developing neuron, vesicle exocytosis is less effective than in mature nerve termini. This was probably due to the lack of rab3a cycling developed during synaptogenesis, and results in efficient exocytosis [27,28]. Further studies, including the measurement of the vesicle exocytosis in CASK-deficient cells, or synaptic plasticity in CASK-knockout mice, will help to understand this model.

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