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CASK point mutation regulates protein–protein interactions and NR2b promoter activity

Tzyy-Nan Huang, Yi-Ping Hsueh *

Graduate Institute of Life Science, National Defense Medical Center, Taipei, 161, Taiwan
The Institute of Molecular Biology, Academia Sinica, 128, Academia Rd., Sec. 2, Taipei 115, Taiwan

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ABSTRACT

Mutations in the CASK gene result in mental retardation and microcephaly in humans, suggesting an important role for CASK in brain. CASK gene knockout in mice causes neonatal lethality, making further elucidation in mouse models difficult. Because CASK was originally identified as a multidomain adaptor protein, identifying a point mutation interrupting a specific protein interaction would be useful in dissecting its molecular function. Here, a Thr-to-Ala mutation in the rat CASK guanylate kinase (GK) domain was shown to reduce interactions among CASK and Tbr-1 and CINAP, two critical brain proteins. The effect is specific: this mutation does not affect CASK dimerization that occurs via the GK domain. The Tbr-1–CASK–CINAP complex regulates expression of the NMDA receptor subunit 2b (NR2b), and we show that this point mutation also affects NR2b promoter activity. The identification of this mutation may make it possible to further dissect the function of CASK in brain.

Introduction

CASK, a membrane-associated guanylate kinase protein, has been reported to play a role in brain development and function in both rodents and humans. In humans, mutations of CASK result in X-linked microcephaly and mental retardation [1]. In mice, CASK gene knockout causes neonatal lethality [2], and reduction of CASK expression in mouse brain leads to a smaller cerebellum [1]. These analyses indicate a critical role for CASK in the nervous system.

CASK, originally identified as a multidomain scaffold protein, contains a CaMK-like domain at the N-terminal region followed by two L27 (LIN-2 and LIN-7 interacting domains), a single PDZ (PSD-95-Dlg-ZO-1) domain, and SH3, protein 4.1-binding, and GK domains. Each CASK domain interacts with specific binding partners [3], and it is thought that CASK interacts with these different partners in different subcellular regions, thus performing unique regional functions [3]. For instance, at the postsynaptic site, CASK contributes to maintenance of dendritic spine morphology. Syndecan-2, a CASK PDZ binding protein, regulates dendritic spinogenesis [4–6]. Through its PDZ domain and protein 4.1-binding motifs, CASK links transmembrane syndecan-2 to the actin cytoskeleton, stabilizing spine morphology [7]. Moreover, CASK is SUMOylated in brain, which reduces the interaction between CASK and protein 4.1 and decreases CASK spinogenic activity [7].

In the nuclei of neurons, CASK interacts with transcription factor Tbr-1 (T-brain-1) and CINAP (CASK-interacting nucleosome assembly protein)/CDA1/DENTT [8]. Cotransfection with CASK enhances the transcriptional activity of Tbr-1, perhaps through recruitment of CINAP, which may remodel the chromatin structure flanking the Tbr-1 binding sites [8,9]. Tbr-1 is critical for brain development. Targeting deletion of the Tbr-1 gene in mice results in abnormal lamination of the cerebral cortex and defects in axonal projection [10]. NMDA receptor 2b (NR2b) and reelin have been identified as Tbr-1 target genes [8,9,11]. The reelin gene encodes an extracellular molecule that is critical to brain development. NMDA receptors are among the most important excitatory neurotransmitter receptors in the mammalian central nervous system. Regulation of NR2b and reelin expression by the Tbr-1–CASK–CINAP protein complex implies a role of this complex in regulating neuronal activity.

These studies have indicated that CASK may play two regulatory roles at the postsynaptic site. One is to physically maintain spine morphology; the other is to regulate expression of the NR2b gene and thus control synaptic activity. These functions may at least partially account for human phenotypes. However, because CASK interacts with more than a dozen proteins through the different protein domains, it is very difficult to dissect its detailed molecular function by simply disrupting its expression. A specific mutation interrupting a specific protein–protein interaction of CASK would be useful for elucidating the physiological

* Corresponding author. Address: Institute of Molecular Biology, Academia Sinica 128, Academia Rd., Sec. 2, Taipei 115, Taiwan. Fax: +886 2 27826085/27827784.
E-mail address: yph@gate.sinica.edu.tw (Y.-P. Hsueh).

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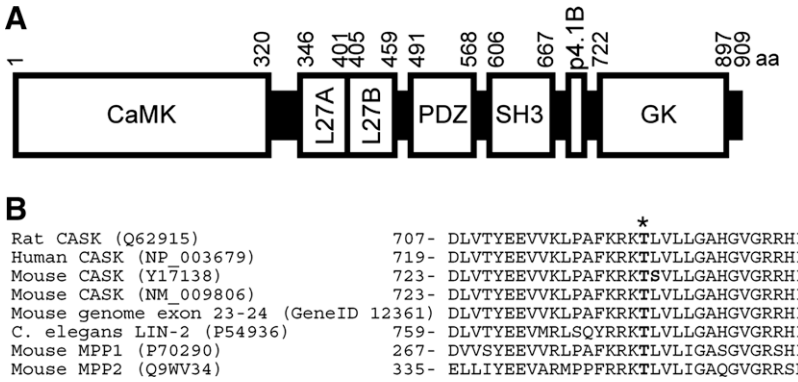


Fig. 1. Domain structure and sequence alignment of CASK. (A) Schematic protein domain structure of CASK. The numbers indicate the amino acid residues at the beginning and end of each domain of rat CASK (Accession No. Q62915). (B) The alignment of the amino acid sequences around the residue T724, marked by an asterisk. GenBank accession numbers for each sequence are indicated.

significance of an individual interaction of the CASK protein. Here, we provide evidence that a point mutation at the Thr724 residue of the rat CASK protein reduces the interaction between CASK and Tbr-1 and CINAP, thus affecting expression of NR2b. Based on this study, it may be possible to further explore the specific role of the Tbr-1–CASK–CINAP protein complex in microcephaly and mental retardation.

Materials and methods

Antibodies. CASK, Tbr-1, and CINAP rabbit polyclonal antibodies have been described previously [4,8,9]. Other antibodies were purchased from commercial suppliers, as follows: CASK monoclonal antibody, Upstate Biotech; non-immune mouse total IgG, Sigma; Myc-tag 9E10 antibody, Santa Cruz; HA tag 12CA5 antibody, Roche.

Plasmid construction and site-directed mutagenesis. For eukaryotic expression, Tbr-1, CINAP, wild-type, and Myc-tagged CASK expression constructs in the vectors GW1-CMV and SG5 have been described previously [4,7–9]. HA-tagged CASK was generated by inserting CASK cDNA into the vector HA-GW1-CMV. To generate the T724A mutant, the QuickChange™ method (Stratagene) was used, and the pair of oligonucleotides for PCR were 5′-GCCAGCGTTC AAAAGGAAAGCATTAGTCTTATTAGGTGC-3′ and 5′-GCACCTAATAAG ACTAATGCTTTCCTTTTGAACGCTGGC-3′. The results of mutagenesis were verified by DNA sequence analysis.

Cell culture, transfection, immunostaining, immunoprecipitation, and luciferase reporter assay. Transfection of COS cells using Lipofectamine (Invitrogen) was performed as described previously [12]. For cotransfection, half of the amount of each plasmid DNA preparation was used to keep the total DNA amounts equal. One day after transfection, cells were harvested for immunoprecipitation or immunostaining. Immunostaining using primary antibodies recognizing Tbr-1 and CASK and secondary antibodies conjugated with Alexa-488 and -555 (Invitrogen) was performed as described [12], as was immunoprecipitation of CASK [8,13].

For the luciferase assay using Neuro-2A cells, cells were cultured in 12-well culture plates, and transfection was performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). On day later, cells were harvested for the luciferase reporter assay. Equal amounts of cell extract were then used for the Luciferase Assay System (Promega). Transfection efficiency was checked by immunoblotting using Tbr-1 and CASK antibodies.

Results and discussion

When the CASK sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/) were analyzed, a mouse variant (Accession No. Y17138) carrying a serine residue at position 741 was found (Fig. 1B). The corresponding residues in other CASK proteins, whether *Caenorhabditis elegans*, mouse, rat, or human proteins, are leucines (Fig. 1B). Even the mouse genomic sequence (GeneID 12361) encodes leucine (L741) at this position (Fig. 1B). We then speculated that this variation might be caused by a sequence error. However, a highly conserved putative PKA phosphorylation site (-KRKT-) was found flanking L741 (Fig. 1B). Because this sequence is located in the GK domain—the Tbr-1 and CINAP interaction site (Fig. 1A)—we then wondered whether this sequence might be involved in the interaction with Tbr-1 and CINAP. Because the rat CASK gene (Accession No. Q62915) is used in this report, we note that the corresponding position of this Thr residue is T724 in rat CASK protein. A T724A mutant of rat CASK was then used to test the interaction among CASK and Tbr-1 and CINAP.

Co-immunoprecipitation using CASK antibodies from COS cells cotransfected with Tbr-1 and wild-type CASK or T724A mutant was performed. The results of immunoblotting analysis showed that CASK antibodies precipitated lesser amounts of Tbr-1 in the presence of the T724A mutant (Fig. 2A). Calibrated by the protein amounts of precipitated CASK, the T724A mutant precipitated ~60% of the Tbr-1 proteins compared with those of wild-type CASK (Fig. 2A). This finding suggested that the T724A mutation reduces the interaction between CASK and Tbr-1. The effect of the T724A mutation on the interaction between CASK and CINAP was also examined by co-immunoprecipitation. Similar to Tbr-1, lesser amounts of CINAP protein were co-precipitated with T724A mutant proteins compared with wild-type CASK (Fig. 2B). Note that the reduction of the interaction between the T724A mutant and CINAP was greater than that of the interaction between T724A mutant and Tbr-1 (Fig. 2A vs. B), suggesting that the T724A mutation has a stronger inhibitory effect on the interaction between CASK and CINAP.

Because CASK forms homodimers through the intermolecular interaction between the CASK GK and SH3 domains, we then wondered whether the T724A mutation might also affect CASK dimerization. The interactions between HA-tagged CASK and Myc-tagged wild-type CASK and T724A mutants were similar to each other (Fig. 2C). These data suggested that T724A mutation does not contribute to the regulation of CASK dimerization but affects the interactions between CASK and Tbr-1 and CINAP.

Previously, we demonstrated that CASK enters the nucleus of COS cells through interaction with Tbr-1 [9]. The T724A mutation is therefore expected to affect the nuclear translocation of CASK as mediated by Tbr-1 in COS cells, and we used immunofluorescence analysis to examine this issue. When the CASK T724A mutant alone was expressed in COS cells, the mutant protein was

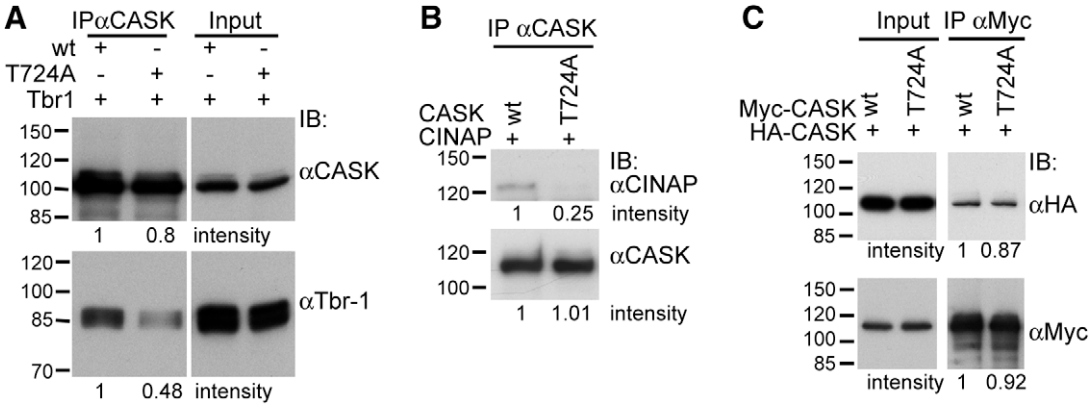
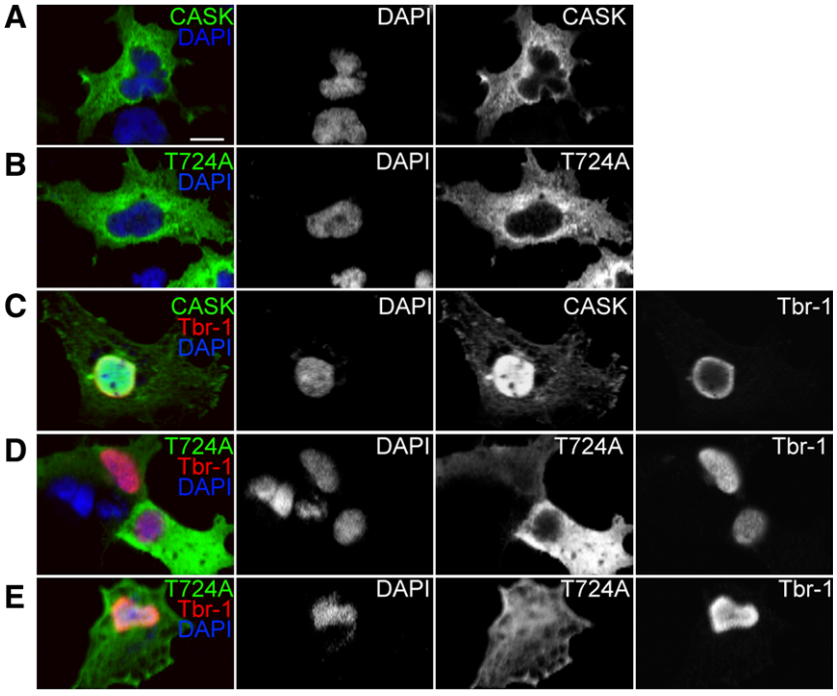


Fig. 2. T724A mutation reduces the interactions of CASK and Tbr-1 and CINAP but not CASK dimerization. COS cells transfected with various expression constructs as indicated were harvested for co-immunoprecipitation using CASK antibodies in (A) and (B) or Myc-tag antibodies in (C). The precipitates and 5% of the extracts for immunoprecipitation (Input) were then analyzed by immunoblotting using antibodies, as indicated. (A) The interaction between CASK and Tbr-1; data presented are representative of three experiments. (B) The interaction between CASK and CINAP; the experiment was repeated three times. (C) Dimerization of CASK; the experiments were repeated twice. For all experiments, the relative precipitated protein amounts of CASK, Tbr-1, and CINAP are shown.

mainly distributed in the cytoplasm, behaving like the wild-type CASK protein (Fig. 3A,B). However, unlike wild-type CASK proteins that entered the nucleus with Tbr-1 (Fig. 3C; quantified in Fig. 3F), the nuclear signal of the T724A mutant was greatly reduced even in the presence of Tbr-1 (Fig. 3D,E). In Tbr-1 and T724A double transfectants, T724A CASK mutant proteins remained in the cytoplasm of 38.4% cells (quantified in Fig. 3F, with an example in Fig. 3D). In 42.9% of double transfectants, the T724A mutant



F

Subcellular distribution of CASK (percentage of total transfected cells, %)

	nucleus	whole cells	cytoplasm
(1) wt (n = 95)	0	7.4	92.6
(2) T724A (n = 100)	0	9	91
(3) wt + Tbr-1 (n = 96)	46.9	44.8	8.3
(4) T724A + Tbr-1 (n = 112)	18.7	42.9	38.4

Fig. 3. T724A mutation reduces Tbr-1-induced nuclear translocation of CASK. (A–E) COS cells were transfected with CASK alone (either wild-type or T724A mutant) or both Tbr-1 and wild-type CASK or T724A mutant, as indicated, and fixed for staining using CASK and Tbr-1 antibodies. CASK was visualized by Alexa-488; Tbr-1 was visualized by Alexa-555. DAPI counterstain was performed to label the cell nuclei. Scale bar, 10 μm. (F) Quantification of a subcellular distribution of wild-type CASK and T724A mutant in the absence or presence of Tbr-1. The distribution patterns of CASK were divided into three groups: (1) nucleus: the majority of CASK immunoreactivity was concentrated in the nucleus, as in the image shown in (C); (2) whole cell: CASK is present in both the nucleus and cytoplasm, as in the image shown in (E); (3) cytoplasm: the majority of CASK was present in the cytoplasm, as in the image shown in (D). Sample sizes (*n*) of each population are indicated.

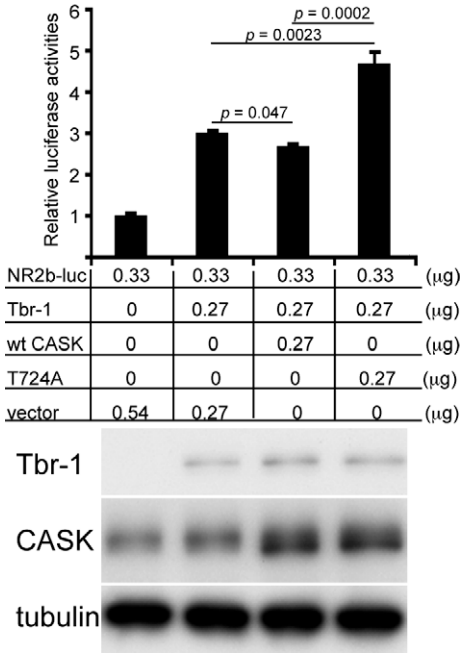


Fig. 4. CASK T724A mutation affects the activity of the NR2b promoter. Neuroblastoma Neuro-2A cells were cotransfected with various plasmids as indicated. Equal protein amounts of cell extracts were used for the luciferase reporter assay. Immunoblotting analyses were performed using antibodies, as indicated, to ensure transfection efficiency. Tubulin served as the internal control. Experiments were repeated twice. Sample size, $n = 6$.

proteins were evenly distributed across the whole cell, including the nucleus and cytoplasm (see example in Fig. 3E). In only 18.7% of cells, T724A mutant proteins were concentrated in the nuclei (Fig. 3F). Consistent with the co-immunoprecipitation study results, these immunostaining data support a reduction in the interaction between Tbr-1 and CASK T724A mutant.

Our previous data showed that Tbr-1, CASK, and CINAP form a transcriptional complex regulating expression of the NR2b gene [8,11]. We thus examined here whether the T724A mutation alters the transcriptional activity of the Tbr-1–CASK–CINAP complex, using a luciferase reporter assay with mouse neuroblastoma Neuro-2A cells. Similar to the previous study, Tbr-1 expression increased activity of the NR2b promoter (Fig. 4). Coexpression of wild-type CASK slightly but significantly reduced induction by Tbr-1 (Fig. 4). Endogenous CINAP and CASK are expressed at low levels in Neuro-2A cells, in which CINAP acts as a negative regulator of NR2b expression rather than as a positive activator in hippocampal neurons [8]. Thus, when CASK T724A mutant proteins were overexpressed, the expected outcome was dissociation of the Tbr-1–CASK–CINAP transcriptional complex and enhanced NR2b promoter activity. Indeed, coexpression of Tbr-1 with the CASK T724A mutant enhanced NR2b promoter activity (Fig. 4). This result supports the inference that the CASK T724A mutation affects formation of the Tbr-1, CASK, and CINAP complex and thus influences NR2b gene expression.

Because NMDAR with the NR2b subunit has a longer duration of activation, there can be more calcium influx from the extracellular space. It is generally believed that NR2b plays a critical role in learning and memory: for example, transgenic mice expressing more NR2b have better learning and memory performance [14]. Regulation of NR2b expression by the Tbr-1–CASK–CINAP protein complex may at least partially account for the mental retardation phenotype in people carrying a CASK mutation [1]. Although, as mentioned, deletion of either CASK or Tbr-1 in mice results in neonatal lethality [2,10], conditional knockout is a possible approach. Here, we provide evidence that the CASK T724A mutation downregulates the interaction of CASK and Tbr-1 and CINAP and affects the NR2b promoter activity. The T724A mutation is thus a very specific alternative for investigating the role of the Tbr-1–CASK–CINAP protein complex in learning and memory. In addition, because T724 is a putative PKA phosphorylation site, it will be interesting to examine whether PKA does phosphorylate and regulate the protein–protein interaction of CASK, thus controlling learning and memory behaviors in mice.

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