

Carom: a novel membrane-associated guanylate kinase-interacting protein with two SH3 domains

Hideki Ohno^{1,2}, Susumu Hirabayashi¹, Ai Kansaku¹, Ikuko Yao¹, Makiko Tajima¹, Wataru Nishimura¹, Hirohide Ohnishi³, Hirosato Mashima², Toshiro Fujita⁴, Masao Omata² and Yutaka Hata^{*1}

¹Department of Medical Biochemistry, Graduate School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan; ²Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; ³Department of Gastroenterology, Jichi Medical School, Tochigi, Japan; ⁴Department of Nephrology and Endocrinology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

MAGI-1 and CASK are membrane-associated guanylate kinases of epithelial junctions. MAGI-1 is localized at tight junctions in polarized epithelial cells, whereas CASK is localized along the lateral membranes. We obtained the KIAA0769 gene product through the yeast two-hybrid screening using MAGI-1 as a bait and named it Carom. Carom has a coiled-coil domain in the middle region, and two src homology 3 domains and a PSD-95/Dlg-A/ZO-1 (PDZ)-binding motif in the C-terminal region. Carom binds to the fifth PDZ domain of MAGI-1 and the calmodulin kinase domain of CASK *in vitro*. MAGI-1 and CASK bind to the distinct sequences in the C-terminal region of Carom, but still compete with each other for Carom binding. The study using a stable transformant of Madine Darby canine kidney (MDCK) cells expressing GFP-Carom revealed that Carom was partially overlapped by MAGI-1 in MDCK cells, which have not yet established mature cell junctions, but became separated from MAGI-1 and colocalized with CASK in polarized cells. Carom was highly resistant to Triton X-100 extractions and recruited CASK to the Triton X-100-insoluble structures. Carom is a binding partner of CASK, which interacts with CASK in polarized epithelial cells and may link it to the cytoskeleton.

Oncogene (2003) 22, 8422–8431. doi:10.1038/sj.onc.1206996

Keywords: epithelial junctions; MAGI-1; CASK; SH3; PDZ

Introduction

Epithelial cell junctions are implicated in the signal transduction that regulates cell proliferation. The disorder in the formation of epithelial junctions results in oncogenesis. Recent studies have revealed a family of proteins named membrane-associated guanylate kinases (MAGUK), which play central roles in the molecular

organization of epithelial junctions. MAGUKs have multiple modules, which mediate protein–protein interactions (Fanning and Anderson, 1999). Tight junctions have ZO-1 and MAGUK with inverted domain organization (MAGI-1) (also called brain angiogenesis inhibitor-associated protein 1) (Stevenson *et al.*, 1986; Anderson *et al.*, 1988; Dobrosotskaya *et al.*, 1997; Shiratsuchi *et al.*, 1998; Ide *et al.*, 1999). hDLG/SAP97 and CASK are also MAGUKs of epithelial cells (Lue *et al.*, 1994; Muller *et al.*, 1995; Hata *et al.*, 1996). These MAGUKs are known to interact with various molecules and are important in the assembly of junctional components.

MAGI-1 has six PSD-95/Dlg-A/ZO-1 (PDZ0–PDZ5), one guanylate kinase (GK), and two WW domains (Dobrosotskaya *et al.*, 1997; Shiratsuchi *et al.*, 1998). A neuronal isoform of MAGI-1, synaptic scaffolding molecule (S-SCAM), binds *N*-methyl-D-aspartate (NMDA) receptor subunits and cell adhesion molecule named neuroligin, and link these proteins to the cytoskeleton and signaling molecules (Hirao *et al.*, 1998). By analogy, MAGI-1 is considered to attach the cytoskeleton and signaling molecules to membrane receptors or cell adhesion molecules. It is reported that MAGI-1 interacts with β -catenin, mNET1, RapGEP, synaptopodin, α -actinin-4, and megalin (Dobrosotskaya and James, 2000; Mino *et al.*, 2000; Dobrosotskaya, 2001; Patrie *et al.*, 2001, 2002). β -Catenin is implicated in the polarized targeting of MAGI-1 (Dobrosotskaya and James, 2000; Nishimura *et al.*, 2000). RapGEP and mNET1 are regulators of small GTP-binding proteins (Mino *et al.*, 2000; Dobrosotskaya, 2001). Synaptopodin and α -actinin-4 interact with MAGI-1 in renal podocytes and link it to the actin cytoskeleton (Patrie *et al.*, 2002). Megalin is an endocytic transmembrane receptor, but is not localized at tight junctions (Christensen and Birn, 2002). Moreover, we have recently reported a novel junctional adhesion molecule that interacts with MAGI-1 and named it junctional adhesion molecule (JAM) 4 (Hirabayashi *et al.*, 2003).

CASK has one calmodulin kinase (Camk), one PDZ, one SH3, and one GK domain (Hata *et al.*, 1996).

*Correspondence: Y Hata; E-mail: yuhammch@med.tmd.ac.jp
Received 3 March 2003; revised 11 July 2003; accepted 15 July 2003

CASK was originally identified as a neurexin-interacting protein and turned out to be a mammalian homologue of Lin-2 (Hata *et al.*, 1996; Hoskins *et al.*, 1996; Dimitratos *et al.*, 1997). It forms a complex with mammalian homologues of Lin-7 (Velis/Mals/mLin-7) and Lin-10 (Mint/X-11) (Butz *et al.*, 1998). The complex is considered to play roles in the transport of NMDA receptors and calcium channels in neurons and potassium channels in epithelial cells (Setou *et al.*, 2000; Maximov and Bezprozvanny, 2002; Olsen *et al.*, 2002). CASK also interacts with syndecans, JAM1, protein 4.1, hDLG, Parkin, and Caskin (Cohen *et al.*, 1998; Hsueh *et al.*, 1998; Martinez-Estrada *et al.*, 2001; Fallon *et al.*, 2002; Lee *et al.*, 2002; Tabuchi *et al.*, 2002). Although the physiological significance of these interactions is not yet clear, CASK may be important for the proper targeting of junctional components and link them to the cytoskeleton (Bierderer and Sudhof, 2001). Moreover, CASK is translocated to the nucleus and may be involved in the regulation of gene transcription (Hsueh *et al.*, 2000).

In this study, we first searched for an MAGI-1-interacting junctional protein to clarify the role of MAGI-1 in the organization of tight junctions. Through a yeast two-hybrid screening, we obtained a protein with two src homology 3 (SH3) domains that we named Carom. The protein was deposited in GenBank under the name KIAA0769 gene product but had not yet been characterized. Carom was not concentrated at tight junctions and separated from MAGI-1 in polarized epithelial cells, although it was partially colocalized with MAGI-1 at immature cell contacts. We subsequently sought to find a Carom interactor and found that CASK interacts with Carom.

Results

Identification of Carom as a MAGI-1-interacting protein

To identify a protein interacting with MAGI-1, we performed a yeast two-hybrid screen of human lung cDNA library, using PDZ domains of MAGI-1 as a bait. We screened 2×10^6 clones and obtained 65 positive clones. Sequence analysis of these clones identified previously uncharacterized genes. Among them, we focused on the clone, pPrey 10474 in this study. The encoded peptide sequence was composed of 509 amino acids and terminated with a PDZ-binding motif (Figure 1a). The search for GenBank database revealed that the amino-acid sequence corresponded to the product of KIAA0769. Human KIAA0769 encodes a putative cytosolic protein with two SH3 domains. We subsequently performed PCR on human lung cDNA to obtain the full length of KIAA0769. We called KIAA0769 protein Carom, because this protein interacts with two molecules sequentially as described later. The constructs of Carom used in this study are summarized (Figure 1b).

Tissue distribution of Carom

To examine which tissues express Carom, we hybridized a multitissue human RNA blot with a Carom cDNA probe. A single 5.5-kb transcript was present in the heart, brain, placenta, liver, skeletal muscle, and pancreas (Figure 2a). Lung and kidney also had a small amount of transcript. We raised a rabbit polyclonal antibody against the C-terminal region of Carom and performed Western blot analysis against various cell

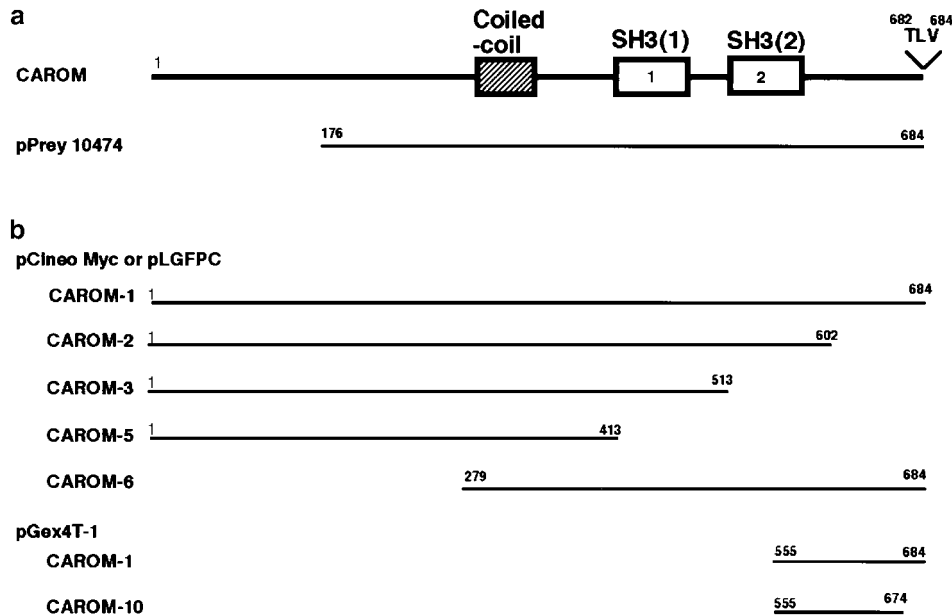


Figure 1 Molecular structure of CAROM and the constructs. **(a)** The molecular structure of Carom. The coiled-coil domain and SH3 domains are depicted. TLV is the PDZ-binding motif (single letter codes indicate amino-acid residues). pPrey 10474 contains 509 amino acids. **(b)** Various constructs of Carom. The constructs used in this study are summarized. The numbers of the first and the last amino-acid residues of each construct are shown

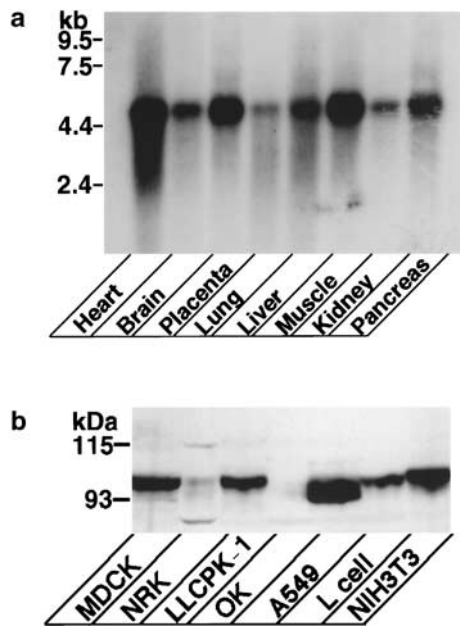


Figure 2 Tissue distribution of Carom. (a) Northern blot analysis of Carom. Uniformly labeled probes corresponding to the full length of Carom were prepared. Blots with 2 μ g of mRNA from each human tissue were hybridized with probes (3 600 000 c.p.m./each probe) and exposed for 24 h. The mobilities of molecular mass standards in kb are indicated in the left. (b) Western blot analysis of various cell lines. NRK, normal rat kidney. OK, opossum kidney. The mobilities of molecular mass standards in kDa are indicated in the left

lines. The affinity-purified antibody detected a 100-kDa protein in Madine Darby canine kidney (MDCK), LLCPK-1, A549, L, and NIH3T3 cells (Figure 2b). The signal was weak in normal rat kidney cells. Opossum kidney cells showed no signal. We used MDCK cells for further studies.

Carom binds MAGI-1 in vitro

To obtain independent evidence for interaction between Carom and MAGI-1, we first performed the coimmunoprecipitation experiment using MDCK cells. As MAGI-1 is highly resistant to the detergent extraction in confluent MDCK cells, we made use of the extracts of about 50% confluent MDCK cells. MAGI-1 was coimmunoprecipitated with Carom (Figure 3Aa). Next,

we expressed various Myc-tagged Carom proteins and HA-tagged MAGI-1 in COS-7 cells and performed the coimmunoprecipitation experiment (Figure 3Ab). HA-MAGI-1 was coimmunoprecipitated with Myc-Carom-1 and -6 (Figure 3Ab, arrows). In contrast, HA-MAGI-1 did not bind to Myc-Carom-2 or -3. Thereby, we reasoned that the C-terminal region is required for the interaction. Subsequently, we conducted pull-downs with GST-fusion protein of the C-terminal region of Carom (GST-Carom-1) and examined which region of MAGI-1 was involved in the interaction (Figure 3Ba). GST-Carom-1 captured Myc-MAGI-1-full and Myc-MAGI-V containing PDZ4 and PDZ5 (Figure 3Bb). GST-Carom-10 lacking the C-terminal PDZ-binding motif did not bind Myc-MAGI-1-V. In the reverse experiment, we prepared 35 S-methionine-labeled Carom by *in vitro* transcription translation, and performed pull-downs with GST proteins containing various PDZ domains of MAGI-1. GST-PDZ4-bound Carom, but other GST proteins did not (Figure 3Bc). Therefore, Carom directly binds to PDZ4 of MAGI-1.

Carom is not concentrated at tight junctions and not colocalized with MAGI-1 in polarized epithelial cells

We examined the subcellular distribution of Carom in confluent MDCK cells. Carom was detected at cell contacts and overlapped with β -catenin and E-cadherin (Figure 4A, a and b). Tight junction marker protein, ZO-1, was not colocalized with Carom. To compare the localization of Carom with that of MAGI-1 directly, we prepared a stable transformant of MDCK cells expressing GFP-tagged Carom (MDCK-Carom cells). GFP-Carom-1 was colocalized with β -catenin and E-cadherin like the endogenous Carom (Figure 4Ba and data not shown). It was not colocalized with MAGI-1 (Figure 4Bb). Thereby, it is unlikely that Carom interacts with MAGI-1 in polarized epithelial cells.

Localization of Carom at cell contacts is mediated by the C-terminal region

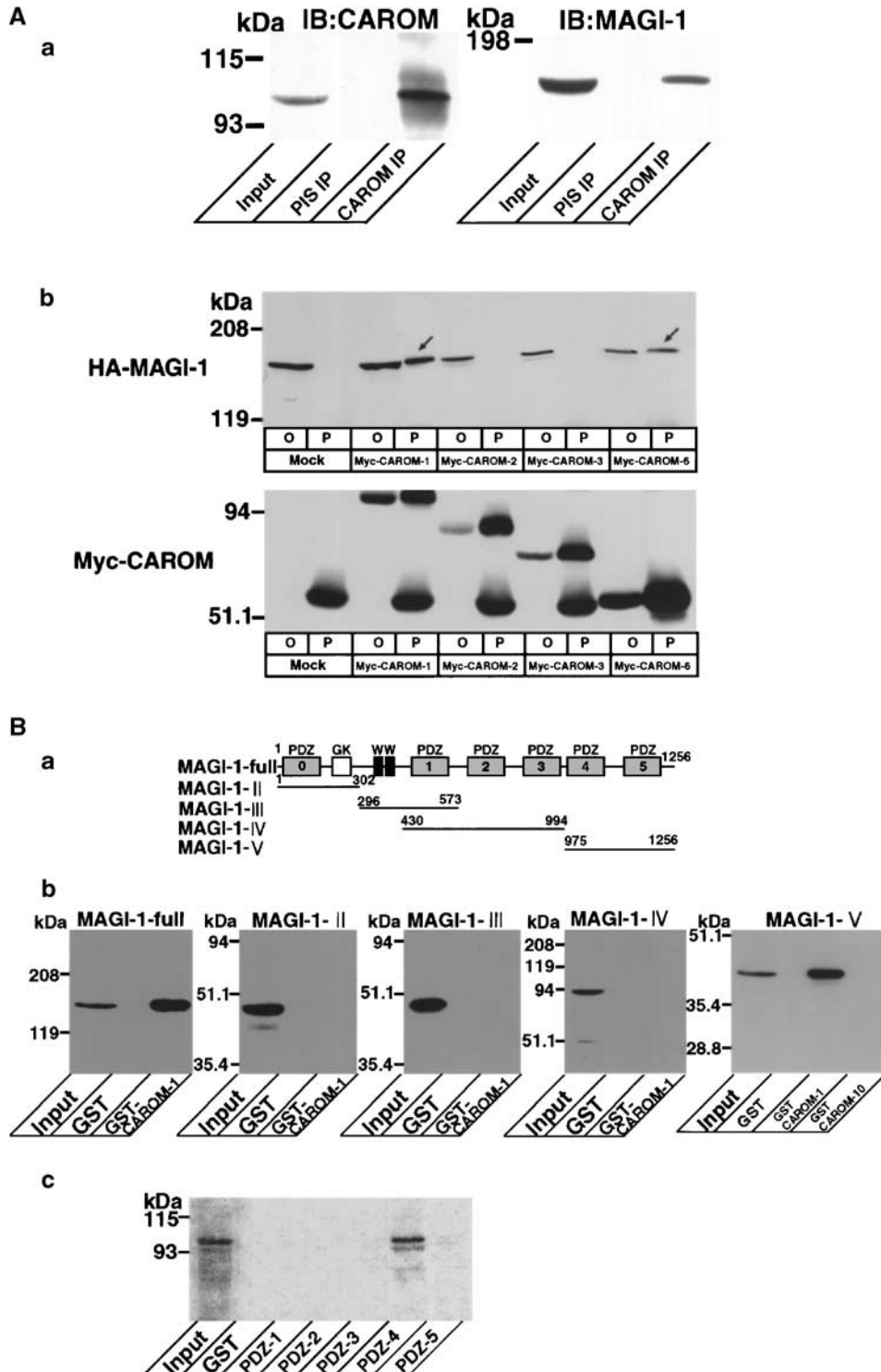
To examine which region determines the subcellular localization of Carom, we generated stable transformants of MDCK cells expressing GFP-Carom-2 and -6.

Figure 3 *In vitro* interaction of Carom with MAGI-1. (A) (a) Coimmunoprecipitation of Carom with MAGI-1 from MDCK cells. The urea extracts of semiconfluent MDCK cells were incubated with either the preimmune serum or the anti-Carom serum. The immunoprecipitates were blotted with anti-Carom and anti-MAGI-1 antibodies. PIS IP precipitates with the preimmune serum. CAROM IP precipitates with the anti-Carom serum. (b) Coimmunoprecipitation experiment of HA-MAGI-1 with various Myc-Carom proteins. COS-7 cells were transfected with pCleo HA-MAGI-1 and various pCleo Myc Carom constructs. The extracts of cells were immunoprecipitated with the mouse monoclonal anti-Myc ascites and the precipitates were immunoblotted with the anti-HA or anti-Myc antibody. (Upper panel) The immunoblot with the anti-HA antibody. (Lower panel) The immunoblot with the anti-Myc antibody. Arrows indicate HA-MAGI-1 proteins in the immunoprecipitates. O, original extracts. P, immunoprecipitates. (B) Carom-interacting region of MAGI-1. (a) The molecular structure of MAGI-1 and schematic drawing of various Myc-MAGI-1 constructs. PDZ domains, GK domain, and WW domains are indicated. The numbers of the first and the last amino-acid residues of each construct are shown. (b) GST-pull-down experiment of various Myc-MAGI-1 proteins with GST-Carom-1. COS-7 cells were transfected with various pCleo Myc MAGI-1 constructs. The extracts of cells were incubated with GST, GST-Carom-1, or GST-Carom-10 immobilized on glutathione Sepharose 4B beads. After the beads were washed, the precipitates were immunoblotted with the anti-Myc antibody. (c) Direct interaction of Carom and MAGI-1. 35 S-methionine-labeled Carom was prepared by *in vitro* transcription and translation, and incubated with various GST constructs of MAGI-1 immobilized on glutathione Sepharose 4B beads. After the beads were washed, the precipitates were analysed by SDS-PAGE and the imaging analyzer. The mobilities of molecular mass standards in kDa are indicated in the left

GFP-Carom-2 lacks the C-terminal region and GFP-Carom-6 covers two SH3 domains and the C-terminal region (Figure 1b). GFP-Carom-2 was distributed in the cytosol and was not accumulated at cell contacts (Figure 4C). GFP-Carom-6 was localized at cell contacts like the full-length Carom (Figure 4C). These findings suggest that the C-terminal region of Carom determines its subcellular localization.

C-terminal region of Carom interacts with CASK

We speculated that some component other than MAGI-1 may bind the C-terminal region of Carom and tether Carom to cell contacts. As Carom has the PDZ-binding motif, we tested the interaction of Carom with PDZ proteins, including CASK, SAP97, Lin-7, and ERBIN. GST-Carom-1 bound CASK but not SAP97, Lin-7, or



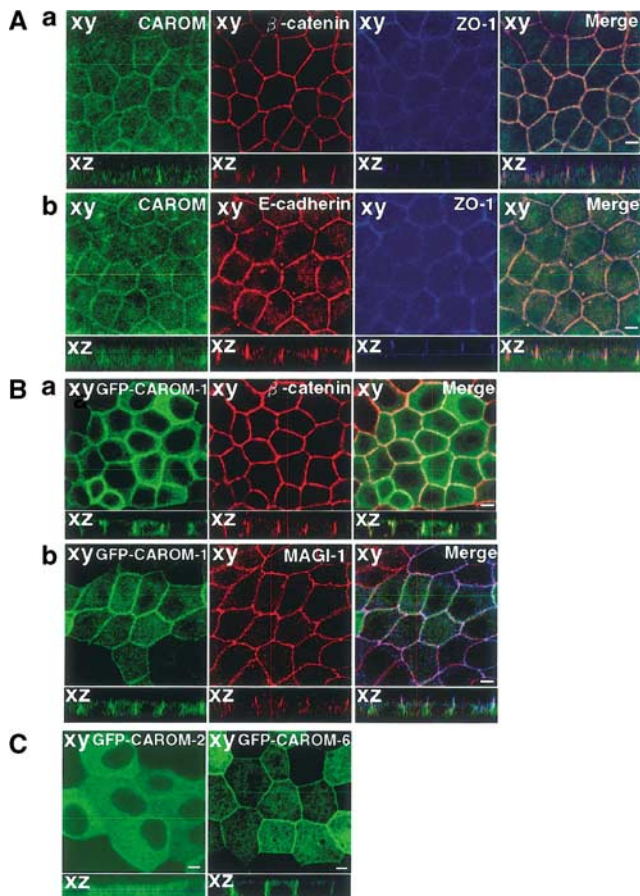


Figure 4 Immunofluorescence analysis of endogenous Carom and GFP-Carom in MDCK cells. (A) The subcellular localization of Carom was compared with those of endogenous β -catenin, E-cadherin, and ZO-1 in MDCK cells. (a) Carom with β -catenin and ZO-1. (b) Carom with E-cadherin and ZO-1. (B) Immunofluorescence analysis of GFP-Carom in MDCK cells. Stable transformants of MDCK cells expressing GFP-Carom were immunostained with either the anti- β -catenin or anti-MAGI-1 antibody. (a) GFP-Carom and β -catenin. (b) GFP-Carom and MAGI-1. (C) Subcellular localization of GFP-Carom-2 and -6 in MDCK cells. Bars, 5 μ m

ERBIN (Figure 5A). We confirmed the interaction using the immunoprecipitation. When the lysates of MDCK cells were incubated with the anti-Carom antibody, the immunoprecipitate contained CASK (Figure 5B). We next determined the Carom-interacting region of CASK. We incubated various Myc-tagged proteins of CASK with either GST or GST-Carom-1 (Figure 5Ca). Unexpectedly, GST-Carom-1 bound the N-terminal region of CASK containing the Camk domain and not the PDZ domain (Figure 5Cb). We also confirmed that CASK bound to GST-Carom-10 lacking the PDZ-binding motif, while MAGI-1 did not bind to GST-Carom-10 (Figure 5Cc). Thereby, CASK and MAGI-1 interact with the different sites of Carom.

CASK inhibits the interaction between Carom and MAGI-1

As CASK and MAGI-1 bind to the distinct regions of Carom, it is possible that Carom binds CASK and

MAGI-1 simultaneously. In the next set of experiments, we tested this possibility. We incubated GST-MAGI-1-PDZ4+5 with GST-Carom-1 immobilized on magnetic beads. GST-MAGI-1-PDZ4+5 was trapped by GST-Carom-1 beads but not by GST beads (Figure 6). However, the addition of GST-CASK-Camk, but not GST inhibited the interaction between GST-MAGI-1-PDZ4+5 and GST-Carom-1 in a dose-dependent manner.

Carom is colocalized with MAGI-1 at immature cell contacts

As described, Carom is colocalized with CASK but not with MAGI-1 in polarized epithelial cells. There remains, however, to determine whether Carom is colocalized with MAGI-1 in MDCK cells that do not have mature cell junctions. Indeed, the result of Figure 3Aa suggests that Carom interacts with MAGI-1 in semiconfluent MDCK cells. We performed calcium switch experiments using MDCK-GFP-Carom cells. Before the treatment, GFP-Carom was not colocalized with MAGI-1 as indicated in the x-z section (Figure 7a, 0 min). When cells were exposed to Dulbecco's modified Eagle's medium (DMEM) containing 1.8 mM CaCl_2 and 5 mM EGTA, cells became dissociated. At this stage, GFP-Carom and MAGI-1 were detected at the same sites in the x-y section (EGTA, 30 min). As cells were thin, we could not evaluate the x-z section. After further incubation, both GFP-Carom and MAGI-1 were hardly detected on the cell surfaces (EGTA, 60 min). Subsequently, cells were switched back to DMEM containing 1.8 mM CaCl_2 . Then, cells started to contact with each other and GFP-Carom and MAGI-1 were accumulated at the cell contacts (Ca^{2+} , 15 min). However, when cells formed wide contacts, GFP-Carom and MAGI-1 were not detected in the same sites, suggesting that GFP-Carom was separated from MAGI-1 (Ca^{2+} , 120 min). We raised the question of whether CASK was localized at the site where GFP-Carom and MAGI-1 were colocalized. To address this question, we attempted to examine the localization of the endogenous CASK, but we could not obtain the clear images for CASK in calcium switch experiments. Thereby, we tried to find dividing cells in which GFP-Carom was colocalized with MAGI-1. Although we could not definitely determine at which stage cells were, both GFP-Carom and MAGI-1 were detected in the middle portion of cell contacts (Figure 7Ba). At this stage, we could not detect CASK at cell contacts (data not shown). We also observed some cells in which GFP-Carom showed linear distribution and MAGI-1 was concentrated at the periphery of cell contacts (Figure 7Bb-1). In such cells, some of GFP-Carom was free of MAGI-1 (Figure 7Bb-1, arrow). MAGI-1 was still colocalized with GFP-Carom (Figure 7Bb-2, arrow heads). CASK was also accumulated at cell contacts and colocalized with GFP-Carom (Figure 7Bb-2, arrows). However, as arrow heads and arrows indicate in Figure 7Bb-2, GFP-Carom was not colocalized with MAGI-1 where it was colocalized with CASK.

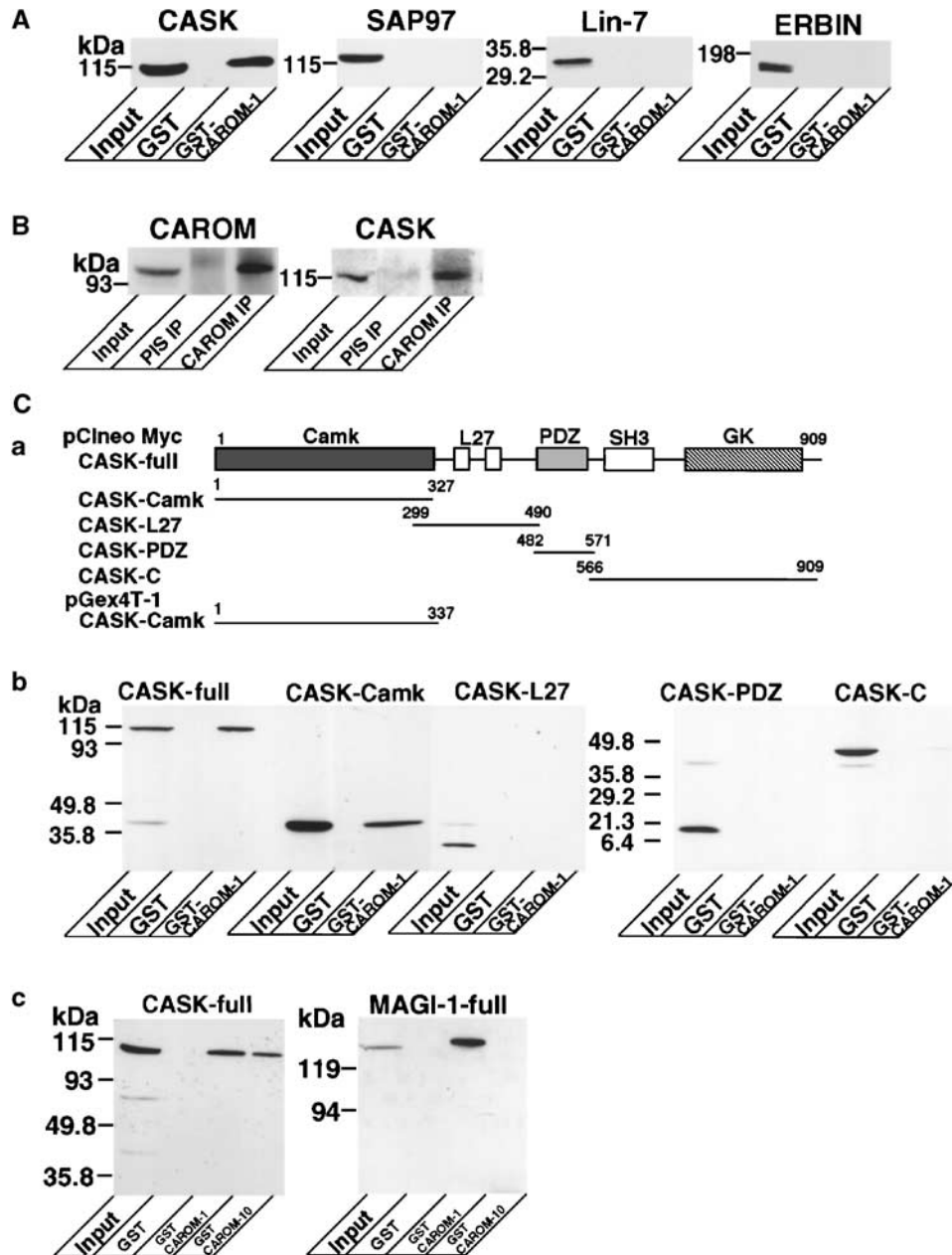


Figure 5 Interaction of Carom with CASK. (A) GST-pulldown experiment of various PDZ proteins with GST-Carom-1. COS-7 cells were transfected with pCneo Myc CASK, SAP97, Lin-7, or ERBIN. The extracts of cells were incubated with either GST or GST-Carom-1 immobilized on glutathione Sepharose 4B beads. After the beads were washed, the precipitates were immunoblotted with the anti-Myc antibody. (B) Coimmunoprecipitation of Carom with CASK from MDCK cells. The urea extracts of MDCK cells were incubated with either the preimmune serum or the anti-Carom serum. PIS IP precipitates with the preimmune serum. CAROM IP precipitates with the anti-Carom serum. (C) Carom-interacting region of CASK. (a) The molecular structure of CASK and schematic drawing of various Myc-CASK constructs. Camk, PDZ, SH3, and GK domains are indicated. The numbers of the first and the last amino-acid residues of each construct are shown. (b) GST-pulldown experiment of various Myc-CASK proteins with GST-Carom-1. COS-7 cells were transfected with various pCneo Myc CASK constructs. The extracts of cells were incubated with either GST or GST-Carom-1 immobilized on glutathione Sepharose 4B beads. After the beads were washed, the precipitates were immunoblotted with the anti-Myc antibody. (c) The interaction of GST-Carom-10 lacking the PDZ-binding motif with CASK. COS-7 cells were transfected with pCneo Myc CASK or pCneo Myc MAGI-1 and the extracts were incubated with GST, GST-Carom-1, or GST-Carom-10 to test the interaction of GST-Carom-10 with CASK. The mobilities of molecular mass standards in kDa are indicated in the left

Carom is triton X-100-insoluble and recruits CASK to the triton X-100-insoluble fraction

During these experiments, we found that Carom was resistant to Triton X-100 extraction in MDCK cells

(data not shown). Carom was also Triton X-100-insoluble in COS-7 cells (Figure 8a). Carom was insoluble even in the extraction with Triton X-100 at 37°C and 25 mM *n*-octyl- β -D-glucoside (data not shown).

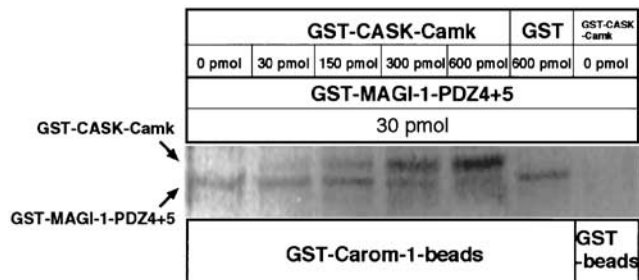


Figure 6 Competition of CASK and MAGI-1 for Carom binding. A measure of 2.5 nmol of GST and GST-Carom-1 were immobilized on 5 mg of magnetic beads. A measure of 30 pmol of GST-PDZ4+5 of MAGI-1 were incubated with 100 μ g of GST-Carom beads in the presence of the increasing amount of GST-CASK-Camk (0, 30, 150, 300, and 600 pmol) (the first to fifth lanes). As control, 600 pmol of GST was added (the sixth lane). GST beads did not trap GST-MAGI-1-PDZ4+5 even in the absence of GST-CASK-Camk (the seventh lane). After the beads were washed, the precipitates were analysed on SDS-PAGE and visualized with Coomassie brilliant blue staining

The treatment with 10 mM methyl- β -cyclodextrin had no effect (data not shown). We expressed various regions of Carom and found that the N-terminal region was insoluble, whereas the C-terminal region was soluble. Next, we expressed CASK with Carom. CASK was Triton X-100 soluble, when expressed alone (Figure 8b). However, CASK became Triton X-100 insoluble, when expressed together with Carom. This finding implies that Carom links CASK to the Triton X-100-insoluble structures.

Discussion

In the present study, we obtained the KIAA0769 gene product as a MAGI-1-interacting protein through a yeast two-hybrid screening and named the protein Carom. Carom has a coiled-coil domain followed by two SH3 domains and the PDZ-binding motif at the C-terminus. The immunoprecipitation study and the GST-pulldown assay using COS-7 cells indicate that Carom directly binds to the fifth PDZ domain (PDZ4) of MAGI-1 by its C-terminal PDZ-binding motif. Although Carom is not concentrated at tight junctions and does not colocalize with MAGI-1 in polarized MDCK cells, we can observe that GFP-Carom and MAGI-1 are colocalized at some stages in the calcium switch experiments and in dividing cells. Thereby, we consider that Carom interacts with MAGI-1 in cells that have not yet formed mature cell junctions.

We next raised a question of which component of cell junctions Carom interacts with in polarized epithelial cells. We speculated that a PDZ protein binds to the PDZ-binding motif of Carom and recruits it to cell junctions, because the C-terminal region of Carom is involved in its polarized targeting. We tested PDZ proteins, including CASK, SAP97, Lin-7, and ERBIN to find that Carom interacts with CASK. The interac-

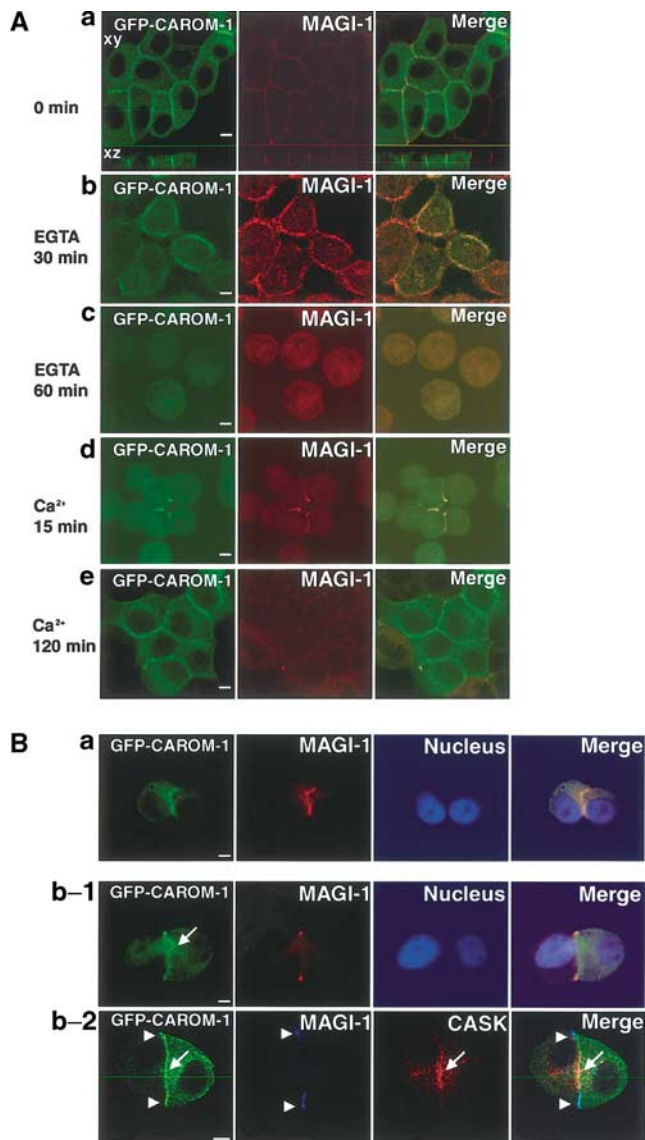


Figure 7 Localization of GFP-Carom, MAGI-1, and CASK in MDCK cells. **(A)** Calcium switch experiments of MDCK-GFP-Carom cells. MDCK-GFP-Carom cells were transferred to DMEM containing 1.8 mM CaCl_2 and 5 mM EGTA. After 60 min, cells were switched back to DMEM containing 1.8 mM CaCl_2 . The cells were fixed at various stages as indicated and immunostained with the anti-MAGI-1 antibody. **(B)** GFP-Carom in cells that have not formed mature cell contacts. **(a)** Cells in which GFP-Carom and MAGI-1 were colocalized at cell contacts. Nuclei were stained with Hoechst 33342. **(b-1)** Cells in which MAGI-1 was concentrated at the periphery of cell contacts and overlapped with GFP-Carom. Some of GFP-Carom was not colocalized with MAGI-1 (arrow). **(b-2)** CASK in the cells that showed the concentration of MAGI-1 at the periphery of cell contacts. Some of the GFP-Carom was colocalized with MAGI-1 like in (b-1) (arrow heads). CASK was detected at cell contacts and colocalized with GFP-Carom (arrow). GFP-Carom was not colocalized with MAGI-1 where it was colocalized with CASK. Bars, 5 μ m

tion between Carom and CASK is supported by the *in vitro* binding experiment, the immunoprecipitation, and the immunocytostaining that reveals the colocalization of Carom and CASK. Unexpectedly, Carom does not bind to the PDZ domain but to the Camk domain of

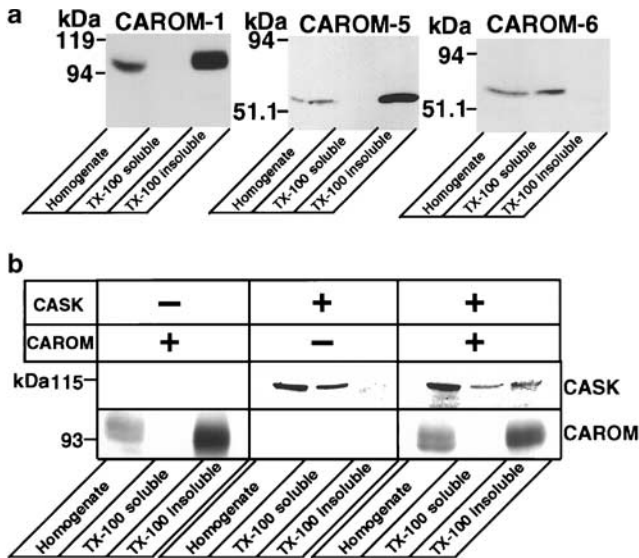


Figure 8 Carom is Triton X-100 insoluble and recruits CASK to the Triton X-100-insoluble structures. (a) Various Carom proteins in the subcellular fractions of COS-7 cells. COS-7 cells were transfected with pCneo Myc Carom-1, -5, or -6, and homogenized in the buffer containing 1.0% (w/v) Triton X-100, and centrifuged at 100 000g for 15 min. The comparable amount of each fraction was immunoblotted with the anti-Myc-antibody. (b) Recruitment of CASK by Carom to the Triton X-100-insoluble structures. COS-7 cells were transfected with pCneo Myc Carom-1 and/or pCneo HA CASK, and the subcellular fractionation was performed. The comparable amount of each fraction was immunoblotted with the anti-Myc and the anti-HA-antibodies. The mobilities of molecular mass standards in kDa are indicated in the left

CASK, and the PDZ-binding motif of Carom is not involved in the interaction with CASK. Thereby, MAGI-1 and CASK bind to the different sequences in the C-terminal region of Carom, implying that MAGI-1, CASK, and Carom can form a tripartite complex. The result of the affinity column experiment, however, did not support this hypothesis. The binding of MAGI-1 to Carom immobilized onto magnetic beads was inhibited by adding CASK. We speculate that binding sites of CASK and MAGI-1 are too close, and that MAGI-1 cannot reach the PDZ-binding motif when the C-terminal region is occupied by CASK. Our observation from the immunocytostaining experiments is consistent with this result. We expressed GFP-Carom in MDCK cells and detected endogenous MAGI-1 and CASK using the antibodies. CASK was not colocalized with GFP-Carom, where MAGI-1 was colocalized with GFP-Carom. These findings together with the *in vitro* competition experiment suggest that Carom separates from MAGI-1 when it is colocalized with CASK.

The *in vivo* significance of the interaction of Carom with MAGI-1 and CASK remains to be tested. Carom is insoluble in the extraction with various detergents. The procedures to disrupt the lipid raft including the extraction with Triton X-100 at 37°C or *n*-octyl- β -D-glucoside and the treatment with methyl- β -cyclodextrin failed to extract Carom, suggesting that Carom is associated with the cytoskeleton. We have found that

CASK is recruited by Carom to the Triton X-100-insoluble structures. Thereby, Carom may link CASK to the cytoskeleton. As MAGI-1 itself is Triton X-100 insoluble, it is unclear whether Carom functions as an adaptor to link MAGI-1 to the cytoskeleton. Carom has two SH3 domains in the C-terminal region. The SH3 domain is a module that binds the proline-rich ligands (Mayer, 2001). As the SH3 domain binds ligands with moderate affinity and selectivity, proteins with the SH3 domain interact with various partners. Therefore, it is likely that Carom interacts with some proteins besides MAGI-1 and CASK through these SH3 domains. In such a model, Carom assembles various proteins under either CASK or MAGI-1 depending on the stages of the cell junction formation and links all these proteins to the cytoskeleton.

Materials and methods

Construction of expression vectors

Various expression vectors used in this study were constructed by conventional molecular biology techniques and the PCR method. pCneo (Promega) and pGex4T-1 (Amersham Pharmacia Biotech) were purchased from the commercial sources. pLGFPC-2, pCneo Myc, pCneo Myc MAGI-1-full, -II, -III, -IV, and -V were described previously (Nishimura *et al.*, 2000). pCneo HA was constructed with ligating the linkers ctaggcccccaacatgtaccatacagcagctcccagactacgctctcagg/aattctctcgagacgtagtctgggacgtctgtatgggtacatgttggggc to *Nhe*I/*Eco*R1 sites of the pCneo vector. The following constructs contain the following amino acids of human MAGI-1: pCneo HA MAGI-1, 1–1256; pBTM116 MAGI-1-8, 429–1256; pGex4T-1 MAGI-1-PDZ1, 430–573; pGex4T-1 MAGI-1-PDZ2, 565–836; pGex4T-1 MAGI-1-PDZ3, 771–994; pGex4T-1 MAGI-1-PDZ4, 975–1104; pGex4T-1 MAGI-1-PDZ5, 1112–1256; and pGex4T-1 MAGI-1-PDZ4 + 5, 975–1256. The following constructs contain the following amino acids of Carom, CASK, and SAP97: pLGFPC and pCneo Myc Carom-1, 1–684 of Carom; pLGFPC and pCneo Myc Carom-2, 1–602; pCneo Myc Carom-3, 1–513; pCneo Myc Carom-5, 1–413; pLGFPC and pCneo Myc Carom-6, 279–684; pGex4T-1 Carom-1, 555–684; pGex4T-1 Carom-10, 555–674; pCneo Myc CASK-full, 1–909 of rat CASK; pCneo Myc CASK-N, 1–490; pCneo Myc CASK Camk, 1–327; pCneo Myc CASK-L27, 299–490; pCneo Myc CASK-PDZ, 482–571; pCneo Myc CASK-C, 566–909; pGex4T-1 CASK-Camk, 1–337; and pCneo Myc SAP97, 1–911 of SAP97. Throughout this study, we describe all constructs that are derived from human MAGI-1 using the term, MAGI-1. pCneo Myc Lin-7 and pCneo Myc ERBIN were described previously (Irie *et al.*, 1999; Ohno *et al.*, 2002).

Antibodies and other reagents

The rabbit anti-Carom antibody was raised against the GST-Carom-1 protein. The mouse monoclonal anti-Myc and anti-HA antibodies were described previously (Nishimura *et al.*, 2000). The mouse monoclonal anti- β -catenin, anti-CASK, and anti-E-cadherin antibodies were obtained from BD Pharmingen and Transduction Laboratories (San Jose, CA, USA). The rat monoclonal anti-ZO-1 antibody and the fluorescent isothiocyanate-conjugated, rhodamine-conjugated, and Cy5-conjugated secondary antibodies for dual labeling were purchased from Chemicon (Temecula, CA, USA). The rabbit

anti-MAGI-1 antibody was raised against the N-terminal region of S-SCAM. Hoechst 33342 and methyl- β -cyclodextrin were obtained from Sigma Aldrich (St Louis, MS, USA).

Yeast two-hybrid screening

Yeast two-hybrid screening was performed using human lung cDNA library (Clontech, Palo Alto, CA, USA) and yeast strain L40. Histidine-selection plates contained 8 mM 3-amino-1,2,4-triazole and 360 mg/l 5-bromo-4-chloro-3-indolyl β -D(-)-galactopyranoside. After a 6-day incubation, blue colonies were picked up for further analysis.

Cell cultures

COS-7, MDCK, phoenix amphi, normal rat kidney, LLCPK-1, opossum kidney, A549, L, and NIH3T3 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin under 5% CO₂ at 37°C. To generate stable transformants of MDCK cells, phoenix amphi cells were transfected with pLGF constructs using the mammalian transfection kit (Stratagene, La Jolla, CA, USA). After 48 h culture, the medium was collected and used as a stock of virus. MDCK cells were infected with the virus, and selected with the medium containing 1 g/l of G418 (Sigma-Aldrich, St Louis, MO, USA). For calcium switch experiments, confluent MDCK cells were washed by phosphate-buffered saline (PBS) and transferred to DMEM containing 1.8 mM CaCl₂ and 5 mM EGTA. After 60 min, cells were returned to DMEM containing 1.8 mM CaCl₂.

Immunocytostaining

MDCK cells were fixed with 4% (w/v) formaldehyde in PBS at room temperature for 15 min and blocked with 50 mM glycine in PBS for 30 min. The samples were incubated with 0.2% (w/v) Triton X-100 in PBS for 15 min and subsequently with 1% (w/v) bovine serum albumin in PBS at room temperature for 30 min. The samples were incubated with various first antibodies and visualized with the appropriate secondary antibodies. The images were obtained by a confocal microscope (Zeiss LSM 510).

Immunoprecipitation

COS-7 cells were transfected with pCneo HA MAGI-1 alone or in combination with various Myc-tagged Carom constructs using the DEAE-dextran method. Cells from two 10-cm plates were homogenized in 400 μ l of the lysis buffer A (20 mM HEPES/NaOH pH 7.4, 100 mM NaCl, and 1% (w/v) Triton X-100) with 6 M urea and centrifuged at 100 000 *g* for 15 min at 4°C. The supernatant was dialysed against 500 ml of 20 mM HEPES/NaOH pH 7.4 and 100 mM NaCl with two exchanges and centrifuged at 100 000 *g* for 15 min at 4°C. The supernatant was incubated with 1.0 μ l of the monoclonal anti-Myc ascites fixed on 7.5 μ l of protein G Sepharose 4 fast flow beads. After the beads were washed, the precipitates were analysed in SDS-PAGE and immunoblotted with either the anti-HA or the anti-Myc antibody. For the immunoprecipitation of endogenous proteins, MDCK cells from four 10-cm plates were collected, homogenized in 800 μ l of buffer A with 6 M urea, and centrifuged at 100 000 *g* for 15 min at 4°C. The supernatant was subsequently processed in the same manner as for the immunoprecipitation from COS-7 cells.

GST-pulldown assay

COS-7 cells were transfected with various Myc-tagged MAGI-1 constructs using the DEAE-dextran method. Cells from one 10-cm plate were homogenized in 200 μ l of the lysis buffer A and centrifuged at 100 000 *g* for 15 min at 4°C. A measure of 175 μ l of the supernatant was incubated with 250 pmol of GST, GST-Carom-1, or GST-Carom-10 fixed on 7.5 μ l of glutathione Sepharose 4B beads. After the beads were washed, the precipitates were analysed in SDS-PAGE and immunoblotted with the anti-Myc antibody. GST-pulldown assays to test the interaction of Carom with CASK, SAP97, Lin-7, and ERBIN were performed in a similar manner. To confirm the direct interaction between MAGI-1 and Carom, ³⁵S-methionine-labeled Carom was prepared with *in vitro* transcription translation (TnT T7 Quick Coupled Transcription/Translation System, Promega) using 5 μ g of pCneo Myc Carom-1 as a template in 250 μ l of the reaction buffer according to the manufacturer's protocol. After the reaction, the total volume was adjusted to 800 μ l by adding 25 mM Tris/HCl pH 8.0, 100 mM NaCl, and 1% Triton X-100, and 120 μ l aliquot was incubated with 250 pmol of GST, GST-MAGI-1-PDZ1, -2, -3, -4, or -5 fixed on 7.5 μ l of glutathione Sepharose 4B beads. After the beads were washed, the precipitates were analysed in SDS-PAGE and the imaging analyzer (FLA-3000, FUJI FILM).

Subcellular fractionation

COS-7 cells were transfected with pCneo Myc Carom-1. Cells from one 10-cm plate were collected and homogenized in 200 μ l of buffer A, and 50 μ l was stocked as original homogenates. The remaining homogenates were centrifuged at 100 000 *g* for 15 min to separate the supernatants and the pellets. The pellets were resuspended in 150 μ l of buffer A. The comparable amount of each fraction was analysed in SDS-PAGE and immunoblotted with the anti-Myc antibody.

Affinity column chromatography

A measure of 2.5 nmol of GST or GST-Carom-1 was covalently coupled to 5 mg of magnetic beads (Epoxy Dynabeads M-270, Dynal) according to the manufacturer's protocol. A measure of 30 pmol of GST-MAGI-1-PDZ4 + 5 was incubated with 100 μ g of beads in 140 μ l of the buffer (50 mM Tris/HCl pH 7.4, 50 mM NaCl, and 0.5% Triton X-100) in the presence of various amounts of GST-CASK-Camk or GST. After vigorous wash, the proteins attached to the beads were analysed by SDS-PAGE and Coomassie brilliant blue staining.

Northern blot

A multiple tissue Northern blot of human tissues (Clontech, Palo Alto, CA, USA) containing 2 μ g of mRNA was hybridized with uniformly labeled probe derived from the full coding sequence of Carom.

Acknowledgements

We thank Dr Gary Nolan (Stanford University) for phoenix amphi cells and Dr Yusuke Nakamura (University of Tokyo) for cDNA of human MAGI-1. We also thank Ms C Rokukawa and Ms M Miyahara-Tenkatsu for skillful technical assistance. GST-CASK-Camk was generated by YH in Dr Thomas C Sudhof's laboratory (University of Texas).

This study was supported by grants-in-aids for Scientific Research (B) and on Priority Areas, and Special Coordination

Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science, and Techno-

logy, and a grant from Yamanouchi Foundation for Research on Metabolic Disorders (2000).

References

- Anderson JM, Stevenson BR, Jesaitis LA, Goodenough DA and Mooseker MS. (1988). *J. Cell. Biol.*, **106**, 1141–1149.
- Bierderer T and Sudhof TC. (2001). *J. Biol. Chem.*, **276**, 47869–47876.
- Butz S, Okamoto M and Sudhof TC. (1998). *Cell*, **94**, 773–782.
- Christensen EI and Birn H. (2002). *Mol. Cell. Biol.*, **3**, 258–268.
- Cohen AR, Woods DF, Marfatia SM, Walther Z, Chisti AH, Anderson JM and Wood DF. (1998). *J. Cell. Biol.*, **142**, 129–138.
- Dimitratos SD, Woods DF and Bryant PJ. (1997). *Mech. Dev.*, **63**, 127–130.
- Dobrosotskaya I, Guy RK and James GL. (1997). *J. Biol. Chem.*, **272**, 31589–31597.
- Dobrosotskaya IY. (2001). *Biochem. Biophys. Res. Commun.*, **283**, 969–975.
- Dobrosotskaya IY and James GL. (2000). *Biochem. Biophys. Res. Commun.*, **270**, 903–909.
- Fallon L, Moreau F, Croft BJ, Labib N, Gu W-J and Fon EA. (2002). *J. Biol. Chem.*, **277**, 486–491.
- Fanning AS and Anderson JM. (1999). *Curr. Opin. Cell. Biol.*, **11**, 432–439.
- Hata Y, Butz S and Sudhof TC. (1996). *J. Neurosci.*, **16**, 2488–2494.
- Hirabayashi S, Tajima M, Yao I, Nishimura W, Mori H and Hata Y. (2003). *Mol. Cell. Biol.*, **23**, 4267–4282.
- Hirao K, Hata Y, Ide N, Takeuchi M, Irie M, Yao I, Deguchi M, Toyoda A, Sudhof TC and Takai Y. (1998). *J. Biol. Chem.*, **273**, 21105–21110.
- Hoskins R, Hajnal AF, Harp SA and Kim SK. (1996). *Development*, **122**, 97–111.
- Hsueh YP, Wang TF, Yang FC and Sheng M. (2000). *Nature*, **404**, 298–302.
- Hsueh YP, Yang C, Kharazia V, Naisbitt S, Cohen AR, Weinberg RJ and Sheng M. (1998). *J. Cell. Biol.*, **142**, 139–151.
- Ide N, Hata Y, Nishioka H, Hirao K, Yao I, Deguchi M, Mizoguchi A, Nishimori H, Tokino T, Nakamura Y and Takai Y. (1999). *Oncogene*, **18**, 7810–7815.
- Irie M, Hata Y, Deguchi M, Ide N, Hirao K, Yao I, Nishioka H and Takai Y. (1999). *Oncogene*, **18**, 2811–2817.
- Lee S, Fan S, Makarova O, Straight S and Margolis B. (2002). *Mol. Cell. Biol.*, **22**, 1778–1791.
- Lue RA, Marfatia SM, Branton D and Chisti AH. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 9818–9822.
- Marinez-Estrada OM, Villa A, Breviaro F, Orsenigo F, Dejana E and Bazzoni G. (2001). *J. Biol. Chem.*, **276**, 9291–9296.
- Maximov A and Bezprozvanny I. (2002). *J. Neurosci.*, **22**, 6939–6952.
- Mayer BJ. (2001). *J. Cell Sci.*, **114**, 1253–1263.
- Mino A, Ohtsuka T, Inoue E and Takai Y. (2000). *Genes Cells*, **5**, 1009–1016.
- Muller BM, Kistner U, Veh RW, Cases-Langhoff C, Becker B, Gundelfinger ED and Garner CC. (1995). *J. Neurosci.*, **15**, 2354–2366.
- Nishimura W, Iizuka T, Hirabayashi S, Tanaka N and Hata Y. (2000). *J. Cell Physiol.*, **185**, 358–365.
- Ohno H, Hirabayashi S, Iizuka T, Ohnishi H, Fujita T and Hata Y. (2002). *Oncogene*, **21**, 7042–7049.
- Olsen O, Liu H, Wade JB, Merot J and Welling PA. (2002). *Am. J. Physiol. Cell Physiol.*, **282**, C183–C195.
- Patrie KM, Drescher AJ, Goyal M, Wiggins RC and Margolis B. (2001). *J. Am. Soc. Nephrol.*, **12**, 667–677.
- Patrie KM, Drescher AJ, Welihinda A, Mundel P and Margolis B. (2002). *J. Biol. Chem.*, **277**, 30183–30190.
- Setou M, Nakagawa T, Seog D-H and Hirokawa N. (2000). *Science*, **288**, 1796–1802.
- Shiratsuchi T, Futamura M, Oda K, Nishimori H, Nakamura Y and Tokino T. (1998). *Biochem. Biophys. Res. Commun.*, **247**, 597–604.
- Stevenson BR, Siliciano JD, Mooseker MS and Goodenough DA. (1986). *J. Cell Biol.*, **103**, 755–766.
- Tabuchi K, Bierderer T, Butz S and Sudhof TC. (2002). *J. Neurosci.*, **22**, 4264–4273.