Structural requirements for calmodulin binding to membrane-associated guanylate kinase homologs

INGO PAARMANN,[1,3] MING F. LYE,[2] ARNON LAVIE,[2] AND MANFRED KONRAD[1]

[1]Max-Planck Institute for Biophysical Chemistry, D-37077 Göttingen, Germany
[2]Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, Illinois 60607, USA

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Abstract

Effector molecules such as calmodulin modulate the interactions of membrane-associated guanylate kinase homologs (MAGUKs) and other scaffolding proteins of the membrane cytoskeleton by binding to the Src homology 3 (SH3) domain, the guanylate kinase (GK) domain, or the connecting HOOK region of MAGUKs. Using surface plasmon resonance, we studied the interaction of members of all four MAGUK subfamilies—synapse-associated protein 97 (SAP97), calcium/calmodulin-dependent serine protein kinase (CASK), membrane palmitoylated protein 2 (MPP2), and zona occludens (ZO) 1—and calmodulin to determine interaction affinities and localize the binding site. The SH3-GK domains of the proteins and derivatives thereof were expressed in *E. coli* and purified. In all four proteins, high-affinity calmodulin binding was identified. CASK was shown to contain a Ca^{2+}-dependent calmodulin binding site within the HOOK region, overlapping with a protein 4.1 binding site. In ZO1, a Ca^{2+}-dependent calmodulin binding site was detected within the GK domain. The equilibrium dissociation constants for MAGUK–calmodulin interaction were found to range from 50 nM to 180 nM. Sequence analyses suggest that binding sites for calmodulin have evolved independently in at least three subfamilies. For ZO1, pulldown of GST-calmodulin was shown to occur in a calcium-dependent manner; moreover, molecular modeling and sequence analyses predict conserved basic residues to be exposed on one side of a helix. Thus, calmodulin binding appears to be a common feature of MAGUKs, and Ca^{2+}-activated calmodulin may serve as a general regulator to affect the interactions of MAGUKs and various components of the cytoskeleton.

Keywords: calmodulin; MAGUK; surface plasmon resonance; CASK; MPP2; SAP97; ZO1

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Membrane-associated guanylate kinase homologs (MAGUKs) have been identified at cell–cell contact sites in organisms from *Drosophila* to man. They have been implicated in the assembly of synapses and tight junctions (Montgomery et al. 2004; Funke et al. 2005), the regulation of neurotransmitter vesicle release (Zordan et al. 2005), and control of humoral immune response (Jun et al. 2003). MAGUKs are multidomain proteins, encompassing at least one PSD-95/SAP90-Dlg-ZO1 (PDZ) domain, a Src homology 3 (SH3) domain, and a guanylate kinase (GK)-like domain (Kim and Sheng 2004; Montgomery et al. 2004; Funke et al. 2005). The MAGUK superfamily consists of four subfamilies, differing in their domain structure. The subfamily comprising synapse-associated protein (SAP) 97/hDlg, SAP90/PSD-95, SAP102/NE-dlg, PSD-93/Chapsyn110, and *Drosophila* Dlg contains two additional N-terminal PDZ domains. Calcium/calmodulin (CaM)-dependent serine protein kinase (CASK), Camguk, and

[3]Present address: Department of Neurochemistry, Max-Planck Institute for Brain Research, D-60528 Frankfurt, Germany.

Reprint requests to: Manfred Konrad, Max-Planck Institute for Biophysical Chemistry, Am Fassberg 11 Turm 5, D-37077 Göttingen, Germany; e-mail: mkonrad@gwdg.de; fax: 49-551-2011074; or Ingo Paarmann, Department of Neurochemistry, Room 210, Max-Planck Institute for Brain Research, Deutschordenstrasse 46, D-60528 Frankfurt, Germany; e-mail: paarmann@mpih-frankfurt.mpg.de; fax: 49-69-96769-441.

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Lin-2 contain a CaMKII domain at the N terminus. Zona occludens (ZO) 1, ZO2, ZO3, and Tamou have two additional N-terminal PDZ domains and an extended C-terminal part. Members of the fourth subfamily, such as p55/membrane palmitoylated protein (MPP) 1, MPP2, Pals2, MPP3, and MPP4, consist mainly of the minimal core for MAGUKs: one PDZ domain, one SH3 domain, and a GK domain. PDZ domains are essential for organization of pre- and post-synaptic plasma membranes through protein–protein interaction (for review, see Kim and Sheng 2004). For PSD-95, dynamic binding of microtubule-associated protein-1a to the GK domain has recently been demonstrated (Reese et al. 2007). Like PDZ domains, GK domains and SH3 domains serve for protein–protein interaction (for review, see Funke et al. 2005). As another main function of the SH3 domains in MAGUKs, the oligomerization of MAGUKs has been proposed (McGee et al. 2001; Yaffe 2002). Besides the PDZ, the SH3, and the GK domains, many MAGUKs contain other protein–protein interaction motifs. Among these are the L27 domain, a heteromultimerization domain (for review, see Funke et al. 2005), and a protein 4.1 binding site in the HOOK region that is located between the SH3 and the GK domains (Lue et al. 1994; Marfatia et al. 1995; Cohen et al. 1998).

The SH3 and the GK domains of MAGUKs show both intramolecular and intermolecular interactions (McGee and Bredt 1999; Shin et al. 2000; Tavares et al. 2001), and it is suggestive that CaM interaction with these sites may modulate the scaffolding properties of MAGUKs by affecting the equilibrium between these two modes of competing intra- and intermolecular interactions in a calcium-dependent manner. Moreover, CaM binding might occur at regions that are important for the association of MAGUKs with other members of the membrane cytoskeleton. In the SAP97 subfamily, high-affinity CaM binding to the HOOK region has been reported (Masuko et al. 1999; Paarmann et al. 2002). In the present study, we have analyzed the structural basis required for molecular interactions between MAGUKs and CaM by surface plasmon resonance (SPR). Our results show CaM binding to members of all four MAGUK subfamilies. In CASK, a Ca^{2+}-CaM site has been identified in the HOOK region, overlapping with the protein 4.1 binding site. ZO1 contains a Ca^{2+}-CaM site within the GK domain. Our findings suggest that CaM binding may represent a general way to modulate the MAGUK-mediated association of proteins within the membrane cytoskeleton.

Results

Expression of recombinant MAGUK C-terminal fragments

GST or MBP fusion proteins of MAGUK constructs were expressed in *Escherichia coli* BL21-CodonPlus and purified via a glutathione sepharose or an amylose resin column, respectively. The GST or MBP moiety was removed by protease cleavage. Since it proved difficult to overproduce large proteins such as full-length MAGUK proteins in bacteria, we first generated constructs encompassing the entire SH3 domain, the HOOK region, and the GK domain, including the C terminus; in ZO1, the C-terminal part was omitted because it is much longer compared with that of other MAGUKs. To map CaM binding sites and to determine sequence motifs for specific MAGUK binding, we successively deleted the SH3 domain, parts of the HOOK region, and the complete HOOK region (Fig. 1). MPP2 constructs without SH3 domain (constructs MPP2-2, MPP2-3, MPP2-4, and MPP2-5) formed inclusion bodies upon bacterial expression and were not further analyzed.

Structural elements of MAGUKs required for CaM interaction

To study the dynamics of the MAGUK-CaM interaction, we made use of SPR spectroscopy. This method allowed us to directly observe the binding of the MAGUK fragments to immobilized GST-CaM in the presence of Ca^{2+} and to assess parameters of association and dissociation kinetics. GST-CaM was immobilized by a sandwich

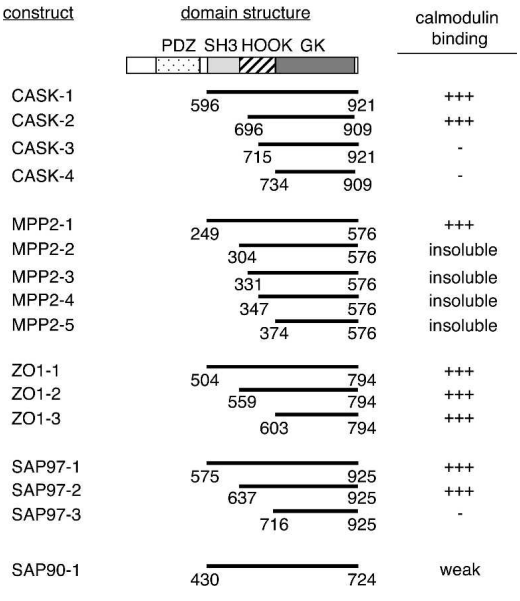

Figure 1. Schematic presentation of MAGUK deletion constructs. Depicted at the *top* is the conserved part of the domain structure common to all MAGUKs, consisting of a PDZ domain followed by an SH3 domain and a GK domain; also labeled is the HOOK region. The numbers indicated for each construct correspond to the positions of the first and the last amino acid, respectively, in the full-length protein. In the *right* column, the calmodulin binding properties are summarized for each construct. (+++) Strong binding; (−) no binding detected.

technique with anti-GST polyclonal antibodies covalently bound via amine coupling to the carboxylated dextran matrix of the sensor chip surface. We observed that the GST fusion protein bound in this way was fixed to the surface matrix in a nearly irreversible manner. Regeneration with 10 mM glycine (pH 2.2), resulted in complete dissociation of all noncovalently bound ligands, while the immobilized antibodies retained almost full binding capacity. To screen for CaM complexation of the various MAGUK fragments to GST-CaM, a single sensor chip was used to detect binding at 1 μM analyte concentration. Figure 2A represents time-dependent GST-CaM interaction analysis of MAGUK fragments containing the SH3 domain, the HOOK region, and the GK domain. The curves obtained for MPP2-1, CASK-1, and SAP97-1 show a perfect overlay, reflecting their similar kinetic behavior and molecular weight. ZO1-1, slightly smaller in size, showed different kinetic properties and gave a smaller maximal response. In contrast to the other MAGUKs, SAP90 exhibited very weak binding, consistent with a previously reported K_D of 5.55 μM for full-length SAP90 (Fukunaga et al. 2005).

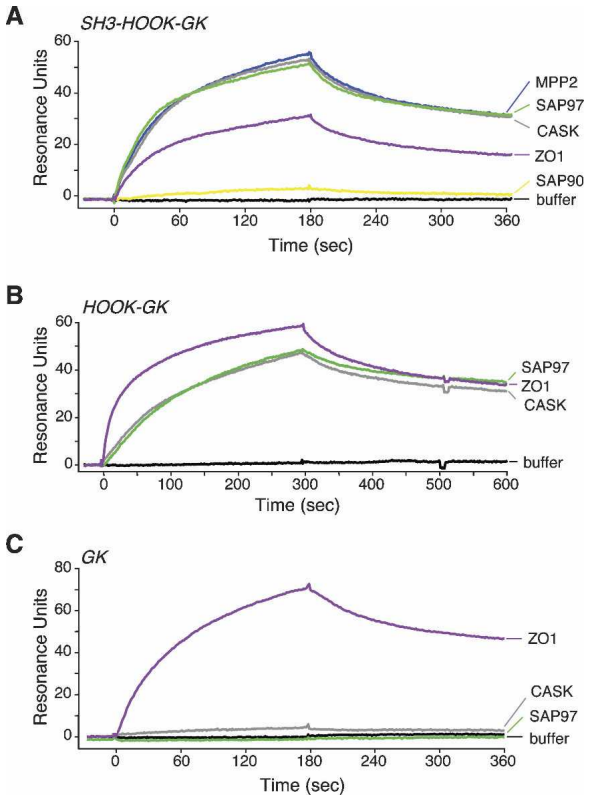

Figure 2. MAGUK domains involved in calmodulin binding. The surface plasmon resonance (SPR) technique was used to detect interaction between MAGUK constructs and CaM. Captured GST-CaM was used as ligand. All constructs were applied at 1 μM. Injection started at 0 sec and was stopped after 180 sec in *A* and *C*, and after 300 sec in *B*. The different MAGUK derivatives and the running buffer are indicated: MPP2 (blue); SAP97 (green); CASK (gray); ZO1 (violet); SAP90 (yellow); and running buffer (black). (*A*) Constructs containing SH3 and GK domains as well as the HOOK region. (*B*) Constructs containing only the HOOK region and the GK domain. (*C*) GK domains only.

Similar experiments, but using constructs lacking the SH3 module, are shown in Figure 2B. The SH3-deleted constructs of CASK and SAP97 exhibit virtually identical CaM binding kinetic properties. ZO1-2 also binds to CaM, but shows a faster association compared with CASK and SAP97. These results reveal that the SH3 domain of the analyzed MAGUK proteins is not required for the interactions with CaM. We next tested constructs carrying deletions of both the SH3 domain and the HOOK region (Fig. 2C). When being devoid of both the SH3 domain and the HOOK region, CASK and SAP97 showed no significant binding. In contrast to CASK and SAP97, the GK domain of ZO1 was still able to interact with CaM.

To obtain comparable data for all MAGUKs, we performed SPR measurements using immobilized GST-CaM and MAGUK proteins that encompassed SH3 domain, HOOK region, and GK domain. Additionally, we analyzed constructs comprising only the HOOK region and the GK domain, or the GK domain alone to study the effects of the SH3 domain and the HOOK region on CaM complexation. The results of the kinetic analyses are summarized in Table 1. The interactions were studied in real time as a function of the protein concentration. Figure 3A shows traces of the change in mass (resonance units) on the sensor chip surface upon passing increasing concentrations of CASK-1 over the antibody-fixed GST-CaM ligand. Analysis of the association phases of the sensorgrams yielded association rate constants for the SH3-HOOK-GK constructs CASK-1, MPP2-1, and ZO1-1 ranging from 1 to 5×10^4 $M^{-1}s^{-1}$, with ZO1 exhibiting an about twofold faster association compared with CASK and MPP2. The dissociation rate constants were in the range from 1.6×10^{-3} s^{-1} to 6.5×10^{-3} s^{-1}. Though ZO1 showed a faster dissociation rate, the resulting K_D is similar for all MAGUKs analyzed, giving values from 1.3 to 1.8×10^{-7} M. In CASK, deletion of the SH3 domain led to both a slower association and dissociation rate, leaving the calculated K_D nearly unaffected. In ZO1, deletion of the SH3 domain caused a three times faster association but had almost no effect on the dissociation kinetics. Overall, ZO1-2 is characterized by a more than 20-times-higher association rate constant compared with CASK-2. The only MAGUK whose GK domain alone was able to bind GST-CaM was ZO1 (construct ZO1-3). In contrast to the other MAGUK members, deletion of the HOOK region of ZO1 had only a minor kinetic effect on the interaction.

Table 1. *Kinetic parameters of CaM interaction with MAGUK constructs*

Construct	k_a (1/mMs)	k_d (1/min)	K_D (nM)
CASK-1	23.1 ± 6.6	0.23 ± 0.07	163.3 ± 7.2
CASK-2	7.1 ± 0.3	0.074 ± 0.005	176 ± 12
CASK-3	—	—	no binding
CASK-4	—	—	no binding
MPP2-1	12.2 ± 2.1	0.125 ± 0.019	175 ± 42
ZO1-1	54.8 ± 9.9	0.413 ± 0.027	129 ± 18
ZO1-2	175.8 ± 9.4	0.545 ± 0.013	51.9 ± 3.3
ZO1-3	110.3 ± 9.2	0.390 ± 0.032	59.7 ± 9.1
SAP90-1	n.d.	n.d.	weak binding
SAP97-1[a]	19.9 ± 8.3	0.16 ± 0.10	122 ± 46
SAP97-2[a]	10.8 ± 2.3	0.20 ± 0.05	334 ± 141
SAP97-3[a]	—	—	no binding

Association rate constants (k_a), dissociation rate constants (k_d), and equilibrium dissociation constants ($K_D = k_d/k_a$) are given. n.d., not determined; —, no binding detectable. Values in the table are the average of four independent series of experiments ± SEM.
[a]Data taken from Paarmann et al. (2002) are included for comparison.

MAGUK sequence motifs for CaM recognition

To understand the structural basis for the MAGUK-CaM interactions, we attempted to identify the MAGUK regions involved. The CaM binding of CASK is strictly Ca^{2+} dependent (Fig. 3B). In CASK, through analysis of deletion constructs (Fig. 3C), the CaM binding site was narrowed down to a 19-amino-acid core region. We identified a small cluster of hydrophobic amino acids and a 1-14 motif as binding motifs (Fig. 4A). The more common Ca^{2+}-dependent 1-5-10 or 1-8-14 CaM binding motifs (Rhoads and Friedberg 1997) were not observed. Remarkably, the CaM binding site contains numerous lysine residues, resulting in a net charge of +6. Not only is this high net charge found in CASK, it is also present in other members of the CASK subfamily of MAGUKs and in the related p55 subfamily (Fig. 4B). The high net charge results mainly from the protein 4.1 binding site overlapping with the CaM binding site, as most clearly seen in CASK. The stepwise deletion approach used to identify the CaM binding site in the HOOK region of CASK could not be applied to MPP2, since we were unable to purify MPP2 constructs lacking the SH3 domain. An explanation for this observation could be that the intramolecular interactions of the SH3 and the GK domains (Tavares et al. 2001) in MPP2 are very hydrophobic. An unmasked hydrophobic surface of the GK domain would undoubtedly affect the solubility and stability of the construct. Therefore, we performed a sequence analysis to detect potential CaM binding sites in MPP2. We identified a 1-8-14 type B CaM binding motif in the first helix of the HOOK region (Fig. 4C).

Taking into account the observed high affinity of CaM binding to ZO1, we confirmed this interaction and its Ca^{2+} dependence by GST-pulldown assays (Fig. 5A). Inspection of the sequence of the ZO1-GK domain allowed us to localize a putative CaM binding site from amino acid positions 735–752 (Fig. 5B) in the so-called LID region of the GK domain of ZO1. This region, which is predicted by homology with the structure of known MAGUKs (McGee et al. 2001; Tavares et al. 2001; Li et al. 2002) to be α-helical, contains an extraordinarily high net charge (+7). In fact, the homology model of the ZO1 SH3-HOOK-GK region that we generated reveals that these conserved basic residues orient in the same general direction (Fig. 5C), consistent with this helix being the CaM binding surface. Interestingly, the putative CaM binding helix is spatially close to the HOOK region, suggesting a possible

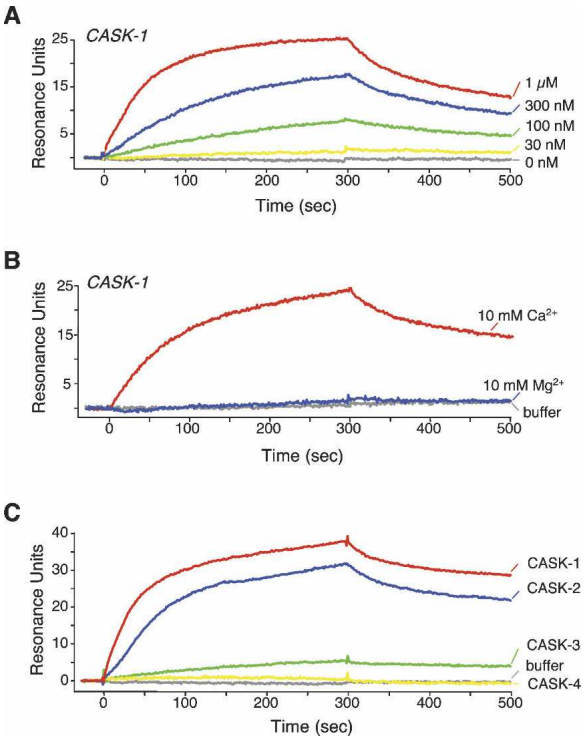

Figure 3. Detailed analysis of the interaction of CASK and calmodulin. (*A*) Determination of the dissociation constant. The interaction kinetics between calmodulin and CASK were measured in the presence of 10 mM Ca^{2+} and 0 mM Mg^{2+} by passing different concentrations of purified CASK-1 protein over immobilized GST-CaM. Injection of CASK-1 started at 0 sec and was stopped after 300 sec. The rate constants (k_a, k_d) were determined from the association and dissociation phases of the sensorgrams using a curve-fitting program for a 1:1 binding model. The equilibrium dissociation constant K_D was calculated according to $K_D = k_d/k_a$. (*B*) Ca^{2+} dependence. Calmodulin binding of CASK-1 at a concentration of 500 nM was measured in the presence of 10 mM $CaCl_2$ or 10 mM $MgCl_2$. Injection was performed as described in *A*. (*C*) Localization of the calmodulin binding site. Individual CASK constructs as indicated were analyzed under identical conditions. Injection of 1 μM of each protein started at 0 sec and was stopped after 300 sec.

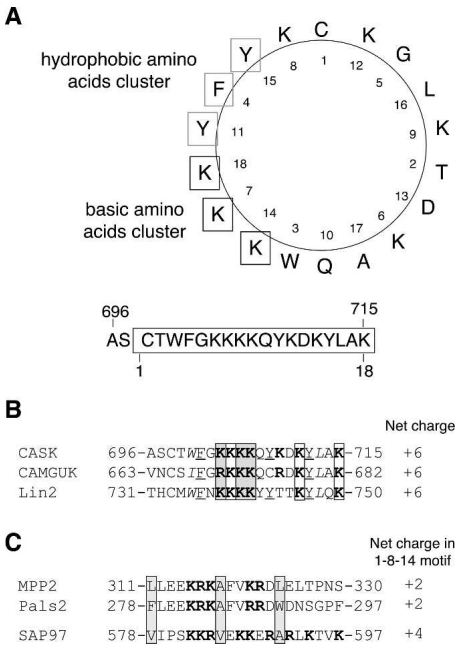

Figure 4. Primary structure analysis of the CaM binding site of CASK. (*A*) Helical wheel representation of 18 residues of the CASK calmodulin binding region. The numbers within the circular plot indicate the positions of the residues in the linear sequence shown *below*. Hydrophobic residues as well as basic amino acids that cluster to one side of the wheel are boxed in gray and black, respectively. *Below* is shown the complete sequence that is crucial for CaM binding according to the interaction analysis of CaM with CASK deletion constructs (Fig. 3B). The part depicted as a helical wheel is boxed. (*B*) Alignment of the CaM binding site of CASK with homologous regions of other members of the CASK subfamily. Hydrophobic cluster, characters underlined; 1-14 motif, italic; basic amino acids, characters in bold; basic cluster, white boxes; and protein 4.1 binding site, gray boxes. The *right* column indicates the net charge of the region between the first and the last amino acid. (*C*) Alignment of putative CaM binding sites in the two p55 subfamily members, MPP2 and Pals2, in comparison with the previously identified site in SAP97. The protein 4.1 binding site and the basic amino acids are indicated as in *B*. The hydrophobic amino acids of the 1-8-14 motif in MPP2 are shown in light gray.

role for the HOOK region in regulating CaM binding. In contrast, in the homologous regions of CASK and SAP97, which do not contain a CaM binding site, such a high net charge is not found (Fig. 5D), and no hydrophobic binding motif could be identified in these regions.

Discussion

For understanding macromolecular complex formation in the cytoskeletal framework, it is essential to analyze the underlying structural features and regulatory mechanisms. CaM, a prototypical calcium sensor protein (Chin and Means 2000), is purported to be an ideal molecule for modulating the interactions of MAGUKs with other cytoskeletal components (Masuko et al. 1999; Paarmann et al. 2002). However, little is known so far about the effect of CaM on MAGUKs. Therefore, in the present study, we investigated the binding of Ca^{2+}-CaM to members of all MAGUK subfamilies.

A CaM binding site of CASK is located in the HOOK region

We noted that the CASK site that interacts with Ca^{2+}-CaM lies in a portion of the HOOK region connecting the SH3 and GK domains. By representing this binding site as a helical wheel (see Fig. 4A) and comparing it to SAP97 and SAP102 (Masuko et al. 1999; Paarmann et al. 2002), a smaller basic amino acid cluster shows up. However, in addition to the basic amino acid cluster, a small cluster of hydrophobic amino acids is found. It is worth mentioning that this region contains numerous lysine residues due to its overlap with the protein 4.1 binding site, resulting in a high net charge. In fact, a high net charge has been cited as a typical feature of CaM binding sites (Stevenson and Calderwood 1990; Vorherr et al. 1993; Rhoads and Friedberg 1997). Apart from the 1-5-10 and 1-8-14 motifs, more divergent Ca^{2+}-dependent CaM binding motifs do exist (Rhoads and Friedberg 1997). Among these motifs are 1-5-16 (CaM kinase kinase) and 1-5-8 (Ca^{2+}-pump) anchor residues (Vetter and Leclerc 2003). In CASK, a 1-14 motif is present (see Fig. 4B).

Putative CaM site in the HOOK region of MPP2

CaM is known to bind positively charged amphiphilic helices with high affinity (O'Neil and DeGrado 1990; Strynadka and James 1990), but net charge, as well as hydrophobic residues at certain positions are also important (Rhoads and Friedberg 1997). Applying these criteria to MPP2, we identified a Ca^{2+}-dependent 1-8-14 type B motif in the first helix (amino acids 311–327) of the HOOK region, which shows similarity to SAP97. Pals2/VAM-1 (Kamberov et al. 2000; Tseng et al. 2001) has a very high homology with MPP2 and can therefore be predicted to bind CaM in a similar manner (see Fig. 4B). However, in the other members of the p55 subfamily, the sequence similarity to the HOOK region of MPP2 is much weaker, though a high net charge in the region between the first helix of the HOOK region and the GK domain is conserved, similar to the CASK subfamily. In contrast to CASK, hydrophobic binding motifs can hardly be identified in this region of p55 subfamily members. Also, this part of the HOOK region, due to its high positive charge, may contribute to CaM binding to MPP2.

CaM site in the GK domain of ZO1

Using SPR, CaM binding to the GK domain was observed. Manual inspection of the amino acid sequence revealed a putative CaM binding site. Analysis of this site (Fig. 5C) by

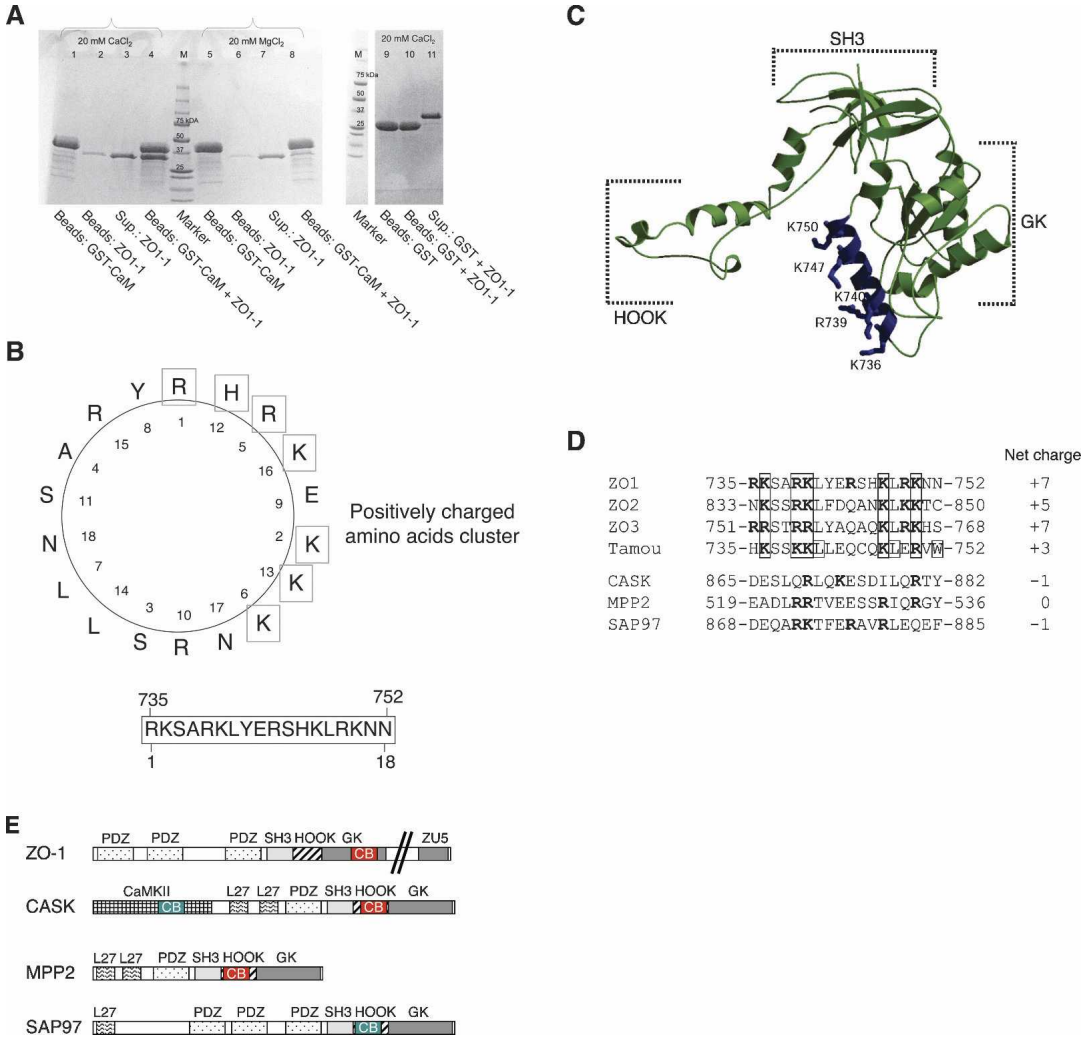

Figure 5. Analysis of the putative CaM binding site of ZO1. (*A*) The interaction between ZO1 and CaM has also been detected by performing pulldown assays. GST-CaM (9.3 nmole) was bound to glutathione sepharose beads in a buffer containing $CaCl_2$ (lanes *1–4*) or $MgCl_2$ (lanes *5–8*). ZO1-1 was added to the washed beads (10 nmole) and left to incubate for 30 min. After three washes to eliminate unbound protein, the beads were applied onto a SDS-PAGE gel. Incubation in the magnesium-containing buffer shows no or very little ZO1-1 bound to GST-CaM (lane *8*). On the other hand, ZO1-1 is observed in the beads fraction when the incubation was done in a calcium-containing buffer (lane *4*). Lanes *1* and *5* are control samples with only GST-CaM. To verify that ZO1-1 does not bind by itself to the glutathione sepharose beads, the protein was incubated with the beads and then washed as was done in the experiments containing GST-CaM. No significant ZO1-1 binding to the beads is observed (lanes *2*,*6*), and most of the protein is in the supernatant (lanes *3*,*7*). The GST alone controls, in the presence of $CaCl_2$, show pure GST protein bound to the beads (lane *9*) and no binding of the ZO1-1 construct (lane *10*); the latter is found only in the supernatant of this control binding experiment (lane *11*). (*B*) Helical wheel representation of 18 residues of the putative calmodulin binding region of ZO1. The numbers within the circular plot indicate the positions of the residues in the linear sequence shown *below*. The positively charged amino acids that cluster to one side of the wheel are boxed in gray. (*C*) Homology model of the SH3-HOOK-GK domains of ZO1 based on the PSD-95 crystal structure (PDB ID 1KJW). The protein is represented as a ribbon diagram in green, with the putative calmodulin binding helix in blue. Conserved basic residues that are boxed in *C* are labeled. Note that these residues all point in the same general direction, consistent with a role in calmodulin binding. (*D*) Alignment of the CaM binding site of ZO1 with homologous regions of other members of the ZO1 subfamily and members of other subfamilies. The conserved hydrophilic residues, forming a positively charged cluster, are boxed. The hydrophobic binding motif of Tamou is indicated in gray. Basic amino acids are depicted in bold letters. (*E*) Domain structure of full-length MAGUK proteins. Known CaM binding sites are indicated in green, putative CaM binding sites identified in this study are depicted in red. CB indicates CaM binding site. HOOK region and ZO-1 are not drawn to scale. ZU5 indicates domain of unknown function present in ZO-1 and Unc5-like netrin receptors (Cirulli and Yebra 2007).

referring to the helical wheel representation reveals an almost perfect positively charged helix, although a hydrophobic binding motif is missing. This is in agreement with the three orders of magnitude weaker binding compared to a perfect amphiphilic helix (O'Neil and DeGrado 1990). A cluster of basic amino acids is conserved in all members of the ZO1 subfamily. Tamou, having a smaller net charge, contains an additional small cluster of hydrophobic residues, which may compensate for the smaller net charge.

According to the crystal structure of the SH3-GK domain of SAP90 (McGee et al. 2001), the HOOK region, being similar in length for ZO1 and SAP90 (Tavares et al. 2001), appears to be in close proximity to the predicted binding helix, thereby hindering CaM binding. Homology modeling indicates that the basic amino acids of the predicted binding helix can indeed be accessed by other proteins (Fig. 5D). In the absence of the SH3 domain, the HOOK region becomes more flexible and therefore interferes to a lesser extent with CaM binding. Therefore, the predicted binding helix is in agreement with our observation that deletion of the SH3 domain of ZO1 leads to a faster association of ZO1 and CaM.

CaM binding as a new common feature of MAGUKs

Our work highlights CaM binding as a novel common feature of the MAGUK family. Whereas previous reports on CaM binding were restricted to the SAP97 subfamily (Masuko et al. 1999; Paarmann et al. 2002) and to the CaMKII domain of CASK (Hata et al. 1996), we identified CaM binding sites in all MAGUK subfamilies (see Fig. 5E). Despite the fact that the CaM binding sites of SAP97 and CASK are located in the HOOK region, they are not found at homologous positions and exhibit no significant sequence similarity. Most strikingly, in ZO1, the CaM binding site is located in a different region, namely, the GK domain. Therefore, it appears that CaM binding sites have evolved independently in at least three MAGUK subfamilies. This may be a strong indication for the critical importance of CaM binding to MAGUKs.

MPP2 is likely to bind to CaM in a way similar to skeletal muscle myosin light chain kinase (Ikura et al. 1992), as suggested by the 1-8-14 motif. CASK contains a hydrophobic 1-14 motif. For both MPP2 and CASK, CaM presumably adopts a collapsed conformation (Vetter and Leclerc 2003). ZO1, however, does not contain these hydrophobic anchor residues. As a consequence, it is not clear if CaM binds in the collapsed conformation or the extended conformation that was found for the potassium channel (Schumacher et al. 2001) and anthrax exotoxin (Drum et al. 2000).

Potential functional role of CaM binding

One well-known mechanism for dynamic regulation of MAGUK function is phosphorylation (Gardoni et al. 2006; Mauceri et al. 2007; for review, see Funke et al. 2005). Phosphorylation of MAGUKs can regulate their synaptic localization, as well as clustering and trafficking of ion channels. Our work suggests that CaM binding represents a second mechanism for dynamic regulation of MAGUK function, CaM being a prototypical calcium sensor protein (Chin and Means 2000). In SAP102, CaM binding is thought to be involved in dimerization with SAP90 (Masuko et al. 1999). The HOOK regions of SAP97, CASK, and p55 contain protein 4.1 binding sites (Lue et al. 1994; Marfatia et al. 1995; Cohen et al. 1998). The CaM binding site of SAP97 is located next to its protein 4.1 binding site (Paarmann et al. 2002), as well as the putative CaM binding site in MPP2, whereas the CaM site of CASK completely overlaps with the protein 4.1 binding site (see Fig. 4B). This suggests that CaM binding competitively interferes with protein 4.1 complexation, implying a modulatory role of CaM in the association of these MAGUKs with the cytoskeleton. In contrast to CASK and SAP97, the binding site in ZO1 is located in the GK domain. Therefore, CaM binding might interfere with binding partners of the GK domain of ZO1 such as occludin (Fanning et al. 1998; Schmidt et al. 2001) which has a 10 times weaker affinity to the GK domain compared with CaM, and α-catenin (Müller et al. 2005). Moreover, the binding sites of occludin and α-catenin on ZO1 (Schmidt et al. 2004; Müller et al. 2005) overlap with the proposed CaM binding site. Therefore, Ca^{2+}-mediated CaM binding to MAGUKs may serve as a general mechanism to modulate their manifold interactions with the cytoskeleton.

Materials and Methods

SPR measurements

The kinetics of association and dissociation reactions of CaM and MAGUK fragments were performed by SPR spectroscopy using a BIAcore 2000 system (BIAcore AB), essentially as described previously (Paarmann et al. 2002). All of the reagents such as amine coupling kit, surfactant P-20, and the CM-5 sensor chip were purchased from BIAcore AB. Four flow cells were placed in a CM-5 sensor chip surface. Using a GST fusion capture kit (BIAcore), anti-GST antibody was immobilized on all flow cells according to the manufacturer's instructions using the amine coupling kit. GST-tagged CaM was then captured in one flow cell. GST protein alone was captured in a different flow cell, which served for online reference subtraction. The running buffer for all experiments, except for determination of the Ca^{2+} dependence of CaM binding, was composed of 10 mM $CaCl_2$, 10 mM dithiothreitol, 10 mM HEPES pH 7.4, 0.15 M NaCl, 3.4 mM EDTA, and 0.05% surfactant P-20. To demonstrate Ca^{2+} dependence, $CaCl_2$ was replaced by 10 mM $MgCl_2$ in the same buffer. Captured GST-CaM was regenerated in 1 M NaCl. Anti-GST antibody on the CM5 surface was regenerated according to the method of the GST fusion kit manual using 1-min pulses of 10 mM glycine, pH 2.2, at flow rates of 10 μL/min.

All solutions used for SPR measurements were filtered (0.22 μm) and degassed.

To determine rate constants of association and dissociation, kinetic measurements were performed at a flow rate of 50 μL/min and adjusting the immobilization level of captured GST-CaM to 300–500 resonance units. All curves were fitted assuming a one-to-one binding model using BIAevaluation 3.1 software. For a review of kinetic analyses using the SPR technique, see Karlsson and Falt (1997).

Homology modeling

Homology modeling of the human ZO1 SH3-GK domain was done using the manual mode in SwissModel (Schwede et al. 2003). The template used was the PSD-95 crystal structure (PDB ID 1KJW). Sequence comparison with multiple MAGUK proteins guided the alignment between ZO1 and PSD-95. Since the HOOK region is missing in the reference structure, this is the region with the highest structural uncertainty in the ZO1 homology model.

GST pulldown

Purified GST-CaM was dialyzed against a solution containing EDTA in order to obtain the calcium-free form. This allowed us to subsequently add either calcium or magnesium and therefore test the effect of these cations on binding to ZO1. For the pulldown assay, GST-beads (~100 μL) were incubated for 15 min at room temperature with GST-CaM (9.3 nmole) in a buffer containing 150 mM NaCl, 10 mM HEPES pH 7.5, and 20 mM of either $CaCl_2$ or $MgCl_2$. After washing to remove any unbound protein, the GST-CaM loaded beads were incubated with 10 nmole of SH3-GK region of ZO1 (designated ZO1-1) for 30 min. Unbound ZO1-1 was removed by three consecutive washes. SDS gel loading buffer was added to the beads, the sample boiled for 7 min, and then resolved by SDS-PAGE.

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