Structural Basis for Nucleotide-dependent Regulation of Membrane-associated Guanylate Kinase-like Domains*

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CASK is a member of the membrane-associated guanylate kinases (MAGUK) homologs, a family of proteins that scaffold protein complexes at particular regions of the plasma membrane by utilizing multiple protein-binding domains. The GK domain of MAGUKs, which shares high similarity in amino acid sequence with yeast guanylate kinase (yGMPK), is the least characterized MAGUK domain both in structure and function. In addition to its scaffolding function, the GK domain of hCASK has been shown to be involved in transcription regulation. Here we report the crystal structure of the GK domain of human CASK (hCASK-GK) at 1.3-Å resolution. The structure rationalizes the inability of the GK domain to catalyze phosphoryl transfer and strongly supports its new function as a protein-binding module. Comparison of the hCASK-GK structure with the available crystal structures of yGMPK provides insight into possible conformational changes that occur in hCASK upon GMP binding. These conformational changes may act to regulate hCASK-GK function in a nucleotide-dependent manner.

Membrane-associated guanylate kinases (MAGUKs) constitute a newly recognized class of proteins found in all animals examined to date. They act as molecular scaffolds for signaling pathway components, regulate synaptic structure and function by mediating specific interactions, and (in Drosophila) act as tumor suppressors. In addition, MAGUKs recruit molecules into localized multimolecular complexes and cluster these complexes at the plasma membrane, such as cell junctions or the apical or basolateral surface, and pre- or post-synaptic sites (1–6). All MAGUK proteins share a similar multidomain structural organization that includes (from the N to C termini) one or three copies of PDZ (PSD-95/SAP-90-Dlg-Zo1) domains, followed by one Src homology 3 (SH3) domain, then followed by a guanylate kinase-like (GK) domain (3). The PDZ domain functions as a protein-protein interaction module and is typically involved in the assembly of supramolecular complexes that perform localized signaling functions at particular subcellular locations (7). Although their specific functions in MAGUKs are not very clear, SH3 domains are found in many other proteins and are recognized as modular protein-protein interaction domains with an affinity for certain proline-rich motifs (8).

The GK domain in MAGUKs shares high sequence similarity with yeast guanylate kinase (yGMPK), a nucleoside monophosphate kinase that converts GMP to GDP using ATP as a phosphate donor. However, no enzymatic activity is present in MAGUK GK domains. Moreover, several protein binding partners of GK domains were found by yeast two-hybrid screens or pull-down assays (see Table III below), and this suggested that the GK domain functions as a protein-binding module. Although the GK domain cannot catalyze phosphoryl transfer, it can bind nucleotides. Binding studies have shown that the GK domain of SAP-90 binds GMP in the micromolar range and ATP in the millimolar range (9). Although a GK domain is present in all MAGUKs, there is remarkable divergence in nucleotide-binding sites among the four MAGUK subfamilies (see Fig. 1 below): Lin2/CASK- and p55-like MAGUKs have nearly intact GMP- and ATP-binding sites; Dlg/SAP-90- and SAP-97-like MAGUKs have conserved GMP-binding sites but lack some residues in their ATP-binding site; the ZO1-like MAGUKs lack some residues in both sites (3). The role of nucleotide binding to the GK domain is unclear in the absence of any enzymatic activity. Based on known nucleotide-dependent conformational changes that were observed in several nucleoside monophosphate kinases, we hypothesized that nucleotides could play a regulatory role in GK function by inducing different conformations between the nucleotide-free and nucleotide-bound forms.

To test this hypothesis we commenced crystallographic studies of GK domains from different MAGUKs. Here we report the 1.3-Å crystal structure of the GK domain of human CASK (hCASK-GK). Comparison of this structure, which is the first MAGUK GK domain elucidated to our knowledge, with the structures of yGMPK with and without GMP reveals important differences in the conformation of the GMP-binding region and the LID region. The structure reveals the reasons hCASK-GK is not able to catalyze phosphoryl transfer. Modeling of GMP into hCASK-GK strongly supports the binding of GMP to hCASK-GK. The hCASK-GK crystal structure and the hCASK-GK-GMP model here reveal how an enzyme has been converted into hCASK-GK strongly supports the binding of GMP to

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‡ The abbreviations used are: MAGUK, membrane-associated guanylate kinase; PDZ, PSD-95/SAP-90-Dlg-Zo1 domain; SH3, Src homology 3; GK, guanylate kinase-like domain; yGMPK, yeast guanylate kinase; hCASK-GK, GK domain of human CASK; r.m.s.d., root mean square deviation; MAD, multil wavelength anomalous diffraction method.
into a protein-binding module and suggest how binding to GMP could regulate the conformational state of this module within multidomain proteins.

EXPERIMENTAL PROCEDURES

Cloning and Recombinant Expression of hCASK-GK—A cDNA encoding the GK domain from human CASK protein (hCASK-GK, Arg-734 to Cys-909) was amplified by the polymerase chain reaction from a pCRII plasmid (Invitrogen Corp.), containing a full-length cDNA of hCASK (10), and cloned into a modified pGEX-2T (Amersham Biosciences, Inc.) E. coli expression vector, which allowed insertion of NdeI/BamHI-restricted fragments into multiple cloning sites of the vector.

Protein Expression and Purification—Escherichia coli BL21(DE3) cells carrying the plasmid that contains the gene encoding the hCASK-GK-GST fusion protein were induced by 0.1 mM isopropyl-β-D-thiogalactopyranoside for 6 h at 25 °C in 2 mM media. Cells were harvested by centrifugation and lysed with lysozyme, and the supernatant after ultracentrifugation was applied to a glutathione S-transferase-Sepharose column pre-equilibrated with 50 mM Tris-HCl, pH 7.5, 200 mM KCl, 5 mM MgCl₂, 1 mM EDTA, and 10 mM dithiothreitol. After washing, the fusion protein was cleaved on the column with thrombin for about 24 h.

The flow-through was collected and further purified to homogeneity by an ion exchange column (HP-WH-QUAT) and gel filtration (Superdex-75). The protein was concentrated by ultrafiltration to 25 mg/ml. Because of the construction of a thrombin cleavage site at the N-terminal of hCASK-GK, the resultant protein after cleavage has four additional amino acids (Gly-Ser-His-Met) at the N terminus. For seleno-methionine-containing protein, the plasmid was transformed into the methionine auxotroph E. coli strain B834(DE3), and the cells were grown and induced as described previously (12). Purification was conducted as with the non-labeled protein. Incorporation of seleno-methionine at all four positions was confirmed by mass spectrometry.

Crystals were obtained by the hanging-drop method at 20 °C. One microliter of 4 mM sodium formate (the reservoir solution) was mixed with an equal volume of 15 mg/ml of the protein in 50 mM Tris-HCl, pH 7.5, 200 mM KCl, 5 mM MgCl₂, 1 mM EDTA, and 10 mM dithiothreitol. Initial crystals were found after 2 weeks. Further crystals were obtained with microseeding. Trigonal crystals appeared overnight and grew to a maximum size of 350 × 350 × 400 μm in 1 week. The Matthews coefficient of 2.73 (55% solvent content) suggested a monoclinic or triclinic symmetry, and it diffracted to 1.7 Å. In addition, a native dataset at 1.31 Å resolution was collected. The data were processed with DENZO/SCALEPACK (14).

The crystal structure of hCASK-GK revealed a trimer with the crystallographic 6-fold symmetry axis. In each monomer, the active site GMPK GMP structure, we could align the two structures once based on very high quality, and the program ARP was successful in automatically building most of the hCASK-GK model (Fig. 3). The glycine-rich ATP binding motif and residues responsible for binding of guanine ring (·), phosphates (§), and Mg²⁺ binding motif of GMP, and for binding of Mg²⁺(+) are boxed as well as the residues involved in catalysis (Arg-135, Arg-146, and Asn-168 in yGMPK, §, and the residue responsible for ATP binding (Arg-131).

RESULTS

Structure Determination of hCASK-GK—Human CASK-GK (Fig. 1) crystallizes in the hexagonal space group P321 with one molecule in the asymmetric unit. The structure (Fig. 2) was solved using the multiwavelength anomalous dispersion method (MAD) (21). Data collection and phasing statistics can be found in Table I. The experimental electron density map was of very high quality, and the program ARP was successful in automatically building most of the hCASK-GK model (Fig. 3). After manual rebuilding with the 1.7 Å MAD dataset, the res-
The core region of hCASK-GK is shown in Fig. 3. The CORE region of hCASK-GK is built by five parallel \( \beta \)-strands and four \( \alpha \)-helices. This region is flanked by the GMP-binding region (red) and the LID region (blue). The former is composed of a four-stranded \( \beta \)-sheet and a single \( \alpha \)-helix, whereas the LID is seven residues shorter than in yGMPK and is composed of a long loop and one \( \alpha \)-helix. The division of hCASK-GK into these three regions was guided by comparing our structure to known structures of yGMPK such that each region can move as a rigid body relative to the other regions. Therefore, our choice of regions is slightly different than those previously chosen for yGMPK, especially for the LID region (22). We have chosen helix a-5 to be a part of the LID region and not the CORE region, because it adopts a quite different conformation and position in hCASK from that in yGMPK.

Comparison between the Structures of hCASK-GK and yGMPK—Comparison of the hCASK-GK structure with the yeast guanylate kinase (yGMPK) and, in a similar manner to yGMPK, especially for the LID region (22). We have chosen helix a-5 to be a part of the LID region and not the CORE region, because it adopts a quite different conformation and position in hCASK from that in yGMPK.

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Overall Structure—The fold of hCASK-GK is similar to that of the yeast guanylate kinase (yGMPK) and, in a similar manner to that of yGMPK, especially for the LID region (22). We have chosen helix a-5 to be a part of the LID region and not the CORE region, because it adopts a quite different conformation and position in hCASK from that in yGMPK.

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residues 774–776 of hCASK-GK. Lysine 774, which is not present in our model, would correspond to Arg-41 of γGMPK. Our inability to model the above loop is consistent with the expectation that this loop would become more ordered once GMP binds to hCASK-GK. Fig. 5B shows the superposition of the residues responsible for GMP binding in GMPK<sub>GMP</sub> and in our hCASK-GK-GMP model. The expected interactions between GMP and hCASK-GK are listed in Table II.

**DISCUSSION**

An impressive amount of information on the structural and functional networks that underlie neuronal activity has recently...
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Several protein binding partners of GK domains were found by yeast two-hybrid screens or pull-down assays (Table III), and this suggested that the GK domain functions as a protein binding module. Remarkably, several MAGUKs and interacting proteins have additional, recently discovered biological functions: GAKIN is a member of the kinesin superfamily of motor proteins, and its interaction with the GK domain seems to play a vital role in the intracellular trafficking of hDlg (28). CASK, a member of the Lin2-like MAGUKs, has a transcription regulation function through interaction of its GK domain with Tbr-1, a T-box transcription factor that is involved in forebrain development (29). The GK domain is also involved in inter- and intramolecular interaction with MAGUK’s SH3 domain, and this intramolecular interaction regulates SAP-97 binding to GKAP and PSD-95/SAP-90 clustering of ion channels (30–33). In addition, it is known that the Drosophila Dlg protein, the prototype member of the MAGUK family, is required in the maintenance of septate junctions and epithelial structure; mutations in the SH3 and GK domains of the protein are oncogenic in larval development, and deletions in the GK domain cause loss of normal growth without affecting epithelial structure (34–36). Similarly, the GK domains of murine Dlg/SAP-97 and of CASK are essential to craniofacial and palatal morphogenesis in mice (37). Because these interactions and functions were shown to be restricted to the GK domains of the respective MAGUK subfamilies, it can be assumed that their specificity is determined by subtle differences in GK domain structures. The high resolution structure of the CASK GK domain we present in this study will allow gains insights into the molecular basis of the distinct mechanisms for the interactions between the GK domains and their binding partners.

Despite having only 27% sequence identity with yGMPK, the structure of hCASK-GK shows that the CORE and GMP-binding regions are markedly similar. The most apparent difference between the two proteins is the LID region, a region that in the enzyme yGMPK contains essential residues for catalysis. Similarly, the essential role of particular arginine residues from the LID region was highlighted in our previous work on thymidylate kinase (11, 38). LID region yGMPK arginines play a role in ATP binding (Arg-131) and in stabilizing the phosphate groups of ATP in the catalytic transition state (Arg-135 and Arg-146). None of the MAGUKs retain all of these three arginines (Fig. 1). The LID region of hCASK-GK has only a single arginine (Arg-869), which the structure-based sequence alignment suggests could correspond to the catalytic Arg-146 of yGMPK. However, the structure of hCASK-GK reveals that this arginine extends toward a totally different direction from that of Arg-146 observed in the structures of yGMPK. This is unlikely due to the lack of nucleotide in our hCASK-GK structure because this residue adopts a similar orientation in both the apo- and GMP-bound yGMPK structures. This shows that the LID region of hCASK-GK is deficient in residues essential for catalyzing phosphoryl transfer. Importantly, our structures show no other residues from other parts of the molecule that could compensate for the lack of these arginine residues. Therefore, our structural analyses yield an explanation for the lack of guanylate kinase activity of the GK domain of MAGUKs.

Although it is now understood why the GK domain cannot catalyze phosphoryl transfer, the question remains: Can it bind ATP or GMP? Our equilibrium binding assays show that hCASK-GK binds GMP with a dissociation constant of 0.3 μM but binds ATP with a dissociation constant greater than 10 mM.2 These results are consistent with our structure analyses that

![Diagram](image_url)
explain the low affinity for ATP. Although hCASK-GK has a nearly complete conserved P-loop motif, several facts suggest that the P-loop is not competent to bind ATP. First, the strictly conserved lysine of the P-loop motif is absent. In hCASK, this residue is an arginine. Alignment of all known guanylate kinase sequences reveals strict conservation of the P-loop lysine. Second, the serine/threonine that follows the lysine in the P-loop motif is replaced by an arginine residue in hCASK. The role of a serine or threonine residue of the P-loop sequence is to participate in ATP binding by donating its hydroxyl side chain as a ligand to the magnesium ion that invariably accompanies ATP. The replacement of such a residue with an arginine in hCASK precludes the ability of coordinating the magnesium ion. But perhaps more importantly, modeling of ATP in the hCASK-GK model (data not shown) reveals that the presence of the larger arginine side chain is not compatible with ATP binding. Third, hCASK-GK does not possess the arginine that would correspond to Arg-131 of yGMPK in the LID region. The function of this residue in yGMPK (and many other ATP-binding proteins) is to bind and position ATP by stacking against the adenine ring. Lastly, main-chain residues 891 and 892 from the CORE region of hCASK-GK, which correspond to residues 169 and 170 in yGMPK and participate in ATP binding, are not at the appropriate position to contribute to ATP binding. Although a conformational change of these residues to a

**FIG. 5.** Modeling of GMP into the hCASK-GK structure. A, stereoview of the GMPK<sub>GMP</sub> (cyan) structure overlaid with the model GMP-bound hCASK-GK (red and purple). For details on model generation see the "Experimental Procedures." The black arrow shows the expected conformational change that would occur in hCASK-GK upon GMP binding. B, superposition of the GMP-binding sites in the GMP-bound model of hCASK-GK (pink) and GMPK<sub>GMP</sub> (cyan). The residues of hCASK-GK are labeled. See also Table II.

**TABLE II**

<table>
<thead>
<tr>
<th>Nucleotide part of GMP making the interaction</th>
<th>Residue in GMPK&lt;sub&gt;GMP&lt;/sub&gt;</th>
<th>Corresponding residue in the hCASK-GK-GMP model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanine ring</td>
<td>Glu&lt;sup&gt;69&lt;/sup&gt;</td>
<td>Glu&lt;sup&gt;802&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Tyr&lt;sup&gt;78α&lt;/sup&gt;</td>
<td>Tyr&lt;sup&gt;810α&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Ser&lt;sup&gt;80&lt;/sup&gt;</td>
<td>Thr&lt;sup&gt;813&lt;/sup&gt;</td>
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<td></td>
<td>Ile&lt;sup&gt;88α&lt;/sup&gt;</td>
<td>Val&lt;sup&gt;83β&lt;/sup&gt;</td>
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<td>Asp&lt;sup&gt;100&lt;/sup&gt;</td>
<td>Glu&lt;sup&gt;833&lt;/sup&gt;</td>
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<td>Ser&lt;sup&gt;84&lt;/sup&gt;</td>
<td>Ser&lt;sup&gt;84&lt;/sup&gt;</td>
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<tr>
<td>Phosphate</td>
<td>Tyr&lt;sup&gt;70&lt;/sup&gt;</td>
<td>Tyr&lt;sup&gt;78&lt;/sup&gt;</td>
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<tr>
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<tr>
<td></td>
<td>Arg&lt;sup&gt;28&lt;/sup&gt;</td>
<td>Arg&lt;sup&gt;771&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Arg&lt;sup&gt;α11&lt;/sup&gt;</td>
<td>No electron density for Lys&lt;sup&gt;774&lt;/sup&gt;</td>
</tr>
<tr>
<td>Indirect interactions</td>
<td>Glu&lt;sup&gt;44&lt;/sup&gt; with Arg&lt;sup&gt;28&lt;/sup&gt;</td>
<td>Glu&lt;sup&gt;777&lt;/sup&gt; with Arg&lt;sup&gt;771&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*These residues made a stacking interaction (3.9 Å) with the adenine ring.*
more productive position for ATP interactions cannot be ruled out, this is unlikely because these residues are in the CORE region, which is rather rigid as exemplified by our comparison to yGMPK (see “Results”). Collectively, the above analysis presents a strong case against the ability of the P-loop sequence of hCASK to act as a true P-loop and bind ATP. Thus, the low affinity binding of ATP to hCASK-GK can only be explained by ATP binding to the GMP-binding site.

Because hCASK-GK has an almost conserved GMP-binding site and it does bind GMP in equilibrium binding assays, we modeled GMP into the structure of hCASK-GK after a movement of the GMP-binding region based on the structure alignment between hCASK-GK and GMPKBlosoya_21, 912–921

**Basis for Regulation of Guanylate Kinase-like Domains**

**TABLE III**

<table>
<thead>
<tr>
<th>Binding partner</th>
<th>MAGUK</th>
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<tr>
<td>Rabphilin3a</td>
<td>hCASK</td>
</tr>
<tr>
<td>Thr-1</td>
<td>CASK (rat)</td>
</tr>
<tr>
<td>SPAR</td>
<td>PSD95</td>
</tr>
<tr>
<td>GKAps, SAPAps,</td>
<td>PSD95, hDg</td>
</tr>
<tr>
<td>GAP41</td>
<td>HDig</td>
</tr>
<tr>
<td>BEGAIN</td>
<td>PSD95/SAP90</td>
</tr>
<tr>
<td>MAP1A</td>
<td>PSD-83</td>
</tr>
<tr>
<td>Occludin</td>
<td>ZO-1 (human)</td>
</tr>
<tr>
<td>SH3 domain of Dg</td>
<td>hCASK</td>
</tr>
<tr>
<td>SH3 domain of hCASK, PSD95, SAP97</td>
<td>hCASK, PSD95, SAP97 (intramolecular interaction)</td>
</tr>
</tbody>
</table>

**REFERENCES**


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