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The Molecular Basis of the Caskin1 and Mint1 Interaction with CASK

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Abstract

CASK is a conserved multi-domain scaffolding protein involved in brain development, synapse formation, and the establishment of cell polarity. To accomplish these diverse functions CASK participates in numerous protein-protein interactions. In particular, CASK forms competing CASK/Mint1/Velis and CASK/Caskin1/Velis tripartite complexes that physically associate with the cytoplasmic tail of neurexin, a transmembrane protein enriched at presynaptic sites. This study shows that a short, linear EEIWVLRK peptide motif from Caskin1 is necessary and sufficient for binding CASK. We also identified the conserved binding site for the peptide on the CASK CaM kinase domain. A related EPIWVMRQ peptide from Mint1 was also discovered to be sufficient for binding. Searching all human proteins for the Mint1/Caskin1 consensus peptide ExIWVxR revealed that TIAM1 contains a conserved EEVIWVRRE peptide that was also found to be sufficient for CASK binding *in vitro*. TIAM1 is well-known for its role in tumor metastasis, but it also possesses overlapping cellular and neurological functions with CASK suggesting a previously unknown cooperation between the two proteins. This new peptide interaction motif also explains how Caskin1 and Mint1 form competing complexes and suggests a new role for the cellular hub protein CASK.

Keywords

peptide-protein interaction; peptide motif; TIAM1; calmodulin kinase; scaffolding protein

Introduction

Calcium/calmodulin-dependent serine protein kinase, or CASK, is a conserved membrane-associated guanylate kinase (MAGUK) protein involved in synapse formation, brain development, and the establishment of cell polarity.[1] CASK was originally discovered as a protein that binds to the C-terminal tail of neurexin, a transmembrane protein enriched at neuronal presynaptic sites.[2] At the same time, mutations in the *C. elegans* CASK homolog, Lin-2, were found to affect vulval development.[3] CASK gene deletion experiments in mice lead to perinatal death suggesting that it is an essential gene in development.[4] This developmental role has been partly rationalized by the fact that CASK localizes to cell

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nuclei and affects gene transcription.[5,1] CASK is primarily known for its function as a neuronal scaffolding protein, however, where it localizes to both pre- and post-synaptic sites and organizes various proteins important for synaptic function.[1,6] In humans, many different CASK mutations lead to mental retardation,[7,8,9,10,11] but the molecular mechanisms are mostly unknown.[6]

Human CASK contains many conserved domains including a calmodulin kinase (CaMK) domain, two tandem Lin-2/Lin-7 (L27) domains, an SH3 domain, a PDZ domain, a poly-lysine Hook motif and a guanylate kinase (GK) domain (Fig. 1). The CaMK domain was originally thought to be an inactive pseudokinase since it contains mutations to the DFG motif, a Mg^{2+} binding sequence thought to be essential for catalysis.[2] Accordingly, the CaMK domain was postulated to have evolved exclusively into a protein-protein interaction domain.[1] This hypothesis was bolstered by the discovery of several proteins that interact directly with the CaMK domain including calmodulin,[2] Mint1/X11α/Lin-10,[12,13] liprin-α2,[14] Caskin1,[15] and carom.[16] Additionally, the GK domain is thought to be catalytically inactive[17] and many other interacting partners have been identified including MALS/Velis/Lin-7,[12,13] CIP98,[18] syndecan,[19] CINAP,[20] parkin,[21] SynCAM,[22] Tbr-1,[5] and others.[1,6] However, a crystal structure of the CASK CaMK domain revealed that ATP could be bound in its active site and biochemical experiments showed that it retained the ability to phosphorylate neurexin despite the absence of Mg^{2+} binding activity.[23] In fact, Mg^{2+} inhibits phosphorylation which has been postulated to have evolved into an adaptive regulatory function of CASK in neurons.[24] To be phosphorylated by CASK, the C-terminus of neurexin must bind to CASK's PDZ domain to compensate for the relatively low enzymatic activity of the CaMK domain.[23] This PDZ/neurexin interaction is reminiscent of other kinase/phosphatase "docking" interactions including CDK-cyclins, MAP kinases, PDK1, GSK1, PP1, and calcineurin which are important for achieving enzymatic specificity.[25] Alternative tripartite complexes of CASK/Velis/Mint1 and CASK/Velis/Caskin1 bind to the cytoplasmic tail of neurexin in vertebrate presynaptic sites, though the function and mechanism of these competing interactions remain unknown.[15]

In this study we have focused on determining the molecular basis through which Caskin1 interacts with CASK. The previously identified CASK interaction domain (CID) of Caskin1 is not recognized as a protein domain in the Pfam[26] or SMART[27] databases and we find experimentally that it is mostly unstructured in solution. We present mutagenesis and binding experiments that show a short, linear peptide motif within the Caskin1 CID is necessary and sufficient for binding CASK. We thoroughly characterized this peptide motif through systematic mutagenesis and deletion studies and have identified the location of the CASK binding pocket. We also discovered related peptide motifs from Mint1 and TIAM1 that are sufficient for binding CASK. These results clarify how Mint1 and Caskin1 compete for binding CASK and suggest how other proteins, like TIAM1, may bind CASK using related motifs.

Results

Characterization of the CASK Interaction Domain (CID) of Caskin1

To characterize the previously identified Caskin1 CID,[15] residues I375 through G471 were cloned, expressed in *E. Coli*, and purified. In agreement with a prediction for intrinsic disorder using PONDR-FIT[28] (Supplemental Fig. 1), the caskin1 CID was found to be largely unstructured in solution at 25 °C by circular dichroism (CD) (Fig. 2a).

The region of CASK encompassing the CaMK domain necessary for binding Caskin1[15] (residues M1-A310) was also cloned, expressed in *E. Coli*, and purified. This recombinant CASK CaMK domain construct was used for all subsequent assays so it is simply referred to

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as CASK throughout the text for simplicity. The binding affinity of the Caskin1 CID/CASK interaction was measured by surface plasmon resonance (SPR) to be in the low micromolar range ($K_d = 7.5 \pm 0.5$ μM) (Fig. 2b).

Caskin1 Residues Necessary for Binding CASK

To determine the specific residues of Caskin1 necessary for binding CASK, a gel filtration binding assay was employed. As shown in Fig. 3A, a Caskin1 fragment that includes the CID and the two Caskin1 SAM domains (I375 through L605) forms a distinct complex when mixed with CASK (Fig. 3a). To find residues important for binding, we targeted conserved residues in the Caskin1 CID. We identified seven conserved regions shown in Fig. 3c, and made a series of seven constructs in which 2–4 residues in the region were changed to alanine. Of the seven mutants tested, only the IWV and LRK triple-alanine mutants affected the interaction with CASK (Fig. 3b). These residues form a contiguous hexapeptide suggesting that they might comprise a short, linear peptide binding motif (Fig. 3c). No similar known motifs were found by the Eukaryotic Linear Motif (ELM) server[29] or Minimotif Miner[30] suggesting that it may be a new interaction motif.

Defining the Minimal Peptide Motif Sufficient for Binding CASK

To determine if the conserved peptide motif is sufficient for binding CASK, an electrophoretic mobility shift assay (EMSA) was employed using super-charged green fluorescent protein (scGFP) fusion proteins. Native gel EMSA experiments using GFP have been utilized in other labs to measure protein-protein interaction affinities.[31,32] We used the negatively-charged scGFP variant developed by the Liu lab[33], because it causes the fusion proteins to predictably migrate toward the cathode. As a positive control, the complete Caskin1 CID (I375-G471) was fused to the C-terminus of scGFP. CASK addition leads to large gel shift of the scGFP-Caskin1 fusion construct towards the anode (Fig. 4a). The negative control experiment mixing only scGFP with CASK does not lead to a gel shift, indicating that the gel shift is specific to Caskin1's CID. Thus, this protein gel shift assay provides a rapid way to assess CASK binding activity.

A series of scGFP-peptide fusion constructs were iteratively screened for their ability to bind CASK. For simplicity, all further scGFP-peptide fusion proteins are referred to by the peptide sequence which was cloned at the end of the same flexible linker. We started with an 11-mer peptide, EEIWVLRKPFA, extending out from the key IWVLRK residues identified previously. The 11-mer actually extends the N-terminus two residues prior to the previously defined CID (I375-G471). As discussed below, this N-terminal extension incorporating the two conserved EE residues has higher affinity for CASK than the shorter CID. We refer to the longer region (E373-G471) as the extended CID.

First, the EEIWVLRKPFA 11-mer peptide was found to be sufficient for binding CASK (Fig. 4a). The C-terminal PFA residues were found to be dispensable, but the N-terminal EE residues could not be removed without impairing binding (Fig. 4a). The fact that the complete original CID (I375-G471) can still bind under the same conditions suggests that some part of the original CID must compensate for the lack of the EE residues. Nevertheless, an 8-mer peptide, EEIWVLRK, is sufficient for CASK binding. The 8-mer could be further whittled down to an EIWVLR hexapeptide sufficient for binding (Fig. 4b). These results indicate that the essential binding determinants are contained within a short stretch of 6 residues.

The EEIWVLRK octapeptide was used as a template for alanine scanning mutagenesis to further define the key side-chains involved in the CaMK interaction (Fig. 4c). Elimination of the W side-chain had the most dramatic effect on binding followed by the neighboring I.

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The mutations to V and R also affect binding, but to a much lesser extent. Thus, the IW sequence forms the core of the binding motif imparting most of the interaction energy.

Determination of Relative Binding Affinities

To determine the relative binding affinities between the minimal peptide motifs and the originally identified Caskin1 CID (I375-G471), a series of competition binding experiments were performed. First, equal amounts of the EEIWVLRK peptide and the I375-G471 fusion constructs were mixed and CASK was titrated from 0 to 30 μM (Fig. 4d). Clearly the EEIWVLRK peptide has a higher affinity for CASK than the original Caskin1 CID presumably due to the inclusion of the additional N-terminal EE residues. Accordingly, an extended Caskin1 CID including residues E373-G471 was then compared to the EEIWVLRK peptide. The EEIWVLRK peptide was found to have an essentially indistinguishable affinity from the extended CID (E373-G471) (Fig. 4e). Lastly, the minimal EIWVLR peptide was compared to the extended CID (E373-G471) and found to have a slightly reduced affinity (Fig. 4f).

Identification of Other Peptides Sufficient for Binding CASK

Caskin1 and Mint1 both bind to CASK, but cannot bind together, suggesting that Mint1 may utilize a similar peptide to bind the same or overlapping site on CASK.[15] We therefore examined the Mint1 sequence for a binding motif similar to Caskin1. A possible EPIWVMRQ binding peptide, which shared significant sequence similarity with the minimal Caskin1 peptide was readily found in the previously identified minimal Mint1 CID.[34] A gel shift assay utilizing the Mint1 EPIWVMRQ showed that this peptide is also sufficient for binding to CASK (Fig. 4g).

As CASK binds to a large number of different proteins, it seems possible that other proteins could utilize the same binding mechanism as Caskin1 and Mint1. We therefore performed a pattern search for all human proteins containing the Caskin1/Mint1 consensus motif ExIWVxR, where × is any amino acid. Using the Protein Information Resource (PIR)[35] we found only one additional protein containing this motif: T-cell lymphoma invasion and metastasis 1 (TIAM1). Specifically, TIAM1 contains a conserved EEVIWVRRE sequence close to the protein C-terminus. A gel shift assay using the TIAM1 peptide showed that this 9-mer peptide is also sufficient for binding CASK (Fig. 4g). We also note that both motifs from Mint1 and TIAM1 are highly conserved, implying functional importance (Fig. 5).

Identification of the Peptide Binding Site on CASK

The interaction of Caskin1 with CASK was previously found to be dependent on the presence of the C-terminal end of the CaMK domain (i.e. residues 275–310) suggesting that this portion of the protein is directly involved in binding.[15] However, deletion of these 35 C-terminal CaMK residues may destabilize the overall structure of the kinase domain and affect distant sites. Accordingly, we sought to identify a specific binding site on the CaMK domain by introducing a single point mutation. Given the nature of the consensus CASK interaction motif, it seemed likely that the peptide binds to a small, complementary, conserved, hydrophobic pocket on the surface of the CASK CaMK domain. Thus, we mapped the evolutionary conservation of surface residues using ConSurf to look for candidate binding sites (Fig. 6a).[36] A conserved, mostly hydrophobic patch with an obvious deep potential binding pocket was readily seen on the C-terminal lobe of the CaMK domain (Fig. 6b). Moreover, the binding pocket appeared large enough to accommodate a Trp side chain – a key element of the peptide motif.

To test the importance of the potential binding site, we introduced mutations into the site. V117, which points directly toward the deep pocket, was changed to D or Q. Both mutations

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V117D and V117Q abolished the ability of CASK to bind the Caskin1, Mint1, and TIAM1 peptides, as well as the original CID (I375-G471) and the extended CID (E373-G471) of Caskin1 indicating that they all bind to the same site (Fig. 6c). The CASK mutants were well behaved and the CD spectra of the mutants were essentially the same as the wild type protein, suggesting that the mutations did not cause global changes in structure (Supplemental Fig. 2).

With the help of the FlexPepDock server,[37] we generated an illustrative model consistent with the mutagenesis data (Fig. 7). For the starting model it was assumed that the indole ring of the key tryptophan inserted deeply into the binding pocket. The surface around the binding pocket has noticeable ridges which seems like it might favor binding of the peptide in a β-hairpin structure though this is obviously uncertain without high-resolution structural studies.

Discussion

These studies demonstrate that a short, linear EEIWVLRK peptide motif from Caskin1 is necessary and sufficient for binding the CASK CaMK domain. Inclusion of at least one of the N-terminal glutamic acid residues was found to be necessary to achieve strong CASK binding even though neither of the glutamic acid residues were originally identified in the minimal Caskin1 CID.[15] The majority of the residues in the motif seem to be present simply to ensure proper interactions with the central hydrophobic residues since only the isoleucine and tryptophan to alanine mutations dramatically impair binding. The importance of these particular hydrophobic residues at the center of the motif is actually a common feature of many peptideprotein interaction motifs.[38] Using this information we were able to locate a conserved binding pocket on the CASK CaMK domain that is responsible for binding to this peptide. CASK is known to autophosphorylate itself on the loop containing S151 and S155[23] adjacent to the binding pocket which suggests that phosphorylation may regulate binding to this site (Fig. 6b).

The fact that most of the residues can be mutated to alanine without loss of binding suggests a broad family of peptide motifs can bind CASK. Discovery of one such peptide motif (EPIWVMRQ) that is sufficient for binding CASK was apparent simply by looking at the amino acid sequence of the previously identified Mint1 CASK minimal binding region. Similar to Caskin1, Mint1's CASK binding region contains no recognizable Pfam/SMART domains and is predicted to be largely disordered using PONDR-FIT[28] (Supplemental Fig. 1). Mint1's minimal binding region was originally defined by a GST pull-down experiment using homologues from *C. elegans* which showed that Mint1's CASK interaction domain could be reduced to a 63-amino acid segment (residues 373–436) which contains the EPIWVMRQ peptide.[34] Pull-down experiments using smaller segments that still contained the motif (i.e. residues 373–392 and 373–402), however, failed to bind CASK. One interpretation is that other residues from the 63-amino acid segment may be involved in directly binding CASK. Alternatively, the analogous Caskin1/CASK micromolar dissociation constant suggests that this is likely a transient interaction which might have simply been missed by the pull-down assay – a common problem in the experimental verification of short, linear peptide binding motifs.[39] As mentioned previously, Mint1 and Caskin1 are known to compete for the same CASK CaMK binding site which is thought to modulate the function of CASK at presynaptic neuronal sites.[15] The presence of common peptide motifs in both proteins provides a consistent molecular explanation for this overlapping interaction. Furthermore, it is easy to explain how convergent evolution can lead to this overlapping interaction given the relatively small number of mutations required for adoption of short, linear binding motifs in general.[39,40] The absence of similar motifs in

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Caskin2, Mint2, and Mint3 is also consistent with the fact that these homologous proteins do not bind CASK.[12,13,15]

Using a relatively conservative search through all human proteins allowing only variation at non-identical residues of the Caskin1/Mint1 consensus motif we found only one additional protein with the motif: TIAM1. Though predictions of functional short, linear peptide motifs are notorious for generating false positives,[39] the highly conserved EEVIWVRRE peptide from TIAM1 bound the CASK CaMK domain *in vitro*. Like the motifs from Caskin1 and Mint1, the motif in TIAM1 is found in the middle of a region with no recognizable Pfam/SMART domains that is also predicted to be largely disordered, though the motif itself is predicted to adopt some structure (Supplemental Fig. 1). TIAM1 was originally discovered as a protein that causes cells to become invasive[41] and is involved in tumor progression and metastasis.[42] Intriguingly, TIAM1 and CASK are both involved in the establishment of cell polarity,[43,44] expressed highly in brain tissue,[2,45] are important for brain development,[6,46] and localize to post-synaptic dendritic spines where they play critical roles in spine development and maintenance.[47,48,49] Given this background and their confirmed *in vitro* interaction it seems quite possible that this interaction occurs *in vivo*. Accordingly, we predict that this TIAM1 and CASK interaction may play an important role in brain development and function.

Materials and Methods

Cloning and Mutagenesis

The template DNA for cloning the Caskin1 (also called KIAA1306) constructs was obtained from Kazusa DNA Research Institute (catalogue number ORK04438). The Caskin1 CID (residues I375 to G471) was cloned into a modified pET-3a vector between MluI and SalI restriction sites containing an N-terminal sequence (MEKTR) for efficient expression and a C-terminal His-tag (RRHHHHHH) for purification. The wild-type Caskin1 CID-SAMs construct (residues I375 to L605) was also cloned into the same modified pET-3a vector containing the same N-terminal sequence (MEKTR) and C-terminal His-tag (RRHHHHHH). The template DNA containing the entire cDNA for CASK (GenBank sequence AF032119) was a gift from Zenta Walther (Yale). Residues 1–310 were cloned into pCDFDuet-1 vector (Novagen) between the EcoRI and SalI restriction sites to yield an expression vector for the CASK CaMK domain containing an N-terminal His tag. Negatively (−30) supercharged green fluorescent protein (scGFP) including an N-terminal 6x His tag, generously provided by the Liu Lab, was cloned into the pBAD-HisA vector (Invitrogen) using NcoI and KpnI sites. An oligomer cassette was inserted using KpnI and HindIII restriction sites adding the sequence for a Gly-Ser linker (GGSGGS) followed by the sequences for the following combination of restriction sites: NotI–AsiSI-PISceI–Asc-FseI. All scGFP-peptide fusion constructs were made by insertion of synthetic oligonucleotide cassettes between the NotI and FseI restriction sites. All mutagenesis reactions were performed by QuickChange (Stratagene/Agilent). All plasmids were verified by DNA sequencing (Genewiz).

Protein Expression and Purification

The Caskin1 CID and CASK expression plasmids were transformed into BL-21 (DE3). Cultures of 2 L were incubated at 37 °C until $OD_{600} \approx 0.6$ and induced with 0.2 μg/mL (final concentration) IPTG for 3–4 hours at 37 °C. Cells were harvested by centrifugation, flash frozen in liquid nitrogen, and stored at −80 °C. Cells were lysed by sonication in lysis buffer (20 mM Tris pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol (BME), 0.5–1 mg/mL lysozyme, 0.1 mM PMSF, and DNase). The lysate was then centrifuged at 15 krpm in a Sorvall SS-34 rotor at 4 °C. Both proteins expressed in the soluble fraction and were bound

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to 2 mL (bed volume) of Ni-NTA Superflow at 4 °C for 1 hour after adding imidazole to 5 mM final concentration. The Ni beads were washed extensively with wash buffer (20 mM Tris pH 8.0, 500 mM NaCl, 5 mM imidazole) and the protein was eluted with 25 mL of elution buffer (20 mM Tris pH 8.0, 500 mM NaCl, 200 mM imidazole). Solid ammonium sulfate was then added to the eluted protein to a final concentration of 1 M and the solution was centrifuged for 30 min at 4000 rpm on a Beckman Allegra benchtop centrifuge. The proteins were then loaded onto a 5 mL HiTrap Phenyl HP column (GE Healthcare). Proteins were eluted using a gradient from high salt (20 mM Tris pH 7.5, 1 M ammonium sulfate) to low salt (20 mM Tris pH 7.5). Both proteins were further purified using a Superdex S200 10/300 GL column using 20 mM HEPES pH 7.5, 500 mM NaCl as the running buffer.

All the wild type and mutant Caskin1 CID-SAMs plasmids were also transformed into BL-21 (DE3) and each protein was expressed as above. After centrifuging in the SS-34 rotor, the proteins were found in the insoluble pellet and dissolved in denaturing binding/wash buffer (20 mM Tris pH 7.5, 6 M urea, 5 mM imidazole) and bound to Ni-NTA. The Ni beads were washed extensively with the denaturing binding/wash buffer and eluted with 25 mL denaturing elution buffer (20 mM Tris pH 7.5, 6 M urea, 200 mM imidazole). The proteins were then refolded by dialysis into refolding buffer (20 mM HEPES pH 7.5, 500 mM NaCl, 1 mM TCEP, 1 mM EDTA) overnight at 4 °C. Imidazole was added to the refolded protein to 10 mM final concentration and each was bound and separately purified using a 5 mL HisTrap column (GE Healthcare) using a gradient of low imidazole (20 mM HEPES pH 7.5, 300 mM NaCl, 10 mM imidazole) to high imidazole (20 mM HEPES pH 7.5, 300 mM NaCl, 300 mM imidazole).

Circular Dichroism (CD) Spectroscopy

The purified recombinant Caskin1 CID (residues I375 to G471 with the N-terminal MEKTR and C-terminal His-tag) was dialyzed into 10 mM Tris pH 7.5, 100 mM NaCl overnight at 4 °C using 3,000 MWCO dialysis tubing. The concentration was determined following dialysis by measuring the absorbance at 280 nm (0.1175) which implied a concentration of 16.8 μM (0.19 mg/mL) using a calculated extinction coefficient of 6,990 $M^{-1}cm^{-1}$ and a molecular weight of 11,361.6 g/mol. The CD spectrum was obtained using a JASCO J-715 Circular Dichroism Spectrophotometer with a Peltier temperature control equilibrated to 25 °C. The data was background corrected with a buffer only control.

The purified recombinant CASK CaMK constructs (wt, V117D, and V117Q) were dialyzed into 20 mM HEPES pH 7.5, 200 mM NaCl, 1 mM EDTA at 4 °C using 10,000 MWCO dialysis tubing. The concentrations were measured by absorbance at 280 nm assuming a calculated extinction coefficient of ~34,880 $M^{-1}cm^{-1}$ and a molecular weight ~36,900 g/mol. The CD spectra were obtained with the same spectrophotometer described above using ~7.5–8 μM protein.

Surface Plasmon Resonance

The purified recombinant Caskin1 CID (residues I375 to G471 with the N-terminal MEKTR and C-terminal His-tag) was dialyzed into 20 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA using 3,000 MWCO dialysis tubing to give a stock solution of approximately 0.2 mg/mL. The Caskin1 construct was immobilized on a Biacore CM5 chip using EDC/NHS cross-linking and a 1:10 dilution of Caskin1 protein stock solution and 10 mM acetate pH 5.5. The chip was then equilibrated with the binding buffer: 20 mM HEPES pH 7.5, 500 mM NaCl, 1 mM EDTA. The recombinant CASK CaMK construct was also dialyzed into binding buffer and fresh DTT (2 mM final concentration) was added immediately prior to the binding experiment. Various concentrations of the CASK CaMK were passed over the chip until equilibrium binding was achieved. The chip was regenerated between each binding

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experiment using a regeneration buffer of 5 mM DTT and 8 M urea followed by a wash step with binding buffer until the response returned to the baseline level. The binding data was fit to a 1:1 steady-state model using Biacore T100 Evaluation software.

Size-Exclusion Binding Assay

A total of 500 μL of purified wt or mutant Caskin1 CID-SAMs (1.8 mg/mL) and CASK-310 (1.8 mg/mL) were applied to a Superdex S200 10/300 GL gel filtration column in 20 mM HEPES pH 7.5, 500 mM NaCl, 1 mM EDTA using an AKTA FPLC run at 0.5 mL/min.

Supercharged GFP Native Gels

Supercharged GFP constructs were transformed into protease deficient ARI814 cells[50] and 25 mL of cell culture was grown in LB media at 37°C until the cell density reached and OD of 0.8. Cells were then transferred to 16°C, induced with 0.2% arabinose, and incubated for an additional 12 hours. After harvesting by centrifugation, cell lysis was carried out in 0.25 mL 20 mM Tris (pH 7.5), 1 M NaCl, 5 mM BME, 5 mM $MgCl_2$ buffer containing lysozyme (5 mg/mL), DnaseI (20 μg/mL), 0.5 mM PMSF, and Complete Protease Inhibitor (Roche). Cells were subjected to three rounds of freeze-thaw followed by two ten second rounds of sonication. The cell lysate was centrifuged at 13,000 rpm for 20 minutes and the pellet discarded. Lysate fluorescence was normalized by transferring 100 μL of lysate into a 96-well clear bottom, black sided plate (Nunc) and measuring the scGFP fluorescence on a Molecular Devices Spectramax M5 plate reader using an excitation wavelength of 488 nm, an emission wavelength of 510 nm, and a cutoff filter of 515 nm. The concentrations of different scGFP fusions in the lysate were normalized by diluting samples with lysis buffer. CASK samples were reduced with DTT (10 mM, final concentration) immediately prior to mixing with lysate. 7 μL of normalized lysate was added to 14 μL of purified CASK protein and 7 μL of 4X RunBlue Native Sample Buffer (Expedeon) and 20 μL was loaded onto a 20% RunBlue 12 –well Native gel (Expedeon). Gels were run at 90 V in 40 mM Tricine, 60 mM Tris buffer for 13–16 hours at 4°C and visualized on a Bio-Rad Molecular Imager FX Pro-Plus using an excitation wavelength of 488 nm and an emission wavelength of 510 nm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MAGUK	membrane-associated guanylate kinase
CaMK	calmodulin kinase domain
L27	Lin-2/Lin-7 doamin
SH3	SRC Homology 3 domain
PDZ	PSD-95/DlgA/zo-1 domain
GK	guanylate kinase
CID	CASK interaction domain

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SPR	surface plasmon resonance
CD	circular dichroism
SAM	sterile alpha motif
scGFP	super-charged green fluorescent protein
EMSA	electrophoretic mobility shift assay

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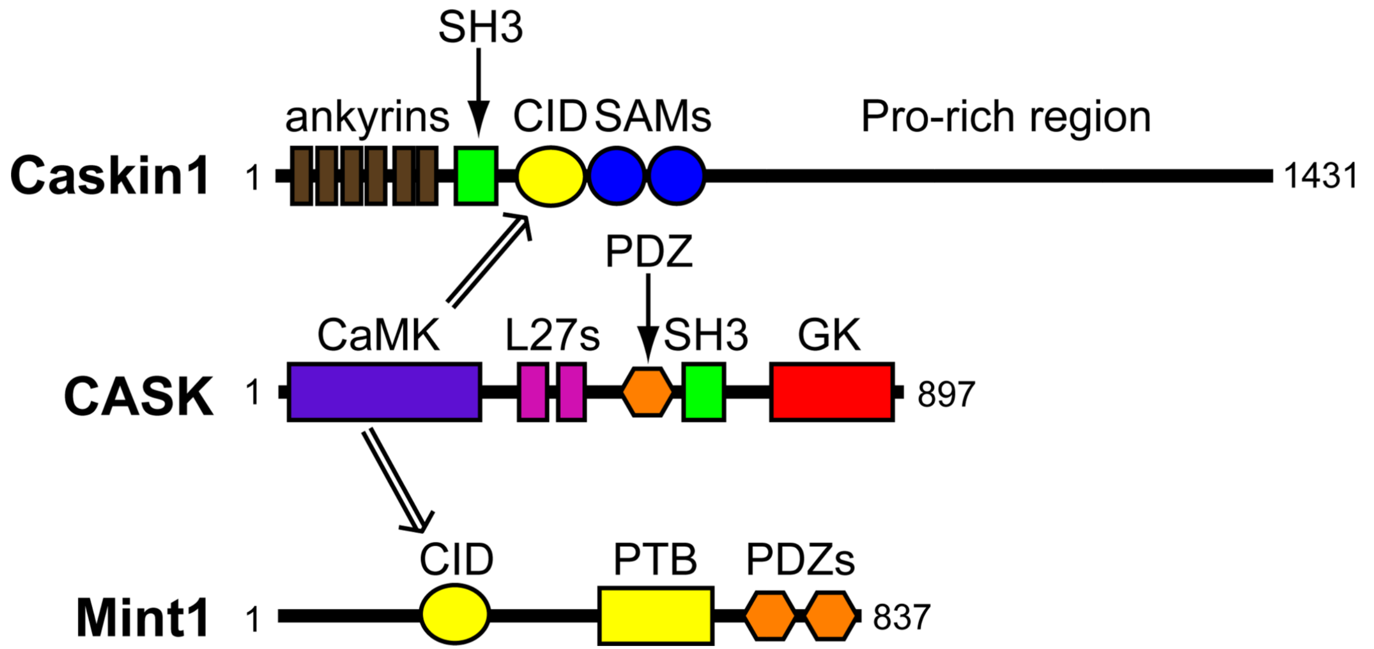

Fig. 1.

Domain arrangement of Caskin1, CASK, and Mint1. The previously identified CASK interaction domains (CIDs) from Caskin1 and Mint1 interact with the Ca/calmodulin kinase (CaMK) domain of CASK. Domain key: CaMK = calmodulin kinase; L27 = Lin-2/Lin-7; PDZ = PSD-95/Dlg/ZO-1; SH3 = Src homology 3; GK = guanylate kinase; ANK = ankyrin; SAM = sterile alpha motif; PTB = phosphotyrosine binding; CID = CASK interaction domain. The protein domains are drawn approximately to scale.

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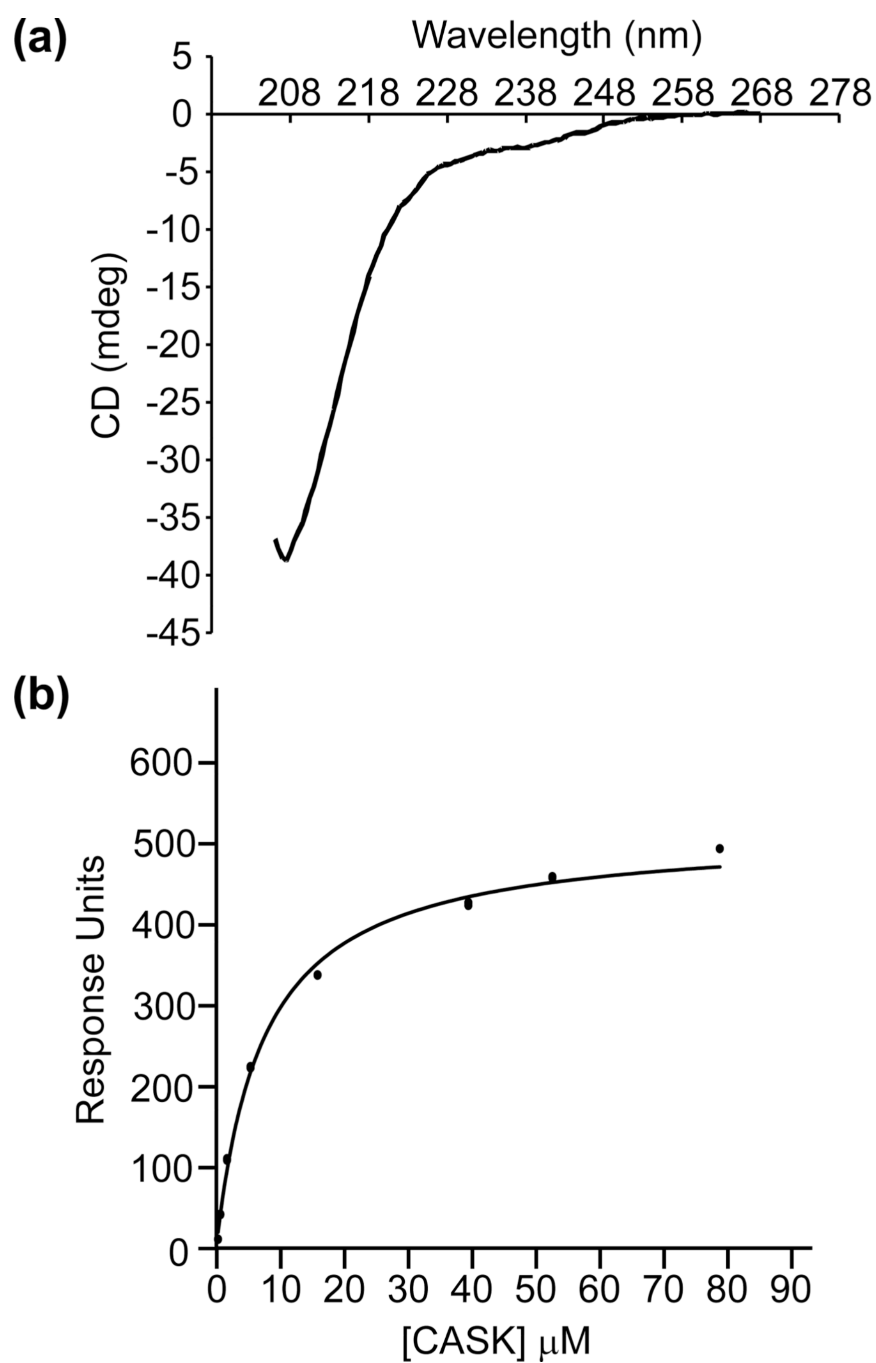

Fig. 2.

Characterization of the previously identified Caskin1 CID. (a) Circular dichorism spectrum of Caskin1 residues I375 to G471 at 25 °C shows that this region is mostly unstructured. (b) Measurement of the Caskin1/CASK binding affinity using surface plasmon resonance. Caskin1 residues I375 to G471 were bound to the chip and various concentrations of the CASK CaMK domain were used in the mobile phase. The binding data was fit to a 1:1 steady-state binding model (line).

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(a)

Caskin1/CASK
Caskin1
CASK
A_{280} (mAU)
Elution Volume (mL)

(b)

all other mutants
LRKtoAAA
IWVtoAAA
A_{280} (mAU)
Elution Volume (mL)

(c)

```
H.sapiens        ----------------------------AGSRAGTEPSLPQGSSSSG---PSAPPEEIWV 377
P.troglodytes    ----------------------------AGSRAGTEPSLPQGSSSSG---PSAPPEEIWV 518
C.familiaris     ----------------------------AGSRAGAEESPPQAGSSSG---PSAPPEEIWV 352
M.musculus       ----------------------------AGSRTGSEPSPPQGGGSLG---PSAPPEEIWV 377
R.norvegicus     ----------------------------AGSRTGSEPSPPQGGGSLG---PSAPPEEIWV 377
G.gallus         ----------------------------TGSR-GADMSPSHLSPSQGGSAAPAPTEEIWV 372
D.rerio          PPPPPHSHLPLFTSFGYNRSPNTPEEPHSGQGSRGSESSPHGSPTPAGGPQGGSSEEIWV 458
                                             :*.    . * .: . : .     ...*****

H.sapiens        LRKPFAGGDRS--GSISGMAGGR--------GSGGHA-----LHAGSEGVKLLATVLSQK 422
P.troglodytes    LRKPFAGGDRS--GSISGMAGGR--------GSGGHA-----LHAGSEGVKLLATVLSQK 563
C.familiaris     LRKPFAGGDRS--GSLSGAAGAR--------SGGGHA-----LHAGSEGVKLLATVLSQK 397
M.musculus       LRKPFAGGDRS--GSLSNVAGGR--------STGGHA-----LHAGSEGVKLLATVLSQK 422
R.norvegicus     LRKPFAGGDRS--GSLSNVAGGR--------STGGHA-----LHAGAEGVKLLATVLSQK 422
G.gallus         LRKPFTGGDRSSLGSTGSVASARSSGSGQSTGSGAHA-----LHAGSEGVKLLATVLSQK 427
D.rerio          LRKPVAGGDRSSVGSSGSVASARSS------GSGQSAGSTSILHAQAEGVKLLATVLSQS 512
                 ****.:*****  **  .. *..*        . *   *     *** :************.

H.sapiens        SVS------ESGPGDSPAKPPEGSAGVARSQ--PPVAHAGQVYGEQPPKKLEPA----SE 470
P.troglodytes    SVS------ESSPGDSTAKPPEGSAGVARSQ--PPVAHAGQVYGEQPPKKLEPA----SE 611
C.familiaris     SVS------ESSLGDSPVKPPEGSTGAARSQ--PPAAHAGQVYGEQPPRKLEPA----SE 445
M.musculus       SVS------ESSPGDSPVKPPEGSSGAARSQ--PPAAHAGQVYGEQPPKKLESASASASE 474
R.norvegicus     SVS------ESSPGDSPVKPPEGSSGAARSQ--PPAAHAGQVYGEQPPKKLESS--SASE 472
G.gallus         ASA------QDAAQDGPAKAQDIPAGSSRSQSVASSPYAPQPPAEQQLKKMEPP----SE 477
D.rerio          AKAKEHLLEQTKSLDHPSHASSNKGPSSRSQSLSSCPLHEQPYGEAVSQRKGEAP---PE 569
                 : :      :    *  . :. .     :***  .. .    *   .*   ::   .    .*
```

Fig. 3.

Caskin1 residues necessary for binding CASK. (a) A gel filtration binding assay using wild-type recombinant proteins shows that the Caskin1/CASK complex peak shifts relative to the Caskin1 and CASK only peaks. (b) Only the Caskin1 IWV and LRK to triple alanine mutants affect the formation of the Caskin1/CASK complex indicating that these residues are necessary for binding. (c) ClustalW sequence alignment surrounding the previously identified Caskin1 CASK interaction region. The sites of the double, triple, and quadruple alanine mutations to conserved residues are highlighted in yellow. The key IWV and LRK residues are enclosed in a red box.

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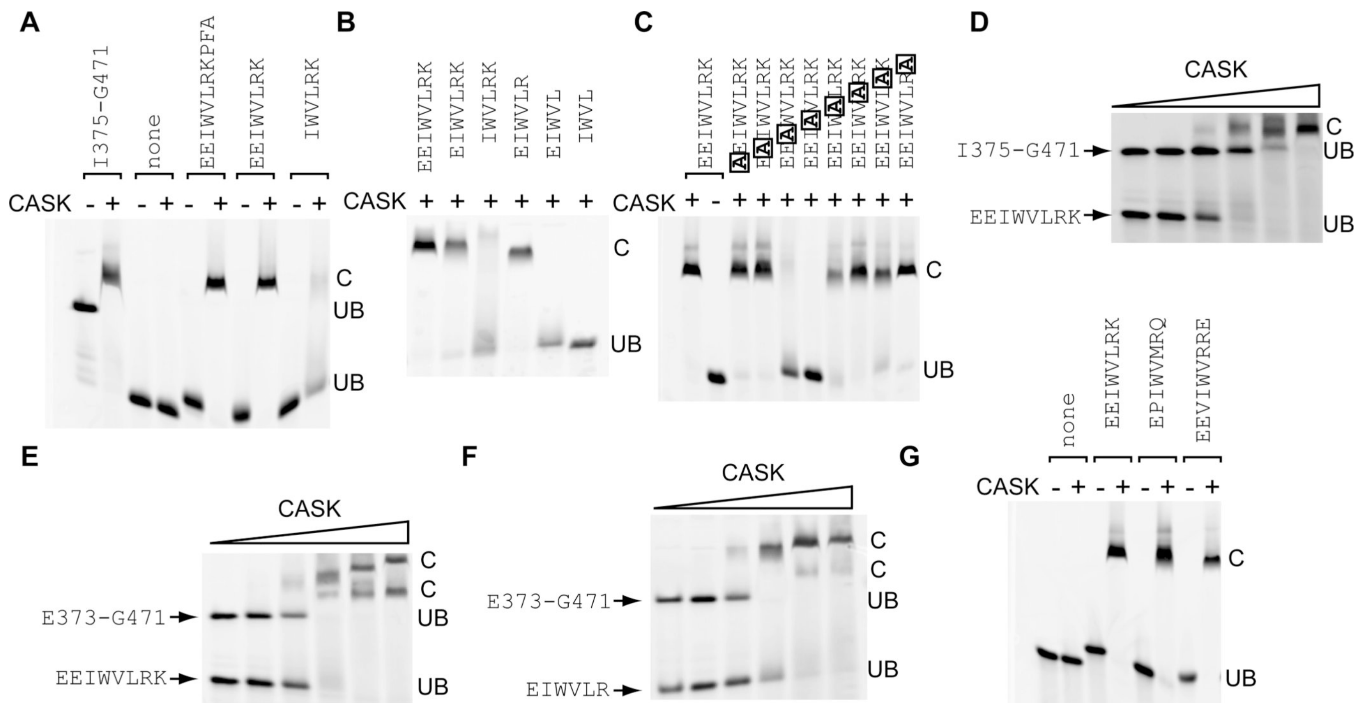

Fig. 4.

Native PAGE scGFP protein-peptide interaction gel shift assays. (a) The previously identified Caskin1 CID residues I375-G471 are confirmed to bind to CASK (30 μM). An EEIWVLRKPFA 11-mer was also found to be sufficient for binding. The C-terminal PFA residues are nonessential as the EEIWVLRK 8-mer is also sufficient. The unbound scGFP fusion protein bands are labeled "UB" and the bands in complex with CASK are labeled "C". (b) The minimum EIWVLR 6-mer peptide was identified by systematically truncating the N-and C-termini of the remaining EEIWVLRK 8-mer. The central IWVL hydrophobic core is insufficient for binding CASK. (c) Alanine-scanning mutagenesis of the EEIWVLRK 8-mer shows that the central IW residues are critical for binding. (d) A competition assay between equal amounts of the EEIWVLRK peptide and the original Caskin1 CID (I375-G471). From left to right, the CASK concentration is 0, 0.3, 1, 3, 10, and 30 μM. The peptide binds with higher affinity as indicated by the absence of the free band at lower CASK concentrations. (e) A competition assay between equal amounts of the EEIWVLRK peptide and the extended Caskin1 CID (E373-G471). The results indicate that the binding affinities of these constructs are similar. (f) A competition assay between equal amounts of the minimal EIWVLR peptide and the extended Caskin1 CID (E373-G471). The results indicate that the extended CID binds with higher affinity. (g) Peptides from different proteins in addition to Caskin1 were screened for CASK binding: Caskin1 = EEIWVLRK; Mint1 = EPIWVMRQ; TIAM1 = EEVIWVRRE.

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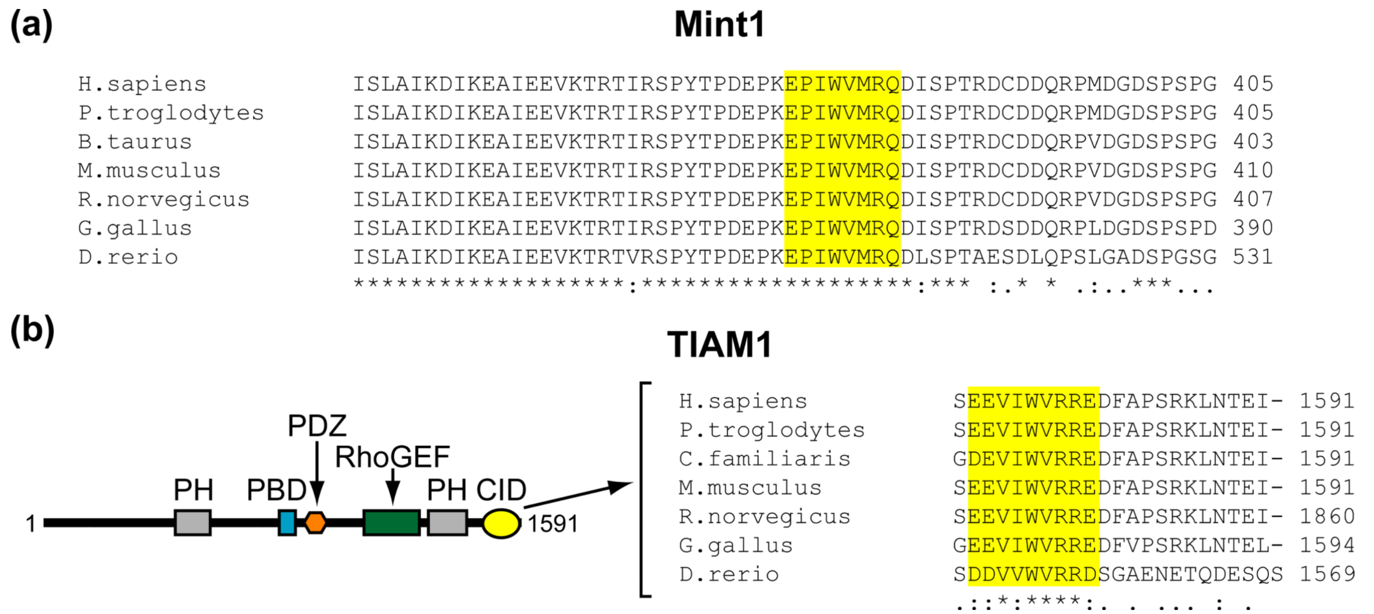

Fig. 5.

The interaction motifs of Mint1 and TIAM1 are highly conserved. (a) A ClustalW sequence alignment of the Mint1 motif is shown. See Fig. 1 for a domain diagram of Mint1. (b) A domain diagram of TIAM1 indicating the location of its putative CID is shown next to a ClustalW sequence alignment of TIAM1 homologues. Domain key: PH = Pleckstrin homology; RBD = Ras-binding domain; RhoGEF = Rho guanine nucleotide exchange factor; PDZ = PSD-95/Dlg/ZO-1.

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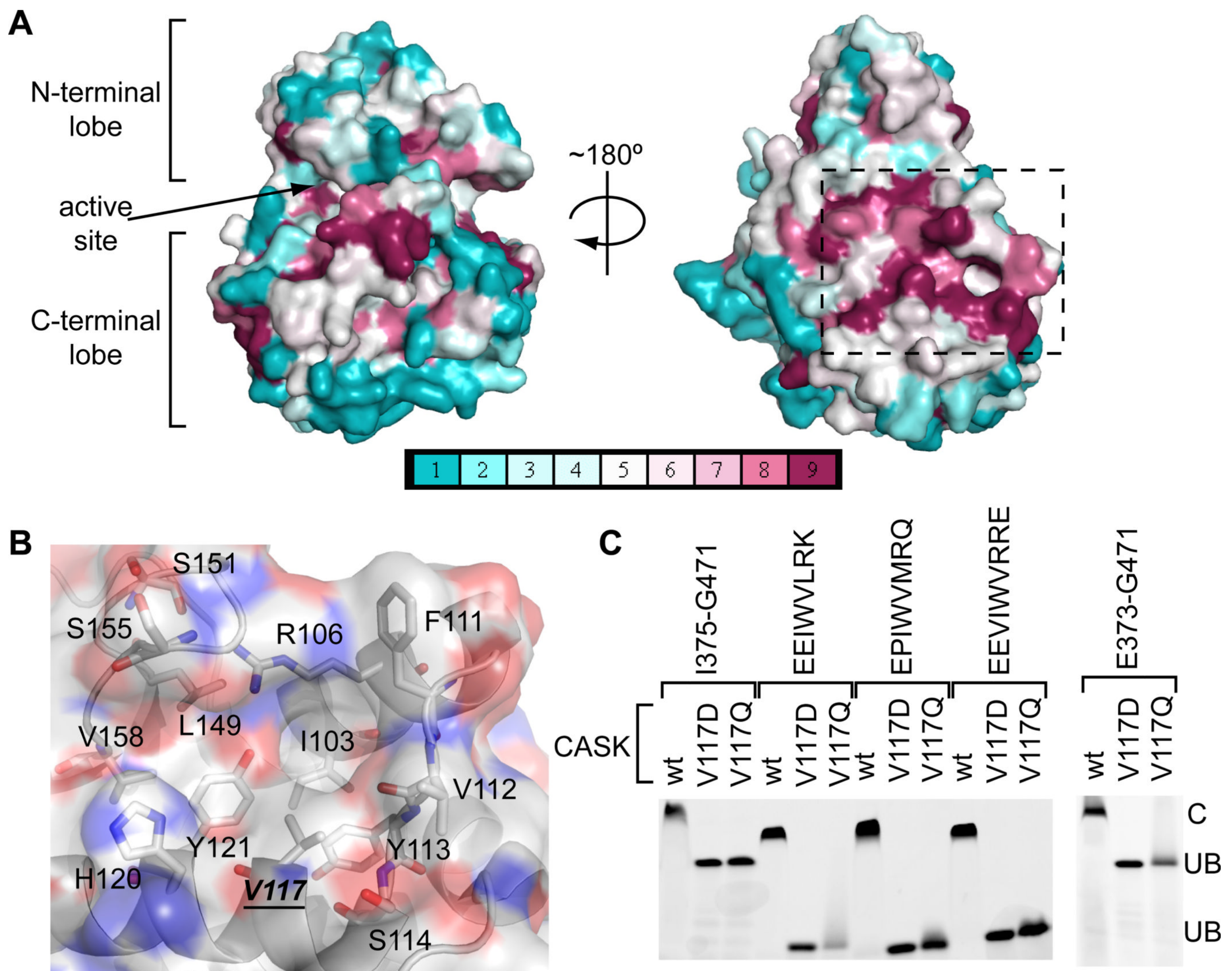

Fig. 6.

Identification of the peptide binding site on CASK. (a) Sequence conservation of surface residues of the CASK CaMK domain (PDB code 3C0G) mapped using ConSurf.[36] A single, large conserved patch on the C-terminal CaMK lobe was identified (dashed box). The surface is shaded from cyan (variable) to purple (conserved). The graphics were made using PyMOL (Schrödinger, LLC). (b) A zoomed in view of the conserved patch (dashed box in panel A) with key residues shown as stick models (carbon = white, nitrogen = blue, oxygen = red). There is a clear binding pocket with I103 forming the back which seems large enough to accommodate the indole ring of the key tryptophan of the Caskin1 peptide motif. The V117 side-chain points directly into this binding pocket, so it was chosen for mutagenesis experiments. (C) Gel shift assays reveal that the V117D and V117Q mutations inhibit the interaction between CASK and Caskin1 I375-G471, EEIWVLRK, EPIWVMRQ, EEVIWVRRE, and Caskin1 E373-G471. The unbound scGFP fusion protein bands are labeled "UB" and the bands in complex with CASK are labeled "C". Note that the E373-G471 series was run on a different gel due to lane limitations.

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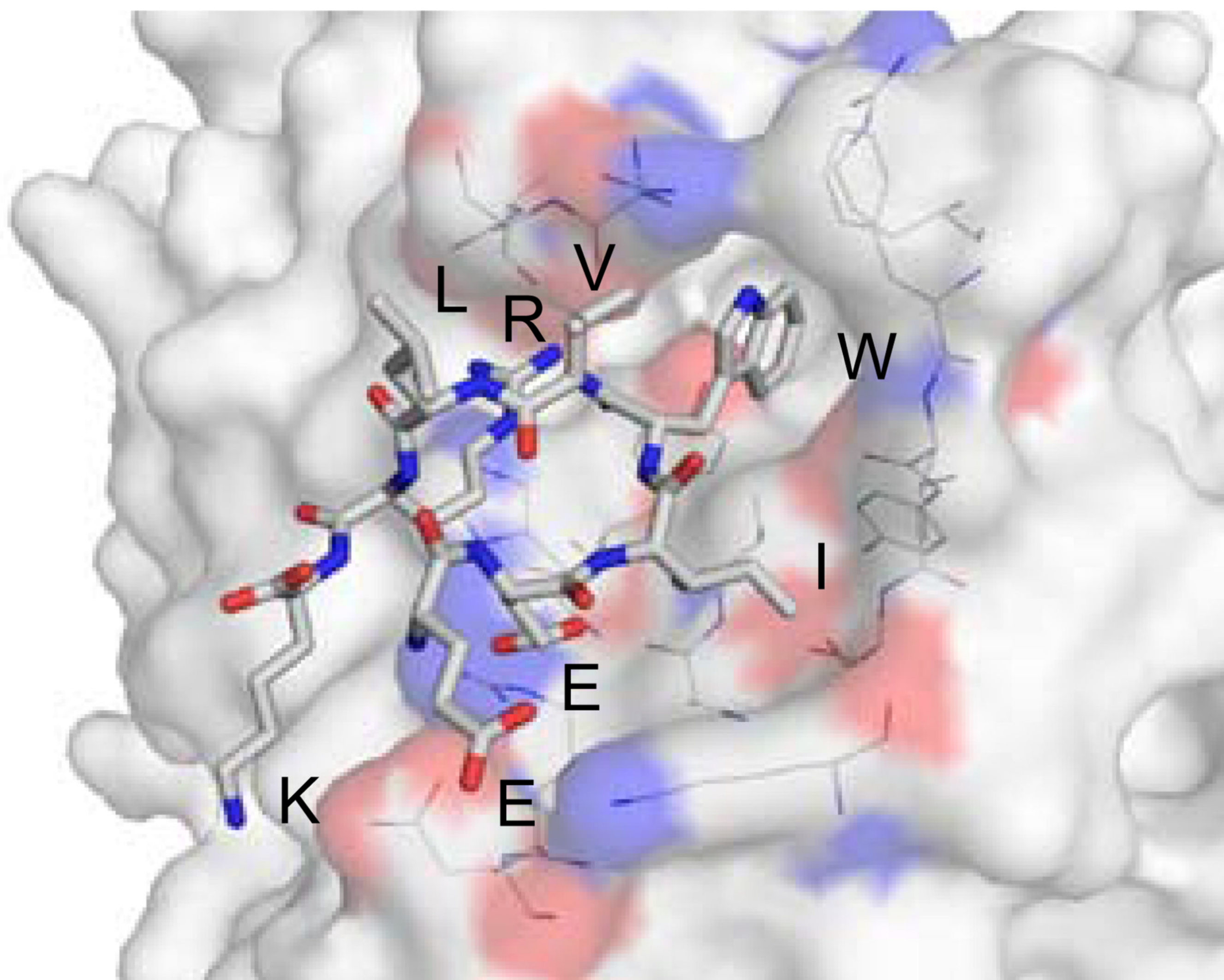

Fig. 7.

An illustrative model the Caskin1 peptide with CASK. The model was created using the FlexPepDock server[37] using a starting model with the tryptophan indole inserted into the central binding pocket. This model was the highest ranked structure after several iterations of docking. Models inconsistent with the experimental data were manually pruned after each iteration. The model is speculative and merely serves to illustrate that the peptide could fit into the identified pocket on the surface of CASK.