Supplementary Data

Supplemental Materials and Methods

Cloning, expression and purification of ZO-1 fragments

Constructs for structure determination. A fragment of ZO-1 (see Fig. 1A) encoding residues 516 to 806 was amplified from the human ZO-1 cDNA (Genbank # NM_175610) by amplification with Taq polymerase using sense primer GCCATATGGGAGATTCTTTCTATATTAGAAC and antisense primer GCGGATCCTCAATCCGCCTTTCCCTCGGA and subcloned into the Nde I/Bam HI sites of pET14b. This SG construct diffracted up to ~3.7 Å and thus a truncation mutation of the U5 region, believed to be a flexible linker based on the previous structure of the homologous PSD-95 MAGUK family member, was made on this construct using the following primers (5'-3'): GCCAGTGTACAGTATGTTCAAACAAAGTTC and GAACTTTGTTTGAACATACTGTACACTGGC. The boundaries of the truncation (SGΔU5; deletion of residues 589-625) were chosen according to the structure-based sequence alignment of rat PSD-95 with the SG sequences of the ZO family across multiple species. The homologous sequences encoding the helix observed in PSD-95 prior to the flexible U5 region (called the HOOK domain in PSD-95 and DLG) was retained and several residues N-terminal to the highly conserved beta-strand that maintains the inter-domain organization were included to ensure an unrestrained linker connection. Additionally, to facilitate heavy atom derivatization for phasing purposes, a cysteine residue (T709C) was introduced into the SGΔU5 construct using the following primers (5'-3'): GCTTTATTAGATGTATGCCCAAATGCAG and CTGCATTTGGGCATACATCTAATAAAGC.

Mutagenesis of the GUK domain. SG constructs with point mutations were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) using the following primers (5'-3'): **TM(A) (K749A/R752A/K753A)** - GAATCTCGGGCTAGTGCCG and CGGCACTAGCCCGAGATTC; **TM(D/E) (K749D/R752D/K753E)** - GAATCTCGGGATAGTGCCG and CGGCACTATCCCGAGATTC; **PM(D/E) (K749D/R752D/K753E/K760E/K763E)** -

CGAGCGATCTCATGAGCTTCGTGAGAATAATCACC and GGTGATTATTCTCACGAAGCTCATGAGATCGCTCG. Following PCR and confirmation of the sequences, the plasmids were transformed into E. coli BL21 (DE3).

Expression and Purification. Cells in 2YT media were grown to an optical density (O.D.) of 0.6 and then induced with 0.1 mM IPTG, and left to grow overnight at 22 °C. For selenomethionine (SeMet)-containing SGΔU5, the cells were grown in M9 minimal media and the protein expressed through methionine biosynthesis inhibition(30) and supplemented with 0.25 g/L $MgSO_4$, 5 g/L glucose, 0.015 g/L $CaCl_2$, and 0.001 g/L each of riboflavin, niacinamide, thiamine, and pyridoxine monochloride. The incorporation of four SeMet sites was confirmed using mass spectrometry. Lysis was accomplished by sonication in a buffer composed of 50 mM Tris-HCl pH 7.5, 1 M NaCl, 10 % glycerol, 1 % Triton, and 1 mM PMSF. After centrifugation at 30k rpm, the supernatant was loaded onto a nickel-affinity column, and the column washed with 500 ml of a buffer composed of 50 mM Tris-HCl pH 7.5, and 1 M NaCl. The bound protein was eluted with an imidazole gradient. Fractions containing the protein were pooled, concentrated, and injected onto a gel-filtration column equilibrated with 50 mM Tris-HCl pH 7.5, 200 mM NaCitrate pH 7.5, and 3 mM DTT (10 mM for the cysteine mutant and SeMet protein). The purity of the protein preparations was confirmed using SDS-PAGE and detected through Coomassie staining.

Supplementary Figure Legends

Fig. S1. The strand following the GUK domain (b6), in addition to making secondary structure interactions with the terminal strand of the SH3 domain (b5), has a conserved tryptophan residue that intercalates into the SH3 domain. The side-chain of Trp799 penetrates a hydrophobic cavity in the SH3 domain built by Tyr520, Val542 and Leu638, all functionally conserved among MAGUK proteins. The view in the zoom panel is rotated by ~180 degrees relative to the upper panel. The coloring scheme follows that used previously for the ZO-1 SH3-GUK module.

Table I. Data collection, phasing, and refinement statistics

A. Data sets used in phasing and calculating the experimental electron density map

Data Set	Native	p-ClHg Benzoic Acid	CH3HgBr Crystal 1	CH3HgBr Crystal 2	Se-Met Crystal 1	Se-Met Crystal 2
Wavelength (Å)	1.0	1.0076	1.0088	1.0077	0.9751	0.9756
Resolution limit (Å)	2.8	3.1	3.5	3.4	2.5	3.3
Space group	P64	P64	P64	P64	P64	P64
No. molecules in ASU	1	1	1	1	1	1
Cell dimensions (Å)						
a = b	126.5	127.6	127.7	128.9	124.7	126.1
c	35.8	36.6	36.4	36.8	35.3	35.76
Reflections observed	28474	40962	31732	61250	81132	60331
Unique reflections	8035	10414	8262	9577	21078	9601
Multiplicity (last shell)	3.5 (3.6)	3.9 (3.3)	3.8 (3.7)	6.4 (6.1)	3.8 (3.8)	6.3 (5.5)
% Completeness (last shell)	97.7 (95.1)	89.4 (76.4)	99.4 (97.9)	98.4 (90.4)	99.4 (96.7)	98.4 (90.3)
R_{sym} (last shell)	3.7 (19.5)	4.6 (49.4)	7.8 (59.8)	5.2 (37.3)	6.4 (59.6)	9.6 (35.5)
I/s (last shell)	20.2 (4.9)	18.0 (1.9)	16.0 (2.5)	22.9 (4.6)	14.9 (2.3)	18.6 (5.9)

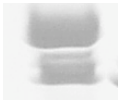
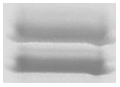
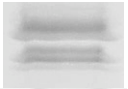
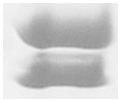
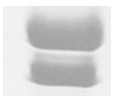
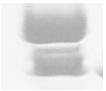
B. MIRAS phasing statistics

Resolution range (Å)	30.0 – 3.0					
No. heavy atom sites		2	2	2	2	1
Mean figure of merit	0.43					

C. Data set used for refinement and refinement statistics

Data Set	Native
Wavelength (Å)	0.9794
Resolution limit (Å)	2.6
Space group	P64
No. molecules in ASU	1
Cell dimensions (Å)	
a = b	125.8
c	35.6
Reflections observed	91205
Unique reflections	10199
Multiplicity (last shell)	8.9 (9.1)
% Completeness (last shell)	99.7 (100.0)
R_{sym} (last shell)	8.5 (58.8)
I/s (last shell)	15.5 (4.4)
Resolution range (Å)	30 - 2.6
R_{work}/R_{free}	20.5/28.7
No. reflections for refinement	9187
No. reflections in test set (%)	1010 (9.9)
No. solvent molecules	73
Average B factor (Å^2)	59.6
rms bond length deviation	0.011
rms bond angle deviation	1.278
Ramachandran plot (residues in):	
most favored regions (%)	90.5
additionally allowed regions (%)	9.0
generously allowed regions (%)	0.5
disallowed regions (%)	0
Residues omitted from final model	684-686

Table II. Binding studies of SG/mutants to glutathione-immobilized calmodulin

Experiment	SG binding to GST-CaM		TM(A) binding to GST-CaM		TM(D/E) binding to GST-CaM		PM(D/E) binding to GST-CaM	
	Gel	Band ratio	Gel	Band ratio	Gel	Band ratio	Gel	Band ratio
1		0.94		0.80		0.75		0.63
2		1.04		0.86		0.67		0.50
3		0.9		0.78		0.70		0.62
Average		0.96		0.81		0.71		0.58
Std. Dev.		0.07		0.04		0.04		0.07

SG: The wild-type sequence of the SH3-GUK module of ZO-1
TM(A): Triple mutant in which K749, R752, and K753 were replaced by alanines
TM(D/E): Triple mutant K749D-R752D-K753E
PM(D/E): Penta mutant K749D-R752D-K753E-K760E-K763E
The top band is GST-CaM. The lower band is SG/mutant.
Band ratio is the ratio of integrated band intensity of SG/mutant versus that of GST-CaM.
The average is calculated from three independent experiments, and the standard deviation is shown.

Figure S1