Crystal-Structure and Biochemical Characterization of Recombinant Human Calcyphosine Delineates a Novel EF-Hand-Containing Protein Family

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Calcypshosine is an EF-hand protein involved in both Ca²⁺-phosphatidylinositol and cyclic AMP signal cascades, as well as in other cellular functions. The crystal structure of Ca²⁺-loaded calcypshosine was determined up to 2.65 Å resolution and reveals a protein containing two pairs of Ca²⁺-binding EF-hand motifs. Calcypshosine shares a highly similar overall topology with calmodulin. However, there are striking differences between EF-hand 4, both N-terminal and C-terminal regions, and interdomain linkers. The C-terminal domain of calcypshosine possesses a large hydrophobic pocket in the presence of calcium ions that might be implicated in ligand binding, while its N-terminal hydrophobic pocket is almost shielded by an additional terminal helix. Calcypshosine is largely monomeric, regardless of the presence of Ca²⁺. Differences in structure, oligomeric state in the presence and in the absence of Ca²⁺, a highly conserved sequence with low similarity to other proteins, and phylogeny define a new EF-hand-containing family of calcypshosine proteins that extends from arthropods to humans.

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Calcium-phosphatidylinositol and cyclic AMP (cAMP) cascades play important roles in the regulation of cell function, proliferation, and differentiation. For example, both cascades trigger cell proliferation in the thyroid, but have opposite effects on thyrocyte differentiation.¹ Cross-regulations between these two cascades may involve the modulation of the receptor–G protein–cyclase or phospholipase C complexes, downstream of intracellular signal disposal systems or reciprocal controls on target enzyme systems.² Proteins regulated by both calcium and cAMP in the intersection of the two cascades are therefore of major interest for the study of regulatory mechanisms that contribute to cell proliferation and differentiation.

Calcypshosine was originally isolated from the canine thyroid cDNA library as a major phosphorylated substrate for protein kinase A in a cAMP-dependent manner, in response to stimulation of canine thyroid cells by thyrotrpin.³ Its synthesis is up-regulated by cAMP and thyrotrpin, which trigger cell proliferation and maintain expression of the differentiated thyrocyte phenotype, and is down-regulated by epidermal growth factor and tumor promotion activator that repress expression of differentiation.⁴ Although the exact function of calcypshosine remains unknown, it is thought to be implicated in the cross-signaling between cAMP and calcium-phosphatidylinositol cascades to coordinate cellular proliferation and differentiation in the thyroid. Furthermore, another member of the calcypshosine

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Abbreviations used: CaM, calmodulin; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N‘-tetraacetic acid; PDB, Protein Data Bank.
family, the R2D5 antigen sharing an 86% amino acid sequence identity with canine calcyphosine, is highly localized to olfactory receptor neurons. R2D5 is highly enriched in a variety of ependymal cells, and its phosphorylation by calmodulin (CaM)-dependent kinase II and protein kinase A may be involved in the cross-talk between cAMP and IP3/Ca2+-mediated pathways in olfactory receptor neurons. Recent biochemical studies revealed an aberrant expression of calcyphosine in pediatric primitive neuroectodermal tumors and ependymomas. Calcyphosine was thus identified as a tumor-specific protein that might be a tumor marker of a new subgroup of ependymomas and as a potential drug target for therapy in pediatric brain tumors.

Calcyphosine has also been detected in other species by immunohistochemistry and Western or Northern blot analysis. It is highly conserved among canine, rabbit, bovine, and human species, but is absent from mouse and five other rodents. In situ hybridization demonstrated that the human calcyphosine gene is localized to the p13.3 region of chromosome 19. Cloning and sequencing of human calcyphosine showed that the 189 encoded amino acids contain one putative site for phosphorylation by CaM-dependent protein kinase and share a sequence similarity with CaM—an important calcium-binding protein involved in the interaction with target proteins that translates the second-messenger calcium into a variety of cellular responses.

Calcyphosine was predicted to contain up to four EF-hand motifs on sequence analysis and was shown to bind calcium ions on autoradiography assay.

Further sequence analysis indicates that calcyphosine and its homologs form a protein family distinct from other calcium-binding protein families. In order to better understand the role of calcyphosine, we therefore initiated studies to elucidate the X-ray structure of calcyphosine. In this work, we reported the crystal structure of recombinant human calcyphosine at 2.65 Å resolution. The structure reveals the nature of calcium-binding EF-hand motifs and shows that calcyphosine is similar in overall folding topology to the structure of CaM, providing deep structural insights into the calcyphosine family.

### Table 1. Data collection and refinement statistics

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<td>Disallowed (%)</td>
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Notes to Table 1:
- SeMet, selenomethionine.
- Native human calcyphosine was expressed in Escherichia coli BL21 (DE3) cells transformed with plasmid pET-28a and purified using Ni-NTA column and Resource Q (GE Healthcare). For preparation of the SeMet derivative of calcyphosine, the pET-28a plasmid was used to transform B834 strain. Cells were grown overnight at 37 °C in minimal M9 medium containing 2% (wt/vol) glucose, 50 μg/ml kanamycin, 30 mg/L SeMet, and 6.5 g/L yeast nitrogen base without amino acid, and induced by IPTG for another 36 h at 16 °C. The purified protein was concentrated using a 10K ultrafiltration membrane (Filtron) to 20 mg/ml in a solution containing 20 mM Tris–HCl (pH 8.0). Crystallization experiments were performed at 16 °C using the hanging-drop vapor diffusion method. Protein solution (1.5 μl) was mixed with 1.5 μl of reservoir solution and equilibrated against 0.2 ml of reservoir solution. Native and SeMet crystals were grown in 0.1 M Pipes (pH 6.3), 20% (wt/vol) polyethylene glycol 8000, and 0.2 M CaCl₂. Crystals were grown for 2 days at 16 °C and disrupted by glass beads for microseeding. The crystals were soaked in a crystallization buffer containing 30% polyethylene glycol 8000 as cryoprotectant prior to X-ray data collection. Preliminary X-ray diffraction analysis of calcyphosine was performed at room temperature using an in-house Rigaku MM-007 X-ray generator (λ = 1.5418 Å) and a Mar345 dbt image plate. X-ray diffraction data from both native and SeMet-substituted crystals were collected at 100 K using ADSC Quantum 315 on beamline BL-5 at the Photon Factory (Tsukuba, Japan) at 2.8 Å and 2.65 Å, respectively. All data sets were processed using HKL2000. The structure was solved by single-wavelength anomalous diffraction. Anomalous Patterson maps were calculated with the program Solve. Experimental phases were calculated to 2.65 Å resolution and improved by solvent flattening with Solve. Several secondary structure elements were evident from the resulting experimental electron density map, enabling the construction of an initial model. Iterative model building was performed with Coot and alternated with refinement against the SeMet-substituted data up to 2.65 Å resolution using CNS. Numbers in parentheses correspond to the highest-resolution shell.

Overall structure of calcyphosine

The crystal structure of recombinant human calcyphosine has been determined by single-wavelength anomalous diffraction and refined to a working R-factor of 22.8% and an Rfree of 27.7% up to a resolution of 2.65 Å (Table 1). One percent of residues are in disallowed regions of the Ramachandran plot.
because the N-terminal part of the structure seems quite flexible and difficult to model. There are two calcyphosine molecules in the asymmetric unit. Each molecule is folded into two globular domains (N-terminal and C-terminal domains) composed of an α-helical structure with 11 α-helices (αA–αK) and 4 short β-strands (Fig. 1a). The helix–loop–helix motifs are arranged in two pairs—an N-terminal pair (EF1 and EF2) and a C-terminal pair (EF3 and EF4)—that are connected by the hinge loop 88–91 and related by an approximate 2-fold axis. The two terminal pairs of EF-hands can be superimposed with a root mean square deviation (r.m.s.d.) of 2.4 Å for all Cα atoms. All calcyphosine EF-hand motifs are involved in binding calcium ions. Furthermore, two pairs of Ca2+-binding EF-hand motifs in close proximity to each other indicate that the two pairs of calcium ions are separated by close distances of 11.1 Å and 12.2 Å, respectively. Aside from the four calcium ions binding to the EF-hand motifs, an

Fig. 1. Overall structure of calcyphosine and the four EF-hand motifs. (a) Ribbon representation with the numbering scheme of the overall structure showing the positions of the high-affinity Ca2+ (red spheres) bound to EF1–EF4 motifs. Regions are color-coded as follows: terminal helix αA and αK, green, EF1, red, EF2, blue, EF3, yellow, EF4, cyan. β-Strands, colored according to different EF-hand motifs, are also labeled β1–β4. Helices and termini are labeled; a dotted line indicates the disordered loop between helices αA and αB. (b) The ribbon diagrams of the EF-hand loops show individual amino acids involved in Ca2+ binding based on their positions (positions 1, 3, 5, 7, and 12 for EF1–EF3, and positions 1, 6, 8, and 13 for EF4) in the EF-hand loops. In this diagram, calcium ions are represented as green spheres. Relevant protein oxygen atoms are shown in red. Dashed lines indicate calcium ligation.
additional calcium ion was found in a position not typically associated with EF-hand domains. Overall, the N-terminal domain appears to be more compact than the C-terminal domain, since the helices that form the C-terminal EF-hand motifs are separated by a greater extent than those in the N-terminal domain. Aside from the four EF-hands, there are three additional helices: the N-terminal helix αA, the C-terminal helix αK, and helix αH within the unusually long linker region between αG and αI. Such a long linker is typically found in the neurocalcin subfamily and in other proteins such as calpain.

Monomeric state of calcyphosine

Many calcium-binding proteins with four EF-hand motifs crystallize as a dimer in the asymmetric unit, while they tend to form monomers or multimers under different conditions in vitro. For example, KChIP3 tends to form dimers and tetramers in a protein-concentration-dependent manner in vitro, while monomeric or dimeric states of GCAP-2 and neurocalcin depend on the presence or on the absence of calcium ions. To determine whether recombinant human calcyphosine exists as a homodimer in solution as it is in the crystal, we performed gel filtration and analytical ultracentrifugation. Recombinant human calcyphosine elutes at a volume corresponding to 31.0 kDa, in the presence of 1 mM CaCl₂ or 1 mM ethylene glycol bis(β-aminoethyl ether) N,N′-tetraacetic acid (EGTA), which is between its monomeric and dimeric molecular masses (Fig. 2a). Bearing in mind that calcyphosine is not a typical globular protein as evidenced by our structure, calcyphosine forms monomers in the presence or in the absence of Ca²⁺.

Fig. 2. Calcypohosine primarily forms monomers in the presence or in the absence of Ca²⁺. (a) Elution profiles of apo-calcyphosine and Ca²⁺-calcyphosine were determined using a Superdex S-75 column (GE Healthcare). The molecular mass standards used were aprotinin (6.512 kDa), ribonuclease A (13.7 kDa), albumin egg (45 kDa), and bovine serum albumin (67 kDa). (b) Analytical ultracentrifugation analysis of apo-calcyphosine and Ca²⁺-calcyphosine. Analytical ultracentrifugation was performed using a Beckman Optima XL-I Ultracentrifuge and a Ti-60 rotor. Purified full-length calcyphosine was equilibrated in a solution containing 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM CaCl₂, or 1 mM EGTA, respectively, to a final concentration with an A₂₈₀ value of 0.8–0.9. EPON double-sector centerpieces containing the protein solution and the sample buffer control, respectively, were centrifuged at 60,000 rpm for 5 h at 20 °C. (c) Hydrophobic interaction chromatography on a phenyl Sepharose column. The Ca²⁺ concentration was 1 mM, and the sample was loaded onto the column at a flow rate of 1 ml/min: 0–26 min with buffer containing 1 mM Ca²⁺, and 26–47 min with buffer containing 1 mM EGTA. A₂₈₀ was monitored. Elution with 1 mM EGTA produced a single peak with a retention time of 12 min corresponding to pure calcyphosine. (d) CD spectra of Ca²⁺-calcyphosine (solid line) and apocalcyphosine treated with 1 mM EGTA (dotted line).
monomers in solution in a calcium-independent manner in vitro. To confirm the gel-filtration results, analytical ultracentrifugation was also performed, and the final data were consistent with molecular masses of 20.6 kDa and 23.2 kDa for Ca\textsuperscript{2+}-loaded calcyphosine and apo-calcyphosine (Ca\textsuperscript{2+}-free calcyphosine), respectively, compared with a calculated value of 21.7 kDa (Fig. 2b). These data indicate that calcyphosine exists as a monomer both in the presence and in the absence of calcium ions, and this raises the question of whether the presence of calcium could induce conformational changes in calcyphosine in vitro.

A number of studies on calcium-binding proteins show that the protein undergoes a conformational change upon Ca\textsuperscript{2+} binding, resulting in the exposure of a hydrophobic patch at the surface of the protein. To examine whether human recombinant calcyphosine undergoes a Ca\textsuperscript{2+}-dependent conformational change, purified calcyphosine was loaded onto a phenyl Sepharose column in the presence of Ca\textsuperscript{2+} and eluted from the matrix with EGTA. Elution results show that the Ca\textsuperscript{2+}-calcysteine bound to the matrix could be eluted with EGTA (Fig. 2c). The secondary structure of the calcyphosine monomer was analyzed by UV–CD spectroscopy, and the resulting spectra for Ca\textsuperscript{2+}-calcysteine and apo-calcysteine were markedly different (Fig. 2d). These results suggest that the calcyphosine monomer changes its conformation in a calcium-dependent manner and is fully functional as a molecular switch.

**Calcium-binding sites**

The classical EF-hand motif is characterized by a loop of 12 residues that are involved in calcium binding.\textsuperscript{20} For many homologous EF-hand proteins, the loop begins with an aspartic acid residue and ends with a glutamic acid residue. For clarity, the position of a residue in the loop is identified in this article with a subscript (e.g., Asp\textsubscript{1}, Glu\textsubscript{12}). Calcium ions bind to canonical EF-hands with high affinity due to the residues at positions 1, 3, 5, 7, and 12. In most EF-hands, a water molecule hydrogen-bonded to a side-chain oxygen from position 7 completes the coordination sphere at one apex, yielding a pentagonal bipyramidal geometry.\textsuperscript{21}

The calcium-binding segments in the N-terminal domain consist of residues 34–45 and 70–81, with a short region of anti-parallel \(\beta\)-sheet interaction between these two calcium-binding loops. In EF1 of calcysteine (Fig. 1b), calcium coordination occurs through the side-chain carboxylates of Asp\textsubscript{34}, Asn\textsubscript{36}, Ser\textsubscript{38}, and Glu\textsubscript{45}; the main-chain carbonyl of Ser\textsubscript{40}; and an assumed water molecule not found in our structure due to low resolution. Calcium ion coordination in EF2 differs between the two calcysteine molecules in the asymmetric unit. In molecule A, calcium coordination occurs through the side-chain carboxylate atoms of Asp\textsubscript{70}, Asn\textsubscript{72}, Ser\textsubscript{74}, and Glu\textsubscript{81}; the main-chain carboxyl of Thr\textsubscript{76}; and an assumed water molecule (Fig. 1b). However, the water molecule in molecule B is replaced by the side-chain carboxylate of Glu\textsubscript{177} from an adjacent symmetry-related calcyphosine molecule, contributing an individual ligand to the coordination sphere. This type of Ca\textsuperscript{2+} coordination is also found in the second EF-hand of the RLC,\textsuperscript{22} in which the seventh ligand is provided by Glu\textsubscript{49} of a symmetry-related ELC molecule, instead of a water molecule found in other canonical EF-hand proteins.

In the C-terminal domain, residues 106–117 and 149–161 form the two functional EF-hand motifs. In EF3 (Fig. 1b), the most notable deviation from the typical EF-hand motif is the involvement of an Asp residue at position 12. The calcium ion is coordinated in typical pentagonal bipyramidal geometry by the side-chain carboxylate atoms of residues Asp\textsubscript{106}, Ser\textsubscript{108}, Asp\textsubscript{110}, and Asp\textsubscript{117}; the main-chain carbonyl of Val\textsubscript{112}; and an assumed water molecule. The sequence of EF4 differs markedly from that of the typical EF-hand. Although the loop of EF4 begins with an aspartic acid residue and ends with a glutamic acid residue, it is composed of 13 residues—a residue longer than the canonical EF-hand. However, a calcium ion is identified in EF4 and coordinated with pentagonal bipyramidal geometry by the side-chain carboxylate atoms of Asp\textsubscript{149}, Asp\textsubscript{154}, and Glu\textsubscript{161}; the main-chain carbonyl of Glu\textsubscript{156}; and two assumed water molecules (Fig. 1b). This type of Ca\textsuperscript{2+}-binding loop is similar to that of AtCBL2 EF1\textsuperscript{23}—a loop with 14 residues but coordinated with pentagonal–bipyramidal geometry by the side-chain carboxylate atoms of Asp\textsubscript{64} and Glu\textsubscript{71}; the main-chain carbonyl of Ser\textsubscript{58}, Ile\textsubscript{62}, and Leu\textsubscript{66}; and a water molecule.

**Comparison of calcyphosine with Ca\textsuperscript{2+}-loaded CaM**

The dendrogram in Fig. 3a depicts the sequence relationship between calcyphosine and a number of other Ca\textsuperscript{2+}-binding proteins with four EF-hand motifs found from a search of the Protein Data Bank (PDB). Based on our analysis, calcyphosine forms a family distinct from those of calcineurin B, revoverin, frequenin, neurocalcin, CaM, and other Ca\textsuperscript{2+}-binding proteins. Calcyphosine exhibits the closest homology to CaM, with approximately 30% sequence identity.

From a comparison of individual domain conformations between calcyphosine and Ca\textsuperscript{2+}-CaM (PDB code 1CLL) (Fig. 3b), a difference arises from the long disordered linker region between residues 122 and 137 in the structure of calcyphosine—a loop that is seven residues longer than the equivalent loop in Ca\textsuperscript{2+}-CaM. Such a long linker region is characteristic of the neurocalcin subfamily. Calcyphosine contains an additional helix (\(\alpha\)A) in the N terminus and a helix (\(\alpha\)K) in the C-terminal region. The conformation varies and affects the degree to which the hydrophobic pocket is exposed. In the C-terminal domain, it is fully exposed; in the N-terminal
domain, it is partly shielded from bulk solvent. These differences reflect a change in global shape. A large body of structural and biochemical data exists to show that the helices of the EF-hands reorient in relation to one another when Ca\textsuperscript{2+} is bound.\textsuperscript{24} From a comparison of interhelical angles between the helices of EF-hands for calcyphosine, Ca\textsuperscript{2+}-loaded CaM, and the compact form of Ca\textsuperscript{2+}-loaded CaM (PDB code 1prw),\textsuperscript{25} the N-terminal EF-hand angles in calcyphosine are 108° and 102°, respectively, which compare well with those of the compact form of Ca\textsuperscript{2+}-CaM but are relatively larger than those of Ca\textsuperscript{2+}-CaM. However, the C-terminal EF4 hand with an unusual calcium-binding form reveals unique interhelical packing information. The interhelical angle of EF4 in calcyphosine is 60°—an obvious departure of up to 25° and 35° from Ca\textsuperscript{2+}-CaM and the compact form of Ca\textsuperscript{2+}-CaM, respectively. Calcyphosine shows a slightly more ‘open’ and ‘deep’ C-terminal

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**Fig. 3.** Comparison of calcyphosine with CaM. (a) Evolutionary relationship of calcyphosine with some of its nearest homologs. The sequences of some typical Ca\textsuperscript{2+}-binding proteins with four EF-hand motifs were aligned using ClustalW, and the output tree file was displayed as a dendrogram using the program TreeView 1.6.6. The proteins are human proteins, unless stated in the dendrogram. (b) Superposition of calcyphosine and CaM (PDB code 1CLL) based on EF-hands 3 and 4. The color scheme is the same as for Fig. 1a, with CaM shown in magenta. All figures were prepared using PyMOL (http://www.pymol.org). (c) Sequence alignment of calcyphosine with CaM. N-terminal additional α-helix αA and C-terminal inserted α-helix αj are shown in blue boxes. Residues that correspond to the target recognition in the C-domain of CaM are highlighted with cyan spheres.
hydrophobic pocket and a strongly contracted N-terminal lobe.

**Novel hydrophobic pocket and its implication in target recognition**

The dominant and striking feature of the calcyphosine structure reported here is the contracted N-terminal domain and a slightly more open and deep C-terminal domain compared with those of the other EF-hand-containing proteins. For the C-domain, the most intriguing is the parallel orientation of additional helix $\alpha_K$ compared with $\alpha_F$, which is lacking from the Ca$^{2+}$-CaM structure. Sequence alignment of calcyphosine with CaM shows a highly conserved C-terminus from residues 154 to 184 in calcyphosine that compares well with residues 133–146 of CaM. The exception is a well-ordered 16-residue insertion from residues 159 to 175 in calcyphosine (Fig. 3c), which constitutes $\alpha_J$ and a hinge residue pair (Met173 and Asn174) that orient helix $\alpha_K$ parallel with $\alpha_F$. The helix $\alpha_K$ is packed against $\alpha_F$ through numerous hydrophobic interactions to assist the formation of the C-terminal hydrophobic pocket. This new conformation dramatically alters the solvent-accessible molecular surface of the molecule compared with that of Ca$^{2+}$-CaM. As a consequence, a large hydrophobic pocket is revealed in the C-terminal domain opposite to the two Ca$^{2+}$-binding EF-hands and made up of 29 residues from five helices.

For most EF-hand-containing proteins, the structural changes within each domain induced by the calcium ions in each of the EF-hand motifs create an open hydrophobic pocket to facilitate simultaneous binding to different targets. However, the N-domain hydrophobic pocket of calcyphosine is almost shielded by helix $\alpha_A$, despite the calcium ions-induced conformational change from closed EF-hands to opened forms. Helix $\alpha_A$ is unique to calcyphosine and is markedly different from the N-terminal $\alpha$-helices found in the recoverin branch of calcium-binding proteins, which are modified with fatty acid groups. In recoverin, the myristoyl group is sequestered into the hydrophobic pocket and hidden from the solvent in the absence of calcium ions. Once Ca$^{2+}$ is loaded, the myristoyl group extrudes from the hydrophobic pocket, leading to the translocation of recoverin from the cytosol to the disc membrane. In calcyphosine, helix $\alpha_A$ is tightly packed on top of the hydrophobic pocket through hydrophobic interactions between residues Met1, Val4, Met8, and Leu11, and those in the hydrophobic pocket. The opposing side of helix $\alpha_A$ is solvent-exposed and dominated by the acidic residues. No biochemical data to date have indicated the role of helix $\alpha_A$ in the target recognition of calcyphosine in the presence of calcium ions.

The most widely understood “wrap-around” mode of interaction with targets such as adenylyl cyclase EF, CaM-dependent kinase CaMKK, smMLCK, CaMKII, the Ca$^{2+}$-activated K$^+$ membrane channel, and the ryanodine receptor RYR1 suggests that CaM recognizes the positively charged amphipathic $\alpha$-helices of its ligands. This model was used as a basis to explore the possibility of calcyphosine adopting a similar target-binding mechanism. The superposition of the N-terminal and C-terminal domains of calcyphosine with the CaM–CaMKK complex shows that the target-binding interface would be completely engrossed by the N-terminal helix $\alpha_A$. In addition, the C-terminal helix $\alpha_K$ blocks the domain-domain linker and would render it unable to wrap around the target peptide unless dramatic conformational changes occur in this area (Fig. 4a).

In addition to the “wrap-around” binding model, CaM is also able to bind target proteins in an extended mode, such that both domains interact with different binding sites of the target. The CaM–EF complex has been well studied in this extended binding mode, in which the C-terminal domain of CaM is Ca$^{2+}$-loaded and adopts a conventional target-recognition mechanism through an exposed hydrophobic pocket, as described above. In contrast, the N-terminal lobe does not contain Ca$^{2+}$ and adopts a closed conformation, which causes it to contact the EF catalytic domain via the exterior surface. This complex structure can be used as a basis...
to examine whether calcyphosine may adopt a similar target-binding mechanism. Superposition of calcyphosine with the CaM–EF complex shows that a slight conformation change in helix αJ or an opposite orientation of the target amphipathic α-helix could make it fit well to this model. Interestingly, in contrast to the closed N-terminal EF-hand motifs in CaM, the hydrophobic pocket of the calcyphosine N-terminal domain is almost shielded by the additional αA helix. Assuming that calcyphosine interacts with target proteins similarly to this type of CaM, the αA helix might protect the calcyphosine hydrophobic pocket against long-term exposure to solvent in the absence of target binding. The hydrophobic pocket colors the molecular surfaces of the CaM–EF complex, and calcyphosine is traced in Fig. 4b. It demonstrates that calcyphosine has a pronounced hydrophobic pocket in the C-terminal domain, which leads to the hydrophobic core of the molecule. In contrast to the acidic patch surrounding the target-recognition site of CaM, the hydrophobic core of calcyphosine is dominated by basic residues, in particular His127, Lys129, and Arg143, which reside in the long linker region and helix αI, respectively.

**Conclusions**

The crystal structure of Ca²⁺-loaded calcyphosine reveals a significant structural similarity to other EF-hand proteins. It has four EF-hand motifs, and each binds Ca²⁺ through canonical interactions characteristic of EF-hand motifs, with the exception of EF4. By comparison with CaM, the most interesting new aspects of the structure of calcyphosine reported here are the additional helices αA and αK, the sharp turn in the domain linker, and a long linker region between residues 122 and 137. A family of proteins related to calcyphosine has been discovered to be highly conserved and distinct from the other EF-hand-containing protein families. Further structural studies of the calcyphosine family
with peptides from target proteins or on direct interaction with target proteins, in combination with further biochemical and cellular assays, will extend our understanding of the role of this protein in cellular signal cascades and other functions.

Accession numbers
Coordinates and structure factors have been deposited in the PDB with accession number 3E3R.

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