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

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Calcyphosine is an EF-hand protein involved in both Ca^{2+}-phosphatidylinositol and cyclic AMP signal cascades, as well as in other cellular functions. The crystal structure of Ca^{2+}-loaded calcyphosine was determined up to 2.65 Å resolution and reveals a protein containing two pairs of Ca^{2+}-binding EF-hand motifs. Calcyphosine 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 calcyphosine 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. Calcyphosine is largely monomeric, regardless of the presence of Ca^{2+}. Differences in structure, oligomeric state in the presence and in the absence of Ca^{2+}, a highly conserved sequence with low similarity to other proteins, and phylogeny define a new EF-hand-containing family of calcyphosine 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.

Calcyphosine 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 thyrotropin.[3] Its synthesis is up-regulated by cAMP and thyrotropin, 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 calcyphosine 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 calcyphosine

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Abbreviations used: CaM, calmodulin; EGTA, ethylene glycol bis(*b*-aminoethyl ether) *N*,*N*′-tetraacetic acid; PDB, Protein Data Bank.

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family, the R2D5 antigen sharing an 86% amino acid sequence identity with canine calcyphosine, is highly localized to olfactory receptor neurons.[5] 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 IP_3/Ca^{2+}-mediated pathways in olfactory receptor neurons. Recent biochemical studies revealed an aberrant expression of calcyphosine in pediatric primitive neuroectodermal tumors and ependymomas.[6] 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.[7] It is highly conserved among canine,[8,9] rabbit, bovine, and human species, but is absent from mouse and five other rodents.[10] *In situ* hybridization demonstrated that the human calcyphosine gene is localized to the p13.3 region of chromosome 19.[11] Cloning and sequencing of human calcyphosine showed that the 189 encoded amino acids contain one putative site for phosphorylation by cAMP-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

Data collection	Native	SeMet (peak)
Space group	$P2_12_12$	$P2_12_12$
Unit cell parameters		
a, *b*, *c* (Å)	*a* = 70.4, *b* = 132.0, *c* = 46.2	*a* = 70.6, *b* = 130.5, *c* = 46.2
α, β, γ (°)	α = 90, β = 90, γ = 90	α = 90, β = 90, γ = 90
Wavelength (Å)	1.0000	0.9792
Resolution (Å)	50.0 (2.9)[a]–2.8	50.0 (2.74)[a]–2.65
Average $I/\sigma(I)$	23.7 (5.0)	21.4 (2.8)[a]
Total reflections	37,953 (3538)[a]	80,048 (3648)[a]
Unique reflections	10,453 (931)[a]	12,799 (1073)[a]
Completeness (%)	94.5 (94.0)[a]	97.7 (83.9)[a]
R_{merge} (%)[b]	7.0 (25.5)[a]	10.3 (28.2)[a]

Refinement	SeMet
Resolution (Å)	50.0–2.65
Number of reflections used in the working set	11,457
Number of reflections used in the testing set	1262
Average *B*-factor (Å^2)	35.1
R-factor (%)[c]	22.8
Free *R*-factor (%)[c]	27.7
r.m.s.d.	
r.m.s.d. bond lengths (Å)	0.010
r.m.s.d. bond angles (°)	1.256
Ramachandran plot	
Most favored (%)	92.0
Allowed (%)	.0
Generously allowed (%)	0.0
Disallowed (%)	1.0

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 R_{free} 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

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 $CaCl_2$. 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 Mar345dtb 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.[12] The structure was solved by single-wavelength anomalous diffraction. Anomalous Patterson maps were calculated with the program Solve.[13] Experimental phases were calculated to 2.65 Å resolution and improved by solvent flattening with Resolve.[14] 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.[15]

[a] Numbers in parentheses correspond to the highest-resolution shell.

[b] $R_{merge} = \sum_i |I_i - \langle I \rangle| / \sum \langle I \rangle$, where I_i is an individual intensity measurement and $\langle I \rangle$ is the average intensity for all reflections *i*.

[c] $R_{work}/R_{free} = \sum ||F_o| - |F_c|| / \sum |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively.

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