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Functional Characterization of the Squid Calexcitin-2, a Calcium and GTP-binding Protein

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Calexcitin, a calcium-binding protein, was previously cloned and functionally characterized in the squid *Loligo pealei*. We now report the cloning of a second form of Calexcitin, Calexcitin-2, found in the squid *Todarodes pacificus* optic lobe. Calexcitin-2 has a significantly different carboxyl terminal region than Calexcitin-1. It lacks the CAAX motif, which is a farnesylation site. The amino acid sequence of Calexcitin-2 shows an 84% identity with Calexcitin-1 and also displays a strong cross immunoreactivity. Western blotting shows that Calexcitin-2 was expressed exclusively in the optic lobe region of squid, but not in other body organs. Regardless of its lack of conserved regions for GTP-binding, Calexcitin-2 shows moderately low affinity GTP-binding and also shows dramatic conformational change induced by GTP-binding. Three possible GTP-binding region mutations, K142A, D144A, and K157A, did not change the GTP binding affinity. This raises the possibility that Calexcitin-2 may have a novel GTP-binding motif.

Key words: Calcium-binding protein, Calexcitin, GTP-binding protein, Memory, Mutagenesis.

Introduction

Calexcitin, a 22 kDa calcium-binding protein, is found in the central nervous systems of marine snails and squid (Nelson *et al.*, 1990). This protein was one of the proteins that were phosphorylated after a light-rotation associative conditioning in the marine snail *Hermissenda*. It was thought to be a component of information processing machinery. When purified and microinjected back into *Hermissenda* photoreceptors, this protein was found to reduce the open times of the voltage-dependent K^+ channels whose conductances (i_A and $i_{Ca\text{-}K+}$) are decreased after light-rotation training in *Hermissenda*. This indicates that Calexcitin may play a role in associative conditioning that is related to information processing (Nelson *et al.*, 1996). Microinjection of Calexcitin into rabbit Purkinje cells, or hippocampal pyramidal cells, also blocks the voltage-dependent K^+ channels. This indicates the functional conservation of Calexcitin among the species (Nelson *et al.*, 1996). So far a single *calexcitin* cDNA sequence is reported from a species of squid, *Loligo pealei*. The cDNA sequence showed a close homology to several Ca^{2+}-binding proteins, particularly SCP-2 (Sarcoplasmic Calcium-binding Protein-2). Our previous results (Nelson *et al.*, 1996) showed that GTP inhibited phosphorylation of Calexcitin by protein kinase C (PKC), implying that this protein might be a GTP-binding protein as well as a calcium binding protein. However, it is still unknown how this protein binds GTP.

Here we report a *calexcitin* gene homologue, *calexcitin-2*, which is isolated from *Todarodes pacificus* that belongs to different suborder than *Loligo*. In this study, we investigate the GTP-binding properties of the Calexcitin-2 protein. We demonstrated *in vitro* that Calexcitin-2 bound GTP with a moderately low affinity and independent of the presence of Ca^{2+}-binding.

Materials and Methods

Cloning of calexcitin-2 gene A polymerase chain reaction (PCR) was used to obtain the portion of *calexcitin* cDNA that is constructed from squid *Todarodes pacificus*. Degenerate primers were designed based on the conserved regions between Calexcitin and SCP2 (Sarcoplasmic Calcium-binding Protein2), such as the second EF-hand motif and an amino-terminal region: CC-1f: 5' ACA TTA CTA CTA GCA ATC ACA ATG 3' (upstream of starting codon); CC-1r: 5' TCA ATA ATG TTG TCA CCN GAN GTY TC 3' (the second EF-hand motif: ^{119}DTSGDNIIH); CC-2f: 5' GAT ACA TCA GGT GAY AAY ATY ATY GA 3' (^{119}DTSGDNIIH); CC-2r: 5' ATG AAT ATA TTA AAA ATA AGG GAT AGG 3' (downstream of stop codon). First-strand cDNA (Pharmacia Biotech, Uppsala, Sweden) obtained from a total RNA (Qiagen, Germany) of the squid optic lobe, was used to

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amplify some portion of the *calexcitin* cDNA. Two specific PCR bands (400 & 254 bp) were obtained by adopting the following PCR procedure: primers: CC-1f & CC-1r; CC-2f & CC-2r, respectively; 94°C, 1 min; 43°C, 1 min; 72°C, 2 min; 35 cycles. Whole *calexcitin* cDNA was obtained by joining the above two fragments by PCR. It was cloned into a pGEM vector (Promega, Madison, USA) and then used to transform *Escherichia coli* DH5α cells. A plasmid containing a 620 base pair insert was sequenced.

GenBank Accession Number The *calexcitin-2* cDNA sequence has been deposited in the GenBank database under accession number AF078951.

Protein Purification *Nde*I and *Xho*I restriction sites were introduced into the ATG starting codon and 3' untranslated region of *calexcitin*-2 cDNA by PCR site-directed mutagenesis (primer A: 5 CAT ATC GAA CTG CATATG GCT GCC CAT CAA CTT 3 (*Nde*I); primer B: 5 CGA TGC GGC CG CTCGAG TAT ATT AAA AAT AAGG 3 (*Xho*I)). The 600 bp PCR products were cloned into *NdeI*/*Xho*I digested pET17b vector (Invitrogen, Netherlands). The recombinant plasmids were transformed into *E. coli* BL21 (DE3) cells. Expression of the Calexcitin-2 protein was induced with 0.1 mM isopropyl β-D-thiogalactoside at 37°C for 1 h. Cultures (400 ml) were pelleted and resuspended in a buffer containing10 mM phosphate buffer, pH 6.5, 1 mM EDTA, 1 mM β-mercaptoethanol and lysed by sonication. Calexcitin-2 proteins were purified by chromatography on a DEAE-Sephacel column and a Mono Q HR5/5 column of fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden). Fractions containing Calexcitin-2 were pooled and stored at -20°C for the subsequent studies. Purity of the preparation was confirmed by SDS-polyacrylamide gel electrophoresis. Concentrations of Calexcitin-2 were determined in 6 M guanidine hydrochloride using a value of molar extinction coefficient, $\varepsilon = 48{,}790$ at 280 nm, calculated from the tyrosine and tryptophan content of the protein (Kwon *et al.*, 1994) and based upon $M_r = 22{,}180$.

GTP-binding analysis GTP-binding to Calexcitin-2 was measured by incubating 1 μg of Calexcitin-2 with various concentrations of [γ-^{35}S]GTP in a 5 μl buffer containing 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ for 30 min at 20°C. The reaction mixture was filtered through a nitrocellulose filter and washed with PBS and the ^{35}S was measured by scintillation counting and autoradiography.

Calcium binding analysis $^{45}Ca^{2+}$ (1 μCi) was incubated with 1 μg Calexcitin-2 for 1 h at 20°C in 5 μl of buffer (50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$). The reaction mixture was filtered through a nitrocellulose filter and washed with PBS. $^{45}Ca^{2+}$ was measured by scintillation counting and autoradiography. For the ^{45}Ca-overlay experiments, 1 μg Calexcitin-2 was analyzed on a 12% SDS polyacrylamide gel, transferred to nitrocellulose, and probed by $^{45}CaCl_2$ (2 μCi, 90 μM) for 1 h at room temperature.

Western blot analysis Samples were analyzed by electrophoresis on a 12% SDS-polyacrylamide gel, followed by blotting onto nitrocellulose, stained by antibody, and visualized with 4-chloro-1-naphthol and hydrogen peroxide. Polyclonal antibodies were raised by injection of recombinant Calexcitin-2 protein (100 μg) into rabbits (Sambrook *et al.*, 1989).

GTP-induced conformational change Conformational change as a function of GTP was monitored by fluorescence spectroscopy (Kim *et al.*, 1995). Recombinant proteins was incubated in 10 mM potassium phosphate, 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and various concentrations of GTP (final pH 6.5) at 25°C. Samples were allowed to equilibrate for 2 h. The tryptophan fluorescence was measured for each sample (Shimadzu RF-5000 fluorescence spectrophotometer) with an excitation at 280 nm and an emission at 330 nm. The protein concentration for the conformational transition was 2 μg/ml.

Results and Discussion

Isolation of calexcitin-2 cDNA The *Calexcitin* gene (*calexcitin-1*) was previously cloned from the squid *Loligo peaeli* optic lobe. It appeared to encode a protein that binds calcium and interacts with neuronal ryanodine receptors (Nelson *et al.*, 1996 & 1999). In this study, we cloned a different *calexcitin* cDNA from the previous one and named it as a *calexcitin-2* cDNA. This *calexcitin-2* cDNA was obtained

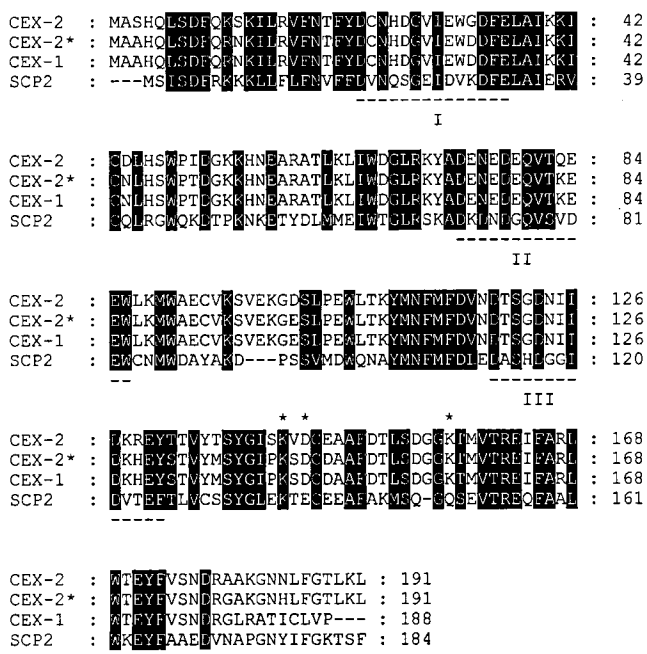

Fig. 1. Sequence comparison among Calexcitins. CEX-2 (Calexcitin-2; GenBank AF078951; *Todarodes pacificus*), CEX-2* (Calexcitin-2; AF080588; *Loligo pealei*), CEX-1 (Calexcitin-1; U49390; *Loligo pealei*)) and SCP2 (sarcoplasmic calcium-binding protein 2: AF014952; *Drosophila melanogaster*). Black bars indicate amino acid residues found among Calexcitins and SCP2. Potential calcium-binding motifs are indicated by horizontal lines and roman numerals (I-III). Amino acid residues, which were subjected to mutational analysis, were labeled by asterisks.

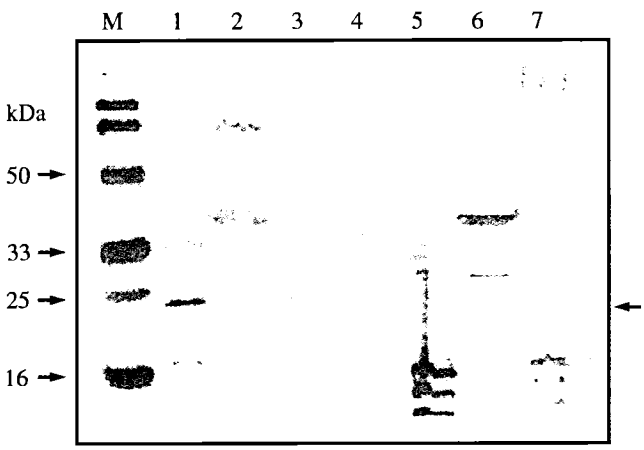

Fig. 2. Specific expression of Calexcitin-2 in the squid optic lobe. Cell extracts (5 μg protein/lane) from each organ were resolved by SDS-PAGE, electrotranferred to nitrocellulose, and then incubated with anti-Calexcitin-2 antibodies raised from rabbits for 1 h at room temperature. The expected size of Calexcitin-2 (22 kDa) is indicated by an arrow. Lane M, size marker; lanel, optic lobe; lane 2, stomach; lane 3, intestine; lane 4, heart; lane 5, kidney; lane 6, muscle; lane 7, gill

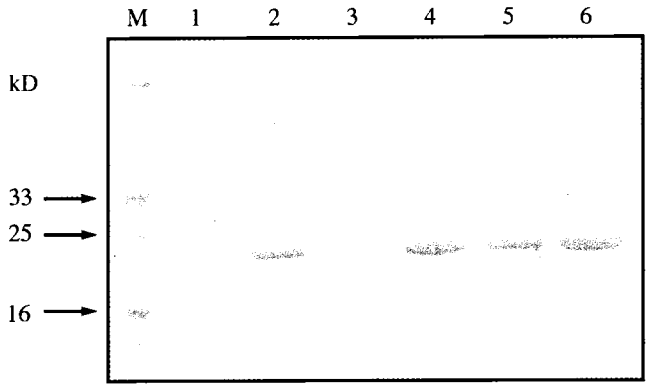

Fig 3. Purification of recombinant Calexcitin-2 in *E. coli*. *E. coli* cells containing *calexcitin-2* cDNA were grown without (lane 1) or with 0.1 mM IPTG (lane 2). Cells containing Calexcitin-2 were lysed and subjected to centrifugation, leading to pellet (lane 3) and supernatant (lane 4). Calexcitin-2 fractions were obtained by DEAE-Sephacel (lane 5) and Mono Q column chromatography (lane 6). Proteins were analyzed by SDS-polyacrylamide (12%) gel electrophoresis and visualized by Coomassie staining.

from the squid *Todarodes pacificus* that belongs to a different suborder than *Loligo*. It had a single base addition, compared to the *calexcitin-1* cDNA, and was predicted to encode 191 amino acid residues compared with the 188 residues of Calexcitin-1 (Fig. 1). The comparison of the predicted Calexcitin-2 with Calexcitin-1 showed an 84% identity (164/188). Surprisingly, the C-terminal region of Calexcitin-2 does not contain the CAAX motif, a farnesylation site, while Calexcitin-1 does ($CLV^{188}P$; Fig. 1). However, three calcium-binding EF-hand motifs were conserved in the *calexcitin-2* gene (Fig. 1; positions 23-36, 73-86 and 119-131). The predicted Calexcitin-2 sequence showed the highest homology to *Drosophila* SCP2 (Sarcoplasmic Calcium-binding Protein-2), a calcium-binding protein that is expressed exclusively in the central nervous system (45% identity and 59% homology; Fig 1; Kelly *et al.*, 1997; Adams *et al.*, 2000). However, it is still unclear whether or not SCP2 is a fruit fly homologue for Calexcitin-2 because an antibody for SCP2 did not cross-react to Calexcitin-2 or vice versa (data not shown). In our screening work, we could not find a *calexcitin-1* cDNA from the *Todarodes pacificus* optic lobe. This implies that Calexcitin-2 is a major form in the optic lobe. However, an analysis of the cDNA clones showed that both *calexcitin-1* and *calexcitin-2* cDNA (92.6% identity to *T. pacificus* Calexcitin-2; Fig. 1) were found in the *Loligo peaeli* optic lobe. This indicates a possible alternative splicing in the *calexcitin* gene. On the other hand, for *Drosophila* SCP2, it is unlikely that there is any corresponding spliced form, since it consists of a long exon V (amino acid residues 82-184; GenBank no. AE003714; Adams *et al.*, 2000).

Localization of Calexcitin-2 To examine the expression of Calexcitin-2 in the squid (*Todarodes pacificus*), we carried out a western blot analysis by using an anti-Calexcitin-2 antibody, which was raised by injection of the purified recombinant Calexcitin-2 into rabbits. Calexcitin-2 proteins were exclusively detected in the optic lobe, but not detected in the gill, heart, intestine, kidney, muscle, or stomach (Fig. 2). However, we cannot exclude the possibility that the 22 kDa band (Fig 2. arrow) contains some Calexcitin-1 protein, because the antibody against Calexcitin-2 still recognizes the Calexcitin-1 protein. Other bands appeared, especially on land 4 and 5, that seem likely to be nonspecific because they appeared as major proteins on coomassie stained gels (data not shown). It is interesting that SCP2, the protein most similar to Calexcitin thus far, is also found only in the nervous system of *Drosophila*. This implies possible conserved roles in brain function.

Purification of Recombinant Calexcitin-2 and its mutants. Induction of *E. coli* that contained *calexcitin-2* cDNA resulted in a major accumulation of a 22 kDa protein, as assessed by SDS-PAGE and Coomassie staining (over 30% among total proteins; Fig. 3). This 22 kDa band was confirmed by cross-reaction with an anti-Calexcitin-1 antibody, before further purification, to be Calexcitin-2. Most of the Calexcitin-2 was recovered from the soluble fraction of the *E. coli* lysates (Fig. 3, lane 4). Calexcitin-2 that was bound to DEAE-Sepharose at pH 6.5 was then eluted at 50 mM NaCl. Most of the contaminating proteins were resolved from Calexcitin-2 by MonoQ column chromatography and yielded over 95% purity, which was assessed by Coomassie staining (Fig. 3, lane 6). All of the mutant Calexcitin-2 were also soluble in *E. coli* after over-expression, and subjected to the same procedure as that of wild type.

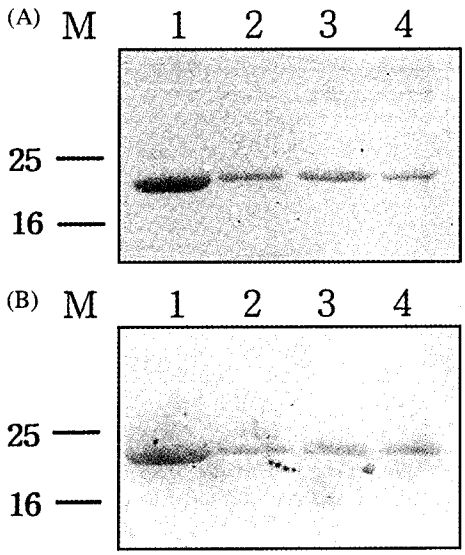

Fig. 4. GTP-binding characteristics of several Calexcitin-2 mutants. Recombinant Calexcitin-2 were resolved by SDS-PAGE and visualized by Coomassie staining (A) or electrotransferred to nitrocellulose and then incubated with [γ-^{35}S]GTP (1 μCi) for 20 min at room temperature (B). The nitrocellulose was washed for 1 h, dried, and subjected to autoradiography. Lane 1, wild type Calexcitin-2; lane 2, K142A; lane 3, D144A; lane 4, K157A. Lane M indicates the size marker (kDa).

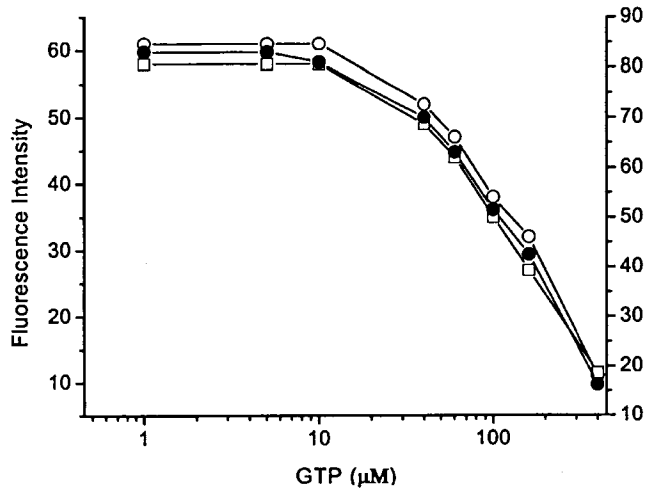

Fig. 5. GTP-induced conformational transitions of wild type Calexcitin-2 (●), K142A (○), and K157A (□) as measured by the decrease in fluorescence emission intensity at 330 nm (ε_{ex} = 280 nm). The fluorescence intensity for K157A was indicated at the right axis of the graph. Samples were equilibrated for 2 h in 10 mM phosphate (pH 6.5), 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 5 mM $MgCl_2$, and various concentrations of GTP at 25°C. The protein concentration was 2 μg/ml. The fluorescence signals for GTP only were between 6 to 11. The graph is representative of three repeated experiments.

GTP-binding properties. Our previous results showed that the addition of GTP at a high concentration (1 mM) inhibited phosphorylation of Calexcitin by protein kinase C (Nelson *et al.*, 1996). However, the GTP-binding characteristics of Calexcitin, and its implication in cell signaling, are still unknown. In order to examine the GTP-binding properties, we first carried out a GTP overlay assay with purified recombinant Calexcitin-2 (Fig. 4B). We found that the Calexcitin-2 bound GTP by overlaying 1 μCi of [^{35}S]GTP. This binding was not interrupted by adding 200 μM of GDP or ATP, indicating GTP-specific (data not shown). A dot blot analysis also showed that GTP-binding to Calexcitin-2 is specific. This is proven by uninterrupted binding of [^{35}S]GTP to Calexcitin-2, with the addition of GDP or ATP to 200 M (data not shown). However, a dot blot analysis showed no saturation of GTP-binding with labeled [^{35}S]GTP, even at a 600 nM concentration. This indicates that the binding affinity for GTP might be much higher than 0.6 μM.

In order to examine the conformational transition of Calexcitin-2 that is induced by GTP-binding, and to further determine the binding affinity constant toward GTP, a Calexcitin-2 sample was subjected to equilibrium conformational transition toward GTP. The transition was then monitored by intrinsic tryptophan fluorescence intensity at 330 nm (Kim *et al.*, 1995). The conformational transition midpoint appears to be around 100 μM of GTP (Fig. 5). This indicates that Calexcitin-2 binds GTP with a moderately low affinity and GTP-binding in turn induces a conformational change of Calexcitin-2. This low affinity GTP-binding is not unusual. Similar GTP-binding is shown in the Dynamin GTP-binding protein (34-100 μM), which is involved in synaptic vesicle endocytosis (Tuma & Collins, 1994; Sweitzer & Hinshaw, 1998). In this assay, GTP-binding to Calexcitin-2 appeared not to be saturated, because a high GTP concentration (>160 μM) resulted in significant signal quenching in intrinsic GTP fluorescence, leading to the complexity of its fluorescence signal. However, specificity for GTP-binding to Calexcitin-2 is still proven by the result that the addition of GDP showed no significant fluorescence signal change of Calexcitin-2, even at 200 μM (17% fluorescence reduction compared to base line) and the addition of ATP showed no fluorescence signal change at 200 μM. Taken together, these results indicate that Calexcitin-2 binds GTP specifically in moderately low affinity.

To determine the binding site for GTP, we introduced three site-directed mutations in Calexcitin-2 and analyzed the GTP-binding affinity for those mutants. Because there is no conserved GTP-binding motif in Calexcitin-2, two possible sites for GTP-binding were changed. The first region contains S^{142}KVD residues, which looks similar to the NKXD conserved region in the GTP-binding protein superfamily. In the region, a Lys residue was reported to play an important role in binding to ribose of GTP and the Asp residue was known to be involved in hydrogen bonding with the guanine of GTP (Bourne *et al.*, 1991). The mutant K142A, or D144A, showed no change in GTP-binding, indicating that the Lys or

Asp residues of Calexcitin-2 might not be involved in GTP-binding (Fig. 4B & Fig. 5; Bourne *et al.*, 1991). Analysis of the second residue from $G^{157}KT$, which is a possible conserved region of GxxxxGKT in small GTP-binding proteins, revealed that the Lys residue is not involved in GTP-binding because the K157A mutant showed wild type GTP-binding characteristics (Fig. 4B & Fig. 5). The Lys residue in the GKT motif is known to stabilize the location of β-phosphate of GTP. Thus, our results imply that an unknown motif in Calexcitin-2 may play a role in GTP-binding.

GTPase activity in Calexcitin-2 was not detected by using [γ-^{32}P]GTP (data not shown). A [γ-^{32}P]GTP-overlay and dot blot analysis showed that Calexcitin-2 might have a low GTPase activity *in vitro*, suggesting that Calexcitin-2 may require some kind of GTPase activating factors *in vivo*. It is interesting to note that the Dynamin GTPase, which has a low GTP-binding affinity, has no intrinsic GTPase activity that is activated by PKC phosphorylation (Robinson *et al.*, 1993). Similarly, the PKC-induced phosphorylation of Calexcitin-2 might increase GTPase activity (Nelson *et al.*, 1996).

In conclusion, in this study we have demonstrated that Calexcitin-2 binds GTP with moderately low affinity and GTP-binding in turn induces a conformational change of Calexcitin-2. Our results suggest that Calexcitin-2 may be a potential target for both GTP and calcium and may play some role in the optic lobe by mediating both calcium- and the GTP-related signal.

Acknowledgments

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