A Potential Dimerization Region of dCAMTA Is Critical for Termination of Fly Visual Response*

Ping Gong, Junhai Han, Keith Reddig, and Hong-Sheng Li

From the Department of Neurobiology, University of Massachusetts Medical School, Worcester, Massachusetts 01605

CAMTAs are a group of Ca\(^{2+}\)/calmodulin binding transcription activators that are implicated in brain tumor suppression, cardiac hypertrophy, and plant sensory responses. The sole fly CAMTA, dCAMTA, stimulates expression of an F-box gene, dFbxl4, to potentiate rhodopsin deactivation, which enables rapid termination of fly visual responses. Here we report that a dCAMTA fragment associated with a full-length protein in co-transfected human embryonic kidney 293 cells. The interaction site was mapped to a region within the DNA-binding CG-1 domain. With this potential dimerization site mutated, the full-length dCAMTA had defective nuclear localization. In transgenic flies, this mutant dCAMTA variant failed to stimulate expression of dFbxl4 and rescue the slow termination of light response phenotype of a dCAMTA null mutant fly. Our data suggest that dCAMTA may function as a dimer during fly visual regulation and that the CG-1 domain may mediate dimerization of CAMTA transcription factors.

Ca\(^{2+}\) ions exert long-term regulation of a broad range of cell activities by altering specific gene expression (1–4), which frequently involves the Ca\(^{2+}\) sensor calmodulin. A group of calmodulin binding transcription activators (CAMTAs)\(^{2}\) have recently been identified in a variety of multicellular organisms. These include six in plants, two in mammals, one in flies, and one in worms (5, 6). They all contain a DNA-binding domain (CG-1 domain) in the N-terminal and two to four IQ motifs in the C-terminal region. Using the fly CAMTA (dCAMTA) as an example, we have demonstrated that the CAMTA transcription activity is stimulated in vivo by direct interaction with calmodulin through the IQ motif region (7). Compared with transcription factors such as the CAMP response element-binding protein and the nuclear factors of activated T cells, which are stimulated through a series of calmodulin-dependent protein kinases or phosphatases (8–10), CAMTAs may respond to Ca\(^{2+}\)/calmodulin in a more rapid and reliable manner.

A human CAMTA, CAMTA1, is expressed specifically in the brain and has been identified as a candidate suppressor of both neuroblastomas and oligodendrogliomas (11–14). The mouse CAMTA2 has been reported to mediate cardiac hypertrophy (15). Thus, it is of both theoretical and clinical significance to characterize the transcription activities of CAMTA.

The CG-1 domain of CAMTA recognizes specific CGCG box-containing DNA sequences (5, 6), and a nearby region activates the transcription (5). Interestingly, the domain architecture of CAMTAs is very similar to that of the NFκB transcription factor precursors p100, p105, and Relish (16, 17). Both groups of proteins have a TIG domain and several ankyrin repeats between the N-terminal DNA-binding domain and the C-terminal regulatory region. Considering that all NFκB transcription factors are either homo- or heterodimers (16–19), it is important to examine whether CAMTAs may also function as dimers.

We have been using dCAMTA as a model to study the transcription activity of CAMTA and have recently demonstrated that dCAMTA stimulates expression of an F-box and leucine-rich repeat gene dFbxl4 both in the fly eye and in transfected human embryonic kidney 293 cells (7). In fly photoreceptor cells, the dCAMTA-promoted dFbxl4 expression is required for rapid deactivation of the G protein-coupled light receptor, rhodopsin. Due to the deficiency of dFbxl4, light responses in the eye of dCAMTA mutant flies terminate much more slowly than wild type (7).

In transfected 293 cells, dCAMTA stimulates expression of a luciferase reporter gene through a dFbxl4 promoter sequence that contains a CGCG box (7). Here we report that in the same system, a CG-1 domain-containing dCAMTA fragment (referred to as CG fragment) had much lower transcription activity than the full-length protein due to defective nuclear transport of the fragment. Remarkably, in co-transfected cells the CG fragment associated with and prevented nuclear transport of a full-length dCAMTA protein. The interaction site was mapped to a region within the CG-1 domain. These data suggest that dCAMTA may dimerize through the mapped region. We further demonstrated that the potential dimerization of full-length dCAMTA protein is required for its nuclear localization and for its visual regulation function in the fly eye (7).

**EXPERIMENTAL PROCEDURES**

**DNA Construction and Mutagenesis**—The plasmid pCMV-dCAMTA-Myc has a full-length dCAMTA cDNA (including 21-bp 5’-untranslated region) inserted at the HindIII/XhoI sites of a pCMV-TAG5C vector (Stratagene). Plasmids pCMV-FLAG-CG and pCMV-FLAG-TA contain the CG and the TA

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fragment, respectively, between the BamHI and EcoRI sites of pCMV-Tag2B (Stratagene). The plasmids for GST-CG and GST-TA fusions were constructed by inserting an EcoRV and a Sall/Eagl fragment of dCAMTA into the SmaI and Sall/NotI sites, respectively, of the vector pGEX-5X-2 (Stratagene). Plasmids for truncated CG fragments were generated by restriction digestion and ligation of the pGEX-5X-CG construct. Site-directed mutagenesis of pGEX-5X-CG was conducted using mutation-containing primers and a QuickChange kit (Stratagene). Mutant fragments were subcloned into a pcDNA3-dCAMTA plasmid to generate a full-length dCAMTA mutant, which was subsequently transferred into the vector pCMV-Tag2B or pCaSpeR-hs.

Cell Transfection and Immunohistochemistry—Using a Polyfect transfection reagent (Qiagen), the plasmids were introduced into 293 cells that were cultured on coated coverslips (2-cm, round) in 6-cm dishes. Two days after transfection, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline, washed twice, and incubated with mouse anti-Myc, rabbit anti-FLAG, or both antibodies (diluted 1:200 in phosphate-buffered saline; Sigma). After washes, fluorescein isothiocyanate- and TRITC-conjugated secondary antibodies (Sigma) were used to label the Myc and FLAG signals, respectively. The stained coverslips were mounted to slides using a 4',6-diamidino-2-phenylindole-containing VectorShield medium (Vector Labs).

Luciferase Assay—A dfbxl4 promoter sequence (−625 to −1+87) was inserted upstream to a luciferase gene in a modified pGL3-basic vector (Promega) from which an intrinsic CGCG box (in the multiple cloning region) had been removed. The construct or the vector (for control) was introduced into 293 cells alone or together with the dCAMTA DNAs. After 24 h, cells were harvested and examined with a luciferase assay system (Promega).

Glutathione-Sepharose Binding Assay—GST fusion proteins were expressed in BL-21 cells and purified with glutathione-Sepharose beads (Amersham Biosciences). Approximately 20 μg of each protein was coupled again to 40 μl of glutathione-Sepharose and incubated with head extracts (in phosphate-buffered saline that contained 1% Triton X-100 and protease inhibitors) of 200 heat-shocked p[hs-dCAMTA];tes2 flies (7) or with 0.5 ml of 293 cell extracts in Tris-buffered saline. After three washes with Tris-buffered saline, proteins were eluted with 20 μl of glutathione in TBS and subjected to SDS-PAGE and Western blotting.

Fly Genetics—Both wild type and tes2 flies had a w1118 background. To generate dCAMTA transgenic flies, a wild type dCAMTA cDNA or mutant variants were subcloned into a pCaSpeR-hs vector, and injected into w; tes2 flies. All flies were raised in an approximate 12-h light/12-h dark cycle. To drive expression of dCAMTA through the heat shock promoter, flies were shocked at late pupal stage by immersing the fly vials in a 37 °C water bath for 1 h. All flies were examined at 1–2 days old.

Real-time Reverse Transcription PCR—Total RNA was extracted from 1- to 3-day-old fly heads using the TRIzol reagent (Invitrogen). A 1-h heat shock was applied to the fly 10 h before the extraction. Real-time reverse transcription PCR was conducted using an ABI PRISM7700 and a SuperScript III Plati-

number One-Step kit (Invitrogen). The raw mRNA level was determined as 2−ΔCT, where CT is the number of cycles required for the SYBR Green fluorescence to cross the threshold of 30 arbitrary fluorescence units. The relative mRNA levels were calculated after setting the raw level of each gene in the wild type fly 100%. The primer pair for dfbxl4 was ctgctacaagaaacgct/ggcagcttgagccaat-ag, which produces a PCR fragment of 189 bp.

Electrophoresis Mobility Shift Assays—The DNA probe CCAA-CAGTGCATGGGCAAGCTGCACCGG (or CGGG)CA-CATTGCGGCAGATGAG was synthesized with overlapping primer pairs using PCR and labeled with 3′-biotin (Pierce). One microgram of GST or GST-CG fragment (with Arg483, 484Glu mutations) was incubated with 10 fmol-labeled probes, either with or without the presence of 2 pmol-unlabeled probes, in 20 μl of binding solution (Pierce lightshift chemiluminescent electrophoretic mobility shift assay kit) for 20 min, and then loaded to 6% PAGE gel. The signal was developed according to the manual included in the kit.

RESULTS

A CG-1 Domain-containing Fragment Localized a Full-length dCAMTA Protein in the Cytoplasm.—In a study for mapping functional domains of dCAMTA, we found that a CG-1 domain-containing fragment (Fig. 1A, CGfragment) was able to stimulate expression of a luciferase reporter gene through a dfbxl4 promoter (7) in transfected human embryonic kidney 293 cells (Fig. 1B). Thus, this fragment may also contain a transcription activation region that is next to the CG-1 domain (5, 15). Nonetheless, the CG fragment displayed a much lower activity of transcription compared with a full-length dCAMTA protein (1.37 ± 0.33 versus 2.93 ± 0.57, Fig. 1B).

To attempt to explain why this fragment is less efficient in transcription activation, we used tag peptide antibodies to examine the distributions of the fragment (with a FLAG tag) and the full-length protein (with a Myc tag) in the transfected cells. The result showed that in contrast to the nuclear localization of the full-length protein, most CG fragment signals were observed in the cytoplasm (Fig. 1C), suggesting that a nuclear localization sequence (NLS) is missing in the fragment. The low level of transcription activity of the CG fragment could have been contributed by the residual amount of proteins in the nuclei. Surprisingly, when the full-length protein and the CG fragment were transfected together into the same cells, both proteins failed to enter the nuclei (Fig. 1D). As a consequence, the luciferase expression level in the co-transfected cells (0.77 ± 0.21) was even lower than that of the CG fragment alone (Fig. 1B). In the cytoplasm, the full-length protein and the fragment appeared to be co-localized in the same areas (Fig. 1D), which could be because of dimerization of these two proteins and because the formed heterodimer was somehow unable to enter the nucleus. As the CG fragment displayed a higher protein
level than the full-length protein in Western blot (Fig. 1E), it is likely that most full-length proteins had been retained in the cytoplasm by the fragment.

To examine whether the CG fragment interacts with its counterpart in the full-length protein, we attempted to pull down FLAG-tagged dCAMTA fragments from 293 cell extracts using the GST-CG fusion protein. The result showed that the FLAG-CG, but not a FLAG-TA fragment, bound to the GST-CG protein (Fig. 2C). In control experiments, the GST-TA fusion protein did not pull down either fragment from the cell extracts (Fig. 2C).

The above observations suggest that a region in the CG fragment may mediate homodimerization of dCAMTA. To map this region, we truncated the GST-CG fusion protein into shorter fragments and tested their binding to the FLAG-CG. The fragments amino acids 274–535 or amino acids 274–509, but not amino acids 274–445, pulled down the FLAG-CG from cell extracts (Fig. 3A). The data indicate that the region amino acids 445–509, which is entirely included in the CG-1 domain, is critical for the interaction between CG fragments. Most residues in this mapped region are conserved among CAMTA proteins from different species (Fig. 3B). We created two adjacent mutations, Arg<sup>483</sup> and Arg<sup>484</sup>, to glutamic acid (E), in the GST-CG fragment. In glutathione-Sepharose

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**FIGURE 1.** A CG fragment of dCAMTA prevented the full-length protein from transporting into the nucleus in co-transfected 293 cells. A, domain architecture of the dCAMTA molecule and the location of the CG fragment. B, dFbxl4 promoter-mediated expression of luciferase in 293 cells that were transfected with a full-length dCAMTA, the CG fragment DNA, or both. The co-transfected cells displayed the lowest luciferase activity. The pCMV vector DNA was used as control. Three sets of data were averaged, and S.E. are shown as error bars. C, in 293 cells transfected separately with relevant DNAs, a full-length dCAMTA protein, but not the CG fragment, is localized in the nucleii. The proteins were stained with antibodies against the tag peptides, and the nucleus was labeled with 4′,6-diamidino-2-phenylindole. Dashed lines indicate the boundaries of cells. D, the full-length dCAMTA protein co-localized with the CG fragment and failed to be transported into the nucleus in the co-transfected cells. E, in co-transfected cells, the CG fragment had higher protein level than the full-length (f.l.). The Western blot was probed with an antibody against the CG fragment (7). Untransfected cells were used as control.
binding assays, these mutations completely disrupted the binding between the GST- and the FLAG-CG fragments (Fig. 3C), suggesting that the residues Arg483 and Arg484 are required for dimerization of the CG fragment.

To test whether the residues Arg483 and Arg484 mediate the CG fragment/full-length protein association in the 293 cells, we introduced a FLAG-tagged R483E,R484E mutant CG fragment into the cell together with the full-length dCAMTA and examined their intracellular distributions. In the co-transfected cells, most fragment signals were still observed in the cytoplasm, whereas virtually all full-length proteins were localized in the nucleus (Fig. 3D). As the mutant fragment failed to prevent the nuclear transport of the full-length protein, the luciferase expression level in these cells was as high as that of the full-length dCAMTA alone (Fig. 3E). Thus, the residues Arg483 and Arg484 are indispensable for the interaction between the CG fragment and the full-length dCAMTA in live cells and could be involved in a potential dimerization of dCAMTA.

The dCAMTA-dCAMTA Interaction Site Is Required for the Transcription Activity and the Physiological Function of dCAMTA in the Fly Eye—In the dCAMTA mutant flies tes1 and tes2, the target gene dFbxl4 has a much reduced expression level (7). To examine whether Arg483 and Arg484 are required for in vivo transcription activity of dCAMTA, we generated transgenic flies that express an R483E,R484E mutant dCAMTA protein in the tes2 background through a heat shock promoter and examined the expression level of dFbxl4. According to real-time reverse transcription PCR, the dFbxl4 mRNA level in these flies was not significantly increased compared with that in tes flies (Fig. 4). In contrast, overexpression of a wild type dCAMTA protein completely recovered the level of dFbxl4 expression. The data indicate that Arg483 and Arg484 are critical for in vivo transcription activity of dCAMTA, probably due to their role in the potential dimerization of dCAMTA.

With insufficient dFbxl4, the light receptor rhodopsin cannot be deactivated promptly in tes photoreceptors, and as a consequence the light response terminates much more slowly than wild type in both ERG and single-cell current recordings (7). This slow termination of light response phenotype was fully rescued by overexpression of wild type dCAMTA proteins. Once the residues Arg483 and Arg484 had been mutated, however, dCAMTA failed to improve the termination speed of light
response in the tes mutant background (Fig. 5). In contrast, mutation of two conserved residues (I1852,1937 to Asn) in the C-terminal region did not significantly impair this visual function of dCAMTA (Fig. 5). Thus, the residues Arg483 and Arg484 are critical for the role of dCAMTA in rhodopsin regulation, which suggests that dCAMTA might need to form dimers to function in fly photoreceptor neurons.

The Potential Dimerization of dCAMTA Is Critical for Its Nuclear Localization—To understand why the potential dimerization site is important for the transcriptional function of dCAMTA, we first examined the effect of R483E,R484E mutations on the DNA binding of dCAMTA. In a electrophoresis mobility shift assay, the mutated CG fragment still bound to and changed the mobility of a biotin-labeled CGCG box-containing DNA probe (7) (Fig. 6A). This binding appeared to be specific because it was blocked by unlabeled probe of high concentration. In addition, changing the CGCG box into CGGG abolished this DNA-protein interaction (Fig. 6A).

As the dimerization did not appear to be critical for dCAMTA binding to specific promoter sequences, we next examined whether it is required for nuclear localization of dCAMTA by expressing a FLAG-tagged full-length mutant protein in 293 cells. Immunostaining of the transfected cells showed that the majority of mutant proteins were localized in the cytoplasm and failed to enter the nuclei (Fig. 6B), suggesting that the Arg483,484-dependent dimerization could be important for appropriate presentation of the NLS. Thus, the mutant dCAMTA protein could be non-functional in the fly eye because of the impaired nuclear localization.

DISCUSSION

In addition to the functions in brain tumor suppression (11–13), cardiac hypertrophy (15), and fly visual regulation, CAMTA transcription factors are implicated in plant sensory responses to various environmental stimuli (6, 21–24). It is of great significance to study the transcription activities of CAMTA.

CAMTA transcription factors stimulate gene expression primarily through binding to CGCG box-containing promoter sequences (5, 6). Here our data suggest that CAMTAs may act as dimers. Given that many transcription factors are assembled into both homo- and heterodimers (16, 25, 26), it is likely that CAMTAs could also heterodimerize with related transcription factors. Because the heterodimers may recognize a promoter sequence different from that of homodimers (25, 27, 28), upon binding to Ca2+/calmodulin, a CAMTA heterodimer could even stimulate expression of genes that lack a CGCG box promoter. Considering this, we may need to look for target genes of CAMTA in a much broader scope.

The dimerization regions of many transcription factors are adjacent to their DNA-binding sites (16, 25, 26, 29). Similarly, dCAMTA appears to dimerize through a region within the
CG-1 domain that is also involved in DNA binding. It has been reported that the CG-1 domain of mouse CAMTA2 mediates its interaction with another transcription factor, Nkx2–5 (15). Through this interaction, CAMTA2 functions as a co-activator of Nkx2–5 and enhances the Nkx2–5-mediated gene expression. Thus, the CG-1 domain may play a pivotal role in the assembly of CAMTA-involved transcriptional complexes. In the future, it would be important to screen for additional CAMTA-interacting factors using a CG-1 domain-containing fragment.

The CG fragment failed to localize in the nucleus of the 293 cell, indicating that the CG-1 domain does not contain an effective NLS. An NLS has been mapped to the C-terminal region of mouse CAMTA2 (15). The observation that the R483E,R484E mutant dCAMTA is defective in nuclear localization may suggest that dimerization of the full-length protein is required for appropriate presentation of the NLS. In co-transfected cells, the CG fragment may have competitively bound to the full-length dCAMTA and prevented dimerization of CAMTA-involved transcriptional complexes. The cytoplasmic localization of the heterodimer suggests that dimerization with a CG fragment cannot expose the NLS in the full-length subunit. The fragment could even mask the NLS of the full-length protein. A similar case is that the P65 and I kBα molecules may play a pivotal role in the assembly of CAMTA-involved transcriptional complexes.

In co-transfected cells, the CG fragment may have competitively bound to the full-length dCAMTA and prevented dimerization between full-length molecules. The cytoplasmic localization of the heterodimer suggests that dimerization with a CG fragment cannot expose the NLS in the full-length subunit. The fragment could even mask the NLS of the full-length protein. A similar case is that the P105 and I kBα molecules bind to the NFκB subunit P65 and mask its NLS (16, 30). Another explanation for the cytoplasmic localization of the heterodimer is that the fragment-full-length dCAMTA interaction may have exposed a strong nuclear export signal that is normally hidden inside the molecule. A nuclear export signal has been identified in the N-terminal region of mouse CAMTA2, close to the CG-1 domain (15). No matter how the CG fragment may have retained the full-length dCAMTA in the cytoplasm, a similar fragment could be used as a dominant inhibitor of mammalian CAMTA activities and to prevent some CAMTA-mediated pathogenic processes such as cardiac hypertrophy.

In summary, our data suggest that dCAMTA may function as a dimer both in vitro and in fly photoreceptor neurons and that the CG-1 domain may mediate the potential dimerization of CAMTA transcription factors.

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REFERENCES