Interaction of Plant Chimeric Calcium/Calmodulin-dependent Protein Kinase with a Homolog of Eukaryotic Elongation Factor-1α*

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A chimeric Ca2+/calmodulin-dependent protein kinase (CCaMK) was previously cloned and characterized in this laboratory. To investigate the biological functions of CCaMK, the yeast two-hybrid system was used to isolate genes encoding proteins that interact with CCaMK. One of the cDNA clones obtained from the screening (LIEF-1α) has high similarity with the eukaryotic elongation factor-1α (EF-1α). CCaMK phosphorylated LIEF-1α in a Ca2+/calmodulin-dependent manner. The phosphorylation site for CCaMK (Thr-257) was identified by site-directed mutagenesis. Interestingly, Thr-257 is located in the putative tRNA-binding region of LIEF-1α. An isoform of Ca2+/dependent protein kinase (CDPK) phosphorylated multiple sites of LIEF-1α in a Ca2+-dependent but calmodulin-independent manner. Unlike CDPK, CCaMK phosphorylated only one site, and this site is different from CDPK phosphorylation sites. This suggests that the phosphorylation of EF-1α by these two kinases may have different functional significance. Although the phosphorylation of LIEF-1α by CCaMK is Ca2+/calmodulin-dependent, in vitro binding assays revealed that CCaMK binds to LIEF-1α in a Ca2+-independent manner. This was further substantiated by coimmunoprecipitation of CCaMK and EF-1α using the protein extract from lily anthers. Dissociation of CCaMK from EF-1α by Ca2+ and phosphorylation of EF-1α by CCaMK in a Ca2+/calmodulin-dependent manner suggests that these interactions may play a role in regulating the biological functions of EF-1α.

Ca2+ is a universal second messenger that regulates diverse developmental and physiological processes in plants (1). One of the major mechanisms decoding the change of intracellular Ca2+ and transducing Ca2+ signal is the action of Ca2+-modulated proteins. Calmodulin (CaM) is a highly conserved and the most widely distributed Ca2+-binding protein (2). CaM is believed to be a primary receptor for intracellular Ca2+ and functions as a regulatory element for its target proteins when activated by Ca2+. One group of CaM-modulated proteins is composed of Ca2+/CaM-dependent protein kinases.

Extensive investigation of protein phosphorylation and dephosphorylation has revealed that protein kinases play key roles in various signal transduction pathways leading to cellular and physiological responses (3). Ca2+/CaM-dependent protein phosphorylation has been implicated in regulating a broad array of biological functions and is believed to play a pivotal role in amplifying and diversifying the action of Ca2+/CaM-mediated signals in animals. A number of different types of Ca2+/CaM-dependent protein kinases, including CaM kinases (CaMKs) I–IV, phosphorylase kinase, and myosin light chain kinase, have been cloned and demonstrated to regulate various cellular processes (4, 5). Some indirect evidence for the existence of Ca2+/CaM-dependent protein phosphorylation has been reported in plants (1). However, until recently, no direct and convincing evidence has been presented. In recent years, three Ca2+/CaM-dependent protein kinase genes having some features similar to the mammalian multifunctional Ca2+/CaM-dependent protein kinase (CaMKII) were cloned from plants (6–8). The apple Ci2+/CaM-dependent protein kinase was isolated using an interaction screening method with labeled CaM as a ligand probe (6). CCaMK from lily and MCK1 from maize were cloned using the polymerase chain reaction screening method with oligonucleotide primers designed based on the conserved regions of mammalian CaMKs (7, 8). However, the sequence similarity between lily CCaMK, maize MCK1, and apple Ca2+/CaM-dependent protein kinase is only observed in their conserved kinase domains. No obvious sequence similarity exists outside their kinase domains. Although these three kinases were directly or indirectly demonstrated to bind CaM in a Ca2+-dependent manner, only biochemical properties for CCaMK have been reported to show that its kinase activity is regulated by Ca2+/CaM (9, 10).

CCaMK has a unique structural feature characterized by the presence of a kinase domain, a CaM-binding domain, and a neural visinin-like Ca2+-binding domain within a single polypeptide (7). Its kinase domain and CaM-binding domain are highly similar to those of mammalian CaMKII. The sequence of its C-terminal domain does not show significant homology to any known protein kinase. This domain has high similarity with a family of neural visinin-like Ca2+-binding proteins (7), and its Ca2+-binding property was confirmed by biochemical characterization (9). In plants, a predominant and widely expressed form of protein kinase is the Ca2+-dependent protein kinase or CaM-like domain protein kinase (CDPK) (11, 12). CDPK contains a kinase domain similar to that of mammalian CaMKII, a junction domain, and a CaM-like domain (11). Although both CCaMK and CDPK contain a kinase domain and a Ca2+-binding domain and have similarity in their overall structures, they distinctly differ in terms of their se-

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‡ The abbreviations used are: CaM, calmodulin; CaMK, Ca2+/calmodulin-dependent protein kinase; CCaMK, chimeric Ca2+/calmodulin-dependent protein kinase; CDPK, Ca2+-dependent protein kinase or calmodulin-like domain protein kinase; CRF2K, corn root Ca2+-dependent protein kinase 2; DTT, dithiothreitol; EF-1α, elongation factor-1α; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.
quences as well as regulation of their kinase activities. There is no striking sequence similarity shared between them except their kinase domains. Unlike the CaM-like domain of CDPK, the Ca$^{2+}$-binding domain of CCaMK contains three EF-hand Ca$^{2+}$-binding sites and has higher similarity to visinin-like proteins than CaM (7). Unlike CCaMK, the full-length CDPK does not bind to CaM, and its kinase activity is Ca$^{2+}$-dependent but does not require exogenous CaM (13). Another difference between these kinases is that CDPK is encoded by a large family of genes and is ubiquitously distributed among plant tissues, while CCaMK is a single copy gene in lily and its expression is developmentally and spatially regulated (7).

Study of protein-protein interactions between a kinase and its target proteins is crucial to illustrate intracellular signal transduction that eventually leads to physiological responses. Isolation and characterization of interacting proteins/substrates of CCaMK is a major step in understanding its in vivo regulation, cellular compartmentalization, and biological functions. In this study, the yeast two-hybrid interaction cloning system was used to isolate cDNAs encoding proteins that interact with CCaMK. Here we report that one of the cDNA clones (LIEF-1a) isolated from the screening is homologous to the eukaryotic elongation factor-1a (EF-1a), and CCaMK phosphorylates LIEF-1c in a Ca$^{2+}$/CaM-dependent manner, but their binding does not require Ca$^{2+}$.

**EXPERIMENTAL PROCEDURES**

**Plant Material—**Lily (Lilium longiflorum Thumb cv. Nellie White) plants were grown in the greenhouse. Various organs and anthers of different stages were collected and immediately frozen in liquid nitrogen for isolation of RNA and proteins.

**Cloning of LIEF-1a Using the Yeast Two-hybrid System—**The two-hybrid cDNA cloning kit was purchased from Stratagene, and the screening procedure was followed as recommended by the manufacturer. The mRNA was isolated from immature lily anthers (flower bud size 0.5–2.5 cm) using standard protocols (14). A lily immature anther cDNA library was constructed in HybriZAP two-hybrid λ vector and expressed as fusion proteins with the activation domain of GAL4 in pPD-GAL4. The full coding region of lily CCaMK was cloned in frame for isolation of RNA and proteins. The mRNA was isolated from immature lily anthers (flower bud size 0.5–2.5 cm) using standard protocols (14).

**Preparation of the Anti-CCaMK Antibody and Western Analysis—**Preparation of the Anti-CCaMK antibody or a polyclonal anti-EF-1 antibody, followed by incubation with the goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad). The immunoblot was developed using an enhanced chemiluminescent substrate (Pierce) and exposed to autoradiography film.

**Preparation of CaM-binding Proteins—**Lily anthers (flower bud size 0.9–2.0 cm) were powdered and homogenized in the extraction buffer (40 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.1% Triton X-100) containing 1 mM CaCl$_2$, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml antipain, 5 µg/ml aprotinin, 5 µg/ml leupeptin, and 5 µg/ml pepstatin. The homogenate was centrifuged at 21,000 × g for 15 min and at 100,000 × g for 1 h at 4 °C. The supernatant was applied onto a calmodulin-Sepharose 4B (Amersham Pharmacia Biotech) column pre-equilibrated with the extraction buffer containing 1 mM CaCl$_2$. The column was then washed extensively with the extraction buffer containing 1 mM CaCl$_2$. The bound proteins were eluted with 50 mM EGTA in 0.1 M HCl (pH 7.5) containing 2.5 mM MgTA, 0.08 M Tween 20, 200 mM NaCl, 0.1% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride and concentrated with a Centricon-30 concentrator (Amicon). Aliquots of eluted proteins were separated on a 12% SDS-polyacrylamide gel and immunoblotted with either the anti-EF-1a antibody or the anti-CCaMK antibody.

**Plasmid Construction and Site-directed Mutagenesis—**The original cDNA clone isolated from the yeast two-hybrid screening encodes the C-terminal portion (amino acids 204–447) of LIEF-1a, and CCaMK phosphorylates LIEF-1c in a Ca$^{2+}$/CaM-dependent manner, but their binding does not require Ca$^{2+}$.
incubated with 4 μg of the anti-CCaMK antibody in the extraction buffer for 4 h at 4 °C with gentle agitation. The beads were collected and washed three times in 1 ml of the extraction buffer. An aliquot of the crude extract containing 800 μg of protein was added to the conjugate of the anti-CCaMK antibody and protein G-agarose beads and incubated with gentle agitation for 4 h at 4 °C. The immunoprecipitate was washed three times with 1 ml of the extraction buffer in the presence of either 1 mM CaCl₂ or 2.5 mM EGTA and subjected to immunoblot analysis with a mouse monoclonal antibody against urchin EF-1α.

Control experiments were carried out by incubation of protein G-agarose beads with either the anti-CCaMK antibody alone or the crude protein extract alone.

RESULTS

Cloning of LIEF-1a1 cDNA—The yeast two-hybrid interaction cloning method was used to isolate genes encoding interacting proteins and/or substrates of CCaMK. Since lily CCaMK is expressed in a stage-specific manner during anther development (7), a cDNA library was constructed using mRNA isolated from lily anthers at the corresponding stages and expressed as fusion proteins with the GAL4 activation domain. The fusion protein of the GAL4 DNA-binding domain/CCaMK was expressed in pBD-GAL4 and utilized as the bait protein to screen the cDNA library. Among the positive clones identified from several rounds of screening, sequence comparison showed that one of these clones has high homology with the eukaryotic elongation factor-1α. The clone is 1016 base pairs long and encodes the C-terminal portion (amino acids 204–447) of LlEF-1a1 (Fig. 1). This cDNA clone was used as a probe to obtain its clone containing the full coding region by screening a cDNA library from which CCaMK was originally isolated (7). The clone coding for the full-length polypeptide was designated as LlEF-1a1 (Fig. 1).

LlEF-1a1 encodes a polypeptide of 447 amino acid residues (Fig. 1), which shares high homology with EF-1αs from other species (Fig. 2). It has very high homology (97.8% similarity and 95.7% identity) with a maize EF-1α and also significant homology with a protozoan parasite (Trypanosoma brucei) EF-1α (82.6% similarity and 76.1% identity) and a rabbit EF-1α (82.2% similarity and 75.9% identity). LlEF-1a1 contains the putative GTP-binding and tRNA-binding regions, and the sequences in these regions are highly conserved among different EF-1αs (Fig. 2).

Expression of CCaMK and EF-1α—Since the visinin-like domain is unique to CCaMK (7), the fusion protein of the visinin-like domain was expressed and purified from E. coli for producing a polyclonal antibody to analyze the expression pattern of CCaMK. CCaMK is specifically expressed only in root tips and anthers, and its expression is undetectable among other organs examined (Fig. 3). The expression of CCaMK is developmentally regulated in anthers. At the early stages, the protein level is very low. CCaMK expression reaches the highest level at the stage when the flower bud size is between 0.9 and 2.0 cm, coinciding with microsporogenesis (18). Its expression
then decreases until it becomes undetectable at the later stages of anther development. On the other hand, EF-1a is a ubiquitous protein and expressed in all of the organs examined. However, its expression level varies dramatically. EF-1a is highly expressed in root tips and anthers at the early stages of development, coinciding with the expression pattern of CCaMK. It appears that both CCaMK and EF-1a are highly expressed in tissues with high mitotic and meiotic activities.

CCaMK binds CaM in a Ca\(^{2+}\)-dependent manner (7, 9). Endogenous CCaMK was obtained from lily anthers by passing the protein extract through a calmodulin-Sepharose column. Interestingly, EF-1a was copurified with CCaMK on the calmodulin-Sepharose column (Fig. 4). However, this result does not demonstrate a direct interaction between CCaMK and EF-1a, since EF-1a was reported to bind to CaM in the presence of Ca\(^{2+}\) (19, 20).

Ca\(^{2+}\)/CaM-dependent Phosphorylation of LlEF-1a by CCaMK—The fact that LIEF-1a1 was cloned using the yeast two-hybrid system indicates that it interacts with CCaMK. There may be different ways in which CCaMK interacts with LIEF-1a1: (a) LIEF-1a1 may physically bind to CCaMK; (b) it may be one of the substrates of CCaMK; and (c) it may serve as an effector of CCaMK. To determine the nature of interaction between CCaMK and LlEF-1a1, we first examined whether CCaMK can phosphorylate LlEF-1a1 because of the identity of CCaMK as a protein kinase. The N-terminal and C-terminal regions of LlEF-1a1 were expressed and purified as GST fusion proteins (mN and mC, Fig. 5A). In the presence of EGTA or Ca\(^{2+}\), CCaMK phosphorylated the mC at the basal level (Fig. 5B). The phosphorylation level was stimulated up to around 25-fold by adding Ca\(^{2+}\) and CaM, indicating that CCaMK phosphorylates the mC in a Ca\(^{2+}\)/CaM-dependent manner. The stimulation of the kinase activity by Ca\(^{2+}\)/CaM is comparable with previous results where other substrates were used (9).
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FIG. 5. Phosphorylation of LlEF-1a1 by CCaMK in a Ca²⁺/CaM-dependent manner. A, schematic diagrams of the N-terminal and C-terminal deletion mutants (mN and mC) of LlEF-1a1. Both were expressed as GST fusion proteins. B, Ca²⁺/CaM-dependent phosphorylation of the mC by CCaMK. The mC (0.2 μg) was incubated in 20 μl of phosphorylation reaction buffer with 0.2 μg of CCaMK in the presence of 2.5 mM EGTA, 0.5 mM Ca²⁺, or 0.5 mM Ca²⁺ plus 1 μM CaM. The reaction was carried out at 30 °C for 2 min and terminated by adding 20 μl of 2.5 × sample buffer and boiling for 5 min. C, left, immunoblot showing the positions of GST, the mN, and the mC, GST, the mN, and the mC were separated by SDS-PAGE and immunoblotted with the goat anti-GST antibody (Amersham Pharmacia Biotech) and visualized with the rabbit anti-goat IgG conjugated with horseradish peroxidase (Sigma). The positions of protein size markers in kDa are shown on the left. Right, the phosphorylation assays were performed as described except that 0.1 μg of the mC was used.

The mN was not phosphorylated by CCaMK even in the presence of Ca²⁺ and CaM (Fig. 5C). The mC was phosphorylated by CCaMK, which agrees with the result of the yeast two-hybrid screening, since the original cDNA isolated from the screening codes for the C-terminal region (204–447) of LlEF-1a1.

Identification of the Phosphorylation Site for CCaMK in LlEF-1a1—CCaMK is similar to the mammalian multifunctional CaMKII in terms of the sequences in their kinase and CaM-binding domains and the regulation of their substrate phosphorylation (7, 9). The phosphorylation sites of CCaMK in LlEF-1a1 were predicted based on the consensus sequence of the minimal motif, RXX(S/T), which is present in majority of the phosphorylation site sequences of the CaMKII protein substrates (5, 21). There are only two putative phosphorylation sites in LlEF-1a1 (Fig. 1). One is Thr-72 in the N terminus.

Since the mN containing Thr-72 was not phosphorylated by CCaMK (Fig. 5C), obviously this site is not the phosphorylation site for CCaMK. The other phosphorylation site is Thr-257 in the putative tRNA-binding region (Figs. 1 and 2). Therefore, Thr-257 in the mC was mutated into Ala to investigate whether Thr-257 is a real phosphorylation site for CCaMK. The result revealed that the mC257A was no longer phosphorylated by CCaMK (Fig. 6), indicating that Thr-257 is indeed the phosphorylation site for CCaMK. Taken together, it is clear that Thr-257 is the only phosphorylation site for CCaMK, which is located in the putative tRNA-binding domain of LlEF-1a1.

Ca²⁺-dependent and CaM-independent Phosphorylation of LlEF-1a1 by CRPK2—In plants, it has been reported that EF-1α can be phosphorylated by CDPK (22). To investigate whether LlEF-1a1 can also be phosphorylated by CDPK, one of CDPK isoforms (CRPK2) isolated from corn root tips was expressed and purified from E. coli. Although CCaMK and CRPK2 show overall structural resemblance (Fig. 7A), there is no significant sequence similarity outside of their kinase domains. CRPK2 is a typical form of CDPK and has extensive and significant homology to other CDPKs (16). The amino acid sequence of CRPK2 shares 75% similarity and 65% identity with the prototype of CDPK, soybean CDPKα, along their three functional domains (kinase domain, junction domain, and CaM-like domain) (11).

Like other CDPKs, CRPK2 showed Ca²⁺-dependent and CaM-independent kinase activity (Fig. 7B). There was no kinase activity in the absence of Ca²⁺, and its kinase activity was not stimulated by CaM. CRPK2 phosphorylated both the mN and the mC in a Ca²⁺-dependent and CaM-independent manner. In fact, CaM slightly decreased the phosphorylation of the mN by CRPK2. The reason may be that CaM masked some of CRPK2 phosphorylation sites by binding to the mN, since CaM binds to EF-1α in the presence of Ca²⁺ (Refs. 19 and 20; Fig. 4).

In an attempt to determine whether CCaMK and CRPK2 phosphorylate the same site, the mC257A, which was not phosphorylated by CCaMK (Fig. 6), was tested for CRPK2 phosphorylation. Fig. 7C showed that CRPK2 phosphorylated both the mC and the mC257A to a similar extent, suggesting that Thr-257 may not be the phosphorylation site for CRPK2. CDPKs were reported to preferentially phosphorylate serine and threonine residues in the four-residue motifs of both RXX(S/T) and KXX(S/T) (12). Besides the two RX(S/T) motifs, there are also a number of KXX(S/T) motifs in LlEF-1a1 (Fig. 1). The mN and the mC cover the full-length LlEF-1a1 and share a stretch of overlapping sequence (amino acids 204–221; Fig. 5A). Since
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CRPK2 phosphorylated both the mN and the mC (Fig. 7B), we studied whether CRPK2 phosphorylated only sites in this overlapping region. There is indeed a threonine (Thr-215) residue located in the motif KXXT in this region. Thr-215 was mutated to Ala in both the mN and the mC. However, both of the site-directed mutants were still phosphorylated by CRPK2 (data not shown). These results indicate that CRPK2 phosphorylates multiple sites of LlEF-1α in a Ca2+-dependent and CaM-independent manner, and its phosphorylation sites are different from that for CCaMK.

Binding of CCaMK to LlEF-1α—To test whether there is direct association between CCaMK and LlEF-1α, in vitro binding assays were carried out, and the results are shown in Fig. 8. CCaMK did not bind to glutathione-Sepharose beads when incubated with GST in the presence of EGTA, Ca2+, or Ca2+ plus CaM, suggesting that CCaMK did not bind to glutathione-Sepharose beads and GST. The protein that migrated at a similar molecular mass as CCaMK (~58 kDa) was only observed when CCaMK was incubated with Ca2+ or the mC in the absence of Ca2+. In the presence of Ca2+ or Ca2+ plus CaM, this protein band disappeared, indicating that its association with the mN and the mC was disrupted. To examine whether the protein bands were CCaMK, a similar experiment was carried out, and the proteins were blotted onto polyvinylidene difluoride membrane for an immunoblot assay with the anti-CCaMK antibody. The result showed that the proteins immunologically reacted with the anti-CCaMK antibody and were indeed CCaMK. These results suggest that CCaMK binds to LlEF-1α in a Ca2+-independent manner, and Ca2+ disrupts their direct and physical interaction.

Association between CCaMK and EF-1α in Lily Anthers—All of the in vitro binding assays mentioned above were carried out using the N-terminal and C-terminal deletion mutants of LlEF-

![Image](image_url)

**Fig. 7.** Phosphorylation of LlEF-1α by CRPK2 in a Ca2+-dependent and CaM-independent manner. A, schematic diagrams showing the functional domain organization of CCaMK and CRPK2 for their structural comparison. Domains of CCaMK were as follows: kinase domain (KD); CaM-binding domain (CD); and neural visinin-like domain (VLD). The three EF-hand Ca2+-binding sites in the neural visinin-like domain are marked. Amino acids are numbered below. Domains of CRPK2 were as follows: N-terminal domain (ND); kinase domain (KD); junction domain (JD); and CaM-like domain (CLD). The four EF-hand Ca2+-binding sites in the CaM-like domain are marked. Amino acids are numbered based on comparison of CRPK2 with soybean CDPKα. B, phosphorylation of LlEF-1α by CRPK2. 0.2 μg each of GST, the mN, and the mC was used in the phosphorylation assays in the presence of 2.5 mM EGTA, 0.5 mM Ca2+, or 0.5 mM Ca2+ plus 1 μM CaM. CRPK2 (0.1 μg) was added into each of the reaction mixtures and incubated at 30°C for 2 min. The positions of protein size markers are indicated on the left. C, phosphorylation of the mC and the mC257A by CRPK2. Left, immunoblot showing that relative equal amounts of the mC and the mC257A were used for the phosphorylation assays. Right, the mC and the mC257A (0.2 μg) were phosphorylated by CRPK2 in the presence of 0.5 mM Ca2+.

![Image](image_url)

**Fig. 8.** In vitro binding assay showing Ca2+-inhibition of binding of CCaMK to LlEF-1α. Left panel, in vitro binding of CCaMK to LlEF-1α. GST, the mN, and the mC (2–8 μg) were incubated with CCaMK (1 μg) in the presence of EGTA, Ca2+, or Ca2+ plus CaM as described under “Experimental Procedures.” Proteins bound to glutathione-Sepharose 4B beads were analyzed by SDS-PAGE and visualized by staining with Coomassie Brilliant Blue. The position of CCaMK is marked with a solid arrow on the left. Right panel, immunoblot showing bound CCaMK in the in vitro binding assays. A binding experiment was performed as described for the left panel. Bound proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and immunoblotted with the anti-CCaMK antibody.
EF-1α undergoes three different post-translational modifications, i.e. methylation, formation of glycerylphosphoryl-ethanolamine, and phosphorylation (23, 33). However, the effects of these post-translational modifications on its translational activity remain unclear. Protein phosphorylation is one of the major mechanisms in regulating cellular and biochemical events. It is also highly involved in protein synthesis through phosphorylation of various components of the translational machinery including EF-1α (33). EF-1α was reported to be phosphorylated by protein kinase C, S6 kinase, and plant CDPK (22, 34, 35). Phosphorylation of EF-1α would facilitate its interaction with aminoacyl-tRNA and decrease its binding to ribosomes, and dephosphorylation is required for the transfer of aminoacyl-tRNA to 80 S ribosomes (33). Phosphorylation of EF-1 by multipotential S6 kinase resulted in stimulation of EF-1 activity (35). However, an in vitro assay indicated that phosphorylation of isolated EF-1α by protein kinase C did not affect its function as an elongation factor (34). Instead of its effect on protein synthesis, phosphorylation of a carrot EF-1α was shown to be pivotal to its function as an activator of phosphatidylinositol 4-kinase (22). These studies indicate that phosphorylation of EF-1α may not necessarily regulate its activity only in protein synthesis and may be involved in mediating its other functions also.

In this report, we demonstrated that a homolog of EF-1α was phosphorylated by a plant Ca2+/CaM-dependent protein kinase in a Ca2+/CaM-dependent manner. The only phosphorylation site for CCaMK is located in the putative tRNA-binding region. This threonine residue is conserved among all EF-1α sequences available in eukaryotes. The functional significance of the phosphorylation and the localization of the phosphorylation site in the tRNA-binding region are currently not known. It is unclear whether the same site can be phosphorylated by the mammalian CaMKII or other protein kinases. We also showed that LIEF-1α was phosphorylated by a CDPK in a Ca2+-dependent but CaM-independent manner, which is consistent with the finding of a previous study (22). The CDPK phosphorylates multiple sites in LIEF-1α, and these sites differ from the phosphorylation site of CCaMK. This suggests that these two kinases have some structural similarities, yet they differ in their regulation of kinase activity. Phosphorylation of EF-1α by CDPK was reported to be crucial for its function as an activator of phosphatidylinositol 4-kinase (22). Since CCaMK and CDPK phosphorylate different sites in EF-1α, it is reasonable to speculate that their phosphorylation may regulate different aspects of its biological functions.

In vitro binding experiments revealed that CCaMK binds to EF-1α in a Ca2+-independent manner, and the addition of Ca2+ disrupts their association. In eukaryotic cells, a large family of protein kinases regulate a variety of cellular processes. How these kinases coordinate their functions is a very challenging and fundamental area in signal transduction. In recent years, progress in this area has revealed that one of the mechanisms for accomplishing the coordination is regulation of their sub-cellular localization through association with their anchoring proteins (36–38). Association between CCaMK and EF-1α may provide a mechanism in the regulation of the kinase activity of CCaMK by localizing it into specific cellular compartments. This hypothesis is supported by the fact that EF-1α is associated with many proteins, including the components of the translational machinery, microtubules, and actin filaments (19, 23, 27, 28, 39, 40). Both CCaMK and EF-1α are highly expressed in similar tissues in lily (Fig. 3). It is very intriguing that targeting CCaMK into cytoskeleton or translational machinery through EF-1α may be one of the mechanisms to localize CCaMK into the neighborhood of its substrates.
The results revealed that CCaMK phosphorylates EF-1α in a Ca\(^{2+}\)/CaM-dependent manner but binds to it in a Ca\(^{2+}\)-independent manner. We propose that CCaMK may be localized in cells through direct association with EF-1α in a Ca\(^{2+}\)-independent manner. The transient kinase/substrate interaction of CCaMK with EF-1α requires Ca\(^{2+}\)/CaM, while EF-1α serves as a substrate for CCaMK. It can be envisioned that CCaMK is compartmentalized into the subcellular domains, where its substrates exist, by association with EF-1α at the resting level of Ca\(^{2+}\) in cells. At this stage in the process, CCaMK is inactive. When Ca\(^{2+}\) concentration increases in cells due to external or internal signals, CCaMK dissociates from EF-1α and subsequently, upon binding Ca\(^{2+}\)/CaM, becomes active to phosphorylate its substrates such as EF-1α or proteins near EF-1α. To some extent, this possible mechanism is analogous to the regulation of another second messenger-regulated protein kinase, cAMP-dependent protein kinase, where activation of the kinase is achieved through the release of the catalytic subunits from the inactive holoenzyme by cAMP (3). Various binding proteins for several protein kinases have been identified. Their interactions are mediated by either kinase domains or other regions and are subject to different types of regulation (41–45). It remains to be investigated how CCaMK mediates its direct association with EF-1α and how Ca\(^{2+}\) disrupts their binding. Future studies will aim at illustrating the functional significance of the phosphorylation of EF-1α by CCaMK and its possible roles in mRNA binding, protein synthesis, and/or other cellular processes.

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