Publications
• Domain analysis of a groundnut CDPK: Nuclear localisation sequence in the junction domain is coupled with nonconsensus calcium binding domains. Ayan Raichaudhuri, Rajasri Bhattacharyya, Shubho Chaudhuri, Pinak Chakrabarti, and Maitrayee DasGupta *J Biol Chem* (2006) 281: 10399-10409

• Oscillation of phosphorylation of an endogenous calcium dependent protein kinase from groundnut (*Arachis hypogea*) Evidences for its role in osmosensory signaling. Ayan Raichaudhuri, Anindita Seal, Shubho Chaudhuri and Maitrayee DasGupta (Manuscript under preparation)
Domain Analysis of a Groundnut Calcium-dependent Protein Kinase

**NUCLEAR LOCALIZATION SEQUENCE IN THE JUNCTION DOMAIN IS COUPLED WITH NONCONSENSUS CALCIUM BINDING DOMAINS**

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The signature of calcium-dependent protein kinases (CDPKs) is a C-terminal calmodulin-like domain (CaMLD) with four consensus calcium-binding sites. A junction domain (JD) joins the kinase with CaMLD and interacts with them through its autoinhibitory and CaMLD binding subdomains, respectively. We noted several CDPKs additionally have a bipartite nuclear localization signal (NLS) sequence as a subdomain in their JD, and this feature is obligatorily coupled with the absence of consensus calcium-binding sites in their respective CaMLDs. These predicted features are substantiated by undertaking investigations on a CDPK (g674779/88) isolated from cultured groundnut (Arachis hypogea) cells. This kinase can bind 3.1 mol of Ca$^{2+}$ under saturating conditions with a considerably high $K_d$ of 392 pm as compared with its canonical counterparts. CD spectroscopic analysis, however, indicates the intracellular structural changes accompanied with calcium binding to be similar to canonical CDPKs. Attesting to the presence of NLS in the JD, the endogenous kinase is localized in the nucleus of osmotically stressed Arachis cells, and in vitro binding assays indicate the NLS in the JD to interact with nuclear transport factors of the importin family. Homology modeling also indicates the feasibility of interaction of importins with the NLS present in the JD of such CDPKs in their activated form. The possible significance of obligatory coupling between the presence of NLS in the junction domain and atypical calcium binding properties of these CDPKs is discussed in the light of the known mechanisms of activation of these kinases.

CDPKs* are unique transducers of calcium signaling in plants and protists that are absent in yeasts and animals (1-3). The core structure of CDPKs has an N-terminal catalytic domain, a C-terminal calmodulin-like regulatory domain (CaMLD), and a junction domain sandwiched between these two domains. As the catalytic domain of CDPKs is most closely related to calmodulin-dependent protein kinases (CaMKs), CDPKs are believed to have arisen from a fusion event between a CaMK and a calmodulin (CaM) gene (1, 4). In CaMKs, the autoinhibitory and calmodulin binding domain is located immediately C-terminal to the kinase domain, and the autoinhibition is released in the presence of calcium and exogenous CaM (10). In CDPKs, the junction domain following the kinase domain is shown to contain subdomains possessing autoinhibitory and CaMLD binding properties. The autoinhibition is relieved by a calcium-dependent intramolecular interaction between the CaMLD and its target in the junction domain (7).

The distinguishing feature in the intramolecular activation of CDPKs is the conserved linkage of the CaMLD with its target in the same polypeptide. Thlimkink/k has been nicknamed as "tertius" region and has been shown to provide a "structural constraint" that is essential for complete activation of a CDPK (9). Any insertion in the tertius results in almost complete disruption in calcium-dependent activation of a CDPK. Thus, in addition to interaction of the CaMLD with its target in the junction domain, intramolecular activation of CDPKs involves a conformational change of the holoenzyme mediated through the tertius.

Genomic sequencing as well as several extensively expressed sequence tag projects indicate the presence of multiple families of CDPKs in various plants (2, 11). They are demonstrated to be involved in regulating ion transport and/or gene expression linked with various cellular processes like cytoskeleton dynamics, stress response, growth and development, and metabolism (3). Such response specificity of individual CDPKs is believed to be generated by their varying subcellular compartmentalizations, varying calcium and lipid sensitivities, and differences in substrate recognition (2).

Here we report the domain analysis of a group of CDPKs, which apart from having the signature features of these kinases also have a bipartite nuclear localization sequence (18-21) in their junction domain. Interestingly, sequence analysis of all CDPKs in the database reveals that the presence of NLS in their junction domains is obligatorily coupled with the presence of nonconsensus calcium binding EF hand loops (22-26) in their respective CaMLDs. We substantiate these predictions by biophysical and biochemical experiments undertaken with an Arachis CDPK that possess these features.

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3. The abbreviations used are CDPK, calcium-dependent protein kinase; CaMK, calcium/calmodulin-dependent protein kinase; CaM, calmodulin; GST, glutathione S-transferase; CaMLD, calmodulin-like domain; NLS, nuclear localization signal; CDPK, death associated protein kinase; IP3, inositol 1,4,5-trisphosphate; NOS, nitric oxide synthase; p38, p38 mitogen-activated protein kinase; PKC, protein kinase C; PMSF, phenylmethylsulphonyl fluoride; JD, junction domain; AT, acetylcholinesterase; N, nitric oxide; CTP, cyclic nucleotide triphosphate; GTP, guanosine 5'-triphosphate; CaM, calmodulin; CaMLD, C-terminal calmodulin-like domain; JD, junction domain; CaMLD, C-terminal calmodulin-like domain; NLS, nuclear localization signal; CaMK, calcium/calmodulin-dependent protein kinase; GST, glutathione S-transferase; CaM, calmodulin; CaMLD, calmodulin-like domain; NLS, nuclear localization signal; CDPK, death associated protein kinase; IP(3), inositol 1,4,5-trisphosphate; NOS, nitric oxide synthase; p38, p38 mitogen-activated protein kinase; PKC, protein kinase C; PMSF, phenylmethylsulphonyl fluoride; JD, junction domain; AT, acetylcholinesterase; N, nitric oxide; CTP, cyclic nucleotide triphosphate; GTP, guanosine 5'-triphosphate; CaM, calmodulin; CaMLD, calcium mechanism.
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EXPERIMENTAL PROCEDURES

Materials

TOPO TA cloning kit was obtained from Invitrogen, pQE expression vectors, nickel-agarose, and anti-RGS-His antibody were obtained from Qiagen (Germany). Enzymes, glutathione-Sepharose 44, Tris, Hepes, glyc erol, Trx-Tn X-100, β-mercaptoethanol, spermine, leupeptin, and IPTG were obtained from Amersham Biosciences PMSE, benzamidine, N-ethylmaleimide, 5'-chloro-1-naphthalene sulfonyl fluoride (W7), anti-tubulin antibody, 1-naphthalene acetic acid, and benzamidine phosphate were obtained from Sigma. Western blotting kit was obtained from Roche Applied Science. Oligonucleotides were obtained from IIT. All other reagents of analytical grade were obtained from SRL (India). 4Ca2+ (185 μM; specific activity 79 mCi/g) was obtained from BRIT, Government of India.

Methods

Cell Cultures and Stress Treatments—Initiation and maintenance of an axum, autotrophic cell suspension culture of Arachis hypogaea are discussed in detail in Ref 27. In brief, the culture is maintained in MS medium (aux:cyt) containing 11.09 μmol liter-1 of benzamidine phosphate, in a gyratory shaker at 100 rpm at 27 °C under a 16/8-h photoperiod at 40 μmol of photonm-2s-1. The cells are subcultured every 7 days by inoculating 3 ml of cells packed at 1 X g.m 50 ml of medium For auxin treatment, the stressed Arachis cells were treated for 1 h with 13.5 μmol liter-1 of 1-naphthalene acetic acid.

Cloning, Expression, and Purification of Arachis CDPK and Other Recombinant Polypeptides—Total RNA was isolated from nontransformed Arachis cells using TRI Reagent (Molecular Research Center). The isolated mRNA was subjected to RT reaction using cDNA synthesis module (Amersham Biosciences) with random hexanucleotide and anchored oligo(dT) primers. The cDNA for a recombinant CDPK was PCR-amplified using primers 5'-GCTCTAGAGTGGCTCCAAATATTCCTCTTGGGGATGCTG-3' that corresponded to the sense sequence of domain I of the catalytic domain and antisense sequence of the fourth calcium-binding site of the CaMLD, respectively. The amplified 1.3-kb fragment was cloned into the BamHl-HindIII sites in the pUC19 backbone was then cloned into the TOPO TA vector from Invitrogen. The sequence named AHCPK2 was submitted to GenBank (gi:67479988) for expressing the JD-CaMLD mul.

For expressing the CaMLD polypeptide of Arabidopsis CDPK, the amplified fragment was cloned into the pQE30 expression vector pQE32 for expressing the CaMLD polypeptide from Arabidopsis CDPK with a N-terminal His6 tag, it was PCR-amplified using primers 5'-GCTCTAGAGCTGATTTCTTGTCC-.3' and 5'-CCCAAGCTTATCATCGCCACTC-3' as reverse primers, respectively, and subcloned into the E. coli BL21 (DE3) cells.

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The JD-CaMLD and the CaMLD polypeptides from Arabidopsis CDPK with a N-terminal His6 tag, it was PCR-amplified using primers 5'-GGGGAATCTATCATCCTGTAAGAGATCTGGGCGG-GGSATCTGGGG-3' as forward and 5'-GCTCTAGAGCTGATTTCTTGTCC-.3' and 5'-CCCAAGCTTATCATCGCCACTC-3' as forward and reverse primers, respectively, and subcloned into the E. coli BL21 (DE3) cells. The recombinant polypeptides were expressed in E. coli BL21 grown in M9, glucose, 0.9% tryptone in the presence of 0.5 mM IPTG for 4–6 h at 25 °C, and the proteins were purified at 4 °C using nickel-nitrilotriacetic acid-agarose (Qiagen) beads according to the manufacturer’s instruction.

The GST-importin fusion proteins (33) were expressed in E. coli BL21 in the presence of 0.5 mM IPTG for 4–6 h at 20 °C, and the proteins were purified at 4 °C using glutathione-Sepharose beads according to the manufacturer’s instruction (Amersham Biosciences). 2 mM dithiothreitol was included in all the solutions throughout the purification procedures. The final preparation of the polypeptides were dialyzed against 20 mM Tris-HCl pH 7.4, and stored in aliquots in −80 °C.

Bioinformatics—Sequences of Arabidopsis CDPK family members were retrieved from the BioMart database (plants, rice, soy, tobacco). CDS sequences containing NLS in their junction domain coupled with noncanoncal EF Hands in their calmodulin-like domains were identified by manually analyzing all entries in the GenBank (www.ncbi.nlm.nih.gov or SwissProt database by using PROSITE (34) Sequences were aligned with ClustalW (35).

The binding properties of CDPKs—The calcium binding property was measured by equilibrium dialysis as described previously (36). For ensuring a calcium-free condition, all buffers and protein solutions were passed through a Chromatob column (Bio-Rad). The stock solutions were diluted to 2.5 mM EDTA overnight, and dialysis bags were heated at 80 °C in 1 mM EDTA, 1 mM NaCl, 20 mM Tris, and both were rinsed with calcium-free water 40 ml (10 μM) of the purified polypeptide was dialyzed for 24 h at 25 °C against 400 μl of a solution of 25 mM MES-KOH, pH 6.0, containing 150 mM KCl and 4Ca2+ (25 μM) in presence of indicated concentrations of CaCl2. After dialysis, 20 μl of the protein solution and the external solution were collected from each of the dialysis sets. The collected solutions were spotted on nitrocellulose membrane (13 mm in diameter), and radioactivity associated was measured. The moles of bound calcium/mole of protein and the concentration of unbound calcium were evaluated from the known concentration of the protein and the initial calcium concentration. The calcium binding data were processed according to the Hill model (37, 38) using Kiplot. The Hill model provides information regarding the degree of cooperativity (Hill constant, A3), the maximum number of calcium-binding sites (A1), and apparent dissociation constant (A2) according to Equation 1,

\[
y = \frac{A_1}{1 + \left(\frac{A_2}{x}\right)^{A_3}}
\]

where y and x denoted the average number of moles of calcium bound/mole of protein and the free calcium concentration, respectively.
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Circular Denaturation—Experiments were performed on Jasco-720 spectropolarimeters. A bandwidth of 1 nm and a scan step size of 0.25 nm were employed with a 1-cm path length. Spectra were recorded at 286 K with 5 μM of the indicated polypeptides in 20 mM Tris-HCl, pH 7.4, in the presence of 1 mM calcium or 5 mM EGTA. Five scans were averaged for each sample, and the spectra for the buffer was subtracted as a blank.

Cell Fractionation—Membrane fractions were isolated and purified from both normal and stressed A. thaliana cells with or without auxin treatment as described previously (28) and according to the procedures described in Refs 12 and 39. In brief, cells were harvested, rinsed with distilled water, ground in a mortar cooled with liquid nitrogen, and stored at −80 °C in small aliquots. Protein inhibitors (0.5 mM PMSF, 1 mM benzamidine, 10 μM leupeptin, and 10 μM aprotinin) were added, gently mixed for 1 h, and then washed three times (15 min each) with 1X Tris-buffered saline containing the indicated concentrations of calcium and EGTA. Binding was done for 4 h at 4°C. Glutathione-Sepharose 4B beads were added, gently mixed for 1 h, and then washed three times (15 min each) with 1X Tris-buffered saline containing the indicated concentrations of calcium and EGTA. The reaction mixture was analyzed in 12% SDS-PAGE (30) and Western-blotted with anti-His or anti-tubulin. Western blotting with anti-His and anti-tubulin was done according to the manufacturer’s instructions.

GST Pull-down Assay—Interactions of the JD-CaMLD, CaMLD, and JD-CaMLD with polypeptides were investigated by this assay following the binding conditions described previously (33, 41) with some modifications. In brief, 300 pmol of polypeptides were incubated in a total reaction volume of 150 μl in 20 mM Tris-HCl, pH 7.4, containing 2 mM β-mercaptoethanol, 10 mM EDTA, 2.5 mM EGTA, 300 mM sucrose, and 0.5 mM PMSF, 1 mM benzamidine, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Membrane and soluble protein fractions were stored at −80°C.

Nuclear extracts were prepared from normal and stressed A. thaliana cells as described in detail previously (29). The final nuclear pellet was resuspended in 20 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 500 mM NaCl, 0.5 mM PMSF, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1% Triton X-100 and stored at −80°C in small aliquots. Protein concentration was estimated by Bradford method (31).

Immunological Techniques—Antibodies against JD-CaMLD polypeptides of AhCPK2 was raised in rabbits. The affinity-purified His-tagged JD-CaMLD polypeptide was subjected to SDS-PAGE. After mild staining of the gel, the appropriate band was excised, crushed, and used for immunization according to standard protocols (40).

Western blotting with anti-AhCPK2 and anti-ABB (29) was done at 1:1000 dilutions using a kit from Roche Applied Science. Western blotting with anti-His and anti-tubulin was done according to the manufacturer’s instructions.
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Figure 2: Domain analysis of CDPKs containing an NLS in their junction domain. A, multiple sequence alignment of the junction domains of CDPKs containing a bipartite NLS as a subdomain. The source and corresponding gi numbers are indicated. The relative positions of the subdomains for nuclear localization, pseudosubstrate autoinhibition, and CaMLD binding in the junction domains are indicated. The determinant amino acids for the bipartite NLS are indicated in red. B, multiple sequence alignment of the calcium binding EF hand loops of the above CDPKs. The residue numbers important for coordination with calcium in the respective calcium binding loops are indicated. C, crystal structures of NLS peptide complexes, the target molecule had four consensus EF hand loops but contains an insertion of 50 amino acids in the border of the catalytic and junction domains. Domain analysis was done using PROSITE. Alignments were developed using ClustalW (35).

RESULTS

Domain Analysis of an A. hypogea CDPK and Its Homologs, Nuclear Localization Sequence in the JD Is Coupled with Nonconsensus EF Hand Loops in the CaMLD. —We developed a 1.3-kb partial cDNA clone (gi:67479988) of an Arabidopsis CDPK (designated AhCPK2) by amplification of RNA prepared from A. hypogea cells that were subjected to osmotic stress (27, 28). The predicted amino acid sequence contains a kinase catalytic domain with subdomains 1 to 10 of Ser/Thr kinases, a junction domain, and a CaMLD with four calcium-binding motifs characteristic of the CDPK family (Fig. 1). Alignment and phylogenetic analysis using the entire length of the deduced AhCPK2 protein sequence showed that it belongs to the branch III cluster of plant and algal CDPKs (3). It showed the highest homology with AtCDPK13, an Arabidopsis CDPK (gi:28090008) with 86% identity and 86% similarity, and OsCDPK, a rice CDPK (gi:36093790) with 83% identity and 92% similarity. The alignment of AhCPK2 with AtCDPK13 and the OsCDPK is shown in Fig. 1.
AhCPK2 also shares 71% identity and 83% similarity with a stress-induced CDPK (AtCPK10) from Arabidopsis (51, 52), which appeared consistent with its expression in the stressed Arabidopsis cells.

In addition to having the core domain arrangement of a canonical CDPK, AhCPK2 contains a bipartite NLS sequence (PROSITE code PS00015) in its junction domain, and the sequence of the second calcium binding EF hand loop (PROSITE code PS00018) in its CaMLD was a deviation from the consensus (Fig. 2, A and B) BLAST search and subsequent domain analysis revealed that several other CDPKs from different species showing strong homology with the Arabidopsis CDPK also have a similar domain composition. As indicated in Fig. 2, a coupling was noticed between the presence of a bipartite NLS in the JDs of CDPKs with the absence of one or more calcium binding EF hand loops in their respective CaMLDs.

Multiple sequence alignment revealed that the position of the NLS in the JD of all these CDPKs was superimposable (Fig. 2A). Canonical bipartite NLS contains the following: (i) two adjacent basic amino acids (Arg or Lys), (ii) followed by a spacer region of any 10 residues, and (iii) followed by at least three basic residues (Arg or Lys) in the next five positions (23). Comparison of the JD sequences revealed that two of the three basic residues in the C-terminal cluster of the bipartite NLS domain are conserved in all CDPKs (alignment not shown). In fact, this cluster overlaps with the known span of the autophosphorylation subdomain of our target group. However, AtCPK13 contained nonconsensus loops in its N-terminal half of the CaMLDs in all CDPKs of our target group. Moreover, AhCPK2 with a nonconsensus EF hand loop in the second calcium binding site was investigated for calcium binding by equilibrium dialysis according to the “Experimental procedures.” The figure represents the best fit to the obtained calcium binding data using the Hill model (37, 38). Under similar conditions, the calcium binding property of the JD-CaMLD polypeptide of AhCPK1 with four consensus EF hands has been investigated as a reference.

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AhCPK2 with a nonconsensus EF hand loop in the second calcium binding site (gi 22655135) was the single exception to the domain coupling phenomenon where, despite having an NLS in the JD, all four calcium binding EF hand loops were predictably functional (Fig. 2B). But unlike any other known CDPK, this kinase was found to have a 26-amino acid insertion just preceding the NLS, between the kinase and the junction domain (Fig. 2C). This evidence in fact further attests to the fact that the presence of an NLS in the junction domain is accompanied by some sort of a compensatory change in the total kinase.

**Calcium Binding Property of the Arabidopsis CDPK—PROSITE analysis indicated the second EF hand loop of the CaMLD of the Arabidopsis CDPK to be nonfunctional.** This is because among the six residues in positions 1, 3, 5, 7, 9, and 12 that are known to coordinate with calcium in a standard 12-residue EF hand loop structure, two residues at positions 1 and 3 are not conserved. The second EF hand loop of the CaMLD of the Arabidopsis CDPK was found to be nonfunctional.
and 5 deviated from the consensus. To understand the effect of such a deviation, we investigated the calcium binding properties of the JD-CaMLD polypeptide of the *Arachis* CDPK, and we compared it with its canonical counterpart obtained from an *Arabidopsis* CDPK AtCPK1 (32) where all four EF hand loops follow the consensus pattern (Fig. 3) Binding of calcium was measured by equilibrium dialysis according to Refs. 36 and 53. The binding parameters were evaluated by analyzing the experimental data based on the Hill model of cooperative binding to equivalent binding sites. The binding capacity of the *Arachis* JD-CaMLD polypeptide was determined to be 3.1 mol of calcium/mol of protein at saturation, and the $K_d$ value was determined to be 992 $\mu M$. Under identical conditions the JD-CaMLD of AtCPK1 could bind 3.8 mol of calcium/mol of the polypeptide with a $K_d$ value of 0.19 $\mu M$. The calcium binding property of the *Arachis* CDPK thus appears to be significantly deviated from those of the canonical CDPKs and is very consistent with the PROSITE prediction of a nonconsensus calcium binding EF hand loop in its CaMLD region. It may be noted that *Plasmodium* CDPK with mutations in the first or second calcium binding loop had its $K_d$ values raised to the 200–330 $\mu M$ range (53), which is similar to the values obtained with the *Arachis* CDPK. The value of the Hill constant (degree of cooperativity) was found to be greater than 1, in calcium binding to *Arachis* JD-CaMLD, indicating that there is a positive cooperativity in calcium binding to this polypeptide. Such cooperativity in calcium binding is a common feature of all calmodulin-like proteins (32, 53).

**Global Fold of the Calcium-responsive CDPK Regulatory Apparatus (32) Remains Unchanged in the *Arachis* CDPK**—The CaMLD polypeptides from canonical CDPKs are known to be highly helical in nature with the helicity increasing further in response to calcium (32, 54). In the corresponding JD-CaMLD polypeptides, interaction of the JD with the CaMLD leads to a further increase in helicity, and this change is attributed to the helical character of the JD as developed as a consequence of its binding to CaMLD as well as the change in spatial distribution of the helices in the CaMLD region that moves from disordered to an ordered structure (32). To understand how the elevation in the calcium binding would affect the characteristic structural response of the *Arachis* CDPK (AhCPK2), we followed the calcium-responsive structural change of the CaMLD and the JD-CaMLD region of this kinase, and we compared it with its canonical counterparts obtained from an *Arabidopsis* kinase AtCPK1 (32), by studying the far-UV CD spectrum of the corresponding polypeptides.

The spectra of CaMLD and the JD-CaMLD polypeptides from AhCPK2 as well as AtCPK1 had dominant contributions from the helical secondary structure with the minima at 208 and 222 nm (Fig. 4). For both the polypeptides with each of these kinases there was an increase in intensity upon addition of calcium. Also, in both cases there was a considerable increase in the helical component of the JD-CaMLD polypeptide as compared with the corresponding CaMLD, which was suggested earlier to be due to the helical character of the junction region in the JD-CaMLD polypeptide (33). This analysis broadly suggests that at saturating concentrations of calcium, the global fold of the regulatory apparatus in the *Arachis* CDPK remains similar to its canonical counterpart despite having wide differences in their calcium binding properties. The activation principle of the *Arachis* CDPK thus appears to follow the known activation principles for CDPKs (7–9).

**Subcellular Localization of the Endogenous *Arachis* CDPK**—The JD-CaMLD region of the *Arachis* CDPK contained unique features that distinguished it from canonical CDPKs (Fig. 2). Therefore, to detect the endogenous polypeptide representing the untagged or native *Arachis* CDPK (AhCPK2) in the cultured *Arachis* cells, we raised polyclonal antibodies against the JD-CaMLD polypeptide of the kinase. This antibody cross-reacted with a single 58-kDa band in the total protein preparation of the *Arachis* cells that showed a characteristic calcium-dependent shift in mobility (Fig. 5A). It failed to cross-react with the 53-kDa CDPK in dry seed extracts of *A. hypogea* that we previously characterized (55, 56), thus indicating the specificity of its interaction (data not shown). AhCPK2 was originally isolated from osmotically stressed cells, we used both normal and stressed cells for our detection experiments. The cells were resolved into soluble and microsomal fractions and/or into a nuclear fraction by isolating the stably bound calcium. The isolated proteins from these fractions were subjected to Western blot analysis using the raised antibody. The cross-reactive 58-kDa polypeptide was localized in the microsomal fractions (Fig. 5B, lanes a and $d$) and the soluble fractions (lanes $b$ and $e$) in both the normal and the stressed cells, indicating the kinase to be distributed in both membrane and soluble fractions. The kinase was not detectable in the nuclear fraction of the normal cells (Fig. 5B, lane $c$), but it was clearly detectable in the nuclear fraction of the stressed cells that were subjected to 0.4 M sucrose for 4 days (Fig. 5B, lane $f$). These *Arachis* cells have a unique stress physiology in the presence of stress they are driven to quiescence, but an hour of auxin treatment completely restores their growth potential (27–29). The subcellular localization of AhCPK2 remains unchanged following auxin treatment in these *Arachis* cells (data not shown). These observations suggest that AhCPK2 responds to stress-geared signals by being localized in the nucleus. Similar dynamism in subcellular localization in response to stress has been noted earlier with McCPK1, a CDPK from *Mesembryanthemum crystallinum* (57). Anti-tubulin antibody cross-reacted with the soluble and the microsomal protein preparations (58) but showed no cross-reaction with the nuclear protein, indicating our fractionation procedures were effective (Fig. 5A). Antibodies raised against the transcription factor AB1 (abscisic acid-insensitive 3) that is induced in presence of auxin in these cells (29) are.
FIGURE 6 Importin binding property of AnCPK2. Importin binding was investigated by GST pull-down assay as described under "Experimental Procedures." GST-tagged proteins were precipitated from the reaction mixtures using glutathione- Sepharose 4B beads, and the importin-bound proteins were detected by Western blot analysis with anti-His or anti-AnCPK2 as indicated. A, the His-tagged JD-CaMLD polypeptide was subjected to binding with GSTalone or the indicated GST-tagged importin (lanes f, g, and h). The compositions of the necessary control reaction mixtures are indicated in the figure (lanes b–e). Lane f contains a standard JD-CaMLD polypeptide without being subjected to binding reactions. The positions of the calcium-bound JD-CaMLD in both panels are indicated by arrowheads and asterisks. Binding was investigated in the presence of the indicated concentrations of calcium (lanes a–c). The compositions of the necessary control reaction mixtures are indicated in the figure (lanes d–g). Lane i contains standard JD-CaMLD, CaMLD, and JD-CaMLDmut polypeptide without being subjected to binding reactions. The positions of calcium-bound polypeptides are indicated by arrowheads. The mutated residues in JD-CaMLDmut are indicated by asterisks C. Binding of GST-importin α with JD-CaMLD of AnCPK2 was investigated in the presence of the indicated concentrations of calcium (lanes a–c) or EGTA (lanes d–f). Subsequent to binding under the indicated conditions, the glutathione beads were subjected to washing in the presence of the indicated concentrations of calcium (lanes a, b, g, and h) or EGTA (lanes c–f). D, the characteristic shift of electrophoretic mobility of JD-CaMLD polypeptide in the presence and absence of calcium is shown in the adenosine panel. E, the His-tagged AnCPK2 polypeptide was subjected to binding with GST-tagged importin α (lanes g–i). The compositions of the necessary control reaction mixtures are indicated (lanes c–e). Binding was investigated in the presence of the indicated concentrations of EGTA (lanes f–g) and calcium (lanes d–g) in the presence (lanes g and i) or in the absence (lanes f and h) of 2 mM W7. Lane i contains standard AnCPK2 polypeptide without being subjected to binding reactions.

Presently, functional annotation available for our target set of CDPKs having an NLS sequence in JD is low, and nuclear localization has not been reported for any of them. In fact, AnCPK2 and ACPK8, members of our target group, have been localized in membranes but not in the nuclear fraction of the stressed Arachis cells.
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importing property for demonstrating the presence of NLS in the JD region of the JD-CaMLD polypeptide from the Arachis CDPK. GST pull-down assays were done to check the binding between GST-tagged importin α1, α2, and α3 proteins from rice (33, 61) and the HisC)-tagged JD-CaMLD polypeptide from the Arachis CDPK. Both anti-His and antibodies raised against the JD-CaMLD polypeptide of AhCPK2 were used for detecting the importin-bound polypeptide by Western blot analysis. Assays were done to check the binding between GST-tagged importin α1, α2, and α3 proteins from rice (33, 61) and the HisC)-tagged JD-CaMLD polypeptide from the Arachis CDPK. Both anti-His and antibodies raised against the JD-CaMLD polypeptide of AhCPK2 were used for detecting the importin-bound polypeptide by Western blot analysis. Assays were done to check the binding between GST-tagged importin α1, α2, and α3 proteins from rice (33, 61) and the HisC)-tagged JD-CaMLD polypeptide from the Arachis CDPK. Both anti-His and antibodies raised against the JD-CaMLD polypeptide of AhCPK2 were used for detecting the importin-bound polypeptide by Western blot analysis.

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identical conditions with the CaMLD polypeptide and the importin α1a. As indicated in Fig 6B, lane a, the CaMLD polypeptide was not detected in association with importin α1a, indicating the observed binding of the JD-CaMLD (Fig 6B, lane a) polypeptide to be specific for the JD region that contains the NLS. To further ascertain that the NLS sequence in the JD is important for binding with importin, we undertook binding assays with a mutated variant of the JD-CaMLD where three important determinants of NLS, namely Arg-256, Arg-257, and Lys-268, have been replaced with Gly, Val, and Leu, respectively. This mutated polypeptide was not detected in association with importin α1a (Fig 6B, lane c), indicating the binding of the JD-CaMLD (Fig 6B, lane a) polypeptide to be specific for the JD region of the kinase. Under identical conditions, GST-importin α1a failed to show any binding with the JD-CaMLD, CaMLD, and the JD-CaMLDα1a polypeptides (Fig 6A, A, lane a, and B, lanes d and e), indicating that the observed bindings of GST-importins were importin-mediated. Thus, the junction domain of AhCPK2 was demonstrated to have a nuclear localization sequence through its ability to interact with the importin proteins of the nuclear transport machinery.

We then checked whether binding of importin to the junction domain was affected by the intramolecular interaction within the JD-CaMLD polypeptide in the presence of calcium. For this, binding reactions were set up with GST-importin α1a and His JD-CaMLD of ASCKP2 in the presence of the indicated concentrations of calcium or EGTA (Fig 6C). As indicated, the presence or absence of calcium did not interfere with the interaction of JD-CaMLD with importin. Depending on the presence of calcium or EGTA during washing, the JD-CaMLD polypeptide was detected in its calcium-bound or unbound form with a characteristic shift of electrophoretic mobility (Fig 6C). Thus, without the constraints of having the catalytic domain around in the JD-CaMLD polypeptide, interaction of the NLS with importin appeared to be calcium-insensitive.

Our attempts to demonstrate importin binding with the whole kinase, where the NLS in the JD was sandwiched between the catalytic and the CaMLD domains, was unsuccessful irrespective of whether we did our binding assays in the presence of saturating concentrations (1 mM) of calcium (Fig 6D, lane a) or in the absence (5 mM EGTA) of calcium (lane c). Most interestingly, when we tested the calcium dependence of the whole kinase-importin interaction, successful binding was noted at low concentrations (10 μM) of calcium (Fig 6D, lane d and f). In addition, we could also detect effective binding of importin with the whole kinase in the presence of 1 mM calcium when W7 was included in the binding reaction (Fig 6D, lane g). It appears that at low calcium concentrations, the CaMLD successfully maneuvered the withdrawal of the autoinhibitory domain from the clutches of the catalytic domain, but it does not interact with JD in a manner that would bury the autoinhibitory domain. Therefore, the adjacent placed NLSs in the JD remain accessible to importins under such conditions. On a similar note, we reasoned that in the presence of saturating concentrations of calcium, interaction of JD with the CaMLD completely sandwiched the NLS between the catalytic and the CaMLD structure and thus hindered its interaction with importin. But under the same conditions in the presence of W7, prohibition of the proper interaction of the JD with the CaMLD allows interaction of importin with the NLS.

Three-dimensional Model of Interaction of Arachis CDPK with Importin—To visualize the interaction of importin with the full-length kinase, we developed a model for the binary complex between the whole structure of the Arachis CDPK and importin through homology modeling and docking. No protein structure is available with JD and CaMLD covalently linked. Therefore, we took recourse in the available three-dimensional structures of binary complexes of calmodulin and target peptides. Considerable efforts have gone into understanding target recognition by CaM using synthetic peptide analogs of CaM binding regions (43, 44, 62–64). Generally, the peptide can be positioned in the peptide-binding channel of CaM in two relative orientations, parallel or antiparallel depending on whether the N-terminal region of the peptide is closer to the N-terminal or the C-terminal domain of CaM/CaMLD. As discussed under "Experimental Procedures," the parallel mode of binding appeared to be more plausible for interaction of JD with CaMLD. The alignments of sequences of the target peptide and CaM in 1CKF with the JD and CaMLD sequences of Arachis CDPK are shown in Fig 7B and C, respectively. Next, we developed a model for the active conformation of the Arachis CDPK catalytic domain using the crystal structure (Protein Data Bank code 1K1T (66) of DAPK (24% identity and 37% similarity) (Fig 7A) and joined it with the JD-CaMLD to construct the complete structure (Fig 7A).

The surface area that is buried as the peptide forms a complex with CaM in 1CKF is 3448 Å², but in our model of JD-CaMLD it is much smaller (1687 Å²). It is possible that because JD is covalently linked to the N-terminal end of CaMLD in CDPK, the CaMLD binding region (the helical part of JD) cannot go deep into the channel, thus remaining partially exposed. In our model, the CaMLD binding region is shared evenly between the two domains, the interface area being 915 and 773 Å² with the N- and C-terminal domains, respectively. However, more residues (His-371, Leu-372, Ala-375, Tyr-378, Phe-379, Leu-392, Ala-395, Val-415, Ile-423, Phe-428, and Met-431, for a total of 16) are contributed by the C-terminal domain than the N-terminal domain (Ala-289, Asp-290, Phe-291, Leu-292, Glu-295, Tyr-301, Ile-303, Lys-306, and Val-389, for a total of 13). Thus, it is consistent with the prevailing understanding about JD and CaMLD interaction, where the C-terminal lobe is documented to be involved in direct binding of the target in JD (33). We have compared the residues (Fig 7C asterisks) in CaMLD of Arachis CDPK that are...
NLS in JDs and Nonconsensus EF Hands in CDPKs

found to be interacting with its JD in our model with the residues (black circles) involved in target interaction in CaM in ICKK (14) and also the residues (open circles) involved in JD interaction in CaMLD or the structure 1561 (66). As can be seen, there is a considerable overlap in the residues and regions of CaMLD that appeared to be interacting with JD in our model with those found to be interacting in similar structures.

The proposed structure (Fig. 8A) has two domains with the terminal catalytic (blue) and CaMLD (green) domains linked by JD, which has mainly an irregular structure (red) with a helical region (yellow) that interacts with the CaMLD. The NLS region of the JD (Fig. 8A, red) is exposed in the structure and appears amenable to interact with its cognate receptors or importins. The active site of the kinase (Fig. 8A, magenta) is spatially away from the NLS, and consequently the binding of importin at the NLS may not block the catalytic function of the activated kinase. The importin–CDPK complex structure (Fig. 8B) also shows that importin binding should not interfere with the kinase activity. It may be mentioned that although there is considerable sequence similarity (Fig. 7B), we did not use the NLS in 1PN as template when building this region. Still, NLS was modeled to have extended, nonregular conformation, as observed for the NLS peptide in 1PN. Additionally, GRAMM (57), which is a surface-recognition program, also identifies NLS as the docking site in AbCPK2, and the overall fitting is very snug. Although it is difficult to comment on the exact conformation of NLS and the relative orientation of the kinase and CaMLD domains in the absence of importin, the model in Fig. 8B depicts the feasibility of a successful interaction of the Arabidopsis CDPK with importins and is consistent with our biochemical data, where binding of several importins has been demonstrated with the kinase.

**DISCUSSION**

We note that the presence of nucleotide localization sequences in the junction domain of CDPKs is obligatorily coupled with nonfunctional EF hand loops in their respective CaMLDs (Fig. 2). Thus this subgroup of noncanonical CDPKs represents a unique and discrete case where interdomain compensatory changes are accompanied with a domain evolution (67, 68). The precise question that arises is why atypical calcium binding property was a primary requisite for acquiring a functional NLS in junction domains of CDPKs. We discuss our results on a CDPK from A. hypogea, a member of our target group of noncanonical CDPKs, and propose a possible explanation for such obligatory domain coupling in the light of the known mechanism of activation of canonical CDPKs (7–9, 32).

The Arabidopsis CDPK has a deviation from the consensus in its second calcium-binding site and has been shown to have significantly low affinity for calcium (Kd 392 μM) as compared with a canonical CDPK (Kd 0.19 μM) (32). However, despite having wide differences in their calcium binding properties (Fig. 3), the change in secondary structure for the Arabidopsis CDPK at saturating concentrations of calcium was exactly similar to its canonical counterparts (Fig. 4) (32). These observations are very similar to an earlier report on a Plasmodium CDPK where mutations in calcium-binding sites of the N-terminal lobe of its CaMLD significantly decreased its affinity for calcium, but the accompanied change in secondary structure of these mutant kinases resembled the wild type at saturating calcium concentrations (1–10 mM).

Because the characteristic calcium-dependent structural response in the CaMLDs is reported to be associated with the regulatory apparatus of canonical CDPKs (7–9), the principle of activation for the Arabidopsis CDPK appears to remain unchanged. Canonical CDPKs are activated by the cumulative effect of two calcium-responsive structural changes. (i) At low calcium levels high affinity binding sites in the C-terminal lobe are occupied and it interacts with the JD (15). When calcium levels rise to fill the two weaker binding sites in the N-terminal lobe, it triggers a conformational change through the tether region. Such interdomain complex interactions form a collapsed structure that leads to exposition of the catalytic site (9, 32). Because in our target group of CDPKs the nonconsensus calcium-binding sites are detected predominantly in the N-terminal lobe, the tether-mediated activation through its low affinity calcium-binding sites is most likely to be inactivated at subsaturating concentrations of calcium. Under similar conditions the interaction of JD with the C-terminal lobe of CaMLD with high affinity binding sites is unlikely to be affected (55).

In the light of this known mechanism of activation of CDPKs, we next discuss the atypical calcium binding properties of the Arabidopsis CDPK in relation to its possible role in the functional exposition of the NLS present in its JD. A known mechanism for regulating the activity of NLS is masking it in order to prevent its recognition by importins (69, 70). It is interesting to note that by virtue of having the NLS in the junction domain with a partial overlap with the autoinhibitory sequence, our target group of CDPKs can have their NLS masked by the catalytic domain in the inactive form of the kinase. Such interdomain rearrangements allow the functional exposure of the NLS sequence in the JD and its subsequent interaction with the nuclear transport factors (Fig. 6D). Through similar binding assays, we could not detect any interaction of importin with the Arabidopsis CDPK at saturating concentrations of calcium. Under such conditions the low affinity calcium-binding sites are occupied in the N-terminal lobe, which triggers a conformational change that appears to bury the JD in the CaMLD in such a manner that the NLS becomes inaccessible to importins (Fig. 6D). Such a proposition was confirmed when we found importin to interact with the Arabidopsis kinase at saturating concentrations of calcium in presence of W7 that prevented the interaction of JD with the CaMLD (Fig. 5D). The discrete differences in importin binding with the Arabidopsis CDPK at saturating and subsaturating calcium concentrations suggest the kinase to have an intermediate structure at low calcium concentration that is capable of interacting with importin. Such a proposition for a calcium-dependent intermediate structure is consistent with the observations made by Zhao et al. (53) on a Plasmodium CDPK. They noticed that conformational change in the wild type kinase was complete at subsaturating concentrations of calcium (100 μM), but in mutant kinase where calcium-binding sites were defective in the N-terminal lobe of its CaMLD, the full conformational change required a very high calcium concentration (53). This clearly indicated that CDPKs with nonfunctional EF hand motifs in their N-terminal lobe gradually attain their requisite change in structure in response to an increase in calcium concentration.

Finally, it appears that by virtue of having atypical calcium binding properties, our target group of CDPKs can sense a specific low calcium signature that helps in functional exposition of the NLS in the JD because of noncanonical interaction between the JD and its CaMLD. In response to the same signature, canonical CDPKs with four conserved calcium-binding sites are expected to have successful intramolecular interaction of JD with their respective CaMLDs, and this would prevent access of importin to its JD. This probably satisfies the coupling between

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A nonconsensus calcium-binding site in a CDPK with the presence of an N-S in its JD is important that importin binding compensates for the absence of the tether constant and provides the extra force for activation of these noncanonical kinases. This is significant because it would ensure that the kinase did not become active without ensuring its localization inside the nucleus.

It may be noted that the calcium dependence of the enzymatic activity of the Arabidopsis CDPK could not be studied as it failed to phosphorylate any known exogenous substrate. Most interestingly, AtCPK10, similar to the Arabidopsis CDPK, is associated with stress-dependent signaling events and is a member of our target group, also failed to phosphorylate any exogenous substrate. The evidence suggests that this subgroup of noncanonical CDPKs is active against unique substrates that have yet to be identified. It was interesting to note that a basic loop, which is believed to be a characteristic feature of the DAPK family (based on which the homology model of the Arabidopsis CDPK in Fig. 8A was built), is partially conserved in the basic loop, which is believed to be a characteristic feature of the DAPK family (Fig. 7A). This loop is believed to have a role in strictly restricting substrate phosphorylation any exogenous substrate. Most interestingly, AtCPK10 phosphorylates any exogenous substrate.

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