14-3-3 proteins activate a plant calcium-dependent protein kinase (CDPK)

Lorenzo Camoni[1,a], Jeffrey F. Harper[b], Michael G. Palmgren[a,*]

[a] *Institute of Molecular Biology, University of Copenhagen, Øster Farimagsgade 2A, DK-1353 Copenhagen K, Denmark*
[b] *Department of Cell Biology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA*

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Abstract Plants and protozoa contain a unique family of calcium-dependent protein kinases (CDPKs) which are defined by the presence of a carboxyl-terminal calmodulin-like regulatory domain. We present biochemical evidence indicating that at least one member of this kinase family can be stimulated by 14-3-3 proteins. Isoform CPK-1 from the model plant *Arabidopsis thaliana* was expressed as a fusion protein in *E. coli* and purified. The calcium-dependent activity of this recombinant CPK-1 was shown to be stimulated almost twofold by three different 14-3-3 isoforms with 50% activation around 200 nM. 14-3-3 proteins bound to the purified CPK-1, as shown by binding assays in which either the 14-3-3 or CPK-1 were immobilized on a matrix. Both the 14-3-3 binding and activation of CPK-1 were specifically disrupted by a known 14-3-3 binding peptide LSQRQRSTpSTPNVHMV (IC_{50} = 30 μM). These results raise the question of whether 14-3-3 can modulate the activity of CDPK signal transduction pathways in plants.

Key words: 14-3-3 protein; Calcium-dependent protein kinase; Signal transduction

1. Introduction

The 14-3-3 proteins are small highly conserved eukaryotic proteins which have been implicated in regulating multiple cellular enzymes, including protein kinases [2,13]. 14-3-3 proteins can bind and modulate the activity of protein kinase C [1,3] and Raf kinase [11,14,22,30]. In addition, they bind wee1 kinase [17] and mitogen-activated protein kinase (MEK) kinases [12]. In plants, 14-3-3 proteins are known to regulate enzymes such as nitrate reductase [6,26] and plant plasma membrane H^+-ATPase [7], but regulation of plant protein kinases as well as other signal pathways elements by 14-3-3 proteins has not been demonstrated.

Calcium-dependent protein kinases (CDPKs) are a unique family of kinases defined by a C-terminal calmodulin-like regulatory domain. CDPKs have been identified in plants and protozoa, but have not been described in fungal or animal systems [15,18,29]. The plethora of CDPK isoforms observed in *Arabidopsis* [18], and the comparable paucity of potential calmodulin-dependent kinases or PKC-like gene homologs, has led to the suggestion that the majority of calcium-regulated protein kinase activity in plants occurs through CDPKs.

In plants, changes in cytosolic Ca^{2+} concentrations are implicated in multiple signal transduction pathways, including light regulated development, plant pathogen defense and responses to abiotic stresses such as cold or drought [10]. Although physiological processes regulated by CDPKs have remained largely elusive, a number of proteins phosphorylated by these kinases have been identified [31]. In addition, there are a few examples in which there is evidence that a CDPK can regulate the activity of a protein, including a chloride channel in the guard cell tonoplast [28], the nodulin 26 channel localized in the symbiosome membrane of soybean root nodules [21], and nitrate reductase [5].

Here we demonstrate that 14-3-3 proteins can bind and activate CPK-1, a CDPK isoform from the model plant system *Arabidopsis thaliana.* This is the first biochemical evidence for a plant kinase activity stimulated by a 14-3-3 protein. Our results raise the possibility that 14-3-3 proteins can further activate CDPK signal transduction pathways in plants.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (specific activity 3000 Ci mmol^{-1}) was purchased from Dupont (Boston, MA, USA). Bovine brain myelin basic protein was from Sigma. Phosphopeptides were synthesized by Schaefer-N (Copenhagen, Denmark). Phosphoserine-Raf-259 peptide (LSQRQRSTpSTPNVHMV) corresponds to amino acids 251–256 of Raf-1 and mimics a 14-3-3 protein binding motif [27]. In the peptide sequence, pS designates a phosphoserine residue.

2.2. Protein expression and purification

The *Arabidopsis thaliana* CDPK isoform CPK-1 [18] was expressed in *Escherichia coli* as a fusion protein sandwiched in between glutathione-*S*-transferase at the N-terminus and six consecutive histidines at the C-terminus. This fusion protein was purified through an affinity purification protocol as previously described [16].

Arabidopsis thaliana 14-3-3 isoforms GF-14-ω, GF14-ψ and GF14-φ, fused in their N-termini to a Met-Arg-Gly-Ser-6×His tag, were expressed in *E. coli* and purified as described [7]. The 14-3-3 cDNA clones [24] were a kind donation by Robert Ferl (University of Florida, Gainesville, FL, USA).

2.3. Kinase assay

The activity of purified CDPK (100 ng) was assayed in a total volume of 50 μl containing 20 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 1 mM EGTA, 1.1 mM $CaCl_2$ (phosphorylation buffer) plus 0.5 mg/ml BSA and 0.04 mg/ml myelin basic protein (MBP). In this buffer the free calcium level is estimated to be higher than 100 μM [16]. Reactions were started by the addition of 100 μM ATP and 1 μCi [γ-^{32}P]ATP. After 15 min incubation, reactions were terminated by the addition of Laemmli sample buffer and samples were subjected to 12% SDS-PAGE. After staining with Coomassie Blue, the MBP phosphorylation level was determined by autoradiography. Alternatively, reactions were stopped by spotting samples onto phosphocellulose filter paper Whatman P81 (2×2 cm) and immersing it into 75 mM phos-

*Corresponding author. Present address: Department of Plant Biology, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark. Fax: (45) (35) 28 3333. E-mail: palmgren@biobase.dk

[1]Present address: Dipartimento di Biologia, Università di Roma 'Tor Vergata', Via della Ricerca Scientifica, I-00133 Roma, Italy.

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phoric acid (10 ml per reaction). Filters were washed as previously described [16] and radioactivity was quantified by scintillation counting.

2.4. Overlay binding assay

Overlay assay was modified after [25]. CDPK (1 μg) was immobilized to glutathione-Sepharose beads and incubated for 30 min with 50 μl of phosphorylation buffer, 100 μM ATP and 25 μCi [γ-^{32}P]ATP. Beads were washed five times with TBS and ^{32}P-autophosphorylated CDPK was eluted with glutathione as already described. Purified recombinant *Arabidopsis thaliana* 14-3-3 isoforms GF-14-ω, GF14-ψ and GF14-φ were subjected to SDS-PAGE and blotted onto nitrocellulose. Next, proteins on the blot were renatured overnight at 4°C with renaturation buffer: 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT, 2.5% fatty acid-free milk and 0.05% Tween-20. The nitrocellulose membrane was incubated with the ^{32}P-labelled CDPK (5×10^5 cpm/ml) in renaturation buffer for 1 h at room temperature. After extensive washings in renaturation buffer without milk, the blot was autoradiographed without intensifying screens for 24 h at −80°C.

2.5. Glutathione-Sepharose resin binding assay

Binding of 14-3-3 proteins to resin-bound CDPK was performed according to [22] with minor modifications. CDPK (100 ng) was immobilized to glutathione-Sepharose beads and incubated for 30 min at 4°C with 1 μg of purified *Arabidopsis thaliana* isoform GF-14-ω in 50 μl buffer containing 20 mM Tris-HCl (pH 7.0), 1 mM DTT, and 5 mM $MgCl_2$. Where indicated, 40 μM phosphoserine-Raf-259 peptide was included. Beads were washed five times with TBS-Tween 0.05%. After elution with 30 μl 50 mM Tris-HCl (pH 8.0) and 10 mM glutathione, proteins were subjected to 12% SDS-PAGE and 14-3-3 protein was identified by immunoblotting.

2.6. Gel electrophoresis and protein immunoblotting

Protein was precipitated with 10% (w/v) trichloroacetic acid before solubilization in an SDS cocktail at room temperature, and aliquots were subjected to SDS-PAGE [20]. Proteins were transferred electrophoretically to a nitrocellulose membrane (Millipore) for immunostaining. A polyclonal anti-BMH1 antiserum kindly provided by G.P.H. van Heusden (Leiden University, The Netherlands), and an anti-14-3-3 protein antiserum raised against a barley 14-3-3 protein which was a kind gift from C. Andersen and D. Collinge (The Royal Veterinary and Agricultural University, Frederiksberg, Denmark) were used as primary antibodies. The secondary antibody was conjugated to alkaline phosphatase (Dianova, Hamburg, Germany) and the protein gel blots were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

2.7. Protein

Protein was measured essentially according to Bradford [9] with bovine γ-globulin as standard.

3. Results

3.1. 14-3-3 protein binds CDPK isoform CPK-1 in vitro

To test whether CDPK interacts with 14-3-3 protein an overlay assay was used. Recombinant *Arabidopsis thaliana* 14-3-3 protein isoforms GF-14-ω, GF14-ψ and GF14-φ were subjected to SDS-PAGE, blotted onto nitrocellulose and allowed to renature. The nitrocellulose membrane was incubated with ^{32}P-labelled CPK-1 and subjected to autoradiography. Fig. 1A shows that CDPK bound strongly to all three 14-3-3 isoforms. Binding specificity was indicated by controls performed with BSA and multiple molecular weight standards which failed to show any detectable binding to CPK-1 (not shown).

To independently confirm that CPK-1 binds to 14-3-3 protein a complementary assay was employed. In this case the glutathione *S*-transferase (GST)-tagged CPK-1 protein was immobilized by binding to glutathione-agarose beads, and then tested for its ability to bind soluble GF14-ω 14-3-3.

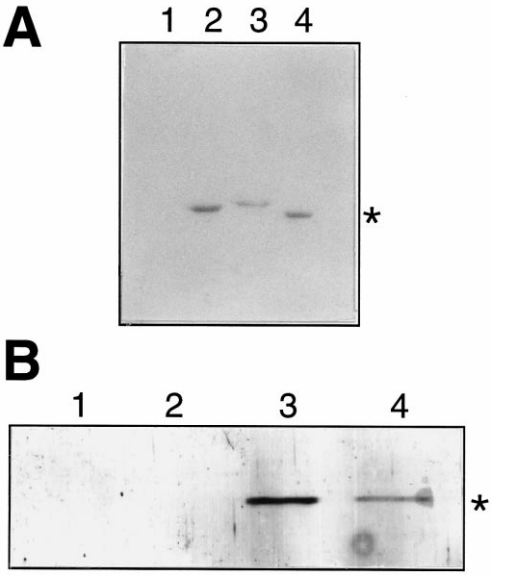

Fig. 1. In vitro association between 14-3-3 protein and CDPK. A: Binding of radiolabeled CDPK to 14-3-3 protein immobilized on a nitrocellulose membrane. The 14-3-3 protein and standard molecular weight protein markers were run on SDS-PAGE and blotted onto a nitrocellulose membrane. After coating of the membrane with milk proteins the blot was incubated with radiolabeled CDPK and bound CDPK (indicated by an asterisk) was visualized by autoradiography. Lane 1: standard molecular weight markers; lane 2: GF14-ω; lane 3: GF14-ψ; lane 4: GF14-φ. B: Binding of 14-3-3 protein to CDPK immobilized on agarose beads. GST-tagged CDPK was immobilized on an agarose resin and subsequently incubated with recombinant 14-3-3 protein. After washing away unbound protein, bound 14-3-3 protein (indicated by an asterisk) was visualized by subjecting the beads to SDS-PAGE and immunoblotting using a 14-3-3 protein antiserum. Lane 1: CDPK alone; lane 2: GF14-ω alone; lane 3: CDPK plus GF14-ω; lane 4: CDPK plus GF14-ω including 40 μM Raf-phosphopeptide.

The retention of 14-3-3 protein on the beads was shown by eluting all proteins with glutathione and detecting the level of bound 14-3-3 by Western blot analysis (Fig. 1B). Control experiments were performed to ascertain that the 14-3-3 protein was unable to bind GST alone (not shown).

A 14-3-3 protein binding site in the Raf-1 kinase involves a phosphorylated serine at position 259. A phosphopeptide corresponding to this region (phosphoserine-Raf-259 peptide) binds 14-3-3 proteins with high affinity [27]. The addition of phosphoserine-Raf-259 peptide as a competing substrate for 14-3-3 protein to the resin assay above significantly reduced (about 50%) binding activity (Fig. 1B, lane 4). This observation suggests that binding of 14-3-3 protein to CDPK involves the same residues of 14-3-3 protein as those involved in binding other target proteins.

3.2. Effect of 14-3-3 protein on CDPK activity

To investigate the functional consequence of a 14-3-3 interaction between CDPK, we analyzed the phosphorylation activity of CDPK in the presence of Ca^{2+} using myelin basic protein (MBP) as a substrate. As shown in Fig. 2A, MBP was readily phosphorylated by CPK-1 and phosphorylation was significantly increased by the addition of 14-3-3 protein. 14-3-3 itself was not phosphorylated by CPK-1.

In more quantitative assays, all three 14-3-3 isoforms tested stimulated CPK-1 activity between 1.5- and 1.9-fold, as shown

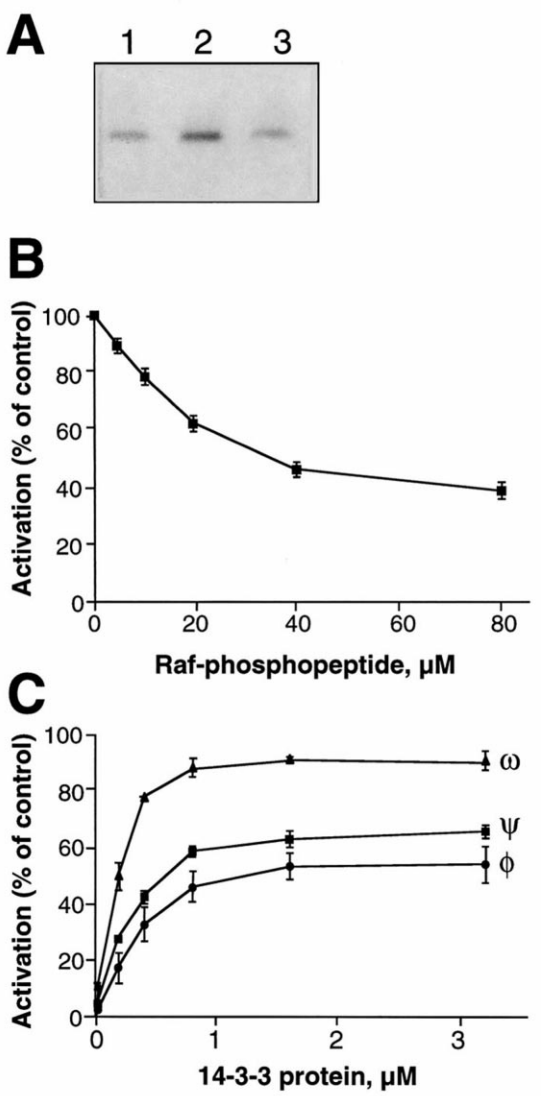

Fig. 2. Stimulation of CPK-1 by 14-3-3 protein. Myelin basic protein was used as a substrate for the phosphorylation reaction. Following phosphorylation the samples were subjected to SDS-PAGE and autoradiography. A: Effect of GF14-ω 14-3-3 protein (200 nM) on CDPK activity. Lane 1: CDPK alone; lane 2: CDPK plus GF14-ω (200 nM); lane 3: CDPK plus GF14-ω (200 nM) including 40 μM Raf-phosphopeptide. B: Dose dependent effect of Raf-phosphopeptide on CDPK activity in the presence of GF14-ω 14-3-3 protein (200 nM). The experiment was repeated two times with equivalent results. C: Dose dependent effect of various *Arabidopsis* 14-3-3 proteins on CDPK activity. The experiment was repeated three times with equivalent results.

in Fig. 2B. Activation was half maximal at 0.2 μM 14-3-3 protein. The degree of stimulation was dependent on the 14-3-3 isoform tested, with the greatest stimulation observed with GF14-ω 14-3-3.

Since the phosphoserine-Raf-259 peptide could be used to disrupt binding of 14-3-3 protein to CDPK (Fig. 1B, lane 4), we tested the ability of this peptide to inhibit 14-3-3 stimulation of CPK-1 activity. In control assays without 14-3-3 added, the phosphoserine-Raf-259 peptide did not inhibit the calcium-dependent activation of CPK-1. However, as shown in Fig. 2C, this peptide inhibited 14-3-3 stimulation with half maximal inhibition at 30 μM.

4. Discussion

We have identified a protein-protein interaction between 14-3-3 proteins and a CDPK. This association was demonstrated in two ways. First, binding studies showed that CDPK isoform CPK-1 could bind to 14-3-3 proteins immobilized on nitrocellulose membranes, and conversely, 14-3-3 protein could bind to CPK-1 immobilized on agarose beads. Second, kinase assays showed that submicromolar levels of 14-3-3 could further stimulate a calcium activated kinase activity. Both the binding and functional assays were specifically inhibited by low micromolar concentrations of a known 14-3-3 binding peptide (phosphoserine-Raf-259). This indicates that CPK-1 and a 14-3-3 can form a specific high affinity interaction which stimulates kinase activity.

The 14-3-3 binding site in CPK-1 has not been determined. However, the N-terminal domain contains the sequence R^{80}TESKP which is very similar to the consensus sequence RS*X*S*X*P (in which the underlined serine is phosphorylated) that was recently identified as a 14-3-3 binding motif [27]. Thus, the R^{80}TESKP sequence may account for the interaction between 14-3-3 proteins and CPK-1. However, alternative binding sites are possible since there are examples of non-consensus 14-3-3 binding sequences, such as in a T cell antigen receptor [23] and the glycoprotein Ib-IX-V complex [4]. Nevertheless, the 14-3-3 binding motif above is thought to require a phosphoserine for high affinity binding [27]. Since the CPK-1 used here was produced in *E. coli*, it is likely that any phosphoserines within the kinase result from autophosphorylation. Thus, the target 14-3-3 binding site in CPK-1 probably overlaps with an autophosphorylation site. Although CPK-1 undergoes autophosphorylation [8], these sites are still undetermined.

In vitro assays using purified proteins showed that the calcium-dependent kinase activity of isoform CPK-1 was further stimulated almost two-fold by submicromolar levels of 14-3-3. All 3 tested 14-3-3 isoforms activated CPK-1, although slight differences in their potency were observed. This suggests that the ability to stimulate CPK-1 is a general feature of 14-3-3 proteins. Maximum stimulation of Raf-1 required an additional cofactor present in cellular extracts [22]. Similarily, 14-3-3 stimulation of a plasma membrane H^+-ATPase in plants was only observed in the presence of fusicoccin [7]. Although fusicoccin did not enhance the 14-3-3 stimulation of CPK-1 (data not shown), we cannot exclude the possibility that other potential cofactors are required for optimal stimulation of the recombinant CDPK by 14-3-3.

There was no stimulatory effect by 14-3-3 protein in the absence of Ca^{2+}. Thus, 14-3-3 does not merely mimic calcium, but rather provides an additional stimulation once the enzyme is activated. This suggests that activation by Ca^{2+} and 14-3-3 protein proceeds by different mechanisms.

The mechanism of calcium activation is proposed to involve intramolecular binding between the C-terminal calmodulin-like domain and the neighboring junction domain [19]. The junction domain contains a pseudosubstrate autoinhibitor and provides the connection between the kinase catalytic domain and the calmodulin-like domain. Activation occurs as a result of calcium binding to the calmodulin-like domain and inducing a conformational change in the junction which disengages the autoinhibitor.

The mechanism by which a 14-3-3 further stimulates a cal-

cium activated CPK-1 is unclear. Our working hypothesis is that 14-3-3 stimulation involves a binding interaction with CPK-1's N-terminal domain, since this domain contains a consensus 14-3-3 binding motif, as well as a site for autophosphorylation (Binder, Harper and Sussman, unpublished). Interestingly, the N-terminal domains of CDPKs are highly variable, and CPK-1 is currently the only *Arabidopsis* isoform to display a consensus 14-3-3 binding motif in this region. Thus, the observed 14-3-3 stimulation of CPK-1 may represent a unique regulatory feature only present in a subset of CDPK isoforms.

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