Phosphorylation of plant actin-depolymerising factor by calmodulin-like domain protein kinase

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Abstract The actin-depolymerising factor (ADF)/cofilin group of proteins are stimulus-responsive actin-severing proteins, members of which are regulated by reversible phosphorylation. The phosphorylation site on the maize ADF, ZmADF3, is Ser-6 but the kinase responsible is unknown [Smertenko et al., Plant J. 14 (1998) 187–193]. We have partially purified the ADF kinase(s) and found it to be calcium-regulated and inhibited by *N*-(6-aminohexyl)-[^{3}H]5-chloro-1-naphthalenesulphonamide. Immunoblotting reveals that calmodulin-like domain protein kinase(s) (CDPK) are enriched in the purified preparation and addition of anti-CDPK to in vitro phosphorylation assays results in the inhibition of ADF phosphorylation. These data strongly suggest that plant ADF is phosphorylated by CDPK(s), a class of protein kinases unique to plants and protozoa. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Actin-depolymerizing factor; Calmodulin-like domain protein kinase; Cofilin; Phosphorylation; Higher plant; Cytoskeleton

1. Introduction

The actin cytoskeleton is a dynamic network in all eukaryotic cells. It is involved in a variety of morphogenetic events (e.g. cell division, cell expansion, root hair and pollen tube growth), and responds to biotic and abiotic stimuli (e.g. pathogen attack, light, cold, and water stress) [2–6]. The actin filaments have the ability to break down into monomeric or G-actin and to reform into filamentous or F-actin. This dynamic process occurs by depolymerisation of actin from the pointed ends and polymerisation at the barbed ends. The state of any given actin filament will depend on the balance between the polymerisation and depolymerisation rates and many actin-binding proteins can modulate these rates [7].

One such group of proteins is the actin-depolymerising factor (ADF)/cofilin family, members of which are found in animals, fungi, plants and protists. In general, the ADF or cofilin group of proteins binds both G-actin and F-actin and modulates its dynamics; it does this by severing actin filaments thereby providing more filament ends for polymerisation and by increasing the dissociation rate constant at the pointed end and providing more monomer for assembly at the barbed ends [8]. The plant ADF/cofilins share approximately 22–44% identity with other members in the group [9]. The plant ADFs are encoded by relatively large multigene families. In *Arabidopsis*, for example, there are at least 13 genes. Members of these gene families are differentially expressed. In particular there are vegetative and pollen-specific ADFs in maize [10]. The biochemical characteristics of three plant ADF/cofilins have been determined: *Arabidopsis* ADF1, the maize vegetative ADF, ZmADF3, and the lily pollen ADF, LlADF1 [11–13]. Recent data suggest that there are fundamental differences in the activities of the pollen and vegetative isoforms [13].

Members of the ADF/cofilin group of proteins are regulated by phosphorylation, are sensitive to pH, and are inhibited by phosphoinositide 4,5-bisphosphate [1,8,12,14,16]. These data suggest that these proteins can be activated/deactivated as part of signal transduction cascades, and hence are able to contribute to the regulation of the actin cytoskeleton in response to environmental stimuli and developmental cues.

ADF/cofilin is phosphorylated on Ser-3 in animal cells [15] or its equivalent Ser-6 in plants [1]. Phosphorylation inhibits the activity of ADF/cofilin in metazoans and plants [1,16]. The metazoan ADF kinase has been identified as LIM kinase [17,18]. ADF/cofilin is the only known substrate for LIM kinases of which there are two forms that are differentially regulated by the Rho family of GTPases [17,18]. However, these LIM kinases do not phosphorylate plant ADF [8], and moreover LIM kinases have no homologues in plants, therefore the kinase(s) that is responsible for phosphorylating ADF in plants remains to be determined.

We have previously reported that plant ADF can be phosphorylated by a calcium-stimulated protein kinase on Ser-6 [1]. In this study we show that phosphorylation of maize ADF on Ser-6 is by calmodulin-like domain protein kinase (CDPK), a class of protein kinases unique to plants and protists.

2. Materials and methods

2.1. Protein purification

Cell suspension cultures of French bean (*Phaseolus vulgaris* L. cv. Immuna 1.1) were harvested as described in [1]. Extracts were prepared, and then further purified as described in Section 3. ZmADF3, zmadf3-3, and zmadf3-4 were expressed and purified by expression as fusion proteins with a His tag, and purification by passing over Ni-NTA spin columns (Qiagen, UK). The His tag was subsequently removed by thrombin cleavage, as described in [1]. Actin was purified from rabbit skeletal muscle as described in [19].

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Abbreviations: W7, *N*-(6-aminohexyl)-[^{3}H]5-chloro-1-naphthalenesulphonamide; ADF, actin-depolymerising factor; CDPK, calmodulin-like domain protein kinase

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2.2. Phosphorylation reactions

Phosphorylation of ZmADF3, zmadf3-3, and zmadf3-4 was carried out essentially as described in [1]. The reaction mixture contained 1.3 μM microcystin LR, 57 mM MOPS pH 7.5, 11 mM $MgCl_2$, 200 μM $CaCl_2$, 20 μM ATP, 2 μg ZmADF3, 3.3 μCi γ[^{33}P]ATP (NEN Life Science Products), and extract containing 0.14 nmol/min protein kinase activity. Where indicated, 10 mM EGTA, 1 mM *N*-(6-aminohexyl)-[^{3}H]5-chloro-1-naphthalenesulphonamide (W7), or anti-CDPK was also included in the incubation. The final volume of the incubation was 50 μl. The incubation was stopped after 15 min by binding all the protein onto 10 μl StrataClean™ resin (Stratagene) according to the manufacturer's instructions. The resin was collected by slow speed centrifugation for 1 min, and the pellet washed in 50 mM MOPS to remove the unbound γ[^{33}P]ATP. The StrataClean resin was then loaded directly onto a 12% SDS polyacrylamide gel.

2.3. Electrophoresis

One-dimensional SDS–PAGE was as described in [20]. Transfer of proteins from gels to PVDF or nitrocellulose and immunostaining were as described in [21], using anti-CDPK [22] or anti-ZmADF3 [23], and anti-rabbit peroxidase-conjugated immunoglobulin (Sigma, UK). Proteins on PVDF membrane were stained with Coomassie brilliant blue R250. Two-dimensional gel electrophoresis was as described in [24].

3. Results

3.1. Protein extraction and partial purification of CDPK

We have previously shown that ZmADF3 can be phosphorylated by a crude protein extract, and that this phosphorylation is stimulated in the presence of calcium [1]. The major source of calcium-stimulated protein phosphorylation in plants is from CDPKs [25] raising the possibility that this class of kinase might be responsible for ADF phosphorylation. Cell suspension cultures of French bean (*P. vulgaris* L. cv. Immuna 1.1) were harvested as described in [1]. Extracts were prepared, and then further purified using standard chromatographic procedures for CDPK: successive steps of anion exchange chromatography (DEAE cellulose), hydrophobic interaction chromatography (octyl agarose) and dye affinity chromatography (blue agarose) [26]. Immunoblotting using antibody raised against the calmodulin-like domain of CDPK, which is highly conserved throughout many CDPKs [22], identified a single band in the 40–70% ammonium sulphate fraction and in the CDPK preparation (Fig. 1). The data show that CDPK has been significantly enriched during the purification as the majority of other proteins have been removed. Further purification of the CDPK was not possible due to low yields.

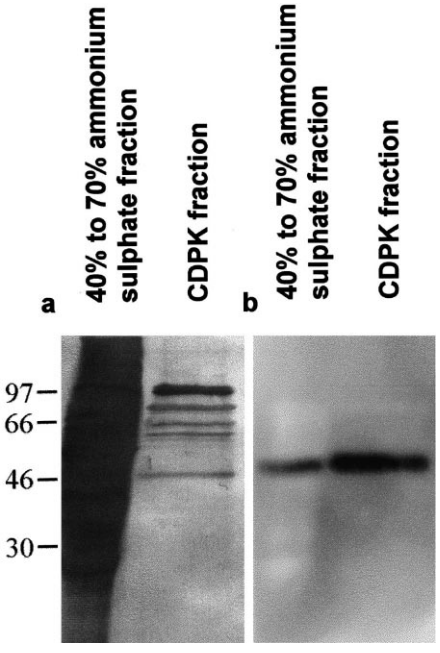

Fig. 1. Partial purification of CDPK. Anti-CDPK identifies a single band in the 40–70% ammonium sulphate fraction and in the CDPK preparation. A Coomassie-stained gel (a) and equivalent immunoblot (b) stained with anti-CDPK. The results show that CDPK is enriched by the purification strategy.

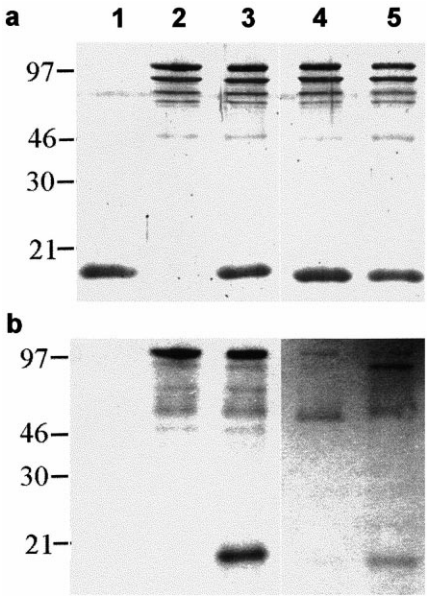

Fig. 2. In vitro phosphorylation assays. A Coomassie blue-stained gel blot (a) and respective autoradiograph (b). Lane 1, ZmADF3 only; lane 2, CDPK preparation; lane 3, ZmADF3 and CDPK preparation; lane 4, ZmADF3 and CDPK preparation with EGTA; lane 5, ZmADF3 and CDPK preparation with W7. The results suggest that ZmADF3 is phosphorylated by CDPK.

3.2. Phosphorylation of ZmADF3 by CDPK preparation

The CDPK preparation was used to phosphorylate ADF. Incubation of purified ZmADF3 with the CDPK preparation and γ[^{33}P]ATP resulted in the phosphorylation of ZmADF3, as shown by analysis on SDS–PAGE, followed by electroblotting onto PVDF and autoradiography. This phosphorylation was abolished in the presence of EGTA (Fig. 2). When the incubation was carried out in the presence of 1 mM W7, a calmodulin antagonist [27,28], the amount of phosphorylation was greatly reduced. These data strongly suggest that CDPK phosphorylates ZmADF3.

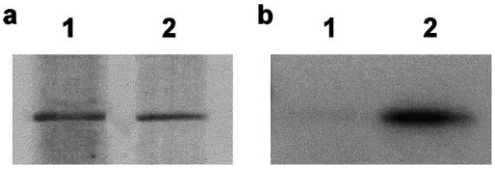

Fig. 3. ZmADF3 is phosphorylated by CDPK. In vitro phosphorylation assays using the CDPK preparation in the presence (lane 1) and the absence (lane 2) of anti-CDPK. A Coomassie blue-stained gel blot (a) and respective autoradiograph (b). The results show that anti-CDPK inhibits the phosphorylation of ZmADF3.

3.3. Anti-CDPK inhibits phosphorylation of ZmADF3

It is possible that other kinases have co-purified with the CDPK in the CDPK preparation. In order to establish whether the CDPK in this preparation is the kinase that is phosphorylating ZmADF3, anti-CDPK was added to the phosphorylation reaction. The results show that anti-CDPK abolishes the ADF-phosphorylating activity of the preparation (Fig. 3). The antibody was raised to the calmodulin-like domain of CDPK [22]. This strongly suggests that ZmADF3 is phosphorylated by CDPK(s).

3.4. Ser-6 is the CDPK phosphorylation site

In order to determine whether CDPK phosphorylates ZmADF3 at Ser-6, as described in [1], the ability of the CDPK preparation to phosphorylate each of the three recombinant proteins (ZmADF3, zmadf3-3, and zmadf3-4) was tested. The recombinant proteins zmadf3-3 and zmadf3-4 differ from ZmADF3 in that Ala and Asp replace Ser-6 in zmadf3-3 and zmadf3-4 respectively, both of which prevent phosphorylation at this site. Neither zmadf3-3 nor zmadf3-4 could be phosphorylated above a background level by the CDPK preparation (Fig. 4). Therefore we conclude that the phosphorylation of ZmADF3 by CDPK is on Ser-6.

To determine the extent of the phosphorylation of the recombinant ZmADF3 by the kinase in our in vitro assays we fractionated 3 μg of γ-^{33}P-phosphorylated ZmADF3 on 2-D gels, immunoblotted the gels using anti-ZmADF3 and exposed the blots to film. The immunoblot shows two major spots which are immediately adjacent to each other, having similar M_r, but differing slightly in p*I*. The γ[^{33}P] was associated with only the spot with the more acidic p*I* (Fig. 5). The proportion of phosphorylated ZmADF3 was estimated densitometrically to be 33% of the total ZmADF3 protein. This value is in line with other studies carried out in vitro using recombinant protein and kinase [29]. The minor spots with more acidic p*I* also visible on the gels are likely to represent other phosphoforms of ADF as there is minor ADF phosphorylation on the zmadf3-3 and zmadf3-4 proteins which lack Ser-6 (see Fig. 4). This has also been seen to occur in phosphorylation experiments using recombinant mammalian cofilin [29].

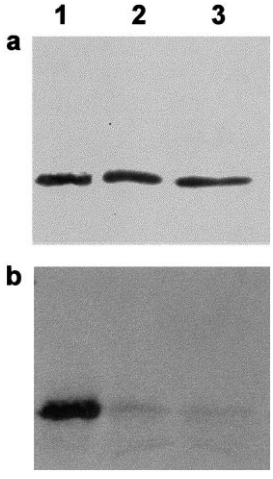

Fig. 4. Ser-6 is the major CDPK phosphorylation site. Coomassie brilliant blue-stained 1-D gel (a) and corresponding autoradiograph (b) of the phosphorylation of ZmADF3, zmadf3-3, and zmadf3-4. Lane 1, ZmADF3; lane 2, zmadf3-3; lane 3, zmadf3-4. These data show that the CDPK phosphorylation site is on Ser-6.

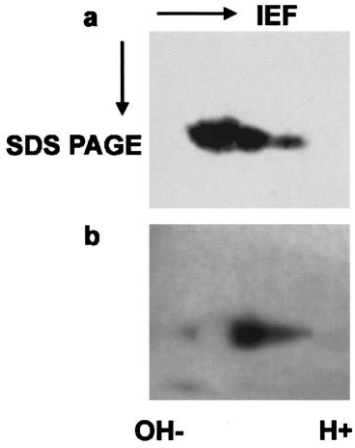

Fig. 5. 2-D gel immunoblot of phosphorylated ZmADF3 stained with anti-ZmADF3 (a) and corresponding autoradiograph (b). The immunoblot shows two major spots, only the more acidic of which is phosphorylated (compare a with the autoradiograph in b). Densitometric scans reveal that 33% of the recombinant ZmADF3 is phosphorylated by CDPK under these conditions.

4. Discussion

In previous papers we have shown that maize ADF3 can increase in vitro actin dynamics and that it can be phosphorylated on Ser-6 by a crude plant protein extract [1,23,30]. Here we report that ZmADF3 is phosphorylated on Ser-6 by CDPK. We have shown that ZmADF3 phosphorylation is calcium-dependent, and that phosphorylation of ZmADF3 is inhibited by addition of anti-CDPK to the phosphorylation assay. Moreover, the CDPK phosphorylation site is shown to be Ser-6.

CDPKs are the main contributors to calcium-stimulated protein phosphorylation in plants, and make up one of the largest protein kinase families in plants (over 30 genes encoding CDPKs have been identified in *Arabidopsis*). They consist of four domains: a regulatory calmodulin-like domain, a junction or autoinhibitory domain, a kinase domain and a variable domain which may be responsible for their subcellular location and/or their substrate specificity [25,31]. Several substrates for CDPKs have been identified using biochemical approaches suggesting potential regulatory roles in gene expression [32], metabolism [33], and ion transport [34]. Many of the experiments to date have used purified or partially purified CDPK preparations and these are likely to contain more than one isoform of CDPK. Sheen [35] showed that individual isoforms can have specific roles. One CDPK out of six kinases tested (two of which were distinct CDPKs) turned on the expression of a stress-, calcium- and abscisic acid-responsive reporter gene implicating this CDPK as a positive regulator of stress signal transduction [35]. Recently, a two-hybrid screen using McCDPK1 revealed a specific interacting substrate further emphasising the likelihood of specific roles for individual CDPKs [36]. It remains to be determined whether a particular isoform of the CDPK family phosphorylates ADF and regulates its activity.

Very little is known about the regulation of actin dynamics in plants. So far, ADF and profilin are the only actin-modu-

lating proteins to be extensively characterised in plants. However, there are many examples of changes in actin organisation in response to specific stimuli [2–6]. If ADF is a major regulator of such plant actin dynamics in vivo, we would expect it to be under the control of signal molecules which are known to result from both environmental and developmental factors. One such signal molecule is calcium, for which a role as a second messenger in signalling processes in plants is well established [37]. Calcium ion fluxes have been correlated with a large number of stimuli (cold, light, hormones, touch, fungal elicitors). Consistent with this is the fact that many cellular processes require calcium (guard cell movement, pollen tube growth, root hair tip growth, cytoplasmic streaming) and all these processes require the activity of a dynamic actin cytoskeleton. We would predict, therefore, the proteins controlling actin dynamics to be responsive to the local calcium signatures. In addition to regulation of ADF phosphorylation by specific CDPKs, different ADF isoforms could be phosphorylated at different calcium concentrations by the same CDPK. Lee et al. [38] have shown that the sensitivity to calcium of some CDPKs depends on the substrate that is being phosphorylated. Phosphorylation of ADF3 by CDPK is on Ser-6, and we have previously shown that a mutation at this site which mimics phosphorylation abolishes the functional activity of ZmADF3. Therefore CDPK phosphorylation of maize ADF3 provides a way by which control of ADF activity can be linked to calcium signalling, and through which actin reorganisation can respond to environmental and developmental signals.

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