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Multisite phosphorylation of 14-3-3 proteins by calcium-dependent protein kinases

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Abstract

Plant 14-3-3 proteins are phosphorylated at multiple sites *in vivo*; however, the protein kinase(s) responsible are unknown. Of the 34 CPK (calcium-dependent protein kinase) paralogues in *Arabidopsis thaliana*, three (CPK1, CPK24 and CPK28) contain a canonical 14-3-3-binding motif. These three, in addition to CPK3, CPK6 and CPK8, were tested for activity against recombinant 14-3-3 proteins χ and ϵ . Using an MS-based quantitative assay we demonstrate phosphorylation of 14-3-3 χ and ϵ at a total of seven sites, one of which is an *in vivo* site discovered in *Arabidopsis*. CPK autophosphorylation was also comprehensively monitored by MS and revealed a total of 45 sites among the six CPKs analysed, most of which were located within the N-terminal variable and catalytic domains. Among these CPK autophosphorylation sites was Tyr⁴⁶³ within the calcium-binding EF-hand domain of CPK28. Of all CPKs assayed, CPK28, which contained an autophosphorylation site (Ser⁴³) within a canonical 14-3-3-binding motif, showed the highest activity against 14-3-3 proteins. Phosphomimetic mutagenesis of Ser⁷² to aspartate on 14-3-3 χ , which is adjacent to the 14-3-3-binding cleft and conserved among all 14-3-3 isoforms, prevented 14-3-3-mediated inhibition of phosphorylated nitrate reductase.

Keywords

14-3-3 protein; *Arabidopsis*; calcium-dependent protein kinase; kinase assay; protein–protein interaction; tyrosine phosphorylation

INTRODUCTION

14-3-3 proteins are a class of regulatory/effector proteins that modulate client protein function through phosphorylation-dependent associations [1]. The *Arabidopsis* genome encodes 15 different 14-3-3 isoforms, 13 of which are transcriptionally expressed [2]. These proteins are expressed in all organs of *Arabidopsis*, from seedlings to developing seed [3,4], and along with proteins containing forkhead-associated domains are the only known phosphoprotein-binding proteins in plants [5]. Thus the functional role of 14-3-3 proteins is

AUTHOR CONTRIBUTION

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centred on their ability to bind and influence phosphoproteins. Biochemical evidence suggests that several metabolic enzymes are altered upon phosphorylation and subsequent 14-3-3 binding, including nitrate reductase and SPS (sucrose-phosphate synthase) [6–8]. Additionally, *in planta* evidence supports the role of 14-3-3 proteins in these metabolic pathways [4,9].

14-3-3 proteins are phosphorylated on multiple residues in several different species [10–13], suggesting that while 14-3-3 proteins bind and influence phosphoproteins, they themselves may be regulated via phosphorylation. In *Arabidopsis*, there are six reports of *in vivo* 14-3-3 phosphorylation, collectively identifying 17 phosphorylation sites on eight different isoforms [11–16]. Many of these phosphorylation events were confirmed by independent investigations, supporting their existence. However, some sites have yet to be confirmed, suggesting that they may be less prevalent or artefactual. Nevertheless, there is strong evidence of 14-3-3 phosphorylation in plants. Despite knowledge of these phosphorylation events, a general understanding of the kinases responsible and the biochemical effect of these post-translational modifications is lacking.

The *Arabidopsis* CPK (calcium-dependent protein kinase) family consists of 34 different isoforms and four distinct subgroups based upon sequence similarity [17]. These kinases become active during intracellular calcium bursts (for a review see [17]). Increases in intracellular calcium concentrations have been correlated with changes in protein phosphorylation, some of which are probably due to CPKs [18]. Like 14-3-3 proteins, CPKs regulate enzymes involved in carbon and nitrogen metabolism. Most notably is the ability of CPKs to phosphorylate NR (nitrate reductase) [6], sucrose synthase [19] and SPS [20] *in vitro*.

Early reports characterized the inhibition of NR from *Spinacia oleracea*, in which phosphorylation of NR by a CPK was followed by binding of an inhibitor protein, later characterized to be multiple 14-3-3 isoforms (most notably 14-3-3 ω and 14-3-3 χ) [21–23]. Similarly, phosphorylation of NR at Ser⁵³⁴ by CPK17 and inhibition by 14-3-3 isoforms was also described using recombinant *Arabidopsis* proteins [6]. *In vitro* experiments have also revealed that SPS is phosphorylated by a CPK [20,24], and 14-3-3 binding can modulate SPS activity [8,25]. Interestingly, 14-3-3 proteins have also been shown to influence CPK1 activity. Kinase activity assays using *Arabidopsis* CPK1 demonstrated that 14-3-3 binding enhanced phosphorylation activity against a bovine myelin basic protein [26]. This interaction was later confirmed *in vivo* when tandem affinity purification of 14-3-3 ω co-purified CPK1 [27]. Recently, it was demonstrated that CPK3 phosphorylates 14-3-3 proteins *in vitro* at a conserved serine residue upon treatment of a sphingoid long-chain base, phytosphingosine [28]. Collectively, these results suggest a relationship between 14-3-3 proteins, CPKs and CPK clients in the regulation of carbon and nitrogen metabolism. On the basis of previously described 14-3-3–CPK associations, we proposed that CPKs directly phosphorylate 14-3-3 proteins to modulate 14-3-3 function on CPKs and potentially other clients. Owing to their previously described phosphorylation and phylogenetic divergence, 14-3-3 isoforms χ and ϵ were studied in this investigation. We demonstrate in the present study that this relationship may be more complex than previously thought, as 14-3-3

proteins may not only affect CPKs and their clients, but are themselves phosphorylated at multiple positions by CPKs in a specific manner.

EXPERIMENTAL

Recombinant protein expression, purification and quantification

All proteins were expressed and purified as described previously [29]. The primer pairs and TAIR accession numbers for all gene constructs are listed in Supplementary Table S1 (at <http://www.biochemj.org/bj/459/bj4590015add.htm>). After purification, kinases were dialysed against storage buffer [20 mM Tris/HCl (pH 7.5), 10 mM MgCl₂, 1 mM CaCl₂ and 1 mM DTT]. Protein was quantified using a modified Bradford assay using BGG (bovine γ -globulin) as a standard (Bio-Rad Laboratories). Proteins were stored in 50% glycerol at – 20°C until *in vitro* kinase assays were performed.

In vitro kinase assays

Target substrates were incubated with no kinase and heat-inactivated kinases (95°C for 10 min) as controls. Reaction mixtures were incubated at 30 °C for 1 h in 40 μ l of kinase buffer [20 mM Hepes-KOH (pH 7.4), 5 mM MgCl₂, 1 mM DTT, 2 mM ATP and 0.2 mM CaCl₂]. Reactions were stopped by adding 6 $\times$ sample buffer [0.36 M Tris/HCl (pH 6.8), 10% (w/v) SDS, 30% (v/v) glycerol, 600 mM DTT and 0.15 mM Bromophenol Blue] and heated for 10 min at 65°C. The entire reactions were resolved 7 cm into an SDS/PAGE gel (13% gel) and stained with PQD (Pro-Q Diamond) as described previously [30]. CPK autophosphorylation assays were treated identically as the *in vitro* kinase assays, but in the absence of substrates. CPKs were then incubated with and without the addition of λ -PPase (λ -phosphatase) (200 units/reaction) and analysed by PQD staining. *In vitro* kinase assays for MS analysis were treated similarly to those stained with PQD. Substrate (0.7 μ M) and kinase (0.3 μ M) concentrations were increased 5-fold in order to increase peptide identifications and protein coverage in MS analysis. SDS/PAGE gels were stained for 16 h with colloidal CBB (Coomassie Brilliant Blue) G-250 [31]. Protein bands corresponding to kinase and substrate molecular masses were excised from SDS/PAGE gels and were processed using a previously described in-gel digestion procedure [29]. Additionally, CPK autophosphorylation assays were digested using in-solution digestion procedures [32]. Tryptic peptides were freeze-dried and stored at – 80 °C until MS analysis. All *in vitro* kinase assays were performed in biological triplicate.

MS analysis

Freeze-dried tryptic peptides were prepared for MS analysis as described previously [29]. Samples were analysed on a LTQ Orbitrap XL ETD (Thermo Fisher Scientific). Peptides were initially bound to C₈ Captraps (Michrom Bioresources) before being eluted using a 25 min acetonitrile gradient (5– 35 % acetonitrile). During the gradient peptides were continually separated using a 'Magic C18' [200 Å (1 Å = 0.1 nm), 5 μ m bead, Michrom Bioresources] fused silica nanospray column. Precursor masses were scanned using the following settings: analyser, FTMS (Fourier Transform Mass Spectrometer); mass range, normal; resolution, 60000; scan type, positive; data type, centroid; and scan ranges, 200– 2000 *m/z*. The top seven masses from the precursor scan were selected for data-dependent

acquisition. Data-dependent scan settings were the following: analyser, ion trap; mass range, normal; scan rate, normal; and data type, centroid. Charge state screening and monoisotopic precursor selection were enabled. Unassigned charge states and masses with a charge state of one were not analysed. The 'data-dependent decision tree' option was enabled as previously described [33], allowing for fragmentation of peptides using CID (collision-induced dissociation) or ETD (electron transfer dissociation).

Database searching

Acquired spectra were searched against a custom database containing the *Arabidopsis* 14-3-3 isoforms and CPKs. All entries were concatenated to a randomized database as a decoy and searched using SEQUEST with Thermo Proteome Discoverer version 1.3.0.339. Proteome Discoverer search parameters included a mass range of 200–5000 Da, positive-mode polarity, signal-to-noise ratio of 3, and a minimum peak count of one. SEQUEST search parameters were static modification of cysteine-carboxyamidomethylation, variable modifications of methionine-oxidation, and phosphorylation of serine, threonine and tyrosine residues. Other search parameters included two missed tryptic cleavage sites, and precursor and fragment ion tolerances of 1.0 Da and 1000 p.p.m. respectively. However, the final precursor ion tolerance was 1.5 p.p.m. after filtering [34]. The peptide filter of 'charge state versus Xcorr' was enabled. Additional filters included a peptide score (SequestNode-Probability) of 13.01, a maximum C_n of 0.04 [35], and a minimum of two peptides per protein. Phosphopeptides that passed these filters were analysed further using the phosphoRS algorithm of Proteome Discoverer [36]. Phosphopeptides with a pRS Score ≥ 50 and a pRS Site Probability of $\geq 75\%$ were reported. No decoy proteins were identified.

Phosphopeptide quantification

Phosphopeptides identified by LC–MS/MS from both 14-3-3 isoforms χ and ϵ were quantitatively compared by integrating the absolute area of the precursor masses. Precursor masses were integrated using a mass deviation of 1.5 p.p.m. The top three peaks from each raw file were manually analysed and integrated using Thermo Xcalibur Qual Browser version 2.0®. Additional Qual Browser settings included the following: y-axis, absolute; smoothing enabled; type boxcar/points 7; baseline subtraction disabled; and mass tolerance, 1.5 p.p.m. Data generated for each run was manually exported from Qual Browser into a Microsoft Excel spreadsheet for further analysis. Using a Tukey range test the ability of kinases to phosphorylate their target 14-3-3 substrates was statistically assessed. Differences in the extent of phosphorylation were considered to be significant if they had a P value <0.01 .

Fluorescence localization

CPKs (3, 6, 24 and 28) and 14-3-3 isoforms (χ and ϵ) were cloned into YFP expression vectors [37]. *Agrobacterium tumefaciens* GV3101 strains containing CPK and 14-3-3 isoforms were infiltrated into 5–6-week-old *Nicotiana benthamiana* leaves for transient expression as described previously [38]. Confocal images were taken of *N. benthamiana* epidermal cells 3 days post-infiltration. Images of leaf epidermal cells for localization assays were acquired as described previously [38,39].

NR activity assays

Arabidopsis NR was purchased from the Nitrate Elimination Company. Phosphorylation of NR was achieved through the addition of CPK28 and ATP to a final concentration of 40 nM and 1 mM respectively. The reaction was incubated at 25°C for 1 h on a rotary agitator. Phosphorylation of Ser⁵³⁴ on NR was confirmed using MS/MS. Anion-exchange chromatography was performed on pNR (phosphorylated NR) using a HiTrap Q HP column (Amersham Biosciences), as described by Lambeck et al. [6]. The final pNR concentration was measured at 413 nm with a molar absorption coefficient of 120 mM⁻¹ cm⁻¹ [6]. All NR activity assays were performed in 100 µl of assay buffer [50 mM Tris/HCl, 50 mM KCl, 5 mM MgCl₂ and 0.2 mM CaCl₂ (pH 7.0)]. The final concentrations of pNR and protein additions (BSA, 14-3-3 χ or 14-3-3 χ -S72D) were 10 nM and 1 µM respectively. pNR was incubated in the presence and absence of the protein substrate at 30 °C for 10 min with slight agitation. NADH was supplemented to a final concentration of 100 µM. The reaction was initiated with 1 mM potassium nitrate. The decrease in A₃₄₀ was measured continuously at 30°C using a Bio-Tek Synergy HT plate reader (Bio-Tek Instruments) in clear 96-well plates. Each assay included seven technical replicates per reaction and was performed in biological triplicate.

RESULTS

In vitro phosphorylation of 14-3-3 χ and 14-3-3 ϵ by CPKs

To determine whether CPKs can phosphorylate 14-3-3 isoforms χ and ϵ , a series of kinase assays were performed using phylogenetically distinct CPKs (1, 3, 6, 8, 24 and 28); all four CPK subgroups were represented. Three of these CPKs (1, 24 and 28) were selected because they contain the canonical 14-3-3-binding motif R-S/T-X-S-X-P, where 'X' represents any amino acid and phosphorylation of the underlined serine residue completes the motif [40]. Co-incubation of 14-3-3 proteins with CPKs revealed 14-3-3 phosphorylation as assessed by PQD staining (Figure 1). Interestingly, two prominent bands were detected by PQD staining, suggesting the presence of multiple 14-3-3 phosphorylation sites. Negative controls of 14-3-3 alone and 14-3-3 incubated with denatured CPKs showed substantially reduced PQD staining. To assess CPK specificity, phosphorylation assays were also performed using BGG. BGG revealed undetectable levels of phosphorylation, as visualized by PQD (results not shown). These results suggested that CPKs have the ability to phosphorylate 14-3-3 χ and 14-3-3 ϵ . To confirm this we analysed these 14-3-3 proteins by MS after tryptic digestion.

MS analysis of 14-3-3 phosphorylation by CPKs (1, 3, 6, 24 and 28) identified a total of seven phosphorylation sites on isoforms χ and ϵ . Three sites were unique to 14-3-3 χ (Ser⁸⁸, Ser¹²⁵ and Thr¹⁵⁶), two to 14-3-3 ϵ (Thr¹⁸ and Thr²⁴⁴) and one site was common to both isoforms (Ser⁷² χ and Ser⁶⁵ ϵ). Thr²⁴⁴ phosphorylation was previously identified from *in vivo* phosphoproteomics screens [11,13]. Phosphorylation site locations were compared with all *Arabidopsis* 14-3-3 isoforms using ClustalW and WebLOGO algorithms (Figure 2). Interestingly, conservation of these amino acid residues varied from being conserved (all 14-3-3 isoforms) to no conservation (one 14-3-3 isoform). These results imply that although some phosphorylation sites may be general to all 14-3-3 isoforms, others could be isoform-specific.

The structural location of these phosphorylation sites was determined by *in silico* homology modelling to assess the potential effect of 14-3-3 phosphorylation on function. These results revealed several phosphorylation sites within 14-3-3 functional domains. Phosphorylation of Ser⁷² on χ , corresponding to Ser⁶⁵ on ϵ , is of interest because this serine residue is conserved among *Arabidopsis* 14-3-3 isoforms and its location lies near a residue involved in creating the 14-3-3-binding cleft (Figure 2, front view number 2). Likewise, phosphorylation of Thr¹⁵⁶ on χ occurs near a binding-cleft residue. Two other interesting phosphorylation sites occurred near the N- and C-terminal regions of 14-3-3 ϵ (Thr¹⁸ and Thr²⁴⁴). The N-terminus has previously been shown to be involved in dimerization [37,41], whereas the C-terminus has previously been described as a ‘gate’, influencing client–protein associations [42]. The precise function of several of these phosphorylation events remains to be determined; however, the impact of one phosphorylation site was investigated below.

Autophosphorylation of CPKs includes Tyr⁴⁶³ for CPK28

Autophosphorylation activity of CPKs was assessed by incubating them in a standard CPK assay mixture lacking exogenous substrates (Figure 3). CPKs were incubated with and without λ -PPase to assess autophosphorylation activity. The addition of λ -PPase reduced phosphorylation and increased the electrophoretic mobility of all CPKs during SDS/PAGE (Figure 3). The observed kDa mobility mass shifts were probably due to the removal of multiple phosphate groups; similar mobility shifts have been observed previously [43]. Recombinant 14-3-3 χ (unphosphorylated) and ovalbumin (a known phosphoprotein) were included as negative and positive controls respectively. As expected, limited PQD staining was observed for 14-3-3 χ , whereas ovalbumin had a similar staining pattern to the aforementioned CPKs.

CPK autophosphorylation sites were determined using MS/MS. In total, 45 autophosphorylation sites were mapped on the six different CPKs, 39 of which were previously unidentified. The autophosphorylation sites identified are depicted on the four CPK domains: the NTVD (N-terminal variable domain), the autoinhibitory J (junction) domain, the PKCD (protein kinase catalytic domain) and the CBEF-hand (calcium-binding EF-hand) domain (Figure 4 and Supplementary Table S2 at <http://www.biochemj.org/bj/459/bj4590015add.htm>). Although phosphorylation sites were observed across the domains of the six CPKs analysed, the NTVD and PKCD were comparatively hyperphosphorylated on specific CPKs. For instance, the NTVD of CPK1 alone contained ten autophosphorylation sites, of which only one site was conserved among the CPKs (CPK1-Thr¹⁰⁷ and CPK6-Thr⁴⁹). In contrast, only four phosphorylation sites were observed on all six CPKs’ CBEF-hand domains.

CPK autophosphorylation site occupancy was compared in a relative manner by spectral counting. Under standard autophosphorylation conditions, 11 sites had higher counts of phosphorylated compared with non-phosphorylated peptides ($P < 0.05$) (Figure 5 and Supplementary Table S3 at <http://www.biochemj.org/bj/459/bj4590015add.htm>). In contrast, seven sites were stoichiometrically underphosphorylated ($P < 0.05$). The remaining 28 phosphorylation sites had statistically equivalent counts of phosphorylated compared with non-phosphorylated peptides. A comparison with a previous study of CPK

autophosphorylation, revealed six overlapping sites on CPK1 (Thr⁶⁴, Thr¹⁰⁷, Ser¹³⁷ and Ser¹⁵¹) and CPK28 (Ser²²⁸ and Ser³¹⁸) [44]. In addition to these six sites, 18 additional phosphorylation sites were identified on these two CPKs. The higher number of autophosphorylation sites identified in the present study was aided by the use of a mass spectrometer with dual fragmentation capabilities (CID and ETD).

Serine and threonine were the most abundant autophosphorylated amino acids with 33 and 11 sites respectively. Additionally, CPK28 was autophosphorylated on Tyr⁴⁶³ (Figure 4 and Supplementary Figure S1 at <http://www.biochemj.org/bj/459/bj4590015add.htm>); no other CPK exhibited phosphorylation on this residue or tyrosine phosphorylation in general (Figure 6A). Phylogenetic alignment of CPK protein sequences revealed CPK28 to be distinct, suggesting that tyrosine phosphorylation might be unique to subgroup IV (Figure 6B). Another interesting characteristic of CPK28 was autophosphorylation of Ser⁴³, which is located within the canonical 14-3-3-binding motif R-S/T-X-S-X-P (Figure 4). Phosphorylation of Ser⁴³ completes this 14-3-3-binding motif and would theoretically promote a 14-3-3-CPK28 interaction.

Relative efficiency of the different CPK isoforms to phosphorylate χ and ϵ

To evaluate the efficiency of 14-3-3 phosphorylation by CPKs a technique termed dMS (differential MS) was used. dMS measures ion current for either precursor or product ions (also referred to as selected reaction monitoring) to compare relative intensities of a given analyte. The high resolution and mass accuracy of the Orbitrap allows for decisive precursor ion separation facilitating accurate quantification by dMS [45]. In the present study, we determined the relative levels of phosphorylation by integrating the measured chromatographic intensities of the identified 14-3-3 phosphopeptide masses. Using the theoretical monoisotopic mass of the identified 14-3-3 tryptic phosphopeptides and a p.p.m. window of 1.5, we compared the ability of CPKs to phosphorylate specific residues on 14-3-3 isoforms χ (Ser⁷² and Ser⁸⁸, Ser¹²⁶ and Thr¹⁵⁶) and ϵ (Thr¹⁸, Ser⁶⁵ and Thr²⁴⁴). These phosphopeptides were initially identified from MS/MS and the site localization algorithm phosphoRS before being selected for dMS analysis.

Although all CPKs were capable of phosphorylating 14-3-3 isoforms χ (Ser⁷² and Ser⁸⁸, Ser¹²⁶ and Thr¹⁵⁶) and ϵ (Thr¹⁸, Ser⁶⁵ and Thr²⁴⁴), the highest levels of phosphorylation was consistently achieved with CPK28 (Figure 7). The exception is Ser⁶⁵ on 14-3-3 ϵ which was phosphorylated at comparable levels by both CPK3 and CPK28. Interestingly, of the CPKs tested, CPK3 is the most phylogenetically related isoform to CPK28 (Figure 6B). This was the only CPK able to phosphorylate a 14-3-3 phosphorylation site at similar levels to CPK28. As a control, a randomly selected 14-3-3 tryptic peptide from both isoforms was compared among all *in vitro* kinase assays, to ensure that differences in phosphorylation were not due to digestion or loading differences. As Figure 7 indicates, there were no significant differences among these ‘loading control’ peptides when evaluated using dMS. These results imply that CPK28 is the most efficient at phosphorylating 14-3-3 isoforms χ and ϵ *in vitro*. However, these results also suggest that CPK3 is equally efficient at phosphorylating Ser⁶⁵ on 14-3-3 ϵ . Taken together these results suggest that CPK3 and CPK28 are strong candidates for phosphorylating 14-3-3 isoforms *in vivo*.

14-3-3 and CPK isoforms have unique and similar cellular localization patterns

Fluorescence localization assays using 14-3-3 and CPK isoforms were performed to further characterize these proteins *in planta*. *Agrobacterium*-mediated transient expression of CPK-YFP (3, 6, 24 and 28) and 14-3-3-YFP (χ and ϵ) in *N. benthamiana* epidermal leaves demonstrated that specific CPKs have unique subcellular localization patterns (Figure 8). CPK3-YFP and CPK6-YFP were primarily localized to the nucleus and cytosol/nucleus respectively, in agreement with previous CPK localization results [46]. However, CPK24-YFP and CPK28-YFP were localized only to the nucleus. Both 14-3-3-YFP fusion proteins localized to both the cytosol and nucleus. Nuclear localization of 14-3-3 and CPK isoforms was confirmed using the fluorescent DNA-specific stain DAPI. These results imply that CPK-14-3-3 interactions could be influenced by subcellular distribution. Specifically by CPKs, as no visual differences were observed in localization patterns between the 14-3-3 isoforms χ and ϵ (Figure 8).

The 14-3-3 χ -S72D phosphomimic lacks the ability to inhibit pNR

To investigate the functional role of 14-3-3 phosphorylation by CPKs, 14-3-3-mediated NR inhibition assays were performed with a phosphomimetic mutant of 14-3-3 χ , constructed by mutating Ser⁷² to Asp⁷². The Ser⁷² phosphorylation site on 14-3-3 χ was studied because of its proximity to the 14-3-3-binding cleft and conservation among *Arabidopsis* 14-3-3 isoforms (Figure 2). Furthermore, this was the only analogous site identified to be phosphorylated by CPKs on both 14-3-3 isoform χ (Ser⁷²) and ϵ (Ser⁶⁵). In *Arabidopsis*, 14-3-3-mediated inhibition of NR is controlled by phosphorylation of Ser⁵³⁴ and subsequent 14-3-3 binding [6]. In order to phosphorylate NR before activity assays, recombinantly expressed *Arabidopsis* NR was incubated with CPK28 under standard *in vitro* kinase assay conditions. Using MS/MS it was confirmed that CPK28 phosphorylated Ser⁵³⁴ (results not shown). pNR was then purified to homogeneity using anion-exchange chromatography. To control for the effect of nonspecific protein-protein interactions, NR activity assays were performed using BSA as a control. Co-incubation of NR with BSA enhanced NR activity by an average of 15% (Figure 9). As described previously, incubation of the native 14-3-3 χ inhibited pNR activity [6]. The initial velocity of NR was reduced by an average 37% upon the addition of 14-3-3 χ . Interestingly, the 14-3-3 χ -S72D phosphomimic was unable to inhibit pNR activity and had a similar effect on NR activity as the BSA control (Figure 9). A previous study reported that 14-3-3 χ inhibited pNR by as much as 70% [6]. The discrepancy between the previous and present study is noteworthy, but could be attributed to the phosphorylation efficiency of Ser⁵³⁴ on NR, as different CPKs were used in the NR phosphorylation assays. Nevertheless, the results of the present study confirm previously reported 14-3-3-mediated inhibition of pNR and demonstrate that phosphomimetic mutagenesis of Ser⁷² on 14-3-3 χ prevents 14-3-3-mediated inhibition of pNR.

DISCUSSION

The 14-3-3/CPK relationship has been previously established as being both indirect and direct; indirect in the context of 14-3-3 binding to phosphorylation clients of CPKs and direct in the form of 14-3-3-CPK interactions [8,21–26]. We extend upon this by reporting CPK phosphorylation of 14-3-3 proteins. Previous biochemical data documented the ability

of 14-3-3 isoforms (ω , ψ and ϕ) to bind CPK1 and demonstrated that 14-3-3 proteins increase CPK1 activity *in vitro* [26]. Camoni et al. [26] also described the inability of CPK1 to phosphorylate these 14-3-3 isoforms. The discrepancy between the present study and that previous study could be due to kinase–client specificity between CPK and 14-3-3 proteins or the sensitivity and accuracy of current phosphoprotein/peptide detection methods. Certainly, the fact that one of the identified phosphorylation sites has previously been identified *in vivo* suggests that these data have biological relevance. Nevertheless, *in vivo* confirmation of the six remaining phosphorylation sites is necessary, and their elusiveness may reflect the convergence of kinase–substrate co-expression and subsequent CPK activation, i.e. elevated intracellular calcium levels.

Phosphorylation of plant 14-3-3 proteins was initially reported in developing rapeseed using 2D gels coupled to PQD [10], then later by phosphoproteomic surveys in *Arabidopsis* [11–15]. At present, eight different *Arabidopsis* 14-3-3 isoforms have been found phosphorylated on 17 sites from a comprehensive collection of *in vivo* plant phosphoproteomic data [data retrieved from P³DB (<http://www.p3db.org>); Supplementary Table S4 <http://www.biochemj.org/bj/459/bj4590015add.htm>] [47]. Despite evidence of multisite *in vivo* 14-3-3 phosphorylation, there remains only one known kinase (SnRK2.8) capable of phosphorylating these 14-3-3 isoforms, although none of these reported sites have been confirmed *in vivo* [48]. In the present study we identify the *Arabidopsis* CPK family as candidates for 14-3-3 phosphorylation, most notably Ser²⁴⁴ (14-3-3 ϵ), a previously identified *in vivo* site [11,13].

The significance of 14-3-3 phosphorylation in plants is unclear; only one study describes a putative function. Phosphorylation of maize 14-3-3 protein (GF14-6) on Tyr¹³⁷ by human IGF-1 (insulin-like growth factor 1) receptor decreased the binding affinity for an immobilized H⁺-ATPase phosphopeptide [49]. Tyr¹³⁷ is conserved in all *Arabidopsis* 14-3-3 isoforms, except π . Furthermore, Tyr¹³⁷ of tobacco 14-3-3c forms a hydrogen bond with the phosphate group of a C-terminal H⁺-ATPase phosphopeptide in co-crystallization assays [41]. Although these results provide a framework for future 14-3-3 phosphorylation studies, the biological relevance is unclear as human IGF-1 has no orthologue in plants.

The 14-3-3 motif that interacts with the phosphate group of client phosphopeptides has been characterized [41,50]. Discrete amino acids within this binding motif are conserved within animal, fungal and plant 14-3-3 isoforms (Figure 2). These residues consist of a series of basic amino acids (in *Arabidopsis* 14-3-3 χ ; Lys⁵⁸, Arg⁶⁵ and Arg¹³⁸) and a tyrosine residue (in *Arabidopsis* 14-3-3 χ ; Tyr¹³⁹), which create a tertiary structure capable of binding phosphoproteins. Interestingly, two of the identified phosphorylation sites (Ser⁷² χ or Ser⁶⁵ ϵ) are seven amino acids away from one of these conserved arginine residues. In the present study we demonstrate that mutation of Ser⁷² to aspartate on 14-3-3 χ perturbs 14-3-3-mediated inhibition of pNR. It is tempting to speculate that other phosphorylation sites within the binding motif may similarly interfere with 14-3-3 function. Alternatively, phosphorylation could stabilize binding of 14-3-3 client proteins through electrostatic interactions (e.g. formation of salt bridges).

The N- and C-terminal regions of 14-3-3 proteins have important, but different, roles. One characteristic these termini share is a lack of sequence conservation among isoforms, suggesting they may provide specificity among the 14-3-3 protein family. N-terminal truncations of 14-3-3 proteins have been shown by BiFC (bimolecular fluorescence complementation) and two-hybrid assays to be directly involved in dimerization [37,51]. In the present study we demonstrate phosphorylation of the N-terminal α -helix of 14-3-3 ϵ (Thr¹⁸). Curiously, Thr¹⁸ is not conserved among any *Arabidopsis* 14-3-3 isoforms. The C-terminal helices 8 and 9 of 14-3-3 proteins bind divalent cations (e.g. Mg²⁺; Figure 2) and biochemical evidence suggests that this interaction influences client protein associations [42,52]. One of the phosphorylation sites identified, Thr²⁴⁴, on 14-3-3 ϵ was mapped to the extreme C-terminus. Functionally, Thr²⁴⁴ phosphorylation could effect the entry or exit of 14-3-3 client proteins by effecting divalent cation binding. Furthermore, 14-3-3 ϵ Thr²⁴⁴ phosphorylation was previously identified *in vivo* from two studies [11,13].

The identification of tyrosine phosphorylation continues to emerge from plant phosphoproteomic studies [13,53–55], yet the number of tyrosine kinases remains limited [54,56]. Consequently, there remains a divide between the identified phosphorylation sites and their associated kinase(s). For instance, CPK9 was phosphorylated on Tyr⁴⁷⁰ in nitrogen-deficient seedlings resupplied with ammonium, but the kinase responsible is currently unknown [57]. *In vivo* phosphoproteomic studies revealed that five additional CPKs (16, 18, 19, 28 and 33) are phosphorylated on tyrosine residues [11,13,57]. Recently, CPK4 and CPK34 were shown to be autophosphorylated on serine, threonine and tyrosine residues [58] and we also demonstrate that CPK28 is autophosphorylated on Tyr⁴⁶³ within the EF-hand domain (Figure 4). Although CPKs have been historically classified as serine/threonine kinases, the report of serine/threonine and tyrosine dual-specificity challenges this dogma. Whether tyrosine phosphorylation of CPKs *in vivo* is a consequence of autophosphorylation remains unclear, but should be considered based upon proximity of the phosphorylation sites and recently described tyrosine phosphorylation activity [58].

One well-established regulatory mechanism of 14-3-3 proteins is their ability to influence client protein subcellular localization patterns. Cytoplasmic retention of transcription factor BZR1 and bZIP transcriptional activator RSG upon phosphorylation and 14-3-3 binding has been described [59–61]. Isoform-specific immunocytochemistry and GFP fusions have demonstrated that 14-3-3 isoforms can and do partition themselves in different subcellular locations [62]. For instance, 14-3-3 χ is largely identified in the nucleus, whereas 14-3-3 ϵ is largely identified in the cytoplasm and/or plasma membrane [62]. CPK isoforms also localize in different subcellular organelles such as the cytosol, nucleus, plasma membrane, etc. [63]. Consistent with a previous report, we have found that CPK3 and CPK6 are both localized to the cytoplasm [46], whereas CPK24 and CPK28 are both localized to the nucleus (Figure 8). 14-3-3 proteins are able to interact with other proteins through canonical binding motifs, non-canonical binding sites and phosphorylation status of the target proteins [64]. Although CPKs 24 and 28 both contain a canonical 14-3-3-binding motif, there is no evidence of interaction with 14-3-3 isoforms. However, regulation and/or interaction of other CPK isoforms with 14-3-3 proteins has been reported [26,28], indicating potential interactions between similar co-localized proteins with 14-3-3 proteins [64]. Whether 14-3-3

proteins associate with these CPKs *in vivo* to control subcellular localization is currently the focus of ongoing studies. Nevertheless, it is tempting to speculate that a subcellular sequestration mechanism exists between 14-3-3 and CPK, similar to the aforementioned transcription factors.

Although the significance of 14-3-3 phosphorylation in plants remains in its infancy, the network topology between 14-3-3 proteins and CPKs continues to expand. *In vitro* phosphorylation of *Arabidopsis* 14-3-3 isoforms (specifically χ and ϵ) by CPK isoforms adds another level of complexity to previously established protein–protein interactions. 14-3-3 ϵ phosphorylation on Thr²⁴⁴ by CPKs effectively identified a kinase capable of phosphorylating a previously identified *in vivo* site [11,13]. Three of the CPKs assayed contain a canonical 14-3-3-binding motif (CPK1, CPK24 and CPK28) suggesting that upon phosphorylation these proteins could interact *in vivo*. Indeed, MS analysis of CPKs revealed CPK28 autophosphorylation on the canonical 14-3-3-binding motif (Ser⁴³). Moreover, CPK28 was also identified to be autophosphorylated on Tyr⁴⁶³ confirming that CPKs can function as dual-specificity kinases [58]. Analysis of CPK phosphorylation efficiencies on 14-3-3 isoforms (χ and ϵ) revealed CPK3 and CPK28 to be the most prolific kinases against these targeted substrates. An *in vitro* reconstitution enzyme activity assay suggests that phosphorylation of Ser⁷² on 14-3-3 χ negatively affects the ability of 14-3-3 to inhibit pNR. Taken together, the results of the present study demonstrate preferential associations between 14-3-3 proteins and specific CPKs, providing a framework for targeted physiological studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BGG	bovine γ -globulin
CBB	Coomassie Brilliant Blue
CID	collision-induced dissociation
CPK	calcium-dependent protein kinase
dMS	differential MS
ETD	electron transfer dissociation
IGF-1	insulin-like growth factor 1

NR	nitrate reductase
NTVD	N-terminal variable domain
PKCD	protein kinase catalytic domain
pNR	phosphorylated NR
λ-PPase	λ-phosphatase
PQD	Pro-Q Diamond
SPS	sucrose-phosphate synthase

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Figure 1. The 14-3-3 isoforms χ and ϵ are phosphorylated *in vitro* by CPKs
 Phosphoproteins were detected with PQR. Total protein was detected with colloidal CBB.
 Denatured (den.) kinases were added as a control to indicate that phosphorylation of the
 14-3-3 isoforms resulted from CPK activity. Approximately 1 μ g of total protein was loaded
 into each respective lane. The molecular mass in kDa is indicated.

Figure 2. Conservation and location of the 14-3-3 phosphorylation sites identified
 14-3-3 isoforms were aligned using the ClustalW program of SDSC Biology Workbench. WebLogo diagrams were generated surrounding the identified 14-3-3 phosphorylation sites and are numerically labelled [65]. Blue columns highlight the identified 14-3-3 χ and 14-3-3 ϵ phosphorylation site(s) and the analogous residues across *Arabidopsis* 14-3-3 isoforms. Green columns indicate homologous 14-3-3 amino acids involved in binding a phosphopeptide [49]. The blue, green and black letters in the alignment indicate fully conserved, strong conservation and non-conserved amino acid residues. The PDB structure 3E6Y [66] was directly compared with the 14-3-3 χ protein sequence to determine the structural location of identified phosphorylation sites. The visual molecular dynamics program was used to generate 3D structures. Yellow asterisks indicate the 3D location of the

phosphorylation sites. Numbers in the WebLOGO diagrams correspond to the numbers on the 3D structures. Green, α -helical; blue, coil; and purple, turns.

Figure 3. CPKs are autophosphorylated

All six CPKs are capable of autophosphorylation. Addition of λ -PPase ($+\lambda$) reduced phosphorylation as determined by a reduction in PQR staining signal and/or molecular-mass shifts. As negative and positive controls for PQR staining, 14-3-3 χ ($-$) and ovalbumin ($+$) were treated in an identical manner. Approximately 1 μ g of total protein was loaded into each respective lane. The molecular mass in kDa is indicated.

Figure 4. Site-specific autophosphorylation of *Arabidopsis* CPKs

Linear domain structures of individual CPKs have been created using PROSITE (<http://prosite.expasy.org>) and phosphorylation sites were determined using MS/MS. The four different protein domains of the CPK family are illustrated: NTVD, N-terminal variable domain; PKCD, protein kinase catalytic domain; J, junction domain; and CBEF-hand, calcium-binding EF-hand domain. Dark boxes within the CBEF-hand domain represent individual EF-hand domains. Sites indicated by asterisks were also identified by Hegeman et al. [44].

Figure 5. Site-specific abundance of CPK autophosphorylation

Site occupancy of the various autophosphorylated CPK peptides is shown by comparing unphosphorylated (peptide) and phosphorylated (phosphopeptide) peptides. The histogram depicts statistically significant autophosphorylation sites ($P < 0.05$). Spectral counts for both the phosphopeptide (white) and unmodified peptide (grey) were plotted. Lower-case letters and superscript numbers indicate the phosphorylation site and amino acid residue respectively. Lower-case 'm' and 'c' indicated methionine-oxidation and cysteine-carboamidomethylation modifications respectively.

Figure 6. CPK phosphorylation residue frequency and a phylogenetic protein comparison
(A) The total number of identified phosphorylation sites for each CPK is displayed in the histogram. The frequency of serine, threonine and tyrosine phosphorylation for each CPK is indicated by grey white and black respectively. **(B)** A phylogenetic tree was generated from CPK full-length protein sequences and labelled with their corresponding phylogenetic subgroups (Sub I–IV) [40].

Figure 7. dMS revealed specificity of CPKs for 14-3-3 isoforms χ and ϵ
 (A) dMS of 14-3-3 χ phosphopeptides. (B) dMS of 14-3-3 ϵ phosphopeptides. Lower-case letters and superscript numbers indicate the phosphorylation site and amino acid residue respectively. The intensities of monoisotopic masses were integrated to determine the relative levels of phosphorylation among CPKs. The peptide mass deviation for the precursor mass was set at 1.5 p.p.m. Differences in the extent of phosphorylation were considered statistically significant if $P < 0.01$ and are labelled with letters. A randomly

selected loading control' peptide was chosen from each 14-3-3 isoform and is not significantly different among samples.

Figure 8. 14-3-3 isoforms and CPK isoforms display unique localization patterns *in planta*
 Images were taken of *N. benthamiana* epidermal cells that have been infiltrated (3 days post-infiltration) with *A. tumefaciens* (strain GV3101) containing the indicated 14-3-3 proteins and CPKs fused to YFP. Panels display YFP fluorescence (YFP), DAPI and bright-field (BF) images. White scale bars represent 10 μ m. Images were processed using Adobe Photoshop version 7.0. Contrast levels were uniformly adjusted for all confocal images. Experiments were performed in biological triplicate.

Figure 9. Comparison of binding activity for 14-3-3 χ and 14-3-3 χ -S72D phosphomimic using *Arabidopsis* NR

NR activity was monitored after co-incubation with recombinant 14-3-3 (both wild-type and S72D phosphomimic). NR activity was determined by spectrophotometric monitoring of NADH depletion. Initial velocities were calculated relative to the initial velocity of pNR in the absence of a protein substrate. To control for non-specific inhibition, NR was incubated with BSA. Values are expressed as the mean $\pm$ S.E.M. for $n = 7$ technical replicates. The

histogram represents one independent assay which is representative of three biological replicates.