Impact of Ca$^{2+}$ on structure of soybean CDPK$\beta$ and accessibility of the Tyr-24 autophosphorylation site

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Calcium signaling is recognized to control many key aspects of plant growth and development, including responses to biotic and abiotic stresses. To a large extent, calcium signaling is mediated by calcium-binding proteins and many different mechanisms are potentially involved in transduction of the signals.$^1$ One important mechanism involves protein phosphorylation mediated by the calcium-dependent protein kinases (CDPKs or CPKs). The CDPKs are unique to plants, being found in terrestrial plants, green algae, and certain protists (ciliates and apicomplexans) and are classified as Ser/Thr protein kinases.$^{2,3}$ There have been 2 recent advances that impact our understanding of the CDPKs. First, it was reported that several CDPKs, including soybean CDPK$\beta$ and AtCPK4 and AtCPK34, are dual-specificity kinases able to autophosphorylate on Ser, Thr, and Tyr residues.$^4$ Second, structural studies were recently performed with the full-length CDPKs from several protist species. The initial studies involved CDPKs from the apicomplexan parasites Toxoplasma gondii and Cryptosporidium parvum,$^3$ which first demonstrated that calcium binding results in a large conformational change whereby the CaM-like domain refolds and translocates from the substrate binding site to the opposite side of the kinase domain, where it interacts with residues of both lobes of the kinase domain. The subsequent study$^5$ extended the structural analysis to CDPK3 of Plasmodium falciparum which strengthened the notion that this novel mechanism could be universal to most members at least of the CDPK superfamily. For the present study, the most critical point to note is that when complexed with calcium, the CaM-like domain and autoinhibitory (Junction) domain is not “free-floating” but rather interacting with the kinase domain remote from the substrate binding site. The interactions with the kinase domain could have implications for the accessibility of auto-phosphorylation sites to protein phosphatases. We explore this possibility with specific reference to the Tyr-24 autophosphorylation site of soybean CDPK.$\beta$

The CDPKs are directly activated by Ca$^{2+}$ and do not require exogenous calmodulin for activity$^7$ because the proteins have a CaM-like domain that generally contains 4 Ca$^{2+}$-binding EF-hands (Fig. 1A). The CaM-like domain is adjacent to the autoinhibitory (Junction) domain that blocks the kinase active site in the absence of Ca$^{2+}$. Calcium binding to the EF-hands of the CaM-like domain results in an interaction with the junction domain that removes it from the active site and relieves the inhibition of kinase activity. Once activated by calcium binding, the CDPKs can also autophosphorylate, which may further impact kinase activity. Several studies have shown that CDPKs can autophosphorylate on Ser/Thr residues in a Ca$^{2+}$-dependent manner,$^7,9,12$ which is consistent with their classification as Ser/Thr kinases. However, we recently reported$^*8$ that several CDPKs could autophosphorylate on Ser, Thr and also Tyr residues. The specific autophosphorylation sites identified for soybean CDPK$\beta$ are demonstrated schematically in Figure 1A. Of particular note is the single site of Tyr autophosphorylation (Tyr-24) at the beginning of the kinase domain, which appears to attenuate kinase activity as the Y24F directed

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A Tyr residue at the start of the kinase domain is strictly conserved among the CDPKs and thus could be a common autophosphorylation site. In the present study, we developed new custom antibodies that recognize CDPK\(\beta\) autophosphorylation site. In the present study, we developed new custom antibodies that recognize CDPK\(\beta\) when phosphorylated at the Tyr-24 site. The sequence used as antigen is shown in Figure 1A. The anti-pY24 antibodies cross reacted with the wild type GmCDPK\(\beta\) but not the Y24F directed mutant, which establishes their specificity (Fig. 1B, left panel). As previously reported,\(^4\) the Y24F directed mutant autophosphorylates on Thr residues (Fig. 1B) but Tyr autophosphorylation is almost completely eliminated suggesting that Tyr-24 is the major (if not sole) site of Tyr autophosphorylation. AtCPK4 cross reacted strongly with the anti-pY24 antibodies which established Tyr-25 of AtCPK4 as at least 4 of the 11 residues in the antigen sequence are conserved.

Figure 1. (A) Schematic representation of the primary structure of soybean CDPK\(\beta\), the positions of known autophosphorylation sites, and the sequence surrounding the Tyr-24 phosphate used to prepare the anti-pY24 antibodies. Antibodies were produced by GenScript (Piscataway, NJ, USA) and affinity purified to remove antibodies that recognize the unphosphorylated peptide sequence. (B) Specificity of the anti-pY24 antibodies for the Tyr-24 site in CDPKβ, and confirmation of phosphorylation of the homologous Tyr-25 site in AtCPK4 and perhaps Tyr-68 in AtCPK34. The recombinant proteins were produced as N-terminal His\(_6\) fusion proteins in E. coli and affinity purified, and analyzed for autophosphorylation status by probing with anti-phosphothreonine (anti-pThr), anti-phosphotyrosine (anti-pY), or anti-pY24 antibodies also as previously described.\(^4\)

We wanted to determine whether the protist CDPK structures were relevant to soybean CDPK\(\beta\). Soybean CDPK\(\beta\) shares 36% (171/478) primary sequence identity and 56% (267/481) sequence similarity to the T. gondii structure in Figure 2. A portion of the N-terminal domain (red) is shown connecting to the kinase domain (tan) with the Tyr-51 side chain in yellow/orange. The Junction domain is in magenta, and the CaM-like domain in purple. Note the proximity of Tyr-346 and Arg-445 to Tyr-51. Tyrosine-346 is located in the CH1 polypeptide, which contains the autoinhibitory (Junction) domain and connects the kinase domain to the beginning of EF1, while Arg-445 is located in the CH2 polypeptide, which connects the N - and C-terminal EF lobes. Both segments are predicted to undergo huge conformational changes when fully loaded with calcium and associate with the “back side” of the kinase domain. As illustrated this nearly covers Tyr-51 at the beginning of the kinase domain.

Figure 2. Three-dimensional crystal structure of T. gondii CDPK\(\beta\) in the presence of calcium enlarged to show the structural space surrounding Tyr-51, which is homologous to the Tyr-24 phosphate site in CDPK\(\beta\). Note that Tyr-51 is in close proximity to Arg-445, as is Tyr-346, emphasizing the close distance between the N-terminal domain and the Junction/ CaM-like domain.
domain (residues 321–461) in purple. The modeled structures are consistent with the large conformational changes that have been documented with the protist CDPKs. With respect to the orientations of the kinase domain presented in Figure 3, in the absence of calcium the CaM-like domain is located underneath the kinase domain, blocking access to the active site. However, in the presence of calcium, the entire domain moves above the kinase domain and as a result, the active site is accessible to substrates but the kinase domain now interacts with the entire CDPK activation domain in a new way. In particular, the beginning of the kinase domain is ‘protected’ by the CaM-like domain. This is illustrated more clearly in Figure 4, which shows an expanded view of the structural region surrounding Tyr-24 at the beginning of the CDPKβ kinase domain. The potential for shielding of Tyr-24 from autophosphorylation and phosphotyrosine-24 from protein phosphatases is certainly suggested. We know that autophosphorylation on all residues, including Tyr, is strictly Ca²⁺-dependent. Therefore, potential shielding of Tyr-24 from autophosphorylation is not observed and the possible basis for this is discussed below. However, the effect of Ca²⁺ on accessibility to protein phosphatases has not been examined.

As a result of the structural studies, we investigated the impact of free Ca²⁺ on accessibility of tyrosine autophosphorylation sites to removal by the recombinant protein tyrosine phosphatase, PTP1B. As shown in Figure 5, the presence or absence of free Ca²⁺ had little impact on the ability of PTP1B to specifically dephosphorylate GmCDPKβ at the Tyr-24 site. In other experiments, we confirmed that PTP1B did not affect recognition by anti-phosphothreonine antibodies (data not shown), as would be expected. These results confirmed that the anti-phosphotyrosine antibodies were indeed specifically recognizing phosphotyrosine residues in the CDPKs, and that free Ca²⁺ did not affect accessibility of the major site of Tyr autophosphorylation. Since calcium has no direct effect on PTP1B activity, the results obtained suggest that phosphorylated Tyr-24 was fully accessibility to the phosphatase despite the large Ca²⁺-induced conformational changes of the CDPK that are likely occurring. That phosphotyrosine-24 was accessible and not irreversibly buried is also suggested by our previous observation that CDPKβ could be affinity purified using immobilized anti-phosphotyrosine antibodies. However, these experiments were performed in a buffer containing no added Ca²⁺ and 0.1 mM EDTA, suggesting that free Ca²⁺ was probably quite low and perhaps in the sub-micromolar range. Because Tyr-24 is the major, if not sole, site of tyrosine autophosphorylation, these earlier results are consistent with the accessibility of phosphotyrosine-24, at least in a low [Ca²⁺] environment.

Finally, it is also interesting that 3 of the 5 autophosphorylation sites identified in CDPKβ were clustered in, or close to, the N-terminal domain. This clustering was noted before in a broader survey study and is particularly interesting given that in some cases the CDPK N-terminus might function in substrate specificity. Because structural studies indicate the potential for protection from protein phosphatases as a result of latching of the CaM-like domain to the kinase domain in the presence of calcium, the accessibility of other autophosphorylation sites to protein phosphatases should be considered in future studies.

To summarize and put these results into a larger context, we are beginning to realize that several families originally classified as Ser/Thr protein kinases are actually dual-specificity kinases with the capacity to autophosphorylate (and perhaps trans-phosphorylate) on Ser, Thr and Tyr residues. This was initially demonstrated for several members of the large receptor-like kinase family in Arabidopsis and a similar situation appears to be emerging with the CDPK family. One clear conclusion is that we do not have an adequate understanding of the kinase domain motifs that confer (or predict) dual specificity. Much remains to be done in this entire area but with respect to
the autophosphorylation of the CDPKs it will be important to take into account the new structural information from protist CDPKs and determine how the large conformational changes impact substrate specificity and regulation by autophosphorylation. One fundamental question is whether the structural models determined for the protist CDPKs applies to higher plant CDPKs and determine how the large conformational changes take into account the new structural information from protist CDPKs, since they form distinct clades. However, assuming the models are universal, it is essential to consider the implications for regulation of the active (calcium loaded) form of CDPK. For example, it is possible that the exact positioning of the CaM-like domain and autoinhibitory (Junction) domain with the kinase domain may be directly affected by autophosphorylation of the Tyr residue at the start of the kinase domain or residues within the N-terminal domain. This could be part of the basis for the impact of autophosphorylation on kinase activity and substrate specificity. However, the extent to which the CDPK structures recently resolved by X-ray crystallography reflect the complete story remains to be determined. It should be remembered that the deduced structures probably reflect the most stable states or perhaps the only form that can crystallize. Other forms may also be present in solution, even transiently. For example, the extent to which the autoinhibitory (Junction) and CaM-like domain interact with, and dissociate from, the kinase domain is not known. We speculate that there may be dynamic changes—conformational breathing and flexing—that allow access of N-terminal residues to be autophosphorylated and for protein phosphatases to access phosphotyrosine-24 even in the presence of calcium, and that this may explain the results obtained in the present study. This is an important area for further study, as dynamic reversibility of autophosphorylation (or the lack thereof) would have important implications for the overall regulation of CDPK activity in vivo.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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