FEBS Letters 580 (2006) 4325–4331

Calmodulin isoform-specific activation of a rice calmodulin-binding kinase conferred by only three amino-acids of OsCaM61

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Received 25 May 2006; accepted 29 June 2006

Available online 7 July 2006

Edited by Julian Schroeder

Abstract **The kinase activity of a Ca^{2+}/calmodulin (CaM)-binding serine/threonine protein kinase from rice (*Oryza sativa*) (OsCBK) has been reported to be unaffected by OsCaM1 binding. In this study, we examined whether other rice CaMs can stimulate OsCBK. It was observed that OsCaM61 stimulated OsCBK in a Ca^{2+}-dependent manner. In addition, Ala^{111}, Gly^{123} and Ser^{127} were identified as critical residues for OsCBK activation. Mutational study and fluorescent spectroscopy analysis indicated that CaM-binding affinity does not correlate with the kinase activity and that these key amino-acids in OsCaM61 play a vital role in suitable changes of OsCBK conformation for kinase activation.**

Keywords: Calmodulin isoform-specific activation; Calmodulin-binding protein kinase; Dissociation constant; Tryptophane intrinsic fluorescence

1. Introduction

Calcium plays vital roles in many biological processes as a universal second messenger [1]. Calmodulin (CaM) is a ubiquitous intracellular Ca^{2+} receptor involved in transducing a variety of extracellular signals [2]. Since CaM itself has no enzymatic activity, it plays its role by regulating CaM-binding proteins. Previous studies have demonstrated that the CaM-target interactions can be affected due to small changes in the amino-acid composition [3–6] and post-translational trimethylation [7]. The key domain of CaM for different target activations may be distinct, which may explain for its multiple biochemical functions.

Higher plants contain multiple CaM isoforms with divergent protein sequences in a single species [8], which may be distinct in their target-activating abilities [9–16], binding characteristics [12,17–20], and physiological functions [21–25]. CaM is highly conserved in both composition and length (149 amino-acids). However, OsCaM61 (accession number: U37936), a CaM isoform from rice, has a 38 amino-acid extension in the C-terminus containing a polybasic domain and a prenylation site (CVIL) in addition to the 149 amino-acids CaM domain (Fig. 1). In addition, the N-terminal of OsCaM61 shares only 85% identity with that of the conserved isoform, OsCaM1 (accession number: AF441190) [26,27]. Thus, OsCaM61 is a novel CaM isoform that differs from the conserved CaMs not only in length but also in composition.

CaM-binding protein kinases (CBKs) have been identified to be different from calcium-dependent protein kinases because of their CaM-regulation [12,28–34]. Although most of them can be stimulated by CaM [12,29], *Orzya sativa* CBK (OsCBK) (accession number: AF368282) is not activated by the binding of OsCaM1 [34].

In this paper, we showed that OsCaM61, unlike OsCaM1, activated OsCBK in a Ca^{2+}-dependent manner. While the extended C-terminal domain was not required for the modulation, Ala^{111}, Gly^{123} and Ser^{127} of OsCaM61 were critical for its regulation on OsCBK activity. Furthermore, the single and triple mutations of OsCaM1/OsCaM61 in these three residues showed similar binding affinities to OsCBK as the wild type OsCaM1/OsCaM61. Previous studies suggest that there is a positive correlation between the binding preferences and the activation profiles in CaM-target enzymes interactions [10,12,18,19]. However, our data indicate that CaM binding affinity does not correlate with the stimulatory effects during the OsCaM61-OsCBK interaction. The fluorescence spectroscopy analysis in the presence of different OsCaMs suggests that OsCBK undergoes conformational changes upon OsCaM61 binding and that these key amino-acids in OsCaM61 play a vital role in the activator-induced conformational changes of OsCBK.

2. Materials and methods

2.1. Expression constructs for CaMs

Total RNAs were isolated from rice and soybean seedlings with TRIZOL (Invitrogen, Carlsbad, CA), and first strand cDNAs were synthesized from total RNAs using RNA Reverse Transcription System (Promega, Madison, WI). The coding regions of OsCaM1, OsCaM61, OsCaM61N (the N-terminal 149 amino-acids of OsCaM61) and GmCaM4 (a divergent CaM isoform from soybean, accession number: L01433 [25]) were amplified with primers as follows: OsCaM1 forward and reverse primers: 5′-GCGGGATCCATGGCGGACCAGCTCACCGACGAG-3′ and 5′-GCGGGATCCTCACTTGGCCATCATGACCTTAAC-3′; OsCaM61 forward and reverse primers: 5′-GCGGGATCCATGGCGGACCAGCTCTCCGAA-3′ and 5′-GCGGGATCCTTACAGGATCACGCACTTCTGGCCACGC-3′; OsCaM61N forward and reverse primers: 5′-GCGGGATCCATGGCGGACCAGCTCTCCGAA-3′ and 5′-GCGGGATCCTTACTTGGCCATCATGCAC-3′; GmCaM4 forward and reverse primers: 5′-TGTCCCCTAAACACCAAACC-3′/5′-GCGGGATCCATGGCAGATATCCTGAGTG-3′ and 5′-GCGGGATCCTCATCGAACGGTCATCATC-3′. The resulting fragments

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Abbreviations: CaM, calmodulin; CBK, CaM-binding protein kinase; OsCBK, *Orzya sativa* CBK

0014-5793/$32.00
doi:10.1016/j.febslet.2006.06.090

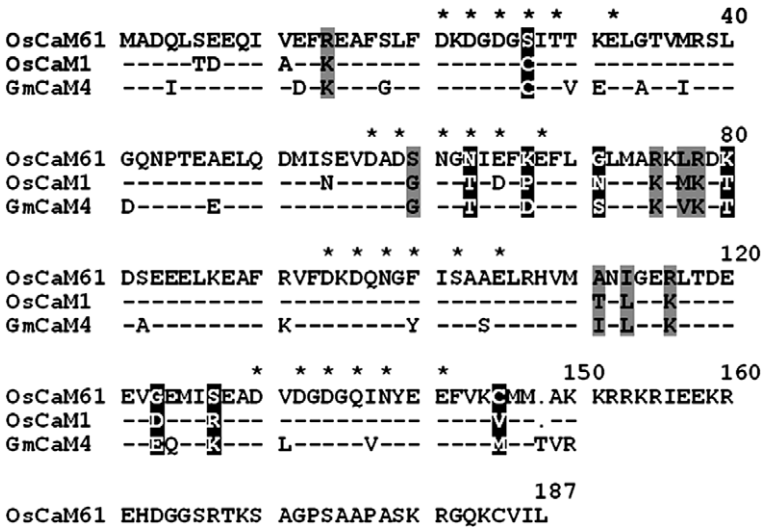

Fig. 1. Comparison of the sequences of OsCaM61, OsCaM1 and GmCaM4. Deduced amino-acid sequences of OsCaM61 (U37936), OsCaM1 (AF441190) and GmCaM4 (L01433) were aligned. Identities between OsCaM61 and other CaMs are indicated by dashes (−). Residues marked with *asterisks* (∗) correspond to Ca^{2+}-binding ligands. Gaps introduced for optimal alignment are indicated by periods (.). Compared with OsCaM61, the conserved amino-acid substitutions are lightly shaded and the non-conserved substitutions are heavily shaded.

were cloned into the *Bam*HI site of pEa expression vector by removing N-terminal tags of pET32a. Mutants were constructed using the PCR-based QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) with pEa-OsCaM1/pEa-OsCaM61. All constructs were verified by DNA sequencing.

2.2. Protein purification

Escherichia coli BL21 (DE3) cells introduced with the recombinant plasmids above were used for CaM purification as the standard protocol [34]. All procedures for OsCBK expression and purification were as previously described [34]. A baculovirus/Sf9 insect system was used for OsCBK expression, and the protein was purified using Ni-NTA resin column and CaM-Sepharose 4B column (Amersham Biosciences, Piscataway, NJ).

2.3. Autophosphorylation and substrate phosphorylation assay of OsCBK

OsCBK autophosphorylation was carried out at 30 °C for 30 min in a 100 μl reaction mixture containing 25 mM Tris–HCl (pH 7.5), 0.5 mM DTT, 10 mM magnesium acetate, 100 μM ATP, 10 μCi [γ-^{32}P]ATP (5000 Ci/mmol) and either 1 μM CaM/0.5 mM $CaCl_2$ or 2 mM EGTA. 15 μg of OsCBK was used in 100 μl of the reaction mixture. Substrate phosphorylation was performed in the same reaction mixture containing 1 mg/ml histone IIIs (Sigma, Louis, MO, Product No. H5505). The reactions were initiated by adding OsCBK, terminated by adding 5× SDS sample buffer, and analyzed by SDS–PAGE (10% gels). After being stained with 0.1% Coomassie Brilliant Blue, the gel was vacuum-dried and exposed to X-ray film at −70 °C. For the activation assay of OsCBK by OsCaMs, aliquots (10 μl) were removed and applied to Whatman (Richmond, CA) P81 phosphocellulose filters (2 cm × 2 cm squares). The filters were washed for four times with 10 min each time in 75 mM phosphoric acid, rinsed in 100% ethanol and air-dried. [^{32}P]P_i incorporation was determined by a Beckman LS 6500 liquid scintillation counter (Fullerton, CA) [34].

2.4. CaM binding assays

Binding assays using biotinylated CaMs were performed as previously described [12]. For binding affinity assay, the CaM cDNAs cloned into pEa were used to prepare ^{35}S-labeled CaM [12], and CaM binding affinity analyses were carried out as previously described [12,13,29,35–37]. 4 pmol purified OsCBK was separated by SDS–PAGE and then electrophoretically transferred onto nitrocellulose filters and incubated overnight with different concentrations of ^{35}S-labelled CaM with 1 mM $CaCl_2$. After the filters were washed with a solution containing 25 mM Tris–HCl (pH 7.5), 50 mM NaCl and 1 mM $CaCl_2$, the radioactivity of the bound CaM on each filter and the free CaM in the incubation buffer collected before washing were measured by the liquid scintillation counter. Average background counts were subtracted from the counts in OsCBK protein samples when calculating the specific binding. The dissociation constants (K_ds) were calculated using SPSS Enzyme Kinetics (SPSS Inc.). All experiments were performed three times in duplicate.

2.5. Fluorescence spectroscopy

Steady-state fluorescence measurements were performed at 20 °C with a Perkin–Elmer Life Sciences LS51 luminescence spectrometer (Boston, MA). OsCBK (2.5 μM) was incubated at 20 °C for 30 min in a buffer containing 50 mM MOPS (pH 7.5), 150 mM KCl and either 0.5 mM $CaCl_2$ or 2 mM EGTA. Subsequently, each OsCaM was added to a final concentration of 5 μM. An excitation wavelength of 295 nm was used to avoid interference from tyrosine residues, and the fluorescence emission spectra in the range of 305–425 nm were recorded.

3. Results and discussion

3.1. Ca^{2+}-dependent gel mobility shifts of OsCaMs and GmCaM4

To investigate the abilities of different OsCaMs on OsCBK activation, the proteins of OsCaM1, OsCaM61, GmCaM4 and several mutated OsCaMs were expressed in *E. coli* and purified with phenyl-Sepharose CL-4B column (Amersham Biosciences) using Ca^{2+}-dependent hydrophobic interaction chromatography. During the purification, all CaMs showed heat-stability, and the CaM proteins bound to the column in the presence of Ca^{2+} and were eluted when EGTA was added. To further confirm the quality of CaMs, we investigated the Ca^{2+}-dependent mobility shift of the purified proteins. We found that all the CaMs migrated more rapidly in the presence of Ca^{2+} than in the absence of Ca^{2+}, which is a typical characteristic of CaM [23,38]. The mobility shift profiles of OsCaM1, OsCaM61, OsCaM61N and GmCaM4 were in accordance with those reported in previous studies [27,39] (Supplemental Fig. 1). Similar results were obtained when all the mutated CaMs were subjected to this assay (data not shown).

3.2. Upregulation of OsCBK kinase activity by OsCaM61 in a Ca^{2+}-dependent manner

Data from our previous study have demonstrated that OsCaM1 binds to OsCBK with K_d 30 nM, but it fails to activate this kinase [34]. In the present study, we observed that while GmCaM4, a divergent CaM isoform from soybean, failed to modulate OsCBK, both autophosphorylation and substrate phosphorylation exhibited an approximately 4-fold increase in the presence of Ca^{2+}/OsCaM61 (Fig. 2A). However, this stimulatory effect was completely blocked in the presence of the Ca^{2+} chelator, EGTA (Fig. 2A, Table 1). These results indicate that OsCBK is activated by OsCaM61 in a Ca^{2+}-dependent manner. Moreover, OsCaM61 activated OsCBK in a dose-dependent manner (K_a = 46.9 nM) (Fig. 3). In addition, when histone IIIs was used as a substrate of OsCBK in the presence of OsCaM61, the V_{max} was increased by approximately 10-folds relative to that of OsCaM1 (Table 1).

3.3. Critical amino-acid residues of OsCaM61 for OsCaM61-specific activation of OsCBK

Because the N-terminal 149 amino-acids of OsCaM61 share high identity with those of OsCaM1, the extra 38 amino-acids in the C-terminus of OsCaM61 are considered to be the possible region responsible for the difference in OsCBK modulation between these two rice CaMs. Our results showed that OsCaM61N, in which the C-terminal 38 amino-acid residues were deleted, activated OsCBK with the maximal activation and kinetic constants similar to those activated by OsCaM61 (Figs. 2B, 3, Table 1). Thus, the difference between OsCaM1 and OsCaM61 on OsCBK activation is likely due to

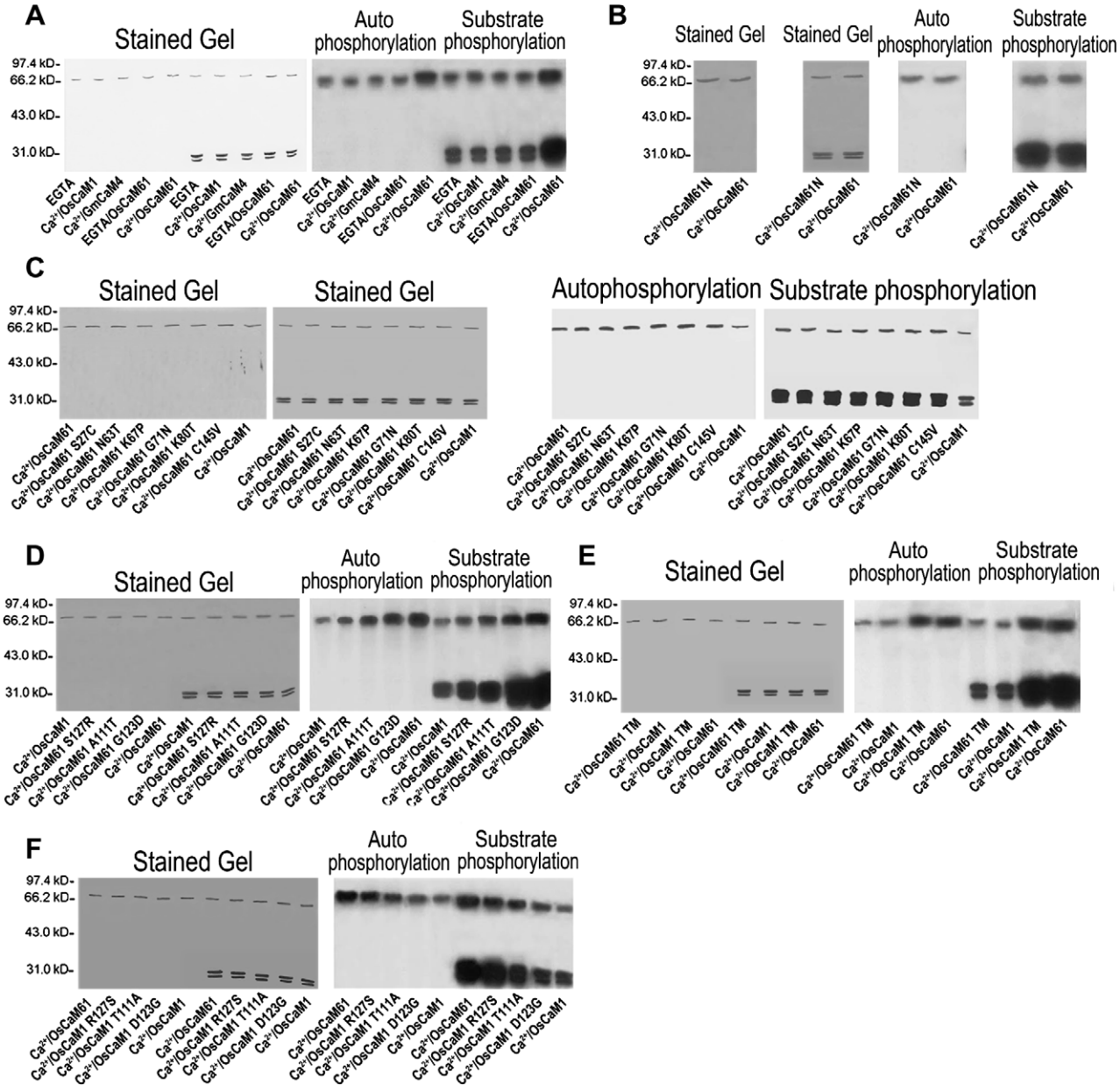

Fig. 2. Effects of CaMs on OsCBK phosphorylation. OsCBK autophosphorylation and substrate phosphorylation assays using histone IIIs as the substrate were performed in the presence of different CaMs as labeled and resolved by SDS–PAGE.

Table 1
Summary of OsCaMs interactions with OsCBK

OsCaMs	Autophosphorylation	Substrate phosphorylation				Binding
	Maximal activation (%)	Maximal activation (%)	K_m (Histone IIIs) (μg ml^{-1})	V_{max} (nmol min^{-1} mg^{-1})	K_a (nM)	K_d (nM)
OsCaM1	–	–	50.0 ± 1.6	1.8 ± 0.7	–	30
OsCaM61	100	100	18.7 ± 4.5	17.7 ± 1.3	46.9 ± 2.2	76.1 ± 3.5
CaM61 mutants						
OsCaM61N	103	108	16.6 ± 2.3	17.3 ± 0.9	41.7 ± 3.5	9.1 ± 0.7
A111T	65	68	29.1 ± 2.7	11.4 ± 0.7	77.9 ± 1.6	71.3 ± 5.1
G123D	72	77	23.7 ± 0.8	14.2 ± 1.9	58.8 ± 1.4	69.7 ± 2.4
S127R	42	39	36.8 ± 3.3	8.6 ± 1.1	125.3 ± 3.8	67.2 ± 3.5
OsCaM61-TM	–	–	51.4 ± 0.8	2.5 ± 0.7	–	72.6 ± 4.3
CaM1 mutants						
T111A	27	26	32.9 ± 1.6	10.6 ± 1.2	121.7 ± 2.7	27.4 ± 1.8
D123G	19	22	38.1 ± 0.8	7.8 ± 0.8	84.1 ± 2.2	32.0 ± 1.5
R127S	59	57	27.3 ± 1.3	15.3 ± 1.1	73.5 ± 3.4	24.3 ± 0.9
OsCaM1-TM	88	91	16.4 ± 0.7	19.4 ± 2.1	43.0 ± 2.6	34.2 ± 2.6

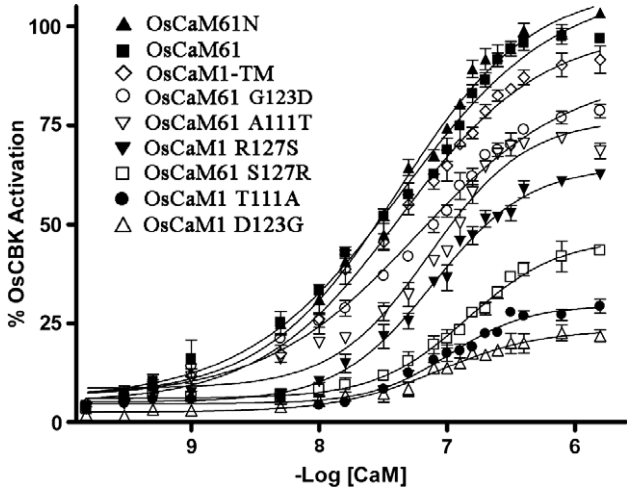

Fig. 3. Histone IIIs as the substrate was phosphorylated with OsCBK in the presence of different OsCaMs under the standard phosphorylation conditions, except that increasing concentrations of OsCaMs ($10^{-9.8}$–$10^{-5.8}$ M) were used.

the different amino-acid compositions between OsCaM1 and the N-terminal region of OsCaM61.

Since both OsCaM1 and GmCaM4 did not stimulate OsCBK in contrast to OsCaM61, the sequence alignment of the three CaMs was compared. A total of 16 amino-acid residues in OsCaM61, including eight conservative substitutions (Arg^{14}, Ser^{60}, Arg^{75}, Leu^{77}, Arg^{78}, Ala^{111}, Ile^{113}, and Arg^{116}) and eight non-conservative ones (Ser^{27}, Asn^{63}, Lys^{67}, Gly^{71}, Lys^{80}, Gly^{123}, Ser^{127}, and Cys^{145}), were found to be different from those in both OsCaM1 and GmCaM4 (Fig. 1). Normally, conservative substitutions exert minimal effects on the protein function because the side chain of the new amino-acid may be functionally similar to that of the amino-acid it replaces. Therefore, eight non-conservative site-specific mutants were designed to identify the critical amino-acid(s) for the isoform-specific activation of OsCBK. In addition, in OsCaM61, a hydrophobic amino-acid, Ala^{111} was mutated to be a hydrophilic one, Thr, which is within OsCaM1.

Our phosphorylation assays showed that six OsCaM61 mutants, S27C, N63T, K67P, G71N, K80T and C145V, stimulated OsCBK as efficiently as OsCaM61 (Fig. 2C), whereas three other OsCaM61 mutants, A111T, G123D and S127R, displayed significantly reduced abilities to activate OsCBK compared with OsCaM61. The abilities of these three OsCaM61 mutants to activate OsCBK followed such a decreasing order: G123D, A111T and S127R (Fig. 2D, Table 1). In addition, the concentration of each OsCaM61 mutant required for the half-maximal activity of OsCBK was higher than that of OsCaM61 (Table 1). While each of the above mutants retained some ability to activate OsCBK, OsCaM61 A111T/G123D/S127R (named OsCaM61-TM) failed to activate this kinase (Fig. 2E, Table 1). Thus, we conclude that the combination of Ala^{111}, Gly^{123} and Ser^{127} contributes to the OsCaM61-specific activation of OsCBK.

3.4. Activation of OsCBK by OsCaM1 mutants

Since minor differences were observed in the sequence alignment between OsCaM1 and OsCaM61, we hypothesized that replacing Thr^{111}, Asp^{123} and Arg^{127} in OsCaM1 with Ala, Gly and Ser, respectively, may generate a mutant form of OsCaM1 with OsCBK activation properties similar to those of OsCaM61. Concomitantly, OsCBK would be subjected to phosphorylation in the presence of different mutant forms of OsCaM1. To address this issue, we compared the OsCBK phosphorylations in the presence of three individual mutants and a triple mutant of OsCaM1. Our data showed that all the three individual mutants enhanced the activity of OsCBK in an increasing order of OsCaM1 D123G, T111A and R127S (Fig. 2F, Table 1), while the triple mutant, OsCaM1-TM (OsCaM1 T111A/D123G/R127S), was found to be almost as effective as OsCaM61 on OsCBK regulation (Figs. 2E, 3, Table 1). These observations further confirm the critical role of these three amino-acid residues for OsCaM61-specific activation of OsCBK.

As a small and conserved protein with a relatively simple structure, CaM modulates many target enzymes. A possible explanation for the multiple biochemical function of CaM is that it regulates different target enzymes via different key domains. Previous studies have documented the important roles of the central helix [3], Ca^{2+}-binding sites [4,5], helix 6 and

helix 6–helix 7 linker [6], C-terminal helix [9] and the domain 1 of CaM [40,41] in the target enzyme stimulation. The results from the present study suggest the important role of helix 6 and helix 7 in OsCaM61-OsCBK interaction. Together with previous studies, these help to understand why CaM with a simple structure can regulate so many targets.

3.5. Binding characteristics of OsCaMs to OsCBK

Although the above data showed a key role of the three amino-acids in OsCaM61-specific activation of OsCBK, the underlying mechanism remains unknown. One possibility is that these mutant sites may modify the binding affinity of OsCaM1/OsCaM61 to OsCBK, thus affecting the kinase phosphorylation. To test this hypothesis, we examined the binding ability of OsCaMs to OsCBK. The biotinylated CaM binding assays showed that all OsCaMs bound to OsCBK in a Ca^{2+}-dependent manner (Supplemental Fig. 2), although their binding affinities to OsCBK differed from each other. As listed in Table 1, OsCaM61 had an 8-fold higher K_d than OsCaM61N (Fig. 4A and B), but these two forms had similar ability to stimulate OsCBK (Fig. 2B, Table 1). These results suggest that the binding affinity of these CaMs to OsCBK does not correlate with OsCBK activation. This is also supported by the observation that although the mutated forms of OsCaM61 and OsCaM1 differed on OsCBK regulation, they had similar K_ds compared with OsCaM61 and OsCaM1, respectively (Fig. 4, Table 1).

Conserved and divergent CaM isoforms may have different affinities to CaM binding proteins due to their specific recognition sequences [19]. Previous studies have suggested a positive correlation between the target binding preferences and the activation profiles: CaM isoform that serves as an activator of target enzymes generally possesses higher affinities than the other CaM isoforms that are unable to stimulate the enzymes [10,12,18,19]. However, our data indicate that the binding affinities of OsCaMs to OsCBK do not correlate with OsCBK activation. Thus the results lead us to explore alternative explanation for the OsCaM61-specific activation of OsCBK.

3.6. Fluorescence changes of OsCBK induced by different OsCaM forms

It has been well known that the change of enzyme activity is concomitant with conformational shift, which may be monitored spectroscopically via Trp fluorescence [42]. In the present study, the spectrum of OsCBK had a higher fluorescence intensity (2.2-fold) in the presence of Ca^{2+}/OsCaM61, as well as a blue shift of the maximum emission (from 373 nm to 355.5 nm). The fluorescence intensity did not change and the λ_{max} shifted very little when OsCaM1 was added (Fig. 5A). It has been shown that there are six Trp residues in OsCBK [34]. However, no Trp was found in either OsCaM61 or OsCaM1 (Fig. 1). Therefore, the changes of the spectra may be due to the conformational change of OsCBK. In addition, the spectrum of OsCBK was not changed by OsCaM61 in the presence of EGTA (Fig. 5A), suggesting that the activated (Ca^{2+}-binding) OsCaM61 induce the conformational change of OsCBK. Moreover, OsCBK showed various degrees of Trp spectrum changes when it bound to the activated individual mutants of OsCaM61 and OsCaM1 (Fig. 5B, C). In addition, similar OsCBK spectra were observed upon OsCaM1-TM binding and OsCaM61 binding, whereas OsCBK spectrum

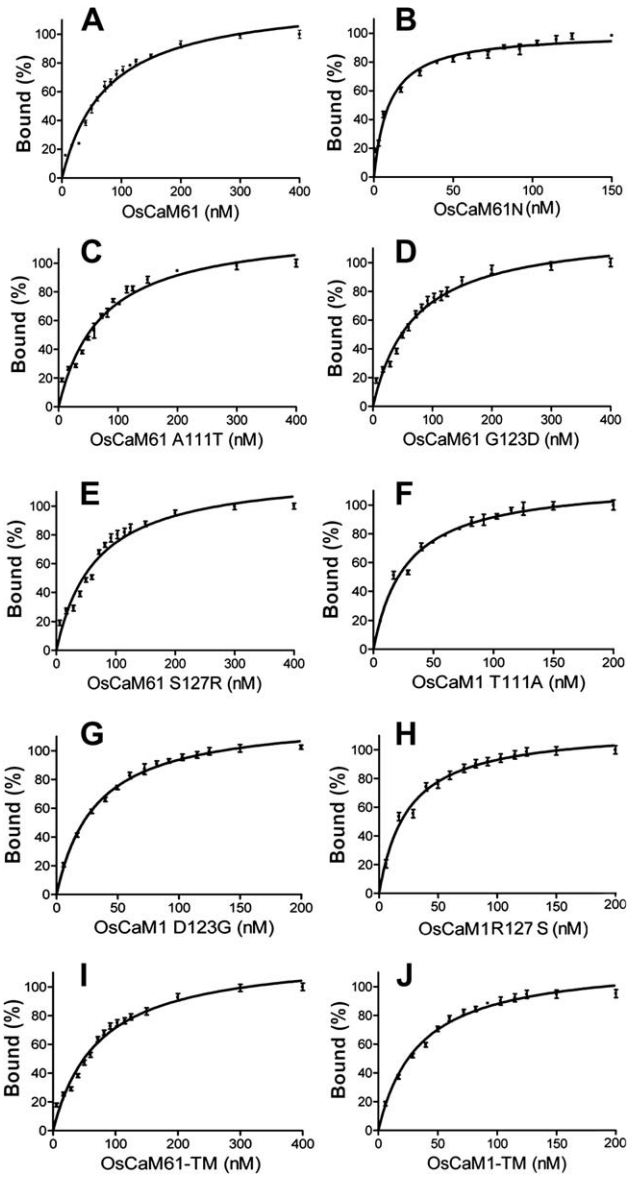

Fig. 4. Kinetics of OsCBK binding to CaMs. The saturation curves of ^{35}S-labeled CaMs binding to OsCBK were generated as follows. OsCBK protein (4 pmol) was transferred onto a nitrocellulose filter and incubated with different amounts of ^{35}S-labelled CaM. After the filter was washed in buffer without [^{35}S]CaM, the radioactivity of the filter was measured using a liquid scintillation counter.

did not change upon OsCaM61-TM binding (Fig. 5D). These data indicate that OsCBK undergoes conformational changes in the presence of Ca^{2+}-binding OsCaM that severs as an activator.

In conclusion, three amino acids in OsCaM61, Ala^{111}, Gly^{123} and Ser^{127} play a vital role in kinase activation and the intrinsic Trp fluorescence spectrum changes of OsCBK. However, these three residues may not be directly involved in the binding of OsCaM1/61 to OsCBK. Our results provide an understanding of the specificity of Ca^{2+} signals in plants through different Ca^{2+} sensors. Further studies are required on the interactions between OsCBK and calmodulin isoforms and the biological significance of the isoform-specific activation of this kinase, which may be achieved with nuclear

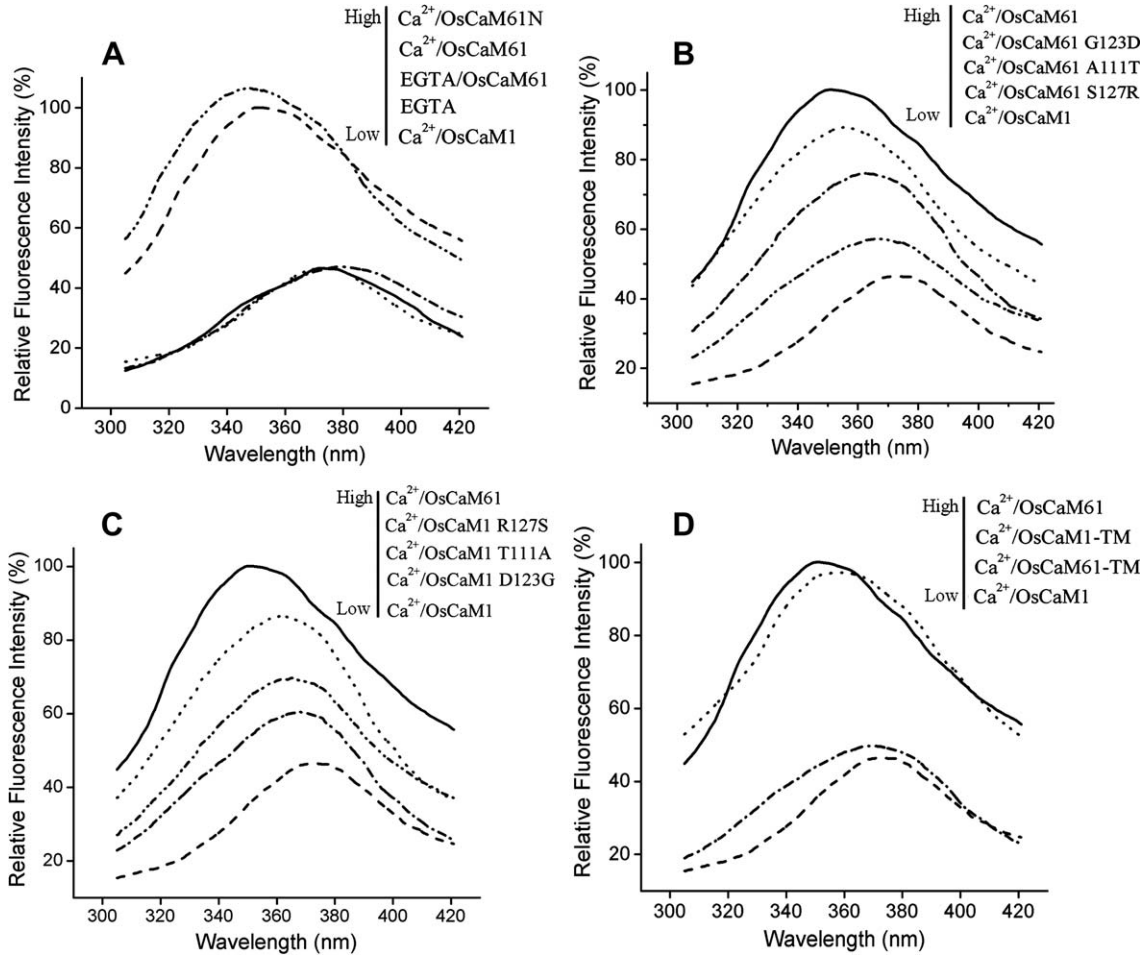

Fig. 5. Steady-state fluorescence assays of OsCBK. Emission spectra of OsCBK in the presence of EGTA or OsCaMs as labeled in figures. An excitation wavelength of 295 nm was used and the fluorescence emission spectra in the range of 305–425 nm were recorded. All curves were corrected for the fluorescence of the buffer containing the same concentration of OsCaMs.

magnetic resonance analysis, *in vivo* interaction, transgenic studies and functional analysis.

Acknowledgements: This work was supported in part by Major State basic Research Program (2005CB120803), National Natural Science Foundation of China (Grant No. 30470848) and the Program for Changjiang Scholars and Innovative Research Team in University to Y.-T. Lu.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.06.090.

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