Arabidopsis KRP1–KRP7 interact with active cyclin D2 (CYCD2)/CDKA and CYCD2/CDKB complexes to a similar extent, they inhibit kinase activity to a different extent. Our results suggest that inhibitory activity is related to the binding ability between KRP proteins and cyclin/CDK complexes, but secondary and tertiary structure may be also involved. These data provide the first evidence that KRP1–KRP7 inhibit kinase activity associated with plant-specific CDKB.

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1. Introduction

In all eukaryotes, the cell cycle is controlled by a family of serine/threonine protein kinases, which consists of a cyclin-dependent kinase (CDK) catalytic subunit and a cyclin regulatory subunit. CDK activation requires binding to a cyclin and phosphorylation of the T-loop by CDK-activating kinase (CAK). The activity of CDKs is suppressed through interaction with other cell cycle regulators including CDK inhibitors (CKIs) [1]. The activity of CDKs is also regulated by inhibitory phosphorylations and stimulatory dephosphorylations at the specific sites within their N-terminal regions. These negative and positive modifications modulate the activity of CDKs, thereby coordinating control of cell cycle progression.

Mammalian CKIs have been classified into two families based on their structural features and CDK targets [2]. The four members of the INK4 family (p16INKnak, p15INKnak, p18INKnak, and p19INKnak) contain ankyrin repeats and specifically bind with CDK4 and CDK6 monomers, inhibiting their kinase activity. In contrast, the three members of the Cip/Kip family (p21Cip, p27Kip, and p57Kip) form ternary complexes with a broad range of cyclin/CDK complexes, including cyclin D/CDK4,6, cyclin E/CDK2, cyclin A/CDK1,2, and cyclin B/CDK1. These complexes are involved in the control of both the G1/S and G2/M transitions, thus the Cip/Kip family is thought to regulate both cell cycle control points [3]. Additionally, the Cip/Kip family plays a further role by participating in the assembly of cyclin D/CDK4,6 complexes without inhibiting their kinase activity [4,5].

Two major types of plant CDKs are the principal mediators of cell cycle control. A-type CDKs contain the canonical PSTAIRE-motif in their cyclin-binding regions and act like CDK1 from mammals and CDC28/Cdc2 from yeasts. B-type CDKs are divided into two subgroups depending on the presence of PPTALRE (CDKB1) or PPTTLRE (CDKB2) motif. Plant-specific features of CDKs include their strictly regulated expression, which peaks during the G2 phase [6,7]. Based on sequence similarity and expression patterns, three major classes of cyclins, A-, B-, and D-types, are found in plants [6,8]. A-type cyclins (CYCAs) are broadly involved during S-M phase, and B-type cyclins (CYCBs) control the G2/M transition probably in association with both CDKA and CDKBs. D-type cyclins (CYCDs) are thought to act mediators linking extracellular and developmental signals to the cell cycle [9,10]. However, limited information is available on regulatory mechanisms governing the kinase activity of plant CDK/cyclin complexes.

Plants genes encoding proteins displaying similarities to the CDK-binding/inhibitory domain of mammalian Cip/Kip proteins have been identified in Arabidopsis, Chenopodium rubrum, pea (Pisum sativum), cotton (Gossypium hirsutum), rice (Oryza sativa), maize (Zea mays) and tobacco (Nicotiana tabacum). The Arabidopsis genome contains seven CKIs genes that are similar to p27Kip1, these are designated Kip-related proteins 1–7 (KRP1–KRP7) [11–14]. Yeast two-hybrid assays and in vitro binding assays have shown that all of the Kip-related protein (KRP) interact with CYCDs but not with CDKBs, and that some of the KRP interacts with CDKA, whereas others do not. KRP1 (also known as ICK1; interactor/inhibitor of Cdc2 kinase) and KRP2 (ICK2) have been shown to inhibit in vitro histone H1 kinase activity of p13Suc1-associated CDK/cyclin complexes prepared from plant tissues [12,15]. Overexpression of the Arabidopsis KRP1, KRP2, KRP6 (ICK4), C. rubrum (ICKCr), or N. tabacum (NtKIS1a) CKIs

Abbreviations: KRP, Kip-related protein; CDK, cyclin-dependent kinase; CK1, CDK inhibitor; ORF, open reading frame

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produces similar phenotypes in Arabidopsis [13–17], including reduced overall growth, serrated leaves, and abnormal flowers.

Currently, little is known regarding the specific functions of plant CKIs. As they share very little sequence similarity with mammalian CKIs outside the C-terminal conserved region [11,13,18], one fundamental issue concerns the structure–function relationships and inhibitory properties of each plant CKI towards different cyclin/CDK complexes. It has been shown previously that expression of N-terminal truncated KRP1 in Arabidopsis significantly enhanced the protein’s stability and resulted in a more severely abnormal phenotype than that observed following overexpression of wild-type KRP1 [19]. In contrast, expression of KRP1 with a deletion of the C-terminal conserved region resulted in plants that were morphologically indistinguishable from wild-type [19,20], consistent with findings that the C-terminal deletion attenuated the interactions of KRP1 with CDKA and CYCD3 [15].

To examine whether Arabidopsis KRPhs have distinct functions, we have analyzed in vitro kinase assays performed using CYCD2/CDKA and CYCD2/CDKB complexes produced in insect cells and purified KRP proteins. Interestingly, the cyclin/CDK complexes were inhibited differently by individual KRP proteins, and were very weakly affected by KRP5. Binding assays showed that all of the KRP proteins bind CYCD2/CDKA and CYCD2/CDKB complexes to a similar extent, suggesting that inhibitory activity is attributable to more than simply binding activity. Although an interaction between KRP proteins and CDKB has never been described, we found that KRP proteins can also bind to CYCD2/CDKB complexes and inhibit their in vitro kinase activity.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis MM2d cells were obtained from Dr. James Murray (University of Cambridge) and cultured at 25 °C in a modified Linsmaier and Skoog medium [21].

2.2. In vitro binding assays

Open reading frame (ORFs) encoding KRP1–7 were amplified by PCR with primers incorporating restriction sites suitable for ligating the products into the pSPUTK vector (Stratagene, La Jolla, CA), which encodes the hemagglutinin (HA)-epitope tag. All PCR-generated clones were verified by sequencing. Full construction details are available from the authors.

In vitro transcription/translation was performed using the rabbit reticulocyte lysate (Promega, Madison, WI). Histidine (His)-tagged CYCD2 (CYCD2;1), CDKA (CDKA;1), and CDKB (CDKB;2;1) were purified from insect cells with Ni²⁺-TALON metal affinity resin (BD Biosciences Clontech, Palo Alto, CA), and incubated with 5 μl of each in vitro-translated HA-KRP protein at 4 °C for 2 h with gentle mixing. After three washes in pull-down assay buffer, the bound proteins were detected using a BAS analyzer (Fuji Film, Tokyo, Japan). To assay for the inhibitory activity of KRPhs, purified GST or GST–KRP fusion proteins were added to the kinase reactions before the addition of a substrate and 32P-ATP.

2.4. Insect cell culture and baculovirus infection

Insect cells (Sf9) were cultured and infected with baculovirus as described [22,23]. N-terminal His-tagged fusions of CYCD2, CDKA, and CDKB were generated by ligating appropriate PCR products into pFastBac HTb (Invitrogen, Carlsbad, CA). Recombinant bacmid, an intermediate plasmid, was isolated and transfected into Sf9 cells using a liposome-mediated transfection kit (Invitrogen).

3. Results and discussion

3.1. In vitro kinase assays using cyclin/CDK complexes

We have previously produced FLAG-tagged tobacco CYCD3/CDKA complexes in insect cells using a baculovirus expression system; these complexes are able to phosphorylate histone H1 and the retinoblastoma-related protein in vitro [22,23]. Here, Arabidopsis His-CDKA (CDKA;1), His-CDKB (CDKB;2;1), and His-CYCD2 (CYCD2;1) were expressed in insect cells. We found that the purified CYCD2/CDKA and CYCD2/CDKB complexes were able to phosphorylate histone H1, while CYCD2, CDKA, CDKB, and mixtures of CYCD2 and CDKA or CYCD2 and CDKB had no kinase activity (Fig. 1). Thus, CYCD2/CDKA and CYCD2/CDKB complexes were probably activated by endogenous insect-cell CAK, as observed with mammalian cyclin D/CDK4 complex [24]. However, mixtures of CYCD2 and CDKA or CYCD2
and CDKB were inactive even though these are the likely components of active complexes.

To examine the relative kinase activities of CYCD2/CDKA and CYCD2/CDKB, we added roscovitine, a CDK inhibitor, to kinase reactions containing CYCD2/CDKA and CYCD2/CDKB complexes. As CYCD2/CDKA showed stronger kinase activity than CYCD2/CDKB, 2.5-fold more of the latter was used to ensure equivalent levels of kinase activity in both samples before the addition of roscovitine. Although roscovitine differentially inhibits kinase activity derived from each class of CDK [25], similar inhibitory effects were seen on both CYCD2/CDKA and CYCD2/CDKB complexes (Fig. 1). Kono et al. showed that Arabidopsis B2-type CDK (CDKB2;1) can interact with CYCD1;1 and CYCD4;1 in vitro, and that the CYCD4;1/CDKB2;1 complexes purified from insect cells exhibit H1 kinase activity [26]. Thus, we provide evidence that CYCD2;1/CDKB2;1 complexes produced in insect cells also show in vitro histone H1 kinase activity.

3.2. KRPs interact more strongly with cyclin/CDK complexes than with monomers

Yeast two-hybrid assays and in vitro binding assays have shown that all of the KRPs interact with D-type cyclins (CYCD1, CYCD2 and CYCD3) but not with CDKBs, and that some of the KRPs interact with CDKA, whereas others do not [12,13,15,18]. To examine whether the KRPs are able to directly interact with cyclin/CDK complexes, we carried out in vitro pull-down assays. KRP proteins tagged with three tandem N-terminal HA epitopes (HA-KRPs) were synthesized by in vitro transcription/translation. Arabidopsis His-CDKA, His-CDKB, and His-CYCD2 were expressed in and purified from insect cells. In addition, His-tagged CYCD2/CDKA and CYCD2/CDKB complexes were purified from insect cells co-expressing these proteins. The individually expressed proteins and complexes were tested for KRP binding using in vitro binding assays. Complex formation was analyzed by Western blotting with anti-HA antibodies following purification using His-affinity columns. Under our experimental conditions, all of the KRP proteins bound to both CYCD2/CDKA and CYCD2/CDKB complexes, but not to CDKA, CDKB, or CYCD2 alone (Fig. 2). Importantly, the KRP proteins were not able to bind to mixtures of CYCD2 and CDKA or CYCD2 and CDKB, implying that KRP proteins bind only to active complex (Fig. 1). Thus, we conclude that KRP proteins bind more strongly to cyclin/CDK complexes than their component monomers. By analogy with the p27Kip1 protein, in which the CDK-binding/inhibitory domain peptide interacts with target sites in both the cyclin and CDK by binding to a groove between the two proteins [27], it seems likely that the homologous C-terminal conserved regions of KRPs possess similar binding properties; thus, affinity for the complex is stronger than for monomers.

3.3. Inhibition of CYCD2-associated kinase activity by KRPs

To investigate whether all of the KRPs have CDK inhibitory activity, we used in vitro kinase assays to test their ability to inhibit CYCD2/CDKA and CYCD2/CDKB complexes. Aliquots of CYCD2/CDKA and CYCD2/CDKB complexes were incubated with different amounts of GST or GST-tagged KRP proteins (Fig. 3). The addition of 1–10 pmol of GST-KRP proteins inhibited kinase activity in a dose-dependent manner, while corresponding amounts of GST alone had no effect. Interestingly, both CDKA- and CDKB-associated kinase activities were very inhibited by GST-KRP proteins.
weakened by the addition of GST-KRP5. The addition of increased amounts of GST-KRP proteins resulted in greater reduction of kinase activity; however, 15–40% of the control kinase activity was retained even at the maximum amounts of KRP5 tested. While similar inhibitory patterns have been observed [12,15,28] in previous studies measuring KRP1, KRP2, or their maize orthologs, this is the first comparative analysis of all of the KRP5. Because previous studies using yeast two-hybrid assays have suggested that KRP proteins are unable to bind to CDKB [13,15,18], the ability of KRP to inhibit CDKB kinase activity has not been previously reported, and our findings thus provide the first evidence that KRP proteins bind and inhibit CYCD2/CDKB complexes produced in insect cells. By contrast, the interaction analysis and kinase activity measurements showed that CDKA, but not CDKB, was inhibited by KRP2 in vitro and in vivo [29]. One possible explanation is that CYCD2/CDKB complexes are not formed in vivo and CDKB interacts with other types of cyclins including B-type cyclins [30].

Interestingly, the GST–KRP1 and -KRP7 proteins inhibited CYCD2/CDKB kinase activity more than CYCD2/CDKA activity (Fig. 2), whereas the other KRP proteins inhibited kinase activity in both complexes to a similar degree. Yeast two-hybrid assays have shown that the C-terminal conserved domain of KRPs is required for interactions with both CDKA and CycD3, while the N-terminal domain suppresses these interactions [15]. Consistent with these results, overexpression of KRP1 reduced endoreduplication and cell size in Arabidopsis trichomes; this function was dependent on the C-terminal domain and was negatively regulated by the N-terminal domain [20]. Additionally, deletion of the C-terminal 29 residues completely abolished the effects of KRP1 overexpression on plant growth and morphology [19]. We have found that deletion of the KRP2 C-terminal region resulted in loss of binding to CYCD2/CDKA complexes and loss of inhibition of kinase activity (unpublished data). Thus, the C-terminal conserved region is important for interacting with cyclin/CDK complexes; however, all of the KRP proteins bind to cyclin/CDK complexes to similar extent (Fig. 1), suggesting that binding alone is not responsible for inhibitory activity. KRPs share very little sequence similarity outside their conserved C-terminal region, suggesting that secondary and tertiary structure may be involved in determining the extent of inhibition.

3.4. Inhibition of reconstituted CYCD2-associated kinases by KRPs

We have previously established reconstituted kinase assays using GST-cyclin fusions. Here, purified His-CYCD2 bound to His-bind resin was incubated with extracts of exponentially growing Arabidopsis MM2d cells. The reconstituted His-CYCD2 was recovered and assayed for histone H1 kinase activity. As well as inhibiting CYCD2-associated kinases from insect cells (Fig. 3), KRP proteins also inhibit the activity of these kinases from plant cells (Fig. 4). In addition, when a p13Sac1 column was used to purify Cdc2-like protein kinases, we found that KRP proteins inhibited kinase activity associated with p13Sac1 (Fig. 4). Given that p13Sac1 is associated with various cyclin/CDK complexes, all of the KRP proteins have the potential to inhibit other cyclin/CDK complexes. Our finding that GST-KRP5 inhibited p13Sac1-associated kinases more strongly than CYCD2-associated kinases from insect cells also suggests that KRP5 may be able to effectively inhibit kinases other than CYCD2-associated kinases. Alternatively, KRP5 may act as an assembly factor for certain cyclin/CDK complexes, in a manner similar to mammalian p21Cip1 and p27Kip1 [4,5].

In proliferating cells, CDKBs phosphorylate KRPs, triggering destruction of the latter through proteasomal degradation. This phosphorylation might change the conformation of KRPs, thereby interfering with their binding to CDKA [29]. On the other hand, in cells triggered to enter endoreduplication cycles, CDKB-associated kinase activity declines, resulting in a stabilization of the KRPs which allows them to bind and inhibit CDKA-associated kinase. The KRP concentration, however, is likely not high enough to inhibit the CDK/cyclin complexes that drive S-phase entry, so that cells may re-enter the S phase without an intervening mitosis. KRP expression is upregulated during cell cycle exit, and CDK/cyclin complexes controlling the entry into S phase become inhibited, resulting in a complete cell cycle arrest. Thus, KRP5 are thought to be important for adjusting CDK activity in proliferating cells, as well as for switching between different cell cycle programs, such as entering endoreduplication cycles or executing cell cycle exit [31]. Recent studies showed that mammalian Cip/Kip family plays additional roles outside of the nucleus: they are involved in the regulation of actin dynamics and cell migration [32]. It will be of interest to examine whether KRP functions other than cell cycle regulation also operate in plants.

4. Conclusion

We have revealed several properties of Arabidopsis KRPs. All of the KRPs inhibited the kinase activity of CYCD2/CDKA and CYCD2/CDKB complexes and reconstituted CYCD2-associated kinases. Thus, KRPs have a broader spectrum of inhibitory effects than previously recognized, suggesting that they might affect cell cycle progression at both the G1/
S and G2/M transitions. However, individual KRP proteins have different inhibitory activity towards CYCD2-associated kinases, suggesting that each KRP has distinct functions, and that the activities of CDK/cyclin complexes are precisely modulated by KRPs. Thus, elucidation of the mechanisms regulating the levels of KRPs in plants may provide considerable insight into these novel functions of CKIs during plant development.

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