Thesis of Ph.D

Novel cyclin-dependent kinase inhibitor protein from Medicago truncatula

Aladár Pettkó-Szandtner

Supervisors: Horváth Gábor
and Dudits Dénes

Institute of Plant Biology
HAS, Biologcal Research Center
Szeged 2006.

Introduction and aims

We have long been fascinated by the beauty and intricacy of cell division. Only during the last two decades have the building blocks of a molecular system and the machinery that regulates the cell cycle been revealed. The central component of the molecular machinery for regulating the cell cycle is the cyclin-dependent kinase (CDK). The CDK itself is subject to multiple layers of control. Specific CDKs are activated by forming complexes with their cyclin (Cyc) partners, and are required at different phases of the cell cycle. In addition to cyclin binding, the activity of CDKs is also regulated by CDK inhibitors, the status of phosphorylation in CDKs and ubiquitin-mediated proteolysis of these proteins. It is now clear that plants use the same CDK-based machinery as animals and yeast to control the cell division cycle. Cell cycle regulators sharing sequence and functional similarities with the animal counterparts have been identified in plants. Despite the similarity at the fundamental level, plants have many obvious differences from animals in terms of the regulation of cell proliferation during growth and development.

In order to cope with their sessile life style, plants display developmental characteristics permitting a flexible response to environmental factors. Plant development is mainly postembryonic, and cell division is restricted to meristematic regions. Endoreduplication resulting from DNA replication without subsequent mitotic cell divisions is widespread in differentiated cells. Most plant cells are totipotent and are able to change their fate at any moment in response to external signals. Due to the rigid cell wall, cell migration does not play any role in development in contrast to the development of many animal tissues and organs. In plants, cell cycle progression can respond to hormonal and stress factors such as auxins, abscisic acid and osmotic shock which are all known to induce Ca^{2+}-dependent signalling pathways. The role of the Ca^{2+}-status in the division of plant

cells has been studied in a limited number of experimental systems. Among the Ca^{2+}-binding sensory proteins, the calcium-dependent protein kinases (CDPKs) are of particular interest because these calmodulin like domain protein kinases (CPKs) play a role in the transmission of stress and hormonal signals.
In my thesis I present the results which attempt to answer the following questions:
Which are the interacting partners of the different alfalfa CDKs in yeast two-hybrid system?
Which of the known cell cycle components are able to interact with the newly identified proteins?
Which factors can influence the expression of the CDK inhibitor gene?
Is the KRPMt a potent inhibitor of the *Medicaco* CDKs?
What are the functional consequences of the phosporylation of the CDK inhibitor protein?
What can be the role of the Ca^{2+}-dependent kinases and the inhibitor protein in the crosstalk between the Ca^{2+}-dependent signal pathways and the control of the cell cycle?
On which amino acid residue is the KRPMt phosphorylated, and what does it cause?

Results and discussion

In this thesis I report the cloning of an alfalfa CDK inhibitor (*KRPMt*) cDNA that encodes an active inhibitor protein with a differential effect on various kinase complexes. Phosphorylation of the KRPMt protein by an alfalfa MsCPK3 recombinant kinase results in the increase of KRP function *in vitro*. This finding supports a hypothesis that modulation of CDK activities by inhibitor protein as a substrate of the Ca^{2+}-dependent kinase provides a molecular mechanism for crosstalk between Ca^{2+}-signalling and cell cycle control.

We used the yeast two-hybrid system to identify genes encoding proteins able to interact with the PSTAIRE-type Medsa;CDKA;1 alfalfa kinase. Among such clones we identified a cDNA clone that encoded an alfalfa protein with characteristic peptide motifs previously found in cyclin-dependent kinase inhibitors from *A. thaliana*. The conceptual translation of the longest open reading frame predicted a polypeptide of 224 amino acids with a molecular weight of 25.3 kDa. Based on similarities in structural organization of KRPMt to the *Arabidopsis* KRP protein family, and the 6 conserved motifs previously described, this alfalfa protein could be identified as the Kip-related protein of *Medicago truncatula* (KRPMt). Searching the GenBank database revealed that KRPMt shows the highest amino acid similarity (51%) and identity (36%) to the *Arabidopsis* protein KRP4. During the in-depth analysis of KRP and KRP-like sequences available in the public databases, we found an additional motif, which is present in the *Arabidopsis* KRP3 and KRP 4 as well as the rice KRP 1, KRP 3, *Lycopersicum* KRP 1, KRP 2 and Euphorbia KRP 4 sequences. This motif is unique to *KRP* genes and in the case of *Medicago* includes the following amino acids: RSARETTPVHLI.

The expression of the *Medicago* inhibitor gene was analysed in various plant organs and cell suspension cultures. This gene was active in all analysed tissues including cultured alfalfa cells where the gene expression showed a gradual increase during the ageing of the suspension culture. The *KRPMt* gene exhibited a characteristic response to ABA resulting in considerable transcript accumulation. Exposure of cell culture to 100 μM ABA resulted in an 8.6-fold increase in *KRPMt* transcript accumulation at 2 hours after hormone treatment. These high mRNA levels were detected in the treated cells after 1 or 2 days to the lower extent. The NaCl treatments also activated the *KRPMt* gene significantly. A 14.9-fold increase in transcript accumulation could be observed after moderate salt stress.

We tested the binding specificity of the alfalfa inhibitor with several *Medicago* CDKs and cyclins. The full length KRPMt protein was shown to interact with a variant of PSTAIRE kinases, namely Medsa;CDKA;1 and various D-type cyclins. The strongest interaction was detected between the inhibitor and the Medsa;CycD3 proteins. Interestingly, we found that the spectrum of interaction partners was significantly altered when the truncated inhibitor protein lacking the N-terminal region of 96 amino acids was analysed in the yeast two-hybrid system. This alteration of the KRPMt protein extended its capability to interact with additional partners such as the Medsa;CDKA;2 kinase and two mitotic cyclins (Medsa;CycB2;3 and Medsa;CycB2;2). As we knew previously, the Medsa;CycD4, which is a strong interacting partner with the KRPMt protein can interact with both alfalfa B-type CDKs. Therefore we checked with a pull-down assay whether the recombinant KRPMt can interact with the A and B type CDK complexes. Our results clearly show that the recombinant KRPMt is able to pull-down both type of CDKs, moreover the Medsa;CDKB2;1 which is the strongest interacting partner of the Medsa;CycD4 can be quantitatively purified with His_6-KRPMt.

We tested the response of specific kinase complexes immunoprecipitated from extracts of cultured alfalfa cells. The degree of inhibition of kinase activity in the $p13^{suc1}$ fraction and complex of PSTAIRE kinase pair (Medsa;CDKA;1 /A1;2) was similar to the concentrations used with the recombinant KRPMt protein. It is an unexpected finding that the Medsa;CDKB1;1 complex was not inhibited, but even activated in some cases in the histone H1 kinase activity assay. The Medsa;CDKB2;1 mitotic kinase turned out to be the most sensitive target. In the presence of 50 pmol of inhibitor protein, only 10% of the histone H1 phosphorylation activity was retained. In addition to differences among the sensitivity of various CDK kinase complexes, the inhibitory function was different in complexes that were isolated from S-phase or G2/M phase cells. Using histone

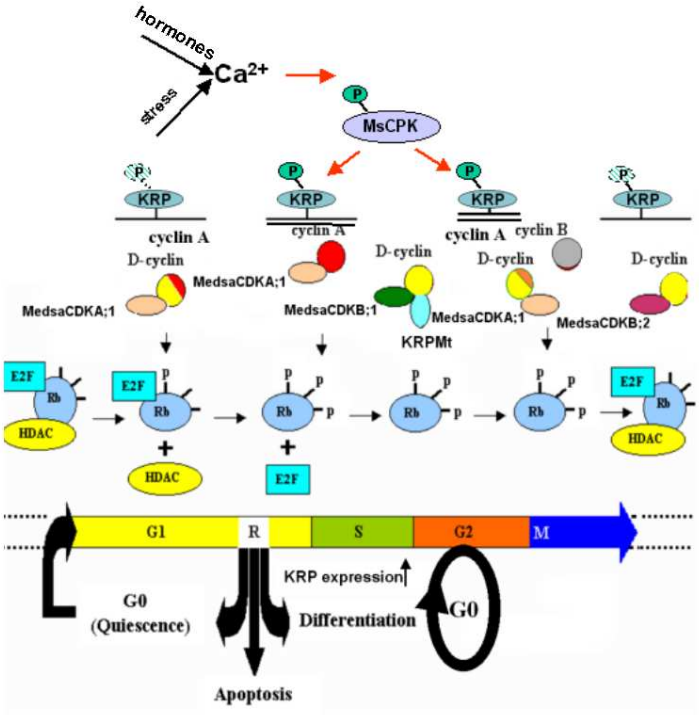
H1 as a phosphorylation substrate, the kinase complex immunoprecipitated by anti-Medsa;CycA2 was significantly inhibited when the G2/M cells were analysed. This cyclin is known to interact with Medsa;CDKA;1. In contrast, the complex isolated from partially synchronized S phase cells failed to respond to the inhibitor protein *in vitro* . In the case of the Medsa;CDKA;1/A;2 complex, the G2/M phase extracts were also found to be more sensitive; however, 24% inhibition could be seen the in protein fraction immunoprecipitated from S-phase cells. Since the retinoblastoma-related proteins (RBRs) are primary targets for CDKs, we tested the phosphorylation of the recombinant MsRBR in the presence of the KRPMt protein. The alfalfa RBR protein can be phosphorylated by this kinase and there was no difference in inhibitory function of KRPMt when histone H1 or MsRBR C-terminal protein fragment were used as a substrate in the Medsa;CDKA;1/A;2 kinase reaction *in vitro.* These studies demonstrate the differential influence of KRPMt protein on activities of various CDKs, emphasizing a multiple role of the inhibitor protein with functional selectivity towards the cell cycle regulatory complexes.

The calmodulin-like domain protein kinase (MsCPK3) phosphorylates the full length recombinant KRPMt protein. After 1 hour kinase reaction approximately 0.2% of the total recombinant protein was phosphorylated by the CPK protein. The ATP and Ca^{2+}-dependent phosphorylation of KRPMt inhibitor significantly reduced the histone H1 phosphorylation function of the immunoprecipitated Medsa;CDKA;1/A;2 complex. Comparison of the effectiveness between the non-phosphorylated and phosphorylated KRPMt indicated an essential increase in inhibitory function as the result of this protein modification. The non-phosphorylated recombinant protein was also found to be active in these assays. The increased inhibitory effect of the phosphorylated KRPMt can be observed in small concentrations of the post-translationally modified protein. A relatively small proportion of the phosphorylated protein was able to reach maximum inhibition.

Phosphopeptide mapping of the KRPMt protein by 2D separation on a thin-layer cellulose plate was performed and the isolated peptides were subsequently hydrolysed. From these experiments we were able to conclude that MsCPK3 and MedsaCDKA;1 phosphorylates the KRPMt protein on one or more N-terminal serines. Phosphorylation by both types of CDK proteins occurs, despite the fact that *in silico* predictions were not able to find any CDK phosphorylation site motifs. However, the phosphorylation of CDKB1;1 is likely to be a possible degradation signal.

7. We were able to prove that the 91st serine residue of KRPMt is phosphorylated. The MsCPK3 kinase was able to phosphorylate the full length KRPMt and the inhibitor protein fragment which contains amino acids 88-223. The KRPMt fragment which lacks the first 96 amino acids was not a potent substrate of the calmodulin-like domain protein kinase. We produced the site directed mutant variants of the 91st serine (alanine, aspartate, glutamate). Whith these mutants we assume that the phosphorylation of this serine residue plays an inportant role in the activation of the KRPMt protein; however, the unphosphorylated form is also active.

We propose a hypothetical model based on results of interaction studies between different *Medicago* CDKs, cyclins and KRP in yeast two-hybrid and pull-down assays, as well as *in vitro* phosphorylation and inhibitor studies.



Cell cycle model of alfalfa. Different kinases and cyclins are expressed and interact in different phases of the cell cycle. The D-type cyclins can interact with the inhibitor KRPMt, but the activity of Medsa;CDKB1;1, which is also able to interact with Medtr;CycD4, is not reduced in the presence of the KRPMt protein. In contrast, both the Medsa;CDKA;1/A;2 and the Medsa;CDKB2;1 kinase complexes are strongly inhibited by this regulatory protein. Phosphorylation of the alfalfa RBR protein can also be affected by the inhibitor. Ca^{2+}-dependent protein kinase (MsCPK3) and the CDK inhibitor (KRPMt) could link Ca^{2+}-signalling and cell cycle control in plants. Model illustrating the role of CDK inhibitors and calmodulin-like domain protein kinases in crosstalk between Ca^{2+} signalling and regulation of cell cycle progression in plants.

Symbols: activation; → inhibition; ⊥ enhanced inhibition. ⫠

List of pulications

1. **Pettkó-Szandtner A.**, Mészáros T., Horváth G.V., Bakó L., Csordás-Tóth É., Blastyák A., Zhiponova M., Miskolczi P. , Dudits D.(2006) Activation of an alfalfa cyclin-dependent kinase inhibitor by calmodulin-like domain protein kinase The Plant Journal (in press)
IF: 6.367

2. Miroslava K. Zhiponova, **Aladár Pettkó-Szandtner***, Éva Stelkovics, Zsuzsanna Neer, Sándor Bottka, Tibor Krenács, Attila Fehér, Dénes Dudits, and László Szilák (2006) Mitosis-specific promoter of the alfalfa cyclin-dependent kinase gene (Medsa;CDKB2;1) is activated by wounding, and ethylene, in a non-cell division dependent manner. Plant Physiology (in press) * contributed equally
IF: 5.881

3. Fülöp, K., **Pettkó-Szandtner, A.,** Magyar, Z., Miskolczi, P., Kondorosi, É., Dudits, D. and Bakó, L. (2005) The *Medicago* CDKC;1-CYCLINT;1 kinase complex phosphorylates the carboxy-terminal domain of RNA polymerase II and promotes transcription. The Plant Journal, 42: 810-820
IF: 6.367

4. Lendvai Á., **Pettkó-Szandtner A.**, Horváth G.V., Csordás-Tóth É., Györgyey J. and Dudits D. (**2004**) The number of retinoblastoma genes may reflect phylogenetic divergence of dicot and monocot plants. *Comparative Biochemistry and Physiology* 137/A: S248
IF: 1,556

5. **Pettkó-Szandtner A.**, Csordás-Tóth É., Mészáros T., Horváth G.V:, Bakó L. and Dudits D. (**2004**) Medicago CDK inhibitor. *Comparative Biochemistry and Physiology* 137/A: S248-S253
IF: 1.556

6. Mészáros, T., Miskolczi, P., Ayaydin, F., **Pettkó-Szandtner, A.,** Peres, A., Magyar, Z. Horvath, G.V., Bakó, L., Fehér, A. and Dudits, D. (2000) Multiple cyclin-dependent kinase complexes and phosphatases control G2/M progression in alfalfa cells. Plant Mol. Biol. 43: 595-605.
IF: 3.510

7 Horváth, G.V., **Pettkó-Szandtner, A.,** Nikovics, K., Bilgin, M., Boulton, M., Davies, J.W., Gutierrez, C. and Dudits, D. (1998) Prediction of functional regions of the maize streak virus replication-associated proteins by protein-protein interaction analysis, Plant Molecular Biology, 38: 699-712
IF: 3.510

9. **Pettkó-Szandtner A.** and Horváth G. V. (2005) Detection of protein-protein interactions: The yeast two-hybrid system. In: Basic Techniques in Molecular Biology, University of Debrecen (Editor: V. Dombrádi) (In Hungarian)

10. P. Miskolczi, A. Pettkó-Szandtner, G.V. Horváth, D. Dudits, A. Fehér, J. Györgyey A novel plant cyclin /Medicago cyclin D patent; EP 00870133.6 (16 June 2000) PCT/EP01/06771 (patent)

11. Lendvai Á., **Pettkó-Szandtner A**., Csordás-Tóth É., Horváth G. V., Györgyey J. and Dudits D.* Dicot and Monocot plants differ in the complexity of Retinoblastoma-related Protein Subfamilies Plant Molecular Biology (submitted)

12. Bihari,Z., **Pettkó-Szandtner,A**., Csanadi,G., Balazs, M., Bartos,P., Kesseru,P., Kiss,I. and Mecs,I.Characterization of a novel n-alkane-degrading strain, Acinetobacter haemolyticus AR-46 Applied Microbiology and Biotechnology (submitted)