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ROLE OF SERINE/THREONINE PHOSPHATASES AND CYCLIN-DEPENDENT KINASES IN ALFALFA CELL DIVISION

Ph.D. Thesis

by

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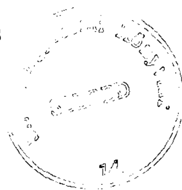


Introduction

Reversible protein phosphorylation is a fundamental mechanism in the regulation of a large number of biological functions. Achievement of such control requires co-ordinated action of the interconverting enzymes: protein kinases and protein phosphatases (PPases). In eukaryotic cells, four major classes of serine/threonine-specific PPases (PP1, PP2A, PP2B and PP2C) have been identified according to the biochemical criteria. Catalytic subunits of PP1 and PP2A are encoded by two related gene families. These two phosphatases can be distinguished by differential sensitivity towards non-protein inhibitors such as okadaic acid (OA) or endothall (ET). A high degree of homology amongst members of the same family, and a high degree of evolutionary conservation between organisms as divergent as mammals and yeast, implies that these enzymes are involved in fundamental cell functions. During mitotic regulation, PPases take part in changing the dephosphorylation status of specific phosphoprotein substrates including cell division related kinases, which have been linked to the processes of chromosome condensation, nucleolar dispersion, nuclear envelope breakdown and certain aspects of mitotic spindle formation. For example, downregulation of cdc2 kinase at the onset of mitosis by PP2A has been shown in a variety of eukaryotes excluding plants. It is known that several homologues of cdc2 kinase ($p34^{cdc2}$) exist in a variety of higher plants. Considering the activity profiles of the alfalfa kinases (cdc2MsA/B; MsD; MsF) defined patterns can be recognised that show a high activity of cdc2MsA/B in the G2 phase and an activity peak for cdc2MsF in mitotic cells. The special role of cdc2MsF kinase in mitosis was further emphasised by the localisation of this kinase in preprophase band (PPB), perinuclear ring in early prophase, mitotic spindle, and phragmoplast. Different aspects of the regulation of cell cycle kinases have been extensively analysed, although the role of PPases in the regulation of plant division-related kinases has not been fully uncovered,

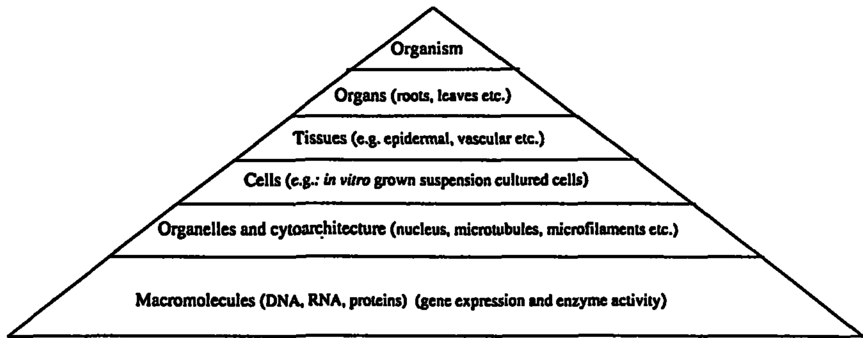
yet. Treatment of plant cells with phosphatase inhibitors caused severe cytological abnormalities, indicating the involvement of these enzymes in basic cellular functions

In the work presented in this thesis, we used a herbicide (Endothall: ET) to study the effects of PPase inhibition on mitotic regulation in cultured alfalfa cells. ET is a potent and specific inhibitor of PP2A and PP1. It is particularly useful for intact plant cell studies, since it is water soluble and can be taken up both by suspension culture cells and by different tissues of intact plants, whereas other phosphatase inhibitors do not appear to penetrate all types of cells. We have analysed the concentration-dependent effects of ET on growth parameters of whole plants and of suspension cultured cells and then we have analysed the concentration-dependent effects on chromosome condensation and the effect of low concentrations of ET at G2/M border from the point of cytoskeleton (both microfilaments and microtubules) and nuclear entry into mitosis in relation to changes in the kinase activities of different alfalfa $p34^{cdc2}$-related kinases. Finally, immunolocalisation patterns of these kinases were determined and interrelation between phosphatases and kinases during cell division was discussed.

Aims of Studies

The main aim of our studies was to uncover the role of serine/threonine phosphatases and their interaction with division-related kinases during plant cell division. The experimental approaches were based on the use of a cell permeable inhibitor endothall (ET) to find out the contribution of phosphatases. *Medicago sativa* was chosen as the model system due to availability of specific phosphatase and kinase antibodies and experiences gathered in our laboratory during the last 15 years of research on this model system.

The objectives of the study included through analysis of these regulatory elements by starting from the "organism" level, scaling down to "macromolecular" level according to the pyramid of levels of organization of living things (see figure below).



Analysis of the inhibitory potency of the phosphatase blocker endothall on alfalfa proteins was aimed at the very beginning of the experiments and it is analysed both at *in vitro* and at *in vivo* levels.

Under the light of above-mentioned top-down approach, aims of the studies, in details, were as follows:

-To analyze concentration-dependent effects of ET on root, shoot and leaf morphology and development of *in vitro* explants (*at organism/organs/tissue level*).

-To characterize concentration-dependent effects of ET on the growth of suspension cultured cells (*at the cellular level*).

-To investigate the dynamics of nuclear cycle (chromosome condensation/decondensation events, nuclear membrane and nucleolar dynamics) during cell division under the effect of phosphatase inhibition (*at subcellular/organellar* level).

-To study the relationship between phosphatases and the cytoskeletal organization (microfilaments and microtubules) during division (*at subcellular/organellar level*).

-To determine the consequences of phosphatase inhibition on division-related genes' expression patterns and on enzyme activities of division-related kinases.

The analysis of the division-related kinases included determination of their immunolocalization patterns, as well (*at macromolecular level*).

Materials and Methods

1) Plant material and ET treatment

Cell suspension culture of *Medicago sativa*, was maintained in Murashige-Skoog liquid medium supplemented with 0.2 mg/l kinetin and 1 mg/l 2,4-D. The culture was diluted 1:5 in fresh medium, weekly. For analysing the effect of endothall (ET) on growth, increasing concentrations of the inhibitor dissolved in DMSO were added into subculturing medium. Packed cell volumes were measured daily by settling 2 ml of culture in 2ml glass pipettes. Experiments on alfalfa plants were done by culturing the explants on NM medium-soaked tissue papers in thin glass tubes. 0, 1, 10 50μM ET were added into medium and explants were cultured for 1.5 weeks.

2) Synchronization of the culture and endothall treatment

The highest synchronization efficiencies were obtained according to the following optimised procedure: Synchronization started with 1:4 dilution of a 7-d old suspension culture. After 12h, 10mM hydroxyurea was added to the medium. Incubation continued for 36 h and then the drug was removed by washing the cells three times with fresh medium (3x20 min) before resuspending the culture in the original volume using three times diluted conditioned medium obtained from a 5-day-old culture. Endothall is then added at the 6th or 8th h of synchronous growth according to how quickly majority of the cells passed the S-phase. Samples were collected at various time intervals for mitotic index determination, flow cytometry, kinase assay and microtubule immunolocalisation.

3) Flow cytometry

The best results with the lowest coefficient of variances (CV) were obtained with the following optimised conditions: 100μl (packed cell volume) of cells were digested in 2ml of the *enzyme mixture* [4% cellulase Y-C or RS, 2% pectinase, 1% driselase and 1% macerozyme R-10 in a buffer containing 3mM MES, 0.6mM Na_2HPO_4, 6.8mM $CaCl_2$, 0.35M mannitol and 0.35M sorbitol, pH 5.6]. Partial digestion of the cell walls was done on a rotary shaker using 4ml tubes during a period of 1h at 25°C. The enzyme was then removed by replacing with fresh culture medium (MS) without addition of any osmoprotector like sorbitol. Swollen cells were then burst by gently pipetting in *nucleus isolation buffer* (45mM $MgCl_2$, 30mM sodium citrate, 20mM MOPS, pH 7.0 and 0.1% Triton-X-100 as detergent) and were fixed with a 2% formaldehyde. Samples were filtered from 25μm metal mesh and stained with 1μg/ml ethidium bromide or propidium iodide and analysed with Becton Dickinson flow activated cell sorter with built-in CellQuest and ModFit software.

4) Labelling of actin filaments

Since fine actin filaments are sensitive to aldehyde fixation, we have used a modified method which excludes chemical fixation. A dense drop of cells was incubated 5min in actin stabilizer buffer which was composed of 50mM PIPES pH 6.9, 2.5mM $MgSO_4$, 5mM EGTA, 0.02% Nonidet P40, 5% DMSO, 300μM MBHE (3-maleimidobenzoic acid N-hydroxysuccinamide ester). Cells were then resuspended in 0.2μM Alexa (fluorochrome)-conjugated Phalloidin and 100ng/ml DAPI (4', 6-diamidino-2phenylindole HCl) in actin stabilizer buffer. They were immediately loaded onto slides and covered with coverslips and were mounted with nail varnish to prevent drying.

5) **Immunocytology and determination of mitotic index**

A dense drop of cells were fixed 1h at 23°C in *MTSB* (50mM PIPES pH 6.9, 5mM $MgSO_4$, 5mM EGTA), washed with the buffer and digested 20 min with 2ml of *enzyme mixture* (1% w/v Cellulase Onozuka R-10, 1% v/v Pectinase in MTSB. Following washing with MTSB, cells were attached to Poly-L-Lysine- or Vectabond-coated slides and were extracted 20 min with 0.5% Triton-X-100. Rat anti-tubulin antibody or alfalfa kinase antibodies were applied as 1/200 dilution in MTSB for 15h at 4°C. FITC-conjugated secondary antibodies were used for 1h at 23°C with 1/200 dilution after washing of the primary antibody. Before last wash of secondary antibody, nuclei were stained with DAPI (100ng/ml) and cells were mounted with Citifluor. For mitotic index determination cells were fixed 15h at 4°C with 4% formalin in 0.01M phosphate buffered saline (pH 7.2). Nuclear material was stained with DAPI (100ng/ml) and mounted as before.

6) **Confocal laser scanning and immunofluorescence microscopy**

Cytological analyses were done by using a Zeiss Axiovert 135 M fluorescence microscope (equipped with laser scanning and detection facilities). Pinhole of the laser-scanning microscope was set to 100 and scanning durations of 4 or 8sec with 2 times averaging were used for most of the observations. When fluorescence intensities were not very strong or when the appropriate laser wavelength was not available for the dye of interest, CCD camera connected to the microscope was used to obtain the image of the stained or labelled cell/tissue. To reach the maximum possible magnification and resolution, majority of the observations has been captured using oil-immersion Plan NeoFluar 100x /1.30 objective. Saved pictures were coloured artificially either by combining red, green and blue channels.

7) Phosphatase assay of PP1 and PP2A

Three-day-old cultures were treated for 3h with 0, 0.3, 1, 3, 10, 30 and 100μM ET, respectively. Proteins were extracted on ice by grinding the cells with a glass rod in sampling tubes with quartz sand. The *homogenisation buffer* was 50mM Tris-HCl pH 7.5, 1mM EDTA, 1mM EGTA, 0.1% β-mercaptoethanol, 5mM benzamidine, 0.2mM PMSF, 4μg/ml leupeptin. After centrifugation supernatants were diluted in *Buffer A* (50mM Tris-HCl pH7.5, 0.1mM EDTA, 0.1% β-mercaptoethanol) supplemented with 1% (w/v) bovine serum albumin. 10μl of the diluted sample was mixed with 10μl Buffer A, containing 0.03% Brij35 and with the addition of either 10U/μl inhibitor 2 (for PP2A assay) or 3nM okadaic acid (for PP1 assay) or without any additive (total phosphatase assay). Following incubation of the samples 15 min at 30°C, 10μl of freshly prepared *substrate cocktail* (3mg/ml ^{32}P-phosphorylase *a*, 5mM caffeine in Buffer A) was added for a duration of 10sec and the reaction was stopped using 10% w/v trichloroacetic acid (TCA) and the precipitate was pelleted by centrifugation and the radioactivity was counted by Cserenkov radiation upon dilution of the supernatant in 0.25M NaOH. Phosphatase assay using immunoprecipitation was essentially the same except antibodies of anti-PP1 and anti-PP2A were used to separate the two types of proteins.

9) Kinase assay of alfalfa division-related CDKs

Proteins were extracted by grinding samples with quartz sand in *extraction buffer* (25mM Tris-HCl, pH 7.5, 75mM NaCl, 15mM EGTA, 15mM *p*-nitrophenyl phosphate, 60mM ß-glycerophosphate, 1mM DTT, 0.1% Nonidet P-40, 0.2 mM Na_3VO_4, 0.5mM NaF, 1mM PMSF, leupeptin/aprotinin/antipain (10μg/ml, each), 5μg/ml both pepstatin and chymostatin) 100μg of total protein in extraction buffer from each sample was incubated with 100μl of 25% (v/v) p13^{suc1}-Sepharose beads overnight at 4°C on a rotary shaker. After incubation, beads were washed three times with RIPA buffer and once with *kinase assay buffer* (50mM Tris-

HCl, pH7.5, 15mM $MgCl_2$, 5mM EGTA, 1mM DTT). Kinase reaction mixture consists of 1mg/ml histone H1 in kinase assay buffer and 10μCi of γ-^{32}P-ATP in 30μl of total volume. Kinase reaction was started by the addition of 30μl of reaction mixture to the washed $p13^{suc1}$-Sepharose beads and stopped after 15min at 28°C with the addition of 7.5μl 5x SDS sample buffer. The samples were analysed by 10% SDS-PAGE and autoradiography.

For immunoprecipitation, equal amounts of protein samples in extraction buffer were incubated at 4°C for 1h with 5-10μg/ml anti-C-terminal alfalfa antibodies. Then, 50μl of 50% (v/v) suspension of ProteinA-Sepharose beads was added and shaken gently for 1h at 4°C. Thereafter the beads were washed three times with RIPA buffer and once with kinase assay buffer. Histone H1 kinase reaction was carried out as described above.

10) RNA preparation and gel blot analysis

Frozen alfalfa cultured cells were homogenized under liquid nitrogen in a small mortar with 0.5 ml of phenol (equilibrated to pH 4.9 with 3M potassium acetate). After diluting with 1% SDS, the samples were incubated for 15 min at 65°C and then centrifuged for 10 min in a centrifuge at 4°C. The supernatant was extracted with phenol-chloroform twice and precipitated with one-quarter volume of 8M LiCl during an overnight incubation at 4°C. The precipitates were dissolved in 100μl of diethyl pyrocarbonate-treated water, and a second LiCl precipitation was applied to remove residual DNA contamination. Total RNA was quantified by optical density at 260nm and the filters were examined under UV light to verify the efficiency of transfer and to test the quality and quantity of loaded RNA samples. Hybridization of the filters was performed in Rapid-hyb buffer at 65°C. Radiolabelled probes were generated by random-primed ^{32}P-labelling from fragments containing the coding, 3'-end gene specific region of cdc2MsF and Medsa;Cyc2A genes and from fragments containing the 3'-nontranslated regions of histone H3-1.

Results

Endothall (ET) as a cell permeable inhibitor of serine/threonine (ser/thr) protein phosphatases was used in the experiments. Using alfalfa cultured cells as the primary model system, different concentrations of the inhibitor were tested both *in vitro* and *in vivo* from the point of phosphatase inhibition. The effects of inhibitory concentrations of ET were analysed macroscopically by observing the growth behaviour of plant cultures and microscopically by analysing the subcellular dynamics of chromosomes, microtubules and microfilaments. Interrelation between phosphatases and kinases was investigated by screening the division-related genes' expressions and kinase activities whose immunolocalisation patterns have also been analyzed.

1) Endothall is a concentration dependent inhibitor of alfalfa protein phosphatases 1 and 2A

Effect of ET on protein phosphatase activity was analysed both *in vitro* and *in vivo*. The activities of PP1 and PP2A were assayed specifically. We found that increasing concentrations of ET resulted in a dose-dependent decrease of alfalfa PP1 and PP2A activities.

2) Endothall blocks the growth of *in vitro* plant cultures

Different concentrations of ET were tested on both alfalfa and tobacco explants and on alfalfa suspension cultured cells. Plantlets displayed reduced root and leaf growth, and the growth of alfalfa suspension cells was blocked in a dose-dependent manner.

3) Endothall causes abnormal chromosome condensation during early mitosis

Effects of low and high concentrations of ET on chromosome condensation were analysed. High concentrations (10, 50μM) led to hypercondensed chromosomes. Low concentration

(1μM), however, resulted in retardation in mitotic entry and accumulation of cells before exit from mitosis while exhibiting normal chromosome formation.

4) Effect of low concentration of endothall on microfilaments

Microfilament dynamics were analysed upon application of 1μM ET on nonfixed cells of alfalfa suspension cultures. Treatment did not affect normal interphase cortical actin skeleton but resulted in abnormal phragmoplast maturation and abolishment of cell plate formation with a resultant binucleate cells after karyokinesis.

5) Accelerated microtubule restructuring in mitotic cells exposed to low dose of endothall

1μM ET was applied at early G2 phase and microtubular dynamics were analysed using immunolabelling methods. ET-treatment resulted in abolishment of preprophase band formation, increase in the number of nuclei with prophase cage, premature polarization of the spindle and abnormal phragmoplast maturation.

6) Regulation of cell division-related kinase complexes and serine/threonine phosphatases

The activity of immunoprecipitated kinase complexes and mRNA expression pattern of selected division-related genes were screened in ET-treated cells. Results revealed the importance of phosphatases on the transcriptional and post-translational regulation of division-related kinases.

7) Immunolocalisation of alfalfa cell division-related kinases

The immunolocalisation patterns of alfalfa division-related kinases were determined by indirect immunolocalisation. Cdc2MsF kinase was localized on preprophase band, spindle and

phragmoplast. Cdc2MsD displayed cytoplasmic and nucleolar signal, whereas cdc2MsA/B was found mainly in nucleoli and partly in nucleoplasm and cytoplasm.

Conclusions

Paul Green, the eminent plant cell biologist, once remarked that there is only one question in biology: "How do they do that?" Answering this question in any field of biology requires a combined effort that makes use of the advantage of different tools and experimental techniques in addition to a systematic experimental approach.

In our case the challenging question was (and still is) that "How do they divide?" Within the context of this question, our specific aim was to understand the role and importance of phosphorylation/dephosphorylation reactions during plant cell division. We have tried to use a range of biochemical and cytological tools and experimental methods, as well as a systematic approach (see Aims of Studies) to unravel, at least in part, the mystery of plant cell division. The outline of conclusions after discussing our results can be summarized as follows:

1) For proper development of alfalfa roots and leaves and for growing of suspension cultured cells normal functioning of serine/threonine phosphatases PP1 and PP2A is essential.

2) Chromosome condensation events during early mitosis involve both PP1 and PP2A functions.

3) Normal arrangement of microtubules and microfilaments of dividing cells requires PP2A activity.

4) PP1 and PP2A may affect the transcriptional regulation of cell division-related kinases and cyclins.

5) PP2A has a post-translational regulatory role on the activities of cdc2MsF and cdc2MsD kinases but not on cdc2MsA/B kinases.

6) Subcellular immunolocalisation patterns of alfalfa division-related kinases differ significantly. This finding suggests that they may have unique roles during alfalfa cell division.

7) Immunolocalisation results of cdc2MsF kinase that localizes on division-related cytoskeleton structures and whose activity is being regulated by PP2A suggest a functional link between regulation of cytoskeleton dynamics and cdc2MsF / PP2A activity during cell division.

Although our conclusions are far from solving the complex puzzle of plant cell division, we believe that our results stimulate further experiments to highlight the possible role of individual protein phosphatases and kinases and their interacting partners in the control of cell division cycle of higher plants.

List of Publications

► Presented thesis is based on the following articles:

1) Inhibition of serine/threonine-specific protein phosphatases causes premature activation of cdc2MsF kinase at G2/M transition and early mitotic microtubule organisation in alfalfa.
F.Ayaydin, E.Vissi, T.Mészáros, P.Miskolczi, I.Kovács, A.Fehér, V.Dombrádi, P.Gergely, D.Dudits
The Plant Journal (2000), 23, 85-96

2) Multiple Cyclin-dependent kinase complexes and phosphatases control G2/M progression in alfalfa cells.
T.Mészáros, P.Miskolczi, *F.Ayaydin*, A.Pettko-Szandtner, A.Peres, Z.Magyar, V.G.Horváth, L.Bakó, A.Fehér, D.Dudits
Plant Molecular Biology (2000), 43, 595-605.

3) The Protein Phosphatases and Their Functions in Plants
E.Vissi, É.C.Tóth, *F.Ayaydin*, E.Kókai, P.Gergely, D.Dudits, V.Dombrádi
Proc. NATO/FEBS on Protein modules in cellular signaling (Accepted)

► Optimisation of the majority of the experimental procedures presented in "Materials and Methods" of the thesis has been achieved during the following work: (Contribution of each article is indicated in parentheses)

4) Immunolocalisation of Novel Annexin Encoded by a Stress and Abscisic Acid Responsive Gene in Alfalfa
I.Kovács, *F.Ayaydin*, A.Oberschall, I.Ipacs, S.Bottka, S.Pondor, D.Dudits and É.C.Tóth
The Plant Journal (1998), 15, 185-197. (Immunolocalisation of proteins on alfalfa suspension cultures)

5) Partial Synchronization of Cell Division in Cultured Maize (*Zea mays L.*) Cells: Differential Cyclin, Cdc2, Histone and Retinoblastoma Transcript Accumulation During the Cell Cycle
A.Peres, *F.Ayaydin*, K.Nikovics, C.Gutierrez, G.V.Horváth, D.Dudits and A.Fehér
Journal of Experimental Botany (1999), 50, 1373-1379. (Synchronisation of cultured cells)

6) Nuclear Localization of a Hypoxia-inducible Novel Non-Symbiotic Hemoglobin in Cultured Alfalfa Cells
C.Seregélyes, L. Mustardy, *F.Ayaydin*, L.Sass L.Kovács, G.Endre, N. Lukács, I.Kovács, I.Vass, G.B.Kiss, G.V.Horváth and D.Dudits
FEBS Letters (2000), 482, 125-130. (Synchronisation of alfalfa cells, mitotic index determination and flow cytometry)

7) Exogenous Auxin and Cytokinin Dependent Activation of CDKs and Cell Division in Leaf Protoplast-Derived Cells of Alfalfa
T.Pasternak, P.Miskolczi, *F.Ayaydin*, T.Mészáros, D.Dudits and A.Fehér
Plant Growth Regulation (Accepted) (Characterization of nuclear morphology)

8) Cell Cycle Phase-Specific Activation of Maize Streak Virus Promoters
K.Nikovics, J.Simidjieva, A.Peres, *F.Ayaydin*, T.Pasternak, M. Boulton, J.Davies, D.Dudits and G.V.Horváth
Molecular Plant-Microbe Interactions (Accepted) (Immunohistochemistry, synchronisation and Confocal laser scanning microscopy)