

RESEARCH PAPER

CK2 phosphorylation weakens 90 kDa MFP1 association to the nuclear matrix in *Allium cepa*

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Abstract

MFP1 is a conserved plant coiled-coil protein located on the stroma side of the chloroplast thylakoids, as well as in the nuclear matrix. It displays species-specific variability in the number of genes, proteins, and expression. *Allium cepa* has two nuclear proteins antigenically related to MFP1 with different M_r , pI , distribution, and expression, but only the 90 kDa MFP1 protein is a nuclear matrix component that associates with both the nucleoskeletal filaments and a new category of nuclear bodies. The 90 kDa AcMFP1 migrates in two-dimensional blots as two sets of spots. The hypo-phosphorylated forms ($pI \sim 9.5$) are tightly bound to the nuclear matrix, while high ionic strength buffers release the more acidic hyper-phosphorylated ones ($pI \sim 8.5$), suggesting that the protein is post-translationally modified, and that these modifications control its attachment to the nuclear matrix. Dephosphorylation by exogenous alkaline phosphatase and phosphorylation by exogenous CK2, as well as specific inhibition and stimulation of endogenous CK2 with heparin and spermine and spermidine, respectively, revealed that the protein is an *in vitro* and *in vivo* substrate of this enzyme, and that CK2 phosphorylation weakens the strength of its binding to the nuclear matrix. In synchronized cells, the nuclear 90 kDa AcMFP1 phosphorylation levels vary during the cell cycle with a moderate peak in G₂. These results provide the first evidence for AcMFP1 *in vivo* phosphorylation, and open up further research on its nuclear functions.

Key words: *Allium cepa*, cell cycle, CK2 phosphorylation, MFP1, nuclear matrix.

Introduction

The nuclear matrix (NM) is the filamentous network that provides the scaffold for dynamic nuclear organization and function (Wasser and Chia, 2000; Shumaker *et al.*, 2003; Bettinger *et al.*, 2004; Kiseleva *et al.*, 2004; Nalepa and Harper, 2004). In plants, it comprises a peripheral layer with associated pore complexes, an internal network of branched filaments, a dense nucleolar skeleton, and nuclear bodies (Moreno Díaz de la Espina, 1995; Yu and Moreno Díaz de la Espina, 1999). Biochemically, the NM is defined as the residual insoluble fraction after removal of chromatin and soluble proteins from the nucleus. Its protein composition is complex and depends not only on the physiological conditions, cell cycle phase, or differentiation state, but also on the isolation procedure (Moreno Díaz de la Espina, 1995; Calikowski *et al.*, 2003). The multiple proteins display specific nuclear distribution (Moreno Díaz de la Espina, 1995; Gindullis and Meier, 1999; Harder *et al.*, 2000; Moreno Díaz de la Espina *et al.*, 2003). Some of them are plant-specific coiled-coil proteins such as NMCP1 and MFP1 (Meier *et al.*, 1996; Masuda *et al.*, 1997), structural elements such as the functional homologues of NuMA and lamins (Li and Roux, 1992; McNulty and Saunders, 1992; Mínguez and Moreno Díaz de la Espina, 1993; Yu and Moreno Díaz de la Espina, 1999; Blumenthal *et al.*, 2004), MAR-binding proteins (Meier *et al.*, 1996; Fujimoto *et al.*,

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2004), transcription factors, components of nuclear complexes, and phosphatases (Nalepa and Harper, 2004).

MFP1 is a protein with dual localization in the NM and in the chloroplast (Gindullis and Meier, 1999; Jeong *et al.*, 2003; Samaniego *et al.*, 2006). Orthologues of MFP1 are present in several plant species, but its functionality is not yet understood (Harder *et al.*, 2000; Jeong *et al.*, 2003, 2004). The sequenced proteins from tomato, *Arabidopsis*, and tobacco contain an N terminus with two conserved hydrophobic domains (which are part of a chloroplast signal peptide), a central coiled-coil rod domain, a C-terminal DNA binding domain, and many CK2 phosphorylation motifs. Although the homology amongst species is not high, some domains are highly conserved (~90%), showing their relevance on the protein functionality, as are two of the consensus sequences for CK2 signalling (Meier *et al.*, 1996; Harder *et al.*, 2000). Recently it has also been reported that MFP1 is associated with nucleoids in *Arabidopsis* chloroplasts, suggesting a function for MFP1 at the interface between nucleoids and the thylakoid membranes (Jeong *et al.*, 2003, 2004).

Sera against AtMFP1 detected an 80 kDa protein in onion with similar M_r and pI values to MFP1 (R Samaniego, C de la Torre, S Moreno Díaz de la Espina, unpublished results). In addition, the anti-LeMFP1 serum detected an immunologically related 90 kDa nuclear protein, associated to the nucleoskeletal network of filaments that was highly enriched in a new category of nuclear bodies (Samaniego and Moreno Díaz de la Espina, 2000; Samaniego *et al.*, 2001). The 90 kDa AcMFP1 migrates as an alignment of spots in two-dimensional (2-D) electrophoresis gels, suggesting the existence of post-translational modifications, most probably phosphorylation, although other alternatives could not be excluded. The 90 kDa AcMFP1 presents two different fractions according to their resistance to high salt extraction during the NM preparation (Samaniego *et al.*, 2001). All these data together suggest that the protein is post-translationally modified, most likely by phosphorylation.

Apart from its structural roles, the NM filament network is a platform for intranuclear protein–protein and protein–nucleic acids interactions (Ahmed, 1999; Fujimoto *et al.*, 2004) involved in the organization of replication, transcription/splicing, and remodelling complexes (Samaniego *et al.*, 2002). It also serves as tracks for intranuclear transport and nuclear signalling (Meier, 2001; Nalepa and Harper, 2004). Phosphorylation regulates the association of numerous proteins to the NM, such as coiled-coil proteins like NuMA (Saredi *et al.*, 1997), lamins (Hachet *et al.*, 2004; Takano *et al.*, 2004), and plant lamin-like proteins (Rose *et al.*, 2003; Blumenthal *et al.*, 2004), nucleolin (Gotzman *et al.*, 1997), chromatin remodelling proteins (Reyes *et al.*, 1997; Bérubé *et al.*, 2000), CK2 (Ahmed *et al.*, 2000), Cdc14B (Nalepa and Harper, 2004), and the retinoblastoma protein (Rb) (Mancini *et al.*, 1994; Claudio *et al.*, 2002).

Protein kinase CK2 is a highly conserved and ubiquitous protein serine/threonine kinase involved in gene expression, growth, and cell cycle progression in plants (Riera *et al.*, 2001a, b). CK2 is involved in signal transduction in the NM and phosphorylates different intrinsic nuclear protein substrates such as topoisomerase II and RNA polymerases (Zhang *et al.*, 1998; Ahmed, 1999; Yu *et al.*, 2001). The NM is also an important target for CK2 nuclear signalling (Wang *et al.*, 2003). At present only two substrates for CK2 have been reported in the plant NM: the pea functional homologues of lamins (Li and Roux, 1992; Blumenthal *et al.*, 2004) and tomato MFP1 (Meier *et al.*, 1996).

In this work, the nature of the association of the 90 kDa AcMFP1 to the NM and the role of CK2 phosphorylation on the process were investigated. The results demonstrate that the 90 kDa AcMFP1 is a phosphoprotein whose binding to the NM depends on its phosphorylation state. The solubility of the 90 kDa AcMFP1 is regulated by exogenous CK2 *in vitro* and can be modified by specific stimulation and inhibition of the endogenous CK2, suggesting that this protein kinase regulates the protein binding to the NM *in vivo*. In meristematic cells the *in vivo* hyperphosphorylation of the protein occurs mainly at the G₂ phase of the cell cycle.

Materials and methods

Isolation of nuclei

Allium cepa L. var. francesa bulbs were used to isolate nuclei. Bulbs were grown in filtered tap water at room temperature for at least 2 d, and root meristems were collected and homogenized with a high-speed Ultra-Turrax (8000 g). Nuclear isolation was performed using 30 µm pore filters and low-speed centrifugation as previously described (Moreno Díaz de la Espina, 1995).

Nuclear matrix extraction with high-salt buffers

This was performed as described by Yu and Moreno Díaz de la Espina (1999). Briefly, fresh isolated nuclei were incubated in cytoskeleton buffer (CSK; 10 mM PIPES pH 6.8, 100 mM KCl, 300 mM sucrose, 3 mM MgCl₂, 20 mM DTT, 1 mM EGTA, 1.2 mM PMSF, 2 mg ml⁻¹ aprotinin) containing 0.5% Triton X-100. After 5 min incubation, nuclear-soluble and membrane-associated proteins were removed by centrifugation at 1400 g for 10 min and collected in the supernatant (S1). The pelleted structures containing the nuclear insoluble proteins (F1) were digested with 200 µg ml⁻¹ RNase-free DNase I in digestion buffer (DB; 10 mM PIPES pH 6.8, 50 mM KCl, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 20 mM DTT, 1 mM EGTA, 1.2 mM PMSF, 2 mg ml⁻¹ aprotinin, 0.5% Triton X-100) for 30 min at room temperature. Then, 1 M in order ammonium sulphate was added drop-wise to a final concentration of 0.25 M in order to remove the DNA and its associated proteins (S2). Pellets (F2) containing NM with loosely bound proteins were further extracted with 4 M NaCl to a final concentration of 2 M. This extraction released the outer proteins (S3) and revealed the NM. Except when mentioned otherwise, all steps were performed at 4 °C.

Nuclear matrix extraction by the LIS method

This was performed as previously described (Moreno Díaz de la Espina, 1995) as follows. Purified nuclei were incubated in 100 µg ml⁻¹ DNase I diluted in DB for 30 min at room temperature,

then extracted in 25 mM LIS (3,5-diiodosalicylic acid lithium salt) buffer (5 mM HEPES-NaOH pH 7.4, 0.25 mM spermidine, 2 mM EDTA-KOH pH 7.4, 2 mM KCl, 1.2 mM PMSF, 2 mg ml⁻¹ aprotinin) for 15 min in ice. A second incubation was achieved in 200 µg ml⁻¹ DNase I dissolved in DB.

Protein analysis

For one-dimensional (1-D) analysis, proteins from each fraction and its corresponding supernatants after 10% trichloroacetic acid precipitation were dissolved in 2× sample buffer (0.125 M TRIS-HCl pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.002% bromophenol blue). SDS-PAGE was carried out in 10% acrylamide gels as described previously (Yu and Moreno Díaz de la Espina, 1999).

For two-dimensional (2-D) analysis, samples were washed in a non-salt buffer (distilled deionized water, 0.5% Triton X-100, 20 mM DTT, 1 mM PMSF, 2 mg ml⁻¹ aprotinin), dissolved in a low volume of SDS buffer (<2% SDS, 0.125 M TRIS-HCl pH 6.8, 10% 2-mercaptoethanol, 20% glycerol, 0.002% bromophenol blue), sonicated at 22.5 kHz for 5 s and boiled for 5 min. Samples were then diluted in 2-D buffer (9.8 M urea, 2% pharmalytes pH 3–10, 5% Triton X-100, fresh 100 mM DTT) until the final ratio of SDS:Triton X-100 was 1:8, and the final SDS concentration was lower than 0.25%.

2-D SDS-PAGE was performed as previously described (Mínguez and Moreno Díaz de la Espina, 1993) using pharmalytes pH 3–10. Isoelectric focusing (IEF) immobilized dry strips of 7 cm length and pH 3–10 (BioRad) were rehydrated, and samples were loaded on the strips and run at 300 V for 3 h/up to 1400 V in 3.5 h/1400 V for 13 h/2000 V for 2 h. Afterwards, the strips were equilibrated in SDS buffer and run in 10% acrylamide gels using a vertical BioRad mini-protean system. pI markers (Pharmacia) were used to confirm the establishment of the pH 3–10 gradients during the IEF.

Immunoblotting and quantification of band densities

After electrophoresis, proteins were transferred to nitrocellulose membranes in 50 mM TRIS pH 8.2, 200 mM glycine, 20% methanol, 0.1% SDS, at 100 V for 1 h at 4 °C. Protein band positions were determined by staining with Ponceau red. After being washed in 0.05% Tween-20 in PBS and blocked in 5% non-fat milk, the membranes were incubated from 2 h to overnight with anti-MFP1288 antiserum at 1:300–1000 dilution and a peroxidase-coupled anti-rabbit secondary antibody (Amersham) at 1:15 000 dilution. The 288 antiserum was raised against amino acids 236–439 of LeMFP1 (Meier *et al.*, 1996). This region lies in the central coiled-coil domain of MFP1, which shows about 40% amino acid identity between species (Harder *et al.*, 2000). The reaction was revealed by the ECL system from Amersham. Determination of M_r values for the reactive bands and spots was done with a Molecular Dynamics ImageQuant Densitometer system.

For heparin and alkaline phosphatase (AP) comparative experiments, total protein concentration of the different samples was estimated by Bradford's method and confirmed by Ponceau red staining. In every line, 8 µg of total protein was loaded. In both cases, determination of the pI and density quantification for reacting bands were calculated in densitometrically scanned films from at least three different experiments, using the *Quantity One* quantification software (Bio Rad).

Alkaline phosphatase dephosphorylation

F2 fractions obtained as previously described were suspended in 100 µl DB buffer containing 1 M diethylamine pH 9.9 and increasing concentrations of 4, 18, 36, and 54 U of calf intestine AP (Roche, Mannheim, Germany), and incubated for 1 h at 35 °C. Buffers without the enzyme or containing boiled enzyme were used as controls. After the incubations, samples were suspended in cold DB buffer and 4 M NaCl was smoothly added to a final 2 M

concentration. After 15 min incubation in ice, samples were centrifuged at 1400 g for 15 min, and NM and S3 fractions collected for densitometrical analysis.

Exogenous CK2 phosphorylation

Extracted NM fractions were incubated for 1 h at 30 °C with 500 U of CK2 (New England, Biolabs) in CK2-buffer (20 mM TRIS-HCl pH 7.5, 50 mM KCl, 10 mM MgCl₂, 200 µM ATP or [γ -³²P]ATP), or without CK2 as the control reaction. Samples were then extracted again with 2 M NaCl, and the corresponding supernatant (S4) and pellet (NM2) collected for densitometrical analysis. Films were exposed for 5 min for immunoblot, and 24–48 h for ³²P autoradiography.

Endogenous CK2-specific inhibition and stimulation

For CK2 inhibition, fresh isolated nuclei were incubated with 100, 200, 300, and 400 nM heparin concentrations diluted in the reaction mixture (50 mM HEPES, 150 mM NaCl, 10 mM MgCl₂, 1 mM PMSF, 2 mg ml⁻¹ aprotinin), for 1 h at 35 °C. No heparin was added to the control reaction mixture. Then NM extraction was performed as previously described, except for the DNase treatment step, where buffers were supplemented with heparin. S3 supernatants were collected for further densitometrical analysis.

For CK2 stimulation, fresh isolated nuclei were incubated in CSK buffer containing 1 mM spermine and 2 mM spermidine for 5, 10, 15, and 20 min at room temperature. Control nuclear samples were incubated for 20 min in CSK buffer. Then NM extraction was performed as previously described, and S3 supernatants collected for further densitometrical analysis.

Statistical analysis

Alkaline phosphatase and endogenous CK2 inhibition and stimulation assays are based on quantifiable modifications in the area (mm²) and optical density (a.u.) of densitometrically scanned bands from immunoblots. Original data from at least three independent experiments were used in all cases to assess the effect of phosphorylation on protein extractability by 2 M NaCl. A block design was used to filter out the variability amongst experiments. Data were analysed by ANOVA followed by a Newman–Keuls multiple range test for separation of significantly different means. The level of significance was 0.05. Results in Figs 1E and 3B are presented in percentages of control data (see Results).

Immunofluorescence

Isolated nuclear and NM fractions were fixed in 2% paraformaldehyde in PBS buffer (pH 7.4) containing 0.5% Triton X-100 for 30 min, centrifuged at 690 g for 10 min and washed in the same buffer 1× 30 min. Pellets were suspended in 20 mM glycine for quenching aldehyde groups and blocked with 2% BSA in PBS with 0.05% Tween-20 for 30 min. Anti-MFP1 serum 288 was added to the blocking buffer to a final concentration of 1:30–50, incubated for 1 h at room temperature, and washed 2× 30 min in PBS with 0.05% Tween-20. Pellets were incubated with A488-coupled anti-rabbit secondary antibody (Molecular Probes) for 45 min at room temperature and washed 2× 30 min. Except when mentioned, all steps were performed at 4 °C and with constant shaking. The labelled fractions were finally layered on multi-well slides, air dried, stained with 1 µg ml⁻¹ DAPI (4',6' diamidino-2-phenylindole) to check the DNA extraction, and mounted with Vectashield. Samples were examined in a confocal microscope (Leica TCS-SP2-AOBS), using the Leica confocal software and Adobe Photoshop to process the images.

Electron microscopy

Root segments were processed and embedded in LR White resin as described by Moreno Díaz de la Espina (1995). Post-embedding

immunogold labelling was performed using an anti-MFP1 288 anti-serum at 1:30 dilution and 10 nm gold-conjugated anti-rabbit secondary antibody (Sigma) (1:50). Pre-embedding labelling of NMs and preparation of resinless nucleoskeletons was performed as in Yu and Moreno Diaz de la Espina (1999). Samples were contrasted with uranyl acetate and examined in a Jeol 1230 electron microscope at 80 kV.

Cell synchronization and BrdU incorporation

For cell cycle analysis, onion bulbs cultured in the dark at 25 °C in continuous aeration, by bubbling air at 10–20 ml ml⁻¹, were submitted to a 14 h continuous treatment with 0.75 mM hydroxyurea (HU) (Sigma) freshly prepared at 25 °C, followed by chase periods of 2, 5, and 7 h in water. Samples treated for 14 h with 0.75 mM HU were used for very early S analysis. Cell synchronization was controlled by flow cytometry and metabolic labelling of roots with 10⁻⁴ M BrdU (Sigma) followed by immunodetection with anti-BrdU antibody (Becton Dickinson), as described in Samaniego *et al.* (2002).

Meristematic root segments synchronized at different cell cycle positions were fixed for 20 min at 4 °C in 4% (v/v) paraformaldehyde in TRIS buffer (10 mM TRIS pH 7.5, 10 mM EDTA, 100 mM NaCl) containing 0.1% Triton X-100. After fixation, samples were washed 3× with TRIS buffer and homogenized in lysis buffer (15 mM TRIS pH 7.5, 2 mM EDTA, 80 mM KCl, 20 mM NaCl, 0.1% Triton X-100) with an Ultra-Turrax T20 (25N-8G, IKA). Homogenates were filtered through a 30 µm nylon-mesh. The nuclear suspensions were then centrifuged at 1000 g for 15 min at 4 °C, and the pellets suspended in lysis buffer until analysis. For estimation of the DNA content by flow cytometry, isolated nuclei were incubated with RNase (Boehringer-Mannheim) at 30 µg ml⁻¹ for 30 min. Nuclei were then stained with 100 µg ml⁻¹ propidium iodide (Sigma) for at least 5 min at room temperature. Flow-cytometry analysis was performed with an EPICS XL analyser (Coulter) equipped with an argon laser tuned at 488 nm, and fluorescent signals from propidium iodide-labelled nuclei collected by a 620 nm band-pass filter.

Results

The 90 kDa AcMFP1 protein is a nuclear phosphoprotein associated with the intranuclear network and highly enriched in nuclear bodies

Immunofluorescence and high resolution immuno EM with anti-LeMFP1 serum 288 in *Allium cepa* proliferating cells showed that the 90 kDa AcMFP1 is a nuclear protein that decorates the intranuclear filament network that links the nucleolus with the nuclear envelope, and is highly enriched in a type of nuclear body (Fig. 1A, B). When DNA-digested nuclei were submitted to sequential extraction to elute soluble proteins and digested chromatin, a fraction of the protein was removed from the nuclei using high ionic strength (2 M NaCl) buffers, while a second one resisted the extraction and remained tightly bound to the NM as demonstrated by immunoblotting (Fig. 1C) and immunofluorescence and immunogold stainings with the same serum (Fig. 1A, B; Samaniego *et al.*, 2001).

Dephosphorylation affects the 90 kDa AcMFP1 protein association to the NM

The 90 kDa AcMFP1 displayed up to 11 different phosphorylation states in 2-D western blots (Fig. 4, Samaniego

et al., 2001). To investigate the effects of phosphorylation in the binding of the protein to the NM, isolated chromatin-depleted nuclei were incubated with increasing concentrations of AP, and the soluble fraction of the protein was removed by NaCl.

The present results showed that the levels of the 90 kDa AcMFP1 bound to the NM were progressively increased after AP incubation. The higher the AP concentrations used, the higher the levels of 90 kDa MFP1 were recovered in the NM fraction, reaching a maximum of 160% compared with the control (100%) after incubation with 54 U AP (Fig. 1D, E). Consequently, the solubilized 90 kDa AcMFP1 collected in the S3 supernatant progressively decreased to reach a minimum value of 70% in relation to controls (Fig. 1D, E). Both results suggest that the hypophosphorylated 90 kDa MFP1 protein fraction remained tightly bound to the NM, while the association of the phosphorylated form is weaker, allowing for this form to be partially removed by high ionic strength. H1 histone levels, quantified as loading control, showed no significant variations amongst the different conditions (Fig. 1E).

Exogenous CK2 modifies the solubility of the 90 kDa AcMFP1 protein

To investigate whether CK2 phosphorylation of the 90 kDa MFP1 modifies its association with the NM, an attempt was made to elute the matrix-bound protein from nuclear matrices by exogenous CK2 phosphorylation and a later high-salt buffer treatment (Fig. 2A). After CK2 incubation, several protein bands were solubilized by 2 M NaCl extraction, by contrast to untreated controls (Fig. 2B, bottom). Western blots demonstrated the release of a fraction of the 90 kDa AcMFP1 after CK2 treatment, but not in controls without CK2 (Fig. 2B, top).

To demonstrate that the change in solubility was indeed due to phosphorylation, [γ -³²P]ATP was used as a source of phosphate groups. At least five different bands in the NM fraction incorporated ³²P, became partially extracted by NaCl, and were collected in the S4 supernatant (Fig. 2C). One of them corresponded to the 90 kDa AcMFP1, as demonstrated by the recognition of the corresponding radioactive band by the anti-MFP1 serum by western blot (Fig. 2C). Control reactions without added exogenous CK2 showed no detectable ³²P bands. As expected, in these reactions the 90 kDa AcMFP1 was exclusively detected in the NM2 fraction, and not in the corresponding S4 control fraction (Fig. 2C).

Stimulation and inhibition of endogenous CK2 activity modifies 90 kDa AcMFP1 solubility

To check if endogenous CK2 had the same effect on the binding of the 90 kDa AcMFP1 to the NM, assays of specific CK2 stimulation by spermine and spermidine (Li and Roux, 1992; Westmark *et al.*, 2002; Tuteja *et al.*,

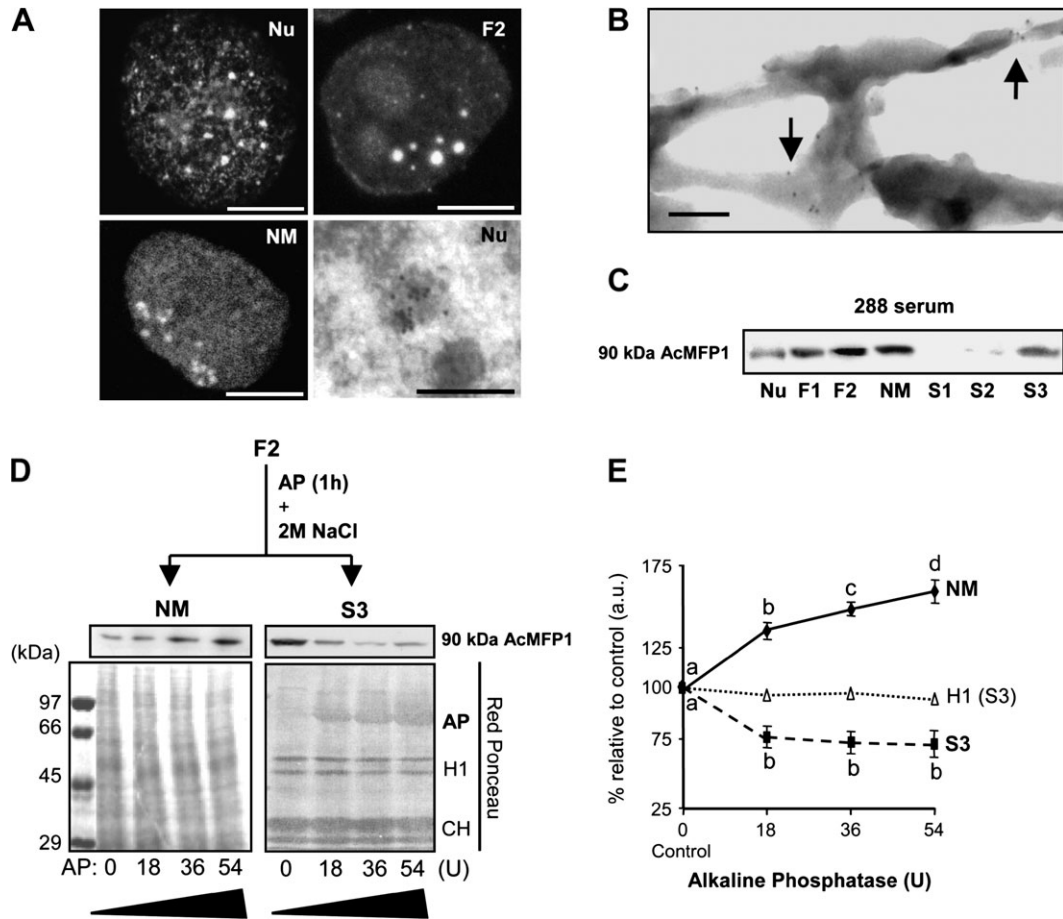


Fig. 1. Nuclear distribution of 90 kDa MFP1 and effect of alkaline phosphatase (AP) dephosphorylation on its association to the NM. (A, B) Topological distribution of the 90 kDa AcMFP1. (A) The protein appeared associated with the intranuclear network of filaments and was highly enriched in nuclear bodies in intact (Nu) and DNase-digested (F2) nuclei and also in nuclear matrices (NM) after incubation with 288 serum against LeMFP1 and confocal microscopy. The EM-immunolabelling reveals the high accumulation of the protein in a nuclear body analogous to those revealed by immunofluorescence with the same serum. (B) EM image of a resinless section showing the association of the 90 kDa AcMFP1 to filaments of the nucleoskeleton after pre-embedding labelling with 288 serum (arrows). (C) Western blots showing the sequential extraction of the 90 kDa AcMFP1 during NM preparation. The protein extracted with NaCl is recovered in the S3 supernatant, while a second fraction is retained in the insoluble nuclear matrix pellet. (D, E), Effect of alkaline phosphatase dephosphorylation on the 90 kDa AcMFP1 association to the NM. (D) Aliquots from F2 fractions of the same experiment were incubated with increasing AP concentrations (18, 36, and 54 U with 0 U as control), and further extracted with 2 M NaCl. The protein collected in both the S3 supernatant and the NM fraction was analysed by western blot with 288 antiserum. AP dephosphorylation produces a progressive increase in the NM-associated protein, and a parallel decrease in the released protein (S3). As loading control, the nitrocellulose membranes stained with Ponceau red before immunoblot are shown. The increasing AP concentrations are evident in the S3 membrane, as well as H1 histone variants (H1) and core histones (CH). (E) Representation of the means and the standard deviations of the bands optical density \times area (y) from three independent experiments, against the four AP treatments (x). Letters show significant differences ($P < 0.05$) when compared with the control value (=100%) and the previous one. Scale bars: in confocal microscopy images=10 μ m; in EM images=200 nm.

2003) and of heparin inhibition were performed and their effects on protein extraction from NM were analysed.

The levels of extracted phosphorylated protein in S3 fractions progressively increased with the time of incubation with CK2 stimulators, reaching a maximum increase of 150% relative to control (100%), after 20 min incubation (Fig. 3). Opposite effects were recorded when fresh nuclei were incubated with increasing heparin concentrations. A reduction of the extracted phosphorylated protein levels in S3 was achieved only with heparin concentrations higher than 300 nM. The lowest level of phosphorylated protein relative to control was 70%, achieved with 400 nM

heparin incubation (Fig. 3). In both assays, H1 histone levels in nuclei did not undergo significant variations compared with the respective control reactions (Fig. 3B).

pI values of the 90 kDa AcMFP1 protein

The experimental isoelectric point (pI) of the hyper-phosphorylated and hypo-phosphorylated 90 kDa AcMFP1 forms were analysed in 2-D blots of nuclei and NM fractions, respectively. Specific 2-D buffers containing different combinations of urea, non-ionic detergents, and reducing agents, but lacking charged detergents such as SDS, proved to be useless to solve the 90 kDa AcMFP1.

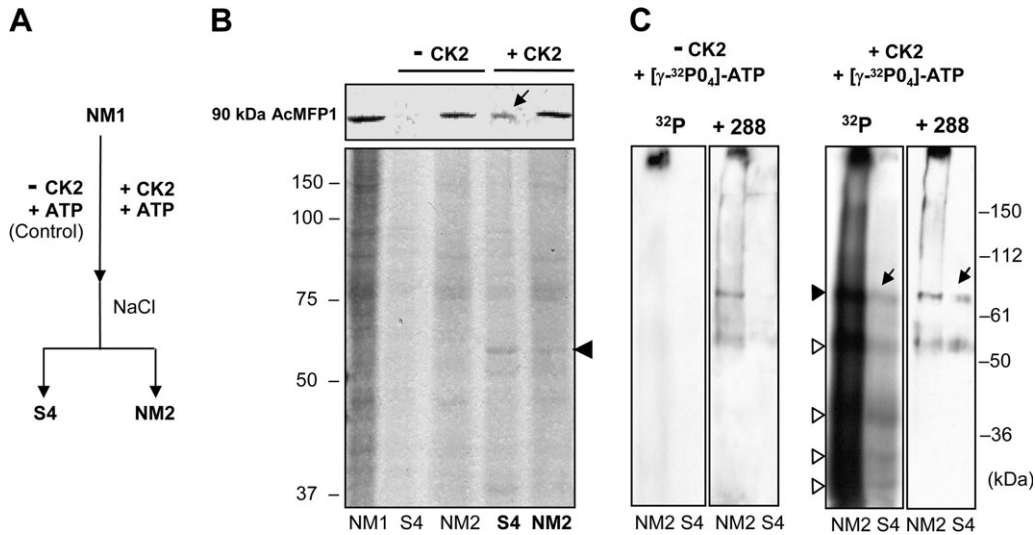


Fig. 2. Phosphorylation by exogenous CK2 releases a fraction of 90 kDa MFP1 from the NM. (A) Scheme of the performed experimental assay. NM fractions prepared by 2 M NaCl extraction (NM1) were incubated with ATP and exogenous CK2, followed by a second 2 M NaCl extraction to release the CK2 phosphorylated proteins from the NM. The corresponding S4 supernatant and NM2 fractions were collected for further analysis. Control reactions were performed in the same conditions but without adding CK2. (B) Detection of the phosphorylated 90 kDa AcMFP1 in the S4 supernatant (top) by western blot with 288 serum. Incubation with CK2 produced phosphorylation of a fraction of the NM-associated 90 kDa MFP1 that was extracted by the second NaCl extraction and is recovered in the S4 supernatant (arrow), while the most insoluble fraction remained matrix-bound. In the controls without CK2 there is no release of the protein from the NM (bottom). Coomassie blue staining of a replica gel. The arrowhead points to the exogenous CK2. (C) The same experimental assay performed with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. At least five major NM proteins incorporate ^{32}P after CK2 incubation, became partially extractable by NaCl, and were collected in the S4 supernatant (arrowheads). One of them corresponds to the 90 kDa AcMFP1 band as detected by western blot in the same membrane with 288 serum (arrows).

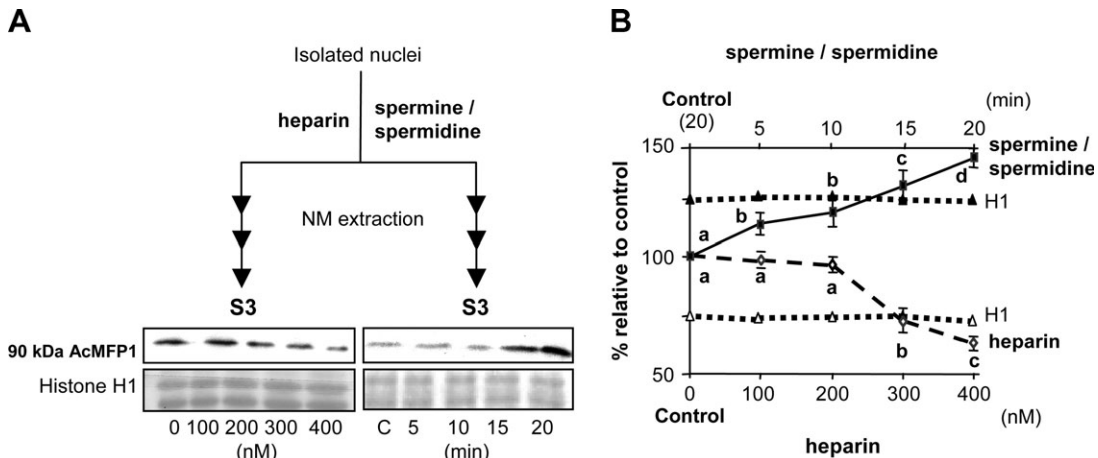


Fig. 3. Effect of endogenous CK2 inhibition and stimulation on the 90 kDa MFP1 association with the NM. (A) For inhibition of the nuclear endogenous CK2, freshly isolated nuclei were treated with increasing heparin concentrations (100, 200, 300, and 400 nM with 0 nM as control), while for stimulation they were treated with either 1 mM spermine or 2 mM spermidine for increasing periods of time (5, 10, 15, and 20 min, using an incubation of 20 min without polycations as control). Immediately after treatment NMs were prepared by high ionic strength extraction and the S3 supernatants collected for analysis of the levels of phosphorylated 90 kDa protein solubilized by the 2 M NaCl treatment. The detected levels of AcMFP1 in S3 progressively decreased when heparin concentration increased, and increased with longer incubation times with polycations. H1 histone stained with Ponceau red is shown as loading control. (B) Representation of the means and the standard deviations of the bands optical density \times area (y) from three independent experiments, against the different treatments: spermine/spermidine (top x); heparin (bottom x). Letters show significant differences ($P < 0.05$) when compared with the control values (=100%). Control H1 histone levels did not vary significantly.

For this reason, a double-solubilization method that highly improved the efficiency of solubilization of other NM-associated proteins was optimized (Szekely *et al.*, 1995) and resolved almost 100 well isoelectric-focused spots in the present NM fractions after silver staining (Fig. 4A).

The method consisted of a preliminary solubilization in buffers containing up to 2% SDS, compatible with sample heating, followed by a dilution in a specific 2-D buffer to obtain a final 1:8 ratio between SDS and a non-ionic detergent that completely removes the SDS from the protein

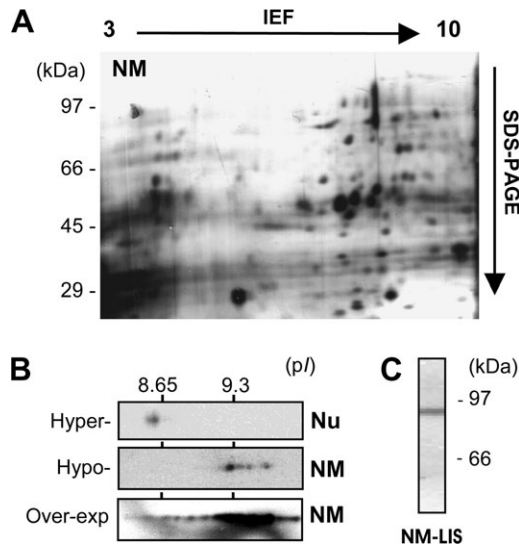


Fig. 4. Detection of the different phosphorylation states of 90 kDa MFP1 in nuclear and NM proteins separated by 2-D electrophoresis. (A) Silver staining of total NM proteins obtained by the double-solubilization method, separated by 2-D electrophoresis through a 3–10 pH gradient. (B) Western blots from nuclear (Nu) and NM proteins separated by 2-D with serum 288-detected 90 kDa spots with different pI values. Top: in the nuclear fraction two spots with a pI \sim 8.5 were detected that correspond to the hyper-phosphorylated states of the protein. Middle: in the NM fraction four hypo-phosphorylated states of the protein were detected with a pI \sim 9.5. Bottom: over-exposure of films of the NM fractions allowed the traces of the hyper-phosphorylated forms with pIs between 8.5 and 9.0 to be detected. (C) 1-D western blot of NM proteins obtained by the LIS method clearly revealed the 90 kDa AcMFP1 band which is not resolved in 2-D blots derived from the same extraction method (not shown).

surfaces. The double-solubilization method includes the use of reducing agents, SDS, and heat, which break inter- and intramolecular hydrogen bridges and cancel hydrophobic interactions, favouring the complete denaturation of 90 kDa MFP1 protein and preventing aggregation with other nuclear proteins during IEF, as reported for nucleolin (Gotzman *et al.*, 1997). Besides, the non-ionic detergent completely removes the SDS from the proteins' surfaces, avoiding a false migration during IEF. Confidence in the method is guaranteed as pI markers solved in the same conditions migrated to their expected positions in the pH gradient.

2-D blots of proteins solubilized by this method revealed that the experimental pI range of 90 kDa AcMFP1 forms is basic (9.7–8.5), by contrast to the slightly acidic pI reported for other MFP1 proteins (\sim 5.5), including the 80 kDa AcMFP1 (R Samaniego, C de la Torre, S Moreno Diaz de la Espina, unpublished results). 2-D blots from NM fractions revealed four hypo-phosphorylated forms of the protein in the range of 9.3–9.7 (Fig. 4B). The pI values of the hyper-phosphorylated forms could not be obtained from the S3 fractions, due to the high salt concentration of the samples (>2 M), 8-fold being the highest compatible with IEF. The analysis of blots from nuclear fractions allowed the iden-

tification of the hyper-phosphorylated forms as two spots at approximately 8.5 pI, more acidic than those of the hypo-phosphorylated forms (Fig. 4B).

The more basic hypo-phosphorylated forms (pI 9.5) would not be detected in the nuclear fractions not pre-treated with high ionic strength buffers (Fig. 4B). In the NM fractions they were probably solved due to the 2 M NaCl cancellation of the ionic interactions between the hypo-phosphorylated protein and the NM (Friso and Wikström, 1999). For the same reason, the 90 kDa AcMFP1 could not be solved by the double-solubilization method from LIS–NMs that were prepared by a mild LIS detergent extraction without using high ionic strength buffers (not shown). Nevertheless it was detected by 1-D western blot analysis in both NaCl- and LIS-prepared NMs, as standard 1-D SDS-PAGE buffers at a concentration higher than 4% can be heated to produce a good solubilization of the protein (Figs 1C, 4C).

The 90 kDa AcMFP1 protein has a phosphorylation peak in the G₂ of proliferating cells

The *in vitro* results obtained here demonstrated that phosphorylation is involved in the binding of the 90 kDa MFP1 protein to the NM, and suggested that endogenous CK2 is involved in the regulation of this binding *in vivo*. To check whether the 90 kDa AcMFP1 protein might experience cell cycle specific phosphorylation *in vivo*, nuclear and S3 fractions from synchronized meristematic cell populations were analysed at four different stages in the cell cycle.

Meristematic root cells were synchronized by a 14 h treatment with 10^{-4} M HU which arrests the cell cycle shortly after S phase initiation but before new DNA chain elongation takes place. It allows 70% of meristematic cells to continue the replication process synchronously after their release from the drug (Pelayo *et al.*, 2001). The levels of 90 kDa MFP1 were analysed at four stages in the cell cycle: 1 h before release from HU (very early S) and 2 h (early-S), 5 h (late-S), and 7 h (G₂) after release (Fig. 5A). Cycle position of cells was controlled by flow cytometry (Fig. 5A) and further corroborated by analysing the frequencies of replicating nuclei and the specific distribution of replication foci characteristic for each subperiod of the S phase (Samaniego *et al.*, 2002), after *in vivo* BrdU incorporation and later immunolabelling (data not shown).

The levels of total nuclear 90 kDa MFP1 did not vary significantly through the cell cycle (Fig. 5B). However, slightly retarded hyper-phosphorylated forms of the 90 kDa MFP1 were seen in western blots from late-S and G₂ cells. The levels of hyper-phosphorylated protein, released by high ionic strength buffer extraction significantly increased from late-S reaching a peak in G₂, as detected in the S3 supernatants, where only the hyper-phosphorylated protein forms are detected (Fig. 5B).

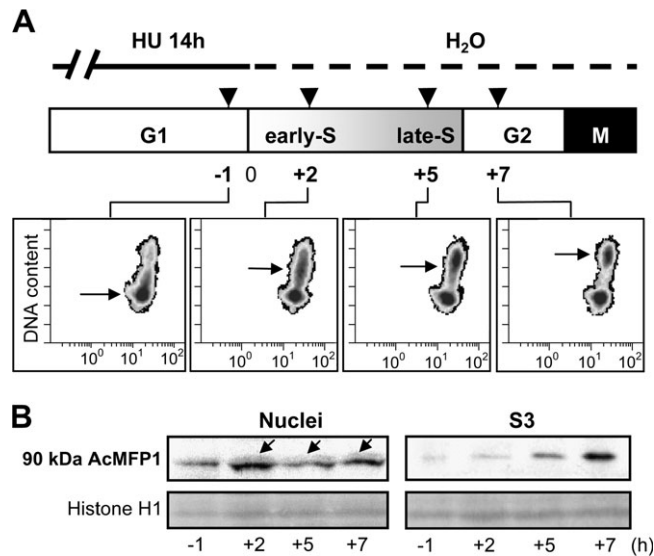


Fig. 5. Variations in the 90 kDa MFP1 phosphorylation levels through the cell cycle. (A) Experimental design used for the synchronization of proliferative cells. The bar represents the relative durations of the cell cycle compartments in the experimental conditions used. The continuous line corresponds to the duration of the HU treatment and the discontinuous one to the times of recovery from HU in water. Arrowheads mark the points at which samples were taken for analysis. Also shown is flow cytometry analysis displaying the changes in DNA-content distribution recorded in the cell population arrested in very early-S (-1), and at different points after their release from the treatment, early-S (+2), late-S (+5), and G₂ phases (+7) (arrows). After continuous 14 h HU treatment, accumulation of cells with 2C DNA contents and a fall in the 4C content were observed. The progression through S phase of the wave of synchronized cells after the release of the treatment (arrows) becomes evident between the +2 and +5 h of recovery. At +7 h, the wave of synchronized cells had reached G₂. (B) Levels of the 90 kDa AcMFP1 protein in both nuclear and S3 fractions at each period of the cell cycle. The hyper-phosphorylated states are distinguishable as slower 90 kDa bands (arrows) in the nuclear fractions corresponding to the late-S and the G₂ phases, and also in the S3 supernatants after high-salt treatment, where only hyper-phosphorylated states are detectable. H1 histones stained with Ponceau red are shown as loading control.

Discussion

90 kDa MFP1 is a phosphoprotein with a basic pI

The results demonstrate that the 90 kDa AcMFP1 is a phosphoprotein, since it incorporates detectable radioactive phosphate groups *in vitro*. Besides, its migration in 2-D blots as an alignment of spots with slight differences in their net charges reveals the existence of different phosphorylation states of the protein.

The present results of 2-D western blots using a double-solubilization method allowed the estimation of the experimental pI values of the most insoluble forms of the protein, which could not be achieved by the standard procedures, as reported for other NM proteins (Szekely *et al.*, 1995), and confirmed the reliability of the pI values previously calculated for the 90 kDa MFP1 proteins (Samaniego *et al.*, 2001).

The procedure combining reducing agents, SDS, and heat, which breaks inter- and intra-molecular hydrogen bridges and cancels hydrophobic interactions, favours the

complete denaturation of the 90 kDa MFP1 proteins and prevents aggregation to other nuclear proteins during IEF, as reported for nucleolin (Gotzman *et al.*, 1997). The more basic hypo-phosphorylated protein forms (pIs ~9.5) were more tightly bound to the NM than the hyper-phosphorylated ones (pIs ~8.5). These were released from the NM by high-salt buffers, probably because their higher number of charged phosphates makes them more hydrophilic.

The pI values of the 90 kDa MFP1 proteins are closer to those of unrelated nuclear DNA-binding proteins (Bickmore and Sutherland, 2002) than to those of the MFP1 proteins themselves, including the 80 kDa AcMFP1, with slightly acidic pIs (~5.5) (Jeong *et al.*, 2004; R Samaniego, C de la Torre, S Moreno Diaz de la Espina, unpublished results), and NAC proteins (4.9–7.0), a group of seven plant NM coiled-coil proteins that have been suggested to functionally replace lamins in plants (Blumenthal *et al.*, 2004). This could be due to sequence differences between them, as MFP1 sequences are not highly conserved amongst species (Harder *et al.*, 2000).

Phosphorylation by CK2 regulates the binding of the 90 kDa AcMFP1 to NM

Phosphorylation plays a role in modulating the functionality and NM association of many nuclear proteins, such as chromatin-associated proteins, transcription factors, Rb proteins, or nucleoskeletal proteins such as lamins and their associated proteins and NuMA (Mancini *et al.*, 1994; Saredi *et al.*, 1997; Dechat *et al.*, 1998; Moir *et al.*, 2000; Hachet *et al.*, 2004; Kappes *et al.*, 2004; Takano *et al.*, 2004).

Phosphorylation modulates the strength of the binding of the 90 kDa MFP1 to the NM, without affecting its intranuclear distribution, since removal of the hyper-phosphorylated states by NaCl does not significantly change its distribution in the NM, and neither its association to the intranuclear matrix filament network nor to NBs (see Fig. 1A).

Incorporation of phosphates induces a lower protein affinity for the NM, possibly because negative charges reduce the electrostatic interactions between the NM-associated 90 kDa MFP1 and the intrinsic interacting proteins in the NM. This mechanism was first reported for vertebrate lamins more than two decades ago (Gerace and Blobel, 1980). Now many other NM proteins undergoing this regulation are known, among them chromatin remodelling proteins (Reyes *et al.*, 1997; Bérubé *et al.*, 2000), NuMA (Saredi *et al.*, 1997), the p73 polypeptide from the p53 family (Ben-Yehoyada *et al.*, 2003), and some transcriptional regulators (De Lucía *et al.*, 2001). In all cases, hypo-phosphorylation correlates with a strong association to the NM, and hyper-phosphorylation with a looser binding or even protein release.

CK2 is a ubiquitous multifunctional Ser/Thr kinase active in the cytoplasm and nucleus of proliferating and

differentiated eukaryotic cells. It is essential for cell cycle progression and viability, is one of the most conserved enzymes (Zhang *et al.*, 1998; Riera *et al.*, 2001b), and is also involved in cellular signaling (Yu *et al.*, 2001). The NM along with chromatin is a preferential target for CK2 in the nucleus (Wang *et al.*, 2003) and phosphorylates a variety of intrinsic proteins in these structures, altering their DNA- and NM-binding properties (Barz *et al.*, 2003; Kappes *et al.*, 2004). CK2 undergoes rapid modulations in its association to the NM by its own phosphorylation and also by sulphhydryl interactions via its β -subunit (Zhang *et al.*, 1998; Riera *et al.*, 2001a; Yu *et al.*, 2001). CK2 phosphorylation changes the chromatin and NM binding properties of proteins (Barz *et al.*, 2003; Kappes *et al.*, 2004).

In plants, CK2 is involved in vital processes such as light-regulated gene expression and cell cycle progression (Riera *et al.*, 2001b). It is essential for initiation and organization of meristematic activity (Espunya and Martínez, 2003), which correlates with the highest levels of 90 kDa MFP1 expression (R Samaniego, C de la Torre, S Moreno Diaz de la Espina, unpublished results). Amongst the nuclear CK2 substrates identified in plants (Tuteja *et al.*, 2001; Riera *et al.*, 2001a, b; Ferraris *et al.*, 2002; Krohn *et al.*, 2002, 2003; Stemmer *et al.*, 2002; Ivanov *et al.*, 2003; Tuteja, 2003; Daniel *et al.*, 2004), only pea lamin-like proteins (Li and Roux, 1992), MFP1 (Meier *et al.*, 1996; Jeong *et al.*, 2004), and the 90 kDa AcMFP1 (Samaniego *et al.*, 2001) are intrinsic components of nuclear matrices. As far as is known, this is the first evidence for the involvement of endogenous CK2 in the binding of a protein to the plant NM.

The 90 kDa MFP1 is clearly phosphorylated by CK2 as it incorporates γ - ^{32}P *in vitro* and showed shift banding after incubation with exogenous CK2. Besides, the results of specific stimulation and inhibition of the endogenous CK2 demonstrate that the 90 kDa MFP1 is a substrate of this enzyme. This is in agreement with the results for the chloroplast MFP1 in *Nicotiana tabacum*, which showed that CK2 phosphorylation of its C terminus modulates the DNA-binding activity of the protein (Jeong *et al.*, 2004), and also with the presence of several consensus sites for the CK2 protein kinase in MFP1 proteins (Meier *et al.*, 1996; Harder *et al.*, 2000). Although attempts to sequence AcMFP1 proteins from a genomic onion library have failed and the sequences of the 80 kDa and 90 kDa proteins are still unknown, the results of double immunoprecipitation of AcMFP1 proteins with sera against the tomato (288) and *Arabidopsis* (OSU 91) proteins suggest the conservation of MFP1 proteins in onion (R Samaniego, C de la Torre S Moreno Diaz de la Espina, unpublished results).

The multiple distribution pattern of the 90 kDa MFP1 in nuclear bodies and aggregates associated with the NM filaments network, and the results of its fractionation during NM isolation, suggest that the nuclear pool of the 90 kDa MFP1 has different levels of phosphorylation and

is in a dynamic, cell cycle-regulated equilibrium depending on their phosphorylation by CK2. The NM would contain a loosely bound hypo-phosphorylated 90 kDa MFP1 protein pool, extractable with high ionic strength buffers that would represent a low phosphorylated transient form that is not detected by shifting, while the NM-tightly bound protein pool is hypo-phosphorylated and resists high ionic strength extraction. The last one can be removed from the NM by *in vitro* incubation with exogenous CK2.

The 90 kDa AcMFP1 is differently phosphorylated along the cell cycle, probably by CK2

Cell cycle-dependent phosphorylation of nuclear proteins, including CK2 phosphorylation, regulates three major nuclear processes: DNA-binding, protein redistribution, and assembly/disassembly of macromolecular complexes or nuclear subdomains, affecting in all cases the protein function(s) (Saredi *et al.*, 1997; Joaquin and Watson, 2003; Kamemura and Hart, 2003; Albert *et al.*, 2004; Allard *et al.*, 2004).

The detected release of the matrix-associated protein in G₂ suggests the existence of a cell cycle-dependent disassembly of the 90 kDa MFP1 in response to a phosphorylation event due to CK2 in late S and G₂ phases. Cyclic phosphorylation patterns have been reported for many NM proteins. Most of them have a peak in G₁, such as Rb (Mancini *et al.*, 1994) and nuclear chromatin- and NM-binding proteins (Minc *et al.*, 1999; Qiao *et al.*, 2001; Miccoli *et al.*, 2003). Nevertheless coiled-coil proteins of the NM with sequence similarity to MFP1 such as lamins (Moir *et al.*, 2000) and NuMA (Saredi *et al.*, 1997) have phosphorylation peaks in G₂ that regulate their disassembly before the cell enters into mitosis.

Considering the DNA-binding activity of MFP1 proteins (Meier *et al.*, 1996; Jeong *et al.*, 2003), the high resolution localization of MFP1 in the perichromatin regions of onion nuclei, where decondensed DNA accumulates (Samaniego *et al.*, 2001), its association with nucleoids in chloroplasts (Jeong *et al.*, 2003), and taking into account that *in vitro* CK2 phosphorylation of chloroplast NtMFP1 inhibits DNA binding of the protein (Jeong *et al.*, 2004), a possible CK2 phosphorylation role modulating the DNA-binding activity of the nuclear MFP1 cannot be discounted.

The 90 kDa MFP1 is not likely to be a basic component of the expansible core filaments of the NM, as the animal EAST (Wasser and Chia, 2000), skeletor (Walker *et al.*, 2000), enaptin, and nuance proteins seem to be (Padmakumar *et al.*, 2004). Instead, its distribution, the modulation of its binding to NM by CK2 phosphorylation and its transient expression in non-proliferating cells depending on tissue and cell type (Samaniego *et al.*, 2001; R Samaniego, C de la Torre, S Moreno Diaz de la Espina, unpublished results)

suggest that the 90 kDa MFP1 might act as an NM protein involved in DNA binding to this structure.

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References

- Ahmed K. 1999. Nuclear matrix and protein kinase CK2 signalling. *Critical Review of Eukaryotic Gene Expression* **9**, 329–336.
- Ahmed K, Davis AT, Wang H, Faust RA, Yu S, Tawfic S. 2000. Significance of protein kinase CK2 nuclear signaling in neoplasia. *Journal of Cellular Biochemistry Supplement* **35**, 130–135.
- Albert AL, Lavoie SB, Vincent M. 2004. Multisite phosphorylation of Pin1-associated mitotic phosphoproteins revealed by monoclonal antibodies MPM-2 and CC-3. *BMC Cell Biology* **5**, 22.
- Allard S, Masson JY, Cote J. 2004. Chromatin remodelling and maintenance of genome integrity. *Biochimica et Biophysica Acta* **1677**, 158–164.
- Barz T, Ackermann K, Dubois G, Eils R, Pyerin W. 2003. Genome-wide expression screens indicate a global role for protein kinase CK2 in chromatin remodelling. *Journal of Cell Science* **116**, 1563–1577.
- Ben-Yehoyada M, Ben-Dor I, Shauland L. 2003. c-Abl tyrosine kinase selectively regulates p73 nuclear matrix association. *Journal of Biological Chemistry* **278**, 34475–34482.
- Bérubé NG, Smeenk CA, Picketts DJ. 2000. Cell cycle-dependent phosphorylation of the ATRX protein correlates with changes in nuclear matrix and chromatin association. *Human Molecular Genetics* **9**, 539–547.
- Bettinger BT, Gilbert DM, Amberg DC. 2004. Actin up in the nucleus. *Nature* **5**, 410–415.
- Bickmore W, Sutherland HE. 2002. Addressing protein localization within the nucleus. *EMBO Journal* **21**, 1248–1254.
- Blumenthal SSD, Clark GB, Roux SJ. 2004. Biochemical and immunological characterization of pea nuclear intermediate filament proteins. *Planta* **218**, 965–975.
- Calikowsky TT, Meulia T, Meier I. 2003. A proteomic study of the *Arabidopsis* nuclear matrix. *Journal of Cellular Biochemistry* **90**, 361–378.
- Claudio PP, Tonini T, Giordano A. 2002. The retinoblastoma family: twins or distant cousins? *Genome Biology* **3**, 30121–30129.
- Daniel X, Sugano S, Tobin EM. 2004. CK2 phosphorylation of CCA1 is necessary for its circadian oscillator function in *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA* **101**, 3292–3297.
- Dechat T, Gotzmann J, Stockinger A, Harris CA, Talle MA, Siekierka JJ, Foisner R. 1998. Detergent-salt resistance of LAP2 α in interphase nuclei and phosphorylation-dependent association with chromosomes early in nuclear assembly implies functions in nuclear structure dynamics. *EMBO Journal* **17**, 4887–4902.
- De Lucía F, Lorain S, Scamps C, Galisson F, Machold J, Lipinski M. 2001. Subnuclear localization and mitotic phosphorylation of HIRA, the human homologue of *Saccharomyces cerevisiae* transcriptional regulators Hir1p/Hir2p. *Biochemical Journal* **358**, 447–455.
- Espunya MC, Martínez MC. 2003. *In situ* hybridization analysis of protein kinase CK2 expression during plant development. *Physiologia Plantarum* **117**, 573–578.
- Ferraris JD, Williams CK, Persaud P, Zhang Z, Chen Y, Burg MB. 2002. Activity of the TonEBP/OREBP transactivation domain varies directly with extracellular NaCl concentration. *Proceedings of the National Academy of Sciences, USA* **99**, 739–744.
- Friso G, Wikström L. 1999. Analysis of proteins from membrane-enriched cerebellar preparations by two-dimensional gel electrophoresis and mass spectrometry. *Electrophoresis* **20**, 917–927.
- Fujimoto S, Matsunaga S, Yonemura M, Uchiyama S, Azuma, T, Fukui K. 2004. Identification of a novel plant MAR DNA binding protein localized on chromosomal surfaces. *Plant Molecular Biology* **56**, 225–239.
- Gerace L, Blobel G. 1980. The nuclear envelope lamina is reversible depolymerized during mitosis. *Cell* **19**, 277–287.
- Gindullis F, Meier I. 1999. Matrix attachment region-binding protein MFP1 is localized in discrete domains at the nuclear envelope. *The Plant Cell* **11**, 1117–1128.
- Gotzmann J, Eger A, Meissner M, Grimm R, Gerner C, Sauermann G, Foisner R. 1997. Two-dimensional electrophoresis reveals a nuclear matrix-associated nucleolin complex of a basic isoelectric point. *Electrophoresis* **18**, 2645–2653.
- Hachet V, Kocher T, Wilm M, Mattaj IW. 2004. Importin alpha associates with membranes and participates in nuclear envelope assembly *in vitro*. *EMBO Journal* **23**, 1526–1535.
- Harder PA, Silverstein RA, Meier I. 2000. Conservation of matrix attachment region-binding filament like protein 1 among higher plants. *Plant Physiology* **122**, 225–234.
- Ivanov KI, Puustinen P, Gabrenaite R, Vihinen H, Ronnstrand L, Valmu L, Kalkkinen, Makinen K. 2003. Phosphorylation of the polyvirus capsid protein by protein kinase CK2 and its relevance for virus infection. *The Plant Cell* **15**, 2124–2139.
- Jeong SY, Peffer N, Meier I. 2004. Phosphorylation by protein kinase CKII modulates DNA-binding activity of a chloroplast nucleoid-associated protein. *Planta* **219**, 298–302.
- Jeong SY, Rose A, Meier I. 2003. MFP1 is a thylakoid-associated, nucleoid-binding protein with a coiled-coil structure. *Nucleic Acids Research* **17**, 5175–5185.
- Joaquin M, Watson RJ. 2003. Cell cycle regulation by the B-Myb transcription factor. *Cellular and Molecular Life Sciences* **60**, 2389–2401.
- Kamemura K, Hart GW. 2003. Dynamic interplay between O-glycosylation and O-phosphorylation of nucleocytoplasmic proteins: a new paradigm for metabolic control of signal transduction and transcription. *Progresses in Nucleic Acid Research and Molecular Biology* **73**, 107–136.
- Kappes F, Damoc C, Knippers R, Przybylski M, Pinna LA, Gruss C. 2004. Phosphorylation by protein kinase CK2 changes the DNA binding properties of the human chromatin protein DEK. *Molecular and Cellular Biology* **24**, 6011–6020.
- Kiseleva E, Drummond SP, Goldberg MW, Rutherford SA, Allen TD, Wilson KL. 2004. Actin- and protein 4.1-containing filaments link nuclear pore complexes to subnuclear organelles in *Xenopus* oocyte nuclei. *Journal of Cell Science* **117**, 2481–2490.
- Krohn NM, Stemmer C, Fojan P, Grimm R, Grasser KD. 2003. Protein kinase CK2 phosphorylates the high mobility group domain protein SSRP1, inducing the recognition of UV-damaged DNA. *Journal of Biological Chemistry* **278**, 12710–12715.
- Krohn NM, Yanagisawa S, Grasser KD. 2002. Specificity of the stimulatory interaction between chromosomal HMGB proteins and the transcription factor Dof2 and its negative regulation by CK2-

- mediated phosphorylation. *Journal of Biological Chemistry* **277**, 32438–32444.
- Li H, Roux SJ.** 1992. Casein kinase II protein kinase is bound to lamina-matrix and phosphorylates lamin-like protein in isolated pea nuclei. *Proceedings of the National Academy of Sciences, USA* **89**, 8434–8438.
- Mancini MA, Shan B, Nickerson JA, Penman S, Lee WH.** 1994. The retinoblastoma gene product is a cell cycle dependent, nuclear matrix-associated protein. *Proceedings of the National Academy of Sciences, USA* **91**, 418–422.
- Masuda K, Xu ZJ, Takahashi S, Ito A, Ono M, Nomura K, Inoue M.** 1997. Peripheral framework of carrot cell nucleus contains a novel protein predicted to exhibit a long α -helical domain. *Experimental Cell Research* **232**, 173–181.
- McNulty AK, Saunders MJ.** 1992. Purification and immunological detection of pea nuclear intermediate filaments: evidence for plant nuclear lamins. *Journal of Cell Science* **103**, 407–414.
- Meier I.** 2001. Subnuclear trafficking and the nuclear matrix. *Nuclear Import and Export in Plants and Animals* **3**, 1–15.
- Meier I, Phelan T, Gruissem W, Spiker S, Schneider D.** 1996. MFP1, a novel plant filament-like protein with affinity for matrix attachment region DNA. *The Plant Cell* **8**, 2105–2115.
- Miccoli L, Biard DS, Frouin I, Harper F, Maga G, Angulo JF.** 2003. Selective interactions of human kin17 and RPA proteins with chromatin and the nuclear matrix in a DNA damage- and cell cycle regulated manner. *Nucleic Acids Research* **31**, 4162–4175.
- Minc E, Allory Y, Worman HJ, Courvalin JC, Buendía B.** 1999. Localization and phosphorylation of HP1 proteins during the cell cycle in mammalian cells. *Chromosoma* **108**, 220–234.
- Mínguez A, Moreno Díaz de la Espina S.** 1993. Immunological characterization of lamins in the nuclear matrix of onion cells. *Journal of Cell Science* **106**, 431–439.
- Moir RD, Spann TP, López-Soler RI, Yoon M, Goldman AE, Khuon S, Goldman RD.** 2000. The dynamics of the nuclear lamins during the cell cycle: relationships between structure and function. *Journal of Structural Biology* **129**, 324–334.
- Moreno Díaz de la Espina S.** 1995. Nuclear matrix isolated from plants cells. *International Review of Cytology* **162**, 75–139.
- Moreno Díaz de la Espina S, Samaniego R, Yu W, de la Torre C.** 2003. Intermediate filament proteins with nuclear functions: NuMA, lamin-like proteins and MFP1. *Cell Biology International* **27**, 233–235.
- Nalepa G, Harper JW.** 2004. Visualization of a highly organized intranuclear network of filaments in living mammalian cells. *Cell Motility and the Cytoskeleton* **59**, 94–108.
- Padmakumar VC, Abraham S, Braune S, Noegel AA, Tunggal B, Karakesisoglou I, Korenbaum E.** 2004. Enaptin, a giant-binding protein, is an element of the nuclear membrane and the actin cytoskeleton. *Experimental Cell Research* **295**, 330–339.
- Pelayo HR, Lastres P, de la Torre C.** 2001. Replication and G₂ checkpoints: their response to caffeine. *Planta* **212**, 444–453.
- Qiao F, Moss A, Kupfer GM.** 2001. Fanconi anemia proteins localize to chromatin and the nuclear matrix in a DNA damage- and cell cycle-regulated manner. *Journal of Biological Chemistry* **276**, 23391–23396.
- Reyes JC, Muchardt C, Yaniv M.** 1997. Components of the Human SWI/SNF complex are enriched in active chromatin and are associated with the nuclear matrix. *Journal of Cell Biology* **21**, 263–274.
- Riera M, Peracchia G, de Nadal E, Arino J, Pages M.** 2001a. Maize protein kinase CK2: regulation and functionality of three beta regulatory subunits. *The Plant Journal* **25**, 365–374.
- Riera M, Peracchia G, Pages M.** 2001b. Distinctive features of plant protein kinase CK2. *Molecular and Cellular Biochemistry* **227**, 119–127.
- Rose A, Gindullis F, Meier I.** 2003. A novel alpha-helical protein, specific to and highly conserved in plants, is associated with the nuclear matrix fraction. *Journal of Experimental Botany* **54**, 1133–1141.
- Samaniego R, de la Torre C, Moreno Díaz de la Espina S.** 2002. Dynamics of replication foci and nuclear matrix during S phase in *Allium cepa* L. cells. *Planta* **215**, 195–204.
- Samaniego R, Jeong SY, Meier I, Moreno Díaz de la Espina S.** 2006. Dual localization of MAR-binding, filament-like protein 1 in *Arabidopsis*, tobacco and tomato. *Planta* (in press).
- Samaniego R, Moreno Díaz de la Espina S.** 2000. Organisation and composition of the plant nuclear matrix: characterisation and subcellular distribution of a MAR-binding protein: Ac-MFP1. *Cellular and Molecular Biology Letters* **5**, 264–266.
- Samaniego R, Yu W, Meier I, Moreno Díaz de la Espina S.** 2001. Characterization and high-resolution distribution of a matrix attachment region-binding protein (MFP1) in proliferating cells of onion. *Planta* **212**, 536–546.
- Saredi A, Howard L, Compton DA.** 1997. Phosphorylation regulates the assembly of NuMA in a mammalian mitotic extract. *Journal of Cell Science* **110**, 1287–1297.
- Shumaker DK, Kuczmarski ER, Goldman RD.** 2003. The nucleoskeleton: lamins and actin are major players in essential nuclear functions. *Current Opinion in Cell Biology* **15**, 358–366.
- Stemmer C, Schwander A, Bauw G, Fojan P, Grasser KD.** 2002. Protein kinase CK2 differentially phosphorylates maize chromosomal high mobility group B (HMGB) proteins modulating their stability and DNA interactions. *Journal of Biological Chemistry* **277**, 1092–1098.
- Szekely L, Jiang WQ, Pokrovskaja K, Wiman KG, Klein G, Ringertz N.** 1995. Reversible nucleolar translocation of Epstein-Barr virus-encoded EBNA-5 and hsp70 proteins after exposure to heat shock or cell density congestion. *Journal of General Virology* **76**, 2423–2432.
- Takano M, Koyama Y, Ito H, Onogi H, Hagiwara M, Fukurawa K, Horigome T.** 2004. Regulation of binding of lamin B receptor to chromatin by SR protein kinase and cdc2 kinase in *Xenopus* egg extracts. *Journal of Biological Chemistry* **279**, 13265–13271.
- Tuteja N.** 2003. Plant DNA helicases: the long unwinding road. *Journal of Experimental Botany* **54**, 2201–2214.
- Tuteja N, Beven AF, Shaw PJ, Tuteja R.** 2001. A pea homologue of human DNA helicase I is localized within the dense fibrillar component of the nucleolus and stimulated by phosphorylation with CK2 and cdc2 protein kinases. *The Plant Journal* **25**, 9–17.
- Tuteja N, Reddy MK, Mudgil Y, Yadav BS, Chandok MR, Sopori SK.** 2003. Pea DNA Topoisomerase I is phosphorylated and stimulated by casein kinase 2 and protein kinase C. *Plant Physiology* **132**, 2108–2115.
- Walker DL, Wang D, Jin Y, Rath U, Wang Y, Johansen J, Johansen KM.** 2000. Skeletor, a novel chromosomal protein that redistributes during mitosis provides evidence for the formation of a spindle matrix. *Journal of Cell Biology* **151**, 1401–1412.
- Wang H, Yu S, Davies AT, Ahmed K.** 2003. Cell cycle dependent regulation of protein kinase CK2 signaling to the nuclear matrix. *Journal of Cellular Biochemistry* **88**, 812–822.
- Wasser M, Chia W.** 2000. The EAST protein of *Drosophila* controls an expandable nuclear endoskeleton. *Nature Cell Biology* **2**, 268–275.
- Westmark CJ, Ghose R, Huber PW.** 2002. Phosphorylation of *Xenopus* transcription factor IIIA by an oocyte protein kinase CK2. *Biochemical Journal* **362**, 375–382.

- Yu S, Wang H, Davis, Ahmed K.** 2001. Consequences of CK2 signalling to the nuclear matrix. *Molecular and Cellular Biochemistry* **227**, 67–71.
- Yu W, Moreno Díaz de la Espina S.** 1999. The plant nucleoskeleton: ultrastructural organization and identification of NuMA homologues in the nuclear matrix and mitotic spindle of plant cells. *Experimental Cell Research* **246**, 516–526.
- Zhang P, Davis A, Ahmed K.** 1998. Mechanisms of protein kinase CK2 association with nuclear matrix: role of disulfide bond formation. *Journal of Cellular Biochemistry* **69**, 211–220.