A hyperphosphorylated form of RNA polymerase II is the major interphase antigen of the phosphoprotein antibody MPM-2 and interacts with the peptidyl-prolyl isomerase Pin1

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SUMMARY

The monoclonal antibody MPM-2 recognizes a subset of M phase phosphoproteins in a phosphorylation-dependent manner. It is believed that phosphorylation at MPM-2 antigenic sites could regulate mitotic events since most of the MPM-2 antigens identified to date have M phase functions. In addition, many of these proteins are substrates of the mitotic regulator Pin1, a peptidyl-prolyl isomerase which is present throughout the cell cycle and which is thought to alter its mitotic targets by changing their conformation. In interphase cells, most MPM-2 reactivity is confined to nuclear speckles. We report here that a hyperphosphorylated form of the RNA polymerase II largest subunit is the major MPM-2 interphase antigen. These findings were made possible by the availability of another monoclonal antibody, CC-3, that was previously used to identify a 255 kDa nuclear matrix protein associated with spliceosomal components as a hyperphosphorylated form of the RNA polymerase II largest subunit. MPM-2 recognizes a phosphoepitope of the large subunit that becomes hyperphosphorylated upon heat shock in contrast to the phosphoepitope defined by CC-3, whose reactivity is diminished by the heat treatment. Therefore, these two antibodies may discriminate between distinct functional forms of RNA polymerase II. We also show that RNA polymerase II large subunit interacts with Pin1 in HeLa cells. Pin1 may thus regulate transcriptional and post-transcriptional events by catalyzing phosphorylation-dependent conformational changes of the large RNA polymerase II subunit.

Key words: RNA polymerase II, MPM-2, CC-3, Phosphoprotein, Pin1, Heat shock

INTRODUCTION

RNA polymerase II (RNAP II) is a large multisubunit enzyme found in two forms according to the level of phosphorylation of its largest subunit, designated either IIa (unphosphorylated, apparent molecular mass 210 kDa) or IIo (multiphosphorylated, apparent molecular mass 240 kDa) (Woychik and Young, 1990; Young, 1991). Phosphorylation sites of IIo are located in its C-terminal domain (CTD) composed of multiple conserved heptapeptide repeats of the consensus sequence YSPTSPS whose number depends on the complexity of the organism (Corden, 1990). Various studies suggest that the IIa form interacts with the promoter to form a stable preinitiation complex and that entry to initiation of transcription is accompanied by CTD phosphorylation (Corden, 1993; Dahmus, 1994; O’Brien et al., 1994; Payne et al., 1989; Zawel and Reinberg, 1992). Upon completion of the transcript, the IIo form must be dephosphorylated to regenerate the IIa form and complete the cycle (Dahmus, 1996). In addition, it has been recently proposed that the CTD acts as a platform to couple mRNA processing to transcription as it was shown to recruit splicing, polyadenylation and capping factors to the transcription complex (Cho et al., 1997; McCracken et al., 1997; Steinmetz, 1997). It has been proposed that modulation of the phosphorylation state of the CTD may regulate its association with transcription and mRNA processing factors, and may involve multiple kinases and phosphatases (Archambault et al., 1998; Dahmus, 1994, 1996).

A few monoclonal antibodies (mAbs) have been produced that recognize different phosphoepitopes on the CTD of the RNAP II (Patturajan et al., 1998). One of these, mAb CC-3, recognizes a hyperphosphorylated form of the enzyme which associates with the nuclear matrix and is largely confined to splicing factor-enriched nuclear speckles (Bisotto et al., 1995; Chabot et al., 1995; Vincent et al., 1996). Besides its reactivity with RNAP II largest subunit in interphase cells, mAb CC-3 recognizes a set of phosphorylated proteins in mitotic cells. Actually, this antibody was initially selected for its strong general reactivity with mitotic cells of different species (Thibodeau et al., 1989; Thibodeau and Vincent, 1991). When eukaryotic cells divide, they undergo profound architectural and functional modifications that have been correlated with a dramatic increase in the level of protein phosphorylation (Caponi et al., 1986; Karsenti et al., 1987; Lohka et al., 1987;
Nigg et al., 1996). This increase results from the activation of a protein kinase cascade, at the head of which sits CDK1 kinase which forms a protein complex with mitotic cyclin B (Nurse, 1990). Several laboratories have used mAbs recognizing subsets of M phase phosphoproteins to identify mitotic regulators and effectors (Davis et al., 1983; Butschak et al., 1995; Kurtyama, 1989). The most characterized of these antibodies, mAb MPM-2, was raised against mitotic HeLa cells and selected for its preferential reactivity with mitotic versus interphase cells (Davis et al., 1983; Vandré et al., 1984). Because MPM-2 antigens are found in important mitotic structures such as centrosomes, kinetochores, spindle fibers, chromosomes scaffolds and the midbody (Taagepera et al., 1993; Vandré et al., 1984, 1986, 1991), it is currently believed that phosphorylation at MPM-2 antigenic sites could regulate mitotic processes. The functional importance of the MPM-2 antigens is further suggested by the inhibition of entry into (Kuang et al., 1989) or exit from mitosis (Davis and Rao, 1987) when cells are microinjected with this antibody.

The strong general reactivity of mAbs MPM-2 and CC-3 with mitotic cells of different species and the fact that MPM-2 staining of interphase cells is restricted to discrete extranuclear spots in the nucleus (Vandré et al., 1984) prompted us to compare the reactivity of both reagents. In this study, we report the identification of a hyperphosphorylated form of RNAP II largest subunit as the major MPM-2 antigen of interphase cells. The immunoreactivity of MPM-2 against this protein is found in both mitotic and interphase cells and is mediated by a phosphopeptiope different from that recognized by CC-3, suggesting that these antibodies may react with functionally distinct subsets of RNAP II. Incidentally, our results indicate that phosphorylation of these CTD phosphoacceptor sites is inversely modulated during a heat shock. As many MPM-2 antibodies are known to be substrates of the phosphorylation-dependent peptidyl-prolyl isomerase (PPIase) Pin1, a novel isomerase that specifically bind to Pin1. This isomerase may thus regulate MPM-2- and CC-3-reactive forms of RNAP II large subunit.

immunofluorescence
Cells were cultured overnight on glass coverslips, washed with phosphate buffered saline (PBS) and fixed for 20 minutes at ~20°C in 100% methanol, followed by rinses in PBS. After the incubation with the primary antibodies MPM-2 and CC-3, cells were stained with a Cy3-conjugated anti-mouse IgG1 antibody and a FITC-conjugated anti-mouse IgG2a antibody (Caltag, San Francisco, CA). In some experiments, cells were counterstained with 0.1 μg/ml 4’6-diamidino-2-phenylindole (DAPI) to reveal DNA. All antibodies were incubated for 1 hour at room temperature in 0.5% bovine serum albumin (BSA) diluted in PBS. After a few PBS washes, slides were mounted with p-phenylenediamine-PBS in glycerol and viewed with a DAS Leitz microscope equipped with epifluorescence optics.

Sample preparation, immunoprecipitation and immunoblotting
For whole cell extracts, mitotic or unsynchronized HeLa cells were directly solubilized in SDS-sample buffer (63 mM Tris-HCl, pH 6.8, 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol) and boiled for 6 minutes. For immunoprecipitation experiments, HeLa cells were homogenized at 4°C with TD buffer (50 mM Tris-HCl, pH 7.55, 150 mM NaCl, 5 mM EDTA, 50 mM sodium fluoride, 0.5% sodium deoxycholate, 0.5% Triton X-100, 150 mM okadaic acid, 2 mM PMSF, 2 mM sodium orthovanadate, 5 μg/ml each of leupeptin, pepstatin and 10 μg/ml of antipain). Extracts were spun at 16000 g for 5 minutes for clarification and then incubated with the primary antibody for 90 minutes at room temperature followed by an additional 2-hour incubation with goat anti-mouse IgG (whole molecule)-agarose (Sigma-Aldrich, Oakville, Ontario). The beads were washed 3 times using TD buffer and the immunoprecipitated complex was then solubilized in SDS-sample buffer as described above. For some experiments (immunodepletion, Fig. 2C), the beads were preincubated with the primary antibody in PBS before incubation with HeLa cell extracts for 90 minutes at room temperature. The depleted supernatants and the immunocomplexes were solubilized with 1 volume of 2X SDS-sample buffer before boiling for 6 minutes. Samples were resolved by SDS-PAGE on a 4% acrylamide gradient gel and transferred to nitrocellulose membranes. The blots were blocked with 1% blocking reagent (Boehringer Mannheim, Laval, Québec) diluted in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.65, 150 mM NaCl) containing 0.05% Tween-20 for 60 minutes at room temperature. Samples were probed overnight with the primary antibody in the same solution at 4°C. The membranes were washed 20 minutes in TBS-T. A peroxidase-conjugated anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was used at 1:10000 for chemiluminescence detection (Boehringer Mannheim, Laval, Québec). The mouse monoclonal antibody (mAb) MPM-2 (IgG1) was purchased from DAKO (Carpinteria, CA). mAb CC-3 (IgG2a) was obtained after immunization of a Balb/C mouse with pharyngeal regions isolated from 3-day-old chick embryos in a search for developmental markers (Thibodeau and Vincent, 1991). mAb POL3/3 (IgG1) recognizes RNAP II largest subunit in a conserved region, located outside the CTD and was kindly provided by E. K. F. Bautz (Krümer et al., 1980).

MATERIALS AND METHODS

Cell cultures
HeLa and PtK2 cells (American Type Culture Collection, Rockville, MD) were cultured in Iscove’s modified Dulbecco’s medium (Canadian Life Technologies, Burlington, Ontario) supplemented with 10% FBS (Medicorp, Montréal, Québec) and kept at 37°C in 5% CO2. Mitotic HeLa cells were obtained by incubating cells with nocodazole (Sigma-Aldrich, Oakville, Ontario) at a final concentration of 0.04 μg/ml for 16 hours. Heat shock treatment was carried out by incubating HeLa cells for 45 minutes at different temperature (from 41°C to 47°C) in the same medium as above.

Antibodies
The mouse monoclonal antibody (mAb) MPM-2 (IgG1) was purchased from DAKO (Carpinteria, CA). mAb CC-3 (IgG2a) was obtained after immunization of a Balb/C mouse with pharyngeal regions isolated from 3-day-old chick embryos in a search for developmental markers (Thibodeau and Vincent, 1991). mAb POL3/3 (IgG1) recognizes RNAP II largest subunit in a conserved region, located outside the CTD and was kindly provided by E. K. F. Bautz (Krümer et al., 1980).
mitosis, allows the visualization of staining associated with the molecular mass range. Immunofluorescence microscopy of whereas the major MPM-2 reactive bands are found in a lower concentrated in the high molecular mass region of the gel. In mitotic HeLa cells, the CC-3 immunoreactive material is abundant during mitosis (Davis et al., 1983). We have selected to react with a set of phosphorylated proteins which are reactive with a major MPM-2 antigen is 116 kDa protein in the case of MPM-2. A hyperphosphorylated form of the RNA polymerase II largest subunit is the major protein recognized by MPM-2 during interphase.

RESULTS

Comparison of MPM-2 and CC-3 immunoreactivity

mAb MPM-2 was raised against mitotic cells and was shown to react with a set of phosphorylated proteins which are abundant during mitosis (Davis et al., 1983). We have selected mAb CC-3 for its strong immunoreactivity with mitotic cells and this antibody was also shown to react with a family of mitotic phosphoproteins with apparent molecular mass ranging from 55 to >250 kDa (Thibodeau and Vincent, 1991). The immunoblot reactivities of MPM-2 and CC-3 on mitotic proteins show rather different patterns (Fig. 1A), although the existence of common reactive species could not be excluded. In mitotic HeLa cells, the CC-3 immunoreactive material is concentrated in the high molecular mass region of the gel whereas the major MPM-2 reactive bands are found in a lower molecular mass range. Immunofluorescence microscopy of metaphase Ptk2 cells, which do not round up when entering mitosis, allows the visualization of staining associated with the mitotic structures (Fig. 1B). Both MPM-2 and CC-3 react with the centrosomes and the mitotic spindle. The contour of the chromosomes is labelled but this is more evident with CC-3. Some diffuse staining is also observed throughout the cytoplasm with both antibodies.

In interphase cells, MPM-2 staining is concentrated in discrete spots in the nucleus (Fig. 1D and see Vandé et al., 1984). Some diffuse staining is also observed in the nucleoplasm apart from the nucleoli. A very similar fluorescent pattern is obtained with CC-3 (Fig. 1D) and was previously shown to colocalize with the non-snRNP splicing factor SC-35 in the so-called nuclear speckles (Bisotto et al., 1995). Double-labelling interphase HeLa cells with CC-3 and MPM-2 shows that the nuclear colocalization is almost perfect (Fig. 1D). Longer exposures of MPM-2-mediated fluorescence are however required to compensate for its relatively weak strength in cells grown at 37°C (see below). As shown on the merged images, slight differences are observed in some cells in which some MPM-2 staining is still present in the cytoplasm. Presumably, these cells have just quitted telophase for interphase as suggested by the stronger MPM-2 staining in late telophase cells (data not shown). In addition, occasional MPM-2 reactive dots are not labelled by CC-3 (arrows in Fig. 1D). The immunoblot reactivities of both antibodies on unsynchronized cell extracts show a major reactive species around 240 kDa (Fig. 1C). The CC-3-reactive peptide, formerly named p255 (Bisotto et al., 1995), was identified as a hyperphosphorylated form of the largest subunit of RNA polymerase II (Vincent et al., 1996). Both antibodies also react with a minor species: an unidentified 180 kDa protein in the case of CC-3 (see below) and a faint 116 kDa protein in the case of MPM-2.

A hyperphosphorylated form of the RNA polymerase II largest subunit is the major protein recognized by MPM-2 during interphase.

To investigate the possibility that the major MPM-2 antigen is also RNA II large subunit, immunoprecipitations of HeLa cell homogenates were carried out with both antibodies and the immunoprecipitated fractions were reciprocally revealed by immunoblotting (Fig. 2A). mAbs CC-3 and MPM-2 immunoprecipitate the same 240 kDa protein which is also recognized by mAb POL3/3, an antibody specific to the largest subunit of RNA polymerase II whose epitope is located in a conserved region outside the phosphorylated CTD (Krämer et al., 1980). POL3/3 is a phospho-independent antibody and can thus recognize the two forms of the RNA II largest subunit: the 210 kDa hypophosphorylated form (IIa) and the 240 kDa hyperphosphorylated form (IIo) (Krämer et al., 1980; Dubois et al., 1994a). The minor IIa bands seen in both CC-3 and MPM-2 immunoprecipitated fractions on the POL3/3 immunoblot is most probably due to slight dephosphorylation of the IIo form after immunoprecipitation (Fig. 2A). The 180 kDa band recognized by CC-3 on immunoblots (Fig. 1C) is also immunoprecipitated by CC-3. The identity of this protein is not yet known, but it is not thought to be a degradation fragment since it is neither recognized by POL3/3 nor by any other antibody against the RNA II large subunit (Fig. 2A; see also Vincent et al., 1996).

As expected from their phosphodependence, incubation of the immunoprecipitated fractions with alkaline phosphatase...
abolished both CC-3 and MPM-2 reactivities on their own immunoprecipitate (Fig. 2B). POL3/3 in turn, clearly reveals the conversion from the IIo to the IIa form in each immunoprecipitated fraction. In the POL3/3 immunoblots of the dephosphorylated fractions, a faint band (arrow in Fig. 2B) migrating faster than the IIa band may correspond to isoform IIb, a breakdown product of RNAP II largest subunit not found in vivo (Corden, 1990; Kim and Dahmus, 1986). To further substantiate the identity of the antigens, both antibodies were shown to immunodeplete a lysate of its POL3/3-reactive RNAP IIo (Fig. 2C). These results confirm that the major MPM-2 interphase antigen is a hyperphosphorylated form of the RNAP II largest subunit localized to the nuclear speckles, as is the CC-3 antigen.

The MPM-2 and CC-3 epitopes of RNAP IIo are different

As it was previously observed that the CC-3 phosphoepitope of RNAP IIo diminished markedly in HeLa cells subjected to a heat shock (Dubois et al., 1997), we analyzed the reactivity of both antibodies at different temperatures to compare the behaviour of their respective epitopes. Surprisingly, MPM-2 immunoblot signal increases at higher temperatures as the CC-3 signal decreases (Fig. 3). The 180 kDa CC-3 reactive band is also diminished in heat-shocked HeLa cells. This experiment demonstrates that MPM-2 and CC-3 recognize different subsets of phosphorylated RNAP II large subunit in defining two distinct epitopes that are inversely modulated during heat shock.

The peptidyl-prolylisomerase Pin1 associates with RNAP IIo

PPIases are ubiquitous enzymes catalyzing rotation about the peptide bond preceding a proline residue and are thought to be important in protein folding, activity, assembly and/or transport (Schmid, 1995). Three distinct families of PPIases are known: the cyclophilins, the FKBPs and the parvulins (Dolinski and Heitman, 1997). Pin1, a member of the parvulin family, has been identified in a yeast two-hybrid screen as a protein that interacts with the essential NIMA protein kinase and suppresses its mitosis-promoting activity (Lu et al., 1996). Pin1 preferentially isomerises proline residues preceded by
RNA polymerase II large subunit interacts with Pin1

It appears to play an important role in regulation of mitotic progression by directly interacting with different mitosis-specific phosphoproteins including NIMA kinase, Cdc25 phosphatase, Wee1 and Plk1 (Lu et al., 1996; Shen et al., 1998; Crenshaw et al., 1998). Many of these Pin1-binding proteins are also recognized by mAb MPM-2 (Shen et al., 1998). For this reason, and because Pin1 localizes to nuclear speckles in interphase cells (Lu et al., 1996), we investigated whether it interacts with RNAP II. Moreover, the CTD of RNAP II contains multiple pS/pT-P sites which may be targets for this isomerase. Pin1 cDNA was obtained by RT-PCR and cloned into the pET19b+ vector allowing the expression of a N-terminal His-tagged protein. Once immobilized on a His-bind resin (Ni²⁺), His-tagged Pin1 was incubated with a HeLa cell homogenate solubilized in a CHAPS-containing buffer. As seen on the CC-3 and MPM-2 immunoblots of Fig. 4, the IIo form of the RNAP II was retained on the Pin1 column and the POL3/3 immunoblot revealed that most, if not all, Pin1-binding species were of the hyperphosphorylated form. The unidentified CC-3-reactive p180 also interacts with the PPIase. Controls were carried out by saturating a His-bind resin with an irrelevant bacterial His-tagged protein and incubating with a HeLa cell homogenate as for Pin1. Samples from the initial homogenate, flow through, washes and eluted fractions were subjected to immunoblot with mAb POL3/3. The specificity of the association between Pin1 and the RNAP II was confirmed since neither IIo nor IIa forms were retained on the control resin.

The same experiments were carried out with homogenates from heat shocked HeLa cells (Fig. 4). The heat treatment increases the overall phosphorylation levels of IIo (Dubois et al., 1997) as well as its MPM-2 antigenicity (Fig. 3). The MPM-2 signal is weak at lower temperatures but increases as the heat shock temperature reaches 43°C. When probed with CC-3, the reactivity of subunit IIo and of the 180 kDa progressively decreases with increasing temperature.
Fig. 4. Pin1 interacts with the hyperphosphorylated form of the RNAP II largest subunit. Unsynchronized (>90% interphase) or heat-shocked HeLa cell homogenates were incubated with a His-binding resin saturated with the His-tagged fusion protein Pin1, washed and eluted. Samples obtained from the initial homogenates, the flowthrough, the washes and the eluted fractions were subjected to SDSPAGE and immunoblotting with MPM-2, CC-3 and POL3/3. The RNAP IIo form was specifically retained on the Pin1 column (E1). Samples from the eluted fractions contained 4 times the number of cell equivalents as the other fractions. No RNAP II species was retained by an irrelevant His-tagged fusion protein (Control). The p180 also seems to interact with Pin1 as revealed by CC-3 in the eluted fractions. IB Abs: immunoblotting antibodies, +: total HeLa cell homogenate, -: flowthrough, W: washes, E: eluted fractions.

DISCUSSION

In the last decade, the study of mitosis has been mainly directed to the identification and characterization of kinases and phosphatases modulating the mitotic process. However, less is known about the targets of these regulatory enzymes and the way they perform cell division. Monoclonal antibodies that recognize phosphoepitopes appearing during the G2/M transition have been proposed to represent remarkable probes to identify and characterize these targets, as the phosphoproteins bearing the antigenic determinants are likely to contain a common site phosphorylated by a single or related kinases. mAb MPM-2 was originally prepared against mitotic HeLa cell extracts and was shown to react with a large family of mitotic proteins in a phosphorylation-dependent manner (Davis et al., 1986). A few of these phosphoproteins have been identified to date and comprise mitotic regulators and effectors (see the Introduction for detailed references).

A key finding of the present study is that a hyperphosphorylated form of RNAP II largest subunit represents the major interphase protein reactive with MPM-2. From the observation that mAbs MPM-2 and CC-3 showed a similar punctate staining of interphase nuclei and a strong increase in the immunofluorescence of mitotic cells, we hypothesized that both monoclonals may react with related antigens. The identity of the major interphase MPM-2 antigen as RNAP IIo was demonstrated by reciprocal immunoprecipitation with CC-3, previously shown to react with a hyperphosphorylated form of RNAP II in interphase cells (Vincent et al., 1996) and by immunodepletion by MPM-2. The results were corroborated by immunoblotting with the RNA polymerase II large subunit specific mAb POL3/3. We have also observed that MPM-2 reacts with RNAP IIo in CC-3-immunoprecipitates prepared from mitotic cells (data not shown).

Results from many laboratories favored a broad epitope definition for MPM-2, suggesting that the MPM-2 epitope overlaps with the phosphorylation consensus sequences of many kinases (Taagepera et al., 1994; Westendorf et al., 1994; Ding et al., 1997). Several protein kinases including MAPK (Kuang and Ashorn, 1993), MEK (Taagepera et al., 1994), cdc2 (Westendorf et al., 1994) and ME-kinase-H, a kinase activated by cdc2 (Kuang and Ashorn, 1993), have been implicated in the phosphorylation of the MPM-2 epitope. In an attempt to identify a physiological kinase phosphorylating the MPM-2 epitope, Che et al. (1997) constructed a substrate formed by a fusion protein between glutathione S-transferase and a 19-residue peptide containing two representative MPM-2 epitope sequences (LTPLQ and LSPMK) overlapping with two potential MAPK phosphorylation sites. They found that the two MPM-2 epitope sequences could be phosphorylated by MAPK but not by cdc2 nor ME kinase-H, a kinase extracted from Xenopus eggs that generated MPM-2 reactivity on multiple polypeptides (Kuang and Ashorn, 1993). These results suggest that the MPM-2 epitope sequence is only part of the phosphorylation consensus sequence and that additional structural information is required for recognition and phosphorylation by ME-kinase H or other mitotic kinases (Che et al., 1997). It cannot be excluded that the epitope sequences tested in this assay were not the type phosphorylated by such kinases. This would imply that subtypes of the MPM-2 epitope exist, that the antibody could not discriminate. These results also provide an explanation for the selective phosphorylation of a small subset of MPM-2 antigens by MAPK and the selective recognition of only some MAPK substrates by MPM-2 (Kuang and Ashorn, 1993). In line with this deduction, MAPK might be responsible for the acquisition of the MPM-2 epitope by RNAP IIo. The consensus sequence for MAPK phosphorylation, PXS/TP, exists integrally in the heptapeptide repeats of the CTD (YSPTSPS) and MAPK family members have been involved in the enhanced phosphorylation of the CTD of RNAP II upon serum stimulation of quiescent cells (Dubois et al., 1994b) or in response to heat-shock and chemical treatments (Trigon and Morange, 1995; Venetianer et al., 1995). In heat-shocked cells, the CTD kinase associated with the general transcription factor TFIIH is impaired concomitantly with the decrease of the CC-3 epitope while the overall phosphorylation of the RNAP II large subunit is increased by a MAPK (Dubois et al., 1997). It is tempting to relate the augmentation of MPM-2 epitope reactivity that we observed...
upon a heat treatment with this heat-shock induced CTD-kinase activity. mAbs MPM-2 and CC-3 may thus discriminate between two distinct forms of RNAP IIo, phosphorylated by a stress-activated MAPK and the TFIIF-associated kinase, respectively. These distinct phosphorylation sites may correspond to different functions of the CTD in transcription or in pre-mRNA processing.

Yaffe et al. (1997) found that Pin1, a PPlase involved in the regulation of mitosis, binds and regulates members of a highly conserved set of mitotic phosphoproteins that overlaps with antigens recognized by MPM-2. When incubated with a mitotic extract, agarose beads containing a glutathione S-transferase (GST)-Pin1 fusion protein precipitated a large number of proteins reacting with MPM-2 in immunoblot (Yaffe et al., 1997). Pin1 preferentially isomerizes proline residues preceded by phosphorylated serine or threonine in mitotic phosphoproteins. It has been suggested that phosphorylation at these specific S/T-P sites creates a binding site for Pin1, which induces conformational changes by catalyzing prolyl isomerization (Yaffe et al., 1997). In interphase cells, Pin1 localizes to the splicing factor-enriched nuclear speckle domain (Lu et al., 1996), which is also labelled by CC-3 (Bisotto et al., 1995) and MPM-2 antibodies (Fig. 1D). When incubated with interphase extracts, GST-Pin1 only precipitated a few MPM-2-reactive polypeptides including a major species around 240 kDa (Yaffe et al., 1997; Shen et al., 1998). From our observations, we anticipated that this Pin1-binding interphase protein might be the RNAP II largest subunit. This prediction was directly verified using a recombinant His-tagged Pin1 protein immobilised on a resin. When incubated with homogenates of HeLa cells, the resin specifically retained RNAP IIo, indicating that Pin1 interacts with a complex containing the hyperphosphorylated form of the polymerase. Further experiments will be needed to verify whether this interaction is direct or not. The Far Western analysis performed by Shen et al. (1998) using GST-Pin1 as a probe indicates that two major bands appearing on both sides of the 200 kDa marker are bound by the Pin1 probe on G1-phase cell extracts (see Fig. 2A in Shen et al., 1998). These bands may correspond to IIo and p180, shown here to be retained on the His-tagged Pin1 column.

Another PPlase (human SRcyp/CASP10) has been shown to interact with the CTD of RNAP II (Bourquin et al., 1997). This enzyme, and its rat homologue matrix CYP (Mortillaro and Berezney, 1998), are members of the cyclophilin family and show 93% identity in amino acid sequence. SRcyp/CASP10 was identified using the two hybrid-system as a protein interacting with the CTD (Bourquin et al., 1997). In addition to a prolylsorase cis-trans domain related to the immunophilins/cyclophilins PPIases, this protein contains a serine/arginine-rich (SR) domain, required for interaction with the CTD, similar to that found in the SR protein family of pre-mRNA splicing factors (Bourquin et al., 1997). The matrix CYP is also a SR-rich protein, and a fusion protein containing the cyclophilin domain of matrix CYP exhibits cyclosporin A-sensitive peptidyl-prolyl cis-trans isomerase activity typical of this family of PPIases (Mortillaro and Berezney, 1998). Like RNAP IIo and Pin1, both enzymes localize to the nuclear speckles. Although suspected, the interaction of SRcyp/CASP10 with the phosphorylated form of the CTD has not been demonstrated formally (Bourquin et al., 1997). Our results indicate that Pin1 interacts specifically with the hyperphosphorylated form of RNAP II. This finding is not surprising given the remarkably high content of pS/pT-P dipeptides in the phosphorylated CTD and the fact that Pin1 preferentially recognizes and isomerizes proline residues preceded by phosphorylated serine or threonine residues in synthetic peptides when compared to their unphosphorylated counterparts (Yaffe et al., 1997). Using such synthetic peptides, Yaffe et al. (1997) demonstrated the failure of members of the cyclophilin (Cyp18) and FKBP (FKBP12) families to effectively catalyze the isomerization of the pS/pT-P bond. In contrast, their catalytic activity was increased towards peptides containing S/T-P or Y/pY-P bonds, suggesting the existence of a substrate specificity distinguishing Pin1 from the cyclophilin and FKBP families (Yaffe et al., 1997). It would thus be interesting to compare the phosphorylation dependence of the CTD-interacting PPlases as they could represent complementary factors involved in the modulation of CTD conformation and therefore, in the regulation of its interactions with many such partners. The CTD was indeed demonstrated, in the recent years, to function as a platform for the assembly of multiprotein complexes that proceed to the capping, the splicing, the cleavage and the polyadenylation of pre-mRNA (Corden and Patturajan, 1997; Neugebauer and Roth, 1997; Steinmetz, 1997). By catalyzing phosphorylation-dependent conformation changes of the CTD, Pin1 may be a new important player in such complexes. Our results further suggest that the biological function of Pin1 is not restricted to a role in regulation of mitotic progression, as generally proposed.

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