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Multisite phosphorylation of Pin I-associated mitotic phosphoproteins revealed by monoclonal antibodies MPM-2 and CC-3

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Published: 01 June 2004

Received: 28 October 2003

BMC Cell Biology 2004, 5:22

Accepted: 01 June 2004

This article is available from: <http://www.biomedcentral.com/1471-2121/5/22>

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Abstract

Background: The peptidyl-prolyl isomerase Pin I recently revealed itself as a new player in the regulation of protein function by phosphorylation. Pin I isomerizes the peptide bond of specific phosphorylated serine or threonine residues preceding proline in several proteins involved in various cellular events including mitosis, transcription, differentiation and DNA damage response. Many Pin I substrates are antigens of the phosphodependent monoclonal antibody MPM-2, which reacts with a subset of proteins phosphorylated at the G2/M transition.

Results: As MPM-2 is not a general marker of mitotic phosphoproteins, and as most mitotic substrates are phosphorylated more than once, we used a different phosphodependent antibody, mAb CC-3, to identify additional mitotic phosphoproteins and eventual Pin I substrates by combining affinity purification, MALDI-TOF mass spectrometry and immunoblotting. Most CC-3-reactive phosphoproteins appeared to be known or novel MPM-2 antigens and included the RNA-binding protein p54^{nrb}/nmt55, the spliceosomal protein SAPI55, the Ki-67 antigen, MAP-1B, DNA topoisomerases II α and β , the elongation factor hSpt5 and the largest subunit of RNA polymerase II. The CC-3 mitotic antigens were also shown to be Pin I targets. The fine CC-3- and MPM-2-epitope mapping of the RNA polymerase II carboxy-terminal domain confirmed that the epitopes were different and could be generated in vitro by distinct kinases. Finally, the post-mitotic dephosphorylation of both CC-3 and MPM-2 antigens was prevented when cellular Pin I activity was blocked by the selective inhibitor juglone.

Conclusion: These observations indicate that the mitotic phosphoproteins associated with Pin I are phosphorylated on multiple sites, suggesting combinatorial regulation of substrate recognition and isomerization.

Background

One of the biggest challenge in cell biology and cancer research is still to understand how cells divide and proceed to the equal partition of their genetic material in each daughter cell. The mechanisms underlying mitosis and

cytokinesis are tightly controlled and reversible protein phosphorylation plays a major role in this regulation [1]. Early and late mitotic events are dependent on protein phosphorylation by multiple serine and threonine kinases of the NIMA, Polo and Aurora families at the head

of which sits the Cdk1/cyclin B complex [1-5]. The nature of most protein substrates of these kinases is still unknown so that the precise roles they play in the regulation of mitosis and cytokinesis remain to be clarified.

A few monoclonal antibodies (mAbs) have been raised in different laboratories against mitotic cell extracts and shown to react with subsets of proteins that are phosphorylated upon entry into mitosis [6-9]. The most characterized of these antibodies, mAb MPM-2, was selected for its preferential reactivity towards mitotic versus interphase cells [6] and shown to react with a phospho-epitope present on a set of proteins concentrated in the centrosomes, the kinetochores, the mitotic spindle and the midbody [6,10], reinforcing the idea that the structural rearrangements observed during mitosis are controlled by phosphorylation events. After twenty years of continuous use as a mitotic marker in scores of laboratories and insistent efforts to characterize the epitope(s) and the kinases involved- it is now clear that many MPM-2 antigens are important mitotic regulators and effectors. They include the Cdc25 phosphatase [11], the Cdk1-inhibitory Wee1 and Myt1 kinases [12-14], the NIMA kinase [15], the microtubule associated-proteins MAP-1 and MAP-4 [16,17], DNA topoisomerase II α and β [18], p42mapk [19], and the Cdc27 component of the anaphase-promoting complex (APC) [20]. Phosphorylation of the MPM-2 antigenic sites is thought to be functionally important as the MPM-2 antibody inhibits oocyte maturation upon microinjection and neutralizes mitosis promoting factor activity from M-phase extracts [21].

The biological relevance of MPM-2 phosphoepitopes was further reinforced when Shen and coworkers [22] showed that the peptidyl-prolyl isomerase (PPIase) Pin1 could bind and regulate many mitotic phosphoproteins also recognized by MPM-2. PPIases are ubiquitous enzymes catalyzing the cis-trans isomerization of the peptide bond preceding a proline residue and are thought to be involved in protein folding, protein assembly, protein trafficking or in the direct regulation of protein activity [23]. Pin1 is unique among prolyl isomerases in that it specifically targets proline residues preceded by a phosphoserine (pS-P) or a phosphothreonine (pT-P) [24-26]. Pin1 is a ubiquitously expressed protein that is essential for cell cycle progression in yeast and in mammalian cells [27]. Furthermore, Pin1 has been shown to interact with the essential mitotic kinase NIMA and to suppress its mitosis-promoting activity [27]. It is now believed that Pin1 acts as an essential mitotic regulator since, in addition to NIMA, it binds other MPM-2-reactive proteins with important mitotic functions including Cdc25 [22,28], Myt1, Wee1, Plk1 and Cdc27 [22]. In the recent years, it was postulated that the analysis of the phosphorylated sites recognized by both Pin1 and MPM-2 might be

a good starting point for a better understanding of the general role of phosphorylation in the mitotic processes. These efforts, mainly orchestrated by K.P. Lu and collaborators, have led to the elaboration of a novel post-phosphorylation regulatory mechanism, in which the isomerase Pin1 induces conformational changes into targeted proteins that have been first phosphorylated by proline-directed kinases [29,30].

The regulative action of Pin1 may not be confined to mitotic processes. Recent results have shown that DNA damage induces an interaction between Pin1 and p53 depending on specific pS-P motifs in p53 [31-33], indicating that phosphorylation-dependent prolyl isomerization is a signalling mechanism also operating in the genotoxic response [34]. Interestingly, it was shown that efficient binding of the p53 protein to Pin1 requires phosphorylation at all three sites of p53 involved in the interaction [32,33].

Previous work from our laboratory has shown that, in human interphase cells, Pin1 interacts with two transcription-related proteins in a phosphorylation-dependent manner: the hyperphosphorylated form of RNA polymerase II largest subunit Rpb1 [35], and a phosphorylated form of the transcription elongation factor hSpt5 [36]. Moreover, studies in yeast provided genetic evidence that Ess1, the yeast homolog of Pin1, interacts with the transcription machinery as most genetic suppressors of *ess1* mutants encode proteins involved in transcription [37] consistent with the earlier observation that *ess1* mutants are defective in mRNA processing [38,39]. These results also indicate that Pin1 operates at multiple levels.

The discovery of the association of Pin1 with Rpb1 and hSpt5 was made possible thanks to the availability of a monoclonal antibody (mAb CC-3) selected for its strong phospho-dependent reactivity with mitotic cells and with nuclear speckles of interphase cells [7]. The interphase CC-3 antigens located in the nuclear speckles were identified as Rpb1 and hSpt5 [35,36] but the numerous CC-3 mitotic antigens have not been identified yet. As CC-3 behavior was somewhat reminiscent of that of MPM-2, a comparison of both mAbs initially revealed that they share the hyperphosphorylated form of Rpb1 as their major interphase antigen [35]. However, their epitope on Rpb1 appeared to be different since upon a heat shock, MPM-2 reactivity was increased whereas that of CC-3 was diminished, suggesting that both antibodies may discriminate between distinct functional forms of RNA polymerase II [35,40].

In the present work, MALDI-TOF mass spectrometry and immunoblotting were combined to identify the major CC-3 mitotic antigens. They included a few proteins

involved in transcription and/or mRNA maturation and most of them also appeared to be known or novel MPM-2 antigens. The fine CC-3- and MPM-2-epitope mapping of the carboxy-terminal domain (CTD) of Rpb1 confirmed that the epitopes are different and can be generated *in vitro* by distinct kinases. Pulldown experiments suggest that the CC-3 mitotic antigens are also Pin1 targets. Finally, incubation of HeLa cells with juglone, an irreversible Pin1 inhibitor [41], prevented the dephosphorylation of both mitotic MPM-2 and CC-3 epitopes. These observations suggest that Pin1 might promote M phase exit by stimulating the dephosphorylation rate of the mitotic regulators and/or effectors on multiple sites revealed by mAbs CC-3 and MPM-2.

Results

MAb CC-3 is a mitotic marker

CC-3 and MPM-2 are phosphodependent mouse monoclonal antibodies strongly reactive with mitotic cells [6,7]. In interphase cells, the reactivity of both antibodies is confined to discrete nuclear speckles and has been previously shown to be mainly mediated by phosphoepitopes located on the CTD of Rpb1 [35]. The immunoblot reactivities of CC-3 and MPM-2 on mitotic extracts reveal that their mitotic antigens are much more numerous and the overall patterns are sufficiently distinct to expect that CC-3 and MPM-2 epitopes are different [35]. In addition, the comparison of CC-3 and MPM-2 immunofluorescence reactivities at different stages of the cell cycle reveals subtle differences between otherwise similar patterns. For example, the centrosomes of interphase cells [35] as well as the midbodies during cytokinesis (data not shown) are labelled more intensely with MPM-2.

Since several MPM-2 antigens identified up to now play an important role in cell division, it appeared relevant to identify the mitotic phosphoproteins recognized by mAb CC-3. The CC-3-antigens were affinity-purified from mitotic extracts and separated by one-dimensional SDS-PAGE. The major bands were submitted to in-gel tryptic digestion and processed for MALDI-TOF mass spectrometry (Table 1). They were identified as the proliferation-associated Ki-67 protein, the microtubule-associated protein MAP-1B, the spliceosomal protein SAP155 and the RNA-binding protein p54^{nrb}/nmt55. As suitable antibodies were available, the nature of the two latter was confirmed by immunoblotting (Fig. 1A). NMT-5, a polyclonal rabbit antibody raised against the middle part of the p54^{nrb}/nmt55 protein [42] and an antibody to SAP155 [43] were used to confirm the presence of slower migrating forms of both proteins in the mitotic CC-3 immunoprecipitate (Fig. 1A, lower panels). These CC-3-reactive bands most probably represent mitosis-specific phosphorylated forms of p54^{nrb}/nmt55 and SAP155. Interestingly, the former was also detectable in the MPM-

2 mitotic immunoprecipitate (Fig. 1A) and may thus represent a novel MPM-2 antigen. Recently, p54^{nrb}/nmt55 was shown to bind to the CTD of Rpb1 [44]. To eliminate the possibility that it merely co-purified with CC-3-reactive Rpb1, p54^{nrb}/nmt55 was immunoprecipitated from mitotic extracts with the anti-p54^{nrb}/nmt55 antibody and immunoblotted. As shown on figure 1B, the p54^{nrb}/nmt55 band reacted with both CC-3 and MPM-2 antibodies. In addition, it has been possible to generate the CC-3-epitope by incubating recombinant p54^{nrb}/nmt55 with a mitotic extract and ATP (Proteau A. et al., unpublished results).

Since the CC-3 immunoreactivity in the high Mr region is rather complex [35], alternative methods to MALDI-TOF mass spectrometry were used to identify other CC-3-antigens. A few candidate proteins in the 170–220 kDa range of SDS gels were already suspected because they had been described as MPM-2 antigens (DNA topoisomerases II α and β) or because they reacted with mAb CC-3 in interphase cells (Rpb1 and hSpt5). Thus, immunoblots using anti-DNA topoisomerase II α and β showed reactive bands in the MPM-2 as well as in the CC-3 mitotic immunoprecipitates. Likewise, Rpb1 and hSpt5 also proved to be mitotic CC-3- and MPM-2-antigens (Fig. 1A). Therefore, it turns out that most of the CC-3 mitotic antigens identified up to now are also MPM-2 reactive: p54^{nrb}/nmt55, Rpb1 and hSpt5 as novel mitotic MPM-2 antigens and MAP-1B, Ki-67 and DNA topoisomerases II as already known MPM-2 antigens. The spliceosomal protein SAP155 is the sole mitotic antigen specifically recognized by CC-3 identified to date. These results prompted us to meticulously compare the nature of the CC-3 and MPM-2 epitopes on a common mitotic and interphase antigen, the CTD of Rpb1.

Fine CC-3 and MPM-2 epitope mapping on the CTD of Rpb1

To map the MPM-2 and CC-3 epitopes of Rpb1, recombinant GST-tagged fragments of the CTD of human Rpb1 were used (Fig. 2A). The N-CTD construct encodes the amino-terminal 26 repeats of the CTD, eighteen of which being identical to the consensus heptad YSPTSPS. The C-CTD construct corresponds to the carboxy-terminal 26 repeats and includes only three heptads conform to the consensus sequence. When the fragments were incubated with a mitotic extract and ATP, CC-3-reactivity was strongly induced on the C-CTD peptide but less intensely on the N-CTD (Fig. 2B). These results are consistent with a previous observation that starfish cdc2 kinase could generate a strong CC-3 signal on the C-terminal part of the CTD [45]. On the other hand, the MPM-2 epitope was weakly generated using the mitotic extract but a strong signal was obtained using the Cdk9/cyclinT1 kinase (Fig. 2B). Here, the reactivity appeared stronger on the N-CTD.

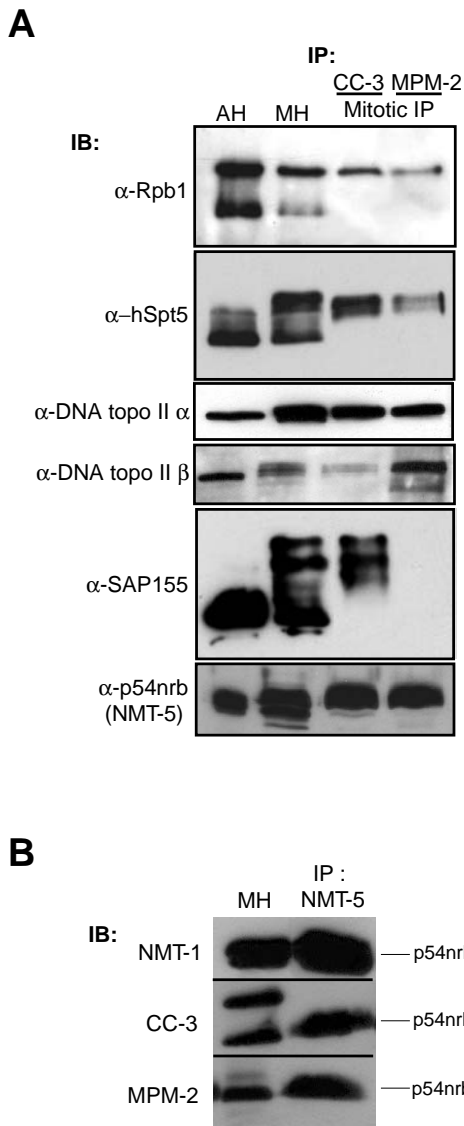


Figure 1
Confirmation of the identity of some CC-3 antigens by immunoblotting. (A) Mitotic cell extracts were immunoprecipitated with either CC-3 and MPM-2 and immunoblotted with different antibodies against proteins suspected to be CC-3 antigens. Lysates from asynchronous (AH) and mitotic (MH) HeLa cells were also electrophoresed and immunoblotted. Both CC-3 and MPM-2 immunoprecipitates contained hyperphosphorylated forms of Rpb1 and hSpt5, the α and β isoforms of DNA topoisomerase II and p54^{nrb}/nmt55. However, only CC-3 is able to precipitate mitotic SAPI55. (B) Mitotic cell extracts were immunoprecipitated with antibody NMT-5, a rabbit polyclonal raised against p54^{nrb}/nmt55 and immunoblotted with MPM-2, CC-3 and NMT-1, a monoclonal to p54^{nrb}/nmt55. Note that a still unidentified mitotic antigen migrating more slowly than p54^{nrb}/nmt55 in the mitotic HeLa cell sample is reactive with CC-3 in this region of the gel (asterisk). IP: immunoprecipitating mAb, IB: immunoblotting mAb.

In both cases however, the epitopes seemed somewhat repetitive as CC-3 and MPM-2 reacted with both fragments incubated with the mitotic kinases or with Cdk9, respectively (Fig. 2B). As expected, neither the unphosphorylated GST-CTDs (arrows in Fig. 2B), nor the phosphorylated GST alone (data not shown) were reactive with the antibodies.

The fine epitope characterization of the antibodies was carried out by probing immobilized CTD peptides with CC-3 and MPM-2 (Fig. 3A). These peptides contained four YSPTSPS heptad repeats phosphorylated at serines 2 and/or 5 since the CTD repeats are thought to be predominantly phosphorylated at these positions [46-48]. In agreement with previous observations [49], CC-3 was shown to recognize the CTD repeats phosphorylated on Serine-2 and the reactivity was decreased by 50% when both Serine-2 and Serine-5 were phosphorylated (Fig. 3A,3B). On the other hand, the strongest MPM-2 signal was obtained when both Serine-2 and Serine-5 were phosphorylated. Surprisingly, at the lowest peptide concentration, MPM-2 reacted only with the unphosphorylated CTD form and the reactivity remained constant at higher peptide concentrations. These results, independently corroborated in Dr Buratowski's laboratory (personal communication), were unexpected since MPM-2 does not react with unphosphorylated recombinant N-CTD and C-CTD (Fig. 2B). In all likelihood, the short CTD peptides used in this assay do not perfectly imitate the structural complexity of an integral CTD. One possible explanation is that MPM-2 does not recognize the phosphoserine but a proline conformation affected by the phosphorylation of a serine residue. It has to be mentioned that a preincubation of the immobilized CTD peptides with recombinant Pin1 protein decreased the reactivity of both antibodies with the phosphorylated peptides but not that of MPM-2 with the unphosphorylated peptide (data not shown).

CC-3 antigens are also targets for the cis/trans peptidyl-prolyl isomerase Pin1

The preceding results support the idea that CC-3 and MPM-2 are two different mitotic markers with distinct substrate specificities to phosphorylated serines or threonines. The peptidyl-prolyl isomerase Pin1 specifically isomerizes the pS/pT-P peptide bond. It is composed of an amino-terminal WW domain responsible for the binding to pS/pT-P sites on substrates and a unique peptidyl-prolyl isomerase domain (PPIase) that specifically cis/trans isomerizes pS/pT-P bonds [26]. Pin1 binds to a subset of mitotic phosphoproteins, many of which are also recognized by MPM-2 [22]. A pull-down experiment was carried out to verify whether Pin1 could also bind to CC-3 mitotic antigens (Fig. 4A). Recombinant Pin1 was immobilized on a resin and further incubated with mitotic HeLa cell

Table 1: Novel CC-3 mitotic antigens identified by MALDI-TOF-MS and ProFound research engine

| Identified CC-3 mitotic antigens | Accession number | Theoretical molecular weight | Number of matched peptides | Measured peptides | Min. sequence coverage (%) | Estimated Z score | Probability |
|----------------------------------|------------------|------------------------------|----------------------------|-------------------|----------------------------|-------------------|-------------|
| Ki-67 long form | 14746822 | 360.9 kDa | 37 | 86 | 11 | 2.04 | 1.0e+000 |
| Ki-67 short form | 14746812 | 321.5 kDa | 19 | 32 | 4 | 1.59 | 1.0e+000 |
| MAP-1B, isoform I | 5174525 | 271.8 kDa | 22 | 86 | 11 | 1.13 | 1.0e+000 |
| SAP155 | 14042921 | 146.5 kDa | 15 | 56 | 11 | 0.78 | 0.99e-000 |
| P54 ^{nrb} /nmt55 | 13124797 | 54.2 kDa | 8 | 31 | 15 | 1.96 | 1.0e+000 |

homogenates prior to washes and elution. Western blots clearly showed that both groups of mitotic antigens were depleted from the extract by incubation with recombinant Pin1. To confirm that Pin1 binding was mediated by its WW domain, a Pin1 recombinant protein mutated in this domain (W34A) was shown to be less effective than the wild type protein and a mutant of the PPIase domain (C113A) in pulling down mitotic CC-3 and MPM-2 antigens (Fig. 4A). However, traces of Pin1 substrates were still visible in the W34A eluted fraction despite a final 60 mM imidazole wash. This binding could be due to non-specific binding activity of the Nickel column although an irrelevant protein (Pbp5), loaded on Nickel beads as a control His-tagged fusion protein, retained less mitotic proteins than the W34A mutant (Fig. 4A, right panel). Actually, the main band retained by the His-tagged Pbp5-Nickel column was p54^{nrb}/nmt55, whose sequence contains a stretch of histidine residues in its N-terminal extremity [50]. Alternatively, the presence of Pin1 substrates in the W34A eluted fraction could be either due to residual binding activity of the mutated WW domain or to the intact catalytic domain of the construct as it was shown that the PPIase domain can also bind, although at a lower affinity, to the same pS/pT-P-containing substrates [30]. A Coomassie blue stained gel of the eluted fractions showed that an equivalent amount of Pin1, mutant proteins and Pbp5 were used in each pulldown experiment (Fig. 4B).

To verify whether the interaction between Pin1 and the CC-3- and MPM-2-reactive mitotic phosphoproteins was direct and driven by the WW domain, immunopurified CC-3 and MPM-2 antigens were subjected to a blot overlay experiment using Pin1 and the WW mutant as probes (Fig. 4C). The specificity of the Pin1 overlay was confirmed by the absence of reactivity of the molecular weight marker bands (Fig. 4C, lane M). In addition, a more robust and complex reactivity was noticed, as expected, on a mitotic extract compared to an interphase extract when equivalent protein amounts were loaded (Fig. 4C, lanes 1 and 2). The strong binding of Pin1 to mitotic CC-3 and MPM-2 immunoprecipitated antigens suggests that Pin1 association with these phosphoproteins is direct (Fig. 4C, lanes 5 and 6). Both CC-3 and MPM-2 interphase immu-

noprecipitates (lanes 3 and 4) were also processed and the result confirmed that Rpb1 and at least one other interphase protein, most probably hSpt5, interact directly with Pin1 (Fig. 1 and [36]). The WW mutant W34A showed a marked reduction of binding in the blot overlay experiment (Fig. 4C, middle panel), supporting the view that CC-3 mitotic antigens are, like MPM-2 antigens, direct targets of Pin1 and that this interaction is driven by the WW domain. Finally, it has to be noticed that the SAP155 protein, which appeared to be a CC-3-positive and a MPM-2-negative antigen (Fig. 1A) was pulled down by Pin1 as revealed by Western blot (Fig. 4D), suggesting that the MPM-2-immunoreactivity may not be a prerequisite for Pin1 binding. In addition, DNA topoisomerase II α , shown to be a common antigen to both antibodies (Fig. 1), was also specifically pulled down by Pin1 (Fig. 4D).

CC-3 and MPM-2 antigens are not dephosphorylated after mitosis in the presence of juglone, a Pin1 specific inhibitor

Juglone is a naphthoquinone which specifically inhibits the members of the parvulin PPIase family [41]. We asked whether Pin1 inhibition by juglone might affect the phosphorylation level of CC-3 and MPM-2 antigens upon mitosis exit. Mitotic HeLa cells were harvested after nocodazole treatment and incubated for a 5-hour period in the presence or absence of juglone. In juglone-free medium, the cells were observed to adhere to the plastic dish whereas in juglone-containing medium, the cells stayed rounded and unattached (data not shown). Western blots of the resulting samples are shown in figure 5. As expected, the immunoreactivity of both antibodies decreased when mitotic cells were chased into G1 in the absence of juglone (Fig. 5: compare lanes 1 to lanes 2). In the juglone-treated cells however, the phosphorylation level of most mitotic CC-3 and MPM-2 antigens remained unaltered (Fig. 5, lanes 3 and 4). Moreover, a Western blot using antibody POL3/3, a non phosphodependent mAb against Rpb1, showed that the hyperphosphorylated form of Rpb1 was the only detectable species after a 5-hour incubation in the presence of the inhibitor. Thus, juglone might favor CC-3 and MPM-2 phosphoantigen accumulation at the exit of mitosis, possibly by making substrates less susceptible to phosphatase activity in the absence of Pin1.

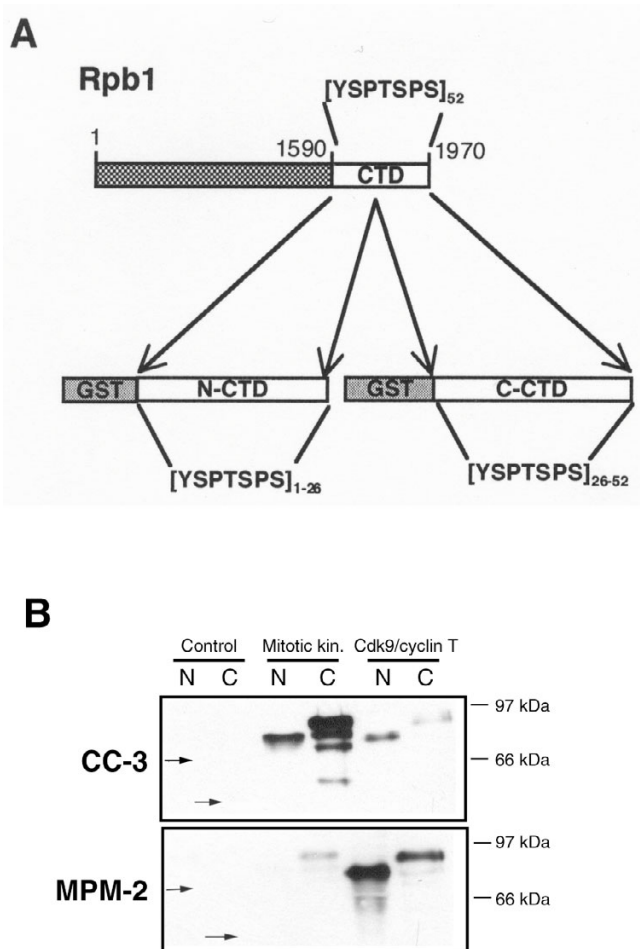


Figure 2
The CC-3 and MPM-2 epitopes of recombinant Rpb1 are generated by different kinases. (A) Schematic representation of the N- and C- moieties of the CTD of Rpb1 fused to GST tags for in vitro phosphorylation experiments. The N-CTD comprised the first 26 repeats of the consensus YSPTSPS and the C-CTD comprised the more degenerated last 26 repeats. (B) N-CTD and C-CTD recombinant proteins were phosphorylated with a mitotic extract or with Cdk9/cyclin T1 and subjected to SDS-PAGE and immunoblotting with CC-3 and MPM-2. Arrows indicate the position of unphosphorylated N-CTD and C-CTD controls, revealed by Ponceau red staining prior to immunostaining. Mitotic kinases strongly enhanced CC-3-reactivity in the C-CTD fragment whereas Cdk9/cyclin T1 generated more efficiently the MPM-2 epitope, especially in the N-CTD fusion protein. N: N-CTD, C: C-CTD.

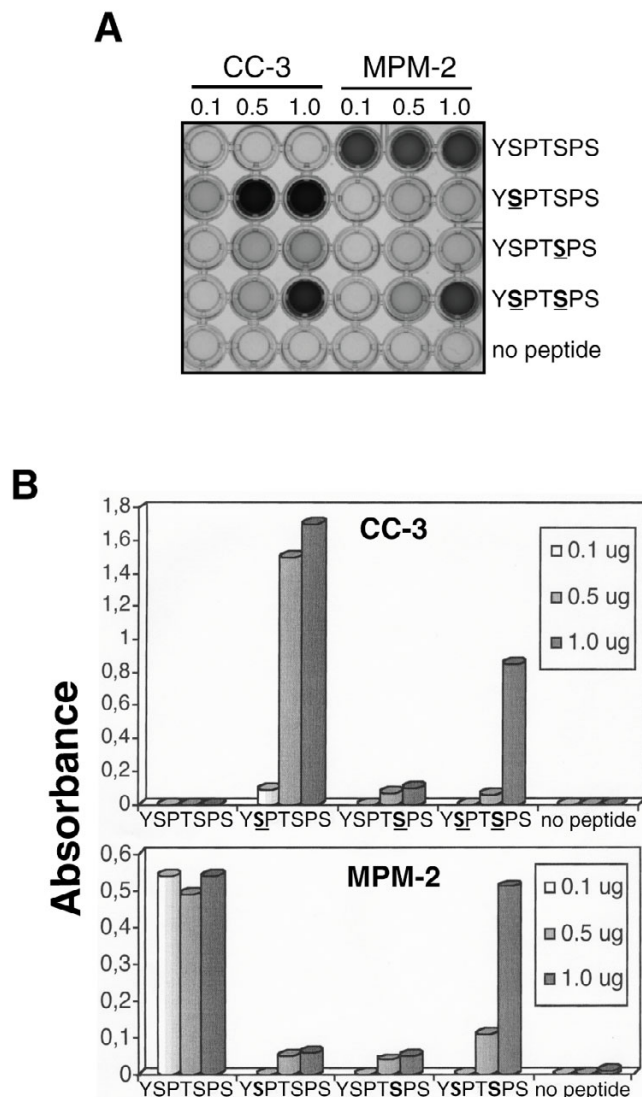


Figure 3
Fine mapping of CC-3 and MPM-2 epitopes on Rpb1 consensus heptad repeats. (A) Biotinylated CTD peptides were immobilized on a streptavidin-coated ELISA plate at three different concentrations and incubated with MPM-2 and CC-3. These peptides contained four YSPTSPS repeats, phosphorylated either on the serine residues at position 2 (YSPTSPS), at position 5 (YSPTSPS) or at both positions (YSPTSPS). An unphosphorylated peptide was used as a control (YSPTSPS). CC-3 was mainly reactive with peptides phosphorylated on serine 2 of the consensus repeat whereas MPM-2 reacts preferentially with the peptide phosphorylated on both serines 2 and 5. Surprisingly, MPM-2 also reacted with the unphosphorylated peptide in a concentration independent manner (see text). (B) Histogram of the CC-3 and MPM-2 reactivities shown in (A).

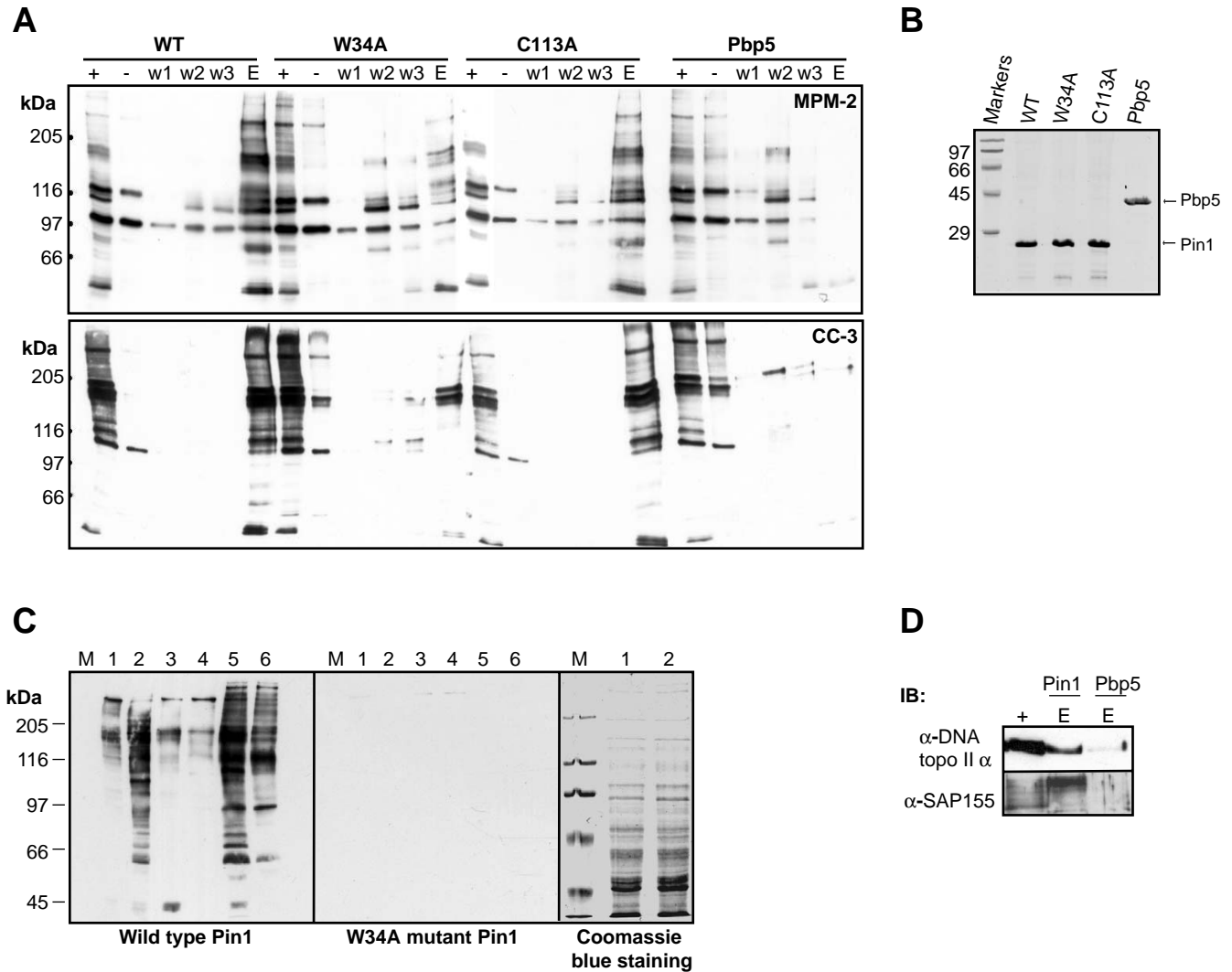


Figure 4

The direct interaction of Pin1 with CC-3 and MPM-2 mitotic antigens involves its WW domain. (A) Mitotic HeLa cell homogenates were incubated with histidine-binding resins saturated with different his-tagged proteins. WT: wild type Pin1; W34A: a Pin1 protein mutated in the WW domain; C113A: a Pin1 protein mutated in the PPlase domain; Pbp5: an irrelevant protein used as a control. Aliquots from the initial lysate (+), the flowthrough (-), the washes (w1 to w3) and the eluted fractions (E) were subjected to SDS-PAGE and immunoblotting. The Pin1 WT and C113A resins retained more MPM-2 and CC-3 mitotic antigens than the WW mutant W34A and the Pbp5 control resins. (B) Coomassie blue staining of a 15% acrylamide gel of the fractions eluted from the different resins in (A) showing that the initial amounts of the bound his-tagged proteins were similar. (C) Blot overlay experiments using either the wild type or the WW mutant Pin1 protein as a probe showing the direct interaction of wild type Pin1 with CC-3 interphase (lane 3) and mitotic (lane 5) antigens as well as with MPM-2 interphase (lane 4) and mitotic (lane 6) antigens, obtained by immunoprecipitation. Lanes 1 and 2 correspond to total homogenates prepared from asynchronous and mitotic HeLa cells respectively. Pin1 did not bind to the Mr markers (lane M) nor to the major bands of the total homogenates (lanes 1 and 2) visualized by Coomassie blue staining (right panel) but did bind strongly to the CC-3 and MPM-2 antigens. No binding was observed when the W34A mutant protein was used as a probe (middle panel). (D) Pin1 specifically binds to SAPI55 and DNA topoisomerase II α . Same experiment as that described in (A) except that the eluted fractions from the his-tagged Pin1 and Pbp5 resins were immunoblotted with antibodies to DNA topoisomerase II α and SAPI55.

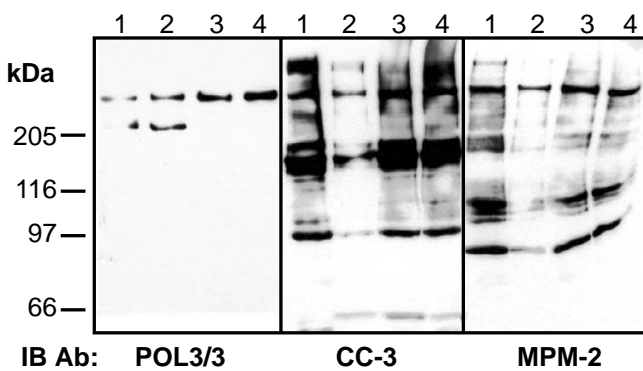


Figure 5
Juglone, a Pin1 inhibitor, prevents exit from mitosis and dephosphorylation of CC-3 and MPM-2 mitotic antigens. Mitotic HeLa cells were withdrawn from nocodazole-containing medium and incubated for 5 hours in the absence (lane 2) or in the presence of juglone at 10 (lane 3) or 20 $\mu\text{g/ml}$ (lane 4). The cells were then solubilized in sample buffer and processed for immunoblotting with CC-3, MPM-2 and POL3/3, a monoclonal antibody recognizing both hypo- and hyperphosphorylated forms of Rpb1. Lane 1 represents a sample of the cells removed before the 5-hour incubation and thus corresponds to $t = 0$. The juglone treatment prevented the dephosphorylation of the MPM-2 and CC-3 antigens after removal of nocodazole. The POL3/3 immunoblot revealed that Rpb1 became hyperphosphorylated after incubation with juglone (lanes 3 and 4) whereas the 5-hour chase in the absence of juglone (lane 2) allowed the cells to recover the usual 1:1 ratio of hypo- to hyperphosphorylated forms of Rpb1.

Discussion

Many observations strongly support the idea that mAb CC-3 reacts with a phosphodependent epitope different from that recognized by MPM-2. First, in immunofluorescence studies of HeLa cells, MPM-2 labelled additional structures when compared to CC-3. Second, the Western blot patterns of both antibodies showed major differences [35]. Third, some proteins may react exclusively with either CC-3 or MPM-2 as illustrated by the CC-3-exclusive SAP155 antigen. Fourth, a heat shock treatment of HeLa cells increased MPM-2 immunoreactivity of Rpb1 whereas that of CC-3 was decreased [35,40]. Finally, the experiments using the CTD recombinant fragments and peptides of Rpb1 showed that MPM-2 and CC-3 epitopes can be differentially generated in vitro by distinct kinases. Altogether, these observations indicate that the CC-3 and MPM-2 phosphoepitopes are different and that a single mitotic phosphoprotein may be phosphorylated on several sites by different kinases, suggesting a combinatorial regulation of the mitotic events. Multisite phosphorylation of proteins is a common mechanism to increase the

regulatory potential of proteins [51] and was envisaged a few years ago for the regulation of cell division by Stukenberg and coworkers [52] after they have systematically undertaken the identification of mitotic phosphoproteins using a screening procedure based on either electrophoretic mobility shifts or MPM-2-epitope recognition. Their study revealed that MPM-2 recognition was not a hallmark of all mitotic phosphoproteins and that no common motifs existed among the substrates that are phosphorylated during mitosis. On the other hand, most of the proteins they have identified contained several S-P or T-P groupings and displayed multiple phosphorylated forms [52]. This observation could explain the variety of immunoreactivities noticed with both CC-3 and MPM-2 and the association of their antigens with Pin1 as seen in the overlay experiments. More recently, Stukenberg and Kirschner [53] found that a Pin1-catalyzed conformational change - monitored among other assays by MPM-2 differential reactivity- was located in such a "SP-rich" region on Cdc25. Whether Pin1 catalyzes similar conformational changes on other mitotic (or interphase) substrates remain to be demonstrated but mAb CC-3 certainly constitutes an additional probe to reach this objective.

A striking feature of the major CC-3 antigens identified in our study is that many of them localize to the nucleus in interphase cells and are associated with transcriptional and/or pre-mRNA processing events. This finding is consistent with one conclusion of Stukenberg and coworkers [52] that is, a large percentage of the proteins phosphorylated during mitosis are transcriptional and translational regulators. Recently, the splicing factor SAP155 was shown to be highly phosphorylated in mitotically arrested COS-1 cells and HeLa cells compared to asynchronous cells [54]. The phosphorylated sites lie in a TP-rich domain that mediates binding to NIPP1, a splicing factor that functions also as a PP1 phosphatase regulator. The use of the Cdk inhibitor roscovitine and the in vitro phosphorylation of SAP155 with Cdk1 strongly support the idea that Cdk1/cyclin B is the major SAP155 mitotic kinase. The relevance of NIPP1-SAP155 interaction may contribute to the mitotic silencing of splicing [54]. These results support the present finding that SAP155 is mitotically phosphorylated in mitosis on the mAb CC-3-epitope and that Cdk1 could be the major CC-3 mitotic kinase (see below). Among the CC-3-reactive proteins that we have identified, the splicing and transcription-associated protein p54^{nrb}/nmt55 and the human transcription elongation factor hSpt5 appeared to be novel MPM-2 antigens. The former possibly corresponds to a previously described MPM-2-reactive p55 protein shown to be threonine phosphorylated upon entry in mitosis [55]. The p54^{nrb}/nmt55 protein contains one putative Cdk1 consensus site (QNHTPRK) in its most N-terminal part and several T-P pairs in a proline-rich

domain in its carboxy terminus [50]. This protein contains two RNA recognition motifs within a 320-aa stretch also found in the splicing factor PSF. Its expression was shown to be down regulated in a subset of breast tumors [42]. The mouse homolog of p54^{nrb}/nmt55, NonO, was independently isolated as a DNA binding transcription factor [56], able to enhance the association of many DNA-binding proteins to their targets [57]. Interestingly, the p54^{nrb}/nmt55 and PSF proteins of HeLa cells have recently been shown to specifically bind the CTD of Rpb1 and it has been suggested that these proteins could provide a direct link between transcription and pre-mRNA processing [44]. This property could be the cause of the purification of p54^{nrb}/nmt55 by CC-3-affinity since the CTD, as shown on figure 1, contains several CC-3 epitopes. However, we think that p54^{nrb}/nmt55 is a bona fide CC-3 antigen and that it did not merely co-purify as a Rpb1 partner because it was shown to be CC-3-reactive in Western blots when immunoprecipitated from mitotic extracts with the anti-p54^{nrb}/nmt55 antibody. In addition, we have recently been able to generate the CC-3-epitope on a recombinant p54^{nrb}/nmt55 protein in vitro using ATP and a mitotic extract as a source of kinases (Proteau A., unpublished results).

The repetitive nature of the CTD, which harbors an impressive series of phosphorylatable S-P or T-P pairs, makes Rpb1 one of the more complex CC-3 and MPM-2 antigens. The results described in figure 2 revealed a stronger CC-3 affinity for the GST-CTDs phosphorylated by a mitotic extract than by Cdk9/cyclinT1, as opposed to MPM-2 reactivity. Maybe most importantly, the generation of the CC-3 epitope by the mitotic extract was stronger on the C-CTD, which possesses only three consensus YSPTSPS repeats. As starfish Cdk1 kinase could generate a strong CC-3 signal on the C-terminal part of the CTD in vitro [45], this observation could mean that Cdk1 is the major kinase of our mitotic extract involved in phosphorylation of the CC-3 epitope. This segment of the CTD contains mostly heptads diverging from the consensus at position 7 and it was shown by Zhang and Corden [58] that the best substrate for Cdk1 was a CTD peptide with a lysine at this position (YSPTSPK). It has previously been shown that TFIIF also increased CC-3 immunoreactivity at the C-terminal half of the CTD in vitro [45] and Cdk7, a component of TFIIF, preferentially phosphorylates synthetic heptapeptides containing a lysine at position 7 [59]. Altogether, these observations suggest that the CC-3 epitope can be generated by a phosphorylated serine at position 2 of the consensus repeat, as demonstrated by the ELISA reactivity of the (YSPTSPS)₄ peptides and in a previous study [49], but the affinity of the antibody is most probably enhanced by the natural context of the non canonical repeats of the C-CTD and presumably, by a similarly permissive environment in the mitotic antigens. In

line with this suggestion, it has to be noticed that mAb H5, also directed to Serine-2 of the CTD [49], revealed a single reactive band corresponding to Rpb1 when blotted on mitotic cell extracts (not shown). Therefore, in spite of the identical specificity of H5 and CC-3 towards the Serine-2 of the CTD, CC-3 clearly recognizes a different motif surrounding the pS-P bond since it reacts with many more mitotic phosphoproteins.

The Cdk9-induced MPM-2 epitope appeared stronger in the highly conserved N-terminal part of the CTD in agreement with a previous report showing that the Cdk9 kinase complex preferentially phosphorylates this portion of the CTD [60]. In addition to demonstrating the different nature of both epitopes, these observations support the view that whichever kinases are involved, MPM-2 and CC-3 could react with functionally distinct isoforms of hyperphosphorylated Rpb1. Remarkably, the loss of the CC-3 epitope and the concurrent increase of MPM-2 reactivity noticed after a heat shock [35,40], could mean that the bulk of phosphorylated serine residues shift from the C-terminal half of the CTD to the N-terminal part under these conditions. Work is under progress to verify if the stress-induced hyperphosphorylation of the CTD contributes to the appearance of the MPM-2 epitope. It has to be kept in mind that such a net increase in the phosphorylation level of Rpb1 could result from the stress-induced activation of kinases [61] and/or inactivation of a CTD phosphatase [62]. According to the ELISA results showing a stronger MPM-2 and a weaker CC-3 reactivity when the CTD peptides are phosphorylated on both serines 2 and 5, it could also be that the heat shock treatment increases the phosphorylation level of Rpb1 on both serines 2 and 5, providing an alternative explanation to the fact that MPM-2 immunoreactivity is increased by this treatment and that of CC-3 is decreased. Whether the heat shock-induced alterations of the CTD phosphorylation contribute to the modifications of gene expression characteristic of the heat shock response remains to be explored.

As CC-3 and MPM-2 antigens were shown to be targets of the peptidyl-prolyl isomerase Pin1, it was of interest to examine their dephosphorylation at the exit of mitosis. It has indeed been shown that Pin1 can alter the dephosphorylation rate of some of its binding partners [63-65]. When nocodazole-arrested mitotic cells were chased into G1 in the presence of juglone, a Pin1 inhibitor [41], we observed that most CC-3 and MPM-2 antigens remained in the phosphorylation state contrary to the situation in juglone-free medium. In addition, the cells treated with the inhibitor remained rounded and did not attach to the culture dish. Because juglone potentially interacts at other levels [66], these results should be interpreted with caution, but they are consistent with the recent suggestion that Pin1 could regulate the activity of phosphoproteins

distributed at different mitotic structures by controlling the accessibility of pS/pT-P motifs to phosphatases [67]. Further experiments using more specific Pin1 inhibitors such as those recently discovered by Uchida and collaborators [68] will help to resolve this issue.

Conclusions

We provided evidence that another monoclonal antibody, CC-3, recognizes a different epitope borne by a subset of mitotic phosphoproteins which, for the most part, are also MPM-2 antigens and Pin1-reactive, suggesting the existence of a combinatorial regulative mechanism. The availability of different phosphorylation-state-specific antibodies to mitotic antigens may thus be crucial to elucidate the functional impact of multisite phosphorylation of regulators and effectors on cell division.

Methods

Cell cultures and synchronization

HeLa cells (American Type Culture Collection) were cultured in Iscove's Modified Dulbecco's Medium (Gibco) supplemented with 10% FBS (Mediocrp) at 37°C in 5% CO₂. Mitotic HeLa cells were obtained by incubating the cells with nocodazole (Sigma-Aldrich) at a final concentration of 0.08 µg/ml for 16 hours. In some experiments, mitotic HeLa cells were harvested, washed twice with PBS and incubated in medium supplemented or not with juglone (Sigma-Aldrich) for 5 hours at 37°C. Stock solutions of juglone were freshly prepared in 0.1N NaOH and used at a final concentration of 10 or 20 µg/ml.

Antibodies

MAb CC-3 (IgG_{2a}) was obtained after immunization of a Balb/C mouse with pharyngeal regions isolated from 72-hour chick embryos in a search for developmental markers [7,69]. The mouse monoclonal antibody MPM-2 (IgG₁) was purchased from Upstate Biotechnology. MAb POL3/3 (IgG₁) recognizes Rpb1 in a conserved region located outside the CTD and was kindly provided by E. K. F. Bautz [70]. NMT-5 (rabbit polyclonal antibody) and mouse monoclonal NMT-1 were raised against the splicing cofactor p54^{nrb}/nmt55 and were kindly provided by A. M. Traish [42]. The anti-SAP155 is a rabbit polyclonal antibody kindly provided by R. Reed [43]. The anti-hSpt5 monoclonal rat antibody raised against a C-terminal fragment of the human Spt5 protein was a gift from H. Handa [71]. The anti-DNA topoisomerase II α (8D2) and anti-DNA topoisomerase II β (5A7) were kindly provided by M. Tanaka [72]. The anti-Pin1 polyclonal antibody was raised by immunizing a rabbit with purified full-length His-tagged recombinant Pin1 protein [35].

Sample preparation and immunoprecipitation

For whole cell extracts, interphase and mitotic HeLa cells were directly solubilized in sample buffer (63 mM Tris pH

6.8, 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol) and boiled for 6 minutes. For immunoprecipitation, mitotic or interphase cells were homogenized at 4°C in IP buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.2% NaF, 0.8% SDS, 1 mM EDTA, 1X protease inhibitor cocktail (Roche Diagnostics) and 40 nM microcystin). The lysates were sheared with a 25G needle several times and boiled for 5 minutes. To neutralize the SDS and to allow antigen-antibody interactions, one volume of Triton buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.2% NaF, 4% Triton X-100, 1 mM EDTA, 1X protease inhibitor cocktail and 40 nM microcystin) was added to the lysates and cleared by centrifugation at 16000 g for 5 minutes. Lysates were incubated for 90 minutes at room temperature with the primary antibodies first coated on goat anti-mouse IgG (whole molecule)-agarose (Sigma-Aldrich). The beads were washed in the same buffer followed by antigen solubilization in electrophoresis sample buffer.

Immunoblots

Samples were resolved by 7% or 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The blots were blocked with 1% blocking reagent (Roche Diagnostics) diluted in Tris-buffered saline (TBS-T) (10 mM Tris pH 7.65, 150 NaCl and 0.05% Tween-20) for 60 minutes at 37°C and probed overnight with the primary antibodies at 4°C. Peroxydase-conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories) were used for chemiluminescence detection (Roche Diagnostics). The molecular mass markers used were: myosin heavy chain (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

MALDI-TOF-MS analysis

The purification of CC-3 mitotic antigens was carried out by immunoprecipitation of mitotic cell extracts solubilized in the IP buffer as described above. The purified antigens were separated on a 9% SDS-PAGE gel, Coomassie blue stained and relatively enriched protein bands were excised and subjected to in-gel trypsin digestion as described [73]. Mass spectrometry analysis and protein purification were performed at the Centre de protéomique de l'est du Québec (Dr G. G. Poirier, dir.). An aliquot (1 µl) of the peptide extract was mixed with 1 µl saturated solution of α-cyano-4-hydroxycinnamic acid matrix (10 mg/ml) prepared in TFA (Trifluoroacetic acid 0.1%, Acetonitrile 50%) and submitted to MALDI-TOF mass spectrometry using a Voyager DE PRO mass spectrometer (Applied Biosystems). Molecular masses of the tryptic peptide profiles were used to search by peptide fingerprinting the National Center for Biotechnology Information (NCBI) database using the ProFound software

(ProteoMetrix). Defined search parameters were as follows: monoisotopic mass tolerance 0.20 Da, 1 missed cut allowed, cysteine as S-carbamidomethyl-derivative and oxidation of methionine allowed. The reported results were selected on the basis of restriction to species *Homo sapiens*.

In vitro phosphorylation

The Cdk9/cyclin T1 complex has been kindly provided by Dr O. Bensaude and was prepared from baculovirus-infected Sf9 cells as described [74]. For the mitotic extract, nocodazole-treated cells were prepared as above and homogenized in 3–5 pellet volumes of extraction buffer (50 mM Tris pH 7.4, 250 mM NaCl, 1 mM EDTA, 50 nM NaF, 1 mM DTT, 0.1% Triton X-100, 1X protease inhibitor cocktail and 40 nM microcystin). The lysate was solubilized with a 25G needle and spun for 30 minutes at 16 000 g. The GST-N-CTD and the GST-C-CTD fusion proteins were produced from a pGEX-3X (Pharmacia) construction kindly provided by Dr M. Vigneron [45] and purified on glutathione-Sepharose (Sigma-Aldrich). The GST-CTD recombinant proteins were phosphorylated *in vitro* with the mitotic extract for 60 minutes at 30°C in a final assay volume of 25 µl containing 50 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 2 mM DTT, 40 mM β-glycerophosphate, 20 mM p-nitrophenylphosphate, 0.1 mM sodium vanadate, 20 nM microcystin, 0.7 mM ATP, 5 µl of mitotic extract and 0.2 µg of N-CTD or C-CTD. Alternatively, the constructs were phosphorylated by Cdk9/cyclin T1 in glycerophosphate buffer (20 mM sodium glycerophosphate pH 7.3, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 10% glycerol) with 1 mM ATP for 60 minutes at 30°C. The phosphorylated peptides were purified on glutathione Sepharose resin, eluted with sample buffer and subjected to SDS-PAGE.

CC-3 and MPM-2 reactivity of synthesized CTD peptides

For analysis of CC-3 and MPM-2 reactivity of the CTD, biotinylated CTD peptides containing four consensus repeats (YSPTSPS) phosphorylated in all repeats at the indicated serines were used (a kind gift of Dr S. Buratowski [75]). Peptides were coupled to streptavidin-coated 96-well plates (Pierce) and processed for ELISA according to the instructions of the manufacturer except that TBS-T was used as the washing buffer and that 1% blocking reagent (Roche Diagnostics) in TBT-T was used to block the plate and to dilute the CC-3 and MPM-2 antibodies. Peroxydase-conjugated anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories) was used at 1/1000. The colorimetric reaction was developed using ABTS tablets (Roche Diagnostics) and the resulting absorbances were recorded at 405 nm with a Vmax kinetic microplated reader from Molecular Devices.

Pin1 pulldown experiments

His-tagged Pin1 recombinant protein was cloned and purified as described [35]. His-tagged Pin1 mutants W34A and C113A were kindly provided by Dr O. Bensaude. Equivalent amounts of wild-type and mutant Pin1 proteins and of an irrelevant his-tagged protein (Pbp5) used as a control were loaded onto distinct histidine-binding columns (Novagen) and incubated for 1 hour at 4°C with mitotic HeLa cell lysates prepared in fresh CHAPS buffer (25 mM Tris pH 7.5, 150 mM NaCl, 0.5% CHAPS, 0.2% NaF, 2X protease inhibitor cocktail without EDTA and 40 nM microcystin) and clarified at 16 000 g for 30 minutes at 4°C. The resins were then washed three times with CHAPS buffer containing increasing imidazole concentrations (10 mM, 35 mM and 60 mM). Bound fractions were eluted with sample buffer 2X and heated for 5 minutes at 95°C. Volumes corresponding to the same cellular equivalents were subjected to Western blots.

Pin1 blot overlay

Different samples were separated on a 10% SDS-PAGE gel and transferred on nitrocellulose. The membrane-bound proteins were renatured overnight at 4°C in Blotto (5% lyophilized skim milk dissolved in PBS (pH 7.1) containing 0.2% Tween-20, 1 mM DTT and 1 mM PMSF) and incubated for 2 hours at the same temperature with 1.3 µg/ml of wild-type or W34A mutant Pin1 proteins. The membranes were washed 3 × 15 minutes in Blotto without DTT and PMSF, incubated with the anti-Pin1 antibody (1/10 000 dilution) for 1 hour at room temperature and processed for chemiluminescence as described above.

Authors' contributions

ALA participated in the design of the experiments, carried out most of the experiments and wrote the first draft of the manuscript. SBL participated in the analysis of the phosphorylation level of many CC-3-antigens (hSpt5, Rpb1, DNA topoisomerases). MV participated in the design and the coordination of the study in addition to write the final version of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We want to thank Drs Olivier Bensaude and Marie-Françoise Dubois for providing the constructs of the Pin1 mutants. We also thank Dr Minkyu Kim and Dr Stephen Buratowski for the kind gift of the biotinylated CTD phosphopeptides. This research was supported by the Natural Sciences and Engineering Research Council of Canada (Grant I22625-01 to M.V. and studentships to A.L.A. and S.B.L.).

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