



HAL
open science

Mitosis-specific MPM-2 phosphorylation of DNA topoisomerase II alpha is regulated directly by protein phosphatase 2A

Alexandre E Escargueil, Annette K Larsen

► **To cite this version:**

Alexandre E Escargueil, Annette K Larsen. Mitosis-specific MPM-2 phosphorylation of DNA topoisomerase II alpha is regulated directly by protein phosphatase 2A. *Biochemical Journal*, 2007, 403 (2), pp.235-242. 10.1042/BJ20061460 . hal-00478669

HAL Id: hal-00478669

<https://hal.science/hal-00478669>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

The mitosis-specific MPM-2 phosphorylation of DNA topoisomerase II alpha is directly regulated by protein phosphatase 2A

Alexandre E. ESCARGUEIL and Annette K. LARSEN*

Group of Cancer Biology and Therapeutics, INSERM U673 and Université Pierre et Marie Curie, Hôpital Saint-Antoine, Paris, France

* To whom correspondence should be addressed.

Tel: 331 49 28 46 12; Fax: 331 42 22 64 29; e-mail: akraghlarsen@aol.com

Postal address: Group of Cancer Biology and Therapeutics, INSERM U673, Kourilsky Research Center, Hôpital Saint-Antoine, 184, rue du Faubourg Saint-Antoine, 75572 Paris Cedex 12, France

Running title: PP2A regulation of mitotic topollalpha phosphorylation

Key words:

DNA topoisomerase II, Protein Phosphatase PP2A, Mitosis, MPM-2, Phosphorylation

This manuscript contains 23 pages and 4 figures

SUMMARY

Recent results suggest a role for topoisomerase II alpha (topollalpha) in the fine-tuning of mitotic entry. Mitotic entry is accompanied by the formation of specific phosphoepitopes such as MPM-2 that are believed to control mitotic processes. Surprisingly, the MPM-2 kinase of topollalpha was identified as protein kinase CK2, otherwise known as a constitutive interphase kinase. This suggested the existence of alternative pathways for the creation of mitotic phosphoepitopes, different from the classical pathway where the substrate is phosphorylated by a mitotic kinase. We now report that topollalpha is colocalized with both CK2 and protein phosphatase 2A (PP2A) during interphase. Simultaneous incubation of purified topollalpha with CK2 and PP2A had minimal influence on the total phosphorylation levels of topollalpha, but resulted in complete disappearance of the MPM-2 phosphoepitope due to opposite sequence preferences of CK2 and PP2A. Accordingly, short-term exposure of interphase cells to okadaic acid, a selective PP2A inhibitor, was accompanied by the specific appearance of the MPM-2 phosphoepitope on topollalpha. During early mitosis, PP2A was translocated from the nucleus while CK2 remained in the nucleus until prometaphase thus permitting the formation of the MPM-2 phosphoepitope. These results underline the importance of protein phosphatases as an alternative way of creating cell-cycle specific phosphoepitopes.

INTRODUCTION

Deregulation of cell cycle controls (checkpoints) is universal in human tumors and represents a leading cause of genetic instability [1-3]. The G2 checkpoint controls mitotic entry of damaged cells and is triggered by a wide range of endogenous, environmental and pharmacological injuries resulting in genotoxic lesions [2,4,5]. Accordingly, abrogation of the G2 checkpoint is accompanied by enhanced mitotic cell death and an increased incidence of genetic alterations among the surviving daughter cells [6,7]. Interestingly, unscheduled activation of mitotic kinases has also been associated with Alzheimer's disease where the accumulation of mitotic phosphoepitopes such as MPM-2 is a hallmark of neurofibrillary tangles [8,9].

Traditionally defined, mitosis is initiated with the first visible signs of chromosome condensation [10]. Chromosome condensation is associated with extensive phosphorylation of proteins involved in the regulation of chromatin structure on mitosis-specific phosphorylation sites [11-13]. Among these, the MPM-2 epitope appears to be particularly important, since its presence on mitotic chromosomes is closely associated with the condensed state [14]. The principal MPM-2 kinases include the mitotic kinases Cdk1 (Cdc2), polo-like kinase (plk) and NIMA [15-20]. The major chromosomal protein recognized by the MPM-2 antibody has been identified as the α isoform of DNA topoisomerase II (topoII α) [11]. This protein plays several essential roles during late G2 and early mitosis [for recent reviews, see 21-23] including premitotic chromosome individualization, chromosome condensation and decatenation of intertwined sister chromatids [24-29].

Unexpectedly, a combination of *in vitro* and *in vivo* studies unambiguously demonstrated that the MPM-2 epitope on Ser-1469 of topoII α was created by protein kinase CK2 (CK2), otherwise known as a constitutive interphase kinase [30]. These findings were even more surprising since the major interphase phosphoepitope on Ser-1525 of topoII α is also created by CK2 [30,31]. Together, these observations suggested the existence of alternative pathways for the creation of mitotic phosphoepitopes different from the classical pathway, where the substrate is phosphorylated by a mitotic kinase.

We now report that the MPM-2 phosphorylation of topoll α is directly regulated by protein phosphatase 2A (PP2A) that prevents the appearance of the Ser-1469 MPM-2 epitope, but not the Ser-1525 phosphorylation site, during interphase. These studies suggest a novel paradigm for the creation of cell-cycle specific phosphorylation sites based on the sequence specificity of protein phosphatases toward their substrates.

EXPERIMENTAL

Purified enzymes

Human topoll α was overexpressed in *Saccharomyces cerevisiae* and purified as previously described [30], CK2 was kindly provided by Dr. Claude Cochet (Grenoble, France) while PP2A was purchased from Upstate Biotechnology (distributed by Euromedex, Mundolsheim, France).

Cell Culture

HeLa and LLP-CK cells were grown in Dulbecco's modified Eagle's Medium (Sigma) supplemented with 10% fetal calf serum (Gibco Life Technology), 60 μ g/ml penicillin G and 100 μ g/ml streptomycin sulfate. For mitotic arrest, cells were incubated overnight in the presence of 75 ng/ml nocodazole. For arrest in the G1/S phase of the cell cycle, cells were incubated for 24 h in the presence of 5 μ M aphidicolin or synchronized by a double thymidine block. Briefly, cells were incubated with 2 mM thymidine for 16 h followed by 8 h recovery and a second thymidine exposure for 16 h. The efficiency of synchronization was assessed by flow cytometry using a Epics XL/MCL flow cytometer equipped with an argon laser to give 488 nm light and the cell cycle distribution was analyzed by Multicycle software (Phoenix Flow Systems, San Diego, CA). Nocodazole increased the fraction of G2/M cells from 8 % to more than 80 % compared to the unsynchronized cells. The mitotic index was also determined by microscopic analysis of propidium iodide-stained cells and ranged from 70 to 90 % for nocodazole-blocked cells. Both aphidicolin and thymidine reduced the fraction of S phase cells from 37 % to approximately 20 % compared to the unsynchronized cells. After aphidicolin or thymidine removal, the fraction of S phase cells reached 40 % by 1 h and more than 80 % by 4 h. For *in vivo*

immunoprecipitation of protein kinase CK2, HeLa S3 cells were released in fresh medium for 1 or 4 h after aphidicolin block.

Immunofluorescence

HeLa cells or LLP-CK cells expressing green fluorescent labeled topoll α [32] were grown on coverslips and then fixed in 4% paraformaldehyde. Antigens were revealed by immunolabeling [33] using primary goat antibody (1:25 dilution) directed against the α subunit of CK2 (Santa Cruz Biotechnology, Santa Cruz, CA) or primary rabbit antibody (1:25 dilution) directed against PP2A (Santa Cruz Biotechnology). Secondary antibodies were donkey anti-goat or anti-rabbit IgGs tagged with Cy3 (1:200 dilution; Jackson ImmunoResearch, Bar Harbor, ME). Nuclear DNA was counterstained with To-Pro 3 (Molecular Probes, Eugene, OR) and the images were collected using a Radiance 2000 MP confocal microscope (BioRad Laboratories, Hemel Hempstead, UK).

CK2 immunoprecipitation and topoll α phosphorylation assay

Mitotic- or G1/S-synchronized HeLa cells were lysed in a buffer containing 50 mM Tris-HCl pH 7.5, 10 mM EGTA, 4 mM MgSO₄ (TEM), 1% CHAPS, 200 mM NaCl, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, 2 μ g/ml pepstatin A, 200 nM microcystin-LR (Calbiochem) and 200 nM okadaic acid (Sigma). Cellular extracts (250 μ g) were incubated for 3 h at 4°C under rotation in the presence of a polyclonal antibody directed against the regulatory beta subunit of CK2 (kindly provided by Dr. Claude Cochet, Grenoble, France) diluted 200 times. Extracts were incubated with 40 μ l of protein A-sepharose beads for 30 min at 4°C. The beads were washed three times with 500 μ l of lysis buffer. Then, 500 ng of purified human topoll α diluted in TEM buffer containing 0.5 mM ATP was added to 15 μ l of beads and incubated for 30 min at 37°C. For the total phosphorylation assay, 5 μ Ci of ³²P-ATP was added to the unlabeled ATP. Reactions were stopped by adding 30 μ l of 2x SDS-PAGE loading buffer. Samples were then subjected to electrophoresis and the results were revealed by Western blot analysis or by autoradiography.

Influence of okadaic acid on the MPM-2 phosphorylation of topoll α

HeLa cells were synchronized by a double thymidine block. After synchronization, cells were released for 1 h in fresh medium containing 0, 50 or 500 nM okadaic acid and the cells were extracted with high salt buffer containing 750 mM NaCl, 2 mM EDTA, 0.75% NP-40, 50 mM Tris-HCl pH 8, 5 µg/ml protease inhibitors, 200 nM microcystin-LR and 200 nM okadaic acid. Topoll α was immunoprecipitated as previously described [28]. The relative levels of MPM-2 phosphorylation were revealed by Western blot and densitometry analysis (phosphoimager Storm 840, Molecular Dynamics).

Preparation of phosphorylated human topoll α , K15F and R20A peptides

MPM-2 and radiolabeled phosphorylated topoll α were prepared by incubation of topoll α with CK2 in the presence of 20 µM ATP containing 5 µCi 32 P-ATP. After phosphorylation, the ATP was separated from topoll α using a Sephadex G-50 column (5'-3' Inc., Boulder, CO). The purified phosphorylated topoll α was used as substrate for the dephosphorylation experiments. Radiolabeled phosphopeptides were prepared as previously described [30].

Dephosphorylation of the MPM-2 epitope by extracts from unsynchronized cells

Unsynchronized HeLa cells were extracted with a lysis buffer containing 100 mM KCl, 2 mM EGTA, 0.2% NP-40, 5 mM MgSO $_4$, 50 mM Tris-HCl pH 7.5, 5 µg/ml aprotinin, 5 µg/ml leupeptin and 2 µg/ml pepstatin A. Cellular extracts from 10 4 cells were added to 300 ng of MPM-2 positive topoll α in a buffer containing 20 mM Tris-HCl pH 7.5, 5 mM MgCl $_2$, 0.1 mM MnCl $_2$, 1 mM DTT, 50 ng/µl heparin, protease inhibitors and increasing amounts of protein inhibitor-2 (Upstate Biotechnology) or okadaic acid. Reactions were performed in a final volume of 20 µl for 30 min at 30°C and stopped by adding 2x SDS-PAGE loading buffer. Samples were then subjected to electrophoresis and the results revealed by Western blot analysis.

Incubation of topoll α in the simultaneous presence of CK2 and PP2A

Topoll α (200 ng) was incubated in the presence of 5 ng CK2 and 0.05 units PP2A (corresponding to 25 ng protein) in a buffer containing 20 mM Tris-HCl pH 7.8, 7 mM MgCl $_2$, 50 mM KCl, 1 mM DTT, 100 ng/µl BSA and 20 µM unlabeled ATP or

GTP. For the total phosphorylation assay, 5 μCi ^{32}P -ATP was added to the unlabeled ATP. To determine the influence of manganese, 0.1 mM MnCl_2 was added to the reaction mixture. Reactions were performed and subjected to Western blot analysis as described above.

PP2A dephosphorylation of K15F and R20A phosphopeptides

Radiolabeled phosphopeptides (200 μM) were incubated with increasing amounts of PP2A in a buffer containing 20 mM Tris-HCl pH 7.8, 7 mM MgCl_2 , 50 mM KCl, 1 mM DTT and 100 ng/ μl BSA (10 μl final volume). To determine the influence of manganese, 0.1 mM MnCl_2 was added. Reactions were performed for 30 min at 30°C and stopped by addition of the Tris-Tricine gel loading buffer (Bio-Rad). Peptides were subjected to Tris-Tricine gel electrophoresis and the results were analyzed by densitometry.

Peptide phosphorylation in the presence of CK2 and PP2A

The K15F or R20A peptides (200 μM) were incubated in the presence of 5 ng CK2 and 0.05 units PP2A (corresponding to 25 ng protein) in a buffer (10 μl final volume) containing 20 mM Tris-HCl, pH 7.8, 7 mM MgCl_2 , 50 mM KCl, 1 mM DTT, 100 ng/ μl BSA and 20 μM ATP including 5 μCi ^{32}P -ATP. To determine the influence of manganese, 0.1 mM MnCl_2 was added. Reactions were performed and analyzed by Tris-Tricine gel electrophoresis as described above.

RESULTS

CK2 is colocalized with topoll α during interphase and is intrinsically able to induce the MPM-2 phosphoepitope

CK2 is an ubiquitous kinase that is active throughout the cell cycle [34]. To explain the selective formation of the MPM-2 phosphoepitope on topoll α during mitosis, we explored different hypothesis. First, the two enzymes might not colocalize during interphase. Although topoll α is phosphorylated by CK2 during interphase [31,35,36], and forms molecular complexes with CK2 [37,38], it was still possible that this interaction concerned only a minor fraction of the two enzymes. The cellular localization of topoll α and CK2 was determined in LLP-CK cells expressing a

functional, enhanced green fluorescent-labeled topoll α [32]. The results (Fig. 1A) showed that both enzymes were predominantly nuclear in interphase cells (panels a and b). Furthermore, topoll α and CK2 showed substantial colocalization (indicated by yellow in panel d). Similar results were obtained when both enzymes were localized by immunolabeling in HeLa cells (data not shown).

Although a substantial part of topoll α and CK2 colocalized during interphase, it was possible that only the mitotic form of the kinase was able to create the MPM-2 phosphoepitope. In support of this hypothesis, it has been reported that CK2 is phosphorylated on multiple sites by Cdk1 during mitosis resulting in substantial conformational and functional alterations [39,40] that might potentially influence its sequence specificity. To explore this possibility, CK2 was immunoprecipitated from HeLa cells synchronized in the G1, S or M phase of the cell cycle, and incubated with purified human topoll α in the presence of radiolabeled ATP or GTP. The results showed that CK2 isolated from interphase (Fig. 1B, lanes 3 and 4) or mitotic cells (Fig. 1B, lane 2) was equally able to phosphorylate topoll α (left panel) and to induce the MPM-2 epitope (right panel). Similar levels of phosphorylation were observed in the presence of ATP and GTP, which is a hallmark of CK2 (results not shown). Together, these results indicate that interphase CK2 is intrinsically able to create the MPM-2 phosphoepitope on topoll α .

PP2A potently dephosphorylates the MPM-2 site on topoll α in vitro

The phosphorylation status of a protein is the result of a balance between kinase-mediated phosphorylation and phosphatase-mediated dephosphorylation. To establish if the MPM-2 phosphoepitope on topoll α was a preferred substrate for protein phosphatases during interphase, cellular extracts were prepared from interphase HeLa cells and their ability to dephosphorylate the MPM-2 phosphoepitope on purified human topoll α was evaluated (Fig. 1C). In the absence of phosphatase inhibitors (lane 2) or in the presence of protein inhibitor-2, a specific inhibitor of protein phosphatase 1 (PP1, lanes 6 to 8) the cellular extracts completely removed the MPM-2 phosphoepitope. Similar studies were carried out in the presence of okadaic acid, a marine-sponge toxin that is a potent inhibitor of PP2A and, to a lesser degree, of PP1 [41]. The results showed that the MPM-2 phosphatase was completely inactive in the presence of okadaic acid (lanes 3-5)

even at concentrations as low as 10 nM (lane 3). Further studies showed that the MPM-2 phosphatase was inhibited with an IC_{50} of 1.5 nM, which is within the same dose range as previously reported for the inhibition of PP2A using the reference substrate, phosphorylated myosin light chain [41] (results not shown).

PP2A is colocalized with topoll α during interphase

To determine if PP2A might also influence the phosphorylation of topoll α in living cells, the cellular localization of PP2A was determined by fluorescence microscopy (Fig. 1D). As previously reported, PP2A was predominantly nuclear during interphase [42] and colocalized partly with topoll α (Fig 1D panel d) and CK2 (data not shown).

Cellular exposure to okadaic acid is accompanied by specific induction of the MPM-2 epitope on topoll α during interphase

To establish if the MPM-2 phosphoepitope on topoll α was also a substrate for PP2A in living cells, G1/S synchronized HeLa cells were incubated with different concentrations of okadaic acid. Since prolonged treatment with okadaic acid may induce a premature mitotic state [43], incubation with okadaic acid was carried out at concentrations and exposure times, where no significant alterations were observed with regard to cell cycle distribution, Cdk1 activity, DNA condensation or the overall formation of MPM-2 antigens as determined by flow cytometry (data not shown). Incubation of cells with low (50 nM) or high (500 nM) concentrations of okadaic acid for 1 h was accompanied by a dose-dependent increase in the proportion of MPM-2 reactive topoll α as determined by Western blot analysis after immunoprecipitation with a topoll α -directed antibody (Fig. 1E, upper panel and Fig. 1F, left) without any detectable changes in the specific activity of CK2 (data not shown). In comparison, no major changes in the overall MPM-2 reactivity were observed for total cell extracts (Fig. 1E, lower panel and Fig. 1F, right). The specific increase of the MPM-2 phosphoepitope on topoll α strongly suggest that PP2A may play a direct role in the regulation of the MPM-2 epitope during interphase.

PP2A preferentially dephosphorylates the MPM-2 epitope on human topoll α

In addition to the MPM-2 phosphoepitope on Ser-1469, CK2 also creates the major interphase phosphorylation site on Ser-1525 of topoll α [30,31]. To establish if

PP2A shows a sequence preference toward one of these phosphoepitopes, topoll α was phosphorylated by CK2 in the presence of radiolabeled ATP, and the ability of purified PP2A to dephosphorylate the labeled substrate was evaluated in the absence or presence of Mn^{2+} , a potent phosphatase activator [44]. The results (Fig. 2A) showed that PP2A was only able to efficiently dephosphorylate topoll α in the presence of Mn^{2+} (Fig. 2A left panel, compare lanes 1 and 3). In marked contrast, PP2A completely dephosphorylated the MPM-2 phosphoepitope even at low Mn^{2+} concentrations (Fig. 2A right panel, compare lanes 1 and 2). To further establish the sequence preference of PP2A, we selected two polypeptides; K15F, containing Lys-1517 to Phe-1531 that includes the major Ser-1525 phosphorylation site [30,31] and R20A, containing Arg-1466 to Ala-1485 that includes the MPM-2 Ser-1469 phosphorylation site of topoll α [30]. The results (Fig. 2B) showed that Mn^{2+} is required for efficient dephosphorylation of the Ser-1525 phosphorylation site on peptide K15F. In clear contrast, PP2A efficiently dephosphorylated the Ser-1469 MPM-2 epitope on peptide R20A even without Mn^{2+} .

PP2A efficiently dephosphorylates the MPM-2 phosphoepitope on topolla even in the presence of CK2

The findings described above strongly suggest that the MPM-2 phosphoepitope is preferentially dephosphorylated by PP2A while previous studies have indicated that Ser-1525 is preferentially phosphorylated by CK2 [30]. We therefore wished to establish how the simultaneous presence of catalytically active CK2 and PP2A influenced the phosphorylation pattern of topoll α . For this, purified topoll α was incubated with CK2 and radiolabeled ATP in the absence or presence of PP2A (Fig. 2C). The protein phosphatase had hardly any influence on the overall phosphorylation of topoll α (Fig. 2C, left panel). In marked contrast, the MPM-2 phosphoepitope was completely removed under the same conditions (Fig. 2C, right panel). Furthermore, the MPM-2 phosphoepitope was not formed in the absence of ATP or GTP, but was favored in the presence of okadaic acid (results not shown). Therefore, the observed effects are consistent with competing kinase and phosphatase activities, but not with physical blocking of the MPM-2 site by PP2A.

For further confirmation, the same experiment was carried out with the K15F and R20A peptides instead of full-length topoll α (Fig. 2D). The results showed that the

K15F peptide containing the major Ser-1525 phosphorylation site was only influenced by PP2A in the presence of high concentrations of Mn^{2+} . In contrast, the MPM-2 epitope on R20A is clearly reduced even at low concentrations of Mn^{2+} (Fig. 2D). These results demonstrate how the opposing sequence preference of CK2 and PP2A can prevent the appearance of the MPM-2 epitope on topoll α during interphase without notable influence on the other CK2-phosphorylation sites.

PP2A leaves the nucleus in early prophase while CK2 remains nuclear until prometaphase

To determine when the MPM-2 phosphoepitope appears on topoll α , the cellular localization of CK2 and PP2A was determined by fluorescence microscopy (Fig. 3). During interphase, PP2A was predominantly nuclear and colocalized partly with both topoll α and CK2 (Fig. 1E). PP2A started to leave the nuclear compartment at mitotic onset (Fig. 3, series 2-4) and was in general not present in early mitotic nuclei (Fig. 3, series 2) or during late prophase (Fig. 3, series 3). During prometaphase, PP2A was diffused in the cytoplasm without any detectable association with mitotic chromosomes (Fig. 3, series 4).

In comparison, CK2 remained nuclear during early mitosis (Fig. 3, series 6-7) and only left the nuclear compartment at prometaphase when the nuclear membrane is disrupted (Fig. 3, compare series 7 (late prophase) and 8 (prometaphase)). These results indicate that CK2 can phosphorylate topoll α during early mitosis in the absence of PP2A resulting in the formation of the mitotic MPM-2 phosphoepitope. At the same time, topoll α became progressively associated with the condensing chromosomes (as indicated by turquoise on the overlay in Fig. 4A, panels d and h). Interestingly, CK2 never became chromosome-associated (as indicated by the absence of purple-pink on the overlay in Fig. 4A, panels d and h) and colocalized preferentially with the nucleoplasmic pool of topoll α during prophase (as indicated by yellow on the overlay in Fig. 4A, panel d). These findings suggest that CK2 predominantly induced the MPM-2 phosphoepitope on nucleoplasmic topoll α which then associated with the mitotic chromosomes.

DISCUSSION

We here present an alternative model to account for the regulation of a cell-cycle-specific phosphorylation site, in this case the mitotic MPM-2 phosphoepitope on topoisomerase II α , a major regulator of mitotic chromosome structure. Although topoisomerase II α was the only chromosome-associated protein that served as substrate for the MPM-2 kinase activity of CK2, up to 10 potential substrates were identified in total mitotic extracts. The two proteins which most consistently served as substrates for the MPM-2 kinase activity of CK2 were topoisomerase II α and a protein with a molecular mass of ~110 kDa [30]. Although the identity of the latter protein is not known, its molecular weight corresponds to another major CK2 substrate, nucleolin, which like topoisomerase II α forms stable complexes with CK2 [45] and undergoes mitosis-specific phosphorylation [46]. Therefore, the proposed model is likely relevant for several proteins besides topoisomerase II α .

The onset of mitotic chromosome condensation is accompanied by a burst of phosphorylation of chromatin-associated proteins. In most cases, the formation of the phosphorylation sites can be defined by a sequential model where competing kinases and phosphatases directly regulate the activation state of a mitotic kinase (such as Cdk1) that, once activated at the G2/M interphase will act on its substrates (such as the condensins). In contrast to this classical model, it is the final substrate, topoisomerase II α , that is subject to competing kinase and phosphatase activities as outlined in Fig. 4B. During interphase, CK2 is colocalized with topoisomerase II α in the nucleus and is intrinsically able to induce the formation of the MPM-2 phosphoepitope. However, the additional colocalization with PP2A ensures that the MPM-2 site on Ser-1469 is kept unphosphorylated due to a combination of strong PP2A activity and weak CK2 activity toward this residue. Simultaneously, topoisomerase II α is phosphorylated by CK2 on Ser-1525 because of strong CK2 activity combined with weak PP2A activity. At mitotic onset, the subcellular distribution of CK2 and PP2A is altered (Fig 4B) without marked change in their expression levels [34,47]. PP2A is rapidly excluded from the nucleus while CK2 remains nuclear until nuclear membrane break-down in prometaphase. Consequently, the existing equilibrium between the MPM-2 kinase and MPM-2 phosphatase activities is disrupted, resulting in extensive MPM-2 phosphorylation of topoisomerase II α .

A major question is why the MPM-2 epitope on topoisomerase II α is subject to such complex regulation. Topoisomerase II α seems to play a role in the fine-tuning of mitotic timing.

Recent findings show that overexpression of topoll α is accompanied by accelerated cell cycle transit in G2 and early mitosis [48] while RNAi-mediated topoll α downregulation delays G2 and early mitosis [49]. A possible explanation for this activity is the capacity of topoll α to recruit the major mitotic kinase Cdk1 to nuclear chromatin thus providing the kinase access to chromosomal proteins that are essential for further chromosome condensation. This is important, since Cdk1 by itself is not a DNA binding protein, and thus not able to associate with nuclear chromatin in the absence of topoll α [28].

A second question concerns the biological function of the MPM-2 phosphoepitope. Although it is clearly established that the MPM-2 phosphoepitope is formed on numerous proteins during mitosis and that this is causally linked to different mitotic functions, the exact role of the MPM-2 modification is not well understood. The MPM-2 phosphoepitope is formed on a serine or threonine residue next to a proline. The peptidyl-prolyl-*cis-trans*-isomerase Pin1 shows almost the same substrate specificity as the MPM-2 antibody and has been shown to bind to the mitotic form of topoll α [30,50]. Interestingly, Pin1 has also been shown to bind and modulate Cdc25, an other key regulator of mitotic entry [51]. The findings for Cdc25C suggests that binding of Pin1 to the MPM-2 site of topoll α might alter its structure, function and/or stability thereby regulating the mitotic functions of topoll α .

It is intriguing that the expression of topoll α , CK2 and PP2A is frequently altered in human tumors. Topoll α is overexpressed in several tumor types including lung and breast cancer and high levels of topoll α expression is generally associated with more aggressive tumor behavior [52,53]. CK2 is also overexpressed in many tumors such as breast, lung, and malignant melanomas and is generally considered as a cellular survival factor [54]. Interestingly, genetic studies in yeast suggest that CK2 facilitate G2 checkpoint override following DNA damage [55]. In contrast, current evidence suggest that PP2A is a tumor suppressor. Loss-of-function mutations of the structural subunit of PP2A have been described in lung and breast cancer as well as in melanoma [56] and the PP2A inhibitor okadaic acid act as a tumor promoter. Recent results indicate that downregulation of PP2A activity might be a requirement for the G2 to M transition [47].

Taken together, our findings document the functional and spatial interaction between topoll α , CK2 and PP2A during the different phases of the cell cycle. The

direct regulation of the MPM-2 phosphoepitope on topol α by PP2A underlines the importance of protein phosphatases in the regulation of key cellular functions.

ACKNOWLEDGEMENTS

The authors thank Dr. Virginie Poindessous for careful reading of the manuscript. This work was supported by grants from Association pour la Recherche sur le Cancer (ARC) grant n^o 4659 and Fondation Recherche Médicale (FRM). A. E. E. was a fellow of FRM and the Federation of European Biochemical Societies (FEBS).

REFERENCES

- 1 Hartwell, L. H., and Kastan, M. B. (1994) Cell cycle control and cancer. *Science* **266**, 1821-1828
- 2 Paulovich, A. G., Toczyski, D. P., and Hartwell, L. H. (1997) When checkpoints fail. *Cell* **88**, 315-321
- 3 Cahill, D. P., Lengauer, C., Yu, J., Riggins, G. J., Willson, J. K., Markowitz, S. D., Kinzler, K. W., and Vogelstein, B. (1998) Mutations of mitotic checkpoint genes in human cancers. *Nature* **392**, 300-303
- 4 Boddy, M. N., Furnari, B., Mondesert, O., and Russell, P. (1998) Replication checkpoint enforced by kinases Cds1 and Chk1. *Science* **280**, 909-912
- 5 Samuel, T., Weber, H. O., and Funk, J. O. (2002) Linking DNA damage to cell cycle checkpoints. *Cell Cycle* **1**, 162-168
- 6 Lau, C. C., and Pardee, A. B. (1982) Mechanism by which caffeine potentiates lethality of nitrogen mustard. *Proc. Natl. Acad. Sci. USA* **79**, 2942-2946

- 7 Bozko, P., Sabisz, M., Larsen, A. K., and Skladanowski A. (2005) Cross-talk between DNA damage and cell survival checkpoints during G2 and mitosis: pharmacologic implications. *Mol. Cancer Ther.* **4**, 2016-2025
- 8 Vincent, I., Rosado, M., and Davies, P. (1996) Mitotic mechanisms in Alzheimer's disease? *J. Cell Biol.* **132**, 413-425
- 9 Vincent, I., Jicha, G., Rosado, M., and Dickson, D. W. (1997) Aberrant expression of mitotic cdc2/cyclin B1 kinase in degenerating neurons of Alzheimer's disease brain. *J. Neurosci.* **17**, 3588-3598
- 10 Leblond, C. P., and El-Alfy, M. (1998) The eleven stages of the cell cycle, with emphasis on the changes in chromosomes and nucleoli during interphase and mitosis. *Anat. Rec.* **252**, 426-443
- 11 Taagepera, S., Rao, P. N., Drake, F. H., and Gorbsky, G. J. (1993) DNA topoisomerase II alpha is the major chromosome protein recognized by the mitotic phosphoprotein antibody MPM-2. *Proc. Natl. Acad. Sci. USA* **90**, 8407-8411
- 12 Giet, R., and Glover, D. M. (2001) *Drosophila* aurora B kinase is required for histone H3 phosphorylation and condensin recruitment during chromosome condensation and to organize the central spindle during cytokinesis. *J. Cell Biol.* **152**, 669-682
- 13 Takemoto, A., Kimura, K., Yokoyama, S., and Hanaoka, F. (2004) Cell cycle-dependent phosphorylation, nuclear localization, and activation of human condensin. *J. Biol. Chem.* **279**, 4551-4559
- 14 Rieder C. L., and Cole R. W. (1998) Entry into mitosis in vertebrate somatic cells is guarded by a chromosome damage checkpoint that reverses the cell cycle when triggered during early but not late prophase. *J. Cell Biol.* **142**, 1013-1022

- 15 Westendorf, J. M., Rao, P. N., and Gerace, L. (1994) Cloning of cDNAs for M-phase phosphoproteins recognized by the MPM2 monoclonal antibody and determination of the phosphorylated epitope. *Proc. Natl. Acad. Sci. USA* **91**, 714-718
- 16 Ye, X. S., Xu, G., Pu, R. T., Fincher, R. R., McGuire, S. L., Osmani, A. H., and Osmani, S.A. (1995) The NIMA protein kinase is hyperphosphorylated and activated downstream of p34cdc2/cyclin B: coordination of two mitosis promoting kinases. *EMBO J.* **14**, 986-994
- 17 Kumagai, A., and Dunphy, W. G. (1996) Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from *Xenopus* egg extracts. *Science* **273**, 1377-1380
- 18 Renzi, L., Gersch, M. S., Campbell, M. S., Wu, L., Osmani, S. A., and Gorbsky, G. J. (1997) MPM-2 antibody-reactive phosphorylations can be created in detergent-extracted cells by kinetochore-bound and soluble kinases. *J. Cell Sci.* **110**, 2013-2025
- 19 Stukenberg, P. T., Lustig, K. D., McGarry, T. J., King, R. W., Kuang, J., and Kirschner, M. W. (1997) Systematic identification of mitotic phosphoproteins. *Curr. Biol.* **7**, 338-348
- 20 Logarinho, E., and Sunkel, C. E. (1998) The *Drosophila* POLO kinase localises to multiple compartments of the mitotic apparatus and is required for the phosphorylation of MPM2 reactive epitopes. *J. Cell Sci.* **111**, 2897-2909
- 21 Larsen, A. K., Escargueil, A. E., and Skladanowski, A. (2003) From DNA damage to G2 arrest: the many roles of topoisomerase II. *Prog. Cell Cycle Res.* **5**, 295-300
- 22 Swedlow, J. R., and Hirano, T. (2003) The making of the mitotic chromosome: modern insights into classical questions. *Mol. Cell* **11**, 557-569

- 23 Clarke, D. J., Vas, A. C., Andrews, C. A., Diaz-Martinez, L. A., and Gimenez-Abian, J. F. (2006) Topoisomerase II checkpoints. *Cell Cycle* **5**, 1925-1928
- 24 Gimenez-Abian, J. F., Clarke, D. J., Devlin, J., Gimenez-Abian, M. I., De la Torre, C., Johnson, R. T., Mullinger, A. M., and Downes C. S. (2000) Premitotic chromosome individualization in mammalian cells depends on topoisomerase II activity. *Chromosoma* **109**, 235-244
- 25 Adachi Y., Luke M., and Laemmli U. K. (1991) Chromosome assembly in vitro: topoisomerase II is required for condensation. *Cell* **64**, 137-148
- 26 Bojanowski, K., Maniotis, A. J., Plisov, S., Larsen, A. K., and Ingber, D. E. (1998) DNA topoisomerase II can drive changes in higher order chromosome architecture without enzymatically modifying DNA. *J. Cell. Biochem.* **69**, 127-142
- 27 Gimenez-Abian J. F., Clarke D. J., Mullinger A. M., Downes C. S., and Johnson R. T. (1995) A postprophase topoisomerase II-dependent chromatid core separation step in the formation of metaphase chromosomes. *J. Cell Biol.* **131**, 7-17
- 28 Escargueil, A. E., Plisov, S. Y., Skladanowski, A., Borgne, A., Meijer, L., Gorbsky, G. J., and Larsen, A. K. (2001) Recruitment of cdc2 kinase by DNA topoisomerase II is coupled to chromatin remodeling. *FASEB J.* **15**, 2288-2290
- 29 Losada, A., Hirano, M., and Hirano, T. (2002) Cohesin release is required for sister chromatid resolution, but not for condensin-mediated compaction, at the onset of mitosis. *Genes Dev.* **16**, 3004-3016
- 30 Escargueil, A. E., Plisov, S. Y., Filhol, O., Cochet, C., and Larsen, A. K. (2000) Mitotic phosphorylation of DNA topoisomerase II alpha by protein kinase CK2 creates the MPM-2 phosphoepitope on Ser-1469. *J. Biol. Chem.* **275**, 34710-34718

- 31 Wells, N. J., Addison, C. M., Fry, A. M., Ganapathi, R., and Hickson, I. D. (1994) Serine 1524 is a major site of phosphorylation on human topoisomerase II alpha protein in vivo and is a substrate for casein kinase II in vitro. *J. Biol. Chem.* **269**, 29746-29751

- 32 Tavormina, P. A., Côme, M. G., Hudson, J. R., Mo, Y. Y., Beck, W. T., and Gorbsky G. J. (2002) Rapid exchange of mammalian topoisomerase II alpha at kinetochores and chromosome arms in mitosis. *J. Cell Biol.* **158**, 23-29

- 33 Iborra F. J., Jackson D. A., and Cook P. R. (2001) Coupled transcription and translation within nuclei of mammalian cells. *Science* **293**, 1139-1142

- 34 Bosc, D. G., Luscher, B., and Litchfield D. W. (1999) Expression and regulation of protein kinase CK2 during the cell cycle. *Mol. Cell. Biochem.* **191**, 213-22.

- 35 Ackerman, P., Glover, C. V., and Osheroff, N. (1988) Phosphorylation of DNA topoisomerase II in vivo and in total homogenates of *Drosophila* Kc cells. The role of casein kinase II. *J. Biol. Chem.* **263**, 12653-12660

- 36 Cardenas, M. E., Dang, Q., Glover, C. V., and Gasser, S. M. (1992) Casein kinase II phosphorylates the eukaryote-specific C-terminal domain of topoisomerase II in vivo. *EMBO J.* **11**, 1785-1796

- 37 Bojanowski, K., Filhol, O., Cochet, C., Chambaz, E. M., and Larsen, A. K. (1993) DNA topoisomerase II and casein kinase II associate in a molecular complex that is catalytically active. *J Biol Chem.* **268**, 22920-22926

- 38 Redwood, C., Davies, S. L., Wells, N. J., Fry, A.M., and Hickson I. D. (1998) Casein kinase II stabilizes the activity of human topoisomerase IIalpha in a phosphorylation-independent manner. *J. Biol. Chem.* **273**, 3635-3642

- 39 Bosc, D. G., Slominski, E., Sichler, C., and Litchfield D. W. (1995) Phosphorylation of casein kinase II by p34cdc2. Identification of

- phosphorylation sites using phosphorylation site mutants in vitro. *J. Biol. Chem.* **270**, 25872-25878
- 40 Litchfield, D. W., Luscher, B., Lozeman, F. J., Eisenman, R. N., and Krebs, E. G. (1992) Phosphorylation of casein kinase II by p34cdc2 in vitro and at mitosis. *J. Biol. Chem.* **267**, 13943-13951
- 41 Bialojan, C., and Takai, A. (1988) Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. *Biochem. J.* **256**, 283-290
- 42 Turowski, P., Fernandez, A., Favre, B., Lamb, N. J., and Hemmings, B. A. (1995) Differential methylation and altered conformation of cytoplasmic and nuclear forms of protein phosphatase 2A during cell cycle progression. *J. Cell Biol.* **129**, 397-410
- 43 Yamashita, K., Yasuda, H., Pines, J., Yasumoto, K., Nishitani, H., Ohtsubo, M., Hunter, T., Sugimura, T., and Nishimoto, T. (1990) Okadaic acid, a potent inhibitor of type 1 and type 2A protein phosphatases, activates cdc2/H1 kinase and transiently induces a premature mitosis-like state in BHK21 cells. *EMBO J.* **9**, 4331-4338
- 44 Cai, L., Chu, Y., Wilson, S.E., and Schlender, K.K. (1995) A metal-dependent form of protein phosphatase 2A. *Biochem. Biophys. Res. Commun.* **208**, 274-279
- 45 Li, D., Dobrowolska, G., and Krebs, E. G. (1999) Identification of proteins that associate with protein kinase CK2. *Mol Cell Biochem.* **191**, 223-8.
- 46 Gulli, M. P., Faubladiere, M., Sicard, H., and Caizergues-Ferrer, M. (1997) Mitosis-specific phosphorylation of gar2, a fission yeast nucleolar protein structurally related to nucleolin. *Chromosoma* **105**, 532-41.

- 47 Klingler-Hoffmann, M., Barth, H., Richards, J., König, N., and Kinzel, V. (2005) Downregulation of protein phosphatase 2A activity in HeLa cells at the G2-mitosis transition and unscheduled reactivation induced by 12-O-tetradecanoyl phorbol-13-acetate (TPA). *Eur. J. Cell Biol.* **84**, 719-732
- 48 Skladanowski, A., Côme, M. G., Sabisz, M., Escargueil, A. E., and Larsen, A. K. (2005) Down-regulation of DNA topoisomerase II α leads to prolonged cell cycle transit in G2 and early M phases and increased survival to microtubule-interacting agents. *Mol. Pharmacol.* **68**, 625-634
- 49 Sakaguchi, A., and Kikuchi A. (2004) Functional compatibility between isoform α and β of type II DNA topoisomerase. *J. Cell Sci.* **117**, 1047-1054
- 50 Shen, M., Stukenberg, P. T., Kirschner, M. W., and Lu, K. P. (1998) The essential mitotic peptidyl-prolyl isomerase Pin1 binds and regulates mitosis-specific phosphoproteins. *Genes Dev.* **12**, 706-720
- 51 Stukenberg, P. T., and Kirschner, M. W. (2001) Pin1 acts catalytically to promote a conformational change in Cdc25. *Mol. Cell* **7**, 1071-1083
- 52 Giaccone, G., van Ark-Otte, J., Scagliotti, G., Capranico, G., van der Valk, P., Rubio, G., Dalesio, O., Lopez, R., Zunino, F., Walboomers, J., et al. (1995) Differential expression of DNA topoisomerases in non-small cell lung cancer and normal lung. *Biochim. Biophys. Acta* **1264**, 337-346
- 53 Koren, R., Rath-Wolfson, L., Ram, E., Itzhac, O. B., Schachter, B., Klein, B., Gal, R., and Dreznik, Z. (2004) Prognostic value of Topoisomerase II in female breast cancer. *Oncol. Rep.* **12**, 915-919
- 54 Ahmed, K., Gerber, D. A., and Cochet, C. (2002) Joining the cell survival squad: an emerging role for protein kinase CK2. *Trends Cell Biol.* **12**, 226-230
- 55 Toczyski, D.P., Galgoczy, D.J., and Hartwell, L.H. (1997) CDC5 and CKII control adaptation to the yeast DNA damage checkpoint. *Cell* **90**, 1097-1106

56 Ruediger, R., Pham, H.T., and Walter, G. (2001) Disruption of protein phosphatase 2A subunit interaction in human cancers with mutations in the A alpha subunit gene. *Oncogene* **20**, 10-15

FIGURE LEGENDS

Fig. 1. **Interaction between CK2 and topolla in interphase cells.** (A) Immunofluorescence microscopy reveals partial colocalization (indicated by yellow in panel d) between EGFP-topoll α (indicated in green in panels a and d) and the catalytic CK2 α subunit (indicated in red in panels b and d) during interphase. DNA counterstaining (indicated in blue in panels c and d) shows that the colocalization of the two enzymes preferentially occurs in low condensed chromatin domains. (B) *In vitro* phosphorylation assays reveal that mitotic and non-mitotic forms of CK2 are equally able to phosphorylate topoll α . Left panel, total topoll α phosphorylation, right panel, MPM-2 phosphorylation of topoll α . Control lanes 1 show the absence of topoll α phosphorylation in an immunoprecipitate obtained with a non-immune antibody. Lanes 2 show topoll α phosphorylation by CK2 immunoprecipitated from nocodazole-blocked HeLa cells (M phase). Lanes 3 and 4 show topoll α phosphorylation by CK2 immunoprecipitated from HeLa cells blocked by aphidicolin and released in fresh medium for 1 or 4 h (S phase), respectively. (C) The MPM-2 epitope on topoll α (lane 1) disappears in the presence of HeLa cell extracts (lane 2). Incubation with increasing amounts of okadaic acid (10, 20 and 50 nM; lanes 3, 4 and 5, respectively) completely inhibits the MPM-2 phosphatase activity in the cellular extracts while incubation with increasing amount of PI-2 (50, 100 and 500 nM; lanes 6, 7 and 8, respectively) has no effect. (D) Immunofluorescence microscopy reveals partial colocalization (indicated by yellow in panel d) between EGFP-topoll α (indicated in green in panels a and d) and the catalytic PP2A subunit (indicated in red in panels b and d) during interphase. DNA counterstaining (indicated in blue in panels c and d) shows that the colocalization of the two enzymes preferentially occurs in low condensed chromatin domains. (E) HeLa cells were synchronized by double thymidine block. After synchronization, cells were released for 1 h in fresh medium containing 0, 50 or 500 nM okadaic acid (lanes 1, 2 and 3, respectively). The

upper panel shows MPM-2 positive topoll α after specific immunoprecipitation, the lower panel shows MPM-2 positive proteins in total cell extracts. (F) The relative intensities of MPM-2 positive topoll α (left panel) and MPM-2 positive proteins in total cell extracts (right panel), obtained as described in (E), were quantified by densitometry analysis. Columns, average of 3 independent experiments; bars, standard errors. White columns, cells released in drug free medium for 1 h; hatched columns, cells released for 1 h in medium with 50 nM okadaic acid; grey columns, cells released for 1 h in medium with 500 nM okadaic acid.

Fig. 2. PP2A shows high specificity toward the MPM-2 phosphoepitope on Ser-1469. (A) Comparison of the ability of PP2A to dephosphorylate ^{32}P -labeled topoll α (left panel) and the topoll α MPM-2 epitope (right panel) reveals strong preference of PP2A toward the Ser-1469 residue. Left panel, PP2A is only able to dephosphorylate topoll α (lane 1) in the presence of Mn^{2+} (lane 3) but not in the absence of Mn^{2+} (lane 2). Right panel, PP2A completely dephosphorylates the MPM-2 phosphoepitope (lane 1) even in the absence of Mn^{2+} (lane 2). (B) Comparison of the capacity of PP2A to dephosphorylate ^{32}P -labeled peptides confirms the strong sequence preference towards the Ser-1469 residue compared with Ser-1525. The R20A sequence corresponds to the region surrounding the MPM-2 phosphoepitope on Ser-1469 while the K15F sequence corresponds to the region surrounding the major phosphorylation site on Ser-1525. (C) CK2-mediated phosphorylation of topoll α in the absence (lane 1) or presence (lane 2) of PP2A. Left panel, total phosphorylation levels of topoll α , as revealed by ^{32}P -labeling, are not affected by the presence of PP2A. Right panel, the formation of the MPM-2 epitope on topoll α , as revealed by Western blot analysis, is strongly inhibited when PP2A is present. Similar results were obtained in the presence and absence of Mn^{2+} . (D) PP2A shows high preference for the Ser-1469 residue compared to the Ser-1525 residue. Lanes 1, peptide phosphorylation in the absence of PP2A, lanes 2, peptide phosphorylation in the presence of PP2A. CK2 phosphorylation of Ser-1469 on R20A is strongly inhibited in the presence of PP2A (right panel) while CK2 phosphorylation of Ser-1525 on K15F is less inhibited by PP2A (left panel) even in presence of Mn^{2+} .

Fig. 3. PP2A is excluded from the nucleus in early prophase while CK2 remains nuclear until prometaphase. Immunofluorescence reveals that PP2A (left panels, red) disappears quickly from early mitotic nuclei while CK2 (right panels, red) stays associated with a non-chromosomal fraction until complete chromosome condensation and nuclear membrane breakdown. Series 1 to 4 show a rapid loss of PP2A during early prophase. Series 5 to 8 show gradual changes of CK2 localization until prometaphase. DNA counterstaining (left and right panels, blue) shows that neither PP2A or CK2 are associated with precondensed or condensed chromatin and that CK2 tends to surround the chromosomes.

Fig. 4. Localization of CK2 and PP2A in early mitosis. (A) Immunofluorescence reveals colocalization (yellow, d) between GFP-topoll α (green, a and d) and the catalytic CK2 α subunit (red, b and d) in early mitosis (top panel). Colocalization is observed for the non-chromosomal fraction of GFP-topoll α as revealed by DNA counterstaining (blue, c). In prometaphase (bottom panel), topoll α (green, e) is fully associated with the condensed chromosomes (blue, g) while CK2 (red, f) shows a diffused pattern surrounding condensed chromosomes. No yellow is seen after superimposition (g). (B) Model for the cell cycle-dependent interaction of CK2 with topoll α . The CK2-mediated MPM-2 phosphorylation of topoll α is directly regulated by PP2A during interphase. The translocation of PP2A from the nuclear compartment during early mitosis allows CK2 to phosphorylate the Ser-1469 MPM2 site. Black lines represents preferential residues targeted by either CK2 or PP2A. Dashed lines represents weaker substrates targeted by either CK2 or PP2A.

Figure 1

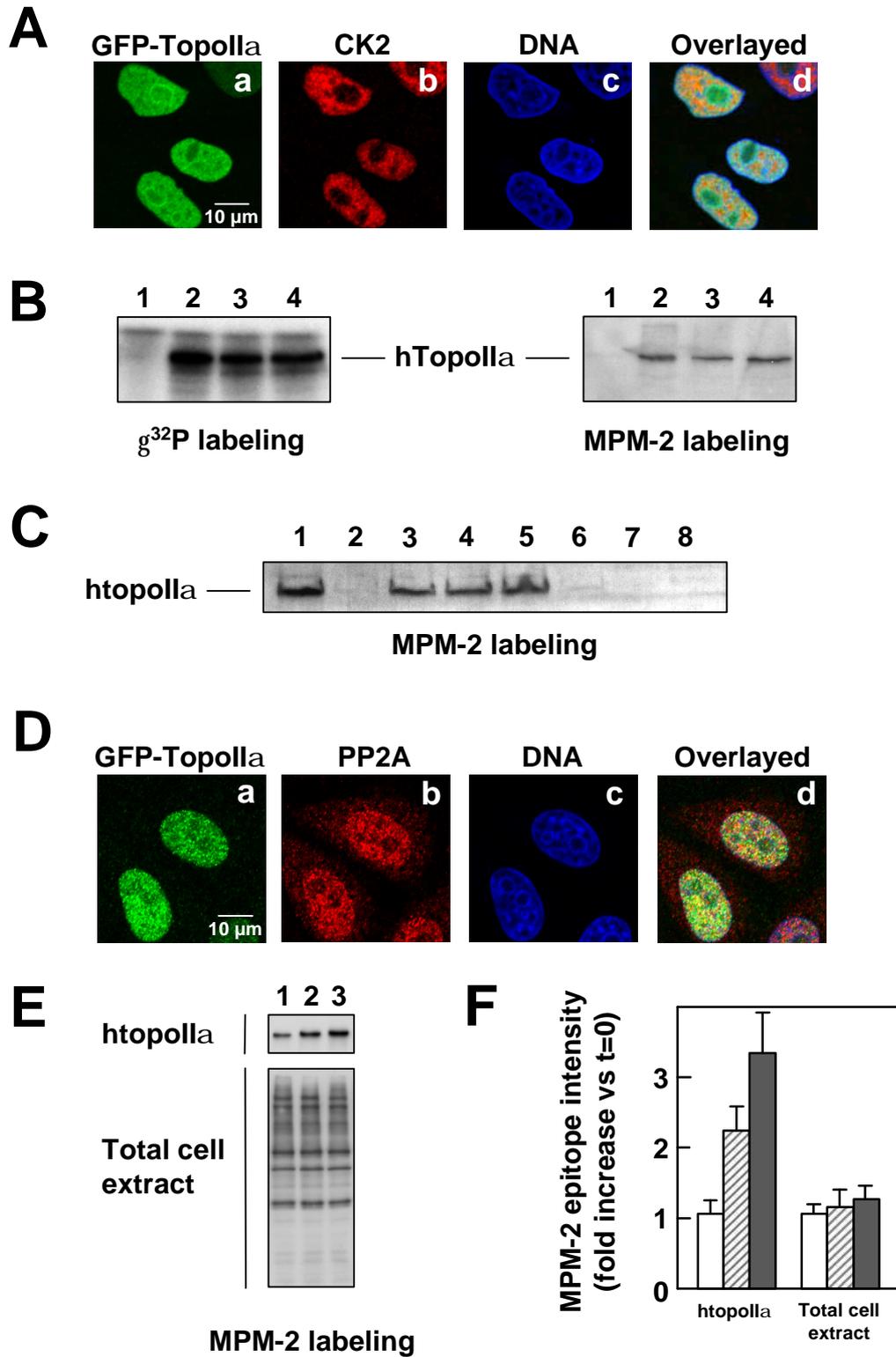


Figure 2

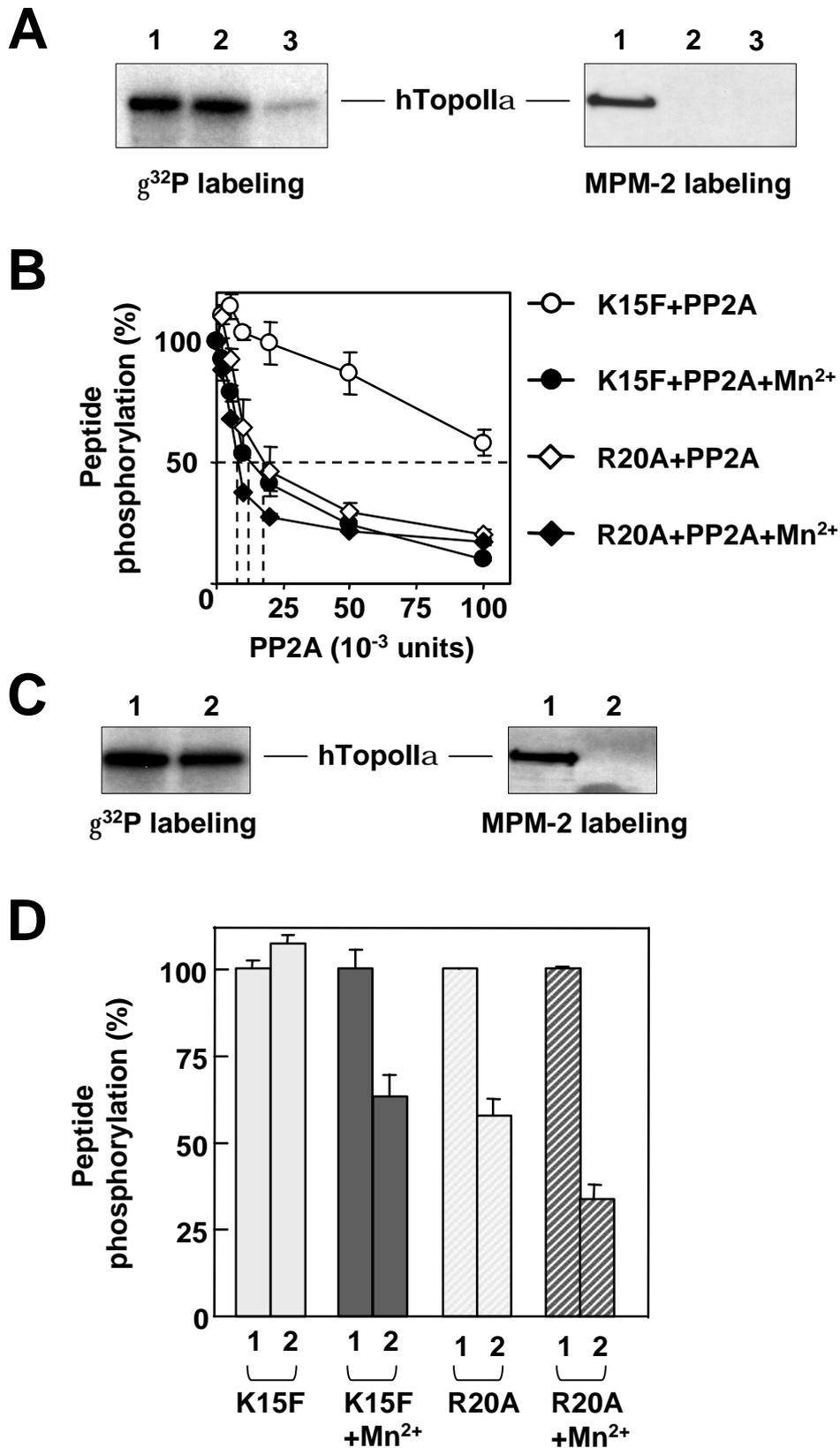


Figure 3

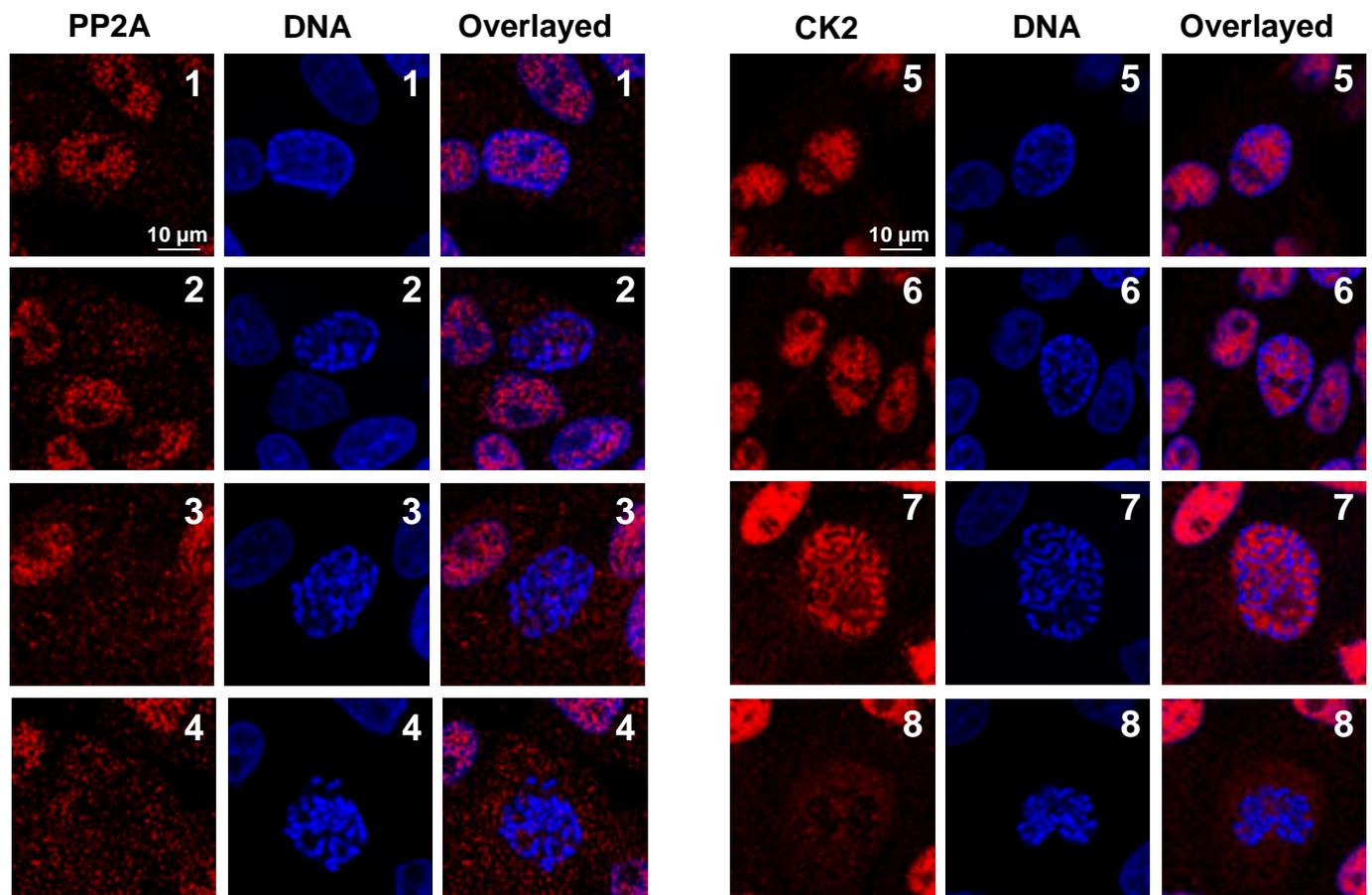


Figure 4

