Identification of a novel mitotic phosphorylation motif associated with protein localization to the mitotic apparatus

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Summary

The chromosomal passenger complex (CPC) is a crucial regulator of chromosome, cytoskeleton and membrane dynamics during mitosis. Here, using liquid chromatography coupled to mass spectrometry (LC-MS), we identified phosphopeptides and phosphoprotein complexes recognized by a phosphorylation-specific antibody that labels the CPC. A mitotic phosphorylation motif {PX[G/T/S][L/M]S(*P*)P or WGLS(*P*)P} was identified by MS in 11 proteins, including FZR1 (Cdh1) and RIC8A – two proteins with potential links to the CPC. Phosphoprotein complexes contained the known CPC components INCENP, Aurora-B (Aurkb) and TD-60 (Rcc2, RCC1-like), as well as SMAD2, 14-3-3 proteins, PP2A and Cdk1 (Cdc2a), a probable kinase for this motif. Protein sequence analysis identified phosphorylation motifs in additional proteins, including SMAD2, PLK3 and INCENP. Mitotic SMAD2 and PLK3 phosphorylation was confirmed using phosphorylation-specific antibodies, and, in the case of Plk3, phosphorylation correlated with its localization to the mitotic apparatus and the midbody. A mutagenesis approach was used to show that INCENP phosphorylation is required for its localization to the midbody. These results provide evidence for a shared phosphorylation event that regulates localization of crucial proteins during mitosis.

Supplementary material available online at
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Key words: Phosphorylation, Mitosis, Proteomics, Chromosomal passenger complex

Introduction

A number of recent studies have focused on identifying the phosphoproteomes present in whole-cell lysates (Beausoleil et al., 2004; Stover et al., 2004; Kim et al., 2005; Olsen et al., 2006) and, although hundreds to thousands of novel phosphorylation sites have been identified, we still do not have a complete grasp on the total number of possible phosphorylation events and their regulation (Steen et al., 2006). In addition, identification of thousands of phosphorylation sites poses problems in how to interpret the functional significance of individual phosphorylation events. These problems can be partially alleviated by analyzing alterations in the phosphoproteomes and signaling pathways under different cellular conditions (Yang et al., 2006; Olsen et al., 2006). Another approach is to analyze a subset of the phosphoproteome to place the identified phosphorylation sites into an important cellular context. As an example of this latter approach, Nousiainen et al. analyzed the phosphoproteome of the mitotic spindle and identified hundreds of specific phosphorylation sites with the potential to regulate various mitotic functions (Nousiainen et al., 2006).

In this paper, we took a more focused approach to identify specific phosphorylation motifs on proteins that potentially interact with or regulate the chromosomal passenger complex (CPC), a crucial regulator of chromosome, cytoskeleton and membrane dynamics throughout mitosis (Adams et al., 2001). The CPC derives its name from its transient association with chromosomes (Earnshaw and Bernat, 1991), localizing to kinetochores during the early stages of mitosis and at the midzone, cleavage furrow and midbody as cells progress through mitosis. The CPC contains Aurora-B (Aurkb) and other proteins, including INCENP, that regulate Aurora-B activity and localization (Vader et al., 2006). Aurora-B kinase phosphorylates crucial substrates throughout mitosis and controls a number of mitotic events, ranging from the phosphorylation of histone H3 and displacement of heterochromatin protein-1 (Cbx5) from mitotic chromosomes (Hirota et al., 2005; Fischle et al., 2005) to the execution of cytokinesis at the end of mitosis (Carmena and Earnshaw, 2003).

To maintain genomic integrity and ensure the proper completion of mitosis, the activity of the CPC itself must be tightly regulated. Regulated gene expression and altered post-translational modifications prevent premature activation before mitosis begins, and several phosphorylation sites on the CPC have been identified that regulate CPC formation and activity (reviewed in Vagnerelli and Earnshaw, 2004; Vader et al., 2006). During the early stages of mitosis, a balance between

Journal of Cell Science

CPC kinase activity and protein phosphatases might prevent premature entry into anaphase. Aurora-B can physically associate with protein phosphatase 2A (PP2A) (Sugiyama et al., 2002), and it has recently been shown that PP2A along with Shugoshin (SGOL1) counters phosphorylation of kinetochore proteins such as cohesin before anaphase onset (Tang et al., 2006). Interactions between the CPC and spindle checkpoint proteins also play important roles in preventing premature entry into mitosis, before the chromosomes are properly positioned in the spindle, perhaps by the ability of the CPC to sense lack of spindle tension (Vagnerelli and Earnshaw, 2004). Aurora-B kinase activity is controlled by regulated protein degradation by the anaphase-promoting complex/cyclosome (APC/C) and is physically associated with FZR1 (Cdh1), an essential regulator of the APC/C, during mitosis (Stewart and Fang, 2005).

Although significant research activity has focused on the function and regulation of the CPC recently, we do not have a complete understanding of the complex regulatory mechanisms that control this essential mitotic complex. Here, we identify mitotic phosphoprotein complexes, which, by immunofluorescent staining, were found to behave identically to the CPC. Analysis of the phosphoprotein complexes by reversed-phase liquid chromatography (RPLC) coupled to mass spectrometry (MS) identified three known CPC components, INCENP, Aurora-B and TD-60 (Rcc2, RCC1-like), as well as other proteins that could be novel CPC components, regulatory factors or binding proteins. A phosphorylation motif on some of these proteins, including INCENP and polo-like kinase 3 (PLK3), might play a role in regulating protein localization and/or interactions with the CPC. The identification of novel proteins that interact with the CPC as well as post-translational modifications on known and potential CPC components provides further insight into the composition and regulation of this important protein complex.

Results

The progesterone receptor (PR) is a known substrate of cyclin-dependent kinases that phosphorylate the PR on several residues, including serine 190 (Moore et al., 2007). A monoclonal antibody (MAb P190) generated against a phosphopeptide sequence containing this phosphoserine, VLPRGLS(*P*)PARQLL, has proven to be a useful tool to detect phosphorylated PR (Clemm et al., 2000). However, in human cell lines, which do not express detectable levels of PR, we found that the P190 antibody cross-reacts with additional phosphoproteins exclusively during mitosis. In human cell lines, including HeLa and human mammary epithelial cells (HMECs), P190 bound to mitotic structures, with immunoreactivity localized to kinetochores, during prophase and metaphase; with the midzone after sister chromatids separate; and with the midbody at the completion of mitosis (Fig. 1A). With the exception of midbody staining, little immunoreactivity was observed in interphase cells. Following overnight treatment with nocodazole, many of the cells were arrested in prophase and showed high levels of P190 immunoreactivity on kinetochores (Fig. 1B). After nocodazole removal, many of the arrested cells progressed through mitosis and P190 immunoreactivity moved from kinetochores to the midzone and finally to the midbody. Some of the cells, particularly those shown in the middle panel of Fig. 1A, did not exhibit mitotic characteristics and were clearly not labeled by the P190 antibody. To confirm that this immunoreactivity coincided with CPC localization, we labeled cells with Aurora-B (Fig. 1C) and INCENP (Fig. 1D) to demonstrate that P190 immunoreactivity localizes with these integral CPC proteins in both HeLa cells and HMECs. This localization pattern was restricted to human cell lines, because we observed little reactivity with cell lines derived from other species, including mouse (Fig. 1E) and pig (not shown).

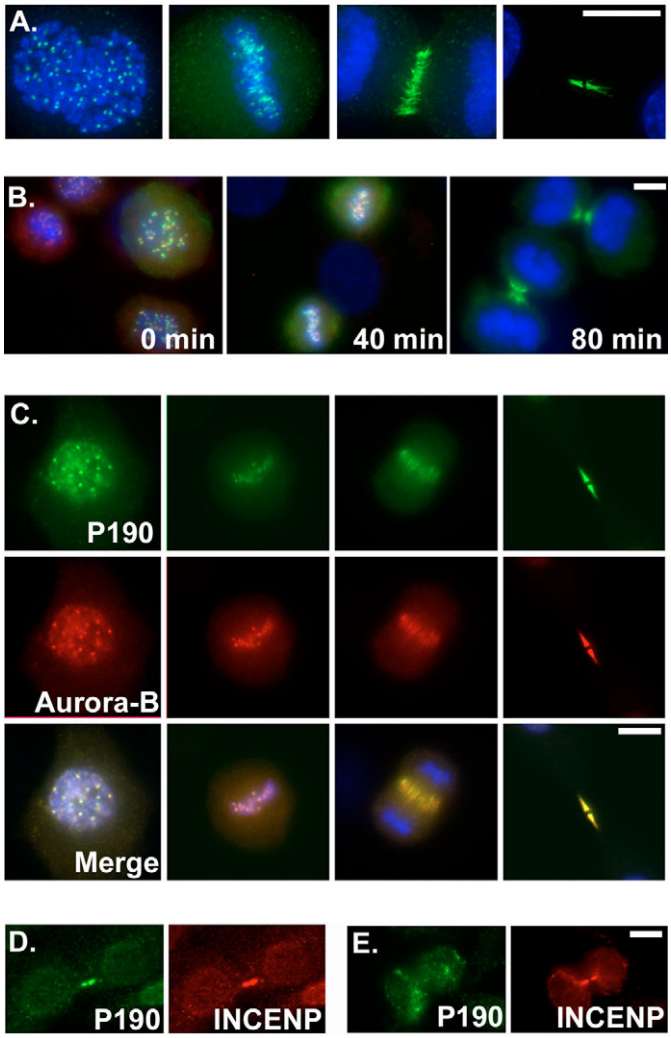

Fig. 1. P190 immunostaining colocalizes with the CPC in human cells. (A) Immunofluorescence staining of mitotic HeLa cells shows P190 immunoreactivity (green) on kinetochores from prophase to metaphase, in the midzone during anaphase to telophase and in the midbody at the completion of mitosis. With the exception of residual midbody staining, no P190 immunoreactivity is observed during interphase. (B) Nocodazole treatment results in a large number of cells in prophase that progress through mitosis after nocodazole has been removed for 40 or 80 minutes. (C) Dual immunofluorescence shows that P190 (green) colocalizes with Aurora-B (red) throughout mitosis. (D,E) P190 immunoreactivity (green) colocalizes with INCENP (red) in HMECs (D) but not in mouse RAW macrophages (E). DNA is labeled with DAPI (blue). Bars, 10 μm.

Western blot analysis demonstrated that the P190 phosphorylation-specific antibody detects several major protein

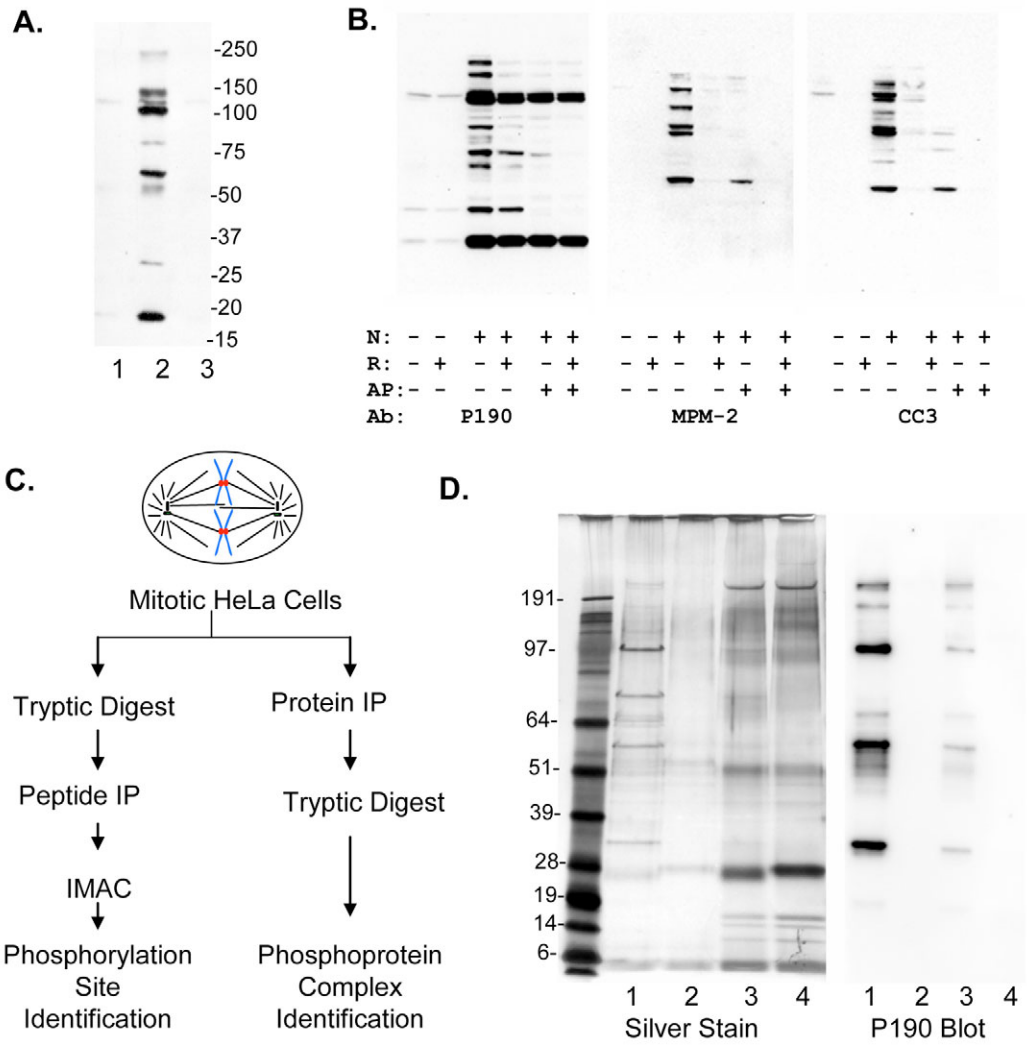

Fig. 2. Recognition of multiple mitotic phosphoproteins by P190. (A) On western blots, P190 recognizes several phosphoproteins in nocodazole-arrested cells (lane 2) but not in unsynchronized (lane 1) or thymidine-treated (lane 3) cells. Molecular weight markers are shown in kD. (B) P190 recognizes a subset of mitotic phosphoproteins that are distinct from those recognized by MPM-2 and CC3 and that show differential sensitivity to CDK inhibition and phosphatase treatments. Shown are western blots on lysates from cells treated with nocodazole (N), roscovitine (R) or alkaline phosphatase (AP), as indicated, and probed with P190, MPM-2 or CC3 antibodies. (C) Strategy for identifying protein complexes and phosphorylation sites containing the P190 phosphoepitope. (D) Immunoprecipitation with P190 (lanes 1 and 3) or control antibody (lanes 2 and 4) eluted with 8 M urea (lanes 1 and 2) or boiling in SDS gel loading buffer (lanes 3 and 4).

bands in mitotic HeLa cell extracts, ranging in size from ~18-225 kD (Fig. 2A). The antibody recognized the same protein bands in HMECs but not in cell lines derived from other species (data not shown), suggesting that these phosphoproteins are common to human cell lines. With increased exposure times, the blots showed that there are multiple minor immunoreactive bands as well (Fig. 2B). Comparative western blots using two other mitotic phosphorylation-specific antibodies, MPM2 (Davis et al., 1983) and CC-3 (Thibodeau and Vincent, 1991), showed that P190 reacts with a distinct subset of mitotic phosphoproteins (Fig. 2B). In addition, P190 reacted with kinetochores in prophase and metaphase; therefore, the phosphoepitope is probably distinct from the 3F3/2 epitope, which is only associated with unattached kinetochores (Campbell and Gorbsky, 1995).

To determine potential kinases that phosphorylate the P190-reactive proteins, we treated mitotic cell extracts with the cyclin-dependent kinase (Cdk) inhibitor roscovitine and observed that many of the P190-reactive proteins were affected by this treatment (Fig. 2B). By comparison, most of the MPM2 and CC-3 immunoreactivity was abolished by these treatments. These results suggest that Cdks and possibly other kinases are responsible for the phosphorylation of the P190-reactive proteins during mitosis. Although most of the P190-reactive bands were affected by addition of phosphatase to cell lysates prior to electrophoresis, three bands were resistant, suggesting that the phosphatase does not have access to the phosphorylation sites on the intact protein. The lower phosphatase-resistant polypeptide was also not efficiently immunoprecipitated (Fig. 2D), suggesting that this

phosphoepitope is inaccessible to both phosphatases and the antibody in the intact protein.

We next performed two types of LC-MS-based analyses to determine the phosphoepitope and protein complexes recognized by P190, as outlined in Fig. 2C. In the first set of experiments, we analyzed phosphopeptides using a modification of a two-step affinity purification procedure described for phosphotyrosine-containing peptides (Zhang et al., 2005). Following tryptic digestion of nocodazole-arrested HeLa cell lysates, peptides were immunoprecipitated with P190, MPM2, CC3 or anti-GFP, which served as our negative control. Eluted peptides were further purified using immobilized metal-ion affinity chromatography (IMAC) as a second phosphopeptide-enrichment step. To affinity purify whole-protein complexes, immunoprecipitations (IPs) were performed on mitotic cell lysates. Shown in Fig. 2D is a silver-stained gel and corresponding western blot showing specific enrichment of several protein bands in the P190 IPs but not control IPs.

To identify phosphopeptides, LC-MS/MS was performed and the resulting spectra were analyzed using SEQUEST (Eng et al., 1994; Yates et al., 1995). To eliminate false positive identification of phosphopeptides that results from the inefficient fragmentation of phosphopeptides during MS/MS (DeGnore and Qin, 1998), we applied a rigorous filtering criteria (see Materials and Methods) and manual confirmation of spectra (Yang et al., 2006). The majority of phosphopeptides identified (492 out of 504 phosphopeptide spectra) had several amino acids in common with the P190 antigen and were present in 11 proteins (Table 1 and supplementary material Table S1 for a complete list of phosphopeptide variants). Eight of the corresponding proteins contained phosphopeptides with the sequence PX[G/S/T]LS(*P*)P, one protein had the sequence PMGMS(*P*)P and two proteins contained the sequence WGLS(*P*)P. Phosphopeptides from four proteins had no discernible similarities to the P190 antigen. However, given the low number of identified phosphopeptide spectra by MS analysis, these phosphopeptides are present in lower abundance (Liu et al., 2004).

The two most abundant motif-containing phosphopeptides were from 60S ribosomal protein L12 (RPL12) and splicing factor 1 (SF1). Two of the proteins containing the motif, FZR1 and RIC-8, both have reported functions that overlap with the CPC: FZR1 is a key regulator of the APC/C that reportedly associates with Aurora-B (Stewart and Fang, 2005) and RIC-8 is required for localization of heterotrimeric G-proteins to the cleavage furrow (Couwenbergs et al., 2004; David et al., 2005; Wang et al., 2005). Several proteins with unknown or poorly characterized functions were also identified. Phosphopeptide IPs with MPM2, CC3 and anti-GFP (supplementary material Table S2) showed no overlap with the phosphopeptides immunoprecipitated by P190. The phosphopeptide IP for MPM2 produced no peptides that passed our selection criteria. CC3 identified 27 phosphoproteins, including TACC3 – a protein that is phosphorylated by Aurora-A (Aurka) and localizes to centrosomes (Kinoshita et al., 2005). We did not identify any obvious amino acid patterns in the phosphopeptides immunoprecipitated by MPM2 or CC3.

To determine the composition of phosphopeptide complexes, we next performed LC-MS-based analyses on whole-protein IPs from nocodazole-arrested HeLa cell lysates using the P190 antibody and a non-specific control antibody (anti-GFP) (Fig. 3B). Equivalent samples to those shown on the western blot and silver stain were digested with trypsin and analyzed by LC-MS/MS. Two biological replicates were performed and any proteins identified by a single peptide were excluded from further analysis to reduce non-specific peptides. From this, we identified 213 proteins exclusively in the P190 IP plus an additional 69 proteins with greater than fourfold enrichment relative to the control IP calculated by the ratio of total peptide (spectra) counts. Table 2 contains a partial list of these proteins. For a complete listing of the enriched proteins, see supplementary material Table S3. Complete datasets containing peptide sequences of all identified proteins can be downloaded at http://ncrr.pnl.gov/data.

In the P190 IP, we identified three known CPC components: INCENP, Aurora-B and TD-60 (Table 2). Ten of the eleven proteins identified in the phosphopeptide IP with P190 phosphorylation motifs were also present in the whole protein

Table 1. Phosphopeptides identified following peptide IP

Protein	Phosphopeptide	Phosphopeptide IP	Protein IP
FZR1	K.RSS*PDDGNDVS**PY**S**LS*P**VSNK	22	82
RIC8A	R.VIQ**P**MGM**S*P**R.G	9	141
ABLIM1	R.QSLGESPR**T*LS*P**TPSAEGYQDVR.D	13	269
RPL12	K.IG**PL**G**LS*P**K.K	157	34
VGLL4	K.NSLDASR**P**A**GLS*PT***LTPGER.Q	4	7
ZADH2	R.LIVIGFISGYQT**PT**G**LS*P**VK.A	2	2
DKFZP434K1815	K.RPDDV**P**LSLS*PSKR.A	27	62
KIAA0182	K.AGGPAIPSHLLSTPY**PF**G**LS*P**SSVVQDSR.F	6	10
SEC16L	R.ADSGPTQP**PL**S**LS*P**APETK.R	14	494
SF1	K.YACGLW**GLS*P**ASR.K	220	132
NKAP	R.IGELGAPEVW**GLS*P**K.N	18	0
THRAP3	R.ES*VDSRDSS*HSR.E	5	86
TBC1D15	K.KDPYT*ATMIGFSK.V	3	0
TRMT1	K.QERLSET*S*PASR.I	2	0
IFIT5	K.KLSSPSNY*K.L	2	0

Most of the phosphopeptides passing the screening criteria outlined in Fig. 3C had amino acids, including phosphorylated serine, in common (bold) with the P190 antigen. The total number of phosphopeptide spectra identified in the phosphopeptide IP and the total number of peptide spectra identified in the protein IP are shown. Minor variations in these phosphopeptides occurred in some cases due to alterations in peptide length or due to the presence of multiple phosphorylation sites; these are shown in supplementary material Table S1; . indicates trypsin cleavage site.

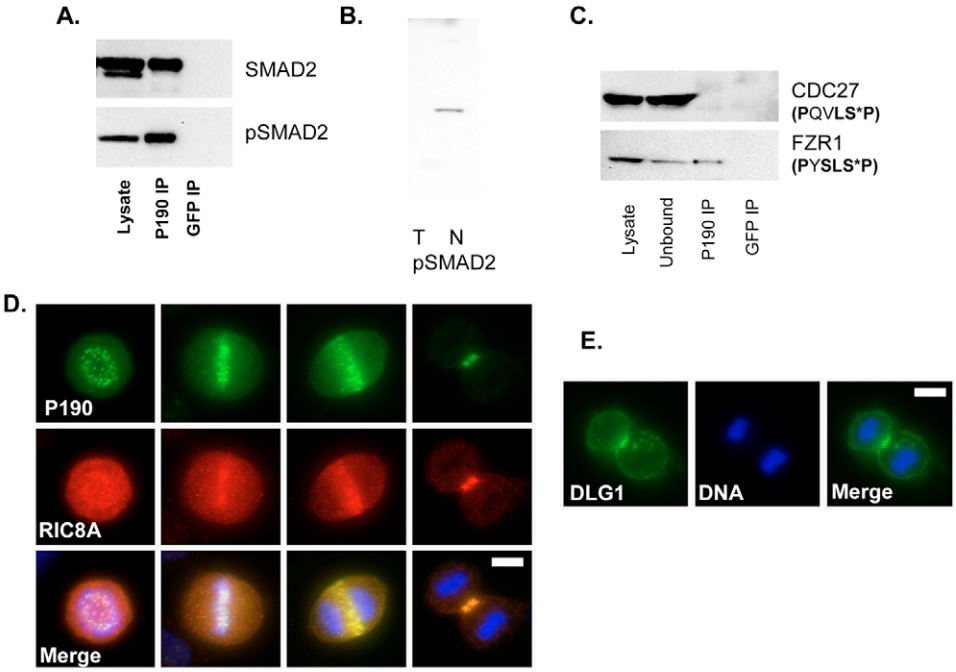

Fig. 3. Validation of proteomic results. (A) SMAD2 and phospho-SMAD2 are specifically immunoprecipitated from HeLa mitotic cell lysates using the P190 antibody. (B) SMAD2 phosphorylation on the P190 motif is increased in nocodazole (N)-arrested versus thymidine (T)-arrested HeLa cells. (C) FZR1 is immunoprecipitated by P190, but CDC27, which contains a related motif, is not. (D) RIC8A (red) faintly colocalizes with P190 (green) during prophase and metaphase, and the amount of colocalization increases at later stages of cell division. (E) DLG1 (green) localizes to the cleavage furrow. DNA is labeled with DAPI (blue). Bars, 10 μm.

IPs (Table 1), providing further confirmation that these are authentic P190-interacting proteins. By contrast, only one of the four non-motif phosphoproteins was found in the whole-protein IP. The most abundant protein identified in our analysis was IRSp53 (BAIAP2), which interacts with Rac and CDC42 to regulate actin cytoskeleton dynamics (Miki et al., 2000). COPII vesicle components (SEC13, SEC16L, SEC23 and SEC24) were also highly enriched, as were the proteasome inhibitor PI31 (PSMF1) and the serologically defined colon cancer antigen 3 (SDCCAG3). Proteins that form complexes with splicing factors

Table 2. Additional proteins of interest in P190 IPs

Protein description	P190 peptides	Control peptides	Protein function	P190 motif
INCENP	2	0	CPC component	**PRTLSP**
Aurora-B	34	1	CPC component	
TD-60	9	0	CPC component	
SMAD2	61	0	TGF-β signaling	**PTTLSP**
SMAD4	6	0	TGF-β signaling	
STRAP	4	0	TGF-β signaling	
SKP1	5	0	SCF ubiquitin ligase	
FBX7	6	0	SCF ubiquitin ligase	
Condensin	5	0	Chromosome condensation	
CDK1	2	0	Mitotic kinase	
NUMA	2	0	Spindle regulation	
PP2A	9	1	Phosphatase	
HDAC2	5	1	Chromatin regulation	
HDAC3	4	0	Chromatin regulation	
TBL1XR1	21	0	Chromatin regulation	
Clathrin heavy chain	66	2	Vesicle trafficking	
Clathrin light chain	3	0	Vesicle trafficking	
SEC23	558	0	Vesicle trafficking	
SEC24	378	0	Vesicle trafficking	PGI**GLSP**
SEH1L	218	15	Vesicle trafficking	
SIPA1	2	0	RAP1 GAP	**PRGLSP**
KLF12	6	0	Transcription	**PRGLSP**
CRTC3	75	1	Transcription	**PLTLSP**
PSMF1	273	0	Proteasome regulation	**PIGTSP**
BAIAP2	704	0	Actin regulation	**PKSLSP**
DLG1	8	0	Membrane dynamics	**PSSLSP**
SDCCAG3	158	0	Unknown	**PAGTSP**

Shown is a subset of the proteins identified by mass spectrometry in P190 IPs; the total numbers of peptide spectra identified in the P190 versus the control IP are shown. Those proteins containing a potential P190 motif are indicated. Amino acids in common with the P190 antigen are shown in bold. For a complete list of proteins see supplementary material Table S3.

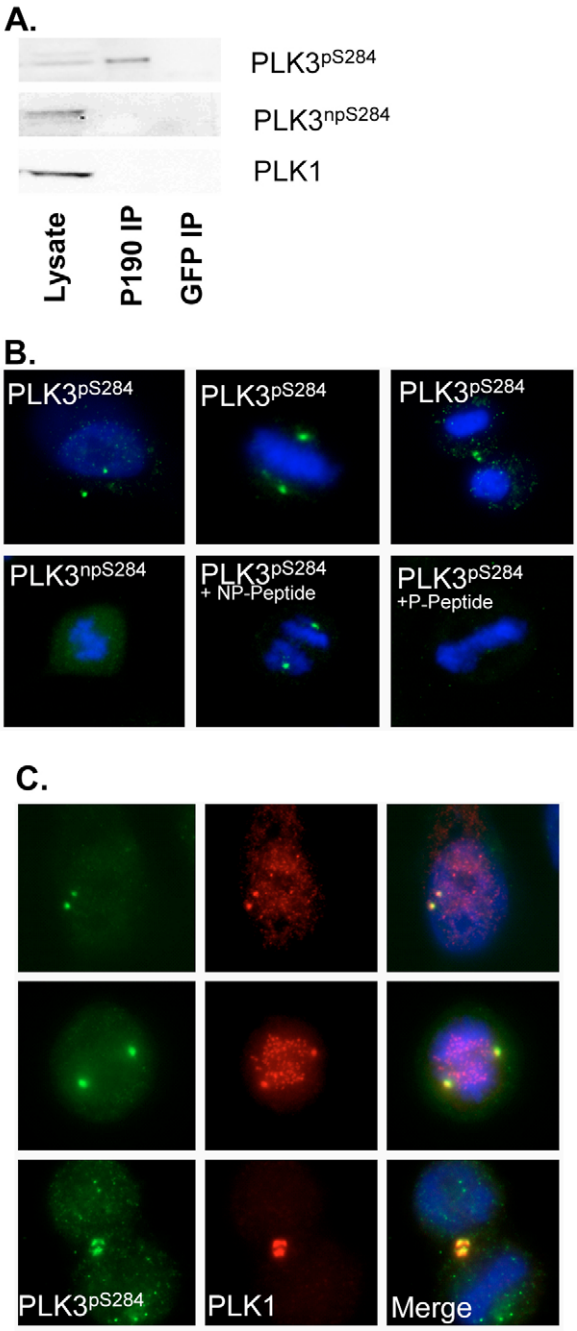

Fig. 4. Identification of the phosphorylation motif in PLK3. (A) Phosphorylated PLK3 ($PLK3^{pS284}$) but not non-phosphorylated PLK3 ($PLK3^{npS284}$) or PLK1 is present in P190 IPs. (B) Phosphorylated PLK3 localizes to centrosomes, spindle poles and midbodies during mitosis. By contrast, non-phosphorylated PLK3 is more diffusely distributed than this during mitosis. The staining pattern of phosphorylated PLK3 is unaffected by blocking with the non-phosphorylated peptide (NP-Peptide) but disappears in the presence of the phosphorylated peptide (P-Peptide). (C) Phosphorylated PLK3 (green) localization partially overlaps with PLK1 (red) at centrosomes, spindle poles and midbodies, and with the CPC at midbodies.

and ribosomes made up a large portion of the proteins present in the P190 IP (see supplementary material Table S3), suggesting that these protein complexes are immunoprecipitated as a result of the phosphorylation sites present on SF1 and RPL12. Other proteins and protein complexes of potential interest in the P190 IP include Cdk1 (Cdc2a), SCF ubiquitin ligase complex components [SKP1 (SKP1A) and FBX7 (FBXO7)], histone deacetylases, several 14-3-3 proteins, NuMA, clathrin, PP2A, casein kinase, and the TGFβ signaling components, SMAD2, SMAD4 and STRAP.

Based on our phosphopeptide IP data, we concluded that the P190 antibody preferentially recognizes proteins containing sequences of **P**X[**G/S/T**]**LS**(*P*)**P** (bold represents consensus motif amino acids). To determine whether other proteins present in the P190 IP contain this motif, we searched the amino acid sequences of each protein in the IP and found several proteins with this potential phosphorylation motif. No additional proteins containing WGLSP or methionine in place of the leucine were identified. The most interesting of the motif-containing proteins is the CPC component INCENP, which contains the sequence **PRTLSP**. Other proteins with potential phosphorylation sites include SMAD2 (**PTTLSP**), BAIAP2 (**PKSLSP**), DLG1 (**PSSLSP**), Krueppel-like factor 12 (KLF12; **PRGLSP**), signal-induced proliferation-associated protein 1 (SIPA1; **PRGLSP**) and transducer of regulated CREB protein 3 (CRTC3; **PLTLSP**). SEC24 (PGI**GLSP**), PSMF1 (**PIGTSP**) and SDCCAG3 (**PAGTSP**) contain related motifs, which could account for their high abundance in the protein IP. Two of these sites, in INCENP (Nousiainen et al., 2006) and in CRTC3 (Olsen et al., 2006), were identified in global phosphoproteomic analyses, whereas the SMAD2 site was identified in a focused analysis on SMAD2 phosphorylation (Kretzschmar et al., 1999), providing evidence that these are actual phosphorylation sites in vivo.

To validate our proteomic data, we independently confirmed that both SMAD2 and FZR1 were enriched in P190 IPs using western blot analyses of P190 IPs. As shown in Fig. 3A, a subset of SMAD2 protein is immunoprecipitated by the P190 antibody. The SMAD2 antibody detected a doublet in whole-cell lysates but only the higher molecular weight band was specifically present in the P190 IP. Using an available antibody that recognizes SMAD2 phosphorylated on any of three closely spaced sites, including the putative P190 motif (PTTLSP) (Kretzschmar et al., 1999), we confirmed that the SMAD2 present in the P190 IP is phosphorylated (Fig. 3A). Because P190 phosphorylations occur during mitosis, SMAD2 should be phosphorylated on this site primarily during mitosis as well. To confirm this, we performed a western blot on control and nocodazole-treated cell extracts, and only found detectable phosphorylated SMAD2 in the nocodazole-arrested lysates (Fig. 3B). We next demonstrated that FZR1 is specifically present in the P190 IPs but a separate APC/C regulator, CDC27, which contains an amino acid sequence (**PQVLSP**) that is similar but not identical to the P190 motif, is not recognized by the P190 antibody (Fig. 3C).

To determine whether the proteins identified by LC-MS localize to the mitotic apparatus, we performed immunofluorescence with several commercially available antibodies. As expected, both Aurora-B (Fig. 1B) and INCENP (Fig. 1C) colocalized with P190. Phospho-SMAD2 was highly enriched in mitotic cells, confirming that phosphorylation occurs during mitosis, but little immunoreactivity was present at regions containing the CPC (data not shown). RIC8A colocalized with P190 and the CPC at later stages of mitosis

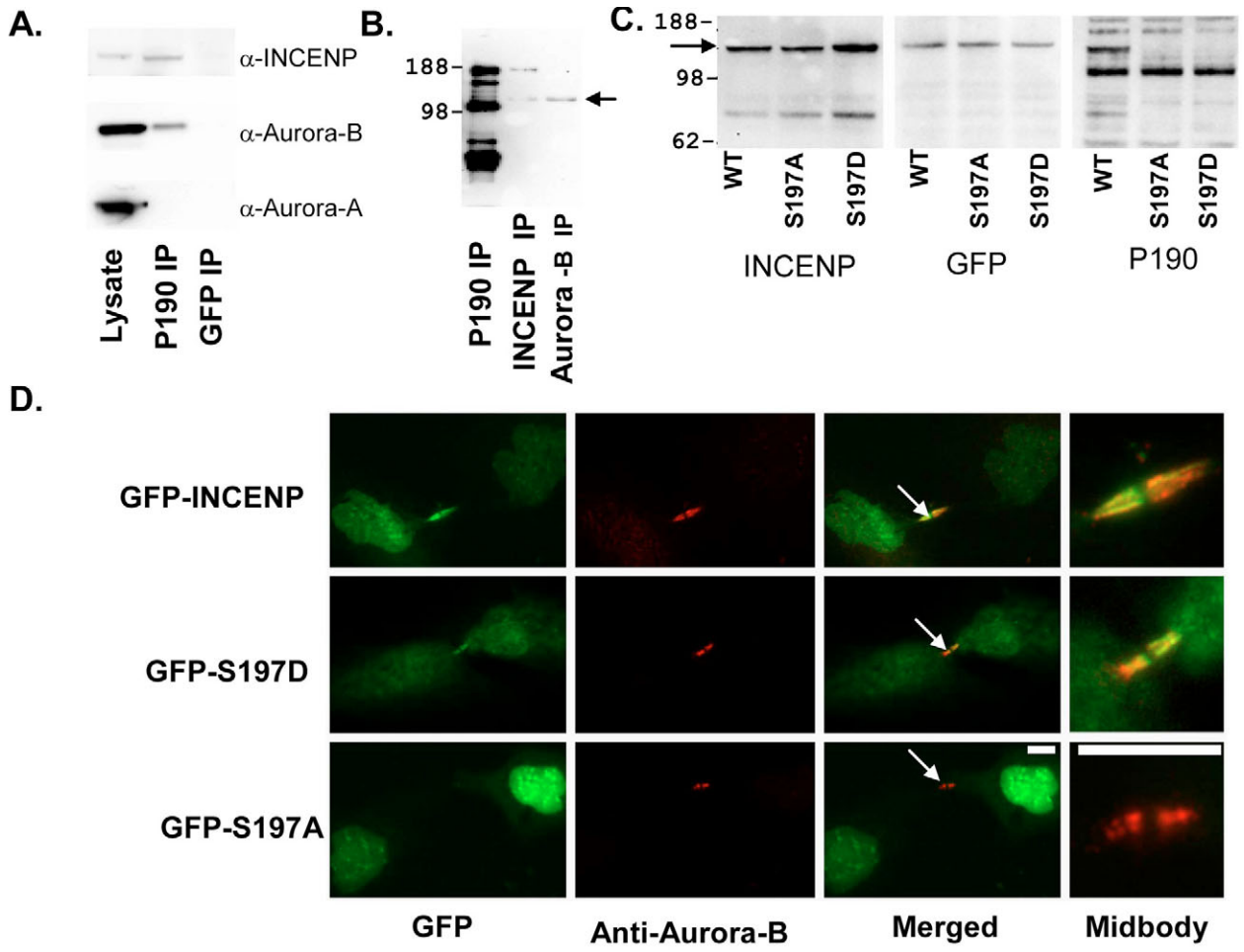

Fig. 5. Confirmation of INCENP as a P190-reactive phosphoprotein. (A) INCENP and Aurora-B are specifically immunoprecipitated from HeLa mitotic cell lysates using the P190 antibody. (B) P190 recognizes bands corresponding to the molecular weight of INCENP (arrow) in both INCENP and Aurora-B IPs. (C) Site-directed mutagenesis of serine 197 to alanine (S197A) or aspartate (S197D) prevents P190 binding. The arrow shows the location of GFP-INCENP. (D) Mutation of serine 197 to alanine, but not to aspartate, prevents INCENP from properly localizing to the midbody. The arrow denotes the midbody in the merged panel and the region containing the midbody was enlarged to clearly demonstrate that S197A (green) is not present in the midbody containing Aurora-B (red). Bars, 10 μm.

(Fig. 3D). Faint colocalization was observed during prophase but this increased at metaphase chromosomes, the midzone and cleavage furrow, and the midbody as cells progressed through mitosis. This localization of RIC8A is consistent with previously published studies showing that RIC8A is required for the localization of heterotrimeric G-proteins to the cleavage furrow (Couwenbergs et al., 2004; David et al., 2005; Wang et al., 2005). DLG1 also localized to the cleavage furrow as the cells divided (Fig. 3E). These results demonstrate that several of the P190-reactive proteins identified by LC-MS and western blot analysis are phosphorylated during mitosis and colocalize to regions containing the CPC. Immunofluorescence with other antibodies, including FZR1 (data not shown), did not show conclusive overlap with P190 staining, perhaps because these antibodies do not distinguish between the phosphorylated and total pools of protein.

To determine whether additional phosphoproteins existed, we searched for motif-containing proteins using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST). Most of the proteins identified in this search were present in our IP analyses; however, we also identified PLK3, which was intriguing based on its similarity to PLK1, a protein that localizes to the CPC and interacts with INCENP (Goto et al., 2006). The PLK3 motif was slightly different in that it contained a leucine between the presumed phosphorylated serine and the proline, but it contained more sequence similarity to the P190 peptide in other regions (**LPRGLSPARQLL** for P190 versus **LP**A**LS**L<u>L</u>**PARQLL** for PLK3; bold represents consensus amino acids; underlined represents additional amino acids). To confirm the phosphorylation of PLK3, we generated antibodies that recognize PLK3 that is either phosphorylated ($PLK3^{pS284}$) or non-phosphorylated ($PLK3^{npS284}$) on serine 284. Using these antibodies, we determined that phosphorylated PLK3, but not non-phosphorylated PLK3 or PLK1, was immunoprecipitated by P190 (Fig. 4B). We then performed immunofluorescence to demonstrate that phosphorylated PLK3 localized to spindle poles, midzones and midbodies (Fig. 4C). This localization pattern could be disrupted by pre-incubation with the phosphorylated peptide, confirming the antibody specificity.

Colocalization experiments revealed that the distribution of phosphorylated PLK3 partially overlaps with PLK1 at centrosomes and midbodies (Fig. 4D). These results suggest that this site-specific phosphorylation plays a role in targeting PLK3 to specific cellular regions during mitosis.

We next set out to confirm that INCENP is immunoprecipitated by P190 because of the presence of this specific phosphorylation motif. Whereas numerous peptides were identified for Aurora-B (34), only two peptides were identified for INCENP (Table 2). First, to confirm that INCENP was specifically present in P190 IPs, we performed western blot analyses on P190 and control IPs using an INCENP antibody. As shown in Fig. 5A, both Aurora-B and INCENP were immunoprecipitated by P190 but not control antibodies, demonstrating that the Aurora-B–INCENP complex is recognized by the P190 antibody. As a control, we repeated this with Aurora-A and observed no Aurora-A immunoreactivity in the P190 IP. To indirectly show that INCENP is phosphorylated on the P190 motif, we performed IPs with INCENP and Aurora-B antibodies and probed these with P190. As shown in Fig. 5B, P190 recognized a band corresponding to the molecular weight of INCENP in both the INCENP and Aurora-B IPs, indicating that INCENP is phosphorylated and that this phosphorylation provides the basis for the presence of both INCENP and Aurora-B in the IP. In the INCENP IP, there is an additional high molecular weight protein recognized by P190, suggesting that INCENP interacts with an additional P190-immunoreactive protein of unknown identity.

To determine whether the phosphorylation of INCENP on serine 197 is necessary for antibody recognition, we generated GFP-tagged wild-type and mutant proteins. As shown in Fig. 5C, P190 recognized wild-type INCENP but did not bind to INCENP mutants in which the serine was mutated to alanine (S197A) or aspartate (S197D). From this, we concluded that serine 197 is phosphorylated on INCENP during mitosis and that this phosphorylation provides the basis for recognition by the P190 antibody. A probable explanation for why this phosphopeptide was not detected in the P190 phosphopeptide IP is that trypsin will cleave INCENP within the motif because of the presence of an arginine (P**R**TLSP). We next tested whether the phosphorylation of INCENP is important for its localization during mitosis by performing localization experiments on GFP-tagged wild-type and mutant INCENPs. As shown in Fig. 5D, both wild-type INCENP and the mutated S197D localized normally to the midbody at the completion of mitosis, whereas S197A failed to localize to the midbody, suggesting that this phosphorylation does play a role in localizing INCENP to the mitotic apparatus.

Discussion

Progression through mitosis requires the orchestration of multiple cellular events involving alterations in chromosomal, cytoskeletal and membrane dynamics to ensure faithful segregation of genetic material and other vital cellular components. The complexity of this process and the necessity to minimize errors to prevent cell death or the uncontrolled proliferation associated with cancer, requires spatial and temporal coordination between multiple cell signaling pathways. Although many of the kinases and specific phosphorylation events that regulate mitotic progression have been identified, our understanding of this fundamental cellular process is still evolving. Our aim here was to identify and characterize a subset of the mitotic phosphoproteome to increase our understanding of the molecular events that regulate mitosis and place the identified phosphorylation events into an important cellular context. In this paper, we identified a novel shared mitotic phosphorylation motif present on a number of proteins with both known and unknown functions; this motif might be involved in regulating the distribution and/or activity of these proteins during mitosis.

Among the proteins identified in our analysis was the integral CPC component INCENP – a crucial regulator of CPC activity and organization (Carmena and Earnshaw, 2006). We conclude, based on the crossreactivity of the P190 antibody with wild-type but not mutant INCENPs, that INCENP is phosphorylated on a P190 motif-containing serine 197. Interestingly, this site is not conserved in other mammalian INCENPs, providing a potential regulatory mechanism that is unique to humans. Nousiainen et al. showed that INCENP that co-purifies with mitotic spindles is phosphorylated on this site, providing confirmation that this is an authentic INCENP phosphorylation site (Nousiainen et al., 2006). However, in that report, it was one of several hundred total phosphorylation sites identified. Our results help to place this site-specific phosphorylation into a cellular context by demonstrating that the phosphorylation occurs during mitosis and might play a role in regulating INCENP localization. This phosphorylated INCENP is associated with Aurora-B, as evidenced by their co-immunoprecipitation with P190, INCENP and Aurora-B antibodies. We also detected TD-60 in P190 IPs by LC-MS, suggesting that this known CPC component (Mollinari et al., 2003) is in the same protein complex as phosphorylated INCENP and Aurora-B.

PLK1 has overlapping functions with the CPC, and a subset of the cellular pool of PLK1 localizes to the CPC and directly associates with INCENP (Goto et al., 2006). We found no PLK1 in our IPs applying either LC-MS or western blot analyses, indicating that the phosphorylated INCENP complexes do not contain Plk1. Interestingly, however, PLK3 was present in the P190 IPs because of the presence of a specific phosphorylation site. Total PLK3 has a diffuse distribution with some localization to centrosomes, mitotic spindles and midbodies (Wang et al., 2002). This localization to the mitotic apparatus is dependent upon the polo-box domains (Jiang et al., 2006). Our results suggest that phosphorylation of PLK3 in a region located between the polo-box and kinase domains leads to increased PLK3 localization to the mitotic apparatus.

Only some of the phosphorylated peptides immunoprecipitated by P190 contained conventional CDK phosphorylation motifs {S(*P*)PX[R/K/H]}, explaining the differential susceptibility of P190-reactive proteins to CDK inhibition (Fig. 2). Other motifs present in these peptide sequences include CaM kinase family phosphorylation motifs [RXXS(*P*)] and any of the many serine/proline-dependent protein kinases. This suggests that the motif is not a recognition motif for an individual kinase but might be a structural motif that is accessible to kinases and potentially other binding proteins. However, if different kinases are responsible for the phosphorylation of different P190-reactive proteins, these kinases are activated within the same time-frame, because cell synchronization studies revealed no

differences in the relative phosphorylation of individual protein bands on western blots (data not shown). The existence of motif variants as observed for PLK3, SF1 and possibly other proteins such as SEC24 suggests that there might be additional mitotic phosphoproteins than those identified here. Furthermore, because the structural features required for antibody and kinase recognition might be different, additional proteins might exist that are phosphorylated by kinases but not recognized by the antibody.

Many of the P190 phosphorylation sites identified here are in the vicinity of additional serine and threonine residues that are phosphorylated or could potentially be phosphorylated. For example, some of the phosphopeptides from actin binding LIM protein 1 (ABLIM1) contain phosphorylation sites at the +2 and –2 positions in addition to the phosphoserine at position 0 within the motif, and Nousiainen et al. reported multiple phosphorylations on INCENP near to and including the P190 phosphorylation motif located at position 197 (Nousiainen et al., 2006). Most of the FZR1 phosphopeptides were also phosphorylated at the –13 position at a site containing SS(*P*)P, a consensus PLK1-binding site (Elia et al., 2003) that is a known regulator of the APC/C (Eckerdt and Strebhardt, 2006). Proteins containing phosphorylation sites with arginine at the –3 position within the motif could be 14-3-3-interacting proteins, which were also detected in our LC-MS analysis. These findings suggest that regions containing the P190 motifs could be the subject of complex regulation through the convergence of multiple phosphorylation events and interactions with phosphorylation-dependent binding partners that serve to regulate the spatially and temporally controlled progression through mitosis.

Some of the proteins identified here reportedly are either targets or regulators of the CPC. Among these is FZR1, a crucial regulator of the APC/C that is essential for mitotic progression and targeted degradation of mitotic substrates such as cyclin B (King et al., 1995) and securin (Zur and Brandeis, 2001). Growing evidence suggests that the APC/C is regulated by Aurora-B at the onset of mitosis (Morrow et al., 2005) and that it regulates Aurora-B by targeting it for degradation at the end of mitosis (Stewart and Fang, 2005). This Aurora-B degradation is mediated by FZR1, providing a link between these proteins (Visintin et al., 1997). HDAC3 functions as transcription repressor during interphase, but de-acetylates histone H3 during mitosis to create the preferred Aurora-B phosphorylation site that results in HP1 dissociation as chromosomes condense (Li et al., 2006).

SMAD2, along with the closely related SMAD3, reportedly interacts with FZR1 and the APC/C to control the targeted degradation of a subset of proteins (Nourry et al., 2004). SMAD2 is a key component of the TGFβ signaling pathway that shuttles to the nucleus following phosphorylation of the C-terminus where it regulates genes involved in maintaining tissue homeostasis (ten Djike and Hill, 2004). The P190 motif in SMAD2 is part of a series of three serine/proline residues that are not affected by TGFβ but instead are phosphorylated by Ras-activated MAP kinases to override the anti-proliferation effects of TGFβ (Kretzschmar et al., 1999). Here we show that these sites are also phosphorylated during mitosis and that this could play a role in regulating SMAD2 interactions with the APC/C and the CPC. This altered phosphorylation could also be involved in the molecular switch that results in TGFβ acting as a tumor suppressor to prevent cancer and a tumor promoter as cancer progresses (Iyer et al., 2005).

Although a number of the proteins identified in the P190 IPs have described functions independent of a role in mitosis, many have been reported to localize to the mitotic apparatus and/or have secondary roles during mitosis. For example, several ribosomal and nucleolar proteins undergo dynamic interactions with chromosomes during mitosis (Van Hooser et al., 2005), and splicing factors have a putative function in mitotic spindle assembly via a poorly understood mechanism (Blencowe, 2003; Kittler et al., 2004). mRNA and ribosomes could also be actual components of the mitotic apparatus (Alliegro et al., 2006; Pederson, 2006). Clathrin heavy chain, which plays a role in vesicle trafficking during interphase but localizes to the kinetochore during mitosis where it regulates kinetochore fiber stability (Royle et al., 2005), might also play a role in cytokinesis (Warner et al., 2006). The COPII complex is involved in vesicle trafficking; however, a member of this complex, SEC13, localizes to the kinetochore during mitosis and might be required for mitotic progression (Loiodice et al., 2004). Therefore, although at first glance many of these proteins might not be considered serious candidates for mitotic regulators or as CPC-interacting proteins, there is mounting evidence to suggest that many proteins have mitotic functions distinct from their well-characterized interphase functions.

The results presented here provide insight into a potential regulatory phosphorylation event shared by several known mitotic regulators. Several proteins with unknown functions were also identified as either containing the phosphorylation site or associating with these phosphoproteins. Future work that is focused on addressing potential mitotic functions for these novel proteins might lead to the identification of new mitotic regulators. In addition, functional characterization of the phosphorylation sites in these proteins might shed light on the role that this site-specific phosphorylation plays in regulating protein localization, function and mitotic progression.

Materials and Methods

Cell culture

HeLa cells were grown in Opti-MEM media (Invitrogen) supplemented with 4% heat-inactivated fetal bovine serum (FBS), streptomycin and penicillin. The human mammary epithelial cell (HMEC) line 184A1 (Stampfer and Yaswen, 1993) was maintained in DFCI-1 medium (Band and Sager, 1989). Mouse C2C12 cells and RAW macrophages were maintained in DMEM with 10% FBS, streptomycin and penicillin. Cells were arrested at the G2/M transition by overnight incubation in medium with 40 ng/ml nocodazole or at the G1/S transition by incubation in medium with 1 mM thymidine.

Immunoprecipitations

IP beads were prepared by incubating 25 μg of primary antibody, P190 (progesterone receptor Ser190 antibody; Labvision), mouse anti-GFP (Rockland Immunochemicals) MPM2 (Upstate) or CC3 (Upstate) with 200 μl of magnetic Dynabeads M-280 sheep anti-mouse IgG (Invitrogen) for 2 hours at room temperature. Beads were washed and primary antibodies were crosslinked to the beads using DMP (Pierce) according to the manufacturer's instructions. Non-adherent mitotic cells (~40×10^6 per experiment) were centrifuged and washed once with PBS. Cells were resuspended (4×10^6 cells/ml) in lysis buffer (50 mM HEPES, 150 mM NaCl, 0.5% NP-40, pH 7.4 with Halt protease and phosphatase inhibitors; Pierce), incubated for 15 minutes on ice and centrifuged at 14,000 ***g*** in a table top microcentrifuge at 4°C. Lysate was divided in half and incubated for 4 hours with P190 or control IP beads. Beads were washed four times for 10 minutes each with lysis buffer and each time beads were separated from lysis buffer using a magnet. Proteins were eluted by incubation in 8 M urea, 50 mM NH_4HCO_3 for 30 minutes at 37°C. Any remaining bound proteins were eluted by boiling beads in SDS sample buffer. Peptide IPs were performed using a modified procedure (Zhang et al., 2005). Basically, washed mitotic cells were lysed in 8 M urea supplemented with

phosphatase inhibitors, reduced with 10 mM DTT for 1 hour at 56°C and alkylated with 55 mM iodoacetamide for 45 minutes at room temperature. Trypsin (100:1 substrate:trypsin ratio; Promega) was added, and proteins were digested overnight at room temperature and purified as described below prior to performing the phosphopeptide IP.

Protein digestion and peptide analysis by the nRP-HPLC-μESI-MS/MS

IP samples were digested with modified trypsin (Promega). Samples were concentrated using a Speed Vac, acidified with glacial acetic acid to pH ~3, and desalted with a 360×200 μm fused silica column packed with 8 cm of 5-20 μm irregular sized C18 beads (YMC ODS-AQ, Waters). The desalted peptides were concentrated to ~20 μl using a Speed Vac. Aliquots (~1-5 μl) of the desalted peptides were loaded onto a 360×75 μm fused silica pre-column packed with 5 cm of 5-20 μm irregular sized C18 beads (YMC ODS-AQ, Waters), washed with 0.1% HOAc, connected to the analytical column [360×20 μm, 15-cm long monolithic column (Luo et al., 2006)] and analyzed by a combination of a nano-HPLC/micro-electrospray ionization on a LTQ mass spectrometer (Thermo Electron, San Jose, CA).

Immobilized metal-ion affinity chromatography (IMAC)

Peptides were desalted as described previously (Ficarro et al., 2002) with the exception that the 1-hour methylation reaction was repeated once to ensure complete methylation. Samples were resuspended in 0.1% HOAc (100 μl), loaded onto a reversed-phase desalting column (360 μm OD ×200 μm ID fused silica; Polymicro Technologies), washed with 0.1% HOAc and eluted to the Fe (III)-activated IMAC (360 μm OD ×200 μm ID fused silica) column packed with 9 cm POROS 20 MC (Applied Biosystems) with 20 μl of 40% acetonitrile (MeCN) in 0.1% HOAc. The IMAC column was washed with 30 μl of 25/74/1 ACN/water/HOAc containing 100 mM NaCl, 40 μl 0.1% acetic acid, and eluted with 25 μl of 20 mM ascorbic acid to a pre-column (360 μm O.D. ×75 μm I.D. fused silica; PolymicroTechnologies). The pre-column was washed with 40 μl of 0.1% HOAc and connected to the analytical column for LC-MS/MS analysis on a LTQ mass spectrometer.

LC-MS/MS parameters on the LTQ mass spectrometer

Samples were analyzed by nanoflow HPLC-microelectrospray ionization on a LTQ mass spectrometer (Thermo Electron, San Jose, CA). The HPLC gradient (A=100 mM acetic acid in water, B=80% MeCN/100 mM HOAc in water) was 0-40% B for 150 minutes, 40-60% B for 20 minutes, 60-100% B for 15 minutes, 100-0% B for 10 minutes and 0% B for an additional 85 minutes to equilibrate the column. Peptides were eluted from the reversed-phase column to the mass spectrometer through the etched emitter tip (Kelly et al., 2006) on the analytical column.

Database search and peptide identification

All MS/MS spectra recorded were searched against the Human International Protein Index (IPI) database (Version 3.05; www.ebi.ac.uk/IPI) using SEQUEST software (Eng et al., 1994; Yates et al., 1995). For peptides, all MS/MS data were searched against the protein database without enzyme restriction. For peptide IP, a differential modification of +80 Da (phosphate group) on serine, threonine and tyrosine was included for the phosphopeptide SEQUEST search and a static modification of +57 Da (carboxyamidomethylation) on cysteine was also used in the search. Only fully tryptic phosphopeptides were considered with Xcorr cutoff 1.4, 2.3 and 3.0 for 1+, 2+ and 3+ charge state peptides, respectively. Only those phosphopeptides present in multiple copies were considered for further analysis. MS/MS spectra for phosphopeptides passing these filtering steps were then manually confirmed. The number of peptide (spectra) counts was used to estimate the relative abundance of proteins (Liu et al., 2004).

Antibodies and immunostaining

Primary antibodies used were P190 (phospho-Progesterone Receptor Ser190 clone 1154; Labvision), Aurora-B, Aurora-A and FZR1 (BD Biosciences), DLG1 (Stressgen), RIC8A (Imgenix), SMAD2 and phospho-SMAD2$^{S245/250/255}$ (Cell Signaling), INCENP (Sigma-Aldrich), and MPM2 and CC-3 (Upstate). Antibodies recognizing phosphorylated PLK3 on serine 284 were generated by Pacific Immunology (Ramona, CA) by injecting rabbits with the synthetic peptide CIKQVHYTLPASLS(*P*)LPAR. Non-phosphorylation-specific antibodies were removed by purification on a column containing the non-phosphorylated version of the peptide and these were shown to recognize both endogenous and bacterially expressed non-phosphorylated PLK3. Phosphorylation-specific antibodies were then affinity purified using the phosphorylated peptide. Primary antibodies were detected with the corresponding secondary antibody, either Alexa-Fluor-488 goat anti-mouse or Alexa-Fluor-594 goat anti-rabbit (Invitrogen) for immunofluorescence or HRP-conjugated antibodies (Pierce).

Site-directed mutagenesis of GFP-tagged INCENPs

A vector encoding full-length INCENP was purchased from Invitrogen. Site-directed mutagenesis of serine 197 to alanine or aspartate was performed using the QuickChange II kit (Stratagene) and corresponding custom primers (Invitrogen). INCENP and each mutant were than amplified by PCR to add *Bsr*GI and *Bam*HI sites for insertion into the pEGFP-C1 vector (Clontech). Sequencing was performed to ensure each clone contained the correct sequence.

Microscopy

Fluorescence microscopy was performed as described previously (Stenoien et al., 1999). Images except Fig. 1A were acquired with a Nikon TE2000 inverted microscope using Metamorph software (Molecular Devices, Downington, PA) and a 60× 1.4 N.A. plan apo lens. Fig. 1A was generated by acquiring a *z*-series of focal planes using a Deltavision microscope (Applied Precision, Issaquah, WA) and deconvolved with the Deltavision software constrained iterative algorithm to generate high-resolution images. Final images were assembled using Adobe Photoshop with equivalent adjustments in background and brightness for each image panel.

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