Role of Polo-like kinase 1 in the regulation of the action of p31^{comet} in the disassembly of mitotic checkpoint complexes

Sharon Kaisari[a], Pnina Shomer[a], Tamar Ziv[b], Danielle Sitry-Shevah[a], Shirly Miniowitz-Shemtov[a], Adar Teichner[a], and Avram Hershko[a,1]

[a]Department of Biochemistry, The Rappaport Faculty of Medicine, Technion–Israel Institute of Technology, 31096 Haifa, Israel; and [b]Department of Biology, Smoler Proteomics Center, Technion–Israel Institute of Technology, 32000 Haifa, Israel

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The Mad2-binding protein p31^{comet} has important roles in the inactivation of the mitotic checkpoint system, which delays anaphase until chromosomes attach correctly to the mitotic spindle. The activation of the checkpoint promotes the assembly of a Mitotic Checkpoint Complex (MCC), which inhibits the action of the ubiquitin ligase APC/C (Anaphase-Promoting Complex/Cyclosome) to degrade inhibitors of anaphase initiation. The inactivation of the mitotic checkpoint requires the disassembly of MCC. p31^{comet} promotes the disassembly of mitotic checkpoint complexes by liberating their Mad2 component in a joint action with the ATPase TRIP13. Here, we investigated the regulation of p31^{comet} action. The release of Mad2 from checkpoint complexes in extracts from nocodazole-arrested HeLa cells was inhibited by Polo-like kinase 1 (Plk1), as suggested by the effects of selective inhibitors of Plk1. Purified Plk1 bound to p31^{comet} and phosphorylated it, resulting in the suppression of its activity (with TRIP13) to disassemble checkpoint complexes. Plk1 phosphorylated p31^{comet} on S102, as suggested by the prevention of the phosphorylation of this residue in checkpoint extracts by the selective Plk1 inhibitor BI-2536 and by the phosphorylation of S102 with purified Plk1. An S102A mutant of p31^{comet} had a greatly decreased sensitivity to inhibition by Plk1 of its action to disassemble mitotic checkpoint complexes. We propose that the phosphorylation of p31^{comet} by Plk1 prevents a futile cycle of MCC assembly and disassembly during the active mitotic checkpoint.

cell cycle | protein degradation | mitosis

The Mad2-binding protein p31^{comet} plays an important role in the inactivation of the mitotic checkpoint, but the mode of its regulation is not sufficiently understood. The mitotic (or spindle assembly) checkpoint is a surveillance system that ensures the fidelity of chromosome segregation in mitosis by preventing anaphase initiation until all chromosomes are correctly attached to the mitotic spindle (reviewed in refs. 1–5). The active mitotic checkpoint causes the accumulation of a Mitotic Checkpoint Complex (MCC), an inhibitor of the ubiquitin ligase Anaphase-Promoting Complex/Cyclosome (APC/C) that targets for degradation cyclin B and the anaphase inhibitor securin. MCC contains the checkpoint proteins Mad2, BubR1, and Bub3, bound to the APC/C activator Cdc20. The assembly of MCC is initiated in the active checkpoint by the conversion of Mad2 from an open (O-Mad2) to a closed (C-Mad2) conformation. C-Mad2 binds to Cdc20 and the Cdc20–Mad2 subcomplex associates with BubR1-Bub3 to form MCC. When the checkpoint is turned off, MCC is disassembled, APC/C regains activity and initiates anaphase by targeting securin and cyclin B for degradation.

The pathways of the disassembly of MCC, essential for the inactivation of the mitotic checkpoint, are surprisingly complex. MCC exists in free and APC/C-bound forms, and the disassembly of MCC in both pools is required for the release of APC/C from checkpoint inhibition (6). The disassembly of APC/C-bound MCC requires the ubiquitylation of the Cdc20 (7) and of BubR1 (8) components of MCC while free MCC is disassembled by an ATP-requiring process (9) that involves the participation of p31^{comet} (10). This protein, also called CMT2, was discovered as a Mad2-binding protein that promotes progress through late mitosis (11). Subsequently, it was found that the structure of p31^{comet} is remarkably similar to that of Mad2 and that it specifically binds to the closed conformation of Mad2 (12). An important action of p31^{comet} is the release of Mad2 from free MCC jointly with the AAA-ATPase TRIP13 (13, 14). It is thought that, in this process, p31^{comet} targets the ATPase to MCC, by binding both TRIP13 and the C-Mad2 component of the mitotic checkpoint complex (15, 16). This system also dissociates the subcomplex Mad2–Cdc20 (13). In this process, C-Mad2 is converted back to O-Mad2 (16), thus terminating the checkpoint mechanism for MCC assembly.

The question arises whether the disassembly of MCC is subject to regulation. Thus, for example, regulation may decrease a futile cycle of simultaneous MCC assembly and disassembly during the active mitotic checkpoint. Currently available information on this problem is scanty and contradictory. It has been reported that, in extracts of *Xenopus* eggs, the phosphorylation of p31^{comet} on residues S4, T6, and T179 by IKKβ kinase increases its binding to Mad2 and accelerates exit from mitosis (17). The general significance of this observation is not clear since these phosphorylation sites in *Xenopus* p31^{comet} are not conserved in its mammalian homologs. In human cells, mitotic phosphorylation

Significance

The mitotic checkpoint system ensures the accuracy of chromosome segregation in mitosis. It detects incorrect attachment of chromosomes to the mitotic spindle and promotes the assembly of a Mitotic Checkpoint Complex (MCC). MCC inhibits the ubiquitin ligase Anaphase-Promoting Complex/Cyclosome (APC/C), whose action is required for chromosome separation. The disassembly of MCC is required for the inactivation of the mitotic checkpoint, but the regulation of MCC disassembly is not sufficiently understood. Here, we show that the mitotic protein kinase Polo-like kinase 1 (Plk1) regulates MCC disassembly by inhibitory phosphorylation of p31^{comet}, an important component of the disassembly machinery. These results reveal an important mechanism to regulate MCC levels in the mitotic checkpoint.

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[1]To whom correspondence may be addressed. Email: hershko@tx.technion.ac.il.

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of p31^{comet} on S102 by an unidentified protein kinase has been observed (18, 19). In contrast to the observations in the *Xenopus* system, it was reported that S102 phosphorylation decreases the binding of p31^{comet} to Mad2 and slows down exit from mitosis (19). Here, we examined the question of the regulation of the disassembly of mitotic checkpoint complexes and found that the phosphorylation of p31^{comet} by Polo-like kinase 1 (Plk1) was involved in this process.

Results

Influence of Mitotic Protein Kinases on the Disassembly of Free Mitotic Checkpoint Complexes. We have first asked whether the disassembly of free mitotic checkpoint complexes is regulated in the cell cycle. For these experiments, we followed the disassembly of the subcomplex Mad2–Cdc20 (MC), rather than that of MCC. Mechanisms of dissociation of MC are similar to those of MCC (13), but MC does not bind to APC/C (20) and is thus not subject to the action of the pathway that dissociates APC/C-bound MCC (6–8). In the experiment shown in Fig. 1*A*, we compared the rate of the disassembly of recombinant MC in extracts derived from checkpoint-arrested or from asynchronously growing HeLa cells. We found that the release of Mad2 from MC is lower in "checkpoint extracts" than in "asynchronous extracts." We furthermore observed that staurosporine, an inhibitor of many protein kinases (21), stimulated the dissociation of the MC subcomplex in extracts from checkpoint-arrested cells, but not in extracts from asynchronous cells (Fig. 1*A*). This suggested the existence of a regulatory mechanism in which the action of some protein kinase(s) present in checkpoint extracts decreases the rate of the disassembly of MC. Further experiments showed that staurosporine stimulates the release of Mad2, not only from the MC subcomplex, but also from the complete

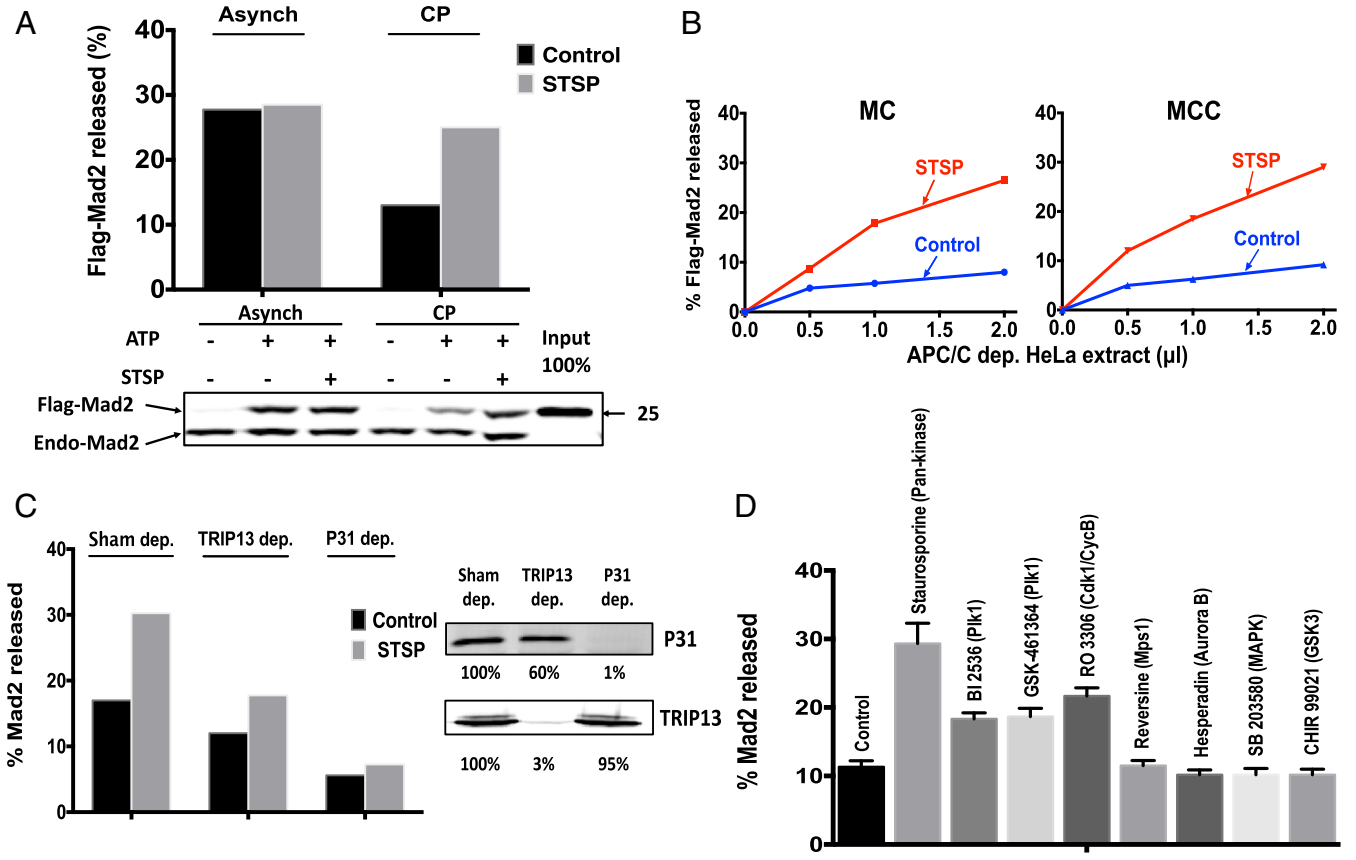

Fig. 1. Release of Mad2 from mitotic checkpoint complexes by the action of the p31-TRIP13 system is suppressed by mitotic kinases. (*A*) Influence of staurosporine on MC dissociation in extracts from checkpoint-arrested (CP) and asynchronously growing (Asynch) HeLa cells. Extracts were assayed for the disassembly of recombinant MC as described under *Materials and Methods*. Where indicated, the following additions were made: ATP, 2 mM together with 10 mM phosphocreatine and 100 μg/mL creatine phosphokinase; Staurosporine (STSP), 10 μM. Reactions without ATP were supplemented with 0.4 mg/mL hexokinase and 10 mM 2-deoxyglucose. Endo-Mad2, endogenous Mad2 from extracts. (*B*) Staurosporine stimulates the release of Mad2 from both Mad2–Cdc20 subcomplex (MC, *Left*) and from complete recombinant mitotic checkpoint complex (MCC, *Right*). APC/C-depleted (APC/C dep) extracts from nocodazole-arrested HeLa cells, at the indicated volumes, were assayed for Mad2 release from either MC (*Left*) or MCC (*Right*) recombinant complexes. Where indicated, STSP was added at 10 μM. (*C*) The stimulatory effect of staurosporine on the disassembly of Cdc20-Mad2 depends upon both p31 and TRIP13. Immunodepletion of mitotic HeLa cell extracts by anti-p31 and anti-TRIP13 was carried out as described under *Material and Methods*. Sham treatment was under similar conditions, with nonimmune rabbit IgG. (*Right*) Samples (20 μg of protein) of supernatants were immunoblotted for the indicated proteins. Numbers below blots indicate the percentage of residual proteins following immunodepletion. Samples (80 μg of protein) of immunodepleted or sham-treated extracts were incubated in the absence or presence of staurosporine (10 μM) and assayed for the disassembly of MC, as described under *Materials and Methods*. (*D*) Influence of selective protein kinase inhibitors on the disassembly of MC in extracts from checkpoint-arrested cells. Extracts from nocodazole-arrested HeLa cells were assayed for the disassembly of MC as described under *Materials and Methods*. Where indicated, the following additions were made: 10 μM staurosporine (Sigma S4400); 200 nM BI-2536 (APEXBIO A3965); 500 nM GSK 461364 (APEXBIO A8411); 10 μM RO 3306 (Sigma SML0569); 1 μM Reversine (APEXBIO A3760); 1 μM Hesperadin (APEXBIO A4118); 10 μM SB 203580 (Calbiochem 559389); 3 μM CHIR 99021 (APEXBIO A3011). All protein kinase inhibitors were dissolved in DMSO and were added at a final concentration of 0.5% DMSO. Controls without inhibitors were supplemented with a similar concentration of DMSO. Error bars represent SEM (n = 3). Protein kinases inhibited by each compound are indicated in parentheses.

(BubR1-containing) MCC (Fig. 1*B*), suggesting that a similar phosphorylation mechanism regulates the disassembly of the different mitotic checkpoint complexes. The latter experiment was done with checkpoint extracts from which APC/C had been removed by immunodepletion, to prevent the binding of MCC to APC/C.

Since the liberation of free Mad2 from mitotic checkpoint complexes is known to be carried out by the joint action of the Mad2-binding protein p31^{comet} and the AAA-ATPase TRIP13 (13, 14), we next asked whether this, or some other unknown system, is the target of regulation by inhibitory phosphorylation. For this purpose, we subjected extracts from checkpoint-arrested cells to immunodepletion by antibodies directed against TRIP13 or p31, as well as to "sham" immunodepletion with nonimmune IgG. Examination of the extents of immunodepletion (Fig. 1 *C*, *Right*) showed that 97% and 99% of TRIP13 or p31, respectively, were removed from checkpoint extracts. The immunodepletion of TRIP13 decreased partially both basal and staurosporine-stimulated release of Mad2 from MC while immunodepletion of p31 inhibited much more markedly these processes (Fig. 1*C*). This difference between effects of immunodepletion of TRIP13 and p31 may be due to a large excess of TRIP13 over p31 in extracts of HeLa cells (~100 nM TRIP13 vs. ~5 nM p31), that results in a larger effective concentration of residual TRIP13 than of p31 following immunodepletion. In both cases, however, the marked decrease in staurosporine-stimulated disassembly of MC following immunodepletion suggested that the activity of the p31-TRIP13 system is inhibited by staurosporine-sensitive protein kinases in checkpoint extracts.

Since staurosporine inhibits ~70% of protein kinases (21), it was possible that it acts on this system by inhibiting several protein kinases active in checkpoint-arrested cells. To gain some information on the identity of protein kinases that may be involved, we tested the action of several selective inhibitors of specific protein kinases on the dissociation of MC by checkpoint extracts. As shown in Fig. 1*D*, the release of Mad2 was significantly stimulated by the inhibitor of Polo-like kinase 1 (Plk1) BI-2536 (22) although the extent of stimulation was less than that obtained with staurosporine. Similar effects were observed with another Plk1 inhibitor, GSK-461364 (23). Significant partial stimulation was also observed with Ro-3306, a selective inhibitor of Cdk1/cyclin B (24). By contrast, several selective inhibitors of other mitotic kinases, tested at concentrations that essentially completely inhibit protein kinase action, had no significant influence on MC dissociation. These included reversine, an inhibitor of Mps1 protein kinase (25), hesperadin (inhibitor of Aurora B) (26), SB 203580 (inhibitor of MAP kinase) (27), and CHIR99021 (inhibitor of GSK3) (28) (Fig. 1*D*). Inhibitors of Bub1 kinase BAY-320 (29) and BNPP1 (23) also had no effect on this process (*SI Appendix*, Fig. S1*A*). These data suggested that the action of mitotic protein kinases Plk1 and Cdk1 is involved in the regulation of the disassembly of mitotic checkpoint complexes. The joint supplementation, at maximally effective concentrations, of inhibitors of Plk1 and Cdk1 resulted in additive stimulation of the disassembly of MC (*SI Appendix*, Fig. S1*B*), suggesting that these protein kinases may have different targets in this system.

Action of Mitotic Protein Kinases on Components of the Purified p31-TRIP13 System. To better define the protein kinases and their targets involved in the regulation of the disassembly of mitotic checkpoint complexes, we used a system composed of purified p31 and TRIP13, which promotes ATP-dependent release of Mad2 from the complexes (13). For this purpose, we used recombinant Plx1, an evolutionarily conserved *Xenopus* homolog of mammalian Plk1, to which it is functionally similar (30, 31) (henceforth termed "Plk1"). Addition of increasing concentrations of Plk1 progressively inhibited the dissociation of MC by the purified p31-TRIP13 system (Fig. 2*A*). Inhibition of this system by Plk1 required its protein kinase activity, as shown by the observations that its action in the disassembly of both MC and MCC is prevented by the selective inhibitor

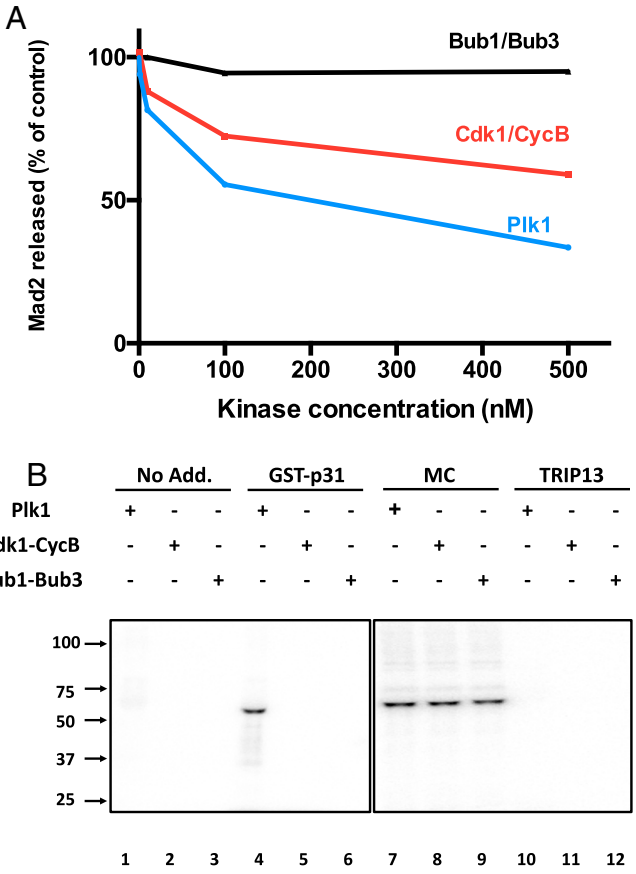

Fig. 2. Action of protein kinases on components of the purified p31-TRIP13 system. (*A*) Effects of protein kinases on the disassembly of Cdc20-Mad2 by the purified p31-TRIP13 system. The recombinant protein kinases were added at the indicated concentrations to a reaction mixture similar to that described for MC disassembly (*Materials and Methods*), except that extract was replaced by TRIP13 (50 nM) and his6-p31 (0.6 nM). The plot shows values of Mad2 release from MC, relative to incubation without protein kinases. (*B*) Phosphorylation of components of the p31-TRIP13 system by mitotic protein kinases. The phosphorylation of the following recombinant proteins—GST-p31 (0.4 pmol), TRIP13 (0.4 pmol), and MC (10 pmol)—was determined in a reaction mixture that contained, in a volume of 10 μL: 25 mm Tris·HCl (pH 7.2), 15 mM MgCl2, 1 mM DTT, 1 mg/mL BSA, 60 mM β-glycerol phosphate, 15 mM p-nitrophenyl phosphate, and 0.06 mM ATP that contained 2.5 μCi [γ-^{32}P]ATP. The indicated protein kinases were supplemented at the following concentrations (suitable for the phosphorylation of known substrate proteins): 80 nM Plk1; 0.8 nM Bub1-Bub3; and 1.6 nM Cdk1-Cyc B. Following incubation at 37 °C for 30 min, samples were subjected to SDS/PAGE. The incorporation of ^{32}P-phosphate into the relevant proteins was detected by phosphor storage analysis. Electrophoretic migration positions of molecular size marker proteins (kDa) are indicated on the left side.

BI-2536 (*SI Appendix*, Fig. S2*A*) or by N172A mutation that renders it catalytically inactive (*SI Appendix*, Fig. S2*B*). Purified Cdk1-cyclin B also inhibited the disassembly of MCC, although to a lesser extent than Plk1 (Fig. 2*A*). These results are in agreement with the effects of selective inhibitors of Plk1 and Cdk1 in crude extracts from checkpoint-arrested cells (Fig. 1*D*). Also in agreement with the lack of effect of Bub1 inhibitors (*SI Appendix*, Fig. S1), we found that recombinant purified Bub1-Bub3 had no influence on the activity of the TRIP13-p31 system (Fig. 2*A*).

To gain some notion on the targets of protein kinases in this system, we examined the possible phosphorylation of different components of this system by different protein kinases. As shown in Fig. 2*B*, GST-p31 was phosphorylated by Plk1 (lane 4), but not to a detectable extent by Cdk1-cyclin B or by Bub1-Bub3 (lanes 5

and 6, respectively). All these three protein kinases phosphorylated the Cdc20 component of MC (Fig. 2*B*, lanes 7 to 9, M_r ~55 kDa), but none phosphorylated significantly TRIP13 (lanes 10 to 12, M ~60 kDa). These results showed that p31 can be phosphorylated by Plk1 but did not indicate that phosphorylation is functional: i.e., that it affects p31 activity.

To further examine the interaction of Plk1 with p31, extracts from checkpoint-arrested HeLa cells were incubated with GST-p31, and binding of p31 to endogenous human Plk1 was examined following isolation with glutathione beads. Plk1 from HeLa extracts bound to GST-p31, but not to control GST (*SI Appendix*, Fig. S2*C*, lanes 1 and 2). Since binding in extracts could be due to indirect interactions, we also tested binding to purified recombinant Plx1, and, again, specific binding to GST-p31 was observed (*SI Appendix*, Fig. S2*C*, lanes 4 and 5). These findings showed that Plk1 binds to p31 directly and with high affinity but still did not prove that binding was functional in the inhibition of p31 activity.

P31^{comet} Is the Target of Plk1 Action. To identify which component of the p31-TRIP13 system is the functional target of Plk1 action, a two-stage experiment was performed, in which Plk1 was first incubated with one of the components to allow its phosphorylation. Subsequently, Plk1-catalyzed phosphorylation was terminated by the inhibitor BI-2536, which was followed by the supplementation of the missing components. Then, a second incubation was carried out for the determination of the release of Mad2 from MC. As shown in Fig. 3*A*, prior incubation of Plk1 with p31 (followed by the addition of TRIP13 and MC in the second incubation) strongly inhibited the release of Mad2 (lane 6), compared with a similar incubation without Plk1 (lane 2). By contrast, prior incubation of Plk1 with TRIP13 (lane 7) or with the substrate MC (lane 8) had no influence on MC dissociation. These observations indicate that the phosphorylation of p31 by Plk1 is the process that inhibits the disassembly of the checkpoint complex. In experiments of similar design, we found that Cdk1-cyclin B acts on the Mad2-Cdc20 substrate to inhibit its dissociation (*SI Appendix*, Fig. S2*D*, lane 8 vs. lane 4).

Since p31 binds to C-Mad2 in mitotic checkpoint complexes, it was possible that the phosphorylation of p31 decreases its binding to C-Mad2 in mitotic checkpoint complexes, as suggested by Date et al. (19). We used coimmunoprecipitation to examine the possible influence of phosphorylation by Plk1 on the binding of p31 to MC. We could not detect any significant influence of Plk1 action on p31-MC binding, assayed either by the amounts of Mad2 or Cdc20 immunoprecipitated with anti-p31 (*SI Appendix*, Fig. S3, lanes 2 and 3) or by the amounts of p31 immunoprecipitated with anti-Cdc20 (*SI Appendix*, Fig. S3, lanes 5 and 6).

Sites of Phosphorylation of p31 by Plk1. To identify the sites of p31 phosphorylation, extracts from checkpoint-arrested HeLa cells were incubated with GST-p31 in the presence of ATP, followed by isolation with glutathione-agarose and analysis by mass spectrometry of tryptic peptides. As shown in Table 1, the phosphorylation of serine 102 of p31 was consistently detected in all analyses while phosphorylation of serine 246 was detected in one out of four analyses. The phosphorylation of S102 of p31 was previously reported (18, 19), but the responsible protein kinase was not identified. Examination of the sequence of amino acid residues surrounding S102 (Table 1) showed no resemblance to the proposed consensus motif of Plk1 phosphorylation (D/E-X-S/T-Φ-X-D/E) (32). However, we found that the phosphorylation of S102 of p31 by HeLa cell extracts was prevented by the selective Plk1 inhibitor BI-2536 (Table 1). This could indicate phosphorylation by Plk1 at a nonconsensus site (18, 33), or to be due to an indirect mechanism, such as the activation of another protein kinase by Plk1. This problem was examined by the analysis of p31 phosphorylation sites following phosphorylation by purified Plk1. We found that, in this case, too, S102 was

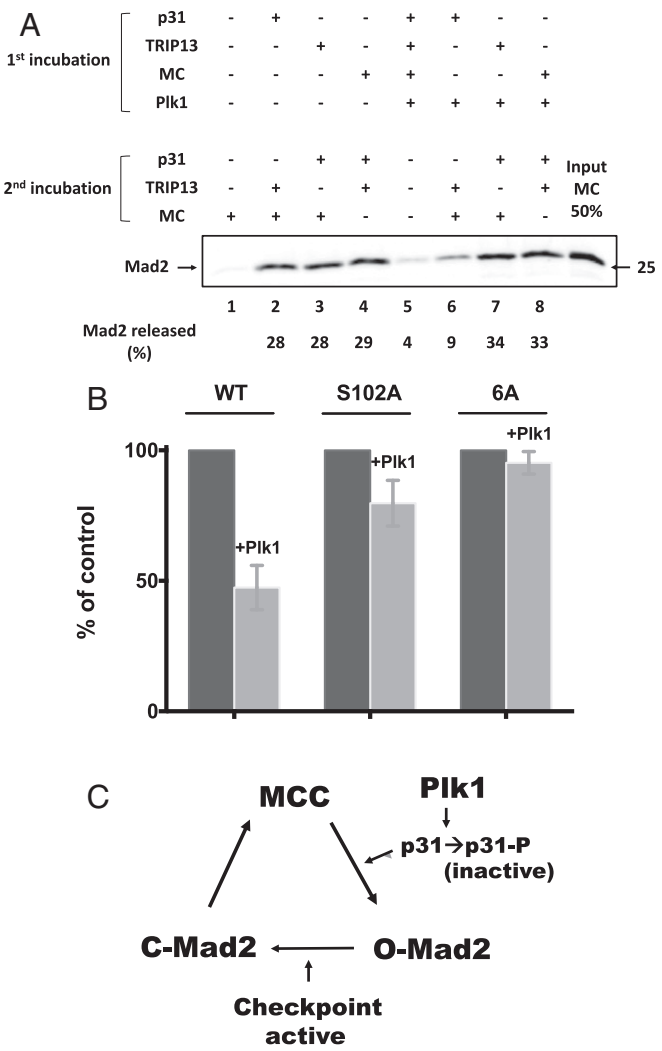

Fig. 3. Phosphorylation of p31^{comet} by Plk1 inhibits the disassembly of MC. (*A*) Disassembly of MC is inhibited by the action of Plk1 on p31, but not on TRIP13 or MC. In the first, phosphorylation incubation, his-p31, TRIP13, or MC (at concentrations similar to those in Fig. 2*A*) were incubated (at 30 °C for 30 min) with or without 100 nM Plk1 as specified in the figure, and with a reaction mixture containing ATP similar to that described in *Materials and Methods*. Subsequently, Plk1 action was stopped by the addition of BI-2536 (200 nM), and samples were allowed to stay for 10 min on ice. Afterward, the missing components were added as specified in the figure, and the mixtures were subjected to another incubation at 30 °C for 30 min (2nd incubation). The reaction mixtures were then subjected to anti-Cdc20 immunoprecipitation, and the supernatants were immunoblotted for released Mad2 as described in *Materials and Methods*. Quantitation of the percentage of Mad2 dissociation is shown below. (*B*) Effects of mutations in p31 phosphorylation sites on its sensitivity to Plk1 action in the disassembly of MC. GST-p31 derivatives (5 nM) were subjected to incubation in the presence or absence of Plk1 (100 nM), followed by BI-2536 addition and a second incubation with TRIP13 and MC as described in *A*. The data represent mean percentage of Mad2 dissociation compared with values obtained with wild-type GST-p31 in the same experiment. Error bars represent SEM (n = 5). Without Plk1 treatment, the mean activities of mutant GST-p31 proteins to stimulate the disassembly on MC were as follows (percent of the activity of wild-type GST-p31): S102A, 99%; 6A, 81%. (*C*) Schematic model of the regulation of MCC disassembly by Plk1-mediated phosphorylation of p31^{comet}. See *Discussion*.

phosphorylated by Plk1 (Table 1). Purified Plk1 also phosphorylated five other sites of p31, none of which had the Plk1 consensus phosphorylation sequence. The phosphorylation of all these sites was also inhibited by BI-2536 (Table 1). We conclude

Table 1. Sites of phosphorylation of p31 by mitotic HeLa cell extract or by purified Plk1 and sensitivity to inhibition by BI-2536

Phosphorylation by					
Plk1		HeLa extract			
Control	+BI-2536	Control	+BI-2536	Sequence	Phosphorylated residue of p31
+	0	+	0	FYRKP$_p$**S**PQAEEM	S102
+	0	0	0	PRAT$_p$**T**EVSSR	T117
+	0	0	0	ATTEV$_p$**S**SRKCQQA	S120
+	0	0	0	PPLMG$_p$**T**VVMA	T211
+	0	0	0	GHKLTV$_p$**T**LSCGRP	T244
+	0	*	0	LTVTL$_p$**S**CGRP	S246

*Phosphorylation of S246 was observed only once in four analyses.

Wild-type GST-p31 (2 μg of protein) was incubated with mitotic HeLa cell extract (800 μg of protein) or with Plk1 (100 nM), in the absence or presence of 200 nM BI-2536, in a reaction mixture that contained, in a volume of 100 μL: 50 mM Tris·HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM DTT, 10% (vol/vol) glycerol, 1 mg/mL BSA, and 2 mM ATP (at 30 °C for 1 h). Samples were then bound to 10 μL of Glutathione Sepharose 4 Fast Flow beads (GE Healthcare), by agitation at 20 rpm for 2 h at 4 °C, and then beads were washed four times with phosphate-buffered saline containing 0.5% Nonidet P-40. Subsequently, GST-p31 was eluted from beads in a volume of 20 μL containing 50 mM Tris·HCl (pH 8), 150 mM NaCl, 0.5% (vol/vol) Nonidet P-40, and 20 mM glutathione, and samples were electrophoresed on 4 to 12% Bis-Tris protein gels (Nupage, Invitrogen). Following staining with Coomassie Brilliant Blue G-Colloidal Concentrate (Sigma B2025), the protein bands corresponding to the electrophoretic migration position of GST-p31 were excised and subjected to tryptic digestion and mass spectrometric analysis. Bold letters indicate phosphorylated amino acid residues.

that Plk1 phosphorylates p31 on S102 and on five additional sites. The phosphorylation of the additional sites was possibly not detectable in HeLa cell extracts due to the opposing action of protein phosphatases.

To determine the functionality of phosphorylations at the sites described above, we generated mutants of GST-p31 in which S102 or S/T residues of all six phosphorylation sites (including S102) were converted to A. Mutant proteins were expressed and purified, and their action on the activity of the purified p31-TRIP13 system was determined with or without Plk1 phosphorylation. As shown in Fig. 3*B*, while Plk1 inhibited more than 50% of the activity of wild-type p31 under these conditions, it inhibited only ∼20% of the activity of the S102A mutant protein. As expected, a mutant in which all six phosphorylation sites were converted to Ala ("6A") was completely insensitive to inhibition by Plk1. The decreased sensitivity of the S102 mutant to inhibition by Plk1 indicates that a significant part of Plk1 action is due to phosphorylation at S102 of p31. The phosphorylation of some additional site(s) of p31 appears to be responsible for further inhibition. The identity of the additional functional phosphorylation sites was not determined in the present study.

Discussion

In this study we asked whether the disassembly of free MCC is regulated in the cell cycle and, if so, by what molecular mechanisms. We initially found that the rate of the release of Mad2 from checkpoint complexes was lower in checkpoint extracts than in asynchronous extracts (Fig. 1*A*). This was due to the inhibition of this process by mitotic protein kinases, as indicated by its stimulation by staurosporine, an inhibitor of many protein kinases (Fig. 1 *A* and *B*). Immunodepletion of p31 and TRIP13 abolished most of the staurosporine-stimulated Mad2 release (Fig. 1*C*), suggesting their involvement in a process regulated by protein kinases. Examination of the effects of a variety of selective protein kinase inhibitors (Fig. 1*D* and *SI Appendix*, Fig. S1) showed significant effects only of inhibitors of Plk1 and Cdk1-cyclin B although stimulation by these agents was less than that obtained by staurosporine. This difference may be due to inhibition by staurosporine of both Plk1 and Cdk1-cyclin B, which act on different components on this system (see below).

Next, we used purified preparations to better define the roles of components suggested by experiments with extracts. The activity of the purified p31-TRIP13 system to disassemble MC was inhibited by Plk1 and Cdk1-cyclin B (Fig. 2*A*), in agreement with the effects of kinase inhibitors in extracts (Fig. 1*D*). Several lines of evidence suggested that p31^{comet} was the target of the action of Plk1: (*i*) Direct phosphorylation experiments showed that p31^{comet} is phosphorylated by Plk1, but not by Cdk1-cyclin B or Bub1-Bub3 (Fig. 2*B*); (*ii*) p31^{comet} binds endogenous Plk1 in extracts of HeLa cells or recombinant purified Plk1 (*SI Appendix*, Fig. S2*C*); (*iii*) Most conclusively, in a functional two-stage experiment, prior phosphorylation by Plk1 of p31^{comet}, but not of TRIP13 or MC, inhibited the disassembly of MC (Fig. 3*A*). A similar two-stage functional experiment showed that the Cdk1-cyclin B acts in this system by phosphorylating the Cdc20 component of MC (*SI Appendix*, Fig. S2*C*). Since Plk1 and Cdk1-cyclin B act on different components, they may have complementary action on the disassembly of mitotic checkpoint complexes.

Examination by mass spectrometry of the sites of p31^{comet} phosphorylated by mitotic HeLa cell extracts showed phosphorylation of S102 (Table 1), as also observed previously (18, 19). The sequence of amino acid residues around S102 (Table 1) shows no resemblance to the originally proposed Plk1 consensus sequence of D/E-X-S/T-Φ-X-D/E (32). We found, however, that S102 phosphorylation by checkpoint extracts was completely inhibited by the highly selective Plk1 inhibitor BI-2536 (Table 1). Moreover, purified Plk1 also phosphorylated the S102 site in p31^{comet} (Table 1). It thus seems that the S102 residue of p31^{comet} is a nonconsensus Plk1 phosphorylation site, as is often observed in many other cellular proteins (18, 33). Purified Plk1 also phosphorylated p31^{comet} in five other sites (Table 1). These additional phosphorylations were not detected with HeLa extracts, possibly due to the opposing action of phosphatases.

Fig. 3*C* summarizes our proposal on the role of Plk1-promoted p31 phosphorylation in the mitotic checkpoint. When the mitotic checkpoint is active, MCC assembly is initiated by the conversion of O-Mad2 to C-Mad2. At the same time, the disassembly of MCC and the conversion of C-Mad2 back to O-Mad2 are prevented by the phosphorylation of p31 by Plk1. This mechanism inhibits a futile cycle and supports the maintenance of high levels of MCC during active mitotic checkpoint.

In many cases, polo-like kinases bind with high affinity to phosphorylated proteins by their polo-box domains (34). The priming protein kinase is often a Cdk, that phosphorylates S/T-P sequences favored for polo-box binding. However, Cdk1-cyclin B does not phosphorylate p31^{comet} (Fig. 2*B*) and neither does Bub1-Bub3, that can also act on S/T-P sequences (see, for example, ref. 35). Thus, at present, we have no evidence for a priming phosphorylation for the action of Plk1 on p31^{comet}.

An important unsolved problem is the mechanism by which phosphorylation of p31^{comet} inhibits the activity of the p31^{comet}–TRIP13 system to disassemble mitotic checkpoint complexes. In contrast to the report of Date et al. (19), we could not find any influence of phosphorylation of p31^{comet} on its binding to Mad2 in MC (*SI Appendix*, Fig. S3). It should be noted that, while we assayed binding in a purified system, Date et al. (19) followed p31^{comet}-Mad2 binding in extracts , in which indirect interactions may occur. It is also possible that phosphorylation of p31^{comet} affects another process, such as its binding to TRIP13 or the rates of the formation or dissociation of the p31^{comet}–TRIP13-substrate complex involved in the disassembly of mitotic checkpoint complexes (15, 16, 36). Further investigation is required to examine these possibilities.

Another unsolved problem is the timing of the dephosphorylation of p31 upon exit from the mitotic checkpoint. At this stage, the rapid disassembly of mitotic checkpoint complexes may precede the degradation of Plk1, which takes place at the onset of anaphase (37). This problem is similar to that of the regulation of Cdc20 activity by phosphorylation: It has been shown that the Bub1-Plk1 complex phosphorylates Cdc20 and inhibits its activity, a process that contributes to APC/C inhibition in mitotic checkpoint (23). In this case, too, Cdc20 has to be dephosphorylated and be converted to the active form in exit from checkpoint before the degradation of these protein kinases in anaphase (37, 38). It is possible, for example, that both p31 and Cdc20 are dephosphorylated and are converted to their active forms by a phosphatase that is activated upon exit from mitotic checkpoint. Such a mechanism may resemble the regulation of the action of phosphatase PP2A-B55 in mitosis, under control of Greatwall/Mastl protein kinase (39, 40).

Materials and Methods

Assay of Release of Mad2 from MC or MCC. Reaction mixtures contained in a volume of 25 μL: 50 mM Tris·HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM DTT, 10% (vol/vol) glycerol, 1 mg/mL BSA, 10 mM phosphocreatine, 100 μg/mL creatine phosphokinase, and 2 mM ATP. Unless otherwise specified, reactions also included HeLa cell extracts (80 μg of protein) and MC or MCC (100 nM). Where indicated, extracts were replaced by recombinant purified p31 and TRIP13 at concentrations specified in figure legends. Reactions were incubated at 30 °C for 30 min, and then samples were mixed with anti-Cdc20 beads (5 μL) and were agitated at 1,400 rpm for 2 h at 4 °C to bind complexes containing Cdc20 or free Cdc20. The samples were centrifuged, and the supernatants were passed through 0.45-μm centrifugal filters to remove residual beads. The release of Mad2 to supernatants from anti-Cdc20–bound MC complexes was determined by immunoblotting with anti-Mad2 antibody. Results were expressed as the percentage of Mad2 released from MC or MCC.

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1. A. Musacchio, E. D. Salmon, The spindle-assembly checkpoint in space and time. *Nat. Rev. Mol. Cell Biol.* **8**, 379–393 (2007).
2. P. Lara-Gonzalez, F. G. Westhorpe, S. S. Taylor, The spindle assembly checkpoint. *Curr. Biol.* **22**, R966–R980 (2012).
3. L. Jia, S. Kim, H. Yu, Tracking spindle checkpoint signals from kinetochores to APC/C. *Trends Biochem. Sci.* **38**, 302–311 (2013).
4. A. Musacchio, The molecular biology of spindle assembly checkpoint dynamics. *Curr. Biol.* **25**, R1002–R1018 (2015).
5. C. Alfieri *et al*., Molecular basis of APC/C regulation by the spindle assembly checkpoint. *Nature* **536**, 431–436 (2016).
6. E. Eytan, D. Sitry-Shevah, A. Teichner, A. Hershko, Roles of different pools of the mitotic checkpoint complex and the mechanisms of their disassembly. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 10568–10573 (2013).
7. S. K. Reddy, M. Rape, W. A. Margansky, M. W. Kirschner, Ubiquitination by the anaphase-promoting complex drives spindle checkpoint inactivation. *Nature* **446**, 921–925 (2007).
8. D. Sitry-Shevah, S. Kaisari, A. Teichner, S. Miniowitz-Shemtov, A. Hershko, Role of ubiquitylation of components of mitotic checkpoint complex in their dissociation from anaphase-promoting complex/cyclosome. *Proc. Natl. Acad. Sci. U.S.A.* **115**, 1777–1782 (2018).
9. S. Miniowitz-Shemtov, A. Teichner, D. Sitry-Shevah, A. Hershko, ATP is required for the release of the anaphase-promoting complex/cyclosome from inhibition by the mitotic checkpoint. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 5351–5356 (2010).
10. A. Teichner *et al*., p31^{comet} Promotes disassembly of the mitotic checkpoint complex in an ATP-dependent process. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 3187–3192 (2011).
11. T. Habu, S. H. Kim, J. Weinstein, T. Matsumoto, Identification of a MAD2-binding protein, CMT2, and its role in mitosis. *EMBO J.* **21**, 6419–6428 (2002).
12. G. Xia *et al*., Conformation-specific binding of p31(^{comet}) antagonizes the function of Mad2 in the spindle checkpoint. *EMBO J.* **23**, 3133–3143 (2004).
13. E. Eytan *et al*., Disassembly of mitotic checkpoint complexes by the joint action of the AAA-ATPase TRIP13 and p31(comet). *Proc. Natl. Acad. Sci. U.S.A.* **111**, 12019–12024 (2014).
14. K. Wang *et al*., Thyroid hormone receptor interacting protein 13 (TRIP13) AAA-ATPase is a novel mitotic checkpoint-silencing protein. *J. Biol. Chem.* **289**, 23928–23937 (2014).
15. S. Miniowitz-Shemtov, E. Eytan, S. Kaisari, D. Sitry-Shevah, A. Hershko, Mode of interaction of TRIP13 AAA-ATPase with the Mad2-binding protein p31^{comet} and with mitotic checkpoint complexes. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 11536–11540 (2015).
16. Q. Ye *et al*., TRIP13 is a protein-remodeling AAA+ ATPase that catalyzes MAD2 conformation switching. *eLife* **4**, e07367 (2015).
17. M. Mo, A. Arnaoutov, M. Dasso, Phosphorylation of *Xenopus* p31(^{comet}) potentiates mitotic checkpoint exit. *Cell Cycle* **14**, 3978–3985 (2015).
18. B. Hegemann *et al*., Systematic phosphorylation analysis of human mitotic protein complexes. *Sci. Signal* **4**, rs12 (2011).
19. D. A. Date, A. C. Burrows, M. K. Summers, Phosphorylation regulates the p31Comet-mitotic arrest-deficient 2 (Mad2) interaction to promote spindle assembly checkpoint (SAC) activity. *J. Biol. Chem.* **289**, 11367–11373 (2014).
20. S. Kaisari, D. Sitry-Shevah, S. Miniowitz-Shemtov, A. Hershko, Intermediates in the assembly of mitotic checkpoint complexes and their role in the regulation of the anaphase-promoting complex. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 966–971 (2016).
21. M. W. Karaman *et al*., A quantitative analysis of kinase inhibitor selectivity. *Nat. Biotechnol.* **26**, 127–132 (2008).
22. P. Lénárt *et al*., The small-molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1. *Curr. Biol.* **17**, 304–315 (2007).
23. L. Jia, B. Li, H. Yu, The Bub1-Plk1 kinase complex promotes spindle checkpoint signalling through Cdc20 phosphorylation. *Nat. Commun.* **7**, 10818 (2016).
24. L. T. Vassilev *et al*., Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 10660–10665 (2006).
25. S. Santaguida, A. Tighe, A. M. D'Alise, S. S. Taylor, A. Musacchio, Dissecting the role of MPS1 in chromosome biorientation and the spindle checkpoint through the small molecule inhibitor reversine. *J. Cell Biol.* **190**, 73–87 (2010).
26. S. Hauf *et al*., The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *J. Cell Biol.* **161**, 281–294 (2003).
27. K. Burkhard, P. Shapiro, Use of inhibitors in the study of MAP kinases. *Methods Mol. Biol.* **661**, 107–122 (2010).
28. C. A. Thorne *et al*., GSK-3 modulates cellular responses to a broad spectrum of kinase inhibitors. *Nat. Chem. Biol.* **11**, 58–63 (2015).
29. A. P. Baron *et al*., Probing the catalytic functions of Bub1 kinase using the small molecule inhibitors BAY-320 and BAY-524. *eLife* **5**, e12187 (2016).
30. A. Kumagai, W. G. Dunphy, Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from Xenopus egg extracts. *Science* **273**, 1377–1380 (1996).
31. H. A. Lane, E. A. Nigg, Cell-cycle control: POLO-like kinases join the outer circle. *Trends Cell Biol.* **7**, 63–68 (1997).
32. H. Nakajima, F. Toyoshima-Morimoto, E. Taniguchi, E. Nishida, Identification of a consensus motif for Plk (Polo-like kinase) phosphorylation reveals Myt1 as a Plk1 substrate. *J. Biol. Chem.* **278**, 25277–25280 (2003).
33. A. Santamaria *et al*., The Plk1-dependent phosphoproteome of the early mitotic spindle. *Mol. Cell. Proteomics* **10**, 004457 (2011).
34. A. E. H. Elia, L. C. Cantley, M. B. Yaffe, Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. *Science* **299**, 1228–1231 (2003).
35. Z. Tang, H. Shu, D. Oncel, S. Chen, H. Yu, Phosphorylation of Cdc20 by Bub1 provides a catalytic mechanism for APC/C inhibition by the spindle checkpoint. *Mol. Cell* **16**, 387–397 (2004).
36. C. Alfieri, L. Chang, D. Barford, Mechanism for remodelling of the cell cycle checkpoint protein MAD2 by the ATPase TRIP13. *Nature* **559**, 274–278 (2018).
37. C. Lindon, J. Pines, Ordered proteolysis in anaphase inactivates Plk1 to contribute to proper mitotic exit in human cells. *J. Cell Biol.* **164**, 233–241 (2004).
38. W. Qi, H. Yu, KEN-box-dependent degradation of the Bub1 spindle checkpoint kinase by the anaphase-promoting complex/cyclosome. *J. Biol. Chem.* **282**, 3672–3679 (2007).
39. D. M. Glover, The overlooked greatwall: A new perspective on mitotic control. *Open Biol.* **2**, 120023 (2012).
40. A. Castro, T. Lorca, Greatwall kinase at a glance. *J. Cell Sci.* **131**, jcs222364 (2018).