

Mammalian Polo-like Kinase 1 (Plk1) Promotes Proper Chromosome Segregation by Phosphorylating and Delocalizing the PBIP1·CENP-Q Complex from Kinetochores

Received for publication, November 3, 2014, and in revised form, February 9, 2015. Published, JBC Papers in Press, February 10, 2015, DOI 10.1074/jbc.M114.623546

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Background: Plk1 is a protein kinase that localizes to subcellular structures called kinetochores to promote mitotic progression.

Results: Plk1 interacts with and regulates a kinetochore-associated PBIP1·CENP-Q complex.

Conclusion: This is a tightly regulated, cell cycle-dependent process, the deregulation of which leads to improper chromosome segregation and mitotic progression.

Significance: This event is likely critical for maintaining genomic integrity and preventing aneuploidy.

Mammalian Plk1 is critically required for proper M phase progression. Plk1 is self-recruited to prekinetochores/kinetochores by phosphorylating and binding to the Thr-78 motif of a kinetochore scaffold protein, PBIP1 (also called CENP-U/50), which forms a stable complex with another kinetochore component, CENP-Q. However, the mechanism regulating Plk1 localization to this site remains largely unknown. Here, we demonstrate that the PBIP1·CENP-Q complex became hyperphosphorylated and rapidly delocalized from kinetochores as cells entered mitosis. Plk1 phosphorylated the CENP-Q subunit of the PBIP1·CENP-Q complex at multiple sites, and mutation of nine Plk1-dependent phosphorylation sites to Ala (9A) enhanced CENP-Q association with chromatin and prolonged CENP-Q localization to kinetochores. Conversely, mutation of the nine sites to phospho-mimicking Asp/Glu (9D/E) residues dissociated CENP-Q from chromatin and kept the CENP-Q(9D/E) mutant from localizing to interphase prekinetochores. Strikingly, both the 9A and 9D/E mutants induced a defect in proper chromosome segregation, suggesting that both timely localization of the PBIP1·CENP-Q complex to prekinetochores and delocalization from kinetochores are critical for normal M phase progression. Notably, although Plk1 did not alter the level of PBIP1 and CENP-Q ubiquitination, Plk1-dependent phosphorylation and delocalization of these proteins from kinetochores appeared to indirectly lead to their degradation in the cytosol. Thus, we propose that Plk1 regulates the timing of the delocalization and ultimate destruction of the

PBIP1·CENP-Q complex and that these processes are important not only for promoting Plk1-dependent mitotic progression, but also for resetting the timing of Plk1 recruitment to prekinetochores in the next cell cycle.

Polo-like kinases constitute a conserved subfamily of Ser/Thr protein kinases that play pivotal roles in cell proliferation (1–5). In mammalian cells, at least four Polo-like kinases (Plk1–4) that exhibit distinct expression patterns and cell functions have been isolated (6). Among them, Plk1 has been shown to play a critical role in proper M phase progression by localizing to multiple subcellular structures, such as centrosomes, kinetochores, and the midbody (7, 8). A growing body of evidence suggests that Plk1 interacts with a large number of cellular proteins through its C-terminal non-catalytic domain, called the Polo-box domain (9). The ability of Plk1 to localize to multiple subcellular locations is thought to stem from its capacity to interact with its distinct binding targets already present at these sites. Hence, understanding the underlying mechanism by which the localization of Plk1-binding targets themselves is regulated will be important for gaining new insights into how Plk1 recruitment to specific subcellular locations is temporally and spatially regulated.

It has been demonstrated that Plk1 localization to late interphase and early mitotic kinetochores is regulated primarily by the ability of Plk1 itself to phosphorylate the Thr-78 motif of a kinetochore scaffold protein, PBIP1 (also called MLF1IP and CENP-U/50), and bind to it (10) through a mechanism called self-priming and binding (11, 12). Unlike non-self-priming and binding, which require a priming kinase other than Plk1, this unusual self-promoted mechanism appears to be pivotal for quickly amassing Plk1 at kinetochores and cooperatively regu-

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lating Plk1-dependent biochemical processes at these sites (11–13). Ironically, however, the level of PBIP1 at late interphase and early mitotic kinetochores diminishes precipitously as an increasing amount of Plk1 is recruited to these sites (10). This observation suggests that Plk1 also binds to protein(s) other than PBIP1 at the late stages of the cell cycle and that the localization of Plk1 to kinetochores is dynamically regulated as cells proceed through the cell cycle.

PBIP1 was proposed to be a component of the CENP-O complex, which includes CENP-O, CENP-P, CENP-Q, CENP-R, and CENP-U (14–16). Studies have suggested that PBIP1 interacts with many other kinetochore components either directly or indirectly (17–19). Interestingly, PBIP1 interacts directly with CENP-Q *in vitro*, and this interaction is required for their mutual stability (19). This observation suggests that PBIP1 and CENP-Q form a subcomplex of the CENP-O complex proposed by Okada *et al.* (15) or that they form a heteromeric complex distinct from the multisubunit CENP-O complex. Remarkably, Plk1 phosphorylates CENP-Q via its interaction with the self-primed phospho-Thr-78 motif of PBIP1 (19), suggesting that Plk1 regulates CENP-Q function only after the formation of a heterotrimeric Plk1·PBIP1·CENP-Q complex. However, the mechanism by which Plk1 regulates the PBIP1·CENP-Q complex and the physiological significance of this event during the cell cycle remain unknown.

In this study, we show that the PBIP1·CENP-Q complex localizes to early interphase prekinetochores, but precipitously delocalizes from late interphase/early mitotic kinetochores. Subsequent investigation of this process revealed that Plk1 that is self-recruited to the phospho-Thr-78 motif of PBIP1 phosphorylates the CENP-Q subunit of the PBIP1·CENP-Q complex at multiple sites to induce the complex's dissociation from kinetochores without disassembling the complex. Analysis of Plk1-dependent CENP-Q phosphosite mutants uncovered that either prolonged or impaired localization of the complex to kinetochores induces a defect in proper chromosome segregation during mitosis. Thus, the level of Plk1-dependent CENP-Q phosphorylation regulates the dynamic localization/delocalization of the PBIP1·CENP-Q complex to/from kinetochores, and deregulating this process results in chromosome missegregation, which may ultimately lead to aneuploidy, a hallmark of cancer.

EXPERIMENTAL PROCEDURES

Plasmid Construction—All of the constructs expressing FLAG-fused CENP proteins (CENP-A, CENP-H, CENP-M, CENP-N, CENP-T, CENP-I, CENP-K, CENP-L, CENP-O, CENP-P, CENP-Q, CENP-R, CENP-S, and CENP-U/PBIP1) and untagged PBIP1 were described previously (10, 19). pEGFP-C1-PBIP1(WT) and its respective K308A/K316A mutant (pKM1365 and pKM2475) were generated by inserting a corresponding BglII-XhoI fragment into the pEGFP-C1 vector (Clontech, Palo Alto, CA) digested with the same enzymes.

Lentiviral constructs expressing WT PBIP1 (pKM542) or the PBIP1(K308A/K316A) mutant (pKM2989) were constructed by inserting a BglII-XhoI fragment containing the *PBIP1* allele into a pHR'-CMV-SV-puro vector (a gift of Chou-Zen Giam, Uniformed Services University of the Health Sciences,

Bethesda, MD) digested with BamHI and Sall. The lentiviral construct expressing the PBIP1(T78A) mutant was described previously (10). A lentiviral construct expressing GFP-fused CENP-Q (pKM1463) was cloned by inserting an AgeI (end-filled)-XhoI fragment containing GFP-CENP-Q into a pHR'-CMV-SV-puro vector digested with BamHI (end-filled) and Sall. Other lentiviral constructs expressing PBIP1-GFP (pKM1516) and unfused CENP-Q (pKM1541), CENP-Q(9A) (pKM2730), or CENP-Q(9D/E) (pKM2774) were also similarly generated. The 9A mutant contains Ala residues in place of Thr-123, Thr-135, Ser-138, Ser-139, Ser-248, Ser-249, Ser-253, Ser-255, and Thr-256 residues. The 9D/E mutant contains mutations T123E, T135E, S138D, S139D, S248D, S249D, S253D, S255D, and T256E.

To generate a lentivirus-based shRNA construct targeting the CENP-Q 3'-UTR (pKM2576), sequence 5'-GGAATTGCCTTAAGGATCT-3' was used to generate a pLKO.1-puro vector-based construct as described previously (10). Lentivirus-based shRNAs targeting PBIP1 and Plk1 have been reported previously (10). The bacterial His-PBIP1·His-MBP-CENP-Q(9A)⁴ (pKM2790) construct was generated in a manner similar to that described for His-PBIP1·His-MBP-CENP-Q (pKM1653) (19).

RT-PCR Analysis—HeLa cells harvested at the indicated time points were subjected to RT-PCR analysis using primers 5'-TGTGGACTGTCTCTCTCTTCAACT-3' (forward) and 5'-TCATCCCTGGTCAAGGAGCTTCTC-3' (reverse) for PBIP1 (expected size of 980 bp) and primers 5'-ATCCCTGAGCTGAACGGGAAG-3' (forward) and 5'-GAGGGGAGATTCAGTGTGGTG-3' (reverse) primers for GAPDH (expected size of 480 bp).

Cell Culture, Synchronization, Transfection, and Virus Generation and Infection—Both HeLa and 293T cells were cultured as recommended by the American Type Culture Collection (Manassas, VA). To synchronize HeLa cells at the G₁/S boundary, cells were treated with 2 mM thymidine (Sigma) for 18 h, released for 9 h, and then treated with another 2 mM thymidine for 16 h. The resulting cells were released into either fresh medium or medium containing 660 nM nocodazole to trap the cells in mitosis. Where indicated, asynchronously growing cells were treated with nocodazole for 16 h to enrich mitotic cells.

Lentivirus production was carried out as described previously (10). Depletion of the protein of interest was achieved by infecting HeLa cells with lentiviruses expressing an appropriate shRNA. To generate a pool of HeLa cells stably expressing various PBIP1 and CENP-Q forms, HeLa cells were infected with lentiviruses expressing the gene of interest for 1 day and then treated with 2 μg/ml puromycin for 2–3 days to select the lentivirus-integrated population. All of the lentiviral expression constructs bear silent mutations that confer resistance against their respective shRNA. The HeLa cells stably expressing PBIP1 or PBIP1(T78A) were described previously (10).

To prevent proteasomal degradation of cellular proteins, cells were treated with 10 μM MG132 for the indicated times. To inhibit Plk1 kinase activity, cells were treated with a small

⁴The abbreviations used are: MBP, maltose-binding protein; Ub, ubiquitin; DT, double-thymidine.

molecule inhibitor, BI 2536 (20), at a final concentration of 100 nM for the indicated times. To inhibit protein synthesis, HeLa cells were treated with 100 μ g/ml cycloheximide for the indicated times.

Cell Fractionation—Cells were washed with PBS and resuspended in buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 10 mM NaF, 1 mM Na₂VO₃, and protease inhibitors). Triton X-100 was added to a final concentration of 0.1%, and the cells were left on ice for 5 min. Cytoplasmic proteins (S1) were separated from nuclei (P1) by centrifugation at 1300 \times *g* for 4 min. Isolated nuclei (P1) were washed once with buffer A and lysed in buffer B (3 mM EDTA, 0.2 mM EGTA, and 1 mM DTT). After incubation for 10 min on ice, soluble nuclear proteins (S2) were separated from chromatin (P2) by centrifugation at 1700 \times *g* for 4 min. Isolated chromatin was washed once with buffer B and spun down at high speed (10,000 \times *g* for 1 min). Chromatin was resuspended in SDS sample buffer (62.5 mM Tris-Cl (pH 6.8), 2% SDS, 10% glycerol, and 5% β -mercaptoethanol) and sheared by sonication. After reconstituting the resulting S1, S2, and P2 fractions to the same volume, an equal volume of each fraction was mixed with SDS sample buffer and separated by SDS-PAGE.

Immunoprecipitation and Immunoblot Analysis—Cells were lysed in 50 mM Tris-Cl (pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40, 5 mM EGTA, 1.5 mM EDTA, and 1% *p*-nitrophenyl phosphate and sonicated for 10 s. After centrifugation at 14,000 \times *g* for 10 min, the resulting supernatant was subjected to immunoprecipitation in a manner similar to that described previously (10).

For immunoblot analysis, samples were electrophoresed on an SDS-polyacrylamide gel, transferred to a PVDF membrane, and analyzed as described previously (21). Signals were detected with an ECL detection system (Pierce).

In Vitro Kinase Reaction—HA-fused WT Plk1 and its kinase-inactive K82M mutant (8) were purified from Sf9 cells. The His-PBIP1-His-MBP-CENP-Q or His-PBIP1-His-MBP-CENP-Q(9A) complex was expressed in *Escherichia coli* BL21 and purified using an His affinity column. Kinase reactions were carried out in kinase reaction buffer (50 mM Tris-Cl (pH 7.5), 10 mM MgCl₂, 2 mM DTT, and 2 mM EGTA) supplemented with 10 μ M ATP (10 μ Ci of [γ -³²P]ATP; 1 Ci = 37 GBq). Reaction mixtures were incubated at 30 °C for 30 min, followed by termination with SDS sample buffer.

Mass Spectrometry Analysis—To determine Plk1-dependent *in vitro* phosphorylation sites on CENP-Q, the purified His-PBIP1-His-MBP-CENP-Q complex was reacted with either WT Plk1 or the kinase-inactive mutant Plk1(K82M) purified from Sf9 cells essentially as described previously (19). To determine the *in vivo* phosphorylation sites on CENP-Q, 293T cells cotransfected with Myc-CENP-Q and untagged PBIP1 constructs were treated with nocodazole for 16 h and then subjected to anti-Myc immunoprecipitation. The resulting *in vitro* reaction and *in vivo* immunoprecipitation samples were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Appropriate CENP-Q bands were excised and then subjected to mass spectrometry analysis. Coexpression of PBIP1 was important not only for stabilizing CENP-Q, but also for promoting Plk1-dependent CENP-Q phosphorylation (19).

Ubiquitination Assay—Cells transfected with His₆-ubiquitin (Ub) (22), and other indicated constructs were lysed in 1 ml of buffer C (6 M guanidinium Cl, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris-Cl (pH 8.0), 0.2% Triton X-100, 10 mM β -mercaptoethanol, and 5 mM imidazole) and then incubated with 50 μ l of nickel-agarose beads for 2 h at room temperature. The beads were precipitated and washed once with 1 ml of buffer C at room temperature for 5 min, once with 1 ml of buffer D (8 M urea, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris-Cl (pH 8.0), 0.2% Triton X-100, 10 mM β -mercaptoethanol, and 5 mM imidazole), and finally three times with 1 ml of buffer E (8 M urea, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris-Cl (pH 6.3), 0.2% Triton X-100, 10 mM β -mercaptoethanol, and 5 mM imidazole). Following the washes, ubiquitinated proteins were eluted with elution buffer (0.15 M Tris-Cl (pH 6.7), 30% glycerol, 0.72 M β -mercaptoethanol, 5% SDS, and 200 mM imidazole) and then analyzed.

Indirect Immunofluorescence Microscopy and Quantification—Immunostaining was carried out as described previously (21). Secondary antibodies, including Alexa Fluor 488 (green)- and Texas Red (red)-conjugated antibodies, were purchased from Invitrogen. Cells grown on a coverslip were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and then blocked with 5% bovine serum albumin prior to the addition of primary antibodies. To stain chromosomes, cells were treated with PBS containing 0.1 μ g/ml Hoechst 33258 (Sigma).

Confocal images were acquired at 512 \times 512 pixels and 12-bit resolution using a Zeiss LSM 710 system mounted on a Zeiss Axiovert 100M microscope. To quantify the fluorescence signal intensities, images of unsaturated fluorescence signals were used. The fluorescence intensity for a particular signal was determined after subtracting the background signal intensity using Zeiss AIM confocal software.

RESULTS

Cell Cycle-dependent Regulation of the PBIP1-CENP-Q Complex—It has been suggested that PBIP1 forms a complex with other centromeric components, such as CENP-O, CENP-P, CENP-Q, and CENP-R (15, 18). To determine whether PBIP1 preferentially interacts with one of the CENP proteins, we carried out co-immunoprecipitation experiments. Two other components, CENP-H and CENP-M, which belong to a CENP-A nucleosome-associated complex, were also included for comparison. The results showed that CENP-Q efficiently interacted with PBIP1 (Fig. 1A). No other components tested exhibited a significant level of interaction with PBIP1 under the same conditions. This finding supports our previous observation that PBIP1 and CENP-Q bind to each other to form a stable complex (19).

To investigate how the PBIP1-CENP-Q complex is regulated during the cell cycle, HeLa cells arrested at the G₁/S boundary by double-thymidine (DT) treatment were synchronously released into fresh medium. Samples harvested at different time points after release were examined by immunoblot analysis. At the G₁/S boundary (DT0), PBIP1 migrated in several tiers (Fig. 1B) due to multiple phosphorylations by Plk1 and other unknown kinases as described previously (10). At DT8, PBIP1 became further phosphorylated, exhibiting the most slowly

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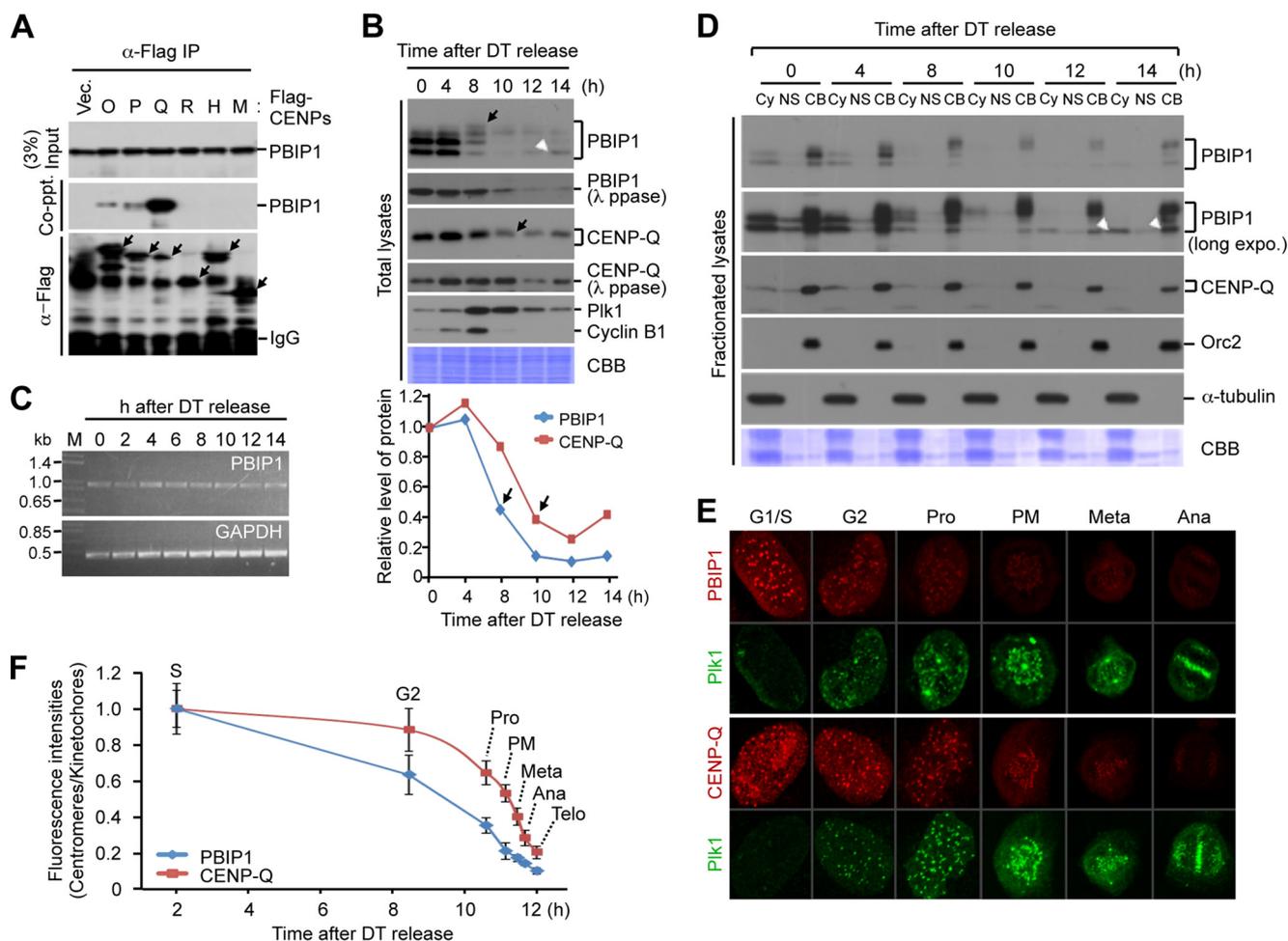


FIGURE 1. The PBIP1-CENP-Q complex becomes hyperphosphorylated and unstable during the late stages of the cell cycle. *A*, HeLa cells transfected with the control vector (*Vec.*) or the indicated constructs were subjected to co-immunoprecipitation (*IP*) analysis. *Letters* denote centromeric proteins CENP-O, CENP-P, CENP-Q, CENP-R, CENP-H, and CENP-M. *Arrows* mark the full-length form of each ligand immunoprecipitated. *Co-ppt.*, co-precipitate. *B*, HeLa cells released synchronously from a DT block were harvested at the indicated time points and then subjected to immunoblot analysis. Membranes were stained with Coomassie Brilliant Blue (*CBB*) as a loading control. The graph indicates the levels of PBIP1 and CENP-Q quantified from the immunoblots. *Arrows* indicate the time points at which PBIP1 and CENP-Q were hyperphosphorylated. The *white arrowhead* indicates underphosphorylated PBIP1 that reappeared in the next cell cycle. λ *ppase*, λ -phosphatase. *C*, HeLa cells harvested at the indicated time points after DT release were subjected to RT-PCR analysis to quantify the levels of PBIP1 and GAPDH transcription. *D*, cells harvested as described for *B* were fractionated and analyzed by immunoblot analysis. *Cy*, cytosolic; *NS*, nuclear soluble; *CB*, chromatin-bound. α -Tubulin and Orc2 served as markers for cytoplasmic and chromatin-bound fractions, respectively. *E*, HeLa cells synchronously released from a DT block were fixed and immunostained with the indicated antibodies. *F*, the signal intensities of PBIP1 and CENP-Q that were localized at kinetochores were quantified from at least 15 confocal images/time point. *Error bars* indicate S.D. of >60 measurements/time point (an average of four kinetochore measurements/cell). *Pro*, prophase; *PM*, prometaphase; *Meta*, metaphase; *Ana*, anaphase; *Telo*, telophase.

migrating form (Fig. 1*B*, upper arrow). PBIP1 remained hyperphosphorylated in DT10–DT12, before its fastest migrating form (*white arrowhead*) reappeared in the following G_1 phase (*i.e.* DT14 sample) (Fig. 1*B*). Treatment of total lysates with λ -phosphatase resulted in the complete collapse of the mobility shift (Fig. 1*B*), thus making it easier to determine the total amount of PBIP1 present in the lysates. Notably, PBIP1 appeared to be least abundant at DT10, the stage in which Plk1 remained high (Fig. 1*B*). The steady-state level of PBIP1 mRNA remained unchanged throughout the cell cycle (Fig. 1*C*), suggesting that PBIP1 is regulated at a post-transcriptional level. As expected, if PBIP1 and CENP-Q functioned as a complex, CENP-Q also exhibited a hyperphosphorylated and slow-migrating form at the late stages of the cell cycle. Interestingly, the timing of CENP-Q phosphorylation (which appeared to peak at DT10) (Fig. 1*B*, lower arrow) was somewhat slower than that of PBIP1. This is in line with the view that Plk1 phosphorylates

CENP-Q after being recruited to the PBIP1 Thr-78 tether through the self-priming and binding mechanism (19).

To examine whether the phosphorylation of PBIP1 and CENP-Q alters the biochemical property of these proteins and influences their ability to associate with kinetochores, a simple cell fractionation experiment was carried out as described previously (23). The results showed that at DT0, PBIP1 was abundantly present in both cytosolic and chromatin-bound fractions, but was sparsely present in the soluble nuclear fraction (Fig. 1*D*). As cells progressed through the cell cycle, cytosolic PBIP1 rapidly disappeared, whereas chromatin-bound PBIP1 declined at a slower rate (Fig. 1*D*). At the late stage of the cell cycle (DT10–DT12), the total amount of PBIP1 was at a low level, but the remaining PBIP1 was predominantly chromatin-bound (Fig. 1*D*). At DT14, a fast-migrating PBIP1 form (Fig. 1*D*, *white arrowheads*) reappeared in both cytosolic and soluble nuclear fractions. These findings suggest that, overall, PBIP1

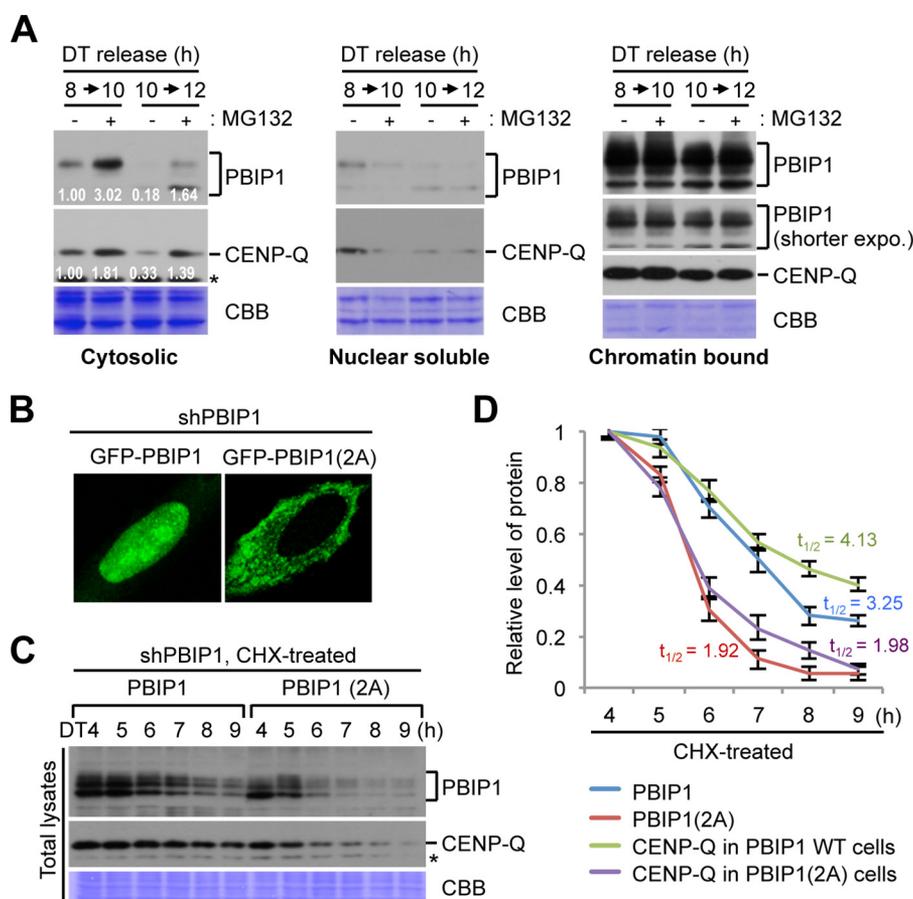


FIGURE 2. The cytosolic PBIP1-CENP-Q complex is sensitive to protease-dependent degradation. *A*, HeLa cells released from a DT block for 8 or 10 h were treated with either dimethyl sulfoxide (–) or 10 μ M MG132 (+) for 2 h. Samples were harvested, fractionated, and then subjected to immunoblot analysis. The asterisk indicates nonspecific cross-reacting protein. *CBB*, Coomassie Brilliant Blue. *B*, HeLa cells expressing either GFP-PBIP1 or PBIP1(K308A/K316A or 2A) were depleted of endogenous PBIP1 (PBIP1 shRNA (*shPBIP1*)) and then subjected to confocal analyses. *C* and *D*, HeLa cells expressing either wild-type PBIP1 (untagged) or PBIP1(K308A/K316A or 2A) were depleted of endogenous PBIP1. The cells were arrested with a DT block, released for 4 h, and then treated with cycloheximide (*CHX*). Samples harvested at the indicated time points after release were analyzed. The levels of proteins detected in the immunoblots were quantified using ImageJ (*D*). The asterisk (*C*) indicates cross-reacting protein. *Error bars* (*D*) indicate S.D. from three independent experiments.

becomes unstable at the late stage of the cell cycle and that cytosolic PBIP1 is more vulnerable to degradation than chromatin-bound PBIP1. Fractionation patterns and abundances of CENP-Q were similar to those of PBIP1, although the phosphorylation-dependent slow migration of the former was less prominent compared with the latter (Fig. 1*D*). Orc2 and α -tubulin served as markers for the chromatin-bound and cytosolic fractions, respectively.

We then immunostained HeLa cells released from a DT block to investigate whether the changes in the levels of chromatin-associated PBIP1 or CENP-Q are reflected in the extent of the localization of these proteins to kinetochores. In good agreement with an earlier observation (10), PBIP1 fluorescence intensity was prominent at early interphase prekinetochores, but was precipitously diminished as cells entered mitosis (Fig. 1, *E* and *F*). PBIP1 was hardly detectable at prometaphase/metaphase kinetochores. The localization of CENP-Q largely mirrored that of PBIP1, although the disappearance of CENP-Q signals from kinetochores appeared to be moderately retarded in comparison with that of PBIP1 signals (Fig. 1, *E* and *F*). Because Plk1 began to localize to late interphase prekinetochores and reached its highest level at early mitotic kinetochores (Fig. 1, *E* and *F*), these findings suggest that the degree of

PBIP1 or CENP-Q localization inversely correlates with that of Plk1 localization.

The Cytosolic PBIP1-CENP-Q Complex Is Vulnerable to Proteasome-dependent Degradation—The rapid disappearance of PBIP1 and CENP-Q at the late stage of the cell cycle (Fig. 1*D*) prompted us to examine whether these proteins are degraded via the 26 S proteasome pathway. To investigate this possibility, HeLa cells released from a DT block for 8 h (DT8) or 10 h (DT10) were treated with a proteasome inhibitor (MG132) for 2 h, and the resulting cells were lysed and fractionated as in Fig. 1*D*. The results showed that cytosolic PBIP1 and CENP-Q were substantially stabilized by the MG132 treatment, whereas both soluble nuclear and chromatin-bound PBIP1 and CENP-Q were not (Fig. 2*A*). Taken together with the results shown in Fig. 1*D*, these observations suggest that the cytosolic PBIP1-CENP-Q complex is readily susceptible to proteasomal degradation.

To corroborate the finding that cytosolic PBIP1 is more vulnerable to proteasomal degradation compared with nuclear PBIP1, we compared the stability of the GFP-fused, nuclear localization-defective PBIP1(K308A/K316A) mutant (2A) with that of WT PBIP1 (Fig. 2*B*). To this end, HeLa cells expressing each of the respective RNAi-insensitive PBIP1 constructs were

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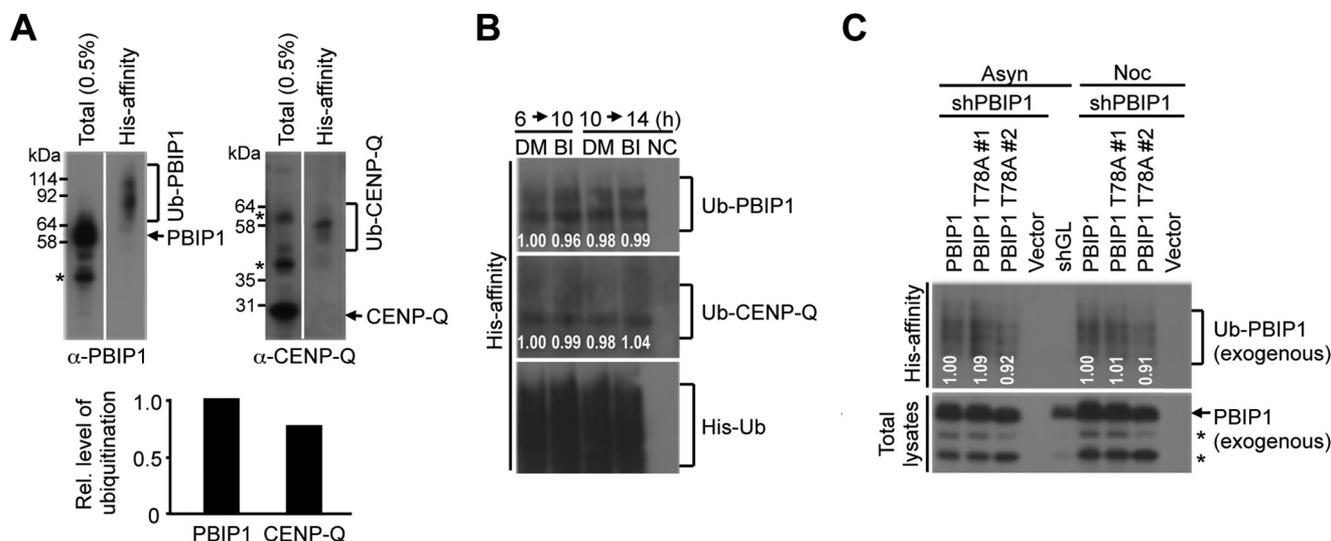


FIGURE 3. PBIP1 and CENP-Q are ubiquitinated in a manner that does not require Plk1. *A*, 293T cells transfected with His-Ub were subjected to nickel affinity purification, followed by immunoblot analysis with anti-PBIP1 (left) or anti-CENP-Q (right) antibodies. The whole cell lysates (0.5%) and affinity-purified samples were from the same membranes. Ubiquitinated, slow-migrating PBIP1 and CENP-Q forms (bracket) were quantified by ImageJ (graph). Asterisks indicate cross-reacting proteins. *B*, 293T cells released from the first thymidine block were transfected with His-Ub or left untreated (NC). After the DT block and release for 6 or 10 h, cells were treated with either dimethyl sulfoxide (DM) or BI 2536 (BI) for 4 h and then analyzed as described for *A*. Numbers indicate the relative levels of protein signals quantified with ImageJ. *C*, HeLa cells stably expressing wild-type PBIP1 (untagged) or the PBIP1(T78A) mutant were depleted of control luciferase (Gaussia luciferase shRNA (shGL)) or endogenous PBIP1 (PBIP1 shRNA (shPBIP1)). The resulting cells were then transfected with His-Ub, treated with nocodazole (Noc) for 20 h or left untreated (asynchronous (Asyn)), and then analyzed as described for *A*. Two independent PBIP1(T78A) mutant-expressing cell lines (1 and 2) were used. Asterisks indicate nonspecific cross-reacting proteins. Numbers indicate relative protein signal intensities.

first depleted of endogenous PBIP1 (PBIP1 shRNA). As the cells were released from a DT block, they were treated with cycloheximide and harvested at different time points for immunoblot analyses. The results showed that WT PBIP1 exhibited a half-life of ~3.25 h, whereas the nuclear localization-defective PBIP1(K308A/K316A) mutant exhibited a substantially shorter half-life of ~1.92 h under the same conditions (Fig. 2, *C* and *D*). As expected, if PBIP1 and CENP-Q functioned as a complex, the half-life of endogenous CENP-Q in the PBIP1(2A)-expressing cells (~1.98 h) was significantly shorter than that of CENP-Q in the WT PBIP1-expressing cells (~4.13 h) (Fig. 2, *C* and *D*).

Plk1 Kinase Activity Does Not Alter PBIP1 and CENP-Q Ubiquitination Levels—Because the levels of PBIP1 and CENP-Q are precipitously diminished at the late stage of the cell cycle, where Plk1 becomes abundant (Fig. 1*B*), we investigated whether PBIP1 and CENP-Q are ubiquitinated and, if so, whether Plk1-dependent phosphorylation of these proteins alters their levels of ubiquitination. To this end, 293T cells transfected with His-Ub were treated with MG132 and then subjected to His affinity purification to analyze Ub-conjugated PBIP1 and CENP-Q. We observed that a similar fraction of both PBIP1 and CENP-Q was detected as a ubiquitinated, slow-migrating form compared with the respective levels of PBIP1 and CENP-Q in the total lysates (Fig. 3*A*). However, treatment of cells with the Plk1 inhibitor BI 2536 (100 nM) (20) for up to 4 h failed to alter the PBIP1 and CENP-Q ubiquitination levels (Fig. 3*B*). In addition, the PBIP1(T78A) mutant, defective in Polo-box domain binding and subsequent Plk1-dependent phosphorylation, exhibited an undiminished level of PBIP1 ubiquitination in both asynchronous and nocodazole-arrested cells (Fig. 3*C*). These results strongly suggest that a fraction of both PBIP1 and CENP-Q is degraded through a ubiquitination-dependent

degradation pathway, but that the activity of Plk1 or its binding to PBIP1 is not required for Ub-mediated degradation of the PBIP1·CENP-Q complex.

Plk1 Phosphorylates CENP-Q at Multiple Sites—Next, we examined whether Plk1 directly phosphorylates and regulates the PBIP1·CENP-Q complex during the cell cycle. To examine these possibilities, we first monitored the mobility of PBIP1 and CENP-Q when cells released from a DT block into a nocodazole-containing medium were treated with BI 2536. We observed that BI 2536 treatment eliminated the several tiers of slow-migrating PBIP1 forms (which began to appear at the 9-h time point) (Fig. 4*A*, upper arrow), while it annihilated less distinct but clearly slow-migrating CENP-Q forms (which began to appear at the 11-h time point) (Fig. 4*A*, middle arrow). Treatment of total lysates with λ-phosphatase completely eliminated slow-migrating PBIP1 and CENP-Q forms (Fig. 4*A*, second and fourth panels), strongly suggesting that Plk1 phosphorylates PBIP1 and CENP-Q. Therefore, we carried out mass spectrometry analyses to determine Plk1-dependent phosphorylation sites on the PBIP1·CENP-Q complex using both *in vitro* kinase-reacted samples and *in vivo* immunoprecipitates from 293T cell lysates cotransfected with Plk1 (see “Experimental Procedures”). From these analyses, we identified nine CENP-Q residues (Thr-123, Thr-135, Ser-138, Ser-139, Ser-248, Ser-249, Ser-253, Ser-255, and Thr-256) that were phosphorylated in both *in vitro* and *in vivo* samples (Fig. 4*B*), suggesting that Plk1 phosphorylates these sites. All nine phosphorylation sites were clustered in two regions (residues 123–139 and residues 248–256) of the protein (Fig. 4*B*). As expected, although mutation of some of these nine residues to Ala exhibited a mild effect, mutation of all nine sites almost completely eliminated Plk1-dependent CENP-Q phosphorylation *in vitro* (Fig. 4*C*). Subsequent analysis with three phospho-specific antibodies (phospho-Ser-138, phos-

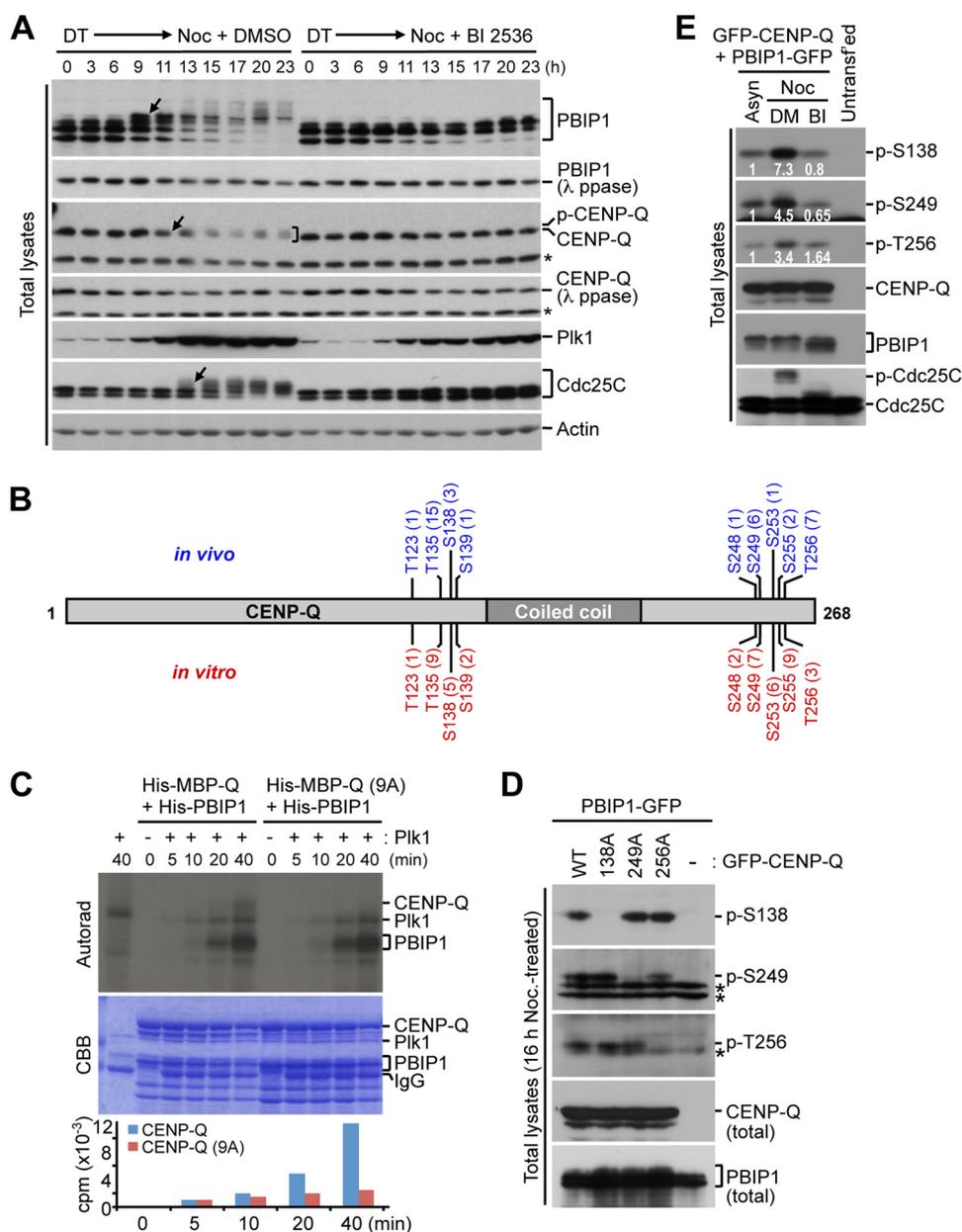


FIGURE 4. Plk1 phosphorylates CENP-Q at multiple sites. *A*, HeLa cells released from a DT block into a nocodazole (Noc)-containing medium were treated with either control dimethyl sulfoxide (DMSO) or BI 2536. Samples were harvested at the indicated time points, treated with λ -phosphatase (λ ppase) where indicated, and immunoblotted. Arrows indicate the first appearance of slow-migrating PBIP1, CENP-Q, and Cdc25C forms after DT release. The bracket in the third panel indicates slow-migrating CENP-Q forms, which disappeared in λ -phosphatase-treated lysates (fourth panel). Asterisks indicate cross-reacting protein. *B*, the diagram depicts nine Plk1-dependent CENP-Q phosphorylation sites identified from both *in vivo* immunoprecipitates from cotransfected 293T cells (upper part) and *in vitro* kinase reaction products (lower part) (see "Experimental Procedures"). Numbers in parentheses indicate phosphopeptide counts containing the indicated residues from mass spectrometry analysis. *C*, bacterially expressed PBIP1-CENP-Q complexes were reacted with (+) or without (-) Plk1 for the indicated times. Samples were separated by SDS-PAGE, stained with Coomassie Brilliant Blue (CBB), exposed, and quantified (graph). Q, CENP-Q. *D*, 293T cells were cotransfected with PBIP1-GFP and either WT GFP-CENP-Q or the indicated mutant construct and treated with nocodazole for 16 h. The resulting cells were harvested and subjected to immunoblot analysis. Asterisks indicate cross-reacting proteins. Note that in the absence of cotransfected CENP-Q, the level of PBIP1 was noticeably diminished (fifth lane). *E*, 293T cells cotransfected with the indicated constructs were left untreated (asynchronous (Asyn)) or were treated with nocodazole for 16 h. The cells were additionally treated with either control dimethyl sulfoxide (DM) or BI 2536 (BI) during the last 2 h of the nocodazole treatment. Untransfected (Untransfected) cells served as controls. The resulting samples were immunoblotted with the indicated antibodies. Numbers indicate relative signal intensities.

pho-Ser-249, and phospho-Thr-256) (Fig. 4D) that we were able to generate revealed that phosphorylation at these sites increased severalfold under nocodazole-treated (M phase) conditions, in which Plk1 is abundant (8). Furthermore, treatment of these cells with BI 2536 greatly diminished the level of all of these phosphopeptides (Fig. 4E).

Plk1-dependent Phosphorylation Alters the Ability of CENP-Q to Associate with Chromatin—Because both PBIP1 and CENP-Q are phosphorylated at the late stages of the cell cycle and delocalized from kinetochores (Figs. 1 and 4), we examined whether the level of phosphorylation alters the stability of the PBIP1-CENP-Q complex. In a co-immunoprecipi-

Plk1-dependent Regulation of the PBIP1-CENP-Q Complex

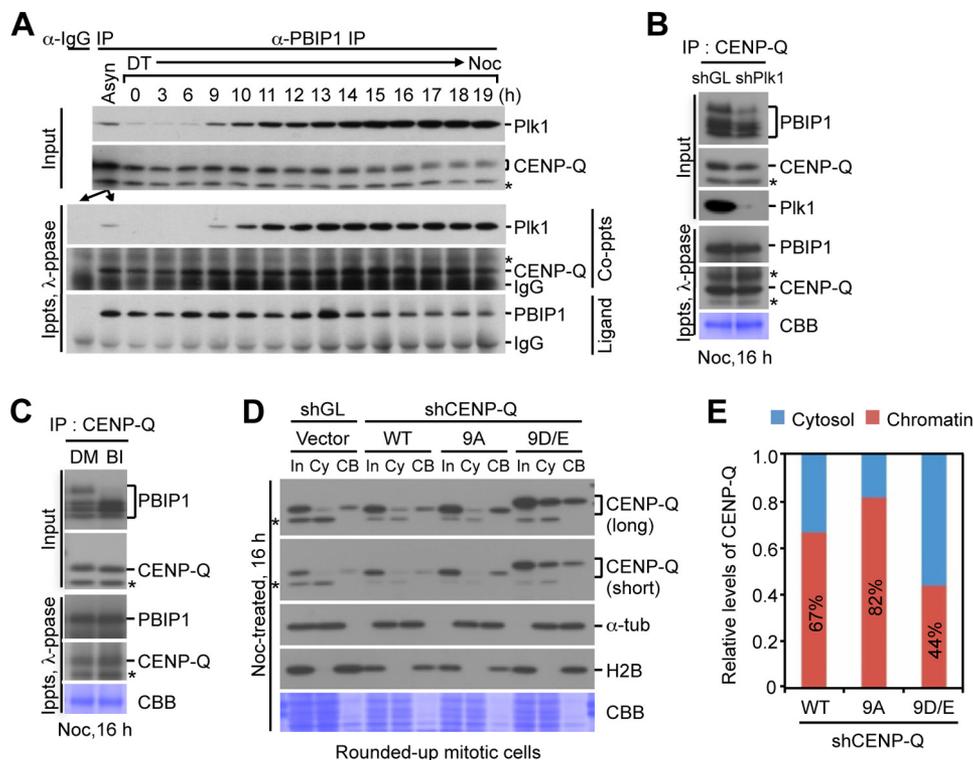


FIGURE 5. Plk1 dissociates CENP-Q from chromatin without disrupting the PBIP1-CENP-Q complex. *A*, HeLa cells released from a DT block into a nocodazole (Noc)-containing medium were harvested and subjected to immunoprecipitation (IP) analyses. To better assess the level of the PBIP1-CENP-Q complex, immunoprecipitates (ippts) were treated with λ -phosphatase (λ ppase) prior to SDS-PAGE. Asterisks indicate cross-reacting proteins. *Asyn*, asynchronous (untreated); *Co-ppts*, co-precipitates. *B*, HeLa cells silenced for either control luciferase (*Gaussia* luciferase shRNA (*shGL*)) or Plk1 shRNA (*shPlk1*) were treated with nocodazole for 16 h before harvest. Immunoprecipitates were treated with λ -phosphatase and then subjected to immunoblot analyses. Asterisks indicate cross-reacting proteins. *CBB*, Coomassie Brilliant Blue. *C*, HeLa cells treated with nocodazole for 16 h were additionally treated with either control dimethyl sulfoxide (*DM*) or BI 2536 (*BI*) during the last 2 h of the nocodazole treatment. The resulting samples were analyzed as described for *B*. Asterisks indicate cross-reacting proteins. *D* and *E*, rounded-up mitotic HeLa cells expressing the indicated constructs silenced by either control luciferase or CENP-Q RNAi (*shGL* or *shCENP-Q*, respectively) were prepared. The resulting samples were fractionated and analyzed (*D*) in a manner similar to that described for Fig. 1*D*, and the levels of CENP-Q in the cytosolic (Cy) and chromatin-bound (CB) fractions were quantified (*E*). Because only rounded-up mitotic cells were used, no soluble nuclear fraction was prepared. α -Tubulin (α -*tub*) and histone H2B served as markers for cytosolic and chromatin-bound fractions, respectively. Asterisks indicate cross-reacting proteins. *In*, input.

tation analysis, we observed that PBIP1 efficiently interacted with CENP-Q throughout the entire cell cycle, regardless of the degree of either protein's phosphorylation (Fig. 5*A*). Consistent with the previous findings that Plk1 binds to the self-primed Thr-78 site of PBIP1 and forms a heterotrimeric complex with PBIP1 and CENP-Q (10, 19), co-precipitation of Plk1 was also evident as early as DT9 (Fig. 5*A*). These findings suggest that the formation of the PBIP1-CENP-Q complex occurs efficiently, even under conditions in which both subunits are hyperphosphorylated. Furthermore, the depletion or inhibition of Plk1 failed to significantly alter the level of the interaction between PBIP1 and CENP-Q (Fig. 5, *B* and *C*), supporting the view that Plk1-dependent phosphorylation does not alter the efficiency of forming the PBIP1-CENP-Q complex.

Next, we examined whether Plk1-dependent CENP-Q phosphorylation alters the ability of the PBIP1-CENP-Q complex to associate with chromatin. To this end, we generated the CENP-Q(9A) mutant, in which the nine Plk1-dependent Ser/Thr residues were replaced with Ala, and then examined the ability of this mutant to associate with chromatin using HeLa cells in which endogenous CENP-Q was silenced by RNAi. Because a previous study suggested that CENP-Q forms a homooctameric complex (24), depleting endogenous CENP-Q would be important to avoid a potential dominant-negative effect. The

phospho-mimicking CENP-Q(9D/E) mutant, in which the nine Ser/Thr residues were substituted with Asp/Glu (see "Experimental Procedures"), was also included for comparative analysis. To maximize the effect of phosphorylation of CENP-Q, fractionation was carried out using rounded-up mitotic cells that were prepared after release from a DT block for 10.5 h. The results showed that 67% of WT CENP-Q was found to be chromatin-bound (Fig. 5, *D* and *E*). Under the same conditions, ~82% of the CENP-Q(9A) mutant was co-fractionated with chromatin, whereas only ~44% of the CENP-Q(9D/E) mutant was co-fractionated with chromatin. These observations suggest that Plk1-dependent phosphorylation negatively regulates the ability of CENP-Q to associate with chromatin.

Plk1 Regulates the Timing of CENP-Q Dissociation from Kinetochores, and a Failure in This Process Leads to Chromosome Missegregation—To further examine whether the differential abilities of the CENP-Q(9A) and CENP-Q (9D/E) mutants to associate with chromatin are reflected in the level of their localization to kinetochores, we immunostained cells expressing WT CENP-Q or the indicated mutant after depleting endogenous CENP-Q. As expected, exogenously expressed WT CENP-Q prominently localized to interphase prekinetochores, but greatly delocalized from metaphase kinetochores (Fig. 6, *A* and *B*). Under these conditions, the corresponding CENP-

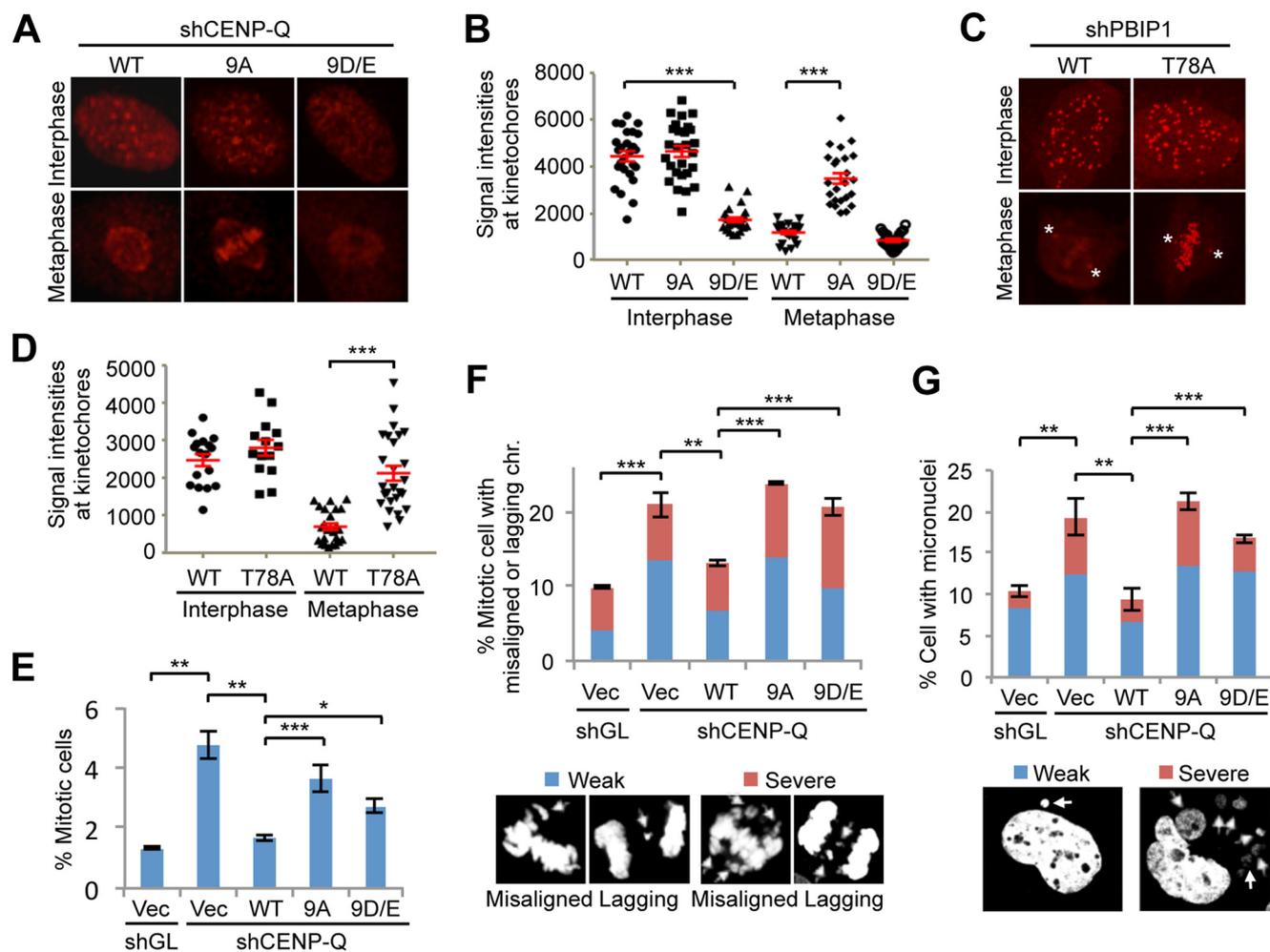


FIGURE 6. Either prolonged or impaired localization of CENP-Q to kinetochores induces a defect in proper chromosome segregation and mitotic progression. *A*, HeLa cells expressing the indicated CENP-Q constructs were depleted of endogenous CENP-Q and subjected to immunostaining analyses. *shCENP-Q*, CENP-Q shRNA. *B*, fluorescence intensities were quantified from 20 randomly chosen kinetochores (four kinetochores/cell) for each sample. Error bars indicate S.E. *C* and *D*, HeLa cells expressing the indicated PBIP1 constructs were depleted of endogenous PBIP1 (PBIP1 shRNA (*shPBIP1*)). The resulting cells were immunostained (*C*), and PBIP1 signals at the interphase prekinetochores and mitotic kinetochores were quantified from >14 cells for each sample (*D*). Error bars indicate S.E. *E–G*, HeLa cells generated in a manner similar to that described for *A* were arrested with a DT block, released into fresh medium for 15 h, fixed, and stained with DAPI to visualize DNA morphologies. The percentages of cells with mitotic morphologies (*E*), misaligned or lagging chromosomes (*F*), or micronucleated DNA (*G*) were quantified. More than 500 cells were quantified from three independent experiments. Error bars indicate S.D. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (unpaired two-tailed *t* test). Arrows indicate misaligned or lagging chromosomes (*F*) or micronucleated chromosomes (*G*). *Vec*, control vector; *shGL*, *Gussia* luciferase shRNA.

Q(9A) mutant appeared to be recruited normally to interphase prekinetochores and remained localized even at metaphase or early anaphase kinetochores (Fig. 6, *A* and *B*). In contrast to the CENP-Q(9A) mutant, its phospho-mimicking CENP-Q(9D/E) mutant failed to localize to both interphase prekinetochores and mitotic kinetochores (Fig. 6, *A* and *B*). These findings suggest that Plk1-dependent CENP-Q phosphorylation promotes the delocalization of the PBIP1-CENP-Q complex from kinetochores and that this event is important for determining the timing of the complex's delocalization from this site. Consistent with this finding, the PBIP1(T78A) mutant, defective in Plk1 Polo-box domain binding (10), exhibited a sustained localization to mitotic kinetochores (Fig. 6, *C* and *D*).

Depletion of PBIP1 function leads to mild mitotic arrest and chromosome missegregation (10). Thus, we examined whether the alterations in CENP-Q localization caused by the 9A or 9D/E mutation influence normal mitotic progression using CENP-Q shRNA cells expressing either the control vector or the indicated CENP-Q constructs. We observed that, in comparison to the cells

expressing WT CENP-Q, cells expressing either the control vector or CENP-Q(9A) exhibited a modest level of mitotic arrest, with a substantially increased fraction of cells exhibiting lagging chromosomes and micronucleated morphology (Fig. 6, *E–G*). Strikingly, CENP-Q RNAi cells expressing CENP-Q(9D/E) also exhibited a significantly increased mitotic population with lagging chromosomes and micronuclei (Fig. 6, *E–G*), although the severity of the 9D/E-associated defect was somewhat lower compared with that of the 9A mutation. These findings suggest that either impaired delocalization or improper localization of CENP-Q, which is caused by the 9A or 9D/E mutation, respectively, can cause a similar defect in proper M phase progression.

DISCUSSION

Mechanism Underlying the Recruitment/Delocalization of the PBIP1-CENP-Q Complex to/from Kinetochores—It has been shown that PBIP1 is recruited to interphase prekinetochores as early as late G₁, but that it delocalizes from this site as cells enter mitosis (10). PBIP1 and CENP-Q form a stable complex that

Plk1-dependent Regulation of the PBIP1·CENP-Q Complex

appears to associate with various outer kinetochore components (15, 18, 19). Consistent with these observations, CENP-Q also abundantly localized to interphase prekinetochores, but localized significantly less to mitotic kinetochores (Fig. 1).

It has been shown that Plk1 efficiently phosphorylates and binds to the Thr-78 motif of PBIP1 through a self-priming and binding mechanism (10, 12) and then phosphorylates the CENP-Q subunit of the PBIP1·CENP-Q complex (19). Consistent with this, inhibition of Plk1 activity greatly diminished hyperphosphorylated, slow-migrating PBIP1 (but not the moderately phosphorylated PBIP1 forms because of the presence of Plk1-independent phosphorylation) and appeared to eliminate slow-migrating CENP-Q forms (Fig. 4A). Although detection of the slow-migrating CENP-Q forms was somewhat difficult because it required a prolonged gel migration (as shown in Figs. 1B and 4A), Plk1 clearly phosphorylated PBIP1-associated CENP-Q at multiple sites (Fig. 4B). Taken together, these observations suggest that Plk1 regulates the PBIP1·CENP-Q complex by phosphorylating both PBIP1 and CENP-Q in a sequential manner.

The first hint that Plk1 may regulate the subcellular localization of the PBIP1·CENP-Q complex came from the observation that the PBIP1(T78A) mutant, resistant to Plk1-dependent self-priming and binding (10), exhibited a prolonged localization to mitotic kinetochores (Fig. 6, C and D). Subsequent studies revealed that the CENP-Q(9A) mutant, lacking the nine Plk1-dependent phosphorylation sites, exhibited a prolonged localization to mitotic kinetochores, whereas the CENP-Q(9D/E) mutant, bearing phospho-mimicking, negatively charged residues at the nine sites, remained dissociated even from interphase prekinetochores, where CENP-Q is normally not phosphorylated (Fig. 6, A and B). These findings strongly suggest that Plk1 directly regulates the PBIP1·CENP-Q complex by multiply phosphorylating CENP-Q via the PBIP1 phospho-Thr-78 tether and that Plk1-dependent phosphorylation of CENP-Q determines when the complex is unloaded from mitotic kinetochores. We were not able to examine the effect of the PBIP1(T78D) or PBIP1(T78E) mutation because the resulting mutants failed to constitutively bind to the Plk1 Polo-box domain (10).

How does Plk1 induce the dissociation of the PBIP1·CENP-Q complex from mitotic kinetochores? One possibility is that, because dissociated PBIP1 and CENP-Q subunits are unstable (19), Plk1-dependent CENP-Q phosphorylation may disassemble the PBIP1·CENP-Q complex by inducing the dissociation of the complex into each subunit. However, the PBIP1·CENP-Q complex remained stable even in mitosis, where both subunits were hyperphosphorylated (Fig. 5A). In addition, depletion or inhibition of Plk1 did not alter the level of the interaction between PBIP1 and CENP-Q (Fig. 5, B and C). These findings suggest that Plk1 may instead induce the dissociation of the entire PBIP1·CENP-Q complex by disrupting the interaction between the complex and other component(s) at the mitotic kinetochores. Although we were not able to further delineate this event because of the multitude of interactions between CENP-Q and other kinetochore components (Fig. 7A), our results support the view that the PBIP1·CENP-Q complex assembles at early interphase prekinetochores prior to the

appearance of Plk1. As the level of Plk1 increases at the late stage of the cell cycle, Plk1 phosphorylates and binds to PBIP1 Thr-78 and then subsequently phosphorylates the CENP-Q subunit of the PBIP1·CENP-Q complex to delocalize the complex from mitotic kinetochores (Fig. 7B).

Plk1 Regulates the Stability of the PBIP1·CENP-Q Complex by Altering Its Level of Chromatin Association, but Not Ubiquitination—Our data showed that underphosphorylated PBIP1 forms, which were generated independently of Plk1 activity (Fig. 4A), appeared to be stable in both cytoplasmic and chromatin-bound fractions (Fig. 1D). In contrast, Plk1-dependent hyperphosphorylated PBIP1 forms, which were detected at the late stages of the cell cycle, were rapidly degraded in the cytosol, although they appeared to be somewhat stable when bound to chromatin (Fig. 1D). Notably, the level of chromatin-bound, hyperphosphorylated PBIP1 forms steadily declined as cells proceeded through the cell cycle (Fig. 1D), suggesting that Plk1-dependent phosphorylation negatively regulates the ability of PBIP1 to associate with chromatin. Consistent with this view, the level of the PBIP1·CENP-Q complex sharply declined at mitotic kinetochores, where Plk1 activity was high (Fig. 1, E and F) (10). These observations suggest that the overall level of the PBIP1·CENP-Q complex is regulated through two successive processes: a rapid, cytosolic degradation, followed by a slow, Plk1-dependent, delocalization-induced degradation.

To determine the underlying mechanism by which Plk1 influences the stability of the PBIP1·CENP-Q complex, we first examined whether Plk1 alters the ubiquitination level of the complex. However, neither the inhibition of Plk1 nor the loss of the Thr-78-dependent Plk1-PBIP1 interaction significantly changed the level of PBIP1 and CENP-Q ubiquitination (Fig. 3, B and C). These observations suggest that Plk1 does not directly generate phosphodegron(s) for PBIP1 and CENP-Q ubiquitination and that it may rather contribute to the stability of PBIP1 and CENP-Q indirectly. In this regard, it is important to note that chromatin-associated PBIP1 and CENP-Q were significantly more stable than cytosolic PBIP1 and CENP-Q (Fig. 1D) and that Plk1-dependent phosphorylation induced the dissociation of the PBIP1·CENP-Q complex from kinetochores (Fig. 6). These findings led us to speculate that simple dissociation of the PBIP1·CENP-Q complex from kinetochores to the nucleoplasm and cytosol may destabilize the complex. Thus, we propose that Plk1-dependent phosphorylation and delocalization of the PBIP1·CENP-Q complex from kinetochores ultimately lead to the down-regulation of the complex in the cytosol (Fig. 7B). Furthermore, eliminating the PBIP1·CENP-Q complex from mitotic kinetochores could be important for allowing the cells to accumulate unphosphorylated complex early in the next cell cycle and to reprogram Plk1-dependent cellular processes at this site.

Role of the PBIP1·CENP-Q Complex in the Regulation of Plk1 Function—Okada *et al.* (15) proposed the existence of the CENP-O complex composed of CENP-O, CENP-P, CENP-Q, CENP-R, and CENP-U/PBIP1 largely based on their localization dependences. However, the exact subunits constituting this complex are debatable (17, 18), and the composition of the complex is not known. Previous studies show that the depletion of PBIP1 or CENP-Q in HeLa cells (10, 15, 18, 25) or the knock-

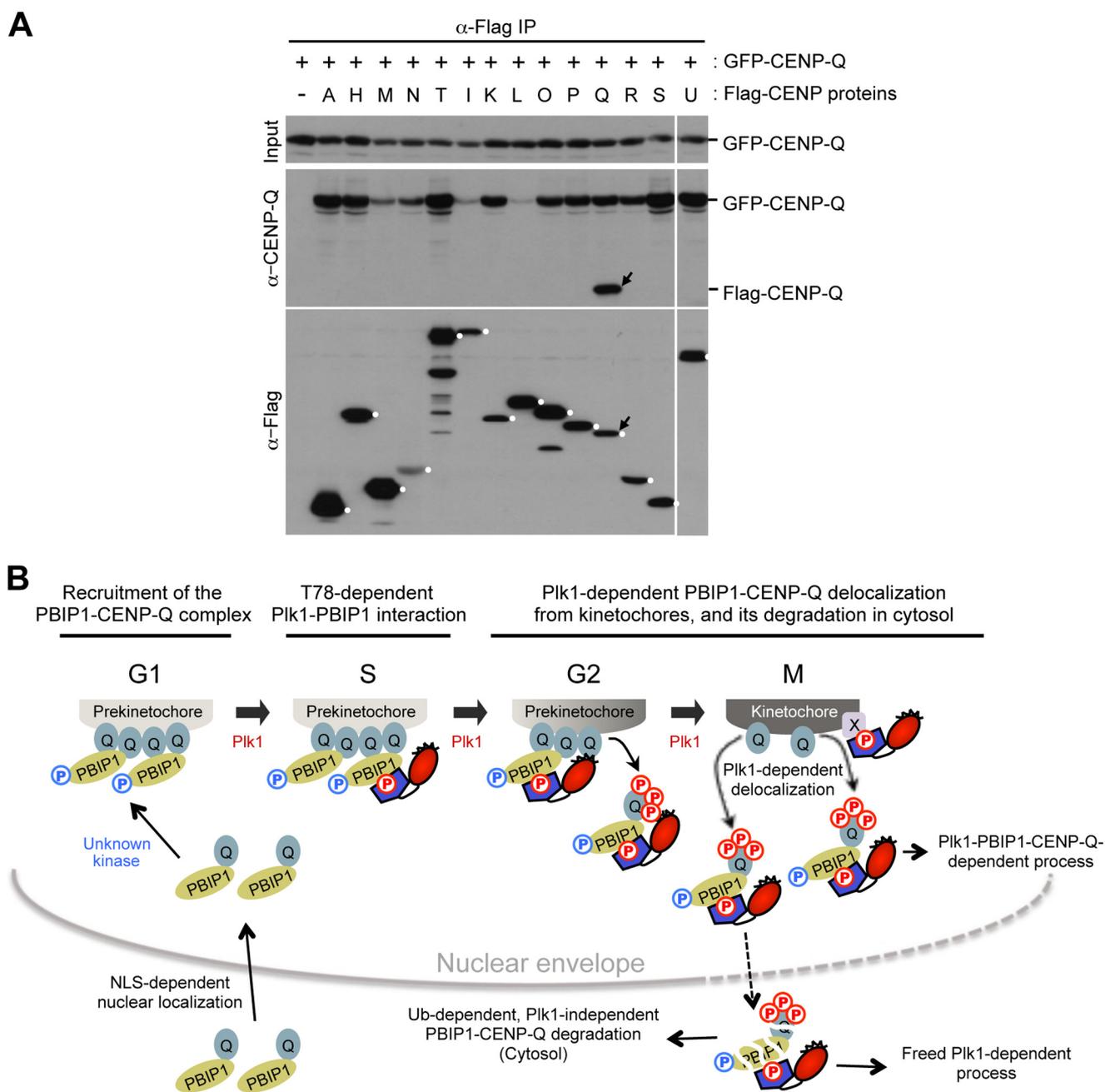


FIGURE 7. Interaction between CENP-Q and other kinetochore proteins and a model illustrating the cell cycle-dependent regulation of the PBIP1-CENP-Q complex. *A*, 293T cells transfected with the indicated constructs were subjected to co-immunoprecipitation (IP) analyses. Letters denote centromeric proteins from CENP-A to CENP-U/PBIP1. Dots mark the full-length form of each immunoprecipitated ligand. Arrows indicate the same FLAG-CENP-Q protein detected by both anti-CENP-Q and anti-FLAG antibodies. *B*, early in the cell cycle, such as in the G₁ phase, the PBIP1-CENP-Q complex is recruited to interphase prekinetochores through its intrinsic nuclear localization activity and its ability to interact with outer centromere components (15, 18, 19). As Plk1 becomes abundant in the late S and early G₂ phases, Plk1 phosphorylates PBIP1 at Thr-78 and binds to the resulting phospho-Thr-78 motif (10), thus consequently promoting its own recruitment to interphase prekinetochores. In the late G₂ and M phases, PBIP1 phospho-Thr-78-bound Plk1 becomes active and phosphorylates the CENP-Q subunit of the PBIP1-CENP-Q complex (19), thereby inducing the dissociation of the complex from late interphase and early mitotic kinetochores. Plk1 does not appear to alter the level of Ub-dependent degradation of the PBIP1-CENP-Q complex. However, because the cytosolic PBIP1-CENP-Q complex is much more susceptible to degradation than the chromatin-associated complex, Plk1-dependent dissociation of the complex from kinetochores may ultimately lead to degradation of the complex in the cytosol. This Plk1-dependent down-regulation of the chromatin-associated PBIP1-CENP-Q complex may help reset the kinetochore cycle and allow newly synthesized complexes to re-accumulate at this site in the following cell cycle. CENP-Q has been proposed to form an octameric complex (24). However, because the binding stoichiometry of the PBIP1-CENP-Q complex is not known, the complex is depicted as a dimer for simplicity. NLS, nuclear localization signal.

out of CENP-O, CENP-P, or PBIP1 in DT40 cells (15) leads to a moderate level of mitotic arrest. Interestingly, the loss of Thr-78-dependent PBIP1 function, which disrupts Plk1 binding, induces a mitotic defect similar to that associated with the depletion of PBIP1, CENP-Q, or any one subunit of the

CENP-O complex (10). Hence, although the role of the entire CENP-O complex has yet to be further investigated, one of the main functions of the complex is to recruit Plk1 to the PBIP1-CENP-Q subcomplex and to promote Plk1-dependent event(s) during M phase progression.

Plk1-dependent Regulation of the PBIP1-CENP-Q Complex

How does the PBIP1-CENP-Q complex promote Plk1-dependent processes at kinetochores? It has been demonstrated that Plk1 phosphorylates the Thr-78 motif of PBIP1 to recruit itself to late interphase and early mitotic kinetochores (10). Here, we showed that Plk1-dependent phosphorylation of the CENP-Q subunit induced the delocalization of the PBIP1-CENP-Q complex from mitotic kinetochores and that it did so without disrupting the Plk1·PBIP1-CENP-Q trimeric complex (Fig. 5A). Strikingly, both the localization-deficient, phospho-mimicking CENP-Q(9D/E) and localization-proficient CENP-Q(9A) mutations caused a similar defect in chromosome segregation and mitotic progression (Fig. 6, E–G). These findings suggest that proper formation of the Plk1·PBIP1-CENP-Q complex needs to occur at prekinetochores/kinetochores, presumably to enrich Plk1 at these sites, and that the timing of the trimeric complex's delocalization is carefully choreographed to coordinate various events that are important for M phase progression. The timely released PBIP1-CENP-Q complex may serve as a diffusible scaffold that promotes Plk1-dependent phosphorylation of its substrates present at or around kinetochores. Alternatively, because the cytosolic PBIP1-CENP-Q complex is more susceptible to degradation, Plk1 could ultimately be liberated from the PBIP1-CENP-Q scaffold and then could freely interact with its substrates, which are important for mitotic progression. Although the two possibilities described above are not necessarily mutually exclusive, we favor the latter scenario that proposes a role of the PBIP1-CENP-Q complex as a temporary scaffold for Plk1 function. In support of our view, the level of kinetochore-localized Plk1 steadily increases until prometaphase (10), the stage where the PBIP1-CENP-Q complex is already significantly delocalized from kinetochores (Fig. 1, E and F). This is likely because of the appearance of other Plk1-binding kinetochore proteins, such as Bub1 (26), which can interact with and recruit Plk1 liberated from the temporary PBIP1-CENP-Q scaffold.

In this study, we investigated the mechanism underlying Plk1-dependent regulation of the PBIP1-CENP-Q complex and the significance of this event during the late stage of the cell cycle. Ironically, early in the cell cycle, Plk1 first phosphorylates and binds to the phospho-Thr-78 motif of PBIP1 to recruit itself to kinetochores. Thus, the kinetochore-localized PBIP1-CENP-Q complex functions not only as a transient platform for initial Plk1 recruitment, but also as a target of Plk1-mediated biochemical processes. Perhaps differential Plk1 activities are required to trigger these two-step events in an orderly manner. Further understanding of how the PBIP1-CENP-Q complex itself is initially recruited to prekinetochores prior to Plk1 recruitment to the PBIP1-CENP-Q complex and what Plk1 does to promote M phase progression after binding to and/or being liberated from the transient PBIP1-CENP-Q tether will require the identification of additional proteins and/or Plk1 substrates whose function is contingent upon the presence of the PBIP1-CENP-Q complex.

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J. Biol. Chem. 2015, 290:8569-8581.

doi: 10.1074/jbc.M114.623546 originally published online February 10, 2015

Access the most updated version of this article at doi: [10.1074/jbc.M114.623546](https://doi.org/10.1074/jbc.M114.623546)

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