Ki67 Antigen Contributes to the Timely Accumulation of Protein Phosphatase 1γ on Anaphase Chromosomes*

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Background: Ki67 is a widely used cell proliferation marker whose cellular functions, however, remain elusive.

Results: Ki67 interacts with protein phosphatase 1γ (PP1γ) and modulates its localization in anaphase.

Conclusion: Ki67 is a novel regulator of PP1γ localization.

Significance: This study shows a novel example of spatial and temporal control of dephosphorylation events on anaphase chromosomes.

Ki67 is a protein widely used as cell-proliferation marker, with its cellular functions being hardly unveiled. In this paper, we present the direct interaction between Ki67 and PP1γ, a protein phosphatase showing characteristic accumulation on anaphase chromosomes via the canonical PP1-binding motif within Ki67. In cells depleted of Ki67, PP1γ is targeted to anaphase chromosomes less efficiently. Additionally, overexpression of Ki67, but not a mutant form without the ability to bind PP1γ, induced ectopic localization of PP1γ on metaphase chromosomes. These observations demonstrate that Ki67 is one factor that defines the cellular behavior of PP1γ in anaphase. To explore the specific roles of the subset of PP1γ recruited on chromosome via its interaction with Ki67 (PP1γ-Ki67), endogenous Ki67 was replaced with a Ki67 mutant deficient in its ability to interact with PP1γ. Although no obvious defects in the progression of mitosis were observed, the timing of dephosphorylation of the mutant Ki67 in anaphase was delayed, indicating that Ki67 itself is one of the substrates of PP1γ-Ki67.

Ki67 is a nuclear protein originally identified from a Hodgkin lymphoma-derived cells line (1). Ki67 is expressed in all phases of the cell division cycle, but its expression level is strongly down-regulated in the resting G0 phase (2, 3). This characteristic makes Ki67 an excellent marker for cell proliferation (4). Because higher proliferation rates are characteristic of cancer cells, Ki67 has been widely used as a prognostic marker in many types of cancer. Using Ki67 in combination with other cell proliferation markers, such as BCL2 (5) or Aurora kinase A (6), should lead to a more accurate prognosis. Despite this increasing value of Ki67 as a clinical marker, surprisingly little is known about its cellular function.

Ki67 is localized mainly in nucleolus during interphase. In early G1 phase, a certain population of Ki67 is localized also on heterochromatic DNA regions (7, 8). Within the nucleolus, Ki67 is localized predominantly in the dense fibrillar component, one of three nucleolar substructures free from the RNA polymerase I transcription apparatus, thereby suggesting a role for Ki67 in late rRNA processing (8, 9). However, another study showed that a small but significant amount of Ki67 co-localizes with components of the RNA polymerase I transcription apparatus and newly synthesized rRNA in vivo, arguing for a role for Ki67 in the early steps of rRNA synthesis (3). These likely functions of Ki67 during ribosome biogenesis may explain the tight correlation of cell proliferation and Ki67 expression. Separately, our previous studies, as well as other studies, identified specific interactions between Ki67 and HP1 proteins (10, 11), suggesting the involvement of Ki67 in the organization of chromatin.

Another conspicuous feature of Ki67 is its localization during mitosis. Ki67 is localized around mitotic chromosomes in a layer, as if it is decorating the chromosomes from outside (12). This characteristic localization was also observed with the homologous protein in mice (13), rat to kangaroos (14), and frogs.4 To elucidate the mitotic function of Ki67 at the perichromosomal layer, its interactive proteins during mitosis have been explored. A mitosis-specific interaction between Ki67 and a kinesin-like motor protein, Hklp2, was initially identified (15). This interaction contributes to the formation and maintenance of the bipolar spindle (16). A mitotically favored interaction between Ki67 and RNA-binding protein NIFK has also been identified (17), but the physiological function of this interaction is currently unknown. Ki67 is hyper-phosphorylated in metaphase and dephosphorylated in later stages (18). Although recent phospho-proteomic analyses identified actual phosphorylation sites on Ki67 (19–22), any link between individual phosphorylation events and specific cellular processes has not been revealed.

For the ordered progression of mitosis, the spatial and temporal control of phosphorylation events on key mitotic substrates is necessary. In the process of mitotic exit, the removal of phosphorylation from a broad range of substrates occurs in a coordinated manner, and this allows for the assembly of a functional interphase nucleus with an intact nuclear envelope and
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reorganized chromatin. Protein phosphatase 1 (PP1), along with protein phosphatase 2A (23, 24), is one of the major serine/threonine-specific phosphatase families involved in mitotic exit by counteracting mitotic kinases (25). Among three PP1 isoforms in mammals (PP1α, β/δ, and γ), PP1γ is considered to play an important role for chromatin decondensation and/or reactions taking place on chromatin, such as nuclear envelope assembly, as a subset of PP1γ targets on chromosomes in early anaphase (26). PP1γ has many interaction partners, all of which are regulatory subunits important for targeting PP1γ to diverse subcellular structures and for restricting its activity and/or specificity (27). In the context of targeting on anaphase chromosomes, a subset of PP1γ is known to do this by forming a complex with Repo-MAN (28). The Repo-MAN-PP1γ complex was shown to contribute to chromatin decondensation (29), although the relevant substrate to be dephosphorylated during the reaction has not been identified. Given the complexity of the mitotic exit process, the existence of PP1γ-targeting subunits other than Repo-MAN is naturally hypothesized (30).

In the present study, we showed that Ki67 directly interacts with a subset of PP1γ to form a complex and also contributes to modulating the cellular behavior of PP1γ in anaphase as a targeting subunit of the enzyme complex. Using newly generated antibodies recognizing a Ki67 that is phosphorylated within its Ki67-repeat domain, we characterized the timely dephosphorylation of Ki67 in anaphase. The dephosphorylation of Ki67 was delayed when its interaction with PP1γ was disrupted, indicating that Ki67 itself is one of the substrates of PP1γ that is recruited on chromosomes via its interaction with Ki67. We discuss the possibility that Ki67 might contribute to the progression of anaphase/telophase by serving as a platform not only for PP1γ but also for various biological activities to target to chromosomes.

**EXPERIMENTAL PROCEDURES**

**Purification of hPP1γ**—A cDNA fragment encoding hPP1γ (NM_002710) was created by PCR with KOD-plus polymerase (TOYOBO) using cDNAs prepared from HeLa cells, such that attB1 and attB2 sequences were introduced at the 5′- and 3′-ends, respectively. The fragment was cloned first into pDONR221 (Invitrogen) via the BP reaction mediated by BP clonase (Invitrogen) and then transferred to pGEX-Dest, a vector modified from pGEX6P3 (GE Healthcare) using the Gateway vector conversion system (Invitrogen), via the LR reaction mediated by LR clonase (Invitrogen) to generate pMT449. hPP1γ fused to the C terminus of GST (GST-hPP1γ) was purified from *Escherichia coli* BL21DE3(pLysS) cells transformed with pMT449 after culturing for 10 h at 20 °C in LB medium supplemented with 1 mM MnCl2 and 0.1 mM isopropyl 1-thio-β-d-galactopyranoside. GST-hPP1γ was liberated by the cells on sonication in sonic buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT) supplemented with 0.3 mM PMSF, with a subsequent addition of 1% Triton X-100, and finally trapped by glutathione-Sepharose 4B (GE Healthcare). After washing the resin extensively with sonic buffer, hPP1γ was chayed with the PreScission Protease (GE Healthcare) from the resin according to the manufacturer’s protocol. hPP1γ, at this point in the PreScission buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT), was loaded onto a Hitrap Q (GE Healthcare) column equilibrated with 50 mM sodium phosphate buffer (pH 8.0) containing 50 mM NaCl. hPP1γ was eluted by increasing the concentration of NaCl to ~440 mM. The eluted hPP1γ was concentrated using a microcon YM-30 (Millipore) to a final concentration of ~1 mg/ml, aliquoted, snap-frozen in liquid nitrogen, and stored at −80 °C.

**In Vitro Binding Assay**—A cDNA fragment encoding residues 130–175 of human Ki67 (NP_001139438.1) was amplified by PCR with KOD-plus polymerase (TOYOBO) using a full-length construct of human Ki67 (31) as a template, such that attB1 and attB2 sequences were introduced at the 5′- and 3′-ends, respectively. The fragment was first cloned into pDONR221 (Invitrogen) via the BP reaction mediated by BP clonase (Invitrogen) to generate pMT477. Point mutations were introduced in pMT477 so that the canonical PP1-binding motif, RVSF, found at residues 145–148 was mutated to RASA, RVD, and RAVF, thereby resulting in constructs pMT478, pMT503, and pMT504, respectively. Each construct was subjected to the LR reaction mediated by LR clonase (Invitrogen) using pGEX-Dest as a destination vector to generate pYK5, pYK6, pMT505, and pMT506. *E. coli* BL21DE3(pLysS) was transformed by each of these constructs or pGEX6P1 (GE Healthcare), cultured at 37 °C until the A600 became ~0.4, cooled down to 20 °C, and then further cultured at 20 °C in the presence of 0.1 mM isopropyl 1-thio-β-d-galactopyranoside for 10 h to induce the expression of GST-Ki67(130–175) and its point mutants or GST alone. Expressed proteins were liberated from cells as described above for GST-hPP1γ and trapped by glutathione-Sepharose 4B (GE Healthcare). These beads were equilibrated with CSF-XB (100 mM KCl, 2 mM MgCl2, 0.1 mM CaCl2, 50 mM sucrose, 10 mM HEPES, pH 7.7, 5 mM EDTA) plus 0.1% Triton X-100, and incubated with purified hPP1γ at 4 °C for 1 h. After washing the beads three times with CSF-XB plus 0.1% Triton X-100, the bound proteins were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue.

**Cell Culture**—HeLa cells were cultured in DMEM containing 10% fetal FBS. HeLa cells stably expressing YFP-PP1γ and trapped by glutathione-Sepharose 4B (GE Healthcare). These beads were equilibrated with CSF-XB (100 mM KCl, 2 mM MgCl2, 0.1 mM CaCl2, 50 mM sucrose, 10 mM HEPES, pH 7.7, 5 mM EDTA) plus 0.1% Triton X-100, and incubated with purified hPP1γ at 4 °C for 1 h. After washing the beads three times with CSF-XB plus 0.1% Triton X-100, the bound proteins were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue.

**Phosphopeptide Antibody**—The phospho-Ki67 antibodies were raised in rabbits against synthetic phosphopeptide CKKRPQ(pT)PEKEA, which had been conjugated to keyhole limpet hemocyanin. The resulting sera were absorbed thoroughly with the Hitrap NHS-activated HP column (GE Healthcare) conjugated with the non-phosphopeptide (CKKRPQTPEKEA) and then subjected to the column conjugated with the antigen phosphopeptide. After washing the column extensively with PBS, the antibodies, which were trapped by the column, were eluted with 0.1 M glycine (pH 3.0), immediately neutralized with appropriate amount of 1 M Tris (pH 8.0), and dialyzed against 50% (w/v) glycerol in PBS.

5 The abbreviations used are: PP1γ protein phosphatase 1 γ; CKRD core of Ki67-repeat domain; CDK cyclin dependent kinase.
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_Journal of Biological Chemistry_—HeLa cells were treated with 100 ng/ml of nocodazole for 16 h, collected by shake-off and subsequent centrifugation, and reseeded in the pre-warmed culture medium containing nocodazole. Cells were further treated with 0.1% DMSO or 10 μM RO-3306 (Millipore) for 10 min, washed with ice-cold PBS supplemented with 0.3 mM PMSE, and snap-frozen in liquid nitrogen. Cells were lysed in extraction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 0.1% Nonidet P-40, 1 mM DTT) supplemented with Complete Protease Inhibitor Mixture (Roche Applied Science), PhosSTOP (Roche), and 0.25 units/μl of benzamidase nuclease (Novagen), for 30 min on ice. Cell extracts were collected as supernatants after centrifugation at 15,000 × g for 10 min at 4 °C. Typically, 2 × 10⁶ cells were extracted with 200 μl of extraction buffer. Four micrograms of antibodies were cross-linked to 20 μl of Dynabeads Protein A (Invitrogen) using dimethyl pimelimidate (Sigma) and used for immunoprecipitation from 80 μl of cell extracts. After incubation on ice for 1 h with occasional agitation, beads were washed three times with extraction buffer supplemented with Complete Protease Inhibitor Mixture (Roche) and PhosSTOP (Roche) using a magnet. For the final wash, sample tubes were replaced with new ones to avoid contamination by proteins bound nonspecifically to tubes. Immunoprecipitated proteins were detached from the beads by boiling for 3 min with 4 μl of concentrated sample buffer (0.25 M Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.02% bromphenol blue) containing 0.1 M DTT and retrieved using a magnet. Samples were electrophoretically separated on a SuperSep Ace 5–20% gradient gel (Wako) and blotted onto Immobilon-P (Merck Millipore). The following antibodies were used as primary antibodies at the indicated dilutions or concentrations: anti-phospho-Ki67 (0.25 μg/ml), anti-Ki67 mAb (1:4,000, NA-59, Merck Millipore), and anti-PP1γ (1:2,000, sc-6108, Santa Cruz). In addition to several of these antibodies, an anti-β-tubulin mAb (1:5,000, AC-15, Santa Cruz) and an anti-phospho-histone H3 (Ser-10) mAb (1:4,000, 6G3, Cell Signaling) were used for the analysis shown in Fig. 5C.

**Live Cell Observation**—Cells were cultured in 35-mm glass-bottom dishes (IWAKI) and mounted on an inverted microscope (IX-71 DeltaVision CORE system; Olympus and Applied Precision) equipped with a humidified environment chamber (MI-IBC, Olympus) to maintain a temperature of 37 °C and the CO₂ concentration at 5%. Cells were imaged using a 40/1.00 UplanApo objective (Olympus) and a Cool Snap HQ2 CCD camera (Photometrics) from a single focal plane every 1.5 or 3 min. SoftWorx software (Applied Precision) was used for controlling image acquisition. For quantification of the accumulation of fluorescent molecules on anaphase chromosomes (Figs. 2 and 3, A–C), the average fluorescence intensity on chromosomal area was divided by that of whole cell area.

**Immunofluorescence and Data Analysis**—Immunofluorescence specimens were prepared essentially as described previously (14) except for the use of Prolong-Gold (Invitrogen) as an anti-fade mounting reagent. The following antibodies were used as primary antibodies at the indicated dilutions or concentrations: anti-phospho-Ki67 (1 μg/ml), anti-Ki67 mAb (1:1,000, NA-59; Merck Millipore), DMIα (1:10,000, Sigma), and anti-phospho-histone H3 (Ser-10) mAb (1:1,000, 06-570; Merck Millipore). Images were acquired with the same system used for live cell observation except for the use of a ×40/1.00 UplanApo objective (Olympus) for Fig. 6. Images were acquired as Z stacks with 0.2- or 0.5-μm spacing, processed by iterative constrained deconvolution and shown as their projections. For quantifications, maximum intensity projections of the Z stacks spanning 4– (Fig. 5A) or 10-μm (Fig. 6) thickness around the focal planes were used. Chromosomal areas were defined relying on the Hoechst staining using the “wand” tool in ImageJ. The average background intensity was obtained from the area devoid of cells. At each area of interest, the mean fluorescence intensity was measured, subtracted by the average background intensity, and multiplied by the area. The obtained values were normalized for comparison (Fig. 5A) or directly compared (Fig. 6).

**RNAi**—The sequences for siRNAs used were as follows: siGL2, 5’-CGUAGCGGAAUACUUCCA-3’; si31 (Ki67), 5’-GGAAGGUCUACAGAGUUCAGAAU-3’; si32 (Ki67), 5’-UCUGGAAGACCUGCCUUAA-3’; siRepo-MAN, 5’-UGACAGACUUGACCAGAAATT-3’ (32). siRNA transfections were performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. Cells were analyzed 48 h after siRNA transfection unless stated otherwise. For the immunoblot analysis shown in Fig. 5C, some cells were treated with 100 ng/ml of nocodazole for the last 16 h.

**Plasmid Construction**—We previously cloned the full-length cDNA of human Ki67 (short form, NP_001139438.1) into the XhoI site of pEGFP-C3 (Clontech) to generate pMT308 (31). The cDNA was transferred to the Sall site of pmCherry-C1 (Clontech) to generate pMT544. Point mutations were introduced in pMT544 so that the RVSF motif, found at residues 145–148, was exchanged to RASA to generate pMT547. To render these constructs resistant to si31 without changing the coding amino acids, the nucleotide sequence 5′-GCA AGG TCT ACA GAG TTC AGG AAT A-3′ was mutated to 5′-GCA AGC ACC GAG TTC AGG AAT A-3′, resulting in constructs pMT557 and pMT556.

**Replacement of Endogenous Ki67 with mCherry-Ki67**—Cells were seeded at 10⁵ cells per 35-mm dish 1 day before transfection with pMT557 or pMT556. Plasmid transfection was carried out using FuGENE HD (Promega) with a smaller amount of plasmid than suggested by the manufacturer’s protocol. Briefly, 800 ng of plasmid was complexed with 2.4 μl of FuGENE HD in 60 μl of Opti-MEM for 5 min at room temperature and added dropwise on cells. One day after transfection, the cells were trypsinized, collected by centrifugation, resuspended with 4 ml of culture medium, and subjected to reverse transfection with siRNA using Lipofectamine RNAiMAX (Invitrogen). For the subsequent live cell observations, 24 pmol of siRNA was complexed with 4 μl of Lipofectamine RNAiMAX in 400 μl of Opti-MEM for 10 min at room temperature, and mixed with 2 ml suspension of cells that had been prepared as described above. The cells were then seeded on a 35-mm glass-bottom dish (IWAKI). For the subsequent immunofluorescence observations, siRNA transfection was carried out in a quarter quantity and cells were seeded on poly-l-lysine-coated coverslips in wells of 24-well cell culture plates. One day after siRNA transfection, cells were subjected to synchronization as illustrated in Fig. 6, A and E; cells were treated with 2 μM thymidine for 16 h,
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**RESULTS**

**Ki67 Interacts with PP1γ via Its Canonical PP1-binding Motif—**

The structural comparison between human Ki67 and marsupial chmadrin (14) effectively highlighted the regions conserved throughout evolution. We noticed that one region corresponding to residues 142–173 of human Ki67 (NP_001139438.1) contained a canonical PP1-binding motif, RVXF (33). We also noticed that Ki67 showed a significantly high similarity to Repo-MAN, a protein known to play a role in targeting PP1 to anaphase chromosomes (28), throughout the region (Fig. 1A). To test whether the region of Ki67 interacts with PP1γ as well as other proteins bearing the RVXF motif, we performed a pull-down assay using a bacterially expressed PP1γ and Ki67 fragment (residues 130–175) fused to GST. PP1γ was pulled down efficiently with GST-Ki67(130–175) but not with GST alone, indicating a specificity of this interaction (Fig. 1A, lane 3). However, this interaction was abolished when the motif was mutated to RASA, thus indicating that the interaction was mediated by the motif (Fig. 1A, lane 4).

released in normal medium for 7 h, treated with 10 μM RO-3306 for another 2 h, and released again in normal medium. For the experiment shown in Fig. 6, A–D, 10 μM MG-132 (Millipore) was included for 2 h in the final step to arrest cells in metaphase. The efficient replacement of endogenous Ki67 with mCherry-Ki67 was verified by the observation that the positive immunostaining of Ki67 was, in most cases, accompanied with the positive fluorescence of mCherry. Cells with exceptionally strong staining of total Ki67 were concluded to have evaded from the analysis.

**Estimation of the Expression Level of mCherry-Ki67 Relative to That of Endogenous Ki67—** HeLa cells were seeded on glass-bottom dishes with grid (Matsunami, D111505) for enabling us to identify particular cells throughout this experiment. One or 2 days after transfection with pMT557, cells were treated sequentially with RO-3306 (at 10 μM for 3 h) and MG-132 (at 10 μM for 2 h) to increase the metaphase population. The fluorescence images of mCherry-Ki67 (RASA) in metaphase cells, whose places on the grid had been recorded, were obtained in a certain condition identical to that for other live imaging experiments. Cells were then fixed with formaldehyde and processed for immunofluorescence with mAb against Ki67 (NA-59). The staining intensity of each cell was plotted against the original fluorescence intensity of mCherry-Ki67 (RASA) recorded in live cells. After drawing the regression line, the value of staining intensity corresponding to endogenous Ki67 was determined by extrapolation and used for normalization. Depending on the normalized regression line, the correlation between the fluorescence intensity of mCherry-Ki67 in live cells and its amount relative to endogenous Ki67 was estimated.
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Ki67 Is Involved in Ensuring the Appropriate Behavior of PP1γ in Anaphase—To explore the physiological significance of the interaction between Ki67 and PP1γ, we first examined the possible contribution of Ki67 on the behavior of PP1γ by utilizing HeLa cells stably expressing YFP-PP1γ (YG cells) (26). Although a good population of YFP-PP1γ is found in the cytoplasm throughout mitosis, a certain population localizes at kinetochores in metaphase (not seen clearly in the resolution obtained here), and an accumulation to the chromosomal regions occurs at the transition from early to late anaphase (Fig. 2A) (26). Upon depletion of Ki67 with two different siRNAs (si31 or si32), the characteristic accumulation of YFP-PP1γ on chromosomes in late anaphase was weakened in common (Fig. 2B and not shown). We also examined the localization of YFP-PP1γ in cells depleted of Repo-MAN (Fig. 2C), whose contribution to the event has been previously reported (28). Quantitative evaluation of the images illustrated that Ki67 does contribute to the accumulation of PP1γ on anaphase chromosomes as Repo-MAN (Fig. 2D). The contribution of Ki67 was specific because the weakened accumulation of PP1γ caused by si31 transfection was rescued by mCherry-Ki67 resistant to the siRNA (Fig. 3, A and C). The rescue was not observed with mCherry-Ki67 (RASA) (Fig. 3, B and C), indicating that Ki67 recruits PP1γ via its RVXF motif.

Apart from these observations, we noticed that YFP-PP1γ became accumulated on metaphase chromosomes upon increasing the amount of mCherry-Ki67. In the case shown in Fig. 3D, the expression of mCheerry-Ki67 as ~2.7 times more as endogenous Ki67 caused clear accumulation of YFP-PP1γ on metaphase chromosomes. This ectopic localization of YFP-PP1γ was not caused by equivalent or even higher amounts of mCherry-Ki67 (RASA) (Fig. 3D). Ki67 clearly has an activity to regulate the localization of PP1γ in vivo via its RVXF motif, but the activity seems to be repressed in metaphase by unknown mechanism. The possibility that Ki67 might regulate the kinetochores localization of PP1γ in metaphase was not addressed in this study.

Characterization of Ki67 Phosphorylation in the Ki67-repeat Domain—We generated antibodies against phosphorylated Ki67 to be used as a tool to help identify a function of the observed interaction between Ki67 and PP1γ. Among many identified phosphorylation sites in Ki67 (19–22), we focused on the sites within the Ki67-repeat domain. The Ki67-repeat domain is composed of 16 moderately conserved repeats (Fig. 4A). Each repeat is comprised of ~130 amino acids, including a highly conserved 20-amino acid tract that will be hereafter referred to as CKRD for the “core of Ki67-repeat domain” (Fig. 4B). The CKRD is assumed to be central in the domain because it appears repeatedly without any intervening residues in marsupial chmadrin (AA78329.1) and amphibian Ki67 (AA121193.1). The threonines residues in the middle of CKRD, immediately followed by a proline, are consensus phosphorylation sites for mitotic kinases such as CDK and MAPK. After confirming through existing mass spectrometry data that these residues could be actually phosphorylated in cells (19–22), we raised antibodies against the synthetic phosphopeptide C-KRRPQ(pT)PKEKA, a peptide whose amino acid tract is similar to the consensus of CKRD. The amino acid sequences that are identical or 91% identical to the peptide occur once or three times, respectively, within the domain (Fig. 4B).

The antibodies were affinity purified and used for immunofluorescence of HeLa cells (Fig. 4C). Cells in prometaphase and metaphase were brightly stained with a perichromosomal staining pattern similar to those obtained with pan-Ki67 antibodies. The staining intensity declined rapidly during the progression of anaphase and almost disappeared in interphase. Prophase cells containing condensing chromosomes were only weakly stained. The antibodies seemed to recognize a subset of
Ki67 existing dominantly in prometaphase and metaphase. Essentially the same observations were obtained in hTERT-RPE1 cells, which are normal retinal pigmented epithelial cells immortalized by telomerase (Fig. 4D). The perichromosomal staining observed in HeLa cells was lost upon Ki67 depletion by siRNA (Fig. 5A) or upon treatment of cells with RO-3306 (Fig. 5B), an inhibitor of Cdk1 kinase. Immunoblotting using the same antibodies detected mitosis-specific protein bands that were sensitive to both Ki67-siRNA and RO-3306 (Figs. 5C and 1C, respectively). The bands were sensitive also to Purvalanol A, another inhibitor of Cdk1 kinase.4 Taken together, we concluded that the antibodies indeed recognized a subset of Ki67 that was phosphorylated by Cdk1 during mitosis.

The antibodies generated in this study allowed for the analysis of the extent and timings of Ki67 phosphorylation and dephosphorylation in the CKRD. The timing of these events was largely similar to the same events on histone H3 at Ser-10 when judged by co-staining by immunofluorescence (Fig. 5D), as well as similar events in a broad range of other mitotic phosphoproteins reported so far (36).

PP1γ-Ki67 Is Responsible for the Timely Dephosphorylation of Ki67 Itself in Anaphase—Because Ki67 dephosphorylation (Figs. 4, C and D, and 5D) and accumulation of PP1γ on chromosomes (Fig. 2A) apparently occur at a similar time during mitotic progression, we examined the possibility that Ki67 itself might be the substrate of PP1γ-Ki67. To this end, we generated an experimental setting where endogenous Ki67 was replaced with mCherry-Ki67 deficient in PP1γ association (RASA mutant), used a wild-type (RVSF) version as a control (experimental schemes diagrammed in Fig. 6, A or E), and monitored the Ki67 dephosphorylation profile in metaphase or anaphase cells with antibodies against phosphorylated Ki67 as described above. In metaphase cells, mCherry-tagged Ki67 proteins, both wild type and RASA mutant forms, targeted the chromosome periphery, similar to endogenous Ki67 (Fig. 6B). In cells arrested in metaphase by sequential treatments with RO-3306

![FIGURE 3. Ki67 modulates the behavior of PP1γ via its RVXF motif. A and B, HeLa cells stably expressing YFP-PP1γ, in which endogenous Ki67 had been replaced with mCherry-Ki67 (WT) (A) or mCherry-Ki67 (RASA) (B) as described under “Experimental Procedures,” were filmed over time from metaphase to telophase with 3-min intervals. C, quantitative analysis of YFP-PP1γ accumulation on anaphase chromosomes as described in the legend for Fig. 2D. D, overexpression of wild-type mCherry-Ki67 (WT), but not mCherry-Ki67 (RASA), caused the ectopic localization of YFP-PP1γ on metaphase chromosomes. The estimated expression levels of mCherry-Ki67 (WT or RASA) relative to endogenous Ki67 were written at the upper left. Bars, 15 μm.](http://jbc.asm.org/content/jbc/289/33/22882.full)
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and the proteasome inhibitor MG-132 (see the “Experimental Procedures”), the amount of phosphorylated populations of Ki67 on chromosomes, as determined by fluorescent staining signal, increased in correlation with the expression levels of ectopically expressed Ki67, irrespective of the wild type or RASA mutant (Fig. 6, B and C). Even in conditions where mCherry-Ki67 (wild-type) was overexpressed at levels where endogenous PP1γ is recruited ectopically on metaphase chromosomes, as in Fig. 3D, mCherry-Ki67 remained phosphorylated,4 indicating that recruitment of PP1γ through its interaction with Ki67 on chromosomes was not sufficient for its functioning as a Ki67 phosphatase. The activation of PP1γ that is normally observed at the onset of anaphase (25) should also be necessary.

In anaphase cells, by contrast, we observed a striking difference in transition profiles of the phosphorylation status between mCherry-Ki67 (wild-type) and mCherry-Ki67 (RASA); the former seemed to be dephosphorylated similar to endogenous Ki67 upon anaphase onset, whereas the latter tended to remain phosphorylated even in later stages of anaphase (Fig. 6, B). The phosphorylation of Ki67 mediated by PP1γ is recruited on chromosomes via its interaction with Ki67. Of note, the phosphorylation of histone H3 at Ser-10 was properly lost in most cases in the same sets of cells examined (Fig. 6f), thereby suggesting that dephosphorylation of Ki67 and Ser-10 in histone H3 occur by distinct mechanisms.

To identify a functional significance of the timely dephosphorylation of Ki67 mediated by PP1γ-Ki67 in anaphase, we examined the appearance of anaphase cells in which endogenous Ki67 had been depleted (Fig. 2B) or replaced with mCherry-Ki67 (RASA) (Fig. 3B). Although the chromosomal targeting of YFP-PP1γ in anaphase was disrupted in these settings, we did not necessarily detect a significant abnormality in the global behavior of segregating chromosomes (Figs. 2B and 3B) or in the following reformation of nuclei.4

DISCUSSION

In this study, we demonstrated that Ki67 interacts with PP1γ both in vitro and in vivo and directs the localization of PP1γ on anaphase chromosomes. However, Ki67 is not required for the localization of PP1γ in interphase nuclei,4 for which other targeting factors such as NOM1 and RRP1B have been identified (37, 38). The contribution of another PP1-binding protein, Repo-MAN, for the anaphase localization of PP1γ has been already reported (28). Interestingly, Ki67 and Repo-MAN
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**FIGURE 5. Specificity of phospho-Ki67 antibodies.** A, evaluation of the specificity of immunofluorescence signal obtained with phospho-Ki67 antibodies. Left, the relative intensities of immunofluorescence signal in metaphase HeLa cells 48 h after transfection with control (siGL2) or Ki67-specific (siKi67) siRNAs were measured. Chromosomal signal was reduced to 22% upon depletion of Ki67 with siKi67 (si31), thereby indicating that most of this signal was derived truly from the Ki67 protein. However, the cytoplasmic signal was reduced only to 54% upon depletion of Ki67, suggesting that at least part of the signal was derived from unrelated proteins that were unwantly recognized by the antibodies. The proteins marked with a single asterisk in the following immunoblot (C) are candidates for such proteins, as these bands did not disappear upon depletion of Ki67. Error bars indicate S.E. (n = 8). Right, similar results were obtained using another siRNA specific to Ki67 (si32). B, CDK activity is necessary for maintaining phosphorylation of the CKRD. The staining of phospho-Ki67 was resistant to treatment with nocodazole (+ noc), but sensitive to subsequent treatment with the CDK inhibitor RO-3306 (+ RO-3306). C, evaluation of the specificity of phospho-Ki67 antibodies by immunoblotting. HeLa cells were transfected with control siRNA (siGL2) or two different siRNAs against Ki67 (si31 or si32) for 48 h. For the last 16 h, cells were mock-treated (A; asynchronous) or treated with nocodazole (M; mitotic). Cells were collected and treated with a buffer containing benzonase nuclease and divided in supernatants (S) and precipitates (P) by centrifugation. All fractions were subjected to immunoblot analyses using phospho-Ki67 antibodies and antibodies against the other indicated proteins. Note that the phospho-Ki67 signal, with the exception of these signals marked with asterisks, was mitosis-specific and sensitive to Ki67 knockdown. The identities of bands marked with asterisks were not clarified. D, immunofluorescence of HeLa cells with phospho-Ki67 antibodies and anti-phospho-histone H3 (Ser-10) antibody. DNA was counterstained with Hoechst 33342. Bars, 15 μm.

shows striking sequence similarity in the region surrounding the PP1 binding motif (69% identity and 78% similarity in 32 amino acids, Fig. 1A), suggesting that the interaction of these two proteins with PP1γ might be regulated by similar mechanisms. As for the interaction of Repo-MAN with PP1γ, this interaction was shown to be negatively regulated by phosphorylation at Thr-412, a threonine residue that is 17 amino acids downstream of the PP1 binding motif (32) and corresponds to Thr-165 in human Ki67 (Fig. 1A). Additionally, this phosphorylation appears to be mediated by CDK. As naturally expected from these observations, the interaction between Repo-MAN and PP1γ occurs preferentially in anaphase when CDK activity declines (32). However, whether the interaction between Ki67 and PP1γ is negatively regulated by the phosphorylation of Ki67 at Thr-165 by CDK has not been determined. Our immunoprecipitation experiment instead demonstrated that this interaction occurred even in metaphase and was not stimulated upon inhibition of CDK activity (Fig. 1C).

To identify the relevance of recruiting PP1γ on anaphase chromosomes through its interaction with Ki67 (PP1γ-Ki67), it is necessary to first identify the specific substrates that need to be dephosphorylated. Taking advantage of newly raised phosphopeptide antibodies and a Ki67 mutant deficient in PP1γ-binding ability, we revealed that the CKRDs of Ki67 itself contain the substrate residues (Fig. 6). Following this finding, we worked to reveal the relevance of Ki67 phosphorylation within the CKRDs during mitosis using Ki67 mutants mutated in the predicted phosphorylation sites. To date we have not observed
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FIGURE 6. Timely dephosphorylation of Ki67 in anaphase depends on its interaction with PP1γ. A–D, Ki67 is phosphorylated in metaphase irrespective of its ability to interact with PP1γ. A, experimental scheme. Endogenous Ki67 was replaced with mCherry-Ki67 (WT or RASA mutant), and the cells were synchronized in metaphase (see details under “Experimental Procedures”), fixed with formaldehyde, and processed for immunofluorescence.

any marked abnormalities in the cellular behaviors of those particular mutants.4 In addition to the phosphorylation sites in the CKRD tracts, there are many sites within or outside of the Ki67 repeat domain that might also be subjected to dephosphorylation by PP1γ. These sites may possibly be more relevant in determining the behavior of Ki67. The sites within the C-terminal region of Ki67 (the LR domain) are especially worth scrutinizing as this domain plays a primary role in the chromosome targeting of Ki67 (14, 31) likely through its DNA-binding activity (18). Furthermore, we have shown that the LR domain possesses the activity to change the higher order chromatin structure (11, 14). It is intriguing to examine whether this activity is utilized for the assembly of mitotic chromosomes. Given that the activity of the LR domain was inactivated by PP1γ, Ki67 may be a candidate for “RCA” (regulator of chromosome architecture), a yet unidentified factor that cooperates with the condensin complex to preserve the mitotic chromosome architecture until metaphase and is inhibited by PP1γ at anaphase onset (29). To test this hypothesis, it is important to further dissect the biochemical activity of the LR domain and its possible regulation through phosphorylation.

We tried to identify any defects in the progression of anaphase in cells from which endogenous Ki67 was replaced with its mutant deficient in PP1γ-binding ability (RASA mutant) to begin to discover the potential substrates of Ki67–PP1γ. Although the accumulation of PP1γ on anaphase chromosomes was weakened in the setting, the global behavior of chromosomes was not always affected. Neither the nuclear pore complex assembly nor the formation of daughter nuclei was apparently affected.4 These rather mild phenotypes are consistent with those observed in cells from which Ki67 was knocked down (16). One plausible explanation is that a subset of PP1γ was recruited on chromosomes independently on Ki67 and functioned redundantly with Ki67–PP1γ. Whether RepoMAN–PP1γ is sufficient for such a redundancy remains elusive. Considering that clear homologues of Ki67 exist only in verte-
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brates, it might be reasonable to speculate that cells possess molecules that are functionally redundant with Ki67. Actually, the activity of Ki67 in regulating the spindle dynamics via modulating the localization of Hk2p, a kinesin-like motor protein, is dispensable in cells with full activity of Eg5, another kinesin-like motor protein (16). We presume that Ki67 might contribute to the quality of mitosis as a hidden "safeguard" whose significance might be concealed even in Ki67-depleted cells until sensitized by depleting/inactivating the redundant molecules.

At the periphery of each mitotic chromosome there is a specialized chromosome domain referred to as the perichromosomal layer (39, 40). This layer is visible by electron microscopy (41) and its constituents, including Ki67, can be isolated together with the chromosomes (39). The particular function of this layer in the progression of mitosis has been discussed (39, 40). This layer might be to provide a "platform" for certain molecules/activities to accumulate and that Ki67 might be dispensable in cells with full activity of Eg5, another kinesin-like motor protein (16). We presume that Ki67 might contribute to the timely accumulation of PP1γ on anaphase chromosomes. As an extension of this finding, we speculate that one general function of the perichromosomal layer might be to provide a "platform" for certain molecules/activities to accumulate and that Ki67 might be an important constituent of this platform. As an exemplification, we have already identified another enzyme, an acetyltransferase, which targets to mitotic chromosomes and is dependent on Ki67 for this targeting.4 To date, many proteins have been reported to localize to the surface of mitotic chromosomes (39, 40, 42). It will be interesting to examine which of these proteins and their biological activities are localized in a manner dependent on Ki67. This knowledge would possibly deepen our understanding of the metaphase-anaphase transition in the cell cycle and also on the fine-tuning of events occurring around chromosomes in anaphase and/or telophase, such as nuclear envelope/nuclear pore complex assembly and chromatin decondensation.

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