Human Tim/Timeless-interacting Protein, Tipin, Is Required for Efficient Progression of S Phase and DNA Replication Checkpoint*

Received for publication, June 12, 2006, and in revised form, November 13, 2006 · Published, JBC Papers in Press, November 13, 2006, DOI 10.1074/jbc.M605596200

Naoko Yoshizawa-Sugata and Hisao Masai

From the Genome Dynamics Project, Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan

Tipin was originally isolated as a protein interacting with Timeless/Tim1/Tim (Tim), which is known to be involved in both circadian rhythm and cell cycle checkpoint regulation. The endogenous Tim and Tipin proteins in human cells, interacting through the N-terminal segment of each molecule, form a complex throughout the cell cycle. Tipin and Tim are expressed in the interphase nuclei mostly at constant levels during the cell cycle, and small fractions are recovered in the chromatin-enriched fractions during S phase. Depletion of endogenous Tipin results in reduced growth rate, and this may be due in part to inefficient progression of S phase and DNA synthesis. Knockdown of Tipin induces radioresistant DNA synthesis and inhibits phosphorylation of Chk1 kinase caused by replication stress, as was observed with that of Tim. Knockdown of Tipin or Tim results in reduced protein level and relocation to the cytoplasm of the respective binding partner, suggesting that the complex formation may be required for stabilization and nuclear accumulation of both proteins. Furthermore, both Tipin and Tim may facilitate the accumulation of Claspin in the nuclei under replication stress, whereas nuclear localization of Tipin and Tim is unaffected by Claspin. Our results indicate that mammalian Tipin is a checkpoint mediator that cooperates with Tim and may regulate the nuclear relocation of Claspin in response to replication checkpoint.

During the course of DNA replication, the genome may be highly susceptible to damages derived from environmental or naturally occurring hazards (1–4). Cell cycle checkpoint is a safeguard system against these threats to maintain genomic integrity. In eukaryotic cells, inhibition of DNA replication by depletion of the cellular nucleotide pool, or by certain forms of DNA damage, leads to activation of the replication checkpoint mediated mainly by ataxia telangiectasia-mutated and Rad3-related kinase ATR7 and its downstream molecules (5). The key function of this signaling pathway is to stabilize the arrested replication forks and to transduce the downstream signals required for the preservation of the forks until the damage is removed. One of the most important targets of ATR is the checkpoint effector kinase, Chk1 (6), which is required for activation of various effector functions, including fork preservation and suspension of further S phase progression (7–9).

Recent studies revealed a group of proteins that affect ATR-dependent Chk1 activation. Budding yeast Tof1 was reported to be required for stable arrest of replication forks in the presence of hydroxyurea (HU) and is required for activation of Rad53 (10–12). Fission yeast Swi1, which is required for replication fork-pausing in mating type switching and is a functional homolog of Tof1 (13), was reported to be essential for full activation of Cds1 and stabilization of replication forks during S phase (14–16). In mammals, Timeless/Tim1/Tim (Tim), originally identified as an ortholog of a fruit fly circadian clock gene (17–19), share structural similarity to Swi1/Tof1. Recently, human Tim was reported to be a mediator of replication and intra-S phase checkpoint (20).

Claspin has been isolated as a Chk1-interacting protein that is essential for phosphorylation of Chk1 by ATR during replication and DNA damage responses in *Xenopus* egg extracts (21). The yeast ortholog of Claspin, Mrc1, is also required for activation of Rad53/Cds1 effector kinases under replication stress (22, 23). In addition, budding yeast Mrc1 was shown to be required for stable arrest of replication forks and was implicated as a component of a fork pausing complex together with Tof1 protein (11, 24).

Fission yeast Swi3 was first isolated as a gene required for mating type switching and was later shown to be essential for replication pausing at the *mat1* locus (25). Swi3 forms a complex with Swi1 (26, 27), and the genetic analyses suggested that Swi3 and Swi1 may function in the same epistasis for the replication checkpoint responses (15, 27). Swi3 as well as Swi1 is required for stalling replication forks not only at *mat1* locus (13) but also at rDNA repeats (28). Its budding yeast counterpart, Csm3, was originally identified as a factor required for proper chromosome segregation (29) and was shown to be involved in sister chromatid cohesion and to bind to Tof1 (30). Csm3 was reported to belong to the same epistasis group with Tof1 and histone H2A serine 129 in response to DNA damages

* This work is supported by grants-in-aid for scientific research “A” and scientific research “C” (to N. Y.) from the Ministry of Education, Science, Sports and Culture, and the Astellas Foundation for Research on Metabolic Disorders (to H. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
1 To whom correspondence should be addressed: Genome Dynamics Project, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Hon-komagome, Bunkyo-ku, Tokyo 113-8613, Japan. Tel.: 81-3-5685-2264; Fax: 81-3-5685-2932; E-mail: hmasai@rinshoken.or.jp.
2 The abbreviations used are: ATR, ataxia-telangiectasia-mutated and Rad3-related kinase ATR; Csm3, minichromosome maintenance; siRNA, small interfering RNA; PCNA, proliferating cell nuclear antigen; PBS, phosphate-buffered saline; DAPI, 4,6-diamidino-2-phenylindole; BrdUrd, bromodeoxyuridine; RDS, radioresistant synthesis; PIPES, 1,4-piperazineethanesulfonic acid; FACS, fluorescence-activated cell sorter.
Tipin Is Essential for Replication Checkpoint

(31). Recently, it was proposed that Csm3, Tof1, and Mrcl are components of a replisome progression complex together with other fork factors, including MCM, Cdc45, and GINS (32). In mammalian cells, Tipin, which was identified as a Tim-interacting protein (33), is regarded as an ortholog of Swi3/Csm3, but its molecular function has not been precisely determined.

In this study, we analyzed the functions and molecular dynamics of human Tipin protein during cell cycle progression and replication checkpoint responses. Our results show that Tipin is essential for replication checkpoint responses and that the Tipin-Tim complex may be required for proper nuclear accumulation of the Claspin molecule.

EXPERIMENTAL PROCEDURES

Cell Culture and Cell Cycle Synchronization—HeLa cells and U2OS cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. For synchronization, cells were arrested at the G1/S boundary by incubation in the presence of 2.5 mM thymidine for 14–16 h twice with a 9-h interval of growth without the drug. Otherwise, cells were arrested at mitosis by 50 ng of nocodazole per ml for 10 h after an interval of growth without the drug. For post-extraction procedure, PBS-washed cells were fixed with 3.5% paraformaldehyde/PBS for 20 min at room temperature, washed three times with PBS for 10 min, and lysed with PHEM containing 0.5% Triton X-100 for 1 min. The cells were fixed with methanol at −20°C for 5 min, immediately dried, and then washed with phosphate-buffered saline (PBS).

—Transfection of small interfering RNA (siRNA) duplexes was conducted by using Oligofectamine (Invitrogen). All siRNA oligonucleotides were purchased from Japan Bio Services Co. Ltd. (Saitama, Japan). The sense strands of the three different siRNAs for Tipin are as follows: Tipin1, 5’-AGGUCAUGAGGCUGAGAgdTdT-3’; Tipin2, 5’-GAUGGUGAAGGACUGCGCdTdT-3’; and Tipin3, 5’-AAGGAGGAGUCAAUAGAUAgdTdT-3’. siRNA for Claspin is CCUCUGCUUGAGGACUGGdTdT-3’. The sequences of control siRNA and siRNA for Cdc7 were reported previously (34).

Absence of Tim (anti-Tim) antibody (anti-Tim) was by immunizing two animals with glutathione S-transferase-tagged full-length human Tipin, and rabbit anti-Tim antibody (anti-Tim-C) antibody was generated by immunizing two animals with the C-terminal half (603–1208 amino acids) of the human Tim. Rat anti-Tim antibody (anti-Tim-N) was also generated by immunization of two Lewis/CrlCrj rats (donated by Charles River Breeding Laboratories, Japan) with the N-terminal half (1–573 amino acids) of human Tim. All antibodies were affinity-purified using HiTrap H+-hydroxysuccinimide-activated HP (GE Healthcare). Antibodies against human Cdc7, anti-huCdc7-1, were described previously (35). Rabbit anti-Cdt1 antibody was a gift from Katsuyuki Tanai (MBL). Antibodies from commercial sources were as follows: Chk1, Chk2, PCNA, and lamin B (Santa Cruz Biotechnology); α-tubulin and FLAG M2 (Sigma); Chk1 S317P, Chk1 S345P, and Chk2 T68P (Cell Signaling Technology); Claspin (Bethyl Laboratories); and Myc (clone 4A6, Upstate, or A-14, Santa Cruz Biotechnology).

DNA Constructions—Human Tim and human Tipin cDNA were subcloned into pME18S-FLAG and pME18S-myc vectors, respectively, generating FLAG-tagged Tim and Myc-tagged Tipin. PCR-amplified fragments encoding deletion derivatives of human Tipin were subcloned into pME18S-myc as shown in Fig. 3B. The constructs 1 and 1+2 of Tim were generated by deleting the C-terminal segments from pME18S-FLAG-Tim. The constructs 2, 4, and 5 of Tim were generated on FLAG-pcDNA3.1, and construct 3 of Tim was generated on pME18S-FLAG by subcloning the corresponding restriction fragments.

Immunofluorescence Staining—Preparation of cells for immunofluorescence staining was performed as described previously (36) with some modification. Briefly, for pre-extraction procedure, HeLa cells grown on coverslips were washed twice with PHEM buffer (60 mM PIPES, 25 mM HEPES-KOH, pH 6.9, 10 mM EGTA, and 2 mM MgCl2) at room temperature and lysed with PHEM containing 0.5% Triton X-100 for 1 min. The cells were fixed with methanol at −20°C for 5 min, immediately dried, and then washed with phosphate-buffered saline (PBS). For post-extraction procedure, PBS-washed cells were fixed with 3.5% paraformaldehyde/PBS for 20 min at room temperature, washed three times with PBS for 10 min, and lysed with 0.5% Triton X-100 for 5 min. For immunostaining, the coverslips were blocked with 3% bovine serum albumin for 30 min and incubated with primary antibodies in AB buffer (0.1 M PIPES-KOH, pH 7.2, 1 mM MgSO4, 1 mM EGTA, 1.83% L-lysine, 1% bovine serum albumin, and 0.1% NaN3) for 1 h at 37°C. After washing three times with PBS for 3 min, the cells were incubated with secondary antibodies (Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch); Alexa 488-conjugated anti-rabbit antibody (Molecular Probes); and 1 μg of DAPI per ml) for 45 min at 37°C. The coverslips were then washed three times with PBS for 3 min and mounted with PBS containing 90% glycerol, 2.5% 1,4-diazabicyclo[2.2.2]octane (Sigma). Samples were observed under a fluorescence microscope (Axiohot, Carl Zeiss) equipped with a Hamamatsu ORCA-ER CCD camera. Images were processed with software Aquacosmos (Hamamatsu Photonics).

Cell Fractionation—Cells were fractionated into Triton X-100-soluble and Triton-insoluble fractions by the procedure described previously (34). The whole cell extracts were prepared by resuspending the cells in SDS-PAGE sample buffer.

Immunoprecipitation—Cells were extracted with lysis buffer (20 mM HEPES-NaOH, pH 7.4, 1 mM EDTA, 0.1 mM EGTA, 2 mM MgCl2, 150 mM NaCl, 1 mM Na2VO4, 20 mM NaF, 5% glycerol, 1% Nonidet P-40, 1 μg/ml pepstatin A, 1.5 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.4 μM Pefabloc SC). After rotation for 30 min at 4°C, the lysate was centrifuged for 20 min at 15,000 × g, and the supernatant was recovered (Nonidet P-40 extracts). Before immunoprecipitation, the extracts were cleared by incubation with protein A- and protein G-Sepharose and centrifuged for 3 min at 11,000 × g. The cleared extracts were used for immunoprecipitation with antibodies as indicated.

DNA Content and Incorporation of BrdU—Cells were incubated with bromodeoxyuridine (BrdU) and analyzed by fluorescence-activated cell sorter (FACS) as described previously (34).

Radioresistant DNA Synthesis (RDS) Assay—RDS after ultraviolet (UV) light irradiation was assayed as reported previously (37) with some modifications. Briefly, 6 × 105 HeLa cells or 3 × 105 U2OS cells in a 6-cm dish were transfected with siRNA and incubated in medium containing 10 nCi of [14C]thymidine per ml (GE Healthcare), and incubation was continued for 40 h.
The cells were then divided into two dishes containing nonisotopic medium and cultured for 2 h. One dish was irradiated with UVC (2 or 5 J/m²) and the other was mock-treated. After 1 h, 10 μCi of [3H]thymidine (GE Healthcare) per ml was added, and incubation was continued for 1.5 h. Finally, cells washed two times with PBS and harvested by scraping into a solution containing 0.1 M NaCl, 10 mM EDTA were lysed by 1 M NaOH, 20 mM EDTA, pH 8.0, and high molecular weight DNA was precipitated by addition of 10% trichloroacetic acid. Acid-insoluble DNA was trapped on a GF/C glass filter, and radioactivity was measured by scintillation counter.

RESULTS

Tipin Is a Nuclear Protein and Associates with Chromatin during S Phase—Tipin, originally isolated as a Tim (an ortholog of yeast Tos1/Swi1)-binding protein, is a likely mammalian ortholog of budding yeast Csm3 and fission yeast Swi3, which may be involved in replication checkpoint pathway (10, 16). To investigate the roles of Tipin in replication checkpoint regulation in mammalian cells, we have generated specific antibodies against human Tipin and Tim proteins. All rabbit (anti-Tipin and anti-Tim-C) and rat (anti-Tim-N) polyclonal antibodies (see “Experimental Procedures”) raised in this study have been successfully used to detect endogenous human Tipin and Tim proteins by immunoblot analysis (Fig. 1A). Among these antibodies, the first determined the expression levels of Tipin and Tipin proteins during the cell cycle. HeLa cells were synchronously released into the cell cycle from arrest at the G1/S boundary by double thymidine block or at mitosis by nocodazole block. As shown in Fig. 1A, lanes 1–4, Tipin proteins by immunoblot analysis (Fig. 1A). Using these antibodies, we first determined the expression levels of Tipin and Tim proteins during the cell cycle. HeLa cells were synchronously released into the cell cycle from arrest at the G1/S boundary by double thymidine block or at mitosis by nocodazole block. As shown in Fig. 1B, the Claspin protein level dramatically decreased in S phase and increased in M to G1 phase, as observed previously (23, 42). Tim protein is expressed at a constant level during the cell cycle, and no mobility shift was observed. It was reported previously that Tim expression was high in S, G2, and M and low in G0 and G1 (20). This discrepancy may be due to the method of cell cycle synchronization protocol (released from G0 after serum starvation versus release from G1/S or mitosis block), or to the different cell types used. In contrast, a mobility-shifted form of Tipin was detected at mitosis (nocodazole block), which may be caused by phosphorylation (Fig. 1B, lane 7), and its level slightly increased during S phase (Fig. 1B, lanes 2–4).

Next we analyzed cellular localization of Tipin and Tim during cell cycle progression. HeLa cells, synchronously released from G1/S arrest by double thymidine block, were fractionated into Triton X-100-soluble fraction containing cytosol and nucleoplasm and Triton-insoluble fractions enriched with chromatin-bound and nuclear matrix-bound proteins (Fig. 1C). Successful fractionation was confirmed by the exclusive presence of tubulin and lamin B proteins in Triton-soluble and Triton-insoluble fractions, respectively. Cdt1 was detected predominantly during late M to G1 phase, and PCNA was detected in Triton-insoluble fractions only during S phase, confirming the cell cycle synchronization. Detection of Cdt1 during the second S phase (Fig. 1C, lanes 10–12) is because of loss of synchrony at the second cell cycle stage after release. Tipin and Tim were detected in the Triton-soluble fractions throughout the cell cycle, but they were detected in the insoluble fractions in early to mid-S phase, coincident with PCNA protein (Fig. 1C, lanes 14–16). This result suggests that Tipin and Tim proteins may associate with chromatin only during S phase.

We next examined localization of endogenous Tipin and Tim proteins by indirect immunofluorescence microscopy. In the formaldehyde-fixed samples, both proteins were mainly detected in nuclei rather uniformly except for nucleoli (Fig. 2A). Under these conditions, the staining patterns of Tipin and Tim were quite similar. When the soluble proteins were pre-extracted by detergent prior to fixation, only a limited fraction of cells showed positive nuclear signals for Tipin with a wide variety of signal strength and staining patterns (Fig. 2B, panels a and e). Double staining of the pre-extracted cells with anti-Tipin and anti-PCNA antibodies revealed that Tipin and PCNA exhibited similar staining patterns (Fig. 2B, panels b and f). This is in agreement with the results of immunoblot analysis, showing the association of Tipin and PCNA with chromatin-enriched fractions only during S phase (Fig. 1C). Similarly, detergent-resistant signals for Tim were similar to those of PCNA (Fig. 2C). In newborn daughter cells in G1, phase (Fig. 2, B and C, cells highlighted by circles), signals were very weak for Tipin, Tim, and PCNA.

Complex Formation between Tipin and Tim—Because Tipin is a Tim-binding protein, we analyzed complex formation between Tipin and Tim. The Nonidet P-40 extracts of HeLa cells were used for immunoprecipitation with anti-Tipin antibody, and the immunoprecipitate was examined by immunoblotting with anti-Tim antibody. The full-length Tim protein was detected in the immunoprecipitate but not in that of the preimmune serum (Fig. 3A, bottom panel) (data not shown). Reciprocally, the immunoprecipitates with anti-Tim-N antibody contained Tipin (Fig. 3A, top panel). The complex formation was analyzed also in the Nonidet P-40 extracts prepared from synchronized cells (Fig. 3A, lanes 3–11). Interactions were observed throughout the cell cycle, suggesting that Tipin and Tim form a complex at all the stages of the cell cycle.

We then tried to determine the domains of Tipin and Tim required for the complex formation. We have constructed deletion mutants of FLAG-tagged Tim proteins as shown in Fig. 3B and transiently coexpressed them with Myc-tagged full-length Tipin in 293T cells. Immunoprecipitation with anti-Myc antibody coimmunoprecipitated the Tim fragments 1, 2, and 1+2 (Fig. 3C, lanes 11–13) but not the Tim fragments 3, 4, or 5 (lanes 14–16). On the other hand, in coexpression of deletion derivatives of Myc-tagged Tipin and FLAG-tagged full-length Tim, fragments a + b, a + b + c, and b but not a, c, or d of Tipin were detected in the immunoprecipitates with anti-FLAG antibody (Fig. 3D). Data shown above indicate that the amino acid residues 1–267 or 267–573 of Tim and amino acid residues 67–143 of Tipin can bind to Tipin and Tim, respectively. Both segments contain amino acid sequences conserved throughout evolution (Fig. 3D), suggesting that this interaction may be conserved.

Depletion of Tipin Inhibits Cell Growth—To explore the functions of Tipin, the protein was depleted from cells by RNA interference. Three different siRNA oligonucleotides specific for Tipin were designed and introduced into HeLa or U2OS cells, and the expression was monitored by Western blotting. Each transfected siRNA significantly reduced the level of Tipin
protein (Fig. 4A) (data not shown). To assess the effects of knockdown of Tipin on cell growth, the increase of cell numbers was monitored 24, 48, and 72 h after transfection (Fig. 4B). As we and other groups reported previously (34, 38), depletion of Cdc7 greatly inhibited cell growth. Treatment with Tipin siRNA also decreased cell growth, whereas treatment with Tim siRNA did not have a significant effect on cell growth (Fig. 4B and phase contrast images in Fig. 4D). As shown in Fig. 4D, cell death was abundantly observed in Tipin or Cdc7-siRNA-treated cells, although there were no significant changes in cellular or nuclear morphology. In contrast, viability and cell morphology were not affected in Tim-depleted cells.

FIGURE 1. Tipin and Tim are associated with chromatin enriched during S phase. A, evaluation of the antibodies developed in this study. Twenty micrograms of whole cell extracts of HeLa cells, run on 10% SDS-polyacrylamide gel, were blotted and reacted with anti-Tim-N (lanes 1 and 2), anti-Tim-C (lanes 3 and 4), or anti-Tipin (lanes 5 and 6) antibodies. Antibodies used for detection were crude antisera (C) or affinity-purified antibodies (P). B, the expression of Tipin and Tim proteins during the cell cycle. HeLa cells were arrested at the G1/S boundary by double thymidine block (Thy) or at mitosis with nocodazole block (Noc) and were synchronously released into cell cycle. The Nonidet P-40 extracts (15 μg) were prepared at each time point. DNA content histograms of each sample are also shown. AS, asynchronously growing cells. C, cell cycle oscillation of cellular localization of Tipin and Tim proteins. HeLa cells, released from the G1/S boundary as in A, were separated into Triton X-100-soluble and -insoluble fractions. B and C, each fraction was analyzed by Western blotting using specific antibodies against the proteins indicated. The Tim antibody used was anti-Tim-C.
Depletion of Tipin Slows Down S Phase Progression—The FACS analysis data above suggest that Tipin is required for normal progression of S phase. Therefore, we examined the effects of knockdown of Tipin on S phase progression. HeLa cells were synchronized at the G1/S boundary by double thymidine block after transfection of siRNA and then were released into the cell cycle (Fig. 5A). In control cells, S phase was completed by 9 h after release, and most of the cells entered mitosis or the next G1 phase by 12 h (Fig. 5B). In Tipin siRNA-treated cells, mid-to-late S transition was retarded (compare the cell populations at 6–9 h after release). In control cells at 9 or 12 h after release, S phase population was 12 or 7.3%, respectively, whereas that in Tipin-depleted cells was 29 or 18% (Fig. 5C, open symbols). In accordance with the delay in S phase, timing of the increase in G1/M population was also delayed; at 9 h, 81% of control cells were in G1/M phase and 62% in Tipin-depleted cells (Fig. 5C, closed symbols). This result is consistent with slight accumulation of mid-to-late S phase cell population in Tipin siRNA-treated asynchronously growing cells (Fig. 4C). We also measured the uptake of BrdUrd after thymidine block release in the same set of the experiments (Fig. 5, A and D). The population of BrdUrd-incorporating cells in Tipin siRNA-treated cells was slightly reduced at 3 and 6 h after release compared with the control cells, and it increased at 9 and 12 h (Fig. 5E). These results are consistent with the idea that depletion of Tipin reduces the efficiency of DNA synthesis (Fig. 4E).

The delay in S phase progression was more directly shown by chasing the BrdUrd-incorporating S phase cells (Fig. 5F). At 4 h after release from BrdUrd labeling, the population of BrdUrd-positive G1 cells was 12.5% in control cells, whereas that in Tipin-siRNA cells was 3.4%. This delayed entry into the next cell cycle may be caused by slow progression of S phase and not by inhibition of mitosis, because S phase cells are still predominant at 4 h, whereas at 8 h G1 cell population significantly increased (21.4%).

Tipin Is Essential for Replication Checkpoint—In fission yeast, Swi1 and Swi3 have been reported to be required for DNA replication stress checkpoint responses and for activation of Cds1 kinase (14, 27). Tof1 and Csm3 of budding yeast have also been known to be involved in DNA replication checkpoint responses as well as in activation of Rad53 kinase (10). In mammalian cells, Tim protein was reported to be required for checkpoint responses induced by UV light or HU treatment (20). Therefore, we next examined the role of Tipin in S phase checkpoint responses. HeLa cells were transfected with Tipin siRNA and incubated with 10 mM HU for the indicated times. In control cells, Ser-317 and Ser-345 phosphorylation of Chk1 was detected at 15 min after HU (Fig. 6A). In Tipin-depleted cells, both Ser-317 and Ser-345 signals in response to HU were significantly reduced at all the time points until 60 min. In another experiment, HeLa cells were transfected with Tipin siRNA and incubated with HU or irradiated by UVC (Fig. 6B). In Tipin-depleted cells, signals for phosphorylated Chk1 protein in response to HU or UV light were reduced (Ser-317) or almost completely lost (Ser-345), whereas phosphorylation of Thr-68 of Chk2 was not affected (Fig.

Next, we analyzed the cell cycle profile to examine whether Tipin knockdown blocks a specific stage of the cell cycle. Cells depleted of Tipin did not show any significant accumulation of cells at a particular cell cycle stage, but slight reduction in G1 population and accumulation in late S phase cells were observed (Fig. 4C). Similar effects were observed also with Tim siRNA treatment and are generally shared with the effect of Cdc7 depletion (39).

To assess the effects of knockdown on DNA synthesis, thymidine incorporation was examined in Tipin-depleted cells. The relative level of thymidine incorporation in Tipin-siRNA-treated cells was significantly reduced (55.5% that of control siRNA; Fig. 4E). A similar result was observed in BrdUrd-pulse labeling experiments (Fig. 5D, top panels) (data not shown). These results indicate that DNA synthesis is inhibited in Tipin-depleted cells.

**FIGURE 2.** Cellular localization of Tipin and Tim proteins. A, immunostaining of Tipin and Tim proteins in HeLa cells fixed with 3.5% paraformaldehyde followed by post-extraction with detergent (see “Experimental Procedures”). The antibodies used were anti-Tipin (panel a) and anti-Tim-C (panel b). DNA was costained with DAPI (panels c and d). B and C, HeLa cells pre-extracted with detergent and fixed with methanol were immunostained with anti-Tipin (B) or anti-Tim-C (C) antibody. Cells were costained with antibody against PCNA (panels b and f) and DAPI (panels d and h). Circles (panels a–d in B and panels e–h in C) indicate newborn daughter cells in G1 phase, which show very little signal for Tipin, Tim, and PCNA. Tipin and Tim are detected on PCNA-positive nuclei in most of the Triton X-100 pre-extracted cells. However, the cell indicated by an arrowhead (panels e–g in B) is positive for Tipin but is negative for PCNA.

**FIGURE 3.** Depletion of Tipin Slows Down S Phase Progression. A, the population of S phase cells was significantly reduced (55.5% that of control siRNA; Fig. 4). Next, we analyzed the cell cycle profile to examine whether Tipin knockdown blocks a specific stage of the cell cycle. Cells depleted of Tipin did not show any significant accumulation of cells at a particular cell cycle stage, but slight reduction in G1 population and accumulation in late S phase cells were observed (Fig. 4C). Similar effects were observed also with Tim siRNA treatment and are generally shared with the effect of Cdc7 depletion (39).

To assess the effects of knockdown on DNA synthesis, thymidine incorporation was examined in Tipin-depleted cells. The relative level of thymidine incorporation in Tipin-siRNA-treated cells was significantly reduced (55.5% that of control siRNA; Fig. 4E). A similar result was observed in BrdUrd-pulse labeling experiments (Fig. 5D, top panels) (data not shown). These results indicate that DNA synthesis is inhibited in Tipin-depleted cells.
Tipin Is Essential for Replication Checkpoint

6B, lanes 5 and 6). Treatment with Tim siRNA resulted in very similar effects on phosphorylation of Chk1, as reported previously (data not shown; see Ref. 20). We also found similar results in Tipin siRNA-transfected U2OS cells (data not shown).

DNA damage such as ultraviolet light or ionizing radiation temporally arrests DNA synthesis and reduces the bulk of DNA synthesis until the damage is repaired. Defects in this intra-S phase checkpoint are known to induce RDS. Depletion of Tim...

FIGURE 4. Depletion of Tipin reduces cell growth. A, knockdown of Tipin protein expression by three different siRNAs. HeLa cells were collected at 50 h after the first transfection. Twenty micrograms of the whole cell extracts were analyzed by immunoblot with anti-Tipin or anti-a-tubulin antibodies. C, 1, 2, and 3 stands for control siRNA, Tipin1, Tipin2, and Tipin3 siRNA, respectively (see "Experimental Procedures"). B, increase of cell numbers after transfection with various siRNA. Upper panel, viable cells were counted at 24, 48, or 72 h after transfection of siRNA for Tipin (open triangle), Tim (gray circle), Cdc7 (gray square), and control (open circle). Bars represent standard deviations. Lower panel, the whole cell extracts containing 20 μg of proteins from the above siRNA-treated HeLa cells (at 72 h after transfection) were analyzed by immunoblot with the antibodies indicated. C and D, cell cycle profiles (C) and phase contrast images (D) of the HeLa cells at 72 h after transfection of siRNA indicated. E, DNA synthesis is inhibited in Tipin siRNA-treated cells. HeLa cells were transfected with siRNA for control or Tipin and incubated with [3H]thymidine for 1.5 h and harvested. Thymidine incorporation was normalized by the14C incorporation. Data are expressed as percentage of control siRNA-treated cells. The average of three independent experiments is presented together with a standard deviation. B–E, Tipin1 siRNA was used for Tipin knockdown.

FIGURE 3. Complex formation between Tipin and Tim proteins. A, interaction of endogenous Tipin and Tim proteins. Nonidet P-40 extracts (100 μg) of asynchronously growing HeLa cells (AS, lane 2) or of cell cycle-synchronized HeLa cells (lanes 3–11), prepared as in Fig. 1A, were immunoprecipitated (IP) with anti-Tim-N (upper and middle panel) or anti-Tipin (lower panel) antibody, and the immunoprecipitates were analyzed by Western blotting using anti-Tipin (upper panels), anti-Tim-C (middle), or anti-TimN (lower panel) antibody. Input (lane 1) represents 15 μg of the starting extracts used for immunoprecipitation in lane 2. Blotting of Tipin in the anti-Tipin immunoprecipitates is not shown, because it overlaps with the strong signal for IgG at 50 kDa. Thy, released from double thymidine block; Noc, released from nocodazole block. B, schematic drawings of Tipin and Tim deletion derivatives used in C. Gray boxes indicate the regions with amino acid (aa) sequence similarity among various species. The three gray boxes in Tim overlap with Tim homology domains 1, 2, and 3 reported previously (17). The black vertical bars indicate predicted nuclear localization signals. The solid lines above the boxes indicate the segment on Tim and Tipin shown in this study to be sufficient for the interaction of the two proteins, respectively. C and D, delineation of Tim-Tipin interacting domains. The Myc-tagged full-length (indicated as FL) Tipin protein and FLAG-tagged Tim fragments (C) or the FLAG-tagged full-length (indicated as FL) Tim and Myc-tagged Tipin fragments (D) were coexpressed in 293T cells. At 48 h after transfection, cells were harvested, and the Nonidet P-40 extracts were prepared. The immunoprecipitates prepared with anti-Myc antibody (C) or anti-FLAG antibody (D) were analyzed by immunoblotting (IB) with the antibodies indicated. D, long exposure of the boxed segment is also presented to show the Myc-Tipin fragment α–c (middle panel), ν (lanes 8, 9, 17, and 18 of C and lanes 8 and 16 of D) indicates transfection of pcDNA3.1(−) vector as a negative control. A, C, and D, asterisks indicate the bands derived from IgG.
Depletion of Tipin and Tim delays S phase progression and reduces the rate of DNA synthesis.

A, time course of the experiment. siRNA-transfected (TF) HeLa cells were synchronized at the G1/S boundary by double thymidine block, released, and collected at 3-h intervals. BrdUrd (10 μM) was added for 30 min before the harvest except for the cells harvested at the time of release (0 h). The bottom bar indicates time after release (hrs). B, DNA content of siRNA-treated cells at the times indicated after the release. C, populations of S phase and G2/M phase cells in B were calculated by ModFit and plotted. D and E, BrdUrd incorporation of siRNA-treated cells after the release. D, x axis and y axis represent DNA contents and BrdUrd incorporation, respectively. E, populations of BrdUrd-incorporating cells (encircled in D) at each time point after release are presented. Open circle, control siRNA; closed triangle, Tipin siRNA. F, HeLa cells transfected with siRNA were cultured for 48 h and incubated with 30 μM of BrdUrd for 30 min. Cells were washed two times with PBS and then cultured in fresh medium and harvested at the indicated times. In the middle and bottom panels, large and small encircled areas represent, respectively, total BrdUrd-positive population and BrdUrd-positive G1 phase cells that completed S phase and mitosis. The numbers in % represent the fraction of the G1 cells out of the total BrdUrd-positive cells. siRNA used for Tipin knockdown was Tipin1 (B–E) or Tipin3 (F).
and Claspin was reported to promote RDS (20, 40). We next examined whether Tipin is required for suppression of RDS. We first measured thymidine incorporation at various times after UV irradiation in Tipin-siRNA-transfected cells (Fig. 6C). Compared with control siRNA cells, Tipin depletion leads to a delay in decline of thymidine incorporation (at 30 min) and to increased incorporation at later times. The relative DNA synthesis after UV irradiation did indeed increase in Tipin-depleted cells (Fig. 6D, 155% of control siRNA-treated cells), indicating that RDS is induced in the absence of Tipin. The extent of increase of RDS was comparable with that reported previously for UV light-induced RDS in cells lacking other replication and checkpoint proteins (20, 41, 42). These results show that Tipin is also required for the intra-S phase checkpoint pathway.

Nuclear Localization of Tipin and Tim Is Interdependent—Knockdown of Tipin or Tim protein by siRNA resulted in clear reduction of the staining signals of each protein (Fig. 7, A, panel b, and B, panel c). Although Tipin is present exclusively in nuclei in control siRNA-treated cells (Fig. 7A, panel a), the nuclear Tipin signals were significantly reduced, and weak cytosol Tipin signals were detected in Tipin siRNA-treated cells (Fig. 7A, panels c and d). Similarly, in Tipin siRNA-treated cells, the nuclear Tim signals substantially decreased and diffused into cytosol compared with control cells showing exclusively nuclear signals (Fig. 7B, panels b and n). The reduced nuclear signals are at least partly because of the decreased expression levels of Tim and Tipin in cells treated with siRNA for their respective partner proteins (Fig. 7D, lanes 1, 2, 5, and 6). However, it is also obvious that they leak out into cytoplasm in the absence of their partner proteins (Fig. 7, A, panel o, and B, panel n).

Thus, the stability of both proteins may increase through the complex formation, and nuclear localization of Tim and Tipin is interdependent. In contrast, Claspin depletion did not affect intensities and localization of Tipin and Tim signals (Fig. 7, A and B, panel d).

Next, we examined detergent-resistant nuclear signals of Tim protein to analyze their potential association with chromatin. As shown in Fig. 2C, Tim exhibited varied staining patterns, such as diffused signals in whole nuclei, strong and uniform nuclear staining except for nucleoli, and discrete fine nuclear foci (Fig. 2C, panels a and e, and Fig. 7C, panel a). All these signals mostly disappeared not only by knockdown of Tim itself (Fig. 7C, panel b) but also by that of Tipin (Fig. 7C, panel c). Likewise, Tim siRNA also abolished detergent-resistant Tipin signals (data not shown). These results suggest that association of Tipin and Tim with chromatin or some insoluble structures may also be interdependent with each other.

Tipin and Tim May Facilitate Nuclear Localization of Claspin in Response to Replication Stress—Claspin and yeast homolog Mrc1, a mediator protein for DNA damage and replication checkpoint (21, 43, 44), may also be involved in progres-
sion of S phase and monitoring replication fork apparatus (11, 24, 40, 43, 45, 46). On the basis of the functional similarity between Tipin-Tim and Claspin, we analyzed the interrelation among these proteins by knockdown experiments. Claspin and Mrc1 proteins accumulate and bind to replicating chromatin in S phase, and this chromatin association is further stimulated by replication stress signals such as HU or aphidicolin treatment (23, 24, 43). Consistent with this, the immunostaining signals for Claspin significantly increased after HU treatment (Fig. 7E, panel b), and they were significantly reduced by Claspin-siRNA (Fig. 7E, panel c). Interestingly, both Tipin-siRNA and Tim-siRNA treatment lead to similar reduction of Claspin staining (Fig. 7E, panels d and e). Because reduction of nuclear signals of Claspin was not obvious by Tipin or Tim siRNA in asynchronous culture (data not shown), Tim and Tipin may regulate nuclear accumulation of Claspin specifically in response to replication stresses. In addition, HU-dependent accumulation of Claspin in the detergent-insoluble fraction was significantly inhibited by depletion of Tipin protein (Fig. 7D, lane 8). Consistent with the immunostaining data, insoluble Claspin in asynchronous culture was not affected by Tipin siRNA (Fig. 7D, lane 6). Thus, Tipin knockdown inhibited the nuclear accumulation and possibly chromatin association of Claspin specifically under replication stress.

**DISCUSSION**

DNA replication forks detect replication stress and emit signals for activating downstream checkpoint responses. Replication stress such as HU or UV light induces the ATR-Chk1 pathway, which stabilizes the arrested forks to prevent their collapse and induces downstream effector actions, including suspension of cell cycle progression. Recent studies indicate that the proteins constituting the replication forks play important roles in checkpoint responses. MCM7 and Cdc6,
factors in pre-RC, have been implicated in activation of a checkpoint kinase (41, 47), and MCM2 has been shown to be phosphorylated in response to replication stress (48). Recent reports indicate that Tof1/Swi1 and Csm3/Swi3 are likely to be components of the moving replication fork complexes. The Swi1-Swi3 complex has been named fork protection complex because of its predicted roles in stabilizing the arrested replication forks (11, 27). Tipin, a Tim-binding protein, is a candidate ortholog of Csm3/Swi3 in mammals, and in this study, we have characterized its role in cell cycle progression and checkpoint responses.

Antibodies we have raised in this study can specifically recognize the endogenous Tipin and Tim proteins. Immunoblot analyses revealed that both Tipin and Tim associate with chromatin-enriched fractions in S phase but not during G2-M-G1 phases, as was observed with other replication checkpoint proteins (12, 14, 43, 49). In contrast to dynamic change of its association with insoluble structures, the expression level of total Tipin protein does not oscillate dramatically during the cell cycle, only with a slight increase during S phase. Immunofluorescence microscopy analyses at higher magnification revealed that a fraction of Tipin and Tim colocalized with PCNA especially in late S phase (Fig. 2, B and C, panel c), whereas the majority of them are only adjacent to the PCNA foci.3 Furthermore, detergent-resistant Tipin signal was detected in some nuclei where PCNA is completely absent (Fig. 2B, panels e–g, arrowhead). This may reflect a role of Tipin in functions other than replication fork regulation, such as sister chromatid cohesion as reported in yeasts (30).

Consistent with the previous report on ectopically expressed Tipin and Tim (33), endogenous Tipin and Tim coimmunoprecipitated. This interaction was detected throughout the cell cycle. We also found that recombinant Tipin and Tim proteins expressed in insect cells form a complex and that the complex could be purified (data not shown), indicating that Tipin and Tim form a stable complex both in vivo and in vitro. Deletion analyses revealed that the interaction involves amino acids 67–143 of Tipin and amino acids 1–267 or 267–673 of Tim. These segments overlap with the conserved segments of Tipin and Tim (17, 26), indicating that the Tipin-Tim complex is highly conserved through evolution.

Knockdown of Tipin in mammalian cells leads to slow growth. Cell cycle analyses showed that S phase progression is retarded in cells depleted of Tipin protein, suggesting the requirement of Tipin for efficient S phase progression. Yeast Mrc1 was shown to be required for efficient S phase progression, and mammalian Claspin was reported to regulate the cell growth positively (40, 50). The Tipin-Tim complex also may somehow facilitate the S phase progression through its interaction with Claspin and/or replication fork machinery.

Our data also showed that Tipin, like Tim, is essential for activation of Chk1 kinase in response to HU or UV light treatment. In contrast, HU- or UV-induced activation of Chk2 kinase was not affected by Tipin depletion. Furthermore, RDS was observed in Tipin siRNA-treated cells, as well as in Tim or Claspin siRNA-treated cells. Thus, Tipin, in conjunction with Tim, may be required specifically for the ATR-Chk1 pathway in the replication checkpoint induced by HU or UV light.

The results in studies with yeasts suggest that Tof1/Swi1, Csm3/Swi3, and Mrc1 proteins may function at the replication forks while interacting with each other (32). Therefore, we examined the functional interactions between Tipin, Tim, and Claspin. The total protein levels of Tipin and Tim were significantly reduced after knockdown of their respective binding partners. Immunostaining analyses revealed that knockdown of either Tipin or Tim leads to significant loss of the nuclear signals of both proteins and relocation of some of them to cytoplasm. Furthermore, detergent-resistant nuclear signals of both protein were mostly abolished by depletion of the partner proteins. These results suggest that the Tipin-Tim complex formation promotes stability and association with chromatin or other insoluble structures of both proteins. It is noteworthy that knockdown of Tipin or Tim also significantly suppressed the replication stress-induced nuclear accumulation of Claspin without affecting the total protein level of Claspin. In contrast, knockdown of Claspin has no effect on both the protein level and localization of Tipin and Tim. Thus, the Tipin-Tim complex may regulate chromatin loading of Claspin in response to replication fork stress.

Although the cellular effects of depletion of Tipin and Tim are similar, some differences have been noted. First, both immunostaining and immunoblot results suggest that Tim may have higher affinity to chromatin or insoluble structures than Tipin. The population of Tipin in the detergent-insoluble fraction is substantially limited compared with that of Tim (Fig. 1C). Detergent-resistant staining signals of Tim are brighter than those of Tipin, and they are detected in nuclei throughout the cell cycle at least at a low level. In transient transfection assays, Tipin and Tim are mainly expressed in cytosol and nuclei, respectively (50), and coexpression of both proteins results in nuclear localization of the complex (33). We therefore speculate that nuclear localization of Tipin may be directed by Tim. Indeed, Tim carries multiple putative nuclear localization signals (Fig. 3B). Second, Tipin knockdown resulted in a more severe inhibition of cell growth and lower cell viability than did Tim knockdown, although a similar extent of S phase retardation was observed. The molecular basis for the differential roles of Tipin and Tim in cell growth and survival requires more detailed analyses of their functions.

In summary, we have shown that Tipin and Tim form a stable complex throughout the cell cycle and associate with detergent-resistant replication foci during S phase. Depletion of either protein retards the S phase progression and abolishes the activation of replication checkpoint. The Tipin-Tim complex may facilitate the loading of Claspin to chromatin under replication stress. Further studies will be needed to unravel the mechanisms by which the Tipin-Tim complex and Claspin contribute to S phase progression and mediate the checkpoint responses.

Acknowledgments—We thank Chika Taniyama for conducting the experiment in Fig. 1C, Naoko Kakusho for conducting the experiment in Fig. 6A and excellent technical assistance, Jung Min Kim and Ai Ishii for initial experiments with Tipin siRNA and for useful information. We thank all the members of our laboratory for helpful discussions.

3 N. Yoshizawa-Sugata and H. Masai, unpublished data.
