Sil Phosphorylation in a Pin1 Binding Domain Affects the Duration of the Spindle Checkpoint§

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***SIL* is an immediate-early gene that is essential for embryonic development and is implicated in T-cell leukemia-associated translocations. We now show that the Sil protein is hyperphosphorylated during mitosis or in cells blocked at prometaphase by microtubule inhibitors. Cell cycle-dependent phosphorylation of Sil is required for its interaction with Pin1, a regulator of mitosis. Point mutation of the seven (S/T)P sites between amino acids 567 and 760 reduces mitotic phosphorylation of Sil, Pin1 binding, and spindle checkpoint duration. When a phosphorylation site mutant Sil is stably expressed, the duration of the spindle checkpoint is shortened in cells challenged with taxol or nocodazole, and the cells revert to a G₂-like state. This event is associated with the downregulation of the kinase activity of the Cdc2/cyclin B1 complex and the dephosphorylation of the threonine 161 on the Cdc2 subunit. Sil downregulation by plasmid-mediated RNA interference limited the ability of cells to activate the spindle checkpoint and correlated with a reduction of Cdc2/cyclin B1 activity and phosphorylation on T161 on the Cdc2 subunit. These data suggest that a critical region of Sil is required to mediate the presentation of Cdc2 activity during spindle checkpoint arrest.**

The *SIL* gene was discovered because of its involvement in a recurrent interstitial deletion associated with the development of T-cell acute lymphoblastic leukemias (1). Following the structural characterization of this gene, genetic and biochemical studies were begun to investigate Sil function. A *SIL* knockout mouse dies during embryonic development between embryonic days 8.5 and 10.5 (18). Sil null embryos 7.5 to 8.5 days old are reduced in size and display delayed development. In addition, they show failure of midline formation and randomization of cardiac looping. Detailed analysis of this phenotype has implicated Sil as a positive regulator of the sonic hedgehog pathway governing neural tube and notochord development (17). Several of these features were also observed in zebrafish Sil null embryos (11).

SIL encodes a 150-kDa protein without clear homology to other known functional protein families or motifs (2). Sequence analysis suggests that Sil is a nonglobular protein. This is a characteristic of proteins that seem to be able to diverge quickly during evolution, and it is not seen, in general, in proteins that have catalytic activity (E. Koonin, National Center for Biotechnology Information, personal communication). *SIL* is expressed in essentially all proliferating tissues: its expression is highest in fetal thymus, bone marrow, and fetal liver and minimal in adult muscle and brain (16). In tissue culture,

SIL mRNA expression is upregulated upon serum stimulation of quiescent fibroblasts. In contrast, contact inhibition, serum starvation, or proliferation arrest induced by terminal differentiation causes a decrease of *SIL* mRNA to undetectable levels (16). These observations have suggested a possible role for Sil in cellular proliferation. Consistent with such a role, *SIL* is overexpressed in epithelial cancers and correlates with the histopathologically defined mitotic index and metastasis (8). Bioinformatic analysis of gene expression data of lung cancers revealed that *SIL* is coregulated with mitotic regulatory genes (32).

Mitosis is a highly complex phase of the cell cycle in which the duplicated chromosomal complement is evenly partitioned between two daughter cells. This phase of the cell cycle is biochemically regulated by the transient activation of the Cdc2 kinase complexed with mitotic cyclins (31). Besides binding to the cyclin subunit, activation of Cdc2 requires the phosphorylation of a conserved threonine (T161 in human) located in the T loop. In addition, the kinase activity of Cdc2 is negatively regulated by phosphorylation on T14 and Y15 that constrains the complex in an inactive state during G₂ (21, 22, 30). Upon G₂/M transition, the peptidyl prolyl isomerase Pin1 (36, 42) activates the protein phosphatase Cdc25 that releases the inhibitory phosphorylation on T14 and Y15 and allows full activation of the Cdc2 complex (23). While the activation of mitotic cyclins and Cdc2 drives mitosis until metaphase, anaphase onset and mitotic exit require the downregulation of cyclin/Cdc2 complex along with other anaphase inhibitors (4, 35). One well-characterized pathway regulating mitotic exit involves the activation of the anaphase promoting complex (APC) that targets mitotic cyclin for proteasomal degradation (10, 13). Regulating cyclin/Cdc2 activity is not only crucial to cell cycle progression but also essential during spindle checkpoint activation (6, 40). In fact, defects in chromosome alignment during metaphase trigger the mitotic checkpoint, thereby

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blocking anaphase onset by preserving a high cyclin B1/Cdc2 activity and by preventing APC activation (33).

In the present paper we describe the cell cycle-dependent phosphorylation of Sil and show how this modification affects the duration of the spindle checkpoint.

MATERIALS AND METHODS

Plasmid construction and in vitro mutagenesis. Using standard PCR techniques, a Flag tag was inserted after the start codon in murine *SIL* cDNA. *SIL* deletion mutants were made by high-fidelity PCR. Point mutations of putative phosphorylation sites were inserted using a Quik Change mutagenesis kit (Stratagene) and verified by DNA sequencing. Cluster II phosphorylation sites (T574, S643, S656, S664, T686, S699, and S760) and cluster III phosphorylation sites (S985, S1006, S1028, S1086, and S1233) were mutated to alanine. All cDNAs were cloned into the BamHI/EcoRI sites of pCDNA3 (Invitrogen), pCEFL, or pCDNA4/TO (Invitrogen). GST-Sil-IIP (encoding positions 1738 to 2552 of the Sil sequence N-terminally fused to glutathione *S*-transferase [GST]) and GST-Sil-IIIP (encoding positions 3103 to 3969 of the Sil sequence N-terminally fused to GST) were constructed by PCR using wild-type murine *SIL* or *SIL* point mutated in the cluster II or III phosphorylation sites as templates. An expressed sequence tag containing human Pin1 cDNA (ATCC 555784; IMAGE clone no. 298671) was purchased from the American Type Culture Collection. A single clone was isolated and verified by double-strand sequencing. The GST-Pin1 fusion protein was made by cloning Pin1 cDNA EcoRI/NotI in pGEX 4T (Pharmacia). An N-terminally hemagglutinin (HA)-tagged Pin1 was made by PCR, verified by sequencing, and cloned into pCDNA3. The WW domain deletion mutant (amino acids [aa] 1 to 57) and the peptidyl prolyl isomerase (PPI) domain deletion mutant (aa 46 to 164) were constructed by PCR and verified by sequencing. The EcoRI/XhoI fragments were cloned in pGEX 4T in order to obtain GST-WW and GST-PPI fusion proteins. For plasmid-mediated RNA interference (RNAi), an oligonucleotide encoding nucleotides 624 to 642 of human *SIL* was synthesized and cloned into pSUPER vector (3).

GST purification and GST pull-down assay. In order to purify GST fusion proteins, *Escherichia coli* DH5 α cells transformed with GST plasmids were induced at an optical density at 600 nm of 0.6 with 0.4 mM IPTG (isopropyl- β -D-thiogalactopyranoside) for 2 h at 37°C. Cellular extracts were made by sonication and clarified at 20,000 $\times g$ for 20 min. GST fusion proteins were purified by binding to glutathione-conjugated beads (Pharmacia). For GST pull-down assays, protein lysates (100 to 200 μ g of total protein) were incubated with 5 μ M GST fusion proteins conjugated to beads. After 2 h of incubation at 4°C, beads were washed extensively with washing buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 10% glycerol, 1% Triton X-100, 100 mM NaF, 50 mM β -glycerophosphate, 1 mM dithiothreitol [DTT]), boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and analyzed by immunoblotting (34). Densitometric analysis of Western blots was performed with IMAGEJ software (W. S. Rasband, ImageJ, U.S. National Institutes of Health, Bethesda, Md.; <http://rsb.info.nih.gov/ij/>).

Cell culture and transfection. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin sulfate. HeLa cells were transfected with Lipofectamine Plus (Invitrogen) using standard protocols; for a 60-mm plate 2.5 μ g of total plasmid DNA was used. To generate stable cell lines, Flag-Sil IIP cloned into pCDNA4 TO was transfected into tTa HeLa cells (Clontech). Stable transfectants were selected with 200 μ g/ml zeocine (Invitrogen). Flag-Sil was cloned into murine stem cell virus plasmid and transfected into tTa HeLa cells. Stable clones were selected with 2 μ g/ml puromycin.

In vitro dephosphorylation. Cell lysates (400 μ g of protein content) prepared from metaphase-arrested HeLa cells were immunoprecipitated with 5 μ g of anti-Sil AP-243 antibody (16) at 4°C for 4 h. The immune complexes were washed three times with lysis buffer and incubated in protein phosphatase buffer (50 mM Tris-HCl, 0.1 mM Na₂EDTA, 5 mM DTT, and 0.01% Brij 58) supplemented with 5 U/ μ l λ phosphatase (New England Biolabs) and 2 mM MnCl₂ at 30°C for 20 min. As a control, one sample was incubated without the enzyme. For λ phosphatase inactivation, one sample was incubated with a mix of phosphatase inhibitors (4 mM Na-orthovanadate, 4 mM Na₂EDTA, 100 mM NaF, and 50 mM β -glycerophosphate). After dephosphorylation, samples were collected by centrifugation, boiled with sample buffer, and resolved on a 6% SDS-PAGE gel.

Cell cycle analysis. HeLa cells were synchronized in S-phase with a double thymidine block. In brief, exponentially growing cells were treated with 2 mM thymidine for 17 h and then were released from the arrest by being washed twice with phosphate-buffered saline. Cells were grown in fresh medium for 9 h and

then retreated with 2 mM thymidine for 16 h. Prometaphase-synchronized cells were obtained by a double thymidine block followed by a 4-h release and then were treated with nocodazole block or taxol for the indicated times. Alternatively, asynchronous cultures of HeLa cells were incubated with 100 ng/ml nocodazole or taxol, and prometaphase cells were purified by mitotic shake-off. Cell cycle distribution was determined by propidium iodide staining and fluorescence-activated cell sorting (FACS) analysis (16).

Cdc2/cyclin B1 kinase assay. Cell lysates (80 μ g of proteins) were incubated with 1 μ g of anti-cyclin B1 antibody (H-433; Santa Cruz) and 30 μ l of protein A-conjugated agarose beads (Santa Cruz) at 4°C for 4 h. After being washed three times with lysis buffer and once with reaction buffer, the immunoprecipitated complex was collected and incubated at 25°C in 30 μ l of kinase reaction mixture containing 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 1 mM DTT, 10 μ M ATP, 5 μ Ci of [γ -³²P]ATP, and 0.5 mg/ml of histone H1 for 30 min. The reaction was terminated by the addition of 10 μ l of 4 $\times$ SDS-PAGE sample buffer and boiling for 5 min. GST-IIP and GST-IIIP (1 μ g) were phosphorylated with 1 U of recombinant Cdc2/cyclin B1 complex (New England Biolabs). For in vivo inhibition of Cdc2, prometaphase-arrested cells were incubated at 37°C for 30 min in the presence of 100 ng/ml nocodazole and 28 μ M roscovitine.

Phosphorylation of GST-Sil. GST-Sil IIP, GST-Sil IIIP, mutant GST-Sil IIP, and mutant GST-Sil IIIP on beads were added to 5 μ Ci of [³²P]ATP, 10 μ M ATP, and 20 mM MgCl₂ in EB buffer (80 mM β -glycerophosphate, pH 7.3, 20 mM EGTA, 15 mM MgCl₂, 10 mM DTT, 1 mg/ml ovalbumin, and 1 $\times$ protease inhibitors [10 μ g/ml each of leupeptin, chymostatin, and pepstatin; Chemicon, Temecula, CA]). One microliter of mitotic or interphase *Xenopus* oocyte extract (a kind gift from Ira Daar, NCI-Frederick) (50 mg/ml) was added, and after incubation for 2 h at room temperature, the reaction was terminated by adding 8 μ l of 5 $\times$ SDS sample buffer. Samples were run on 12.5% SDS-polyacrylamide gels (Criterion, Bio-Rad) and analyzed by phosphorimaging (Storm, Molecular Dynamics).

Cdk activating kinase (CAK) assay. Immunoprecipitates of Cdc2/cyclin B1 on beads were added to 30 ng of recombinant CDK7/cyclin H complexes or 5 μ l of EB buffer in the presence of 588 μ M ATP in a total volume of 10 μ l of EB buffer and incubated for 90 min at room temperature. A total of 1.5 μ Ci of [γ -³²P]ATP and 1.6 μ g of histone H1 (Roche) were added, and after incubation for 15 min at room temperature, the assay was terminated by adding 8 μ l of 5 $\times$ SDS sample buffer.

Immunofluorescence. HeLa cells were fixed with 70% ice-cold methanol and stained at room temperature with AP243 antibody using a standard protocol (16). For mitotic index determination, bright condensed nuclei of DAPI (4',6'-diamidino-2-phenylindole)-stained cells were scored as mitotic.

Antibodies and reagents. The following antibodies were used: anti-cyclin B1 (H-433), cyclin A (H-432), green fluorescent protein (GFP; FL), and HA-probe (F7) (all from Santa Cruz); MPM-2 Upstate Biotech); anti-Plk (cocktail; Zymed); anti-phospho-histone H3 (Ser10) (Upstate Biotech); anti-FLAG M2 (F1804) and anti- α -tubulin clone DM1a (both from Sigma); anti-Cdc2 (ab-1; Calbiochem); and phospho-Cdc2 (Tyr15) and phospho-Cdc2 (Thr161) (both from Cell Signaling Technology). For Cdc2 T-161 staining, a custom phospho-specific antibody was also used. Nocodazole, taxol, propidium iodide, and thymidine were from Sigma. For Sil detection, the affinity-purified AP243 anti-Sil antibody was used (16). This antibody was raised against a peptide located at the C terminus of the Sil protein, which does not contain any putative phosphorylation site.

RESULTS

Sil is phosphorylated in mitosis and following spindle checkpoint activation. The cyclical pattern of accumulation of Sil (16) is reminiscent of the behavior of several mitotic proteins, thus suggesting a possible role for Sil in mitosis. Since a number of mitotic proteins are posttranslationally regulated during G₂/M, we looked for such evidence of modification of the Sil protein. HeLa cells were synchronized by a double thymidine block-and-release protocol, and the mitotic cells were purified by shake-off. Western blot analysis showed that mitotic Sil migrated as a slower band compared to interphase Sil, suggesting a mitosis-specific posttranslational modification (see Fig. S1 in the supplemental material). A similar observation was made when HeLa cells were synchronized in promet-

FIG. 1. Sil is phosphorylated during mitosis. (A) For Sil phosphorylation analysis, interphase (I) or mitotic (M) HeLa cell extracts were run on a 6% gel and probed with anti-Sil AP243 antibody. (B) For in vitro dephosphorylation, 200 μ g of mitotic HeLa cell extracts was immunoprecipitated with 1 μ g of Sil antibody. Immunoprecipitated beads were incubated with 0.5 μ l of λ phosphatase (λ) or with λ phosphatase and a mixture of phosphatase inhibitors (i) or were mock incubated with buffer. Pellets were analyzed by Western blotting followed by staining with anti-Sil AP243 antibody.

aphase with either nocodazole treatment (Fig. 1A) or taxol (data not shown). The slower mitotic band was restored to the interphase migration pattern when phosphatase inhibitors were omitted from the lysis buffer or when sodium fluoride and

β -glycerophosphate were replaced by sodium orthovanadate (data not shown). These data suggested that phosphorylation was responsible for the altered electrophoretic mobility. When mitotic Sil was immunopurified and dephosphorylated in vitro by incubation with lambda phosphatase, the mitotic form could be converted to the interphase form. Inclusion of phosphatase inhibitors in the dephosphorylation reaction mixture prevented this conversion (Fig. 1B). Thus, Sil appears to be phosphorylated in unperturbed mitosis and following the activation of the mitotic checkpoint.

The murine Sil sequence possesses 20 (S/T)P sites in three distinct regions that represent putative phosphorylation sites of mitotic kinases (Fig. 2A). Serine and threonine residues of (S/T)P sites contained between residues 567 and 704 (region II) were mutated to alanine (mutant Sil IIp). Serine 760, given its proximity and its conservation in Sil sequences, was also included in this set of mutations (see Fig. S2 in the supplemental material). In addition, since four out of five (S/T)P sites in region III are conserved in mouse and human Sil, these residues were mutated as well (mutant Sil IIIp). Given the high number of putative phosphorylation sites contained in regions II and III, we decided to mutate all the sites in each of the two

FIG. 2. Analysis of Sil phosphorylation sites. (A) Schematic of (S/T)P regions. The top bar represents the mouse Sil sequence with the (S/T)P sites indicated as lollipops. Circles are shaded with reference to the degree of conservation across species: in white are the residues conserved in up to 35% of the known Sil sequences, in black are sites conserved in at least 75% of the Sil sequences, and in gray are sites whose degree of conservation was between 36 to 74%. (B) Western blot analysis of the pattern of phosphorylation of Sil point mutants. HeLa cells transfected with empty vector (lane 1), Flag-Sil (lane 2), mutant Flag-Sil IIp (lane 3), mutant Flag-Sil IIIp (lane 4), or mutant Flag-Sil II-IIIp (lane 5) were synchronized in prometaphase with nocodazole. Samples were immunoprecipitated with anti-Flag antibodies and were analyzed by Western blotting using the anti-Sil AP243 antibody. (C) Recombinant GST-Sil IIp (IIp) or GST-Sil IIIp (IIIp), wild type (W) or mutated in the putative phosphorylation sites (M), was phosphorylated by *Xenopus* interphase or mitotic oocyte extracts. The arrows point to full-length GST fusions: the black arrow marks GST-Sil IIp, and the gray arrow indicates GST-Sil IIIp. (D) GST-Sil IIp or GST-Sil IIIp, wild type (W) or mutated in the putative phosphorylation sites (M), was phosphorylated using purified Cdc2/cyclin B1. As a negative control GST (G) was included. Arrows are as described for panel C. (E) In vivo inactivation of Cdc2. Prometaphase-arrested cells were treated with 28 μ M roscovitine (+) or mock treated (–) for 90'. Extracts were analyzed by Western blotting using anti-Sil AP243 antibody.

FIG. 3. Sil interacts with Pin1. (A) GST (G) or GST-Pin1 beads (P) were incubated with 250 μ g of G₁/S (I) or 100 μ g of mitotic extract (M) at 4°C for 2 h. Bound fractions were immunoblotted with anti-Sil antibody. (B) Mitotic extracts were incubated either with λ phosphatase (λ) or with λ phosphatase and a mix of phosphatase inhibitors (I) or mock incubated with buffer for 15 min at 25°C. The extracts were probed for Pin1 binding in a GST pull-down assay. As a control for dephosphorylation, 15 μ g of extracts was run on a 6% gel and immunoblotted (input). (C) Full-length Pin1 fused to GST (P), the Pin1 WW domain fused to GST (WW), or the PPI GST (PPI) were incubated with 150 μ g of mitotic extract for 2 h at 4°C. As a control, 10% of the input (i) was loaded. (D) HeLa cells were transfected with HA-tagged Pin1 and Flag-tagged Sil. Following a single thymidine block and release (6 h), cells were treated with nocodazole for 12 h. Lysates were subjected to immunoprecipitation with anti-Flag antibody. The Western blot was probed with anti-HA antibody (for Pin1 detection) and anti-Sil AP243 antibody.

clusters and proceed in the analysis of sets of mutations rather than analyze all the possible combinations of single point mutations in each individual cluster. We first evaluated the contribution of each region to the phosphorylation pattern of Sil. Mutant Sil IIp, a mutant Sil IIIp, and a mutant Sil (Sil II-IIIp) bearing a combination of the two sets of mutations were transiently expressed in HeLa cells and then immunoprecipitated from cells synchronized in prometaphase. Since phosphorylated Sil migrates as a broad band, the phosphorylation of the point mutants was roughly assessed by the width of the band resolved by SDS-PAGE (Fig. 2B). Mutants in clusters II and III each showed a reduction in the thickness of the bands (Fig. 2B, lanes 3 and 4), indicating that both regions contained true phosphorylation sites. The combination of the mutations in clusters II and III yielded a protein migrating as a narrow band (Fig. 2B, lane 5), suggesting that (S/T)P sites contained in IIp and IIIp represent many of the mitotic phosphorylation sites. To gain further evidence, interphase or mitotic extracts from *Xenopus* oocytes were assayed for their ability to phosphorylate region IIp and region IIIp individually. While interphase and mitotic extracts were equally capable of phosphorylating region IIIp, region IIp was phosphorylated only by mitotic extract (Fig. 2C). Moreover, point mutation of the (S/T)P sites contained in region IIp abolished mitotic phosphorylation (Fig. 2C). Similar results were obtained (Fig. 2D) using purified Cdc2/cyclin B1, the major kinase complex active during mitosis. Region IIp (Sil IIp) and IIIp (Sil IIIp) could be phosphor-

ylated, but point mutation of the (S/T)P sites contained within each abolished phosphorylation (Fig. 2D). In vivo treatment of prometaphase-arrested cells with roscovitine, a Cdc2 inhibitor, reduced the extent of the mitotic shift typical of phosphorylated Sil, suggesting that in vivo Cdc2 activity is required for maintaining fully phosphorylated mitotic Sil (Fig. 2E).

Sil interacts with Pin1. A subset of mitotic phosphoproteins is regulated by interaction with the peptidyl prolyl isomerase, Pin1 (34). Pin1 exerts a pleiotropic control over mitosis (26), selectively isomerizing a peptide bond shared by phosphorylated serine or threonine followed by a proline residue (39). To establish whether Sil would interact with Pin1, we employed a GST pull-down assay. Glutathione beads bound to either GST or a GST-Pin1 fusion were incubated with protein extracts prepared from HeLa cells synchronized either in G₁/S or in prometaphase. Western blot analysis of the GST-bound fraction revealed that when mitotic extracts were used, Sil was specifically pulled down by GST-Pin1 (Fig. 3A). In contrast, incubation with G₁/S (Fig. 3, I) extracts did not produce any detectable binding, suggesting that only mitotic Sil is able to interact with Pin1. The specificity of the interaction seems to depend on Sil phosphorylation. In fact, when mitotic extracts were dephosphorylated in vitro and then subjected to a GST pull-down assay, Sil-Pin1 binding was abolished (Fig. 3B). The presence of phosphatase inhibitors prevented Sil dephosphorylation, thus protecting Sil interaction with Pin1 (Fig. 3B).

Pin1 is a modular protein composed of a WW domain be-

FIG. 4. Amino acid residues 567 to 704 of Sil are required to bind Pin1. (A) Schematic representation of mouse Sil sequence and the deletion mutant used. The top bar represents the murine Sil sequence with the relative positions of the putative (S/T)P phosphorylation sites. The bars below represent the Sil deletion mutants used in the GST pull-down assay: shaded areas indicate the region comprising the (S/T)P sites; lines connecting bars mark the sequence that has been deleted. (B) GST pull-down assay of 293T cell lysates transfected with either full-length Sil or a panel of Sil deletion mutants. Lysates from nocodazole-synchronized cells were incubated with either GST (G) or GST-Pin1 beads (P). The bound material was analyzed by Western blotting; 10% of the input was loaded as a reference.

lieved to function as a protein interaction domain targeting phosphorylated proteins and a C-terminal PPI domain representing the catalytic portion of the enzyme. The WW domain is usually sufficient to mediate the interaction with target proteins (27). The WW or the PPI domains of Pin1 were fused to GST and tested for the ability to pull down Sil from mitotic extracts. The WW domain was found to be as effective as the full-length Pin1 in precipitating Sil, thus demonstrating that this domain is required for Sil binding (Fig. 3C, W). No Sil could be detected in PPI (Fig. 3C, PI) precipitates.

To verify the Sil-Pin1 interaction *in vivo*, HeLa cells were transfected with Flag-tagged Sil alone or in combination with HA-tagged Pin1. After 24 h of transfection, cells were synchronized in prometaphase with nocodazole, and protein extracts were immunoprecipitated with anti-Flag antibody. HA-Pin1 could be detected in Sil immunoprecipitates only when Sil and Pin1 were coexpressed, proving that the two proteins can exist in a complex *in vivo* (Fig. 3D).

Mapping of Pin1 binding sites on Sil. Since the Sil-Pin1 interaction is dependent on Sil phosphorylation, Sil deletion mutants that selectively lacked one or more of the three phosphorylation clusters were designed and tested for their ability to bind Pin1 in a GST pull-down assay (Fig. 4A). Deletion of region III (Sil $_{\Delta 959-1119}$) had no effect on the affinity, while partial deletion of the region II (Sil $_{\Delta 567-704}$) completely abolished Sil binding to Pin1, indicating that this region contained sites critical for the interaction. A contribution of region I could be ruled out since Sil $_{1-421}$, a truncated protein containing only cluster I, was unable to bind Pin1. In addition, Sil $_{\Delta 567-704/959-1119}$ that combined deletion of cluster II and III but retained cluster I displayed negligible Pin1 binding (Fig. 4B).

This deletion mutant analysis had indicated that the Pin1

binding site on Sil mapped to region II. The Sil proteins point mutated in the putative phosphorylation sites, as previously described, were tested for the ability to bind Pin1. The GST pull-down assay showed that mutation of the cluster IIp greatly reduced the affinity of the Pin1-Sil complex (Fig. 5). Mutation in cluster III of Sil did not significantly affect the affinity for Pin1, while the combination of the point mutations of regions II and III may have affected the interaction with Pin1 more than the IIp mutation alone. This observation raises the possibility that region III may contain low-affinity secondary binding sites for Pin1, while region II may represent a prominent Pin1 binding site.

Sil phosphorylation affects the spindle checkpoint. Since Sil is phosphorylated following mitotic checkpoint activation, we tested whether mitotic checkpoint activation of cells overexpressing either wild-type Sil or Sil mutated in the putative phosphorylation sites contained in region II and region III would respond to the checkpoint activation. HeLa cells were first transiently transfected with either phosphorylation mutants of Sil or wild-type Sil and then synchronized by nocodazole treatment. The mitotic index of cells overexpressing the different Sil constructs was monitored in immunofluorescence experiments by scoring the nuclear morphology of cells stained with Sil antibody (Fig. 6A). Expression of mutant Sil IIp had the most dramatic effect. Over 17 h of treatment, only 25% of the cells expressing mutant Sil IIp synchronized in mitosis compared to 55% of vector-transfected or wild-type Sil-overexpressing cells (Fig. 6B). Mutation of cluster IIIp may have somewhat affected the efficiency of prometaphase synchronization. Combined mutation of clusters II and III was able to impair the checkpoint response although to a lesser extent than mutation of IIp (perhaps, again, a function of differential mu-

FIG. 5. Point mutations of putative phosphorylation sites of Sil and Pin1 binding HeLa cells transfected with empty vector (lane 1), Flag Sil (lane 2), Flag Sil IIp (lane 3), Flag Sil IIIp (lane 4) or Flag Sil II-IIIp (lane 5) were synchronized in prometaphase with nocodazole. Cell lysates were incubated with GST-Pin1. Samples were analyzed by Western blotting and probed with Sil AP243, anti- α -tubulin, or anti-GFP antibody. GFP was used as a cotransfection marker to show comparable efficiency of transfection in the different samples. Blots were developed at lower sensitivity in order to stain only the overexpressed proteins and reduce the staining of the endogenous Sil (lane 1). The percentage of Sil bound to GST-Pin1 (% bound) was calculated as the ratio of the intensity of the Sil band present in the GST-Pin1 pellet divided by the intensity of Sil present in the corresponding input.

tant protein stability or folding resulting in a decrease of the presumed dominant-negative activity). One possible explanation for the lower efficacy of the II-IIIp mutant compared to the Sil IIp could be that the accumulation of several point mutations might have affected the general activity/folding of this protein.

To understand the role of the phosphorylation sites contained in region II, we generated cell lines stably expressing a Flag-tagged Sil IIp phosphorylation mutant (clones 35, 58, and 66) as well as wild-type Flag-Sil (clones S2 and S9). The level of ectopic Sil expression was measured by immunoprecipitating cellular extracts using an anti-Flag antibody (Fig. 7A). In line with the observation of the transient expression experiments, cell lines expressing mutant Sil IIp (e.g., clone 58) had a limited ability to synchronize in metaphase following treatment with either nocodazole (Fig. 7B and C) or taxol (not shown), while cell lines expressing wild-type Sil (e.g., clone S2) would activate the mitotic checkpoint to an extent comparable to the parental cell line. The drop in mitotic synchronization correlated with decreased MPM2 staining displayed by the cell lines expressing mutant Sil IIp (Fig. 7A). Of note, the decreased percentage of cells capable of metaphase arrest seemed to correlate with the amount of mutant Sil IIp expressed in the cell lines (Fig. 7A and B). Despite the differences in mitotic index, all the cell lines displayed similar initial accumulation in G_2/M following a 17-h treatment with microtubule inhibitors, thus suggesting that the lower mitotic index of cells expressing mutant Sil IIp (e.g., clone 35) was not due to early cytokinesis (Fig. 7D). In addition, when the cells expressing mutant Sil IIp (clone 35) were first synchronized with nocodazole and then released from the block, they exited mitosis and accumulated with a G_1 DNA content, albeit with

FIG. 6. Transient overexpression of Sil phosphorylation mutants alters the response to nocodazole treatment. (A) Double staining of a cytospin preparation of nocodazole-arrested HeLa cells, transiently transfected with wild-type Sil (wtSil) or Sil mutated in the putative phosphorylation sites contained in region IIp (Sil IIp). Nuclei were stained with DAPI (blue), and Sil-overexpressing cells were stained in red by the anti-Sil AP243 antibody and a tetramethyl rhodamine isothiocyanate-conjugated secondary antibody. Bright-stained nuclei correspond to prometaphase arrested cells. (B) Wild-type Sil and a panel of Sil point mutated in the putative phosphorylation sites (Sil IIp, Sil IIIp, and Sil II-IIIp) were transiently transfected in HeLa cells. As a control, a group of cells transfected with empty vector was included (mock). After 24 h, cultures were treated with nocodazole for 17 h. Positively transfected cells showing bright Sil staining in immunofluorescence were scored for the mitotic index. In the histogram graph, each bar represents the mean count of at least 200 cells of four independent samples; the error bar indicates the standard deviation.

slower kinetics compared to the S2 cells (Fig. 8A and B). In the absence of spindle checkpoint activation, all the cell lines progressed into mitosis at a similar rate (see Fig. S3A in the supplemental material), thus implying that the reduced ability of the mutant Sil IIp cell lines to arrest in prometaphase could reflect a difference in the maintenance of the mitotic checkpoint rather than a general difference in cell cycle kinetics.

To study in detail the influence of the Sil phosphorylation mutant on the kinetics of the mitotic spindle checkpoint, the stable cell lines expressing Flag-Sil or mutant Flag-Sil IIp were presynchronized by a double thymidine block and then, 4 h after the G_1/S release, treated with taxol. Under these conditions cells would progress into mitosis and respond to the activation of the mitotic checkpoint in a synchronized fashion (Fig. 9A). Interestingly, the cell lines expressing either wild-type Sil or mutant Sil IIp were able to activate the mitotic checkpoint in similar ways, as evidenced by the similar rate of accumulation of prometaphase-arrested cells (assessed by nu-

FIG. 7. Stable expression of Flag-Sil and Flag-Sil Iip. Analysis of HeLa cells stably transfected with wild-type Flag-Sil (clones S2 and S9) or mutant Flag-Sil Iip (clones 35, 58, and 66). V represents a polyclonal collection of cells transfected with empty vector. Stable clones were analyzed for their ability to arrest in prometaphase following a 17-h treatment with nocodazole. (A) Lysates prepared from the different clones were analyzed by Western blotting for total Sil content, tubulin (tub) as a reference, cyclin B1 (cycB1), and MPM2 as a marker for G₂/M. The relative expression of Flag-Sil was evaluated by immunoprecipitating cell extracts with Flag-specific antibody followed by Western blot analysis using the anti-Sil AP243 antibody. (B) Mitotic index was assessed by DAPI staining of cytopsin preparations. Histograms represent the count of at least 300 cells from three independent experiments. (C) DAPI staining of a cytopsin preparation of two representative samples. (D) Cell cycle profiles of cell lines expressing wild-type Sil (S2) or mutant Sil Iip (35) or mock transfected with empty vector (V). Cells were treated for 17 h with taxol or nocodazole.

clear morphology) measured up to 10 h after the release from the G₁/S block). However, after 12 h the pattern of mitotic accumulation started to diverge: cells expressing mutant Sil Iip (clone 58) displayed a faster decline in the mitotic index compared to the Flag-Sil-expressing cell line or the vector-expressing control cell line (Fig. 9A). By 20 h, cells expressing mutant Sil had, for the most part, escaped the mitotic block. The majority of cells had reformed nuclei and regained adherence to the plate (Fig. 9B), although cells still had a 4N DNA content, thus indicating that these cells had not undergone cytokinesis (Fig. 9C). Of note, cells expressing Sil Iip displayed levels of apoptosis comparable to cells expressing wild-type Sil, thus ruling out that the decreased mitotic index observed in the cell line expressing mutant Sil could be due to increased apoptosis of prometaphase-arrested cells (see Fig. S3B in the supplemental material).

The Cdc2/cyclin B1 kinase activity mirrored the mitotic index pattern: the Flag-Sil-expressing cell line displayed accumu-

lation of active Cdc2/cyclin B1 peaking at 12 h and maintaining relatively high activity thereafter. In contrast, cells expressing mutant Sil Iip showed a considerable drop in Cdc2/cyclin B1 activity after reaching a peak at 10 h (Fig. 9D). Accordingly, the mitotic phosphorylation level, represented by the MPM2 staining, decreased with the drop in mitotic index and Cdc2/cyclin B1 activity (Fig. 9E; the decrease in MPM2 staining is particularly evident in the 16-, 18-, and 20-h time points). The escape from the checkpoint arrest did not seem to involve the activation of the APC, since the levels of known APC targets like cyclin B1 (Fig. 9E) and Polo-like kinase (see Fig. S4 in the supplemental material) revealed no differences among the cell line subclones, mutant or wild type. Thus, cells expressing a Sil phosphorylation mutant escape the mitotic checkpoint by downregulating Cdc2 activity in the presence of normal protein levels of cyclin B1.

Cdc2 activity is regulated by its association with the cyclin subunit as well as by the inhibitory phosphorylation of tyrosine

FIG. 8. Kinetics of mitotic exit of prometaphase-arrested cells expressing Flag-Sil and Flag-Sil IIP. Prometaphase-arrested cells (time zero) expressing wild-type Sil (clone S2) or mutant Sil IIP (clone 35) were released into G₁. At different times, samples were analyzed by FACS. (A) Table reporting the percentage of G₂/M cells at different times from the release of the mitotic block. (B) Cell cycle profiles determined by FACS analysis.

15 and threonine 14, sites that are dephosphorylated during the G₂/M transition, and additionally by the activating phosphorylation of threonine 161 (30). Since, in the context of spindle checkpoint activation, cells expressing mutant Sil IIP displayed a drop in Cdc2/cyclin B1 activity in the absence of downregulation of cyclin B1 and Cdc2, we analyzed the phosphorylation state of tyrosine 15 and threonine 161. While the tyrosine 15 phosphorylation pattern of the mutant Sil IIP-expressing cells displayed little, if any, difference compared to the wild-type Sil-expressing cells, analysis of threonine 161 revealed a difference (Fig. 9E). Cells expressing wild-type Sil or mutant Sil IIP activated the mitotic checkpoint and displayed threonine 161 phosphorylation, but as cells expressing the mutant Sil escaped the checkpoint, threonine 161 phosphorylation decreased in concert with the reduction of Cdc2/cyclin B1 activity (Fig. 9E).

When Cdc2/cyclin B1 complexes were immunopurified from cells at early or late stages (12 h and 19 h after G₁/S release), the sample from a mutant Sil IIP (clone 58) taken at 19 h showed a reduced amount of T-161 phosphorylation and reduced activity compared to the control wild-type clone (S2 sample). Incubation with purified CAK (19) partially restored Cdc2/cyclin B1 activity of complexes purified from clone 58 (Fig. 10A). Thus, at least in part, loss of Cdc2 activity in cells expressing mutant Sil IIP correlated with decreased T-161 phosphorylation and could be reversed by rephosphorylating T-161 using recombinant CAK. As shown in Fig. 10B, at 19 h, not only was the amount of T-161 phosphorylation reduced in

cells expressing the mutant Sil IIP, but the overall amount of Cdc2 complexed with cyclin B1 was also decreased. The reduction of Cdc2 associated with cyclin B1 may account for the incomplete reactivation by CAK of the Cdc2/cyclin B1 complex.

Sil knock-down impairs the spindle checkpoint response. To further substantiate the physiological role of Sil and its involvement in the mitotic checkpoint, we took advantage of plasmid-mediated RNA interference (3). Efficient suppression of Sil expression could be achieved by cotransfecting a puromycin resistance vector and then selecting cells with that antibiotic (Fig. 11A) or by using a double transfection protocol (see Fig. S6A in the supplemental material). Short-term downregulation of Sil (up to 48 h) did not have an effect on the cell cycle, while long-term silencing of Sil (72 to 96 h) affected cell survival and cell cycle distribution (see Fig. S6B in the supplemental material). Since in the first 48 h of RNAi there was not a change in the cell cycle profile, we designed the experiment not to exceed this window. HeLa cells were transfected with the RNAi Sil vector and, after 24 h, were challenged with taxol or nocodazole for an additional 18 h. The spindle checkpoint activation, measured by the mitotic index and MPM2 staining, showed a reduced metaphase arrest in cells with suppressed Sil expression (lanes S) compared to the control mock-transfected population (lanes V) (Fig. 11B). Similar results were obtained when either taxol or nocodazole was used. The apoptotic index (Fig. 11B) and G₂/M accumulation (Fig. 11C) of Sil-downregulated cells was comparable to control cells. Moreover, cell cycle

FIG. 10. Characterization of the Cdc2/cyclin B1 complexes during prolonged spindle checkpoint activation. The clone S2 (expressing wild-type Sil) and clone 58 (expressing mutant Sil IIP) cell lines were synchronized by double thymidine block and, after a 4-h release, were treated with taxol (as described in the legend of Fig. 5). Samples were taken at 12 h or 19 h after release from the G₁/S block. (A) Cdc2/cyclin B1 complexes purified from either S2 cell line (wild type) or the 58 cell line (IIP) were preincubated with CAK or mock incubated (–CAK). Cdc2 activity was assayed and reported in the histogram graphs as arbitrary units. (B) Western blot analysis of Cdc2 and cyclin B1 in the cell lysates and in immunopurified complexes (IP Cyc B1). Blots were stained for Cdc2 and a phospho-specific antibody for its phosphorylation on threonine 161 (T-161).

profiles of untreated cells or thymidine-blocked cells did not show relevant differences between Sil-suppressed cells and control samples (see Fig. S7A in the supplemental material). As in the previously described experiments using phosphorylation-mutant Sil proteins, Sil-suppressed cells showed lower Cdc2/cyclin B1 activity that correlated with reduced phosphorylation of Cdc2 T-161 (Fig. 11A). A second, distinct, independent and inducible Sil RNAi construct yielded consistent results (A. Eraz and S. Izraeli, unpublished results). Thus, Sil silencing affected the spindle checkpoint response.

Overexpression of Pin1 and the spindle checkpoint. Since the checkpoint arrest depends on Sil phosphorylation in the IIP cluster, we assessed whether overexpression of Pin1, known to promote dephosphorylation of its substrates by isomerizing the peptide bond preceding the phosphorylated residues, would affect the spindle checkpoint. HA-tagged Pin1 was transiently transfected in HeLa cells expressing either wild-type Flag-Sil (clone S2), the Sil IIP mutant (clone 58), or the parental cell line. After 24 h cells were blocked with nocodazole for an additional 19 h. The transfected cells were analyzed by FACS for cell cycle distribution, and the mitotic cells were

defined by phosphorylated histone 3 staining. Pin1 overexpression in the different cell lines did not reveal any change in the previously demonstrated spindle checkpoint responses (Fig. 12).

DISCUSSION

Mitotic phosphorylation of Sil could be observed in purified preparations of mitotic cells and upon mitotic checkpoint activation induced by drugs affecting microtubule dynamics. The phosphorylation could be easily detected by SDS-PAGE analysis since the hyperphosphorylated Sil shows delayed migration that is sensitive to protein phosphatase treatment. Of the 20 (S/T)P sites present in the Sil sequence, we have mapped 7 putative (S/T)P phosphorylation sites by site-directed mutagenesis located in the central cluster of phosphorylation sites (region II) that seem to be specifically phosphorylated by Cdc2/cyclin B1 and by mitotic extracts. These sites seem to be primarily involved in the interaction with Pin1 and in the regulation of the spindle checkpoint response.

We have shown that mitotic Sil is able to interact with Pin1.

FIG. 9. Flag-Sil IIP-expressing cells are unable to sustain a prolonged mitotic checkpoint arrest. Double thymidine block and release experiment of cell lines expressing Flag-Sil (clone S2), Flag-Sil IIP (clone 58), or the empty vector (V). Four hours after release from the thymidine block, cells were treated with taxol. At the indicated times, samples were collected and analyzed. (A) Mitotic index of DAPI stained samples. Time zero represents the time of release from the thymidine block. The data reported represent the mean of a triplicate experiment; for each data point at least 200 cells were counted. Independent experiments in which selected time points were analyzed confirmed the trend. (B) Phase-contrast pictures of clone S2 cells and clone 58 cells taken at different times after release from thymidine block and taxol treatment. (C) FACS analysis of samples taken at 12 or 19 h after G₁/S release. (D) Cdc2/cyclin B1 kinase activity assayed using histone H1 as a substrate. (E) Western blot analysis of MPM2 (arrows highlight three prominent MPM2-positive proteins whose intensity of staining decreases over time in the clone 58 cell line), cyclin B1 (Cyc B1), c-Cbl (as a loading control), Cdc2, and Cdc2 phosphorylation on tyrosine 15 (Y-15) and threonine 161 (T-161).

FIG. 11. Plasmid-mediated RNAi of Sil. HeLa cells were transfected with pSUPER-Sil or the empty vector. After 24 h, cells were treated with the indicated microtubule inhibitors for 18 h. (A) Western blot analysis of samples treated with taxol, stained for Sil, c-Cbl (as a loading control), cyclin B1 (Cyc B1), Cdc2, Cdc2 phosphorylation on threonine 161 (T-161), MPM2, and Cdc2/cyclin B1 kinase assay. (B) The histogram represents the mitotic index (black bars), MPM2 staining (gray bars), and apoptotic index (white bars) of cells transfected with empty vector (V) or pSUPER-Sil (S). As a control, the apoptotic index of untreated cells (NT) is also reported. The values reported in the table represent the mean of two independent experiments performed in duplicate. (C) FACS profile of cells transfected with empty vector (blue) or pSUPER Sil (red), selected with puromycin, and then treated for 18 h with taxol.

Pin1 is a recently identified PPI that regulates mitosis by specifically interacting with a number of mitotic phosphoproteins (39). Upon binding to the target protein, Pin1 is believed to specifically catalyze the *cis/trans* isomerization of the peptide bond shared by a phosphorylated serine or threonine residue and a proline. The phosphorylation-dependent conformational change caused by the *cis/trans* isomerization is thought to affect the activity of the substrate protein (36, 41, 42). The interaction between Sil and Pin1 could be observed by GST pull-down assay or coimmunoprecipitation of the overexpressed proteins. Overexpression of Pin1 by itself or in combination with wild-type or mutant Sil did not alter the Sil-related spindle checkpoint effects. It is possible that, given the slow kinetics of the release from the checkpoint arrest, the isomerization of the phosphorylated peptide bond is not kinetically limiting. The interaction is cell cycle regulated by the mitotic phosphorylation of Sil, as demonstrated by a binding experiment using *in vitro* dephosphorylated Sil or point mutants of Sil in the putative phosphorylation sites. These experiments suggest that region II may contain the Pin1 binding sites. In fact, deletion of

region II abolished Pin1 binding, and mutation of its putative phosphorylation sites considerably reduced the binding. Phosphorylation of region III could also play a role in the interaction, since its combination with the point mutation of region II may contribute to further reduce the affinity to Pin1. This may explain the modest effect that point mutation of region III exerted on the mitotic checkpoint response.

We have provided evidence that mitosis-specific phosphorylation of region II of Sil is required for the maintenance of the spindle checkpoint. The expression of a Sil protein mutated in its putative Pin1 relevant phosphorylation sites (Sil Iip) interferes with the ability of the cell to maintain the spindle checkpoint. The mitotic spindle checkpoint controls chromosome segregation by blocking anaphase onset until the kinetochores of each chromosome pair achieve stable attachment to the mitotic spindle (14, 24). Essential components of the spindle checkpoint are conserved during evolution and include proteins like Mad1, Mad2, Bub1, Bub3, BubR1, CENP-E, and Mps1 (29). These proteins constitute the core components that assemble on kinetochores in response to lack of tension or

FIG. 12. Spindle checkpoint response in cells overexpressing HA-tagged Pin1. Parental HeLa cells or HeLa cells expressing wild-type (clone S2) or mutant Iip (clone 58) Sil were transfected with HA-tagged Pin1 (HA-Pin1) or the empty vector. After 24 h, samples were treated with nocodazole for 19 h. The histogram reports the cell cycle distribution (percentage of cells in each phase of the cell cycle) determined by FACS analysis of samples stained with propidium iodide and with an anti-phosphorylated histone 3 antibody (H3) in order to discriminate G_2 from M phase.

attachment to the mitotic spindle (29). Upon checkpoint activation, the release of Mad2 from the kinetochores and its binding to Cdc20/Fizzy result in the inhibition of the APC (9, 15). APC activation regulates anaphase onset by targeting Pds1 (4) and cyclin B1 (10) for ubiquitin-dependent proteasomal degradation. Therefore, spindle checkpoint activation enforces a unique biochemical state in which the high level of Cdc2/cyclin B1 activity and an inactive APC arrest cells at prometaphase. Under conditions that should enforce the spindle checkpoint, mutants in checkpoint genes like Mad2 (7, 28) or Bub3 (20) fail to inhibit APC activity, leading to cyclin B1 degradation (and other targets) and unscheduled mitotic exit. Equally critical for the maintenance of a mitotic state is the control of the Cdc2 subunit. In budding yeast, along with the stabilization of the cyclin subunit, the Cdk/cyclin complex can be regulated by the opposing activity of the Cdc14 phosphatase and by binding of the cyclin-dependent kinase inhibitor Sic1 (38). In fission yeast, the Cdc14 homologue Clip1/Fpl1 regulates Cdc2 activity by dephosphorylating tyrosine 15 (5, 37). Moreover, there is evidence suggesting that Cdc2 threonine 161 is dephosphorylated during anaphase (12, 25). How Cdc2 regulation may also affect the mitotic spindle checkpoint has not been addressed.

Here, we have shown that a group of phosphorylation sites of Sil, required for Pin1 binding, is also critical in regulating checkpoint arrest. Our data suggest that expression of a mutant Sil affects the duration of the checkpoint arrest. The escape from the spindle checkpoint is associated with a progressive decline in Cdc2 activity and a decreased level of general mitotic phosphorylation, as measured by staining of MPM2 epitopes. Similar results could be obtained by knocking down endogenous Sil levels by plasmid-mediated RNAi. Unlike the cases where checkpoint components have been mutated, cells expressing mutant Sil escape the prometaphase block without activating the APC and revert to a G_2 -like state without degrading cyclin B1. Mutant Sil Iip expression downregulates the

kinase activity of the cyclin B1 complex, apparently by regulating the phosphorylation of threonine 161 on the Cdc2 subunit. Given the lack of evidence of physical association of Sil to Cdc2/cyclin B1 (data not shown), we speculate that Sil could regulate the phosphorylation level of Cdc2 by affecting the activity of the CAK and/or phosphatases active during mitosis. Since CAK activity in cellular extracts of mutant Sil Iip-expressing cells arrested at prometaphase was comparable to CAK activity measured in cells expressing wild-type Sil (see Fig. S5 in the supplemental material), it seems more plausible that Sil affects dephosphorylation of Cdc2 on threonine 161. Dephosphorylation of threonine 161 may represent a novel way to escape the spindle checkpoint, which suggests the existence of two parallel pathways regulating Cdc2/cyclin B1 activity during the spindle checkpoint induction. One pathway would involve the inhibition of APC activity by the mitotic spindle checkpoint, while a second pathway would require Sil phosphorylation and control the activity of Cdc2 through the regulation of threonine 161 phosphorylation.

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