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Functional Homology among Human and Fission Yeast Cdc14 Phosphatases*

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Budding and fission yeast Cdc14 homologues, a conserved family of serine-threonine phosphatases, play a role in the inactivation of mitotic cyclin-dependent kinases (CDKs) by molecularly distinct mechanisms. *Saccharomyces cerevisiae* Cdc14 protein phosphatase inactivates CDKs by promoting mitotic cyclin degradation and the accumulation of a CDK inhibitor to allow budding yeast cells to exit from mitosis. *Schizosaccharomyces pombe* Flp1 phosphatase down-regulates CDK/cyclin activity, controlling the degradation of the Cdc25 tyrosine phosphatase for fission yeast cells to undergo cytokinesis. In the present work, we show that human Cdc14 homologues (hCdc14A and hCdc14B) rescued *flp1*-deficient fission yeast strains, indicating functional homology. We also show that hCdc14A and B interacted *in vivo* with *S. pombe* Cdc25 and that hCdc14A dephosphorylated this mitotic inducer both *in vitro* and *in vivo*. Our results support a Cdc14 conserved inhibitory mechanism acting on *S. pombe* Cdc25 protein and suggest that human cells may regulate Cdc25 in a similar manner to inactivate Cdk1-mitotic cyclin complexes.

In all eukaryotic cells, entry into mitosis is regulated by the activation of cyclin-dependent kinases (CDKs)[1]; in contrast, mitotic exit depends on the inhibition of CDK activity and a reversal of CDK phosphorylation events (1, 2). In the budding yeast *Saccharomyces cerevisiae*, the phosphatase Cdc14 is essential for antagonizing this mitotic CDK activity, allowing cells to exit from mitosis and to coordinate cytokinesis events (3, 4). Thus, Cdc14 promotes mitotic cyclin destruction and CDK inactivation by dephosphorylating the APC activator Cdh1/Hct1, the CDK inhibitor Sic1, and the transcription factor Swi5 (4, 5). It has recently been shown that Cdc14 has many other CDK substrates that regulate a variety of other cellular events involved in mitotic progression, such as mitotic spindle dynamics and ribosomal DNA and telomere segregation. Thus, Cdc14 dephosphorylates the inner centromere-like protein Sli15-lpl1 to regulate its interaction with Aurora B and survivin early in anaphase, directing the protein complex to the spindles to stabilize them (6). Additionally, it has been shown that Cdc14 is also required to resolve ribosomal DNA segregation by promoting the enrichment of condensins, which are required for chromosome condensation (7–9). Finally, Cdc14 plays an important role in its own full activation because it stimulates mitotic exit network activity, probably through the dephosphorylation of certain mitotic exit network components, thus enhancing the mitotic exit function of the mitotic exit network (10, 11). Cdc14 activity is regulated, at least in part, by control of its cellular location. Cdc14 is sequestered in the nucleolus, where it remains inactive through association with its inhibitor Net1/Cfi1 for most of the cell cycle up to metaphase. During anaphase, Cdc14 is released from the nucleolus and localizes to the nucleus and cytoplasm, being able to dephosphorylate its own substrates (3, 12, 13).

In the yeast *Schizosaccharomyces pombe*, the Cdc14 homologue Flp1 (14), also called Clp1 (15), negatively regulates Cdc2 during mitotic exit despite not being strictly required for cyclin degradation and exit from mitosis (14, 15). The mechanisms by which Flp1 accomplishes mitotic CDK inactivation appear to be different from those of Cdc14. Flp1 does not dephosphorylate the Rum1 and Ste9/Srw1 proteins, homologues of Sic1 and Hct1 of *S. cerevisiae*, respectively. Instead, it antagonizes mitotic CDK activity by promoting the phosphorylation of the conserved tyrosine residue Tyr^{15} in Cdc2, in part by down-regulating the Cdc25 phosphatase, which dephosphorylates Tyr^{15} (16, 17). Once Cdc25 has been dephosphorylated by Flp1, it rapidly becomes unstable as *S. pombe* cells exit from mitosis. This Flp1-driven indirect down-regulation of CDK activity is required for cytokinesis to proceed in a timely fashion (16). Although in *S. pombe* cells Flp1 localizes predominantly to the nucleolus during the G_1 and S phases, it also resides at the spindle pole bodies. During prophase, Flp1 is released from the nucleolus and localizes to the nucleus, mitotic spindle, the spindle pole bodies, and the medial ring (14, 15). Thus, the cellular localization of Flp1 is slightly different from that of its budding yeast counterpart, and its activity would probably also be regulated in different ways. It is still unclear whether the release of Flp1 from the nucleolus alone is sufficient for its activation, but Flp1 activity could also be regulated by phosphorylation changes because Flp1 is phosphorylated during mitosis (14, 15).

In humans there are two Cdc14 homologues, hCdc14A and hCdc14B (18), whose possible functions in cell cycle progression are poorly understood. Although hCdc14A and hCdc14B are structurally related proteins (19) and share 50% sequence identity, they have noteworthy differences; hCdc14B has a unique 54-amino acid N-terminal fragment, and the two proteins have different C-terminal domains. *In vitro* studies have shown that hCdc14A, which localizes to centrosomes during interphase, dephosphorylates the substrates of serine-proline- or threonine-proline-directed kinases (20–22). One of these substrates is hCdh1, and this hCdc14-directed dephosphorylation recon-

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[1] The abbreviations used are: CDK, cyclin-dependent kinase; GST, glutathione *S*-transferase; DAPI, 4′,6-diamidino-2-phenylindole; FACS, fluorescence-activated cell-sorting.

stitutes active APC^{Cdh1} *in vitro* (21), indicating that hCdc14A could bring about mitotic CDK inactivation. This hCdc14A protein has been shown to primarily regulate centrosome replication *in vivo* (22, 23). In addition, hCdc14A is located at the central spindle during anaphase, where it seems to be involved in the spatial regulation of the Aurora B kinase, a key regulator of chromosome segregation and cytokinesis (24). Thus, deregulation of hCdc14A phosphatase leads to centrosome separation, chromosome segregation, and cytokinesis defects (22–24). hCdc14B localizes to the nucleolus during interphase but not during mitosis (21–23), and so far no function has been ascribed to this homologue.

Because it has previously been reported that *S. pombe* Flp1 phosphatase induces the degradation of the fission yeast Cdc25 protein (16, 17), it would be of general interest to know whether human Cdc14 homologues interact with and dephosphorylate Cdc25, a protein phosphatase family of CDK inducers (25). To gain insight into this issue, here we analyze whether or not hCdc14 homologues are able to interact with and dephosphorylate *S. pombe* Cdc25, perhaps to reverse the CDK-mediated activation of this mitotic inducer. We show that the expression of either hCdc14A or hCdc14B in the fission yeast is able to rescue defects associated with the lack of the phosphatase-encoded *flp1*$^+$ gene in these cells. Although the two human phosphatases interact *in vivo* with Cdc25, only hCdc14A dephosphorylates Cdc2-dependent Cdc25 phosphate residues. We propose that hCdc14A would act like the *S. pombe* homologue Flp1 by dephosphorylating the mitotic inducer Cdc25.

MATERIALS AND METHODS

Strains, Plasmids, and Fission Yeast Methods—Standard methods were used for manipulation of DNA. The media, growth conditions, and standard molecular biology and genetic methods have been described previously (26, 27). The *S. pombe* strains used in this study were from A. Bueno's laboratory. The strains involving the *flp1*$^+$ deletion have been described previously (14). Plasmid constructions were made using standard molecular biology techniques. The hCDC14A and hCDC14B cDNAs (gifts from J. Lukas) were PCR-amplified with oligonucleotides containing NdeI and NotI at the 5′ and 3′ ends, respectively. Site-directed mutagenesis was carried out with QuikChange (Stratagene), according to manufacturer's protocols. To construct the GST derivatives, the PCR products were cut with NdeI and NotI and cloned into the pREPKZ *S. pombe* expression plasmid, which carries the thiamine-inducible *nmt1* promoter (28). All constructs were sequenced using automated sequencing (Genomic Facility, Universidad de Salamanca, Centro de Investigación del Cáncer). The GST-Flp1 construct has been described elsewhere (16). The *S. pombe cdc25*$^+$ open reading frame was cloned into the pGEX-KG vector (a gift from S. Moreno). Fission yeast cells were transformed by the lithium acetate transformation protocol (27). For the induction of the *nmt1* promoter, a culture growing exponentially in medium containing thiamine (10 μM) was washed twice and resuspended in medium without thiamine, as described previously (14).

Cell Extracts, Western Blot Analyses, Immunoprecipitation, and Kinase Assays—For Western blotting, whole-cell lysates were prepared from cells growing exponentially. Cells were collected by centrifugation. Cellular pellets were kept on ice and processed by vortexing with glass beads in lysis buffer (10 mM Na_2HPO_4, pH 7.0, 1% Triton X-100, 0.1% SDS, 10 mM EDTA, and 150 mM NaCl, with inhibitors (20 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM sodium orthovanadate)). Cell extracts were clarified by two successive centrifugations, and protein concentrations were measured with the BCA protein assay kit (Pierce). Protein extracts (75 μg) were resolved on standard SDS-PAGE gel, transferred to nitrocellulose, and probed with rabbit affinity-purified anti-Cdc25 and anti-Cdc2 polyclonal antibodies. Mouse TAT1 anti-tubulin monoclonal antibody was used to detect tubulin as a loading control. The secondary antibodies used were goat anti-rabbit or goat anti-mouse conjugated to horseradish peroxidase. Immunoblots were developed using the ECL kit (Amersham Biosciences). For immunoprecipitation, soluble protein extracts were obtained as described above in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 400 mM NaCl, 1 mM EDTA, 1 mM 2-β-mercaptoethanol, 10% glycerol, 50 mM NaF, 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride) containing a complete protease inhibitor mixture (Roche Applied Science). For Cdc2-Cdc13 complex immunoprecipitation, 750 μg of soluble protein were incubated for 1 h on ice with 2 μl of SP4 anti-Cdc13 polyclonal antibody, followed by a 2-h incubation at 4 °C with 20 μl of protein A-Sepharose beads (Amersham Biosciences). The beads were washed five times with 1 ml of immunoprecipitation buffer and resuspended in kinase buffer. Kinase assays were performed with H1 histone (Calbiochem) as a substrate for immunoprecipitated mitotic CDK complex as described previously (29, 30).

In Vitro Phosphatase Reactions—Cdc25 was expressed and purified from bacteria as a GST-Cdc25 fusion protein. GST-hCdc14A and GST-hCdc14B and their phosphatase-inactivating mutants (in which Cys^{278} and Cys^{314}, respectively, were mutated to serine (GST-hCdc14-CS)) were expressed and purified from a *S. pombe* Δ*cdc25 cdc2-3w* strain. GST purification has been described previously (31). The CDK substrate GST-Cdc25 was incubated with 2 units of cyclin B-Cdc2 human complex (New England Biolabs) in kinase buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, and 50 mM ATP) in the presence of 0.15 μCi of [γ-^{32}P]ATP for 30 min at 30 °C. Samples were then washed three times with 1 ml of phosphatase buffer (50 mM imidazole, pH 6.9, 1 mM EDTA, and 1 mM dithiothreitol), and GST-hCdc14 was added and incubated for 45 min at 30 °C. Reactions were stopped by the addition of loading buffer and boiling (5 min at 95 °C), and the proteins were resolved by SDS-PAGE and visualized by autoradiography.

Flow Cytometric Analysis and Immunofluorescence Microscopy—Approximately 10^7 cells were collected by centrifugation, washed once with water, fixed in 70% ethanol, and processed for flow cytometry or DAPI staining, as described previously (26). Microtubule staining was accomplished as described previously (32, 33), using TAT1 anti-tubulin antibody (1:100) (32). Staining of Myc_{12}-Cdc25 and GST-hCdc14 proteins was performed with anti-Myc and anti-GST specific antibodies, respectively. Fluorescence images were collected using a Zeiss Axioplan 2 microscope with ×40, ×63, or ×100 objectives and a digital camera (Hamamatsu ORCA-ER) and processed with Improvision software. The percentage of cells in anaphase B was estimated by counting cells with two nuclei and an elongated spindle.

RESULTS

Human Cdc14 Proteins Complement the S. pombe flp1$^+$ *Deletion*—To determine whether or not human Cdc14 proteins were functionally equivalent to their *S. pombe* homologue Flp1, we performed complementation analyses in mutant yeast strains lacking *flp1*$^+$ gene. Because a null mutant of the *S. pombe flp1*$^+$ gene is viable (14, 15), we used a double mutant *S. pombe* Δ*flp1 cdc25-22* strain in an attempt to determine whether the expression of hCdc14 proteins might rescue the cell division cycle defect shown by this mutant at 32 °C due to the lack of *flp1*$^+$ (16). The hCDC14A and hCDC14B open reading frames or their mutant forms (in which Cys^{278} and Cys^{314} of hCDC14A and hCDC14B, respectively, were changed to serine (hCDC14-CS)) were placed under the control of the *nmt1* promoter in the pREPKZ *S. pombe* expression plasmid. As a control, the *flp1*$^+$ open reading frame was also cloned into this plasmid. Upon transformation into the Δ*flp1 cdc25-22* mutant, cells were plated on medium containing 2 μM thiamine. Cells expressing moderate levels of GST-hCdc14A and GST-hCdc14B, as tested by Western blot (data not shown), were able to rescue the viability of these cells at 32 °C, as did GST-Flp1 (Fig. 1*A*). Transformants containing the catalytically phosphatase-inactive hCDC14A form did not rescue this Δ*flp1 cdc25-22* double mutant at 32 °C. Although cells expressing hCdc14B-CS grew better than the GST-transformed Δ*flp1 cdc25-22* negative control, they did not, however, rescue viability to the same extent as the catalytically active hCdc14B. A similar result was obtained when transformant cells were plated on medium containing a lower thiamine concentration (0.2 μM) (data not shown).

Although the *S. pombe* Δ*flp1* mutant is viable, it has been shown that it is advanced in mitosis and divides at reduced size (14, 15). This phenotype is somehow stronger when cells grow at 37 °C, the temperature at which they tend to form clumps. Thus, we next examined whether or not *S. pombe* Δ*flp1* cells

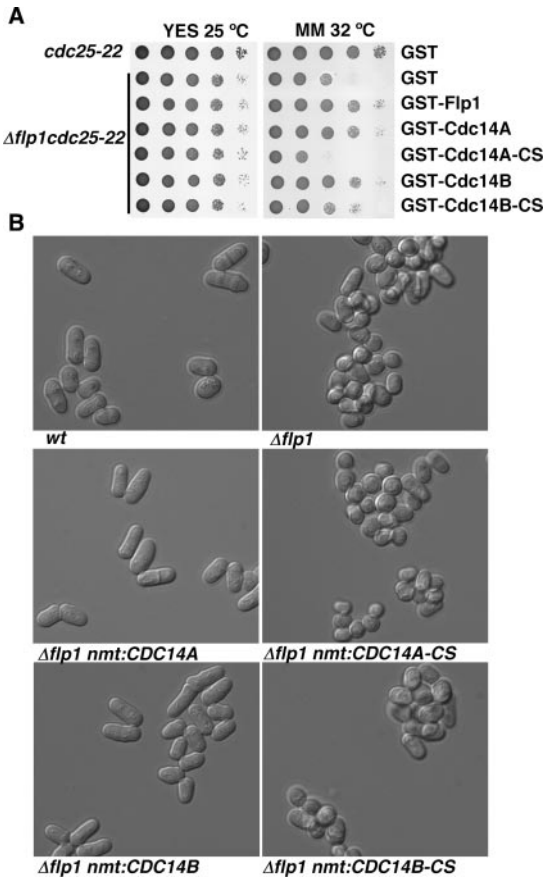

FIG. 1. **Complementation of Δ*flp1* mutant cells by human Cdc14 proteins.** *A*, *S. pombe* Δ*flp1 cdc25-22* mutant cells were transformed with pREPKZ empty vector or pREPKZ expression vector containing GST-*flp1*$^+$, GST-hCDC14A, GST-hCDC14A-CS, GST-hCDC14B, or GST-hCDC14B-CS and grown on yeast extract with supplement (*YES*) or minimum media (*MM*)-Leu and 2 μM thiamine to induce moderate protein expression levels at 25 °C or 32 °C, as indicated. *cdc25-22 flp1*$^+$ cells were also transformed with pREPKZ empty vector. *B*, microscopy images of wild-type, Δ*flp1*, and Δ*flp1* cells expressing the two hCdc14 proteins and their catalytically inactive forms. *S. pombe* cells lacking *flp1*$^+$ gene were transformed as described above, and protein expression was induced by growing them in minimum media-Leu and 0.2 μM thiamine media for 20 h at 37 °C.

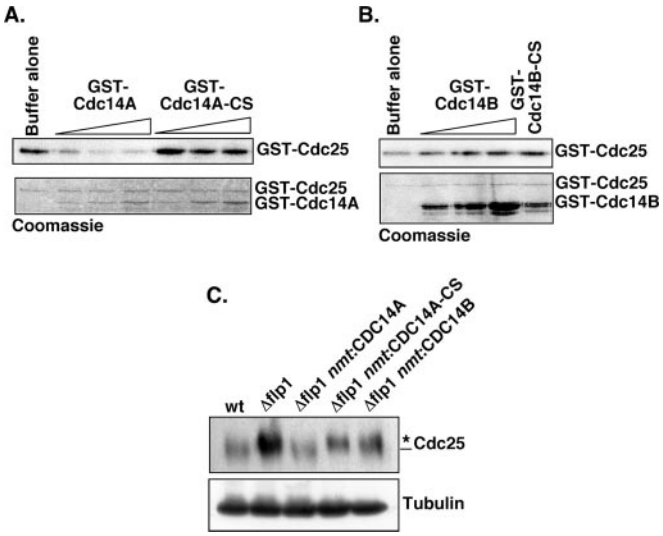

FIG. 2. **Human Cdc14A can dephosphorylate *in vitro* and *in vivo* the Cdc2-dependent phosphorylation of Cdc25.** *A* and *B*, GST-Cdc25 purified from *Escherichia coli* was phosphorylated *in vitro* by Cdc2-cyclin B complex and subsequently incubated with increasing amounts of GST-hCdc14A, GST-hCdc14A-CS, GST-hCdc14B, GST-hCdc14B-CS, or buffer alone at 30 °C for 45 min as described under "Materials and Methods." Reactions were separated by SDS-PAGE and analyzed by Coomassie Blue staining (*bottom panels*) and autoradiography (*top panels*). *C*, extracts from wild-type *S. pombe* cells, Δ*flp1* cells, or Δ*flp1* cells expressing hCdc14A, hCdc14A-CS or hCdc14B were prepared and analyzed after SDS-PAGE separation by immunoblotting with anti-Cdc25 polyclonal antibody. The *asterisk* indicates the position of the hyperphosphorylated Cdc25 protein. Detection of tubulin with anti-tubulin antibody was used as control.

reach a normal *S. pombe* cellular size when expressing hCdc14 proteins. A Δ*flp1* strain was transformed with either pREPKZ vector alone as control or the same vector containing one of the two hCDC14 cDNAs or their phosphatase-inactive variants. Transformants were grown at 37 °C in medium containing 0.2 μM thiamine for 20 h. Control cells showed the characteristic semi-wee phenotype of Δ*flp1* cells; the cells expressing either hCdc14A or hCdc14B protein, but not their inactive variants, were able to reverse that phenotype, resulting in cells that were slightly longer than the controls (wild-type, 11.3 ± 1.21 μm; Δ*flp1*, 4.3 ± 0.52 μm; Δ*flp1 nmt:*hCDC14A, 12.1 ± 1.84 μm; Δ*flp1 nmt:*hCDC14A-CS, 4.5 ± 0.62 μm; Δ*flp1 nmt:* hCDC14B, 11.6 ± 1.56 μm; and Δ*flp1 nmt:*hCDC14B-CS, 4.4 ± 0.62 μm (measured in minimum media)) and no longer formed clumps (Fig. 1*B*). Taken together, these observations clearly suggest that both hCdc14A and hCdc14B are capable of complementing the lack of *flp1*$^+$ gene.

hCdc14A Dephosphorylates S. pombe Cdc25 Protein—*S. pombe* Flp1 has been shown to interact *in vivo* with Cdc25 and to reverse some Cdc2-dependent phosphorylation events (16, 17). To determine whether human Cdc14 homologues were able to interact with and dephosphorylate *S. pombe* Cdc25, we first tested whether recombinant GST-hCdc14 could dephosphorylate recombinant GST-Cdc25, which was *in vitro* phosphorylated by active Cdc2-cyclin B complex. GST-hCdc14A, but not its phosphatase-dead version, GST-hCdc14A-CS, was able to remove radioactive phosphate from GST-Cdc25 in a dose-dependent fashion (Fig. 2*A*). However, recombinant GST-hCdc14B did not reverse this Cdc2-dependent phosphorylation state (Fig. 2*B*).

It has been shown that Cdc25 electrophoretic mobility changes as a function of its phosphorylation state (14, 34); thus, using Western blot analysis with anti-Cdc25 antibody, we next examined the phosphorylation state of Cdc25 in *S. pombe* Δ*flp1* mutant cells that expressed either of the two forms of hCdc14 phosphatase. Deletion of the *flp1*$^+$ gene results in the accumulation of hyperphosphorylated Cdc25 protein, regardless of the stage of the cell cycle (16). As shown in Fig. 2*C*, when Δ*flp1* mutant cells expressed hCdc14A, but not the phosphatase-dead version, hCdc14A-CS, both the phosphorylation state (in accordance with its electrophoretic mobility) and the protein levels of Cdc25 were comparable to those of wild-type *S. pombe* cells. Again, the expression of hCdc14B was not able to modify the band shift of Cdc25 in Δ*flp1* cells. These results suggest that hCdc14A directly or indirectly dephosphorylates Cdc25 when expressed in *S. pombe* yeast cells.

Although both hCdc14A and hCdc14B phosphatases are able to complement the lack of the *flp1*$^+$ gene, only hCdc14A dephosphorylates Cdc25 protein *in vitro* and *in vivo*, suggesting that the molecular mechanisms by which they rescue Δ*flp1* defects are probably different and that hCdc14A behaves functionally, at least in part, like the *S. pombe* Flp1 protein when expressed in these cells.

Both hCdc14A and hCdc14B Interact in Vivo with Fission Yeast Cdc25—Having observed that Cdc25 is a substrate of hCdc14A, we wished to determine whether or not these two proteins interact *in vivo*. Both GST-hCdc14A and the catalytically inactive form, hCdc14A-CS, which is assumed to bind

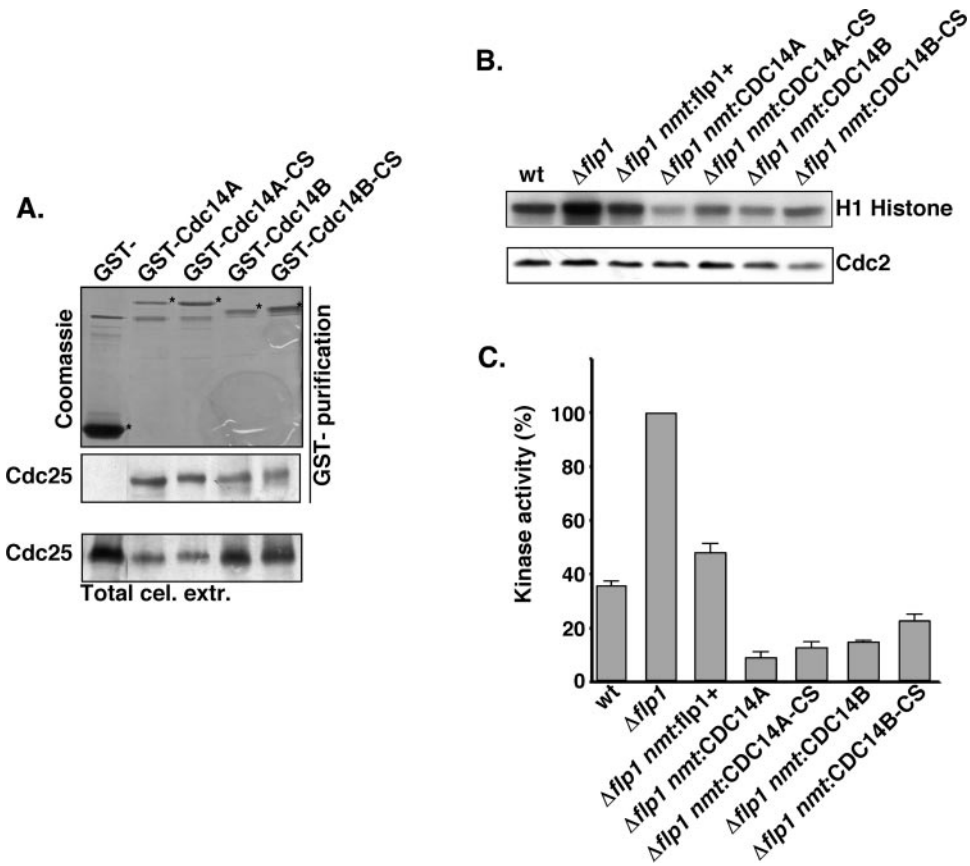

FIG. 3. **Both hCdc14A and hCdc14B interact with Cdc25.** *A*, GST−, GST-hCdc14A, GST-hCdc14A-CS, GST-hCdc14B, and GST-hCdc14B-CS proteins were expressed under the control of the *nmt1* promoter in a Δ*flp1* strain. After 20 h of induction in media lacking thiamine, samples were collected, and the proteins were purified with glutathione-Sepharose beads. Samples were processed by SDS-PAGE, and proteins were either detected by Coomassie Blue staining (*top panel*) or immunoblotted with anti-Cdc25 antibody (*middle panel*). *Asterisks* indicate the position of the predicted size of the fusion proteins. The *bottom panel* shows Cdc25 detection in whole-cell extracts prepared from the same samples before carrying out purification of the GST fusion protein. *B*, Δ*flp1* cells expressing different hCdc14 forms have lower levels of Cdc2/Cdc13-associated H1 kinase activity than Δ*flp1* cells. Cdc2-Cdc13 complexes were immunoprecipitated with SP4 anti-Cdc13 polyclonal antibody from wild-type, Δ*flp1* cells, and Δ*flp1* cells expressing GST-Flp1, GST-hCdc14A, GST-hCdc14A-CS, GST-hCdc14B, or GST-hCdc14B-CS protein extracts. Cells were grown for 16 h in media lacking thiamine to induce the expression of fusion proteins. Immunoprecipitated samples were processed for histone H1 kinase activity as described under "Materials and Methods." Total cell extracts were immunoblotted for Cdc2 detection as control. *C*, the relative kinase activity was normalized to the quantity of Cdc2 protein loaded and expressed as a percentage of that obtained with Δ*flp1* cell extracts. The plot represents the results from two independent experiments.

with higher affinity to its substrates (35), were expressed in and purified from a Δ*flp1* strain. Although hCdc14B phosphatase does not seem to dephosphorylate the Cdc2-dependent phosphates of Cdc25, we also expressed and purified both GST-hCdc14B and its corresponding catalytically inactive mutant, GST-hCdc14B-CS. By Western blot analysis, we observed that both hCdc14A and hCdc14B phosphatases, but not GST alone, associated with Cdc25. With regard to the two CS mutants, these showed the same levels of interaction as their corresponding catalytically active forms (Fig. 3*A*). Similar results were obtained when cells were grown in medium containing 2 μM thiamine to reduce the expression levels of the GST fusion proteins in the cell (data not shown).

As has been shown, Flp1 dephosphorylates and inactivates Cdc25, and as consequence of this, *S. pombe* cells lacking *flp1*$^+$ gene accumulate hyperphosphorylated Cdc25, which in turn leads to higher levels of mitotic CDK kinase activity in these cells (16, 17). To examine whether the hCdc14A-dependent Cdc25 dephosphorylation observed before underlies an inactivation of the Cdc2-Cdc13 complex, we expressed GST-hCdc14A and GST-hCdc14A-CS in *S. pombe* Δ*flp1* cells and analyzed Cdc2 activity through kinase assays with immunoprecipitated mitotic Cdc2 kinase from cell cultures at 16 h after the start of induction, a time of induction with no consequences in the cell cycle profile as checked by FACS analysis. Expression of GST-hCdc14A induced the decay of high levels of mitotic CDK activity associated with Δ*flp1* cells (Fig. 3, *B* and *C*). This analysis also shows that cells expressing GST-hCdc14A-CS reach a lower level of CDK activity as compared with Δ*flp1* cells. In addition, expression of GST-hCdc14B or GST-hCdc14B-CS in Δ*flp1* cells also decreased the activity of Cdc13-associated H1 kinase (Fig. 3, *B* and *C*). Low Cdc2/Cdc13 levels after the expression of hCdc14A and hCdc14B could be the direct consequence of their interaction with fission yeast Cdc25, preventing it from activating the Cdc2-cyclin complex.

Since hCdc14B does not appear to dephosphorylate Cdc2-phosphorylated Cdc25, we next examined the possible effect of expressing GST-hCdc14B on the *in vivo* location of Cdc25, with which it interacts. Interestingly, we found that in those cells, Cdc25 protein is observed exclusively in the cytoplasm, as opposed to wild-type cycling cells, in which Cdc25 accumulates in the nucleus (Fig. 4). Because the Cdc2-Cdc13 mitotic complex is located in the nucleus throughout the cell cycle, the effect of hCdc14B excluding Cdc25 from the nucleus may contribute to its ability to alter cellular Cdc2/Cdc13 activity. By contrast, Cdc25 appears both in the cytoplasm and in the nucleus in cells expressing GST-hCdc14A, and this Cdc25 signal is also of low intensity in many of these cells (Fig. 4).

Overexpression of hCdc14A and hCdc14B Phosphatases in S. pombe—We were also interested in studying the effect of over-

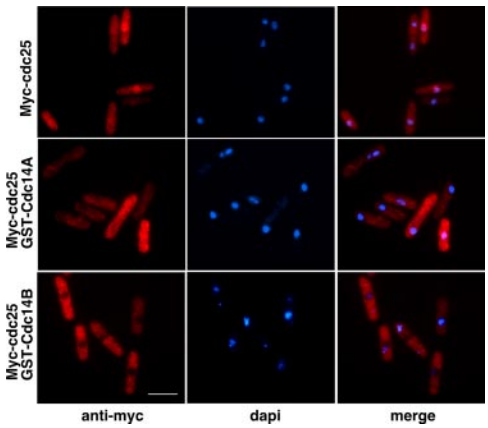

FIG. 4. **Subcellular localization of Cdc25 protein in *S. pombe* cells overexpressing hCdc14A and hCdc14B.** Asynchronously growing cultures of Myc-cdc25 cells expressing pREPKZ empty vector (*top panels*) or pREPKZ-GST-hCdc14A (*middle panels*) or pREPKZ-GST-hCdc14B (*bottom panels*) for 18 h were subjected to indirect immunofluorescence microscopy using anti-Myc antibodies. Cells were stained with DAPI to visualize DNA. *Bar*, 10 μm.

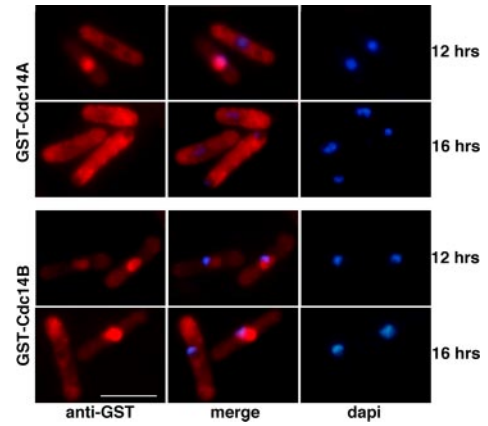

FIG. 5. **Localization of hCdc14A and hCdc14B in *S. pombe* cells.** After growth in culture media lacking thiamine for 12 and 16 h at 32 °C, cells overexpressing GST-hCdc14A or GST-hCdc14B were fixed with paraformaldehyde and analyzed by immunofluorescence using anti-GST antibodies and DAPI staining. Note that GST-hCdc14B seems to be preferentially associated with a non-DAPI-stained region of the nucleus, possibly the nucleolus. *Bar*, 10 μm.

expression of human Cdc14 phosphatases on *S. pombe* cells. Thus, a wild-type *S. pombe* strain was transformed with pREPKZ vector alone or with the same vector containing the hCDC14A or hCDC14B cDNAs. Examination of the subcellular localization of hCdc14A and hCdc14B by indirect immunofluorescence revealed that the proteins are localized mainly to the nucleus after 12 h of induction, although hCdc14A and hCdc14B were observed both in the nucleus and in the cytoplasm after longer induction periods (Fig. 5).

The transformants thus obtained were grown for 24 h at 32 °C in culture media without thiamine to induce the *nmt1* promoter. As a reference, we also used a pREPKZ-*flp1*+ transformant. The expression of these proteins was tested by Western blot analysis with anti-GST antibodies (data not shown), and cells were examined by FACS analysis and fluorescence microscopy. As shown in Fig. 6, FACS analysis revealed an aberrant DNA profile of hCdc14-overproducing cells, similar to the one observed for the overexpression of Flp1. Thus, at 20 h in thiamine-depleted medium, a significant change was observed in the DNA content profile of hCdc14-overexpressing cells. This thiamine-depleted incubation time led to the appearance of cells with both less and more than a 2C DNA content when expressing hCdc14A or with a DNA content higher than 2C when expressing hCdc14B. The DNA profile of the control wild-type cells expressing GST alone was unchanged even after 24 h in the thiamine-depleted medium. It therefore seems that overexpression of either hCdc14A or hCdc14B could lead to mitotic defects.

To investigate whether these aberrant DNA profiles were correlated with structural changes in nuclear morphology and/or spindle structure, microscopic analysis of hCdc14A, hCdc14B, and Flp1-overexpressing cells double-stained with DAPI and anti-tubulin was carried out after 22 h of culture in the absence of thiamine. Overexpression of Flp1 causes cells to become elongated and delayed in the G_2 phase, and, upon prolonged induction, these elongated and single nucleated *S. pombe* cells eventually form a mitotic spindle, enter mitosis, and, in some cases, septate without segregating the chromosomes (15, 16). These effects clearly differ from phenotypes of *S. pombe* cells overexpressing either GST-hCdc14A or GST-hCdc14B (Fig. 7*A*). It is noteworthy to mention that the observed phenotype was strikingly different for the two human Cdc14 proteins. When cells overexpressed hCdc14B, the nuclei appeared fragmented and anomalously distributed throughout the cell. Many of these cells did not show spindle staining, perhaps due to breakage or incomplete formation (Fig. 7*A*). The same effect was observed when cells overexpressed hCdc14B-CS (data not shown). In the case of hCdc14A overexpression, altered states of chromatin compaction, chromosome decondensation, and missegregation were observed (Fig. 7, *A* and *B*; Table I). The tubulin staining revealed a higher fraction of anaphase spindles in hCdc14A-overexpressing cells (5.8% anaphase cells, $n = 90$) as compared with wild-type cells (3% anaphase cells, $n = 60$). In addition, whereas all wild-type cells found in anaphase had a normal morphology, most of the hCdc14A-overexpressing cells in anaphase (86%) had abnormal, decondensed, and entangled chromosomes. Thus, these cells, which displayed a characteristic anaphase spindle, also had some elongated nuclear structures with their chromatin fibers apparently extending along the spindle or with a single nucleus at the center or at one end of the mitotic spindle (Fig. 7*B*). These aberrant phenotypes are in accordance with the FACS analysis. Again, overexpression of the phosphatase-dead hCdc14A-CS caused the same effect (data not shown).

DISCUSSION

The Cdc14 family of phosphatases is conserved in most if not all eukaryotes and is considered a major player in controlling the mitotic exit and cytokinesis antagonizing the action of mitotic CDKs (4, 5, 16, 17, 22, 23, 36, 37). The molecular mechanisms by which Cdc14 regulates CDK inactivation at the end of mitosis are best understood in *S. cerevisiae*, in which additional roles of Cdc14 during late mitotic stages have been found (6–9). The mechanisms by which the *S. pombe* Cdc14 homologue Flp1 accomplishes mitotic CDK inactivation and other cellular processes are quite different from those of the budding yeast Cdc14 (16, 17). Much less is known about the two human Cdc14 homologues, hCdc14A and hCdc14B, which have been shown to play a role in mitosis, and some of which localize to mitotic structures (centrosomes and central spindle) (22–24). Here we investigated the possibility that human Cdc14 homologues might share some functional mechanisms with the *S. pombe* Flp1 phosphatase.

Unlike *S. cerevisiae* Cdc14, Flp1 is not essential for mitotic exit, and a null mutant of the *S. pombe flp1*$^+$ gene is viable but divides at a reduced size and has defects in septation (14, 15). Both hCdc14A and hCdc14B are able to complement the lack of *flp1*$^+$ in *S. pombe* cells, as shown by the double mutant *S. pombe* Δ*flp1 cdc25-22*, which was able to grow at 32 °C when it

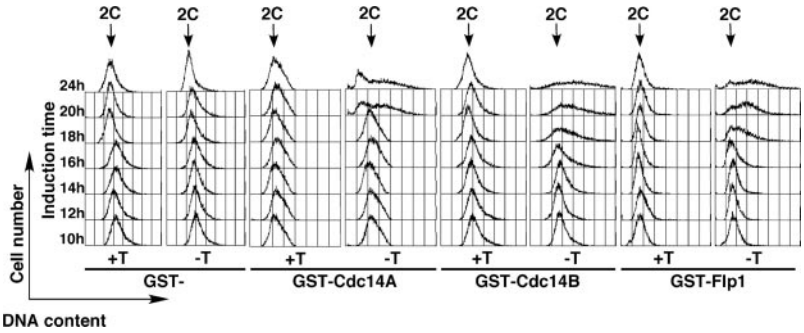

FIG. 6. **Flow cytometry analysis of *S. pombe* cells overexpressing hCdc14A, hCdc14B, and Flp1 proteins.** *S. pombe* cells transformed with pREPKZ empty vector or pREPKZ expression vector containing GST-hCDC14A, GST-hCDC14B, or GST-*flp1*+ were grown in liquid culture media with (+*T*) or without (−*T*) thiamine for 24 h at 32 °C. Samples were collected at the indicated time points, and 10^7 cells were fixed with ethanol and stained with propidium iodide, and their DNA content was determined by flow cytometry.

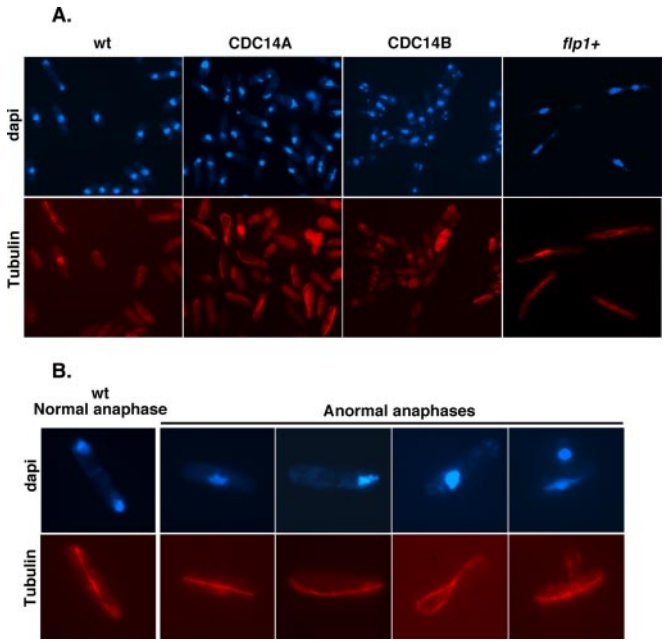

FIG. 7. **Nuclear and spindle structure of *S. pombe* cells overexpressing hCdc14A, hCdc14B, and Flp1 proteins.** *A*, *S. pombe* cells overexpressing hCdc14A, hCdc14B, or Flp1, after growth in culture media lacking thiamine for 22 h at 32 °C, were fixed with paraformaldehyde and analyzed by immunofluorescence using antitubulin antibodies (TAT1) and DAPI staining. *B*, several hCdc14A-overexpressing cells with decondensed chromosomes or displaced nuclei (abnormal anaphases) are shown. A wild-type anaphase (*wt*) is shown in the *left panel*.

TABLE I
Aberrant phenotypes of Cdc14A-overexpressing cells
A total of 224 cells were measured.

One-end nucleus	65	
Decondensed anaphase	40	
Irregular segregation	30	
Septation before segregation	3	

expressed Flp1 or either of the two human Cdc14 homologues ectopically. Moreover, when Δ*flp1* cells expressed hCdc14A or hCdc14B, they rescued their size defects. These results suggest that, under the conditions studied, human Cdc14 phosphatases may provide Flp1-like activity in fission yeast *flp1* mutant strains. It is known that hCdc14A is able to rescue the *cdc14-1*^{ts} cell cycle arrest of *S. cerevisiae* (18), suggesting that hCdc14A is the functional homologue to the yeast Cdc14. Because it is known that the two yeast homologues Cdc14 and Flp1 differ in the molecular mechanisms through which they accomplish mitotic CDK inactivation, it is possible that human Cdc14 proteins might be able to dephosphorylate different substrates to rescue both budding and fission yeast Cdc14 mutants.

It is known that Cdc14 phosphatases specifically remove phosphate groups placed in site by Ser/Thr-Pro-directed kinases (4, 16, 17, 22). Here we show that hCdc14A interacts with *S. pombe* Cdc25 protein *in vivo* and that hCdc14A dephosphorylates *S. pombe* Cdc25 protein, the only Flp1 substrate described so far (16, 17), *in vitro* and *in vivo*. Our *in vitro* dephosphorylation assays demonstrate that hCdc14A removes at least some Cdc2-dependent phosphate residues on Cdc25. The increased stability of Cdc25, which is characteristic of cells lacking the *flp1*$^+$ gene, was reversed to normal levels when these cells expressed hCdc14A. It has been demonstrated that Flp1 is required for Cdc25 ubiquitination and later degradation (16, 17), and our data suggest that hCdc14A may dephosphorylate Cdc25, also probably acting to deprotect it from degradation, as does Flp1. hCdc14B does not dephosphorylate Cdc25 *in vitro*, even though it does interact with Cdc25 *in vivo*. *S. pombe* Δ*flp1* cells expressing hCdc14B phosphatase do not modify the high levels of hyperphosphorylated Cdc25 present in this strain. Although we cannot exclude the possibility that hCdc14B could dephosphorylate some Cdc25 phospho-amino acids undetected under our experimental conditions, these results suggest that the molecular mechanisms through which the two human Cdc14 proteins complement the lack of *flp1*$^+$ in *S. pombe* are most likely different.

We also show here that overexpression of hCdc14A decreases the high levels of mitotic CDK activity present in Δ*flp1 S. pombe* cells to a striking extent. This is probably due to the inactivation and/or degradation of Cdc25, as suggested above. However, when the cells expressed hCdc14B, which appears not to dephosphorylate Cdc25, or when either hCdc14A or hCdc14B catalytically inactive versions were expressed, CDK activity was also reduced. These surprising results could be due to the interaction of these proteins with Cdc25. Thus, catalytically inactive hCdc14 phosphatases bind to Cdc25 and, as a consequence, may sequester Cdc25 and change its proper cellular location, as we found for hCdc14B, which causes Cdc25 to be excluded from the nucleus. A second possibility is that the hCdc14/Cdc25 interaction might also prevent Cdc25 from interacting with mitotic CDK complex, abolishing the positive feedback loop, or with other potential kinases able to stimulate Cdc25 activity. All these possible mechanisms result in Cdc25 inactivation, which in turn results in the reduction of mitotic

CDK activity. Alternatively, it is possible that these effects might be independent of Cdc25 and that other proteins could be affected by hCdc14 expression, also leading to a loss of Cdc2/Cdc13 activity. Distinguishing among these alternatives will be addressed in future studies.

We also report that overexpression of hCdc14A and hCdc14B in fission yeast results in mitotic abnormalities. Overexpression of hCdc14A produces a high percentage of cells in late anaphase but retains unsegregated chromosomes lying in the spindle midzone or at one end of the spindle. The result is an unequal chromosome segregation, producing cells with anomalous DNA contents, as observed in the FACS analyses. Defective chromatin condensation and chromosome segregation have been reported following overexpression of Hrp1, a chromodomain ATPase protein involved in the correct chromosome segregation in *S. pombe* (38). Other *S. pombe* mutant proteins that play a role in chromatin condensation, such as Swi6 or Top2, also show these cytological defects (39, 40). Human Cdc14A has been shown to interact with and dephosphorylate the inner centromere-like protein, a passenger protein involved in the coordination of chromosome segregation with cytokinesis, probably contributing to its relocalization from kinetochores to the central spindle in anaphase (24). It is possible that overexpressed hCdc14A could interact with and prevent the correct function of *S. pombe* proteins involved in condensation or chromatin segregation.

The defects shown by cells overexpressing hCdc14B give rise to elongated cells with fragmented nuclei anomalously distributed between daughter cells and the spindles disrupted in most cases. A similar phenotype has been reported previously following the overproduction of mouse type 1 protein phosphatases in *S. pombe* cells (41), and it is also consistent with the chromosome segregation defects shown by type 1 protein phosphatase mutants in several organisms (42–45). Although the overexpression of the two hCdc14 phosphatases leads to defects in chromosome distribution during mitosis, their phenotypes are quite different, again suggesting the existence of different substrates for each of the two human phosphatases. In addition, it is clear that these mitotic defects are independent of phosphatase activity because we observed that overexpression of the catalytically inactive forms was able to cause the same effects. These results could be explained in terms of interactions with fission yeast proteins, which probably differ between the two hCdc14 homologues, affecting their function in *S. pombe*. It is also worth mentioning that the Flp1 overexpression phenotype observed in *S. pombe* cells is also induced by overexpression of the Flp1 phosphatase-dead variant (16).

Both hCdc14A and hCdc14B phosphatases are able to rescue some Δ*flp1* defects, but only hCdc14A can replace (at least to some extent) the *S. pombe* Flp1 phosphatase function acting through Cdc25. We propose that human Cdc14A, the phosphatase that shares some *S. cerevisiae* Cdc14 function (18, 21, 24), would act like the *S. pombe* homologue Flp1, dephosphorylating the major mitotic inducer Cdc25. This suggests that it could perform a similar function through some of the human Cdc25 homologues.

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