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Association of yeast SIN1 with the tetratrico peptide repeats of CDC23

(chromatin structure/protein–protein interaction/transcriptional repression/chromosome segregation)

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ABSTRACT **The yeast SIN1 protein is a nuclear protein that together with other proteins behaves as a transcriptional repressor of a family of genes. In addition, *sin1* mutants are defective in proper mitotic chromosome segregation. In an effort to understand the basis for these phenotypes, we employed the yeast two-hybrid system to identify proteins that interact with SIN1 *in vivo*. Here we demonstrate that CDC23, a protein known to be involved in sister chromatid separation during mitosis, is able to directly interact with SIN1. Furthermore, using recombinant molecules *in vitro*, we show that the N terminal of SIN1 is sufficient to bind a portion of CDC23 consisting solely of tetratrico peptide repeats. Earlier experiments identified the C-terminal domain of SIN1 to be responsible for interaction with a protein that binds the regulatory region of *HO*, a gene whose transcription is repressed by SIN1. Taken together with the results presented here, we suggest that SIN1 is a chromatin protein having at least a dual function: The N terminal of SIN1 interacts with the tetratrico peptide repeat domains of CDC23, a protein involved in chromosome segregation, whereas the C terminal of SIN1 binds proteins involved in transcriptional regulation.**

SIN1/SPT2 is a non-histone chromatin component in yeast that was first described as a negative transcriptional regulator of a family of genes (1–3). In addition, *sin1* mutants are defective in proper segregation of chromosome III but not chromosome V during mitosis (2). Detailed study has indicated that SIN1 functionally interacts with the C-terminal domain of RNA polymerase II (4), and that under certain conditions it can act as a positive transcriptional regulator (2). Analysis of the predicted amino acid sequence revealed an internal amino acid domain that bears sequence and potential structural similarity to mammalian HMG1 (2, 5, 6). SIN1 is known to be able to bind DNA nonspecifically (2). Recently, experimental evidence has been presented showing that the HMG-like domain can mediate SIN1 DNA binding (7).

In an effort to determine the biochemical basis for these disparate phenotypes, we used the yeast two-hybrid system (8–10) to identify proteins that directly associate with SIN1. Here we demonstrate that the N terminus of SIN1 is able to directly interact with the tandem tetratrico peptide repeat (TPR) domain of CDC23, a protein that is required for proper chromosome segregation.

MATERIALS AND METHODS

Plasmids and Yeast Strains. Plasmid pBTM116/SIN1 was constructed by subcloning the *Eco*RI fragment from pGEX-3X/SIN1 (11) containing the SIN1 coding region into the *Eco*RI site of pBTM116 (10). This construct contains the complete LexA protein coding sequence fused in-frame to the entire SIN1 coding sequence under the control of the yeast ADH1 promoter. pBTM116/SIN1 was transformed into yeast strain CTY10-5d (*MATa ade2, trp1-901, leu2-3, 112, his3-200, gal4, gal80, URA3::lexA op-lacZ*) (10). The pGAD libraries (9) were screened using the yeast two-hybrid system. Plasmid p519 was isolated from the libraries and was found to contain the entire *CDC23* coding region. Plasmid pSS527 was constructed by PCR amplification of the entire *CDC23* coding region using p519 as a template, and its insertion between the *Pst*I and *Bam*HI sites of pBluescript. The following plasmids were constructed by insertion of portions of CDC23 that had been amplified by PCR into pBluescript. In each plasmid, a stop codon was placed after the *CDC23* coding region. pSS918 contains DNA coding for amino acids 1–215 of CDC23 inserted between the *Bam*HI and *Hin*cII sites of pBluescript. pSS825 contains DNA coding for amino acids 1–254 of CDC23 inserted into the *Xba*I site of pBluescript. pSS761 contains DNA coding for amino acids 294–571 of CDC23 inserted between the *Sac*I and *Xba*I sites of pBluescript. Each construct was verified by DNA sequencing. The primers and templates used for the PCR amplifications of CDC23 were: Amino acids 1–215, forward primer CGGGATCCAGAACCATGAATG-ACGACAGC, reverse primer GCTCTAGAGTGCTAACC-CTAAATCGG, template pSS527; amino acids 1–254, forward primer as above, reverse primer GCTCTAGAACTCCAGC-CAGCAGGAC, template pSS527; amino acids 294–571, forward primer from within the vector, reverse primer GCTCTA-GATACCTTCCAGAAGTTCTTCCAC, template pSS634 (similar to pSS761, but does not contain a stop codon following the *CDC23* coding region).

Plasmids coding for the glutathione-*S*-transferase (GST)/SIN1 fusions illustrated in Fig. 2*a* were synthesized by amplification of portions of the *SIN1* gene in a PCR reaction, and subsequent subcloning into the vector pGEX-3X as described (11, 12). The peptide spanning amino acids 100–162 of SIN1 was translated from a nucleotide sequence amplified from primers GCGGATCCGGTTTAAGAGGTCTATTG and C-CGAATTCAACCTGGCTTGTTAAAATG. The peptide spanning amino acids 224–304 was translated from a nucleotide sequence amplified from primers GACGGATCCAATC-AAGATACCAGGATG and CCGAATTCTTGCCATTTC-CTCCTCTT.

DNA Sequencing. DNA sequencing of plasmids isolated from the pGAD libraries was performed by the Biological Services of the Weizmann Institute of Science using an Applied Biosystems 373 DNA Stretch Sequencer and dideoxy *Taq* terminators.

Oligonucleotide Synthesis. Oligonucleotides were synthesized by Biotechnology General (Kiryat Weizmann, Rehovot, Israel).

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Abbreviations: TPR, tetratrico peptide repeat; GST, glutathione-*S*-transferase; UAS, upstream activating sequence.

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Coupled Transcription/Translation. Radiolabeled [^{35}S]methionine-labeled CDC23 protein and its derivatives were synthesized in a coupled T3 transcription/translation system (TNT; Promega).

***In Vitro* Binding Assay.** Swelled glutathione (50 μl) agarose beads (Sigma) were suspended in binding buffer (50 mM Tris·Cl, pH 8.0/50 mM KCl/50 mM NaCl/5 mM $MgCl_2$/10% glycerol/0.5 mM EGTA/0.5 mM EDTA/0.1% Triton X-100/1 mM phenylmethylsulfonyl fluoride/1 mM dithiothreitol). Crude bacterial extract from *E. coli*, producing either GST/SIN1 fusions or GST alone (11, 12), were mixed with the glutathione agarose beads for 1 hr at 4°C. After washing the beads six times with 400 μl of binding buffer, the beads were incubated for 3 hr with 10 μl of the TNT reaction. Following another six rounds of washing with binding buffer, the bound radiolabeled peptides were eluted with 25 μl of binding buffer containing 100 mM glutathione (pH 8.0). The eluted peptides were subjected to SDS/PAGE and autoradiography.

RESULTS

The Two-Hybrid System Indicates That SIN1 and CDC23 Can Interact *in Vivo*. The coding region of *SIN1* was subcloned downstream and in-frame to LexA in plasmid pBTM116 (10). The resulting plasmid was transformed into yeast strain CTY10-5d (10), which contained two LexA operators in a UAS-less promoter upstream of a lacZ gene. Western blot analysis using anti-SIN1 antibodies directed against extracts from this transformed yeast strain confirmed high levels of expression of the LexA/SIN1 fusion protein (data not shown). The *Saccharomyces cerevisiae* pGAD libraries (9) were transformed into the yeast expressing the LexA/SIN1 fusion protein. About 350,000 colonies were screened for β-galactosidase activity, of which 12 were positive. Nine of the library plasmids recovered caused β-galactosidase expression independent of the LexA/SIN1 fusion and were discarded. The inserts in the three remaining plasmids were partially sequenced and compared against the GenBank/EMBL sequence databases. One plasmid was found to contain an in-frame GAL4 activating domain/CDC23 fusion encompassing the entire CDC23 coding region.

SIN1 and CDC23 Can Interact *in Vitro*. To determine whether the SIN1 and CDC23 proteins can interact directly, we synthesized SIN1 and CDC23 *in vitro* and asked whether they could bind each other. To do so, the entire *CDC23* coding region was amplified from the cloned library DNA using PCR and then subcloned into pBluescript such that the first *CDC23* codon was immediately downstream of the T3 promoter. Radiolabeled CDC23 was synthesized from this plasmid in a coupled transcription/translation system. SIN1 was expressed in *E. coli* as a fusion protein to GST. GST/SIN1 was bound to glutathione-agarose beads and was mixed with the radiolabeled CDC23. After washing, the proteins were eluted from the beads with glutathione, separated by SDS/PAGE, and autoradiographed. As can be seen in Fig. 1, the radiolabeled CDC23 bound the GST/SIN1 fusion protein, whereas it did not bind GST alone. This *in vitro* experiment demonstrates that the SIN1 and CDC23 molecules can interact with each other directly, without an intermediary yeast protein.

The N terminal of the SIN1 Molecule Is Sufficient to Interact with CDC23. Previous work has defined several hypothetical functional domains in SIN1 based on genetic, structural and biochemical considerations (Fig. 2*a*) (2, 6, 7, 12, 13). We therefore synthesized GST/sin1 fusions that contain portions of the SIN1 molecule based on the functional domains that have been suggested, bound them to glutathione-agarose beads, and asked whether the radiolabeled CDC23 molecule would bind the partial SIN1 molecule. As seen in Fig. 3, only peptides that contained the N-terminal domain of SIN1 (ami-

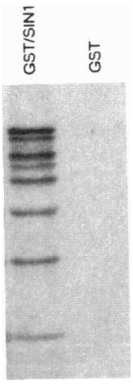

FIG. 1. Association between SIN1 and CDC23 proteins *in vitro*. GST or GST/SIN1 fusion proteins were immobilized on glutathione agarose beads and incubated with [^{35}S]methionine-labeled CDC23. After washing, bound proteins were eluted with glutathione, resolved by SDS/PAGE, and analyzed by autoradiography. The multiple bands represent partial CDC23 molecules that result from premature transcriptional and translational termination, internal transcriptional initiation, and RNA and protein degradation.

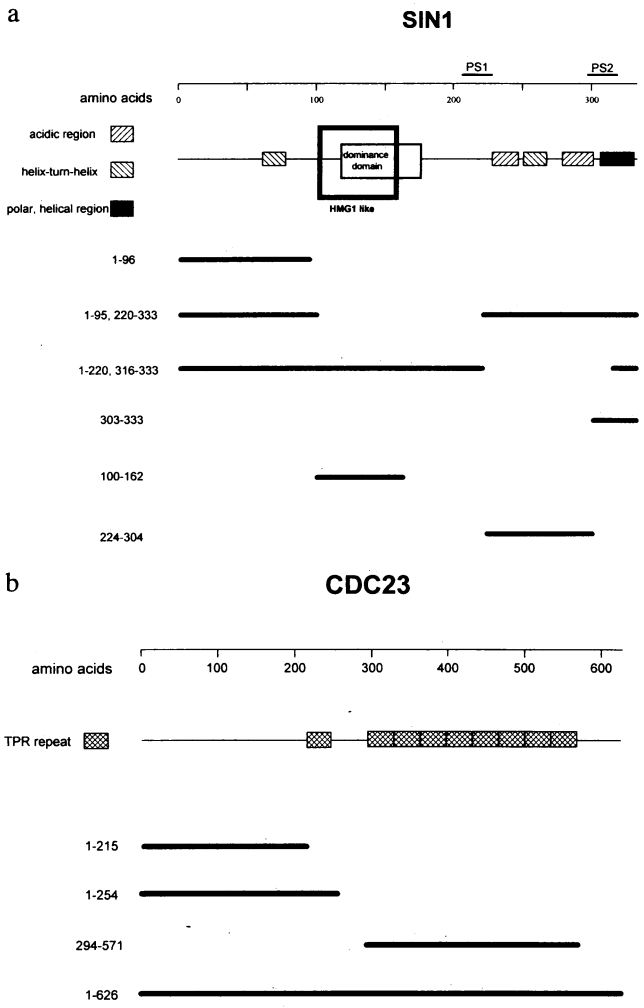

FIG. 2. Salient features of the SIN1 and CDC23 molecules. (*a*) SIN1. The functional regions are those defined by Lefebvre and Smith (13). PS1 and PS2 represent the synthetic peptides against which the SIN1 antibodies were raised. The lower part of the figure illustrates the portions of SIN1 that were produced as GST fusion proteins. (*b*) CDC23. The TPR repeats (14) are noted. The lower part of the figure illustrates the portions of CDC23 that were produced in the transcription/translation reactions.

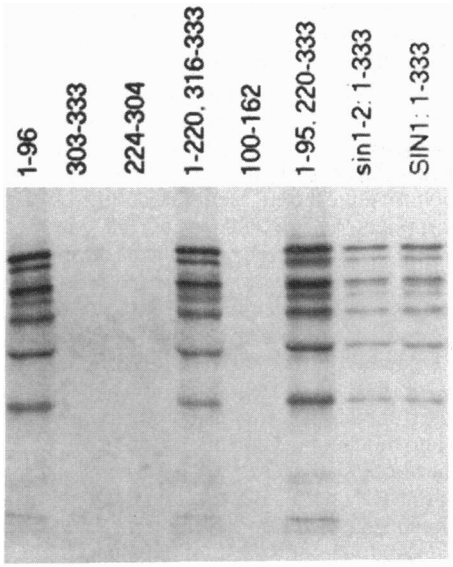

FIG. 3. Association of the N terminal of SIN1 with CDC23. GST/SIN1 fusions illustrated in Fig. 2*a* were bound to glutathione agarose beads and tested for CDC23 binding as described in Fig. 1. The *sin1–2* point mutation near the C terminus of SIN1 (11) does not affect the SIN1–CDC23 interaction.

no acids 1–96) bound the CDC23, whereas SIN1 molecules containing solely the "HMG domain" (amino acids 100–162), the "acidic domain" (amino acids 224–304), or the C terminal (amino acids 303–333) of SIN1 did not bind CDC23.

TPR Repeats 2–9 in CDC23 Are Sufficient to Mediate Binding to SIN1. As shown in Fig. 2*b*, CDC23 contains 9 TPR units (14). The first one is flanked by other sequences, whereas TPR units 2–9 are contiguous. With this structure as a guide, we designed peptides that were examined for interaction with SIN1. Employing PCR, we amplified portions of the *CDC23* gene and subcloned them into pBluescript. Using transcription/translation, we synthesized various radiolabeled peptides containing amino acids 1–215, amino acids 1–254 (including the N terminus and the first TPR unit), or amino acids 294–571 (containing only the block of contiguous TPR units 2–9). These polypeptides were tested for SIN1 binding. The left panel of Fig. 4 shows that only the protein transcribed from the full-length *CDC23* gene and the peptide that contained TPR units 2–9 specifically bound SIN1. This indicated that the TPR units 2–9 themselves are responsible for the interaction with SIN1. To finally prove this point, we used the SIN1 N-terminal domain/GST fusion protein to bind the various CDC23 peptides. As shown in Fig. 4, the N terminal of SIN1 and the entire SIN1 molecule bound the CDC23 peptides identically.

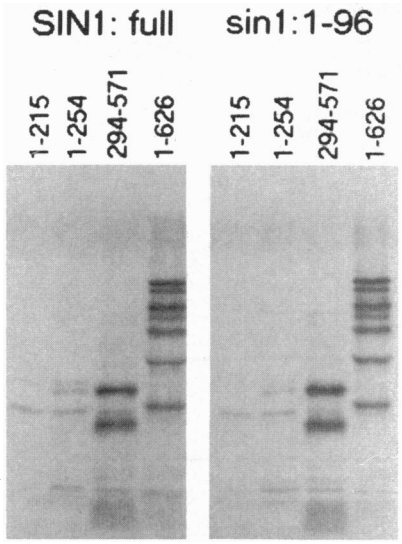

FIG. 4. *In vitro* binding between the TPR repeats of CDC23 and SIN1. CDC23 molecules were translated from portions of the *CDC23* gene shown in Fig. 2*b*. These peptides were tested for binding to full-length SIN1 (*left*) or the N terminal of SIN1 amino acids 1–96 (*right*) as described in Fig. 1.

DISCUSSION

It has been proposed that TPR repeats are responsible for protein–protein interactions in cellular processes ranging from transcription to mitosis (14–17). However, only recently have TPR repeats been shown to directly interact with other proteins. The TPR repeats of CDC27 interact with CDC23 (15) and the TPR repeats 1–3 of CYC8 interact with TUP1 (17). Our results indicate that the TPR repeats of CDC23 interact with the N terminal of SIN1. We note that this region of SIN1 contains a potential helix–turn–helix (13), which may be required for this association, as has been previously suggested (17).

CDC23 has been shown to be part of a large complex that includes CDC16 and CDC27 (15). This complex is required for the onset of anaphase by facilitating sister chromatid separation at the metaphase to anaphase transition. Human homologues of the CDC16 and CDC27 proteins colocalize to the centrosomes and the mitotic spindle (18). A 20S complex containing CDC27 and CDC16 (and presumably CDC23) homologues has been isolated from *Xenopus* egg extracts, which catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B (19). CDC23 and CDC16 in yeast have been shown to be required for the ubiquitin mediated proteolysis of the B-type cyclin CLB2, which is necessary for exit from mitosis (20). Taken together, these and other data suggest that CDC23 is part of a complex that promotes proteolysis of proteins that hold sister chromatid together, and of cyclin B during anaphase and G1.

Because *sin1* mutants are defective in chromosome segregation, we believe that it is particularly significant that SIN1 binds CDC23. This observation leads us to speculate that the SIN1 molecule may serve as a scaffold upon which CDC23 and possibly other molecules involved in chromosome segregation are attached. Inappropriate tethering of the SIN1 molecule to the chromatin, as can be the case in *sin1* mutants, would result in improper chromosome segregation. Alternative explanations for the SIN1–CDC23 interaction are possible as well, such as the CDC23 targeting of SIN1 for degredation.

Previous work on *SIN1* has concentrated on its role in transcriptional regulation. Although SIN1 does not bind DNA in a sequence specific manner, it does participate in the transcriptional regulation of a family of genes including *HO*, *INO1*, and the glucocorticoid receptor when expressed in yeast (2, 4, 11, 13, 21). In earlier work, we showed that the C terminus of the SIN1 molecule interacts with a protein that binds the *HO* promoter (11, 12). We suggested that the transcriptional regulation of *HO* and other genes by SIN1 may be mediated by sequence-specific DNA-binding proteins such as the one we identified.

Various components of the chromatin affect a number of different cellular functions including recombination (21), mitosis (18), and transcription (22). We find it especially interesting that two distinct domains of the SIN1 molecule appear to be involved in two different cellular functions. The domain of SIN1 associating with CDC23 is found at the N terminus of the molecule, whereas the C terminus associates with a protein that binds the regulatory region of *HO*. A central portion of the SIN1 molecule has sequence and potential structural similarity to mammalian HMG1, and presumably mediates SIN1 binding to DNA. We believe that it is likely that SIN1 is involved in

both transcriptional repression and the control of chromosome segregation by serving as an anchor in the chromatin for molecules that are involved in controlling both processes. This affords the cell the possibility to conserve valuable space in the chromatin by assigning two roles to the same molecule. Further experiments are required to determine how the respective proteins that interact with SIN1 regulate transcription and mitotic chromosome segregation.

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