The Activity of Cdc14p, an Oligomeric Dual Specificity Protein Phosphatase from *Saccharomyces cerevisiae*, Is Required for Cell Cycle Progression*

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The essential CDC14 gene of the budding yeast, *Saccharomyces cerevisiae*, encodes a 62-kDa protein containing a sequence that conforms to the active site motif found in all enzymes of the protein tyrosine phosphatase superfamily. Genetic studies suggest that Cdc14p may be involved in the initiation of DNA replication, but its precise cell cycle function is unknown. Recombinant Cdc14p was produced in bacteria, characterized, and shown to be a dual specificity protein phosphatase. Polyansions such as polyglutamate and double-stranded and single-stranded DNA bind to Cdc14p and affect its activity. Native molecular weights of 131,000 and 189,000 determined by two independent methods indicate that recombinant Cdc14p self-associates in *vivo* to form active oligomers. The catalytically inactive Cdc14p C283S/R289A mutant is not able to suppress the temperature sensitivity of a cdc14–1* mutant nor replace the wild type gene in *vivo*, demonstrating that phosphatase activity is required for the cell cycle function of Cdc14p. A distinctive COOH-terminal segment (residues 375–551) is rich in Asn and Ser residues, carries a net positive charge, and contains two tandem 21-residue repeats. This COOH-terminal segment is not required for activity, for oligomerization, or for the critical cell cycle function of Cdc14p.

Genetic analyses of temperature-sensitive CDC mutants as well as biochemical studies using *Xenopus* oocytes have revealed that reversible protein phosphorylation is a major mechanism for regulating cell cycle progression (1). Transitions in the cell cycle are coordinated by changes in the activity of kinases and phosphatases and in the phosphorylation state of their target proteins. CDC genes as well as a number of other genes that have been linked to cell cycle progression in *Saccharomyces cerevisiae* encode protein kinases or protein phosphatases. The CDC14 gene sequence has confirmed several errors in the original sequence (2) that when corrected result in a larger predicted open reading frame encoding a 62-kDa protein. We demonstrate herein that Cdc14p is an oligomeric, dual specificity phosphatase that is completely dependent on an active site cysteine for activity. We have shown that its phosphatase activity is required for its cell cycle function and have also found that the Asn/Ser-rich COOH-terminal domain is not required for activity, for oligomerization, or for the critical cell cycle function executed by Cdc14p.

**EXPERIMENTAL PROCEDURES**

*Yeast Strains and Media*—The following yeast strains were obtained from the Yeast Genetic Stock Center: T–41 (MATa ade1 ade2 ura1 his7 lys2 tyr1 gal1 cdc14–1), YHP2 (MATa ura3–52), and BJS465 (MATa trp1 ura3–52 leu2–Δ1 his3Δ200 pep4::HIS3 prb1ΔL6R can1 GAL¹). Strain DBY746 (MATa his3–Δ1 leu2–3 leu2–112 ura3–52 trpl–129 gal1) and CG219–2 (MATa ura3–52 gal1) were provided by G. Kohlhaw. Strains YLC1 (MATa trp1–129 gal1 cdc14–1) and YLC11 (MATa ura3–52 cdc14–1) were generated for this study using strains T–41, DBY746, and CG219–2. Strain DBYLCS5 (MATa his3–Δ1 leu2–3 leu2–112 ura3–52 trpl–129 gal1 cdc14–1 HIS3), which requires plasmid pYL56 (CEN6 ARSH4 URA3 CDC14) for viability, was prepared as described below. YPD, SD, and sporulation media were prepared as described (15, 16). Unless indicated otherwise, yeast were grown at 30°C. Yeast transformations were performed using a lithium acetate protocol (16).

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Cloning of CDC14—A λ phage genomic clone (17) of *S. cerevisiae* (70468) that contains a fragment (~7 kb) of chromosome VI and the CDC14 gene was obtained from the American Type Culture Collection. Phage DNA was digested with Smal and XhoI to yield a 2.6-kb fragment containing the CDC14 gene (2). This clone was inserted into pBluescript II KS (+) (Stratagene) to create pBSC15. To express Cdc14p, pBSC15 was used to create pBSC16 in which Cys<sup>235</sup> of the Cdc14p coding sequence is replaced by Ser. The authenticity of all plasmids carrying inserts generated by PCR or modified by site-directed mutagenesis was verified by nucleotide sequencing.

**Yeast Plasmids**—The wild type CDC14 gene and five mutant alleles were cloned into the XhoI site of pBluescript II KS (+) that was used as a plasmid vector for *S. cerevisiae* and transformed into the strain DBYLC5 (CEN6 ARSH4 URA3 TRP1 HIS3 ARSH4). The expression plasmid pET-GSTx-Cdc14 was made using the sequence CDC14 was amplified by PCR using the lambda clone shuttle vector pIde130 (Novagen). Complementary oligonucleotides encoding the HA epitope and the EcoRI-compatible overhangs at their 5′- and 3′-ends were used to anneal and ligate into pYX112 to create pET-GSTx-Cdc14. The pET-GSTx-Cdc14 plasmid was in frame with the corresponding fragment derived from pYL55 to create plasmid pXL107, which encodes HA-Cdc14p (C283S/R289A).

**Construction of the CDC14 Deletion Mutant**—The CDC14 gene was disrupted by the one-step gene replacement procedure (20) to create strain DBYLC5. A fragment containing the HIS3 gene was inserted into the StuI/EcoRV sites within the plasmid pYL56 to create pYL57, which was digested with *Pvu*I and inserted into a linear fragment containing the CDC14 gene. The HIS3 insertion deleted base +465 to base +1473, equivalent to 336 amino acid residues (61%) of the CDC14 coding region. Strain DBY746 (MATa his3 leu2 ura3 trp1 gal1) was first transformed with pY56, which carries the CDC14 and URA3 genes, and then transformed with linearized pYL57 carrying the disrupted CDC14 gene and plated on media to select for URA<sup>+</sup> and HIS<sup>+</sup> cells carrying the disruption. PCR was used to verify the disruption of the CDC14 gene in DBYLC5.

**Plasmid Shuttle Assay**—A plasmid shuttle assay (21) was used to ascertain whether plasmids bearing various CDC14 mutants could replace the pYL56 plasmid (CEN6 ARSH4 CDC14 URA3), which is required for viability of the strain DBYLC5 (MATa his3 leu2 ura3 trp1 gal1 CDC14:HY3). The assay employs 5-fluoroorotic acid (5-FOA) for negative selection of the pYL56 plasmid. Strain DBYLC5 cells were transformed with a second plasmid containing one of the five CDC14 mutants (pYL2, pYL3, pYL4, pYL10, or pYL55). Trp<sup>+</sup> transformants were selected and either directly plated or grown overnight in liquid media at 30 °C before streaking on negative selection media (SD plates supplemented with leucine, uracil, and 5-FOA). Growth on 5-FOA plates demonstrated the ability of a cd14 mutant to complement the null mutation.

**Construction of Plasmids for Expression of Glutathione S-Transferase (GST) Fusion Proteins**—Thrombin-cleavable GST fusion proteins of wild type and mutant Cdc14p were expressed in *E. coli* under the control of the T7 polymerase promoter using pET-GST vectors, which were constructed as follows. A fragment containing the GST coding sequence, a glycy linker, and a thrombin cleavage site was obtained from pGEX-2T (22) by PCR and inserted into the BamHI-EcoRI sites of pET21b to create pET-GST, which encodes GST containing an 11-residue T7 epitope tag at its NH<sub>2</sub> terminus. An XhoI fragment containing the entire CDC14 coding region was removed from pBSC15 and cloned into pET-GST to create pET-GST-Cdc14. The expression plasmid pET-GST-Cdc14 was made using a PCR-based strategy to replace a segment (bases +1123 to +1473) from the open reading frame of pET-GST-Cdc14 with the sequence TAAAGATATCCGCCGC, which creates a stop codon at amino acid position 375. Plasmids for expression of the Cdc14p mutants were prepared by replacing a P7MI/AflI fragment from pET-GST-Cdc14 and pET-GST-Cdc14(1–374) with a corresponding fragment derived from pBSC16.

When treated with thrombin, fusion proteins expressed from the pET-GST vectors described above were cleaved at internal sites within Cdc14p as well as the site within the linker. The pET-GSTx vectors, which have a different sequence flanking the thrombin cleavage site, produce higher yields of intact Cdc14p upon thrombin cleavage; these expression vectors were constructed as follows. A DNA fragment encoding GST, the glycine linker, and the thrombin cleavage site from pGEX-KT (22) was generated by PCR and cloned into the NdeI and BamHI sites of pET-21a to create pET-GSTx. Using PCR, the entire coding sequence for Cdc14p was amplified and inserted into the BamHI-XhoI sites of pET-GSTx to create pET-GSTx-Cdc14. The pET-GSTx-Cdc14(1–374) was constructed by excising a fragment of the yeast plasmid pYL3 (see above) and inserting it into pET-GSTx-Cdc14.

**Plasmids for Expression of Epitope-tagged Cdc14p**—Cdc14p mutants containing an NH<sub>2</sub>-terminal epitope tag from human influenza virus hemagglutinin (HA) were constitutively overexpressed in the strain DBYLC5 containing the trpl<sup>−</sup> ura3<sup>−</sup> trp1<sup>−</sup> his3<sup>−</sup> and encodes Cdc14p-(1–374); pYL4 harbors an insert in which the segment containing a stop codon at position 375 (bases 1122 to 1125) was deleted and replaced by a 14-nucleotide fragment containing released Cdc14p was made 1 and 300 mM in phenylmethylsulfonyl fluoride and NaCl, respectively. Fractionation of the released Cdc14p was made using a Mono Q HR5/5 column using a Pharmacia FPLC system. After washing with 5 ml of buffer A, the column was eluted at a flow rate of 1 ml/min with a 25-min linear gradient from 0 to 500 mM NaCl in buffer A followed by a 5-min linear gradient from 500 to 1000 mM NaCl in buffer A. GST-Cdc14p was recovered in three or four overlapping peaks, which eluted between 300 and 370 mM NaCl. Fractions from all of the peaks were combined, since the GST-Cdc14p from each fraction was indistinguishable with respect to SDS-PAGE mobility and activity.

**Thrombin Cleavage**—GST fusion proteins were expressed using pET-GSTx vectors described above except that prior to affinity purification nucleic acids were removed from bacterial lysates by treatment with polyethyleneimine according to the method of Burgess (24). After the Mono Q separation, purified GST fusion protein was bound to glutathione-agarose beads and treated with thrombin (3000 NIH units/mg) for 80 min at room temperature using a protease-substrate mixture containing 1:1000 (w/v). After collection, the bead supernatant containing released Cdc14p was washed with 300 and 300 ml in phenylmethylsulfonyl fluoride and NaCl, respectively.

**Preparation of Phosphorylated Substrates**—Myelin basic protein (MBP) (Life Technologies, Inc.), casein (Sigma), RCML (Life Technologies), Raytide (Oncogene Science), RR-Src (Life Technologies), or angiotensin (Sigma) was radiolabeled and phosphorylated on tyrosine residues using recombinant GST-tyrosine kinase as described previously (25).
Phosphoamino acid analysis of substrates confirmed the presence of phosphoryrosine and the absence of phosphothreonine residues.

MBP, casein, histone H1 (Sigma), and Leu-Arg-Ag-Ala-Ser-Leu-Gly (Kemptide) (Sigma) were phosphorylated on serine or threonine residues using the catalytic subunit of bovine cAMP-dependent protein kinase (26) as described by Kishimoto et al. (27). Phosphoamino acid analysis showed that MBP and histone H1 contained phosphoserine/threonine residues, whereas only phosphoserine was detected in casein.

Phosphatase Assays—Activity with pNPP was measured at 30 °C as described (25) in reaction buffer containing 50 mM imidazole, pH 6.9, 1 mM DTT, 1 mM EDTA, 20 mM pNPP, and 200–1000 ng of enzyme. Assays with 5–10 μM phosphorylated protein and peptide substrates were performed at 30 °C in buffer containing 50 mM imidazole, pH 6.6, 1 mM DTT, 1 mM EDTA, and 20–200 ng of enzyme as described (25).

Generation of Polyclonal Antibodies against Cdc14p—Cdc14p prepared as described above was further purified by electrophoresis on 12% SDS-polyacrylamide gels. Gel slices containing only full-length Cdc14p were fragmented by passage through a 21-gauge needle as described (28) and injected subcutaneously into a rabbit without adjuvant. Standard antigen injection and serum collection protocols were used (28).

Antiserum was affinity-purified essentially as described by Olmsted (29) except that the antigen, GST-Cdc14p, was immobilized on poly-vinylidene difluoride membrane following SDS-PAGE. Affinity-purified antibodies were conjugated to 1 mg antibody using an ultrafiltration device (Amicon) and stored at 4 °C. For immunoprecipitation, anti-Cdc14p antibodies were purified from serum using protein A-Sepharose (28).

Immunoprecipitation and Immunoblotting of Cdc14p and HA-Cdc14p—To immunoprecipitate Cdc14p from yeast, cells (strain BJ5465) were grown to mid-log phase (0.5–1.5 × 10^6 cells/ml) collected by centrifugation, washed in cold buffer H (50 mM imidazole, pH 7.0, 2 mM EDTA, 5 mM benzamidine, 5 mM phenylmethylsulfonyl fluoride containing 5 μg/ml each leupeptin, aprotinin, and pepstatin), and re-suspended in buffer H (250 mM/liter of yeast culture). Cells were mixed with an equal volume of glass beads and lysed by vortexing eight times for 30 s each. The lysate was centrifuged at 4 °C for 10 min at 15,000 × g, and Cdc14p was immunoprecipitated from the supernatant by the addition of anti-Cdc14p antibodies (100 μg/ml) for 2 h at 4 °C followed by incubation with protein A-Sepharose (200 μl/mg antibody added) for 1 hour. After washing with buffer H containing 1% (v/v) Triton X-100, beads were treated with 2 × SDS sample buffer. For immunoprecipitation of HA-tagged proteins, cell cultures (strain YLC11 transformed with pYLY105 or pYLY107) were grown to mid-log phase, harvested as described above, lysed by sonication, and centrifuged at 4 °C for 15 min at 15,000 × g. An aliquot of supernatant containing about 1 mg of protein was mixed with 5 μg of anti-hemagglutinin 12CA5 monoclonal antibody (Boehringer Mannheim) for 1 h at 4 °C and treated with protein A-Sepharose.

Immunoprecipitates were separated on SDS gels and electrophoresed to polyvinylidene difluoride membranes (Millipore) that were blocked with TBS buffer (1% [w/v] dry milk). Membranes were probed with affinity-purified anti-Cdc14p antibodies (0.5 μg/ml) for 2 h. Blots were visualized with the ECL system (Amersham Corp.) and donkey anti-rabbit IgG-horseradish peroxidase conjugates according to the manufacturer's protocols.

Molecular Weight Determinations—Native molecular weights were determined from Ferguson plots derived from the results of nondenaturing gel electrophoresis performed with running gels containing four different polyacrylamide concentrations (30). The Svedberg equation was used to calculate native molecular weights of proteins using their Stokes radii, sedimentation coefficients, and partial specific volumes. Stokes radii were determined by gel filtration chromatography on a Sephacryl S300 (Pharmacia) column (1.5 × 97 cm) eluted in 50 mM Tris, pH 8.0, 300 mM NaCl, 2 mM EDTA, 0.1% (v/v) β-mercaptoethanol at a flow rate of 15 ml/h. The Stokes radii of unknowns were estimated using a plot of Ke versus the log Stokes radii of six standards. Sedimentation coefficients were estimated using glycerol density gradient sedimentation as described (31). Linear 15–50% (v/v) glycerol gradients in 10 ml of buffer containing 50 mM imidazole, pH 7.0, 300 mM NaCl, 1 mM EDTA, and 1 mM DTT were formed and centrifuged in a SW41 Ti rotor at 41,000 rpm (sed = 288,000) for 24 h at 4 °C. The following protein standards were used for calibration of the gradient: bovine catalase (Ke,0.2 = 11.3), bovine serum albumin (Ke,0.2 = 4.3), rabbit aldolase (Ke,0.2 = 7.4), and horse cytochrome c (Ke,0.2 = 1.9). Partial specific volumes were calculated from the amino acid composition as described by Cohn and Edsall (32).

RESULTS

Data from this study (Fig. 1), the yeast genome sequencing project, and several other laboratories (14, 34) have confirmed that the original nucleotide sequence (GenBank™ M61194) reported for the CDC14 gene (2) contains several errors. The sequence (GenBank™ D50617) reported by Eki et al. (34) is identical to that reported here. The sequence (GenBank™ D55715) of Shirayama et al. (14) differs in having a C in place of G at position +352 in Fig. 1, which results in substitution of Pro for Ala at amino acid residue 118. This difference may be attributed to allelic variation.

The corrected sequence contains an open reading frame that is much larger than that which originally reported (2). As shown in Fig. 1, the CDC14 gene contains a 1653-bp open reading frame that encodes a protein of 551 amino acids with a predicted molecular mass of 61,906 Da and a calculated pI of 8.0. Sequences adjacent to the putative initiator codon give a good match to the consensus sequence thought to be required for efficient translation initiation in yeast (35). The location of the putative ATG start codon at a site where 20 of the 30 upstream nucleotides are adenines is not uncommon for yeast genes, which frequently contain A-rich 5′-leader sequences (35). The sequence, TATAAT, located at −63 to −58, fits the consensus for eukaryotic TATA boxes that typically precede the transcription start site by 20–30 nucleotides. The length of the deduced protein sequence is consistent with the size (1.9 kb) of the CDC14 transcript previously determined by Northern analysis (2).

The sequence, HCKAGLGRGRT, located at residues 282–291 of Cdc14p (Fig. 1), fits the consensus sequence, HCXAGXXR(S/T), found at the active site of all protein tyrosine phosphatases (3–5). This phosphatase superfamily can be divided into distinct subfamilies, those that are tyrosine-specific and those that exhibit dual specificity because they are also capable of hydrolyzing phosphoserine/threonine residues. Aside from a 20-residue segment encompassing the active site motif, data base searches reveal that Cdc14p exhibits little similarity to the tyrosine-specific phosphatases and does not contain the 240-residue conserved domain that is found in this group of enzymes (3, 4). Previous sequence comparisons (25) suggested that Cdc14p might be a dual specificity enzyme because it is most closely related to BVP, a dual specificity phosphatase encoded by an insect virus.

The schematic diagram in Fig. 1B illustrates the structural organization of Cdc14p. Sequence alignments (25) with BVP and two other closely related homologs suggest that there is a 170-residue core structure that contains the highly conserved active site. Like most other protein tyrosine phosphatases, the catalytic core appears to be flanked by N- and COOH-terminal noncatalytic segments that exhibit no significant similarities to sequences of known proteins. The sequence 375–551 from the COOH terminus of Cdc14p has several notable features including an unusually high content of Asn (13.6 mol %) and Ser (15.3 mol %) residues and a large net positive charge (calculated pI = 10.1). As shown in Fig. 1C, the basic, Asn/Ser-rich COOH-terminal segment contains two internally homologous 21-residue repeats (33% sequence identity) located at residues 400–420 and 423–443. The structural and/or functional significance of these tandem repeats is unknown.

Detection of Endogenous Cdc14p in S. cerevisiae—Endogenously expressed Cdc14p was detected in asynchronous cultures of a protease-deficient strain of yeast (BJ5465) using

anti-Cdc14p antibodies. Immunoprecipitates prepared from crude cell lysates were separated on SDS gels and immuno-
blotted using affinity-purified anti-Cdc14p antibodies. As
shown in Fig. 2, a single cross-reactive band with a mobility
identical to that of recombinant Cdc14p was observed in yeast
extracts. The identity in size of the recombinant and en-
dogenous enzymes is fully consistent with the assignment of
the start codon and the size of the open reading frame deduced
from the nucleotide sequence shown in Fig. 1.

Expression and Purification of GST-Cdc14p—
Cdc14p was
expressed as a cleavable GST fusion protein in
E. coli
BL21
(DE3) cells carrying the pET-21b GST-Cdc14 plasmid. As
shown in Fig. 3,
lane 2,
isopropyl thioc-
b-
D-galactopyranoside
induced the expression of protein with an apparent molecular
weight of 90,000, which is in agreement with the calculated
value of 90,359. Nearly all of the fusion protein was recovered
in the soluble fraction (Fig. 3,
lane 4)
of bacterial lysates,
yielding approximately 15 mg/liter of culture. After glutathi-
one-agarose affinity purification, the yield of GST-Cdc14p was
about 5 mg/liter of culture (Fig. 3,
lane 5). Affinity-purified
GST-Cdc14p was subjected to Mono Q FPLC ion exchange
chromatography as described under “Experimental Proce-
dures” to reduce the level of a contaminant identified as GST by
immunoblotting and to remove a significant quantity of bacte-
rial nucleic acids that were not eliminated by affinity purifica-
tion (Fig. 3,
lane 6). To ensure complete removal of nucleic acid
from the protein used for the size and subunit analyses de-
scribed below, bacterial lysates were also treated with polyeth-
yleneimine prior to affinity purification.

Phosphatase Activity of GST-Cdc14p—
GST-Cdc14p dephos-
phorylated Tyr(P)-MBP, Tyr(P)-casein, Ser/Thr(P)-MBP, and
Ser(P)-casein in a reaction that was linear with respect to time
(Fig. 4, A and B) and the amount of enzyme added (data not

Fig. 1. Nucleotide sequence of CDC14 and the predicted amino acid sequence of Cdc14p. A, amino acids are given in single letter code
and are numbered on the right. The nucleotide sequence is also numbered on the right beginning with the first base of the ATG initiator codon.
A putative TATA-box motif is shown in boldface type. The highly conserved active site motif is enclosed in a box. This sequence is identical to that
reported by Eki et al. (34) and can be found at the following accession numbers: GenBank 50617TM, PIR S56283. B, schematic diagram illustrating
the structural organization of Cdc14p. The positions of a conserved catalytic core (solid black box) and an Asn/Ser-rich COOH-terminal domain
(shaded) are shown. The position of the highly conserved active site region and the locations of two 21-residue tandem repeats (hatched boxes) are
indicated. C, sequence alignment of the two internally homologous tandem repeats from Cdc14p.
Cdc14p is an Oligomeric Dual Specificity Phosphatase

The substrate pNPP was hydrolyzed at 30 °C with a pH optimum of 6.9, $K_m$ of 4 mM, and $k_{cat}$ of 1.7 s⁻¹. GST-Cdc14p ($k_{cat}/K_m = 480 \text{M}^{-1}\text{s}^{-1}$) hydrolyzes pNPP more effectively than three other dual specificity enzymes, GST-BVP, GST-cdc25p, and VH6, which have $k_{cat}/K_m$ values of 17, 16, and 1.6 M⁻¹ s⁻¹, respectively, but is about 7-fold less efficient than VHR, which has a $k_{cat}/K_m$ of 3200 M⁻¹ s⁻¹ (25, 36–38). The 140-fold higher $k_{cat}/K_m$ value measured for the T cell protein tyrosine phosphatase³ is typical of the tyrosine-specific enzymes, most of which have $k_{cat}/K_m$ values at least 2–3 orders of magnitude larger than those of the dual specificity enzymes (39, 40).

Cdc14p was separated by GST by thrombin cleavage to compare the activities of the carrier-free recombinant enzyme and the fusion protein. Enzyme generated by thrombin cleavage is herein designated as rCdc14p to denote that it contains four NH₂-terminal residues (GSGS) not found in the native protein. When expressed on a molar basis, the activities of rCdc14p and the fusion protein toward pNPP were nearly identical (data not shown). In contrast, the activity of rCdc14p toward both Tyr(P)- and Ser/Thr(P)-MBP was about 1.6-fold higher than that for the fusion protein (data not shown), indicating that presence of GST at its NH₃ terminus reduces the activity of rCdc14p toward protein substrates by about one-third.

GST-Cdc14p C283S, a mutant in which the Cys located in the putative active site region was replaced by Ser, exhibited no detectable activity with all substrates that were tested (Table I). The lack of activity with this mutant shows that Cys²⁸³ is essential for activity and confirms that the observed phosphatase activity is catalyzed by GST-Cdc14p rather than a contaminating bacterial enzyme. These results demonstrate that Cdc14p is capable of dephosphorylating phosphotyrosine and phosphoserine/threonine residues and is a dual specificity phosphatase.

Effect of Salt Concentration on Activity—The activity of GST-Cdc14p is highly sensitive to ionic strength. Increasing NaCl concentration results in the reduction of activity toward both pNPP and protein substrates (data not shown). At 170 mM NaCl, phosphatase activity with pNPP and Tyr(P)-MBP was about 50% of that measured in low ionic strength assay buffer. At 400 mM NaCl, the activity was 36 and 14% of control with pNPP and Tyr(P)-MBP, respectively.

Inhibitors and Activators of Phosphatase Activity—The effect of classic phosphatase inhibitors and other potential effectors on the activity of GST-Cdc14p was examined using three different substrates as shown in Table II. GST-Cdc14p was insensitive to tetratetanol, which inhibited three other dual specificity phosphatases, respectively. The fusion protein was not sensitive to either NaF, which inhibits the protein tyrosine phosphatases, or 100 mM okadaic acid, an inhibitor of both type 1 and type 2A protein Ser/Thr phosphatases. In contrast, sodium vanadate and sodium tungstate, which are potent inhibitors of both protein tyrosine and dual specificity phosphatases, effectively inhibited GST-Cdc14p (Table II). These findings in combination with the aforementioned effects of the Cys²⁸³ mutation support our identification of Cdc14p as

³ G. Taylor and H. Charbonneau, unpublished observations.
Cdc14p is an Oligomeric Dual Specificity Phosphatase

Specific activities were determined from assays performed at 30 °C using the indicated quantities of substrates. Reactions with pNPP were carried out in 50 mM imidazole, pH 6.9, 1 mM DTT, and 1 mM EDTA. Assays with protein and peptide substrates were performed in 50 mM imidazole, pH 6.6, 1 mM DTT, and 1 mM EDTA. The concentrations of peptide or protein substrates given below indicate the total concentration of phosphorylated residues. Protein was determined by the method of Bradford (33) using bovine serum albumin as a standard.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration</th>
<th>Activity</th>
<th>Activity</th>
<th>Activity</th>
</tr>
</thead>
</table>
| pNPP            | 20,000 µM     | 1200     | 101      | 0
| Tyr(P)-MBP      | 5 µM          | 31       | 24       | 0
| Tyr(P)-casein   | 5 µM          | 10       | 0        | 0
| Tyr(P)-RR-Src   | 10 µM         | 10       | 0        | 0
| Tyr(P)-Raytide  | 45 µM         | 1        | 0        | 0
| Tyr(P)-antigenin| 5 µM          | 0        | 0        | 0
| Ser/Thr(P)-MBP  | 5 µM          | 8        | 11       | 0
| Ser(P)-casein   | 5 µM          | 7        | 0        | 0
| Ser/Thr-P-histone H1 | 5 µM | 1 | 0 | 0
| Ser(P)-Kemptide | 15 µM         | 0        | 0        | 0

$^a$ Zero indicates that no phosphatase activity was detected in assays in which the lower limit of detection was 0.1–0.2 pmol of phosphate released.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
<th>pNPP</th>
<th>Tyr(P)-MBP</th>
<th>Ser/Thr(P)-MBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 µg/ml</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NaF</td>
<td>20 mM</td>
<td>98</td>
<td>102</td>
<td>78</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>100 mM</td>
<td>101</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Tartrate</td>
<td>5 mM</td>
<td>99</td>
<td>103</td>
<td>84</td>
</tr>
<tr>
<td>Tetramisole</td>
<td>100 µM</td>
<td>100</td>
<td>91</td>
<td>94</td>
</tr>
<tr>
<td>Sodium vanadate</td>
<td>200 µM</td>
<td>5</td>
<td>48</td>
<td>33</td>
</tr>
<tr>
<td>Sodium tungstate</td>
<td>200 µM</td>
<td>6</td>
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<td>0</td>
</tr>
<tr>
<td>ZnCl$_2$</td>
<td>1 mM</td>
<td>94</td>
<td>90</td>
<td>101</td>
</tr>
<tr>
<td>Spermidine</td>
<td>2 mM</td>
<td>94</td>
<td>86</td>
<td>53</td>
</tr>
<tr>
<td>EDTA</td>
<td>2.5 mM</td>
<td>97</td>
<td>108</td>
<td>106</td>
</tr>
<tr>
<td>ATP</td>
<td>1 mM</td>
<td>94</td>
<td>204</td>
<td>186</td>
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<tr>
<td>GTP</td>
<td>1 mM</td>
<td>90</td>
<td>226</td>
<td>199</td>
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<tr>
<td>cAMP</td>
<td>1 mM</td>
<td>47</td>
<td>220</td>
<td>228</td>
</tr>
<tr>
<td>Heparin</td>
<td>20 µg/ml</td>
<td>37</td>
<td>229</td>
<td>ND$^b$</td>
</tr>
<tr>
<td>Polyglutamate</td>
<td>100 µg/ml</td>
<td>34</td>
<td>370</td>
<td>395</td>
</tr>
<tr>
<td>dsDNA$^c$</td>
<td>100 µg/ml</td>
<td>56</td>
<td>387</td>
<td>258</td>
</tr>
<tr>
<td>ssDNA$^c$</td>
<td>50 µg/ml</td>
<td>30</td>
<td>367</td>
<td>156</td>
</tr>
</tbody>
</table>

$^a$ Double-stranded DNA (dsDNA) was linearized pYES vector (5.9 kb).

TABLE II

Effect of Various Compounds on GST-Cdc14p Activity

Phosphatase assays were conducted at 30°C for 15 min using 20 mM pNPP, 5 µM Tyr(P)-MBP, or 5 µM Ser/Thr(P)-MBP as substrate. Values are given as a percentage of control enzyme activity without additions and are the mean of duplicate samples.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentration</th>
<th>pNPP %</th>
<th>Tyr(P)-MBP</th>
<th>Ser/Thr(P)-MBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 µg/ml</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NaF</td>
<td>20 mM</td>
<td>98</td>
<td>102</td>
<td>78</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>100 mM</td>
<td>101</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Tartrate</td>
<td>5 mM</td>
<td>99</td>
<td>103</td>
<td>84</td>
</tr>
<tr>
<td>Tetramisole</td>
<td>100 µM</td>
<td>100</td>
<td>91</td>
<td>94</td>
</tr>
<tr>
<td>Sodium vanadate</td>
<td>200 µM</td>
<td>5</td>
<td>48</td>
<td>33</td>
</tr>
<tr>
<td>Sodium tungstate</td>
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<td>6</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>ZnCl$_2$</td>
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<td>94</td>
<td>90</td>
<td>101</td>
</tr>
<tr>
<td>Spermidine</td>
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<td>94</td>
<td>204</td>
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<td>ssDNA$^c$</td>
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<td>367</td>
<td>156</td>
</tr>
</tbody>
</table>

$^a$ Double-stranded DNA (dsDNA) was linearized pYES vector (5.9 kb).

a M13 DNA (7.2 kb) was the source of single-stranded DNA (ssDNA).

b Double-stranded DNA (dsDNA) was linearized pYES vector (5.9 kb).

c M13 DNA (7.2 kb) was the source of single-stranded DNA (ssDNA).

A major protein peak (A$_{280}$) containing rCdc14p as confirmed by SDS-PAGE (data not shown) was observed. Both Tyr(P)- and Ser/Thr(P)-MBP were bound by heparin-agarose under conditions used for assays (data not shown), indicating that the influence of polyanions may also be explained in part by their ability to interact with these basic substrates.

The COOH-terminal Asn/Ser-rich Domain Is Not Required for the Activity of Cdc14p—As shown in Table I, truncated GST-Cdc14p(1–374) in which residues 375–551 had been removed was active with all three substrates tested. When compared on a molar basis, the full-length and truncated enzymes had comparable activities toward the phosphoprotein substrates tested. In contrast, the truncated enzyme hydrolyzed pNPP at a rate that was about 10-fold lower than that of the full-length enzyme (Table I). Truncation of the fusion protein reduced the $V_{max}$ with pNPP but had little effect on $K_m$. These data demonstrate that residues 375–551 from the Asn/Ser-rich COOH-terminal segment are not required for phosphatase activity in vitro and suggest that this noncatalytic domain has little influence on activity toward artificial protein substrates.

Recombinant Cdc14p Is an Oligomer—To estimate its size, thrombin-cleaved rCdc14p was analyzed by gel filtration chromatography on a Sephacryl S300 column as shown in Fig. 5A. A major protein peak (A$_{280}$) containing rCdc14p as confirmed by SDS-PAGE (data not shown) coeluted with a peak of pNPPase activity (Fig. 5). SDS-PAGE also showed that the major protein peak (Fig. 5A) is due to the presence of lower molecular weight fragments derived from Cdc14p. Variable amounts of these fragments (about 5–20% of the total protein) were generated by internal cleavage during thrombin treatment of GST-Cdc14p but not GST-Cdc14p(1–374). Calibration of the S300 column with molecular weight standards gave an apparent molecular weight of 248,000, suggesting that rCdc14p self-associates to form multimers, since its calculated molecular weight is 62,200. The oligomeric nature of rCdc14p was confirmed by determining its native molecular weight using two different methods. Nondenaturing gel electrophoresis gave an estimated molecular weight of 131,000, while the value calculated from the sedimentation coefficient and Stokes radius was 169,000 (Table III). Although the values differ significantly, these independent measurements confirm the oligomeric state of rCdc14p.

When rCdc14p(1–374) was separated on the same column (Fig. 5B), a single symmetrical protein peak also coeluted with phosphatase activity. The apparent molecular weight estimated from its elution position was 65,000, a value that is significantly larger than that expected for the monomer. The
native molecular weight values of 89,700 and 84,800 (Table III) obtained from nondenaturing electrophoresis and calculated from hydrodynamic parameters, respectively, are consistent and indicate that the truncated enzyme associates to form a homodimer. Furthermore, chemical cross-linking with the reagent, dimethyl suberimidate, confirmed that rCdc14p-(1–374) is a dimer (data not shown). Cross-linking experiments with the full-length enzyme were consistent with the presence of a dimer, but the pattern of products was complicated by the presence of the low molecular weight fragments (data not shown). With both full-length and truncated enzymes, molecular weights estimated by gel filtration did not agree with those obtained from nondenaturing electrophoresis and calculated from sequence. Positions are generally considered to be less reliable than those obtained by the other methods employed in this study.

Analysis of the Function of cdc14 Mutants—A series of active site and truncation mutants could complement a null mutation as shown by plasmid shuffle assays (Fig. 6). The ability of Cdc14p to oligomerize in vitro indicates that it may also function as a multimer in vivo. Multimer formation could lead to intragenic complementation between the temperature-sensitive protein encoded by the genome and the plasmid encoded mutants. To avoid this potential complication, we determined whether the active site and truncation mutants could complement a null mutation in the essential CDC14 gene using the plasmid shuffle technique (21). As described under “Experimental Procedures,” we created strain DBYLC5 (MATa trp1 gal1 cdc14–1) to determine whether they could rescue growth at the restrictive temperature (37 °C).

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tant encoding inactive enzyme in which Cys283 has been replaced by Ser were constructed to determine whether the phosphatase activity is essential. Several studies have shown that dual specificity and tyrosine-specific phosphatases in which the active site Cys residue has been replaced can bind their phosphorylated substrates (4, 41). If the key substrate(s) of Cdc14p is inhibitory to cell cycle progression in its phosphorylated state, then the ability of a Cdc14p C283S mutant to bind this substrate at a critical point in the cell cycle could be functionally equivalent to dephosphorylating it (4). In this way, the full-length C283S mutant could retain its ability to rescue the temperature sensitivity and complement the null mutant by virtue of its ability to sequester the phosphorylated substrate. In this regard, we found that GST-Cdc14p C283S immobilized on glutathione-agarose is capable of binding phosphorylated substrates and that GST-Cdc14p C283S inhibited the activity of GST-Cdc14p in a concentration-dependent manner (data not shown). Thus, the Cdc14p C283S mutant has the potential to mimic active enzyme by sequestering its phosphosubstrate. To eliminate or minimize the possibility of phosphosubstrate binding, we also constructed a cdc14 double mutant containing C283S and R289A replacements. Arg289 in Cdc14p corresponds to a critical active site Arg, which is involved in binding the phosphate group of the substrate (3, 5). Since the neutral Ala side chain at position 289 should be incapable of interacting with the phosphate, we reasoned that this replacement should reduce or eliminate the ability of Cdc14p to bind its substrate.

To demonstrate that active site mutants are stable and not degraded when expressed in yeast, HA-tagged wild type protein and double mutant, Cdc14p C283S/R289A, were overexpressed under the control of the constitutive triose phosphate isomerase promoter. The ability of cdc14-1 cells overexpressing HA-tagged Cdc14p to grow at the restrictive temperature (Fig. 6) demonstrated that the presence of the NH2-terminal epitope did not significantly affect the function of the protein and confirmed that overexpression of Cdc14p did not seriously affect growth (data not shown). In contrast, introduction of the plasmid encoding the mutant HA-Cdc14p C283S/R289A into cdc14-1 yeast failed to suppress the temperature sensitivity of this strain (Fig. 6). As shown in Fig. 7, comparable amounts of HA-Cdc14p and HA-Cdc14p C283S/R289A were immunoprecipitated with the HA-specific 12CA5 monoclonal antibody, ruling out the possibility that failure to rescue was due to enhanced degradation of the active site mutant.

As shown by plasmid shuffle assays (Fig. 6), both the C283S single and C283S/R289A double mutants failed to complement the cdc14 null mutation, suggesting that these inactive mutants are not able to function in vivo. The introduction of a plasmid carrying the cdc14 double mutant was also unable to rescue the temperature sensitivity of the cdc14-1 strain (Fig. 6). Interestingly, in this strain, the single C283S mutant was capable of supporting growth at the restrictive temperature. The data compiled in Fig. 6 and the evidence that the double mutant is stably expressed in cells demonstrate that the phosphatase activity of Cdc14p is required for its ability to support growth and to perform a critical step in cell cycle progression.

DISCUSSION

The Cdc14 gene of S. cerevisiae encodes a 62-kDa protein containing the sequence, HCKAGLRTG, that fits the consensus active site motif contained in all enzymes of the protein tyrosine phosphatase superfamily. Enzymatic characterization of the recombinant protein expressed in E. coli demonstrated that Cdc14p is a dual specificity phosphatase. Cys283 within the conserved active site region of Cdc14p is essential for ac-
Cdc14p is an Oligomeric Dual Specificity Phosphatase

activity, suggesting that its mechanism of catalysis is similar to that utilized by other protein tyrosine phosphatases (3, 5). Although the number of substrates tested in this study is limited, it is clear that Cdc14p exhibits substrate selectivity (Table I). A notable feature of Cdc14p is its ability to hydrolyze phosphoserine/threonine and phosphothreonine residues at comparable rates (Table I). This finding suggests that yeast proteins containing only phosphoserine/threonine must be given serious consideration as potential physiologic substrates of Cdc14p.

We have shown that the phosphatase activity of Cdc14p is essential for the viability of yeast and for the critical cell cycle function executed by this enzyme. To address this issue, we employed inactive C283S mutants, which have a one-atom replacement in the side chain of residue 283. As shown by the x-ray structure of a PTP1B mutant (42), replacement of the active site Cys with Ser has little effect on enzyme conformation. Thus, this mutation should specifically eliminate activity without perturbing other potential functions of the enzyme. Inactive mutants, Cdc14p C283S and Cdc14p C283S/R289A, were unable to complement the null cdc14 mutant. In contrast, Cdc14p C283S but not Cdc14p C283S/R289A was able to rescue the temperature sensitivity of a cdc14–1Δ17 strain (Fig. 6). Suppression of temperature sensitivity by the C283S active site mutant was unexpected and most likely results from intragenic complementation due to the formation of thermostable mixed multimers composed of the temperature-sensitive enzyme and the C283S mutant. However, there are other plausible explanations that have not been excluded by our data. Shirayama et al. (14) recently found that C283A, A285L, and R289P single mutants were each unable to suppress temperature sensitivity. We did not examine single mutations at Ala285 or Arg289, but the discrepancy in results obtained with our Cys mutants may be attributed to the difference in the amino acid chosen as a replacement (Ser versus Ala). Nevertheless, our conclusions regarding the requirement for phosphatase activity agree with those of Shirayama et al. (14).

The Asn/Ser-rich COOH-terminal segment (residues 375–551) of rCdc14p is not required for phosphatase activity because its removal has little or no effect on activity measured in vitro. We have also shown that this Asn/Ser-rich COOH-terminal region is not required for the function of Cdc14p because it was not required for rescuing the temperature sensitivity of the cdc14–1Δ17 strain or complementation of the null mutant. While we are not able to assign a functional role for this noncatalytic segment, it could be involved in mediating nonessential cell cycle functions of Cdc14p or in carrying out functions of the enzyme that are unrelated to its role in cell cycle regulation. In other protein tyrosine phosphatases, noncatalytic sequences such as this are involved in regulation by targeting enzymes to specific subcellular locations or by modulating their catalytic activity (3, 4). A truncation mutant, in which the first 124 NH2-terminal residues were removed, was expressed in bacteria and in insect cells. Although most of the recombinant enzyme produced in these systems was found in particulate fractions, the soluble protein was inactive, suggesting that residues 1–124 are required for folding into an active form of the enzyme.

Determination of the native molecular weight of rCdc14p using two different techniques demonstrated that the recombinant enzyme is active as an oligomer in vitro. The molecular weight of 131,000 determined by nondenaturing electrophoresis suggests a homodimer, whereas the value calculated with hydrodynamic parameters (Table III) is about 29% greater than that expected for a dimer but less than that of a trimer. With these discrepancies, we are unable to reach definitive conclusions regarding the subunit composition of rCdc14p. Determination of the subunit composition and the concentration dependence of oligomerization will require more detailed biophysical analyses. With rCdc14p (1–374), the native molecular weight values (Table III) are in good agreement and are corroborated by cross-linking studies indicating that the truncated enzyme is a homodimer. The ability of Cdc14p (1–374) to oligomerize suggests that the Asn/Ser-rich COOH-terminal segment is not required for self-association.

To the best of our knowledge no other dual specificity or nonreceptor protein tyrosine phosphatase has been shown to self-associate to form oligomers. Bilwes et al. (43) have shown that the membrane-proximal domain of the receptor tyrosine phosphatase, RPTPα, exists as a dimer in crystals and oligomerizes in solution. However, in this case, oligomerization is thought to result in the inhibition of phosphatase activity and to serve as a means of regulating activity. The dependence of oligomerization on Cdc14p concentration was not analyzed in this study. We have not yet identified nondenaturing conditions that give subunit dissociation; therefore, we are unable to determine whether oligomerization is required for activity. Thus far, we have no evidence that Cdc14p exists as a multimer in the cell, but the fact that oligomerization occurred under conditions similar to physiologic ionic strength and pH suggest that it is feasible. If Cdc14p is able to form dimers or higher order oligomers in vivo, its multimeric state will permit sensitive regulation via allosteric interactions with effector molecules. Such regulatory features could prove to be advantageous for a protein involved in cell cycle regulation.

The activity of Cdc14p was inhibited by increasing NaCl concentration and was significantly affected by polyionic compounds. Direct interactions with positively charged substrates (e.g. MBP) appears to account for some effects of polyanions. However, the binding of Cdc14p to immobilized heparin and double or single-stranded DNA shows that polyanions can affect activity by binding directly to the enzyme. The potential involvement of Cdc14p in initiation of DNA replication and its ability to bind polyanions including nucleic acids indicate that a careful investigation of its DNA-binding properties is warranted.

Sequence comparisons confirm our previous findings (25) that Cdc14p exhibits little or no significant similarity to VH1 and its mammalian homologs (PAC1, VH6, MKP1, etc.). VH1-like dual specificity phosphatases such as MKP1, MKP2, and PAC1 have been shown to dephosphorylate and inactivate MAP/ERK kinases (44, 45). The activity of Cdc14p toward phosphorylated ERK2 (46) was several orders of magnitude lower than that observed with artificial substrates, confirming that it is not a MAP kinase phosphatase. We believe that Cdc14p, like Cdc25p, is the prototype for a distinct class of dual specificity enzymes with specific roles in controlling cell cycle progression.

As with many other cell cycle proteins from yeast, there is considerable evidence that Cdc14p homologs are found in metazoan organisms. Residues 1–364 of Cdc14p exhibits 32% sequence identity with a putative phosphatase encoded by gene C17G10.5 (GenBankTM U28739) on chromosome III of the nematode, Caenorhabditis elegans, and by four expressed sequence tags corresponding to this gene. Human placenta cDNAs isolated in this laboratory and several human expressed sequence tags encode two different proteins with 32 and 36% sequence identity to Cdc14p. This degree of sequence similarity between yeast and human proteins suggests that Cdc14p is conserved among eukaryotes. It will be important to determine

4 L. Hao and H. Charbonneau, unpublished observations.
whether these metazoan homologs have a cell cycle function like that of Cdc14p from S. cerevisiae.

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REFERENCES

The Activity of Cdc14p, an Oligomeric Dual Specificity Protein Phosphatase from Saccharomyces cerevisiae, Is Required for Cell Cycle Progression

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