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Characterization of the Net1 Cell Cycle-dependent Regulator of the Cdc14 Phosphatase from Budding Yeast*

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In the budding yeast *Saccharomyces cerevisiae*, the multifunctional protein Net1 is implicated in regulating the cell cycle function of the Cdc14 protein phosphatase. Genetic and cell biological data suggest that during interphase and early mitosis Net1 holds Cdc14 within the nucleolus where its activity is suppressed. Upon its transient release from Net1 at late anaphase, active Cdc14 promotes exit from mitosis by dephosphorylating targets in the nucleus and cytoplasm. In this paper we present evidence supporting the proposed role of Net1 in regulating Cdc14 and exit from mitosis. We show that the NH_2-terminal fragment Net1(1–600) directly binds Cdc14 *in vitro* and is a highly specific competitive inhibitor of its activity (K_i = 3 nM) with five different substrates including the physiologic targets Swi5 and Sic1. An analysis of truncation mutants indicates that the Cdc14 binding site is located within a segment of Net1 containing residues 1–341. We propose that Net1 inhibits by occluding the active site of Cdc14 because it acts as a competitive inhibitor, binds to a site located within the catalytic domain (residues 1–374), binds with reduced affinity to a Cdc14 C283S mutant in which an active site Cys is replaced, and is displaced by tungstate, a transition state analog known to bind in the catalytic site of protein-tyrosine phosphatases.

The Cdc14 phosphatases are a conserved subset of dual specificity enzymes (1–3) of the protein-tyrosine phosphatase (PTP)[1] family (4–7). The essential Cdc14 phosphatase from budding yeast (1, 8–10), *Saccharomyces cerevisiae*, is involved in driving cells from late anaphase into G_1 of the subsequent cell cycle, a series of events known as exit from mitosis (for review, see Refs. 11–13). The onset of mitosis occurs when cyclin-dependent kinases are activated following their association with mitotic cyclins. Exit from mitosis requires the inactivation of cyclin-dependent kinases, a process that involves the ubiquitination and subsequent destruction of cyclins and other regulatory proteins. The anaphase-promoting complex/cyclosome (APC/C) is a tightly regulated multisubunit ubiquitin ligase that first initiates anaphase, then exit from mitosis, by targeting proteins for degradation in an ordered and tightly coordinated fashion (for review, see Ref. 14). In budding yeast, inactivation of the mitotic cyclin-dependent kinase (Cdc28) occurs by two processes, the APC/C-dependent ubiquitination and subsequent destruction of B-type cyclins (Clb1–6) and the synthesis of the Clb/Cdc28 inhibitor Sic1 (12, 13). Cdc14 drives both of these processes by dephosphorylating at least three targets: Hct1, Swi5, and Sic1 (8, 10). Cdc14 dephosphorylates inhibitory sites and thereby activates the APC/C regulator Cdh1/Hct1 so that a subset of mitotic cyclins, Clb2 and Clb3, is targeted for ubiquitination by the APC/C (8, 10). Swi5 is a zinc finger transcription factor that is required for expression of the *SIC1* gene. The dephosphorylation of Swi5 permits its translocation from the cytoplasm to the nucleus where it can activate Sic1 transcription (8). The Sic1 protein itself may be protected from premature degradation when dephosphorylated by Cdc14 (8).

Net1 (also known as Cfi1) is a core component of the nucleolar RENT complex that regulates Cdc14 during the cell cycle (for review, see Refs. 15–17). From G_1 to anaphase, Net1 sequesters Cdc14 in the nucleolus, where its access to substrate is limited, and its phosphatase activity is suppressed (18–20). At late anaphase, Cdc14 is transiently released from Net1 permitting the active phosphatase to reach targets in the nucleus and cytoplasm (18, 19). The RENT complex appears to have multiple functions besides Cdc14 regulation including roles in maintaining the integrity of the nucleolus (20, 21) and sequestering Sir2 to tandem rDNA repeats (21). The NAD-dependent histone deacetylase activity of Sir2 silences rDNA chromatin and represses recombination among tandem rDNA repeats, a deleterious process that leads to senescence of cells (22–24).

The mechanism by which Cdc14 is released from Net1 is not yet clear, but it is dependent in part on activation of a signaling pathway known as the mitotic exit network (9, 11, 12). When the dividing nucleus spans the bud neck during late mitosis, the mitotic exit network pathway is activated, and a signal is propagated which promotes release of Cdc14 (25, 26). The APC/C-mediated destruction of the anaphase inhibitor Pds1 is not only necessary for sister-chromatid separation but also for the subsequent release of Cdc14 (27–29). However, Pds1 degrada-

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[1] The abbreviations used are: PTP, protein-tyrosine phosphatase; APC/C, anaphase-promoting complex/cyclosome; SC, synthetic complete; GST, glutathione *S*-transferase; PEI, polyethyleneimine; HCdc14, human homologs of yeast Cdc14; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; MBP, myelin basic protein; pNPP, *p*-nitrophenyl phosphate; Tyr(P)-MBP, myelin basic protein phosphorylated on tyrosine residues.

tion and Cdc14 release are not sufficient for exit from mitosis unless the Clb5 cyclin has also been destroyed by the APC/C at the metaphase to anaphase transition (28). Thus, multiple controls ensure that exit from mitosis occurs only after chromosome segregation and correct partitioning of the dividing nucleus to the mother and daughter cells (25, 26, 28–30).

Previous studies have not determined whether Net1 on its own is sufficient to inhibit Cdc14 activity. In this paper we have characterized the direct effect of Net1 on Cdc14 activity *in vitro*. We have shown that a 600-residue NH_2-terminal fragment of Net1 alone binds the catalytic domain of Cdc14 and acts as a potent and specific competitive inhibitor of its activity toward physiologic substrates. We have defined a segment of Net1 spanning residues 1–341 which contains the Cdc14 binding site and fully inhibits phosphatase activity. We also provide evidence that Net1 acts by occluding the active site of Cdc14.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—Plasmids were constructed using standard cloning and polymerase chain reaction techniques as briefly outlined below. The authenticity of all plasmids was verified by DNA sequencing. For two-hybrid analyses, the coding sequence of the *CDC14* gene was cloned into the pGBDU-C1 vector (31) to create pGBDU-CDC14, which encodes a fusion protein with the *GAL4* DNA binding domain. Using polymerase chain reaction, the *NET1* coding sequence was inserted into pGAD-C1 (31) to produce pGAD-NET1, which expresses a fusion protein with the *GAL4* activation domain. Restriction fragments derived from pGAD-NET1 were used to construct a series of six pGAD plasmids encoding fusion proteins comprised of Net1 truncation mutants containing residues 1–601, 1–341, 1–207, 92–341, 342–1189, and 92–1189. A pGAD vector encoding a fusion protein with Net1(1–146) was constructed using a fragment amplified by polymerase chain reaction. The complete *SWI5* coding sequence and codons 1–600 of *NET1* were amplified by polymerase chain reaction and inserted into pET21a to generate the pET-SWI5-His_6 and pET-NET1(1–600)-His_6 expression plasmids. A restriction fragment from pGAD-Net1(1–341) was inserted into pET21a to give pET-NET1(1–341)-His_6 expression plasmid. All of these pET expression plasmids encode proteins bearing NH_2-terminal T7 tags.

Yeast Two-hybrid Screens and Assays—A two-hybrid screen was performed by transforming the yeast strain PJ69–4A (31) with the pGBDU-CDC14 plasmid expressing the Cdc14 bait protein. This strain was then transformed with each of three *S. cerevisiae* Y2HL genomic libraries (31), and about 2×10^6 colonies with each library were screened for activation of the *HIS3* and *ADE2* reporter genes (see below). After establishing that reporter activation was dependent on the presence of both bait and target proteins, plasmids from positive clones were isolated and sequenced. This screen identified several positive clones containing 5′-coding sequences from the yeast YJL076W (*NET1*) gene.

Two-hybrid assays were used to assess the ability of Net1 truncation mutants to interact with Cdc14. After transformation of the yeast strain PJ69–4A (31) with the pGBDU-CDC14 plasmid and a pGAD vector encoding one of the Net1 truncation mutants (see above) cells were plated on medium permitting detection of reporter gene activation. Ade^+ cells were identified by growth on SC medium lacking adenine, leucine, and uracil, whereas His^+ cells grew on SC medium lacking histidine, leucine, and uracil but containing 1 mM 3-aminotriazole. Activation of the *lacZ* reporter gene was assessed by performing liquid β-galactosidase assays as described (31).

Protein Expression and Purification—Yeast GST-Cdc14 and GST-Cdc14(1–374) were overexpressed in *Escherichia coli* BL21 (DE3) cells as described (1) except that lysates were treated with polyethyleneimine (PEI) prior to affinity purification. Using a stock solution of 5% (w/v) PEI at pH 8, lysates were adjusted to a final PEI concentration of 0.15% (w/v) and centrifuged at $10,000 \times g$ for 10 min at 4 °C. The pellet was resuspended in 1 volume of lysis buffer containing 500 mM NaCl, mixed and clarified by centrifugation ($10,000 \times g$ for 10 min) prior to purification using glutathione-Sepharose (1). The GST-HCdc14A (1–379) and GST-HCdc14B (1–418) fusion proteins were expressed in *E. coli* BL21 (DE3) cells from pET-GST vectors (1) and purified from PEI-treated extracts as described above. The TC45 splice variant of the human T cell phosphatase was expressed and purified using the method of Hao *et al.* (32). Purified recombinant VHR and PPT1 protein phosphatases were provided by Drs. Zhong-Yin Zhang and S. Rossie, respectively.

E. coli BL21 (DE3) cells transformed with expression plasmids encoding Net1(1–600)-His_6, Net1(1–341)-His_6, and Swi5-His_6 were grown at 37 °C in LB medium containing 100 μg/ml ampicillin until the A_{600} was about 0.7. Following the addition of 50 μM isopropyl-1-thio-β-D-galactopyranoside, cells expressing Net1(1–600)-His_6 and Swi5-His_6 were grown for 14–16 h at room temperature, whereas cells producing Net1(1–341)-His_6 were induced with 400 μM isopropyl-1-thio-β-D-galactopyranoside and grown for 4 h at 30 °C. Swi5-His_6 and Net1(1–600)-His_6 were purified directly from extracts using His-Bind resin (Novagen) according to protocols from the manufacturer, whereas lysates containing Net1(1–341)-His_6 were treated with PEI as described above before purification on His-Bind resin.

For further purification, Net1(1–600)-His_6 (1–2 mg) was dialyzed with buffer A (50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% (v/v) 2-mercaptoethanol), loaded on a Mono Q HR 5/5 column, and eluted with a 25-ml linear gradient from 0 to 500 mM NaCl in buffer A using a fast protein liquid chromatography system (Amersham Pharmacia Biotech). Peak fractions were combined, dialyzed with 50 mM imidazole, pH 6.6, 1 mM EDTA, 0.1% (v/v) 2-mercapthoethanol for phosphatase assays or with 25 mM Tris, pH 7.4, 2.6 mM KCl, 137 mM NaCl, 0.1% (v/v) 2-mercaptoethanol for *in vitro* binding assays and concentrated with Centricon-10 (Amicon) membranes. Net1(1–341)-His_6 was purified further on a Sephacryl S300 HR 1 × 80-cm column (Amersham Pharmacia Biotech) equilibrated in 50 mM imidazole, pH 6.6, 350 mM NaCl, 1 mM EDTA, 0.1% (v/v) 2-mercapthoethanol and eluted at a flow rate of 0.5 ml/min using the fast protein liquid chromatography system. Peak fractions were combined and concentrated by either ultrafiltration with a Centriprep-10 (Amicon) membrane or dehydration in a dialysis bag covered with dry Sephadex G-25. Sic1-Myc-His_6 was expressed as a fusion protein with maltose-binding protein and purified as described (33).

The Cdc28 cyclin-dependent kinase was immunopurified from yeast as a complex with either the Clb2 or Cln2 cyclin carrying epitope tags derived from human influenza virus hemagglutinin (HA) as described (34). Yeast with copies of either *GAL1-HA-CLB2* or *GAL1-HA-CLN2* genes were grown in medium containing 2% galactose to induce expression and disrupted with glass beads. The HA-Clb2·Cdc28 and HA-Cln2·Cdc28 kinase complexes were immunopurified from extracts using 12CA5 monoclonal antibodies cross-linked to protein A-Sepharose beads (34).

In Vitro Binding Assays—GST, GST-Cdc14, and GST-Cdc14 C283S affinity matrices were prepared by adding 0.5 nmol of each protein to 200 μl of a 50% slurry of glutathione-Sepharose in binding buffer B (25 mM Tris, pH 7.4, 137 mM NaCl, 2.6 mM KCl, 0.1% (v/v) 2-mercaptoethanol). Each mixture was incubated for 30 min at 4 °C in a final volume of 1 ml buffer B. The supernatant was removed, and each of the three affinity matrices was suspended in buffer B and split into two aliquots of equal volume. 1 nmol of Net1 in buffer B was added to one aliquot of each affinity matrix (final volume of 1 ml of buffer B), whereas the second sample of affinity resin received an equal volume of buffer B without Net1. After mixing by inversion for 30 min at 4 °C, the resin was centrifuged and washed four times with 1 ml of buffer B containing 0.01% (v/v) Triton X-100. Washed beads were suspended in 50 μl of 2 × SDS-PAGE loading buffer, boiled for 5 min, and an aliquot (20 μl) was separated on a 12% SDS-polyacrylamide gel. Proteins were visualized by staining the gel with Coomassie Blue. Densitometry was used to estimate the relative amounts of Net1 fragment and GST-Cdc14 proteins in the SDS gels assuming that the two proteins have equal staining with Coomassie dye. Images of gels dried between translucent membranes were obtained from a scanner (Astra 2200, Umax) used in the transparency mode and analyzed using ImageQuant software (Molecular Dynamics).

Preparation of ^{32}P-labeled Substrates—Swi5 and histone H1 were phosphorylated with [γ-^{32}P]ATP using the partially purified HA-Clb2·Cdc28 complex, whereas Sic1 was phosphorylated with the HA-Cln2·Cdc28 kinase (see above). Myelin basic protein (MBP) (Life Technologies, Inc.) was phosphorylated on tyrosine using GST-*lyn* kinase as described (35). Radiolabeled casein phosphorylated on Ser residues was provided by Dr. S. Rossie. Protein substrate concentrations given herein represent the total concentration of phosphorylated residues used in each assay.

Phosphatase Assays—The phosphatase activity of yeast Cdc14, HCdc14A, HCdc14B, VHR, and TC45 toward pNPP and protein substrates was measured as described (1, 32) in reactions carried out at 30 °C for 5–40 min in buffer containing 50 mM imidazole, pH 6.6, 1 mM EDTA, 1 mM dithiothreitol, and 0.5 mg/ml bovine serum albumin. Assays with radiolabeled Sic1, Swi5, histone H1, and Tyr(P)-MBP substrates were performed in a total volume of 30 μl; 50-μl reactions were

TABLE I

Two-hybrid interaction of Net1 or Net1 mutants with Cdc14

Yeast two-hybrid assays (31) were used to assess the ability of Net1 or Net1 truncation mutants to interact with Cdc14. Yeast (PJ69–4A) expressing a Cdc14 fusion protein with the *GAL4* DNA binding domain (pGBDU-CDC14) were transformed with pGAD plasmids encoding Net1 or Net1 truncation mutants fused with the *GAL4* DNA activation domain. Protein-protein interactions were detected by selection for *ADE* and *HIS* prototrophy and measurement of *lacZ* expression using liquid β-galactosidase assays.

Activation domain fusion protein	*ADE2* activation	*HIS3* activation[a]	*LacZ* activation	
			β-Gal activity[b]	Fold activation[c]
			Miller units	
pGAD-C3 alone	−	−	3.2	1.0/−
Net1	+	−	4.5	1.4/+
Net1(1–601)	+	+	12.4	3.8/+
Net1(1–341)	+	+	8.0	2.5/+
Net1(342–1189)	−	−	3.1	1.0/−
Net1(1–207)	+	−	3.5	1.1/−
Net1(1–146)	−	−	2.1	0.6/−
Net1(92–1189)	+	+	4.5	1.4/+
Net1(92–341)	−	−	3.1	1.0/−

[a] Transformants were screened for activation of the *HIS3* gene on SC-His-Leu-uracil medium containing 1 mM 3-aminotriazole.

[b] β-Galactosidase activities are the mean of values obtained in triplicate. These data are representative of the results obtained from four different experiments.

[c] Values differing from control by more than 0.5 Miller unit were statistically significant indicating activation of the *lacZ* gene as shown with a plus (+) sign.

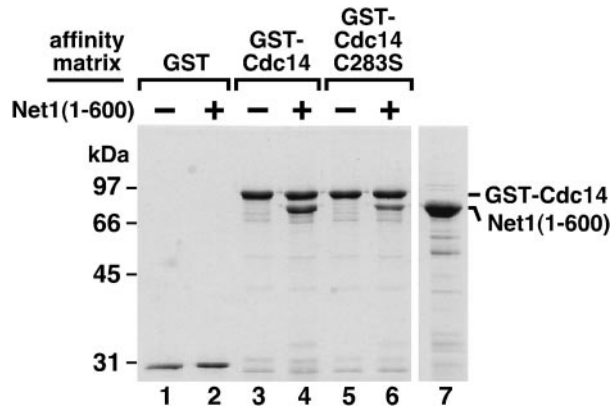

FIG. 1. **Net1(1–600) binds Cdc14 *in vitro*.** GST, GST-Cdc14, and GST-Cdc14 C283S affinity matrices were prepared by binding 0.25 nmol of each protein to glutathione-Sepharose beads. Purified Net1(1–600)-His_6 (1 nmol) was incubated for 30 min at 4 °C with each affinity matrix in 1 ml of buffer B. After washing with buffer B containing 0.01% (v/v) Triton X-100, an aliquot (20%) of each Net1(1–600)-His_6-treated matrix (*lanes 2*, *4*, and *6*) and the untreated controls (*lanes 1*, *3*, and *5*) were mixed with sample buffer and separated on 12% SDS-polyacrylamide gels. An aliquot (7 μg) of purified Net1(1–600)-His_6 used in these studies was also subjected to SDS-PAGE (*lane 7*). Protein was visualized by staining with Coomassie Blue. The positions of molecular mass markers are shown on the *left*, and the locations of GST-Cdc14 and Net1(1–600)-His_6 are indicated on the *right*. These results are representative of those obtained in multiple experiments.

employed for pNPP and casein. The final assay buffer for yeast PPT1 contained 56 mM Tris, 36 mM imidazole, pH 7.2, 1.8 mM EDTA, 1.2 mM EGTA, 0.1% 2-mercaptoethanol, 0.5 mg/ml bovine serum albumin, and 6 μM phosphocasein.

Limited Proteolysis of Net1(1–600)-His_6—Digestion with *Staphylococcus aureus* V8 protease (0.15 μg) was performed for 60 min at room temperature with 75.5 μg Net1(1–600) in 0.5 ml of buffer containing 40 mM ammonium bicarbonate, pH 7.8, 4 mM Tris, 22 mM NaCl, 0.4 mM KCl, 3% (v/v) glycerol, and 0.02% (v/v) 2-mercaptoethanol. Cleavage with 1.5 μg of endoproteinase Arg-C (stored at −20 °C in water) was carried out at 30 °C as described above for staphylococcal V8 protease except that the digestion buffer contained 80 mM ammonium bicarbonate at pH 8. Aliquots (50 μl) of each digest were precipitated with trichloroacetic acid as described previously (32) and analyzed on 12% SDS-polyacrylamide gels. The remainder of each reaction (equivalent to 1 nmol of the Net1(1–600) fragment) was stopped by adding soybean trypsin inhibitor to a final concentration of 1 mg/ml and used for *in vitro* binding assays with affinity matrices containing 0.25 nmol of GST-Cdc14 or GST (see above). For amino acid sequencing, proteolytic fragments were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes as described (36). Protein bands on the membrane were stained with Coomassie Blue, excised, and analyzed by automated gas phase sequencing.

Determination of the Mode of Inhibition and K_i—Because of its high affinity for Cdc14, the Henderson method (37) for tight binding inhibitors was used for the analysis of Net1 inhibition. For these experiments, the activity of GST-Cdc14 (5 nM) was measured at six different substrate concentrations ranging from 0.27 to 17 μM Tyr(P)-MBP. For each concentration of substrate, six assays were performed with Net1(1–600) concentrations between 0 and 100 nM. For each substrate concentration, the value $I_t/(1 - v_i/v_0)$ was plotted on the *y* axis *versus* v_0/v_i on the *x* axis, where I_t is the total Net1(1–600) concentration, and v_i and v_0 are the velocities with and without inhibitor, respectively. This plot generates a series of lines for each concentration of substrate which were fit by unweighted linear regression using a fixed intercept value of 5 nM, which was the total enzyme concentration (37). The K_i and mechanism of inhibition were determined from a replot of the slopes of these lines *versus* substrate concentration.

RESULTS

Net1 (1–600)-His_6 Alone Binds Cdc14 Directly—In addition to a genetic screen described by Shou *et al*. (18), we and others (19) also identified Net1 in a yeast two-hybrid screen for regulators or substrates of Cdc14 (Table I). Along with the immunolocalization and genetic studies (18–20), these two-hybrid results support the notion that Net1 and Cdc14 interact *in vivo*, but they fail to establish whether this interaction is direct or mediated by other proteins. To determine whether there is a direct interaction between Net1 and Cdc14 *in vitro*, we used binding assays employing recombinant GST-Cdc14 (1) and Net1(1–600)-His_6. This NH_2-terminal fragment of Net1 was used to preclude problems associated with the bacterial expression of the 1,189-residue full-length protein. Sequence analysis of Net1 clones obtained in our two-hybrid screens indicated that the NH_2-terminal half of Net1 (residues 1–641) interacted with Cdc14. A two-hybrid assay confirmed that a fragment of Net1 containing residues 1–601 could interact with Cdc14 (Table I, third row).

Purified GST-Cdc14 or GST alone was immobilized on glutathione-Sepharose beads and incubated with a 4-fold molar excess of Net1(1–600). SDS-PAGE analysis (Fig. 1) revealed that Net1(1–600) was retained by the GST-Cdc14 affinity support but not by immobilized GST alone. The molar ratio of GST-Cdc14 to Net1(1–600) on the affinity matrix (Fig. 1, *lane 4*) was estimated to be 1:1 using densitometric analysis of the SDS-gel. This experiment corroborates results from two-hybrid analyses and demonstrates that *in vitro* Net1(1–600) binds GST-Cdc14 with high affinity and in a manner that is not dependent on other proteins.

Net1 Is a Potent Inhibitor of Cdc14 Activity—Evidence that Net1 negatively regulates Cdc14 activity includes the ability of a recessive *net1* mutant to bypass the requirement for Tem1 and Cdc15 in promoting exit from mitosis (18), an elevation of Cdc14 activity in *net1* mutants (18), the ability of Net1 overproduction to suppress cell cycle defects induced by Cdc14 overexpression (19, 20), and the inactivation of GST-Cdc14 by immunopurified Net1 (18). To corroborate these findings and to determine whether Net1 alone directly inhibits Cdc14 activity, we measured the effect of purified Net1(1–600) on GST-Cdc14 activity *in vitro*.

The transcription factor Swi5 and the Cdc28 inhibitor Sic1, which are physiologic Cdc14 substrates (8), were employed to evaluate the efficacy of Net1 as an inhibitor. Both substrates

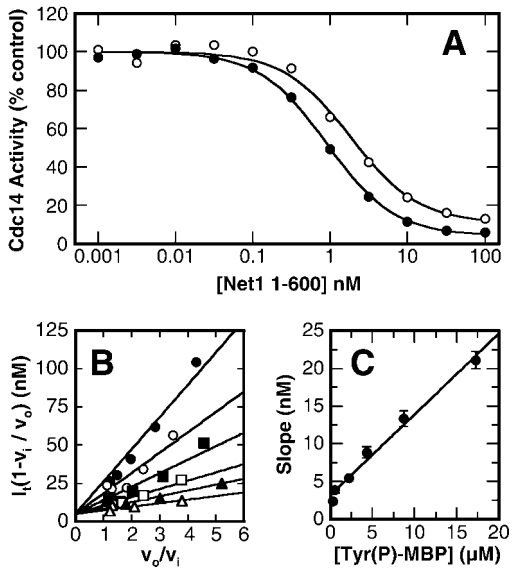

FIG. 2. **Net1(1–600) is a potent competitive inhibitor of Cdc14 activity.** *Panel A*, the phosphatase activity of 4 nM and 1 nM GST-Cdc14 with 3 μM phospho-Sic1 (○) and 2 μM phospho-Swi5 (●), respectively, was measured in the presence of the indicated concentrations of recombinant Net1(1–600)-His_6. Phosphatase activity is plotted as the percent of the control value measured in the absence of Net1(1–600)-His_6. Each point is the mean of three to five determinations. Inhibition curves were obtained using nonlinear regression analysis to fit the data to an equation for a rectangular hyperbola. Protein was estimated by the method of Bradford (49) using bovine serum albumin as standard. *Panel B*, the mode of inhibition for Net1(1–600)-His_6 was determined using the methods for tight binding inhibitors described by Henderson (37). GST-Cdc14 activity was determined in the presence of varying Net1(1–600)-His_6 concentrations at the following Tyr(P)-MBP substrate concentrations: 0.27 μM (△), 0.54 μM (▲), 2.2 μM (□), 4.3 μM (■), 8.7 μM (○), and 17 μM (●). For each substrate concentration, $I_t/(1 - v_i/v_0)$ *versus* v_0/v_i was plotted where I_t is the total Net1(1–600)-His_6 concentration, and v_i and v_0 are the velocities measured with and without inhibitor, respectively. Each data point is the mean of three determinations. *Panel C*, the K_i was derived from a replot of the slopes (●) (± S.E.) obtained for each line in *panel B versus* Tyr(P)-MBP concentration. The *y* intercept of this plot yields the K_i value.

are phosphorylated *in vivo* by the Cdc28 protein kinase in a complex with either mitotic or G_1 cyclins, respectively (33, 38). For use in inhibition assays, recombinant Sic1 and Swi5 were phosphorylated *in vitro* using immunopurified complexes of Cln2·Cdc28 and Clb2·Cdc28 kinases, respectively. Net1(1–600) potently inhibited the dephosphorylation of both Sic1 and Swi5 by GST-Cdc14 with IC_{50} values of 2 and 1 nM, respectively (Fig. 2*A*). Net1(1–600) also inhibited activity toward three artificial substrates, pNPP, histone H1 phosphorylated on Ser/Thr residues (data not shown) and as shown below, Tyr(P)-MBP. We observed a dependence of IC_{50} values on the total GST-Cdc14 concentration over a range from 1 to 34 nM, indicating that Net1 binds in a stoichiometric manner, which is consistent with a dissociation constant of 10 nM or less.

The Noncatalytic Domain of Cdc14 Is Not Required for Net1 Binding and Inhibition—The Asn/Ser-rich COOH-terminal domain (residues 375–551) of yeast Cdc14 is not required for its activity or its role in promoting cell cycle progression (1). To assess the role of this noncatalytic domain in binding Net1, *in vitro* binding and inhibition studies were performed with GST-Cdc14(1–374), a fully active mutant of Cdc14 containing the catalytic domain. The amount of Net1(1–600) bound by immobilized GST-Cdc14(1–374) was comparable to that observed with equivalent quantities of the full-length GST-Cdc14 affinity matrix (data not shown). Using Tyr(P)-MBP as an artificial substrate (1), Net1(1–600) inhibited both GST-Cdc14 and GST-Cdc14(1–374). The IC_{50} value measured with the catalytic domain, Cdc14(1–374), was about 1.5-fold greater than that for the full-length enzyme (data not shown). Similar results were obtained using pNPP as substrate. These data show that Net1 binds to a site residing within the catalytic domain and indicate that the Asn/Ser-rich COOH-terminal domain has little influence on the affinity of Cdc14 for Net1.

Effect of Ionic Strength on Net1 Inhibition—Because of the substantial reduction in GST-Cdc14 activity in the presence of more than 100 mM monovalent salt (1), the inhibition studies described above were performed in low salt buffers having nonphysiologic ionic strength. Therefore, the sensitivity of GST-Cdc14 to Net1(1–600) was also measured in buffer having an ionic strength comparable to that in the cell. In the presence of 120 mM KCl, the IC_{50} value for Net1(1–600) increased from 5.6 to 74 nM (data not shown). The 10-fold reduction in affinity of Net1 for Cdc14 was not accompanied by changes in the shape of the inhibition curve.

Net1 Is a Specific Inhibitor of Yeast Cdc14 Activity—To assess its specificity for Cdc14, we examined the ability of Net1(1–600) to inhibit other enzymes of the PTP family. The activity of the dual-specific phosphatase VHR (39) and the TC45 variant of the tyrosine-specific T cell phosphatase (32) were not affected by the addition of Net1(1–600) at concentrations 300-fold greater than those giving half-maximal inhibition of GST-Cdc14 (data not shown). Likewise, up to 1 μM Net1(1–600) had no effect on the activity of yeast PPT1, a PP5-like enzyme of the Ser/Thr phosphatase family (40).

The two human homologs of yeast Cdc14 were also not inhibited by Net1 (data not shown). At concentrations up to 0.3 μM, Net1(1–600) had no effect on GST-HCdc14A (1–379) and GST-HCdc14B (1–418)[2], fusion proteins containing the catalytic domains from each of the human Cdc14 phosphatases (2). At 3 μM Net1(1–600), HCdc14A and HCdc14B were stimulated 1.5- and 2.5-fold, respectively. Under conditions identical to those employed with yeast Cdc14, there was no detectable binding of Net1(1–600) to affinity matrices containing the HCdc14A and B catalytic domains (data not shown). Thus, Net1 not only fails to inhibit, but is also incapable of binding the human enzymes. These data indicate that Net1 exhibits a high degree of specificity for yeast Cdc14.

Net1 Is a Competitive Inhibitor of Cdc14—To determine the type of inhibition and K_i value, the Henderson method (37) for analyzing tight binding inhibitors was used with Net1(1–600). A representative experiment using Tyr(P)-MBP as substrate is shown in Fig. 2, *B* and *C*. The linear increase in slope with increasing substrate concentration shown in the slope replot of Fig. 2*C* indicates that Net1(1–600) is a competitive inhibitor of Cdc14. The intercept of the slope replot yields a K_i value of 3 nM. A similar K_i was obtained using pNPP as substrate (data not shown).

To evaluate the possibility that Net1 binds at the active site, we examined the effect of sodium tungstate on the interaction with Cdc14. Tungstate, a competitive inhibitor of PTPs including yeast Cdc14 (1), binds within the active site of these enzymes. Pretreatment with sodium tungstate reduced in a concentration-dependent manner the amount of Net1(1–600) that was bound to a GST-Cdc14 affinity matrix (Fig. 3, *A* and *B*). These results show that Net1 and tungstate binding are mutually exclusive and suggest that access to the phosphatase active site is necessary for the interaction with Net1. The reduced affinity of Net1(1–600) for the Cdc14 C283S active site mutant is consistent with this conclusion. Under identical conditions, the amount of Net1(1–600) bound to an affinity matrix

[2] L. Has, T. Despande, Y. Liu, G. S. Taylor, C. Baskerville, and H. Charbonneau, manuscript in preparation.

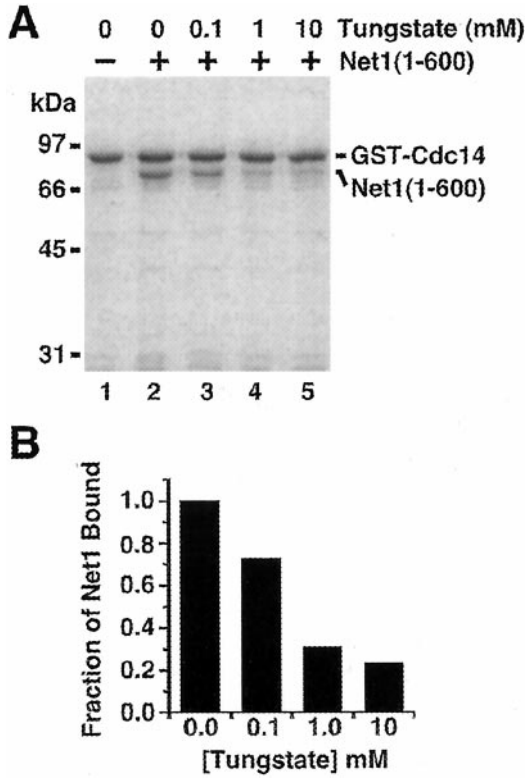

FIG. 3. **Sodium tungstate blocks the binding of Net1(1–600) to Cdc14.** *Panel A*, an *in vitro* binding assay was performed as described for Fig. 1 except that the GST-Cdc14 affinity matrices were incubated with 0.1, 1.0, and 10 mM sodium tungstate for 20 min prior to the addition of 1 nmol of Net1(1–600)-His_6 (*lanes 3–5*). The incubation with Net1(1–600)-His_6 and subsequent washing steps were done in the presence of the indicated concentration of sodium tungstate. After washing, the affinity matrices were collected and separated on a 12% SDS-polyacrylamide gel. *Lane 1* shows the GST-Cdc14 affinity matrix alone, and *lane 2* shows the amount of Net1(1–600)-His_6 bound in the absence of sodium tungstate. Protein was visualized by staining with Coomassie Blue. The positions of molecular mass markers are shown on the *left*, and the locations of GST-Cdc14 and Net1(1–600)-His_6 are indicated on the *right. Panel B*, the relative amount of Net1(1–600)-His_6 bound to the GST-Cdc14 affinity matrix in the presence of the indicated concentrations of sodium tungstate. The relative amount of bound Net1(1–600)-His_6 was estimated by densitometry of the SDS-gel in *panel A* as outlined under "Experimental Procedures."

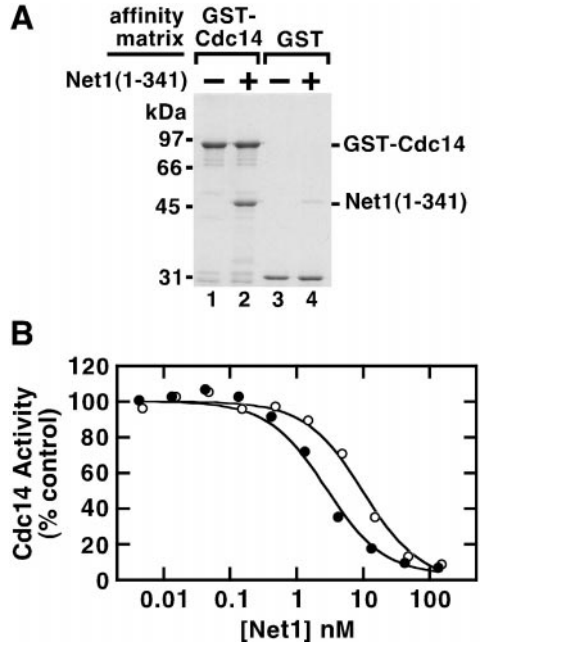

FIG. 4. **Net1(1–341) binds and inhibits Cdc14 *in vitro*.** *Panel A*, purified Net1(1–341)-His_6 (1 nmol) was incubated for 30 min at 4 °C with GST and GST-Cdc14 affinity matrices containing 0.25 nmol of each protein. Affinity matrices and Net1(1–341) were mixed in 1 ml of buffer B. After washing with buffer B containing 0.01% (v/v) Triton X-100, the affinity matrices, both those mixed with Net1(1–341)-His_6 *(lanes 2* and *4)* and untreated controls (*lanes 1* and *3*), were treated with sample buffer and resolved on 12% SDS-polyacrylamide gels that were stained with Coomassie Blue. The positions of molecular mass markers are shown on the *left*, and the positions of GST-Cdc14 and Net1(1–341)-His_6 are indicated on the *right. Panel B*, the relative phosphatase activity of 2.5 nM GST-Cdc14 with 3.2 μM Tyr(P)-MBP as substrate was measured in the presence of the indicated concentrations of recombinant Net1(1–600)-His_6 (●) and Net1(1–341)-His_6 (○). Phosphatase activity is given as the percent of the control value measured in the absence of Net1 fragment, and each data point is the mean of at least three determinations.

comprised of the GST-Cdc14 C283S mutant was 54% of that bound to a matrix formed with the wild type enzyme (compare *lanes 4* and *6* of Fig. 1).

Identification of an NH_2-terminal Net1 Fragment That Binds and Inhibits Cdc14—To delineate further the Cdc14 binding region of Net1 a series of truncation mutants lacking both NH_2- and COOH-terminal sequences was analyzed using yeast two-hybrid assays. The Net1(1–341) truncation mutant interacted with Cdc14 as shown by activation of all three reporter genes in the two-hybrid system (Table I, fourth row). To assess its functional properties and confirm the two-hybrid results, Net1(1–341)-His_6 was expressed in *E. coli*. An *in vitro* binding experiment performed in a manner comparable to that used for Net1(1–600) showed that purified Net1(1–341) was bound to the GST-Cdc14 affinity matrix (Fig. 4A, *lane 2*). A relatively small but detectable amount of Net1(1–341) was retained by GST alone (Fig. 4A, *lane 4*). The addition of 300 mM NaCl eliminated the association of Net1(1–341) with GST without a significant reduction in binding to GST-Cdc14 (data not shown). This effect may be explained by the ability of salt to suppress the aggregation of Net1(1–341) and thereby prevent the retention of small amounts of insoluble protein on the affinity matrices. Using Tyr(P)-MBP as substrate, the IC_{50} for Net1(1–341) was 9.7 nM, about 3-fold higher than that measured for Net1(1–600) under identical conditions (Fig. 4*B*). These results demonstrate that Net1(1–341) retains the ability to bind and fully inhibit Cdc14 albeit with a modest reduction in affinity compared with Net1(1–600).

Among 11 truncation mutants examined, no NH_2-terminal fragments shorter than Net1(1–341) were shown conclusively to bind Cdc14. In two-hybrid assays, Net1(1–146) was unable to associate with Cdc14, and the results with a segment comprised of residues 1–207 were interpreted as being negative because only one of the three reporter genes was activated (Table I, sixth and seventh rows). Consistent with these results, a 27-kDa product obtained from limited cleavage by the Arg-C protease failed to bind a GST-Cdc14 affinity matrix despite containing as many as the first 242 residues from the NH_2 terminus of Net1 (see below). In addition, a preparation of ^{35}S-labeled Net1(1–242) which was produced using the reticulocyte *in vitro* translation system also failed to bind a GST-Cdc14 affinity matrix. The failure to express His-tagged Net1(1–276) and Net1(1–207) in *E. coli* precluded the analysis of these fragments in binding and inhibition assays.

Net1 truncation mutants with 112 or more residues deleted from the NH_2 terminus failed to interact with Cdc14 in two-hybrid assays (data not shown). The Net1(92–1189) mutant interacted with Cdc14 (Table I, eighth row), whereas deletion of the same 91-residue segment from Net1(1–341) abrogated the interaction (Table I, ninth row). These results indicate that the

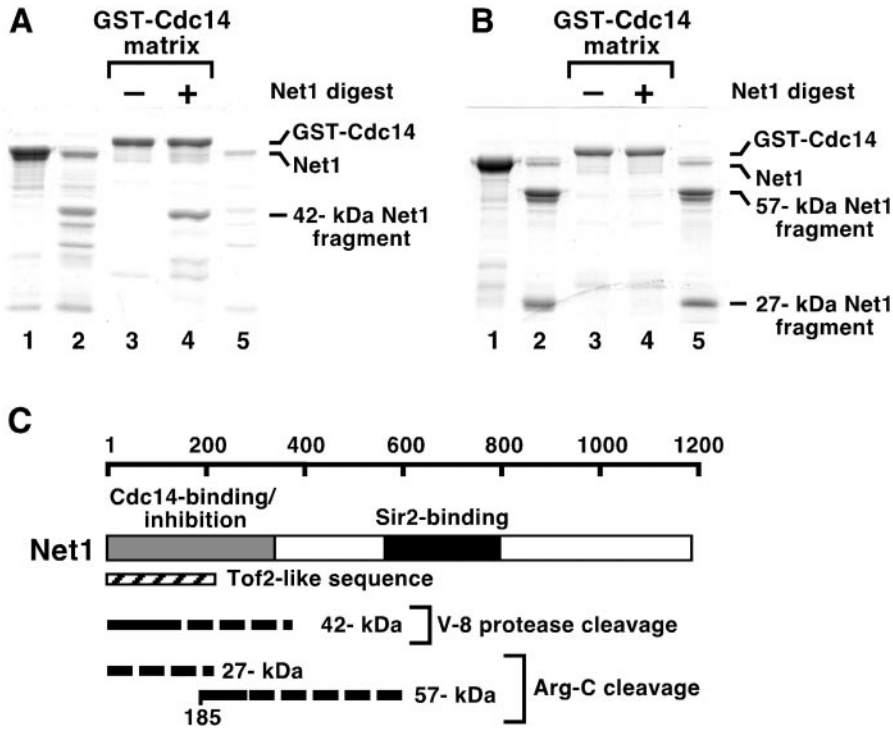

FIG. 5. **Analysis of proteolytic fragments of Net1(1–600).** *Panel A*, Net1(1–600)-His_6 was digested with staphylococcal V8 protease for 60 min at room temperature using a protease:Net1weight ratio of 1:500. Aliquots of an untreated control (*lane 1*) and the digested sample (*lane 2*) were precipitated with trichloroacetic acid and separated on a 12% SDS-polyacrylamide gel. The remaining sample was treated with soybean trypsin inhibitor and analyzed for its ability to bind to a GST-Cdc14 affinity matrix (see Fig. 1). *Lane 3*, washed GST-Cdc14 matrix without added V8 digest; *lane 4*, washed GST-Cdc14 matrix mixed with the V8 digest; *lane 5*, digested material not bound by the affinity matrix. *Panel B*, Net1(1–600)-His_6 was digested with Arg-C protease for 60 min at 30 °C using a protease:Net1 weight ratio of 1:50. Samples of untreated (*lane 1*) and digested (*lane 2*) Net1(1–600)-His_6 were analyzed as above for *panel A*. An *in vitro* Cdc14 binding assay was performed on an aliquot of the Arg-C digest as in *panel A*. *Lane 3*, washed GST-Cdc14 matrix without added Arg-C digest; *lane 4*, washed GST-Cdc14 matrix mixed with the Arg-C digest; *lane 5*, digested material not bound by the affinity matrix. Protein was visualized by staining with Coomassie Blue. In *panels A* and *B*, the positions of GST-Cdc14, Net1(1–600)-His_6, and proteolytic fragments are indicated on the *right*. *Panel C*, schematic diagram illustrating the structural organization of full-length Net1 (*upper bar*) and the location of the major fragments (*black lines*) derived from limited proteolysis. The NH_2 termini of fragments were mapped by amino acid sequence analysis, whereas the *dashed lines* indicate that the locations of COOH termini were deduced from size as estimated by SDS-PAGE and were not determined directly. The *shaded box* within the *upper bar* delineates the location of sequences containing the Cdc14 binding site, and the *black box* denotes the position of the Sir2 binding region as determined by Cuperus *et al.* (41). The *cross-hatched bar* below indicates the location of Net1 sequences displaying a high degree of sequence similarity to Tof2.

first 91 residues at the NH_2 terminus of Net1 may not be necessary for Cdc14 binding, but they may influence the stability or folding of Net1, particularly in the context of the smaller Net1(1–341) fragment.

Limited Proteolytic Cleavage of Net1(1–600)—To examine its domain and structural organization, recombinant Net1(1–600) was subjected to limited proteolysis using trypsin, chymotrypsin, subtilisin, endoproteinase Arg-C, and *S. aureus* V8 proteases. Utilizing low protease to substrate ratios (1:500–1:2,000) typically employed in limited proteolysis studies, Net1(1–600) was degraded rapidly by trypsin, chymotrypsin, and subtilisin, providing no evidence for canonical protease-resistant domains (data not shown). However, digestion with Arg-C and V8 proteases released in modest yield fragments exhibiting partial resistance to proteolysis (Fig. 5, *A* and *B*). These products were tested for their ability to bind GST-Cdc14 using *in vitro* binding assays as described above. A major 42-kDa fragment and multiple minor products from the V8 protease digest were bound by the GST-Cdc14 affinity matrix (Fig. 5*A*) but not by GST alone (data not shown). The amino acid sequence of the 42-kDa fragment matched that of the NH_2 terminus of recombinant Net1(1–600) (Fig. 5*C*). The COOH-terminal residue of this fragment is unknown, but its size (estimated from SDS-PAGE mobility) suggests that it could extend up to Glu-369.

Cleavage with endoproteinase Arg-C yielded three major products, a 27-kDa fragment and two polypeptides migrating as a doublet around 57-kDa (Fig. 5*B*). The NH_2 termini of the 27-kDa fragment and the major component of the doublet were determined by sequence analysis, but the location of their COOH termini was based on size estimates derived from SDS-gel mobilities. The 27-kDa fragment is derived from the NH_2 terminus and could extend from residue 1 to either Arg-216 or Arg-241 (Fig. 5*C*). The 57-kDa product was generated by cleavage after Arg-184, and its large size suggests that it contains residues 185–600 of Net1 (Fig. 5*C*). Because neither product binds GST-Cdc14 (Fig. 5*B*), it appears that Arg-C cleavage disrupts a contiguous sequence surrounding Arg-184 which is necessary for an interaction with Cdc14 (Fig. 5*C*). These studies are fully consistent with results from truncation mutagenesis showing that residues 1–341 encompass a Cdc14 binding region.

DISCUSSION

Current models (15–18) posit that Net1 retains inactive Cdc14 in the nucleolus during interphase and early mitosis until the transient release of the active phosphatase at late anaphase triggers exit from mitosis. The intrinsic biochemical properties of Net1(1–600) which we have observed *in vitro* fully support this model. Nanomolar concentrations of Net1(1–600) alone fully inhibited the activity of Cdc14 through a direct interaction that was not dependent on other proteins. The effect of Net1(1–600) was specific to Cdc14 and observed with all substrates examined including the physio-

logic targets, Swi5 and Sic1. Importantly, Net1(1–600) retained its ability to act as a high affinity inhibitor (IC_{50} = 70 nM) when buffers approximating physiologic ionic strength and pH were employed. These findings demonstrate that Net1 alone has the capacity to regulate all cellular functions requiring Cdc14 by sequestering the phosphatase from the nucleoplasm to the nucleolus and fully inhibiting its activity. Our results also suggest that post-translational modifications of the type commonly observed in eukaryotes are unlikely to be required for inhibition because Net1(1–600) was produced in bacteria. However, post-translational modification or the binding of other protein ligands could influence the affinity of Net1 for Cdc14 and may mediate the release of Cdc14 at late anaphase as suggested previously (18).

Results from *in vitro* binding, inhibition assays, and limited proteolytic cleavage demonstrate that residues 1–341 of Net1 are sufficient for both binding and inhibiting Cdc14 (Fig. 5*C*). Two-hybrid assays suggest that the first 91 residues of Net1 are not essential for binding, but the possibility that this segment is needed for inhibition has not been excluded. Net1 also sequesters Sir2 within the nucleolus where it is involved in rDNA silencing. Cuperus *et al.* (41) have shown that residues 566–801 of Net1 interact with an NH_2-terminal fragment of Sir2 (Fig. 5*C*). Their results together with our data establish that Sir2 and Cdc14 bind to distinct, non-overlapping sites within the linear sequence of Net1 and show that neither protein is required for binding of the other (Fig. 5*C*). Additional structure-function analyses of Net1 will be required to define the minimal sequence(s) required for binding and inhibiting Cdc14.

Several findings suggest that Net1 inhibits by occluding the active site of Cdc14. Net1 acts as a competitive inhibitor of Cdc14, indicating that inhibitor and substrate binding are mutually exclusive. The Net1 binding site has been mapped to a position within the conserved domain of Cdc14 (residues 1–374) which encompasses the entire active site region (residues 279–291). The structures (4, 42–45) of several different PTPs reveal that tungstate, a general PTP inhibitor that mimics the transition state, is bound within the active site in a manner similar to the phosphate of substrates. Sodium tungstate prevents Net1 binding in a concentration-dependent manner (Fig. 3), suggesting that contacts within the Cdc14 active site are required for binding. The significant reduction in Net1 affinity which occurs upon replacement of the Cdc14 active site cysteine also supports the idea that binding involves the catalytic site. Several studies indicate that replacement of the active site Cys can induce local changes in the conformation and properties of PTP catalytic sites (46, 47). For example, a *Yersinia* PTP mutant, in which the analogous Cys is replaced by Ser, exhibits a 10-fold reduction in affinity for tungstate (47). Although we propose an interaction within the catalytic cleft of Cdc14, it is likely that contacts with flanking surfaces are also involved. Interactions with adjacent surfaces could account for the selectivity of Net1 for the yeast enzyme over human Cdc14 and other PTPs despite the high degree of sequence similarity in their catalytic sites. These data favor a model in which Net1 binds at the active site, but we cannot rule out the possibility that it binds to a distinct inhibitory site and competes indirectly through long range interactions that result in conformational changes at the substrate binding site. Additional studies will be required to delineate further the precise location of the Net1 binding site.

Residues 1–220 of Net1 exhibit 30% sequence identity to residues 1–233 of another yeast protein Tof2 (Fig. 5*C*). Other than its ability to interact with DNA topoisomerase I (48), little is known about the function of Tof2. Although the Tof2-like segment is contained within the Cdc14 binding region defined by these studies (Fig. 5*C*), it is not yet clear whether these sequences are involved directly in binding and/or inhibition. The lack of binding observed with the 27-kDa cleavage product of Net1, which spans most if not all of these conserved sequences, suggests the Tof2-like region may not have a direct role in regulating Cdc14.

The *Caenorhabditis elegans*, *Drosophila*, and human genomes and the sequence data bases contain no open reading frames exhibiting significant sequence similarity to Net1. The lack of an extant Net1 homolog in metazoans is surprising because Cdc14 phosphatase genes are found in the *C. elegans*, *Drosophila*, zebrafish, chicken, mouse, and human genomes. This observation coupled with the insensitivity of human Cdc14 to Net1 suggests that the regulation of metazoan and budding yeast Cdc14 differ. In contrast to fungi, the nuclear membrane and nucleolus of metazoans break down during mitosis. As a result of its disassembly, higher eukaryotes may not employ the nucleolus as a site to regulate exit from mitosis. It will be important to determine how Cdc14 activity and its potential function in mitotic exit are controlled in metazoans.

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