Cdi1, a Human G1 and S Phase Protein Phosphatase That Associates with Cdk2

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Summary

We used the interaction trap, a yeast genetic selection for interacting proteins, to isolate human cyclin-dependent kinase interactor 1 (Cdi1). In yeast, Cdi1 interacts with cyclin-dependent kinases, including human Cdc2, Cdk2, and Cdk3, but not with Cdk4. In HeLa cells, Cdi1 is expressed at the G1 to S transition, and the protein forms stable complexes with Cdk2. Cdi1 bears weak sequence similarity to known tyrosine and dual specificity phosphatases. In vitro, Cdi1 removes phosphate from tyrosine residues in model substrates, but a mutant protein that bears a lesion in the putative active site cysteine does not. Overexpression of wild-type Cdi1 delays progression through the cell cycle in yeast and HeLa cells; delay is dependent on Cdi1 phosphatase activity. These experiments identify Cdi1 as a novel type of protein phosphatase that forms complexes with cyclin-dependent kinases.

Introduction

In eukaryotes, the cell cycle proceeds through two major checkpoints, one before the transition from G1 to S, the other before G2 to M. Many events that result in progression through the G2 checkpoint are intracellular, such as the completion of DNA synthesis (Enoch and Nurse, 1991). By contrast, many events that result in passage from G1 to S and S to G2/M are extracellular (Pardee, 1989). In yeast, progress through both checkpoints depends on a cyclin-dependent kinase (Cdk), encoded in Schizosaccharomyces pombe by cdc2* and in Saccharomyces cerevisiae by CDC28 (reviewed by Nasmyth, 1993). Cell cycle progression in mammals requires multiple Cdns. Cdc2 (Lee and Nurse, 1987) is required for the G2 to M transition (Riabowol et al., 1989). The G1 to S transition requires another Cdk, Cdk2 (reviewed by Pagano et al., 1993; Tsai et al., 1993). Recently, more Cdns have been isolated, including Cdk3, Cdk4, and Cdk5. Although the function of these proteins is not known, it is widely thought that they may also regulate progression through particular parts of the cell cycle (Meyerson et al., 1992).

As the name suggests, Cdk activity depends on association with cyclins, regulatory molecules synthesized at different times during the cell cycle that complex with Cdns. Availability of cyclins often regulates Cdk activity; in S. cerevisiae, the rate-limiting step for the G1/S transition is thought to be the accumulation of G1-specific cyclins, including the products of the CLN1, CLN2, CLN3, HSC26, and CLB5 genes (reviewed by Nasmyth, 1993). Mammalian cells also contain a number of cyclins during G1 to S (cyclins D1, D2, D3, and E; reviewed by Sherr, 1993), and it is likely that changes in their level result in changes in the activity of Cdns present at that time. For example, accumulation of cyclin E and the formation of active cyclin E–Cdk2 complexes are necessary for cells to pass from G1 to S (Tsai et al., 1993; Ohtsubo and Roberts, 1993).

Cdk activity is also regulated by protein modification. In S. pombe, during G2, Cdc2 complexed with cyclin B is phosphorylated by the Wee1 and Mik1 tyrosine kinases on Tyr-15 at the ATP-binding site (reviewed by Nillar and Russell, 1992). The phosphorylated complex cannot hydrolyze ATP until it is dephosphorylated by the Cdc25 tyrosine phosphatase (see Moreno et al., 1989); dephosphorylation occurs after DNA synthesis is complete and allows entry into mitosis. A similar mechanism apparently operates in higher cells (Dunphy and Kumagai, 1991; Gautier et al., 1991; Parker and Piwnica-Worms, 1992). Cdc2 is also modified on at least one other residue, Thr-160, by Cdc2-activating kinase (see Solomon et al., 1993; Poon et al., 1993; Fesquet et al., 1993). Thr-160 phosphorylation is required for binding of cyclins to Cdns and for full Cdk activity (Ducommun et al., 1991; Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993). Although the relevance of phosphorylation to events at G1 to S is not clear, Cdk2, which is required for the G1 to S transition, is modified on the equivalent residues (Gu et al., 1992). The cyclins complexed with Cdns also show intricate patterns of phosphorylation, although the significance of these modifications is not known (see, for example, Hall et al., 1993; Koff et al., 1992).

These regulatory proteins are modified by other regulators. For example, in S. pombe and probably in mammalian cells, at G2/M the Wee1 tyrosine kinase is negatively regulated by the Nim1 product, perhaps in response to signals that DNA replication is complete (see Coleman et al., 1993, and references therein). In S. cerevisiae, at G1/S, the activity of G1 cyclins is negatively regulated in response to a proliferation-inhibiting stimulus, α factor. In this case, Fus3 (Ellen et al., 1990) phosphorylates Far1 (Chang and Herskowitz, 1990), which in turn binds to Cln1 Cdc28 and Cln2–Cdc28 complexes and inhibits their activity (reviewed by Nasmyth, 1993); Fus3 also inhibits CLN3 by an unknown mechanism (Ellen et al., 1990). In mammalian cells, the upstream regulatory proteins operate at G1/S and G2/M are currently not well understood. The understanding of mammalian Cdk regulators...
and their upstream regulators is important, however, because these proteins will likely help modulate Cdk activity by coupling it to signals inside and outside the cell. Modulation of Cdk activity during G1 is of particular interest because it is during this period that cells typically decide whether to commit to a new round of passage through the cell cycle, to withdraw temporarily from the cycle and enter a nondividing resting state, or to withdraw permanently from the cell cycle and terminally differentiate (Pardee, 1989). Failures in the mechanisms that govern these G1 decisions may underlie some kinds of aberrant differentiation and cancer.

To understand further mammalian G1 decisions, we have sought to isolate proteins involved in the process. To this end we developed a selection (the interaction trap) for genes encoding proteins that bind to known proteins, a selection that relies on work by Fields and Song (1989). We used the trap to isolate a protein, Cdk interactor 1 (Cd1), that binds human Cdks. Cd1 has weak sequence similarity to known tyrosine and dual specificity phosphatases, but is different enough to suggest that it belongs in a novel sequence class. Sequence similarity is most pronounced in an 11 residue region that spans the putative active site. In vitro, Cd1 dephosphorylates tyrosines in model substrates. Cd1 phosphatase activity is inhibited by chemical inhibitors and abolished by mutation of a critical cysteine in the active site. When overexpressed, Cd1 retards cell cycle progression in yeast and mammalian cells. Cell cycle retardation depends on phosphatase activity. Cd1 mRNA is maximally expressed during G1 and G1/S, and the protein forms stable complexes with a Cdk, Cdk2, operative at this time. Cd1 does not increase the kinase activity of tyrosine-phosphorylated cyclin-Cdk2 complexes. These experiments are consistent with the idea that the Cd1 phosphatase might affect cell cycle progression either by controlling phosphorylation of Cdk2-associated proteins or by acting together with Cdk2 complexes on common substrates.

Results

Interaction Trap

To isolate Cd1, we developed a general transcription-based selection for protein-protein interactions. This interaction trap is an outgrowth of our attempts to use the modular nature of transcription activators (Brent and Ptashne, 1985; Ma and Ptashne, 1988; Triezenberg et al., 1988) to detect protein-protein interactions in yeast. It incorporates a seminal suggestion by Fields and Song (1989) (see Discussion), who, together with other groups, have also developed effective methods for transcription-based interaction cloning (Chien et al., 1991; Dalton and Treisman, 1992; Durfee et al., 1993; Vojtek et al., 1993). Development of the trap, shown in Figure 1a, required careful attention to three classes of components: a fusion protein that contains a LexA DNA-binding domain and that is known to be transcriptionally inert (the "bait"); reporter genes that have no basal transcription and that are bound by the bait; and the proteins encoded by an expression library, which are expressed as chimeras and whose amino termini contain an activation domain and other useful moieties (the "prey"). Relevant design criteria are detailed in Experimental Procedures.

Isolation of Cd1

We isolated Cd1 as described (see Experimental Procedures). We rescued library plasmids from cells in which both reporter genes showed galactose-dependent transcription, assigned the plasmids to three different classes by restriction mapping, identified plasmids from each class that contained the longest cDNA inserts, and verified by Western analysis with anti-epitope antiserum that the plasmids directed the synthesis of fusion proteins (data not shown). Detailed restriction mapping and partial DNA

![Figure 1. Interaction Trap](image-url)
 sequencing showed that two of the recovered cDNA classes were previously identified genes CS11hs and CS21hs, the human homologs of S. pombe suc' product (Richardson et al., 1990). Sequence of the third cDNA class showed it to be a previously unidentified gene. We called this gene CD11 and its protein product Cd11. We expressed activation-tagged Cd11 in a panel of EGY48-derived strains containing different baits to test the reproducibility and specificity of its interaction (Figure 2a). As judged by the LEU2 and lacZ transcription phenotypes, Cd11 interacted specifically with LexA-Cdc2 and did not interact with LexA-c-Myc-C-term, LexA-Max, LexA-Bicoid, LexA-Cln3, or LexA-Fus3 (Figure 2a). Cd11 did, however, interact with other CdkS (see below). Specificity of the Cd11-Cdc2 interaction was confirmed by physical criteria: in vitro, anti-LexA antisera precipitated epitope-containing proteins from yeast extracts that contained LexA-Cdc2 and Cd11, but not from extracts that contained LexA-Bicoid and Cd11 (Figure 2b, lane 5); the mobility of the precipitated proteins was identical to that of immunoreactive Cd11 bands in extracts of yeast that contained tagged Cd11 but were not precipitated (Figure 2b, lanes 3 and 7).

The Cd11 Protein

CD11 was isolated from 12 different library plasmids that contained cDNAs of four different lengths. Sequence analysis revealed that all CD11 cDNA inserts contained an open reading frame, and inspection of the sequence of the longest cDNAs revealed an ATG with a perfect match to the consensus translation initiation sequence (PucC/GATGG; Kozak, 1986) (Figure 3a). Careful analysis of the size of CD11 mRNA in HeLa cells (data not shown) revealed that this ATG occurs between 15 and 45 nt from the 5' end of the CD11 message, suggesting that the longest cDNAs spanned the entire open reading frame, which spans 212 codons. Since isolated CD11 cDNAs by definition encoded proteins that interacted with Cdc2, the sizes of CD11 cDNA inserts from this hunt necessarily localized the portion of the protein sufficient for interaction with Cdc2 to the carboxy-terminal ~170 amino acids of Cd11. The Cd11 sequence (Figure 3a) revealed previously identified protein motifs. First, at the amino terminus, 19 out of 35 amino acids are either proline, glutamic acid, serine, or threonine. Proteins that contain these stretches, called PEST sequences, are degraded rapidly (Rogers et al., 1986), and this stretch of Cd11 is more enriched in these amino
acids than the carboxyl termini of the yeast G1 cyclins, in which the PEST sequences contribute to their rapid degradation (see Nasmyth, 1993). Second, the Cd1 sequence contains potential sites for phosphorylation by casein kinase II and protein kinase C and a weak match to a site for phosphorylation by CdcP (see the legend to Figure 3a).

Third, Cd1 shows weak similarity to protein phosphatases such as human protein tyrosine phosphatase 1 (Figure 4a; Charbonneau et al., 1989). The similarity is strongest in residues 138–148, which show a good match to the Cdk consensus site (Z-S/TP-X-Z, where X is polar and 2 generally basic; Moreno and Nurse, 1990) at 162.

Protein Phosphatase Activity of Cd1

We tested the ability of Cd1 to dephosphorylate phosphotyrosine-containing substrates. We altered the Cd1 codon corresponding to the catalytic cysteine (Pot and Dixon, 1992) in the tyrosine phosphatase active site consensus

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**Figure 3.** CDP Coding Sequence and Deduced Protein Sequence

The EcoRl adaptor, poly(T) tail, and the Xhol site at the 3' end are not shown. Sequence contains six potential casein kinase II phosphorylation sites (S/T-X-X-D/E; Pinna, 1990) at 10, 14, 15, 60, 116, and 170; three potential protein kinase C sites (S-X-P; Woodgett et al., 1966) at 170, 162, and 200; and a weak match to the Cdk consensus site (Z-S/TP-X-Z, where X is polar and 2 generally basic; Moreno and Nurse, 1990) at 162.

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**Figure 4.** Comparison of Cd1 to Other Protein Tyrosine and Dual Specificity Phosphatases

(a) Comparison of Cd1 and human placental protein tyrosine phosphatase 1B (PTP1B; Charbonneau et al., 1989). Sequences were aligned by the GAP algorithm (Pearson and Lipman, 1986). Vertical lines indicate identity, colons and periods indicate conserved changes. Highly conserved residues found in all phosphatases are indicated by asterisks (Freeman et al., 1982).

(b) Comparison of the active site region of Cdp1 to the active site region of other tyrosine phosphatases as aligned by Gautier et al. (1991). The top block includes human T cell protein tyrosine phosphatase (HsTCPTP; Cool et al., 1989), human protein tyrosine phosphatase 1B (PTP1B), human leukocyte common antigen-related protein domain 1 (HLSF1); Gurr et al., 1992; human leukocyte common antigen-related protein domain 2 (HLSF1); Gurr et al., 1992; and vaccinia virus (VH1) (Russell et al., 1991a; tyrosine and dual specificity phosphatases. The bottom block of Cd1 and human protein tyrosine phosphatase 1B (PTP1B) are also included. The similarity is strongest in residues 138–148, which show a good match to the Cdk consensus site (Z-S/TP-X-Z, where X is polar and 2 generally basic; Moreno and Nurse, 1990) at 162.
Cdil, a Cdk2-Interacting Protein Phosphatase

795

805

800

795

To one encoding serine (see Experimental Procedures). We produced Cdil and Cdil-C140S in Escherichia coli as glutathione S-transferase (GST) fusions and purified them to ~90%-95% homogeneity, as judged on Coomassie-stained SDS protein gels (data not shown). We used the purified proteins in phosphatase assays with model substrates. First, we utilized the chromogenic substrate para-nitrophenyl phosphate (pNPP; Sigma). Wild-type Cdil efficiently hydrolyzed pNPP. Hydrolysis increased linearly as a function of Cdil concentration (Figure 5a). Cdil-C140S did not catalyze detectable pNPP hydrolysis (Figure 5a). Sodium vanadate, an inhibitor of the known phosphotyrosine-specific phosphatases (Charbonneau and Tonks, 1992), effectively blocked hydrolysis by wild-type Cdil (Figure 5a). In 2 hr incubations, 1 molecule of GST–Cdil catalyzed hydrolysis of ~140 pNPP molecules (1.2 mol/min). On a molar basis, Cdil was 8-12 times more active than similarly purified full-length human Cdc25C (data not shown).

We then tested the ability of Cdil to dephosphorylate a peptide substrate. For this purpose we labeled a peptide (Raytide; Oncogene Sciences, Incorporated, Manhasset, New York) with c-Src tyrosine kinase and [γ-32P]ATP. We incubated labeled peptide in the presence or absence of Cdil and measured released 32P04 (see Experimental Procedures). Results with this substrate were similar to those with pNPP. Wild-type Cdil efficiently removed phosphate from the peptide. Sodium vanadate inhibited dephosphorylation. Cdil-C140S displayed no activity (Figure 5b). We also tested whether Cdil functioned as a serine/threonine phosphatase by determining whether it could remove phosphates from 32P-labeled H1 histone phosphorylated by CdcP; in our hands, it did not (data not shown). The above results demonstrate that Cdil is a protein tyrosine phosphatase and suggest that it is not active against serines and threonines.

Cdil Expression in HeLa Cells

Because it interacts with Cdc2, we explored the possibility that the Cdil phosphatase might affect cell cycle progression. We first examined Cdil expression during the cell cycle. We synchronized exponentially growing adherent HeLa cells by two cycles of treatment with high concentrations of thymidine to arrest them in late G1, followed by release from this block by exposure to normal concentrations of thymidine, measured their progression through the cell cycle by fluorescence-activated cell sorting (Figure 6a), and isolated RNA at different times. These cells entered S phase more slowly after release from block than reported for suspension HeLa cells (Lew et al., 1991). As is shown in Figure 6b, expression of Cdil mRNA peaks during late G1 and early S. Accumulation of Cdil protein follows the same pattern, as judged by Western blots with anti-Cdil antiserum (data not shown). In these experiments, two reference mRNAs showed the expected time of expression: cyclin E mRNA was expressed late in G1 and disappeared by late S phase (Lew et al., 1991), while cyclin B1 mRNA was expressed later, appearing in S phase and disappearing in M (Pines and Hunter, 1989).

This timing of expression suggested that Cdil phosphatase might function during late G1, during the G1 to S transition, or during early S. To test this idea, we stably transfected HeLa cells with a plasmid that expressed Cdil from the Moloney murine leukemia virus long terminal repeat and includes the neo resistance gene. Compared with the vector, the Cdil-expressing construction did not yield a reduced number of G418-resistant transformants. However, fluorescence-activated cell sorting analysis of DNA in Cdil-expressing transformants showed a significant and reproducible increase in the proportion of cells in G1 or early S relative to control populations (Figure 6c), suggesting that Cdil overexpression may retard passage through these phases of the HeLa cell cycle.

Cdil Expression in Yeast Cells

Cdil also inhibited cell cycle progression in yeast. Cultures of S. cerevisiae that expressed Cdil from the Moloney murine leukemia virus virus long terminal repeat and includes the neo resistance gene. Compared with the vector, the Cdil-expressing construction did not yield a reduced number of G418-resistant transformants. However, fluorescence-activated cell sorting analysis of DNA in Cdil-expressing transformants showed a significant and reproducible increase in the proportion of cells in G1 or early S relative to control populations (Figure 6c), suggesting that Cdil overexpression may retard passage through these phases of the HeLa cell cycle.
Cdil-expressing cells were larger and, when stained with DAPI, that the nuclei of some of the largest cells were not condensed (data not shown). Less than 10% of the cells in Cdil-expressing populations showed buds, as opposed to 40% of the cells in the control population, suggesting that more of the cells in the Cdil-expressing population were in G1 and that Cdil overexpression causes a retardation in passage through the cell cycle similar to its effect in mammalian cells (Figure 7b).

We examined two models for Cdil inhibition of cell cycle progression. First, because Cdil interacts with Cdc28 (see below), its growth retardation phenotype might be due to sequestration of yeast Cdc28 into protein complexes that were not competent to cause the cell to traverse G1 and early S. If so, then the phenotype should be reversible by co-overproducing the human Cdc2 against which Cdil was selected. Second, because Cdil is a protein phosphatase, its inhibitory effect might be dependent on its phosphatase activity. To test these ideas, we expressed wild-type Cdil and the Cdil-C140S mutant in cells that

![Figure 6. CD/l Expression in HeLa Cells](image)

(a and b) Timing. We synchronized adherent HeLa cells by a double thymidine block (Lew et al., 1991) and collected aliquots every 3 hr after release. (a) Fluorescence-activated cell sorting analysis of DNA content, which shows that cells reentered the cell cycle 9 hr after release. (b) Northern blot analysis of RNAs. RNA from each aliquot was run on a formaldehyde-agarose gel and blotted onto nylon as described (Ausubel et al., 1987-1993). The blot was probed with random-primed DNA probes from Cdil, human cyclin E, human cyclin B1, and human glyceraldehyde-phosphate-dehydrogenase (GAPD), a normalization control. Lane labels show times in hours after release.

(c) Effect. HeLa cells were transfected either with pBNCdi (see Experimental Procedures), which directed the synthesis of Cdil, or with vector alone and were analyzed as described in Experimental Procedures. The G1 midpoint is defined as the mode of the distribution of each graph; the modes on the two panels are of different heights (272 counts for cells transformed with the vector, 101 counts for cells that contain Cdil); the broadened peak in the Cdil cells reflects the increased proportion of the population containing approximately 1 x DNA content. Results of a typical run are shown.

![Figure 7. Effect of Cdil on Yeast Cell Growth](image)

(a) Growth rates of cells that expressed Cdil. S. cerevisiae W303 that carried the indicated combinations of Cdil and/or Cdc2 expression vectors were grown in the indicated media and growth monitored by OD as described in Experimental Procedures. Note that the Cdil and Cdc2 expression plasmids together caused some growth inhibition even in glucose medium, which we attribute to leaky expression from the GAL1 promoter derivative on the pJGC1 expression plasmid (J. G., unpublished data). Closed triangles, expression vectors only; open squares, Cdc2; open circles, wild-type Cdil; open triangles, Cdil-C140S; closed squares, Cdc2 and wild-type Cdil; closed circles, Cdc2 and Cdil-C140S.

(b) Budding index. Cells that expressed Cdil or contained the expression vector were grown for 8 hr in galactose as described above. Cells (400 from each population) were examined by phase-contrast microscopy, and the budding index was calculated as the percentage of budded cells in each population.
contained Cdc28, with and without overexpressed native human Cdc2.

Figure 7a shows that coexpression of Cdc2 did not rescue Cdi-dependent growth inhibition. The Cdi–C140S mutant did not cause growth inhibition, whether it was expressed alone or together with Cdc2 (Figure 7a). Unexpectedly, coexpression of Cdc2 with wild-type Cdi increased the severity of the Cdi-dependent growth inhibition by about 2-fold (Figure 7a). These experiments suggest three conclusions. First, because expression of excess Cdc2 does not rescue Cdi1 growth retardation, they suggest that this growth inhibition cannot be explained by simple sequestration of Cdc28 into nonfunctional complexes. Second, because Cdi1-dependent growth inhibition is strictly dependent on the phosphatase activity of Cdi, these results suggest that the inhibition is due to diphosphorylation of Cdi substrate(s). Finally, because Cdi1-dependent growth inhibition is heightened by coexpression of Cdc2, these results suggest a functional genetic interaction between Cdi1 and Cdc2 in addition to the physical interaction.

Cdi1 Activity In Vitro

We considered two explanations for the genetic interaction between Cdi1 and Cdk2 suggested by the yeast coexpression experiments. First, just as Cdc2 is reported to phosphorylate Cdc25 and increase its phosphatase activity (Hoffman et al., 1993), we thought that Cdc2 might phosphorylate Cdi1 and increase its activity. We thus tested whether known Cdk2 could phosphorylate Cdi1 in vitro. We found that neither Cdc2 nor Cdk2 complexes isolated from HeLa cells nor cyclin B1–Cdk2 complexes reconstituted from pure proteins expressed in Sf9 cells (a gift of B. Gabrielli and H. Piwnica-Worms) could phosphorylate GST–Cdi1, although these complexes phosphorylated histone H1 (data not shown). Second, we asked whether Cdi1 activated Cdk2. For this purpose we used a cyclin B1–Cdk2 complex phosphorylated in vitro on Tyr-15 by the human Wee 1 tyrosine kinase (a gift of B. Gabrielli and H. Piwnica-Worms). Treatment of this complex with GST–Cdi1 did not increase its histone H1 kinase activity, while treatment with GST–Cdc25C did (data not shown). This suggested that Cdi1 is unable to remove the inhibitory phosphate from Tyr-15 and to activate the kinase. Thus, although Cdi1 phosphatase and Cdk2 interact, these results suggest that neither protein is a substrate for the other.

Cdi1 Interaction with Cdk2 in Yeast

Since Cdi1 is normally expressed during G1 and its overexpression retards the G1 to S transition, it seemed possible that it might normally form complexes with Cdk2 other than Cdc2. To determine which Cdk2 might be Cdi1 partners, we used the interaction trap to measure the ability of Cdi1 to interact with a panel of different bait proteins. This panel included LexA derivatives of Cdc2 proteins from yeast (Lorincz and Reed, 1984), humans (Lee and Nurse, 1987), and flies (Jimenez et al., 1990; Lehner and O'Farrell, 1990), LexA derivatives of human Cdk2 (Ellidge and Spotswood, 1991; Tsi et al., 1991) and Cdk3 (Meyerson et al., 1992), and LexA derivatives of the less closely related human Cdk4 (Hanks, 1987) and yeast Fus3 (Ellion et al., 1990).

Table 1 shows that activation-tagged Cdi1 stimulated transcription from these baits to different levels. Cdi1 activated strongly in strains that contained the human Cdc2 bait against which it was selected, less strongly in strains that contained S. cerevisiae Cdc28, human Cdk2, or human Cdk3 baits, and only weakly in strains that contained DmCdc2, one of the two Drosophila Cdc2 homologs. In strains that contained human Cdk4, DmCdc2c, or Fus3 baits, Cdi1 did not activate at all (Table 1). All kinases with which Cdi1 interacted contained the so-called PSTAIRE motif, a stretch of amino acids that includes the Cdk catalytic site (Meyerson et al., 1992). Cdi1 did not interact with Cdk4, which has a similar sequence in this region (PISTVRE; Meyerson et al., 1992), or with Fus3, which has no apparent similarity to this region. Cdi1 did not interact with DmCdc2c, which contains a PSTAIRE motif but which differs substantially at other residues. This experiment suggested that the partners of Cdi1 in mammalian cells may be other PSTAIRE-containing Cdk2 present when Cdi1 is, such as Cdk2.

Association of Cdi1 with Cdk2 in HeLa Cells

To explore the Cdi1–Cdk2 interaction, we used anti-Cdi1 antisera (tested as described in Experimental Procedures) and other antisera that specifically recognized Cdc2 and Cdk2 (gifts of L.-H. Tsai and E. Harlow) to precipitate complexes containing these proteins from 35S-labeled HeLa cell extracts. Precipitated proteins were separated on SDS gels (Figure 8a). Cdi1 antisera brought down major protein bands with apparent molecular masses of 21 kd and 33 kd (Figure 8a, lane 3). These bands were absent from precipitates that used Cdi1 preimmune serum (Figure 8a, lane 2) and nonspecific rabbit serum (lane 6). The 21 kd band corresponded to Cdi1; its mobility was the same as that of in vitro translated Cdi1 and of immunoprecipitated in vitro translated Cdi1 (see Experimental Procedures). The 33 kd band had a similar mobility to one of the two major bands immunoprecipitated from HeLa

<table>
<thead>
<tr>
<th>Bait</th>
<th>Species</th>
<th>β-Galactosidase Activity</th>
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<tr>
<td>LexA Cdc2</td>
<td>Human</td>
<td>1240</td>
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<tr>
<td>LexA–Cdk2</td>
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</tr>
<tr>
<td>LexA–Cdk3</td>
<td>Human</td>
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<td>LexA–Cdc2</td>
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<td>LexA–Cdc2c</td>
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<td>&lt;2</td>
</tr>
<tr>
<td>LexA–Fus3</td>
<td>S. cerevisiae</td>
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Table 1. Differential Interaction of Cdi1 with Cdc2 Family Members
Cells and Cdk's

Discussion

Passage of eukaryotic cells through different phases of the cell cycle is dependent on Cdk's. Interacting regulatory proteins connect the activity of the Cdk's to signals generated inside the cell and to events in the extracellular environment (Sherr, 1993). To understand better cell cycle regulation in humans, we developed a general yeast transcription genetic selection and used it to isolate proteins that touch Cdk's and might modulate their activity or function with them during the cell cycle. This selection, the interaction trap, relies on the fact that eukaryotic transcription activators are often composed of discrete DNA-binding and activation domains and that the activation domain can be brought to the promoter by protein–protein interactions (Brent and Ptashne, 1985; Ma and Ptashne, 1988; Fields and Song, 1989). It is especially dependent on the two-hybrid system proposed by Fields and Song (1989) in that our use of transcription to detect proteins that interact with DNA-bound baits incorporates their suggestion that potential interacting proteins could be detected if they carried activation domains. Several techniques that make use of this suggestion have been developed in a number of labs (Chien et al., 1991; Dalton and Treisman, 1992; Durfee et al., 1993; Voitek et al., 1993).

As demonstrated here and in Zervos et al. (1993), a number of features make the trap particularly useful. First, the reporters have qualitatively different phenotypes: the LexAop–LEU2 reporter provides a selection, allowing large numbers of potential interactors to be easily surveyed, while the LexAop–lacZ reporter provides a simple secondary test to reject adventitious LEU* colonies. Second, the reporters have different promoters, which may eliminate false positive proteins that interact with the promoter instead of the bait. Third, the reporter phenotypes differ in their sensitivity, which affects the affinity of the bait–prey interactions they can detect. The LexAop–LEU2 reporter responds to interactions with an apparent Kd of about 10^-6 M, while the different LexAop–lacZ reporters respond with different sensitivities; which lacZ reporter is used is determined by the investigator. Fourth, the activation region on the library proteins is relatively weak, to avoid restrictions in the spectrum of recovered proteins that might be caused by the strong, semitoxic activation domains of GAL4 and VP16 (Gill and Ptashne, 1988; Triendenberg et al., 1988; Berger et al., 1992). Finally, conditional expression from the library vector allows the Leu

Figure 8. Cdl1-Associated Proteins in HS-Labeled HeLa Cell Extracts

Proteins were immunoprecipitated from HeLa cell extracts and analyzed as described in Experimental Procedures.

(a) Molecular mass markers (M) (lane 1), preimmune serum (lane 2), and anti-Cdl1 (lane 3), anti-Cdc2 (lane 4), anti-Cdk2 (lane 5), and nonspecific rabbit (NR) (lane 6) antisera.

(b) Partial proteolysis of proteins indicated amounts of S. aureus V8 protease as described (see Experimental Procedures). Lanes 1–3, in vitro translated (IVT) Cdc2; lanes 4–6, p33 band from anti-Cdc1 immunoprecipitations; lanes 7–9, in vitro translated (IVT) Cdk2.
and LacZ phenotypes to be unambiguously ascribed to the library protein, diminishing the number of library plasmids that must be excluded by subsequent tests. While useful schemes with some of these attributes have recently been described (e.g., Vojeck et al., 1993), none has the entire set.

Because this interaction hunt used a relatively insensitive lacZ reporter, it identified only three proteins, all of which associate strongly with human Cdc2. Two had previously been identified: the CKS1 and CKS2 proteins (Richardson et al., 1990), the human homologs of the S. pombe suc1" product. Cdi1, the protein reported here, contains 212 amino acids, of which the carboxy-terminal 170 are sufficient for interaction with Cdc2. Cdi1 has consensus sites for protein kinase C and casein kinase II. The amino-terminal 35 residues of the protein comprise a PEST sequence (Charbonneau and Tonks, 1992). From sequence, Cdi1 is more different from known nonreceptor tyrosine and dual specificity phosphatases, including similarity to a stretch of amino acids that defines the active site (Charbonneau and Tonks, 1992). In vitro, Cdi1 possesses tyrosine phosphatase activity on model substrates such as pNPP and tyrosine-phosphorylated peptides. Cdi1 activity is inhibited by chemical inhibitors and by a mutation that eliminates a cysteine in the catalytic site. These facts establish that Cdi1 represents a novel class of human protein phosphatase, which, like S. cerevisiae Cdc14 and Cdc25, may function in cell cycle progression (Miliar and Russell, 1992; Wan et al., 1992).

In HeLa cells that express Cdi1 under the control of a retroviral promoter, the proportion of cells in the population in G1 or early S is increased, suggesting that Cdi1 overexpression delays progression beyond these stages of the cell cycle. This fact is consistent with the time of its expression, which is strikingly specific for the cell cycle stage: in synchronized populations, Cdi1 mRNA is highest during G1 and at the G1 to S transition. Expression of Cdi1 also retards cell cycle progression in S. cerevisiae; again, Cdi1 increases the proportion of cells in G1, suggesting that expression retards the G1 to S transition. Retardation requires Cdi1 phosphatase activity and is enhanced by overexpression of Cdc2. These results indicate that cell cycle retardation by Cdi1 is not likely due to sequestration of Cdc28 by Cdi1 into inactive complexes and suggest a functional genetic interaction between Cdi1 and Cdk2.

As measured by interaction in yeast, Cdi1 interacts with different Cdns with very different affinities. All Cdns with which Cdi1 interacts strongly (Cdc2, Cdk2, and Cdk3) contained a distinctive set of amino acids in the active site, the so-called PSTAIRE motif (Meyerson et al., 1992). To identify those proteins complexed with Cdi1 in human cells, we immunoprecipitated Cdi1-associated proteins from HeLa cells. The apparent molecular mass of one of these (33 kd) suggested that it might be the Thr-160-phosphorylated form of Cdk2. We analyzed this protein by partial V8 proteolysis and confirmed that the protein was Cdk2. We were unable to demonstrate an association of Cdi1 with other Cdns in HeLa extracts. These experiments demonstrate that Cdi1 associates with Cdk2, a kinase thought to govern the G1 to S transition, and thus further emphasize that Cdi1 may influence this transition.

The most obvious model for Cdi1 function, given its association with Cdk2, is that Cdi1 function might modify Cdk2, dephosphorylating the Tyr-15 equivalent and stimulating kinase activity. However, since we cannot activate tyrosine-phosphorylated Cdk2-containing complexes by treatment with Cdi1, we do not think it removes tyrosines from Cdk2. A second model is that Cdi1 dephosphorylates other Cdk2-associated proteins, such as cyclins, thus indirectly affecting Cdk activity; this idea receives support from the fact that complex patterns of cyclin A, B1, D1, and E phosphorylation exist (see, for example, Hall et al., 1993; Koff et al., 1992). A third model is that Cdi1 functions with other members of Cdk2-containing complexes on common substrates. This idea is consistent with the observed functional interaction between Cdi1 and Cdk2 in yeast in which coexpression of Cdc2 increases Cdi1-dependent cell cycle retardation. In this view, the proteins complexed with Cdi1 may provide the regulatory domain that Cdi1 apparently lacks. Identification of the Cdi1 substrate(s) will help distinguish among these ideas and in understanding the function of this Cdk2-associated phosphatase during G1 and early S.

**Experimental Procedures**

**Bacteria and Yeast**

Manipulation of E. coli and DNA was by standard methods (Ausubel et al., 1987-1993). "Sure" mcrA3 (mr, hsdRMS, mcrBC) endA1 supC44 thr-1 gyrA95 recA1 leu2-3,112 recA1 araD1 araE180 uvrD1-Tn5 (kan R) uvrC1

**E.coli**

Avidin beads were prepared as described (Hanks, 1987; Meyerson et al., 1992). Analyses of the Cdi1 present in the lysate were performed by alpha scanning. These results indicate that cell cycle retardation by Cdi1 is not likely due to sequestration of Cdc28 by Cdi1 into inactive complexes and suggest a functional genetic interaction between Cdi1 and Cdns.

**Cdk2-Interacting Protein Phosphatase**

799

**Interaction Trap**

Detailed map, sequence, and methodological information is available on request and from the Massachusetts General Hospital (MGH) Molecular Biology Internet Gopher server. Baits were produced constitutively from a 2u HIS3 plasmid under the control of the ADH1 promoter and contained the LexA terminus of the cyclin A, B1, D1, and E phosphorylation exist (see, for example, Hall et al., 1993; Koff et al., 1992). A third model is that Cdi1 functions with other members of Cdk2-containing complexes on common substrates. This idea is consistent with the observed functional interaction between Cdi1 and Cdk2 in yeast in which coexpression of Cdc2 increases Cdi1-dependent cell cycle retardation. In this view, the proteins complexed with Cdi1 may provide the regulatory domain that Cdi1 apparently lacks. Identification of the Cdi1 substrate(s) will help distinguish among these ideas and in understanding the function of this Cdk2-associated phosphatase during G1 and early S.

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al., 1989) was a gift of K. Visvanathan. These proteins contained two amino acids (EF) between the last amino acid of LexA and the bait moieties. The Drosophila Cdc2 bait (Lemmer and O’Farrell, 1990) were gifts of R. Finley. LexA–UAS3 (Elion et al., 1990) and LexA–Cln3 (Cross, 1988) were made analogously except they were cloned as BamHI fragments and thus contained five amino acids (EPFGI) between LexA and the fused moieties. All these fusions contained the second codon to the stop codon of each bait. LexA–C–Myc–C–term (which contains the carboxy-terminal 176 amino acids of human C–Myc), LexA–Max (which contains all of the human Max coding sequence), and LexA–Bicoid (amino acids 2–160), a gift of R. Finley, have been described (Zervas et al., 1993; Golend and Brent, 1992).

Reporters

The LexAop–LEU2 construction replaced the yeast chromosomal LEU2 gene. The other reporter, pB1840, one of a series of LexAop–Gal1–lacZ genes (Brent and Ptashne, 1985; Kamens et al., 1990; S. Hanese, unpublished data), was carried on a 2μ plasmid. Basal reporter transcription was extremely low, presumably owing both to the removal of the entire upstream activating sequence from both reporters and to the fact that LexA operators introduced into yeast promoters decrease their transcription (Brent and Ptashne, 1984). Reporters were chosen to differ in sensitivity. The LEU2 reporter contained three copies of the high affinity LexA-binding site found upstream of E. coli colE1 (Ebina et al., 1983; Kamens et al., 1990), which presumably bind a total of six dimers of the bait. In contrast, the lacZ gene contained a single lower affinity operator (Brent and Ptashne, 1984) that binds a single dimer of the bait. The operators in the LEU2 reporter were closer to the transcription start point than they were in the lacZ reporter. These differences in the number, affinity, and operator position all contribute to that fact that the LEU2 reporter is more sensitive than the lacZ gene. Construction of the EGY148 selection strain will be detailed elsewhere (E. G., unpublished data).

Expression Vectors and Library

Library proteins were expressed from pJG4–5, a member of a series of expression plasmids designed to be used in the interaction trap and to facilitate analysis of isolated proteins. These plasmids carry the 2μ replicator and the TRP1 marker. pJG4–5, whose construction will be described elsewhere, directs the synthesis of fusion proteins. Proteins expressed from this vector possess the following features: galactose-inducible expression so that their synthesis is conditional, an epitope tag to facilitate detection, a nuclear localization signal to maximize intranuclear concentration to increase selection sensitivity, and an activation domain derived from E. coli (Ma and Ptashne, 1967). Library proteins were expressed from pJG4–5, a member of a series of expression plasmids designed to be used in the interaction trap and to facilitate analysis of isolated proteins. These plasmids carry the 2μ replicator and the TRP1 marker. pJG4–5, whose construction will be described elsewhere, directs the synthesis of fusion proteins. Proteins expressed from this vector possess the following features: galactose-inducible expression so that their synthesis is conditional, an epitope tag to facilitate detection, a nuclear localization signal to maximize intranuclear concentration to increase selection sensitivity, and an activation domain derived from E. coli (Ma and Ptashne, 1967).

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Mammalian Expression

We grew HeLa cells on plates and transfected them (Ausubel et al., 1987–1993) either with a DNA copy of pBNCod1 (J. G., unpublished data), a derivative of a neo resistance retroviral expression vector (Morgenstern and Land, 1990) that directs synthesis of native Cdc1 under the control of the Moloney murine leukemia virus promoter, or with vector alone. Cdc1 expression did not diminish the number of G418-resistant cells recovered. We rescued individual (about 20) clones from each transfection and grew them on plates in Dulbecco’s modified Eagle’s medium plus 10% calf serum. We suspended cells from four Cdc1-transfected and four control-transfected clones in 225 μl of 30 mg/ml trypsin in 3.4 mM citrate, 0.1% Nonidet P-40, 1.5 mM spermine, and 0.5 mM Tris and incubated them on a rotator for 10 min at room temperature. We then added 188 μl of 0.5 mg/ml of trypsin inhibitor and 0.1 mg/ml of RNAase A, vortexed the suspension, added 100 μl of 0.4 mg/ml of propidium iodide and 1 mg/ml spermine, and incubated the samples for 30 min at 4°C. For each sample, we analyzed 10,000–20,000 events in a Becton-Dickinson fluorescence-activated cell sorter using the CellFIT cell cycle analysis program (version 2.01.2).

Cdc1 and Cdc1–C140S

We made the Cdc1 Cys–140 to Ser mutation (Cdc1–C140S) by polymerase chain reaction with overlapping primers and Vent polymerase, essentially as described (Hanes and Brent, 1991), changing TGC at position 140 to TCT. Mutagenesis was verified by sequencing the entire mutant gene. We introduced EcoR1–Xhol fragments containing the wild-type and C140S coding sequences into a modified pGEX2T vector and expressed and purified the GST fusion proteins essentially as described (Ausubel et al., 1987–1993). We performed pNPP assays on these proteins in 500 μl of 50 mM Tris–HCl (pH 7.2), 2 mM EDTA, 0.1% (v/v) 8-mercaptoethanol, 20 mM pNPP (Sigma) containing the indicated amount of protein at 30°C. After 2 hr, we stopped the reactions by adding 50 μl of 5 M NaOH and measured OD405. For peptide assays, we labeled Raytide (Oncogene Science), at its tyrosine residue with [γ–32P]ATP and c-Src tyrosine kinase (Oncogene Science) as described by the supplier. For phosphatase assays, we used 10© cpm of radioactive [γ–32P]tyrosine–Raytide in 50 nl reactions as described in Figure 5 and by Streuli et al. (1990).

Antibodies and Immunoprecipitation

Rabbit polyclonal antibody to GST–Cdc1 was produced by East Acres Biologicals, Incorporated (Southbridge, Massachusetts) according to standard methods (Harlow and Lane, 1988). Specificity of the antiserum was demonstrated by immunoprecipitation of in vitro translated Cdc1 (TNT coupled reticulocyte lysate system. Promega) GST–Cdc1 blocked precipitation of this labeled product. Programmed lysate (5 μl) was separately precipitated with 10 μl of Cdc1 antisera and 10 μl of Cdc1 preimmune serum in 600 μl of phosphatase-buffered saline in the presence or the absence of an excess (20 μg) of GST–Cdc1 protein. Metabolic labeling and precipitations from subconfluent HeLa cells were performed essentially as described in Harlow and Lane.
(1988) except that cells were lysed in 250 mM NaCl, 50 mM HEPES-KOH (pH 7.5), 5 mM EDTA, 0.1 mM NaVO₃, 50 mM NaF, 0.1% Triton, 1 mM phenylmethanesulfonyl fluoride, 25 µg/ml leupeptin, 25 µg/ml pepstatin for 30 min at 4°C. In control precipitations, GST-Cdk1 blocked precipitation of Cdk1 and associated proteins. Pellets were run on 11% SDS protein gels that were analyzed either on a Molecular Dynamics image analyzer or by fluorography. For V8 mapping, bands were excised and partially digested in the gel with Staphylococcus aureus V8 protease as in Harlow and Lane (1988). Products, together with those resulting from digestion of in vitro translated Cdkp and Cdil, a CdkP-interacting protein phosphatase, were separated on 7.5% SDS–polyacrylamide gels, dried, and visualized on the image analyzer.

Acknowledgments

We are very grateful to those named in the text for gifts of reagents and to Andrew Mendelsohn, Russ Finley, Joanne Kamens, Steve Hanes, Matt Meyerstern, Enrico Lees, John Little, Michaela Poggenku, Giulio Draetta, Josh Kaplan, Robin Wharton, Stanley Fields, Jun Ma, and Richard Treisman for useful discussions, comments, or both on the manuscript. J. G. was supported by the Human Frontier Science Project (HFSP), E. G. by the National Institutes of Health, and R. B. by the Pew Scholar's program and by an American Cancer Society faculty research award. Work was supported by the HFSP.

Received August 24, 1992; revised October 20, 1992.

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GenBank Accession Number

The number for the sequence reported in this paper is U02681.