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Mutations at Sites Involved in Suc1 Binding Inactivate Cdc2

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***suc1*$^+$ encodes an essential cell cycle regulator of the fission yeast *Schizosaccharomyces pombe*. Its product, a 13-kDa protein, interacts with the Cdc2 protein kinase. Both positive and negative effects on cell cycle progression have been attributed to Suc1. To date, the exact mechanisms and the physiological role of the interaction between Suc1 and Cdc2 remain unclear. Here we have studied the molecular basis of this association. We show that Cdc2 can bind Suc1 or its mammalian homolog directly in the absence of any additional protein component. Using an alanine scanning mutagenesis method, we analyzed the interaction between Cdc2 and Suc1. We show that the integrity of several domains on the Cdc2 protein, including sites directly involved in catalytic activity, is required for binding to Suc1. Furthermore, Cdc2 mutant proteins unable to bind Suc1 (but able to bind cyclins) are nonfunctional when overexpressed in *S. pombe*, indicating that a specific interaction with Suc1 is required for Cdc2 function.**

The mechanisms regulating cell cycle progression are starting to be elucidated in detail (for a review see references 7 and 38). Several proteins, initially isolated from yeast cells but later identified in higher eukaryotic species as well, have a direct role in controlling cell cycle progression. Among them, the Cdc2 protein kinase plays a central regulatory role. Cdc2 is a 34-kDa serine/threonine protein kinase; it associates with two distinct polypeptides, cyclin and Suc1. It has been suggested that this kinase is activated at specific stages of the cell cycle through binding to distinct cyclin subunits (37). The formation of a complex between Cdc2 and cyclin at a certain stage of the cell cycle is dependent upon the accumulation of the specific cyclin and posttranslational modifications. The active kinase complex would then phosphorylate critical substrates for each cell cycle stage. In this model, the degradation of the cyclin subunits, probably through a ubiquitin-dependent protease (15), would shut off the kinase activity by releasing inactive monomeric Cdc2.

The most extensively studied Cdc2 kinase complex is the maturation-promoting factor, present in both meiotic and mitotic cells. Maturation-promoting factor is a complex of the Cdc2 catalytic subunit and cyclin B, a component which accumulates and binds Cdc2 during the G_2 phase of the cell cycle (13, 30). The Cdc2-cyclin B complex is kept inactive until the end of G_2 by phosphorylation at Tyr-15, a residue located within the ATP binding site of the protein (17). In *Schizosaccharomyces pombe*, tyrosine dephosphorylation of Cdc2 leads to the activation of the kinase complex (1, 16) and entry into mitosis. Substitution of Tyr-15 with phenylalanine causes cells to undergo mitosis prematurely, since they do not complete DNA replication before undergoing mitosis. For chicken cells (25) it has been found that, in addition to Tyr-15, Thr-14 is also phosphorylated; phosphorylation at both residues may have a role in keeping the Cdc2 kinase inactive until DNA replication is completed. Indeed, in both *Xenopus* and HeLa cells, the expression of Cdc2 with a double mutation, T14A Y15F, causes premature mitotic events (26, 36). Additional sites of phosphorylation of Cdc2 have been identified. In chicken embryo fibroblasts, during the G_1 and S phases, Cdc2 is phosphorylated at Ser-277 (25). Although this residue is conserved in all Cdc2 homologs, its phosphorylation has not been demonstrated in yeast cells. An additional site of threonine phosphorylation has been identified at amino acid 167 (Thr-161 in vertebrate Cdc2) (15a). This site corresponds to a site conserved in all known protein kinases (2). Mutations of this threonine generate functionally inactive proteins unable to bind cyclins (10).

In addition to the cyclins, it has been found that in fission yeast cells, a 13-kDa protein can specifically coimmunoprecipitate with Cdc2 (4). This protein is the product of the *suc1* gene, which was identified as a plasmid suppressor of some, but not all, temperature-sensitive *cdc2* mutations (21). Both G_1/S and G_2/M functions of *cdc2* are rescued by Suc1 overproduction. *suc1* encodes an essential function, since germinating spores containing a null allele of the gene undergo two or three divisions but then die, mostly with an elongation (Cdc$^-$) phenotype (20, 23). *cdc2* mutants which can be rescued by Suc1 overproduction show a defective interaction with the Suc1 protein in vitro (4). Conversely, in wild-type cells gross overproduction of Suc1 in vivo causes cell cycle arrest (1a). *S. pombe* strains carrying an integrated copy of the *suc1* gene under the control of a strong constitutive promoter have a reduced growth rate, about half that of the wild type (20, 23). Most of the Suc1-overproducing cells have a G_2 DNA content similar to that found in wild-type cells, suggesting that the delay caused by Suc1 overproduction occurs in the G_2 phase rather than the G_1 phase of the cell cycle (20). In addition, it has been found that the addition of Suc1 to G_2-arrested *Xenopus* oocyte extracts inhibits tyrosine dephosphorylation and the subsequent appearance of the histone H1 kinase activity of Cdc2 (11). Similar inhibitory effects on cell division have been obtained by microinjecting the Suc1 protein in rat fibroblasts (40). *suc1* homologs have indeed been isolated in other eukaryotic species. The *Saccharomyces cerevisiae* homolog, *CKS1*, has been isolated as a high-copy-number plasmid suppressor of a temperature-sensitive *CDC28* mutation. Its gene product has also been found to interact with CDC28 (18). Antibodies raised against the *S. pombe suc1* gene product recognize a homologous protein in HeLa cells (9). Recently, two *suc1* or *CKS1* homologs were identified in mammalian cells (41); they can rescue a null mutation of the

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CKS1 gene in *S. cerevisiae*, and their gene products can bind the CDC28 or Cdc2 protein kinase.

Since Suc1 can be expressed at high levels in bacteria and easily purified, it has been possible to purify and characterize Cdc2 complexes from a number of different species by affinity chromatography on Suc1 beads (p13-Sepharose) (4, 12). For example, a complex of cyclin B and Cdc2 can be purified to homogeneity from starfish oocytes by chromatography on p13-Sepharose (29, 39).

In this study, we investigated the molecular basis of the interaction between Cdc2 and Suc1. We show that these two proteins interact in the absence of any other component. We identified regions in the Cdc2 protein which are required for this interaction and show that Cdc2 mutant proteins which are unable to bind Suc1 in vitro are nonfunctional in vivo in *S. pombe*.

MATERIALS AND METHODS

Site-directed mutagenesis. The coding sequence of the human *cdc2* gene was cloned in the pT7F1A expression vector (10). Uridylated single-stranded DNA was prepared as described by Sambrook et al. (43) by M13K07 helper phage infection in *Escherichia coli* BW313. Oligonucleotide-directed mutagenesis was performed as described previously (27, 47), and the colonies obtained were screened by DNA sequencing.

In vitro transcription and translation procedures. In vitro transcription of the human *cdc2* mutations cloned in pT7F1A was performed with a Riboprobe kit (Promega). The transcription mixture contained 1× polymerase buffer (Promega), 0.5 mM ribonucleoside triphosphates, 10 mM dithiothreitol, 0.1 U of RNasin (Promega) per μl, 0.04 U of 7mGpppG (Boehringer), 1.2 U of T7 polymerase per μl, and 0.5 μg of template DNA in a final volume of 10 μl. The reaction was performed at 37°C for 1 h. The reaction mixture was diluted to 100 μl, and the RNA was subsequently extracted by phenol and chloroform-isoamyl alcohol extractions. After ethanol precipitation in the presence of 0.3 M Na acetate (pH 5.4) and 1 μg of glycogen, the RNA was resuspended in 10 μl of nuclease-free water containing 1 U of RNasin.

In vitro translation was performed with rabbit reticulocyte lysate (Promega) in accordance with the instructions of the manufacturer. Trans^{35}S-label was purchased from ICN. The translation mixture was supplemented with 200 mM potassium acetate and 1.2 mM $MgCl_2$. The reaction was performed at 30°C for 90 min, and the reaction mixture was immediately used in the binding assay.

Binding assay. In vitro-translated proteins (5 μl of the translation mixture) were diluted in 1 ml of buffer I (50 mM Tris-HCl [pH 8.0], 250 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 10 μg of soybean trypsin inhibitor per ml, 1 μg of aprotinin per ml, 10 μg of tolylsulfonyl phenylalanyl chloromethyl ketone per ml, and 20 μl of p13-Sepharose beads (4) or CKS2-Sepharose beads (38a) was added. Bovine serum albumin-Sepharose beads were also used in some of the experiments. Incubation was done for 1 h on a rotator at 4°C. The precipitates were washed three times in the same buffer and resuspended in Laemmli sample buffer prior to electrophoresis on 12.5% acrylamide gels. After being stained and treated with Enlightning (DuPont), the gels were dried and exposed to X-Omat AR film (Kodak) for 6 to 12 h.

Expression of mutant proteins in *S. pombe*. The *S. pombe* strains used were wild-type 972 (h^{-s}), SP200 (h^{-s} *leu1-32 ura4 ade210*), SP356 (h^{+n} *leu1-32 ura4 ade6-210 cdc2-33*), temperature-sensitive mutant SP32 (h^{-s} *cdc25-22*), and cold-sensitive strain h^{-s} *nda3*-KM311 (a generous gift from M. Yanagida). Cells were cultured in complete medium (0.5% yeast extract, 3% glucose, 75 μg of adenine per ml) or in minimal medium (34).

Human wild-type and mutant Cdc2 cDNAs were subcloned as *Nde*I-*Bam*HI cassettes in the thiamine-repressible replicative shuttle vector pREP42 (1a, 33). The *ura4* marker was used for selection. *S. pombe* SP200 and SP356 were transformed with pREP42 plasmid constructions. The promoter was kept under repressing conditions by the addition of 4 μM thiamine to the solid and liquid media. Derepressing conditions were obtained by replica plating on solid medium without thiamine or by washing the cells twice in liquid minimal medium and growing them in the absence of thiamine. Induction of the proteins was observed after about 10 h (1a). The phenotypes of the mutants in a wild-type background were analyzed at 27°C 20 h after promoter induction. Complementing activities were tested in temperature-sensitive mutant SP356 grown at 27°C and shifted 10 h after promoter induction to the nonpermissive temperature (36°C) for 16 h.

Cross-linking experiment. Suc1 was expressed and metabolically labeled with Trans^{35}S-label by use of a T7 polymerase-driven system in the presence of rifampin in *E. coli* (4, 45). The protein was purified by gel filtration and Mono-Q chromatography as previously described (4). In vitro-translated Cdc2 was mixed with [^{35}S]methionine-labeled Suc1, and the mixture was incubated at 30°C for 90 min in the presence of rifampin as described previously (24). Reactions were analyzed on 7.5 to 15% gradient sodium dodecyl sulfate (SDS)-polyacrylamide gels.

RESULTS

Suc1 can bind Cdc2 directly. To investigate the interaction between Cdc2 and Suc1, we produced human Cdc2 by in vitro translation and studied its binding to the Suc1 protein expressed in *E. coli*. A human cDNA encoding *cdc2* cloned in the pT7F1A vector (28) was transcribed by use of the T7 RNA polymerase promoter, and its mRNA was translated in vitro in a rabbit reticulocyte lysate. A [^{35}S]methionine-labeled polypeptide of the expected M_r (34,000) was produced (Fig. 1A, lane 1). In addition, a number of lower-molecular-weight products were generated. These products were expressed only in the presence of *cdc2* mRNA. They could be immunoprecipitated (Fig. 1A, lanes 3 and 4) with antisera raised either against the whole Cdc2 protein (G8) (9) or against a peptide modelled upon the carboxy terminus of the human Cdc2 protein (G6) (8). The additional bands were therefore amino-terminal truncations resulting from translation initiation at internal methionine codons (methionine at positions 32, 71, 85, and 100, which would produce species of 30,654, 26,200, 24,456, and 22,706 daltons, respectively). After incubation of the in vitro translation mixtures with the Suc1 protein coupled to Sepharose (p13-Sepharose) (4), the 34,000-M_r band, but not the smaller products, was precipitated (Fig. 1A, lane 5), indicating that a domain within the first 31 amino acids of Cdc2 is necessary, either directly or indirectly, for the interaction between Suc1 and Cdc2. The specificity of the binding was confirmed by the fact that although in this experiment, both the 30,000- and the 34,000-M_r polypeptides were produced in roughly similar

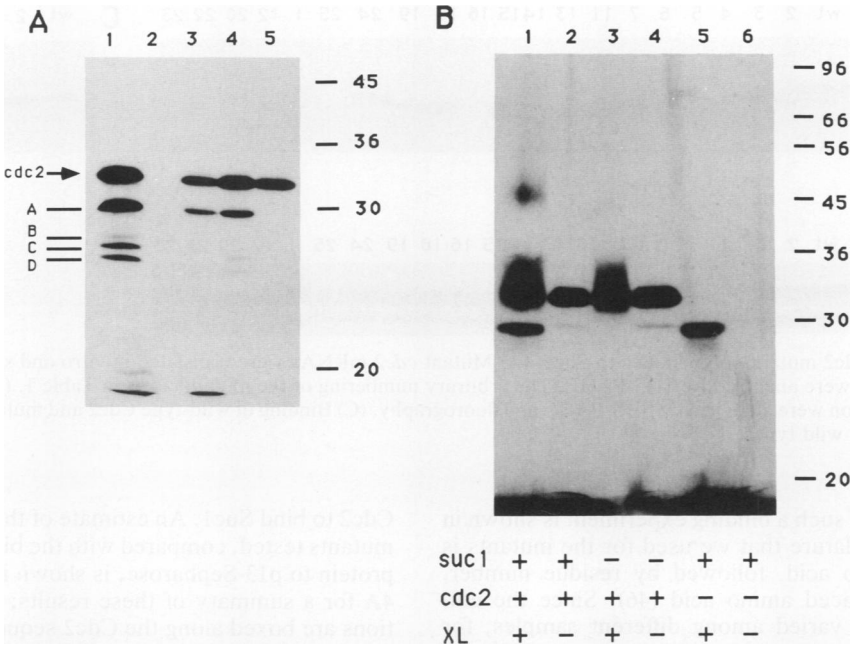

FIG. 1. Binding of in vitro-translated Cdc2 to Suc1. (A) Human *cdc2* mRNA was translated in vitro. Lanes: 1, total translation mixture (A, B, C, and D are amino-terminal truncations of the protein; see the text); 2, control precipitation with Sepharose beads; 3, immunoprecipitation with anti-Cdc2 (whole protein; G8) polyclonal serum; 4, immunoprecipitation with anti-Cdc2 (carboxy terminus; G6) polyclonal serum; 5, precipitation with p13-Sepharose. (B) Cross-linking between in vitro-translated [^{35}S]methionine-labeled Cdc2 and purified recombinant [^{35}S]methionine-labeled Suc1 with dimethyl suberimidate (XL). Additions were as indicated. Reactions were analyzed by 7.5 to 15% SDS-PAGE and fluorography. Numbers on the right are molecular weights in thousands.

amounts, only the 34,000-M_r polypeptide was bound to p13-Sepharose. The apparent molecular weight of the in vitro-translated Cdc2 protein was estimated to be 30,000 by gel filtration on a Superose 12 column (data not shown), consistent with the fact that Cdc2 produced by in vitro translation was present as a monomeric species in the reticulocyte lysate. Most (up to 70%) of the translated protein added to the assay was bound to p13-Sepharose.

To determine the stoichiometry of the interaction between the Suc1 and Cdc2 proteins, we used chemical cross-linking with the homobifunctional imidoester dimethyl suberimidate (44). After incubation of in vitro-translated [^{35}S]methionine-labeled Cdc2 protein and purified recombinant [^{35}S]methionine-labeled Suc1 protein (4), the interaction was analyzed by subjecting the reaction mixtures to SDS-polyacrylamide gel electrophoresis (PAGE) and fluorography (Fig. 1B). In this experiment, the level of amino-terminally truncated forms of Cdc2 was extremely low (the internal initiation of translation is affected by the RNA concentration and the potassium and chloride concentrations and varies among different lysates). In the presence of a cross-linker, a 47,000-M_r species was generated, demonstrating that the Cdc2-Suc1 complex which was formed in vitro under our assay conditions (Fig. 1B, lane 1) consists of Suc1 and Cdc2 in a 1:1 ratio. This result was confirmed by the observation that no such 47,000-M_r band was seen in the absence of either Suc1 or Cdc2. In addition, a 26,000-M_r species was formed as a result of cross-linking of two Suc1 molecules (Fig. 1B, lanes 1 and 5). This dimer did not seem to interact with Cdc2, since a 2:1 Cdc2-Suc1 complex was not observed even after a very long exposure of the same gel (data not shown). Under the conditions used for the cross-linking experiment, the Cdc2 and Suc1 proteins were present at very low concentrations (less than 1 nM) and were bound at a low stoichiometry.

Binding sites for Suc1 on Cdc2. To identify the sites on the Cdc2 protein which are involved in the interaction with the Suc1 protein, we used site-directed mutagenesis followed by the expression of recombinant proteins and in vitro binding assays. A mutagenesis strategy that had been used successfully for the study of the interaction between regulatory and catalytic subunits of the cyclic AMP-dependent protein kinase was used here (14). It consists of mutagenizing clusters of charged amino acids (Glu, Asp, His, Arg, and Lys) to alanine with the aim of disrupting sites involved in interactions with other polypeptides. This rationale stems from two lines of reasoning: (i) in any given protein, charged regions are likely to be exposed to the solvent, and (ii) at least part of the binding domain in each subunit is composed of hydrophilic amino acid residues, which are likely to interact with the solvent when the protein is not bound to its partner. Furthermore, given the important role of Cdc2 phosphorylation in regulating Cdc2 association with cyclins (10), we also mutated Thr-14, Tyr-15, Thr-161, and Ser-277, which have been identified as phosphoacceptor sites in vivo, and analyzed the effects of these mutations on the binding to Suc1.

Twenty cluster mutations and nine phosphorylation site mutants were generated, and each was checked by DNA sequencing. Plasmids encoding the mutant proteins were transcribed, and the mRNAs were translated in a rabbit reticulocyte lysate. Four of the mutants did not give rise to detectable translated protein (perhaps because of inefficient translation and/or instability of the products) and were not tested further. All mutants which gave rise to a translation product were tested for binding to Suc1 with p13-Sepharose

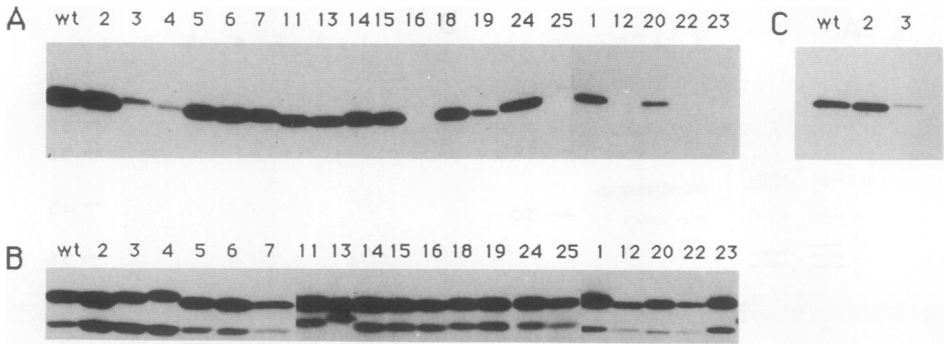

FIG. 2. Binding of Cdc2 mutant polypeptides to Suc1. (A) Mutant *cdc2* mRNAs were translated in vitro and subjected to p13-Sepharose precipitation. Reactions were analyzed by SDS-PAGE. The arbitrary numbering of the mutants is as in Table 1. (B) Total lysates from each in vitro translation reaction were analyzed by SDS-PAGE and fluorography. (C) Binding of wild-type Cdc2 and mutants 2 (Y15F) and 3 (Y15Q) to CKS2-Sepharose. wt, wild type.

(4, 12). An example of such a binding experiment is shown in Fig. 2A. The nomenclature that we used for the mutants is the substituted amino acid, followed by residue number, followed by the replaced amino acid (46). Since the efficiency of translation varied among different samples, for each experiment we always compared the amounts of bound Cdc2 protein with the actual amounts of Cdc2 protein produced in each translation reaction (Fig. 2B). It is evident that a number of mutations profoundly affected the ability of Cdc2 to bind Suc1. An estimate of the percent binding for all mutants tested, compared with the binding of wild-type Cdc2 protein to p13-Sepharose, is shown in Table 1 (see also Fig. 4A for a summary of these results; the Cdc2 cluster mutations are boxed along the Cdc2 sequence, and shaded boxes represent clusters whose integrity was absolutely required for the interaction with Suc1). Starting from the amino terminus of Cdc2, a deletion of the first 31 amino acids completely abolished the ability to bind Suc1 (Fig. 1A, lane 5). Within this sequence we mutated the Cdc2 phosphorylation sites located within GlyXxxGlyThrTyrGly, a region conserved in all protein kinases and other nucleotide binding proteins (19). Mutations which did not allow Cdc2 phosphorylation within the glycine cluster (T14A and Y15F) did not affect the ability of Cdc2 to bind Suc1. On the contrary, a substitution of tyrosine with glutamic acid or glutamine (Y15E or Y15Q, respectively), markedly inhibited binding. Similar results were obtained when the human Suc1 or CKS1 homolog CKS2 (41) was bound to Sepharose and used to

TABLE 1. Binding of Cdc2 mutant proteins to p13-Sepharose[a]

Cdc2 protein	Sequence	% Binding
Wild type	Wild type	100
1	T14A	117
2	Y15F	117
3	Y15Q	7
4	Y15E	7
5	T161V	87
6	T161D	99
7	T161E	101
8	S277E	98
9	S277A	101
10	E2A D3A K6A	124
11	E8A K9A E12A	104
12	K20A R22A H23A K24A	7
13	K33A K34A R36A	68
14	E38A E40A E41A E42A	104
15	R50A E51A	95
16	K56A E57A R59A H60A	5
17a	E57N	100
17b	E57Q	100
18	D68A D73A R75A	87
19	D86A K88A K89A D92A	70
20	R151A	74
21	R158A H162A E163A	60
22	R170A E173A	10
23	R180A D186A	10
	E196A K200A K201A	ND[b]
24	H205A D207A E209A D211A	85
25	R215A R218A	3
26	Deletion of amino acids 233 to 253	10
27	K243A K245A	117
28	D271A K274A R275A	10

[a] Data were normalized to the amount of immunoprecipitable Cdc2 and to a value of 100% in the wild type. Data are the averages from two or three determinations for each mutant.

[b] ND, not determined. There was an insufficient level of translation in the reticulocyte lysate.

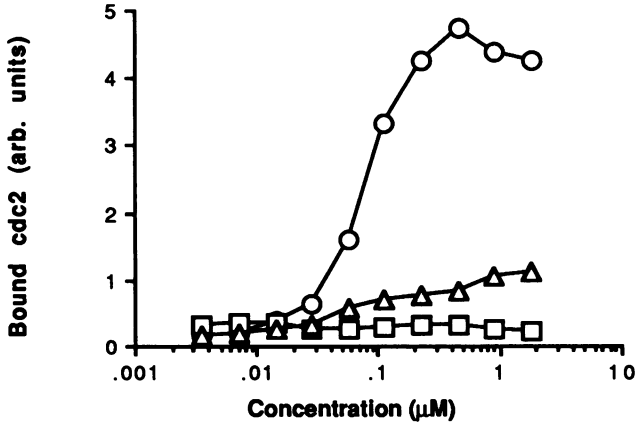

FIG. 3. Quantitative estimate of the binding of human CKS2 to Cdc2 mutant proteins. Wild-type Cdc2 (○), mutant 2 (Y15E) (△), and mutant 16 (K56A E57A R59A H60A) (□) were translated in vitro. Increasing amounts of the human Suc1 homolog CKS2 bound to Sepharose, were mixed with bovine serum albumin-Sepharose to a constant final volume and used for assaying Cdc2 binding. Bound material was quantified after SDS-PAGE and fluorography. arb., arbitrary.

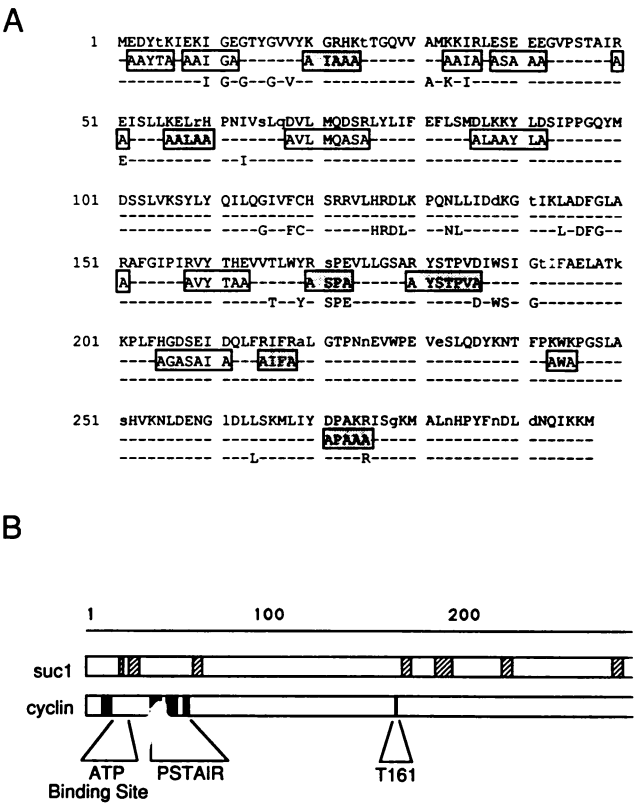

FIG. 4. Cdc2 structural requirements for Suc1 binding. (A) Scheme of the clusters of charged residues (Arg, Lys, His, Asp, and Glu) that were changed to Ala. The complete human Cdc2 sequence is shown in the top line, with conserved amino acids in all Cdc2 homologs shown in uppercase letters. The mutant clusters are shown in the middle line. The residues conserved in all kinases are shown in the bottom line. Shaded boxes represent mutations that severely affect in vitro binding between Cdc2 and Suc1. (B) Schematic linear representation of the Cdc2 sequence and proposed domains of interaction with the regulatory subunits Suc1 (this study) (striped boxes) and cyclins A and B (black boxes) (10).

assay interactions with some of these Cdc2 mutants (Fig. 2C and Fig. 3).

A basic region between amino acids 20 and 24 (mutations K20A, R22A, H23A, and R24A) was also found to be important for binding. This cluster is conserved in all Cdc2 homologs and in some other protein kinases. Mutations at two neighboring conserved clusters present in all Cdc2 homologs did not affect binding. A double mutation (R50A E51A) located within the conserved PSTAIR sequence, a region conserved in all Cdc2 homologs, did not affect binding. On the contrary, Cdc2 with a cluster mutation (K56A E57A R59A H60A) (the affected residues lie at the end of PSTAIR) was unable to bind Suc1. Within this cluster, a substitution (E57N) present in the *Drosophila* Cdc2 cognate protein (Cdc2c) is not able to rescue a temperature-sensitive mutation in *S. pombe cdc2* (31). A mutant carrying the E57N or E57Q substitution but not the other amino acid substitutions present in Cdc2c could interact normally with Suc1, indicating that this mutation alone was not sufficient to inactivate Cdc2 function. Mutations encompassing a region of about 110 amino acids, which includes the conserved threonine phosphorylation site of Cdc2 (Thr-161), did not affect binding to Suc1. The next clusters (amino acids 170 to 173 and amino acids 180 to 186) were found to be important for binding. This result was also confirmed by the finding that strains carrying point mutations of Cdc2 in this region of the molecule show a weakened interaction between Cdc2 and Suc1 (4, 5; reviewed in reference 7). Mutations within a short region (the cluster from amino acids 215 to 218) also resulted in a weakened interaction with Suc1. Furthermore, a 20-amino-acid deletion between amino acids 233 and 253 also severely affected binding. A further cluster mutation (D271A K274A R275A) also inhibited binding between Suc1 and Cdc2.

In summary, several regions of the Cdc2 protein were found to be important for Suc1 binding, most notably the glycine loop of the ATP binding site, the end of the conserved PSTAIR sequence, the conserved S/APE cluster, and a conserved region (domain X) of protein kinases, the functional role of which is still not clear (19). It is interesting to note that while the region between amino acids 187 and 201 had previously been suggested to be involved in Suc1 binding, the role that the amino terminus of Cdc2 may play in Suc1 binding had not been identified genetically. This result might be explained by the fact that mutations within the ATP binding site of Cdc2 might severely alter the functionality of the protein and therefore could not be isolated in conditional temperature-sensitive mutants.

Given the important role of the Cdc2 phosphorylation state on Cdc2 biochemical function, Thr-161 and Ser-277 mutations were also generated, and the binding of the mutant proteins to p13-Sepharose was assayed (Table 1). None of the mutations of Thr-161 (to valine, aspartic acid, or glutamic acid) or Ser-277 (to glutamate or alanine) affected the association with Suc1.

A quantitative estimate of the binding to the mammalian Suc1 homolog CKS2 (41) of wild-type Cdc2 and two of the mutant proteins was performed. Cdc2 proteins with mutation Y15E and a cluster mutation (K56A E57A R59A H60A), both severely affecting the interaction with Suc1 in vitro, were compared with wild-type Cdc2 protein. Wild-type Cdc2 was precipitated by CKS2-Sepharose, even at a low CKS2 concentration (Fig. 3). Fifty percent binding of Cdc2 was obtained with 100 nM CKS2 in the assay. Over a large range of concentrations, neither Y15E or K56A E57A R59A H60A mutant proteins were significantly retained by CKS2-Sepharose.

Expression of Cdc2 mutant proteins failing to bind Suc1 in *S. pombe*. To ensure that the binding deficiency observed in vitro was really of functional and physiological significance, we expressed wild-type human *cdc2* cDNA as well as plasmids encoding mutant proteins which failed to bind Suc1 in *S. pombe*. Overexpression was attained under the control of a thiamine-regulatable promoter (1a, 33). To test whether the proteins were functional, we assessed the ability of the plasmids to rescue strains carrying a temperature-sensitive *cdc2* mutation. In addition, we also tested whether any of the mutant proteins would cause a dominant lethal phenotype when overexpressed in an *S. pombe* wild-type strain. The results are summarized in Table 2. All of the mutant proteins that were unable to bind Suc1 showed a lack of rescuing ability for a temperature-sensitive *cdc2* strain. Mutations Y15E and Y15Q behaved as dominant lethal mutations and caused cell cycle arrest (see Discussion). We also tested mutant proteins showing reduced binding to p13-Sepharose (60 to 80%, compared with the wild type) and found that all of them were able to rescue *cdc2-ts* strains. The mutant proteins that were able to bind Suc1 were also tested in vivo. Some were unable to rescue temperature-sensitive *cdc2-33*

TABLE 2. Phenotypes of Cdc2 mutant proteins defective for Suc1 binding in *S. pombe*

Sequence	Phenotype of protein[a]		Binding to:	
	Wild type	*cdc2-33*	Suc1[b]	Cyclin[c]
pREP42	Wild type	Cdc$^-$	+	+
Wild type	Wee	Wee + elongation[d]	+	−
E8A K9A E12A	Wee	Wee + elongation[d]	−	+
K20A R22A H23A K24A	Wild type	Cdc$^-$	−	+
K56A E57A R59A H60A	Wild type + elongation[d]	Cdc$^-$	−	+
R158A H162A E163A	Wild type	Wild type + elongation[d]	+	+
R170A E173A	Wild type	Cdc$^-$	−	+
R180A D186A	Wild type	Cdc$^-$	−	+
E196A K200A K201A	Wild type	Cdc$^-$	ND[e]	ND
R215A R218A	Wild type	Cdc$^-$	−	+
Deletion of amino acids 233 to 253	Elongation	Cdc$^-$	−	+
D271A K274A R275A	Wild type	Cdc$^-$	−	+
Y15F	Wee[f]	Wee + elongation[d]	+	+
Y15E	Cdc$^-$[g]	Cdc$^-$	−	+
Y15Q	Cdc$^-$[g]	Cdc$^-$	−	+

[a] *cdc2* mutations were cloned in the inducible pREP42 expression vector (1a) and transformed into SP200 (wild-type *cdc2*$^+$ strain) and SP356 (temperature-sensitive *cdc2-33* strain) cells. Cell phenotypes in SP200 were observed at 27°C 20 h after promoter induction. SP356 cells were grown at 27°C and shifted to a nonpermissive temperature (36°C) 10 h after promoter induction, and phenotypes were observed 16 h later. Cdc$^-$, nondividing; wee, dividing, cells half the size of wild-type cells.

[b] Data are from Table 1.

[c] Data are from reference 10.

[d] The presence of a few elongated cells was probably due to plasmid loss.

[e] ND, not determined.

[f] Mitotic catastrophe (42).

[g] Dominant negative phenotype.

strains. When checked for their ability to bind cyclin, they all showed a defective interaction with cyclin (Fig. 4) (10). Mutant proteins with cluster mutations that did not affect either Suc1 or cyclin binding in vitro were fully functional in vivo.

DISCUSSION

In this study, the molecular basis of the Cdc2 association with Suc1 was investigated. We demonstrated that (i) a complex between Cdc2 and Suc1 could be formed in vitro, (ii) several noncontiguous amino acid sequences on Cdc2 were required for binding to Suc1, and (iii) mutations at sites required for Suc1 binding inactivated Cdc2.

We have shown that Cdc2 can bind to Suc1 in the absence of additional protein components. We have also demonstrated that Suc1 can directly bind Cdc2, forming a 1:1 complex. Several studies have shown that some Cdc2 temperature-sensitive mutations can be rescued by Suc1 overexpression. The Cdc2 proteins encoded by these mutations have low kinase activity at the restrictive temperature, but the deficient enzyme can be rescued in vitro by the addition of Suc1 (3, 35). The amino acid substitutions in the mutant proteins are located within the carboxy-terminal part between amino acids 177 and 212 (4, 5, 32). To get a more precise idea of the domains of Cdc2 involved in the interaction with Suc1, we used scanning mutagenesis. The results of our studies are clear. Multiple sites of interaction for Cdc2 on Suc1 exist.

Most of the mutations did not affect the overall structure of the Cdc2 protein, since they did not interfere with Cdc2 binding to cyclins A and B (10) (Fig. 4B). The alanine scanning mutagenesis method has been shown to be very useful in protein-protein interaction studies, and it has clearly been demonstrated that, since alanine does not impose steric or electrostatic effects on neighboring side chains (6), an alanine substitution for a charged amino acid does not affect the overall protein structure. The scheme presented in Fig. 4 shows the cluster mutations which caused a decreased ability to bind Suc1. This study confirms the requirement for domains located in the carboxy-terminal part of Cdc2. In addition, domains located within the amino-terminal part of cdc2 are also required for Suc1 binding.

We investigated the possible role of posttranslational modifications of Cdc2 on binding to Suc1. Since there appears to be a specific role for Cdc2 phosphorylation in the regulation of the association of Cdc2 with cyclins and the activation of Cdc2 (10), we analyzed the consequences of mutating phosphorylation sites on the in vitro association with Suc1. While mutations in the ATP binding site causing the inability of Cdc2 to become phosphorylated at Thr-14 (T14A) and Tyr-15 (Y15F) did not affect binding to Suc1, substitution of Tyr-15 with an acidic residue (Y15E or Y15Q) severely inhibited binding. One hypothesis proposed to explain this observation is that tyrosine-phosphorylated Cdc2 does not bind Suc1 and that substitution of Tyr-15 with a negatively charged residue mimics this phosphorylation, thereby inhibiting binding to Suc1. In vivo, in G_2-arrested *S. pombe* cells Cdc2 is phosphorylated at Tyr-15, while Cdc2 is completely dephosphorylated at mitosis (17), yet we found no differences in Suc1 binding between extracts from G_2-arrested cells (*cdc25-22*) and extracts from cells arrested at mitosis (*nda3-K311*) (10a). Therefore, in *S. pombe* it appears that Suc1 can bind both tyrosine-phosphorylated and dephosphorylated forms of Cdc2; we suggest that the structural alterations in the ATP binding site caused by the removal of a phenolic ring (Y15E or Y15Q mutation), more than phosphorylation itself, cause the inability to bind Suc1. Mutations of Thr-161 to nonphosphorylatable residues severely affects binding to cyclins A and B (10). Conversely, we found that mutations at this site did not alter the ability of Cdc2 to bind Suc1. In chicken cells, Cdc2 is phosphorylated at Ser-277 during the G_1 and S phases of the cell cycle (25). The role of this phosphorylation is not known, but such

phosphorylation also does not appear to regulate Suc1 binding, since Ser-277 substitutions had no effect. Nevertheless, we did not detect any phosphoserine on Cdc2 in *S. pombe* (data not shown). However, it should be pointed out that our in vitro binding assay may not detect subtle differences in affinity between Cdc2 and Suc1; the use of p13-Sepharose, a high-capacity Cdc2 binding matrix, may mask differences in affinity occurring during the cell cycle. To test whether Cdc2 mutant proteins unable to bind Suc1 in vitro were functional in vivo, we expressed the mutant proteins in *S. pombe*. All of the Suc1 binding-defective mutant proteins were unable to rescue temperature-sensitive Cdc2 mutations. In addition, the Y15E and Y15Q mutations caused a dominant lethal phenotype (22) and caused cell cycle arrest in a wild-type *cdc2* background. These results suggest that a Cdc2 protein carrying a Y15E or a Y15Q mutation acts as a dominant lethal protein perhaps by binding and titrating some cellular component away from the wild-type protein. Such a component(s) could be cyclin(s). We are presently investigating the biochemical properties of such altered proteins. It is worth emphasizing that none of the mutations which severely affected Suc1 binding caused any defect in cyclin binding (Fig. 4B) (10). Therefore, the nonfunctionality of their encoded protein (i.e., the inability to rescue a temperature-sensitive mutation of *cdc2*), when expressed in *S. pombe*, can be due either to a Suc1 binding defect or to a direct effect on catalytic activity but does not involve a complete subversion of the structure of the Cdc2 protein.

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