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A Three-Dimensional Model of the Cdc2 Protein Kinase: Localization of Cyclin- and Suc1-Binding Regions and Phosphorylation Sites

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The Cdc2 protein kinase requires cyclin binding for activity and also binds to a small protein, Suc1. Charged-to-alanine scanning mutagenesis of Cdc2 was used previously to localize cyclin A- and B- and Suc1-binding sites (B. Ducommun, P. Brambilla, and G. Draetta, Mol. Cell. Biol. 11:6177–6184, 1991). Those sites were mapped by building a Cdc2 model based on the crystallographic coordinates of the catalytic subunit of cyclic AMP-dependent protein kinase (cAPK) (D. R. Knighton, J. Zheng, L. F. Ten Eyck, V. A. Ashford, N.-H. Xuong, S. S. Taylor, and J. M. Sowadski, Science 253:407–414, 1991). On the basis of this model, additional mutations were made and tested for cyclin A and Suc1 binding and for kinase activity. Mutations that interfere with cyclin A binding are localized primarily on the small lobe near its interface with the cleft and include an acidic patch on the B helix and R-50 in the highly conserved PSTAIRE sequence. Two residues in the large lobe, R-151 and T-161, influence cyclin binding, and both are at the surface of the cleft near its interface with the PSTAIRE motif. Cyclin-dependent phosphorylation of T-161 in Cdc2 is essential for activation, and the model provides insights into the importance of this site. T-161 is equivalent to T-197, a stable phosphorylation site in cAPK. On the basis of the model, cyclin binding very likely alters the surface surrounding T-161 to allow for T-161 phosphorylation. The two major ligands to T-197 in cAPK are conserved as R-127 and R-151 in Cdc2. The equivalent of the third ligand, H-87, is T-47 in the PSTAIRE sequence motif. Once phosphorylated, T-161 is predicted to play a major structural role in Cdc2, comparable to that of T-197 in cAPK, by assembling the active conformation required for peptide recognition. The inhibitory phosphorylation at Y-15 also comes close to the cleft interface and on the basis of this model would disrupt the cleft interface and the adjacent peptide recognition site rather than prevent ATP binding. In contrast to cyclin A, both lobes influence Suc1 binding; however, the Suc1-binding sites are far from the active site. Several mutants map to the surface in cAPK, which is masked in part by the N-terminal 40 residues that lie outside the conserved catalytic core. The other Suc1-binding site maps to the large lobe near a 25-residue insert and includes R-215.

Cdc2 is the catalytic subunit of a protein kinase complex that was first identified through genetic screens of yeast mutants defective in cell cycle progression (17). In yeasts, Cdc2 is required both for DNA synthesis and for entry into mitosis. In higher eukaryotes, different Cdc2-related proteins exist, and they appear to activate distinct cell cycle transitions (48). Activation of the Cdc2 kinase requires a physical association with regulatory subunits called cyclins (10, 28). Cdc2 is, therefore, the prototype of a new class of protein kinases, the cyclin-dependent kinases. Posttranslational events control, in addition to binding to cyclins, the proper timing of the activation of a given cyclin-dependent kinase complex. Phosphorylation of T-167 of Cdc2 (T-161 in higher eukaryotes) by an as yet unidentified protein kinase leads to stabilization of the complex with cyclin (13, 22, 46) and to its activation (9, 50). In *Schizoaccharomyces pombe* and in human cells, phosphorylation of Y-15, a residue located within the ATP-binding site, by the Wee1 protein kinase inactivates Cdc2 (23, 36, 38). The adjacent residue, T-14, is also phosphorylated in higher eukaryotes (32, 41). In vivo, it was previously demonstrated that these phosphorylations ensure that the activation of the Cdc2-cyclin B complex and the subsequent entry into mitosis occur only after DNA replication has been completed (16, 36). At that time, Cdc25, a specific protein phosphatase, dephosphorylates and activates Cdc2 (39).

In human cells, Cdc2 associates with either cyclin A or cyclin B (11, 21, 44, 47). Cdc2 also binds a small (9- to 15-kDa) protein, Suc1, identified in both yeasts and higher eukaryotes (5, 24–26, 49). While association with cyclin is an absolute requirement for the activation of the catalytic subunit, binding to Suc1 does not affect the activity of Cdc2 in vitro. In vivo, Suc1 associates with Cdc2, and when overexpressed it can rescue several Cdc2 mutant strains (25). Interestingly, Suc1 can restore the Cdc2 kinase activity of extracts from those same strains in vitro (4, 40). Additional roles have been ascribed to Suc1. For example, the addition of Suc1 to a *Xenopus laevis* extract prevented the dephosphorylation and activation of the tyrosine-phosphorylated form of cyclin B-Cdc2 (pre-maturation-promoting factor) (15). The tyrosine dephosphorylation of Cdc2 bound to cyclin beads by Cdc25 was also inhibited in vitro by the

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addition of Suc1 (19). The mechanism of this inhibition might rely on Suc1 preventing the interaction of Cdc25 with Cdc2-cyclin B, rather than directly inhibiting Cdc25 catalytic activity, although it was previously reported that the dephosphorylation of reduced, carboxyamidomethylated, and maleylated lysozyme by the Cdc25 phosphatase was inhibited by Suc1 in vitro (18).

Although much is known about the physiological roles of Cdc2 and some of its substrates and upstream regulators, our present knowledge of the biochemical regulation of this protein kinase is still limited. We previously used charged-to-alanine scanning mutagenesis (8, 20) to identify regions in Cdc2 that are important for its interaction with either Suc1 or cyclin (12, 13). In order to understand these interactions better, a model of the Cdc2 catalytic subunit based on the crystal structure of the catalytic subunit of cyclic AMP-dependent protein kinase (cAPK) was constructed (31). We show that the putative cyclin A- and Suc1-binding sites each lie on distinct surfaces in Cdc2. The close proximity of the cyclin-binding site to the catalytic site might explain why cyclin binding is needed for activation of the kinase. The relationship of the two phosphorylation sites to each other and to the Suc1- and cyclin-binding sites is also discussed.

MATERIALS AND METHODS

Binding and kinase assays. The procedure for generating and testing Cdc2 mutant proteins was previously described (12, 13). Briefly, [^{35}S]methionine-labeled Cdc2 proteins were generated by in vitro transcription-translation. Proteins were then tested for Suc1 binding by studying their interaction with Suc1 cross-linked to Sepharose beads. Cyclin binding was assayed with cyclin A cross-linked to Sepharose. The mutants were also tested for their abilities to replace the yeast Cdc2 function in vivo, as described by Ducommun et al. (13).

Cdc2 mutants were immunoprecipitated (44) from a rabbit reticulocyte lysate and incubated with 300 nM bacterially expressed human cyclin A and 100 μg of an interphase *Xenopus* egg extract (13) at 25°C for 30 min. As a control, the same was done with unprogrammed lysate. The immunoprecipitates were washed and assayed for H-1 kinase activity as previously described (44). For quantitation, H1 histone bands were cut out from the sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels and counted in a Beckman liquid scintillation counter. To every value, the counts per minute obtained from unprogrammed lysate were subtracted and values were normalized for the amount of protein synthesized. The amount of protein translated was determined by SDS-polyacrylamide gel electrophoresis (PAGE) and by scanning the gels with a PhosphorImager (Molecular Dynamics).

***S. pombe* growth conditions and extract preparation.** A temperature-sensitive strain (*cdc2-33*) of *S. pombe* (42) was grown at 25°C in YEA medium (3% glucose, 0.5% yeast extract, 75 mg of adenine per liter). Exponentially growing cells (2×10^9 to 4×10^9) were collected by centrifugation and pooled into a single Eppendorf tube. After centrifugation and aspiration of the supernatant, 250 μl of buffer I (4) and 500 μl of glass beads were added to the cell pellet. Cell breakage was performed by vortexing for 5 min at 4°C. After centrifugation for 3 min at 13,000 rpm in a microcentrifuge at 4°C, the soluble fraction was transferred into a new Eppendorf tube and then centrifuged twice for 15 min at 4°C. The protein concentration of the supernatant was determined by the Bradford assay (3, 4a). Immunoprecipitation and kinase assays were performed as described previously (44) with the modification described in the legend to Fig. 7.

FSBA treatment. In vitro-translated Cdc2 (50 μl) was treated with 2 mM *p*-fluorosulfonylbenzoyl adenosine (FSBA) in 2% dimethyl sulfoxide or 2% dimethyl sulfoxide as a control for 30 min at 25°C after depletion of endogenous ATP by spin column filtration (45). Unreacted FSBA was then removed by spin column filtration.

Modeling of Cdc2. The model of Cdc2 was prepared by replacing side chains of a cyclic AMP-dependent protein kinase crystal structure model (Brookhaven Protein Data Bank entry 1CPK) with Cdc2 side chains according to the alignment of Fig. 1. The relative inserts and deletions were modeled initially by using backbone fragments of the correct length extracted from structures in the Brookhaven Protein Data Bank and given Cdc2 sequence. The atomic positions were unchanged in the replacement procedure for the conserved backbone, and Cdc2 side chains followed cAPK side chain positions where possible. A series of energy minimization steps was carried out to eliminate improper atomic overlaps and poor geometry, and simulated annealing was performed to give the regions of insertion and deletion local minima of lower energy. The atoms of insertions and the borders of deletions were first energy minimized against the remainder of the model, holding that remainder fixed. The side chains of conserved regions and all atoms of inserts and deletion borders were then minimized, keeping the C-alpha positions fixed. The insertion and deletion regions were subjected to simulated annealing from 4,000 to 300 K with the remainder of the model held fixed, followed by additional energy minimization of the same. This assembly was subjected to 2,400 conjugate gradient cycles of energy minimization with C-alpha atoms in the conserved regions held fixed. Additional conjugate gradient cycles were run, with conserved C-alpha atoms harmonically restrained to their initial positions, until the maximum gradient was less than 0.0005 kcal/(mol Å). The program X-PLOR (6) was used for all energy minimization and simulated annealing steps. The final model has root mean square (r.m.s.) deviations from ideal bond lengths of 0.009 Å (ca. 0.0009 nm) and those from ideal bond angles of 2.9°. The modeling procedure changed the positions of the C-alpha atoms of regions conserved between cAPK and Cdc2 by an r.m.s. value of 0.19 Å (ca. 0.019 nm). On the basis of 28% sequence identity between cAPK and Cdc2, deviation of the true Cdc2 structure from cAPK is expected to be higher (7) in general, but conclusions about Suc1- and cyclin-binding regions are not affected. The coordinates for the Cdc2 model have been deposited with the EMBL Data Library.

RESULTS

The sequence of Cdc2, one of the smallest protein kinase catalytic subunits, can be easily aligned with the conserved catalytic core of cAPK (Fig. 1). On the basis of this alignment, a three-dimensional model of Cdc2 was built. This model is summarized in Fig. 2. The general folding of the core structure in Cdc2 is probably conserved, and most of the deletions and insertions relative to the catalytic (C) subunit of cAPK are minor. A single large insertion of 25 residues occurs between the G and H helices. With the exception of this large insert, the Cdc2 residues can be easily accommodated within the catalytic core as defined by the C subunit. The small lobe of this core is associated with nucleotide binding, while the large lobe is associated with peptide binding and catalysis (31, 53). On the basis of the

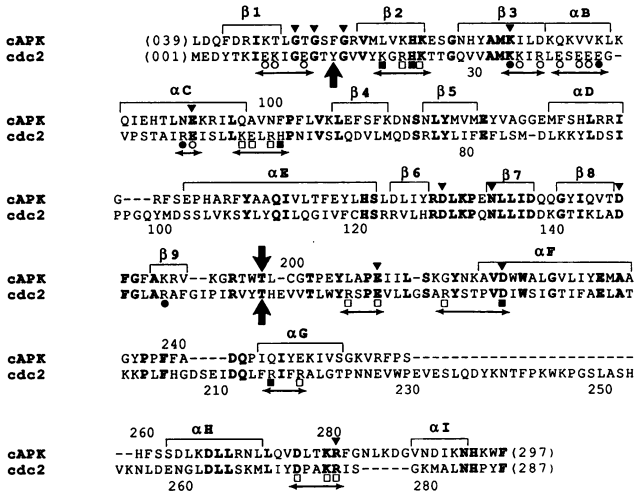

FIG. 1. Sequence alignment of Cdc2 with the catalytic subunit of cAPK. The phosphorylation sites are indicated by thick arrows. Residues whose replacement interfered with either cyclin binding or Suc1 binding are indicated by circles and squares, respectively. Clusters of charged residues that were changed simultaneously are underlined with double-headed arrows. Open symbols indicate clusters of charged residues that were replaced simultaneously. Closed symbols indicate single residues within a cluster whose replacement with Ala impaired binding of cyclin A or Suc1. Residues that are conserved throughout the protein kinase family are indicated by arrowheads. Residues that lie in the indicated helices and β strands are bracketed. Deletions and insertions are indicated by dashes. Highly conserved residues are in boldface type.

conservation of residues at the active site, we assume that the specific residues associated directly with ATP binding and catalysis (G-50, G-52, G-55, K-72, E-91, D-166, N-171, D-184, E-208, D-220, and R-280 in the cAPK sequence) will be spatially conserved in Cdc2 and cAPK.

Cluster mutations. Charged-to-alanine scanning mutagenesis can be used to probe the functional significance of charged residues in a given protein. By this method, all charged residues are changed in clusters of two to four to alanine. The resulting family of mutants can then be scanned for various functional defects. In a previous study, charged-to-Ala scanning mutagenesis was used to identify regions in Cdc2 that were important for binding to cyclins A and B and to Suc1 (12, 13). These cluster mutants are summarized in Fig. 3. All of the cluster mutants that gave a greater than 80% loss in cyclin binding were within the amino-terminal portion of the molecule corresponding to the small lobe. These include the E-8 cluster, the K-33 cluster, the E-38 cluster, and the R-50 cluster. Further single mutants affecting cyclin binding are a deletion, E-41Δ, in the small lobe and R-151A in the large lobe. T-161 is phosphorylated in vivo (22, 32), and replacement of T-161 with either Glu, Asp, or Val inhibits cyclin A binding (13). The locations of these cyclin A-binding mutations on the Cdc2 modeled structure are indicated as closed circles in Fig. 2 and are also correlated with the sequence in Fig. 1. Similar effects of Cdc2 mutations were seen for both cyclins A and B in these earlier studies; however, for simplicity, we measured only cyclin A binding for our further analysis of these mutants.

The cluster mutants failing to bind Suc1 are K-20, K-56, R-170, R-180, R-215, and D-271, and as seen in Fig. 2 (open circles), these clusters lie in both lobes. Deletion of amino acids 233 to 253 also blocks Suc1 binding but has no effect on cyclin binding. Mutations that remove an aromatic residue from position 15, Y-15E and Y-15Q, also prevent Suc1 binding. The inability of Y-15E and Y-15Q mutants to bind Suc1 is intriguing. In vivo Cdc2 can be reversibly phosphorylated on Y-15; Suc1 can bind both tyrosine-phosphorylated and unphosphorylated Cdc2, although its affinity for the tyrosine-phosphorylated Cdc2 seems higher (14). Since the in vitro-translated Cdc2 used for the binding assays is not tyrosine phosphorylated but still binds Suc1 (12), it is plausible that the inabilities of Y-15E and Gln mutants to

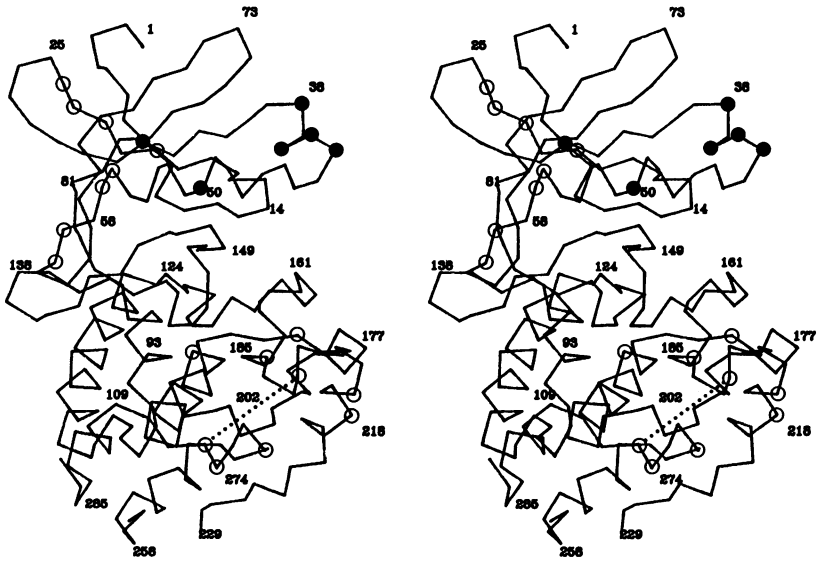

FIG. 2. Model of Cdc2 based on crystallographic coordinates of catalytic subunit of cAPK. The closed circles represent residues whose replacement with Ala interfered with cyclin A binding. Open circles represent sites whose replacement with Ala interfered with Suc1 binding. The position of the 25-residue insert is left as a gap between residues 229 and 224. The conserved ion pair between E-173 and R-275 is indicated by the dotted line.

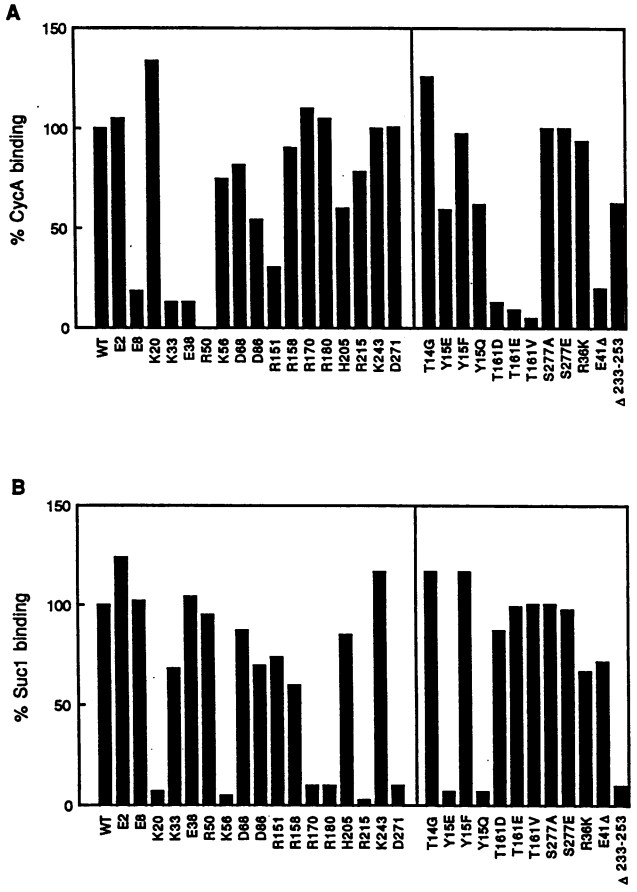

FIG. 3. Binding of Cdc2 cluster mutant proteins to cyclin A and Suc1. (A) Binding of Cdc2 cluster mutants to cyclin A beads (derived from reference 13). Clusters indicated in the figure are as follows: E-2, E-2A–D-3A–K-6A; E-8, E-8A–K-9A–E-12A; K-20, K-20A–R-22A–H-23A–K-24A; K-33, K-33A–K-34A–R-36A; E-38, E-38A–E-40A–E-41A–E-42A; R-50, R-50A–E-51A; K-56, K-56A–E-57A–R-59A–H-60A; D-68, D-68A–D-71A–R-73A; D-86, D-86A–K-88A–K-89A–D-92A; R-158, R-158A–H-162A–E-163A; R-170, R-170A–E-173A; R-180, R-180A–D-186A; H-205, H-205A–D-207A–E-209A–D-211A; R-215, R-215A–R-218A; and D-271, D-271A–K-274A–R-275A. The nomenclature used to describe each single mutant is the amino acid substituted in wild-type (WT) Cdc2 followed by the residue number followed by the amino acid replacement. The values of binding are expressed as percentages of wild-type binding. Δ233-253, deletion of amino acids 233 to 253. (B) Binding to Suc1 beads of the same Cdc2 mutant proteins as in panel A (derived from reference 12). The values are expressed as percentages of wild-type binding.

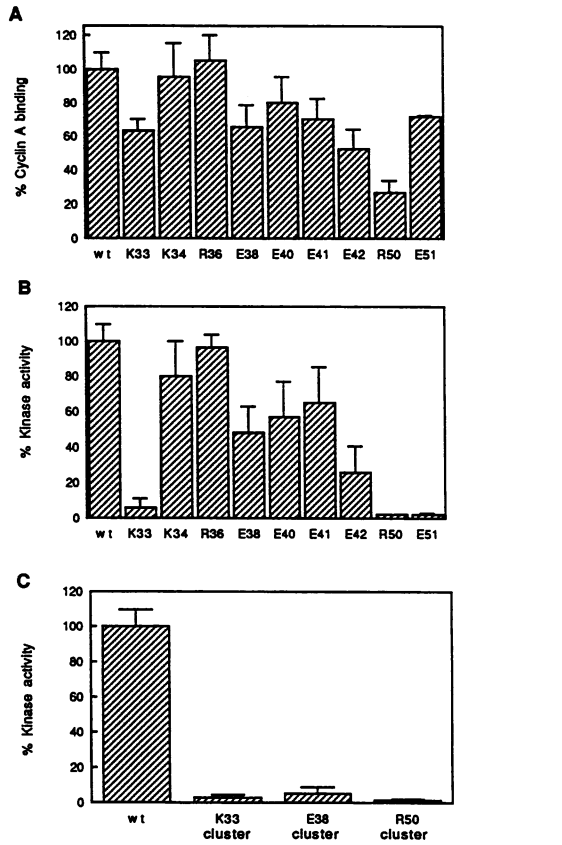

FIG. 4. Single-amino-acid changes from Cdc2 clusters defective in cyclin A binding. (A) Binding to cyclin A-Sepharose. The single-amino-acid substitutions to alanine tested are depicted in the abscissa. The values of binding are expressed as percentages of wild-type binding. (B) Histone H1 kinase activity of the same Cdc2 single mutants as in panel A. The values of binding are expressed as percentages of wild-type activity. (C) Histone H1 kinase activity of the Cdc2 cluster mutants that are defective in cyclin A binding. K-33, K-33A–K-34A–R-36A; E-38, E-38A–E-40A–E-41A–E-42A; R-50, R-50A–E-51A. The values are expressed as percentages of wild-type activity. wt, wild type.

bind Suc1 are due to a structural alteration in the ATP-binding loop of Cdc2, caused by the loss of an aromatic residue, more than to the introduced negative charge or to the lack of tyrosine phosphorylation.

Three of the cluster mutants in the large lobe contain invariant residues that play a structural role in the catalytic subunit of cAPK. The equivalents of E-173 and R-275 (E-208 and R-280 in cAPK) form a buried salt bridge in cAPK. D-186 (homologous to D-220 in cAPK) interacts with the backbone of the catalytic loop (29). It is likely, therefore, that these mutations lead to structural perturbations of the large lobe that indirectly impair Suc1 binding.

In general, the mutations affecting cyclin binding did not influence Suc1 binding and vice versa. The cyclin-binding sites were clustered within the small lobe, with the exceptions of R-151 and T-161, while the Suc1-binding sites were distributed in both the small and the large lobes. In vivo, Cdc2 proteins carrying these sets of mutations were unable to rescue Cdc2-defective strains of yeast (12, 13), suggesting that the interaction with both cyclin and Suc1 is of functional importance for Cdc2, although the two proteins probably play distinct roles. Five invariant residues were found in these clusters. K-33 and E-51 are associated with ATP binding in the small lobe, while E-173, D-186, and R-275 in the large lobe are thought to play a structural role.

To understand better the binding surfaces created by the mutated residues, it was important to establish the contribution of individual mutations within the clusters. Therefore, new mutants with single amino acids changed to alanine were generated. We focused on clusters that seemed to create a well-delineated surface and also tried to split conserved and nonconserved residues.

Cyclin A-binding sites. As summarized in Fig. 4, mutants carrying individual amino acid changes in the clusters show-

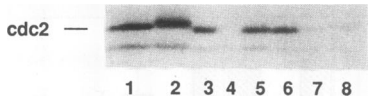

FIG. 5. Binding of FSBA-treated Cdc2 to cyclin A. Lanes: 1, in vitro-translated [^{35}S]methionine-labeled Cdc2 (4 μl); 2, in vitro-translated Cdc2 pretreated with FSBA (4 μl); 3, binding of Cdc2 (8 μl) to cyclin A beads; 4, binding of FSBA-treated Cdc2 (8 μl) to cyclin A beads; 5, binding of FSBA-treated (8 μl) and untreated (8 μl) Cdc2 to cyclin A beads; 6, binding of Cdc2 (8 μl) and unprogrammed lysate (8 μl) to cyclin A beads; 7, binding of Cdc2 (8 μl) to bovine serum albumin (BSA) beads; 8, binding of FSBA-treated Cdc2 (8 μl) to BSA beads.

ing altered cyclin A binding were characterized for both cyclin A binding and activity. With the K-33 cluster, the K-33A mutant showed a slight decrease in its ability to bind cyclin A, while K-34A and R-36A showed no binding defects. The K-33A mutant was also inactive, while K-34A and R-36A were active. Thus, the loss of activity in the cluster can be attributed exclusively to K-33, while the loss of cyclin A-binding activity is only partially due to K-33. K-33 is the residue equivalent to K-72 of cAPK and is directly involved in nucleotide binding (53). To further probe the importance of K-33, Cdc2 was treated with FSBA, a reagent shown to inactivate cAPK by covalent attachment to K-72 (54). FSBA treatment resulted in quantitative modification of Cdc2, as seen by its retarded migration in SDS-PAGE (Fig. 5, lanes 1 and 2). The FSBA-modified Cdc2 was unable to stably interact with cyclin A (lane 4), further demonstrating that K-33 is important for the structural integrity of the cyclin interaction site.

When the four Glu residues in the E-38 cluster were mutated individually, no single residue could be identified as critical for cyclin binding. Although E-42A showed the greatest decrease in both cyclin binding and catalysis, all had a partial effect, leading to the conclusion that this extended acidic surface is important for cyclin binding. In myosin light-chain kinase, this region corresponding to the B helix in cAPK is probably not helical (30).

Within the R-50A–E-51A cluster, the residue affecting cyclin A binding was R-50, although neither single mutant was catalytically active. R-50 is not conserved in the protein kinase family but is present in all cyclin-dependent kinases. E-51, a conserved residue in all protein kinases that ion pairs with K-33, was required for kinase activity but not for cyclin A binding. The loss of activity in the R-50 mutant is probably due indirectly to its inability to bind cyclin A.

Only one charged-to-Ala mutant in the large lobe, R-151A, led to reduced cyclin binding. In cAPK, this residue is a primary ligand for the phospho-T-197 that is analogous to T-161 in Cdc2, and as discussed earlier, mutagenesis of T-161 also disrupts cyclin A binding. The relationship of this surface region of the large lobe to the cyclin-binding regions in the small lobe is clearly critical for Cdc2 function.

Suc1-binding sites. In the experiment shown in Fig. 6, single-amino-acid substitutions within the clusters affecting Suc1 binding were analyzed. The effect of altering these sites on both Suc1 binding and kinase activity is shown. In most cases, the single mutations showed lesser effects than replacing all of the amino acids within each cluster. In many cases, the mutations affecting binding are not conserved in cAPK. The four single mutants in the K-20 cluster all showed slightly reduced binding to Suc1, with H-23 showing the greatest effect. When assayed for kinase activity, K-20A and

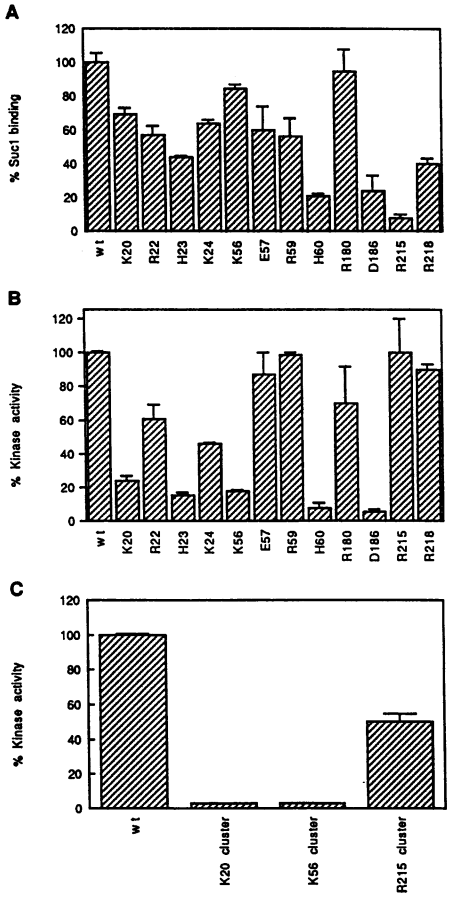

FIG. 6. Single-amino-acid changes from Cdc2 clusters defective in Suc1 binding. (A) Binding to Suc1-Sepharose. The single-amino-acid substitutions to alanine tested are depicted in the abscissa. Values of binding are expressed as percentages of wild-type Cdc2 binding. (B) Histone H1 kinase activity of the same Cdc2 single mutants as in panel A. The values are expressed as percentages of wild-type activity. (C) Histone H1 kinase activity of the Cdc2 cluster mutants found to be defective for Suc1 binding. K-20, K-20A–R-22A–H-23A–K-24A; K-56, K-56A–E-57A–R-59A–H-60A; R-215, R-215A–R-218A. The values are expressed as percentages of wild-type activity. wt, wild type.

H-23A showed the least activity. Within this cluster, it appears that substitution of just one of the four positively charged residues did not dramatically affect the interaction with Suc1. For the K-56 cluster (K-56–E-57–R-59A–H-60A), however, the H-60A substitution showed a strong effect on Suc1 binding; the other single substitutions showed Suc1 binding essentially unchanged compared with that by the wild-type protein. H-60A also had low kinase activity. K-56A had reduced kinase activity but normal Suc1 binding. In the R-180 cluster (R-180–D-186), the D-186A mutation was responsible for inhibition of both Suc1 binding and kinase activity. This residue corresponds to one of the invariant residues (D-220) in the protein kinase family. In the R-215A cluster (R-215–R-218), the R-215A mutant was most defective in binding Suc1, while R-215A and R-218A both showed wild-type kinase activities. The D-271 cluster containing the invariant R-275 that presumably ion pairs with E-173 was not further mutated, nor was the R-170 cluster containing the invariant E-173.

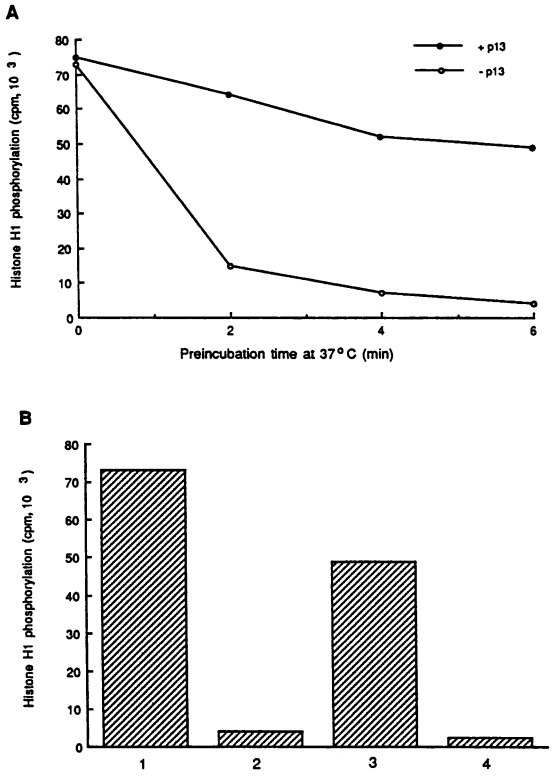

FIG. 7. Effect of Suc1 on temperature-sensitive Cdc2 kinase. (A) The *S. pombe* cyclin B, Cdc13, was immunoprecipitated from a yeast *cdc2-33* strain and incubated at 37°C in the presence or absence of 20 μM Suc1 for 0, 2, 4, or 6 min. After the incubation, immunoprecipitates were washed three times with lysis buffer and once with kinase assay buffer. Histone H1 kinase activity was then assayed at 25°C (see Materials and Methods). (B) Cyclin B immunoprecipitates, prepared as described above, were assayed for histone H1 kinase activity without preincubation or Suc1 addition (bar 1), with preincubation in the absence of Suc1 for 6 min at 37°C (bar 2), with preincubation in the presence of 20 μM Suc1 for 6 min (bar 3), or with preincubation in the absence of Suc1 for 6 min, followed by a further preincubation in the presence of 20 μM Suc1 for 6 min (bar 4).

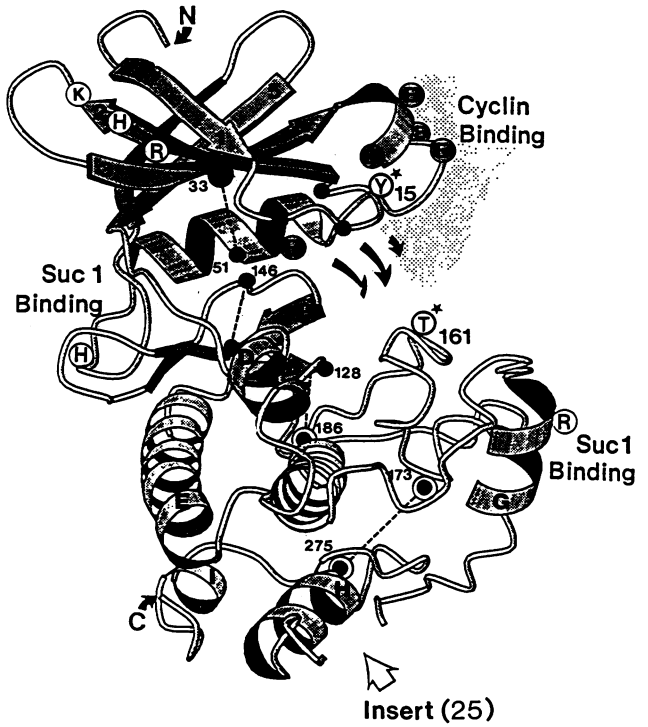

FIG. 8. Ribbon diagram of Cdc2 model. The site where a major 25-residue insert occurs between residues 229 and 254 is shown as a gap and indicated by an open arrow. The two major sites of phosphorylation, Y-15 and T-161, are circled and indicated by stars. Essential residues that are conserved throughout the protein kinase family are indicated by solid circles, and those that interact are connected by dashed lines. Several of these essential residues are numbered. The charged-to-alanine mutants that influence cyclin A binding are shown as shaded circles; those influencing Suc1 binding are shown as open circles. The arrows at the cleft interface indicate the general location of the PSTAIRE sequence that faces the surface containing T-161. The N and C termini are designated N and C.

Although the Suc1-binding sites described in our model mapped to regions distant from the catalytic site, most mutants defective in Suc1 binding, with the exception of R-215A, were also inactive. In order to test whether Suc1 had a specific role in catalysis or simply helped to stabilize the Cdc2 structure, Cdc2 was immunoprecipitated from a strain of *S. pombe* expressing a temperature-sensitive Cdc2 protein (from the *cdc2-33* allele) (40, 42). After immunoprecipitation, Cdc2 was preincubated at 37°C in the presence or absence of Suc1. Kinase activity was then assayed at the permissive temperature (25°C) with histone H1 as a substrate (Fig. 7A). When preincubation was carried out in the absence of Suc1, Cdc2 activity was rapidly lost; however, the addition of Suc1 prior to the preincubation prevented the loss of activity. To test whether Suc1 could be a positive effector, Cdc2 was preincubated in the absence of Suc1 for 6 min. Suc1 was then added, and after an additional 6-min incubation, kinase activity was assayed (Fig. 7B). Cdc2 activity could not be rescued by the readdition of Suc1 under these conditions. Similar results were obtained with a distinct *cdc2*(Ts) mutant (Cdc2-L7) (data not shown). These results indicated that in these Cdc2 temperature-sensitive mutant proteins Suc1 is required for stabilizing Cdc2 but that it does not play a specific role in catalysis.

DISCUSSION

A summary ribbon diagram of the Cdc2 three-dimensional model is presented in Fig. 8. This shows the general features of Cdc2 and localizes the two major phosphorylation sites, the key charged residues that influenced cyclin A or Suc1 binding, and some of the residues that are conserved throughout the protein kinase family. In Table 1, several key residues in Cdc2 are correlated with their functional equivalents in cAPK. Several specific features of this model will be considered. First will be the phosphorylation site that is critical for activation of Cdc2, with particular emphasis on the importance of this region for peptide recognition and its localization relative to the conserved PSTAIRE sequence. The inhibitory phosphorylation sites at T-14 and Y-15 are also discussed. Finally, the general cyclin A- and Suc1-binding sites will be reviewed.

Phosphorylation sites. The cyclin-dependent phosphorylation of T-161 is essential for activation of Cdc2. What can cAPK tell us about this site? The equivalent of T-161 in the C subunit is T-197. Phosphorylation of T-197 is structurally

TABLE 1. Correlation of key residues in Cdc2 and cAPK

Residue(s) in cAPK	Cdc2 equivalent[a]	Description or function in cAPK
Lys-72	Lys-33	Anchors α- and β-phosphates of ATP
Glu-91	Glu-51	Ion pairs with Lys-72
Gly-50, Gly-52, Gly-55	Gly-11, Gly-13, Gly-16	Gly-rich loop between β-strand 1 and β-strand 2; anchors β-phosphate
Asp-166	Asp-128	Catalytic base
Lys-168	Lys-130	Binds to γ-phosphate of ATP and anchors peptide to small lobe
Asp-184	Asp-146	Binds to activating Mg^{2+} that bridges the β- and γ-phosphates
(P)-Thr-197[b]	(P)-Thr-161	On surface of large lobe at edge of cleft; important for recognition of P + 2 and P + 3 regions of peptide substrates
Ser-53, Phe-54	(P)-Thr-14, (P)-Tyr-15	In glycine-rich loop between β-strand 1 and β-strand 2
R-165	R-127	Bivalent ligand to (P)-Thr-197
K-189	R-151	Ligand to (P)-Thr-197; precedes catalytic base D-166
His-87	Thr-47	Ligand to (P)-Thr-197 in closed conformation but not in open conformation

[a] The top group contains conserved active site residues. The bottom group contains phosphorylation sites.
[b] Phosphorylation sites are indicated as (P), where P refers to phosphate.

essential for the active conformation of the C subunit (29, 31). Phosphorylation of T-197 is also important for recognition by the R subunit (34, 52) and may be a critical step in the assembly of the active enzyme (51, 52). This phosphate is a dominant structural feature of the surface of the large lobe at the cleft interface. It binds to three side chains: R-165 preceding the catalytic base, K-189 in the large lobe, and H-87 in the small lobe (Fig. 9A). This accounts for its stability and resistance to phosphatases. This surface also comprises the peptide recognition site for residues in the P + 2 region and beyond. Mutations in the region flanking T-197 and mutations of T-197 itself and the residues that bind to it all generate proteins that are defective in recognition of the R subunit (20, 34, 43). Therefore, phosphorylation of T-197 is not only structurally important but also assembles this surface of the enzyme so that proper recognition of substrates and inhibitors can be achieved.

The corresponding region in Cdc2 containing the two phosphorylation sites near the cleft interface is shown in Fig. 9B. On the basis of the model of Cdc2 and the predicted cyclin-binding sites, cyclin binding will lead to a conformational change at the cleft interface near T-161 that will allow it to be recognized as a substrate for a heterologous protein kinase. Once phosphorylated, T-161 will very likely become an integral feature of this surface, similar to T-197 in cAPK. Both ligands to T-197 in the large lobe of the C subunit are conserved in Cdc2 (Table 1). R-127 preceding the catalytic loop is the equivalent of R-165, and the bivalent interaction of this guanidinium side chain with the phosphate is probably critical for structural stability and may be equally important

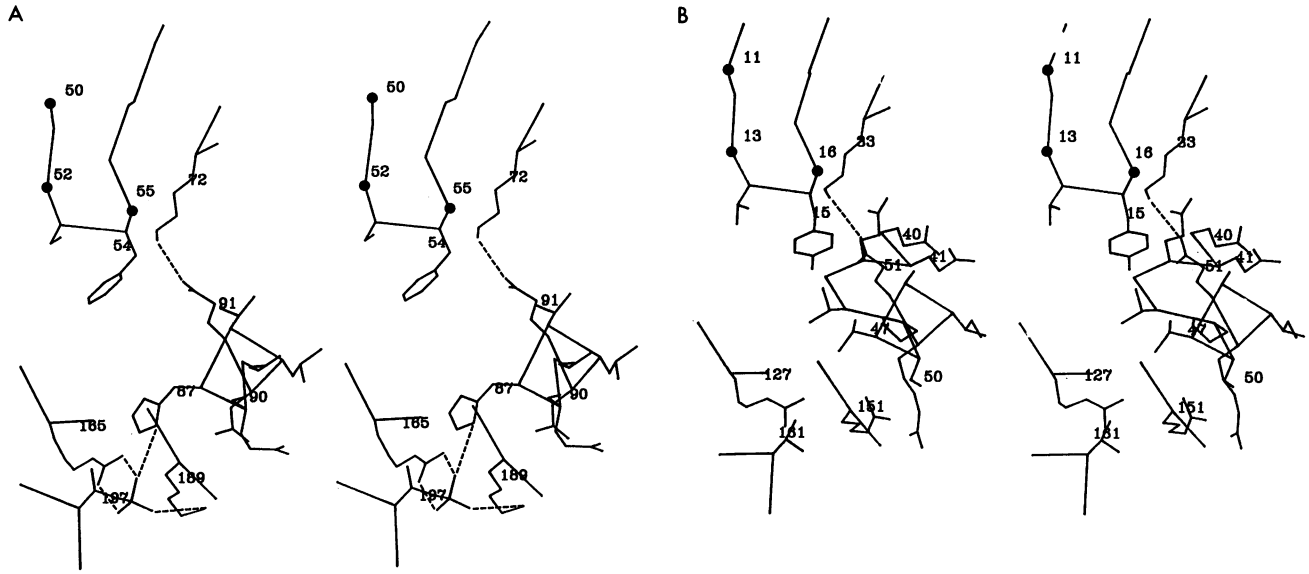

FIG. 9. Stereo view of phosphorylation sites and PSTAIRE sequence in Cdc2 model and corresponding region in catalytic subunit of cAPK. (A) Region surrounding the T-197 phosphorylation site in the catalytic subunit of cAPK. Residues close enough to ion pair are indicated by dashed lines. The α-carbons of the invariant glycines in the glycine-rich loop, 50, 52, and 55, are indicated by black circles and marked. The conserved ion pairing between the invariant Lys and Glu in the small lobe is indicated by a dashed line. K-72 interacts with the α-phosphate of ATP in the ternary complex (53). (B) Corresponding region of Cdc2. T-161 is analogous to T-197 in the C subunit. The PSTAIRE sequence (residues 45 to 51) includes the invariant Glu that ion pairs to K-33. This ion pair, indicated by a dashed line, is a conserved feature of all protein kinases.

indirectly for the proper configuration of catalytic residues at the active site. K-189 in the C subunit is replaced with R-151 in Cdc2, and mutation of R-151 reduces cyclin binding. The equivalent of the third ligand, H-87, in Cdc2 is T-47 in the conserved PSTAIRE sequence motif in the small lobe. Since the region of the peptide C terminal to the phosphotransfer site is of particular importance for peptide recognition by Cdc2 (37), the model predicts that phosphorylation of T-161 will be necessary for proper peptide recognition.

The other important regulatory phosphorylation site in Cdc2 is at T-14 and Y-15. Although T-14 phosphorylation may be important, Y-15 appears to be sufficient for inhibition on the basis of mutational analysis of this site (33). This site is phosphorylated only after Cdc2 has formed a complex with cyclin A or B, and whether Y-15 gets phosphorylated depends on the particular cyclin. G-1 cyclins, for example, do not seem to generate this inhibitory phosphorylation (see reference 35 for a review). The residue corresponding to Y-15 in cAPK is F-54 in the glycine-rich loop of cAPK. As seen in Fig. 9, F-54, H-87, and T-197 in cAPK are all quite close in the tertiary structure, as are Y-15, T-47, and T-161 in Cdc2. The Cdc2 model suggests, furthermore, that phosphorylation of Y-15 is inhibitory because it displaces the PSTAIRE sequence away from the active site at the cleft interface between the two lobes, thereby disrupting interaction between the lobes as well as peptide recognition. On the basis of FSBA labeling experiments, Atherton-Fessler et al. showed that ATP binding is not abolished by Y-15 phosphorylation, and this is also quite consistent with the model (1).

Cyclin A- and Suc1-binding sites. By generating a family of charged-to-Ala mutants of Cdc2, it was possible to screen these mutants for their capacities to bind cyclin A and Suc1. Consider first the mutants defective in cyclin A binding. Those mutations that interfere with cyclin binding are located primarily in the small lobe, and they have been reported to be the same for cyclin B binding. Each residue (E-38, E-40, E-41, and E-42) in the cluster of acidic residues that immediately precedes the PSTAIRE sequence is clearly important for cyclin binding. In addition, R-50A at the end of the PSTAIRE sequence motif and just before the conserved E-51 shows reduced binding for cyclin A. The single mutants, R-50A and E-51A, each abolish activity, but it is R-50A that is important for cyclin A binding. E-51 is the conserved amino residue that ion pairs with K-33, which also is conserved, and the K-33A mutant also shows reduced cyclin binding. Therefore, we believe that the position of R-50 at the surface of the cleft is important for cyclin binding and that when the ion pairing that helps to position this binding is disrupted, cyclin cannot bind properly. These mutations all suggest that cyclin binds primarily to the surface of the small lobe that lies at the edge of the cleft.

Only two residues in the large lobe influence cyclin binding, T-161 (discussed above) and R-151. R-151 lies in β-strand 9 and lies close to the region of the PSTAIRE sequence that approaches the cleft interface. When T-161 is phosphorylated, R-151 probably is one of the side chains that forms an ion pair with the phosphate. Both of these mutants lie on the surface that is opposite to the PSTAIRE region in the small lobe at the cleft interface. From these results we conclude that the cyclins bind near the surface of the B and C helices in the small lobe. This surface faces the large lobe in the cleft and helps to assemble this part of the molecule for phosphorylation and potentially for peptide recognition. Therefore, it is likely that cyclin itself may provide some determinants for peptide recognition. Indeed, some evidence already supports this, since Cdc25 can be phosphorylated by Cdc2-cyclin B but not by Cdc2-cyclin A (27).

Several laboratories have previously shown that T-161 phosphorylation is required for stabilization of the Cdc2-cyclin complex (12, 13, 22, 41, 46). Others have reported that T-161 mutants of Cdc2 are unaffected in their abilities to bind cyclins (1, 9, 50). Despite the apparent controversy, there is general agreement on the order of events, i.e., cyclin binding preceding and being necessary for the phosphorylation of T-161. The assay conditions that we used allowed us to detect differences in affinity for both cyclin A and cyclin B and between phospho-T-161-Cdc2 and unphosphorylated Cdc2. This finding nicely correlates with the structural information provided by the present work.

When the charged-to-Ala mutants were screened for Suc1 binding defects, a different profile emerges. Residues from both the small and the large lobes seem to be involved in the recognition of Suc1, and in contrast to the proposed cyclin-binding sites, these regions are, in general, far from the active site. The single exception is Y-15, where an aromatic residue is required. The cluster of basic residues at the end of β-strand 2 (K-20A, R-22A, H-23A, and K-24A) also causes reduced Suc1 binding when all are replaced with Ala. These residues lie on the back surface of the enzyme away from the active site. In the K-56 cluster, located in the extended loop connecting the C helix with β-strand 4, H-60A is the most disruptive single mutant. This region also lies on the back surface of the enzyme away from the active site. In the C subunit, the equivalent of H-60 is F-102, and this Phe interacts with several aromatic rings that are also conserved in Cdc2. This region in the C subunit is masked in part by the insertion of 40 residues at the N terminus that are not found in Cdc2.

In addition to these clusters in the small lobe, several charged-to-Ala replacements in the large lobe lead to reduced affinity for Suc1. These include three clusters that each contain an invariant residue: R-170–E-173, R-180–D-186, and D-271–K-274–R-275. The essential residue is the last residue in each cluster. These replacements may perturb the general conformation of the large lobe rather than be involved directly in Suc1 binding. Single mutations of the R-180 cluster confirm that D-186, the conserved residue that interacts with the catalytic loop, is responsible for the disruption of Suc1 binding. Clearly, perturbation of the large lobe in a variety of ways influences Suc1 binding. In addition to the above replacements that include essential conserved residues, Suc1 binding is abolished in the R-215A mutant. According to the model, this residue would be exposed on the surface and is likely to be a direct contact point for Suc1, since the R-215A mutation has no effect on activity or cyclin binding. Finally, when the insert between helix G and helix H is deleted, Suc1 binding is reduced. These results suggest that Suc1 may wrap around the surface of the enzyme and make contact with both lobes. Since Cdc2, unlike the C subunit, does not have an extension at its N terminus that precedes the conserved core, it is tempting to think that Suc1 mimics, in part, the interactions that the N-terminal portion of the C subunit makes in cAPK. The N-terminal 40 residues in cAPK begin on the surface of the large lobe and extend all the way up to the conserved core that begins with β-strand 1. These 40 residues thus span a large surface area that extends over both lobes. It does not, however, extend as far as R-280, the equivalent of R-215 in Cdc2. The possibility of more than one binding site for Suc1 cannot be discounted. Nevertheless, when Suc1 is split into two fragments, it appears as though each fragment binds to a different site on

the enzyme, which would be consistent with our mapping results (2). In addition, cross-linking experiments established that Cdc2 and Suc1 form a 1:1 complex (12).

Conclusion. The model described here provides a basis for thinking about the various surfaces of Cdc2, how these surfaces interact with different proteins, and how protein-protein interactions, as well as phosphorylation, might contribute to Cdc2 function. The purpose of any model is to serve as a framework for testing. For the mutants identified here, it should be possible to identify the specific residues that contribute to the countercharge in the subunit pair. Cyclin binding appears to be particularly important for generating a substrate binding site, and its localization near the PSTAIRE sequence and T-161 at the base of the cleft is consistent with such a role. Suc1 clearly plays a role in stabilizing the enzyme, but it is not essential for activity. This is consistent with Suc1 recognition sites being located on surfaces that are away from the active site. It will be important now to determine which sites in Suc1 and in cyclins A and B are important for recognition by Cdc2.

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