Roles of Active Site Residues and the NH$_2$-terminal Domain in the Catalysis and Substrate Binding of Human Cdc25*

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Human Cdc25 proteins are dual specific protein phosphatases that play important roles in cell cycle regulation. In this study, the catalytic mechanism and substrate binding specificity of human Cdc25A and -B proteins were investigated by site-directed and deletion mutagenesis methods. Mutations of the cysteine or the arginine residues in the active site motif abolished the Cdc25 phosphatase activity. However, the cysteine mutation in both Cdc25A and -B created enzymes that still retain the ability to bind their substrates. This allowed us to test the ability of Cdc25A and -B to bind various cyclin-Cdk complexes in vitro. While Cdc25A Cys → Ser could interact with cyclin A-Cdk2, cyclin B-Cdc2, and cyclin E-Cdk2 strongly, Cdc25B mutant was only found to bind to cyclin A-Cdk2 at significant levels. We also identified Arg$^{452}$ and Ser$^{449}$ as two crucial residues that could be directly involved in the molecular interactions between Cdc25 and cyclin-Cdk proteins. Deletion mutagenesis data also indicate that the phosphatase catalytic domains of Cdc25A and -B proteins are located within their carboxyl terminus.

In eukaryotic cells, regulation of the cell cycle is under the control of a tightly regulated network of protein kinases and phosphatases. Phosphorylation/dephosphorylation on Thr$^{14}$, Tyr$^{15}$, and Thr$^{161}$ of Cdc2, the catalytic subunit of the mitosis-promoting factor regulates the kinase activity and therefore ensures the proper timing of mitosis (1–4). Phosphorylation of Tyr$^{15}$ plays an important role in the negative regulation of Cdc2 and is carried out by the protein kinase Weel (5, 6). A stimulatory phosphorylation event is modulated by a Cdk$^{1}$ activation kinase, which phosphorylates Thr$^{161}$ (7, 8). In Schizosaccharomyces pombe, as a protein dual specific phosphatase, Cdc25 acts as a mitotic inducer by removing the phosphate from Tyr$^{15}$ and Thr$^{14}$ on Cdc2 and activating the activity of the mitosis-promoting factor (9–13).

Three different isoform cdc25 genes have been cloned in mammalian or human cells (14, 15). In HeLa cells, human cdc25C is expressed predominantly in the G$_{2}$ phase (14) and has been shown to activate the histone H1 kinase activity of p$^{34}$cdc2-cyclin B complex by dephosphorylation of Tyr$^{15}$ and Thr$^{16}$ in vitro (12, 13, 16). Therefore, it is very likely that Cdc25C functions at the G$_{2}$ to M transition. On the other hand, microinjection of anti-Cdc25A antibodies in G$_{1}$ cells blocks entry into S-phase, suggesting the implication of Cdc25A for the regulation of the S-phase entry (17, 18).

The phosphatase activity of Cdc25 proteins is regulated by extensive phosphorylation of its NH$_{2}$-terminal regulatory domain (16, 17, 19). The Cdc25 stimulatory kinase has been examined and identified to be Cdc2-cyclin B (16, 20). During interphase, Cdc25C has very weak phosphatase activity and becomes much more active at the G$_{2}$-M transition due to its phosphorylation. Human Cdc25A also undergoes a similar phosphorylation event during the G$_{3}$/S transition and exhibits an elevated phosphatase activity (17, 18).

Recently, the catalytic mechanism of the protein-tyrosine phosphatases and dual specific phosphatases have been the subject of many biochemical and biophysical studies (21–31). An invariant active site cysteine residue that is essential for the enzymatic activity has been identified in all the protein-tyrosine phosphatase and dual specific protein phosphatases studied up to now. In many cases, a phospho-enzyme intermediate is present during the enzymatic reaction, indicating the formation of a thiphosphate. Sequence alignments of various tyrosine and dual specific phosphatases suggest that they all contain a putative catalytic domain of about 170 amino acids (22, 32). Within the catalytic domain, there is a highly conserved HCXXXXXXX signature motif. Crystal structures of human protein-tyrosine phosphatase 1B (28, 31) and Yersinia protein-tyrosine phosphatase (33) have suggested that the conserved active site motif that contains the catalytic cysteine forms a phosphate binding loop (P loop) in a conformation that is similar to each other. Most recently, based on crystal structure of Yersinia protein-tyrosine phosphatase (33) and mutagenesis studies of vaccinia H1-related dual specific phosphatases (32), a highly conserved aspartic acid residue was proposed to act as a general acid to protonate the leaving group in the step of the formation of the enzyme-phosphate intermediate.

In this study, we showed that the active site residues residing in the putative P loop of human Cdc25A and -B proteins play important roles in the catalysis and protein interactions between Cdc25 proteins and various cyclin-Cdk complexes. We also revealed the in vitro substrate specificity of Cdc25A and -B regarding different cyclin-Cdk complexes.

MATERIALS AND METHODS

Site-directed and Deletion Mutagenesis in Human cdc25 Genes—

Human cdc25A cDNA was subcloned into pGEX 2T as described by Galaktionov et al. (15). A human cdc25B polymerase chain reaction (PCR)-generated BamHI-HindII fragment was synthesized using full-length cDNA as the template. Compared with the published human cdc25B sequence, this cDNA has a 14-amino acid insertion between residues 67 and 68 and also a 41-amino acid deletion, from residue 154 to 194. This clone represents a minor cDNA form of the cdc25B gene.
Site-specific mutagenesis was performed using PCR by Gene Amp PCR reagent kit with AmpliTaq DNA Polymerase (Perkin-Elmer). A 5′-primer overlapping the initiation codon and containing the BamHI site was used as the mutant 3′-primer (5′-CCCCCGATCCATGGGAGGTT-GCCCCAGCCGGAGCC), whereas a primer (5′-ATGAGATCTCAAGGGATTCGATGAAATG) overlapping both the Cys446 and an adjacent His site was used as the mutagenic 3′-primer. The C446S mutant cdc25B GST fusion protein was constructed by replacing the BamHI/EcoRI fragment of the wild-type cdc25B gene with the BamHI/EcoRI PCR fragment containing the C446S mutation. The H445N, S449A, and E451Q mutations were generated in a fashion similar to C446S. The R452A mutant cdc25B was generated by recombinant PCR technique (34). Briefly, the full-length cdc25B containing the Arg452 to Ala mutation was made by PCR reaction using the heteroduplex formed by two overlapping primers as the template and primed with two external PCR primers. The 1.6-kilobase PCR product containing the full-length cdc25B was digested by BamHI and HindIII and ligated to PGEX 2T treated with the same restriction enzymes. The ΔN-(351–540) Cdc25B, ΔN-(366–540) Cdc25B, and ΔN-(336–523) Cdc25A carboxyl-terminal Cdc25C proteins were also generated by PCR technique. The reactions were primed by primers that contain a BamHI site with the 5′ sequence of the gene and primers that contain the 3′ sequence of the gene, the stop codon, and the HindIII site. To generate cdc25A and cdc25B mutations in ΔN-(351–540) Cdc25B and ΔN-(336–523) Cdc25A, the C446S, C430S and C446S/C430S double mutants were used as the PCR templates. All of the mutations were confirmed by DNA sequencing.

Production and Purification of the Cdc25 GST Fusion Proteins in E. coli and the Thrombin Cleavage of the GST Fusion Protein—The expression and affinity purifications of GST fusion proteins of Cdc25 were performed as described in Refs. 15 and 35.

Bovine thrombin from Calbiochem was used to remove the GST moiety from the ΔN-(351–540) Cdc25B GST fusion protein. The thrombin-cleaved ΔN-(351–540) Cdc25B contains a glycine and serine next to the methionine at the amino terminus. After the thrombin reaction, the protein was further purified by a Superdex 75 HR 10/30 Gel filtration column on a Pharmacia fast protein liquid chromatography system.

pNPP Phosphatase Assays—Phosphatase activity of the purified wild-type and mutant Cdc25 proteins was assayed in 50 mM Tris, 50 mM NaCl, 1 mM EDTA, and 1 mM diethiothreitol, pH 8.0, using p-nitrophenyl phosphate (Sigma) as substrate. Absorbance at 410 nm was monitored on a Hewlett Packard 8452A diode array spectrophotometer.

Cell Culture, Synchronization, and Extracts—HeLa-S3 cells obtained from the American Type Culture Collection were grown in Joklik’s minimal essential medium with 5% calf serum and 1% penicillin/streptomycin in 8-liter spinner flasks. Cell extracts were prepared by a method described before (36). Asynchronous HeLa cells were added with 10 mM hydroxyurea and incubated for 18 h. This treatment allowed 85–90% of the cells to be arrested in early S phase (20, 36).

Immunoblot Analysis and Immunoprecipitation—Protein samples were dissolved in 2 × sample SDS buffer and electrophoresed on an SDS-polyacrylamide gel (37). Proteins were then transferred onto polyvinylidene difluoride PolyScreen membrane (DuPont NEN) by semidyblotting on a MillBlot-SD System (Millipore) as described (36). Antibodies against cyclin A and cyclin B were developed by immunizing rabbits with purified recombinant human cyclin A and B proteins (36). The anti-Cdk2 antisera was generated by injecting rabbits with purified recombinant human cyclin A and B proteins (36). Both C446S (Fig. 2, lane 4) and R452A (Fig. 2, lane 5) failed to dephosphorylate Cdc2 in this experiment.

To test the phosphatase activity of the mutant Cdc25B proteins on cyclin-Cdk complex, the in vivo substrate of Cdc25 proteins, cyclin A-cdc2, or cyclin B-Cdk2 protein kinase complexes was purified from HeLa lysate by immunoprecipitation with anti-cyclin A and anti-cyclin B antibodies. When analyzed on Western blot, by anti-Cdc2 antibody, Cdc2 proteins that associate with cyclin A or cyclin B proteins were found to be mainly the tyrosine-phosphorylated species, the slower migrating band on the SDS gel when compared with the nonphosphorylated Cdc2 marker purified from E. coli (3) (see Fig. 2, A and B, lanes 1 and 2, and B, lanes 1 and 2). Upon incubation with wild-type Cdc25B protein, a fast migrating Cdc2 was detected, indicating the dephosphorylation of Tyr15 on Cdc2 (Fig. 2, A, lane 3, and B, lane 4). Both C446S (Fig. 2, A, lane 4, and B, lane 3) and R452A–Cdc25B proteins (Fig. 2, A, lane 4, and B, lane 5) failed to dephosphorylate Cdc2 in this experiment.
Kinetic parameters of GST Cdc25B wild-type and mutant proteins

Reactions were carried out at 37 °C with p-nitrophenyl phosphate as substrate in 50 mM Tris, 50 mM NaCl, 1 mM EDTA, and 10 mM dithiothreitol. The GST fusion proteins were preincubated at room temperature for 10 min before the reactions.

| Enzyme       | $V_{\text{max}}^a$ | $K_{\text{m}}$ |
|--------------|-------------------|--------------|
| Wild type    | 70 ± 7.5 μmol mg⁻¹ min⁻¹ | 11.4 ± 2.4 mM |
| C446S       | 0                 |             |
| R452A       | 0                 |             |
| H445N       | 107 ± 10          | 308.5 ± 17.3 |
| S449A       | 7.9 ± 0.1         | 5.7 ± 0.1   |
| E451Q       | 51.5 ± 3.5        | 5.4 ± 0.4   |
| ΔN-(351-540) | 366 ± 33          | 5.6 ± 0.2   |
| ΔN-(366-540) | 206 ± 33          | 10.5 ± 2.0  |

*a After affinity chromatography on glutathione-Sepharose, there are two endogenous bacterial proteins and some degradation product of Cdc25A. GST fusion proteins that copurified with full-length protein (13, 20). Protein concentration was determined by the Bio-Rad version of Bradford’s dye binding assay by using bovine serum albumin as standard.

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Negative Cdc25 proteins since they are the substrates of the enzymes.

Ser451 in the Putative Catalytic Loop (P Loop) Plays an Important Role in the Interaction with Cyclin-Cdk Complexes—To investigate the potential roles of the P loop residues in catalysis or substrate binding of human Cdc25 proteins, we replaced serine 449 with alanine and glutamic acid 451 with glutamine in Cdc25B (see Fig. 1). The kinematic parameters of the Cdc25 proteins were determined using pNPP as substrate. The E451Q mutant enzyme showed similar kinetic properties compared with the wild-type enzyme. The replacement of Ser449 caused a 10-fold decrease in the $V_{\text{max}}$ value along with a 2-fold decrease in $K_{\text{m}}$. The $k_{\text{cat}}/K_{\text{m}}$ value of S449A mutant enzyme is reduced 4-fold compared with that of the wild-type enzyme (see Table I).

To test whether Ser449 or Glu451 are important for the interaction between Cdc25 and cyclin-Cdk complex, we generated the C446S/S449A and C446S/E451Q double mutations in human Cdc25B. The C446S mutation should eliminate the phosphate activity of Cdc25 and therefore provide us the opportunity to study the effect of S449A and E451Q mutations on the interaction between Cdc25B and cyclin A-Cdk2 complex. We tested the binding of the double mutant proteins with cyclin A and Cdk2 in Western blots. As shown in Fig. 4, while C446S/E451Q double mutant protein interacts with both cyclin A and Cdk2 proteins, C446S/S449A lost its ability to bind either cyclin A or Cdk2.

In Vitro Substrate Specificity of Human Cdc25A and Cdc25B Proteins—Since the active sites of human Cdc25A and Cdc25B show strong sequence homology (Fig. 1), it is reasonable to expect that replacement of the catalytic Cys430 with serine in Cdc25A will not disturb the interactions between Cdc25A and cyclin-Cdk complexes. This possibility was tested by Western blotting the proteins trapped by the GST fusion protein of C430S Cdc25A using various cyclin and Cdk antibodies. The GST fusion protein of C446S Cdc25B protein was
used in these experiments, to provide us with possible insights into the in vitro substrate specificity of human Cdc25A and -B proteins. Both C430S Cdc25A and C446S Cdc25B proteins exhibited strong interaction with cyclin A and Cdk2 proteins (Fig. 5, A and B). These interactions were observed with lysates prepared from either asynchronous HeLa cells (Fig. 5, A and B, lanes 5 and 7) or HeLa cells arrested in early S phase by hydroxyurea treatment (Fig. 5, A and B, lanes 6 and 8). Interestingly, significant differences between C430S Cdc25A and C446S Cdc25B proteins were observed in their ability to bind Cdc2-cyclin B complexes (Fig. 5, C and D). When 100 μg of GST fusion proteins were incubated with 2 mg of lysates from either asynchronous or hydroxyurea-blocked HeLa cells, strong signals were observed for C430S Cdc25A samples on both anti-cyclin B (Fig. 5C, lanes 4 and 5) and anti-Cdk2 (Fig. 5D, lanes 4 and 5) Western blots. No significant interaction was observed with either anti-cyclin B or anti-Cdk2 antibodies on the Western blots when C446S Cdc25B protein was used in the experiments (see lanes 6 and 7 of Fig. 5, C and D).

We also tested the possible interaction between cyclin E, C430S Cdc25A, and C446S Cdc25B proteins. As observed in Fig. 6, Cdc25A C430S interacted strongly with cyclin E when incubated with HeLa lysates at concentrations of either 4 mg/ml (Fig. 6A, lane 2) or 10 mg/ml (Fig. 6B, lane 1). Under the same experimental conditions, no interactions or very weak interactions between Cdc25B C446S and cyclin E proteins were observed (Fig. 6, A, lane 3, and B, lane 2). These results suggested that when compared with Cdc25B protein, Cdc25A has a much stronger interaction with cyclin E-Cdk2 complex, which is the major kinase present during the G2 phase (42). Significant amounts of cyclin E and Cdk2 proteins were also found to interact with wild-type Cdc25A protein (see Fig. 6, C and D, lane 4).

The Catalytic Domain of Human Cdc25A and -B Resides in the Carboxyl Terminus of the Protein—To elucidate functions of the amino terminus of human Cdc25A and Cdc25B and identify their catalytic domains, two carboxyl-terminal Cdc25A and -B proteins that contain residues 336–523 of Cdc25A (ΔN-(336–523)) and residues 351–540 of Cdc25B (ΔN-(351–540)) were generated by PCR (see Fig. 1), respectively. The sequence alignments of putative catalytic domains of human Cdc25A and -B showed 62–65% identity in their amino acid sequence. Both proteins were expressed in Escherichia coli as GST fusions. As shown in Tables I and II, both proteins retained their catalytic activity against pNPP. We also constructed a 175-amino acid carboxyl terminus of Cdc25B containing residues 366–540 (ΔN-(366–540)). This protein was also fully active in the pNPP assay (see Table I). Furthermore, both carboxyl-terminal catalytic domains of Cdc25A and -B proteins become better catalysts against pNPP, since their $k_{cat}/K_m$ ratios are 3–10-fold higher than that of the wild-type enzymes.

To measure the kinetic parameters of the native catalytic domain of human Cdc25B, the protease, thrombin, was used to remove the GST moiety from the ΔN-(351–540) Cdc25B GST fusion protein. After gel filtration purification on Superdex 75 column, the purity of the native Cdc25B catalytic domain was over 90%. When assayed with pNPP as substrate at pH 8.0, the enzyme showed a maximal velocity of 500 μM/mg⋅min$^{-1}$ and a $K_m$ of 4 mM.

Since it would be interesting to know whether the amino terminus of Cdc25 proteins contributes to the interactions between Cdc25 and the cyclin-Cdk complex, we mutated the catalytic cysteine residue to a serine in the catalytic domain of Cdc25B and tested whether this mutant protein could still interact with cyclin A and Cdk2 proteins. After incubation with HeLa cell lysate at two different concentrations, 4 and 10 mg/ml, we analyzed the results on immunoblots using anti-cyclin A and Cdk2 antibodies. Approximately the same amounts of cyclin A (see Fig. 7, A and B) and Cdk2 (Fig. 7, C and D) were detected for both ΔN-(351–540) and full-length Cdc25B.

**DISCUSSION**

Like all other known protein-tyrosine phosphatases, human Cdc25B carries the active site motif HCXXXnX. Amino acid
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**Fig. 6.** Interactions between Cdc25A and cyclin E or Cdk2. A and B, Western blots developed by anti-human cyclin E antibodies. In A, lane 1 is HeLa cell lysate. Lanes 2, 3, and 4 are the C430S Cdc25A, C446S Cdc25B, and GST proteins incubated with 2 mg of HeLa cell lysates at a concentration of 4 mg/ml. In B, lanes 1, 2, and 3 are the C430S Cdc25A, C446S Cdc25B, and GST proteins incubated with 5 mg of HeLa cell lysates at a concentration of 10 mg/ml. Shown in C and D are the immunoblots of anti-cyclin E and anti-Cdk2 antibodies. Lane 1 is the HeLa cell lysate. Lanes 2, 3, and 4 are GST, C430S, and wild-type Cdc25A proteins that have been incubated with 5 mg of HeLa cell lysate at a concentration of 10 mg/ml.

**Table II**

Kinetic parameters of GST Cdc25A wild-type and mutant proteins.

| Enzyme | $V_\text{max}^a$ | $K_\text{m}$ |
|--------|-----------------|-------------|
| Wild type | $52 \pm 4^b$ | $12 \pm 1.7$ |
| ΔN-(324–524) | $211 \pm 11$ | $11.4 \pm 1$ |

$^a$ The protein concentrations were measured by the same method as in Table I.

$^b$ The extinction coefficient of p-nitrophenolate used to calculated $V_\text{max}$ is $1.72 \times 10^4$ mol·cm$^{-1}$·ml$^{-1}$.

**Fig. 7.** ΔN-(351-540) Cdc25B retains its binding affinity for cyclin A/Cdk2 complex GST fusion proteins of full-length and ΔN-(351-540) Cdc25B were incubated with 2 mg HeLa cell lysates at a concentration of 4 mg/ml. The interaction between the Cdc25B proteins with cyclin A or Cdk2 were tested by Western blot by either anti-cyclin A (A and B) or anti-Cdk2 (C and D). In A and C, lane 1 is the HeLa cell lysates. Lanes 2, 3, and 4 are the GST, GST C446S Cdc25B, and GST ΔN-(351-540) C446S proteins that have been incubated with 2 mg of HeLa cell proteins at concentrations of 4 mg/ml. In B and D, lanes 1, 2, and 3 are the GST, GST C446S Cdc25B, and GST ΔN-(351-540) C446S proteins that were incubated with 2 mg of HeLa cell proteins at concentrations of 4 mg/ml.

**sequence alignment of human Cdc25A, Cdc25B, and Cdc25C shows 15 identical residues (FHCEFSSERGPRMCR) in this region (15). Previous mutagenesis studies in various protein-tyrosine phosphatases suggested that replacement of the cysteine and arginine residues in the active site motif eliminates enzymatic activity completely (9, 10, 27, 43). However, replacement of the histidine residue with alanine in Drosophila Cdc25 (10) or alanine or asparagine in Yersinia protein-tyrosine phosphatase (24) does not apparently affect the catalytic activity of these enzymes. Our mutagenesis results on human Cdc25A and -B indicate that the cysteine and arginine in the putative P loop are also two essential residues for the phosphatase activity. As is the case for other protein tyrosine phosphatases, the histidine in the active site motif is not essential for catalysis. These results agree with the observations of crystal structure of the human protein-tyrosine phosphatase 1B (28) and Yersinia protein-tyrosine phosphatase (33). In these structures, the side chain of histidine residues in the active sites was found not to interact with the cysteine or the phosphate. Therefore, it is unlikely that the imidazole side chain of histidine 445 in human Cdc25B is directly involved in the catalysis process of the enzymatic reaction.

It has been reported that the replacement of catalytic cysteine residues in the generation of inactive enzymes such as cysteine proteases (44), thymidylate synthetases (45, 46), and DNA cytosine methylase (47). Usually, the catalytically inactive mutant enzymes, in which a serine residue replaces the catalytic cysteine, retained their abilities to bind substrates, thus forming nonproductive stable enzyme-substrate complexes. Solving the crystal structure of such a complex can often provide a detailed picture of the molecular interactions between an enzyme and its substrate (31). We believe that such mutants in human Cdc25 proteins could provide an in vitro tool for studying the surface interactions between Cdc25 and cyclin-Cdk complexes. We tested the interaction between the cyclin and Cdk proteins with the C446S and R452A mutant Cdc25B proteins. Our data in this study suggested that the C446S Cdc25B retained its ability to interact with cyclin A and Cdk2 proteins. Since cyclin A protein mainly forms complexes with Cdk2 at the G1/S phase of the cell cycle, it is likely that the cyclin A and Cdk2 proteins bound by C446S Cdc25B protein exist as a cyclin A-Cdk2 complex. Furthermore, our data also suggested that Cdk2 proteins that interacted with Cdc25B are phosphorylated on Thr$^{14}$ and Tyr$^{15}$. These observations are consistent with the idea that Cdc25 proteins are the dual specific protein phosphatases that dephosphorylate the cyclin-Cdk complex during the cell cycle. The fact that Cdc25B R452A lost its affinity with both cyclin A and Cdk2 proteins is consistent with the observations in the structural studies in human protein-tyrosine phosphatase 1B (28) and Yersinia protein-tyrosine phosphatase (33). A charge-charge interaction between the active site arginine and the phosphate oxygen of the substrate was observed in human protein-tyrosine phosphatase 1B and Yersinia protein-tyrosine phosphatase. This interaction may also exist in human Cdc25 and is a determining factor in the substrate binding. The wild-type Cdc25B protein did not trap cyclin A and Cdk2 proteins in our experiment; this may reflect the transient nature of the interaction between an enzyme and its substrate.
In fission and budding yeast, the cdc25 gene has been linked to mitotic control by genetic investigations (48–50). Different from yeast, multiple cdc25 genes have been reported in both mice and humans (14, 15, 51, 52). Although three human Cdc25 proteins show strong homology in their putative catalytic domain, outside this region they are highly divergent, with no obvious sequence similarity. These differences suggest that the human Cdc25 species may function at different stages of the cell cycle and dephosphorylate different cyclin and Cdk complexes (53).

Human Cdc25C protein was found to activate the Cdc2/cyclin B complex and regulate the M phase entry in HeLa cells (12, 14, 16, 20). Microinjection of anti-Cdc25A antibodies into human fibroblasts in G1 blocked cell entry into S-phase (17). Furthermore, Cdc25A is phosphorylated during S-phase by the Cdk2-cyclin E kinase, and this phosphorylation increases its pNPP phosphatase activity 15–20-fold (17). However, until now no detailed studies have been conducted to establish substrate specificity of Cdc25 proteins. In this study, we tried to address this question by using the sytoine mutants of human Cdc25A and -B to probe for their specific interactions with various cyclin-Cdk complexes. Both Cdc25A C430S and Cdc25B C446S bind to cyclin A-Cdk2 complex from HeLa cell lysates. However, only Cdc25A C430S interacts with cyclin B and Cdc2 proteins strongly. Since Cdc2 mainly forms a complex with cyclin A or cyclin B and regulates G2/M transition (1–4), it is very likely that Cdc25A interacts with both cyclin A-Cdc2 and cyclin B-Cdc2 complexes. Furthermore, our data implies that the binding affinity between human Cdc25B and cyclin B-Cdc2 or cyclin A-Cdk2 complexes is much weaker than that of Cdc25A. We also observed strong interaction between Cdc25A and the cyclin E-Cdk2 complex, which is consistent with the in vivo studies results that human Cdc25A most likely acts at G1 or in the early S phase of cell cycle by activating the cyclin E/Cdk2, cyclin A/Cdk2, or cyclin D4/cdk4 kinase (17, 18, 41). Our data here also agree with observations made by Hoffmann et al. (17) that cyclin E/Cdk2 kinase phosphorylates Cdc25A protein and activates its phosphatase activity during S phase of the cell cycle (17). The strong interaction between cyclin E-Cdk2 and wild-type Cdc25A we observed may reflect the existence of this relationship.

Previous studies using Drosophila Cdc25 by Gautier et al. (10) suggested that residues within the putative catalytic loop may be important specificity determinants for the enzyme. Our observations on S449A human Cdc25B also suggested the importance of the putative P loop residues in the enzyme-substrate interaction. Since S449A mutant Cdc25B protein retains at least 10% of the phosphatase in the pNPP assay when compared with the wild-type Cdc25B, it is unlikely that S449A mutation causes a major structural disturbance around the active site region that could cause the loss of interaction between mutant Cdc25B and cyclin A-Cdk2 complex. Instead, the side chain of this serine residue may be directly or indirectly involved in the interface interactions between the Cdc25B and cyclin-Cdk complex. Since this serine residue is highly conserved in all of the Cdc25 proteins but not present in other protein-tyrosine phosphatases or dual specific phosphatases, it may act as one of the residues that define the substrate specificity of Cdc25 protein.

The alignment of all known dual specific protein-tyrosine phosphatase indicates that they all contain a putative catalytic domain of 170 amino acids (32). However, these enzymes are very different from each other in terms of their length and their amino acid sequence outside the putative catalytic domain. The function of these noncatalytic domains remains unknown (32). The fact that Cdc25 is a phosphorylated protein in S, G1, Xenopus, and human (19, 20, 54, 55) suggests the possible regulatory function of the amino-terminal domain of Cdc25. Human Cdc25C undergoes phosphorylation during the cell cycle and is hyperphosphorylated in M phase. The phosphorylated Cdc25C purified from mitotic cells by immunoprecipitation showed a higher pNPP hydrolysis activity than the unphosphorylated Cdc25C from interphase cells (20). Microsequencing of human Cdc25C protein suggested that six of the seven potential S/T-P consensus sites can be phosphorylated by Cdc2-cyclin B, and all of these sites are located at the NH2-terminal part of the protein (16). Phosphorylation of human Cdc25A by Cdk2-cyclin E kinase (17) or Raf1 kinase (56) can also enhance its phosphatase activity.

Our deletion mutagenesis data on human Cdc25A and -B proteins demonstrates that their catalytic domains reside in the carboxyl terminus. Both the GST fusion catalytic domain of Cdc25A and -B are fully active in the pNPP assay when compared with the wild-type GST fusion proteins. Furthermore, the thrombin-cleaved and -purified Cdc25B native catalytic domain is also catalytically active in the pNPP assay. Since the kcat/Km values of the catalytic domains are 3–10-fold higher than that of the full-length Cdc25 proteins, it is likely that the amino terminus has a negative regulatory effect on the Cdc25 phosphatase activity. Our results here also indicated that for Cdc25B protein, the amino terminus is not required for its interaction with cyclin A-Cdk2 complex. The catalytic domain has about the same affinity for cyclin A-Cdk2 complex as the full-length Cdc25B protein. The function of the amino terminus of Cdc25A and the interaction with various cyclin-Cdk complexes is under investigation.

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