Hyperphosphorylation of the N-terminal Domain of Cdc25 Regulates Activity toward Cyclin B1/Cdc2 But Not Cyclin A/Cdk2*

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Cdc25 regulates entry into mitosis by regulating the activation of cyclin B/cdc2. In humans, at least two cdc25 isoforms have roles in controlling the G_{2}/M transition. Here we show, using bacterially expressed recombinant proteins, that two cdc25B splice variants, cdc25B2 and cdc25B3, are capable of activating cyclin A/cdk2 and cyclin B/cdc2, but that mitotic hyperphosphorylation of these proteins increases their activity toward only cyclin B1/cdc2. Cdc25C has only very low activity in its unphosphorylated form, and following hyperphosphorylation it will efficiently catalyze the activation of only cyclin B/cdc2. This was reflected by the in vivo activity of the immunoprecipitated cdc25B and cdc25C from interphase and mitotic HeLa cells. The increased activity of the hyperphosphorylated cdc25s toward cyclin B1/cdc2 was in large part due to increased binding of this substrate. The substrate specificity, activities, and timing of the hyperphosphorylation of cdc25B and cdc25C during G_{2} and M suggest that these two mitotic cdc25 isoforms are activated by different kinases and perform different functions during progression through G_{2} into mitosis.

Regulation of the activity of cyclin-dependent kinases (cdks) underlies cell cycle progression. Cdk are regulated by a combination of factors, including cyclin association, and phosphorylations and dephosphorylations of the cdk subunit. The final step in the activation of cyclin/cdk is catalyzed by a member of the cdc25 family of dual specificity protein kinases, which remove inhibitory phosphates from Thr^{14} and Tyr^{15} of cdk2 and cdc2.

The role and regulation of cdc25 phosphatase has been well defined in yeast and Xenopus egg systems, using a combination of genetics and biochemistry. In Schizosaccharomyces pombe, cdc25 is controlled at the transcriptional, translational, and post-translational levels. The mRNA and protein levels oscillate through the cell cycle, being maximal in G_{2}, but the protein requires further post-translational modification in the form of phosphorylation for maximal activity (1, 2). Xenopus cdc25 levels do not change, but its activity is also controlled by phosphorylation and dephosphorylation (3, 4). In humans, three isoforms of cdc25 exist, cdc25A, -B, and -C. Cdc25A has a role in regulating the G_{1}/S cell cycle phase transition whereas cdc25B and cdc25C regulate the G_{2}/M transition (5–9). The level of mRNA for each isoform changes through the cell cycle, but the protein levels of only cdc25A and cdc25B have been reported to change in a cell cycle-dependent manner (5, 7, 9). All three cdc25s are phosphorylated in vivo, and phosphorylation has been demonstrated to regulate the activity of cdc25A and cdc25C (6, 10, 11).

Whereas the in vivo substrates identified for the cdc25s to date are the Tyr-phosphorylated cyclin/cdk, cdc25 activity has usually been measured in vitro using a range of substrates, most of which are non-physiological e.g. p-nitrophenyl phosphate. The cdc25 phosphatasates utilize this substrate very poorly (12), and there is evidence that the cyclin subunit of the cdk complexes directly modifies the activity of cdc25 (13, 14), a subtlety which would be lost with the artificial substrates. While different cdc25 isoforms have been demonstrated to activate a range of different cyclin/cdk (15–17), there are no data on the relative activity of the cdc25 isoforms toward the different cyclin/cdk, nor data on how the observed phosphorylations of the different cdc25 isoforms affects their activities toward the cyclin/cdk substrates.

In this report, we provide biochemical evidence that two cdc25B splicing variants and cdc25C utilize cyclin A/cdk2 and cyclin B1/cdc2 as substrates with different efficiencies. Using bacterially expressed GST fusion proteins of cdc25B, cdc25C, and catalytic domain constructs, we demonstrate that in vitro two cdc25B splicing variants identified (18) have relatively high activity toward both cyclin A/cdk2 and cyclin B1/cdc2, whereas cdc25C will activate cyclin B1/cdc2 to the same extent as the cdc25Bs only after hyperphosphorylation. The phosphorylation induced increases in activity of all the cdc25s are due to increased binding of the cyclin/cdk. Similar increases in activity were observed for the hyperphosphorylated forms of cdc25B and cdc25C immunoprecipitated from mitotic HeLa cells. Activity assays of chimeric constructs of cdc25B and cdc25C revealed that exchanging the N-terminal regulatory domains significantly modified their catalytic activities. We propose that mitotic hyperphosphorylation modifies the structure of the N-terminal regulatory domain of the cdc25s to allow better access of cyclin/cdk to the catalytic domain.

MATERIALS AND METHODS

Cell Culture and Synchrony—HeLa cells were cultured in RPMI 1640 with 5% bovine serum (Serum Supreme, Biowhittaker). Single and double thymidine block/release synchronies of HeLa cultures were performed as described previously (19).

Production and Expression of GST-cdc25s—Production of GST fusion proteins of wild type and catalytically inactive C/S and CR/SK mutants of cdc25B3 (using the nomenclature of Baldwin et al. (18)) and cdc25C, 2

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1 The abbreviations used are: cdk, cyclin-dependent kinase; GST, glutathione S-transferase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

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where critical Cys and Arg residues have been mutated to Ser and Lys, are described elsewhere (9, 20), and production of GST-cdc25B2 is described elsewhere. The cdc25 chimeras were produced by cutting full-length cdc25B and cdc25C in pUC58R (9) with Acl I, which cuts the vector 5′ of the cloned cDNA and both cdc25s in the N-terminal region of the chimera. The excised Acl I fragments from the two cdc25s were then subcloned into the corresponding sites of the other cdc25s. The fragments were ligated in-frame and encoded chimeric cdc25s from cdc25B3 codon 1–251 and cdc25C 181–472 (designated cdc25BC2), and cdc25C codon 1–180 and cdc25B3 252–580 (designated cdc25CB3). The chimeras were then subcloned in pGEX 2T (Pharmacia) as either an Xba I or Avr II fragment of cdc25B/C into the corresponding sites of cdc25C (9), or BamH I/BamH I fragment of cdc25B/C subcloned in BamH I-digested pGEX 2T. The production of GST fusion proteins of the C-terminal fragments of both cdc25B3 and cdc25C has been described elsewhere (20). The GST fusion proteins were expressed in Eschericha coli DH5α and the induced proteins were extracted by Sarkosyl lysis and affinity purification on glutathione-agarose as described previously (9), modified to include 5 mM DTT in all extraction and washing buffers.

Cdc25 Activity Assays—The activity of the GST-cdc25 fusion proteins was assayed by their ability to activate the Tyr15-phosphorylated forms of cyclin B1/cdk or cyclin A/cdk. The cdc25 activity was quantitated as the increased histone H1 kinase activity of the activated cyclin/cdk. The cyclin/cdkks were isolated by immunoprecipitation with either anti-cyclin B1 or anti-cyclin A monoclonal antibodies from extracts of either G1/S or S/G2 synchronized cells, or mitotic bacteria and purified by glutathione-agarose affinity chromatography. These proteins have previously been shown to be catalytically active GST-cdc25B3 CR/SK mutant. Where indicated, the GST-cdc25s were diluted with elution buffer. In all assays, GST-cdc25BC3 was initially diluted 3-fold to give a similar level of protein as that of the eluted GST-cdc25B and GST-cdc25C.

The activity of HeLa cell cdc25B and cdc25C was assayed by immunoprecipitation from extracts of either G1/S or G2/S synchronized cells and cdc25C, GST fusion proteins of each were expressed in bacteria and purified for each of the cdc25s, C-terminal constructs and two chimeric proteins, but we were unable to purify a full-length GST-cdc25 to give a final volume of 200 μl and incubated at 30 °C for 1 h. The reaction was stopped by the addition of 300 μl of 2 mM NaOH and the turnover of p-nitrophenyl phosphate determined as the increased absorbance at 410 nm using the e of 18,000 M⁻¹ cm⁻¹ for p-nitrophenol.

RESULTS

Production of cdc25B and cdc25C Chimeras—To investigate the intrinsic activities of the two splicing variants of cdc25B and cdc25C, GST fusion proteins of each were expressed in bacteria and purified by glutathione-agarose affinity chromatography. These proteins have previously been shown to be catalytically active GST-cdc25B3 CR/SK mutant. Where indicated, the GST-cdc25s were diluted with elution buffer. In all assays, GST-cdc25BC3 was initially diluted 3-fold to give a similar level of protein as that of the eluted GST-cdc25B and GST-cdc25C.

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The activity of the GST-cdc25 was initially assayed using p-nitrophenyl phosphate as substrate. The catalytic C-terminal regions of the two cdc25B and cdc25C display a high degree of sequence identity and would thus be expected to have similar catalytic properties. When the activities of the GST-cdc25s were compared on an equimolar basis, p-nitrophenyl phosphate was found to have a 4–5-fold greater activity than either cdc25B or cdc25C, which had similar activities with p-nitrophenyl phosphate (Table I). The full-length cdc25B and its isolated C-terminal domain construct had similar activity with this substrate, although this was less than half the activity of the cdc25B. Interestingly, the two full-length chimeras had very different activities from
their parent forms, cdc25BC2 was essentially inactive with this substrate, whereas cdc25CB3 had 3-fold higher activity than cdc25B3, but a similar activity to the cdc25B C-terminal fragments (Table I).

The activity of the GST-cdc25Bs and GST-cdc25C with \( p \)-nitrophenyl phosphate indicated only small differences between the catalytic activities of the bacterially expressed proteins. Others have shown that the cyclin subunit of the in vivo substrates of cdc25, the cyclin/cdkS, can contribute to the activity and possibly specificity of the cdc25 (13, 14). To investigate this possibility, the activity of the GST-cdc25s, chimeras, and C-terminal constructs toward Thr\(^{14} \)Tyr\(^{15} \)-phosphorylated cdc25B/cyclin B1 isolated from S/G2 HeLa cells and Tyr\(^{15} \)-phosphorylated cdk2/cyclin A isolated from G1/S HeLa cells was assayed. The activity of each GST-cdc25 was assayed as a dilution series to account for the differences in the levels of each GST fusion protein expressed in bacteria, and their different relative activities. Cdc25 activity was measured by the activation of immunoprecipitated, Tyr\(^{15} \)-phosphorylated cyclin/cdk, and quantitated in terms of the increased histone kinase activity of the complexes (Fig. 2A).

Cdc25C C-terminal fragments and chimeric cdc25CB3 had the highest relative activities toward both cyclin B1/cdc2 and cyclin A/cdk2 (Fig. 2, B and C). Cdc25B2 and cdc25B3 had similar levels of activity toward cyclin B1/cdc2, but cdc25B2 displayed a 2–4-fold higher activity toward cyclin A/cdk2 than

![Fig. 1 A](A) schematic diagram of the GST-cdc25s and chimeras used. The restriction sites used to construct the chimeras and C-terminal domains, and the codons at which they cut are indicated. The conserved catalytic domain of cdc25B and cdc25C, cdc25B[385–581] and cdc25C[275–473], are shown as differently shaded regions. B, Coomassie Blue-stained SDS-PAGE gel of eluted GST-cdc25s and chimeras used. The strongly staining bands less than 36 kDa are premature termination products of the GST fusion proteins. The minor bands migrating between these and the full-length proteins are truncations in the C-terminal catalytic domain and therefore inactive.

### Table I

<table>
<thead>
<tr>
<th>Activity</th>
<th>µM/min/mg</th>
</tr>
</thead>
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<tr>
<td>Cdc25B2</td>
<td>59</td>
</tr>
<tr>
<td>Cdc25B3</td>
<td>74</td>
</tr>
<tr>
<td>Cdc25C</td>
<td>25</td>
</tr>
<tr>
<td>Cdc25BC2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cdc25CB3</td>
<td>237</td>
</tr>
<tr>
<td>Cdc25B C-terminal</td>
<td>350</td>
</tr>
<tr>
<td>Cdc25C C-terminal</td>
<td>26</td>
</tr>
</tbody>
</table>

This table lists the p-nitrophenylphosphatase activity of the GST-cdc25s.
cdc25B3. Neither GST-cdc25C, cdc25C C-terminal fragments, nor chimeric cdc25BC2 displayed significant activity toward cyclin A/cdk2, although GST-cdc25C and GST-cdc25C C-terminal fragments activated cyclin B1/cdk2 2-3-fold. The difference in the relative activities of the GST-cdc25s toward p-nitrophenyl phosphate and the immunoprecipitated cyclin/cdkks was not a consequence of steric interference of the GST moiety, as removal of the GST moiety with thrombin followed by assay using p-nitrophenyl phosphate or immunoprecipitated cyclin B1/cdk2 gave similar relative activities (data not shown).

**Mitotic Phosphorylation and Activation of the GST-Cdc25s—**
Mitotic hyperphosphorylation of cdc25C is associated with its increased activity using a number of non-physiological substrates (10, 11), and phosphorylation of cdc25B is correlated with its activity in vivo (9). To test the effects of mitotic hyperphosphorylation on cdc25 activity toward cyclin A/cdk2 and cyclin B1/cdk2, the full-length GST-cdc25s and chimeras were phosphorylated in vitro using extracts from mitotic HeLa cells. The phosphorylated cdc25s displayed the characteristically retarded electrophoretic mobilities observed for these proteins in vivo (Fig. 3A; Refs. 9 and 10). In these experiments, all of the GST-cdc25C was shifted to the hyperphosphorylated form as was cdc25CB3, whereas only half of the cdc25B2, cdc25B3, and cdc25BC3 was in the retarded form. The phosphorylated GST-cdc25s were assayed for their activity toward immunoprecipitated S/G2 cyclin B1/cdk2 (Fig. 3B). Hyperphosphorylation of GST-cdc25B2 and cdc25B3 resulted in 2–3-fold and 3–5-fold activation, respectively. Chimeric GST-cdc25BC2 again showed no activity after mitotic phosphorylation, although phosphorylation of the cdc25B3 N-terminal regulatory domain produced a similar change in electrophoretic mobility to that seen in the full-length cdc25Bs (Fig. 3A). GST-cdc25CB3 showed a small increase in activity (no more than 2-fold), whereas phosphorylation of GST-cdc25C produced the greatest increase in activity, to have greater cdc25 activity on a relative mole cdc25 basis than either of the activated cdc25Bs, although this was still less than the activated GST-cdc25CB3 (Fig. 3B, lower panel). The increased histone kinase activity was not due to any histone kinase associated with the phosphorylated GST-cdc25s from the HeLa extracts used for phosphorylation, and no increase in histone kinase activity was associated with mitotic phosphorylation of the catalytically inactive GST-cdc25B3 CR/SK mutant, which was used as the control for this experiment or with GST-cdc25BC2.

To determine whether the mitotic phosphorylation of the...
GST-cdc25s was directly responsible for the increased activities observed, the GST-cdc25s were phosphorylated as in the previous experiment, then incubated either with or without recombinant λ-phosphatase to dephosphorylate the proteins, following which the phosphatase was washed away and the GST-cdc25s assayed for activity using immunoprecipitated S/G2 cyclin B1/cdc2 and G1/S cyclin A/cdk2 as substrates. The λ-phosphatase completely dephosphorylated the GST-cdc25s in each case (Fig. 4A). Interestingly, the hyperphosphorylated GST-cdc25C was poorly stained with the anti-GST antibody, which was also seen using anti-cdc25C antibody (data not shown). The differences in the activity levels of the phospho- and dephospho-forms of the GST-cdc25s for cyclin B1/cdc2 (Fig. 4B) were similar to those seen with the phosphorylated and dephospho-forms of the GST-cdc25s for cyclin B1/cdc2 (Fig. 3B). Interestingly, the mitotic phosphorylation of the GST-cdc25s had little effect on their activity toward the immunoprecipitated cyclin A/cdk2 (Fig. 4B, lower panel). The low degree of cdc25 activation in the experiment shown was due to the relatively high histone kinase activity of the immunoprecipitated cyclin A/cdk2. Despite this high background, GST-cdc25CB3 produced a 5-fold activation, indicating that the lack of hyperphosphorylation induced increase in activity of the GST-cdc25s similar to that seen with cyclin B1/cdc2 was not due to an insensitive cyclin A/cdk2 substrate. In this experiment, anti-cdk2 immunoprecipitates were used, but a similar lack of phosphorylation dependent activation was observed using anti-cyclin A immunoprecipitates (data not shown). The small increase in GST-cdc25B3 activity with cyclin A/cdk2 was consistently observed.

The mechanism by which the mitotic phosphorylation increased the activity of cdc25B and cdc25C was further investigated, taking advantage of the ability of the catalytically inactive cdc25 C/S mutants to form stable complexes with the substrate cdk-cyclin complexes (21). The C/S mutants of GST-cdc25B2, cdc25B3, and cdc25C were hyperphosphorylated with mitotic HeLa extracts as in the previous experiments, and were used to pull down interacting cdk/cyclins from extracts of synchronized S/G2 Molt4 cells. The binding of cdk2 and cdc2 complexes was analyzed by immunoblotting, compared with the unphosphorylated GST-cdc25s as controls. The levels of hyperphosphorylation obtained were equivalent to those obtained in the earlier phosphorylation experiments (Fig. 5). GST-cdc25B2

**Fig. 4.** Hyperphosphorylation of the GST-cdc25s increased their activity with cyclin B1/cdc2 but not cyclin A/cdk2. A, GST-cdc25s were hyperphosphorylated as described in the legend to Fig. 3, and then incubated either with or without λ-phosphatase (λ). The dephosphorylation was detected as an increase in the electrophoretic mobility of the bands immunblotted with anti-GST antibody. B, the activity of the hyperphosphorylated and dephosphorylated GST-cdc25s in A shown as histone kinase activity and quantified as fold increase over the CR/SK control, with either immunoprecipitated cyclin B1/cdc2 or cyclin A/cdk2 as substrate.

GST-cdc25s was directly responsible for the increased activities observed, the GST-cdc25s were phosphorylated as in the previous experiment, then incubated either with or without recombinant λ-phosphatase to dephosphorylate the proteins, following which the phosphatase was washed away and the GST-cdc25s assayed for activity using immunoprecipitated S/G2 cyclin B1/cdc2 and G1/S cyclin A/cdk2 as substrates. The λ-phosphatase completely dephosphorylated the GST-cdc25s in each case (Fig. 4A). Interestingly, the hyperphosphorylated GST-cdc25C was poorly stained with the anti-GST antibody, which was also seen using anti-cdc25C antibody (data not shown). The differences in the activity levels of the phospho- and dephospho-forms of the GST-cdc25s for cyclin B1/cdc2 (Fig. 4B) were similar to those seen with the phosphorylated and unphosphorylated GST-cdc25s in the previous experiment (Fig. 3B). Surprisingly, the mitotic phosphorylation of the GST-cdc25s had little effect on their activity toward the immunoprecipitated cyclin A/cdk2 (Fig. 4B, lower panel). The low degree of cdc25 activation in the experiment shown was due to the relatively high histone kinase activity of the immunoprecipitated cyclin A/cdk2. Despite this high background, GST-cdc25CB3 produced a 5-fold activation, indicating that the lack of hyperphosphorylation induced increase in activity of the GST-cdc25s similar to that seen with cyclin B1/cdc2 was not due to an insensitive cyclin A/cdk2 substrate. In this experiment, anti-cdk2 immunoprecipitates were used, but a similar lack of phosphorylation dependent activation was observed using anti-cyclin A immunoprecipitates (data not shown). The small increase in GST-cdc25B3 activity with cyclin A/cdk2 was consistently observed.

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**Fig. 5.** Hyperphosphorylation increases the affinity for cdc2 but not cdk2-cyclin complexes. GST-cdc25 C/S mutants were hyperphosphorylated as in Fig. 3, incubated in extracts of Molt4 cells synchronized in late S/G2 phase, and the levels of bound cdc2 and cdk2-cyclin complexes assessed by immunoblotting for these proteins. The relative levels of GST-cdc25s are indicated in the GST immunoblot. Lysates of asynchronously growing HeLa cells were also run as controls for the immunoblotted cdc2 and cdk2. The strongly staining band running ahead of the cdk2 doublet is a cross-reactive species.
regulate the activation of different pools of mitotic cyclin/cdk.

This was further supported by the timing and proportion of the cdc25B and cdc25C activated during the cell cycle. Immuno-blotting of HeLa cells synchronized using a double thymidine block/release protocol, which resulted in up to 80% of cells progressing synchronously from early S phase through to mitosis, revealed hyperphosphorylated forms of cdc25B as early as 8 h after release, when cells were in late S/early G2 phase, preceding the activation of cyclin B1/cdc2 (Fig. 7). In contrast, the hyperphosphorylated form of cdc25C was only detected at 10 h simultaneous with the activation of cyclin B1/cdc2. The hyperphosphorylated form accounted for only a small proportion of the total cdc25C. This low proportion is clearly indicated by the apparently similar levels of the underphosphorylated cdc25C in all the time point samples, and cannot be accounted for by incomplete synchrony of the cultures, loss due to dephosphorylation in the extract as the majority of the cdc25B is present in hyperphosphorylated form at 10 h after release (Fig. 7), or the reduced sensitivity of immunoblotting for the hyperphosphorylated form already noted with the in vitro hyperphosphorylated cdc25C (Figs. 4A and 5).

DISCUSSION

The in vitro analysis of cdc25 substrate specificity and activation reported here has provided a biochemical basis for the observed activities of cdc25B and cdc25C in vivo. The catalytic properties of the isolated catalytic domains of these two phosphatases were surprisingly different considering they share over 56% identity and 67% homology of the amino acid sequences in the conserved catalytic domains. The cdc25B C-terminal had the highest activity of all the GST-cdc25s with all of the substrates used, whereas cdc25C C-terminal fragments dephosphorylated p-nitrophenyl phosphate and cyclin B1/cdc2 at only 10% the rate of cdc25B C-terminal fragments and was essentially inactive with cyclin A/cdk2 (Table II). This suggests that some of the differences in activities of the full-length cdc25s is due to the different activities of the catalytic domains. Interestingly, full-length cdc25C and its C-terminal domain construct had similar activities toward p-nitrophenyl phosphate, consistent with a previous report (20), and the cyclin/ cdks, whereas cdc25B2 and cdc25B3 had only 25% the activity of their common catalytic domain construct with all the substrates tested here (Table II). The difference in the catalytic activities of cdc25B2 and similar C-terminal constructs has also been observed by others using p-nitrophenyl phosphate as a substrate (21).

The relative ability of the bacterially expressed GST-cdc25s to bind cyclin A/cdk2 and cyclin B1/cdc2 generally reflected the relative catalytic rates of the GST-cdc25s with cyclin A/cdk2 and cyclin B1/cdc2. Hyperphosphorylation of the GST-cdc25s resulted in increased binding of cyclin B1/cdc2, which would
TABLE II
Relative activity of GST-cdc25s towards different substrates

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<th>Cdc2</th>
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<td></td>
<td>A/ekd2</td>
<td>B/ecd2</td>
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</tr>
<tr>
<td>Cdc25B2</td>
<td>2.4</td>
<td>10</td>
<td>22</td>
<td>2</td>
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<td>3</td>
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<td>1–2</td>
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* pNPP, p-nitrophenylphosphate.

The surprising result that the dramatic increases in binding and catalytic activities with cyclin B1/cdk2 induced by hyperphosphorylation of the GST-cdc25s were not reflected in the binding or activity with cyclin A/cdk2, with the exception of GST-cdc25B3, which showed small increases in both binding and activity for this complex, suggests that interaction of cyclin B1/cdk2 and cyclin A/cdk2 with the cdc25s is very different, and that the conformational changes induced by hyperphosphorylation of the N-terminal domain affects cyclin A/cdk2 access/affinity to the catalytic site only minimally. The reason for this lack of phosphorylation-dependent effect with cyclin A/cdk2 may be due to structural differences between the cyclin A and cyclin B1/cdk complexes and the manner in which these complexes interact with the cdc25s. The cdk5s have a high degree of sequence identity, whereas, outside a few conserved domains, cyclins are quite divergent and are likely to possess very different tertiary structures. Structural differences are also suggested by the range of binding affinities of the cdk inhibitor proteins, e.g., p21CIP1 and p27Kip1, for cyclin A and cyclin B-ckd complexes (24).

Cell cycle analysis of expression of cdc25B and cdc25C shows the expected dramatic increase in cdc25B proteins (cdc25B3 is the most abundant form, although cdc25B2 expression is also increased in G2, G3, and G1 phases of the cell cycle (25)). At this time, cdc25B2 and cdc25B3 are localized to the chromosome where the cyclin B1/cdk2 is also localized (25), and...
the *in vitro* substrate specificity experiments indicate that cyclin A/cdk2 is a good substrate for both cdc25Bs. Thus cdc25B2 and cdc25B3 may first activate cyclin A/cdk2 in G2, then cdc25B3 accumulates in the cytoplasm where it activates part of the pool of cyclin B1/cdk2, which in turn catalyzes increases in microtubule dynamics and increased levels of microtubule nucleation at the centrosome, both of which are blocked by overexpressing catalytically inactive mutants of cdc25B3 (9). The translocation of cyclin B1/cdk2 from the cytoplasm to the nucleus in prophase is correlated with the conversion of the centrosomes to mitotic microtubule organizing centers (26), and thus translocation of cyclin B1/cdk2 must occur after cdc25B3 appears in the cytoplasm. It is unclear as to what proportion of the cyclin B1/cdk2 pool is active when it is translocated, but cdc25C may be hyperphosphorylated by the active cyclin B1/cdk2 translocated into the nucleus, and the former may in turn activate the remainder of the translocated pool of cyclin B1/cdk2. The hyperphosphorylated cdc25C may also act on other, unidentified substrates, but appears not have a role in the activation cyclin A/cdk2.

In summary, we have shown that recombinant cdc25B2 and cdc25B3 have relatively high activity toward Tyr15-phosphorylated cdc2/cyclin B1 and cdk2/cyclin A, with cdc25B2 having a higher activity with cdk2/cyclin A than cdc25B3. Cdc25C possesses only very low or no activity with these substrates *in vitro*. The hyperphosphorylated forms of these proteins all showed increased activity with cdc2/cyclin B1, but little increase in activity with cdk2/cyclin A. This was reflected in the relative binding efficiencies of the cdc25s for the cdk-cyclin complexes. Similar phosphorylation-dependent increases in cdc25 activity were observed for cdc25B and cdc25C *in vivo*. These data indicate that the different cdc25s potentially regulate different pools of cdk/cyclins, and suggest that the phosphorylation dependent activation of the cdc25s is due to conformational changes which result in increased access to the catalytic pocket for the substrates.

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