A Bipartite Substrate Recognition Motif for Cyclin-Dependent Kinases

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Abstract

Cy or RXL motifs have been previously shown to be cyclin binding motifs found in a wide range of cyclin-cdk interacting proteins. We report the first kinetic analysis of the contribution of a Cy motif on a substrate to phosphorylation by cyclin dependent kinases. For both cyclin A/cdk2 and cyclin E/cdk2 enzymes, the presence of a Cy motif decreased the $K_m^{peptide}$ 75 to 120-fold while the $k_{cat}$ remained unchanged. The large effect of the Cy motif on the $K_m^{peptide}$ suggests that the Cy motif and S/T-P-X-K/R together constitute a bipartite substrate recognition sequence for cyclin dependent kinases. Systematic changes in the length of the linker between the Cy motif and the phosphoacceptor serine suggest that both sites are engaged simultaneously to the cyclin and the cdk, respectively, and eliminate a “bind and release” mechanism to increase the local concentration of the substrate. PS100, a peptide containing a Cy motif, acts as a competitive inhibitor of cyclin/cdk complexes with a 15-fold lower $K_i$ for cyclin E/cdk2 than for cyclin A/cdk2. These results provide kinetic proof that a Cy motif located at a minimal distance from the SPXK is essential for optimal phosphorylation by cdks and suggest that small chemicals that mimic the Cy motif would be specific inhibitors of substrate recognition by cyclin-dependent kinases.
Introduction

Timely progression through the cell cycle depends upon the well-orchestrated activation and deactivation of cyclin-dependent kinases. Each of these kinases is active for only a short period of the cell cycle during which time it phosphorylates a number of substrates required for entry into the next phase of the cell cycle. Cyclin A/cdk2 and cyclin E/cdk2 both play a major role in the G1/S transition of the cell cycle by the phosphorylation of various substrates including pRb, E2F, and CDC6 (1,2). Despite their critical role in this process, little is known about how these substrates are targeted to specific cyclin-cdk complexes.

Since the S/T-P-X-K/R consensus phosphorylation site is broadly applicable to all substrates of all cdks, it would be incapable of conferring the substrate specificity seen within a cellular context. An alternate mechanism by which this specificity could be achieved is through the presence of a docking site on the substrate that recruits the appropriate cyclin-cdk to the protein. The resulting high localized concentration of the cyclin-cdk then facilitates the phosphorylation of potential ser/thr phosphorylation sites that have been brought into close proximity. In previous work, we and others have identified a sequence motif present in a number of cellular proteins that interact with cyclin-cdk complexes and could potentially perform this function (3-5). These cyclin-binding (Cy or RXL) motifs have been found in substrates such as E2F and CDC6, activators like Cdc25a, and inhibitors of the p21/p27 family, and are absolutely required for the association of cyclin-cdk complexes with these proteins (3,4,6-8). The importance of this motif in the interaction of these proteins with cyclin-cdks is
further highlighted by the crystal structure of the cdk inhibitor p27 complexed with cyclin A/cdk2 (9). In this structure, the N-terminal half of the inhibitor p27 was shown to associate with cyclin A/cdk2 through two distinct regions of the protein – a C-terminal region buried in the ATP-binding cleft of the cdk2 active site and a N-terminal Cy motif bound to a shallow hydrophobic groove on the surface of the cyclin. Although there is no structural evidence to confirm it, it seems likely that substrates containing a Cy motif would bind in a similar fashion as the inhibitor. The Cy motif of the substrate would bind to the same groove on the cyclin and allow potential phosphorylation sites on the protein to associate with the nearby cdk2 subunit and become phosphorylated.

One substrate which we propose acts in this fashion is the human replication factor, CDC6. This factor is involved in the formation of a pre-replication complex and is required for the initiation of DNA replication (10,11). At the onset of S-phase, mammalian CDC6 is phosphorylated by cyclin A/cdk2 which inactivates it by exporting it from the nucleus into the cytoplasm (7,12,13). Phosphopeptide analysis has shown that this phosphorylation by cyclin A/cdk2 occurs on Ser-54, Ser-74, and Ser-106 (13). This requires the presence of a nearby Cy motif at residues 94-98 as evidenced by the fact that its mutation abolishes phosphorylation at these sites (14).

In this report, we describe the first kinetic analysis of a Cy motif containing substrate to determine the contribution of the Cy motif to the catalytic efficiency of cyclin-cdk complexes. Using a series of peptides derived from CDC6 that contain a consensus SPXK phosphorylation site and either a wild-type or
mutated Cy motif, we show that an intact Cy motif plays a critical role in targeting the peptide to cyclin-cdk complexes. We have also examined the effect of changing the length of the linker between Cy motif and the cdk phosphorylation site to show that both sites must be simultaneously bound to the cyclin/cdk to maximize phosphorylation of the substrate.

Materials and Methods

Expression and purification of cyclin/cdk complexes – Baculoviruses expressing GST-cyclin E, GST-cyclin A, and cdk2 were gifts from Helen Piwnica-Worms. Sf9 cells were coinfectected with the appropriate cyclin/cdk pair and affinity-purified as previously described (15) with the following changes. After affinity binding to glutathione agarose beads, the complexes were cleaved from GST using Novagen’s Thrombin Cleavage Capture Kit.

Kinase assays – Phosphorylation reactions were performed in a total volume of 15 µl containing 50 mM Hepes (pH 7.4), 10 mM MgCl₂, 0.5 mM dithiothreitol, 0.02% Triton X-100, 1 µCi [γ-³²P] ATP, and various concentrations of ATP and peptide dissolved in deionized water. Five different peptide and ATP concentrations were used that ranged from 0.5 x Km to 5 x Km for each substrate. Reactions were initiated by addition of 1 µl of enzyme diluted in reaction buffer and incubated at 30°C for 20 min. Reactions were terminated with 1 µl of 0.5 M EDTA and 10 µl of the reaction mixture spotted onto a 2cm x 2cm square of Whatman P81 phosphocellulose filter paper. Papers were washed in 0.5% H₃PO₄ three times for five minutes, once in 50% EtOH, 0.5%
H₃PO₄ for five minutes, and dried under a heat lamp. Incorporation of [γ-³²P]ATP into the phosphoacceptor peptide was then quantified by liquid scintillation counting of the paper squares. Under these conditions less than 10% of peptide was phosphorylated upon termination of reaction and velocities were linear with respect to both time and enzyme concentration. Assuming steady state kinetics, initial velocity data and ATP concentrations were fitted to the Michaelis-Menten equation using the Kaleidagraph program and kcat and Km were determined. All experiments were done at least twice in duplicate. Protein quantitation was determined by Bio-Rad protein assay.

Peptide Synthesis and purification — The peptides containing a polyglycine linker of either 2 or 6 residues, PS100, and DTM101 were commercially synthesized by Research Genetics, Inc. All other peptides were synthesized using the Trp-LE expression system. Oligonucleotide cassettes based on CDC6 were subcloned into the vector pMM (a gift from Stephen Blacklow) which expresses the peptide as a fusion protein with a trpLE peptide leader sequence. The peptides were then purified to homogeneity as described in Blacklow et al (16). Peptide purity was assessed by HPLC and identity confirmed by MALDI-TOF mass spectroscopy. The sequences of PS100 (a peptide derived from the cdk inhibitor p21) and DTM101 (a p21-derived peptide with a scrambled Cy motif) are ACRRLFGPVDSE and ACRFGRLPVDSE, respectively. The sequences of the CDC6-derived peptides are shown in Fig. 1.

Results
Purification of Enzymes and Substrates – To determine the contribution of the Cy motif to a cyclin/cdk substrate, we constructed a series of recombinant peptide substrates derived from the replication factor, HsCDC6 (11). These peptides all contain a cyclin/cdk phosphorylation site at the N-terminus and either a wild-type Cy motif (CDC6(wt)), a mutated Cy motif (CDC6(mut)), or a null Cy motif (CDC6(null))(Fig 1). We postulated that these peptides would be ideal substrates for this study considering that (1) the N-terminal SPXK is known to be phosphorylated by cyclin/cdk complexes \textit{in vitro} and (2) the phosphorylation of this site \textit{in vivo} is dependent upon an intact Cy motif. The two sites are in close proximity in the amino acid sequence of HsCDC6 (~ 20 residues) allowing a peptide to easily span this region. After expression of these peptides in \textit{E. coli}, they were purified to homogeneity before their use in the kinetic studies (data not shown). Cyclin A/cdk2 and cyclin E/cdk2 were also purified to homogeneity as determined by SDS-PAGE and Coomassie Blue staining (Fig. 2). The identities of the proteins were confirmed by western blotting with the appropriate antibodies (data not shown). Phosphorylation of cyclin A/cdk2 and cyclin E/cdk2 with bacterially expressed CIV1 resulted in a 2-fold increase in velocity suggesting that the purified cyclin/cdk complexes were not completely phosphorylated on Thr160.

Determination of Kinetic Parameters – Using purified enzyme and the peptide substrates, we developed a highly reproducible kinase assay. Phosphorylation of the peptide substrates by both cyclin A/cdk2 and cyclin E/cdk2 followed hyperbolic kinetics and increased linearly as a function of both enzyme
concentration and time when substrate concentrations were not limiting (data not shown). All further experiments were carried out using conditions within this linear range to ensure the results could be interpreted using Michaelis – Menten based equations.

Initial velocities were determined for both cyclin A/cdk2 and cyclin E/cdk2 complexes using our CDC6-based peptides as substrates. These velocities were plotted against ATP concentrations on a double-reciprocal plot using various fixed concentrations of peptide substrate. A representative plot in which cyclin E/cdk2 was used to phosphorylate the CDC6(wt) peptide is shown in (Fig 3A). In all of these plots, the intersecting pattern of initial velocities is consistent with a sequential kinetic mechanism in which both substrates (ATP and peptide) must be bound before any products are released. From this data, however, we are unable to show whether substrate addition is an ordered or random process. 

$k_{cat}$ and $K_m$ for a given substrate/enzyme pair were determined by secondary plots of the slopes and intercepts of the initial velocity lines versus reciprocal substrate concentration (Fig 3, B and C). A summary of the data for all of the enzymes and substrates can be found in Table 1.

The wild-type substrate was efficiently bound by both cyclin A/cdk2 and cyclin E/cdk2 as demonstrated by $K_m$ values of 1.7 $\mu$M and 7.9 $\mu$M, respectively. Upon mutation of the Cy motif in the N-terminus from RRLVF to RAARA, these values increased 75-fold to 145 $\mu$M for cyclin A/cdk2 and 120-fold to 970 $\mu$M for cyclin E/cdk2. These dramatic increases in $K_m$ demonstrate the importance of the Cy motif in targeting substrates to these enzyme complexes. The $K_m$ values
for CDC6(mut) were 27 µM and 165 µM for cyclin A/CDK2 and cyclin E/CDK2, respectively, a 15-fold and 20-fold increase compared to the wild-type peptide. Thus, this mutation produces a partially functional Cy motif, rather than a completely non-functional motif.

In contrast to the Km values for the peptide substrates, The kcat and the KmATP values for the enzymes remained very similar with less than a 4-fold change between substrates. This would suggest that although the Cy motif plays a critical role in increasing the affinity of cyclin/CDK complexes for a particular substrate, it does not significantly increase the efficiency of phosphoryl transfer from ATP to the peptide.

*Competition with Cy-motif containing Peptides* – To further demonstrate that the Cy motif acts as a docking site for the interaction of substrate with enzyme, we tested the ability of a Cy-motif containing peptide, PS100, to inhibit the phosphorylation of our peptide substrates. If the Cy motif truly directs substrates in this manner, then the PS100 peptide is expected to inhibit the phosphorylation of Cy motif-containing substrates such as our CDC6(wt) and CDC6(mut) peptides but unable to inhibit CDC6(null) which lacks a Cy motif. The data are shown in Figures 4A and 4B. The concentration of the substrate peptides had to be adjusted to obtain equivalent phosphorylation by cyclin/CDK complexes, with more of CDC6(null) being used relative to CDC6(wt) or CDC6(mut). Despite this, a comparison of the ratio of the inhibitor to substrate for any given peptide substrate shows that PS100 selectively inhibits the phosphorylation of only Cy motif containing substrates, CDC6(wt) and CDC6(mut), but not that of
CDC6(null). DTM101, a peptide containing a scrambled Cy motif, does not inhibit the phosphorylation of any of the substrates (data not shown), consistent with our previous results that a negative control inhibitory peptide containing a mutation in the Cy motif does not inhibit the phosphorylation of Rb (4).

Considering the unusual shape for the inhibition curve of cyclin A/cdk2 with the CDC6(wt) peptide and PS100, we carried out a systematic inhibition study to determine the mode of inhibition of PS100 for both cyclin A/cdk2 and cyclin E/cdk2 using the CDC6(wt) peptide as the substrate. Lineweaver-Burke plots for these inhibition experiments are shown in Figure 5A and 5B for cyclin E/cdk2 and cyclin A/cdk2, respectively. Visual inspection of these plots shows that PS100 competitively inhibits the phosphorylation of the CDC6(wt) peptide by both cyclin E/cdk2 and cyclin A/cdk2. From these data, we were also able to determine the inhibition constant (Ki) for PS100 which was $7.5 \pm 0.5 \ \mu M$ for cyclin E/cdk2 and $117.5 \pm 11.6 \ \mu M$ for cyclin A/cdk2.

Effects of Linker Length on Substrate Phosphorylation – Previous studies on the mechanism of action of Cy motifs have been unable to determine whether both the Cy motif and the cdk phosphorylation site must be simultaneously engaged with the cyclin/cdk complex or whether the Cy motif binds first to the cyclin in order to increase the local concentration of the substrate around the enzyme and is then released to allow the cdk phosphorylation site to bind the kinase active site (17). To distinguish between these two possibilities, we reasoned that simultaneous engagement of both binding sites would require the Cy motif and the phosphorylation site to be separated by an amino acid linker of sufficient
length to span the 40A° distance from the binding site on the surface of the cyclin to the catalytic site on the cdk. The bind and release mechanism, on the other hand, would be independent of the length of the amino acid linker. To test this hypothesis, we systematically replaced the wild-type amino acid linker (16 residues) connecting the cdk phosphorylation site and Cy motif of our CDC6 peptide with flexible predominantly polyglycine linkers of 2, 6, 12, or 18 residues. Assuming the flexible linkers would extend on the average 4A°/residue, the distance separating the two sites on these substrate peptides would be 8, 24, 48, and 72 A°, respectively. These substrates were made in the context of both the CDC6(wt) and CDC6(null) peptides and then tested for their ability to be phosphorylated by cyclin A/ck2 and cyclin E/ck2. By comparing the phosphorylation of the wild-type versus the null peptides, we were able to specifically determine the contribution of the Cy motif for a given linker length and thus eliminate any artifacts that may arise from differential binding of the shorter peptides to p81 phosphocellulose. As shown in Fig. 6A and 6B, we found that only substrates containing both an intact Cy motif and either the 12 or the 18 residue linker were effectively phosphorylated. Substrates that either lacked a Cy motif or contained a linker that was unable to span the distance from the cdk binding site to the cyclin binding site were phosphorylated extremely poorly. This length dependence of the linker strongly suggests that both the Cy motif and the cdk phosphorylation site must be simultaneously bound to cyclin/ck2 complex to promote its efficient phosphorylation and eliminates the bind and release model of substrate phosphorylation.
Discussion

We have used a series of peptide substrates derived from HsCDC6 to determine the contribution of a Cy motif to the phosphorylation of a substrate by cyclin/cdk complexes. This detailed kinetic analysis of the phosphorylation of these substrates reveals its importance in substrate recognition by cyclin/cdks and provides additional insight into its mechanism of action.

The CDC6 wild type peptide was efficiently phosphorylated \textit{in vitro} by both cyclin E/cdk2 and cyclin A/cdk2 complexes. The measured Km for the peptide was less than 10 µM for both enzymes suggesting the existence of a high affinity interaction between the enzyme and our substrate. This is in contrast to previously characterized substrates whose Km values were no lower than 200 µM – 100-fold greater than our peptide (18). Since these previously characterized substrates contained only the consensus S/T-P-X-K/R phosphorylation site, this reduction in Km for our peptides can likely be attributed to the presence of a Cy motif. Indeed, the presence of this Cy motif makes the wild-type CDC6 peptide the most efficient peptide substrate of cyclin/cdk complexes characterized to date.

The extremely efficient phosphorylation of our CDC6(wt) peptide is surprising considering a study by Solomon \textit{et al.} which defined the sequence requirements of the consensus cdk phosphorylation site (18). They showed that a SPPK phosphorylation site, like that present in CDC6, is phosphorylated at less than 5% of the level of the SPRK phosphorylation site of their wild-type peptide. This decrease in phosphorylation can be attributed to the enzyme’s inability to
tolerate a proline at the third position of the sequence. Their result is consistent with our data for the CDC6(null) peptide which is poorly phosphorylated by cyclin/cdk complexes. Hence, we conclude that the addition of a Cy motif is sufficient to convert a peptide whose phosphorylation site would normally make it a poor substrate into a very efficient substrate, emphasizing the contribution of the Cy motif to the enzyme-substrate interaction. Therefore, substrate recognition by cyclin/cdk's occurs through a bipartite recognition sequence on the substrate consisting of both the cdk phosphorylation site (S/T-P-X-K/R) and the cyclin binding Cy motif.

We had earlier reported that the Cy motif of p21 inhibited the phosphorylation of pRb but not histone H1 (4). Now we show that a Cy motif containing peptide (PS100) is able to selectively inhibit only Cy motif containing substrates. This is consistent with PS100 competing with substrate for the binding site on the cyclin and confirms our model in which the Cy motif targets substrates to the enzyme via a docking site on the cyclin. If the physiological targets of cyclin-cdk's necessarily use the Cy-cyclin interaction, peptides or chemicals that mimic the Cy motif are likely to be specific inhibitors of cdk's and will differ from existing inhibitors that target the ATP binding site. Indeed, preliminary studies show that such peptides lead to the selective killing of only transformed cells in which the E2F pathway has been deregulated (19).

Not much is known about how the specificity of cyclin /cdk complexes is determined. Our results suggest one mechanism by which this specificity could be achieved. The Km for CDC6(wt) was 1.7 µM for cyclin A/cdk2 and 7.9 µM for
cyclin E/cdk2 suggesting that both enzymes have a high affinity for the Cy motif present in this particular peptide. In contrast, CDC6(mut) had a Km of 27 µM for cyclin A/cdk2 but 163 µM for cyclin E/cdk2. Therefore, cyclin A/cdk2 but not cyclin E/cdk2 could effectively phosphorylate the mutant substrate. Thus, although the wild-type Cy motif interacted strongly with both enzymes, mutations could be made in the Cy motif which confer specificity to cyclin A/cdk2 over cyclin E/cdk2. We also observed that the inhibitory PS100 peptide containing the RRLFG Cy motif was a far better inhibitor of cyclin E/cdk2 (Ki = 7.5 µM) than cyclin A/cdk2 (Ki = 117.5 µM). Based on these results, it seems likely that different Cy motifs will preferentially associate with a specific cyclin/cdk complex and thereby target that substrate for phosphorylation by only that enzyme. By studying the effects of linker length on substrate phosphorylation, we have shown that both the Cy motif and the cdk phosphorylation site must be simultaneously bound to the cyclin/cdk complex. Previous work suggests that the purpose of the Cy motif was to increase the local concentration of the substrate around the enzyme (20). Our results suggest that in addition to this role, the Cy motif may also be responsible for orientating specific cdk phosphorylation sites with respect to the active site of cdk2 to further facilitate their phosphorylation – a mechanism that requires the concurrent binding of the Cy motif and cdk phosphorylation site to the enzyme as seen with the CDC6-derived substrates. For example, binding of the Cy motif of a substrate to the cyclin might conformationally restrain the substrate such that only particular cdk phosphorylation sites are accessible to the cdk. In this way, the Cy motif would
not only increase the overall affinity of the cyclin/cdk for the substrate, it would also specify which phosphorylation sites would be targeted by the kinase.

Acknowledgements

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References

14. Delmolino, L., and Dutta, A.
Figure Legends

Fig. 1. Schematic of Substrate Peptides Derived from HsCDC6. Peptides were constructed that spanned the consensus cdk phosphorylation site from residues 74-77 and the Cy motif from residues 94-98. The N-terminal cdk phosphorylation site and the C-terminal Cy motif are highlighted in bold.

Fig. 2. SDS-PAGE of cyclin E/cdk2 and cyclin A/cdk2. Purified Cyclin E/Cdk2 (lane 1) and Cyclin A/Cdk2 (lane 2) were loaded on a 12% gel and the proteins were stained with Coomassie Blue.

Fig. 3. Representative Initial Velocity Patterns and Secondary Plots. (A) Initial velocity pattern was determined for cyclin E/cdk2 using ATP as the varied substrate and the following fixed concentrations of CDC6 wild-type peptide: 2.5 µM (♦), 5 µM (■), 10 µM (▲), 20 µM (χ), and 40 µM (●). (B) Secondary plot of primary slopes vs. reciprocal peptide concentration. (C) Secondary plot of primary intercepts vs. reciprocal peptide concentration.

Fig. 4. Cy-motif containing peptide selectively inhibits the phosphorylation of only Cy-motif containing substrates by (A) cyclin E/cdk2 and (B) cyclin A/cdk2. The Cy Motif Containing peptide PS100 is able to inhibit the phosphorylation of 5 µM CDC6(wt) (♦), 50 µM CDC6(mut) (○) but not 1 mM of CDC6(null) (♦) substrate.

Fig. 5. PS100 competitively inhibits the phosphorylation of the CDC6(wt) substrate by (A) cyclin E/cdk2 and (B) cyclin A/cdk2. Initial velocities were determined in the presence of different fixed concentrations of the PS100 peptide: 0 µM (♦), 6.25 µM (■), 12.5 µM (▲), and 25 µM (●) for cyclin E/cdk2 and 0 µM (♦), 100 µM (■), 200 µM (▲) and 500 µM (●) for cyclin A/cdk2.

Fig. 6. Phosphorylation of peptide substrates by (A) cyclin E/cdk2 and (B) cyclin A/cdk2 is dependent on the length of the linker connecting the N-terminal cdk phosphorylation site and the C-terminal Cy motif. For each given linker length, the velocities were determined for both the wild-type Cy motif (■) and the null Cy motif (♦).
Figure 1

Full-length CDC6

$H_2N$ ———————————— Cy ———————————— COOH

CDC6 (wt)  $H_2N$ — LPPCSPPKQGKKENGPPHSLTLKG RRLVFDNQL — COOH
CDC6 (mut) $H_2N$ — LPPCSPPKQGKKENGPPHSLTLKG RRAAFDNQL — COOH
CDC6 (null) $H_2N$ — LPPCSPPKQGKKENGPPHSLTLKG RAARA DNQL — COOH
CDC6 (wt-linker) $H_2N$ — LPPCSPPK — (X)$_n$ — RRLVFDNQL — COOH
CDC6 (null-linker) $H_2N$ — LPPCSPPK — (X)$_n$ — RAARA DNQL — COOH
Figure 2

66 kDa

45 kDa

31 kDa

Cyclin A

Cyclin E

Cdk2

Dye Front

1 2
Figure 3

A

B

C
Figure 4

Cyclin E/cdk2

% Activity

PS100 (μM)

Cyclin A/cdk2

% Activity

PS100 (μM)
Figure 5

A

$K_i = 7.5 \pm 0.5 \ \mu\text{M}$

B

$K_i = 117.5 \pm 11.6 \ \mu\text{M}$
Figure 6

Cyclin E/cdk2

Cyclin A/cdk2

v (pmol/min)

0 0.5 1 1.5

CDC6 2 6 12 18

Linker Length

Linker Length
Table 1. Kinetic Parameters for cyclin/cdk complexes and CDC6-derived peptides. Units for $K_m$^{ATP} and $K_m$^{peptide} are expressed in mM while units for $k_{cat}$ are in min$^{-1}$.

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<th>CDC6 (wt)</th>
<th>CDC6 (mut)</th>
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<tr>
<td>CyclinE/cdk2</td>
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<tr>
<td>$k_{cat}$</td>
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<td>$K_m$^{peptide}</td>
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