The Role of the Phospho-CDK2/Cyclin A Recruitment Site in Substrate Recognition*  

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Kin-Yip Cheng‡, Martin E. M. Noble‡, Vicky Skamnaki‡, Nick R. Brown†, Ed D. Lowe‡, Luke Kontogiannis‡, Kui Shen†, Philip A. Cole‡, Giuliano Siligardi‡, and Louise N. Johnson††  

From the ‡Laboratory of Molecular Biophysics, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, United Kingdom, the ‡Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and the ‡Diamond Light Source, Chilton, Oxon OX11 0QX, United Kingdom  

Phospho-CDK2/cyclin A, a kinase that is active in cell cycle S phase, contains an RXL substrate recognition site that is over 40 Å from the catalytic site. The role of this recruitment site, which enhances substrate affinity and catalytic efficiency, has been investigated using peptides derived from the natural substrates, namely CDC6 and p107, and a bispeptide inhibitor in which the ϕ-phosphate of ATP is covalently attached by a linker to the CDC6 substrate peptide. X-ray studies with a 30-residue CDC6 peptide in complex with pCDK2/cyclin A showed binding of a dodecamer peptide at the recruitment site and a heptapeptide at the catalytic site, but no density for the linking 11 residues. Kinetic studies established that the CDC6 peptide had an 18-fold lower $K_m$ compared with heptapeptide substrate and that this effect required the recruitment peptide to be covalently linked to the substrate peptide. X-ray studies with the CDC6 bispeptide showed binding of the dodecamer at the recruitment site and the modified ATP in two alternative conformations at the catalytic site. The CDC6 bispeptide was a potent inhibitor competitive with both ATP and peptide substrate of pCDK2/cyclin A activity against a heptapeptide substrate ($K_i = 0.83$ nM) but less effective against RXL-containing substrates. We discuss how localization at the recruitment site ($K_D = 0.4$ μM) leads to increased catalytic efficiency and the design of a potent inhibitor. The notion of a flexible linker between the sites, which must have more than a minimal number of residues, provides an explanation for recognition and discrimination against different substrates.

Protein kinases catalyze the phosphorylation of serine, threonine, or tyrosine residues in target proteins. They provide the signaling pathways by which extracellular signals (hormone, growth factor, etc.) are converted to intracellular responses through changes in metabolism or gene expression. There are over 500 protein kinases in the human genome (1). Cross-talk between differently activated protein kinase signaling pathways is regulated by a variety of mechanisms that target the activity of an individual protein kinase to a select group of substrates. Most protein kinases exhibit specificity for a defined epitope around the site of phosphorylation, as first elaborated for cAMP-dependent kinase (2). Both the mitogen-activated protein kinase (MAPK) and the cyclin-dependent kinase (CDK) families of protein kinases phosphorylate a serine or threonine residue as part of an (S/T)P motif that in CDK2 substrates is enhanced by a preference for basic residue in the P + 3 position (i.e. (S/T)P(X/K/R)). The proline residues act as a powerful discriminator for other kinases (3) but may be insufficient to prevent cross-talk between CDK and MAPK pathways. Further mechanisms are utilized to enhance kinase specificity.

CDK2 in complex with cyclin A is active during S phase of the cell cycle. Its activity is controlled in a temporal fashion through regulated transcription (and subsequent degradation) of the activatory cyclin subunits and the Kin/Cip p21 family of inhibitory proteins (4). Activation of CDK2/cyclin A requires phosphorylation on a threonine residue (Thr$^{160}$ in the human CDK2 sequence) in the activation segment of the kinase by the cyclin-dependent kinase activating kinase CAK1. Levels of cyclin A remain high throughout S and G$_2$ until cells enter mitosis where activation of the anaphase-promoting complex results in ubiquitination and destruction of cyclin A. At G$_2$/M, levels of cyclin B rise, and CDK1/cyclin B initiates mitosis. The activities of the CDKs are governed by the cyclins relative to the phases of the cell cycle.

The cyclins not only serve as activatory subunits but may also function in substrate recognition through direct interaction with substrates. Recruitment sites for phospho-CDK2/cyclin A (pCDK2/cyclin A) substrates that contain an RXL motif (where $X$ is any amino acid) were first observed with the substrates p107 and p130 (5–8) and the CDK2 inhibitors p21 and p27 (9, 10). Many other CDK2 substrates that are important for cell cycle progression contain the recruitment motif RXL or KXL at a site that is remote from the site of phosphorylation (e.g. pRb, p53).

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† To whom correspondence should be addressed: Laboratory of Molecular Biophysics, Dept. of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK. Tel.: 44-1865-275365; Fax: 44-1865-285353; E-mail: louise.johnson@biop.ox.ac.uk.

‡ The atomic coordinates and structure factors (code 2CC1 (CDC6 peptide complex) and 2CCH (CDC6 bispeptide complex)) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

§ The abbreviations used are: CDK, cyclin-dependent kinase; ATP, adenosine 5′-O-3-thiotriphosphate; GST, glutathione S-transferase; AMPPNP, adenosine 5′-(β,γ-imino)triphosphate; MES, 4-morpholineethanesulfonic acid; PEG, polyethylene glycol; PK, pyruvate kinase; PEP, phosphoenolpyruvate; LDH, lactate dehydrogenase; RMSD, root mean square deviation.

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p53, E2F1, human papilloma virus (HPV) replication factor E1, CDC6, endomexin, Myt1, CDC25A (11), the APC substrate-activating subunit Cdh1 (12), p21<sup>WAF1</sup> (13), and BRCA2 (14). Structural studies of pCDK2/cyclin A in complex with the p<sup>27<sub>Ki</sub></sup>-<sup>1</sup> inhibitor (15) and with peptides from p107, pRb, E2F, p53 (16) showed that the RXL recognition site is located on the cyclin A molecule at an exposed non-polar site containing the characteristic MRAIL cyclin sequence that is conserved in cyclins A and E (17). The recruitment site is some 40 Å from the catalytic site (as measured from the Cα of the serine at the substrate phosphorylation site and the Ca of the R of the RXL motif) (18). Mutation of the hydrophobic patch on cyclin A eliminates phosphorylation of substrates that require an RXL motif but not those CDK2 substrates, such as histone H1, that do not contain an RXL motif (19). Fusion of an RXL motif to deletion mutants can restore activity (20).

Recognition of the RXL motif by pCDK2/cyclin A or pCDK2/cyclin E may be sufficiently tight so that stable complexes can be observed as with p107, E2F, and the inhibitor p<sup>27<sub>Ki</sub></sup>-<sup>1</sup>. In other instances there is no stable association of the substrate with CDK2 but the integrity of the RXL (or KXL) motif has been shown to be essential for phosphorylation of target residues such as in the substrates pRb and p53 (19, 21, 22). Paradoxically stronger physical association between the CDK/cyclin and the substrate does not necessarily lead to a more efficient phosphorylation than weaker interaction, as shown with studies E2F/DP1 phosphorylation (23). Mutagenesis experiments with p107 and kinetic experiments with model peptides have shown that one of the roles of the RXL motif is to make a poor substrate with suboptimal consensus phosphorylation motif an effective pCDK2/cyclin A substrate (24, 25). RXL-containing peptides are effective inhibitors of the activity of pCDK2/cyclin A against RXL-containing substrates and have potential therapeutic applications (26–28).

The structural mechanism by which the RXL recognition site on cyclin A promotes substrate phosphorylation by pCDK2 is not understood. There are no conformational changes when the RXL site is occupied (16, 18). The most likely mechanism is that the RXL motif serves as an entropic effector, localizing the CDK close to the substrate leading to an increase in the local substrate concentration (19) but mechanisms that envisage a more direct participation between the RXL and catalytic site are also possible (29). To explore the relationship between the recruitment site and catalytic site, we have carried out structural and kinetic studies with a substrate peptide that contains the recruitment motif. We based our peptide on the naturally occurring CDC6 protein that contains a phosphorylation site twenty amino acids N-terminal to the RXL site. To make direct participation between the catalytic site and the RXL motif significantly increases affinity for substrates and inhibitors, respectively. Kinetic studies show that there is an obligation for a covalent path between the catalytic site and the RXL motif, but no direct stereochemical path is observed in the crystal studies. A model is proposed based on localization effects and a flexible linker, which must be greater than a specified minimal length (about 15–16 residues), to explain the nanomolar potency of the CDC6 bispeptide inhibitor.

**EXPERIMENTAL PROCEDURES**

**Peptides**—The heptapeptide substrate, the modified CDC6 peptide, and the recruitment HTL peptide HTLGRRRLVFDN, were synthesized by Dr. G. Bloomberg (University of Bristol Peptide Synthesis Laboratory) and purified by HPLC. The mass of the CDC6 peptide was confirmed by mass spectrometry and concentration determined by amino acid analysis. The p107 peptide (Fig. 1) was expressed as a GST fusion protein with the vector pGEX-6P from B834 DE3 pl<sub>y</sub>S cells at 18 °C overnight, purified on a glutathione-Sepharose fast flow 4B affinity chromatography column (Amersham Biosciences), and the GST tag cleaved by 3C protease. The cleaved peptide was further purified by reverse phase chromatography on a HPLC column (Jupiter 10–C5 by Phenomenex) followed by lyophilization. The lyophilized peptide was dissolved in aqueous solution, and its molecular mass confirmed by MALDI mass spectrometry. Its concentration was determined by amino acid analysis.

**Synthesis of ATP-conjugated Peptides I and II**—Peptides were synthesized following the same strategy as described previously (33). The amino-alanine-containing peptides were assembled on Wang resin using automated solid-phase peptide synthesis via the Fmoc strategy. The allyloxy carbonyl (alloc) protection of the side chain of amino-alanine was removed by a 2-h treatment under nitrogen with 5 eq of Pd(PPh<sub>3</sub>)<sub>4</sub>, 10 eq of N-methylmorpholine, and 20 eq of acetic acid in chloroform. The side chain of amino-alanine was then bromoacetylated in dimethylformamide for 30 min using symmetric anhydride formed by a 10-min mixing of 10 eq of di-isopropylcarbodiimide and 5 eq of bromoacetic acid in methylene chloride. Trifluoroacetic acid cleavage (with 5% phenol, 5% THF, 5% water, 2.5% ethanedithiol, and 1% triisopropylsilane as scavengers), reverse phase HPLC, and lyophilization gave the bromoacetylated peptides confirmed by MALDI-TOF MS (the long peptide I, calculated [M] 3588, found [M+H]<sup>+</sup> 3588 ± 1; the short peptide II, calculated [M]+993, found [M+H]<sup>+</sup> 993 ± 1). Treatment of the bromoacetylated peptides with ATPγS (1.5 eq; Roche Applied
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The chemical structures of the substrate and inhibitor peptides.

FIGURE 1. The chemical structures of the substrate and inhibitor peptides.

Science) in 0.1 M ammonium acetate buffer (pH 7.0) (~10 mg peptide in 1 ml of solution) overnight at room temperature followed by gel filtration using Bio-Gel P-2 Gel (~15 mg purified over ~50 ml resin) in 0.1 M ammonium acetate buffer (pH 7.0) and lyophilization gave the desired ATP-conjugated peptides confirmed by ESI MS (the long peptide I, calculated [M+H]+ 4034, found [M+H]+ 4034, [M+2H]+ 2018, [M+3H]+ 1346, [M+4H]+ 1009, [M+5H]+ 808, [M+6H]+ 674, and [M+7H]+ 501, 0.2 M ammonium sulfate, 0.1 M citrate/acetate buffer pH 5.6 at 4 °C. Crystals were grown by the sitting drop vapor diffusion method in which 1 ml of reservoir solution contained 10–17% (v/v) PEG monomethylether 5000, 0.2 M ammonium sulfate, and 0.1 M MES pH 7.0 and left on ice for 30 min. Crystals were grown by the sitting drop vapor diffusion method in which 1 ml of reservoir solution contained 10–17% (v/v) PEG monomethylether 5000, 0.2 M ammonium sulfate, and 0.1 M MES pH 7.0 and left on ice for 30 min.

Catalytic site and recruitment site. The model was refined with cycles of restrained refinement and manual rebuilding using O (37). This refinement process was intercalated with 4 rounds of simulated annealing and energy optimization (CNS) (38) using an initial temperature of 2500 K with subsequent cooling step of 25 K per cycle of dynamics.

Crystallization and Crystal Structure of the pCDK2/Cyclin A-CDC6 Bispeptide Complex—The complex was crystallized using the sitting drop vapor diffusion method in which 1 μl of pCDK2/cyclin A (18 mg/ml; 0.28 mM) containing 0.29 mM CDC6 bispeptide was mixed 1 μl of reservoir solution containing 13% PEG monomethyl ether 5000, 0.2 M ammonium sulfate, 0.1 M citrate/acetate buffer pH 5.6 at 4 °C. Three weeks later a crystal was taken and back-soaked in a ammonium sulfate-free solution containing 13% PEG monomethyl ether 5000, 0.1 M citrate/acetate buffer pH 5.6, 15% glycerol, 5 mM MgCl2, 0.58 mM bispeptide overnight before freezing in liquid nitrogen and data collection (Table 1). In a second experiment, magnesium was omitted from the final soak so as to minimize any possible ATPase activity. The pCDK2/cyclin A (18 mg/ml) was co-crystallized with 0.29 mM bispeptide at 4 °C with 14% PEG monomethyl ether 5000, 0.2 M ammonium sulfate, 0.1 M citrate/acetate buffer pH 5.6. Ten weeks later, a crystal was taken and back-soaked in 14% PEG monomethyl ether 5000, 0.1 M citrate/acetate buffer 15% glycerol, 0.58 mM bispeptide for 4–5 min before freezing with liquid nitrogen and data collection (Table 1).

Previously we had observed peptide binding at the CDK2 catalytic site by transferring the crystals, which had been grown with lithium sulfate as the precipitant, to a non-polar solvent in order to strengthen polar interactions (18). We reasoned that the ammonium sulfate in the present crystallization condition, albeit at a concentration of only 0.2 M, could have affected binding of the CDC6 peptide to the catalytic site. Crystals of the ternary complex were grown as described above and back-soaked into a solution that was the same as the original conditions but without ammonium sulfate and which contained 10 map showed the presence of the CDC6 peptide at both the catalytic site and recruitment site. The model was refined with cycles of restrained refinement and manual rebuilding using O (37). This refinement process was intercalated with 4 rounds of simulated annealing and energy optimization (CNS) (38) using an initial temperature of 2500 K with subsequent cooling step of 25 K per cycle of dynamics.

Crystallization and Crystal Structure of the pCDK2/Cyclin A-CDC6 Bispeptide Complex—The complex was crystallized using the sitting drop vapor diffusion method in which 1 μl of pCDK2/cyclin A (18 mg/ml; 0.28 mM) containing 0.29 mM CDC6 bispeptide was mixed 1 μl of reservoir solution containing 13% PEG monomethyl ether 5000, 0.2 M ammonium sulfate, 0.1 M citrate/acetate buffer pH 5.6 at 4 °C. Three weeks later a crystal was taken and back-soaked in a ammonium sulfate-free solution containing 13% PEG monomethyl ether 5000, 0.1 M citrate/acetate buffer pH 5.6, 15% glycerol, 5 mM MgCl2, 0.58 mM bispeptide overnight before freezing in liquid nitrogen and data collection (Table 1). In a second experiment, magnesium was omitted from the final soak so as to minimize any possible ATPase activity. The pCDK2/cyclin A (18 mg/ml) was co-crystallized with 0.29 mM bispeptide at 4 °C with 14% PEG monomethyl ether 5000, 0.2 M ammonium sulfate, 0.1 M citrate/acetate buffer pH 5.6. Ten weeks later, a crystal was taken and back-soaked in 14% PEG monomethyl ether 5000, 0.1 M citrate/acetate buffer 15% glycerol, 0.58 mM bispeptide for 4–5 min before freezing with liquid nitrogen and data collection (Table 1).

Both experiments showed binding at the recruitment site for a dodecamer peptide and weak binding at the catalytic site in which only the modified ATP was clearly located. Two conformations were visible for the ribose and triphosphate in both CDK2 molecules and also two conformations for residues Asp145 and Leu148. The structure for the 1.7-Å data (Table 1) for the pCDK2/cyclin A-bispeptide complex, including the recruitment peptide, ATP, and waters assigned by ARP/wARP, was further refined using TLS (a procedure that allows correlation of internal torsional motion with overall molecular motion in crystals) and isotropic B factors, followed by TLS with anisotro-
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pCDK2 factors with strong restraints toward isotropic values, and finally with TLS and anisotropic B values and hydrogen atoms as determined by REFMAC. At each stage the $R_{free}$ value was carefully monitored. The refinement statistics (Table 1) support the incorporation of anisotropic B values and hydrogen atoms.

Kinetics—Pyruvate kinase (PK), lactate dehydrogenase (LDH), phosphoenolpyruvate (PEP), ATP, NADH, and other chemicals were obtained from Sigma. The pCDK2/cyclin A concentration was determined according to Bradford (39).

Phosphorylation of the peptides was measured by a spectrophotometric assay in which ADP production was coupled to the NADH oxidation by PK and LDH as previously described (40, 41) with minor modifications. The molar extinction coefficient for NADH was assumed to be 6220 M$^{-1}$ cm$^{-1}$ at 340 nm. All reactions were performed at 30°C.

The assay mixture (volume 0.25 ml) for measurement of pCDK2/cyclin A activities with the heptapeptide contained 30 units/ml LDH, 12 units/ml PK, 1 mM PEP, 0.128 mM NADH, 0.5 mg/ml bovine serum albumin, 50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM KCl, 10 mM MgCl$_2$, 2 mM dithiothreitol with the heptapeptide substrate concentration between 0.106–2.13 mM. After incubation for 1–2 min, the reaction was initiated by the simultaneous addition of 0.74 mM ATP and pCDK2/cyclin A (0.48 μg/ml). Aliquots (0.08 ml) were withdrawn at 3, 6, and 9 min and transferred into 0.08 ml of sodium dodecyl sulfate (0.2%). Assays with the heptapeptide substrate were also performed in the presence of 0.75 mM of the recruitment HTL peptide. Control reactions in the absence of peptide substrate were used to monitor any ATPase activity. Reaction rates were found to be linear with enzyme concentration. Measurements with the CDC6 peptide were made using similar conditions. CDC6 peptide concentrations were varied from 0.032–0.48 mM, and reactions were stopped at 13 min with 20 mM triethanolamine pH 8.0, 200 mM EDTA to avoid precipitation of the longer peptides that occurred with SDS. Kinetic data were analyzed with the nonlinear regression program GraFit (42).

The assay mixture for inhibition measurements with the CDC6 bispeptide against the heptapeptide and the p107 peptide substrates was as above except the reaction volumes were 0.08 ml and NADH 0.14 mM. Concentrations of the CDC6 bispeptide inhibitor were 58 nm and 116 nm in experiments with the heptapeptide substrate (0.4–2.2 mM) and 5.3 μM and 10.6 μM in experiments with the p107 peptide substrate (15.7–157 μM). After 1–2 min of incubation, the reactions were initiated by the simultaneous addition of 0.75 mM ATP and pCDK2/cyclin A (1 μg/ml). After 7 min, the reactions were terminated by the addition of 0.08 ml 0.2% SDS solution. The inhibition of pCDK2/cyclin A by the CDC6 bispeptide with respect to ATP was assayed with pCDK2/cyclin A (1 μg/ml), and the ATP concentration varied from 0.016–0.45 mM at 0.166 mM of p107 peptide in the presence of 0.42 μM and 4.2 μM of the CDC6 bispeptide inhibitor. The assay conditions were as above except the reaction volume was 0.1 ml, and the reaction was stopped after 12 min in 0.1% SDS.

The bisheptapeptide, which lacks the RXL motif, was assayed with respect to the heptapeptide under similar conditions to those described above. No inhibition was detected up to concentrations of bisheptapeptide 0.46 mM assayed in the presence of 0.75 mM ATP and 0.135–1.69 mM heptapeptide in a reaction volume of 0.12 ml and reaction time 6 min.

CD Spectroscopy—CD spectra were recorded on a nitrogen-flushed JASCO 1720 spectropolarimeter with multiscanning. Nine scans were used with a scan speed of 20 nm/min in the wavelength range 250–360 nm for the CD titrations of the pre-incubated complex pCDK2/cyclin A with HTL recruitment peptide, p107 peptide, and CDC6 bispeptide, respectively, and for pCDK2 with CDC6 bispeptide. The concentrations of pCDK2/cyclin A and pCDK2 were 6.5 μM, as determined spectrophotometrically using molar extinction coefficients $ε_{280} = 72,115$ M$^{-1}$ cm$^{-1}$ and 37,025 M$^{-1}$ cm$^{-1}$, respectively, as calculated by ProtParam (43). The concentrations of HTL and p107 peptides were determined by weighing taking into account the association of trifluoroacetic acid, while that of CDC6 bispeptide spectrophotically ($ε_{259\,nm} = 15400\,M^{-1}\,cm^{-1}$). All CD spectra were reported in ΔA = (A$_H$ – A$_L$). The spectropolarimeter was calibrated with ammonium 1-camphor-10-sulfonate.

The CD titrations were conducted in a stepwise manner adding small aliquots of 4 μl and 8 μl of ligand stock solution with a Finnipette P.C.R. (volume range of 2–20 μl) directly into the cuvette of 1-cm pathlength containing an initial volume of 520 μl of pCDK2/cyclin A in 1 mM HEPES, 10 mM NaCl, 0.01% (v/v) MTG, 0.3 mM EDTA pH 7.0. Each titration was terminated upon ligand saturation, which occurred before the final volume was greater than 12% of the initial volume, and each CD spectrum was subsequently corrected for dilution.

For a single binding site at equilibrium, the CD, expressed in ΔA, at any wavelength is proportional to the concentration of the bound and unbound species of the host (H) and ligand (L) components. Based on the Beer’s Law (ΔA = εcl) and for a titration carried out in 1-cm path length cell (l = 1) with the concentration at equilibrium of the host [H], ligand [L], and host-ligand complex [HL], ΔA$_{HL}$ = Δε$_{HL}$[HL], ΔA$_H$ = Δε$_H$[H], and ΔA$_L$ = Δε$_L$[L]. The dissociation constant $K_d$ = 1/K was determined with the Origin program by analyzing the differential CD data at single wavelength using a nonlinear regression analysis of Equation 1 below as described in Ref. 44.

$$ΔA = (Δε_{HL} - Δε_H)(((K[H] + K[L]) + 1) - ((K[H] + K[L]) + 1)^2 - 4KK[H][L])/2K + Δε_0[H]$$

(Eq. 1)

where [H], [L], and [HL] are the total concentrations of H, L, and HL, respectively. The differential CD data were calculated by subtracting from each observed spectrum of the host-ligand mixture the equivalent CD contribution of the ligand.
Catalytic site but no peptide binding at the catalytic site. After a crystal back-soak experiment in which the concentration of CDC6 peptide was increased to 0.5 mM, and ammonium sulfate was excluded from the mother liquor, the crystallographic data, gave a structure at 2.7-Å resolution (Table 1) in which a heptapeptide (residues 78QGKKENGPPHS88) was not visible in the electron density map. A 10-Å c axis shrinkage on back-soaking was accompanied by a rotation and translation of the molecules in the unit cell with no significant conformational changes. None of the changes in lattice contacts are close to the catalytic site. Binding at the catalytic site in the back-soaked crystals appears to be due to the higher concentration of CDC6 peptide and the exclusion of ammonium sulfate.

At the pCDK2 catalytic site, the seven N-terminal residues (the phosphorylatable Ser defined as the P + 0 site) bind in an extended conformation similar to the structure of pCDK2/cyclin A-heptapeptide substrate complex (18) and make similar contacts to the enzyme (Fig. 2a). The peptide was less well ordered than the protein as indicated by the high B factors. The discussion refers to the A subunit, which is the better ordered pCDK2 subunit. The side chain of Ser74 (P + 0) is hydrogen-bonded (2.8 Å) to the NZ of the Lys129 side chain and to number of van der Waals contacts. Arg76 (P + 0) is hydrogen-bonded to the NZ of the Lys129 side chain and to the phospho-Thr160 O3 oxygens (2.3 Å). N-terminal to the phosphorylatable residues including Leu148 (8 contacts), Glu162 (9), Val163 (8), Val164 (7), Thr165 (4), and Arg169 (4). (Numbers in parentheses refer to the A subunit, which is the better ordered pCDK2 subunit. The side chain of Ser74 (P + 0) is hydrogen-bonded (2.8 Å) to the NZ of the Lys129 side chain and to the OD2 of Asp157 (2.8 Å), the two key catalytic residues (45).

It is directed toward the γ-phosphate of AMP-PNP (separation Ser74 OG to the γ-phosphorus 5.2 Å) in a manner consistent for phosphor group transfer during kinase catalysis. Pro75 at the P + 1 position docks into the pocket formed by the left-handed conformation Val164 of the activation segment (Fig. 2a) and makes a large number of van der Waals contacts to CDK2 residues including Leu148 (8 contacts), Glu162 (9), Val163 (8), Val164 (7), Thr165 (4), and Arg169 (4). (Numbers in parentheses refer to the A subunit, which is the better ordered pCDK2 subunit. The side chain of Ser74 (P + 0) is hydrogen-bonded (2.8 Å) to the NZ of the Lys129 side chain and to the OD2 of Asp157 (2.8 Å), the two key catalytic residues (45).

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for other shorter recruitment peptides (16) in the vicinity of the RXL motif but the longer peptide makes more extensive interactions than previously observed. These are described in more detail below in the description of the pCDK2/cyclin A-CDC6 bispeptide complex where the data are at higher resolution.

The Structure of the pCDK2/cyclin A-CDC6 Bispeptide Complex—Co-crystallization of the pCDK2/cyclin A complex in the presence of 0.290 mM CDC6 bispeptide showed binding at the recruitment site, adenine binding at the ATP site but no peptide binding at the catalytic site. Two crystal back-soak experiments, one with 5 mM Mg$^{2+}$ and the other in the absence of Mg$^{2+}$, and both with 0.58 mM CDC6 bispeptide and exclusion of ammonium sulfate, gave similar results. There was additional density at the ATP site for the ribose and triphosphate but no clear density for the peptide at the catalytic site or the linker. There were no changes in unit cell dimensions after the back-soak experiments, in contrast to the data for the pCDK2/cyclin A-CDC6 peptide complex.

The 1.7-Å structure of pCDC2/cyclin A in complex with the CDC6 bispeptide (without Mg$^{2+}$) is the highest resolution structure for a pCDC2/cyclin A complex, and we use this structure for the following descriptions (Table 1).

The Catalytic Site—At the catalytic site, the adenine moiety of ATP was bound in a single defined conformation but two alternative conformations for the ribose and triphosphate were apparent. The two ATP molecules were refined with 50% occupancy. One of the ATP conformations was similar to the conformation observed in other pCDK2/cyclin A ternary complexes (18) and in the CDC6 peptide complex, while the other conformation had a change in the C5’-O5’ torsion angle so that the γ-phosphate was directed toward the metal binding aspartate Asp$^{145}$ (Fig. 2b). The alternative conformation is visible at a contour level of 0.2 $e/Å^3$ whereas the standard conformation is only visible at 0.13 $e/Å^3$. In both subunits there is an indication of the sulfur atom of the thio linker but no connecting density for the linker or the peptide. There is a disconnected peak corresponding to the position of the proline residue in peptides bound at the catalytic site. The side chains of the two residues, Asp$^{145}$ and Leu$^{148}$, adopt dual conformations that are correlated with the two conformations of the ATP. The different conforma-

**FIGURE 2. Stereo diagram illustrating the CDC6 peptide bound at the catalytic site of pCDK2 in the pCDK2/cyclin A-CDC6 peptide complex and omit electron density maps demonstrating the dual conformations of the ribose-triphosphate part of ATP in the pCDK2/cyclin A-CDC6 bispeptide complex. a, CDC6 heptapeptide bound at the catalytic site. The electron density within 2.5 Å of the bound peptide is shown. CDK2 Ca atoms for residues in contact with the peptide are shown in yellow and CDC6 peptide Ca atoms in green. AMPPNP with Mg$^{2+}$ in cyan are also shown. b, CDC6 bispeptide ATP moiety. At the end of the refinement, two REFMAC runs were carried out using models from which either the A (alternate) conformation (left panel) or B (standard) conformation (right panel) of ATP and Asp$^{145}$ were omitted. Difference maps based on $mF_o - DF_c$ coefficients were calculated and contoured at 0.1 $e/Å^3$. The electron density indicates the existence of two conformations, in which the A conformation has a slightly higher occupancy. c, two conformations of the modified ATP and CDK2 contact residues superimposed.**
tions of the triphosphate are not because of the lack of Mg\(^{2+}\) because similar conformations were observed for the 2.4-Å resolution complex in the presence of Mg\(^{2+}\). In both experiments, with and without Mg\(^{2+}\) added, there was a small electron density peak intermediate between the Mg\(^{2+}\) position and a water molecule observed in the substrate complexes. This could be a metal scavenged from solutions in the experiment where no Mg\(^{2+}\) was deliberately added or a water molecule.

We considered if the attempt to accommodate the thio linker to the peptide might have led to a distortion of the triphosphate component of the modified ATP. In its extended conformation the linker distance from the amino alanine to the Py-P₂-phosphorous is 5.7 Å (32). In the pCDK2/cyclin A-CDC6 peptide or heptapeptide complexes the corresponding distance (Ser OG to Py) is 5.2 Å. Small changes in torsion angles of the linker to a more folded state can readily allow a distance of 5.2 Å. Indeed in the IRK/bispeptide complex, the distance is 5.0 Å (32, 46).

After equilibration under conditions similar to those employed in the crystallization experiments (pH 5.6), the CDC6 bispeptide molecular mass was checked with mass spectrometry and found to be 4034.44 ± 0.38 Da, confirming that the peptide was unaltered. We also checked if there had been any catalysis of the CDC6 bispeptide by measuring the liberation of ADP on incubating the CDC6 bispeptide with pCDK2/cyclin A. There was no detectable release of ADP after 4 days at pH 7.4. Thus there was no detectable degradation of the CDC6 bispeptide at pH 5.6 and no any catalytic conversion by pCDK2/cyclin A at pH 7.4. We conclude that the lack of peptide at the catalytic site is due to the mobility in the ribose and triphosphate moieties that result in a variety of conformations for the linker and peptide components in the vicinity.

**The RXL site**—At the RXL recruitment site on the surface of cyclin A, the CDC6 bispeptide residues 89–100 (sites P - 7 to P + 4) are bound in an identical manner to those observed with the CDC6 peptide. The details of the intermolecular contacts and the complementary surface charge of cyclin A are shown in Fig. 3, a and b. The leucine of the RXL, Leu\(^{96}\) defined as the P + 0 site, docks into the hydrophobic pocket created by residues Ile\(^{213}\), Leu\(^{214}\), Trp\(^{217}\) (from the cyclin A α-helix), and Gln\(^{234}\) (from the cyclin α3-helix). Its main chain nitrogen makes a hydrogen bond to the side chain of Gln\(^{234}\). The next hydrophobic residue, Val\(^{97}\) (P + 1), bulges out into the solvent, whereas the following residue Phe\(^{98}\) (P + 2) docks into the hydrophobic pocket contacting Met\(^{210}\), Ile\(^{213}\), Arg\(^{250}\), and Leu\(^{253}\), as in the structures previously observed for the binding of the E2F, pRb, and p53 peptides (16). The CDC6 bispeptide residues Leu\(^{96}\) and Phe\(^{98}\) together make 44 non-polar van der Waals contacts with cyclin A residues in the non-polar pocket. The side chain of Arg\(^{250}\) of cyclin A shifts about 4.9 Å to accommodate the carbonyl oxygen of Phe\(^{98}\) (P + 2) and its side chain hydrogen bonds to Phe\(^{98}\) main chain oxygen. The C-terminal residues (Asp\(^{99}\) (P + 3) and Asn\(^{100}\) (P + 4)) make few contacts. Asp\(^{99}\) points into solution while Asn\(^{100}\) forms a few van der Waals contacts with residues Met\(^{210}\) and Arg\(^{250}\) on cyclin A.

Most of the residues N-terminal to the Leu\(^{96}\) (P + 0) make extensive contacts. Arg\(^{95}\) (P - 1) points into solution and makes a few van der Waals interactions with Thr\(^{282}\) and Thr\(^{285}\) on cyclin A α4-helix. Arg\(^{94}\) (P - 2), the crucial arginine of the RXL motif, makes two hydrogen bonds from its side chain nitrogen groups, NH1 and NH2, to Glu\(^{220}\) side chain oxygens OE1 (3.1 Å) and OE2 (2.8 Å). The main chain oxygen and nitrogen of Arg\(^{94}\) hydrogen bond to nitrogen NE2 of Gln\(^{234}\) (2.8 Å) and main chain oxygen of Ile\(^{281}\) (3.0 Å), respectively. These interactions are highly conserved in the other RXL motif-containing peptides (16). In other recruitment peptides, residues at the P - 3 position make no hydrogen bonds but a significant number of van der Waals interactions. In the CDC6 peptide and bispeptide complex structures the residue at the P - 3 position is a glycine that makes a number of van der Waals contacts from the main chain atoms with residues from the α4-helix (Tyr\(^{280}\), Ile\(^{281}\), Thr\(^{282}\), and Asp\(^{283}\)).

The Lys\(^{82}\) (P - 4) exhibits differences from previous observations with the p107 peptide complex (16) (Fig. 3a). Lys\(^{82}\) (P - 4) takes a different path (a shift of 3.5 Å compared with the equivalent residue in p107 peptide) and does not make specific hydrogen bonds. The loss of the two hydrogen bonds appears to be compensated by three hydrogen bonds further upstream with the CDC6 peptide. The main chain nitrogen of Leu\(^{94}\) (P - 5) hydrogen bonds (2.9 Å) to Gln\(^{234}\) oxygen OE1 and Thr\(^{90}\) (P - 6) main chain nitrogen hydrogen bonds (3.3 Å) to Gln\(^{234}\) oxygen OE2. The Gln\(^{234}\) side chain adopts a different conformation and is shifted by 1.9 Å to accommodate these hydrogen bonds. The side chain of Leu\(^{91}\) (P - 5) docks into a minihydrophobic pocket formed by residues Glu\(^{220}\), Val\(^{221}\), and Glu\(^{224}\); consistent with studies with model E2F peptides that had indicated that the P - 5 site might be important for binding (47). Thr\(^{90}\) makes few interactions. Finally His\(^{80}\) (P - 7) forms a number of van der Waals contacts with residues Gln\(^{224}\) (7 contacts), Tyr\(^{225}\) (3), Gln\(^{277}\) (4), and Tyr\(^{280}\) (30). The side chain is buried and achieves a mutual non-polar shielding effect with Tyr\(^{280}\).

The route taken by the CDC6 peptide from the N terminus to the site P - 4 is different to that observed previously with the 14-residue peptide from E2F (16). Despite the specific interactions that both peptides make with the protein, it appears that a variety of conformations can be accommodated on the cyclin.

**Kinetics**—The kinetic results for the assay of pCDK2/cyclin A with the model heptapeptide substrate and the CDC6 peptide are summarized in Table 2. Results for the CDC6 peptide have greater uncertainties because of reduced sensitivity of the coupled enzyme assay for reactions with low k\(_{cat}\) and K\(_m\) values. The heptapeptide substrate has k\(_{cat}\) = 42.7 s\(^{-1}\), but a high K\(_m\) = 612 μM. The CDC6 peptide exhibits a lower k\(_{cat}\) = 6.2 s\(^{-1}\) and a lower K\(_m\) = 34.1 μM, an 18-fold decrease in K\(_m\) from the heptapeptide. The CDC6 peptide has a 2.6-fold increase in the catalytic efficiency (k\(_{cat}\)/K\(_m\)) compared with the heptapeptide substrate. Similar results have been obtained from a detailed steady state solvent viscosimetric study by Lew and co-workers (25) who investigated the effects of the RXL motif for both optimal substrates and non-optimal substrates (e.g. lacking the basic group at P + 3). They showed that fusion of the RXL motif to a non-optimal substrate sequence increased catalytic efficiency by conferring increased binding affinity without affecting k\(_{cat}\) but that with an optimal peptide sequence the RXL motif increased catalytic efficiency by smaller amounts. In the lat-
The kinetic results for the CDC6 bispeptide against the heptapeptide substrate (Table 3) show that it is a potent competitive inhibitor with respect to both heptapeptide and ATP substrates (results not shown) with $K_i$ (apparent) values of 7.9 nM and 5.9 nM, respectively. These values extrapolate to 0.83 nM and 2.0 nM at zero fixed substrate concentration (Table 3). These $K_i$ values are among the highest affinity CDK2 inhibitors known.

To test the contribution of the recruitment site to the high affinity of the CDC6 bispeptide inhibitor, we carried out inhibition studies using the bisheptapeptide substrate that lacks the RXL motif (Fig. 1). There was no detectable inhibition for concentrations up to 460 $\mu$M bisheptapeptide concentrations when competing with 750 $\mu$M ATP (Table 3), supporting the notion that the bispeptide binds weakly at the catalytic site as observed in the crystallographic experiments.

The p107 peptide, like the CDC6 peptide, contains both the RXL motif and a serine phosphorylatable site. The sequence around the serine (SPIS) is non-optimal for a CDK2 substrate, as it lacks a basic residue in the P + 3 position, and phosphorylation is dependent on the RXL motif. Assays with the p107 peptide substrate were complicated by the apparent low $K_m$ for the p107 peptide (~22 $\mu$M), its low $k_{cat}$ and the limits of the sensitivity of the coupled enzyme assay. At low concentrations of p107 substrate, significant substrate depletion occurred, which could give an overestimate of the apparent $K_m$, although checks with time course studies showed the reaction was linear in the substrate range used. For these reasons we do not quote the kinetic parameters with p107 as the variable substrate. These difficulties do not affect the measurements in which ATP was the variable substrate because ATP is replenished in the coupled enzyme assay. The $K_i$ value of the CDC6 bispeptide is 1.9 $\mu$M with ATP as the variable substrate and p107 at 0.166 mM (Table 3). This change in apparent $K_i$ value compared with those for the heptapeptide substrate is discussed below.

3 N. R. Brown and L. N. Johnson, unpublished results.
CD changes of the spectrum of pCDK2/cyclin A indicated at 

spectrum below 280 nm (Fig. 4 (data not shown), whereas CDC6 bispeptide showed a weak effect on the binding of free HTL and p107 peptides were negligible, almost zero up to the concentration of ligand peptides used in the titrations, the CD spectra were consistent with the presence of the ligand within a range of 5 Å. With the concentrations of the ligand and free peptides, the aromatic side chains can be seen as probes in which their CD contributions are perturbed by the binding interface. The crystal structure of the pCDK2/cyclin A-CDC6 peptide complex shows that pCDK2 in the absence of cyclin A has an inactive conformation (49) but nevertheless pCDK2 exhibits some kinase activity and binds the adenine moiety of ATP in a similar mode to the active conformation.

For the titration of pCDK2/cyclin A with HTL and p107 peptides, the magnitudes of the CD changes in the 260–295 nm region were qualitatively similar (Fig. 4, a and b). The CD changes for the titration with CDC6 bispeptide were larger in the 260–280 nm region, which is consistent with the adenylation of the CDC6 bispeptide experiencing a change of environment on binding at the catalytic site (Fig. 4c). The $K_d$ calculated from the CD data at various wavelengths, 260, 269, and 292 nm for the CDC6 bispeptide were similar and indicate that the CDC6 bispeptide has strong binding at both the cyclin A recruitment site and the pCDK2 catalytic site. The stoichiometry of 1:1 is also consistent with CDC6 bispeptide binding to both binding sites of the pCDK2/cyclin A complex (data not shown). The observations of weak binding at the catalytic site monitored by the adenine spectra in the absence of binding at the recruitment RXL ($K_d = 5$ μM), the moderate binding at the recruitment site when there is no binding at the catalytic site ($K_d = 400$ μM), and the tight binding observed for the pCDK2/cyclin A-CDC6 bispeptide complex when both the adenyl and recruitment sites are occupied ($K_d \approx 1–60$ nM) support the view that both binding sites, one provided by pCDK2 and one by cyclin A recruitment site, contribute to the tight binding affinity of the CDC6 bispeptide to pCDK2/cyclin A.

### DISCUSSION

The crystal structure of the pCDK2/cyclin A-CDC6 peptide has shown that the CDC6 peptide bound with seven residues together with AMP/PNP/Mg$^{2+}$ at the catalytic site of pCDK2 and twelve residues at the cyclin A recruitment site. Binding at the catalytic site was only achieved with a high concentration (10 mM) of the CDC6 peptide. There was no electron density for the intervening eleven residues (78QGKENGPH588). The distance between the Ca atoms from the end of the catalytic site lysine to the start of the recruitment peptide His to 24 Å, a distance that could be easily spanned by the eleven residues (Fig. 5). Examination of the crystal lattice shows that there is a clear path for the peptide around the cyclin A molecule and that this path is not obscured by crystal lattice contacts. We conclude that either the CDC6 peptide is bound with these eleven residues disordered or that the CDC6 peptide binds in two separate modes with tight binding at the RXL site and weak binding at the catalytic site. The linker region in the CDC6 peptide contains the KEN motif, which is a site for recognition by the anaphase promoting complex/CDH1 complex for ubiquitination of CDC6 prior to its degradation. Phosphorylation of CDC6 at Ser$^{14}$ and Ser$^{2}$ are important events that protect CDC6 from degradation, thus allowing CDC6 to play its role in the assembly of the prereplication complex during G1 (50).

The kinetic results indicate that there is an 18-fold reduction in $K_m$ for CDC6 peptide that contains the RXL recruitment motif compared with the heptapeptide that does not. Moreover this effect is associated with a covalent linkage between the RXL motif peptide and the substrate peptide because the addition of the 12-residue RXL binding peptide to assay mixtures gave no effects on the kinetic parameters for the heptapeptide sub-

### Table 3

| Inhibitor | Substrate | $k_{cat}$ | $K_m$ | $K_d$ (apparent) | $K_d$ |
|-----------|-----------|----------|-------|-----------------|-------|
| CDC6 bispeptide | HHASPRK (ATP 0.75 mM) | 26.8 ± 3.3 | 450 ± 160 | 7.9 ± 1.9 | 0.83 |
| | ATP (HHASPRK peptide 0.9 mM) | 21 ± 1.6 | 88.5 ± 19 | 5.9 ± 1.3 | 2.0 |
| | ATP (p107 peptide 0.166 mM) | 5.6 ± 0.2 | 39.3 ± 5.9 | 1900 ± 400 |
| Bispeptide | HHASPRK (ATP 0.75 mM) | 31.8 ± 1.4 | 485 ± 53 | 485 NI |

$K_d$ Measurements using Circular Dichroism—To complement the kinetic measurements with direct binding measurements at the recruitment site and the catalytic site, the binding interactions between pCDK2/cyclin A and the HTL peptide, p107 peptide, and CDC6 bispeptide, respectively, were studied by CD in the near UV region (350–250 nm) (48) (Fig. 4). For molecular interactions where aromatic residues are involved in the binding interface, the aromatic side chains can be seen as probes in which their CD contributions are perturbed by the presence of the ligand within a range of 5 Å. With the concentrations of ligand peptides used in the titrations, the CD spectra of free HTL and p107 peptides were negligible, almost zero (data not shown), whereas CDC6 bispeptide showed a weak effect on the binding of free HTL and p107 peptides. The linker region in the CDC6 peptide contains the KEN motif, which is a site for recruitment sites are occupied ($K_d = 400$ μM), the tight binding observed for the pCDK2/cyclin A-CDC6 bispeptide complex when both the adenyl and recruitment sites are occupied ($K_d = 1–60$ nM) support the view that both binding sites, one provided by pCDK2 and one by cyclin A recruitment site, contribute to the tight binding affinity of the CDC6 bispeptide to pCDK2/cyclin A.

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pCDK2/Cyclin A Substrate Recognition

**pCDK2/cyclin A/HTL peptide**

- $K_d = 0.4 \mu M$

**pCDK2/cyclin A/p107 peptide**

- $K_d = 0.16 \text{ M}$

**pCDK2/cyclin A/CDC6 bispeptide**

- $K_d = 20.3 \text{ nM}$

**pCDK2/CDC6 bispeptide**

- $K_d = 5000 \text{ nM}$
The CDC6 bispeptide with the RXL motif was a potent inhibitor that was competitive with both the heptapeptide and ATP substrates with \( K_i \) values 0.83 nM and 2.0 nM, respectively (extrapolated to zero concentration of fixed substrate). The structural results show strong binding at the recruitment site identical to that observed with CDC6 peptide but only the modified ATP, with two alternative conformations, located at the catalytic site. There were no significant conformational changes. Modeling shows that the modified ATP linked peptide could be accommodated at the catalytic site. We note that binding of peptides at the catalytic site in pCDK2/cyclin A is often problematic and high concentrations with back soaks are needed in order to achieve binding. There are relatively few contacts that govern the recognition of peptide at the catalytic site. The major specificity is achieved through the contacts from the proline residue docking into the pocket created by the activation segment. Weak binding at the catalytic site is consistent with the high \( K_m \) of peptide substrates and the lack of inhibition by the bisheptapeptide. Binding of the ATP moiety is consistent with the \( K_D = 5 \mu M \) established by CD for the adenine moiety of the CDC6 bispeptide binding to pCDK2.

The binding of the 12 residues around the RXL motif of the CDC6 peptide has \( K_D = 0.4 \mu M \) determined from CD for the HTL peptide bound at this site. The interactions include those from an additional 4 residues not previously located with other recruitment peptides. These extra residues wrap around the cyclin making contacts especially with Tyr280 and head in a direction toward the pCDK2 catalytic site. Binding at the recruitment site is a crucial factor for the low \( K_i \) value of the CDC6 bispeptide when the pCDK2/cyclin A is assayed against the heptapeptide substrate. We envisage that localization of the CDC6 bispeptide at the recruitment site increases the local concentration of the covalently linked modified ATP, allowing the modified ATP to bind more effectively than if binding were to be achieved by diffusion of free ATP. This binding blocks access for both ATP and peptide substrates at the catalytic site. Thus although the linker region is not well ordered, effective inhibition is achieved.

The apparent flexibility of the linker region has two possible advantages. First, it allows pCDK2/cyclin A to recognize a variety of substrates provided that there is a minimum number of residues between the RXL site and the catalytic site. Analysis of the model shown in Fig. 5 indicates that the minimum number of residues between the Ser at the catalytic site and the Arg of the RXL could be slightly less than the 19 residues of the CDC6 peptide and could be reduced to 15–16 residues by taking out the one turn of helix indicated in Fig. 5. Secondly, the lack of any structure in the linker region implies that there is no energy cost involved in ordering the linker region because the linker could retain its solution conformation.

Schulman et al. (19) introduced the notion that the role of the recruitment site was to provide a high local concentration of substrate through interactions that are distant from the catalytic site. Hence it seems that the effects of the RXL motif for substrate recognition require the RXL and catalytic sites to be covalently linked. But from the crystallography there appears to be no defined stereochemical path between the two.

The studies with the CDC6 bispeptide inhibitor have allowed us to further elaborate on the power of the remote recruitment site. Kinetic experiments showed that the bisheptapeptide was not a good inhibitor of pCDK2/cyclin A exhibiting no inhibition at 460 \( \mu M \) when competing with 750 \( \mu M \) ATP. In contrast, the CDC6 bispeptide with the RXL motif was a potent inhibitor that was competitive with both the heptapeptide and ATP substrates with \( K_i \) values 0.83 nM and 2.0 nM, respectively (extrapolated to zero concentration of fixed substrate). The structural results show strong binding at the recruitment site identical to that observed with CDC6 peptide but only the modified ATP, with two alternative conformations, located at the catalytic site. There were no significant conformational changes. Modeling shows that the modified ATP linked peptide could be accommodated at the catalytic site. We note that binding of peptides at the catalytic site in pCDK2/cyclin A is often problematic and high concentrations with back soaks are needed in order to achieve binding. There are relatively few contacts that govern the recognition of peptide at the catalytic site. The major specificity is achieved through the contacts from the proline residue docking into the pocket created by the activation segment. Weak binding at the catalytic site is consistent with the high \( K_m \) of peptide substrates and the lack of inhibition by the bisheptapeptide. Binding of the ATP moiety is consistent with the \( K_D = 5 \mu M \) established by CD for the adenine moiety of the CDC6 bispeptide binding to pCDK2.

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pCDK2/Cyclin A Substrate Recognition

lytic site. These authors showed by mutating the RXL site on cyclin A, activity with the p107 substrate by pCDK2/cyclin A was abolished but was restored on introducing a p107 specific recognition motif (LYCYEQQL) toward the N-terminal region of the cyclin. They concluded that localization was the key for substrate activity enhancement but that the precise position for localization site was less important. In experiments with a peptide derived from the CDC6 protein, Dutta and co-workers (29) found evidence for a possible more direct interaction. They investigated the effects of different linker spacings between the phosphorylatable serine and the RXL motif. If the number of residues between Lys and Arg was 2 or 6 glycine residues, no activity was observed with the synthetic substrate. But if the linker length was 12 or 18 glycine residues, activity was restored to about 80% of the native CDC6 activity. The fact that activity was not 100% of the native CDC6 substrate could indicate some contribution to binding of amino acid side chains in the linker region, as indeed is observed in our crystallographic experiments for residues 89–94. These values for the number of residues spanning the sites correspond to 15 and 21 residues in our system defined above where we estimate that 15–16 residues is about the minimum number.

CDC2/cyclin A substrates that contain an RXL motif and for which phosphorylation sites have been identified show an apparent minimal distance of 17 residues between the Ser position at the catalytic site and the Arg of the RXL. For example p107 contains two potential pCDK2 phosphorylatable sites (shown in bold) in the sequence (S-SPISVHERYSPTAGAKRRLF) but only the first site (Ser) is phosphorylated and not the second (Ser), the second being only 7 residues from the RXL (24). Analysis of RXL containing substrates suggests that there is no obvious maximal distance between the phosphorylation and recruitment site and indeed the two sites may be on different proteins as in the E2F/DP1 complex.

Thus experimental evidence and our crystallographic observations support the notion that the RXL motif increases the local concentration of the substrate close to the catalytic site, and also indicate that there is a minimal length required between the phosphorylatable site and the RXL motif. The RXL motif provides additional substrate specificity such that only particular phosphorylation sites are accessible for the pCDK2/cyclin A. The power of the RXL site in making the CDC6 bispeptide a potent inhibitor is seen in the experiments where the CDC6 bispeptide was in competition with the RXL motif containing p107 peptide substrate, compared with the hirudin peptide substrate that lacks the motif. The \( K_d \) (apparent) with the p107 peptide substrate was 1.9 \( \mu M \) compared with the hirudin peptide substrate where the \( K_d \) (apparent) was 5.9 \( \mu M \). If we compare these values with the \( K_d \) for binding of p107 peptide and the HTL recruitment peptide at the recruitment site (0.16 \( \mu M \) and 0.4 \( \mu M \), respectively), it appears that what is being measured with the p107 substrate is competition for binding at the recruitment site.

Two ligands (A and B), each of which may bind weakly, can give potent binding when they are covalently linked (A–B) (51). Jencks has explained how the increased affinity can be viewed in terms of a connection Gibbs energy \( \Delta G^\circ \), which is largely, but not entirely, an entropy term. \( \Delta G^\circ \) represents the change in probability of bind-

ing that results from the connection of A and B in A–B. For the CDC6 bispeptide, the measured equilibrium binding constants \( (K_d) \) at the catalytic site for the modified ATP (5 \( \mu M \), at the recruitment site (0.4 \( \mu M \)) are greatly enhanced in the CDC6 bispeptide (\( \sim 2 \mu M \)) when the two components are covalently linked so that the whole is greater than the sum of the parts.

Our present studies have used peptide substrates and not intact protein substrates. The recognition of pCDK2 by its cognate phosphatase KAP occurs almost entirely through a remote docking site (52), whose mutation can prevent activity (53). Recognition is dependent upon the intact tertiary structure of pCDK2 supporting the notion, in this case, of a direct link between the catalytic site and the docking recognition site. Protein kinases can show significant differences with intact protein substrates than peptide substrates giving \( K_m \) values for intact substrates that may be orders of magnitude lower than \( K_m \) for peptide substrates (54, 55). The structural basis for substrate or inhibitor recruitment sites based solely on peptide binding have been identified for a number of other kinases that include p38 MAPK, GSK3, PDK1 (56), and ERK2 (57) but only in two instances of inhibitor binding studies, pCDK2/cyclin A binding part of the p27kip1 inhibitor (15) and PKA binding part of its regulatory inhibitor subunit (58), have more extensive descriptions of the communication between recruitment site and catalytic site been possible. Moreover the Pnt-P2 substrate of the "Drosophila" MAP kinase rolled requires an extensive docking surface that involves more than a linear peptide epitope and which is exploited for regulation through docking site blocking (59).

The use of the recruitment site for substrate specificity is not dissimilar to the well studied system of thrombin exosites. As anticipated by Fenton (60) and first exemplified by the thrombin/ hirudin complex structure (reviewed in Ref. 61), the hirudin inhibitor \( (K_d \sim 10^{-14} \mu M) \) folds with its N-terminal region at the thrombin catalytic site and its C-terminal region interacting through electrostatic and hydrophobic interactions at the exosite \( \sim 15–20 \) Å away. Similar contacts at the exosite are likely to be exploited for the natural substrate fibrinogen. The exosite both confers substrate specificity so that only selected bonds are cleaved but can also change substrate specificity as demonstrated by the thrombin-thrombomodulin complex (62). Thrombin and pCDK2/cyclin A are similar in that in their tight inhibitor complexes (thrombin-hirudin or pCDK2/cyclin A-p27) there is a direct stereochemical path between the catalytic site and remote site, whereas in substrate complexes (thrombin/E region of fibrin (63) or pCDK2/cyclin A-CDC6 peptide) a path has been modeled but not observed directly. The studies with pCDK2/cyclin A and the substrate recruitment peptides and bispeptides have been informative and shown the power of the recruitment site for a potent inhibitor. A structure of pCDK2/cyclin A with a full-length intact protein substrate is necessary to provide definitive information for substrate recognition.

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