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13. ABSTRACT (Maximum 200 Words) While studying the action of p21 on cyclin-cdks a 12 amino acid peptide, the Cy motif, was shown to be sufficient for inhibiting the protein kinase. The Cy motif was subsequently found on several substrates of cdks, where they are part of the substrate recognition sequence. A peptide containing the Cy motif inhibited the cdk competitively. In the last year we have developed an assay for small chemicals that mimic the action of a Cy peptide and used this assay to screen about 12,000 compounds in a synthetic chemical library. In addition, we have discovered that statins inhibit cancer cell proliferation by inhibiting the activating phosphorylation of cdk2. Analysis of the action of p21 on PCNA has also identified a small peptide that is used by p21 to interact with PCNA. Recent studies on the interaction of PCNA with Fen1 and DNA polymerase delta, have however indicated that such a p21 based peptide is unlikely to have differential inhibitory effects on the interaction of PCNA with Fen1 or polymerase delta and so will not be useful therapeutically.				
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INTRODUCTION:

In the introduction to our original grant application we discussed the importance of p53 function in prevention of breast cancer and how p53 exerts part of its tumor suppressor activity through the induction of p21. We and others had observed that the crucial function of p53 for stopping cell proliferation was the induction of p21. In work that formed the foundation of this application, we showed that although p21 interacts with and inhibits both the cyclin-dependent kinase and the replication factor PCNA, the domains of p21 involved in these interactions are separable [1]. Further, the domain that could interact with and inhibit cdk was sufficient to stop cell proliferation. We subsequently discovered that a small peptide derived from p21 was responsible for docking the molecule on the cyclin part of cyclin-cdk [2]. In previous reports we have reported our discovery that similar Cy motifs are present on substrates of cyclin-dependent kinases.

Cy motif on inhibitors of cdks: The Cy motif was conserved in well-known inhibitors of cdks: p21, p27 and p57. Together with our biochemical data, the crystallographic structure of cyclin A-cdk2 complexed with p27 [3] suggests that the Cy motif- cyclin interaction serves as a docking interaction essential for the complete interaction between cdk inhibitors and cyclin-cdk.

Cy motif on substrates of cdks: 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. 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 crystal structure of the cdk inhibitor p27 complexed with cyclin A/cdk2 [3] indicates that the N-terminal Cy motif of p27 is 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.

CDC6, a substrate of cdk2 that is dependent on its Cy motif for phosphorylation by cdk2: 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. 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 [4, 5]. Phosphopeptide analysis has shown that this phosphorylation by cyclin A/cdk2 occurs on Ser-54, Ser-74, and Ser-106 [6]. This requires the presence of a nearby Cy motif at residues 94-98 as evidenced by the fact that point mutation of this sequence abolishes phosphorylation at these sites [7].

Change in focus of work during year 4: Recent studies made it clear to us that one of our objectives, development of p21-based chemicals that would selectively interfere with PCNA interaction with polymerase delta while retaining the interaction with Fen1 was unfeasible. Polymerase delta was reported to have a subunit that contains the same sequence motif as p21 and Fen1 for interacting with PCNA [8-10]. Therefore a chemical that mimics this sequence motif is unlikely to be a specific inhibitor of polymerase delta or of Fen1. In addition a new protein, Dna2, was reported to be as important as Fen1 for processing of Okazaki fragments, and co-operates with Fen1 in the processing [11]. Thus it is unlikely that over-expression of Fen1 alone will have the desired effect of excessive nuclease activity leading to single-stranded regions that would be fatal for the cell.

Simultaneously a better understanding of the Cy motif made it clear that it would be desirable to have small chemicals that mimic the action of the Cy motif and inhibit the cdk. Thus we focused our effort on developing a suitable assay for discovering such chemicals in a high throughput screen. In addition, we noticed that a well known class of therapeutic chemicals, the HMG CoA reductase inhibitors called statins, inhibit cell proliferation by inducing endogenous p21. This appeared to be another promising approach to stop the cell-cycle using chemicals and we decided to study this further in the fourth year.

BODY

Statement of Work:

Technical objective 1. Analysis of the interaction between p21, cyclin and cdk.

Task 1: Months 1-24: Oligonucleotide directed mutagenesis of the portion of p21 in the cyclin binding site to determine what sequence feature is essential for binding to cyclins.

Task 1: Months 1-24: Lineweaver-Burke analysis of the inhibition of cyclin E-cdk2 by intact p21 and by p21 without the cyclin binding site.

Task 2: Months 24-36: Determination of the binding affinity of the cyclin-binding site for cyclins and of the cdk2 binding site for cdk2 and comparison with the binding affinity of intact p21 with the cyclin E-cdk2 holoenzyme.

Task 3: Months 24-48: Creation and testing of mutations of p21 with different affinities for cyclins D1 and E.

Task 4: Months 24-48: Creation and testing of versions of p21 with variation of the distance between the cyclin-binding and the cdk2 binding sites (in cis).

Changed to develop an assay for screening for small chemicals that mimic the action of Cy motif.

Technical objective 2. Analysis of the interaction between p21, PCNA and Fen1.

Task 5: Months 1-12: Determination of which part of PCNA interacts with p21 and with Fen1.

Tasks 6 and 7: Months 24-48: Designing D amino acid based retro-inverse peptides that mimic the PCNA interacting portion of p21. Analysis of their ability to inhibit PCNA in vitro.

Abandoned based on new results.

Task 8: Months 36-48: Testing effect of adding Fen1 to replication reactions or over-expressing Fen1 in MCF-7 cells in culture.

Changed to analyze how chemicals that induce p21 in cells inhibit the cell-cycle.

METHODS.

Plasmid Construction, Expression, and Purification of p21N Cy Mutants. The bacterial expression plasmid pGEX-p21N (residues 1-90) was constructed as described previously [1]. The Stratagene Quikchange system was used to introduce a HindIII site upstream of the region of the gene coding for the Cy motif. Oligonucleotide cassettes flanked by HindIII and BlnI sites and containing either the desired mutation or a randomized codon were inserted into pGEX-p21N. The mutations were identified using standard DNA sequencing. The p21N-ΔCy mutant had a deletion that removed amino acids 17-24 from p21N. Plasmids encoding the mutants were then transformed into

BL21(DE3). The GST fusion proteins were expressed and purified as before [1, 2]. Protein concentrations were determined using the Biorad Protein Assay.

Expression and Purification of Cyclin/Cdk Complexes. All cyclin/cdk complexes used in the in vitro kinase assays were expressed and purified by coinfecting Sf9 with baculoviruses encoding the appropriate cyclin/cdk pair. Cells were harvested 48-72 hours later and purified as described previously [12]. Baculoviruses expressing GST-cyclin E, GST-cyclin A, cdk2, GST-cdk4, and cyclin D1 were all kind gifts from Helen Piwnicka-Worms.

The cyclin and cyclin/cdk complexes used in the in vitro binding assay were expressed in *E. coli*. The genes for cyclin A and cyclin E were cloned using PCR into pHisTrx (a kind gift from Christophe Briand) and expressed as His6-thioredoxin fusion proteins [13]. BL21(DE3) cells carrying this plasmid were grown at 25°C, induced with the addition of IPTG to a final concentration of 1 mM, and harvested 6-8 hours later. The genes encoding Cdk2 and Cdc2 (gift from Mark Solomon) were also PCR cloned in the pMM vector (gift from Steve Blacklow) and expressed from a single promoter as a bicistronic message. BL21(DE3) cells carrying this plasmid were grown at 30°C, induced with IPTG, and harvested 24 hours later. Recombinant bacterial cyclin/cdk complexes were assembled by mixing bacterial lysates containing either Histx-cyclin A or Histx-cyclin E and cdk2 and purified using Ni-NTA beads as described by the manufacturer (Qiagen). Additional information regarding plasmid construction and protein purification is available upon request.

Construction and Expression of Substrate Peptides 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 [14]. 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.

Kinase Assays. For the kinetic measurements, the kinase assays were performed as follows. Phosphorylation reactions were performed in a total volume of 15 μ l containing 50 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.02% Triton X-100, 1 μ Ci [γ - ^{32}P] 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 K_m to 5 x K_m for each substrate. Reactions were initiated by addition of 1 μ l of enzyme diluted in reaction buffer and incubated at 30°C for 20 min. Reactions were

terminated with 1 μ l of 0.5 M EDTA and 10 μ l of the reaction mixture spotted onto a 2cm x 2cm square of Whatman P81 phosphocellulose filter paper. Papers were washed in 0.5% H_3PO_4 three times for five minutes, once in 50% EtOH, 0.5% H_3PO_4 for five minutes, and dried under a heat lamp. Incorporation of [γ - ^{32}P] 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 k_{cat} and K_{m} were determined. All experiments were done at least twice in duplicate. Protein quantitation was determined by Bio-Rad protein assay.

For determining the inhibitory potency of the p21 derivatives, the kinase assays were performed as follows. For cyclin A/cdk2 and cyclin E/cdk2 in vitro kinase assays, the CDC6(wt) peptide was used as the phosphoacceptor substrate at a final concentration of 16 μM . For cyclin D1/cdk4 kinase assays, a substrate peptide derived from the CDC6(wt) peptide in which the SPPK phosphorylation site was changed to SPKK was used at a final concentration 133 μM . The concentration of ATP was 65 μM in these assays. Substrate peptides with the mutant Cy motif were tested for their ability to be phosphorylated by cyclin A/cdk2 or cyclin E/cdk2 using 13 μM of peptide and 85 μM ATP.

In Vitro Binding Assays. Purified GST-p21N mutants were mixed with bacterial lysates containing HisTrx-cyclin A or HisTrx-cyclin E, or with purified HisTrx-cyclin A/cdk2 or purified HisTrx-cyclin E/cdk2. The GST-p21N associated proteins were purified on GSH agarose, washed extensively with a buffer containing 50mM Tris pH8.0, 150 mM NaCl, 10% Glycerol, and 0.01% NP-40, and analyzed using SDS-PAGE. Immunoblotting with anti-His antibodies (Santa Cruz) revealed the presence or absence of cyclins in the GST pull-down.

Cell Cycle Arrest by p21N mutants. Wild-type p21N, p21N- ΔCy , p21N-R19V, and p21N-R19W were cloned in to the mammalian GST-expression plasmid, pEBG [15]. U2OS cells were cotransfected with 5 μg each p21N mutant and 1 μg of a plasmid encoding farnesylated GFP (pEGFP-F, Clontech) using lipofectamine-mediated transfection (Gibco BRL). Cells were harvested 48 hours after transfection and prepared for FACS as described by Jiang et al [16]. A Coulter Epics XL flow cytometer was used to determine the DNA profile of GFP-positive cells which should also express the p21N plasmid. The cell cycle distribution for a given population was determined using the WinCycle DNA Analysis software. All experiments were done in duplicate.

Cell Culture and Flow Cytometry PC3 prostate cancer cells were grown in RPMI media supplemented with 10% heat inactivated fetal bovine serum and antibiotics.

Mevastatin(compactin) was from sigma chemicals and was dissolved in dimethyl sulfoxide (DMSO). Unless indicated 10 μ M of mevastatin was added to subconfluent cells. Control cells were treated with equivalent amount of DMSO. TGF β 1 was from Gibco-BRL. 2 μ g was dissolved in deionized water and 80 pM was added per subconfluent dish. Antibodies to cdk2, cyc E, cyc A, p57, p21, p27, were obtained from Santa Cruz biotechnology . Phosphotyrosine antibody was Upstate biochemicals . Anti RB antibody was the kind gift of Dr E. Harlow (Harvard University).Anti phospho Rb antibody was from Cell Signaling Technologies (Beverly, MA). Radioactive isotopes; $^{32}\gamma$ -ATP, $^{32}\alpha$ -ATP,(32 P) orthophosphate,(3 H)-thymidine and (35 S)-methionine were from PerkinElmer Life sciences

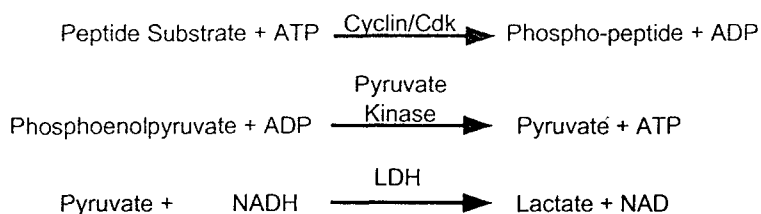
Immunoblots Protein extracts were obtained by treating cells at specified times with lysis buffer (50 mM, Tris pH 7.4 , 0.2% Nonidet P40, 150 mM NaCl, 1 mM EDTA and protease inhibitors). Lysates were clarified by centrifugation and protein concentration determined by the Bradford assay method. For immunoblots, 30 μ g of total cellular protein was loaded per lane and separated by SDS-PAGE.

Immunoprecipitations and kinase assays Cells were treated with lysis buffer supplemented with 50 mM NaF , 1 mM Na 3 V04 and protein concentration determined as above. Equal amounts of lysates (200 μ g- 400 μ g of protein) were immunoprecipitated with either CDK2, cyclin E or cyclin A antibodies. The pellet was washed 3 times with lysis buffer and resuspended in 1x sample buffer. For kinase assays, the pellet was washed twice in lysis buffer and then washed twice in kinase wash buffer(50 mM Tris, pH 7.4, 10 mM MgCl $_2$ and 5 mM MnCl $_2$). The pellet was resuspended in 20 μ l of kinase reaction buffer(50 mM tris pH7.4, 10 mM MgCl $_2$, 5 mM MnCl $_2$, 5 mM DTT, 10 μ M ATP, 0.5 μ Ci $^{32}\gamma$ -ATP, 2 μ g of histone H1 or 200 ng of RBc). The mixture was incubated at 30 $^{\circ}$ C for 30 minutes and the reaction stopped by the addition of equal volume of 2x sample buffer.

35 S-methionine and 32 P04 labeling Subconfluent cells (50%-60%) were grown in the presence or absence of mevastatin for 42 hours. For (35 S)-methionine labeling, the media was removed , cells washed 2x in PBS and incubated in methionine free media with or without mevastatin for 1 hour. Cells were labeled for 4 hours in methionine free media, supplemented with 5% dialyzed fetal calf serum, 25 μ Ci/ml of 35 S- methionine(specific activity, 1175 Ci/mmol) and with or without mevastatin Cells were labeled for 4 hours and proteins extracted with lysis buffer as above. Protein concentration was determined by the Bradford assay method.

For 32 P04 labeling of cellular proteins, cells were incubated in phosphate free media for 2 hours and then in media containing 350 μ Ci/ml of (32 P)orthophosphate for 3hours prior to harvesting.

Screen for Cy-mimetic chemicals: Chemical libraries were screened using a high throughput spectrophotometric method in which ATP hydrolysis by cyclin/cdk complexes is coupled to the following reaction:



Since NADH, but not NAD, absorbs in the ultraviolet region, the velocity of the reaction is monitored by measuring the decrease in absorbance at 340 nm as a function of time.

SUMMARY OF RESEARCH DONE OVER YEARS 1-3

Tasks 1, 2 and 3 and beyond

Takeda D, Wohlschlegel J A, and Dutta A. A bipartite substrate recognition motif for cyclin-dependent kinases. *J. Biol. Chemistry*. 2001; 276, 1993-1997.

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.

Wohlschlegel J A, Dwyer B, Takeda D and Dutta A. A mutational analysis of the Cy motif from p21 reveals sequence degeneracy and specificity for different cyclin-dependent kinases. *Mol. Cell. Biol.* 2001; 21, 4868-4874.

Inhibitors, activators and substrates of cyclin-dependent kinases utilize a cyclin binding sequence, known as a Cy or RXL motif, to bind directly to the cyclin subunit. Alanine scanning mutagenesis of the Cy motif of the cdk inhibitor p21 revealed that the conserved arginine or leucine (constituting the conserved RXL sequence) were important for p21s to inhibit cyclin E/cdk2 activity. Further analysis of mutant Cy motifs showed, however, that RXL was neither necessary nor sufficient for a functional cyclin-binding motif. Substitution of either of these two residues with small hydrophobic residues like valine preserved p21's inhibitory activity on cyclin E/cdk2 while mutations to either polar or charged residues dramatically impaired p21's inhibitory activity. Expressing p21N with non-RXL Cy sequences inhibited growth of mammalian cells providing in vivo confirmation that RXL was not necessary for a functional Cy motif. We also show that the variant Cy motifs identified in this study can effectively target substrates to cyclin/cdk complexes for phosphorylation providing additional evidence that these non-RXL motifs are functional. Finally, binding studies using p21 Cy mutants demonstrated that the Cy motif was essential for the association of p21 with cyclin E/cdk2 but not with cyclin A/cdk2. Taking advantage of this differential specificity toward cyclin E versus cyclin A we demonstrate that cell growth inhibition was absolutely dependent on the ability of a p21 derivative to inhibit cyclin E/cdk2.

Task 5, 6 and 7: Determination of the part of PCNA that interacts with p21 and Fen1.

A report appeared in the Literature [17] that indicated residues QLGI in the inter-domain connecting loop of PCNA was important for the association of PCNA with p21. Plasmids encoding the mutant forms of PCNA described in that report were obtained from the authors. Bacterially produced GST-p21 and to GST-Fen1C (containing the C terminal portion of Fen1 which interacts with PCNA) were bound to glutathione agarose beads and their association with PCNA assayed as described by us [8]. The wild type and mutant forms of PCNA were produced in two ways: (a) by in vitro-transcription translation in rabbit reticulocyte lysates and (ii) by expressing in *E. coli*. PCNA produced by in vitro transcription-translation was labeled with 35S methionine and visualized by fluorography, while the bacterially produced PCNA was visualized by immunoblotting with commercial anti-PCNA antibody.

In vitro transcribed -translated PCNA (wild type and mutants) were bound to glutathione agarose beads coated with GST-p21, GST-Fen1C and GST (negative control). No difference was seen in the binding of the mutants to p21 or Fen1, a result that contradicts with the published report [17]. We reasoned that non-radioactive wild-type PCNA subunits in the rabbit reticulocyte lysate may form heteromers with the radiolabeled mutants and facilitate their association with p21 or Fen1 giving a false

positive result. Wild type and mutant PCNA were therefore synthesized in *E. coli* (which do not contain wild type eukaryotic PCNA), lysates containing the PCNA prepared, and the binding of PCNA to GST, GST-p21 and GST-Fen1C assayed. Now wild type PCNA and several of the mutants successfully bound to p21 and to Fen1. However, in agreement with published results, a mutation that changed the QLGI in the inter-domain loop of PCNA to AAAA, resulted in PCNA that failed to bind to p21 and Fen1. Thus Task 5 was completed (mapping the part of PCNA that interacts with p21 and with Fen1).

Several groups also published exactly which portions of PCNA interact with polymerase delta and with Fen1 [17-20]. The reports provide strong evidence that the same QLGI motif of PCNA is involved in these interactions. Thus it is unlikely that we will succeed in our original goal of obtaining selective mutants in p21 which will spare the PCNA-Fen1 interaction while disrupting the PCNA-polymerase delta interaction. In addition a new protein, Dna2, was reported to be as important as Fen1 for processing of Okazaki fragments, and co-operates with Fen1 in the processing [11]. Thus it is unlikely that over-expression of Fen1 alone will have the desired effect of excessive nuclease activity leading to single-stranded regions that would be fatal for the cell. Hence Tasks 6 and 7 were abandoned and the resources directed to tasks 1-3 that were giving very promising results.

RESULTS AND DISCUSSION FOR YEAR 4

Task 4: Screen for Cy-mimetic chemicals Using the spectrophotometric kinase assay we were able to duplicate results obtained from the well-characterized radioenzymatic assay of Cy-peptide dependent inhibition of cdks [21]. In both assays, velocity was proportional to enzyme concentration as well as substrate concentration. Importantly, PS100, a peptide containing a Cy motif, was able to inhibit the photometric assay with a comparable IC₅₀ value, whereas PS101, a peptide lacking a Cy motif, was unable to significantly inhibit enzymatic activity. Reaction velocity was also unaffected when varying the concentration of pyruvate kinase and LDH, indicating that the coupling reaction was not rate limiting. Therefore we concluded that the photometric assay would be sufficient to use in a high throughput screen for inhibitors of cyclin dependent kinases.

To date we have screened 12,000 out of 16,000 compounds contained in a commercially available chemical library. An example of the results from one plate is presented in [Fig. 1](#). We identified 47 compounds, which showed greater than 40% inhibition of kinase activity. These were subjected to two secondary screens.

First we determined if these chemicals had any inhibitory effect using the radioenzymatic assay which directly measures substrate phosphorylation rather than ATPase activity. Second, we repeated the radioenzymatic assay using another protein

kinase, protein kinase A (PKA), to determine whether the inhibition was specific to cyclin dependent kinases. We confirmed 30 compounds as kinase inhibitors using the radioenzymatic assay. Of the 30, 11 were shown to have specificity for cyclin dependent kinases compared to PKA. For an example, see [Fig. 2](#). Presumably, the 19 compounds that inhibited nonspecifically represent ATP analogues that are able to inhibit a variety of different kinases.

We then performed a tertiary screen measuring phosphorylation of a substrate containing either a wild type or mutant Cy motif. We discovered one of the 11 cyclin dependent kinase specific compounds to inhibit only the Cy motif containing substrate ([Fig. 3](#)). We hypothesize that this compound represents a new class of cyclin dependent kinase inhibitors that function as a Cy motif mimetic.

Task 8: Mechanism by which mevastatin inhibits cancer cell proliferation Inhibitors of the cyclin dependent kinases (CDKs) like p21 can inhibit the kinase in vitro by direct association with active kinase. There are reports in the Literature that statins, inhibitors of HMG-CoA reductase and of cholesterol synthesis, inhibit cell growth through induction of p21 and concurrent inhibition of CDK2. Since statins are widely approved for human use, it is important to dissect the mechanism of this cell growth inhibition in the hope that once we find the exact mechanism, one can find better statins with better cytostatic action that can be used for anti-cancer therapy.

Mevastatin treatment leads to a G1 block and decrease in kinase activity of CDK2
[Table 1](#) shows a cell cycle profile (FACS analysis) of mevastatin treated and untreated cells. As has been reported with lovastatin treatment of PC3 cells ([22]) a significant decrease in the S phase population with a concurrent increase in G1 is notable by 24 hours; a more pronounced G1 block obvious by 36 hours. This inhibition of cell growth is paralleled by a dose dependent effect of mevastatin on the activity of CDK2 ([Fig. 4A](#)). A greater than 90% inhibition of CDK2 is evident at 2.5 μ M concentration of the statin. Immunoblot analysis of cell lysates after 36 hours of mevastatin treatment shows that while the level of the cell cycle regulated proteins; CDK2, p27, p57 remain unchanged, the amount of p21 is increased ([Fig. 4B](#)). Immunoprecipitation of cell lysates with antibodies to CDK2 confirm that while the amount of CDK2 remains relatively unchanged, there is a small but perceptible increase in p21 association with CDK2. Interestingly, this rather modest increase in p21 results in a dramatic inhibition of kinase activity of CDK2 following mevastatin treatment ([Fig. 4C](#)). These experiments confirm previous observations that the increase cellular p21 and the subsequent inhibition of CDK2 parallel the growth inhibitory effect of statins.

Although p21 is induced in PC3 cells following TGF β 1 treatment, there is no concurrent inhibition of cdk2 activity. Treatment of PC3 cells with TGF β 1 results in significant induction of p21, increased association of p21 with CDK2, and yet no noticeable inhibition of kinase activity (Fig. 5) and no inhibition of cell proliferation by FACS analysis (not shown). It is worth noting that the increase in p21 association with CDK2 is more profound in TGF β 1 treated than with mevastatin treated cells (compare with Fig. 4C). This observation, coupled with the discrepancy between p21 associated CDK2 and the accompanying dramatic loss of kinase activity, suggests that a simple association of p21 to CDK2 does not explain the entirety of the observed anti proliferative effect of mevastatin.

Decrease in activating phosphorylation on Threonine 160 of Cdk2 accounts for inhibition of CDK2 upon mevastatin treatment. Because CDK2 regulation involves phosphorylation events, we evaluated the role of phosphorylation in the activity of the kinase in mevastatin treated cells. We analyzed immunoprecipitates of CDK2 for the presence of phosphotyrosine a marker of inhibition and noted no differences in phosphorylation (not shown). However we noted that upon metabolic labeling of cells with (32P) orthophosphate, a pattern of phosphorylation that parallels the in vitro kinase activity of CDK2 following mevastatin treatment was apparent (Fig. 6A). Because this finding could be explained on the basis of a decreased pool of cyclin A/K2 complexes, we repeated these experiments by immunoprecipitation with an antibody to cyclin E that selectively examined CDK2 associated with cyclin E. As shown in Fig. 6B, there is a concurrent decrease in the phosphate content of CDK2 from mevastatin treated cells. To confirm this result, we modified our gel running conditions such that phosphorylated CDK2 runs faster than unphosphorylated cdk2. Immunoblotting cell lysates with anti-CDK2 antibody reveals that mevastatin treatment results in a significant decrease in phosphorylated cdk2 (Fig. 6C). To confirm that this was not due to most of the CDK2 being free of cyclins, the experiment was repeated by specifically immunoprecipitating with anti-cyclin E antibody followed by immunoblotting for the associated CDK2 (Fig. 6D). Here too the amount of unphosphorylated (slower mobility) CDK2 was significantly increased following mevastatin treatment.

This finding could be as a result of 1) decreased levels or activity of the trimeric cyclin-cdk activating kinase (CAK), 2) decrease in levels or activity of a novel CAK or 3) the presence of a phosphatase activity targeted to Thr160 of CDK2 in mevastatin treated cells. We performed western immunoblots to evaluate the levels of CDK7 and cyclin H (components of mammalian CAK) in PC3 cells and noted no changes in their respective cellular levels following mevastatin treatment (not shown). Immunoprecipitation of the

complex with cyclin H antibodies followed by analysis of its kinase activity against exogenously derived glutathione-S transferase (GST) tagged CDK2 showed no inhibition upon mevastatin treatment (Fig. 6E).

We have investigated the possibility that a phosphatase activity is induced following mevastatin treatment. Despite an exhaustive search for this activity under various conditions we have been unable to appreciate any difference in phosphatase activity of mevastatin treated cell-lysates. We are currently left with the possibility that a novel CAK like activity may be suppressed in mevastatin treated cells. This hypothesis is strengthened by recent debates regarding the identity of the physiologic mammalian CAK. Although the trimeric complex of cyclin H, cdk7 and MAT1 has long been felt to be the mammalian CAK [23, 24], the presence of an active monomeric CAK in yeast [25] and the recent evidence of a protein from Hep G2 cells that cross-reacts with anti-yeast CAK antibody [26], suggests that an alternative CAK might indeed exist.

Either way, the effect of statin has to be now tested on a wide range of cancer cells, particularly breast cancer cells, to determine (a) whether the cell cycle is inhibited and (b) whether the inhibition of CDK2 is accompanied by the dephosphorylation of CDK2 on T160. Given the large numbers of statins that are now in clinical use, screening through these might unearth potent inhibitors of the cell-cycle that may be useful for anti-cancer therapy.

KEY RESEARCH ACCOMPLISHMENTS.

Discovered that contrary to current view the phosphoacceptor serine (in the context of SPXK) by itself is an inefficient substrate of cyclin dependent kinases.

The addition of a cyclin binding motif (Cy motif) at a defined distance from the phosphoacceptor serine is essential for making an optimal substrate (with a K_m in the low μM range).

Cy motif based cdk inhibitors may be specific inhibitors of cyclin E-cdk2, a matter of some importance to the 20-30% breast cancers that selectively up-regulate cyclin E levels.

Contrary to current view an RXL is neither necessary nor sufficient to be an effective Cy motif. The exact requirement seems to be for a hydrophobic patch, suggesting that Cy-mimetic chemicals need to be hydrophobic.

Design of a high-throughput screen for Cy-mimetic chemicals.

Discovery that statins, a group of commonly used chemicals in clinical practice, inhibit cdk2 through the inhibition of the activating phosphorylation of cdk2

REPORTABLE OUTCOMES.

Manuscripts

Takeda D, Wohlschlegel JA, Dutta A. A bipartite substrate recognition motif for cyclin-dependent kinases. *J. Biological Chemistry*. 2001, 276:1993-1997.

Wohlschlegel JA, Dwyer BT, Takeda D, Dutta A. Mutational analysis of the Cy motif from p21 reveals sequence degeneracy and specificity for different cyclin-dependent kinases. *Molecular and Cellular Biology*. 2001, 21:4868-4874.

Abstracts

Dutta A, Wohlschlegel J, Dwyer B and Takeda D. The role of cyclin binding Cy motifs on cdk inhibitors and substrates. *Gordon Conference on Molecular Genetic Basis of Cell Proliferation*. July 4- July 9, 1999, New Hampshire.

Dutta A, Wohlschlegel J, Dwyer B and Takeda D. Cyclin binding Cy (RXL or LFG) motifs on cdk inhibitors and substrates provide a novel molecular target for therapy.. *Keystone meeting on the Cell Cycle*. Jan. 8-Jan. 13, 2000. Colorado.

Dutta A, Wohlschlegel J, Dwyer B and Takeda D. Cyclin binding Cy (RXL or LFG) motifs on cdk inhibitors and substrates provide a novel molecular target for therapy. *American Association of Cancer Research, annual meeting*, April 1- April 5, 2000. San Francisco.

Dutta A, Wohlschlegel J, Dwyer B and Takeda D. Cyclin binding Cy motifs on cdk inhibitors and substrates provide a novel molecular target for therapy of breast cancers. *Era of Hope meeting, U.S. Army Medical Research, Breast Cancer Program*. June 8-June 12, 2000. Atlanta.

Wohlschlegel J, Dwyer B, Takeda, D and Dutta A. Biochemical studies of the Cy motifs from the cell cycle inhibitor p21 and the replication factor Cdc6. Cell cycle meeting, May 17-21, 2000. Cold Spring Harbor Laboratory, New York.

Dutta A, Ukomadu C, Wohlschlegel J, Dwyer B and Takeda D. Blocking the cell cycle through p21/WAF1/CIP1: effects of statins and of novel Cy motif based cdk inhibitors. American Association of Cancer Research annual meeting. Mar. 24-28, 2001. New Orleans.

Wohlschlegel J, Dwyer B, Takeda D, and Dutta A. Mutational Analysis of the Cy Motif from p21 Reveals Sequence Degeneracy and Specificity for Different Cyclin-Dependent Kinases. Salk Cell Cycle meeting, June 22-26, 2001. San Diego.

Wohlschlegel J, Dwyer B, Takeda D, and Dutta A. Mutational Analysis of the Cy Motif from p21 Reveals Sequence Degeneracy and Specificity for Different Cyclin-Dependent Kinases. Gordon conference on Molecular Genetic Basis of Cell Proliferation, July 1-5, 2001. New Hampshire.

Presentations that included work supported by this award

Dutta A.

2000

International meeting on "Role of protein phosphorylation in signal transduction", Osaka Bioscience Institute, Osaka, Japan. May 10-12.

University of Colorado Health Sciences Center, Denver, Jan 13

Stanford University School of Medicine, April 6

Seoul National University, Seoul, S. Korea, May 4

Samsung Biomedical Research Institute, Seoul, S. Korea, May 4

Nagoya City University, School of Medicine, Nagoya, Japan, May 8

Nagoya University School of Medicine, Nagoya, Japan, May 8

Nara Institute of Biomedical Science, Nara, Japan, May 9

Osaka University, Dept. of Biology, Osaka, Japan, May 10

Tokyo University Institute of Medical Sciences, Tokyo, Japan, May 15

Vanderbilt University, Oct. 12

2001

U. Connecticut Health Sciences Center, Jan 31

U. of Pennsylvania, Abramson Cancer Center, Feb. 8

Duke University Medical Center, Feb. 22
Massachusetts General Hospital, Cancer Center, May 30
Harvard University, Molecular and Cellular Biology, Oct. 11
Yale University, Oct 18
U. of Chicago, Oct. 24
U. of Nebraska, Dec. 3
U. of Alabama, Dec. 17

Funding applied for based on work supported by this award

RO1 from the National Institutes of Health: "Role of Cy motifs in substrate recognition by CDKs". P.I. Anindya Dutta. Awarded CA89406

Predoctoral Fellowship from the U.S. Army's breast cancer program: "Discovery and development of inhibitors that selectively interfere with cyclin-dependent kinase substrate recognition." P.I. James Wohlschlegel. Awarded.

Predoctoral Fellowship from the Howard Hughes Medical Institute: P.I. Mr. David Takeda. Awarded.

Employment applied for based on work supported by this award

Senior research assistant, Genetics Institute and Millennium Pharmaceuticals. Mr. Brian Dwyer.

Ph.D. program in Biological and Biomedical Sciences of Harvard Medical School. Mr. David Takeda.

CONCLUSIONS FOR THE ENTIRE DURATION OF GRANT

Technical objective 1. Analysis of the interaction between p21, cyclin and cdk.

Task 1: Months 1-24: Oligonucleotide directed mutagenesis of the portion of p21 in the cyclin binding site to determine what amino acid sequence feature is essential for binding to cyclins.

The results, published in [27] showed that RXL of RRLFG is neither necessary nor sufficient for a functional Cy motif. Thus, VRLFG, LRLFG or RRVFG function as effective

Cy motifs. Conversely, **RRLAA** or **RALAG** did not act as a Cy motif. We concluded that the overall hydrophobicity of the peptide sequence was the critical feature of a Cy motif.

Task 1: Months 1-24: Lineweaver-Burke analysis of the inhibition of cyclin E-cdk2 by intact p21 and by p21 without the cyclin binding site.

p21 without a Cy motif was ineffective in inhibiting cyclin E-cdk2 [2]. Lineweaver-Burke analysis of the inhibition of cyclin E/cdk2 and cyclin A/cdk2 by a Cy mimetic peptide indicated that the latter was a competitive inhibitor with a K_i of 7.5 μ M and 117.5 μ M on the two kinases respectively [21]. These results suggest that a Cy mimetic chemical may be a potent and selective inhibitor of cyclin E-cdk2.

Task 2: Months 24-36: Determination of the binding affinity of the cyclin-binding site for cyclins and of the cdk2 binding site for cdk2 and comparison with the binding affinity of intact p21 with the cyclin E-cdk2 holoenzyme.

p21 inhibits Cdk2, kinase with a K_i of 0.5-15 nM [12]. In comparison, the K_i of a Cy motif peptide on cyclin E-cdk2 is 7500 nM [21]. We were not able to get a K_i for p21 without a Cy motif because its inhibitory concentration was too high for the amount of soluble protein we could produce. These results suggest that a Cy mimetic chemical by itself is unlikely to be an effective inhibitor of Cdk2 for therapeutic purposes. Such a chemical might, however have additional effects on the activation of Cdk2 (see results with statin reported above) and could show synergy of inhibition with ATP-mimetic Cdk2 inhibitors.

Task 3: Months 24-48: Creation and testing of mutations of p21 with different affinities for cyclins D1 and E.

In our hands, p21N, the N terminal 90 residues of p21 that functions as a cdk2 and cell cycle inhibitor, failed to inhibit cyclin D1-cdk4 [27]. We therefore focused on creating p21N derivatives that had differential effects on cyclin E-cdk2 versus cyclin A-cdk2. Deletion of the Cy motif of p21N provided such a mutant: it inhibited cyclin A-cdk2 but not cyclin E-cdk2 [27]. This mutant was very useful because it did not inhibit the cell-cycle, implying that inhibition of cyclin E-cdk2 is critical for stopping the cell-cycle. Since Cy-mimetic chemicals show stronger inhibition of cyclin E-cdk2, such chemicals might therefore be useful for stopping cell-cycle progression.

Task 4: Months 24-48: Develop an assay for screening for small chemicals that mimic the action of Cy motif.

We have successfully developed an assay for high throughput screening of small chemicals that inhibit cyclin E-cdk2 specifically by acting as a Cy-mimetic. Screens are ongoing. Such Cy-mimetic chemicals will be vital for testing whether inhibition of Cy-cyclin interaction will have additional effects on cdk2 in cancer cells (e.g. prevention of

activation of cdk2) and whether they will show synergistic effects with ATP-mimetic cdk2 inhibitors in vitro.

Technical objective 2. Analysis of the interaction between p21, PCNA and Fen1.

Task 5: Months 1-12: Determination of which part of PCNA interacts with p21 and with Fen1.

The saddle area of each PCNA subunit (interdomain connector loop) was found to contain a hydrophobic pocket that was the site of interaction with both p21 and Fen1

Tasks 6 and 7: Months 24-48: Designing D amino acid based retro-inverse peptides that mimic the PCNA interacting portion of p21. Analysis of their ability to inhibit PCNA in vitro.

Abandoned based on published results that suggest that such peptides will be equally disruptive for PCNA-Fen1 as for PCNA-polymerase delta interactions.

Task 8: Months 36-48: analyze how chemicals that induce p21 in cells inhibit the cdk2.

Statins were found to be potent inhibitors of the cell-cycle and of cdk2 kinase activity. Surprisingly, although the inhibition is associated with the induction of p21, it is not due to the association of p21 with the cdk2. Instead inhibition of cdk2 phosphorylation on threonine160 (T160) accounts for the cdk2 inhibition. This result will set the stage for analyzing whether better statins can be obtained for inhibiting cdk2 and whether such statins would be useful for anti-cancer therapy.

PERSONNEL RECEIVING SALARY FROM THIS GRANT

Anindya Dutta

Nikhil Wagle

David Takeda

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David Garcia Quintana

Partha Saha

Zhi-hui Hou

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APPENDIX

TABLE & FIGURE LEGENDS

Table 1: FACS analysis of PC3 cells in the absence (UNT) and presence (MEV) of mevastatin. Cells were harvested fixed with ethanol and the DNA stained with propidium iodide. The cells were then subjected to FACS analysis for DNA content. Note the significant decrease in S phase population accompanied by an increased G1 population following mevastatin treatment.

Fig. 1 The results of one plate in the high throughput screen is shown. % inhibition of kinase activity is the (rate of kinase reaction in absence of compound – rate in presence of compound) divided by (rate of kinase activity in the absence of any compound). Well B5 contains a compound that significantly inhibits phosphorylation by cyclin E/cdk2.

Fig. 2 Example of a radioactive kinase assay with cyclin E/cdk2 and with protein kinase A. % inhibition is the same as in Fig. 1. Compound A is a non-specific inhibitor of both cyclin E/cdk2 and of protein kinase A. Compound B is a specific inhibitor of cyclin E/cdk2 relative to protein kinase A.

Fig. 3 Example of a compound that specifically inhibits the phosphorylation of CDC6 based peptide substrates that contain Cy motifs (described in Takeda et al.). % inhibition is measured in a radioactive kinase assay as described in Fig. 1 and 2. All three substrates contain SPXK sequence. Cy(WT) has the rest of the peptide sequence including a Cy motif. Cy(Null) has point mutations in the Cy motif so that it is rendered non-functional. Cy(Truncated) has been truncated 5 residues C terminal to the SPXK so that there is no Cy motif and no linker sequence.

Fig. 4 Abundance of cell cycle regulated proteins and CDK2 complex analysis in PC3 cells. **A)** Immunoblot of lysates of PC3 cells in absence (-) and presence of mevastatin (+). 48 hours after addition of mevastatin, cells were lysed and 30 µg of total protein was loaded per lane. While levels of CDK2, p27 and p57 remain relatively unchanged a significant increase in p21 level is noted. **B)** CDK2 complex from PC3 cells. 200 µg of total cellular protein from PC3 cells was immunoprecipitated with an antibody to cdk2. Immunoblots with antibodies to CDK2 and p21 was performed. Note that CDK2 levels remain unchanged (top), while a small increment in associated p21 is noted (middle). In the bottom panel the activity profile of the immunoprecipitated CDK2 with histone H1 as a substrate shows dramatic reduction in activity.

Fig. 5 TGF β -1 enhances p21 levels and its association with CDK2. **A)** 30 μ g of cell lysates derived from cells grown in absence (-) or presence(+) of 80 pM of TGF β -1 were subjected to immunoblot analysis (left). CDK2 level remains unchanged with a significant induction of p21. **B)** Immunoblots following immunoprecipitation with an anti-CDK2 antibody shows an increased amount of p21 associated CDK2 (middle panel) but no significant reduction in kinase activity (bottom).

Fig. 6 Reduced phosphorylation of CDK2 with mevastatin treatment of PC3 cells.

A) Immunoprecipitation of in vivo phosphate labeled lysates with anti CDK2 antibody. After separation on SDS-PAGE gel, proteins were transferred to nitrocellulose and processed for autoradiography (top) and subsequently immunoblotted with anti-CDK2 antibody. Note the decreased phosphate incorporation in CDK2 with mevastatin treatment. The immunoblot confirms equal loading of the two lanes. **B)** Immunoprecipitation of in vivo phosphate labeled lysate with anti-cyclin E antibody. Note the decreased incorporation of radiolabeled phosphate. A parallel experiment with ³⁵S-methionine labeled lysate, immunoprecipitated with anti-CDK2 antibody. Note similar amount of immunoprecipitated CDK2. **C)** Immunoblot with anti-CDK2 after cell lysate is run under conditions that separate the un-phosphorylated and phosphorylated CDK2. Note decrease in phosphorylated CDK2 following mevastatin treatment. **D)** Same as in C) except that cyclin E associated CDK2 is examined specifically by immunoprecipitating with anti-Cyclin E antibody and immunoblot with anti-CDK2 antibody. Note decrease in phosphorylation of CDK2 associated with cyclin E. **E)** Immunoprecipitation of mevastatin treated and untreated lysates with cyclin H antibody (IP cdk7) and subsequent evaluation for activity against GST-CDK2. The kinase activity is retained in mevastatin treated cells (lanes 2 and 4). Immunoprecipitation with non-immune sera (lanes 1 and 3) serve as negative controls.

Table 1: Inhibition of S phase by mevastatin treatment of PC3 cells

	24 hours			36 hours		
	G0-G1 (%)	S (%)	G2-M (%)	G0-G1 (%)	S (%)	G2-M (%)
UNT ^a	58.4	22.4	19.2	62.3	17.1	20.6
MEV ^b	71.8	7.9	20.3	84.3	3.2	12.5

^a PC3 cells in the absence of mevastatin.

^b PC3 cells in the presence of mevastatin

Representative plot of calculated % inhibition from a Single Plate

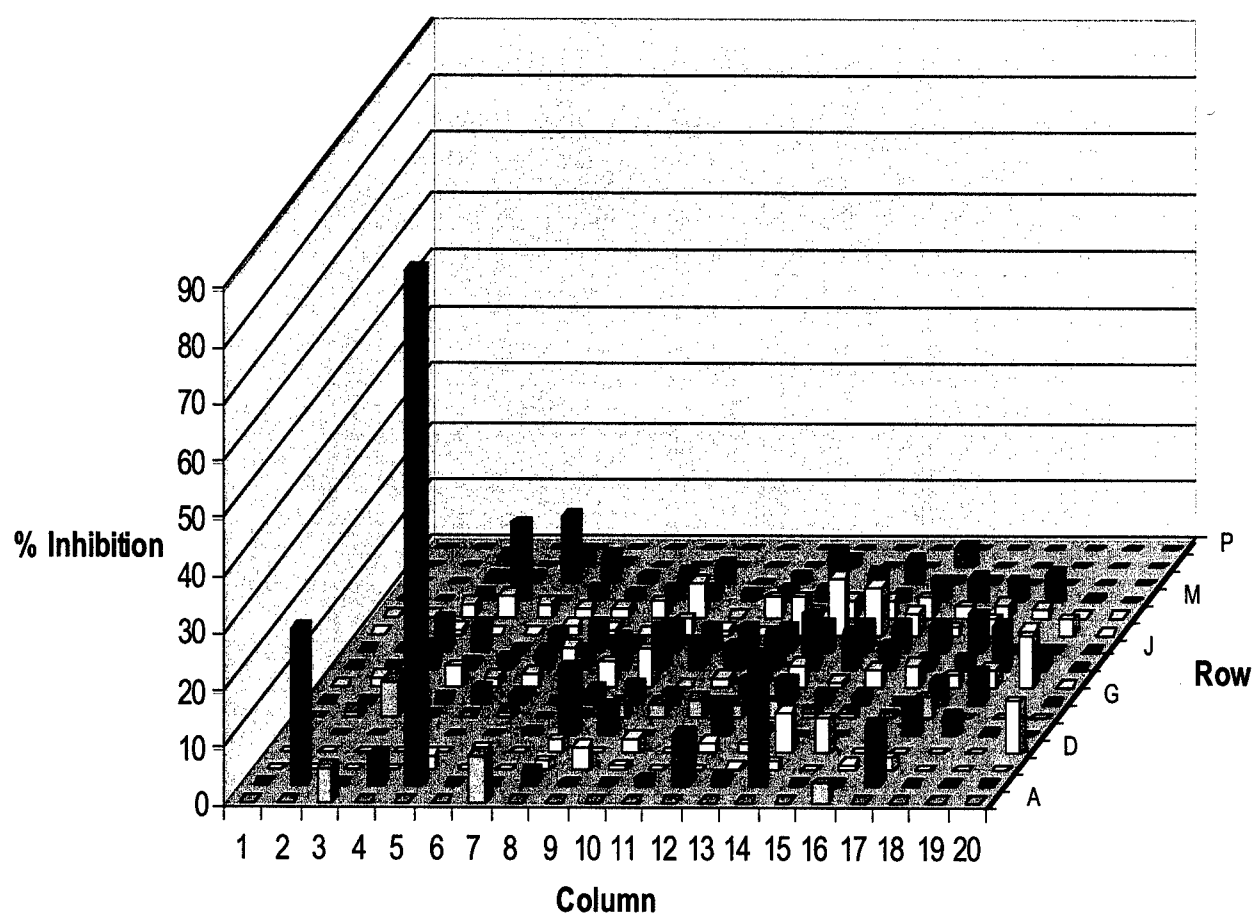


Fig. 1

Example of a compound (B) showing cyclin/Cdk specific inhibition

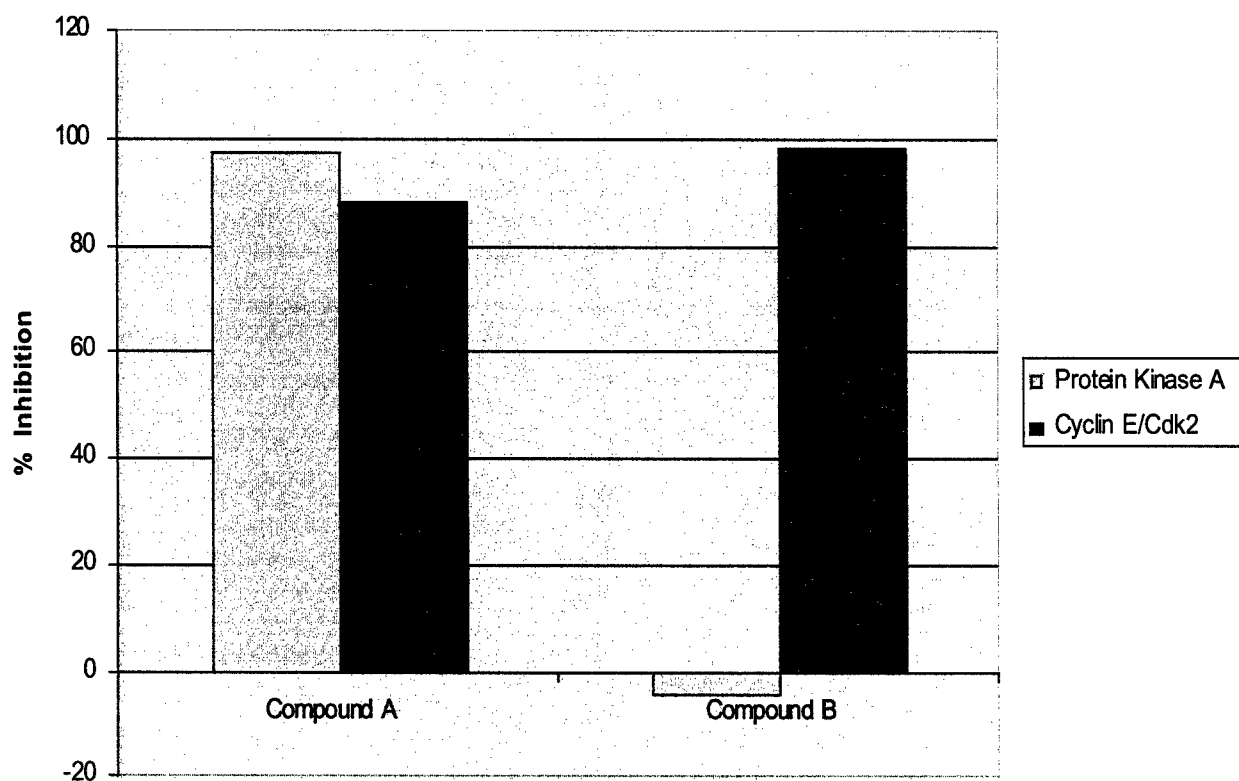


Fig. 2

Compound Z inhibits SPXK substrates with wild type Cy motifs preferentially over other SPXK substrates without Cy motifs

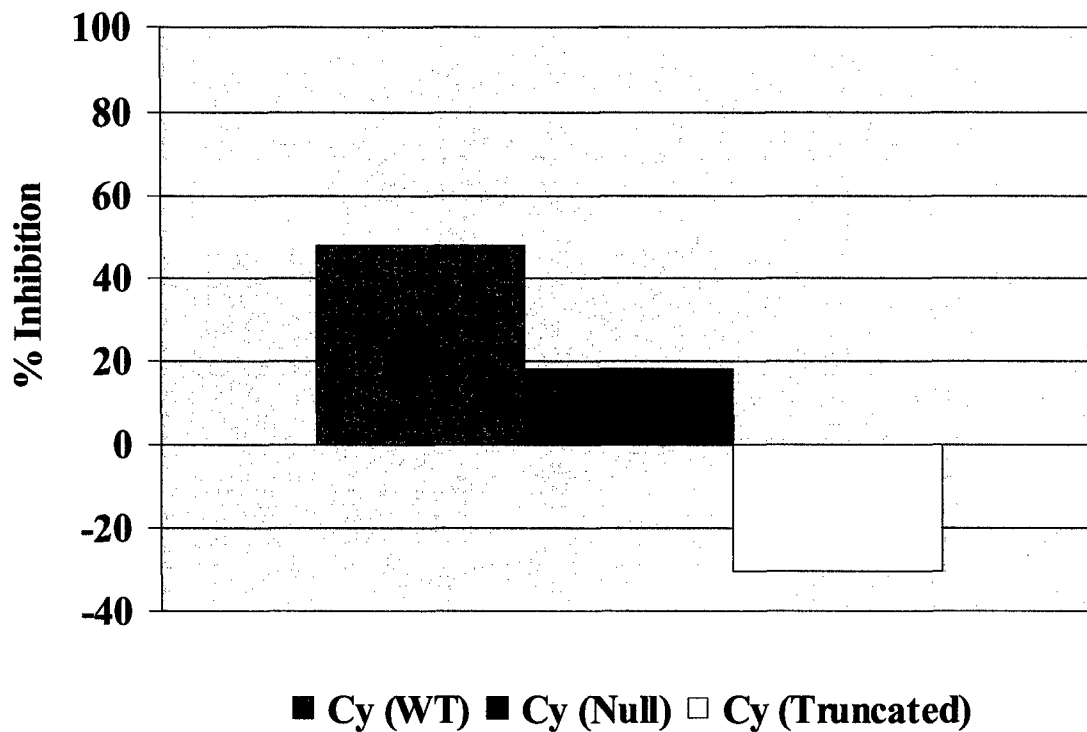
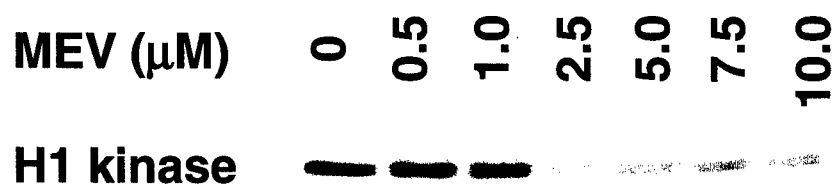


Fig. 3

A)



B)



C)

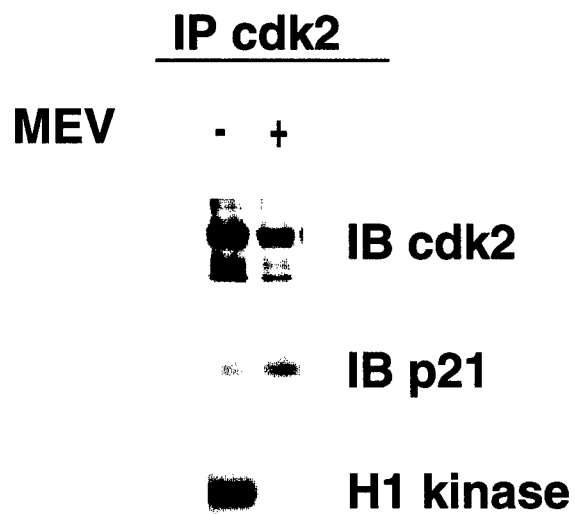


Fig. 4

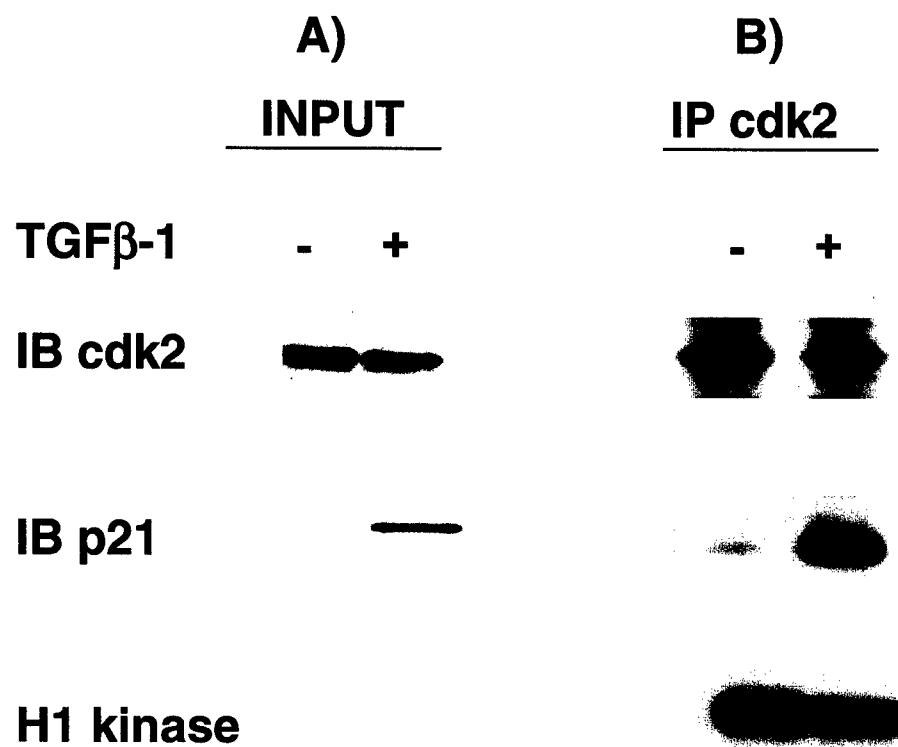


Fig. 5

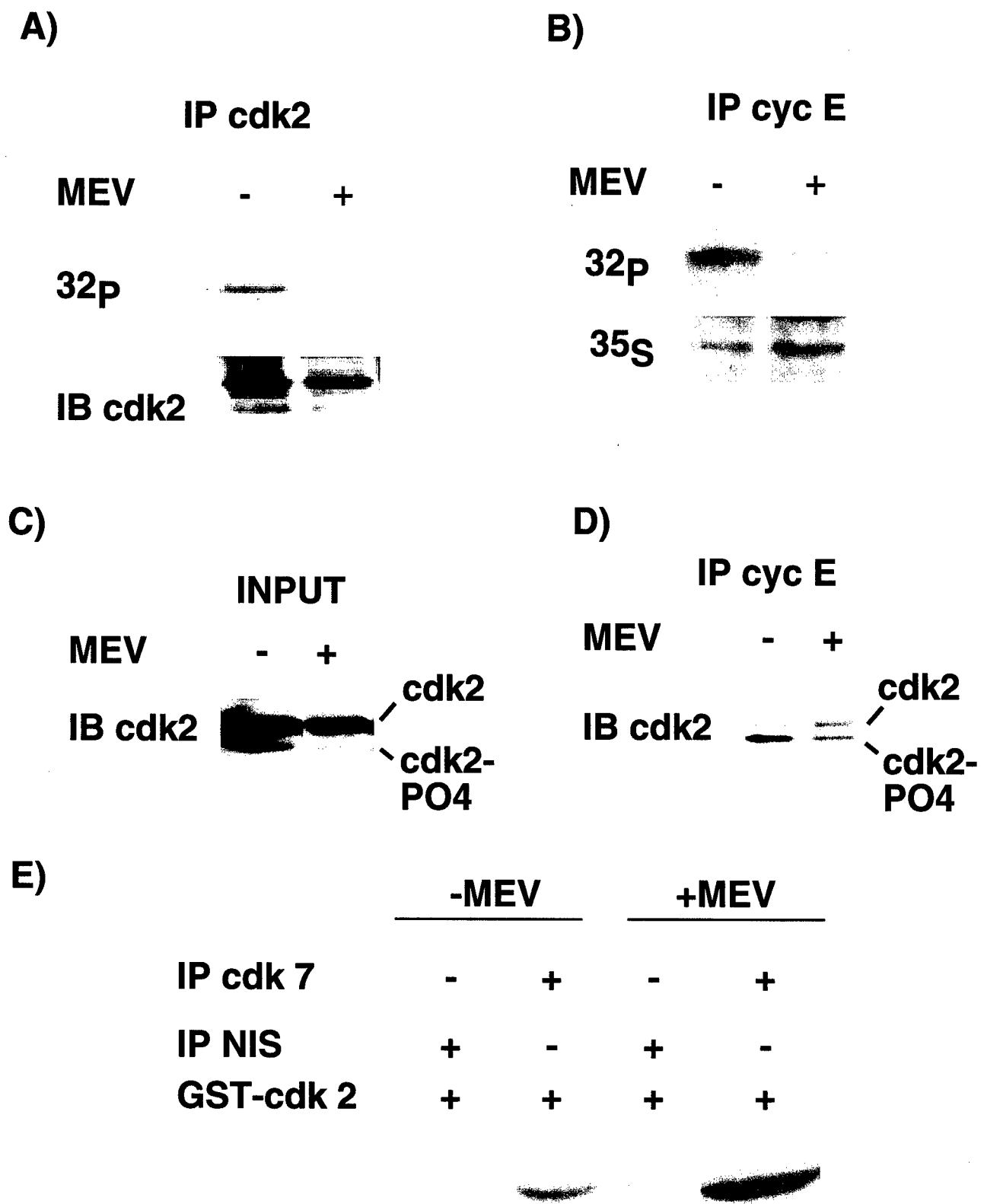


Fig. 6

A Bipartite Substrate Recognition Motif for Cyclin-dependent Kinases*

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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 $K_{m(\text{peptide})}$ 75–120-fold while the k_{cat} remained unchanged. The large effect of the Cy motif on the $K_{m(\text{peptide})}$ suggests that the Cy motif and (S/T)PX(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 a minimal distance from the SPXK is essential for optimal phosphorylation by Cdk and suggest that small chemicals that mimic the Cy motif would be specific inhibitors of substrate recognition by cyclin-dependent kinases.

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-Cdk2¹ 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.

Because the (S/T)PX(K/R) consensus phosphorylation site is broadly applicable to all substrates of all Cdk, 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 motifs (Cy or RXL) have been found in substrates such as E2F and CDC6, activators like Cdc25a and inhibitors of the p21–27 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-Cdk 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 fashion similar to 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 that we propose acts in this fashion is the human replication factor, CDC6. This factor is involved in the formation of a prereplication 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.²

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 the Cy motif and the Cdk phosphorylation site

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¹ The abbreviations used are: Cdk, cyclin-dependent kinases; GST, glutathione S-transferase.

² L. Delmolina and A. Dutta, unpublished result.

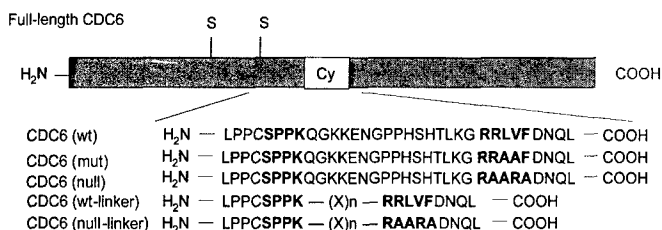


FIG. 1. Schematic of substrate peptides derived from HsCDC6. Peptides were constructed that spanned the consensus Cdk phosphorylation site from residues 74–77 and the Cy motif from residues 94–98. The N-terminal Cdk phosphorylation site and the C-terminal Cy motif are highlighted in **bold**.

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 Pwnica-Worms. Sf9 cells were coinfectected with the appropriate cyclin-Cdk pair and affinity-purified as described previously (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 μ l containing 50 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.02% Triton X-100, 1 μ Ci of [γ - 32 P]ATP, and various concentrations of ATP and peptide dissolved in deionized water. Five different peptide and ATP concentrations were used that ranged from $0.5 \times K_m$ to $5 \times K_m$ for each substrate. Reactions were initiated by the addition of 1 μ l of enzyme diluted in reaction buffer and incubated at 30 $^\circ$ C for 20 min. Reactions were terminated with 1 μ l of 0.5 M EDTA and 10 μ l of the reaction mixture spotted onto a 2- $\times$ 2-cm square of Whatman phosphocellulose P-81 filter paper. Papers were washed in 0.5% H_3PO_4 three times for 5 min, once in 50% EtOH, 0.5% H_3PO_4 for 5 min, and dried under a heat lamp. Incorporation of [γ - 32 P]ATP into the phosphoacceptor peptide was then quantified by liquid scintillation counting of the paper squares. Under these conditions <10% of peptide was phosphorylated upon termination of the 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 k_{cat} and K_m 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 two or six 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 Trp-LE peptide leader sequence. The peptides were then purified to homogeneity as described in Blacklow and Kim (15). Peptide purity was assessed by high performance liquid chromatography and identity was confirmed by matrix-assisted laser desorption/ionization time-of-flight 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 ACRFRGLPVDSE, 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 SPXXK is known to be phosphorylated by cyclin-Cdk complexes *in vitro* and 2) the phosphorylation of this site *in vivo* is dependent upon an intact

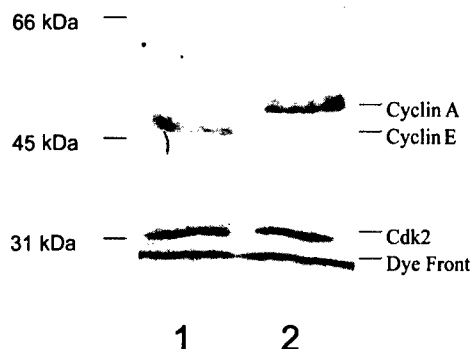


FIG. 2. SDS-polyacrylamide gel electrophoresis of cyclin E-Cdk2 and cyclin A-Cdk2. Purified cyclin E-Cdk2 (lane 1) and cyclin A-Cdk2 (lane 2) were loaded on a 12% gel, and the proteins were stained with Coomassie Blue.

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 *Escherichia 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-polyacrylamide gel electrophoresis 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 Thr-160.

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 that 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 these 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 I.

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 and 7.9 μ M, respectively. Upon mutation of the Cy motif in the N terminus from RRLVF to RAARA, these values increased 75-fold to 145 μ M for cyclin A-Cdk2 and 120-fold to 970 μ 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 μ M and 165 μ M for cyclin A-Cdk2 and cyclin E-Cdk2, respectively,

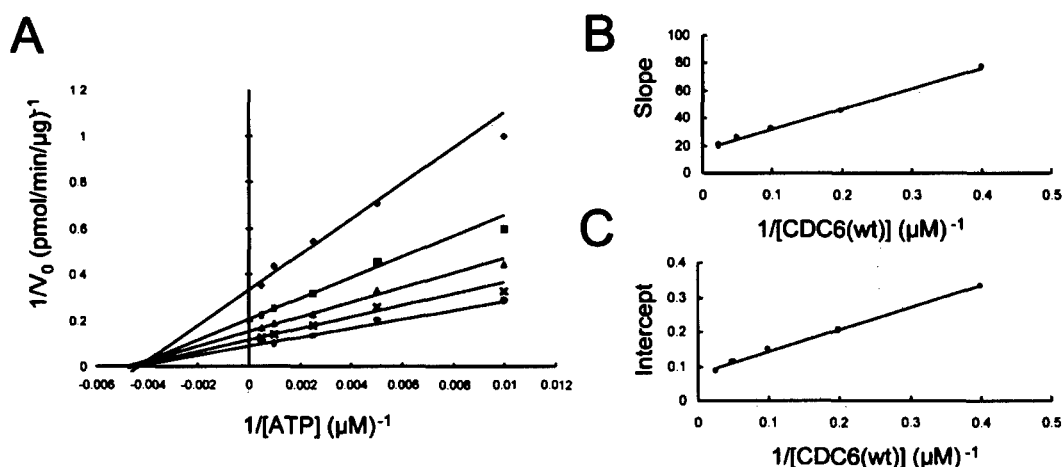


FIG. 3. **Representative initial velocity patterns and secondary plots.** A, initial velocity pattern was determined for cyclin E-Cdk2 using ATP as the varied substrate and the following fixed concentrations of CDC6 wild-type peptide: 2.5 μM ($\blacklozenge$), 5 μM ($\blacksquare$), 10 μM ($\blacktriangle$), 20 μM ($\times$), and 40 μM ($\bullet$). B, secondary plot of primary slopes versus reciprocal peptide concentration. C, secondary plot of primary intercepts versus reciprocal peptide concentration.

TABLE I
Kinetic parameters for cyclin/cdk complexes and CDC6-derived peptides

Units for $K_{m(\text{ATP})}$ and $K_{m(\text{peptide})}$ are expressed in mM while units for k_{cat} are in min^{-1} .

		CDC6 (wt)	CDC6 (mut)	CDC6 (null)
Cyclin E-Cdk2	k_{cat}	86.6 ± 5.0	60.1 ± 11.1	25.0 ± 4.0
	$K_{m(\text{peptide})}$	7.9 ± 0.53	163 ± 34	970 ± 184
	$K_{m(\text{ATP})}$	206 ± 16	165 ± 32	377 ± 18
Cyclin A-Cdk2	k_{cat}	0.82 ± 0.06	2.57 ± 0.16	0.62 ± 0.09
	$K_{m(\text{peptide})}$	1.7 ± 0.33	27 ± 2.4	145 ± 34
	$K_{m(\text{ATP})}$	34 ± 13	84 ± 16	103 ± 16

a 15- and 20-fold increase compared with the wild-type peptide. Thus, this mutation produces a partially functional Cy motif rather than a completely nonfunctional motif.

In contrast to the K_m values for the peptide substrates, the k_{cat} and the $K_{m(\text{ATP})}$ 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 Fig. 4, A and B. 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-Burk plots for these inhibition experiments are shown in Fig. 5, A and B, 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 (K_i) for PS100 which was $7.5 \pm 0.5 \mu\text{M}$ for cyclin E-Cdk2 and $117.5 \pm 11.6 \mu\text{M}$ 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 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 (16). 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 40-Å 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 4 Å/residue, the distance separating the two sites on these substrate peptides would be 8, 24, 48, and 72 Å, 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-Cdk2 and cyclin

FIG. 4. Cy motif-containing peptide selectively inhibits the phosphorylation of only Cy motif-containing substrates by cyclin E-Cdk2 (A) and cyclin A-Cdk2 (B). The Cy motif-containing peptide PS100 is able to inhibit the phosphorylation of 5 μ M CDC6(wt) (●) and 50 μ M CDC6(mut) (○) but not of 1 mM CDC6(null) (◆) substrate.

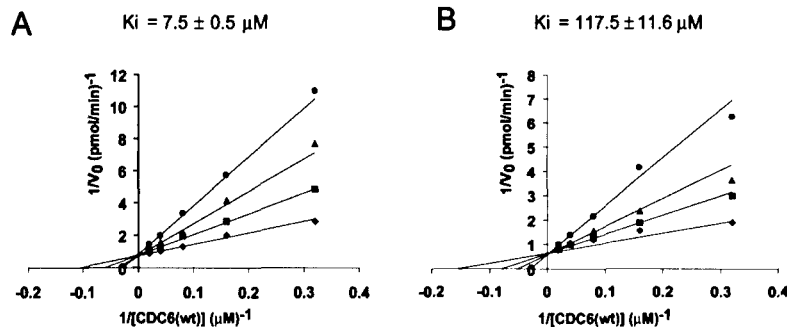
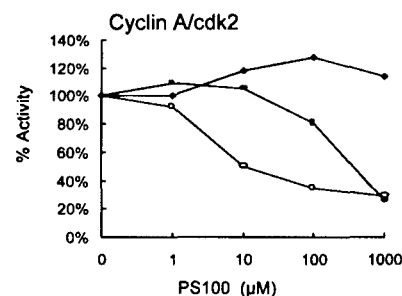
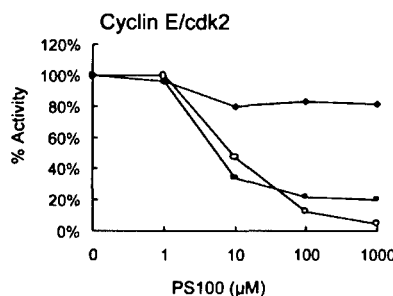


FIG. 5. PS100 competitively inhibits the phosphorylation of the CDC6(wt) substrate by cyclin E-Cdk2 (A) and cyclin A-Cdk2 (B). Initial velocities were determined in the presence of different fixed concentrations of the PS100 peptide: 0 μ M (◆), 6.25 μ M (■), 12.5 μ M (▲), and 25 μ M (●) for cyclin E-Cdk2 and 0 μ M (◆), 100 μ M (■), 200 μ M (▲), and 500 μ M (●) for cyclin A-Cdk2.

E-Cdk2. 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. 6, A and B, 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-Cdk 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 *in vitro* by both cyclin E-Cdk2 and cyclin A-Cdk2 complexes. The measured K_m for the peptide was less than 10 μ M 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 K_m values were no lower than 200 μ M, 100-fold greater than our peptide (17). Because these previously characterized substrates contained only the consensus (S/T)PX(K/R) phosphorylation site, this reduction in K_m 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 *et al.* (17), which defined the sequence requirements of the consensus Cdk phosphorylation site. They showed that a SPPK phosphorylation site, like that present in CDC6, is phosphorylated at <5%

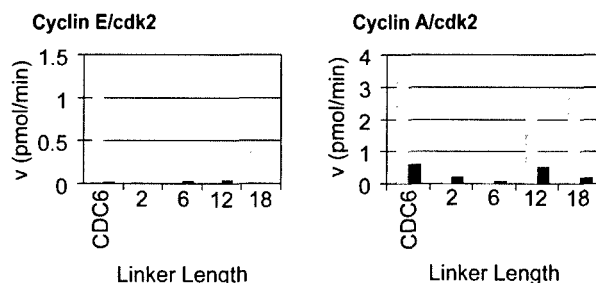


FIG. 6. Phosphorylation of peptide substrates by cyclin E-Cdk2 (A) and cyclin A-Cdk2 (B) is dependent on the length of the linker connecting the N-terminal Cdk phosphorylation site and the C-terminal Cy motif. For each given linker length, the velocities were determined for both the wild-type Cy motif (gray bar) and the null Cy motif (black bar).

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-Cdks occurs through a bipartite recognition sequence on the substrate consisting of both the Cdk phosphorylation site ((S/T)PX(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-Cdks necessarily use the Cy motif-cyclin interaction, peptides or chemicals that mimic the Cy motif are likely to be specific inhibitors of Cdks 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 (18).

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 K_m 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 K_m 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 that 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 ($K_i = 7.5 \mu\text{M}$) than cyclin A-Cdk2 ($K_i = 117.5 \mu\text{M}$). 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 (19). 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 phospho-

rylation 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.

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Mutational Analysis of the Cy Motif from p21 Reveals Sequence Degeneracy and Specificity for Different Cyclin-Dependent Kinases

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Inhibitors, activators, and substrates of cyclin-dependent kinases (cdks) utilize a cyclin-binding sequence, known as a Cy or RXL motif, to bind directly to the cyclin subunit. Alanine scanning mutagenesis of the Cy motif of the cdk inhibitor p21 revealed that the conserved arginine or leucine (constituting the conserved RXL sequence) was important for p21's ability to inhibit cyclin E-cdk2 activity. Further analysis of mutant Cy motifs showed, however, that RXL was neither necessary nor sufficient for a functional cyclin-binding motif. Replacement of either of these two residues with small hydrophobic residues such as valine preserved p21's inhibitory activity on cyclin E-cdk2, while mutations in either polar or charged residues dramatically impaired p21's inhibitory activity. Expressing p21N with non-RXL Cy sequences inhibited growth of mammalian cells, providing *in vivo* confirmation that RXL was not necessary for a functional Cy motif. We also show that the variant Cy motifs identified in this study can effectively target substrates to cyclin-cdk complexes for phosphorylation, providing additional evidence that these non-RXL motifs are functional. Finally, binding studies using p21 Cy mutants demonstrated that the Cy motif was essential for the association of p21 with cyclin E-cdk2 but not with cyclin A-cdk2. Taking advantage of this differential specificity toward cyclin E versus cyclin A, we demonstrate that cell growth inhibition was absolutely dependent on the ability of a p21 derivative to inhibit cyclin E-cdk2.

Progression through the eukaryotic cell cycle requires the activity of a family of kinases known as cyclin-dependent kinases (cdks). cdks are inactive as monomers but become active upon heterodimerization with regulatory subunits known as cyclins. The assembly of these cyclin-cdk complexes is further regulated by the temporal expression of different cyclins so that only certain cyclin-cdk complexes are present during a given phase of the cell cycle. In early G₁ phase of the cell cycle, cyclin D is complexed with Cdk4 or Cdk6 and phosphorylates the retinoblastoma protein (pRb), an early event in the G₁-to-S transition. This is followed by the activation of cyclin E-cdk2 (late G₁) and then cyclin A-cdk2 (S phase), which are responsible for the initiation of DNA replication and progression through S phase. In G₂ phase of the cell cycle, cyclin B-cdc2 begins to accumulate and drives cells through mitosis, at which time the cell cycle is allowed to begin again. These topics have been extensively reviewed (16, 17, 20).

In addition to the temporal control of cyclin-cdk complexes, which restricts their activity to distinct periods in the cell cycle, cells have devised mechanisms for targeting cdks to specific proteins during their window of activity. We first noted this targeting mechanism in structure-function studies of p21; two independent motifs were identified, either of which could target p21 to cyclins and at least one of which was essential for optimal inhibition of kinase activity by p21 (5). This targeting sequence, also known as a Cy or RXL motif, is not limited to inhibitors but also forms the basis of the association of an

activator, the cdc25a phosphatase, with cyclin-cdk complexes (23). Other groups identified a similar targeting sequence on many substrates that associate directly with the cdks (e.g., p107, p130, and Rb) and have inferred that this sequence targets the substrate to the cyclin part of the cyclin-cdk complex (1, 2, 24, 26). Similar Cy motifs have surfaced in the targeting of other cellular proteins to cyclin-cdk complexes, e.g., Myt1 to cyclin B-cdc2 (14), SSeCKS to cyclin D (13), CDC6 to cyclin A-cdk2 (11, 19, 22), human papillomavirus E1 protein to cyclin E-cdk2 (15), and β 3-endonexin to cyclin A (18).

Although the use of Cy motifs as a key mechanism for targeting cellular factors to cdks is now well established, relatively little is known about the exact nature of this motif. Based on sequence conservation among known Cy motifs the core of the Cy motif appears to consist of an arginine and a leucine separated by a single amino acid, giving rise to the moniker RXL motif (Fig. 1). High-resolution structures of the cdk inhibitor p27 complexed with cyclin A-cdk2 and a Cy motif-containing peptide from p107 also complexed with cyclin A-cdk2 suggest that the Cy motif interacts with a hydrophobic groove on the surface of cyclin A (3, 7, 21). It is still not known, however, what amino acids, if any, can be tolerated at different positions of the Cy motif and whether different Cy motifs associate differentially with particular cyclin-cdk complexes. We have recently shown that the presence of a Cy motif on a cdk substrate results in a 100-fold decrease in K_m (25). Such a large contribution to the efficiency of phosphorylation of a substrate makes it likely that most physiological cdk substrates will have a functional Cy motif. Yet a conserved RXL sequence has not been noted in the vicinity of the phosphoacceptor serine in many of the cdk substrates studied, suggesting that an

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	Cy motif				
E2F1	K	R	R	L	D
E2F2	K	R	K	L	D
E2F3	K	R	R	L	E
p107	K	R	R	L	F
p130	K	R	R	L	F
Cdc6	G	R	R	L	V
Myt1	P	R	N	L	S
Cdc25a	P	R	R	L	F
p57	C	R	S	L	F
p27	C	R	N	L	F
p21(N)	C	R	R	L	F
p21(C)	K	R	R	L	I
HPV18 E1	K	R	R	L	F
SSeCKS (1)	L	K	K	L	F
SSeCKS (2)	L	K	K	L	S
b3-endonexin	K	R	S	L	K

FIG. 1. Sequence alignment of known Cy motifs. HPV, human papillomavirus. Conserved residues are in boldface.

RXL sequence is not necessary to form a cyclin-binding Cy motif.

Here we report the results of an extensive mutational analysis of the Cy motif from cdk inhibitor p21. The data indicate that the inhibitory activity of p21 is tolerant of a number of different amino acids in key positions of the Cy motif. We show that this high degree of tolerance is also true for Cy motifs present in cdk substrates. Finally, our results suggest that the mode of binding of p21 with cyclin E-cdk2 is different from its mode of binding with cyclin A-cdk2, and we take advantage of this observation to demonstrate that inhibition of cyclin E-cdk2 is absolutely essential for the growth suppression activity of p21, while the inhibition of cyclin A-cdk2 activity alone is insufficient for this effect.

MATERIALS AND METHODS

Plasmid construction and expression and purification of p21N Cy mutants. Bacterial expression plasmid pGEX-p21N (residues 1 to 90) was constructed as described previously (4). The Stratagene Quikchange system was used to introduce a *HindIII* site upstream of the region of the gene coding for the Cy motif. Oligonucleotide cassettes flanked by *HindIII* and *BspI* sites and containing either the desired mutation or a randomized codon were inserted into pGEX-p21N. The mutations were identified using standard DNA sequencing. The p21N-ΔCy mutant had a deletion that removed amino acids 17 to 24 from p21N. Plasmids encoding the mutants were then transformed into BL21(DE3). The glutathione *S*-transferase (GST) fusion proteins were expressed and purified as before (4, 5). Protein concentrations were determined using the Bio-Rad protein assay.

Expression and purification of cyclin-cdk complexes. All cyclin-cdk complexes used in the *in vitro* kinase assays were expressed and purified by coinfecting Sf9 cells with baculoviruses encoding the appropriate cyclin-cdk pair. Cells were harvested 48 to 72 h later and purified as described previously (8). Baculoviruses expressing GST-cyclin E, GST-cyclin A, cdk2, GST-cdk4, and cyclin D1 were all kind gifts from Helen Pownall-Worms.

The cyclin and cyclin-cdk complexes used in the *in vitro* binding assay were expressed in *Escherichia coli*. The genes for cyclin A and cyclin E were cloned using PCR into pHisTrx (a kind gift from Christophe Briand) and expressed as six-His-thioredoxin (His-Trx) fusion proteins (12). BL21(DE3) cells carrying this plasmid were grown at 25°C, induced with the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM, and harvested 6 to 8 h later. The genes encoding Cdk2 and Cdv1 (gift from Mark Solomon) were also cloned by PCR in the pMM vector (gift from Steve Blacklow) and expressed from a single promoter as a bicistronic message. BL21(DE3) cells carrying this plasmid were grown at 30°C, induced with IPTG, and harvested 24 h later. Recombinant bacterial cyclin-cdk complexes were assembled by mixing bacterial lysates containing either His-Trx-cyclin A or His-Trx-cyclin E and cdk2 and purified using Ni-nitrilotriacetic acid beads as described by the manufacturer

(Qiagen). Additional information regarding plasmid construction and protein purification is available upon request.

Construction and expression of substrate peptides. A plasmid encoding the substrate CDC6(wt) peptide has been described previously (25). Oligonucleotide cassettes were used to replace the Cy motif-encoding region of the CDC6 (wt) peptide expression plasmid with desired sequences allowing expression of CDC6-derived peptides containing either the arginine-to-valine or arginine-to-tryptophan mutations. Peptides were expressed and purified according to previously published methods (S. C. Backlow and P. S. Kim, Letter, Nat. Struct. Biol. 3:758–762, 1996).

Kinase assays. All *in vitro* kinase assays were performed as described previously (25). For cyclin A-cdk2 and cyclin E-cdk2 *in vitro* kinase assays, the CDC6(wt) peptide was used as the phosphoacceptor substrate at a final concentration of 16 μM. For cyclin D1-cdk4 kinase assays, a substrate peptide derived from the CDC6(wt) peptide in which the SPPK phosphorylation site was changed to SPKK was used at a final concentration of 133 μM. The concentration of ATP was 65 μM in these assays. Substrate peptides with the mutant Cy motif were tested for their ability to be phosphorylated by cyclin A-cdk2 or cyclin E-cdk2 using 13 μM peptide and 85 μM ATP.

***In vitro* binding assays.** Purified GST-p21N mutants were mixed with bacterial lysates containing His-Trx-cyclin A or His-Trx-cyclin E or with purified His-Trx-cyclin A-cdk2 or purified His-Trx-cyclin E-cdk2. The GST-p21N-associated proteins were purified on agarose beads coupled to glutathione; washed extensively with a buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, and 0.01% NP-40; and analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoblotting with anti-His antibodies (Santa Cruz Biotechnology) revealed the presence or absence of cyclins in the GST pull-down.

Cell cycle arrest by p21N mutants. Coding sequences for the genes encoding wild-type p21N, p21N-ΔCy, p21N-R19V, and p21N-R19W were cloned into mammalian GST expression plasmid pEBG (23). U2OS cells were cotransfected with 5 μg of the expression plasmid for each p21N mutant and 1 μg of a plasmid encoding farnesylated green fluorescent protein (GFP; pEGFP-F; Clontech) using Lipofectamine-mediated transfection (Gibco BRL). Cells were harvested 48 h after transfection and prepared for fluorescence-activated cell sorting (FACS) as described by Jiang and Hunter (10). A Coulter Epics XL flow cytometer was used to determine the DNA profile of GFP-positive cells, which should also express the p21N plasmid. The cell cycle distribution for a given population was determined using the WinCycle DNA analysis software. All experiments were done in duplicate.

RESULTS

Alanine scanning mutagenesis of Cy motif. To address the role of individual amino acids in the Cy motif-cyclin binding interface, we performed a mutational analysis of cdk inhibitor p21. All our experiments were done with a derivative of p21 containing only the N-terminal 90 amino acids, called p21N. This strategy eliminates a second Cy motif present in the C-terminal half of p21 that is redundant to the first Cy motif in the N-terminal half and so interferes with our functional analysis of the latter (5). Our earlier studies have shown that p21N is sufficient for interacting with and inhibiting cdks and for suppressing cell growth (4). Further, the cdk inhibitors related to p21, p27, and p57 are homologous to p21 only over this N-terminal half, and the crystal structure of the N-terminal half of p27 complexed with cyclin A-cdk2 demonstrates that this region contains all the sequences necessary for interacting with the cyclin-cdk complex.

pGEX-p21N is a bacterial GST expression vector that encodes p21N (residues 1 to 90), which includes the N-terminal Cy motif (residues 19 to 23) and the cdk-interacting domain (residues 53 to 58). Each residue in the core Cy motif from p21 (RRLFG, corresponding to residues 19 to 23) was mutated to alanine by inserting an oligonucleotide cassette into pGEX-p21N. These mutants were then tested for their ability to inhibit the kinase activity of cyclin D10-cdk4, cyclin E-cdk2, and cyclin A-cdk2. We also tested a p21N derivative lacking a

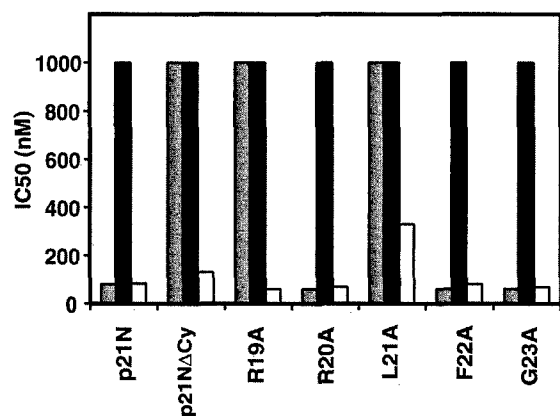


FIG. 2. Alanine scanning mutagenesis of the Cy motif from p21N reveals the importance of R19 and L21 in cdk inhibition. All five residues of the core Cy motif of p21N (RRLFG) were mutated to alanine and tested for their ability to inhibit cyclin E-cdk2 (gray bars), cyclin D1-cdk4 (black bars), and cyclin A-cdk2 (white bars). IC₅₀ are plotted for each mutant.

functional Cy motif, p21N-ΔCy, in which residues 17 to 24, a region overlapping the Cy motif, were deleted. The 50% inhibitory concentrations (IC₅₀) for each of these mutants with different cyclin-cdk complexes are shown in Fig. 2. Wild-type p21N potently inhibited cyclin E-cdk2, whereas deletion of the Cy motif abrogated this inhibitory activity. Similarly, mutations of the conserved arginine and leucine (R19 and L21) to alanine abolished the inhibitory activity of p21N, while mutations in the other positions had little effect, suggesting that R19 and L21 are the critical mediators of cyclin binding. In contrast to what was found for cyclin E-cdk2, the Cy mutants appeared to have little to no effect on p21N's ability to inhibit cyclin A-cdk2, an observation that we address later (see Fig. 7). None of the p21N mutants inhibited cyclin D1-cdk4 at the concentrations tested (up to 1 μM).

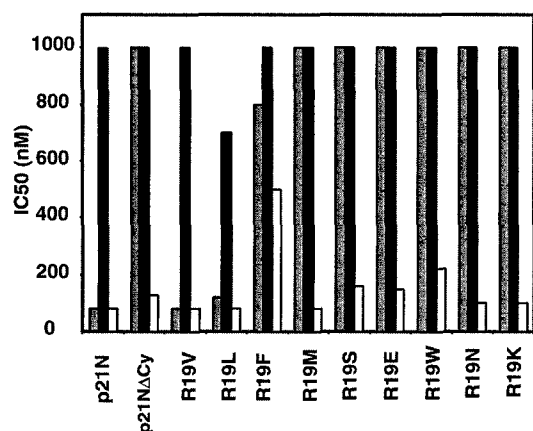
Arginine 19 and leucine 21 mutations. Having shown that R19 and L21 are critical binding determinants for the Cy

motif-cyclin interaction, we created additional mutations at these two sites and monitored their effect on cyclin-cdk inhibition. By inserting mutant oligonucleotide cassettes, in which the codon for either R19 or L21 was randomized, into pGEX-p21N, we created a series of different mutations at each of these two positions. The IC₅₀ for each mutant are shown in Fig. 3.

For both the R19 and L21 positions, we found that only a narrow range of substitutions would allow the p21N mutant to retain its ability to inhibit cyclin E-cdk2. Replacement of R19 with either valine or leucine, both small hydrophobic residues, resulted in functional p21N derivatives with inhibitory activity. On the other hand, insertion of charged or polar residues or large aromatic residues at this position dramatically impaired the ability of p21N to inhibit cyclin E-cdk2 kinase. As for the L21 position, replacement of the leucine with other small hydrophobic residues such as valine or isoleucine preserved p21N's inhibitory activity on cyclin E-cdk2, whereas less-conservative mutations to polar and charged residues abolished it. Although there were strong amino acid preferences for both of these positions, it is important to note that the requirements for arginine and leucine are not absolute, as might be suggested by sequence alignments of known Cy motifs (Fig. 1). In both cases, mutants that maintained the inhibitory activity of p21N were found. As was found with the alanine mutants, none of the R19 or L21 mutants showed a significantly impaired ability to inhibit cyclin A-cdk2 or increased inhibitory activity for cyclin D1-cdk4.

RXL is not sufficient for a Cy motif. In addition to the single-amino-acid substitutions, we also examined a limited set of double mutants. The results from the alanine mutations suggested that residues R20, F22, and G23 were dispensable for the Cy motif-cyclin association. It is possible, however, that each of these residues makes a small but additive contribution to the binding interface that was undetectable using single-amino-acid substitutions but that would become noticeable if we combined two point mutations that had no effect alone. The IC₅₀ of several of these mutants are shown in Fig. 4. For the

A.



B.

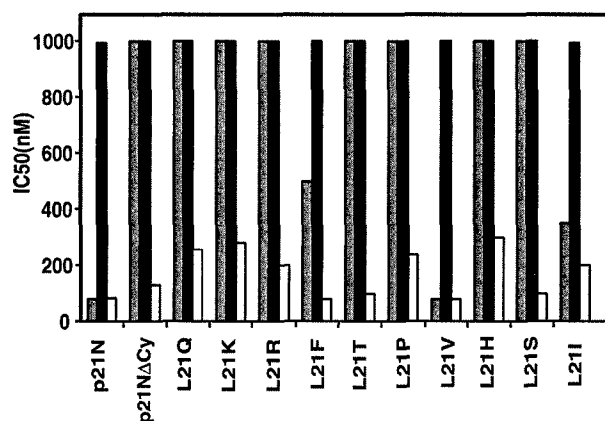


FIG. 3. Mutational analysis of residues R19 and L21 shows limited sequence degeneracy. Residues R19 (A) and L21 (B) were mutated to a number of other amino acids and then tested for their ability to inhibit cyclin E-cdk2 (gray bars), cyclin D1-cdk4 (black bars), and cyclin A-cdk2 (white bars). IC₅₀ are plotted for each mutant.

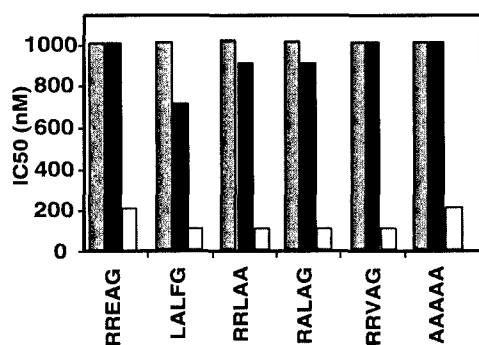


FIG. 4. Analysis of double mutants. A number of double mutations were made in the core Cy motif of p21N and were tested for their ability to inhibit cyclin-cdk complexes. IC₅₀ for each mutant with cyclin E-cdk2 (gray bars), cyclin D1-cdk4 (black bars), and cyclin A-cdk2 (white bars) are plotted for each mutant.

majority of these mutants, we clearly see that the combination of two mutants which had little effect on their own was able to impair p21N's inhibitory activity. For example, mutants RRLAA and RALAG both lost their inhibitory activity on cyclin E-cdk2 even though R20A, F22A, and G23A had no significant effect on their own. From these results, we conclude that the amino acid context in which the RXL residues exist is critical for its high-affinity interaction with the cyclin and that the other positions of the Cy motif play an important role in cyclin binding.

Cell cycle arrest by p21N Cy mutants. To prove that the non-RXL Cy mutants are functional in vivo, we tested a subset of the p21N Cy mutants for their ability to block cell cycle progression in cell culture. Wild-type p21N, p21N-ΔCy, p21N-R19V, and p21N-R19W were expressed from a strong EF1α promoter in EBG plasmids, which were cotransfected with a plasmid encoding farnesylated GFP into U2OS cells. Cells were harvested 40 h later, stained with propidium iodide, and then analyzed by FACS to determine the DNA content of the transfected, GFP-positive cells. The data are shown in Fig. 5. Cells transfected with empty vector showed a normal cell cycle distribution (percentage of cells in G₁ [percent G₁] = 59.7% ± 8.2%), while cells transfected with wild-type p21N were completely blocked in G₁ phase of the cell cycle (percent G₁ = 95.1% ± 1.2%). p21N-R19V, which contains a VXL type Cy

motif, which inhibited both cyclin E-cdk2 and cyclin A-cdk2 in vitro, halted cell cycle progression (percent G₁ = 90.2% ± 5.8%), suggesting that VXL was a functional Cy motif in vivo. p21N-ΔCy, which did not inhibit cyclin E-cdk2 but which did inhibit cyclin A-cdk2 in vitro, did not significantly affect cell cycle distribution (percent G₁ = 58.9% ± 7.9%), suggesting that inhibition of cyclin E-cdk2 was essential for p21N to block cell cycle progression.

Effects of Cy mutants on CDC6-derived peptide substrates. Although Cy motifs have been discovered independently on cdk inhibitors and on substrates, we do not know whether the same attributes of a Cy motif are necessary for docking to the cyclin in the two cases. Further, as pointed out in the introduction, no sequence other than the SPXK sequence has been conserved near the phosphoacceptor serine in traditional cdk substrates, suggesting that Cy motifs on substrates are degenerate in sequence. Because we had variant Cy motifs that were functional in a cdk inhibitor, we could address these issues by testing a few of the variant Cy mutations for their ability to target a substrate for phosphorylation by cyclin-cdk complexes.

We have previously shown that a peptide containing residues 70 to 102 from replication factor CDC6 was a high-affinity substrate for cyclin-cdk complexes and that the efficient phosphorylation of this substrate required an intact Cy motif (25). Using this peptide as a template, we replaced the wild-type Cy motif (RRLVF) with the Cy mutant from p21N (RRLFG) as well as mutants R1V (VRLFG) and R1W (WRLFG). For the negative control, we used the previously characterized CDC6(null) peptide, which contained Cy motif RAARA (25). The rates of phosphorylation of each of these peptides with cyclin A-cdk2 and cyclin E-cdk2 were measured, and the results are shown in Fig. 6. As expected, the CDC6 peptide containing the Cy motif from p21N was phosphorylated very well, 39- and 24-fold better than the null peptide for cyclin E-cdk2 and cyclin A-cdk2, respectively. This shows that although a Cy motif appears nonessential in the interaction of p21N with cyclin A-cdk2, it is required for the efficient interaction of a substrate with this kinase. Likewise, the R19V substrate peptide is phosphorylated 14-fold better than the null peptide by cyclin E-cdk2 and 25-fold better than the null peptide by cyclin A-cdk2. These results are consistent with the

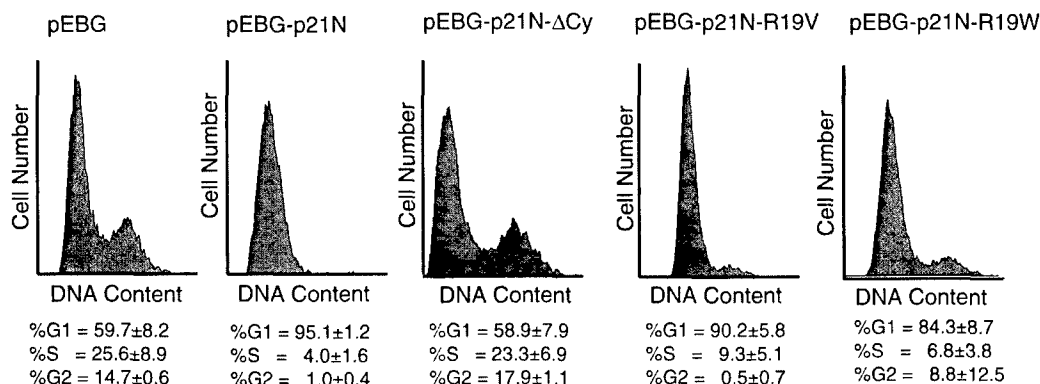


FIG. 5. p21N Cy mutants are able to cause G₁ cell cycle arrest in mammalian cells. Different Cy mutants were overexpressed in U2OS cells and then analyzed by FACS to determine their cell cycle distributions.

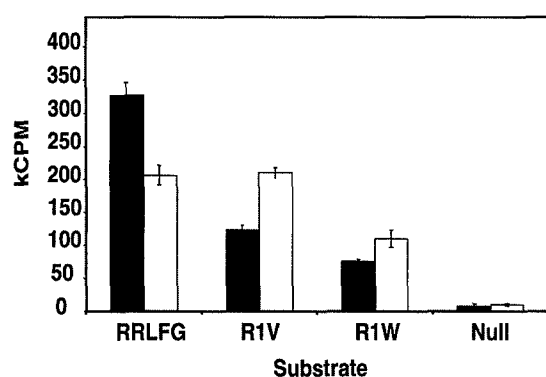


FIG. 6. Analysis of different Cy mutants in substrate peptides shows their ability to target substrates to cyclin-cdk complexes. Phosphorylation of different CDC6-derived substrate peptides by either cyclin E-cdk2 (black bars) or cyclin A-cdk2 (white bars) was measured. Error bars indicate standard deviations from three separate experiments.

FACS data and clearly indicate that non-RXL motifs can act as Cy motifs on substrate.

Gradation of Cy motif activity revealed by R19W mutant. p21N-R19W, which did not inhibit cyclin E-cdk2 in vitro (Fig. 3A), nevertheless halted cell cycle progression (percent $G_1 = 84.3\% \pm 8.7\%$) (Fig. 5). A peptide substrate containing an R19W Cy motif is phosphorylated 9- and 13-fold better than a peptide containing a null Cy motif by cyclin E-cdk2 and cyclin A-cdk2, respectively (Fig. 6). The most likely explanation for these results is that an R19W Cy motif contains a weak cyclin-binding activity such that p21N-R19W is unable to significantly inhibit cyclin E-cdk2 at the highest concentration tested in our in vitro kinase assays ($1 \mu\text{M}$). The cyclin-binding activity of R19W is, however, strong enough to allow p21N-R19W to inhibit cyclin E-cdk2 and suppress cell growth in vivo and to promote the phosphorylation of a substrate by cdk2 in vitro.

Differential binding of p21N Cy mutants to cyclin E-cdk2 and cyclin A-cdk2. In Fig. 2 and 3, we showed that the deletion of the Cy motif of p21N had remarkably little effect on the ability of p21N to inhibit cyclin A-cdk2. This was puzzling considering that the importance of the Cy motif in interactions with cyclin A-cdk2 appears well established, particularly in the crystal structure of p27N complexed with cyclin A-cdk2 (21). One possible explanation was that, despite the crystal structure, the interaction of p21N with cyclin A-cdk2 was not dependent on the presence of a Cy motif, while that with cyclin E-cdk2 was absolutely dependent on the Cy motif-cyclin interaction. To test this hypothesis GST, GST-p21N, GST-p21N- ΔCy , GST-p21N-R19V, and GST-p21N-R19W were tested for their ability to interact with cyclin E and cyclin A in either the presence or absence of cdk2 (Fig. 7).

Only GST-p21N is able to pull down cyclin E alone, suggesting that, although the variant Cy motifs R19V and R19W were functional in other assays, their interactions with cyclin E were not strong enough to survive the washing conditions of a GST pull-down experiment. Consistent with this, GST-p21N-R19V was able to pull down cyclin E-cdk2 complexes while GST-p21N- ΔCy and GST-p21N-R19W failed to do so. Therefore R19V is a functional Cy motif such that its weak interaction with the cyclin, which was not detected when it was incubated

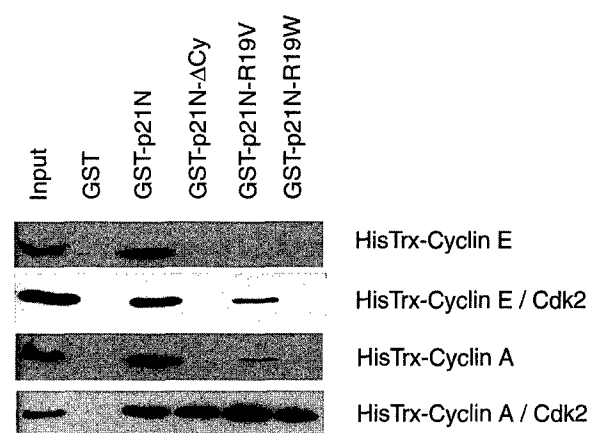


FIG. 7. In vitro binding assays demonstrate differential Cy motif requirements for the binding of p21N to cyclin A-cdk2 and cyclin E-cdk2. Different GST-p21N Cy mutants were tested for their ability to bind cyclin A or cyclin E either alone or in complex with cdk2. Binding reactions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with an anti-His antibody.

with cyclin E alone, was sufficiently strong to allow the rest of p21N to form a stable complex with the cyclin E-cdk2 heterodimer. Even though p21N-R19W was unable to bind cyclin E-cdk2 in these assays, the results of both FACS and substrate phosphorylation data indicate that R19W is capable of functioning as a Cy motif but likely interacts with too low of an affinity for cyclin E to be detected in either the in vitro binding assays or in vitro kinase assays.

We also find that cyclin A alone interacts strongly with wild-type p21N and weakly with p21N-R19V. The fact that both wild-type p21N and p21N-R19V but not p21N- ΔCy bind to cyclin A suggests that the binding of p21N to cyclin A alone requires a functional Cy motif. As discussed earlier, we believe that p21N-R19W contains a partially functional Cy motif and that its inability to interact with cyclin A in this assay is consistent with the affinity of the interaction being lower than that for either the wild-type or R19V Cy motif-cyclin A interaction. This conclusion is supported by the substrate phosphorylation data from Fig. 6, in which the R19W substrate peptide is strongly phosphorylated compared to the null Cy motif peptide but still is phosphorylated less than either the wild-type or R19V peptide.

All of the Cy mutants including p21N- ΔCy are capable of binding to the cyclin A-cdk2 complex. The ability of p21N- ΔCy to pull down cyclin A-cdk2 suggests that the Cy motif-cyclin A interaction is not required for complex formation. Instead, the cdk binding site of p21N is sufficient for its association with cyclin A-cdk2 and occurs regardless of whether or not a functional Cy motif is present. This is in contrast to the association of p21N with cyclin E-cdk2, which required a functional Cy motif in order to form a stable association. The ability of p21N to interact with cyclin A-cdk2 independent of a Cy motif explains why deletion of the Cy motif had no effect on p21N's ability to inhibit cyclin A-cdk2 kinase activity (Fig. 2 to 4).

DISCUSSION

In this study we have used an extensive mutational analysis of cdk inhibitor p21 to define the amino acid requirements of a Cy motif (Fig. 2 to 4). We have further characterized a subset of these Cy mutants to determine their effect on the ability of p21 to inhibit mammalian cell growth (Fig. 5) and their ability to target substrates to cyclin-cdk complexes (Fig. 6). We also show that the Cy motif requirement for kinase inhibition by p21 differs between cyclin E-cdk2 and cyclin A-cdk2 (Fig. 7). Through this detailed characterization of a Cy motif, we have identified a number of interesting features regarding the molecular details of the Cy motif-cyclin interaction.

Based on the sequence alignment of a large number of Cy motifs, Cy motifs have been described as having the pattern ZRXL, where Z and X are predominantly basic residues. Our results suggest that a cluster of basic residues or an arginine and leucine separated by a single amino acid (RXL) should not be the defining characteristic of a functional Cy motif. In our mutational analysis, the conserved arginine (R19 in our study) of a Cy motif can be replaced with a small hydrophobic residue such as valine or leucine with only a small effect on the ability of the mutant protein to inhibit cyclin-cdk activity. This ability to replace a polar amino acid with a hydrophobic residue with little or no loss of activity as well as the importance of having a small hydrophobic residue at residue 21 strongly emphasizes the hydrophobic character of this binding interface. In addition to emphasizing the hydrophobic nature of this binding surface, our data indicate that the amino acid sequence RXL is neither necessary nor sufficient for the formation of a functional Cy motif. The ability of VXL, LXL, or RXV to replace the wild-type RXL with only a small diminishment in p21N's inhibitory activity for cyclin E-cdk2 suggests that RXL is not an absolute requirement for a Cy motif. In Fig. 3, we show that the mutation of wild-type Cy motif RRLFG to either RRLAA or RALAG results in a dramatic decrease in the ability of p21N to inhibit cyclin E-cdk2 even though the RXL pattern is maintained. From this data, we conclude that the defining characteristic of a functional Cy motif is not ZRXL but instead a cluster of hydrophobic residues whose context allows them to adopt the appropriate conformation for interacting with the hydrophobic substrate recognition patch on the surface of the cyclin.

Another interesting feature of cyclin-cdk recognition observed in our study is the differential dependence on the Cy motif-cyclin interaction for p21 to interact with cyclin E-cdk2 versus cyclin A-cdk2. In Fig. 7, we show that the association of p21N with cyclin E-cdk2 requires a functional Cy motif bound to cyclin E to allow subsequent binding of p21N's cdk-binding site to the catalytic cleft of cdk2. The use of the Cy motif as the initial anchor for complex formation, which then allows the cdk-binding site to disrupt the active site of cdk2, was predicted by the high-resolution structure of p27 complexed with cyclin A-cdk2 based on the large number of cdk2 structural rearrangements required by the p27-cdk2 interaction (21). Interestingly, this appears not to be true for the association of p21N with cyclin A-cdk2. Our data indicate that cdk binding by p21N can occur independently of a functional Cy motif. Indeed, all of the Cy motif mutants tested retained their ability to bind and inhibit cyclin A-cdk2. Evidence that this differential Cy motif

requirement for cyclin E-cdk2 and cyclin A-cdk2 is also present *in vivo* can be found in studies looking at the ability of different anti-p21 monoclonal antibodies to immunoprecipitate endogenous p21 complexes from WI-38 fibroblasts (5, 6). In this work, immunoprecipitation of p21 by CP36, an antibody that specifically recognizes the Cy motif, is able to coimmunoprecipitate cyclin A-cdk2 but not cyclin E-cdk2. The most likely explanation for these results is that the antibody disrupts both the p21Cy-cyclin E and p21Cy-cyclin A associations but that p21 is still capable of associating with cyclin A-cdk2 via its cdk-binding site whereas it is unable to bind cyclin E-bound cdk2 using this site since that association is Cy motif dependent.

Although further structural studies will be required to explain the molecular basis for this difference between cyclin A-cdk2 and cyclin E-cdk2, one possibility is that the binding of cyclin A and cyclin E to cdk2 induces different structural rearrangements in the cdk2 molecule thereby altering the interaction of cdk2 with p21 and perhaps other cellular factors. It has already been shown that the binding of a cyclin to cdk2 activates its kinase activity by inducing a number of structural rearrangements in the cdk2 protein including the remodeling of the ATP binding pocket as well as the repositioning of the T loop (9). Although it was believed that these changes only increase the overall catalytic activity of the enzyme, it is possible that these alterations in cdk structure also affect the interaction of cdk2 with p21 and other cellular factors and that the exact natures of the cdk alterations for cyclin A and cyclin E differ.

Although it is well established that different cyclin-cdk complexes including cyclin E-cdk2 and cyclin A-cdk2 have distinct substrate preferences, the molecular basis for this specificity is still unclear. Our results suggest a potential mechanism for generating specificity by targeting proteins to specific cyclin-cdk complexes. By using mutations to modulate the affinity of the Cy motif-cyclin association, we were able to create a series of p21N mutants that are able to inhibit cyclin A-cdk2 but not cyclin E-cdk2. This raises the possibility that cells could use similar mechanisms to selectively target proteins to a specific cyclin-cdk complex. We provide some experimental proof for this in Fig. 6, where we show using initial velocity measurements that an RRLFG Cy motif is a better substrate of cyclin E-cdk2 than of cyclin A-cdk2, but the situation is reversed for VRLFG and WRLFG Cy motifs.

During the mutational analysis, we were able to identify a number of p21 mutants that selectively inhibited cyclin A-cdk2. When a subset of these proteins were expressed in cells, we were able to show that the ability to inhibit cell growth correlated with the ability to inhibit cyclin E-cdk2 but not cyclin A-cdk2. Wild-type p21N and p21N-R19V were both potent inhibitors of cyclin E-cdk2 *in vitro* as well as potent inhibitors of cell growth *in vivo*. In contrast, p21N- Δ Cy lost the ability to inhibit cyclin E-cdk2 *in vitro* as well as the ability to arrest cell growth. Since p21N- Δ Cy is still capable of inhibiting cyclin A-cdk2 kinase activity *in vitro*, it seems likely that p21N's ability to inhibit cell growth depends on its ability to bind and inhibit cyclin E-cdk2. Since we have not identified any mutations that confer selective inhibition of cyclin E-cdk2 but not of cyclin A-cdk2, we are unable to determine whether the inhibi-

TABLE 1. Summary of cyclin E-Cy motif mutant interactions

Cy motif	p21				Substrate promotes phosphorylation
	Inhibition of E/K2 ^a in vitro	Inhibition of cell growth	Association with cyclin E in vitro	Association with E/K2 in vitro	
RRLFG	++	++	++	++	+++
ΔCy	—	—	—	—	—
VRLFG	++	++	—	++	++
WRLFG	—	+	—	—	+

^a E/K2, cyclin E-cdk2.

tion of cyclin A-cdk2 is necessary for p21N's ability to halt cell cycle progression.

Finally, our results, summarized in Table 1, show that the Cy motif-cyclin interaction can have a wider range of affinities than can be appreciated from the conserved RXL sequences identified to date. For substrate phosphorylation, the mutant Cy motifs with the weaker affinities for cyclin E are functional (compared to null Cy motifs), while for the pull-down assay on cyclin E alone only the RRLFG Cy motif is functional. For example, the R19W Cy motif produces a Cy motif-cyclin interaction that is sufficient for phosphorylation of substrates by cyclin A-cdk2 and cyclin E-cdk2 (Fig. 6) and inhibition of cyclin E-cdk2 in vivo (Fig. 5) but is not strong enough to pull down cyclin E or cyclin E-cdk2 in an in vitro binding assay (Fig. 7) or inhibit cyclin E-cdk2 in an in vitro kinase assay at the concentrations tested (Fig. 3A). Appreciation of this dynamic range of the Cy motif-cyclin interaction could be important to those trying to identify physiological substrates for cdk2 in diverse organisms and those seeking pharmacological inhibitors of cdk2 that disrupt the Cy motif-cyclin interactions.

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J.A.W. and B.T.D. contributed equally to this work.

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