A p21\textsuperscript{WAF1/Cip1} Carboxyl-terminal Peptide Exhibited Cyclin-dependent Kinase-inhibitory Activity and Cytotoxicity When Introduced into Human Cells

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ABSTRACT

In the present study, we report the cyclin-dependent kinase (Cdk)-inhibitory activity of a series of p21\textsuperscript{WAF1/Cip1} (p21) peptide fragments spanning the whole protein against the cyclin D1/Cdk4 and cyclin E/Cdk2 enzymes. The most potent p21 peptide tested in our initial peptide series, designated W10, spanned amino acids 139 to 164, a region of p21 that has been found independently to bind to proliferating cell nuclear antigen and also to inhibit Cdk activity. We go on to report the importance of putative \(\beta\)-strand and \(3_\text{ho}\)-helix motifs in the W10 peptide for cyclin-dependent kinase-inhibitory activity. We also describe the cellular activity of W10 and derivatives that were chemically linked to an antennapedia peptide, the latter segment acting as a cell membrane carrier. We found that the W10AP peptide exhibited growth inhibition that resulted from necrosis in human lymphoma CA46 cells. Furthermore, regions in the W10 peptide responsible for Cdk-inhibition were also important for the degree of this cellular activity. These studies provide insights that may eventually, through further design, yield agents for the therapy of cancer.

INTRODUCTION

Cell cycle progression in mammalian cells is regulated by the sequential activation and subsequent inactivation of a series of serine-threonine protein kinases called the Cdks.\textsuperscript{7} Cdks are composed of two subunits: the regulatory unit, cyclin, and the catalytic unit, Cdk (1, 2). There are at least eight cyclin proteins, and they act at different points in the mammalian cell cycle. Cyclins D and E, and probably cyclin C, are required for progression from G\(_1\) to S phase (3–6). Cyclin A also seems to be required for S phase entry (7). Cyclin A and cyclin B, are required for the G\(_2\)-M transition (8). These cyclins complex with Cds, the prototypical member being Cdc2 (Cdk1). Cyclin D1 complexes with Cdk4 and Cdk6, cyclin E and A complex with Cdk2, and cyclins A and B complex with Cdk2 (9).

The Cdks are regulated through cyclin-subunit interaction and phosphorylation and by the binding of a number of low-molecular-weight inhibitor proteins including p21\textsuperscript{WAF1/Cip1} (p21), p27\textsuperscript{Kip1}, p57\textsuperscript{Kip2}, p16\textsuperscript{INK4A}, p15\textsuperscript{INK4B}, Far1, and p53\textsuperscript{SIC1} (2, 10, 11). Human p21\textsuperscript{WAF1/Cip1} was independently isolated by several groups as a Cdk2-interacting protein in a yeast two-hybrid system (\textit{CPI}; Ref. 12), as a p53-inducible transcript that inhibited cellular growth when transfected into cells (\textit{WAF1}; Ref. 13), and as a gene corresponding to a transcript elevated during cellular senescence (\textit{SDI1}; Ref. 14). p21 turned out to be the protein originally reported in Cdk complexes immunoprecipitated from normal human diploid fibroblasts but not from transformed cells (15, 16), and a murine homologue of p21 has also been identified in Cdk2-immune complexes (17, 18). Because of the numerous aliases applied to this Cdk-inhibitor, it is mostly referred to simply as p21. Transcriptional induction of the p21 gene has been linked to G\(_1\) arrest and the inhibition of the cyclin E/Cdk2 kinase after DNA damage in wild-type p53-containing cells (4, 19, 20). Confirmation of this role in p53-dependent G\(_1\) arrest has been presented by Deng et al. (21) and Brugarolas et al. (22), who found that cells from transgenic mice lacking p21 genes exhibited reduced G\(_1\) arrest after exposure to \(\gamma\)-irradiation. p21 can be induced independently of p53 by serum or growth factors (23), and elevated expression of p21 has been found in differentiating cells that lack p53 function (24–27).

p21 shares homology with two other Cdk-inhibitors: p27\textsuperscript{Kip1} (28, 29) and p57\textsuperscript{Kip2} (30, 31). The homology between these inhibitors is especially high in the NH\(_2\) terminus of these proteins, and—consistent with this region being important in Cdk-inhibition—Polyak et al. (28), found that a recombinant truncated p27\textsuperscript{Kip1} protein spanning amino acids 28–79 inhibited cyclin E/Cdk2 activity. In accord with these findings, a number of groups have recently shown that an NH\(_2\)-terminal portion of p21 (amino acids 1–90) inhibited Cdk activity, whereas a separate domain of p21 (amino acids 87–164) bound to PCNA (32–36), a protein involved in DNA synthesis and DNA repair (37, 38). p21-PCNA interaction blocks SV40 DNA replication in vitro, although in some cases sparing the in vitro DNA repair activity of PCNA (39–41). Indeed, our studies and those of others have found that cells lacking p21 exhibit greater sensitivity to some DNA-cross linking chemotherapeutic agents and UV light, possibly because of a DNA repair defect in p21\textsuperscript{−/−} cells (42, 43). In regard to COOH-terminal domain of p21, some groups reported that this region was important for Cdk-inhibitory activity of p21 using peptide fragment or mutant of p21 (44–46). Several reports cite conjugation of p21 peptide segments to the cell internalizing agents, such as the AP homeodomain 16-mer peptide (47), for in-cell studies (46, 48, 49).

To delineate the most potent Cdk-inhibitory peptide region in p21, we synthesized a series of p21 peptide fragments, covering the complete p21 sequence, and measured their inhibitory activity against cyclin D1/Cdk4 and cyclin E/Cdk2 kinases, with the Rbc protein as substrate. Our results show that the most potent Cdk-inhibitory peptide in our p21 peptide series spanned amino acids 139–164 (W10). This region of p21 has been shown to associate with PCNA, and a peptide from this region had previously been shown to inhibit Cdk activity. We explored this peptide further in a mutation analysis and report that a putative \(3_\text{ho}\)-helix motif and a potential \(\beta\)-strand motif within the W10 peptide were important for Cdk-inhibitory activity.

We also report the Cdk-inhibitory activity of the W10 peptide conjugated to the AP carrier peptide (47). The W10AP peptide, when introduced into cells, induced cytotoxicity in the form of necrosis, and we found that the same amino acids that were required for Cdk-inhibition were also required for cell killing.
MATERIALS AND METHODS

Recombinant Proteins. High-performance liquid chromatography purified recombinant p21 protein was a kind gift from Drs. Wade Harper and Steve Elledge (Baylor College of Medicine, Houston, TX). p21 was prepared in bacteria before being stored in 50 mM Tris (pH 7.5), 400 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM DTT, 5 μg/ml leupeptin, and 5 μg/ml aprotinin (12). A plasmid-encoding recombinant GST-Rbc (amino acids 792–928) was kindly provided by Drs. Liang Zhu and Ed Harlow (Massachusetts General Hospital Cancer Center, Charlestown, MA). The GST-Rbc protein was prepared in bacteria before being purified on glutathione Sepharose beads (Pharmacia Biotech). GST-Rbc was stored in distilled water with 10 mM EDTA, 10% glycerol, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 500 μM AEBSF. All of the proteins were stored at −70°C in separate aliquots, and an individual aliquot was thawed only immediately before use.

Synthesis of Peptides. The preparation of the 10 peptides (W1–W10; Fig. 1), spanning the complete amino acid sequence of the human p21 protein, were synthesized and reported previously (50). The COOH-terminal 26-mer mutant peptides, HM1 and AM1 to AM5 (Table 1) were synthesized using Fmoc-based methodology using an ABI/Perkin-Elmer Model 433A synthesizer. Analogous methods were used to prepare the following agents:

(a) peptide W11, corresponding the p21 (141–160)-mer sequence, previously synthesized and evaluated in kinase-inhibitory assays (46);

(b) peptide AP, 16-mer COOH-terminal segment (amino acids 43–58) of the AP homodomain protein (47), which acts as a trans-membrane carrier of our peptides, sequence: RQIKIWFQNRRMKWKK; and

(c) peptide W10AP, the W10 peptide bound at the COOH terminus to AP, sequence: GRKRRQTSMDFYHSKRRLIFSKRKP-RQIKIWFQNRRMKWK.

Two W10AP-mutant peptides were synthesized. These are:

(a) HM1AP, GRKRRQTSGSGSHSKRRIFSKRKP-RQIKIWFQNRRMKWK; and

(b) AM2AP, GRKRRQTSMDFYHSKAAAIFSKRKP-RQIKIWFQNRRMKWK.

The underlined amino acids are the mutated sequence, above. All of the synthetic peptides were purified by high-performance liquid chromatography on a VyDAC C18 column and a gradient of water/acetonitrile containing 0.05% trifluoroacetic acid. The identity of each synthetic peptide was confirmed by fast atom bombardment mass spectrometry and amino acid analysis. Mass spectra were measured in a glycerol or DTT/dithioerythritol matrix on a VG 7070E-HF double focusing mass spectrometer (VG Analytical, Manchester, England). Amino acid analyses were performed at the Protein Structure Laboratory, University of California (Davis, CA).

Cdk Assays. Baculoviral vectors encoding Cyclin D1 and Cdk4 or Cyclin E and Cdk2 were kindly provided by Dr. Gregory Hannon (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). These vectors were used to infect insect Sf9 cells, and cells were subsequently lysed in 50 mM HEPES (pH 7.5), 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 500 μM AEBSF. Five μl of insect cell lysate and 5 μl of the peptide solution (10–1000 μM) were coincubated at 30°C for 30 min. Then 10 μl of a kinase reaction mixture that contained 0.3 μg of GST-Rbc, 30 mM HEPES, 600 μM DTT, 6 mM MgCl2, 6 μg/ml aprotinin, 6 μg/ml leupeptin, and 1.2 mM AEBSF, 7 μM ATP and 10 μCi [γ-32P]ATP (6000 Ci/mmol, NEN) was added. After fifteen min at 30°C, samples were boiled in SDS-gel loading buffer for 5 min and then subjected to electrophoresis on 10% SDS-polyacrylamide gels. For autoradiography, quantitation of 32P, incorporated into substrate, was achieved using a Phosphorimager (Molecular Dynamics, CA).

Cell Culture and Growth Inhibition Assays. The human Burkitt’s lymphoma cell line, CA46, which contains mutant-p53 (51), was grown at 37°C in 95% air/5% CO2 in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) containing 15% FBS (Intergen, Purchase, NY), 2 mM l-glutamine, 50 units of penicillin, and 50 μg/ml streptomycin (Life Technologies, Inc.). For growth inhibition assays, 50,000 cells in exponential growth phase were seeded in a volume of 975 μl RPMI without serum in 24-well plates (Costar, Cambridge, MA) and then 25 μl of distilled water containing increasing concentrations of W10AP peptide was added. After thorough mixing, the plate was incubated at 37°C for 3 h, and then 150 μl of FBS was added to each well. Cells were then incubated for 3 days at 37°C, and, subsequently, cells were counted on a Coulter Counter (Coulter Electronics, Hialeah, FL). Values shown are mean ± SD of three independent experiments that contained duplicate samples within each experiment.

Velocity Flow Cytometry. Exponentially growing CA46 cells were incubated with 6.3 μM of AP or W10AP peptide for 3 h without serum, followed by 72-h incubation with medium containing serum. After this time, cells were analyzed by flow cytometry. Flow cytometry was performed essentially as described previously (51). Briefly, cells were fixed in ice-cold 70% ethanol, washed with PBS (pH 7.4), treated with RNase (Sigma, 500 units/ml) at 37°C for 15 min, and then stained with PI (Sigma, 50 μg/ml). Cell cycle analysis was performed using a Becton Dickinson FACScan flow cytometer. The Cell-Quest software

![Fig. 1. Schematic representation of the domain structure of p21 with synthesized overlapping peptides spanning the complete amino acid sequence of p21. Shown are regions of p21 that participate in binding to and inhibition of cyclin/Cdk complexes. Cyclin binding probably spans amino acids 14–39; Cdk binding probably spans amino acids 42–82 (60). Also shown is a potential domain in p21 that as a peptide, has been shown to bind recombinant p21 protein in ELISA assays (amino acids spanning 21–45; 50). The documented PCNA binding region in p21 (amino acids 139–164) is also shown (53). The two rulers act as a reference and depict the overlapping peptide array used in our studies. The actual amino acid sequence and location of each peptide in the p21 structure is shown in the “Materials and Methods” section.](image-url)
package (Becton Dickinson, Mansfield, MA) was used to analyze data gathered on 15,000 cells for each individual sample.

Electron Microscopy Analysis. For transmission electron microscopy analysis, a cell suspension was washed in PBS, and the cells were fixed in 2.5% glutaraldehyde solution (made in PBS) for 15 min on ice. The fixative was then removed and the pellet was washed in 0.13 M sodium phosphate buffer (pH 7.4). The pellet was postfixed by 1% osmium tetroxide for 1 h and then stained with 2% uranyl acetate for 1 h. The samples were cut 600–800 Å thick on an AO Reichert Ultracut ultramicrotome (Reichert, Germany), and sections were mounted on copper grids. Sections were then stained using lead citrate for 1.5 min. The stained grids were examined in the Zeiss EM10 CA transmission electron microscope (Ziess, Germany).

RESULTS

Effect of Recombinant p21 Protein and p21 Peptide Fragments on Cyclin D1/Cdk4 and Cyclin E/Cdk2 Kinase Activity. We assessed the ability of recombinant p21 protein and p21 peptide fragments covering the complete p21 sequence (Fig. 1) to inhibit cyclin D1/Cdk4 and cyclin E/Cdk2 kinases. In these assays, we used GST-Rhc (Rh amino acids 792–926) as a substrate for each kinase (Fig. 2). Consistent with previous results (52), the p21 protein inhibited both cyclin D1/Cdk4 and cyclin E/Cdk2 to similar degrees (Fig. 2A). The concentration of p21 protein required to inhibit kinase activity to 50% of the control value (IC50) was 0.4 and 0.3 μM for cyclin D1/Cdk4 and cyclin E/Cdk2, respectively (Table 1). Each p21 peptide fragment (W1 to W10) was initially assayed against each kinase at a concentration of 1 μM. Under these conditions, kinase inhibition was observed with peptides W2 to W5, which encompasses the cyclin and Cdk binding sites of p21 (amino acids 15–71). Significantly, peptides W8 to W10, which covered the PCNA binding site of p21 (amino acids 106–184), also exhibited kinase inhibition (Fig. 2). For these active peptides, we performed dose-response curves to establish the relative degree of Cdk-inhibitory potency for each peptide. The most potent peptide in this series was W10 (amino acids 139–164), which contained the PCNA-binding site of p21 (Table 1). We also found that the W10 peptide was approximately 2-fold more potent at inhibiting cyclin E/Cdk2 than cyclin D1/Cdk4; IC50 values for cyclin D1/Cdk4 and cyclin E/Cdk2 were 16 μM and 7 μM, respectively.

Effect of Amino Acid Substitutions on the Cdk-inhibitory Potency of the W10 Peptide. Having established that the W10 peptide was the most potent peptide of the p21 series studied, we set out to define important region(s) within W10 responsible for Cdk-inhibition. To do so, we performed a mutation analysis of this peptide based on a reported crystal structure of a similar p21 peptide (amino acids 139–160) bound to PCNA (Ref. 53; Fig. 3A). In this previous study, the COOH-terminal amino acid residues 152–160 formed a β-strand and interacted with PCNA through ion-pairing and hydrogen-bonding (Fig. 3A). Amino acid residues 146–151 adopted a β7-helical conformation and interacted with PCNA through hydrophobic bonding. Using this analogy, we disrupted the potential β7-helix and β-strand sequences in the W10 peptide by synthesizing mutant peptides. The β7-helix mutant, HM1 (147MTDFY/GSGSG151) exhibited markedly reduced Cdk-inhibitory activity compared with the native W10 sequence. For cyclin D1/Cdk4, the IC50 value for the HM1 peptide was 116 μM compared with 16 μM for the W10 peptide (7-fold reduced activity). For cyclin E/Cdk2, the IC50 value for the HM1 peptide was 25 μM; and for the W10 peptide, the IC50 value was 7 μM (4-fold reduced activity; Fig. 4 and Table 1).

The AM2 peptide (155RRL/AAA157) also exhibited markedly reduced Cdk-inhibitory activity compared with the W10 peptide. For cyclin D1/Cdk4, the IC50 value for the AM2 peptide was 90 μM; and for the W10 peptide, the IC50 value was 16 μM (6-fold reduced activity). For cyclin E/Cdk2, the IC50 value for the AM2 peptide was 128 μM; and for the W10 peptide, the IC50 value was 7 μM (18-fold reduced activity). On the other hand, the single-alanine mutants, AM3 (155RRL/ARL157), AM4 (155RRL/RAL157), and AM5 (155RRL/RRA157) were found to be nearly equivalent to W10 in their ability to inhibit cyclin D1/Cdk4 and cyclin E/Cdk2 (Table 1). The AM1 peptide (142RRQ/AAA144) also exhibited decreased Cdk-inhibitory activity when compared with the W10 peptide. Fig. 4 serves for comparison within each set, in determining the relative effect of various mutations in W10, when assayed with the two separate Cdk/cyclin kinases.

Effect of W10AP-related Peptides on the Growth of Human Lymphoma CA46 Cells and Cdk Activity. To assay the effect of W10 and its mutant peptide analogues on cells, we chemically linked the W10, AM2, and HM1 peptides to a peptide fragment from the homeodomain of AP to act as a cell membrane carrier (Fig. 5A; Ref. 47). The entry of such an AP peptide into cells has been elegantly shown using a fluorescein-labeled AP peptide (48, 54). We assayed the effects of these peptides on the growth of human lymphoma CA46 cells. We initially used a continuous exposure of peptide in the

<table>
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<th>Peptide</th>
<th>Amino acid position</th>
<th>Amino acid sequencea</th>
<th>Cyclin D1/Cdk4 IC50 (μM)b</th>
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[a] Single letter amino acid code.
[b] Concentration required to inhibit kinase activity by 50% of control value.
[c] IC50 value was not reached at maximum concentration used.
[d] W10 peptide with alanine mutation (underlined amino acid sequence).
[e] W10 peptide with mutation disrupting a potential 310-helix motif (underlined amino acid sequence).
presence of medium containing 15% serum and did not observe any cell growth inhibition of CA46 cells (data not shown). However, when we incubated peptides with CA46 cells in serum-free medium for 3 h followed by complete medium we observed a dose-dependent inhibition of growth with the W10AP and the AP peptides (GI50 values of 0.8 μM and 29.5 μM, respectively; Fig. 5B and Table 2). Consistent with intracellular action, the W10 peptide did not show any growth inhibition of CA46 cells up to a concentration of 100 μM (Fig. 5A).

We also found that the AM2AP and HM1AP peptides showed reduced cell growth-inhibitory activity compared with the W10AP peptide (Fig. 5B).

We next verified the in vitro cyclin E/Cdk2-inhibitory activity of the W10AP, AM2AP, HM1AP, and AP peptides using GST-Rbc as substrate (Table 2, second column). The AP peptide by itself exhibited a relatively weak Cdk-inhibitory activity, with an IC50 value of 18.5 μM, in comparison with an IC50 of 0.6 μM for W10AP. Interestingly, conjugation to the carrier peptide, AP, potentiates the inhibitory activity in comparison with the W10 peptide alone (IC50 = 7 μM; Table 1). However, a similar phenomenon was observed also for NH2-terminal p21 peptides conjugated to the AP peptide, by Bonfanti et al. (48). In comparison with W10AP, the two mutant peptide conjugates, AM2AP and HM1AP, exhibited reduced cyclin E/Cdk2 kinase-inhibitory activity. The IC50 values for these peptides were 3.8 μM and 1.8 μM, respectively. Thus, the ratio of the in vitro kinase-inhibitory IC50 values for the W10AP, AM2AP, and HM1AP peptides was 1.0:0.2:0.3, respectively. This ratio is very similar to the ratio of the cell growth-inhibitory effectiveness (GI50) of the same set of three peptide conjugates (1.0:0.3:0.4; Table 2).

Effect of W10AP and AP Peptides on CA46 Cells As Assayed by Flow Cytometry and Electron Microscopy. To further assess the effects of the AP and W10AP peptides on CA46 cells, we performed flow cytometric analysis of cells that had been treated with each peptide at a concentration of 6.3 μM for 75 h. The 6.3-μM peptide concentration was chosen as an intermediate value, above the GI50 for W10AP (0.8 μM) and below the GI50 value of the carrier peptide AP (29.5 μM). We found that W10AP-treated cells exhibited a dramatic change in their SSC profile compared with AP-treated or control cells. This shift in SSC has previously been reported to reflect changes in internal cell granularity consistent with necrosis (55, 56). Supporting the view of necrosis in the treated culture, we found that the degree of PI staining in the SSC-shifted population was also shifted (Fig. 6), most likely reflecting greater chromatin accessibility to PI in the necrotic cells (56). To confirm whether these flow cytometry changes were indeed reflecting necrosis, we assayed the morphology of the cells using electron microscopy (Fig. 7). We indeed found that almost

![Flow Cytometry and Electron Microscopy](image-url)
We describe our findings using a series of p21 peptide fragments covering the complete p21 sequence. We report Cdk-inhibitory activity in p21 peptides spanning amino acids 15–71 (W2, W3, W4, W5) and 106–164 (W8, W9, W10). The most potent peptide tested in our initial peptide series, designated W10, spanned amino acids 139–164, a region of p21 normally bound to PCNA. We go on to report the importance of putative β-strand and 3_10-helix motifs in the W10 peptide for Cdk-inhibitory activity and describe the cellular activity of W10 and derivatives that were chemically linked to an AP peptide sequence to act as cell-membrane carrier (47). We found that the W10AP peptide exhibited potent cytotoxicity in the form of necrosis in human lymphoma CA46 cells and that regions important for Cdk-inhibition were also important for this cytotoxicity.

Although the NH₂ terminus of p21 (amino acids 15–71) was known to contain the Cdk-binding and -inhibitory domain of p21 (40–43, 57, 58), we found that the most potent Cdk-inhibitory peptide tested in our initial p21 peptide series spanned amino acids 139–164 (W10). The Cdk-inhibitory potency of W10 was approximately 35-fold higher than any of the other peptides tested in this series but approximately 23-fold less potent than full-length p21 protein. This region can also bind to PCNA (57, 58); therefore, at first we were surprised by our finding. However, Ball et al. (46) have also found that a p21 peptide fragment spanning amino acids 141–160 was indeed a potent inhibitor of the cyclin D1/Cdk4 kinase. To directly compare our W10 peptide (amino acids 139–164) to the peptide of Ball et al. (46), we synthesized the latter peptide (W11; amino acids 141–160) and assayed it along with W10 against the cyclin D1/Cdk4 and cyclin E/Cdk2 kinases. We found very similar inhibitory activities for the two peptides. Our results agree with Ball et al. (46) and provide new data on their cyclin E/Cdk2 kinase-inhibitory potency. Chen et al. (44) and Adams et al. (45) have also reported that a COOH-terminal peptide of p21 (amino acids 152–158) exhibited Cdk-inhibitory activity. The relative activity of this peptide compared with a complete series of p21 peptides was, however, not fully established in these earlier studies. The fact that the COOH-terminal region of p21 contains a potential Cdk-inhibitory motif is interesting. However, such a region would be expected to be bound to PCNA in exponentially growing cells, titrating out the influence of this region for Cdk inhibition. Whether this COOH-terminal domain of p21 becomes more important for Cdk inhibition in quiescent and/or senescent cells (in which PCNA levels are lowered) remains to be explored.

We went on to investigate the effect of amino acid substitutions on the Cdk-inhibitory activity of the W10 peptide to better define its functionally important region(s). Our mutations were based partly on the reported crystal structure of PCNA bound to a similar p21 peptide (amino acids 139–160; Ref. 53). 45) have also reported that a COOH-terminal peptide of p21 (amino acids 152–158) exhibited Cdk-inhibitory activity. The relative activity of this peptide compared with a complete series of p21 peptides was, however, not fully established in these earlier studies. The fact that the COOH-terminal domain of p21 contains a potential Cdk-inhibitory motif is interesting. However, such a region would be expected to be bound to PCNA in exponentially growing cells, titrating out the influence of this region for Cdk inhibition. Whether this COOH-terminal domain of p21 becomes more important for Cdk inhibition in quiescent and/or senescent cells (in which PCNA levels are lowered) remains to be explored.

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DISCUSSION

Fig. 3. Schematic representation of the crystal structure of a p21 COOH-terminal peptide interacting with PCNA and sequences of mutant W10 peptides used in the present studies. A, structure information, both of these peptides adopted a short 3_10-helix structure followed by a less well-defined COOH-terminal β-strand.

3484

ACTIVITY OF p21 COOH-TERMINAL PEPTIDE ON HUMAN CELLS

Although the NH₂ terminus of p21 (amino acids 15–71) was known to contain the Cdk-binding and -inhibitory domain of p21 (40–43, 57, 58), we found that the most potent Cdk-inhibitory peptide tested in our initial p21 peptide series spanned amino acids 139–164 (W10). The Cdk-inhibitory potency of W10 was approximately 35-fold higher than any of the other peptides tested in this series but approximately 23-fold less potent than full-length p21 protein. This region can also bind to PCNA (57, 58); therefore, at first we were surprised by our finding. However, Ball et al. (46) have also found that a p21 peptide fragment spanning amino acids 141–160 was indeed a potent inhibitor of the cyclin D1/Cdk4 kinase. To directly compare our W10 peptide (amino acids 139–164) to the peptide of Ball et al. (46), we synthesized the latter peptide (W11; amino acids 141–160) and assayed it along with W10 against the cyclin D1/Cdk4 and cyclin E/Cdk2 kinases. We found very similar inhibitory activities for the two peptides. Our results agree with Ball et al. (46) and provide new data on their cyclin E/Cdk2 kinase-inhibitory potency. Chen et al. (44) and Adams et al. (45) have also reported that a COOH-terminal peptide of p21 (amino acids 152–158) exhibited Cdk-inhibitory activity. The relative activity of this peptide compared with a complete series of p21 peptides was, however, not fully established in these earlier studies. The fact that the COOH-terminal region of p21 contains a potential Cdk-inhibitory motif is interesting. However, such a region would be expected to be bound to PCNA in exponentially growing cells, titrating out the influence of this region for Cdk inhibition. Whether this COOH-terminal domain of p21 becomes more important for Cdk inhibition in quiescent and/or senescent cells (in which PCNA levels are lowered) remains to be explored.

We went on to investigate the effect of amino acid substitutions on the Cdk-inhibitory activity of the W10 peptide to better define its functionally important region(s). Our mutations were based partly on the reported crystal structure of PCNA bound to a similar p21 peptide (amino acids 139–160; Ref. 53). Our thinking here was that although the p21 peptide alone has minimal secondary structure in solution (59), it can take up a specific conformation when bound to one of its target proteins, such as PCNA (53). The structure adopted was also similar, at least in the 3_10-helical region, to a proximal NH₂-terminal segment in p27Kip1 that was found inserted into the ATP binding domain of the catalytic cleft of Cdk2 (60). On the basis of X-ray structure information, both of these peptides adopted a short 3_10-helix structure followed by a less well-defined COOH-terminal β-strand.
We hypothesized that, in our studies, the W10 peptide was adopting a structure similar to that seen in the PCNA-p21 peptide crystal structure when it bound to the cyclin E/Cdk2 and cyclin D1/Cdk4 kinases. Indeed, we found peptides with substitutions that disrupted a potential $\alpha$-helix motif ($147\text{ SMTDFY} 151$ changed to $147\text{ SGGSG}$) and a potential $\beta$-strand motif ($153\text{ SKRRLIF} 159$ changed to $153\text{ SKAAAIF}$), and exhibited significantly reduced Cdk-inhibitory activity compared with the native segment W10 peptide (Fig. 4). Less predictably, triple alanine mutation at amino acid positions 142–144 in W10 (mutant peptide AM1) also affected inhibitory potency, possibly destroying ionic interactions with the cyclin/Cdk complex. Our results extend beyond the observations reported in the work of Ball et al. (46) who, using single alanine substitutions, found that the integrity of the $155\text{ RRLIF} 159$ sequence was important for retention of cyclin D1/Cdk4-inhibitory activity. Our data show for the first time that disruption of a potential $\alpha$-helix in the W10 peptide with amino acid substitutions can markedly affect Cdk-inhibitory activity against both cyclin E/Cdk2 and cyclin D1/Cdk4. This region in the W10 peptide was not uncovered in the studies of Ball et al. (46), probably because these workers only used single amino acids substitutions. One possibility is that such single amino acid substitutions may not efficiently disrupt the $\alpha$-helix motif. Again, solution of the X-ray crystal structure of this peptide bound to Cdk would help address this tantalizing possibility.

In our previously published work (50), we failed to observe any cyclin E/Cdk2-inhibitory activity with a number of p21 peptide fragments when using histone H1 as the kinase substrate. The maximum peptide concentration used previously was 30 $\mu$M, for which clear cyclin E/Cdk2 inhibition was observed in the present studies using GST-Rbc as the kinase substrate. We, therefore, compared at the same time the W10 peptide in assays using histone H1 and GST-Rbc as substrates and found that the inhibitory activity of the W10 peptide was 15-fold higher when GST-Rbc was used as the substrate compared with histone H1 (IC$_{50}$ : GST-Rbc $= 5 \mu$M; IC$_{50}$ : histone H1 $= 90 \mu$M; data not shown). Chen et al. (44) found that their p21 peptide fragments were poor kinase inhibitors using histone H1 as a kinase substrate, in accord with our results. Our results and those of others suggest that the substrate used in determining Cdk-inhibitory activity is an important factor in assessing at least peptides in such assays.

In the present study, we also go on to report the effects of the W10
peptide in human cells. To do so, we linked the W10 peptide to the AP peptide sequence of Drosophila to act as a cell membrane carrier (47). We found that the W10AP peptide inhibited the growth of CA46 cells and that substitutions in the W10AP peptide that reduced Cdk-inhibitory activity also reduced growth-inhibitory activity to a similar degree (see Table 2).

These findings suggested that the growth-inhibitory activity of the W10AP peptide series might reflect Cdk inhibition in cells. We performed flow cytometry on the cells and found that the majority of the W10AP-treated culture underwent necrosis after treatment with W10AP. The dominant response of CA46 cells to W10AP at the time of measurement (necrosis) disguised the cell cycle effects of W10AP in CA46 cells. However, earlier studies have shown an accumulation of cells in G1 phase, and in some cases G2 phase also, before cell death with p21 peptides (46, 48, 49, 61) and Cdk inhibitors such as flavopiridol (62–64). Our studies are complemented by similar studies of Bonfanti et al. (48) using flow cytometric DNA analysis of human ovarian cancer cell lines treated with NH2-terminal Cdk-inhibitory peptide segments conjugated to AP carriers. They used, however, different cell treatment protocols and much higher concentrations of peptides for cell treatment. Flow cytometric DNA analysis did not bring to light any phase-specific arrest induced by active concentrations of the NH2-terminal peptide conjugates but, rather, a blockade of the cell cycle progression in all of the phases. This is consistent with the generalized block of activity of the various Cdk isoforms by p21 that act

### Table 2 Comparison of the inhibitory potency of a series of p21 peptides in cyclin E/Cdk2 kinase and CA46 cell growth assays

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC50</th>
<th>GI50</th>
<th>IC50 of W10AP</th>
<th>GI50 of W10AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>W10AP</td>
<td>0.6</td>
<td>0.8</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>AM2AP</td>
<td>3.8</td>
<td>2.6</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>HM1AP</td>
<td>1.8</td>
<td>1.8</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>AP</td>
<td>18.5</td>
<td>29.5</td>
<td>0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>

a IC50 value is the quantity of peptide (μM) required to reduce cyclin E/Cdk2 kinase activity to 50% of the untreated control.

b GI50 value is the quantity of peptide (μM) required to inhibit CA46 cell growth to 50% of the untreated control.

c X represents each p21 peptide tested (W10, AM2, HM1, W10AP, AM2AP, HM1AP, or AP).

Fig. 6. Effect of the W10AP and AP peptides on cell cycle progression of CA46 cells. Exponentially growing CA46 cells were incubated with W10AP or AP peptide (6.3 μM) without serum for 3 h, followed by an additional 72 h of incubation with medium containing serum (15% FBS). Cells were prepared for flow cytometric analysis as described in “Materials and Methods.” A, laser scattering dot-plots of CA46 cells treated with the AP or W10AP peptide. The ordinate represents SSC and the abscissa represents forward scatter. Gates R1 and R2 were set to isolate, for analysis, the two distinct cell populations shown. B, DNA histograms of CA46 cells treated with the AP or W10AP peptides. Upper panel, the DNA histograms of the total population of cells; the other panels, cells in either Gate R1 or in Gate R2. The broken line at 200 designates 2N (diploid) DNA content and the broken line at 400 represents 4N, or doubling of DNA content, in untreated cells.
at various phases of cell cycle progression, and, thus, block cells in both G₁ and G₂ phases.

Necrosis induced by the W10AP peptide in our study may be attributable to Cdk-inhibition because a correlation was uncovered between the degree of Cdk-inhibitory activity and cell death using a series of the W10AP mutant peptides (Table 2). However, other mechanisms must be considered because the same W10 peptide also binds to PCNA. More recent results suggest that p21 may inhibit cell cycle progression by two independent mechanisms: (a) inhibition of cyclin/Cdk complexes; and (b) inhibition of PCNA function resulting in both G₁ and G₂ arrest (34, 49). Also, the COOH-terminal p21 domain may function to capture PCNA and, therefore, to disassemble it from DNA repair sites after DNA repair has been accomplished (65). Furthermore, Chuang et al. (66) recently reported that a peptide with a similar sequence to the COOH-terminal region of p21 protein disrupts the interaction of PCNA with DNA-(cytosine-5) methyltransferase, an enzyme involved in DNA replication and DNA repair. Thus, at the present time, we are cautious about ascribing the necrosis induced by the W10AP peptide solely to Cdk inhibition. Additional studies would be necessary to prioritize the importance of each mechanism in the cytotoxicity observed.

In summary, we describe our findings using a series of p21 peptide fragments covering the complete p21 sequence. We report that the most potent peptide tested in our initial peptide series, designated W10, spanned amino acids 139–164 of p21. We go on to report the importance of putative β-strand and 3₁₀-helix motifs in the W10 peptide for Cdk-inhibitory activity and describe the cellular activity of W10 and derivatives that were linked to an AP peptide sequence to act as cell membrane carrier. We found that the W10AP peptide exhibited potent cytotoxicity in the form of necrosis in human lymphoma CA46 cells and that regions important for Cdk inhibition were also important for this cytotoxicity. The actions of the COOH-terminal domain of p21 are thus complex, with PCNA binding influencing a potential Cdk-inhibitory sequence that requires further exploration. Indeed, these studies provide insights into the actions of a peptide derived from p21 that may, through further design, potentially yield additional Cdk-inhibitory molecules.

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REFERENCES

A p21Waf1/Cip1 Carboxyl-terminal Peptide Exhibited Cyclin-dependent Kinase-inhibitory Activity and Cytotoxicity When Introduced into Human Cells

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