Identification of an Hexapeptide That Binds to a Surface Pocket in Cyclin A and Inhibits the Catalytic Activity of the Complex Cyclin-dependent Kinase 2-Cyclin A*

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The protein-protein complexes formed between different cyclins and cyclin-dependent kinases (CDKs) are central to cell cycle regulation. These complexes represent interesting points of chemical intervention for the development of antineoplastic molecules. Here we describe the identification of an all D-amino acid hexapeptide, termed NBII, that inhibits the kinase activity of the cyclin-dependent kinase 2 (cdk2)-cyclin A complex through selective binding to cyclin A. The mechanism of inhibition is non-competitive for ATP and non-competitive for protein substrates. In contrast to the existing CDKs peptide inhibitors, the hexapeptide NBII interferes with the formation of the cdk2-cyclin A complex. Furthermore, a cell-permeable derivative of NBII induces apoptosis and inhibits proliferation of tumor cell lines. Thus, the NBII-binding site on cyclin A may represent a new target site for the selective inhibition of activity cdk2-cyclin A complex.

The interactions between proteins represent points of chemical intervention for therapeutic gain in the biological processes associated with disease. Because of the specificity of individual protein-protein interactions, the modulation of these complexes is viewed as a putative source of future new highly selective drugs (1–3). The early experience in the design of peptides and small non-peptidic compounds that bind to protein interfaces (2, 4) encouraged the viability of protein-protein interactions as target for drug discovery. In particular, there has been much progress in the discovery of small molecule inhibitors of the unregulated cell growth that characterize cancer cells (5). Proliferation of eukaryotic cells is under control of a series of concerted molecular mechanisms defined as the cell division cycle whose progression is tightly governed by members of the cyclin-dependent kinase family (CDKs)3 (6, 7). Thus, CDKs mediated phosphorylation of the tumor suppressor protein pRb is required for inactivation of the protein and for the subsequent release of E2F transcription factors, thus altering the status of E2F-regulated genes from fully repressed to induced and leading progression through G1 to S phases (8, 9). The pRb-E2F pathway is frequently deregulated in cancer cells. E2F transcription factors are the preferential pRb targets, however, their binding to DNA is also regulated by cdk2-cyclin A activity. E2F phosphorylation by cdk2-cyclin A inhibits its binding to DNA leading to the end of the transcription of E2F-regulated genes produced at the beginning of mitosis (10, 11). It has also been shown that E2F overexpression increases cell proliferation but paradoxically, it simultaneously also triggers apoptosis (12–14). All this information suggests that pharmacologic inhibition of cdk2-cyclin A might be of potential interest as an antineoplastic strategy because cancer cells show an increased activity of the E2F due to pRb inactivation. Furthermore, cdk2-cyclin A complex is also required for S phase progression regulating the pre-replicative complex formation and activation (15, 16). Moreover, cyclin A is revealed as an essential gene because its deletion results in embryonic lethality shortly after implantation (17, 18).

The protein-protein complexes formed between different cyclins and cdks (we will refer to the complexes here as CDKs) are central to cell cycle regulation. These complexes and their natural inhibitors defined as CDK inhibitor proteins (CKIs) have been the object of extensive research. Considerable effort has been focused on the development of ATP-competitive small molecule inhibitors of CDKs (19). However, in general, these compounds have several alternative protein targets that compromise their demanded selectivity. Then, new strategies for the inhibition of CDKs activity through blocking of substrate recruitment (20, 21) and more recently, through interference with protein-protein conformational changes (22) have been proposed. The cyclin recruitment motif (CRM) is a binding domain for a large number of cdk2-cyclin A substrates, as for instance, the transcription factor E2F1 (10), the tumor sup-

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3 The abbreviations used are: CDK, cyclin-dependent kinase; CKI, CDK inhibitor proteins; CRM, cyclin recruitment motif; GST, glutathione S-transferase; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; PARP, poly(ADP-ribose) polymerase; Ac, acetyl; MCM2, minichromosome maintenance 2.
pressors pRb (23) and p53 (24), the CKIs p21\(^{CIP1}\) and p27\(^{KIP1}\) (23, 25) and other substrates as MDM2 (26). All these substrates contain a highly homologous region of 12 amino acids, which includes the sequence RXLYY\(^{\gamma}\), where \(X, Y,\) and \(\gamma\) are any and hydrophobic amino acids, respectively, responsible for the binding to the CRM.

We are currently engaged in a discovery program that targets the complex cdk2-cyclin A with the aim of finding new lead compounds that could be developed in safety and selective antineoplastic drugs. Here we describe the identification of an hexapeptide that inhibits the kinase activity of the cdk2-cyclin A complex through selective binding to cyclin A. The characterization of the inhibitory mechanism revealed that the hexapeptide is non-competitive for either ATP or histone H1. A cell permeable derivative of the hexapeptide induces apoptosis and inhibits proliferation of tumor cell lines.

**EXPERIMENTAL PROCEDURES**

**Preparation of Synthetic Combinatorial Libraries and Individual Peptides**—The dual defined position library, Ac-XXOXXX-NH\(_2\) was synthesized in an iterative format using the process of divide, couple, and recombine in conjunction with simultaneous peptide synthesis. All individual peptides were prepared using simultaneous multiple peptide synthesis. Peptide mixtures and individual peptides were solubilized in H\(_2\)O or 5\% Me\(_2\)SO/H\(_2\)O aliquoted and stored at \(-20\,^{\circ}\)C. Individual peptides were characterized by laser desorption time-of-flight mass spectroscopy analyses and purified by preparative reverse-phase-high performance liquid chromatography.

**Protein Expression and Purification**—Cdk2, cyclin A full-length, and cyclin A fragment (A1 amino acids 1–88; A2 amino acids 1–171; A3 amino acids 1–257; A4 amino acids 1–345; A5 amino acids 171–432; and A6 amino acids 257–432) cDNAs were cloned into pGEX-6P plasmid and a fragment of pRb, fpRb (amino acids 792–928), and p21\(^{CIP1}\) were cloned into pGEX-KG-2T plasmid. All were glutathione S-transferase (GST) fusion proteins and were expressed in *Escherichia coli* BL21(DE3). Cells were grown at 37 \(^{\circ}\)C with shaking (200 rpm) to mid-log phase (\(A_{600} = 0.8\)) Expression was induced by the addition of isopropyl 1-thio-\(\beta\)-\(\delta\)-galactopyranoside at a final concentration of 0.5 mM and the culture was incubated for a further 4 h at room temperature. Bacteria were harvested by centrifugation, and the cell pellet was resuspended in NENT buffer (20 mM Tris, pH 7.4, 250 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 0.1% Tween, and 0.02% sodium azide) overnight at 4 \(^{\circ}\)C. Plates were washed 3 times with 100 \(\mu\)l of washing solution (PBS containing 0.02% Tween and 0.02% sodium azide). Plates were then dried during 2–4 h at room temperature, and stored at 4 \(^{\circ}\)C. Under these conditions, they are stable for 1 month. Assays were performed in a final volume of 60 \(\mu\)l of kinase buffer (25 mM Hepes, pH 7.4 and 10 mM MgCl\(_2\)) containing 4 \(\mu\)g of histone H\(_1\) (Roche Applied Science), 30 \(\mu\)M ATP, 2 mM dithiothreitol, 0.2 \(\mu\)Ci of [\(\gamma\)-\(32\)P]ATP (Amersham Biosciences, 3000 Ci/mmol, 10 mCi/ml), 800 nm GST-cdk2, and 800 nm GST-cyclin A. Assays were carried out in the presence or absence of different concentrations of peptide mixtures to be checked. An inhibitory control was performed adding 800 nm GST-p21 to the reaction media. Mixtures were incubated for 30 min at 37 \(^{\circ}\)C. After incubation, 50 \(\mu\)l of each mixture were filtered in nitrocellulose membranes placed in a dot blot apparatus. Samples were washed with 35 \(\mu\)l of 10\% trichloroacetic acid, and finally with two washes of 100 \(\mu\)l of 10\% trichloroacetic acid followed by 100 \(\mu\)l of H\(_2\)O. After this process, membranes were dried at room temperature. The radioactivity associated to the membranes was detected with a Molecular Imager FX; Bio-Rad Laboratories, Inc.

**Determination of cdk1, cdk2, and cdk6 IC\(_{50}\)**—Assays were performed in duplicate in a 30-\(\mu\)l final volume containing 2 \(\mu\)g of histone H\(_1\) or histone H\(_1\)-derived peptide, pepH1 (PKTP-KKKKL) as a substrate and the CDK complex (25 ng of cdk2-cyclin A, 25 ng of cdk2-cyclin E, 20 ng of cdk1-cyclin B1, or 25 ng of cdk6-cyclin D3), all purchased to Upstate Biotechnology, in kinase buffer (25 mM Hepes, pH 7.4, 10 mM MgCl\(_2\), 2 mM dithiothreitol, 30 \(\mu\)M ATP, and 0.2 \(\mu\)Ci of [\(\gamma\)-\(32\)P]ATP). Reactions were incubated 30 min at 37 \(^{\circ}\)C and then were stopped by transferring 25 \(\mu\)l of each on Whatman P81 phosphocellulose paper. The filters were washed three times with 1% phosphoric acid, air dried, and finally counted in liquid scintillation equipment (Wallac). In the substrate competitive assays the sequence of peptide inhibitor (referred to as pepH1-T3V) is PKVPKKAKKL.

In some assays, the GST-pRb protein fragment, fpRb (amino acids 792–928), was used as a substrate. In these cases, the reaction was stopped by adding 10 \(\mu\)l of Laemmli buffer. Samples were solved in a 10% SDS-PAGE gel and then the gel was dried. The radioactivity associated to the gel was detected with a PhosphorImager (GE Healthcare). In the assays describing a non-competitive action to the CRM of cyclin A, the pep21 (FYHSKRLIFS), derived from p21\(^{CIP1}\) that targets the CRM, was used.

**Immunoprecipitation and CDK Activity Assays**—HCT116 cells were lysed in IP buffer (50 mM Hepes, pH 7.4, 250 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 0.1% Tween 20, 0.1 mM NaF, 10% glycerol, 10 mM \(\beta\)-glycerophosphate, 1 mM dithiothreitol, 0.1 mM Na\(_3\)VO\(_4\), 1 mM phenylmethylsulfonyl fluoride, 0.5 \(\mu\)g/mL aprotinin, 10 \(\mu\)g/mL leupeptin) for 40 min on ice. Lysates (0.25–0.5 mg of protein) were incubated with 1 \(\mu\)g of anti-cdk1 (Santa Cruz sc-54), anti-cdk2 (Santa Cruz sc-163), or anti-cdk4 (Santa Cruz sc-749) overnight at 4 \(^{\circ}\)C. Then, 40 \(\mu\)g of protein A/G-agarose beads (Pierce) were added and samples were incubated for 1 h at 4 \(^{\circ}\)C. After three washes in IP buffer and two in kinase buffer (50 mM Hepes, pH 7.4, 2.5 mM EGTA, 10 mM MgCl\(_2\)) immunoprecipitates were suspended in a final volume of 30 \(\mu\)l of kinase buffer containing 15 \(\mu\)M ATP, 10 \(\mu\)Ci of...
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[^32P]ATP, 2 mM dithiothreitol, and 3 μg of histone H1 (for cdk2 and cdk1) or 3 μg of pRb (for cdk4 assays) for 30 min at 30 °C. Reactions were stopped by the addition of Laemmli buffer. The samples were then electrophoresed on a 12% SDS-polyacrylamide gels and then stained with Coomassie Blue and dried. The radioactivity associated to the gels was detected with a PhosphorImager.

Determination of CK2 Activity—Protein kinase CK2 activity was assayed as described previously (27) using 4 mg/ml β-casein, and β-casein peptide only phosphorylated by CK2 as substrates. One unit of protein kinase activity is defined as the amount that catalyzes the transfer of 1 nmol of phosphate from [γ-^32P]GTP to β-casein per min at 30 °C.

Determination of Mitogen-activated Protein Kinase II Activity—The activity was measured by the incorporation of radioactive phosphate into myelin basic protein, catalyzed by the active mitogen-activated protein kinase 2/Erk2 (recombinant protein expressed in E. coli, catalog number 14-492, Upstate Biotechnology) and the Phospho-Glycogen Synthase Peptide-2 (glycogen synthase kinase 3 substrate), catalog number 12-241 Upstate Biotechnology) following the manufacturer’s protocol.

Determination of Glycogen Synthase Kinase 3β, Glycogen Synthase Kinase 3β Activity—The glycogen synthase kinase 3β, active (recombinant protein expressed in SF21 cells) catalog number 14-492, Upstate Biotechnology) and the Phospho-Glycogen Synthase Peptide-2 (glycogen synthase kinase 3 substrate), catalog number 12-241 Upstate Biotechnology) as a substrate, were used following the manufacturer’s protocol.

Fluorescence Polarization Assays—Fluorescence polarization measurements were made in black 96-well plates using a WallacVictor2 1420 Multilabel HTS counter with an excitation wavelength of 485 nm and observed emission wavelength of 535 nm. The assay was carried out by adding 60 nM of the carboxyfluorescein NBI1-labeled peptide (CF-NBI1) to different concentrations of cyclin A-(171–432) in PBS buffer and incubating the samples during 20 min at 30 °C before reading.

Surface Plasmon Resonance Experiments—All the surface plasmon resonance measurements were performed in black 96-well plates using a WallacVictor2 1420 Multilabel HTS counter with an excitation wavelength of 485 nm and observed emission wavelength of 535 nm. The assay was carried out by adding 60 nM of the carboxyfluorescein NBI1-labeled peptide (CF-NBI1) to different concentrations of cyclin A-(171–432) in PBS buffer and incubating the samples during 20 min at 30 °C before reading.

Viability Assays—Cells were seeded in 96-well plates at 7 × 10^3 cells/well. After 24 h Tat or Tat-NBI1 were added at different concentrations and incubated for 24 h. Following incubation, plates were rinsed with PBS and 100 μl of stock 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (0.5 mg/ml in culture medium) was added. Cells were incubated at 37 °C for 1 h; then 200 μl of Me₂SO were added to each well. Plates were read on a MRX (Dynatech Laboratories) reader after a 1-h incubation, at a wavelength of 570 nm. Results shown are from triplicates in at least three independent experiments.

Flow Cytometry Analysis—Cells were plated in 60-mm² culture dishes at 5 × 10^5 cells/plate 24 h prior treatments. Tat or Tat-NBI1 were added for 24 or 48 h. Cells were fixed with 70% methanol for 2 h at room temperature, washed with PBS, and finally incubated with 50 μg/ml of propidium iodide (Sigma) and 200 μg/ml RNase for 10 min at room temperature. Analysis of DNA content was carried out in a BD Biosciences FACs Calibur. Results shown are from triplicates in at least three independent experiments.

Determination of DNA Synthesis, Thymidine Incorporation—Cells were seeded at 5 × 10^5 cells/plate and allowed to grow for at least 24 h. Tat or Tat-NBI1 was then added for 24 h and [methyl-^3H]thymidine (4 mCi/ml; Amersham Biosciences) was incorporated 1 h before collecting the cells. DNA synthesis was determined measuring the incorporation of [methyl-^3H]thymidine into DNA as described (28).

Cell Cycle Synchronization and Laser-scanning Confocal Analysis—Cells were grown on coverslips for 24 h. G1 synchronization was achieved by maintaining cultures with 0.5% fetal calf serum medium for 24 h. Cells were synchronized at G2 phase with 1.5 mM hydroxyurea for 24 h. For synchronization in M phase, cells were treated with 100 ng/ml nocodazole during 18 h. After synchronization drugs were removed and cells were washed with fresh media and rinsed over 1 h. Treatments with Tat or Tat-NBI1 at 20 μM were then added and time courses were performed.

The DNA cellular content in synchronized cultures was analyzed by laser-scanning cytometry. Cells were fixed with 70% methanol for 2 h at room temperature and washed with phosphate-buffered saline. Then, they were incubated with 50 μg/ml propidium iodide (Sigma) and 200 μg/ml RNase for 10 min. The coverslips were seeded onto a slide using mounting medium containing 25% propidium iodide (100 μg/ml in PBS) and 75% glycerol. Slides were scanned using the ×40 objective and 5 milliwatts of Argon laser power. A red fluorescent threshold and a minimum cell size of 100 pixels identified cells for measurement. At least 10,000 cells were analyzed.

Protein Extraction and Western Blot Analysis—Treated cells were washed twice in PBS, scraped, and lysed in lysis buffer (80 mM Tris-HCl, pH 6.8, 2% (v/v) SDS). Protein concentration was determined by the Lowry method. Lysates (25 μg) were loaded into a SDS-PAGE gel and after that, proteins were transferred to Immobilon membranes. Subsequently, membranes were blocked in 5% nonfat dry milk in TBS buffer for 1 h at room temperature and then incubated with different primary anti-
bodies overnight at 4 °C: anti-phospho(S32)-MCM2 1:20000 (Abcam ab11897), anti-MCM2 1:50 (Abcam ab6153), anti-PARP 1:1000 (BD Pharmingen 556493), and anti-actin clone C4 1:1000 (ICN Biomedicals). After three washes with TBST, membranes were incubated for 45 min with horseradish perox-

Apoptosis Assays—For detection of cellular fragmentation into apoptotic bodies, cells were seeded on coverslips, treated at different times, fixed with 70% methanol, and subjected to DNA staining with propidium iodide.

RESULTS

The crystallographic structure of the complex cdk2-cyclin A shows that cyclin A binds to and interacts with both the N- and C-lobes of cdk2 to form a continuous protein-protein interface (29, 30). The structure of cdk2-cyclin A complexed with the CKI protein p27Kip1 has also been resolved showing the interaction between the CKI substrate and the complex at the CRM
A number of groups have reported the identification of inhibitors of cdk2-cyclin A through the design of molecules that bind to the CRM (20, 32, 33). As stated above, the goal of this study was to identify inhibitors of cdk2-cyclin A complexes but not targeting the ATP binding site nor the CRM. To this aim, we screened combinatorial libraries in the appropriate experimental conditions, thus we adjusted the ATP concentration to be high enough to difficult ATP competition while minimizing the use of $[^{32}P]ATP$. As substrate, we used histone H1 that is phosphorylated directly and without the required binding to the cyclin A CRM that have other substrates as pRb and E2F protein families (32).

Screening of a Dual Defined Position Peptide Combinatorial Library—We initially screened a diversity oriented positional scanning library of trimers of $N$-alkylglycines that have previously allowed the identification of lead compounds in different biological assays in our laboratories (34–37). However, we did not find any suitable lead compound that satisfied the initial goals. Thus, we decided to focus on a putatively more reliable library that encompasses a more step-by-step definition of the functionalities required by the experimental conditions. Thus, we synthesized a dual defined position peptide combinatorial library as initially proposed by Houghten et al. (38). The peptide combinatorial library consisted of 400 separate hexapeptide mixtures having a COOH-terminal amide and an acetylated NH$_2$ terminus. Each mixture (represented by the formula Ac-OOX$_{2}$X$_{4}$-NH$_2$) had the first two positions defined with one of the 20 naturally occurring amino acids (referred to as “O”) and the remaining four positions are close to equimolar mixtures of 19 of the naturally occurring amino acids (referred to as “X,” cysteine was omitted). The peptide mixtures were screened for the ability to inhibit cdk2-cyclin A activity. As shown in Table 1 a number of peptide mixtures assayed were found to have significant inhibitory activity. From these assays the peptide mixture Ac-RWX$_{4}$X$_{2}$-NH$_2$ was selected for further deconvolution. Furthermore, from this initial screening we learned that the presence of positive charges at the NH$_2$ terminus of the peptide mixtures was recognized as one of the characteristic of all of the most active peptide mixtures, thus we decided to proceed with the deconvolution process using a non-acetylated version of the initial library. Moreover, due to the potential interest in performing in vivo studies with the putative inhibitory peptides defined after the deconvolution process, we decided to synthesize the peptide mixtures using the more protease-stable D-stereoisomers of the amino acids (that will be referred here using lowercase letters). Nevertheless, prior to a further deconvolution step we synthesized the peptide mixture rwxxxx-NH$_2$ that was shown to posses an inhibitory activity similar to that of the mixture Ac-RWXXXX-NH$_2$.
To define the third amino acid from the hexapeptide, the library rwoxx-NH₂ (containing 20 different mixtures) was synthesized and screened. The most active mixtures were rwoxx-NH₂, rwlxx-NH₂, and rwfxxx-NH₂ (Table 1). Due to the similar chemical character of the third amino acid, only rwixo-NH₂ was tracked in the iterative process. The Leu-Phe pair is one of the most abundant residue pairs found at the cyclin A CRM (39) (i.e. RXLFY’). Taking into account our goal of finding non-CRM ligands together with the slightly higher activity of the mixture rwixo-NH₂, we selected D-Ile at the third position. A new library was synthesized with the general formula rwixo-NH₂ and the next position was identified. The most active mixtures were rwimxx-NH₂, rwivxx-NH₂, rwirxx-NH₂, rwiixx-NH₂, and rwixo-NH₂ (Table 1). All five showed similar inhibitory activity then, at this point to make the selection we incorporated a secondary assay directed toward kinase selectivity. All five mixtures were screened against the CDK non-related kinases calmodulin-dependent kinase II and p38 SAPK. The mixtures rwixo-NH₂, rwixo-NH₂, rwixo-NH₂, and rwixo-NH₂ but not rwixo-NH₂ showed some inhibitory activity against these two kinases, then D-Met was selected at the fourth position of the hexapeptide. A new set of mixtures, rwimox-NH₂, was synthesized to define the fifth amino acid. The library was screened and four mixtures, rwimox-NH₂, rwimfx-NH₂, rwimxx-NH₂, and rwimcx-NH₂, were shown to have similar IC₅₀ values (Table 1). Thus, in this case the criterion used to define the amino acid was based on the chemical nature of the most active amino acid that defines each sub-library. D-Tyrosine was selected as representative of the aromatic amino acids that appeared to be the most active at this particular position. Finally, a collection of 20 individual hexapeptides was synthesized. The sequences rwimyc-NH₂ and rwimyf-NH₂ with IC₅₀ values of 1.0 ± 0.1 μg/ml were shown to be the most active compounds. The peptides were extensively purified and analyzed by high performance liquid chromatography and mass spectrometry. We noticed that the peptide rwimyc-NH₂ showed a tendency to form dimers in the kinase assay experimental conditions, we then decided to continue our study with the purified rwimyc-NH₂ (peptide NBII). In addition, a scrambled version of NBII (sequence ykrwqm-NH₂, named NBIIr) and a peptide derived from a different study that shares four of six of the amino acids of NBII (sequence ykrwqm-NH₂, named NBIIr) did not inhibit the kinase activity of the cdk2-cyclin A complex in our experimental conditions.

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D-Met was selected at the fourth position of the hexapeptide. A new library was synthesized with the general formula rwixo-NH₂, and the next position was identified. A new set of mixtures, rwimox-NH₂, was synthesized to define the fifth amino acid. The library was screened and four mixtures, rwimox-NH₂, rwimfx-NH₂, rwimxx-NH₂, and rwimcx-NH₂, were shown to have similar IC₅₀ values (Table 1). Thus, in this case the criterion used to define the amino acid was based on the chemical nature of the most active amino acid that defines each sub-library. D-Tyrosine was selected as representative of the aromatic amino acids that appeared to be the most active at this particular position. Finally, a collection of 20 individual hexapeptides was synthesized. The sequences rwimyc-NH₂ and rwimyf-NH₂ with IC₅₀ values of 1.0 ± 0.1 μg/ml were shown to be the most active compounds. The peptides were extensively purified and analyzed by high performance liquid chromatography and mass spectrometry. We noticed that the peptide rwimyc-NH₂ showed a tendency to form dimers in the kinase assay experimental conditions, we then decided to continue our study with the purified rwimyc-NH₂ (peptide NBII). In addition, a scrambled version of NBII (sequence ykrwqm-NH₂, named NBIIr) and a peptide derived from a different study that shares four of six of the amino acids of NBII (sequence ykrwqm-NH₂, named NBIIr) did not inhibit the kinase activity of the cdk2-cyclin A complex in our experimental conditions.
cyclin A complex but does not compete with ATP or with the substrate histone H1.

The Inhibitory Activity of NBII1 on the Complex cdk2-Cyclin A Is Not Mediated by the CRM of Cyclin A—As mentioned above it is remarkable that the inhibitory activity of NBII1 appears to be dependent on the cyclin subunit associated to cdk (Table 2). In fact, we found that NBII1 inhibited more effectively the complex cdk2-cyclin A than cdk2-cyclin E by a factor of 50-fold. This result points to a putative new type of cdk2-cyclin A inhibitors that in addition to being non-ATP competitive they could not be directed to the CRM. To further support this observation, we performed the cdk2-cyclin A kinase assays using a well defined CRM-dependent substrate (fpRb, a fragment of pRb) or a non-CRM-dependent substrate (histone H1). As expected, pep21, a peptide derived from p21Cip1 that targets the CRM inhibited the phosphorylation of pRb but not that of histone H1. Interestingly, when the peptide NBII1 was included in the kinase assays the phosphorylation of both substrates was abolished (Fig. 2A). The double-reciprocal plot of the steady-state kinetic analysis using fpRb as a substrate revealed that peptide NBII1 behaved as a non-competitive inhibitor with respect to fpRb (Fig. 2B).

**NBII1 Binds to Cyclin A but Not cdk2**—To initially characterize the binding site of NBII1 to cyclin A a fluorescent analogue of NBII1 (CF-NBII1) was synthesized. The peptide CF-NBII1 bound to cyclin A with a dissociation constant ($K_d$) of 102 ± 15 nM as determined in a fluorescence polarization assay (Fig. 3A). The binding profile, by means of surface plasmon resonance technology, was also analyzed. To facilitate the analysis, peptide TAT-NBII1 was synthesized, where peptide NBII1 was fused to the well characterized cell-carrier peptide TAT that corresponds to a short basic region comprising residues 48–57 of human immunodeficiency virus type 1 TAT protein (40, 41). TAT-NBII1 was coupled to a CM5-dextran chip at a surface density of 2000 resonance units. Then, increasing concentrations of purified recombinant cyclin A or cdk2 were injected onto the chip surface and the binding subsequently analyzed. Cyclin A (Fig. 3B) but not cdk2 (Fig. 3C) bound to immobilized TAT-NBII1 with association and dissociation rate constants consistent with a $K_d$ value of 52 nM, in good agreement with the $K_d$ value determined in the fluorescence-based binding experiment. The ability of increasing concentrations of NBII1 peptide to compete for the binding of cyclin A bound to the TAT-NBII1 surface was also measured. Results shown in Fig. 3D indicate that when cyclin A was premixed with increasing concentrations of free in solution NBII1 the amount of cyclin A that bound to the immobilized TAT-NBII1 decreased. Consequently, it is demonstrated that peptide NBII1 binds to cyclin A. Furthermore, in a similar experiment where cyclin A was premixed with different concentrations of purified cdk2 (Fig. 3E) minor alterations of binding where found.

With the aim of identifying a structural domain with NBII1 binding capability, the association of different cyclin A fragments to the TAT-NBII1 peptide was analyzed. Thus, six different cyclin A fragments mapping the entire protein sequence were subcloned, expressed in *E. coli* bacteria, and...
purified (Fig. 4A). The binding profile to TAT-NBI1 was analyzed by means of surface plasmon resonance (Fig. 4B). The results suggest that the minimal structural domain that binds to NBI1 is defined at a highly conserved region between amino acids 257 and 345 that comprises helices H9251, H9252, and H9253. These helices form part of the structural domain named the cyclin box (residues 209–310), a region conserved among members of the cyclin family and implicated in the interaction with cdks (30).

**NBI1 Inhibits Cell Cycle Progression and Induces Apoptosis** — We further aimed to study the effect of the hexapeptide on cell viability. To ensure an efficient uptake of NBI1 across cell membranes we employed the TAT-NBI1 peptide in cell based assays. Preliminary studies showed that TAT-NBI1 had the same inhibitory activity as NBI1 in the in vitro cdk2-cyclin A assay (data not shown). Furthermore, immunoprecipitation experiments on cells treated with TAT-NBI1 were performed to analyze the in vivo effect of the peptide on cdk activity. Thus, immunoprecipitation using anti-cdk1, anti-cdk2, or anti-cdk4 antibodies were carried out on cells treated with TAT-NBI1 or TAT control peptide. Then, the different cdk activities were analyzed in the immunoprecipitates. In agreement with the in vitro results (Table 2), we observed that cdk2 and cdk1 activities were inhibited in cells treated with TAT-NBI1, whereas control cdk4 activity was only slightly inhibited (Fig. 5).

Thus, after ensuring that TAT-NBI1 is able to enter the cell and exert its inhibitory activity on cdk2 activity, the effect of different doses of TAT-NBI1 on TAT were studied in a number of tumor cell lines (HCT116, HT29, T98G, and A2780) using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability assay. Results revealed that the hexapeptide decreased cell viability in a dose-dependent fashion (Fig. 6).

Flow cytometric analysis of asynchronously growing tumor cells treated with TAT-NBI1 resulted in a cell cycle arrest mainly at the S phase, clearly accompanied by an increase in the sub-G1 population (Fig. 7). The blockage in S phase was further confirmed in two cell lines (HCT116 and A2780) by measuring the rate of DNA synthesis in the presence of the TAT-NBI1 peptide (Fig. 8A). These effects were correlated with a NBI1-induced in vivo decrease of cdk2-cyclin A activity, as measured by the low rate of phosphorylation of the CDK protein substrate minichromosome maintenance 2 (MCM2), a nuclear protein involved in the onset of DNA replication (Fig. 8B) (42). We subsequently analyzed the effect of TAT-NBI1 and TAT peptide control on cells synchronized at different phases of the cell cycle. Interestingly, TAT-NBI1 did not affect cell cycle progression in G1 cells, whereas in contrast a strong increase in the sub-G1 population was observed when S or G2/M cells were treated with TAT-NBI1 (Fig. 9A). These results indicate that cells in S or G2/M are highly sensitive to the peptide and are in agreement with the fact that cdk2-cyclin A and cdk1-cyclin A/B are the complexes that govern S and G2/M progression and that are strongly inhibited by TAT-NBI1. The treatment of cells (HCT116 and A2780) with the hexapeptide caused apo-
Cyclin A Surface Inhibitors

**FIGURE 8. TAT-NBI1 peptide inhibits DNA synthesis.** A, effect of TAT-NBI1 on DNA synthesis in HCT116 (left panel) and A2780 (right panel) cells. The percentages on the left represent [3H]thymidine incorporation percentage. Cultured cells were treated with 20 μM TAT-NBI1 or TAT peptide control by 24 h, and DNA synthesis was measured by [3H]thymidine incorporation as described under "Experimental Procedures." In both cases, the values are the mean ± S.D. from three independent experiments. B, Western blot analysis of MCM2 phosphorylation. HCT116 (upper blots) and A2780 (lower blots) were treated at 24 h with 20 μM TAT peptide control, or three different doses of TAT-NBI1 (10, 20, and 40 μM). Representative Western blots using specific antibodies, anti-phospho-MCM2, anti-MCM2 total protein, and loading control with anti-actin are shown.

ptosis as observed by caspase 3-dependent cleavage of PARP (Fig. 9B) and by the induction of apoptotic bodies (Fig. 9C).

DISCUSSION

Natural inhibitors of CDKs are either mutated or deleted in primary tumor cells. In an attempt to regulate aberrant cell cycle pathways, new synthetic agents that can restore the functions of altered tumor suppressor proteins, as CKIs, are central targets in current cancer research. Active sites of enzymes such as kinases have challenged drug discovery approaches. However, ATP competitive drug-like compounds have proven difficult to define kinase specificity because most of such enzymes share a high degree of sequence similarity within the ATP active site. Alternative inhibitory pathways were found derived from studies directed to elucidate the structure and activation mechanisms of CDKs (43, 44). Thus, the CRM, a consensus short recognition motif present in many cell cycle regulatory proteins, could interact with a hydrophobic patch on the surface of cyclins (23), inspired the design of new inhibitors. In this sense, peptides derived from the COOH terminus of the CDK inhibitor p21Cip1 (45) or from the transcription factor E2F1 (23, 46) were shown to have antiproliferative effects in multiple cell lines. More recently, Gondeau et al. (22) have described another strategy to inhibit the activity of CDKs by targeting the interface between the cdk and cyclin. Thus, a 22-residue long peptide derived from the amino acid sequence of the α5 helix of cyclin A was shown to bind to the cdk2-cyclin A complex and in turn, it was demonstrated to block the proliferation of tumor cell lines (22). These peptides, besides their own activity, are valuable tools that could drive the discovery of new families of small molecule inhibitors of CDKs (1, 5, 47, 48).

To explore alternate binding sites that may inhibit the activity of CDKs we screened chemical libraries under restrictive assay conditions. The imposition of such screening conditions would limit the putative hits to molecules that could not compete with ATP and could bind to cyclin A and inhibit the productive protein-protein interaction that could render the active cdk2-cyclin A complex. The peptide NBI1 (rwimyf-NH2) was selected as lead compound for the development of this new class of inhibitors (Table 1). The peptide showed an IC_{50} value of 1.1 μM for the inhibition of the activity of the cdk2-cyclin A complex, whereas was less active against a panel of other kinases (Table 2). The kinetic analysis showed that the NBI1 peptide neither competes with ATP nor with CRM substrates as pRb nor with substrates as histone H1 (Figs. 1 and 2). Furthermore, we have shown that the NBI1 peptide selectively binds to cyclin A (Fig. 3). Thus, it is tempting to hypothesize that the NBI1-binding site on cyclin A may represent a new target site for the selective inhibition of activity for the cdk2-cyclin A complex. In contrast to the existing CDKs peptide inhibitors, the hexapeptide NBI1 could disrupt the cdk2-cyclin A complex. The cdk2-cyclin A complex x-ray crystal structure (30) showed that at the complex interface several structural elements from both the cdk2 and the cyclin A subunits adapt each other resulting in a buried surface area larger than 3000 Å². Thus, the NBI1 peptide should recognize and bind to a critical structural element of cyclin A that in turn should induce a putative conformational change that diminishes its affinity to bind to the cdk2 subunit. It would be of interest to identify such a structural element. Human cyclin A aggregates at the concentrations required for structural studies. Nevertheless, the reported x-ray crystal structures of the cdk2-cyclin A complexes were determined using a functional cyclin A domain comprised by residues 173–432 (30, 47). However, this domain is still very unstable for future structural determinations in the absence of the cdk2 subunit.

In an attempt to get a deeper insight into the structural basis of the interaction, we docked the NBI1 peptide onto the cyclin A molecule. Fig. 10 shows the results obtained using the deposited coordinates for the complex cdk2-cyclin A, and a peptide (structure on red in Fig. 10) bound to the CRM (Protein Data Bank code 2C5V), and the results obtained using AutoDock (49) for the peptide NBI1 (structure on blue in Fig. 10). Although it is still speculative, the NBI1 peptide could bind to a cleft on the surface of cyclin A that is important for the binding of cyclin A to cdk2. From the output of the docking procedure, it looks like residues Gln^{223}, Asn^{229} (helix α3), Asn^{312}, Gln^{313} (helix α6); Met^{334} (helix α7); and Lys^{417} (at the COOH-terminal end) could contribute to the definition of this new binding site. These results are in agreement with those obtained from the analysis by surface plasmon resonance of the minimal structural domain that binds to NBI1 (Fig. 4). Interestingly, the docking experiments also explain the selectivity found for NBI1 against different cyclins. As it was shown in Table 2, NBI1 shows a remarkable
selectivity for the cdk2-cyclin A complex, but it is a weak inhibitor of the cdk2-cyclin E complex. Because both cyclins bind to the same Cdk, the only explanation for the different selectivity has to be found in structural differences between both cyclins at the binding region suggested by the docking experiments. Fig. 11 shows a surface representation of cyclin A (Fig. 11A) and cyclin E (Fig. 11B) structures, together with the pose suggested by Autodock for NB11 in cyclin A (Fig. 11C). The figure clearly shows that the nature of the cavity is different in both proteins, and that NB11 cannot make the same contacts with the two proteins. In fact, from the enzyme inhibition data, it seems clear that

FIGURE 9. Effect of TAT-NBI1 peptide on cell cycle and apoptosis. A, TAT-NBI1 affects cell cycle progression. HCT116 cells synchronized at different phases of the cell cycle were treated with TAT-NBI1 or TAT peptide control at 20 μM for 6 h and then, the cell cycle distribution was analyzed by laser-scanning cytometry (black square, cells in G1 phase; empty square, cells in S phase; gray square, cells in M phase and crossed square, cells in sub-G1 phase). Cells were synchronized in G1 (left panel), S phase (middle panel), or mitosis (right panel). B, detection of cleaved PARP in cell extracts. HCT116 or A2780 cells were treated during 24 h with 20 μM TAT control peptide, or three different doses of TAT-NBI1 (5, 10, and 20 μM). Representative Western blots using anti-PARP revealed the presence of PARP cleavage products on the blot (85-kDa PARP fragment). C, TAT-NBI1 peptide induces formation of apoptotic bodies in HCT116 and A2780 cells. Images show HCT116 and A2780 cells subjected to 48 h of TAT-NBI1 or TAT control peptide (40 μM) treatment. Cells were stained with propidium iodide and loss of integrity of nuclear envelope and visualization of apoptotic bodies were detected (white arrows).

FIGURE 10. Surface representation of the cdk2-cyclin A structure and location of the NBI1 binding site on cyclin A. A, surface representation of the structure of cyclin A (soft gray) with a CRM binding peptide in red (coordinates were extracted from Protein data bank code 2CSV). The L-amino acid version of the peptide NB11 (in blue) was docked on the structure of cyclin A. In this docking approach, the peptide backbone of NB11 was kept rigid, whereas all the side chains were defined as flexible using the deftors module. Cyclin A was treated as the macromolecule part of the docking calculation as was kept rigid. B, surface representation of the structure of the cdk2 (soft blue)-cyclin A (soft gray) complex. The surface representation of a CRM-binding and NB11 peptides are shown in red and blue, respectively.
the interaction between NBI1 and cyclin A is more efficient than with cyclin E. On the other hand, cdk1 possesses a very high similarity with cdk2 (66% sequence identity), and it is known that cyclin A can bind, in addition to cdk2, to cdk1. In this context, it is not surprising that NBI1 could also disrupt the cdk1-cyclin A interaction. To the best of our knowledge, the structure of cyclin B1 is not yet known, so it is difficult to explain in detail why NBI1 can inhibit cdk1-cyclin B1 activity. However, results obtained by comparative homology modeling using different software packages (data not shown) have not been able to reveal big differences between cyclin B1 and cyclin A structures, thereby explaining why NBI1 is also able to disrupt the cdk1-cyclin B1 complex. Finally, the low degree of sequence similarity between cdk2 and cdk6 (49% sequence identity) and among cyclin D3 and cyclin A (30% sequence identity) makes it very difficult to structurally interpret the results obtained for the cdk6-cyclin D3 (Table 2).

Supporting the data obtained from the in vitro model systems, NBI1 in its TAT-NBI1 form was shown to have activity in living cells. In the cell cycle S phase, cdk2-cyclin A complexes are important for phosphorylation and inactivation of the E2F/DP1 transcription factor. Inhibition of cdk2-cyclin A results in an elevated E2F concentration leading to S phase arrest and apoptosis. TAT-NBI1 was able to inhibit cell proliferation (Fig. 6) in a concentration- and time-dependent manner, and induce both, specific blockade of cell cycle at S phase (Fig. 7) and apoptosis (Fig. 9) in the glioblastoma (T98G) colon (HCT116) and ovarian (A2780) cancer cells evaluated.

The peptide NBI1 could define a new class of cdk2-cyclin A inhibitors binding at a different site in the cyclin A molecule than other available pharmacological inhibitors. In fact, in contrast to most reported CDKs inhibitors that are competitive to known substrates of CDK complexes, the mechanism of action of NBI1 appeared to be non-competitive to ATP and non-competitive to CRM substrates. The ability of NBI1 to specifically bind to a new binding site on cyclin A and inhibit the formation of the cdk2-cyclin A complex provide new alternatives for drug discovery because the chemical properties for binding are different from active site-directed compounds and would also allow selectivity.

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