Identification and Structure Characterization of a Cdk Inhibitory Peptide Derived from Neuronal-specific Cdk5 Activator*

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The activation of cyclin-dependent kinase 5 (Cdk5) depends on the binding of its neuronal specific activator Nck5a. The minimal activation domain of Nck5a is located in the region of amino acid residues 150 to 291 (Tang, D., Chun, A. C. S., Zhang, M., and Wang, J. H. (1997) J. Biol. Chem. 272, 12318–12327). In this work we show that a 29-residue peptide, denoted as the α5 peptide, encompassing amino acid residues Gln145 to Asp173 of Nck5a is capable of binding Cdk5 to result in kinase inhibition. This peptide also inhibits an active phospho-Cdk2-cyclin A complex, with a similar potency. Direct competition experiments have shown that this inhibitory peptide does not compete with Nck5a or cyclin A for Cdk5 or Cdk2, respectively. Steady state kinetic analysis has indicated that the α5 peptide acts as a non-competitive inhibitor of Cdk5-Nck5a complex with respect to the peptide substrate. To understand the molecular basis of kinase inhibition by the peptide, we determined the structure of the peptide in solution by circular dichroism and two-dimensional 1H NMR spectroscopy. The peptide adopts an amphipathic α-helical structure from residues Ser149 to Arg162 which can be further stabilized by the helix-stabilizing solvent trifluoroethanol. The hydrophobic face of the helix is likely to be the kinase binding surface.

Cyclin-dependent kinases (Cdks) are key regulatory enzymes in the eukaryotic cell cycle. The activation of a Cdk depends on its association with its specific cyclin partners. The activity of these enzymes is further regulated by an intricate system of protein-protein interactions and phosphorylation (1). Members of the Cdk family are closely related by sharing a high level of amino acid sequence identity (40–70%). In contrast, cyclins are a family of molecules of diverse molecular mass and low sequence identity. Sequence alignments have shown that cyclins share a somewhat conserved region of approximately 100 amino acids in the center of the molecule, and this region is called the cyclin box (2). Recent crystal structures of cyclin A and cyclin H have shown that the cyclin box sequence forms a compact 5-helix domain called the cyclin fold (3–5), and that a region of cyclin A, C-terminal to the cyclin box also forms a cyclin fold. However, there is virtually no sequence similarity between the two cyclin fold domains. Theoretical predictions have suggested that other members of the cyclin family also contain two cyclin folds (3, 6).

Unlike other Cdks, Cdk5 activity has been observed only in neuronal and developing muscle cells although the catalytic subunit of the enzyme is present in many mammalian tissues and cell extracts (7–11). Recent experimental evidence has demonstrated that Cdk5 plays important roles in neurite outgrowth (12), patterning of the cortex and cerebellum (13), and cytoskeletal dynamics (9, 14, 15). Loss of regulation of Cdk5 has been suggested to be involved in Alzheimer’s disease (16). Active Cdk5 was first purified from brain extracts as a heterodimer with subunit molecular masses of 33- and 25-kDa, respectively (8, 10). The 33-kDa subunit was later identified as Cdk5, and the 25-kDa activator (neuronal Cdk5 activator, Nck5a) was a novel protein with no sequence similarity to any other known proteins. The 25-kDa subunit was later found to be a proteolytic product of a larger 35-kDa protein (17, 18). An isoform of Nck5a (Nck5ai) with 57% sequence identity to Nck5a has also been identified (19). Despite their functional similarity in terms of binding and activation of a Cdk, the Cdk5 activators share little sequence similarity to cyclins. Moreover, while the activation of the well characterized Cdks such as Cdk1 and Cdk2 by cyclins depends on the phosphorylation of the Cdk at a specific threonine residue, Cdk5 activation by its activator is phosphorylation-independent (20, 21). Recently, the activation domain of Nck5a was precisely mapped to amino acid residues from Glu150 to Asn201 (21, 22). Extensive truncation and site-directed mutation studies of Nck5a, together with computer modeling, strongly suggested that the 142-residue activation domain of Nck5a adopts a cyclin fold structure (3, 22).

In this work, we describe the discovery of a 29-residue Cdk inhibitory peptide which is derived from an internal fragment of Nck5a. This peptide is able to bind to and hence to inhibit the kinase activities of Cdk5-Nck5a and Cdk2-cyclin A complexes in a non-competitive manner. The solution structure of the peptide determined by two-dimensional NMR spectroscopy showed that a large part of the peptide adopts an amphipathic α-helical structure, and this helix is likely to be the main binding surface of the peptide to the enzyme complexes.

MATERIALS AND METHODS

Peptide Synthesis—A 28-residue peptide (ASTSELLRCLGEFLCRCYRLKHALPTD) corresponding to peptide fragment Ala146 to Asp173 of Nck5a was synthesized on an Applied Biosystems model A431. The peptide was then subjected to desalting and lyophilization. The purity of the peptide was determined by high performance liquid chromatography (HPLC).
automated peptide synthesizer using Fmoc-based chemistry (Fig. 1). A 31-residue peptide (DYHEDIHTYLREMEVKCKPKVGYMKKQPDIT) corresponding to the sequence Asp177 to Thr207 of human cyclin A was synthesized in the same manner (Fig. 1). After cleavage from the solid support, the peptides were purified by gel filtration and reverse phase high performance liquid chromatography to a purity greater than 95%. All of the peptide purifications were carried out in 0.1% trifluoroacetic acid/H2O (v/v) as low pH can prevent the oxidation of the sulfhydryl group of the Cys residues in the peptides. Trifluoroacetic acid was removed from the peptide by dissolving the lyophilized peptide powder in pre-chilled Milli-Q water before re-lyophilization. The authenticity of the peptides was checked using mass spectrometry as described earlier (23).

Protein Expression and Purification—GST-Cdk5 and GST-Nck5a were prepared as described previously (20, 22). The N-terminal histidine-tagged Cdk5 (H6-Cdk5) was expressed in and purified from *Escherichia coli* cells (strain M15 from Qiagen). The host cells, harboring a H6-Cdk5-containing expression plasmid, were cultured in 6 liters of tryptone/phosphate-rich medium containing 50 μg/ml of ampicillin and 25 μg/ml kanamycin to *A*600 = 1.0 before induction of protein expression with isopropyl-1-thio-

![Fig. 1. Amino acid sequences of synthetic peptides derived from the N-terminal end of Nck5a (the α<sub>N</sub> peptide) and the cyclin A peptide used in this work. The alignment of the two peptide sequences was derived by aligning cyclin A and the minimal activation domain of Nck5a (for details, see Ref. 22). The black bar above the cyclin A peptide represents the N-terminal α-helix of the protein found in the x-ray structure (3). The dotted region below the α<sub>N</sub> peptide depicts the predicted α-helix of the peptide.](image)

![Fig. 2. Discovery and inhibitory properties of the GST-α<sub>N</sub> fusion peptide. A, dose-dependent inhibition of GST-Cdk5-GST-Nck5a by the bacterially expressed GST-α<sub>N</sub>. The inset shows SDS-PAGE analysis of the purified GST-α<sub>N</sub>, its thrombin digestion product, and GST as a control. The minor bands seen in the GST-α<sub>N</sub> lane are due to proteolytic degradation of GST-α<sub>N</sub>, as cleavage of the fusion peptide by thrombin resulted only one band corresponding to GST. B, reconstituted GST-Cdk5-GST-Nck5a kinase activity (B1), and its inhibition by GST-α<sub>N</sub> (B2), by the α<sub>N</sub> peptide released from thrombin cleavage (B3), and not by GST (negative control, B4). C, inhibition of GST-Cdk5 activity by GST-α<sub>N</sub> both before and after GST-Cdk5 reconstitution with Nck5a. The addition of the GST-α<sub>N</sub> to GST-Cdk5 before reconstitution with GST-Nck5a (GST-Cdk5 + GST-Nck5a, C2) or after reconstitution with GST-Nck5a (GST-Cdk5/GST-Nck5a, C3) results in the same inhibition of kinase activity. C1 serves as a positive control (no GST-α<sub>N</sub> addition).](image)
expressed and purified in essentially the same manner as described for H6-Cdk5. Briefly, the expression plasmid pET21d containing a cyclin A gene lacking the N-terminal 173 amino acids was transformed into BL21(DE3) E. coli cells. The host cells were cultured in LB medium containing 100 μg/ml ampicillin, and cyclin A expression was induced by addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.1 mM. The induction of cyclin A expression was for 3 h at 30 °C. The pelleted cells were resuspended in 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 0.05% Triton X-100, and then lysed in the French press. The subsequent purification of cyclin A using a Ni2+-NTA column was carried out in a manner identical to that described for the purification of H6-Cdk5. The monomeric, active form of cyclin A-H6 was further purified by passing the cyclin A-H6-containing eluent through a Sephacryl S-200 gel filtration column (Amersham Pharmacia Biotech).

**Peptide/Cdk Binding Assay—H6-Cdk5** (about 3 μg) and 15 μg of GST-p25 were premixed in 300 μl of 1 × phosphate-buffered saline with 0.5 mg/ml bovine serum albumin. Some 50-μl quantities of the mixture were taken out to mix with various concentrations of the αN peptide (from serial dilutions made from a 2.0 mM stock solution), and the total volume of the mixture was adjusted to 150 μl using Buffer R (1 × phosphate-buffered saline containing 1 mM EDTA, 1 mM dithiothreitol, 0.6 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml antipain, 5 mg/ml bovine serum albumin). The reaction mixture was incubated at 4 °C for 15 h. GST-Nck5a-H6-Cdk5 complex was then precipitated by the addition of 40 μl of GSH-Sepharose beads pre-equilibrated with 1 × phosphate-buffered saline (50%, v/v). The GSH-Sepharose beads were washed three times with 1 × phosphate-buffered saline buffer, and subsequently resuspended in 20 μl of water and 20 μl of 2 × protein sample treatment buffer. The co-precipitated H6-Cdk5 was detected by SDS-PAGE followed by Western blot using a monoclonal antibody against Cdk5 (22).

The binding of the αN peptide to the GST-Cdk2/cyclin A-H6 complex was studied in a similar manner to that described above for H6-Cdk5. Briefly, 3 μg of GST-Cdk2 and 10 μg of cyclin A-H6 were reconstituted in Buffer R with various concentrations of the αN peptide at 4 °C for 15 h. GST-Cdk2 was then precipitated by the addition of 20 μl of GSH-Sepharose beads. After washing, the co-precipitated cyclin A-H6 was detected by SDS-PAGE followed by Western blot using a monoclonal antibody against cyclin A (Santa Cruz).

**Peptide Inhibition of Cdk5 and Cdk2 Kinase Activity—Cdk5 kinase activity was assayed essentially as described previously (20, 22), except that for each Cdk5 kinase reaction 1 μg of GST-Cdk5 was reconstituted with 2 μg of GST-Nck5a, and 1 μg of GST-Cdk5 was mixed with 3 μg of cyclin A-H6 in Cdk5 assays. The reconstituted complexes were added, in duplicate, to an assay mixture containing 30 mM MOPS, pH 7.4, 10 mM MgCl2, 40 μM of the histone H1 peptide, 50 μM [γ-32P]ATP, and various concentrations of various αN peptide analogs at 30 °C for 30 min before measuring the Cdk5 and Cdk2 kinase activities.

**Steady State Kinetic Experiments—** All assay conditions were the same as in the inhibition assay described above, except that the reaction time was kept at 15 min so that the product formed was less than 5% of the total substrate concentration used. To determine suitable concentrations of the substrate and the inhibitor for the steady state kinetic experiments, the K<sub>m</sub> value of GST-Cdk5-GST-Nck5a complex and a concentration-dependent inhibition profile of the enzyme by the αN peptide were determined. Four different concentrations of the histone H1 peptide (7.5, 10, 15, and 30 μM) were used in the kinetic analysis of the enzyme inhibition. For each substrate concentration, four concentrations of the αN peptide (0, 10, 20, and 30 μM) were used in the inhibition assay.

**CD Experiments—** Concentrations of the αN peptide stock solutions were determined by the UV absorption of the single Tyr residue at 280 nm. For CD measurement, the αN peptide was dissolved in a 20 mM sodium acetate buffer, pH 4.0, containing various concentrations of 2,2,2-trifluoroethanol (TFE). The concentration of the peptide was fixed at 50 μM throughout the experiment. CD spectra were collected at 35 °C on a JASCO J-720 CD spectropolarimeter equipped with a Neslab temperature controller using a cell path length of 1 mm.

**NMR Experiments—** For NMR studies, the αN peptide was dissolved in unbuffered 90% H<sub>2</sub>O, 10% D<sub>2</sub>O, or 99.99% D<sub>2</sub>O containing various concentrations of deuterated TFE-d<sub>3</sub> (0–20%, v/v), and 2 mM deuterated dithiothreitol-d<sub>4</sub> at pH (or pD) 4.0. The concentration of the αN peptide was approximately 2.0 mM. All 1H NMR data were recorded on a Varian INOVA 500 spectrometer at a 1H frequency of 500.11 MHz. Two-dimensional TOCSY and NOESY spectra were acquired with a spectral width of 6000 Hz in both dimensions (24). The "WET" pulse sequence was employed for solvent suppression (25) of the peptide samples in H<sub>2</sub>O. FID data matrices were composed of 2048 (256) × 1024 (512) × 65535 (2048) points.

**RESULTS**

A Peptide Derived from the N-Terminal Region of the Activation Domain of Nck5a Inhibits the Kinase Activity of Cdk5 and Cdk2—In the course of studying the structure and function relationship of the activation domain of Nck5a, we created a series of truncated forms of GST-Nck5a mutants. One such GST fusion mutant, which contains a 29-residue peptide fragment corresponding to residues Gln<sup>145</sup> to Asp<sup>173</sup> of Nck5a (termed αN as it represents the N-terminal α-helix of Nck5a, see "Discussion") was found to inhibit Cdk5 kinase activity in a dose-dependent manner (Fig. 2A). About 50% kinase activity was inhibited at a GST-αN concentration of 5 μM. The inhibition of Cdk5 activity originated solely from the peptide fragment as GST did not have any effect on the kinase activity of the enzyme (Fig. 2B). Release of the peptide from the fusion protein by thromin had no effect on the inhibitory activity (Fig. 2B). Furthermore, the addition of GST-αN before or after the reconstitution of GST-Cdk5 with GST-Nck5a gave rise to the same inhibition profiles (Fig. 2C), suggesting that the peptide does not compete with Nck5a for Cdk5 (see below for more details). We also tested the inhibitory effect of GST-αN toward the GST-Cdk2/cyclin A-H6 complex, and found that GST-αN...
also inhibited the kinase activity of Cdk2. As expected, the inhibition of the Cdk2-cyclin A complex originated from the αN peptide portion of the fusion protein, as seen in the case of Cdk5 (data not shown, see also below for results obtained with the synthetic peptide).

Low expression level (about 1 mg/ml of soluble GST-αN) and poor homogeneity of the GST fusion product prevented us from a detailed characterization of this inhibitory peptide. To overcome these problems, we decided to use a synthetic peptide instead of the GST fusion protein. A 28-residue peptide corresponding to amino acid residues A146 to A273 of Nck5a (abbreviated as the αN peptide) was synthesized and purified to homogeneity. The N-terminal Glu residue (Gln145) was deleted from the synthetic peptide sequence to avoid complication from its cyclization to form glycoglutamate. The titration curves shown in Figs. 2 and A revealed that both the recombinant and synthetic inhibitory peptides inhibit GST-Cdk5-GST-Nck5a complex with similar potencies.

In order to assess whether the αN peptide was a specific inhibitor of Cdk5 and Cdk2, we synthesized a 31-residue peptide corresponding to Asp177 to Thr207 of cyclin A (the cyclin A peptide, Fig. 1), and tested for its ability to inhibit GST-Cdk5-GST-Nck5a. This region of cyclin A was previously shown to align with the αN peptide based on the sequences, the secondary structures, and the functions of the two proteins (22). Despite the fact that the cyclin A peptide could adopt an α-helical conformation similar to that seen in its crystal structure (28), the cyclin A peptide inhibited neither GST-Cdk5-GST-

Nck5a nor GST-Cdk2-cyclin A-H6 (Fig. 3). Therefore, it is likely that the unique amino acid sequence of the αN peptide entails its inhibition of Cdk5 and Cdk2.

In addition, the αN peptide was shown to adopt an amphipathic α-helical structure in solution (see below), we tested the Cdk inhibitory effect of another amphipathic peptide, a 26-residue peptide fragment comprising the calmodulin-binding domain of myosin light kinase (the MLCK peptide) (29, 30). Although MLCK peptide was indeed able to inhibit the activities of both GST-Cdk5-GST-Nck5a and GST-Cdk2-cyclin A-H6, its inhibitory efficiency is significantly lower than that of the αN peptide (Fig. 3). Therefore, it is suggested that the potent and efficient inhibitory activity of the αN Peptide is due to the unique amino acid sequence.

The Binding of the αN Peptide to Cdk5-Nck5a and Cdk2-Cyclin A Complexes Does Not Lead to a Dissociation of Nck5a and Cyclin A—The inhibition of Cdk5 and Cdk2 by the αN peptide shown in Fig. 3 may result from a direct competition of the peptide with GST-Nck5a and cyclin A-H6 for GST-Cdk5 and GST-Cdk2, respectively, or from the binding of the peptide to the binary complexes of GST-Cdk5-GST-Nck5a and GST-Cdk2-cyclin A-H6. To discriminate between these possibilities, we performed direct binding competition experiments. Various concentrations of the peptides were added to a H6-Cdk5-GST-Nck5a mixture, and GST-Nck5a was then precipitated using GSH-agarose beads. The amount of H6-Cdk5 in complex with GST-Nck5a was determined by Western blotting of the enzyme co-precipitated by GSH-agarose beads. The amount of H6-Cdk5 and cyclin A-H6 used in the experiment described in Fig. 4, A and B, were chosen to ensure that their respective antibodies would be in excess. The addition of various amounts of the αN peptide (corresponding to approximately 10, 50, and 90% inhibition of the Cdk5 activity) did not lead to a dissociation of GST-Nck5a from H6-Cdk5 (Fig. 4A), indicating that the αN peptide was able to bind to and hence inhibit the binary complex of H6-Cdk5-GST-Nck5a. Similarly, the inhibition of GST-Cdk2-cyclin A-H6 activity by the αN peptide also resulted from the formation of a ternary complex between the αN peptide and GST-Cdk2-cyclin A-H6 rather than from a direct competition between the αN peptide and cyclin A for Cdk2 (Fig. 4B). The activity of the Cdk5-Nck5a complex could be inhibited immediately upon the addition of the αN peptide (Fig. 2C), further
Cdk5 interaction with respect to its substrate. Dixon plot analysis of the peptide acts as a noncompetitive inhibitor of the enzyme complex by the Nck5a complex by the αN peptide. Four different concentrations of the αN peptide were used in the assay (0 μM, ○; 10 μM, ●; 20 μM, ▲; 30 μM, ▼). The data were fitted by the standard linear least square fitting. B, Dixon plot of the inhibition of Cdk5-Nck5a complex by the αN peptide. The concentrations of the histone H1 peptide used were: 7.5 μM (○), 10 μM (●), 15 μM (▲), 30 μM (▼).

indicates that the αN peptide can bind to and inhibit Cdk5 without the dissociation of its activator.

To further prove that the αN peptide does not compete with Nck5a for Cdk5, we performed a direct competition experiment. First, GST-Cdk5 was reconstituted with various concentrations of its activator. Then, we assayed the inhibition of the reconstituted GST-Cdk5-GST-Nck5a complexes by the αN peptide (50 μM, a concentration which leads to about 90% inhibition of the enzyme, see Fig. 3). If the inhibitory peptide were to compete with Nck5a for Cdk5, the large excess of Nck5a would mask the inhibition of the kinase by the peptide at low concentrations. However, data in Fig. 5 show that the presence of a large excess of GST-Nck5a has no significant effect on the enzyme inhibition profile by the αN peptide, further supporting the contention that the inhibitory peptide does not compete with Nck5a for Cdk5.

**Kinetic Analysis of Cdk5 Inhibition by the αN Peptide**—We also analyzed the kinetic properties of the αN peptide with respect to the kinase substrate, the histone H1 peptide. The double-reciprocal plot shown in Fig. 6A demonstrates that the peptide acts as a noncompetitive inhibitor of the enzyme complex with respect to its substrate. Dixon plot analysis of the data reveals that the $K_i$ for the αN peptide inhibition of the GST-Cdk5-GST-Nck5a complex is approximately 25 μM (Fig. 6B). Linearity of the Dixon plots indicates that the αN peptide is a dead end inhibitor (i.e., the kinase-inhibitor complex is catalytically inactive) (31). This notion is further supported by the near complete inhibition of the kinase by an excess amount (i.e., 400 μM) of the αN peptide (data not shown).

**Structure of the αN Peptide Determined by CD and NMR**—In order to understand the structural basis of Cdk5 and Cdk2 inhibition by the αN peptide, we determined the structure of the peptide by NMR spectroscopy. The $^{1}H$ NMR spectra of the αN peptide in aqueous solution showed reasonable chemical shift dispersion (data not shown). However, careful inspection of the spectra revealed that the majority of resonance in both the amide and aliphatic regions had exceptionally broad line widths for a peptide of only 28 amino acid residues. These results indicated that the peptide might be in equilibrium between multiple conformers or in an aggregated state. A number of amide protons throughout the peptide displayed more than one cross-peak to their $α$ protons in a TOCSY spectrum of the peptide in 90% H$_2$O, 10% D$_2$O, pH 4.0, at 30 °C (data not shown). When the pH of the sample was raised to 4.5 or above, the line widths of the NMR signals broadened further, and the TOCSY spectra were more complicated. Changing the sample temperature (from 8 to 35 °C) or concentration (from 0.5 to 3.5 mM) did not improve the quality of the NMR spectra. CD studies also showed that the molar ellipticity of the peptide at 222 nm remained constant when the concentration of the peptide was varied from 8 μM to 0.2 mM (data not shown). These results indicated that the αN peptide in aqueous solution has multiple conformational states that are exchanging at slow to intermediate rates. Such multi-conformational equilibrium prevented us from a detailed structural characterization of the peptide in aqueous solution, although we were still able to obtain nearly complete backbone assignment of the peptide at pH 4, 35 °C.

To overcome the complications encountered in aqueous solution, we used TFE as a co-solvent for the structural characterization of the peptide. Fig. 7 shows CD spectra of the αN peptide...
at various concentrations of TFE. The CD spectrum of the peptide in the absence of TFE did not show the well defined double minima at 222 and 208 nm which are characteristics of an ordered α-helix in aqueous solution. However, the shape of the CD curve does suggest a measurable population of α-helix (32). For the samples dissolved in 5, 10, 15, 20, and 30% (v/v) TFE/H2O mixtures, the CD spectra showed increasingly clearer double minima at 222 and 208 nm, indicating an increasing amount of ordered α-helix. The CD spectra of the peptide in 5 to 30% TFE (v/v) solution had a common intersection at 204 nm, indicating that the peptide was undergoing a two-state conformational transition (Fig. 7), whereas the CD curve of the peptide in aqueous solution did not join this intersection (Fig. 7). This result further supports the suggestion that the peptide in aqueous solution adopts multiconformational states. The structural transition induced by TFE was effectively complete at a TFE concentration of 30% (v/v). Consequently, detailed structural characterization of the αN peptide was carried out at a TFE concentration of 30%.

The complete assignment of the αN peptide in 30% TFE (pH 4.0, 35 °C) was achieved using standard two-dimensional 1H NMR techniques (24). The CD spectrum in Fig. 2 shows the amide-amide region of the NOESY spectrum of the αN peptide dissolved in 30% TFE/H2O at 35 °C. A number of strong and continuous (i, i + 1) NOEs are observed, and correspondingly labeled in the spectrum.

FIG. 8. Amide-amide region of the NOESY spectrum (mixing time = 150 ms) of the αN peptide dissolved in 30% (v/v) TFE/H2O at 35 °C. A number of strong and continuous (i, i + 1) NOEs are observed, and correspondingly labeled in the spectrum.

The minimal activation domain of Nck5a has previously been mapped to contain 142 amino acid residues spanning residues Asp150 to Asn291 (21, 22). A number of theoretical and experimental studies have suggested that this minimal activation domain of Nck5a adopts a cyclin-fold (3, 6, 22). In this work, we have identified a 29-residue peptide, residues Gln145 to Asp173 of Nck5a, that can inhibit the kinase activities of the Cdk5/αN peptide and Cdk2/cyclin A complexes. Based on our earlier prediction, the sequence of this peptide encompasses the N-terminal α-helix of the cyclin fold (thus the peptide is termed the αN peptide) as well as some flanking amino acid residues at both ends of the helix (3, 4, 22). The inhibition of Cdk5 by the αN peptide supports an earlier study that a 50-amino acid fragment spanning residues 109 to 159 of Nck5a retains partial...
binding capability to Cdk5 (21). Knowing that Nck5a only weakly activates Cdk2 to the basal level, i.e. the activity observed for a Cdk2-cyclin A complex without Thr160 phosphorylation (20, 21), it is surprising that the $\alpha_N$ peptide inhibits Cdk2-cyclin A activity with an even higher potency than in the case with Cdk5-Nck5a inhibition (Fig. 3). In contrast, the corresponding peptide encompassing the N-terminal $\alpha$-helix of cyclin A inhibits neither Cdk2 nor Cdk5 (Fig. 3). In this work, we have investigated the inhibition of Cdk5 and Cdk2 by the $\alpha_N$ peptide, and it would be interesting to know whether the $\alpha_N$ peptide can also inhibit other members of the Cdk family. Further work is in progress on this matter in our laboratories.

Since the $\alpha_N$ peptide was derived from an internal fragment of Nck5a, it is expected that it might act as a noncompetitive inhibitor with respect to the substrate of Cdk5 (Fig. 6). However, it is unusual that the $\alpha_N$ peptide also functions as a noncompetitive inhibitor with respect to Nck5a (Figs. 4 and 5). Our results indicate that the inhibition of Cdk5 by the $\alpha_N$ peptide results from the formation of a ternary complex between the $\alpha_N$ peptide and the Cdk5-Nck5a complex. Presumably, the $\alpha_N$ peptide competes with the corresponding fragment in Nck5a for Cdk5 binding. This suggestion is in agreement with an earlier observation that the removal of 4 amino acid residues from the helical part of the peptide fragment from Nck5a completely abolished the ability of Nck5a to activate Cdk5 (22). Comparison of the crystal structures of cyclin A in complex with Cdk2, and cyclin H, has indicated that the N-terminal helix of various cyclins may function as a relatively independent structural unit with respect to the tightly packed cyclin folds (4, 5, 28). However, this N-terminal helix is dispensable for the activity of cyclins (22, 32, 33–35), although the contacts between the helix and the kinase are not extensive (4). Therefore, we hypothesize that the binding of the $\alpha_N$ peptide dislodges the corresponding N-terminal $\alpha$–helix of Nck5a from Cdk5, thereby inhibiting the activity of the enzyme. The dislocation of the N-terminal helix does not lead to dissociation of the whole activator. Unlike the $\alpha_N$ peptide, the control peptide derived from cyclin A inhibits neither Cdk2-cyclin A nor Cdk5-Nck5a (Fig. 3), suggesting a significant difference between the binding and activation of Cdk2 by cyclin A, on the one hand, and Cdk5 by Nck5a, on the other.

The $\alpha$-helical structure detected by CD spectroscopy for the $\alpha_N$ peptide in aqueous solution (Fig. 6) qualitatively agrees with our earlier prediction that part of the $\alpha_N$ peptide could adopt an $\alpha$-helical conformation (22). The existence of multi-conformational states of the peptide prevented us from a detailed structural determination of the peptide in aqueous solution. Hence, TFE and water were used as a co-solvent to study the structure of the $\alpha_N$ peptide. The peptide segment from Ser149 to Arg162 was found to adopt a stable $\alpha$-helical conformation in aqueous TFE solution. Similar NOE patterns (especially $d_{NN}$ NOE connectivities that were relatively well resolved) have also been observed for the $\alpha_N$ peptide in pure water solution (data not shown), suggesting that the same $\alpha$-helical conformation exists in this solution. It has been observed in numerous cases that TFE can either stabilize unordered $\alpha$-helices in various peptide fragments in aqueous solution or promote the formation of $\alpha$-helices in peptide fragments that have intrinsic propensities to form $\alpha$-helix, but not induce new $\alpha$-helical conformation (for example, see Refs. 30, 32, 36, and 37). Therefore, we suggest that the $\alpha$-helical region observed in the $\alpha_N$ peptide would probably adopt a similar $\alpha$-helix structure in Nck5a. The peptide region found to adopt an $\alpha$-helical conformation has also been predicted to be an $\alpha$-helix in the protein, and this $\alpha$-helix aligns well with the N-terminal $\alpha$-helix of the first cyclin-fold of cyclin A (Refs. 3, 4, and 22, also see Fig. 1). The above notion is further underscored by the fact that the same $\alpha$-helical structure was observed for the cyclin A peptide in solution as the corresponding N-terminal helix in the full-length cyclin A structures (28).

A helical wheel presentation of the $\alpha$-helix found in the $\alpha_N$ peptide is shown in Fig. 9. The height of the boxes indicates the relative intensities of the NOE cross-peaks. The dashed lines indicate the NOEs that are ambiguous due to resonance overlap. Chemical shift index (CSI) data of the $\alpha$ protons are also included (33).
peptide shows that the peptide is amphipathic with 4 Leu and 1 Phe on the hydrophobic face (Fig. 10). Indeed, deletion of part of the N-terminal end of the α-helix completely abolished the inhibitory effect of the peptide. In an earlier study, we have also shown that mutations of the hydrophobic amino acid residues in the α-helix (Leu151, Leu152) to a polar amino acid residue (Asn) greatly reduced the Cdk5 activation ability of Nck5α (22). In the crystal structure of the Cdk2-cyclin A complex, the corresponding N-terminal α-helix of cyclin A makes a significant amount of contacts with various regions (e.g. T-loop and α3 helix) of Cdk2 via hydrophobic interactions (4). It is likely that the hydrophobic face of the peptide forms the major binding area between the αN peptide and Cdk5. This hypothesis was supported by the result shown in Fig. 3 that an unrelated peptide binds to calmodulin in an α-helical conformation with its hydrophobic face forming the main contact area with calmodulin (30, 38, 39). The lower extent and potency of inhibitory activity observed with the MLCK peptide may originate from a large sequence difference in the α-helical as well as the C-terminal random coil region between the MLCK peptide and the αN peptide.

The structure of the αN peptide determined here and the interaction observed between the N-terminal α-helix of cyclin A and Cdk2 (4) suggest that systematic alterations of the amino acid residues in the hydrophobic face of the α-helix and the C-terminal end of the αN peptide may enable us to find peptide inhibitors with higher specificity and/or potency toward various Cdk’s. We note that the present Cdk5 inhibitory peptide was discovered based on the unique regulatory property of the C-terminal random coil region between the MLCK peptide andCdks. This hypothesis was supported by the result shown in Fig. 3 that an unrelated peptide in solution can be promoted by TFE, and the MLCK peptide binds to calmodulin in an α-helical conformation with its hydrophobic face forming the main contact area with calmodulin (30, 38, 39). The lower extent and potency of inhibitory activity observed with the MLCK peptide may originate from a large sequence difference in the α-helical as well as the C-terminal random coil region between the MLCK peptide and the αN peptide.

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