THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 19, Issue of May 9, pp. 12318–12327, 1997

Printed in U.S.A.

Cyclin-dependent Kinase 5 (Cdk5) Activation Domain of Neuronal Cdk5 Activator

EVIDENCE OF THE EXISTENCE OF CYCLIN FOLD IN NEURONAL Cdk5a ACTIVATOR*

(Received for publication, October 7, 1996, and in revised form, January 15, 1997)

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Neuronal Cdk5 activator (Nck5a) differs from other cyclin-dependent kinase (Cdk) activators in that its amino acid sequence is only marginally similar to the cyclin consensus sequence. Nevertheless, computer modeling has suggested that Nck5a contains the cyclin-fold motif recently identified in the crystal structure of cyclin A. In the present study, a number of truncation mutants and substitution mutants of the Nck5a were produced and tested for the Cdk5 activation and Cdk5 binding activity. The active domain of Nck5a determined by using the truncation mutants consists of the region spanning residues 150 to 291. The size of Nck5a active domain is essentially the same as that of cyclin A required for Cdk2 activation (Lees, E. M., and Harlow, E. (1993) *Mol. Cell. Biol.* 13, 1194–1201). The change, or the lack of change, in Cdk5 activation activity observed with a number of substitution mutants may be understood on the basis of structure and function relationship of cyclin A. These results provide support to the previous suggestion (Brown, N. R., Noble, M. E. M., Endicott, J. A., Garman, E. F., Wakatsuki, S., Mitchell, E., Rasmussen, B., Hunt, T., and Johnson, L. N. (1995) *Structure* 3, 1235–1247) that the activation domain of Nck5a adopts a conformation similar to that of cyclin A. They also provide a partial answer to the question of how Nck5a, a non-cyclin, activates a cyclin-dependent kinase.

Progress through animal cell cycle depends on the coordinated actions of a family of cdc2-like kinases (1–5) that are heterodimers of a cdc2-homologous catalytic subunit, called cyclin-dependent kinases (Cdks),[1] and an essential regulatory subunit belonging to the cyclin family (6, 7). Cyclins are molecules of diverse molecular masses, but they share the characteristics of containing a homologous region of approximately 100 residues, called cyclin box (8). The recently elucidated crystallographic structure of an active fragment of cyclin A has shown that the cyclin box (of cyclin A) displays a uniquely folded structure comprising five alpha helices. This structure, cyclin fold, repeats itself in a region extending from the C terminus of the cyclin box. There is little amino acid sequence similarity between the two cyclin folds (9, 10).

Among Cdks, Cdk5 is unique in several respects. Although Cdk5 is present in all mammalian tissues and cell extracts examined (11), brain is the only source where Cdk5 kinase activity has been demonstrated (12, 13). The protein is highly expressed in neurons of the central nervous system (14, 15), whereas most of the other Cdks such as Cdk1 and Cdk2 are essentially undetectable in post-mitotic neurons (11). In parallel with these observations is the finding in the neurons of mammalian brains of two highly homologous Cdk5 activator proteins called neuronal Cdk5 activator (Nck5a) and neuronal Cdk5 activator isoform (Nck5ai) (16–19). Curiously, despite their ability in activating a cyclin-dependent kinase, these two Cdk5 activators show little sequence similarity to members of cyclin family (18, 19). The mechanism of activation of Cdk5 by Nck5a and Nck5ai appears to differ from that of Cdk activation by cyclins. While the activation of Cdk1, Cdk2, or Cdk4 by the respective cyclin has been shown to depend on the phosphorylation of the Cdk subunit on a specific threonine residue by an activating kinase, CAK (20–24), Cdk5 activation by Nck5a or Nck5ai is independent of the phosphorylation of Cdk5 (25).

The present study was initiated to probe the structural domain of Nck5a essential for the activation of Cdk5. We find that the size of the active domain of Nck5a is essentially identical to that of cyclin A. By site-directed mutagenesis, a few residues of the protein important for Cdk5 binding and Cdk5 activation were located. The results are compatible with a previous suggestion from computer modeling that Nck5a may assume a tertiary structure similar to that of cyclin A (9, 26).

MATERIALS AND METHODS

Construction of N-terminal Deletion Mutants of Human p35^{nck5a}—A set of N-terminal deletion of human p35^{nck5a} was generated by the polymerase chain reaction (PCR). PCR primers were designed to match a common C-terminal region and different N-terminal regions with *Bam*HI site and *Eco*RI site flanking the N-terminal and the C-terminal primers, respectively. PCR was carried out in 50 μl of reaction mixture containing 100 ng of double-stranded DNA template, 200 μM deoxynucleoside triphosphate, 2.5 units of Pyrococcus furiosus DNA polymerase (Stratagene) using a DNA thermal cycler (GeneAmp PCR System 2400, Perkin-Elmer) for 30 cycles. The PCR amplified fragments were gel purified with a GenecleanII kit (Bio 101, Inc. from BCH Medical Supplies Co.). After digestion with *Bam*HI and *Eco*RI, the fragments were inserted into *Bam*HI/*Eco*RI linearized pGEX2T vector.

Construction of C-terminal Deletion Mutants of Human p35^{nck5a} Using Bal 31 Nuclease—C-terminal deletions of p35^{nck5a} with Bal 31 were performed according to the published procedures (27–29). Briefly, 30 μg of pGEX2T construct containing p35^{nck5a} insert was linearized at the 3′ end by *Eco*RI. The linearized DNA, after phenol extraction and ethanol precipitation, was resuspended in water containing 500 μg/ml BSA. An equal volume of 2 × concentrated Bal 31 nuclease buffer (40 mM Tris-HCl, 1200 mM NaCl, 24 mM $CaCl_2$, 24 mM $MgCl_2$, and 2 mM EDTA),

* This work was supported by the Research Grant Council of Hong Kong, a Hong Kong University of Science and Technology Infrastructure Grant, and by the Biotechnology Research Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Cdk, cyclin-dependent kinase; Nck5a, neuronal Cdk5 activator; Nck5ai, Nck5a isoform; PCR, polymerase chain reaction; BSA, bovine serum albumin; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; GST, glutathione S-transferase.

prewarmed to 30 °C, together with 2 units of Bal 31 (New England Biolabs) were added. The mixture was then incubated at 30 °C for different periods of time. To obtain multiple C-terminal deletions, an aliquot of sample was withdrawn into EGTA solution (final 20 mM) at every 20-s interval up to 6 min and was heated at 65 °C for 10 min to terminate the Bal 31 reaction. Samples from three time points in every minute were pooled and treated with T4 DNA polymerase (Life Technologies, Inc.), after phenol extraction and ethanol precipitation, according to standard protocols (28, 29). 8-mer *Eco*RI linkers (Promega) were ligated to the repaired ends, and the multiple C-terminal deletion p35^{nck5a} fragments were cut out with *Bam*HI/*Eco*RI and cloned into pGEX2T vector linearized with the same enzymes.

Site-directed Mutagenesis—Three procedures were used to produce the site-directed mutations. For the mutation sites close to either the N or C terminus, mutations were incorporated into the 5′ site of the PCR primers. After PCR reaction, the amplified DNA fragments were ligated into pGEX2T vector. The mutations were verified by DNA sequencing.

The site-directed substitution of Glu-221 with an alanine was performed using a commercial kit (QuikChange™ site-directed mutagenesis kit, Stratagene). Basically, a pair of complementary PCR primers with 30–40 bases was designed that placed the mutation in the middle of the primers. In this case, the up primer is 5′-GGGCTCGGATCAT-GcGCTCCAGGCCGTCC-3′ and the low primer is 5′-GGACGGCCTG-GAGCgCGTGATCCGAGCCC-3′. Parental cDNA inserted in pGEX2T was amplified using Pyrococcus furiosus DNA polymerase with these primers for 15 cycles in a DNA thermal cycler (Perkin-Elmer). After digestion of the parental DNA with *Dpn*I, the amplified DNA incorporated with the nucleotide substitution was transformed into *Escherichia coli* (XL 1- Blue strain). The mutation was confirmed by DNA sequencing.

The alanine substitutions of Leu-222, Gln-223, Leu-232, and Glu-240 were carried out in N145/pGEX2T plasmid according to the methods of Mikaelian and Sergeant (30). Briefly, four PCR primers, with two at one end, one at the other end of the insert, and one containing the substitution nucleotides in the middle of the primer, were grouped for two rounds of PCR amplifications. The final PCR product was digested with *Bam*HI and *Eco*RI and inserted into pGEX2T vector. In the same way, alanine substitution of Arg-153, single asparagine substitition of Leu-151 and Leu-152, and double substitutions of Leu-151/Leu-152 with asparagine in the p25^{nck5a} were also generated. The designed nucleotide substitution was confirmed by DNA sequencing.

Plasmid Purification—Plasmid DNA used in this study was purified either by a standard alkaline lysis miniprep protocol (29) or by Wizard™ Minipreps kit (Promega) according to the procedure recommended by the supplier.

DNA Sequencing—The nucleotide sequences of all the PCR-amplified DNA were determined by the chain termination method using ^{T7}Sequencing™ kit (Pharmacia Biotech Inc.). The DNA was sequenced either in the pBluescript KS$^+$ vector (Strategene) using T3 (5′-GCAAT-TAACCCTCACTAAAG-3′) and T7 primers (5′-TAATACGACTCAC-TATAGGG-3′) or in the pGEX2T vector (Pharmacia) with sense primer (5′-CAGCAAGTATATAGCATGGC-3′) and antisense primer (5′-GGA-GCTGCATGTGTCAGAGG-3′).

GST-fusion Protein Expression—GST-fusion proteins were purified as described previously (19, 25, 31). For expression of the GST-fusion proteins, *E. coli* strain BL21(DE3) was freshly transformed with the DNA constructs, and cells were cultured to A_{600} = 1.2 and then stimulated with 0.2 mM of isopropyl-β-D-thiogalactopyranoside at room temperature overnight. Cells were then lysed with a French press (1100 p.s.i.) in MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4) containing 2 mM DTT, 2 μg/ml antipain, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 10,000 × *g* for 5 min at 4 °C, the GST-fusion proteins were purified on glutathione-agarose (Sigma).

Cdk5 and Cdk2 Kinase Assay—In vitro Cdk5 kinase assay was carried out as described previously (18, 25). Cdk5 and its activator were reconstituted in phosphate-buffered saline containing 1 mM EDTA, 1 mM DTT, and 1 mg/ml BSA at 30 °C for 1 h. The histone kinase activity of the reconstituted Cdk5 complex was measured in 30 mM MOPS, pH 7.4, 10 mM $MgCl_2$, 100 μM histone H1 peptide, and 100 μM [γ-^{32}P]ATP (400 cpm/pmol) at 30 °C for 30 min. The reaction was stopped by addition of acetic acid, and the incorporation of phosphate into the histone H1 peptide was measured by a scintillation counter. An active Cdk2 kinase was reconstituted *in vitro* by incubation of GST-Cdk2 (18 ng), protein A-poly(His) fused cyclin A, partially purified CAK from bovine thymus, 0.5 mM ATP in kinase assay buffer (30 mM MOPS, pH7.4, 10 mM $MgCl_2$, 10 mM β-glycerophosphate, 2 mM sodium fluoride) at 30 °C for 60 min. An aliquot of the reconstituted Cdk2 was used to determine the kinase activity at 30 °C for 30 min in a 30-μl reaction containing the kinase assay buffer, 200 μM [γ-^{32}P]ATP, and 100 μM histone H1 peptide.

FIG. 1. **Amino acid sequence comparison of p35^{nck5a} and p39^{nck5ai}.** The *top* and *bottom* sequences are for human p35^{nck5a} and p39^{nck5ai}, respectively. *Vertical lines* and *dots* indicate the identical and similar amino acid residues between these two proteins. The highly conserved core region of p35^{nck5a}, which was expressed as the GST-fusion protein, is *shaded*.

Cdk5 Binding Assay—Binding of Cdk5 by p25 and other mutated p35 was performed as described previously (19). Briefly, bovine brain (1 kg) was homogenized in buffer containing 25 mM Hepes, pH 7.2, 1 mM EDTA, 1 mM DTT, 0.6 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml aprotinin, 0.3 mg/ml benzamidine, and 0.1 mg/ml soybean trypsin inhibitor. The crude homogenate was centrifuged at 100,000 × *g* for 20 min, and the supernatant (S100) was incubated with either GST-p25 or other mutated GST-p25 in the homogenization buffer containing 150 mM NaCl and 2 mM DTT at 4 °C for 12 h. Cdk5 complexes were then precipitated by the addition of glutathione-beads, and the beads were washed three times with MTPBS containing 2 mM DTT and resuspended in 2 × concentrated SDS protein sample buffer. The bound bovine brain Cdk5 was then analyzed by Western blotting with antibody against Cdk5.

Western Immunoblotting—Proteins were separated by SDS-PAGE (32) and transferred to a polyvinylidene difluoride (Bio-Rad) membrane. The membrane was blocked by 10% skim milk in blotting buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% polyoxyethylenesorbitan monolaurate (Tween 20)) and probed with polyclonal anti-Cdk5 antibody at 2 μg/ml in blotting buffer containing 10% BSA for 1 h at room temperature. Signal was developed with ECL Western blotting kit (Amersham Life Science, Inc.) according to the protocol of the supplier.

RESULTS

The Conserved Core Region of Nck5a Can Fully Activate Cdk5—Mammalian brains contain Cdk5 activators p35^{nck5a} and p39^{nck5ai}, which are specifically expressed in neurons of central nervous tissue (17, 18, 19). Comparison of the amino acid sequences of the two proteins reveals a highly conserved region, expanding approximately 150 amino acid residues (Fig. 1). Earlier studies have identified a number of recombinant truncated forms of Nck5a and Nck5ai that are capable of maximally activating Cdk5; these are p25^{nck5a}, p21^{nck5a}, and p30^{nck5ai} (18, 19, 25). All contain this conserved region except that p21^{nck5a} ends at residue 291, whereas the C terminus of the conserved region of Nck5a is glutamate 292 (Fig. 1). To test whether or not this conserved core region alone can activate Cdk5, this region of Nck5a, starting at amino acid residue Gln-145 and ending at Glu-292, was expressed in *E. coli* as a GST-fusion protein. The fusion protein was purified by affinity chromatography on a glutathione column and then tested for the ability to activate a bacterially expressed GST-Cdk5. Fig. 2*A* shows that the fusion protein was able to activate Cdk5 in

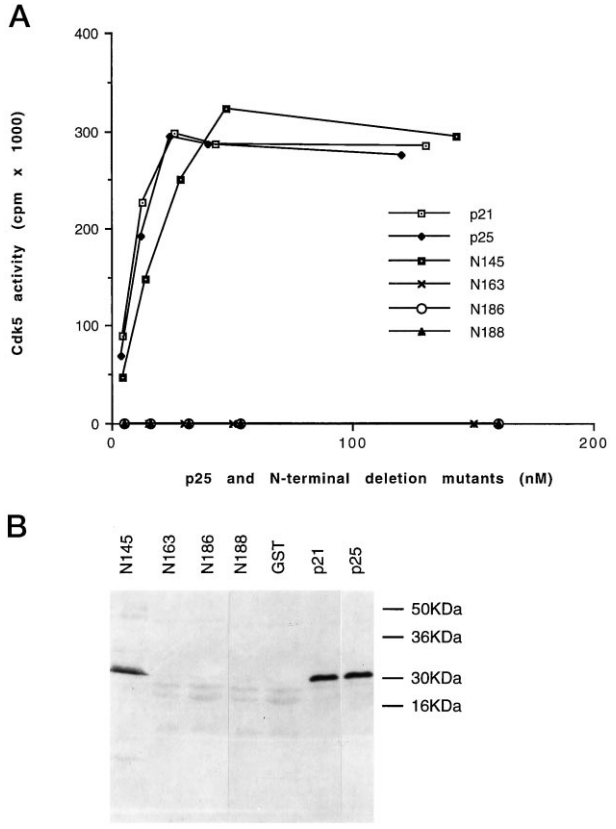

FIG. 2. **Binding and activation of Cdk5 by the N-terminal deletion mutants of p35^{nck5a}.** *A*, dose-dependent activation of Cdk5 by N-terminal deletion mutants. A series of N-terminal deletions together with GST-p25^{nck5a} and GST-p21^{nck5a} were *in vitro* reconstituted with 30 nM GST-Cdk5 as described under "Materials and Methods" in a dose-dependent manner, and the histone kinase activity of these Cdk5 complexes were assayed in 30 mM MOPS, pH7.4, 10 mM $MgCl_2$, 100 μM histone H1 peptide, and 100 μM [γ-^{32}P]ATP (400 cpm/pmol) at 30 °C for 30 min. *B*, binding of N-terminal deletion mutants of p35^{nck5a} to bovine brain Cdk5. The bovine brain 100,000 × *g* supernatant was prepared (see "Materials and Methods"), and the native bovine brain Cdk5 was then precipitated by these N-terminal deleted GST-fusion proteins of Nck5a (see "Materials and Methods"). The precipitated Cdk5 was detected by immunoblot with a polyclonal anti-Cdk5 antibody.

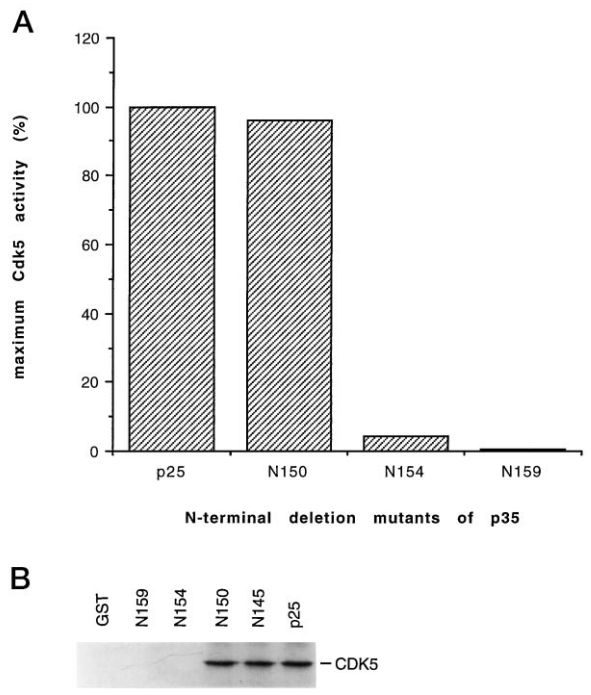

FIG. 3. **Map of the N-terminal boundary of p35^{nck5a} for Cdk5 association and activation between residues 145 and 163.** *A*, maximum Cdk5 activation by p25^{nck5a}, N150, N154, and N159. p25 and three GST-fusion proteins of N-terminal truncations between 145 and 163 were *in vitro* reconstituted, in a dose-dependent manner, with 30 nM of GST-Cdk5. The histone H1 peptide activities of these Cdk5 complexes were assayed as above. The maximum activity of each Cdk5 complex was standardized against that of Cdk5-p25 complex. *B*, binding of N150, N154, and N159 to bovine brain Cdk5. The binding experiment was performed as described under "Materials and Methods" and in the legend of Fig. 2.

a dose-dependent manner to a maximum level of kinase activity (N145 in Fig. 2*A*) comparable with that achieved by p25^{nck5a}. The corresponding region in Nck5ai was also able to fully activate Cdk5 (data not shown). Previous studies have shown that the kinase reconstituted from the bacterially expressed Cdk5 and p25^{nck5a} displays a specific kinase activity similar to or higher than that of the homogeneous preparation of brain neuronal cdc2-like kinase, the heterodimer of Cdk5 and p25^{nck5a}, purified from bovine brain (15, 25). Thus, results of Fig. 2 indicate that the conserved regions of the two Cdk5 activators contain all the structural elements required for Cdk5 activation.

Defining the N-terminal Boundary of the Activation Domain—To examine whether or not the conserved region represented the minimal size of the protein required for Cdk5 activation, a set of N-terminal deletions were generated by PCR amplification and cloned into pGEX2T as described under "Materials and Methods." These GST-fusion proteins were expressed, affinity purified, and tested for their abilities to activate GST-Cdk5. As shown in Fig. 2*A*, while the truncated Nck5a missing the first 144 amino acid residues showed full Cdk5 activation activity, deletion of 162 or more amino acid residues from the N-terminal completely abolished the ability of the protein to activate Cdk5. Thus, at least some of the structural elements essential for Cdk5 activation are located between residues 145 to 162.

As the failure of a Nck5a derivative to activate Cdk5 does not preclude the protein from displaying high affinity association with Cdk5, the ability of the truncated forms of Nck5a to bind Cdk5 has been examined. Bovine brain extract 100,000 × *g* supernatant contains a high amount of the monomeric form of Cdk5, which may undergo high affinity association with various forms of Nck5a derivatives and Nck5a homologous proteins to form active forms of Cdk5 (33). To test the ability of the truncated forms of Nck5a to bind Cdk5, each of the expressed GST-fusion proteins was incubated with an aliquot of bovine brain 100,000 × *g* supernatant and then affinity precipitated by using glutathione beads. After thorough washing of the beads, the precipitated protein was analyzed by Western immunoblot for the existence of Cdk5. Fig. 2*B* shows that Cdk5 coprecipitated with all the activating derivatives of Nck5a but none of the inactive derivatives. The procedure used could detect only high affinity association of Cdk5. The possibility that some of the inactive derivatives of Nck5 could associate weakly with Cdk5 is not ruled out.

To more precisely map the N-terminal boundary of the Cdk5 activation domain, we generated a second set of N-terminal deletion mutants of Nck5a by PCR strategy as outlined above. Fig. 3 shows that deletion of five amino acid residues from the N145 mutant still allowed the remaining Nck5a to associate with Cdk5 and to fully activate the enzyme, but removal of four additional residues abolished the ability of the protein to activate Cdk5 and to undergo high affinity association with Cdk5. Thus, the four amino acid residues, Glu-150, Leu-151, Leu-152, and Arg-153, contain structural elements indispensable for

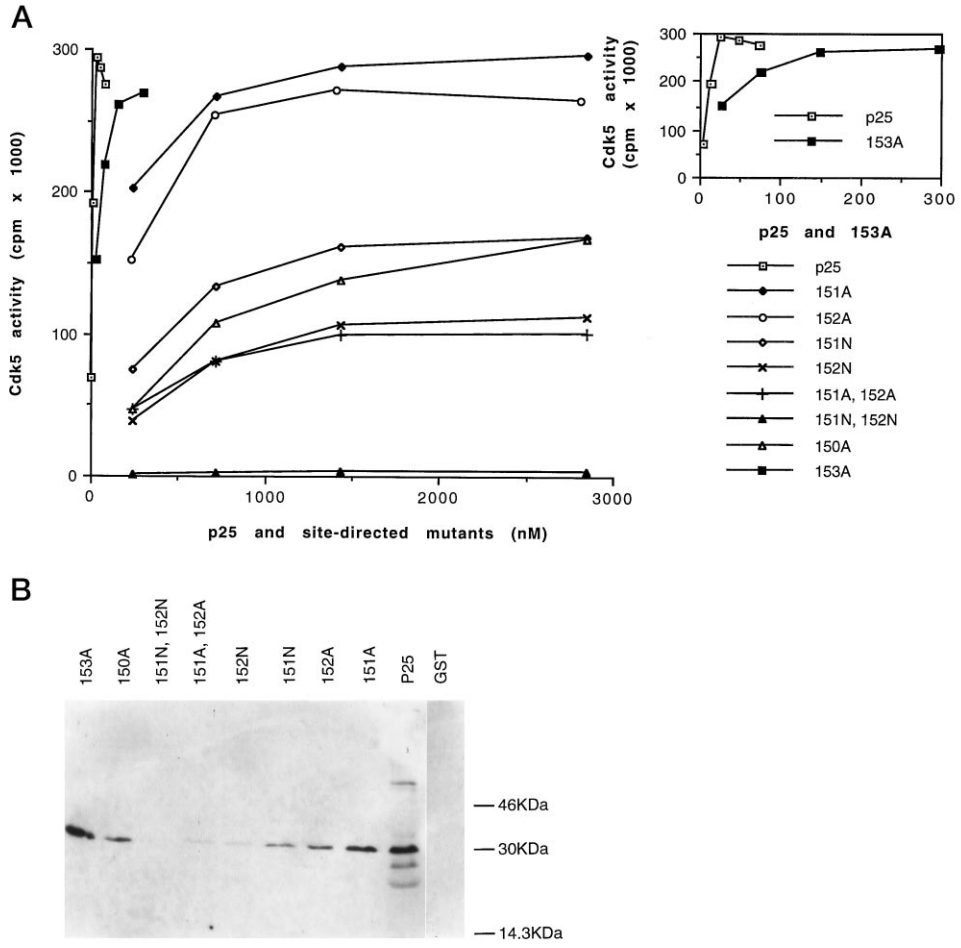

FIG. 4. **Characterization of ^{150}ELLR^{153} of N150^{nck5a} in terms of Cdk5 activation and association by the site-directed mutants in this region.** *A*, dose-dependent Cdk5 activation by the site-directed mutant proteins. Different site-directed mutants in the region of ^{150}ELLR^{153} were generated as GST-fusion proteins (see "Materials and Methods"), which were then used to activate Cdk5 (30 nM) in a dose-dependent manner. The Cdk5 activity of Cdk5-p25 and Cdk5-Ala-153 is amplified in the *inset* of this figure. *B*, Cdk5 binding by these site-directed mutants. Binding of bovine brain Cdk5 by these mutant proteins and immunoblotting using polyclonal antibody against Cdk5 was carried out as described above.

Cdk5 activation and high affinity Cdk5 binding. To further characterize these four N-terminal residues with respect to the contribution to Cdk5 binding and activation, we introduced individual or double amino acid substitutions in this region by site-directed mutagenesis. These mutant proteins were expressed as GST-fusion proteins and used in the Cdk5 binding and activation studies as described above for the truncation protein mutants. As shown in Fig. 4, substitution of Arg-153 by an alanine had little or no effect on the maximal Cdk5 activation of the protein. On the other hand, substitution of Glu-150 by an alanine resulted in 43% reduction in the maximal Cdk5 activation of the protein (Table I). The result suggests that Glu-150 may be considered to be the N terminus of the Cdk5 activation domain of Nck5a, at least in terms of maximal kinase activation.

Both Leu-151 and Leu-152 appear to contribute to Cdk5 activation activity of the protein by participating in hydrophobic interactions. While substitution of Leu-151 or Leu-152 by an alanine had little effect on the ability of the protein to achieve maximal Cdk5 activation, single substitution mutants with asparagine at the position 151 or 152 showed markedly reduced maximal Cdk5 activation of the protein, to 57.2 or 38.1%, respectively (Fig. 4*A* and Table I). The suggestion that the hydrophobic residues Leu-151 or Leu-152 are important for Cdk5 activation was further tested by examining the double substitution mutants of the protein at these positions. When both Leu-151 and Leu-152 were substituted by less bulky alanine, the maximal Cdk5 activation achieved by the mutant protein was reduced to 34.4% of that of the parent protein. The double substitution mutant with hydrophilic asparagine at positions 151 and 152 was found to have completely lost the ability to activate Cdk5 (Table I). The possibility, that the two leucine residues Leu-151 and Leu-152 appear to be critically involved in the Nck5a and Cdk5 interaction because they exist at the immediate N-terminal region of the protein derivatives, has been considered. A number of amino acid substitution analogues of the 25 kDa Nck5a mutated at positions 151, 152, and 153 were constructed, bacterially expressed, and tested for Cdk5 activating activity. It was found that the substitution of arginine at position 153 by an alanine had no effect on the ability of the protein to activate Cdk5. On the other hand, single substitution of the leucine residue at position 151 or 152 by asparagine significantly reduced the maximal activation of Cdk5 by 30 or 50%, respectively. When both leucine residues were substituted together by asparagine, the double substituted protein had no detectable Cdk5 activating activity. These observations have confirmed that the two leucine residues are critically involved in the interaction between Cdk5 and Nck5a.

In addition to comparing the relative maximal Cdk5 activation of the Nck5a protein mutants, an attempt was made to determine the relative affinity of the proteins to Cdk5 on the basis of the dose-dependent kinase activation curves. Bacterially expressed GST-fusion proteins affinity purified by the glutathione column usually contain proteolytic derivatives of the

TABLE I
Summary of substitution mutants for Cdk5 activation

Nck5a and derivatives	Residues substituted		Protein concentration for 50% activation	Maximum activation[a]
	Wild type	Mutant		
			nM	%
p25	p25	NA[b]	9.23	100
Ala-150	Glu-150	Ala-150	520.4	57.1
Ala-151	Leu-151	Ala-151	176.1	100.9
Ala-152	Leu-152	Ala-152	208.2	92.3
Ala-153	Arg-153	Ala-153	24.9	89.5
Ala-151, Ala-152	Leu-151, Leu-152	Ala-151, Ala-152	287.1	34.4
Asn-151	Leu-151	Asn-151	312.0	57.2
Asn-152	Leu-152	Asn-152	432.0	38.1
Asn-151, Asn-152	Leu-151, Leu-152	Asn-151, Asn-152	NA	0
Ala-221	Glu-221	Ala-221	297.8	45.5
Ala-222	Leu-222	Ala-222	120.2	81.9
Ala-223	Gln-223	Ala-223	50.0	98.4
Ala-232	Leu-232	Ala-232	74.7	95.7
Ala-240	Glu-240	Ala-240	54.0	7.0
Ala-288	Asp-288	Ala-288	484.1	26.9
Ala-289	Leu-289	Ala-289	874.7	5.0
Ala-290	Lys-290	Ala-290	58.6	84.0
Ala-291	Asn-291	Ala-291	52.9	83.0

[a] Maximal activation of Cdk5 activated by p25 is taken as 100%.
[b] NA, not available.

fusion protein as well as other proteins that are of bacterial origin. To determine the amount of the intact fusion protein in the sample, an aliquot of the sample was subjected to SDS-PAGE to separate the protein from the contaminant proteins. The Coomassie-stained gel was then analyzed by densitometry to determine the percentage of the intact fusion protein in the sample. The amount of the intact fusion protein in the kinase activation reaction was then calculated and used for the construction of activation dose-dependent curves (Fig. 4). Results of Fig. 4*A* show that the Nck5a mutant proteins, which have the ability to achieve full maximal Cdk5 activation, all display the affinity for Cdk5 similar to the parent protein. On the other hand, mutant proteins achieving significantly lower levels of maximal Cdk5 activation had lower Cdk5 affinity. There appears to be a general correlation between the decrease in maximal Cdk5 activation and that in Cdk5 affinity. It should be stressed, however, that the Cdk5 affinity of the protein determined from the activation curve is very tentative. A portion of the intact fusion protein in the sample might be incorrectly folded. The relative amount of the correctly folded intact fusion protein in the sample probably varied among the mutant proteins and from preparation to preparation of the same protein mutant. The general suggestion, nevertheless, is supported by the results of Fig. 4*B*, documenting the association of the Nck5a mutant GST-fusion proteins with bovine brain Cdk5. With mutant fusion proteins that achieve full maximal Cdk5 activation, amounts of Cdk5 coprecipitated from the brain extract were similar to those from the parent fusion protein, whereas significantly lower amounts of Cdk5 were found in the glutathione beads precipitates with mutant proteins of lower maximal Cdk5 activation.

Cdk2 may be activated by a number of cyclins; its activation by cyclin A is especially well characterized. As shown in Fig. 5*A*, the 25 kDa truncated form of Nck5a is also capable of activating Cdk2. Although the Cdk2 affinity of $p25^{nck5a}$ appears to be somewhat lower than that of cyclin A (Fig. 5*A*, *inset*), the maximal activations of Cdk2 achieved by the two proteins are similar. There is one important difference in Cdk2 activation by the two activators. While Cdk2 activation by cyclin A can be greatly enhanced (more than 20-fold) upon phosphorylation of Cdk2 by CAK, the activation of Cdk2 by Nck5a is independent of CAK. As a result, the maximal Cdk2 activation by Nck5a is only a fraction of that achieved by cyclin A under the optimal activation conditions. The same kinase activation domain of Nck5a appears to be involved in the Cdk2 and Cdk5 activation. Fig. 5*A* shows that the minimal-sized Cdk5 activating derivative, N150, is capable of activating Cdk2, although with markedly decreased activation affinity and maximal activation. The activation of Cdk2 by cyclin A and Nck5a probably involves the same protein domain of Cdk2, as $p25^{nck5a}$ is capable of inhibiting the activation of Cdk2 by cyclin A (in the presence of CAK) in a dose-dependent manner (Fig. 5*B*). Although Cdk2 appears to be activated by Nck5a and the activation involves the same activation domain as that for Cdk5, it is important to note that Cdk5 is not activated by cyclin A either in the presence or in the absence of CAK (Fig. 5*A*).

Defining the C-terminal Boundary of the Activation Domain—To map the C-terminal residues of Nck5a that are necessary for Cdk5 activation, a series of C-terminal deletions were generated by following the time course of a Bal 31 exonuclease treatment of N145 and cloned into pGEX2T vector as detailed under "Materials and Methods." The deleted C-terminal residues were determined by DNA sequencing. The affinity purified GST-fusion proteins were tested for Cdk5 association and the activation of Cdk5. The observation that the conserved core region by itself as well as $p21^{nck5a}$ (25) can fully activate Cdk5 suggests that at least 15 residues from the C terminus of Nck5a could be removed without adverse effect on the ability of the protein to bind to and activate Cdk5. Further deletion of four amino acid residues (residue 288 to 292), however, resulted in a drastically reduced maximal Cdk5 activation (Fig. 6). The marginal Cdk5 activation of the protein could be completely eliminated when an additional nine residues were deleted from the C terminus. Since these C-terminal deletions were generated by Bal 31 exonuclease digestion, the immediate stop codon was disrupted, resulting in the addition of two or three amino acid residues to the C terminus of these fusion proteins. To rule out the possibility that addition of these residues caused the disruption of the Cdk5 activation or Cdk5 binding, stop codon was added immediately to the C terminus of these inactive deletion proteins by PCR. The purified GST-fusion proteins derived from these constructs showed neither Cdk5 activation nor Cdk5 binding activity.

The observation that deletion of four residues from the C terminus of the conserved region of Nck5a resulted in a drastic reduction of the maximal Cdk5 activation, and Cdk5 binding ability of the protein suggests the existence of structural ele-

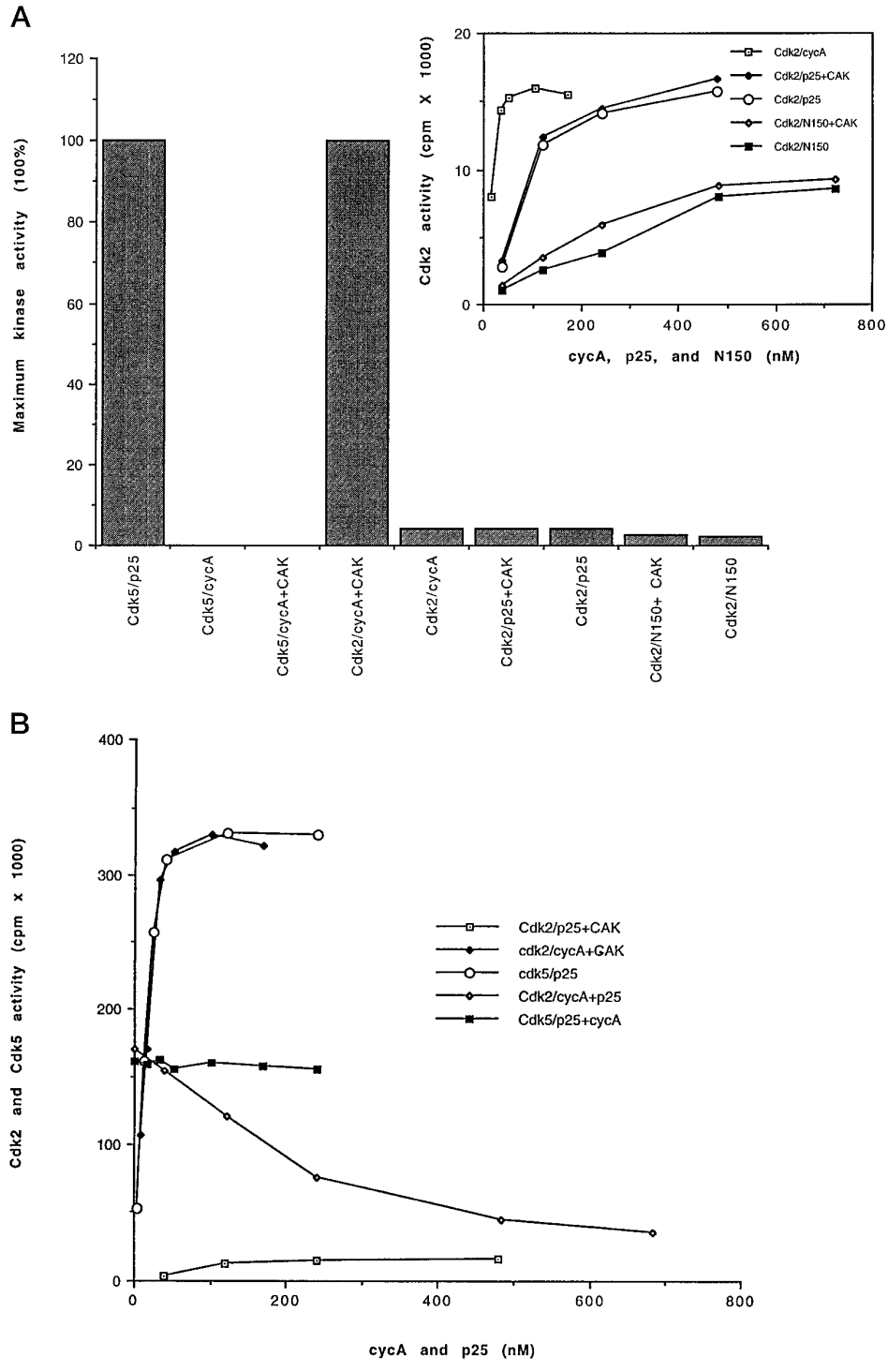

FIG. 5. **Activation of Cdk2 by p25^{nck5a} and cyclin A.** *A*, maximum Cdk5 activation by p25^{nck5a} and cyclin A (*cycA*), and the maximum activation of Cdk2 by cyclin A, p25^{nck5a}, and Nck5a (150–292). The histone H1 activities of Cdk5 and Cdk2, stimulated by different activators, were standardized against that of Cdk5/p25^{nck5a} and Cdk2-cyclin A + CAK. The *inset* shows the dose-dependent activation of Cdk2 by cyclin A, by p25^{nck5a} (with and without CAK), and by Nck5a (150–292) with and without CAK. *B*, dose-dependent inhibition of Cdk2-cyclin A with p25^{nck5a}. Cdk5 was dose-dependent activated by p25^{nck5a}, and Cdk2 (18 ng) was dose-dependent activated by both cyclin A and p25^{nck5a}. While gradually increasing the amount of cyclin A to Cdk5/p25^{nck5a} shows no effect on the kinase activity, a dose-dependent increasing of p25^{nck5a} to Cdk2-cyclin A results in gradual reduction of the kinase activity of Cdk2-cyclin A.

ments within this four residue region important for Cdk5 activation and Cdk5 binding. To define the important structural element more precisely, the four C-terminal residues, Asp-288, Leu-289, Lys-290, and Asn-291, were individually substituted by alanine, and the resultant mutant proteins were analyzed for their abilities to activate Cdk5 and to bind Cdk5. As shown in Fig. 7, substitution of Lys-290 or Asn-291 by alanine did not significantly affect the ability of protein to provide maximal Cdk5 activation, whereas substitution of Leu-289 by alanine drastically reduced the ability of the protein to activate Cdk5. The result suggests that leucine residue at position 289 may define the C boundary for the Cdk5 activation domain of Nck5a in terms of maximal kinase activation. In addition, residue Asp-288 is shown to participate in the kinase activation since

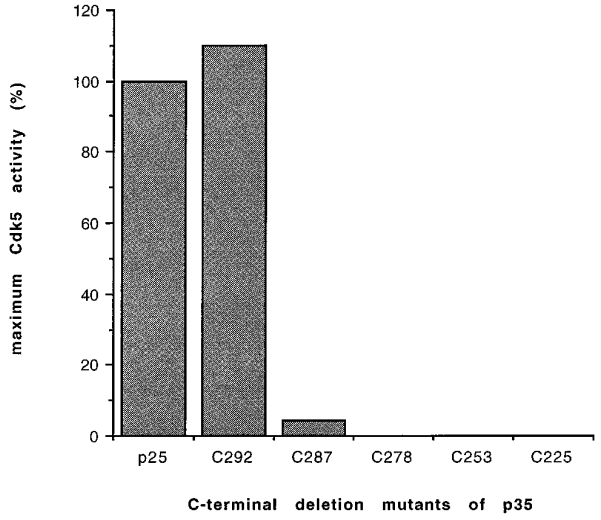

FIG. 6. **Activation of Cdk5 by the C-terminal deletions of Nck5a.** A set of C-terminal deletions were generated by Bal 31 exonuclease digestion. The deleted regions were confirmed by DNA sequencing. The mutant GST-fusion proteins were expressed and *in vitro* reconstituted in a dose-dependent manner with GST-Cdk5, and the histone H1 peptide activities of these Cdk5 complexes were assayed accordingly. The maximum histone kinase activity of each Cdk5 complex was standardized against that of Cdk5-p25 complex.

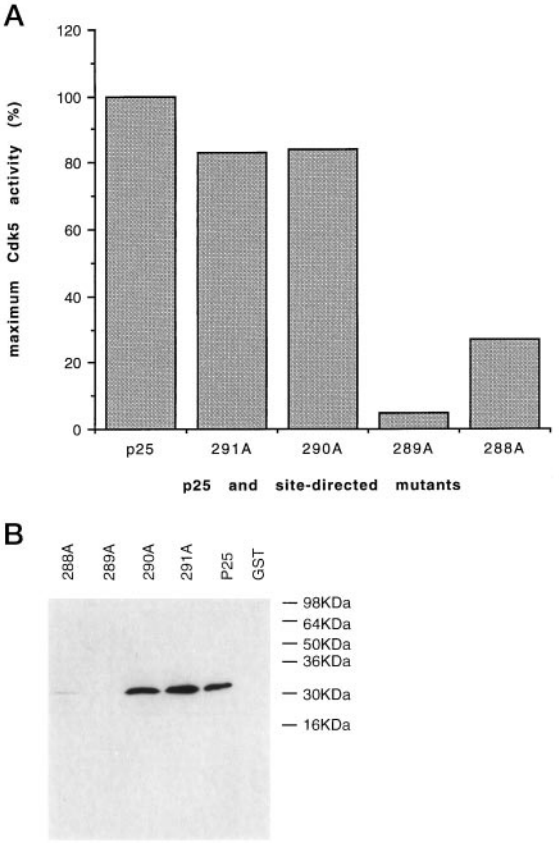

FIG. 7. **Association with and activation of Cdk5 by the single residue substitution mutants of ^{288}DLKN^{291} of N145^{nck5a}.** Alanine was used to substitute ^{288}DLKN^{291} individually as outlined under "Materials and Methods." The maximum Cdk5 activation stimulated by these proteins was standardized against the maximum Cdk5 activity stimulated by p25 (*A*). Bovine brain Cdk5 was precipitated by the these proteins, and the bound Cdk5 was detected by immunobloting with polyclonal antibody against Cdk5 (*B*).

the alanine substitution mutant, D288A, displayed a markedly lower level of maximal Cdk5 activation. Similar to the N-terminal truncation and substitution mutants of Nck5a, the C-terminal mutants showing low levels of maximal Cdk5 activation also displayed decreased Cdk5 binding activity as determined by the amount of Cdk5 coprecipitating with the GST-fusion proteins from the 100,000 × *g* supernatant of bovine brain extract (Fig. 7*B*). No Cdk5 coprecipitation was demonstrated with the mutant proteins that showed maximal Cdk5 activation less than 10% of that of the parent protein.

Mutation of the Cyclin-like Region—Although the overall amino acid sequence similarity between Nck5a and cyclin consensus sequence is very low, we have previously identified a region of about 20 amino acid residues of Nck5a that shows a significant level of sequence similarity to the cyclin consensus sequence (Fig. 8*A*) (18). A number of single alanine substitution mutants of Nck5a at this region were therefore constructed, and the mutant proteins were bacterially expressed and tested for kinase activation activity. Fig. 8 shows that substitution of residue Leu-222, Gln-223, or Leu-232 by alanine had no effect or only slight effect on the ability of the protein to activate Cdk5 and to bind Cdk5. This conserved region is flanked by two glutamate residues, Glu-221 and Glu-240. Single alanine substitution mutants at these two positions have also been tested. As shown in Fig. 8*B*, the substitution of Glu-221 by alanine had only a slight effect on maximal Cdk5 activation, but maximal Cdk5 activation by the Ala-240 mutant was drastically lowered to about 7% of that achieved by the wild-type protein. Unlike the other low activity mutants that showed little or no high affinity Cdk5 binding activity (see Figs. 4 and 7), Ala-240 mutant could bind Cdk5 with high affinity. The dose dependence of Cdk5 activation by the Ala-240 mutant (Fig. 8*B*) suggested that there was only a small decrease in Cdk5 affinity of the protein. The suggestion is supported by the observation that the amount of Cdk5 from bovine brain extract 100,000 × *g* supernatant precipitated with the mutant protein appeared to be similar to that with the wild-type protein (Fig. 8*C*).

The availability of a high affinity but low activation activity mutant of Nck5a suggests the possibility of construction of a dominant negative mutant of the protein. Thus, the ability of the alanine 240 mutant protein to block the activation of Cdk5 by the wild-type protein was examined. Fig. 9 demonstrates that the activation of Cdk5 by a constant level of the wild-type Nck5a could be effectively blocked by the alanine 240 mutant in a dose-dependent manner. The final level of the kinase activity approached that of the maximal activation of the mutant protein. In contrast to Ala-240 mutant, the Ala-289 mutant, which has no Cdk5 activating activity nor high affinity Cdk5 binding activity, had little effect on the activity of Cdk5/p25. Although Cdk2 can also be activated by p25^{nck5a} and the minimal-sized activating Nck5a, Nck5a (150–292), the mutant Nck5a Ala-240 displays essentially no Cdk2 activating activity. As shown in Fig. 9, the activity of Cdk2-cyclin A is only marginally affected by Nck5a Ala-240 mutant. Although the mutant Ala-240 Nck5a shows specific inhibition of Cdk5, the mutant protein also possesses significant, albeit low, Cdk5 kinase activation activity. Thus, additional mutagenesis may be needed to construct an effective dominant negative mutant.

DISCUSSION

One of the salient features of Nclk that distinguishes the enzyme from other cdc2-like kinases is its unique regulatory subunit, the neuronal Cdk5 activator. In addition to its tissue- and cell-type-specific expression, Nck5a does not have sufficient amino acid sequence similarity to the consensus cyclin box sequence to be classified as a cyclin. Although Cdk5 shares a high degree of sequence similarity with other Cdks (34), no cyclin has been demonstrated to activate Cdk5. The observation has raised the question as to how a non-cyclin protein

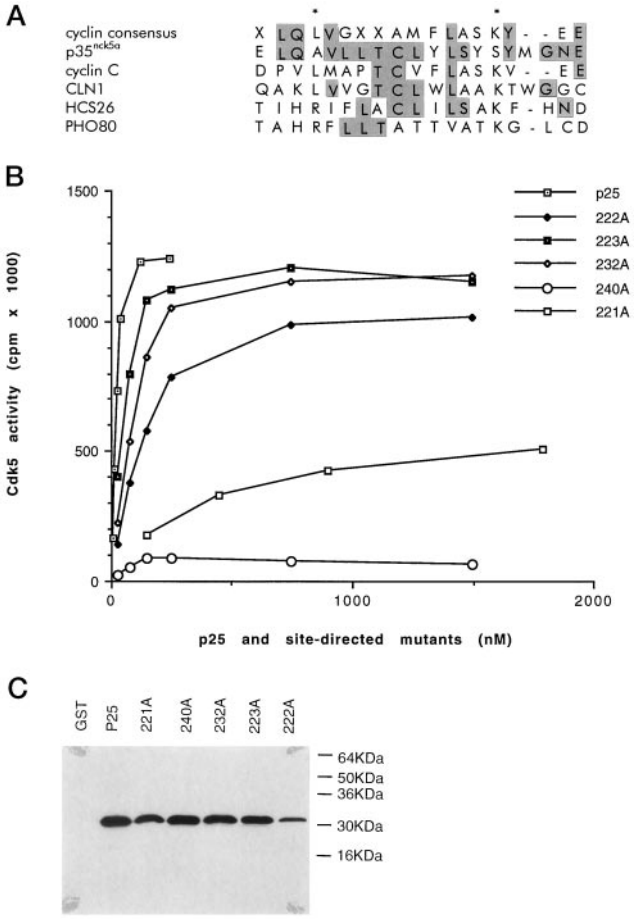

FIG. 8. **Characterization of cyclin homologous region of N145^{nck5a} for Cdk5 activation.** *A*, comparison of Nck5a with cyclin and cyclin-like sequences. The cyclin consensus sequences are derived from cyclins A, B, D, and E; residue *X* indicates non-conserved amino acid for these cyclins and *dash* indicates the introduced gap between residues. The highly conserved residues, Leu and Lys, for all cyclins are indicated with *asterisks*. *Shaded* residues indicate matches to Nck5a. *B*, activation of Cdk5 by the site-directed mutants in this region. Glu-221, Leu-222, Gln-223, Leu-232, and Glu-240, which are indicated by the number underneath, were individually substituted with alanine (see "Materials and Methods") and expressed as GST-fusion proteins that were then used to activate GST-Cdk5 (30 nM) in a dose-dependent manner. *C*, Cdk5 association with these mutants. Binding of bovine native Cdk5 monomer with these mutant GST-fusion proteins was performed as detailed under "Materials and Methods."

carries out Cdk activation activity, such as the Nck5a action on Cdk5 activation.

The crystallographic structure of a truncated and active cyclin A, both in its free and Cdk2-bound states, has been elucidated recently (9, 10). The characteristic structure comprises two repeating subdomains, called cyclin fold, each containing five helices. The two repeating subdomains are sandwiched by two helices, an N-terminal and a C-terminal α-helix. Although there is little sequence similarity between the first and the second subdomains, the tertiary structures of the two repeats are almost superimposable, thus suggesting that the amino acid sequence requirement of the "cyclin fold" is not highly rigid. This suggestion is supported by the observation that proteins such as Rb and TFIIB, which are not related to cyclins either structurally or functionally, appear to contain the cyclin-fold structure (9, 26, 38, 39, 40). Furthermore, Nck5a has been suggested to adopt the cyclin-fold structure on the basis of computer modeling (9, 26). By analysis of the Cdk5 activation and Cdk5 binding activities of a large number of deletion mutants and a few amino acid substitution mutants of Nck5a, the present study provides an experimental support for the suggestion that Nck5a may assume a cyclin-like tertiary structure.

By systematic truncation in combination with mutation of the terminal residues of the truncated forms of the protein, the minimal size of Nck5a capable of fully activating Cdk5 has been determined to be 142 amino acid residues, residue 150 to 291. Sequence alignment of this protein region with the active domain of cyclin A (27), using the Multalin program (41) (Fig. 10), shows that the active domains of the two proteins are essentially of the same size. As expected, this region of cyclin A includes all the amino acid residues that make contacts with Cdk2, as revealed in the crystal structure of the cyclin A-Cdk2 complex (9, 10). Furthermore, the secondary structure of Nck5a predicted by using a neural network algorithm (42) shows that the predicted α-helices of the active domain of Nck5a are located at the regions well matched with those of α-helices in cyclin A. Although the overall sequence similarity between cyclin A and Nck5a is very low, a small region of approximately 20 amino acids has a significant number of identical residues between the two sequences (Fig. 10, *open box region*; Refs. 18 and 19). This region correlates approximately with the α3-helix, which appears to play a pivotal role in maintaining the cyclin-fold structure of cyclin A. The α3-helix of cyclin A serves as the core upon which the other four α-helices of the first of the two repeating subdomains are packed. In addition, the N-terminal α-helix is also packed closely with the α3-helix (9, 10). Thus, it seems that the general structural features of the active domain of Nck5a are compatible with the notion that Nck5a adopts a conformation similar to that of a cyclin fold.

Although many of the amino acid residues of cyclin A identified to be involved in direct contact with Cdk2 or directly involved in intramolecular interactions are not conserved in Nck5a on the basis of the sequence alignment of Fig. 10, analysis of a few amino acid substitution mutants of Nck5a has revealed potential common structural basis of Nck5a and cyclin A in the protein folding and in Cdk activation. For example, Leu-151 and Leu-152, which contribute to the Cdk5 activation by participating in an essential hydrophobic interaction (see Fig. 4 and Table I), are located in the region corresponding to isoleucine 182 of cyclin A (Fig. 10). From the crystal structure, Ile-182 is seen to be in the N-terminal α-helix of cyclin A. The N-terminal helix is packed intimately with the α3-helix and the residue Ile-182 interacts directly with a phenylalanine residue, Phe-152 of Cdk2. This phenylalanine and its neighboring residues are strongly conserved throughout the Cdk family of kinases, including Cdk5. Thus, it may be suggested that the residues Leu-151 and Leu-152 are involved in the interaction with the phenylalanine residue of Cdk5 that is equivalent to Phe-152 of Cdk2. On the other hand, the observation that substitution of Arg-153 of Nck5a to alanine has little effect on the kinase activation activity of Nck5a can also be understood by assuming that Nck5a and cyclin A have similar structure-function relationships in kinase activation. The equivalence of Arg-153 in cyclin A is a threonine residue, Thr-184. Crystal structure of cyclin A shows that Thr-184 has no direct contact with any residue in Cdk2.

While the critical importance of the residues at the N-terminal boundary of the active domain of Nck5a may be understood by the participation of these residues in the kinase binding and activation, residues at the C-terminal boundary appear to correspond to a region of cyclin A important for the protein folding. For example, the observation that mutation of Leu-289 had a large effect on the kinase activation activity of Nck5a may be understood by assuming that this residue is important for the protein folding. The Leu-289 equivalent residue in cyclin A is Tyr-318, which is buried deeply in the hydrophobic core of

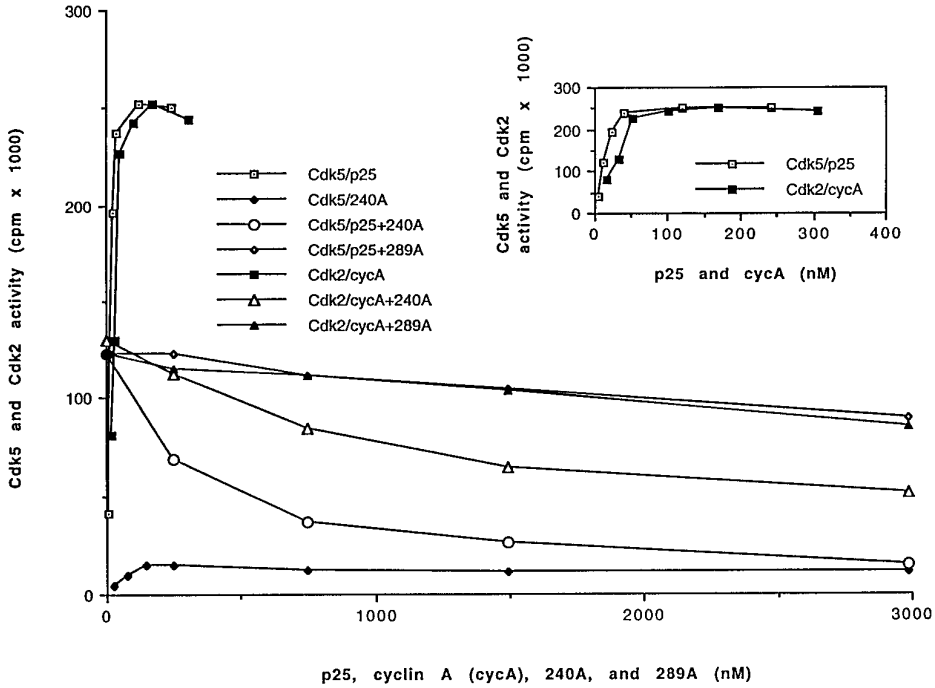

FIG. 9. **Dose-dependent competition of Cdk5 binding of p25^{nak5a} with Ala-240^{nck5a}.** Cdk5 was dose-dependent activated by p25 and Ala-240. A fixed amount of Cdk5 and p25, which lies in the linear range of p25 stimulation curve, was *in vitro* reconstituted with increasing doses of Ala-240, resulting in gradual reduction of Cdk5 activity. The activation curves of Cdk2 and Cdk5 are enlarged in the *inset*.

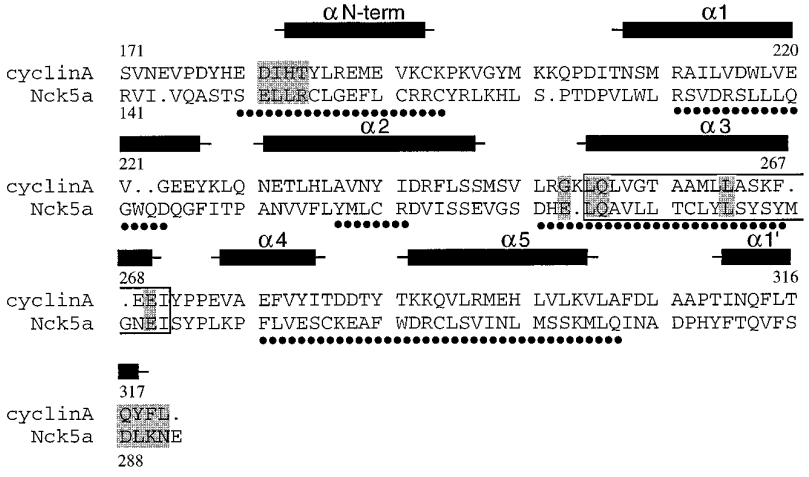

FIG. 10. **Sequence alignment of the conserved core region of Nck5a with that of cyclin A.** The *black bars* above the cyclin A sequences represent the α-helices observed in the crystal structure of cyclin A (9, 10). The *dotted regions* below the Nck5a sequences typify the α-helix predicted for Nck5a. The *shaded regions* represent the Nck5a residues that have been subjected for site-directed mutagenesis studies.

cyclin A, suggesting an involvement in the maintenance of the tertiary structure of the protein. Thus, in addition to defining the active domain of Nck5a, the functional roles of the boundary regions of the active domain are elucidated in the present study. In addition, a number of substitution mutants with mutations within or in proximity to the putative α3-helix region were examined. The results also support the suggestion that Nck5a may have similar general conformation as cyclin A. Two aspartate residues, Glu-221 and Glu-240, which correspond to Gly-232 and Glu-269 of cyclin A, respectively, have been substituted individually by alanine (Fig. 10). It was observed that while substitution of Glu-221 had only little effect, substitution of Glu-240 markedly decreased the kinase activation activity of Nck5a (Fig. 8). Presumably, like Glu-269 in cyclin A, Glu-240 of Nck5a participates in the Cdk5 activation by interacting with Arg-149, equivalent to Arg-150 of Cdk2 (10, 43). Together, these results provide strong support to the suggestion that Nck5a assumes a conformation similar to that of cyclin A (9). Such a suggestion is further supported by the observation that Cdk2 could be activated by p25^{nck5a} and some of the derivatives of Nck5a (35).

While the full activation of Cdks by their respective cyclins typically depends on the phosphorylation of the Cdk by the activating kinase, CAK (20–24), Cdk5 is maximally activated by Nck5a in the absence of Cdk5 phosphorylation (25). The observation has raised the question of whether the unique mechanism of the Cdk5 activation by Nck5a is attributable to Cdk5 or to Nck5a (25). The observation that Cdk2 activation by Nck5a is also independent of CAK suggests the phosphorylation-independent activation of the Cdks is determined by the activator protein. In addition to its phosphorylation-independent activation, Nclk does not appear to be significantly inhibited by the common Cdk inhibitory kinase Wee1 kinase (35, 44), nor by the inhibitor proteins of Cdks, p21^{Cip}, and p27^{kip1} (36, 37). The question of what structural differences between cyclins and Nck5a contribute to the unique regulatory properties of Nck5a is therefore raised. To address this and other related questions, a more in depth characterization of the structure

and function of Nck5a is required.

Poon *et al.* (35), independently, have carried out a study on the Cdk5 active domain of Nck5a and obtained similar results. The minimally sized Nck5a derivative capable of activating Cdk5 determined in their study is the truncated form of residues 150 to 292, essentially the same as that obtained in this study. However, they have found that truncation derivatives with up to 168 residues deleted from the C terminus of Nck5a can still bind Cdk5, a finding significantly different from our observation that only a small region of C terminus deletion can be tolerated in terms of Cdk5 binding. This difference may be attributed to the different experimental approaches used in the two studies. The method used to detect Cdk5 binding in this study was designed mainly to reveal protein derivatives of Nck5a with high affinity Cdk5 binding activity.

In conclusion, although the amino acid sequence of Nck5a has little similarity to those of cyclins, results of the present study support the previous suggestion (19, 26) that Nck5a may adopt a conformation containing the cyclin-fold structure. In addition, a number of amino acid residues in Nck5a have been identified as playing important roles in the kinase activation or protein folding in Nck5a. The success in assigning functions to specific residues on the basis of cyclin A structure has greatly strengthened the suggestion. More importantly, it partly answers the question of how a non-cyclin may activate a cyclin-dependent kinase. The present study, however, does not address the question about the structure-function relationship of Nck5a concerning the unique regulatory properties of the protein. With the knowledge of the crystal structure of T-160 phosphorylated cyclin A-Cdk2 at hand (43), further studies using specially designed Nck5a mutants may be constructed to address the question of why Nck5a activation of Cdk5 is phosphorylation-independent.

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