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Identification of a Common Protein Association Region in the Neuronal Cdk5 Activator*

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Cyclin-dependent protein kinase 5 (Cdk5) depends on the association with neuronal Cdk5 activator (Nck5a) for kinase activity. A variety of cellular proteins have been shown to undergo high affinity association with Nck5a, including three novel proteins, C42, C48, and C53 found by a yeast two-hybrid screen (Ching, Y. P., Qi, Z., and Wang, J. H. (2000) *Gene* 242, 285–294). The three proteins show competitive binding to Nck5a suggesting that they bind at a common site. The binding site has been mapped to a region of 26 amino acid residues (residues 145 to 170) at the N-terminal boundary of the kinase activation domain of Nck5a. This region of Nck5a contains an amphipathic α-helix whose hydrophobic face is involved in Cdk5 activation (Chin, K. T., Ohki, S, Tang, D., Cheng, H. C., Wang, J. H., and Zhang, M. (1999) *J. Biol. Chem.* 274, 7120–7127). Several lines of evidence suggest that Nck5a interacts with the binding proteins at the hydrophilic face of the amphipathic α-helix. First, the Nck5a-(145–170) peptide can bind Cdk5 and Nck5a-binding proteins simultaneously. Second, the association of Nck5a-(145–170) to C48 can be markedly reduced by high ionic strength whereas the interaction between Nck5a and Cdk5 is not affected. Third, substitution of Glu^{157} by glutamine in Nck5a-(145–170) abolishes the peptide's ability to bind to the three Nck5a-binding proteins without diminishing its Cdk5 binding activity.

Cyclin-dependent protein kinase 5, Cdk5,[1] was originally discovered by virtue of its sequence homology to cell cycle regulatory Cdks (1–4). Like cell cycle regulatory Cdks that depend on association with a cyclin for kinase activity (5), Cdk5 is dependent on association with a protein activator for its kinase activity. The first active form of Cdk5 was isolated from mammalian brain and shown to be a heterodimer of Cdk5 and a 25-kDa regulatory protein (6–8). The regulatory subunit was subsequently shown to be a truncated form of a 35-kDa protein now known as neuronal Cdk5 activator, Nck5a (9). Two forms of mammalian Cdk5 activators have been identified, the 35-kDa protein and a 39-kDa protein that is called neuronal Cdk5 activator isoform, Nck5ai (10). Among the mammalian tissues examined, Cdk5 kinase activity was readily demonstrated only in brain extracts (1, 7, 11), and the two activators, p35^{nck5a} and p39^{nck5ai}, were detected virtually exclusively in neurons of the central nervous system (6, 9, 10). Cdk5 and its activators have been suggested to play important regulatory functions in mammalian brain development as well as neuronal activities in mature brains (12, 13). Evidence has been accumulating to suggest that aberrant regulation of Cdk5 may lead to cell death and/or neurodegeneration, thus contributing to various neurodegenerative diseases including Alzheimer's Disease (12–19).

Previously we reported the existence of three protein complex forms of Cdk5 in bovine brain: the monomeric Cdk5, a heterodimer of Cdk5 and p25^{Nck5a}, and a 670-kDa macromolecular protein complex containing Cdk5 and p35^{Nck5a} complex (20). The three forms of Cdk5 have different kinase activities (20). The monomeric Cdk5 has no endogenous kinase activity but can be activated by the addition of bacterial expressed Nck5a. While the heterodimer of Cdk5 and p25^{nck5a} displays high kinase activity, and the macromolecular protein complex containing Cdk5 and p35^{nck5a} has, surprisingly, no kinase activity, nor can it be activated by the addition of its activator (20). This observation suggests that the proteolytic conversion of p35^{nck5a} to p25^{nck5a} may be a mechanism of Cdk5 regulation in neurons. This suggestion is supported by recent studies showing various biological and functional differences between the two forms of Nck5a such as, intracellular localization, protein turnover rate, and the ability to induce cell apoptosis (19).

The fact that Cdk5-p25^{nck5a} exists as a heterodimer whereas Cdk5-p35^{nck5a} is part of a macromolecular protein complex suggests that Nck5a may show high affinity binding to specific cellular proteins (20). Over last few years, several laboratories have reported the identification of specific Nck5a-binding proteins, including neurofilament proteins, retinoblastoma protein, a small GTPase Rac, and β-catenin (21–24). We have used a yeast two-hybrid system to screen for Nck5a-binding proteins, resulting in the identification in a human brain library of 7 Nck5a-binding proteins, including three novel proteins. Full-length clones of these novel Nck5a-binding proteins, called C42, C48, and C53, have subsequently been isolated from a rat brain cDNA library (25).

Although Nck5a and Nck5ai activate a cyclin-dependent protein kinase, they do not appear to contain a cyclin box, a conserved region of the protein's primary structure characteristic of members of the cyclin protein family (26–28). On the other hand, D-type and E-type cyclins have been shown to bind to Cdk5, but the heterodimeric proteins have no observable kinase activity (4, 29). Thus, it has been suggested that the unique function and regulatory properties of Cdk5 arise, to a large extent, from the structure of Nck5a and Nck5ai. The present study is concerned mainly with the examination of the

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[1] The abbreviations used are: Cdk, cyclin-dependent kinase; GSH, glutathione; GST, glutathione *S*-transferase; Nck5a, neuronal Cdk5 activator; Nck5ai, neuronal Cdk5 activator isoform; PCR, polymerase chain reaction.

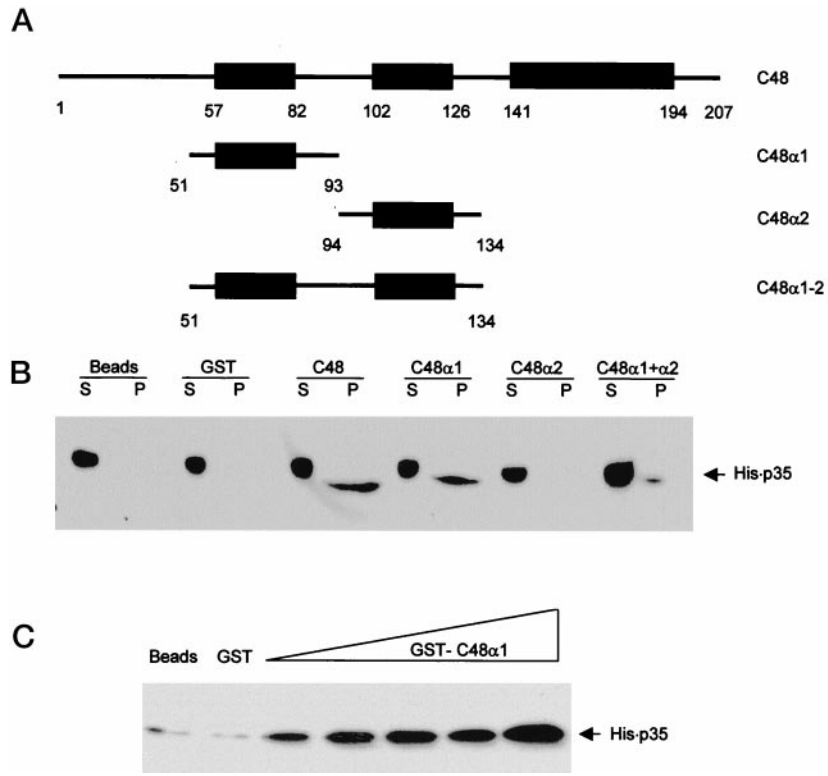

FIG. 1. **Binding of C48 and its deletion mutants with p35^{nck5a}.** *A,* schematic diagram showing the predicted α-helical regions of C48. The predicted helixes of C48 are highlighted with *black boxes*. The C48α1, C48α2, and C48α1–2 constructs are indicated with their, respectively, starting and ending amino acid residue numbers. *B,* mapping of the p35^{nck5a}-binding region in C48. The presence of $(His)_6$-tagged p35^{nck5a} was detected with the p35^{nck5a}-specific antibody (C-19) in both the supernatant (S) as well as in the GSH-agarose-precipitated protein complexes (*P*). *C,* concentration-dependent binding between C48α1 and p35^{nck5a}. Increasing amount of GST-fusion C48α1 (10, 100, 200, 400, and 600 μg) was pre-bound to GSH-agarose beads. 15 μg $(His)_6$-tagged p35^{nck5a} was subsequently added and the C48α1-bound form of $(His)_6$-tagged p35^{nck5a} was analyzed with the p35^{nck5a}-specific antibody (C-19). GSH-agarose beads and GST protein were used as the negative controls.

structural basis of the interactions between Nck5a- and Nck5a-binding proteins. We have found that the three novel Nck5a-binding proteins either share a common binding site or have overlapping binding sites. Using C48 as the model binding protein, a region of Nck5a spanning 26 amino acid residues, has been found to be the minimal sequence required for C48 binding. This region of Nck5a is proximal to the N-terminal boundary of the kinase activation domain (26). We have also characterized the interaction between Nck5a and C48 in detail.

EXPERIMENTAL PROCEDURES

Constructs—GST-fused forms of C48, C42, and C53 expression constructs used in this study were described previously (25).

Construction of GST-fused N-terminal and C-terminal deletion mutants of human p35^{nck5a} followed the procedures described in an earlier study (26). To construct $(His)_6$-tagged or GST-fused Nck5a-(145–170) bacterial expression plasmids, a PCR amplified, *Bam*HI/*Eco*RI-digested fragment was inserted into the pET32a (Novagen) or pGEX4T-2 vector (Amersham Pharmacia Biotech), respectively. C48α1, C48α2, and C48α1–2 fragments amplified by PCR were digested with *Bam*HI and *Eco*RI and subcloned into pGEX4T-2 expression vector for protein expression.

Protein Expression and Purification—Expression of GST- and histidine-tagged proteins and their purification from bacterial cells followed previously described methods (10, 18).

An untagged form of Cdk5 was purified from a thrombin digestion mixture of GST-Cdk5 fusion protein. GST-Cdk5 (5 mg in 200 μl) was incubated with 15 units of thrombin in 50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl and 2.5 mM $CaCl_2$ buffer at 4 °C for 3 h. The digestion mixture was then loaded onto a Superose-12 gel filtration chromatography column (Amersham Pharmacia Biotech) equilibrated with MTPBS buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3). The untagged Cdk5 was eluted with 40 ml of MTPBS buffer at a 0.5 ml/min flow rate. Western blot analysis with a Cdk5-specific antibody (C-8, Santa Cruz Biotech) was used to assay the presence of Cdk5 in each fraction.

In Vitro Binding Assay—GST fusion proteins (30 μg) were incubated with 30 μl of GSH-agarose beads in 500 μl of binding buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3, 1 μg/ml antipain, 1 μg/ml leupeptin, 5 mg/ml bovine serum albumin). After incubation at 4 °C with end-to-end rotation for 1 h, the beads were washed three times with 1 ml of binding buffer and subsequently resuspended in 500 μl of 25 mM Tris-HCl, pH 7.5. About 15 μg of $(His)_6$-tagged proteins were added to the above solution. After incubation at 4 °C with end-to-end rotation for an additional 30 min, the GSH-agarose beads were washed five times with 1 ml of 25 mM Tris-HCl, pH 7.5. The proteins precipitated by beads were then dissolved in 20 μl of SDS-polyacrylamide gel electrophoresis loading buffer and resolved by 10% or 12% SDS-polyacrylamide gel electrophoresis. The precipitated histidine-tagged proteins were then analyzed by Western blotting using a p35^{nck5a}-specific antibody (C-19, Santa Cruz Biotech) or anti-His antibody (Amersham Pharmacia Biotech). The binding competition assay followed the procedure described for the *in vitro* binding assay except that specific competitor proteins were added together with the $(His)_6$-tagged proteins.

In Vitro Kinase Assay—*In vitro* Cdk5 activity was assayed as described previously (25, 26). Two assay conditions were considered to evaluate the effect of the full-length C48 (or the α1 fragment) on Cdk5 activity. Under the first condition, GST-p25^{nck5a} and GST-Cdk5 was co-incubated together with the full-length GST-C48 protein or the GST-C48α1 fragment in the reconstitution step. Under the second condition, GST-p25^{nck5a} was preincubated with GST-C48 full-length protein or GST-C48α1 fragment for 30 min prior to the enzyme reconstitution.

Site-directed Mutagenesis—The site-directed substitution of Glu^{157} with a glutamine was performed using the QuickChange™ Site-directed Mutagenesis Kit (Stratagene). A pair of complementary PCR primers were designed with the mutation in the middle of the primers. The sense primer was 5′-CGCTGCCTGGGTCAATTTCTCTGCCGCC-3′, and the antisense primer was 5′-GGCGGCAGAGAAATTGACCCAG-GCAGCG-3′. The wild type $p25^{145–170}$ in pGEX4T-2 was used as the template for PCR amplification, and the mutation was confirmed by DNA sequencing.

Secondary Structure Prediction—The secondary structure of C48 was predicated by the program PHD (32).

RESULTS

The Nck5a-binding Site of C48 Is Located within a Predicted Helical Region—One of the Nck5a-binding proteins, C48, was chosen as the model protein to study the molecular basis of interactions between Nck5a and its binding proteins. The C48 protein, upon expression in *Escherichia coli* as a GST fusion protein or a $(His)_6$-tagged protein, can be readily purified from the bacterial cell lysate. The predicted secondary structure of C48 contains three α-helices (Fig. 1*A*). Since the last α-helix is not contained in the protein encoded by the C48 DNA fragment originally identified by the yeast two-hybrid screen (25), this

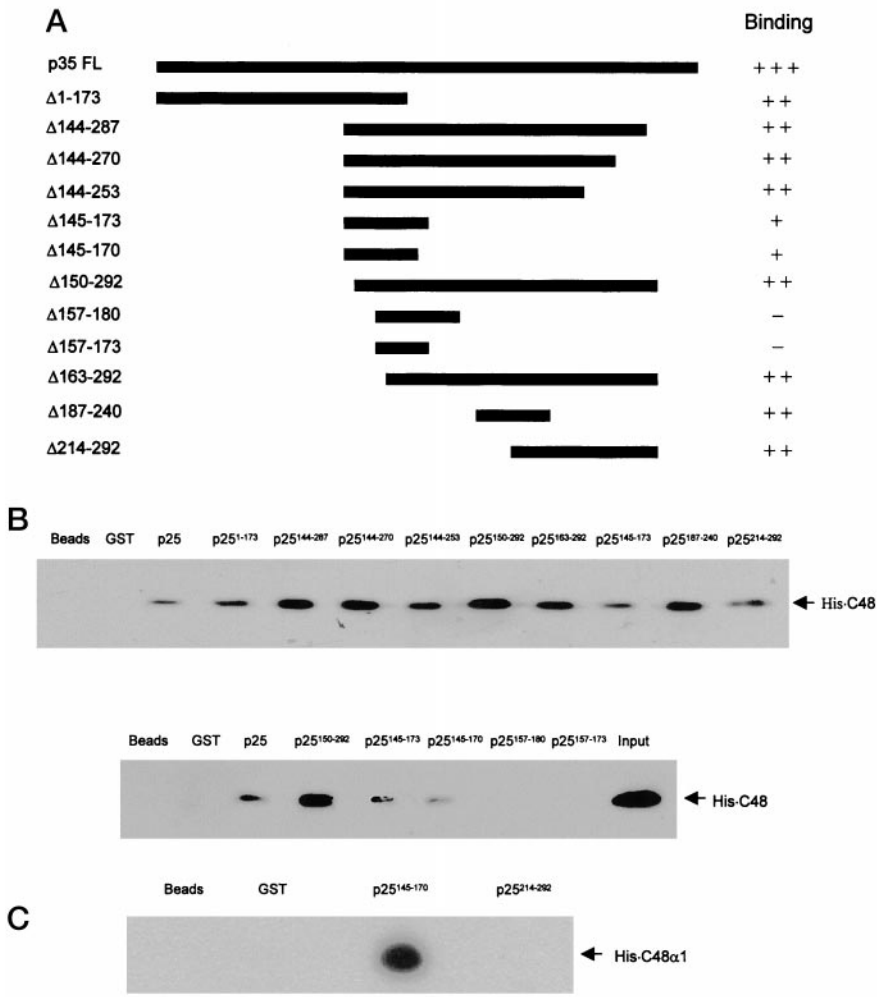

FIG. 2. **Mapping of the C48-binding region in p35^{nck5a}.** *A,* schematic diagram showing the p35^{nck5a} truncation mutants used in the experiment. The C48 binding activities of the p35^{nck5a} mutants are also summarized in the figure. *B,* "pull-down" assay of the binding between p35^{nck5a} mutants and C48. Approximately 30 μg of GST fusion p35^{nck5a} deletion mutants and 15 μg $(His)_6$-tagged C48 protein were used in the assay mixture. The existence of C48 in the GSH-agarose-precipitated protein complexes was detected with a monoclonal anti-His antibody. GSH-agarose beads and GST protein were used as the negative controls and GST-p25^{nck5a} was used as the positive control. *C,* the binding of p25$^{145-170}$ and p25$^{214-292}$ with $(His)_6$-tagged C48α1. About 30 μg of GST-fusion proteins and 15 μg of $(His)_6$-tagged C48α1 were used in the binding assay. The lane labeled "*Input*" represents the loading control of $(His)_6$-tagged C48 protein.

helix is likely not required for Nck5a binding. To further define the Nck5a-binding domain of C48, constructs containing each of the first two α-helix fragments, C48α1 and C48α2, as well as one containing both helixes (C48α1–2) were created and the GST fusion forms of these protein fragments were expressed in *E. coli*. The recombinant protein samples were then tested for their ability to bind to Nck5a. Data in Fig. 1*B* show that $(His)_6$-tagged p35^{nck5a} can bind to GST-C48α1, GST-C48α1–2 as well as the full-length C48, but not to GST-C48α2. This result suggests that the Nck5a-binding site is contained within the first α-helix of C48. To verify the above result, we examined the concentration-dependent binding between the two proteins. The amount of $(His)_6$-tagged p35^{nck5a} found in the complex precipitated by GSH-agarose beads was shown to increase in parallel with the increasing amount of GST-C48α1 used in the binding assay (Fig. 1C).

The C48-binding Site of Nck5a Is Localized within a 26-Amino Acid Residue Fragment—To map the C48-binding site of Nck5a, a series of N-terminal and C-terminal trunction mutants of Nck5a were constructed (Fig. 2*A*). The mutant proteins were expressed as GST fusion proteins in *E. coli* and tested for their ability to bind to C48. Data in Fig. 2*B* show that extensive deletions from either N-terminal or C-terminal ends of Nck5a did not abolish its C48 binding activity. The observation that non-overlapping fragments of Nck5a (*e.g.* Nck5a-(1–173) and Nck5a-(214–292)) could bind to C48 suggests that there are two C48-binding sites in Nck5a. These two sites are designated here as the **a**-site and **b**-site and correspond to the binding sites close to either the N terminus or the C terminus of Nck5a, respectively. The smallest C48-binding fragment that contains the **a**-site is the 26-residue fragment of Nck5a-(145–170). The second site, **b**-site, appears to be localized to a region spanning residues Ser^{214} to Glu^{240}, as both deletion mutants Nck5a-(187–240) and Nck5a-(214–292) show high binding affinity for C48 (Fig. 2).

Although two C48-binding sites were identified in Nck5a using the deletion approach, it was not clear whether both sites are functional in the intact protein. To test this, we resorted to the use of truncation mutants of C48 that contain the Nck5a-binding site. We reasoned that by using a fragment of C48 with the regions not essential for binding eliminated, the possibility of nonspecific binding might be reduced. As C48α1 was the smallest Nck5a binding fragment of C48 available (Fig. 1*B*), it was tested for binding to the two deletion mutants Nck5a-(145–170) and Nck5a-(214–292), which contain the N- and C-terminal C48-binding sites, respectively. Fig. 2*C* shows that only the deletion mutant containing the N-terminal binding site could bind C48α1, suggesting that the C-terminal-binding site does not operate in the intact Nck5a.

Simultaneous Binding of the Nck5a-(145–170) Peptide to Cdk5 and C48—In earlier studies, we showed that the region corresponding to the Nck5a-(145–170) peptide is required for Nck5a to activate Cdk5 (26, 30). The question of whether the binding of C48 will modulate the kinase activity of Cdk5/p25^{nck5a} therefore arises. To address this question, we tested the effect of C48 on Cdk5 kinase activity under two different conditions. Under one condition, Nck5a was preincubated with C48 prior to its mixing with Cdk5. Under the other condition,

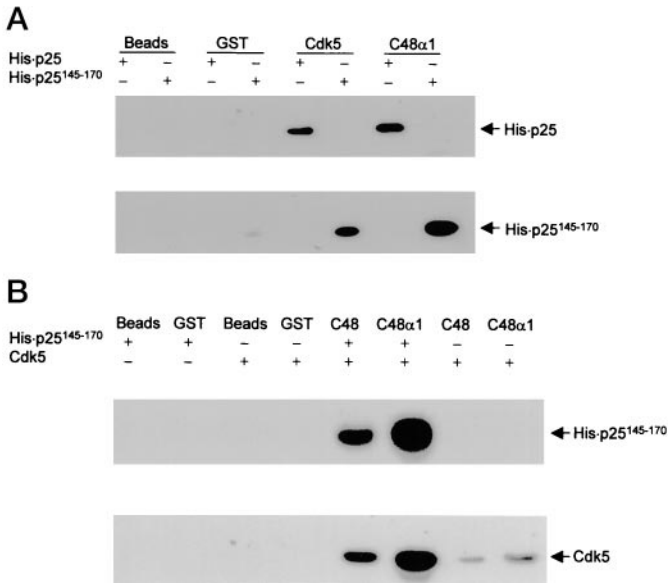

FIG. 3. **Formation of the Cdk5, p25^{nck5a}, and C48 ternary complex.** *Panel A* shows that Cdk5 binds to both p25^{nck5a} and p25$^{145–170}$ from the pull-down assay. The presence of $(His)_6$-tagged p25^{nck5a} and $(His)_6$-tagged p25$^{145–170}$ in the GSH-agarose precipitated protein complexes were detected with a monoclonal anti-His antibody. *Panel B* shows that p25^{nck5a} and Cdk5 form ternary complexes with C48 or C48α1. In this experiment, both untagged Cdk5 and $(His)_6$-tagged p25$^{145–170}$ can be precipitated by the GSH-agarose bound form of GSH-C48 (or GST-C48α1). Whereas very little direct interactions between C48 (or C48α1) and Cdk5 were detected (last two lanes).

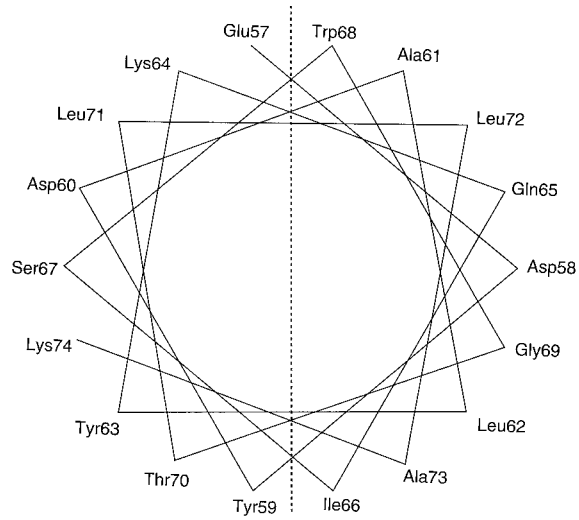

FIG. 4. **Helical wheel presentation of the amphipathic C48 α1 helix.** The hydrophobic and hydrophilic faces of the peptide are separated by a *dashed line*.

C48 was mixed with preactivated Cdk5·Nck5a complex to test its effect on kinase activity. Under both conditions, no change in Cdk5 activity was observed even when the concentration of C48 was increased to as high as 2 mg/ml (data not shown). These results suggest that Cdk5 and C48 do not compete with each other for binding to Nck5a. This observation is also in agreement with an earlier observation that C48 is capable of binding to both free Nck5a and Nck5a in association with Cdk5 (25).

We then examined whether the 26-residue minimal C48-binding peptide of Nck5a (Nck5a-(145–170)) can simultaneously interact with Cdk5 and C48. In these experiments, GST-fused C48 or C48α1 was incubated with both $(His)_6$-tagged Nck5a-(145–170) and Cdk5, and the protein complexes were precipitated with GSH-agarose beads. The precipitates were then analyzed by Western blot for the existence of Nck5a-(145–170) and Cdk5. Since Cdk5 is capable of associating with Nck5a-(145–170) (Fig. 3*A*) but not directly with C48 (Fig. 3*B*), the equivalent amount of Cdk5 together with Nck5a-(145–170) in the C48 affinity precipitates indicates that Nck5a can simultaneously associate with Cdk5 and C48 to form a ternary complex.

C48 and Cdk5 Use Different Mechanisms to Bind to Nck5a—The observations that the minimal C48-binding peptide of Nck5a (Nck5a-(145–170)) can form a ternary complex with Cdk5 and C48 and that C48 protein does not effect the Nck5a-activated Cdk5 kinase activity suggest that C48 and Cdk5 react with different residues in this small peptide fragment. Structural studies has shown that this peptide fragment is likely to form an amphipathic α-helix (30). It is conceivable that the two sides of the amphipathic α-helix may be used to bind to the two different proteins. The association between the Nck5a-(145–170) peptide and Cdk5 is mediated via hydrophobic interactions (26, 27). The binding to C48 is thus likely to involve the hydrophilic face of Nck5a-(145–170). Secondary structure prediction shows that the C48α1 peptide is also amphipathic (Fig. 4). Therefore, we reasoned that the association between C48α1 and Nck5a-(145–170) is likely to be electrostatic in nature.

Two different experiments were carried out to test the notion that the peptide Nck5a-(145–170) uses different faces of its amphipathic α-helix to associate with Cdk5 and C48. One of these examined the effect of salt and non-ionic detergent on the interaction of the peptide with the two proteins. As shown in Fig. 5, the association between C48α1 and Nck5a-(145–170) decreased as the NaCl concentration was increased in the binding reaction buffer. The binding of C48α1 to the Nck5a peptide was essentially abolished when the salt concentration reached 1 M (Fig. 5*A*). On the other hand, 1% Triton X-100 had no effect on the interaction between Nck5a-(145–170) and C48α1. In striking contrast to the C48-Nck5a interaction, the association between Nck5a and Cdk5 was not weakened by high concentrations of NaCl but was abolished completely by 1% Triton X-100 (Fig. 5*B*). These results support the suggestion that, in the region spanning residues Gln^{145} to Ser^{170}, Nck5a interacts with Cdk5 and C48 via the hydrophobic and hydrophilic faces of its amphipathic α-helix, respectively.

Site-directed mutagenesis was carried out as the second approach to test this hypothesis. The hydrophilic faces of the amphipathic α-helices of both Nck5a-(145–170) and C48α1 are rich in charged amino acid side chains (Ref. 30, also see Fig. 4). Thus, it is possible that ionic interactions play important roles in the association between the two proteins, and mutations involving certain charged residues may therefore adversely effect this protein-protein interaction. Although attempts were made to produce mutations at two individual charged residues, only one mutant protein was successfully expressed in *E. coli.* Fig. 6*A* shows that substitution of Glu^{157} by glutamine abolished the ability of the peptide to bind to C48α1. Since Glu^{157} is located at the center of the hydrophilic face of the α-helix, this result strongly implicates the hydrophilic face of the α-helix in the association between C48 and Nck5a-(145–170). In contrast, the association between the mutant peptide and Cdk5 was not adversely effected by this amino acid substitution (Fig. 6*B*). In a previous study, we showed that a dual substitution mutant of Nck5a (where both Leu^{151} and Leu^{152} within the Nck5a-(145–170) region of the activator replaced by asparagines) lost most of its activity due to a severely diminished affinity for Cdk5 (26). This mutant protein was found to bind C48 as well as the wild type protein (results not shown). The differential effects of protein mutations on the interaction of

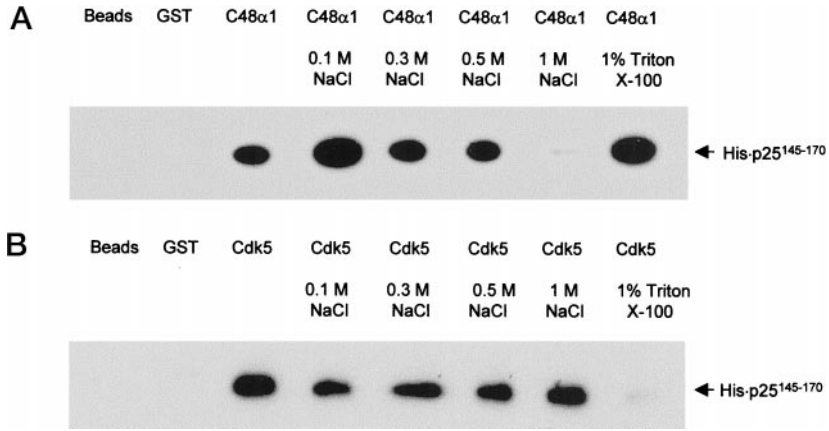

FIG. 5. **Characterization of the interaction of Nck5a-(145–170) with C48α1 and Cdk5.** The interaction of $p25^{145–170}$ with C48α1 (*A*) and Cdk5 (*B*) under different concentrations of NaCl in the presence of 1% Triton X-100. In this experiment, GST-C48α1 was absorbed onto GSH-agarose beads, subsequently about 15 μg of $(His)_6$-tagged $p25^{145–170}$ were added to the GST-C48α1 sample in the presence of various concentration of NaCl, or 1% Triton X-100. The C48α1-bound form of Nck5a-(145–170) was precipitated by GSH-agarose beads and detected by Western blot. The concentrations of NaCl and Triton X-100 indicated in the figure represent the final concentration of the assay mixture.

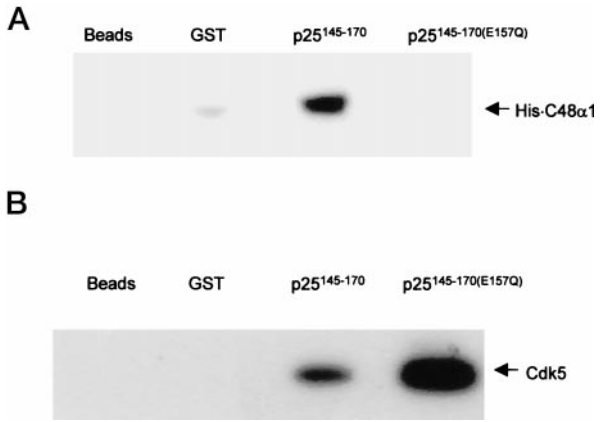

FIG. 6. **Effect of Glu^{157} to Gln mutation of $p25^{145–170}$ on its interaction with C48α1 and Cdk5.** The binding assay was carried out using the conditions described in the legend to Fig. 1. *Panel A* shows the binding of $p25^{145–170}$ and $p25^{145–170(E157Q)}$ with $(His)_6$-tagged C48α1. *Panel B* shows the binding of $p25^{145–170}$ and $p25^{145–170(E157Q)}$ with GST-free Cdk5.

Nck5a with Cdk5 and C48 have provided further support for the suggestion that the binding of Cdk5 and C48 to Nck5a-(145–170) involve the hydrophobic and hydrophilic faces of the amphipathic α-helix respectively.

Nck5a-(145–170) Is the Common Binding Region for $p35^{nck5a}$-associated Proteins—To test whether the C48-binding site of Nck5a is specific for C48, or shared by many of the Nck5a-binding proteins, we examined the interaction of Nck5a-(145–170) with C42 and C53, respectively. Fig. 7 shows that both C42 and C53 bind to Nck5a as well as Nck5a-(145–170) with high affinities. This observation suggests that these three novel Nck5a-binding proteins share the same binding site on $p35^{nck5a}$.

To further test this hypothesis, a competition assay was carried out between these $p35^{nck5a}$-associated proteins (C42 and C53) and C48. In this assay, GSH beads were first precoated with the competing Nck5a-binding protein (GST-C42 or GST-C53) and then incubated with a fixed amount of $(His)_6$-tagged $p25^{nck5a}$ and increasing amounts of $(His)_6$-tagged C48. The samples were then affinity precipitated using GSH-agarose beads and the precipitates analyzed by Western blotting (see Fig. 8*A*). Fig. 8*A* shows that as the amount of competing Nck5a-binding protein C48 was increased in the binding reaction, the amount of $(His)_6$-tagged $p25^{nck5a}$ in the affinity precipitates was reduced, consistent with a competitive binding between C48 and C42 or C53 for Nck5a. Similar results were obtained when C48α1, instead of the full-length C48, or Nck5a-(145–170) was used instead of $p25^{nck5a}$ in the competitive binding assay (Fig. 8, *B* and *C*). These results further support the suggestion that both C42 and C53 compete with C48 for Nck5a binding at the site Nck5a-(145–170). The observation that C48α1, which binds selectively to the **a**-site, can effectively block the binding of C42 and C53 to $p25^{nck5a}$, strongly supports our view that site-**b** does not function as a binding site in intact Nck5a.

Like C48, C42, and C53 bind strongly to Nck5a-(145–170). When the residue Glu^{157} was substituted with Gln, the binding activity of the peptide for these two proteins was mostly eliminated (Fig. 9). This result suggests that the mechanism used by C48, C42, and C53 to bind to Nck5a are similar (*i.e.* through the hydrophilic faces of the two α-helixes). This result further supports the model whereby these three proteins bind to the same region of Nck5a.

DISCUSSION

During the last few years a variety of cellular proteins have been found that undergo specific and high affinity association with Nck5a (21–25). Characterizations of some of these protein-protein interactions have shed light on the function, regulation, and the mechanism of action of Nck5a (24, 31). However, to date there is no study on the biochemical mechanisms of interactions between Nck5a and its binding proteins. The present study is concerned mainly with the molecular basis of the interactions between Nck5a and three novel Nck5a-binding proteins, designated C42, C48, and C53 (25). Upon analyses of the binding characteristics of a large number of Nck5a deletion mutants, a region of 26 amino acid residues, proximal to the N-terminal boundary of the kinase activation domain of Nck5a, was identified as containing the binding site(s) of these Nck5a-binding proteins. The peptide corresponding to this region of Nck5a (designated as Nck5a-(145–170)) can associate with these Nck5a-binding proteins with affinities in the same order of magnitude as those of the full-length Nck5a. Interestingly, this region of Nck5a has also been suggested to contain structural elements essential for the kinase activation (26, 27). This suggestion has been substantiated in the present study by the observation that Nck5a-(145–170) binds Cdk5 with high affinity.

Due to its small size and relative ease of purification of the recombinant protein, C48 was used initially as the model Nck5a-binding protein in these studies. When full-length C48 was used to examine the binding properties of the various Nck5a deletion mutants, the results suggested the existence of two binding sites, one within the 26-residue region (**a**-site), and another in the region of residues 214–240 (**b**-site). Subsequently, the Nck5a-binding site was mapped to within the first α-helix of C48 and a C48 deletion mutant corresponding to this α-helix region, C48α1, could bind only to the **a**-binding site. This observation suggests that only the **a**-binding site can bind C48 in intact Nck5a. This suggestion was further supported by

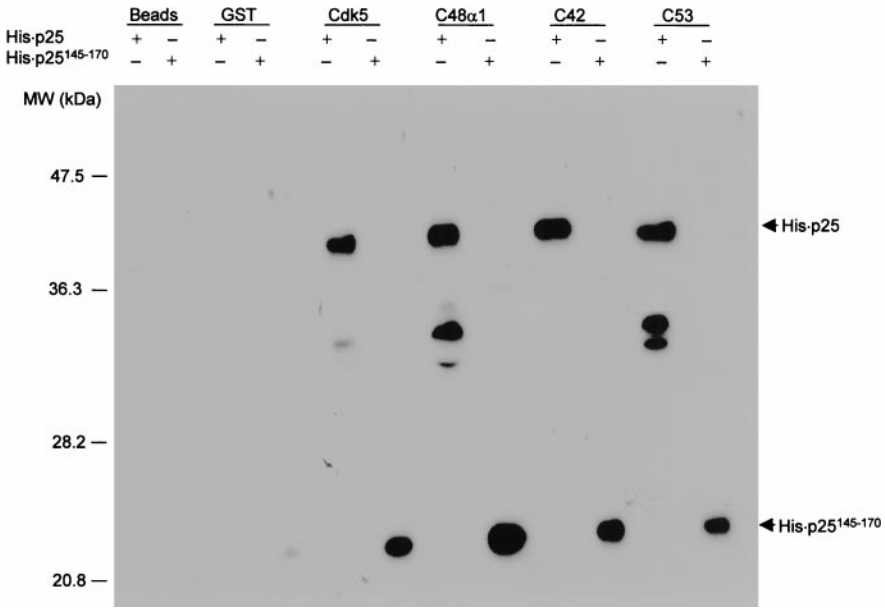

FIG. 7. **The p25^{nck5a}-associated proteins share the same binding site in Nck5a.** The binding assay was carried out under the same conditions as described in the legend to Fig. 1, except that C42 and C53 instead of C48 were used in the experiment (see "Experimental Procedures" for more details).

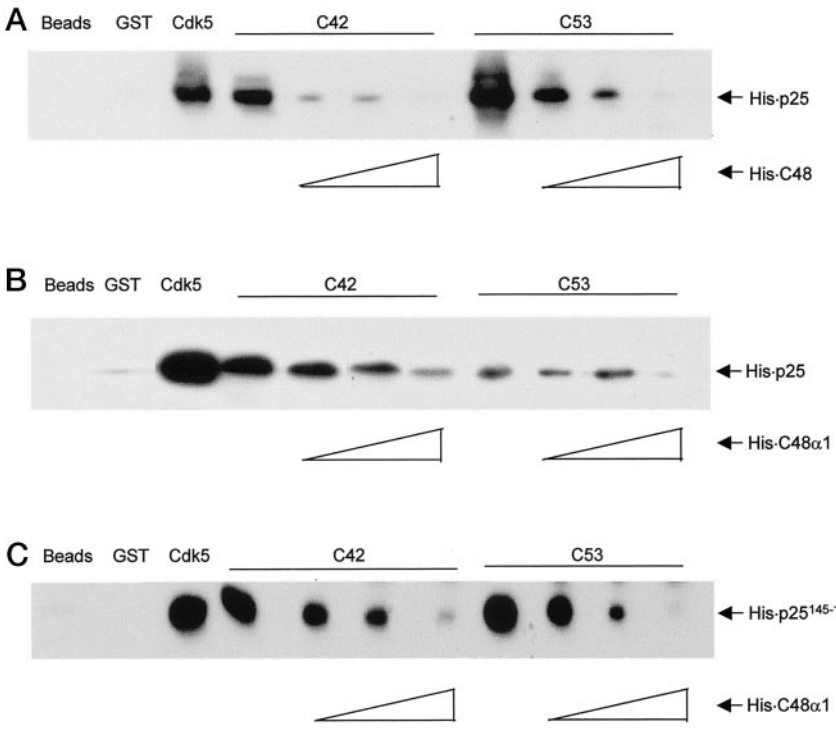

FIG. 8. **p25^{nck5a}-associated proteins compete with each other for the activator.** *A,* C48 competes with C42 and C53 for p25^{nck5a}. Approximately 30 μg of GST-fusion C42 and C53 proteins were prebound to GSH-agarose beads, 15 μg of $(His)_6$-tagged p25^{nck5a} together with increasing amounts of $(His)_6$-tagged C48 (150, 300, and 750 μg) were subsequently mixed with the GST fusion proteins. The amount of $(His)_6$-tagged p25^{nck5a} pulled down by the GST fusion proteins was analyzed by p35^{nck5a}-specific antibody (C-19). GSH beads and GST protein were used as the negative controls and GST-Cdk5 was used as the positive control. *B* and *C,* C48α1 competes with C42 and C53 for p25^{nck5a} and p25$^{145–170}$, respectively. The experimental conditions were identical to those described in *panel A*.

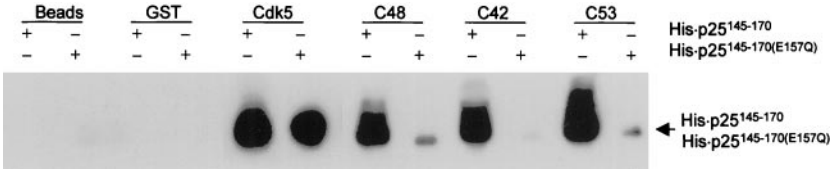

FIG. 9. **The effect of Glu^{157} to Gln mutation of p25$^{145–170}$ on its binding to C42 and C53.** GST-fused C42 and C53 were used to interact with $(His)_6$-tagged p25$^{145–170}$ or $(His)_6$-tagged p25$^{145–170(E157Q)}$. The experiment was performed as described in the legend to Fig. 6.

the observation that the binding of C53 and C42 to p25^{nck5a} can be effectively be blocked by both C48 and C48α1. It is a common practice to use deletion protein mutants to map specific binding sites on a protein molecule. The observation that binding site **b** demonstrated on deletion mutants may not function in the intact protein suggests caution against such artifacts.

The three Nck5a-binding proteins (C42, C48, and C53) used in this study to characterize interactions between Nck5a and its binding proteins show common binding characteristics. They all display high affinity and specific binding to the peptide Nck5a-(145–170) and they compete with each other for bindings to the peptide or intact Nck5a, suggesting that they bind to Nck5a at a common site. The suggestion is further supported by the observation that the interactions of Nck5a with C42, C48, and C53 are similarly affected by the ionic strength and detergent contents of the reaction medium, as well as by site-directed mutation of Nck5a (see below). However, the possibility that C42, C48, and C53 have distinctive but overlapping sites in this region cannot be completely excluded.

In an earlier study (30), we showed that a 29-residue peptide derived from Nck5a displayed potent inhibitory activity toward Cdk5 and Cdk2. This Nck5a-derived inhibitor that spans residues Gln^{145} to Asp^{173} encompasses the amino acid residues of the peptide Nck5a-(145–170). Secondary structure prediction and analysis of the Cdk inhibitory peptide by circular dichroism and two-dimensional ^1H NMR spectroscopy have identified an amphipathic α-helix that spans amino acid residues Ser^{149} to Arg^{162} (30). A number of amino acid side chains at the hydrophobic face of this amphipathic α-helix have been suggested to play important roles in the interaction between Nck5a and Cdk5 (26, 27). Our observation that 1% Triton X-100 completely abolishes the binding of the peptide Nck5a-(145–170) to Cdk5 is consistent with this suggestion (Fig. 5*B*). While the interaction between Cdk5 and Nck5a is dominated by hydrophobic interactions, several lines of evidence suggests that the hydrophilic face of the amphipathic α-helix plays a major role in the association of Nck5a with the Nck5a-binding proteins. First, Cdk5 and the Nck5a-binding proteins do not compete in their interactions with Nck5a-(145–170), and Nck5a-binding proteins can bind to both monomeric Nck5a and Nck5a in the heterodimer Cdk5/Nck5a (25). These observations indicate that Nck5a uses distinct binding sites to interact with Nck5a-binding proteins and with Cdk5. Second, Nck5a-(145–170) loses its ability to associate with the Nck5a-binding proteins in high concentrations of NaCl, whereas the interaction between the peptide and Cdk5 is not adversely affected. On the other hand, while the interactions of the peptide with the Nck5a-binding proteins are totally refractory to the presence of 1% Triton X-100, the peptide-Cdk5 interaction is negated in the presence of the nonionic detergent. These results suggest that, in contrast to the Nck5a-Cdk5 interaction that depends on hydrophobic interactions, the association of Nck5a-binding proteins with Nck5a-(145–170) is dependent on electrostatic interactions. Lastly, substitution of a glutamate, Glu^{157}, that is situated at the center of the hydrophilic face of the α-helix, by glutamine results in almost complete elimination of the interaction of Nck5a-(145–170) peptide with the Nck5a-binding proteins, without interfering with the binding of the peptide to Cdk5. On the other hand, substitution of two leucine residues, Leu^{151} and Leu^{152}, in the hydrophobic face of the amphipathic α-helix by asparagines, previously shown to significantly decrease the interaction between Nck5a and Cdk5, has little effect on the association of Nck5a with the binding proteins.

As the three Nck5a-binding proteins appear to bind at a common site on Nck5a, structural comparison of the three proteins may be expected to reveal a structural motif that is specific for the Nck5a-binding site. However, amino acid sequence alignments have failed to reveal such a structural motif. Perhaps the binding motif depends on structural features other than those in the primary structure. The smallest C48 fragment displaying high affinity binding to Nck5a, C48α1, contains an amphipathic α-helix. It is possible that the binding of C48 to Nck5a involves the hydrophilic face of this protein. Work is in progress to isolate the smallest Nck5a-binding fragments of the three binding proteins so as to define the binding motif.

Cyclin-dependent kinase 5 has many distinct functional and regulatory properties among members of Cdk family. It has been suggested that many of the distinct properties of Cdk5 arise from the unique structure of Nck5a. While all the other known activators of Cdks belong to the cyclin protein family, Nck5a does not contain in its structure a conserved cyclin-box characteristic of cyclins. Structure and function analysis of Nck5a, however, has localized the kinase activation domain of Nck5a to a region of 142 residues, Glu^{150} to Asn^{291} (which is similar in size to cyclin fold of other cyclins) and this region of Nck5a appears to assume a cyclin-box structure (27). These results suggest that the unique structure of Nck5a has evolved to support a number of other functions in addition to the kinase activation. Presumably, some of these functions are manifested in the specific interactions of Nck5a with the various cellular proteins to which it binds. The identification of a specific protein-binding site in Nck5a represents the first attempt in the elucidation of the structural basis of interactions between Nck5a and Nck5a-binding proteins. The binding site is within a subdomain of Nck5a that is important for Cdk5 activation. An amphipathic α-helix in this domain uses its hydrophobic and hydrophilic phases to interact with Cdk5 and the Nck5a-binding proteins, respectively. It should be reiterated that although all three binding proteins studied in the present work appear to bind to this site, it does not indicate that this is a common binding site for all Nck5a-binding proteins as preliminary results have shown that certain other Nck5a-binding proteins bind to Nck5a at distinct sites.[2]

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