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Structural Insights into Cdk5 Activation by a Neuronal Cdk5 Activator

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Although Cdk5 shows high sequence identity to Cdk1 and Cdk2, it can be fully activated by its neuronal activators p35/p25^{nck5a} and p39^{nck5ai} in a phosphorylation-independent manner. To understand structural basis of the Cdk5/p25^{nck5a} activation, the complex is modelled to assume either an obstructed or an opened conformation based on X-ray structures of the unphosphorylated or the phosphorylated Cdk2/cyclin A complex, respectively. Comparison and analysis of the two models, along with mutagenesis studies of p25^{nck5a}, suggest that the opened form represents more closely the structure of active Cdk5/p25^{nck5a}. The results provide a rationale basis for understanding the phosphorylation-independent activation of Cdk5/p25^{nck5a}. © 2001 Academic Press

Key Words: **Cdk5; Nck5a; comparative homology modelling; phosphorylation-independent activation.**

Cdk5 plays important roles in many of the cellular processes occurring within neurons in the central nervous system (CNS) (1). Nck5a (p35^{nck5a} and its proteolytic product p25^{nck5a}) and an isoform p39^{nck5ai} are Cdk5 activator proteins uniquely expressed in CNS neurons (2–5). The activation of Cdk5 by p35/p25^{nck5a} or p39^{nck5ai} is in a phosphorylation-independent manner, which is distinct from that of Cdk activation by cyclins. The mechanism of Cdk activation by cyclins was revealed by crystal structure of Cdk2 in a complex with an active fragment of cyclin A (6–8). Upon binding of cyclin A to Cdk2, the T-loop moves down from an inhibitory position to partially open the substrate-binding site. Further phosphorylation by Cdk-activating kinase (CAK) at Thr-160 results in a fully extended T-loop to allow access of substrates to the active site of the Cdk2 catalytic subunit (9–11). Despite the fact that an analogous Thr-160 residue exists in the Cdk5 T-loop (Ser-159) and that the Cdk5 T-loop and its surrounding residues are highly homologous to those of Cdk1 and Cdk2, full activation of Cdk5/p25^{nck5a} can be achieved without T-loop phosphorylation (12).

We believe that unique structural features of Cdk5/p25^{nck5a} contribute to the phosphorylation-independent activation. However, knowledge of the Cdk5/Nck5a regulatory properties is very limited and experimental structure of Cdk5/p25^{nck5a} has not been reported. Recently, computed Cdk5 structures have been proposed on the basis of non-phosphorylated Cdk2/cyclin A in which the substrate-binding site is partially blocked by the T-loop (13, 14). However, the preferential conformation of the T-loop was not clearly investigated in the activated Cdk5/p25^{nck5a} complex. Is it in a partially obstructing or in a fully opened conformation analogous to that of the T-loop of unphosphorylated or phosphorylated Cdk2/cyclinA complex respectively? To address this question, Cdk5/p25^{nck5a} structure is modelled based on the structure of either phosphorylated or nonphosphorylated Cdk2/cyclin A to investigate the activation mechanism of Cdk5/p25^{nck5a}. Both the molecular modelling analysis and mutagenesis experiments support a fully extended Cdk5 T-loop in the activation complex to maintain an opened substrate-binding site, consistent with that of a phosphorylated Cdk2/cyclinA complex.

MATERIALS AND METHODS

Molecular modelling of the Cdk5/p25^{nck5a} complex and its mutants. The alignment between p35^{nck5a} and cyclin A sequences was done manually. The p35^{nck5a} fragment of residues 141–292 was used for modelling two possible structures of the *Cdk5/p25^{nck5a}* complex. They are designated "obstructed" and "opened" and were computated based on the template structures of the nonphosphorylated and

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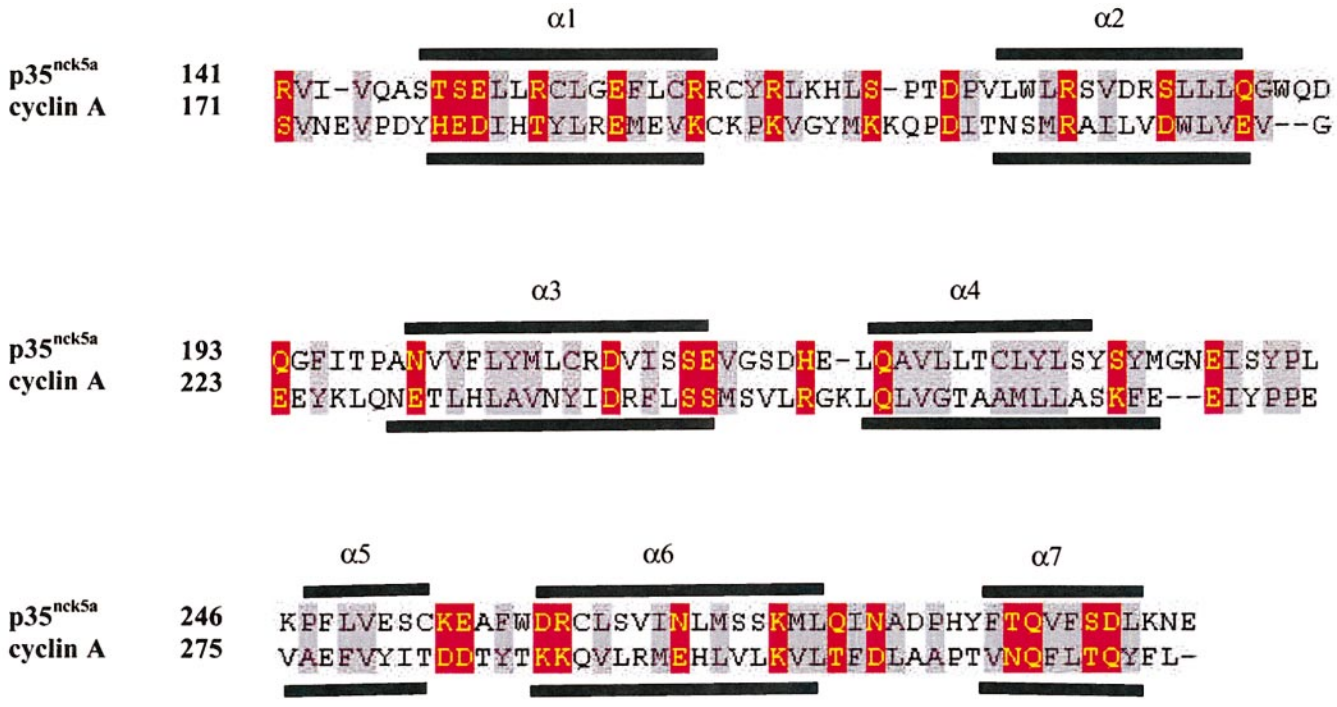


FIG. 1. Schematic diagrams of Cdk5-activation domain of p35^{nck5a} with the corresponding sequence of human cyclin A. Black bars are the seven helices found in cyclin A and the p35^{nck5a} model. The colour scheme is grey for hydrophobic residues and red for polar or charged residues.

phosphorylated Cdk2/cylin A complexes. The corresponding protein data bank codes are 1fin and 1jst, respectively (7, 8). LOOK (Molecular Application Group), which is based on an algorithm that uses self-consistent ensemble optimisation to determine the global minimum structure (15), was used to generate the models. The models were further subjected to energy minimization with a restrained and full conjugate gradient geometry optimisation to refine the loops and side chains using the molecular modelling software SYBYL 6.5 performed on a UNIX workstation model IRIS/indigo (Silicon Graphics Inc., Mountain View, CA). All the final structures were examined using the program ProCheck (16).

Preparation of recombinant proteins. Mutants of p25^{nck5a} were constructed using the QuikChange site-directed mutagenesis kit (Stratagene) with corresponding oligonucleotide primers. Expression and preparation of recombinant proteins were as described previously (12). The active kinase complexes were reconstituted from GST-Cdk5 and GST-p25^{nck5a} or its mutant proteins as described in Qi *et al.* (12). The protein kinase activity was then measured using the substrate peptide HS(9–18): PKTPKKAKKL (12).

Protein binding assays. Mouse brain lysate is a rich source of Cdk5 and is employed for binding assays between Cdk5 and recombinant p25^{nck5a} proteins (17, 18). Mouse brains were homogenized in the buffer of 25 mM Tris–HCl pH 7.5, 20 mM β-glycerophosphate, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 3 μg/ml leupeptin and pepstatin, 0.1 mM benzamidine. The crude homogenate was centrifuged at 100,000*g*. The supernatant was incubated with 10 nM of GST or GST-p25^{nck5a} proteins at 4°C in a total volume of 1 ml. GST fusion proteins were then precipitated with GSH-beads, and the precipitates were analysed by Western blotting with a Cdk5 antibody (C8, Santa Cruz BioTech) or a p35 antibody (C19, Santa Cruz BioTech).

RESULTS AND DISCUSSION

Molecular modelling analysis of Cdk5/p25^{nck5a} complex. Models of the Cdk5/p25^{nck5a} complex was computed based on structures of the Cdk2/cyclinA complexes. The high sequence identity (50%) between Cdk5 and Cdk2 together with the predicted cyclin fold in p25^{nck5a} provides the rationale for the modelling of Cdk5/p25^{nck5a} against CDk2/cyclin A (Fig. 1) (19). Comparison between the "obstructed" and "opened" models of CDk5/p25^{nck5a} (Fig. 2A) revealed differences particularly around the T-loop in both models. The region of residues 157–161 of the "obstructed" model curves upwards, thereby blocking part of the active site of Cdk5 (Figs. 2A and 2B). In the "opened" model, the partial obstruction of the active site is relieved when the T-loop is extended. This is structurally analogous to Cdk2/cyclin A. Phosphorylation of the Cdk2 T-loop results in an unobstructed active site that is 80- to 300-fold more active than a partially obstructed active site present in the unphosphorylated Cdk2/cyclin A (20). The present study aims to investigate which of the two proposed conformations, "obstructed" or "opened" more closely represents the activated Cdk5/p25^{nck5a} complex. The protein interfaces between the T-loop of Cdk5 and p25^{nck5a} of both models are analysed to identify residues that are uniquely involved in protein-protein interactions only in one model but not the other.

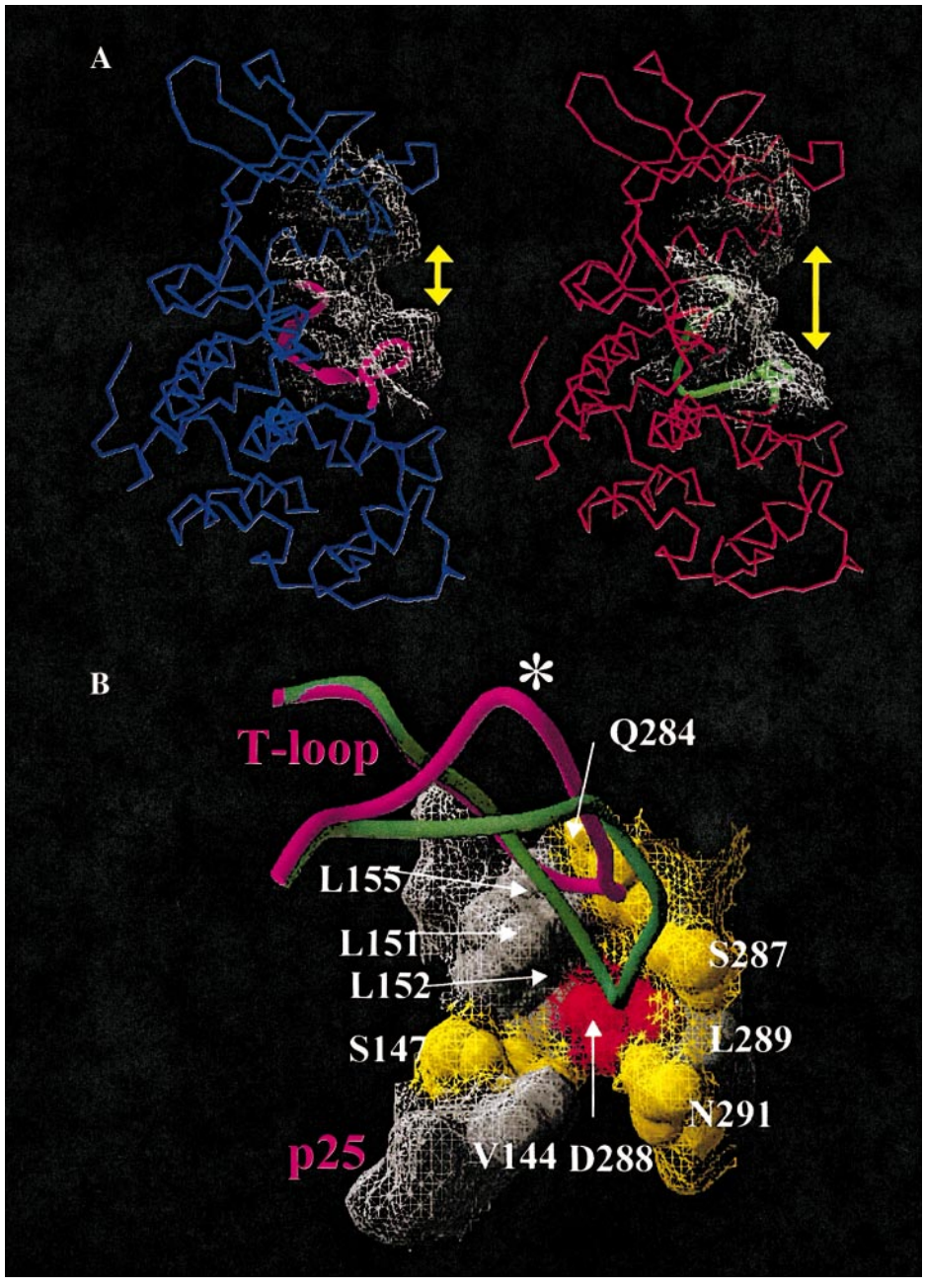


FIG. 2. (A) Comparison of the "obstructed" and "opened" models of Cdk5/p25^{nck5a}. The backbone is shown in the "obstructed" (blue) or the "opened" (red) models. The T-loop is represented as a pink ribbon in the "obstructed" and a green ribbon in the "opened" model. The active site region is shown as transparent wireframe solvent accessible surface (grey) with the entrance as indicated by a yellow arrow. (B) Structural analysis of interactions between the Cdk5 T-loop and p25^{nck5a}. The two models shown in Fig. 2A are superimposed. The root mean squared deviation of the backbone positions of the two p25^{nck5a} models is negligible, and therefore only the "opened" p25^{nck5a} model is shown. The superimposed T-loop of the two Cdk5 models are represented as ribbons and coloured accordingly as Fig. 2A. The white bold asterisk indicates the T-loop region (residues 157–161) of the "obstructed" model (pink) that partially blocks the substrate binding site. In the p25^{nck5a} model, residues flanking the pocket discussed in the text are labelled and shown as space-fill and coloured according to residue types (red for acidic, yellow for polar, and grey for hydrophobic). These residues are overlaid with a wireframe solvent accessible surface that is coloured according to the underlying residues.

Comparison of protein interface of both models. Along the protein-protein interface between Cdk5 and p25^{nck5a}, the T-loops of both models interact extensively with the residues of helices α1 and α7 of p25^{nck5a}. Unlike the "obstructed" structure, the T-loop of the "opened" model extends and bends downwards and its

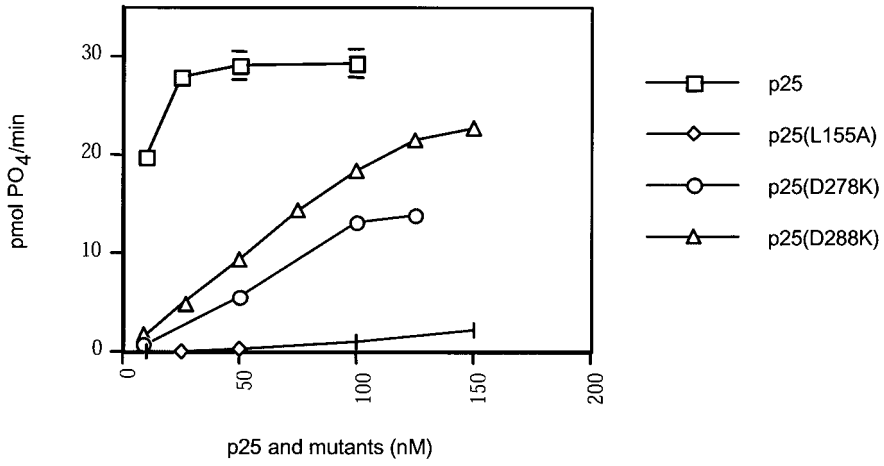


FIG. 3. Activation of Cdk5 by p25^{nck5a} or its mutants. One microgram of GST-Cdk5 was reconstituted with various amounts of GST-p25^{nck5a}, GST-p25^{nck5a}(L155A), GST-p25^{nck5a}(D278K), and GST-p25^{nck5a}(D288K) as indicated. The kinase activity was then determined by phosphorylation of the substrate peptide HS(9–18).

tip descends by about 7Å close to a shallow pocket formed between helices α1 (residues 147–162) and α7 (residues 282–289) of p25^{nck5a} (Fig. 2B). In the "opened" but not in the "obstructed" model, the tip of the T-loop is in close proximity with a series of residues flanking the pocket. These residues are Val-144, Ser-147, Leu-151, Leu-152 and Leu-155 from helix α1, and Gln-284, Ser-287, Asp-288 and Ser-291 from helix α7 of p25^{nck5a}. Generally, the residues from helix α1 that are close to the T-loop are mostly hydrophobic and contribute to hydrophobic interactions with the T-loop. Indeed, when Tang *et al.* replaced Leu-151 and Leu-152, the Cdk5-binding and activating activity was significantly affected (19). The residues from helix α7 are generally polar or negatively charged and contribute to the T-loop stabilization by electrostatic effect. Similarly, when Tang *et al.* replaced Asp-288 and Ser-291 with alanine, the Cdk5-binding and activating activity was significantly affected (19). To provide further experimental support that the protein-protein interaction due to residues flanking the pocket is important for the Cdk5-binding and activating activity, Leu-155 of helix α1 is selected because of its potential involvement in the hydrophobic interaction and Asp-288 of helix α7 is selected for Asp-288→Lys mutation to test the requirement of a negative charge. Further survey of the protein-protein interfaces of the two models identifies residue Asp-278 of p25^{nck5a} as the next candidate residue for mutagenesis study. In Cdk5/p25^{nck5a} complex, the side chain of Asp-278 contributes to an unique non T-loop interaction with Cdk5 only in the "opened" structure but not the "obstructed" model.

Site-directed mutagenesis of p25^{nck5a}. The identified residues, Leu-155, Asp-278, and Asp-288, were subjected to mutagenesis and biochemical Cdk5 activation studies. Upon reconstitution with Cdk5, p25^{nck5a}(L155A), p25^{nck5a}(D278K), and p25^{nck5a}(D288K) displayed impaired Cdk5 activation as compared to the parent p25^{nck5a} protein (Fig. 3). These p25^{nck5a} mutants generally have poor activation of Cdk5 and much more of them were required to reach their maximal activations. Even upon application of sufficient activator proteins, the full activation of Cdk5 by p25^{nck5a}(L155A), p25^{nck5a}(D278K), and p25^{nck5a}(D288K) reached only about 8, 48, and 77% respectively of that obtained with wild type p25^{nck5a} (Fig. 3). The mutation of Leu-155 to Lys almost abolished the Cdk5 activation activity. These observed mutational effects on the Cdk5 activation were strengthened by results from Cdk5 binding assays. Endogenous mouse brain Cdk5 was clearly shown to be precipitated by a small amount of GST-p25^{nck5a} (10 nM), whereas only trace or undetectable amounts of Cdk5 were pulled down by the same amount of the mutant proteins (Fig. 4). Therefore, substitutions of Leu-155, Asp-278 and Asp-288 give rise to severe effects on the binding affinity of the two protein partners as well as the maximal Cdk5 activity.

It was shown in a previous study that helix α1 of p25^{nck5a} forms an amphipathic structure with its hydrophobic region interacting with Cdk5 in the Cdk5/p25^{nck5a} complex (21). The severe effect of the Leu-155 mutation along with the reported effects of mutating neighbouring hydrophobic residues, Leu-151 and Leu-152, indicates that these residues are major contributors to the hydrophobic interaction between helix α1 and Cdk5 (19). The tip region of the Cdk5 T-loop can be seen to descend and move closer to the hydrophobic residues of p25^{nck5a} helix α1, only in the "opened" but not in the "obstructed" model. This implies that the interaction between Cdk5 and p25^{nck5a} in the "opened"

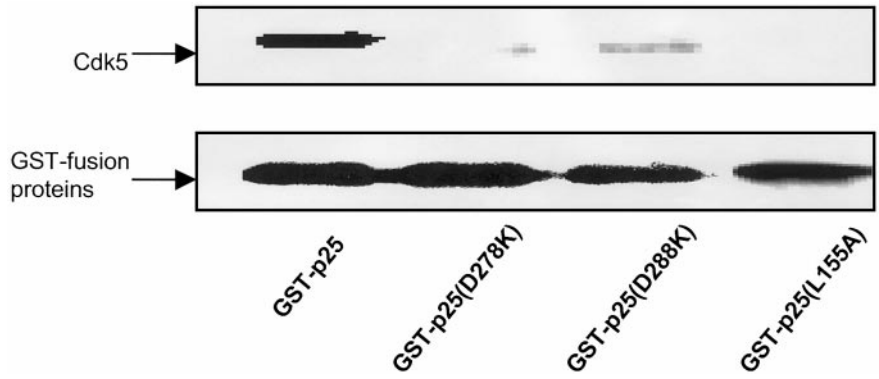


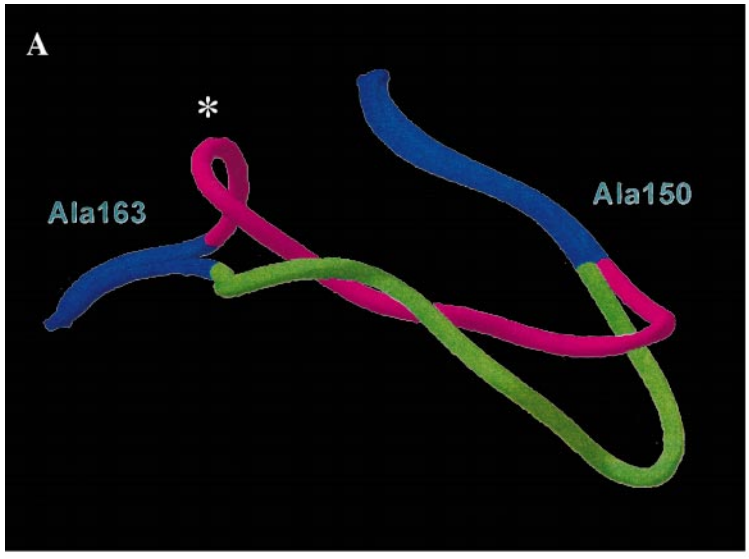
FIG. 4. Binding of Cdk5 to p25^{nck5a} and its mutants. Endogenous Cdk5 of mouse brain lysates was precipitated with GST-p25^{nck5a}, GST-p25^{nck5a}(L155A), GST-p25^{nck5a}(D278K), and GST-p25^{nck5a}(D288K) as indicated. The bait GST-p25^{nck5a} proteins and precipitated Cdk5 were detected by immunoblotting with specific antibodies.

but not the "obstructed" structure is affected by the mutations of these three identified hydrophobic residues in p25^{nck5a}.

In the "opened" model, Asp-278 is predicted to interact with Asn-121, a non-T-loop residue of Cdk5. The average distance between the two side chains in the "opened" and "obstructed" model is 3.5A and 7.8A, respectively. In the "obstructed" model the side chain of Asp-278 bends inwards and away from the protein-protein interface of Cdk5/p25^{nck5a}. The side chain of Asp-278 is a surface residue of p25^{nck5a} that does not contribute to stabilisation of the Cdk5 T-loop. Yet when this residue is mutated, it results in a 40% loss of activation. Asp-278 is required for optimum binding and activation of the Cdk5/p25^{nck5a} complex. The analysis presented here argues that the importance of Asp-278 can only be rationalised if the activated Cdk5/p25^{nck5a} complex adopts an "opened" but not an "obstructed" model. Moreover, in cyclin A the homologous residue to Asp-278 of p25^{nck5a} is alanine (Fig. 1). In fact, the homologous position for majority of the cyclin protein family is occupied by amino acids with smaller side-chains such as alanine and threonine. Only in a few distantly related cyclin members is the position occupied by cysteine, glycine or serine. For members of the p25^{nck5a} protein family, the homologous position in two-thirds of them is occupied by aspartic acid while the remaining is occupied by glutamic acid. Therefore, Asp-278 is a unique residue that is conserved in p25^{nck5a} homologues and required for stabilization of the Cdk5/p25^{nck5a} complex.

When Asp-288 is replaced with lysine, it results in a significant loss in the Cdk5 activation. This is consistent with the equally severe loss of activity due to the Asp-288→Ala mutation reported previously (19). Molecular modelling analysis of electrostatic potential distribution around the p25^{nck5a} pocket indicates that the surface contributed by helix α7 is biased towards negative charges. The base of the pocket is occupied by the side chain of Asp-288. Both Asp-288→Ala and Asp-288→Lys mutations lead to alteration around the pocket, especially the protein surface charge distribution contributed by helix α7 of p25^{nck5a}. Of the two proposed models, when Asp-288 is mutated, we believe that the T-loop in the "opened" model is much more likely to experience the change in the negative charge distribution than the corresponding T-loop region in the "obstructed" model.

Comparison of T-loop stabilising hydrogen bonds of both models. Hydrogen bonding is important for stabilization of the T-loop conformation required for Cdk5/p25^{nck5a} activation (13). To evaluate hydrogen bonding in both the "obstructed" and "opened" models, the two structures are superimposed and the T-loop backbones are compared. The H-bond pattern is generally similar within residues 145–150 and 163–165, which are called the common regions, but are significantly different within residues 151–162, which is called the unique region of the two T-loop conformations. As shown in Fig. 5A the unique region represents the region around the tip of T-Loop. 11 and 13 hydrogen bonds are identified within the unique region of the "obstructed" and "opened" models, respectively. Most of the hydrogen bonds identified in the unique region of the "obstructed" T-loop are not found in the unique region of the "opened" T-loop and vice versa. Transition of the T-loop, particularly in the unique region, from the "obstructed" into the "opened" conformation, will result in a redistribution and a further increase of two hydrogen bonds (Fig. 5B). Interestingly, of the three unique residues in the T-loop of Cdk5 compared to that of Cdk2, two of them contribute to the hydrogen bonding network that stabilizes the T-loop. Cys-157 is involved in forming two hydrogen bonds in the "opened" model but only one in the "obstructed" model. Moreover, Ser-159 is involved in hydrogen bonding only in the "opened" conformation (Fig. 5B). In other words, of the three



B

Hydrogen bonds between the T-loop and the rest part of Cdk5/p25^{nck5a}

	Obstructed Model			Opened Model		
	T-loop	Cdk5/p25^{nck5a}	H-bond	T-loop	Cdk5/p25^{nck5a}	H-bond
Common regions	Ala150	Met237*	1	Ala150	Met237*	1
	Val163	Arg168	2	Val163	Arg168	2
Unique regions	Phe151	Asn121	1	Phe151	Asn121	2
	Gly152	Ala150	1	Gly152	Tyr158	1
	Val155	Tyr179	1	Arg156	Tyr158	1
	Cys157	Lys177	1	Arg156	Asn239*	1
	Tyr158	Glu161	1	Cys157	Arg156	2
	Glu161	Arg50	2	Ser159	Tyr179	2
	Glu161	Arg125	1	Glu161	Arg168	1

FIG. 5. (A) Comparison of the T-loop in the "obstructed" and "opened" models. The ribbon diagram of the T-loop is derived from the superimposed structure discussed in the legend to Fig. 2B. Regions of the T-loop that do not deviate significantly are coloured blue (residues 149–150 and 163–165). Unique regions of the T-loop are coloured pink and green for the "obstructed" and "opened" models, respectively. "*" marks the same region shown in Fig. 2A. (B) Hydrogen bonding patterns of the T-loop in the "obstructed" and "opened" models. Residues marked with "*" are from p25^{nck5a}, and the others are from Cdk5.

residues which differ between the Cdk5 and Cdk2 T-loops, two of them form additional hydrogen bonds to stabilize the Cdk5 T-loop only in the "opened" but not in the "obstructed" model.

In summary, two structure models of Cdk5/p25^{nck5a} were computed based on the templates of Cdk2/cyclin A. The detailed comparative structural analysis of the "opened" and the "obstructed" models has allowed the design of a series of site-directed mutagenesis experiments to probe structure of the activated Cdk5/p25^{nck5a} complex. The previous and current experimental results support the Cdk5/p25^{nck5a} structure with a fully extended T-loop conformation. This is consistent with the assumption that for both Cdk5/p25^{nck5a} and Cdk2/cyclin A, a fully opened substrate-binding site is one of the prerequisites for full activation. It is reasonable to believe that the T-loop conformation of monomeric Cdk5 blocks its substrate binding site in a way similar to that of non-activated Cdk2. Binding of Cdk2 and cyclin A leads to an "obstructed" T-loop and an additional T-loop phosphorylation is required for transition of the T-loop into an "opened" conformation. The current study suggests that the binding between Cdk5 and p25^{nck5a} alone can provide the energy necessary to favour the transition into the "opened" conformation. The comparative hydrogen bonding analysis discussed here suggests that the transition from the "obstructed" into the "opened" T-loop conformation is an energeti-

cally favourable transition. The current study offers a plausible explanation of why the activation of Cdk5/p25^{nck5a} complex is independent of the T-loop phosphorylation.

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