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A novel yeast protein showing specific association with the cyclin-dependent kinase 5

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Abstract The present study describes a significant amino acid sequence homology between neuronal Cdk5 activator (nck5a) and an open reading frame of an unknown gene on the yeast *S. cerevisiae* chromosome III. A cDNA encoding a 25 kDa fragment of this yeast protein, the region containing homologous sequence to nck5a was cloned and expresed in *E. coli* as a glutathine-*S*-transferase fusion protein (GST-p25Y). GST-p25Y was found to block the in vitro activation of Cdk5 by nck5a and to affinity precipitate Cdk5 from bovine brain extract. The observations indicate that the yeast protein is capable of specific and high affinity association with Cdk5.

Key words: Cdk5; Neuronal Cdk5 activator (nck5a); Yeast

1. Introduction

Mammalian brains contain a Cdc2-like kinase [1,2], called neuronal Cdc2-like kinase (Nclk). Nclk is composed of Cdk5, the catalytic subunit and a 25 kDa regulatory subunit. This 25 kDa regulatory subunit is essential for the kinase activity [3,4] and has been indicated to be a proteolytic derivative of a brain- and neuron-specific 35 kDa protein [3,5], named neuronal Cdk5 activator (nck5a, or p35/p25nck5a). More recently, an isoform of the p35nck5a has been identified and cloned [6], named neuronal Cdk5 activator isoform (p39nck5ai). The p39nck5ai shows 57% identity to p35nck5a in amino acid sequence [6]. While all of the well-characterized Cdk activators belong to cyclin family, p35nck5a and p39nck5ai display no overall sequence homology to cyclins except a region of 17 amino acid residues where it shows a low degree of similarity to members of cyclin [3,7]. In addition, although most of the cyclin activation of Cdks has been found to depend on the action of a Cdk activating kinase (Cak), nck5a and nck5ai activation of Cdk5 shows no such dependence on Cak. These results suggest that nck5a and its isoform represent a distinct type of Cdk activator.

In the present study, we show that an amoni acid sequence deduced from an open reading frame (ORF) of an unknown gene on the chromosome III of yeast *Saccharomyces cerevisiae* (a hypothetical 143.8 kDa protein in ERS1-TUP1 intergenic region) [8] contains a region homologous to p35nck5a. A fragment of 25 kDa recombinant protein encoded by this yeast ORF corresponding to the region of 21 kDa functional fragment of p35nck5a (p21nck5a) [3,4] was expressed in *E. coli* and further demonstrated to bind Cdk5 with high affinity. The results are compatible with the notion that p35nck5a defines a new family of kinase activators and suggest that this novel protein may represent an activator protein for Cdk5-like protein kinase in yeast.

2. *Materials and methods*

2.1. *Cloning of the cDNA fragment of the yeast ORF and constructing into the pGEX-2T expression vector*

A cDNA segment encoding residues 636–843 of the ORF was amplified from yeast *S. cerevisiae* genomic DNA library (a gift from Dr. D. Young, University of Galcary) by PCR [3] with primers derived from the reported yeast hypothetical amino acid sequences [8]. The PCR product was inserted into a PCR™II plasmid vector (Invitrogen, San Diego, CA) flanked by *Eco*RI site on both sides. After *Eco*RI digestion, the cDNA of yeast gene fragment was cloned into an expression plasmid vector pGEX-2T linearized at *Eco*RI site. This construction encodes a protein composed of a 26 kDa GST fused to the 25 kDa fragment of the yeast protein (GST-p25Y). Orientation and translational reading frame of this recombinant DNA was confirmed by DNA sequencing.

2.2. *Expression and purification of recombinant proteins from* E. coli

The above recombinant plasmid for GST-p25Y, as well as recombinant plasmids for the 21 kDa fragment of p35nck5a (GST-p21) [3] and Cdk5 (GST-Cdk5) [5] were transformed into the *E. coli* strain BL21(DE3) [9] for expression. Briefly, a freshly transformed bacterial colony carrying each of the recombinant plasmid was cultured into two liters of LB medium containing 100 μg/μl ampiccillin. When OD_{600nm} of the bacterial culture reached 0.6–0.8, the protein expression was induced by 0.4–0.8 mM isopropyl-β-D-thiogalactoside (IPTG) at 22°C for 16 h (GST-p25Y) or 37°C for 3 h (GST-p21 and GST-Cdk5). The bacteria cells were then harvested and fusion proteins expressed were affinity purified by glutathione linked agarose (GSH, from Sigma, St. Louis, MO, USA) as described previously [3].

2.3. *Reconstruction of Nclk kinase activity and competition assay*

The in vitro reconstruction of kinase activity by purified GST-p21 and GST-Cdk5 were carried out as described in [3]. Briefly, 8 pmol of GST-p21 or GST were incubated with 2 pmol of GST-Cdk5 in a kinase assay buffer at 30°C for 2 h for reconstruction, followed by adding 100 μM of histone peptide of the kinase reaction substrate and 100 μM of r-[^{32}P]ATP (400 dpm·mol^{-1}) incubating at 30°C for 20 min. The kinase activity was quantitatively measured as the incorporation of ^{32}P into the histone peptide as described previously [1]. For competition assays, various amount of GST-p25Y or GST (2, 4, 8 and 16 pmol) were mixed individually with constant amount of GST-p21 at 8 pmol; or various amount of GST-p21 (2, 4, 8 and 16 pmol) were mixed individually with constant amount of GST-p25Y or GST at 8 pmol, and then perform the in vitro reconstruction of kinase activity the same as above. The incorporation of ^{32}P into the histone peptide was compared with the activity without GST-p25Y competition.

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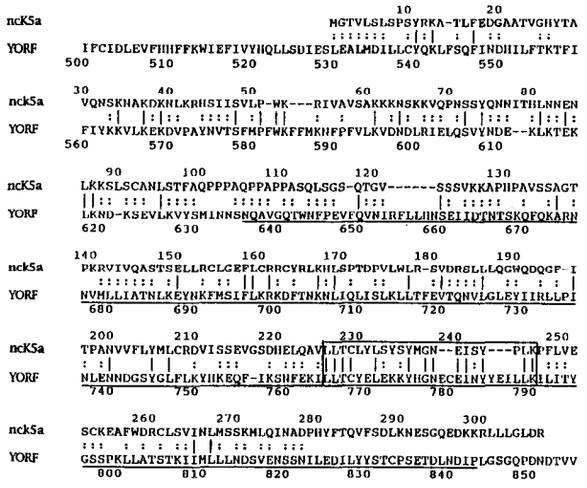
2.4. Western blot analysis of the GST-p25Y binding protein from bovine brain

Native monomeric Cdk5 was partially purified from the 120,000 × *g* supernatant of bovine brain extract by Mono-S ion-exchange and Superose 12 gel filtration as described [1]. The fractions that contain the monomeric Cdk5 were pooled and incubated with GST-p25Y or GST in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT and 2 mM ATP at 22°C for 1 h. Buffer equilibrated GSH beads were added into the reaction and rotated at 4°C for 1 h to affinity bind with GST. The beads were then washed 3 times in the same buffer and 2 times supplemented with 0.35 M NaCl. After boiled in SDS polyacrylamid gel electrophoresis (SDS-PAGE) sample buffer, the bound proteins were resolved by 12% of SDS-PAGE [10] and were electro-transferred to the Immobilon P membrane. The blotted membranes were stained by Coomassie Blue R250 or immunostained by specific antibodies raised against a peptide of Cdk5 internal sequence (Cdk5 I), a peptide of Cdk5 carbonyl terminal sequence (Cdk5 C), and the intact bacterial-expressed Cdk5 isolated from GST-Cdk5 fusion protein.



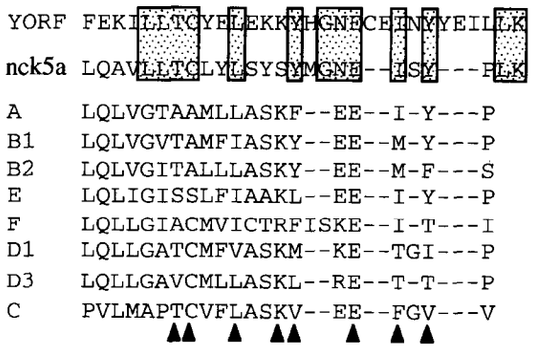


Fig. 1. Amino acid sequence alignment between p35nck5a and a yeast ORF (YORF). (A) Amino acid sequence alignment of p35nck5a and the YORF (500–855). The boxed region shows the highest homology between these two proteins. The underlined region represents the fragment that has been cloned and expressed (p25Y) for characterization. (B) Sequence comparison of the high homology segment among p35nck5a, YORF and members of human cyclin family (A, B1, B2, E, F, D1, D and C). The residues in the shaded boxes indicate identical amino acids in p35nck5a and YORF. The lower arrow heads indicate amino acids in YORF which are also conserved in members of the human cyclin family.

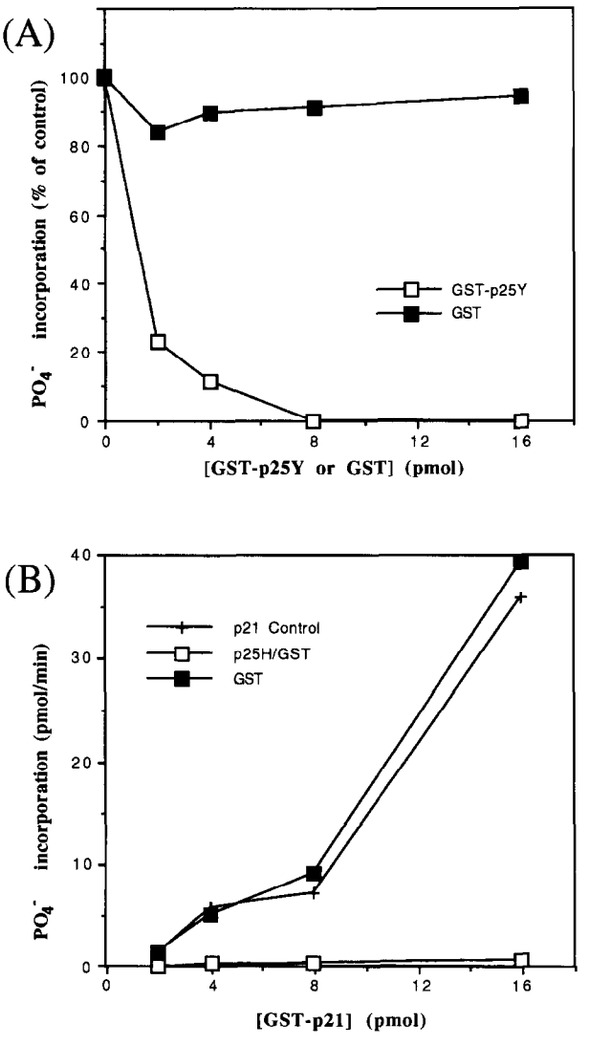


Fig. 2. Competition assay of the GST-Cdk5 activation. (A) The competition assay was performed by mixing 2, 4, 8 and 16 pmol of GST-p25Y (open square) or GST (solid square) individually with 8 pmol of GST-p21 and reconstituting with 2 pmol of GST-Cdk5. The results are plotted as the percentage of phosphate incorporation in the absence of GST-p25Y competition. (B) 2 pmol of GST-Cdk5 was activated by reconstitution with 2, 4, 8 and 16 pmol of GST-p21 in the absence (+) or presence of 8 pmol of GST-p25Y (open square) or GST (solid square). The results are plotted as the amount of phosphate incorporation (pmol/min).

3. Results and discussion

During GenBank searching for proteins similar to p35nck5a, the only homologous sequence found was an open reading frame from an unknown gene on yeast *S. cerevisiae* chromosome III. This yeast open reading frame encodes a hypothetical 143.8 kDa protein of 1226 amino acid residues. Fig. 1A shows the alignment of the homologous region between p35nck5a and residues 530–851 of the yeast open reading frame. The two sequences show 16.3% identity and 42.0% conservation. Although the overall sequence similarity is not high, a region of 21 residues (residues 226–246) in p35nck5a displays a significantly higher degree of sequence identity to the corresponding

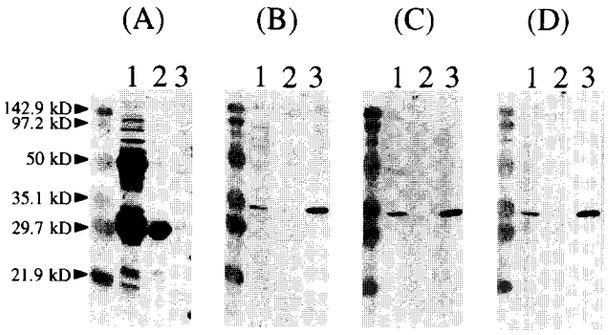


Fig. 3. Western blot analysis of the GST-p25Y binding protein in bovine brain. Partially purified bovine brain extract was incubated with GST-p25Y (lane 1) or GST (lane 2) at 20°C for 60 min and then affinity precipitated by the GSH-agarose beads. The precipitated proteins were analyzed along with the native Cdk5 purified from bovine brain (lane 3). The blots were stained by Coomassie Blue R250 (A) and probed with specific antibodies of Cdk5-I (B), Cdk5-C (C) and the expressed Cdk5 (D).

region of the yeast protein (residues 765–790). In this region, a 62% identity was observed. Significantly, this region of p35nck5a contains most of the 17-residue sequence that is the only region displaying some similarity to cyclin sequences (Fig. 1B). The similarity between p35nck5a and th eyeast ORF in this 17-residue sequence is higher than that between p35nck5a and cyclins and, as shown in Fig. 1B, some sequence similarity can also be observed between yeast ORF and cyclins in the consensus region of cyclin box.

A region of the yeast protein corresponding to the 21 kDa functional fragment of p35nck5a was cloned by PCR and expressed as GST fusion protein in *E. coli* (GST-p25Y). Previous studies have shown that p21nck5a and its GST-fusion protein can associate with Cdk5 to result in an active histone H1 peptide kinase, suggesting that this truncated p21nck5a protein contains the kinase activation domain [3,4]. When the GST-p25Y was incubated with GST-Cdk5, no kinase activity was obtained (results not show), whereas incubation of GST-p21 with the GST-Cdk5 resulted the expected kinase activity.

Although GST-p25Y exhibited no Cdk5 activation activity, it could effect the GST-Cdk5 by blocking the kinase activation by p21nck5a. Thus, the activation of a GST-Cdk5 sample by 8 pmol of GST-p21 could be effectively inhibited by GST-p25Y in a dose dependent manner (Fig. 2A). Complete inhibition was obtained at 8 pmol or more of GST-p25Y. The control GST samples at up to 16 pmol showed little or no inhibition effect on the activation of Cdk5 by GST-p21. Same result can also be observed when activation of 2 pmol of Cdk5 by 2–16 pmol of GST-p21 in the presence of 8 pmol of GST-Cdk5. The GST-p21 activation were significantly inhibited by GST-p25Y, but not GST (Fig. 2B). The effect of GST-p25Y on Cdk5 appears to be specific, since the activation of Cdk2 by cyclin A, in the presence of Cak, was found not to be effected by GST-p25Y (results not shown).

A plausible suggestion for the observed p25Y inhibition of Cdk5 activation by p21nck5a is that p25Y is capable of binding to Cdk5 at the activation site. This binding is of a high affinity, similar to that of the association between Cdk5 and p21nck5a. To demonstrate the high affinity binding of Cdk5 to p25Y, a sample of GST-p25Y was incubated with partially purified Cdk5 monomer from bovine brain, followed by affinity-precipitation of GST-p25Y by GSH beads. Western immunoblot analysis of the precipitate using three antibodies known to react with Cdk5 showed that Cdk5 coprecipitated with GST-p25Y. No Cdk5 was detected in the control samples where bacterial expressed GST was used instead of the GST-p25Y in the incubation (Fig. 3).

In summary, we have identified and cloned a protein fragment from the putative 143.8 kDa yeast protein which containes a region homologous to the recently discovered neuronal-specific Cdk5 activator. Although the similarity between the yeast protein and the two nck5a's is relatively low, a small region containing most of the 17-residue sequence in nck5a and nck5ai that is the only region displaying homology to the consensus sequence of cyclins has much higher degree of similarity to the yeast protein. A recombinant protein fragment of the yeast ORF containing the region equivalent to the 21 kDa activation domain of p35nck5a showed high affinity and specific association with Cdk5. These results suggest that the putative yeast protein may be functionally related to nck5a and nck5ai. We have recently suggested that the nck5a defines a new family of protein kinase activator, the putative yeast protein may be a member of this protein family.

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