Identification of a Neuronal Cdk5 Activator-binding Protein as Cdk5 Inhibitor*

Received for publication, January 16, 2002, and in revised form, February 25, 2002 Published, JBC Papers in Press, March 6, 2002 DOI 10.1074/jbc.C200032200

Yick-Pang Ching‡, Andy S. H. Pang†, Wing-Ho Lam‡, Robert Z. Qi‡, and Jerry H. Wang‡‡

From the ‡Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong, China and the ¶Department of Medical Biochemistry, Faculty of Medicine, University of Calgary, Calgary, Alberta T2N 1N4, Canada

Neuronal Cdc2-like kinase (Nelk) plays an important role in a variety of cellular processes, including neuronal cell differentiation, apoptosis, neuron migration, and formation of neuromuscular junction. The active kinase consists of a catalytic subunit, Cdk5, and an essential regulatory subunit, neuronal Cdk5 activator (p35\textsuperscript{nck5a} or p25\textsuperscript{nck5a}), which is expressed primarily in neurons of central nervous tissue. In our previous study using the yeast two-hybrid screening method, three novel p35\textsuperscript{nck5a}-associated proteins were isolated. Here we show that one of these proteins, called C42, specifically inhibits the activation of Cdk5 by Nck5a. Co-immunoprecipitation data suggested that C42 and p35\textsuperscript{nck5a} could form a complex within cultured mammalian cells. Deletion analysis has mapped the inhibitory domain of C42 to a region of 135 amino acids, which is conserved in Pho81, a yeast protein that inhibits the yeast cyclin-dependent protein kinase Pho85. The Pho85-Pho80 kinase complex has been shown to be the yeast functional homologue of the mammalian Cdk5/p35\textsuperscript{nck5a} kinase.

Cyclin-dependent protein kinases (Cdks)\textsuperscript{1} play critical roles in the regulation of cell division (1). As the name implies, functional Cdks require binding of a cyclin for kinase activity. In addition to depending on cyclin for activity, Cdk activities are regulated by complex mechanisms including protein phosphorylation and association with specific Cdk inhibitors. There are two Cdk inhibitor families: INK4 and Kip/Cip. Crystallography analysis has shown that the INK4 family member p16\textsuperscript{Nkx} causes the kinase inhibition by direct association to Cdk4 (4). On the other hand, p27\textsuperscript{Kip1/Cip1} binds to both Cdk2 and cyclin A in the Cdk2-cyclin A complex to inhibit the kinase activity (5).

Cyclin-dependent kinase 5 is unique among Cdks in many respects. Unlike most other Cdks, Cdk5 has no known function in the cell division cycle but is involved in the regulation of neuronal differentiation and neurocytoskeleton dynamics (6–10). There are two mammalian Cdk5 activators: neuronal Cdk5 activator, p35\textsuperscript{nck5a}, and neuronal Cdk5 activator isoform, p39\textsuperscript{nck5a}, both Nck5a and Nck5ai are expressed predominantly in neurons of central nervous systems (11, 12, 20). While Nck5a and Nck5ai are homologous proteins, they show little or no sequence similarity to cyclins. Members of both Cdk inhibitor families have been tested and shown to have no activity toward Cdk5. Biochemical analysis of Cdk5 in bovine brain extract suggests the existence of an inhibitory factor that exists together with Cdk5 and p35\textsuperscript{nck5a} in a protein complex (13). However, the molecular identity of the factor has not been established. In Saccharomyces cerevisiae, there is a cyclin-dependent protein kinase, Pho85, and its cyclin partner Pho80, which have been shown to be the functional homologues of mammalian Cdk5 and Nck5a, respectively (14). In addition, there is a specific Pho80/Pho85 inhibitor protein, Pho81, whose mammalian functional homologue is not known.

Recently we have used the yeast two-hybrid system to screen for the p35\textsuperscript{nck5a}-associated protein (15). Among the positive clones are three clones, called C42, C48, and C53, whose cDNA sequences are novel (16). In the present study, we show that one of the novel proteins, C42, displays an inhibitory effect on Cdk5 kinase activity. Interestingly, while both the full-length C42 and the C42 fragment obtained from the yeast two-hybrid screen can bind Nck5a, only the full-length protein has Cdk5 inhibitory activity. Using deletion mutants, we have mapped the inhibitory domain of C42 to a 135-amino acid region, which shows a significant homology to the inhibitory region of Pho81.

**MATERIALS AND METHODS**

Cloning and Construction of Plasmids—The DNA fragment encoding full-length C42 (C42-FL) and C42 binding fragment were subcloned into pCMV-Myc (CLONTECH) via EcoRI/NotI and XhoI/KpnI sites, respectively. To generate the deletion mutants of C42, the pGEX-N492 plasmid was digested with NheI for the N492 mutant, filled in at the黏碱i end, and re-ligated. To make the FLAG-p35\textsuperscript{nck5a} expression construct, the DNA encoding p35\textsuperscript{nck5a} was retrieved by digestion with HindIII, filled in, and then cut with BamHI. The BamHI-blunt DNA fragment was ligated into pFLAG-CMV2 (Kodak) via BamHI/SalI sites.

Purification of the Proteins—The glutathione S-transferase (GST) fusion forms of C42, Cdk5, and Cdk2 proteins were purified according to a previous protocol (17). p25, p35, and p39 were subcloned into the pET32 vector, and His-tagged proteins were purified using the nickel-chelate affinity purification. The DNA fragment encoding p35\textsuperscript{nck5a} was retrieved by digestion with HindIII, filled in, and then cut with BamHI. The BamHI-blunt DNA fragment was ligated into pFLAG-CMV2 (Kodak) via BamHI/SalI sites.

Cell Culture and Transfection—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37 °C in a humidified atmosphere at 5% CO\textsubscript{2}. Transient transfection was carried out using LipofectAMINE Plus reagent (Invitrogen). Cells were incubated for 24 h before harvesting. The cells were washed twice in PBS and lysed with 100 μl of lysis buffer (50 mM Heps, pH 7.2, 250 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 5 mM sodium fluoride, 10 μg/ml leupeptin, 1 μg/ml antipain, 2 mM phenylmethylsulfonyl fluoride).
all three proteins were mixed together at the same time, but for the postincubation samples, His6-p35 and GST-Cdk5 were mixed up first before concentration was determined by the Bradford assay (Bio-Rad).

addition of GST-free Cdk5 (1 μg of g5262) released by addition of 2 μg of H11003 Biosciences were added to the mixture and kept on ice for 1 h, beads according to the method described previously (18). Since the GST-C42 protein was incubated with His6-p35 for 30 min followed by addition of GST-Cdk5 and reconstitution for another 30 min (pre-incubation). For co-incubation, all three proteins were mixed together at the same time, but for the postincubation samples, His6-p35 and GST-Cdk5 were mixed up first before addition of GST-C42-FL. The samples were assayed for kinase activity after incubation. The full-length form of the other two novel binding proteins, C42-BF or the GST protein has little effect on Cdk5 activity. The C42 inhibition of Cdk5 depends on the order of addition of C42 to the Cdk5 activation reaction. The kinase inhibition could be readily observed if C42 was incubated with p35nck5a prior to the addition of Cdk5 or if the three proteins, C42, p35nck5a, and Cdk5, were mixed simultaneously. On the other hand, preincubation of Cdk5 with p35nck5a markedly lowered the inhibitory effect of C42 (Fig. 1B). To examine whether p35nck5a could still form a complex with Cdk5 after binding with C42 protein, affinity pull-down experiments were performed. A sample of p35nck5a preincubated with the GST-fused full-length C42 or C42 fragment was tested for the ability to associate with Cdk5. As shown in Fig. 1C, both GST-C42-FL-p35nck5a and GST-C42-BF-p35nck5a preformed complexes were able to pull down Cdk5. In some experiments, a small amount of Cdk5 was found to co-precipitate with the GST control. The reason for this is not clear. In contrast to p35nck5a-bound GST-C42, free GST-C42, either the full-length or the fragment, did not pull down Cdk5 in the affinity binding assay (data not shown). The observation that the full-length C42-p35nck5a complex can bind Cdk5 indicates that C42 inhibition is not due to a competition between C42 and Cdk5 for p35nck5a binding.

Identification of a Neuronal Cdk5 Activator-binding Protein

RESULTS

Inhibition of Nck5a Activating Activity of Cdk5 Kinase by Full-length C42 Protein —The C42 clone obtained from the yeast two-hybrid screen encodes a polypeptide of 112 amino acid residues corresponding to Gln475–Thr700 (the C terminus). This polypeptide displays high affinity Nck5a binding and is referred to as C42-BF. The C42-FL and C42-BF were bacterially expressed in GST fusion forms and tested for their effect on Cdk5 kinase activity. Fig. 1Aii shows that C42-FL inhibited Cdk5 kinase markedly in a dose-dependent manner, whereas the C42-BF or the GST protein has little effect on Cdk5 activity. The full-length form of the other two novel binding proteins, i.e. C48 and C53, were also tested and found to have no Cdk5 inhibitory activity (data not shown). Western immuno blot analysis of the incubation reaction sample showed quantities of Cdk5 and the activator remained unchanged after incubating with increasing amounts of C42-FL protein (Fig. 1Ai1), suggesting that the loss of Cdk5 kinase activity could not be attributed to the possible contamination of proteases in the C42-FL sample.

In vitro Kinase Assay—The Cdk5 inhibitory activity was measured by the Bradford assay (Bio-Rad).

Affinity Binding Assay—The GST fusion form of C42 protein (20 μg) was incubated with His-tagged p35nck5a (1 μg) for 30 min followed by addition of GST-free Cdk5 (1 μg) and incubation for another 30 min at 30 °C. After 20 μl of (50%) GSH-agarose beads (Amersham Biosciences) were added to the mixture and kept on ice for 1 h, beads were washed four times with 1 ml of PBS. The bound proteins were released by addition of 100 μl SDS loading buffer.

In Vitro Kinase Assay—The Cdk5 kinase assay was performed according to the method described previously (18). Since the GST-C42 full-length preparation was contaminated with degraded forms, the molten concentration indicated was a calculated proportion of the GST-C42 protein band to the total protein used. For the protein substrate assay, the reactions were stopped by addition of 100 μl SDS loading buffer, and the proteins were resolved by 15% SDS-PAGE.

Immunoprecipitation and Western Blotting—For immunoprecipitation, antibodies were incubated with transfected cell lysates at 4 °C for 1 h before the addition of protein G-Sepharose beads. For immunoblotting, blots were visualized by enhanced chemiluminescence according to the manufacturer’s procedure (Amersham Biosciences). Anti-Cdk5 (C-8), anti-c-Myc (A-14), and anti-p35 (C-19) antibodies were obtained from Santa Cruz Biotechnologies, and anti-FLAG (M2) antibody was obtained from Sigma.
pressed bacterially and tested for Cdk5 inhibitory activity. As expected to contain the Cdk5 inhibitory domain. To further is within the N-terminal region of C42 (residues 1–157) from residues 22–157 of C42 is refractory to C42 inhibition toward p35\textsuperscript{ncb5a}, that also associates with and activates Cdk5 (20). To further test the C42 inhibition specificity, the effects of C42 on Cdk5 activated by these various forms of activator proteins were examined. As shown in Fig. 2B, C42 is capable of inhibiting Cdk5 irrespective of the form of the activator protein used. An early study had shown that Nck5a, in addition to activating Cdk5, can also activate Cdk2 albeit to an activity level much lower than that achieved by cyclin A (17). Fig. 2C shows that although cyclin A-activated Cdk2 was efficiently inhibited by the full-length C42. In addition to demonstrating the specificity of C42 inhibition toward p35\textsuperscript{ncb5a}, these results rule out the possibility that the loss of kinase reaction is attributed to the existence of protease, ATPase, or phosphatase activity in the C42 sample.

Identification of the N-terminal Inhibitory Region of C42—To map the inhibitory domain of C42, a series of C-terminal deletion mutants were generated, including N492, N360, N168, and N56 (the number refer to the residues left from the N-terminal). All of the mutants were able to inhibit the p35\textsuperscript{ncb5a}/Cdk5 kinase activity except N56 (Fig. 3A) (data not shown for the N492 mutant). The result indicates that the Cdk5 inhibitory domain is contained in the N-terminal region (residues 1–168) of the C42 protein. This region does not contain the sequence corresponding to C42-BF, thus suggesting the existence of separate Nck5a-binding and Cdk5 inhibition domains in C42. S. cerevisiae protein Pho81 displays specific inhibitory activity toward the yeast Cdk5/Nck5a homologue, i.e. Pho85/Pho80. Alignment of C42 and Pho81 has revealed a moderate sequence homology (19% identity and 56.8% similarity). Recently the inhibitory domain of Pho81 has been identified to comprise about 60 residues, residues 645–724 (21). When this domain was compared with the full-length C42 protein sequence, it aligned to the N-terminal region of C42 from residues 22–157 (Fig. 3B). Note that this is within the N-terminal region of C42 (residues 1–168) expected to contain the Cdk5 inhibitory domain. To further test whether the region comprising residues 22–157 of C42 is the Cdk5 inhibitory domain, this portion of C42 was expressed bacterially and tested for Cdk5 inhibitory activity. As shown in Fig. 3, C and D, the GST fusion form of the inhibitory domain can suppress the phosphorylation of both protein and peptide substrates by Cdk5 kinase. In the assay using histone H1 protein as substrate, addition of C42 inhibitory domain had reduced about 60% of the Cdk5 kinase activity (Fig. 3D).

FIG. 2. Specific inhibition of Nck5a by C42 protein. A, GST-Cdk2 (1 \( \mu g \)/His\textsubscript{6}-cyclin A (0.5 \( \mu g \)) and GST-Cdk5 (1 \( \mu g \)/His\textsubscript{6}-p35 (0.5 \( \mu g \)) were reconstituted in the presence of increasing concentrations of full-length GST-C42 protein for 30 min at 30 °C and then assayed for the histone H1 kinase activity. The activity was expressed as percentage of control. B, proteins were reconstituted similar to those in A, but His\textsubscript{6}-p35 (0.5 \( \mu g \)), His\textsubscript{6}-p29 (0.5 \( \mu g \)), and His\textsubscript{6}-p39 (0.5 \( \mu g \)) were used to activate GST-Cdk5. For GST control, the GST-C42 was replaced by an equal amount of GST protein in the assay of His\textsubscript{6}-p39-GST-Cdk5 complex. C, proteins were reconstituted similar to those in A, but GST-Cdk2, instead of GST-Cdk5, was used for reconstitution with His\textsubscript{6}-p35.

DISCUSSION

Previously we have used the yeast two-hybrid screen to isolate three novel p35-binding proteins, named C42, C48, and C53, and showed that they bind to p35\textsuperscript{ncb5a}/Cdk5 complex (16). In this study, we demonstrate that the full-length C42, but not the C42 fragment from the yeast two-hybrid screen, possesses potent inhibitory activity toward Cdk5. We have carefully ruled out a number of artifacts that may give rise to an apparent kinase inhibition, such as the contamination of protease, ATPase, or phosphatase activity in the C42 sample. Affinity precipitation using bacterially expressed proteins indicates that either full-length C42 or C42 fragment can form a ternary complex with Cdk5 and Nck5a. Immunoprecipitation analysis shows that the ternary complexes can also exist in HeLa cell lysates. These results strongly suggest that C42 is a Cdk5 inhibitor. In fact, we have carried out transfection experiments showing that ectopic expression of the full-length C42 in NG108 neuroblastoma cells markedly suppressed the differentiation of the cells in differentiating medium, whereas the expression of C42-BF had significantly less effect.\footnote{Y.-P. Ching, W.-H. Lam, and J. H. Wang, unpublished observation.} The amino acid sequence of C42 shows no sequence similarity to proteins of either CKI family. The mechanism of Cdk5 kinase inhibition of C42 also appears to distinguish the protein from the known CKIs. While CKIs of the Kip/Cip family interact through the kinase catalytic subunit, members of the INK4
family undergo physical interactions with both the kinase and the cyclin subunits. In contrast, C42 appears to inhibit Cdk5 activity by interacting exclusively with the regulatory subunit Nck5a (or Nck5ai). Affinity binding experiments have shown that C42 undergoes high affinity binding to both Nck5a (or Nck5ai) and the Nck5a-Cdk5 complex but not to monomeric Cdk5. The inhibitory activity of C42 shows a strict specificity toward Nck5a or Nck5ai rather than the kinase subunit. This may be demonstrated by using Cdk2 instead of Cdk5 as the inhibition target. Only the p35Nck5a-activated Cdk2 but not the cyclin A-activated Cdk2 can be inhibited by C42.

While there is a good correlation between C42 inhibition activity and the ability of the protein to undergo high affinity binding to Cdk5 activator proteins, the inhibition activity is not solely due to the protein binding. Both full-length C42 and the C42 fragment bind to Nck5a with high affinity, but only the full-length C42 displays kinase inhibitory activity. Despite the fact that the other two novel Nck5a-binding proteins, C48 and C53, share the same Nck5a binding region of C42, they did not exhibit Nck5a inhibitory activity. These observations have led us to speculate that the full-length C42 contains a kinase inhibitory domain in addition to the high affinity Nck5a-binding domain. Deletion analysis has suggested the existence of a kinase inhibitory domain distinct from the Nck5a-binding domain of C42, a suggestion subsequently confirmed from sequence comparison between C42 and the recently identified Cdk inhibition domain in Pho81 (see below). A simple model to account for the existence of both Nck5a-binding and kinase inhibition domains is that while kinase inhibition depends solely on the kinase inhibitory domain, C42 uses the high affinity Nck5a-binding domain to increase the affinity of the inhibitory domain-enzyme interaction. The fact that the inhibitory domain can only inhibit about 60% of the kinase activity may suggest that the Nck5a-binding domain is required for achieving high inhibitory activity. This is also in agreement with the observation that the concentration of the inhibitory domain required for kinase inhibition is about an order of magnitude higher than that of full-length C42.

A number of observations suggest that C42 is related to Pho81, a Cdk inhibitor that is specific for Pho85/Pho80, the yeast homologue of Cdk5/Nck5a. The kinase inhibitor domain of Pho81 has recently been mapped (21). Sequence alignment of C42 and Pho81 has identified a region in C42 showing significant similarity to the kinase inhibitory domain of Pho81. The C42 deletion mutant containing only this region of the protein displayed Cdk5 inhibitory activity. In addition to structural similarity, C42 and Pho81 are similar in their Cdk inhibition mechanisms. Results of complementary assays in yeast suggested that Cdk5 could associate with Pho80 to form an active kinase and that the active kinase responded to Pho81 in yeast growing in low phosphate medium. Like C42, Pho81 exerts its kinase inhibitory activity through interaction with the regulatory subunit Pho80, rather than with Pho85, the catalytic (Cdk) subunit. The kinase inhibition by C42 is strongly dependent on the order of addition of Cdk5 to Nck5a and Cdk5 (Fig. 1B). Intriguingly a ternary complex of Cdk5, Nck5a, and C42 can be formed under either the inhibiting or the noninhibiting conditions. Based on their functional analysis of Pho81 and Pho85/Pho80 interaction in yeast cells, Huang et al. (21) suggested that Pho81 might form either an active or an inactive ternary complex with Pho85/Pho80 kinase in high and low phosphate medium. Thus, from these various lines of evidence, it is suggested that C42 and Pho81 may be grouped into a new family of CKIs.

REFERENCES
1. Morgan, D. O. (1997) Annu. Rev. Cell Dev. Biol. 13, 261–291
2. Deleted in proof
3. Deleted in proof
4. Jeffrey, P. D., Tong, L., and Pavletich, N. P. (2000) Genes Dev. 14, 3115–3125
5. Pavletich, N. P. (1999) J. Mol. Biol. 287, 821–829
6. Dhavan, R., and Tsai, L. H. (2001) Eur. J. Biochem. 269, 1534–1546
7. Deleted in proof
8. Deleted in proof
9. Grant, P., Sharma, P., and Pant, H. C. (2001) Eur. J. Biochem. 285, 419–423
10. Deleted in proof
11. Lew, J., Huang, Q. Q., Qi, Z., Winkfein, R. J., Aebersold, R., Hunt, T., and Wang, J. H. (1994) Nature 371, 423–426
12. Tsai, L. H., Delalle, I., Caviness, V. S., Jr., Chae, T., and Harlow, E. (1994) Nature 371, 419–423
13. Lee, K. Y., Rozales, J. L., Tang, D., and Wang, J. H. (1996) J. Biol. Chem. 271, 1538–1543
14. Huang, D., Patrick, G., Moffat, J., Tsai, L. H., and Andrews, B. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14445–14450
15. Qi, Z., Tang, D., Zhu, X., Fujita Donald, J., and Wang, J. H. (1998) J. Biol. Chem. 273, 2329–2335
16. Ching, Y. P., Qi, Z., and Wang, J. H. (2000) Gene (Amst.) 242, 285–294
17. Tang, D., Chun, A. C. S., Zhang, M., and Wang, J. H. (1997) J. Biol. Chem. 272, 12318–12327
18. Lew, J., Beaudette, K., Litwin, C. M., and Wang, J. H. (1992) J. Biol. Chem. 267, 13383–13390
19. Qi, Z., Huang, Q. Q., Lee, K. Y., Lew, J., and Wang, J. H. (1995) J. Biol. Chem. 270, 10847–10854
20. Tang, D., Yeung, J., Lee, K. Y., Matsushita, M., Matsui, H., Tomizawa, K., Hatase, O., and Wang, J. H. (1995) J. Biol. Chem. 270, 20997–20993
21. Huang, S., Jeffery, D. A., Anthony, M. D., and O’Shea, E. K. (2001) Mol. Cell. Biol. 21, 6695–6705
22. Deleted in proof
23. Deleted in proof

Fig. 3. Mapping of the inhibitory region of C42. A, deletion mutants of C42 were assayed for the inhibition of p35-Cdk5 kinase complex. Different concentrations of the full length (FL), binding fragment (BF), GST control, and the mutants including N360, N168, and N56 were reconstituted similar to proteins in Fig. 2A, and the kinase activity was assayed as described. B, the inhibitory domain of Pho81 was aligned with the full-length sequence of C42 by the ClustalW program. The numbers at the right-hand side of the C42 sequence represent the position of the residue corresponding to the full-length sequence. Asterisks indicate identical residues, and semicolons indicate similar residues. C, the FL fragment C22–157 was tested for the inhibition of p35-Cdk5 kinase complex. Different concentrations of the protein were incubated with the kinase complex, and the activity was measured using histone H1 peptides. D, the inhibition of Cdk5 kinase activity by the C42-N22–157 fragment was assayed using histone H1 protein substrate as described under “Materials and Methods.” The phosphorylation of histone H1 proteins was quantitated by densitometry, and the relative intensity of the signals in percentage was indicated at the bottom of the band.
Identification of a Neuronal Cdk5 Activator-binding Protein as Cdk5 Inhibitor
Yick-Pang Ching, Andy S. H. Pang, Wing-Ho Lam, Robert Z. Qi and Jerry H. Wang

J. Biol. Chem. 2002, 277:15237-15240.
doi: 10.1074/jbc.C200032200 originally published online March 6, 2002

Access the most updated version of this article at doi: 10.1074/jbc.C200032200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 17 references, 9 of which can be accessed free at http://www.jbc.org/content/277/18/15237.full.html#ref-list-1