An Isoform of the Neuronal Cyclin-dependent Kinase 5 (Cdk5) Activator∗

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Neuronal Cdc2-like kinase is a heterodimer of Cdk5 and a 25-kDa subunit that is derived from a 35-kDa brain- and neuron-specific protein called the neuronal Cdk5 activator (p35/p259a) (Lew, J.; Huang, Q.-Q.; Qi, Z.; Winkfein, R. J.; Aebersold, R.; Hunt, T.; and Wang, J. H. (1994) Nature 371, 423–426; Tsai, L. H.; Delalle, I.; Caviness, V. S.; Jr., Chae, T.; and Harlow, E. (1994) Nature 371, 419–423). Upon screening of a human hippocampus library with a bovine Nck5a cDNA, we uncovered a distinct clone encoding a 39-kDa isoform of Nck5a. The isoform, designated the neuronal Cdk5 activator isoform (p39nck5a), showed a high degree of sequence similarity to p35nck5a with 57% amino acid identity. Northern blot analysis detected its mRNA transcript in bovine and rat cerebrum and cerebellum, but not in any other rat tissues examined. In situ hybridization showed that Nck5ai was enriched in CA1 to CA3 of the hippocampus, but absent in the fimbria of hippocampal formation. Among seven cell lines in proliferating cultures, only PC12 and N2A, two cell lines capable of differentiating into neuron-like cells, were found to contain Nck5ai mRNA. A 30-kDa truncated form of Nck5ai expressed as a glutathione S-transferase fusion protein in Escherichia coli was found to associate with Cdk5 to form an active Cdk5 kinase. Thus, the isoform shares many common characteristics with p35nck5a, including Cdk5 activating activity and brain- and neuron-specific expression. Both proteins show limited sequence homology to cyclins, suggesting that they define a new family of cyclin-dependent kinase-activating proteins.

The cell division cycle gene, cdc2, in fission yeast performs rate-limiting functions in both G1/S and G2/M transitions. The protein product of the gene, p34cdc2, is a protein kinase catalytic subunit that associates with specific cyclins to form functional protein kinases, which are activated at discrete phases of the cell cycle (1–11). In mammalian cells, seven Cdc2 homologous proteins have been discovered; most have been shown to depend on cyclin for activity. These Cdc2 homologous proteins are called cyclin-dependent kinases (Cdks) and are individually identified by a numerical system as Cdk1 (Cdc2), Cdk2 up to Cdk7 (12–14). In addition to Cdks, an extended family of cyclins has been demonstrated to exist in mammalian cells. Combinatorial association between members of these two protein groups gives rise to a large number of heterodimeric protein kinases that are called Cdk2-like kinases (15–17).

While most of the Cdc2-like protein kinases are cell cycle regulators, the concept has emerged recently that some of the Cdc2-like kinases may have major functions unrelated to cell division (18). A novel Cdc2-like kinase has been discovered, purified from mammalian brains, and extensively characterized recently (19–22). The purified kinase has been shown to be a heterodimer of Cdk5 and a 25-kDa regulatory subunit that is essential for kinase activity (19). Molecular cloning studies have revealed that the 25-kDa protein is derived proteolytically from a 35-kDa precursor protein that is expressed specifically in brain neurons (23, 24). Since this brain Cdc2-like kinase is considered neuron-specific on account of its regulatory subunit, it has been designated neuronal Cdc2-like kinase, and the regulatory subunits, neuronal Cdk5 activator (Nck5a or p35nck5a or p259nck5a).

In addition to its tissue specificity, p259nck5a possesses certain unique properties that distinguish it from members of the cyclin family. The amino acid sequence of the protein displays minimal homology to cyclins (23). While the activation of Cdk1, Cdk2, and Cdk4 by various cyclins has been shown to depend on the phosphorylation of the Cdks by a specific Cdk-activating kinase (25–30), the activation of Cdk5 by p259nck5a is independent of Cdk5 phosphorylation (31). Northern blot analysis has revealed two populations of mammalian brain mRNA (4.0- and 2.4-kb mRNAs) that hybridize with a Nck5a probe, suggesting the existence of isoforms of Nck5a (23). In this study, we document the discovery, cloning, and preliminary characterization of an isoform of Nck5a. The isoform, a 39-kDa protein, shows 57% amino acid sequence identity to Nck5a. The protein, tentatively designated the neuronal Cdk5 activator isoform (Nck5ai or p39nck5a), is similar to Nck5a in displaying Cdk5 activation activity and brain- and neuron-specific expression, but distinct from Nck5a in cell culture distribution.

MATERIALS AND METHODS

cDNA Library Screen—A 2-year-old female human hippocampus cDNA library (Stratagene) was screened with a randomly labeled probe

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) U34051.

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The abbreviations used are: Cdks, cyclin-dependent kinases; kb, kilobases; Pipes, 1,4-piperazinediethanesulfonic acid; Mops, 4-morpholinopropanesulfonic acid; GST, glutathione S-transferase; DTT, dithiothreitol.
of bovine brain Nck5a according to the protocols of the manufacturer. 10^6 plaques were transferred to Hybond™ nylon membranes (Amer sham Corp.); prehybridized at 42°C with prehybridization buffer containing 20 mM Pipes (Sigma), 0.8 mM NaCl, 50 mM formamide (Life Technologies, Inc.), 0.5% SDS, and 100 μg/ml denatured fragmented salmon sperm DNA for at least 2 h; and hybridized by addition of the probe. Autoradiography of the reconstituted Cdk5 complex was performed as described (23, 31). GST-Cdk5 was incubated with either pGST-p21^ras™ or GST-p30^nckai at room temperature for 2 h in phosphate-buffered saline containing 1 mM EDTA and 1 mM DTT. The histone kinase activity of the reconstituted Cdk5 complex was measured in 30 mM Mops, pH 7.4, 10 mM MgCl2, 100 μM histone H1 peptide, and 100 μM [γ-32P]ATP (1000 cpm/ml) at 30°C for 30 min. The reaction was stopped by addition of acetic acid. Phosphate incorporation into the histone peptide was measured by a scintillation counter.

RESULTS

During the screening of a human hippocampus library with a randomly labeled DNA probe from a bovine Nck5a cDNA encoding the active domain of the protein (23), several clones (9I4, 3I4, 10I4, 15I23, and 7I12) were obtained. Restriction map analysis of the clones suggested that these clones represented two different mRNA transcripts. Partial nucleotide sequences and the deduced amino acid sequences of clones 15I23, 3I4, and 10I4 indicated that these clones encoded a protein that was identical to human Nck5a (24). On the basis of their nucleotide sequences and deduced amino acid sequences, clones 9I4 and 7I12 were found to encode a distinct Nck5a homologous protein. Clone 7I12 appeared to contain the complete protein reading frame. Fig. 1 shows the nucleotide sequence of clone 7I12 and the deduced amino acid sequence of the protein. The protein contains 367 amino acid residues with a calculated M₀ of ~39,000. The assignment of the initiation codon is based on the observation that the sequence flanking the ATG codon contains an A at position −3 and a G at position +4, characteristic of a functional initiation codon (39). A GeneBank™ search with the nucleotide sequence and the protein sequence has shown that this is a novel protein.

The sequence alignment of p39^nckai and the isoform, p39^nckai, is presented in Fig. 2A. The two proteins show 57% amino acid identity in the overall sequence, with a higher level of homology (65% amino acid identity) in the region of the protein corresponding to the 25-kDa subunit of the purified neuronal Cdc2-like kinase. This region of Nck5a contains the Cdk5-activating domain (23). The amino-terminal sequence of 9 amino acid residues and the carboxyl-terminal sequence of 3 residues of the two proteins are identical. The difference in molecular weights of the two proteins can be attributed mainly to the presence of a number of gaps in the Nck5a sequence when the proteins are aligned for optimal homology. Most of these gaps are located close to the protein termini. Like p39^nckai, p39^nckai shows no overall amino acid sequence homology to cyclins. A small region of 17 residues in Nck5a and Nck5ai sequence is the only region with detectable sequence similarity to the equivalent region of the cyclin box consensus sequence (Fig. 2B). Amino acid identity of Nck5a and Nck5ai in this region is 88.2%.

In a previous study, Northern blot analysis of bovine brain...
mRNA using a Nck5a cDNA probe detected a 4-kb mRNA band, whereas human brain was found to contain two populations of mRNA of 4 and 2.4 kb (23). In the present study, mRNAs from bovine cerebrum and cerebellum and rat cerebrum and cerebellum were probed by the randomly labeled Nck5a cDNA. Fig. 3 shows that the two populations of mRNA could be detected in both bovine and rat cerebrum, with the Northern blot intensity of the 2.4-kb transcript much weaker than that of the 4-kb transcript. Bovine and rat cerebellum also contained the transcripts, but at much lower levels, and only the 4-kb transcript was clearly seen on the Northern blot. When the blots of rat and bovine brain mRNAs were probed by a randomly labeled Nck5ai cDNA probe, the same two populations of mRNA of 4.0 and 2.4 kb were detected in bovine and rat cerebrum. However, contrary to what was revealed by the Nck5a cDNA probe, the smaller transcript displayed far higher intensity than the larger transcript on the Northern blot probed by the Nck5ai cDNA (Fig. 3). Weak Northern blot signals of the 2.4-kb transcript could also be detected in bovine and rat cerebellum, whereas no signal was detected at the 4.0-kb mRNA positions (Fig. 3). These observations indicate that the 4.0- and 2.4-kb mRNAs represent Nck5a and Nck5ai transcripts, respectively.

Tissue distribution study in humans has revealed that the mRNA transcript of Nck5ai is expressed specifically in the brain (23). Fig. 3 shows that Nck5a mRNA displays a similar brain-specific expression in rats. Essentially identical tissue expression patterns were observed for the 4- and 2.4-kb transcripts. Both transcripts were found to be expressed exclusively in brains, with levels in the cerebrum markedly higher than those in the cerebellum. No expression of either of the two transcripts was detected in non-neuronal tissues even upon increasing the exposure time by 10 times that used for the experiment of Fig. 3. The tissue distributions of Nck5a and Nck5ai mRNAs are markedly different from that of Cdk5. Previous studies showed that Cdk5 was widely distributed in humans as all human tissues examined were found to contain Cdk5 mRNA. Northern blot analysis of Cdk5 tissue distribution in rats showed that Cdk5 mRNA expression could also be readily detected in all the rat tissues examined (data not shown).

A number of proliferating cell lines were examined previously by Northern blot analysis and Western immunoblot analysis for Cdk5 distribution; all were found to contain Cdk5 mRNA and Cdk5 protein (40). To determine whether the cultured cells contained any of the neuronal Cdk5 activators, several cell lines were examined for the expression of p35 and p39 mRNAs by Northern blot analysis. Fig. 4 shows that none of the cell lines expressed p35 mRNA, with the exception of Jurkat cells, a human T cell line, which showed a very low, nonetheless detectable Northern blot signal. On the other hand, N2A cells, a neuroblastoma cell line, and PC12 cells, a pheochromocytoma cell line that can be differentiated by nerve growth factor treatment into sympathetic neuron-like cells, expressed significant levels of p39 mRNA (Fig. 4B). The level of the mRNA transcript was especially high in PC12 cells. None of the other cell lines, including a glioma cell
line (C6), contained detectable p39\textsuperscript{ndk5ai} transcript. The observation that only cell lines of neural origin or with neuron characteristics express p39\textsuperscript{ndk5ai} suggests that the protein is neuron-specific.

In situ hybridization procedures were used to examine the regional distribution of p39\textsuperscript{ndk5ai} mRNA in the brain of a 3-month-old rat. The result indicated that the messenger transcript was highly expressed in CA1 to CA3 of hippocampal formation, an area enriched in neurons (Fig. 5). The presence of the Nck5ai mRNA in neurons can be clearly seen with a higher magnification of the CA3 region (Fig. SC). Some expression of the p39\textsuperscript{ndk5ai} mRNA could also be seen in the dentate gyrus of the hippocampus. In contrast, no expression of the mRNA was detected in the fimbria hippocampi, an area containing axons of neurons and glial cells. These results on the regional distribution of p39\textsuperscript{ndk5ai} mRNA are compatible with the suggestion that p39\textsuperscript{ndk5ai} is a neuron-specific protein.

The active neuronal Cdc2-like kinase purified from bovine brain contains a 25-kDa subunit (p25\textsuperscript{ndk5ai}) (19), a truncated form of p35\textsuperscript{ndk5ai} (23). Bacterially expressed p25\textsuperscript{ndk5ai} or a further truncated form of 21 kDa (p21\textsuperscript{ndk5ai}) can activate bacterially expressed monomeric Cdk5 to an activity similar to that of the purified neuronal Cdc2-like kinase (31). This observation suggests that p21\textsuperscript{ndk5ai} contains the active domain essential for Cdk5 activation. A CDNA clone encoding a 30-kDa truncated form of p30\textsuperscript{ndk5ai} containing the region corresponding to p25\textsuperscript{ndk5ai} was expressed as a GST fusion protein in E. coli. The expressed fusion protein, GST-p30\textsuperscript{ndk5ai}, was purified by glutathione affinity chromatography and tested for Cdk5 activating activity. Fig. 6 shows that the protein could activate bacterially expressed Cdk5 to a maximal activity about the same as that achieved by a GST-p21\textsuperscript{ndk5ai} fusion protein. Three different preparations of GST-p30\textsuperscript{ndk5ai} were tested for the ability to activate Cdk5; essentially identical levels of maximal Cdk5 activation were obtained. However, the dose dependence of the Cdk5 activation by the bacterially expressed fusion protein varied from preparation to preparation, probably because different preparations contained different proportions of the active protein.

Bovine brain extract contains high levels of monomeric Cdk5. Monomeric brain Cdk5 has been partially purified and shown to associate readily with p25\textsuperscript{ndk5ai} or p21\textsuperscript{ndk5ai} to form active Cdk5 kinases. When the GST-p30\textsuperscript{ndk5ai} fusion protein was incubated with an aliquot of bovine brain extract and then affinity-precipitated by glutathione beads, Cdk5 in the brain extract was found to coprecipitate with the fusion protein as revealed by a specific Cdk5 antibody probe (Fig. 7A). The precipitate displayed good histone H1 peptide kinase activity, suggesting that brain Cdk5 could be activated by the fusion protein (Fig. 7B). In a separate experiment, a monomeric Cdk5 preparation partially purified from bovine brain was incubated with the GST-p30\textsuperscript{ndk5ai} fusion protein for 30 min, and the kinase activity of the sample was then determined. While neither monomeric Cdk5 nor the bacterially expressed fusion protein alone had any kinase activity, incubation of the two proteins together resulted in high histone H1 kinase activity (data not shown). This observation provides direct proof that Nck5ai is capable of activating the native form of Cdk5.

**DISCUSSION**

Until now, Nck5ai has been the only protein known to be capable of reconstituting highly active Cdk5 kinases. In this study, we demonstrate the existence of an isoform of Nck5a, a
39-kDa protein that displays a high degree of amino acid sequence homology to p35nck5a with 57% amino acid identity. Like p35nck5a, this protein, called p39nck5ai, is capable of associating with Cdk5 to form functional kinases of high activity and shows brain-specific mRNA expression as revealed by the Northern blot analysis of its tissue distribution in both bovine and rat tissues. On the basis of detailed immunohistochemical studies and in situ hybridization analysis, p35nck5a has been suggested to be expressed specifically in neurons. At least two lines of evidence support the suggestion that p39nck5ai is also present specifically in animal brain neurons. Among a number of cell lines examined in culture, only N2A cells (a cell line of neural origin) and PC12 cells (a pheochromocytoma cell line capable of differentiating with nerve growth factor into neuron-like cells) were found to contain p39nck5ai mRNA. Significantly, a cell line of glial origin, the C6 glioma cell line, did not show detectable p39nck5ai mRNA signal on its Northern blot. The in situ hybridization patterns of Nck5ai indicate that p39nck5ai is greatly enriched in the CA1 to CA3 zone of hippocampal formation of adult rat brain (a region rich in neurons) and absent in the fimbria hippocampi, where glial cells predominate.

The existence of two brain- and neuron-specific Cdk5 activators has raised the question as to the physiological significance of the isoforms. As the brain is a highly complex organ containing many different cell types, one possible reason for the existence of isoforms of neuronal Cdk5 activators may be that the isoforms have distinct cell-type or brain regional distributions. Alternatively, the isoforms may coexist in the same neurons, but have differential subcellular localizations. We have re-

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cently observed that Cdk5 and p35\textsuperscript{ncdA} are localized in different compartments in neurons of adult rat brains. While Cdk5 is enriched in the axons of central nervous neurons, p35\textsuperscript{ncdA} has been most extensively observed in the cell body.\textsuperscript{2} It is tempting to speculate that p35\textsuperscript{ncdA} may have an axonal localization so that the cell body and axonal function of Cdk5 in neurons are regulated by Nck5A and Nck5Ai, respectively. The situation is somewhat similar to the regulation of Cdc2 by phase-specific cyclins in yeast during cell division cycle progression (10, 11), except that the phase-specific cyclins are temporal specific regulators of Cdc2, whereas Nck5A and Nck5Ai are spatial specific regulators of Cdk5. Work is under way to determine whether p35\textsuperscript{ncdA} indeed has an axonal localization.

The suggestion that p35\textsuperscript{ncdA} and p39\textsuperscript{ncdA} have different functional roles in brain neurons appears to be supported by the observation that the two proteins are differentially expressed in cultured proliferating cells. Of all the cells examined, only the two cell lines with neuronal characteristics and no other proteins have been shown to activate Cdk5, this new family of kinase activators may be specific and unique for Cdk5 or Cdk5-like kinases. While Cdk5 has a wide tissue and cell distribution, only brain- and neuron-specific Cdk5 activators have been identified to date. It seems reasonable to suggest that there are additional members of this protein family that function as Cdk5 activators in non-neuronal cells. If so, degenerate polymerase chain reaction primers derived from some of the most conserved regions of these two neuronal Cdk5 activators may be useful in the search for additional Cdk5 kinase activators.

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