Neuronal cdc2-like kinase (Nck) purified from bovine brain is a heterodimer of Cdk5 and an essential 25-kDa regulatory subunit (Lew, J., and Wang, J. H. (1995) Trends Biochem. Sci. 20, 33-37). The regulatory subunit is an N-terminal truncated derivative of a 35-kDa protein expressed specifically in brain, hence the name neuronal Cdk5 activator, p25/p35\textsuperscript{nck}\textsubscript{a}. In this study, we probe the relationship between the two different forms of Nck\textsubscript{a} and their interaction with and activation of Cdk5 in bovine brain extract. Using protein fractionation procedures in combination with Western blot analysis and protein kinase assay, three forms of Cdk5 have been detected in bovine brain: a monomeric Cdk5 that can be activated by bacterially expressed GST-p21\textsuperscript{ccka}, a heterodimer of Cdk5 and p25\textsuperscript{nck}\textsubscript{a} that displays high kinase activity, and a Cdk5/p35\textsuperscript{nck}\textsubscript{a} complex that is inactive and refractory to GST-p21\textsuperscript{ccka} activation. Analysis of the Cdk5/p35\textsuperscript{nck}\textsubscript{a} complex by gel filtration chromatography indicated that the complex was part of a macromolecular structure with a molecular mass of \(\sim 670\) kDa. When the macromolecular complex was subjected to gel filtration chromatography in the presence of 10% ethylene glycol, the fractions containing both p35\textsuperscript{nck}\textsubscript{a} and Cdk5, although eluting at the same position as control, displayed high kinase activity. The result is compatible with the suggestion that the macromolecular complex contained a kinase inhibitory factor that dissociated from the complex in 10% ethylene glycol.

The animal cell division cycle is controlled by the coordinated actions of a family of protein Ser/Thr kinases, called cdk2-like kinases, which are activated and deactivated at discrete phases of the cell cycle. Members of the cdk2-like kinase family are heterodimers of a cdk2-related protein called cyclin-dependent kinase (Cdk)\textsuperscript{1} and a cyclin. The activity of a cdk2-like kinase in dividing cells is usually dictated by the level of the cyclin subunit as well as the phosphorylation state of the Cdk subunit. In general, the catalytic activities of cell cycle cdk2-like kinases depend on the phosphorylation of a threonine residue (Thr-161 or its equivalent) by a specific CDK-activating kinase, whereas phosphorylation of a threonine or a tyrosine residue at the nucleotide binding loop of Cdk subunit by specific inhibitory kinases results in the inactivation of the kinases (for review, see Refs. 1–10). In addition, cdk2-like kinases undergo functional and regulatory significant associations with a large number of cellular proteins, which include a family of low molecular weight Cdk inhibitory proteins (11–17), transcription factors, and Rb and Rb-related proteins (18–28). In a number of reports, Cdns and cdk2-like kinases have been shown to exist within macromolecular complexes (26–32). While it is logical to suggest that these macromolecular complexes represent the functional and regulatory significant association of Cdns and/or cdk2-like kinases with defined cellular proteins, systematic studies of such complexes have not been carried out.

Recently, a cdk2-like kinase has been discovered and purified from bovine brain as a proline-directed kinase (33, 34) or a tau protein kinase II (35). This enzyme has been implicated in the regulation of neurocytoskeletal proteins, and a loss in the regulation of the enzyme has been suggested to be involved in Alzheimer pathology (34, 36). The purified protein kinase is a heterodimer of a 33-kDa catalytic subunit and a 25-kDa regulatory subunit (33). Molecular cloning has identified the 33-kDa subunit as Cdk5 and the 25-kDa regulatory subunit (33). Molecular cloning has identified the 33-kDa subunit as Cdk5 and the 25-kDa regulatory subunit as a proteolytic derivative of a novel neuron-specific 35-kDa protein (31, 34, 37). The regulatory subunit is essential for the kinase activity and only expresses in the central nervous system. Therefore, it has been designated as the neuronal Cdk5 activator, Nck5a (p25\textsuperscript{nck}\textsubscript{a}/p35\textsuperscript{nck}\textsubscript{a}), and the heterodimeric choi/enzyme as the neuronal cdk2-like kinase, Nck (38–40). Since neurons in adult brain do not divide, Nck is unique in being a cdk2-like kinase not involved in cell division.

As a cdk2-like kinase, Nck is expected to share many common regulatory and functional properties with other family members. Thus, the enzyme is composed of a catalytic subunit and a positive regulatory subunit. All the regulatory phosphorylation sites found in other Cdns are conserved or conservatively substituted (Ser to Thr) in Cdk5 (34, 41). On the other hand, for its neuronal functions, Nck5a may possess unique regulatory properties that distinguish itself from the cell cycle regulatory cdk2-like kinases. For example, while Nck5a displays a cyclin-like activity in that it activates the catalytic subunit, it shows little, or at best, marginal amino add sequence homology to cyclins (37, 41). In addition, the activation of Cdk5 by Nck5a is independent of the Cdk-activating kinase, whereas cyclin activation of Cdns in many cases have been found to depend on the Cdk-activating kinase (38). A comparison of the regulatory properties of Nck with those of the cell cycle regulatory cdk2-like kinases may shed new light on both cell cycle regulation and the control of neuronal functions.
Although the purified bovine brain Nck is composed of Cdk5 and p25\textsuperscript{nck5a}, the intact p35\textsuperscript{nck5a} is the predominant form of the protein in crude brain extract (37). The relationship between the two forms of Nck5a and their interaction with Cdk5 in the brain extract are not known. In this study, we have examined the states of Cdk5 in crude bovine brain extract using protein fractionation procedures in combination with kinase activity and immunological analysis. The results suggest that Cdk5 exists in at least three forms: a free Cdk5 monomer that is readily activated by a bacterially expressed p21\textsuperscript{nck5a}, a heterodimer of Cdk5/p25\textsuperscript{nck5a} that is highly active, and a Cdk5/p35\textsuperscript{nck5a} form in association with a macromolecular complex that is neither enzymatically active nor activatable by the bacterially expressed GST-p21\textsuperscript{nck5a}.

**EXPERIMENTAL PROCEDURES**

**Materials**

DEAE-Sepharose CL-6B, FPLC Mono-S, and Superose-12 columns were obtained from Pharmacia Biotech Inc. (Baie d’Urfé, Hydroxylapatite and molecular weight markers were purchased from Bio-Rad. [(γ-P\textsuperscript{32}P]ATP (4500 Ci/mmol) was obtained from ICN. P81 phosphocellulose was purchased from Whatman. All other chemicals were purchased from Sigma.

**Protein Fractionation Procedures**

Tissue Homogenate—All procedures were carried out at 4°C. 1 kg of fresh bovine brain was homogenized in a Waring blender in 1.5 liters of buffer A (25 mM Hapes, pH 7.2, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.6 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml apro tinin, 2 μg/ml antipain, 0.3 μg/ml benzamidine, 0.1 mg/ml soybean trypsin inhibitor) for a 15-s burst on low speed and then two more times at 15 s on high speed. The crude homogenate was centrifuged at 12,000 × g for 25 min in a Beckman JA-10 rotor, and the supernatant was further centrifuged at 120,000 × g for 30 min in a Beckman Ti-45 rotor.

FPLC Mono-S Column Chromatography—40 mg of the 120,000 × g supernatant was diluted 3-fold with buffer A and injected at 0.5 ml/min onto a Pharmacia Mono-S (HR 5/5) column for initial characterization of Nck5a and Cdk5 from bovine brain. The column was washed with 20 ml of buffer A and eluted with a 30-ml linear gradient (0–0.5 M NaCl) in buffer A (at 0.5 ml/min. 1-ml fractions were collected.

DEAE-Hydroxyapatite Column Chromatography—1.3 liters of the 120,000 × g supernatant was loaded at 2 ml/min onto a 400-ml DEAE-Sepharose CL-6B column that was pre-equilibrated with buffer A. After sample application, the DEAE-Sepharose CL-6B column was loaded with 1 bed volume of buffer A. The DEAE-Sepharose CL-6B column was removed, and the hydroxyapatite column was washed with 500 ml of buffer A. The hydroxyapatite column was eluted with a linear gradient of K\textsubscript{2}HPO\textsubscript{4} (0–400 mM) at 1 ml/min. 12-ml fractions were collected.

FPLC Superose-12 Gel Filtration Column Chromatography—Each of the pooled fractions (fractions I, II, and III) from the hydroxyapatite column were concentrated at 4°C by reverse dialysis against Aquacide phosphate-buffered saline, suspended in 30 ml of lysis buffer (50 mM MOPS, pH 7.4, 30 mM MgCl\textsubscript{2}, 100 μM (γ\textsuperscript{32}P]ATP (300 dpm/ml), and 100 μM histone H1 peptide P\textsubscript{3}KTPKKAKKL\textsubscript{18} as described (42). γ\textsuperscript{32}P]Phosphate incorporation into the substrate peptide was quantitated by liquid scintillation using a Beckman LKB 1215 scintillation counter.

**In Vitro Reconstitution Assays**

Either bacterially expressed GST-p21\textsuperscript{nck5a} (0.1 μg) or bacterially expressed GST-Cdk5 (0.1 μg) was combined with 10 μl of column fraction and incubated at room temperature for 40 min before histone H1 peptide phosphorylating activity was determined as described above.

Expression and Purification of GST-fusion Proteins from Bacteria

GST-fusion proteins were purified as described (43). PGEX-2T recombinant plasmid containing either Cdk5 or p21\textsuperscript{nck5a}, a truncated form of p25\textsuperscript{nck5a} (38), was transformed into Escherichia coli strain DH5α. The host cells were cultured in 4 liters of LB medium containing 100 μg/ml ampicillin at A\textsubscript{590nm} = 0.7 at 37°C. The expression of GST-fusion proteins was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (Life Technologies, Inc.) at room temperature overnight. The cells were harvested by centrifugation at 5,000 rpm for 15 min, washed with 1× phosphate-buffered saline, suspended in 30 ml of lysis buffer (50 mM Tris-Cl, pH 7.4, 2 mM EDTA, 1 mM DTT, 0.25 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml apro tinin, and 1 μg/ml antipain), and lysed by three times using a French press (SLM Aminco, Silver Spring, MD) with the cell pressurized to 16,000 psi. The resulting lysate was centrifuged at 18,000 rpm for 20 min. The supernatant was incubated for 1 h at 4°C with 4 ml of glutathione-agarose (Sigma) previously equilibrated with the same buffer. After loading the slurry onto a 10-ml column, the column was washed with 10 bed volumes of 1× phosphate-buffered saline supplemented with 0.25 mM KCl, 0.1% Tween 20, 1 mM DTT, 0.25 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml apro tinin, and 1 μg/ml antipain and followed by 5 bed volumes of 1× phosphate-buffered saline supplemented with 1 mM DTT. The expressed GST-fusion proteins were eluted by 5 ml reduced glutathione in 50 mM Tris-Cl, pH 8.0, 1× mM DTT.

SDS-Polyacrylamide Gel Electrophoresis, Immunoblots, and Antibodies

SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (44) in 12.5% vertical slab gels. For Western blot analyses, ~30 μg total protein was loaded onto each lane. After electrophoresis, proteins were transferred to Immobilon-P membrane (Millipore). The blots were probed with the indicated primary antibodies, followed by incubation with a 1/5000 dilution of the affinity-purified goat anti-rabbit IgG conjugated with alkaline phosphatase (Jackson Immunoresearch Laboratories Inc.) and detected with 10 ml of 100 mM Tris buffer, pH 9.5, containing 100 mM NaCl, 5 mM MgCl\textsubscript{2}, 0.37 mM p-nitro blue tetrazolium chloride, and 0.41 mM 5-bromo-4-chloro-3-indoly phosphate (BDH). The blots were washed for 10 min with 0.1% Tween 20, 0.1% Triton X-100, 0.1% sodium dodecyl sulfate. Specific rabbit polyclonal antibodies were generated in our laboratory against bacterially expressed p25\textsuperscript{nck5a} and Cdk5 proteins, a C-terminal p25\textsuperscript{nck5a} peptide (QPM\textsubscript{251-255}KPPDKLN-SRESUGDQEN\textsubscript{259}), and an N-terminal p25\textsuperscript{nck5a} peptide (P\textsubscript{109}PASOLQGGS\textsubscript{120}). All were used at a concentration of 2 μg/ml except the latter, which was used at 100 μg/ml.

For the ECL system, the blots were probed with 1/2000 dilution of a rabbit polyclonal Cdk5 (C-8) antibody from Santa Cruz Biotechnology, Inc., followed by incubation with goat anti-rabbit IgG conjugated with horseradish peroxidase and developed as specified by the manufacturer (Amer sham Corp.).

**Protein Concentration Determination**

Protein concentration was determined from A\textsubscript{280nm} values or by Bradford microassay (45).

**RESULTS AND DISCUSSION**

To analyze the relationship between p25\textsuperscript{nck5a} and p35\textsuperscript{nck5a} and their interactions with Cdk5 in brain extracts, we attempted the fractionation of the two forms of Nck5a in a 120,000 × g fraction of the brain extract on various columns. Initial characterization using specific antibodies showed that both Cdk5 and Nck5a were quantitatively absorbed on a hydroxyapatite column or a cation exchanger such as an FPLC Mono S column but negligibly bound to anion exchangers such as DEAE-Sepharose and FPLC Mono Q columns. When a small amount of crude sample (40 mg) of fresh bovine brain 120,000


**Fig. 1. DEAE-hydroxyapatite column chromatography.** Protein fractionation of the 120,000 x g supernatant by DEAE-hydroxyapatite column was carried out as described under "Experimental Procedures." A, ---, A<sub>280nm</sub> value; ○-○, endogenous kinase activity; □-□, kinase activity in the presence of GST-p21<sup>nck5a</sup>, kinase activity in the presence of GST-Cdk5. ---, a linear gradient of 0–0.4 M K<sub>2</sub>HPO<sub>4</sub>. Inset represents the kinase activity at different salt scales indicating the separation of two different activity peaks. B, immunoblot analysis of the eluted proteins with a Nck5a-specific antibody. C, immunoblot analysis of the eluted proteins with a Cdk5-specific antibody. D, immunoblot analysis of the eluted proteins with a Cdk5-specific antibody, C-8, and developed by the ECL system as described under "Experimental Procedures."

Extract was applied to an FPLC Mono S (HR 5/5) column, and the column eluents from a linear gradient of 0–0.5 M NaCl were analyzed by Western blot analyses using Cdk5- and Nck5a-specific antibodies. Cdk5 was found to be eluted from the column over the entire salt gradient, whereas p25<sup>nck5a</sup> and p35<sup>nck5a</sup> were eluted separately, each within a narrow range of the salt gradient. The result was in agreement with a postulate that there were at least three forms of Cdk5 in the crude bovine brain sample: a p35<sup>nck5a</sup>-complexed form, a p25<sup>nck5a</sup>-complexed form, and a form not associated with Nck5a. To analyze the activity states of the different forms of Cdk5, the column eluents were assayed for histone H1 kinase activity in the presence and absence of GST-p21<sup>nck5a</sup>, a bacterially expressed truncated form of p25<sup>nck5a</sup> that is capable of activating a bacterially expressed Cdk5 (38). When the kinase assay was carried out in the absence of GST-Cdk5, a small amount of the sample can be processed, and the separated fractions may be further analyzed. A sample (1,300 ml) of the 120,000 x g fraction was loaded onto a 400-ml DEAE-Sepharose column directly coupled to a 100-ml hydroxyapatite column. After sample application, the columns were disconnected, and the hydroxyapatite column was eluted with a linear gradient of K<sub>2</sub>HPO<sub>4</sub> (0–400 mM). The eluted proteins were analyzed by different column chromatographic procedures, a procedure combining both DEAE-Sepharose and hydroxyapatite column. In comparison with a Mono S chromatography, this procedure has the advantage that a large amount of the sample can be processed, and the separated fractions may be further analyzed. A sample (1,300 ml) of the 120,000 x g fraction was loaded onto a 400-ml DEAE-Sepharose column directly coupled to a 100-ml hydroxyapatite column. After sample application, the columns were disconnected, and the hydroxyapatite column was eluted with a linear gradient of K<sub>2</sub>HPO<sub>4</sub> (0–400 mM). The eluted proteins were analyzed by histone H1 peptide-phosphorylating activity in the presence and absence of GST-p21<sup>nck5a</sup>, bacterially expressed truncated form of p25<sup>nck5a</sup> that is capable of activating a bacterially expressed Cdk5 (38), and immunoblotted with either a Nck5a- or Cdk5-specific antibodies. As shown in Fig. 1A, in the absence of GST-p21<sup>nck5a</sup>, a small peak of endogenous kinase activity eluted at 280 mM K<sub>2</sub>HPO<sub>4</sub>. In the presence of GST-p21<sup>nck5a</sup>, the kinase activity was markedly increased. The peak of the GST-p21<sup>nck5a</sup>-activated kinase activity eluted slightly before that of the endogenous kinase activity (see Fig. 1A, inset). This indicates that the majority of Cdk5 exists in an inactive form but can be activated by GST-p21<sup>nck5a</sup>.

Immunoblot analysis of the eluted proteins with a Nck5a-specific antibody showed two major immunoreactive bands with molecular weights corresponding to p25<sup>nck5a</sup> and p35<sup>nck5a</sup> (Fig. 1B). These two proteins were partially separated by hydroxyapatite column chromatography: the 25-kDa protein eluted between 250 and 330 mM K<sub>2</sub>HPO<sub>4</sub>, whereas the 35-kDa activation.

To further test this suggestion, the 120,000 x g bovine brain extract was analyzed by different column chromatographic procedures, a procedure combining both DEAE-Sepharose and hydroxyapatite column.
The mass of infraction eluted at a volume corresponding to the molecular mass of p25 and became highly active when the kinase assay was carried out in the presence of GST-p21 complexed form, and (iii) a p35 fraction II.

A column was carried out as described under "Experimental Procedures." Activity in the presence and absence of GST-p21 enriched with free Cdk5, p25 fraction II (nos. 36–42), and fraction III (nos. 44–54), which were pooled into three different groups; fraction I (nos. 28–34), fraction II (nos. 36–42), and fraction III (nos. 44–54), were enriched with free Cdk5, p25/Cdk5, and p35/Cdk5 complex, respectively. Each of the fractions was concentrated by the gel filtration column chromatography, did not form a complex. A number of observations, however, argued against the existence of GST-p21/Cdk5 form contained in these fractions is that the two proteins, although co-eluted at the void volume of the column, did not result in kinase activity, suggesting that Cdk5 and p25/Cdk5 exists as a complex. There was a peak of GST-p21/Cdk5-activated kinase activity at an elution volume corresponding to the molecular weight of ~30 kDa. However, this is expected because the separation of the free and p25/Cdk5 complexed Cdk5 forms on the hydroxylapatite column was incomplete, and the fraction II sample contained free Cdk5 (see Fig. 1). Analysis of fraction III by Superose-12 gel filtration chromatography showed that most of p35/Cdk5 and Cdk5 of the sample co-eluted at the void volume of the column (Fig. 4), thus raising the possibility that these proteins were in a complex of over 400 kDa. There was little or no histone H1 kinase activity associated with these fractions in the presence and absence of GST-p21/Cdk5 (Fig. 1). A–D. Together, these results support the existence of three distinct forms of Cdk5 in bovine brain extract: (i) a free form that is inactive but activable by Nck5a, (ii) an active p25/Cdk5 complexed form, and (iii) a p35/Cdk5 complexed form that is neither active nor activable by Nck5a.

To further test the existence of three distinct forms of Cdk5 in bovine brain extract, the hydroxylapatite fractions were pooled into three different groups; fraction I (nos. 28–34), fraction II (nos. 36–42), and fraction III (nos. 44–54), which were enriched with free Cdk5, p25/Cdk5/Cdk5, and p35/Cdk5/Cdk5 complex, respectively. Each of the fractions was concentrated and further analyzed by FPLC Superose-12 gel filtration chromatography. As shown in Fig. 2, A and C, the majority of Cdk5 in fraction I eluted at a volume corresponding to the molecular mass of ~30 kDa and became highly active when the kinase assay was carried out in the presence of GST-p21/Cdk5. This indicates that Cdk5 exists mainly as a monomeric form. When fraction II was analyzed by Superose-12 gel filtration chromatography, a single peak of endogenous kinase activity containing both p25/Cdk5 and Cdk5 eluted at a volume corresponding to the molecular mass of ~60 kDa (Fig. 3). Addition of GST-p21/Cdk5 to the p25/Cdk5/Cdk5-containing fractions did not increase the kinase activity, suggesting that Cdk5 and p25/Cdk5 exists as a complex.

While the existence of GST-p21/Cdk5-activated kinase activity at an elution volume corresponding to the molecular weight of ~30 kDa, suggesting that Cdk5 did not exist as a free form. Similarly, the addition to these column fractions of a bacterially expressed GST-Cdk5, which could be readily activated by GST-p21/Cdk5 (see Figs. 1 and 5), did not result in active kinase, thus arguing against the presence of free p35/Cdk5.
with a suggestion that a low molecular weight inhibitor had been removed from the complex. An alternative explanation that ethylene glycol incubation caused a conformational change of the kinase from an inactive to an active state is unlikely since incubation of the sample in 10% ethylene glycol without subjecting the gel filtration chromatography did not result in activation of the kinase (data not shown). Taken together, our results suggest a potential interaction between a low molecular weight inhibitor and the p35\(^{nck5a}\)-Cdk5 complex to form the inactive high molecular weight complex.

In summary, we observed three distinct Cdk5 molecular species in bovine brain extract: (i) a monomeric Cdk5 that can be activated by Nck5a, (ii) a p25\(^{nck5a}\)-Cdk5 form that is intrinsically active, and (iii) a p35\(^{nck5a}\)-Cdk5 form that is inactive and refractory to Nck5a activation. Further analysis of the fractions containing both p35\(^{nck5a}\) and Cdk5 indicated the presence of an inactive p35\(^{nck5a}\)-Cdk5 high molecular weight complex. A number of recent papers support the presence of the p35\(^{nck5a}\)-Cdk5 high molecular weight complex: (i) the existence of inactive macromolecular complex-containing multiple p21 subunits in insect Sf9 cell lysate where p21 has been suggested to act as a Cdk assembly factor to promote the association of cyclin and Cdk subunits (30), (ii) the identification of Cdk-activating kinase complex as a component of TFIIH (26–28), (iii) the identification of cyclin-dependent kinase binding protein (p15\(^{cdk-BP}\)) as a part of a high molecular weight complex in starfish oocyte lysate (32), and (iv) the existence of additional Cdk5-regulatory subunits (p60, p62, and p180) aside from p25\(^{nck5a}\) and p35\(^{nck5a}\) in rat neuronal cells (31, 40). Although

Fig. 5. Superose-6 gel filtration column chromatography of fraction III. A, protein fractionation of fraction III by a Superose-6 column was carried out as described under "Experimental Procedures." A, ——, A\(_{280\text{nm}}\) value; ●—●, endogenous kinase activity; ○—○, kinase activity in the presence of GST-p21\(^{nck5a}\); —-, kinase activity in the presence of GST-Cdk5. B, immunoblot analysis of the eluted proteins with a Nck5a-specific antibody. C, immunoblot analysis of the eluted proteins with a Cdk5-specific antibody.
the molecular basis for the lack of kinase activity of the macromolecular complex is not known, initial experiments are compatible with the suggestion that the complex contains kinase inhibitory factor(s). Tests are underway to identify a possible low molecular weight inhibitor.

Acknowledgments—We thank Erwin Wirch for excellent technical assistance. We also extend our gratitude to Dr. Jeffrey D. Bjorge for helpful suggestions in the final stage of completion of the manuscript.

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