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Cdk5/p25^{nck5a} Interaction with Synaptic Proteins in Bovine Brain

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Abstract Cyclin-dependent kinase 5 (Cdk5) exists in large multimeric complexes, but its function and binding partners in these complexes are unclear. We explored these issues by chromatographic and immunochemical analyses of Cdk5 and p25^{nck5a} (a neuronal Cdk5 activator) and their associated proteins from bovine brain. Mono-S column enzyme eluates were divided into three fractions and analyzed by gel filtration. The majority of p25^{nck5a} from Mono-S fractions I, II, and III eluted from the gel filtration column at ~60, 200, and 400 kDa, respectively, and Cdk5 was abundant in fractions >400 kDa. We characterized these macromolecular structures by immunoprecipitating p25^{nck5a}, followed by a second immunoprecipitation of remaining unbound proteins using a Cdk5 antibody. The p25^{nck5a} immunoprecipitates showed association with Cdk5. Amphiphysin was detected in the 400-kDa complex and synapsin I in the >400 kDa structure. The Cdk5 immunoprecipitates, however, revealed abundant retained Cdk5 but no remaining p25^{nck5a}, indicating that Cdk5 in macromolecular structures is mostly unassociated with p25^{nck5a}. Thus, we demonstrate: an amphiphysin-associated 400-kDa Cdk5/p25^{nck5a} complex, a synapsin I-associated >400-kDa Cdk5/p25^{nck5a} complex, and nck5a-free Cdk5 complexes (200 to >400 kDa). Amphiphysin acts as a Cdk5/p25^{nck5a} substrate in the 400-kDa complex and we speculate that Cdk5/p25^{nck5a} participates in amphiphysin-mediated endocytosis. J. Cell. Biochem. 78:151–159, 2000.

Key words: cyclin-dependent kinase; Cdk5 activator; phosphorylation; amphiphysin; endocytosis

Cyclin-dependent kinase 5 (Cdk5) and its neuronal Cdk5 activator (nck5a), p25^{nck5a} (an N-terminally truncated form of its precursor protein, p35^{nck5a}), form the neuronal Cdc-2–like kinase (Nclk), a serine/threonine kinase discovered as a homologue of Cdc2 [see Lew and Wang, 1995 and Lee et al., 1997b for review]. Although Cdk5 and Cdc2 have similar catalytic properties, they are distinctly regulated. For example, while nck5a is cyclin-like in that it can activate Cdk5, it has little sequence homology to conventional cyclins [Lew et al., 1994; Tsai et al., 1994]. Furthermore, unlike other Cdks, Cdk5 phosphorylation by Cdk-activating kinase is not required for activation by nck5a [Qi et al., 1995].

In contrast to other Cdks and their cyclins, which are known to govern the eukaryotic cell cycle [Nigg, 1993], Cdk5 activity, which is mostly associated with postmitotic neurons, has been implicated in other cellular processes. For example, Cdk5 activity has been suggested to regulate neurite outgrowth during neuronal differentiation [Nikolic et al., 1996] through modification of Pak1 activity [Nikolic et al., 1998]. Furthermore, Cdk5/p25^{nck5a} phosphorylation of the retinoblastoma protein during a period of increased programmed cell death [Lee et al., 1997a] and increased Cdk5 expression in dying cells [Ahuja et al., 1997] implied a role for the kinase in apoptosis. Deregulation of Cdk5/p25^{nck5a} is also believed to contribute to the abnormal phosphorylation of tau and neurofilament proteins in neurodegenerative disorders such as Alzheimer's disease and amyotrophic lateral sclerosis [see Lew and Wang,

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1995 and Lee et al., 1997b for review; Lee et al., 1999]. While the specific functions of Cdk5/p25^{nck5a} are yet to be defined in these various cellular contexts, it is likely that regulation of the kinase is important in controlling some or all of these events.

Previously, we have shown that Cdk5 in bovine brain exists as a free monomer or as an active heterodimer with p25^{nck5a} [Lee et al., 1996]. However, we also found that Cdk5 can exist as part of a macromolecular structure [Lee et al., 1996; Lee and Johnston, 1997]. Thus, like other Cdks, Cdk5/p25^{nck5a} may associate with other molecules to achieve its regulatory and functional role in the cell. Indeed, we have shown that association of Cdk5/p25^{nck5a} with macromolecular structures that contain neurofilaments likely supports neurofilament phosphorylation [Lee and Johnston, 1997]. Cdk5 can also bind to Munc-18 and syntaxin IA [Halachmi and Lev, 1996; Shuang et al., 1998], and has the ability to phosphorylate synapsin I [Matsubara et al., 1996]. Recently, Cdk5 was suggested to modulate neuronal exocytosis through Munc-18 phosphorylation [Fletcher et al., 1999]. In this study, we wish to further examine the interaction of Cdk5/p25^{nck5a} with proteins in the neuronal synapse through characterization of the large multimeric Cdk5/p25^{nck5a} complexes in brain tissue.

Ion-exchange and gel filtration column chromatographic procedures allowed us to isolate macromolecular structures of Cdk5. By immunoprecipitation of Cdk5 and p25^{nck5a} from high molecular weight Cdk5-containing complexes, we found a Cdk5/p25^{nck5a} complex associated with amphiphysin and another associated with synapsin I. We also observed Cdk5 complexes that are free of nck5a. Association of Cdk5 and/or p25^{nck5a} with amphiphysin and synapsin supports a potential role for the kinase in neuroterminal functions such as endocytosis and exocytosis.

MATERIALS AND METHODS

Preparation of Tissue Extract

Tissue homogenization was carried out as previously described [Lee et al., 1996]. The resulting supernatant after centrifugation of the crude homogenate at 12,000g for 25 min in a Beckman JA-10 rotor was further centrifuged at 120,000g for 30 min in a Beckman Ti-45 rotor, and the final supernatant was designated S_{120}.

FPLC Mono-S Column Chromatography

S_{120} (50 mg) was diluted threefold with buffer A [25 mM HEPES, pH 7.2, 1 mM ethylene-diamine-tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.6 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml aprotinin, 2 μg/ml antipain, 0.3 mg/ml benzamidine 0.1 mg/ml soybean trypsin inhibitor] and loaded into a Pharmacia Mono-S (HR 5/5) column at 0.5 ml/min. Proteins were eluted with a 30-ml linear gradient of NaCl (0–0.5 M NaCl in buffer A) and 1-ml fractions were collected.

FPLC Superose-12 Gel Filtration Column Chromatography

Pooled fractions (I, II, and III) from the Mono-S column were concentrated separately by reverse dialysis against Aquacide II (Calbiochem) at 4°C. 2–3 mg protein in 0.3 ml volume was applied to an FPLC Pharmacia Superose-12 (HR 16/50) gel filtration column equilibrated in buffer A without soybean trypsin inhibitor but with 0.15 M NaCl. The column was run at a flow rate of 0.5 ml/min and 0.5-ml fractions were collected. Molecular weight calibration of the column was performed using a calibration kit from Bio-Rad (bovine thyroglobulin, 670 kDa; ferritin, 440 kDa; bovine gamma globulin, 158 kDa; chicken ovalbumin, 44 kDa; horse myoglobin, 17 kDa; vitamin B-12, 1.35 kDa).

Protein Kinase Assay

Protein kinase assays were performed as described previously [Lee et al., 1996] but kinase activity was measured in 5-μl aliquots of column chromatographic fractions ± 0.1 μg of GST-p25^{nck5a}. Incorporation of γ-^{32}P[ATP] into the substrate peptide was quantitated by liquid scintillation using a Beckman LS 5000CE scintillation counter.

Immunoprecipitation

To determine whether synaptic proteins associate with the Cdk5-containing fractions from the gel filtration column, 200-μg samples of the 200-, 400-, and >400- (from Mono S fraction I) kDa complexes were subjected to immunoprecipitation. Each sample was pre-

cleared with 20 μl of protein A/G plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with 2 μg of nck5a-specific antibody [C-19, Santa Cruz Biotechnology; preadsorbed to 20 μl of protein A/G plus agarose and washed with Tris-buffered saline containing 0.1% Tween-20 (TBST) three times] for 2 h at 4°C on a rotating platform. In some experiments, the supernatant from the nck5a immunoprecipitate was subjected to another immunoprecipitation using 2 μg of Cdk5-specific antibody (DC-17, Santa Cruz Biotechnology; preadsorbed to 20 μl of protein A/G plus agarose and washed with TBST three times). The immunocomplexes were washed extensively with TBST. The presence of nck5a, Cdk5, and associated proteins in the immunoprecipitated samples were determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using specific antibodies against the proteins of interest. Immunoprecipitation of amphiphysin from 250 μg bovine brain S_{120} was carried out using 2 μg of monoclonal amphiphysin antibody from Transduction Laboratories.

Dephosphorylation of Immunoprecipitated Proteins

Immunoprecipitated samples were dephosphorylated by incubating with 10 units of calf intestinal alkaline phosphatase (Pharmacia Biotechnology) in 1× one-phor-all buffer (Pharmacia Biotechnology) for 90 min at 37°C. The immunoprecipitates were then cleared of alkaline phosphatase by extensive washing with TBST and a final wash with kinase assay buffer containing phosphatase inhibitors (microcystin-LR and sodium fluoride).

Assay for Amphiphysin Phosphorylation by Cdk5/p25^{nck5a}

The initial assay for phosphorylation of amphiphysin IP from bovine brain S_{120} by Cdk5/p25^{nck5a} was performed using a bacterially expressed enzyme. GST-Cdk5 and GST-p25^{nck5a} (0.2 μg each) were preincubated for 30 min at 25°C in the presence of 1 mM DTT and 0.25 mg/ml BSA in kinase assay buffer [Lee et al., 1996]. The Cdk5/p25^{nck5a} complex was then incubated with the amphiphysin immunoprecipitate at 30°C for 40 min in the presence of γ-^{32}P[ATP] in kinase assay buffer. The reaction was stopped by adding SDS-PAGE sample buffer and boiling for 5 min. Phosphorylation of amphiphysin was determined by autoradiography of samples run in a 12.5% SDS-PAGE and transferred onto an immobilon-P membrane (Millipore, Bedford, MA).

Assay for phosphorylation of amphiphysin by Cdk5/p25^{nck5a} in the 400-kDa Cdk5 complex was conducted by preparing two sets of nck5a immunoprecipitates. One set was dephosphorylated (to dephosphorylate the nck5a-associated amphiphysin) followed by incubation with the other nck5a immunoprecipitate that was not subjected to dephosphorylation (to maintain an active enzyme). The phosphorylation reaction was allowed to proceed and examined as described above.

SDS-PAGE and Western Blotting

SDS-PAGE was performed according to the method of Laemmli [1970] in 12.5% vertical slab gels. After electrophoresis, proteins were transferred to an immobilon-P membrane and the blots were probed with the primary antibody (anti-nck5a, C-19, Santa Cruz Biotechnology; anti-Cdk5, C-8, Santa Cruz Biotechnology; anti-synapsin I, Calbiochem-Novabiochem, La Jolla, CA; anti-amphiphysin, Transduction Laboratories, Lexington, KY) followed by incubation with the secondary antibody conjugated with horseradish peroxidase (Pierce, Rockford, IL). Immunoreactive proteins were detected using the ECL method and developed as specified by the manufacturer (Amersham Life Science, Arlington Hts, IL).

Miscellaneous Procedures

In vitro reconstitution assays and expression and purification of GST-fusion proteins from bacteria were performed as described previously [Lee et al., 1996]. Protein concentration was determined from A_{280nm} values or by Bradford microassay [Bradford, 1976].

RESULTS

Elution Pattern of Cdk5/nck5a From a Mono-S Column

Bovine brain extracts (S_{120}) loaded onto a Mono-S column were eluted with a linear gradient of 0–0.5 M NaCl over 30 ml and analyzed for histone H1 kinase activity in the presence and absence of GST-p25^{nck5a}, a bacterially expressed truncated form of p35^{nck5a} that is capable of activating Cdk5 [Qi et al., 1995]. As shown in Fig. 1A, a peak of endogenous kinase

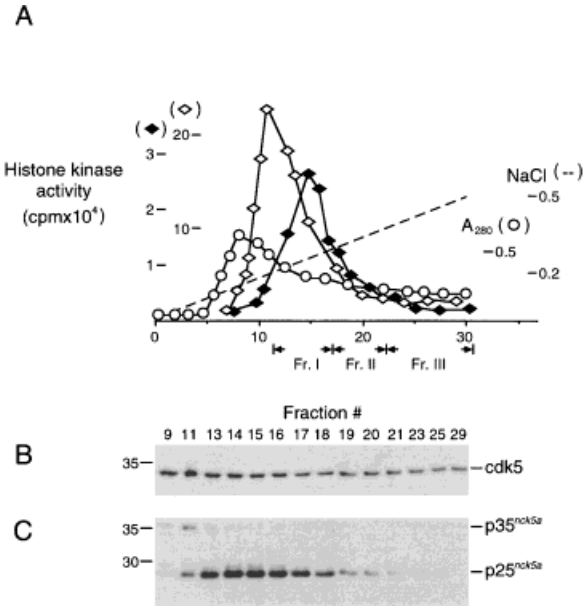

Fig. 1. Elution pattern of Cdk5/p25^{nck5a} from a Mono-S column. Bovine brain extract (S_{120}, 50 mg) was injected into an FPLC Mono-S (HR 5/5) column and proteins were eluted as described in Materials and Methods. **A:** ○—○, A_{280nm} values of the eluted fractions; ◆—◆, endogenous kinase activity of the fraction; ◇—◇ kinase activity in the presence of GST-p25^{nck5a}. Eluted proteins were then analyzed by 12.5% SDS-PAGE and immunoblotted with a Cdk5-specific (DC-17) antibody (**B**) and an nck5a-specific (C-19) antibody (**C**).

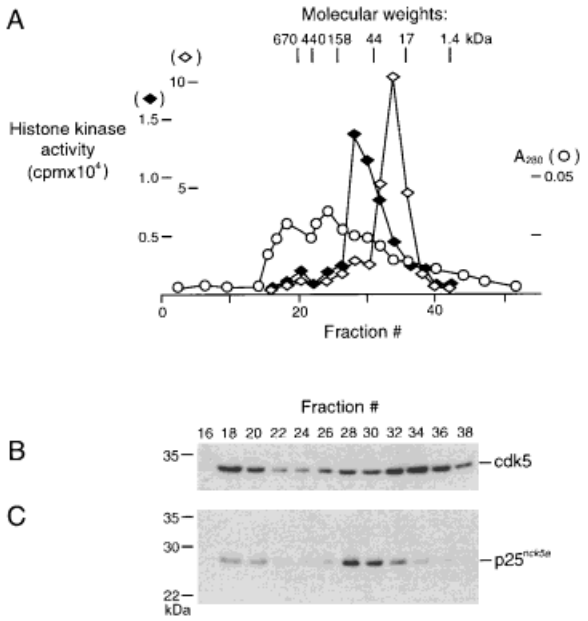

Fig. 2. The endogenous peak of Cdk5/p25^{nck5a} kinase activity from pooled Mono-S fraction I eluted at a molecular weight of ~60 kDa from a Superose 12 column. Fractions 11–17 from the Mono-S column (Fig. 1) were pooled and concentrated using Centricon-10. 2.15 mg total protein in 0.3 ml volume was injected at a flow rate of 0.5 ml/min into a Pharmacia Superose-12 (25 ml) column pre-equilibrated with buffer A (see Materials and Methods) and 1 ml fractions were collected. **A:** ○—○, A_{280nm} values of the eluted fractions; ◆—◆, endogenous kinase activity of the fraction; ◇—◇, kinase activity in the presence of GST-p25^{nck5a}. Column eluates were analyzed by 12.5% SDS-PAGE and immunoblotted with Cdk5 (DC-17) (**B**) and nck5a (C-19) (**C**) antibodies.

activity eluted at 250 mM NaCl, corresponding to the fractions (14 and 15) that contain the most p25^{nck5a} (Fig. 1C). In the presence of exogenous GST-p25^{nck5a}, kinase activity was enhanced up to 50-fold in fractions 9–13, suggesting the presence of abundant free Cdk5 monomers in these fractions. This GST-p25^{nck5a}-induced peak of kinase activity eluted at 220 mM NaCl (Fig. 1A).

We divided the eluates from the Mono-S column into three major fractions (as indicated in Fig. 1A): fraction I, the Cdk5/p25^{nck5a}-enriched, endogenously active and/or GST-p25^{nck5a}-activatable portion, corresponding to fractions 11–17; fraction II, the Cdk5/p25^{nck5a}-containing, less active/activatable portion, corresponding to fractions 18–22; and fraction III, a Cdk5-containing portion corresponding to fractions 23–31 (Figs. 1A–1C). To further characterize Cdk5/p25^{nck5a} in these three fractions, pooled samples were independently loaded into a Superose-12 gel filtration column and evaluated for histone H1 kinase activity and for the presence of Cdk5 and its activator protein, nck5a.

Cdk5/nck5a Elution From the Superose-12 Gel Filtration Column

As shown in Figs. 2A, 3A, and 4A, in the presence of exogenous GST-p25^{nck5a}, a peak of kinase activity at approximately 30–40 kDa, corresponding to fractions containing the highest concentrations of monomeric Cdk5 (Fig. 2B, 3B, and 4B), was observed. In contrast, endogenous peaks of kinase activity eluted at fractions containing the most p25^{nck5a} (Fig. 2C, 3C, and 4C). In Mono-S pooled fraction I, this peak eluted at an apparent molecular size of 60 kDa (Fig. 2A). In fraction II, the apparently lower peak of endogenous kinase activity eluted at approximately 200 kDa (Fig. 3A), a considerably larger structure than the 60-kDa Cdk5/p25^{nck5a} complex in fraction I. Analysis of Mono-S fraction III revealed a modest endogenous kinase activity that eluted over a relatively broad range at approximately 400 kDa

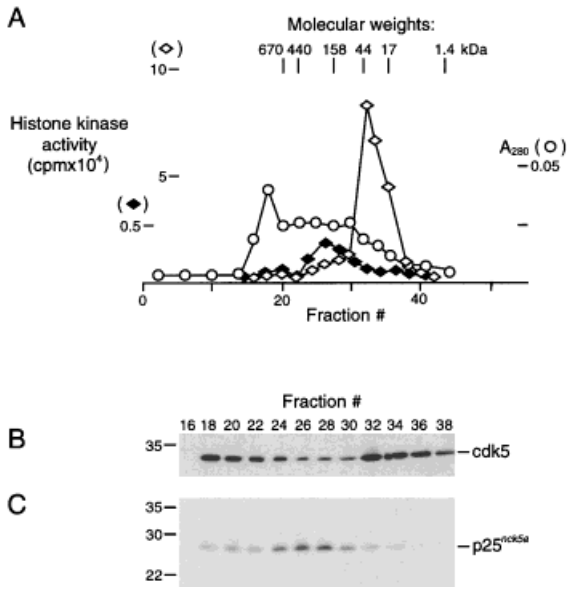

Fig. 3. The endogenous peak of Cdk5/p25^{nck5a} kinase activity from pooled Mono-S fraction II eluted at a molecular weight of ~200 kDa from a Superose-12 column. Fractions 18–22 from the Mono-S column were pooled and concentrated using Centricon-10. 3.0 mg total protein in 0.3 ml volume was injected at 0.5 ml/min into a Pharmacia Superose-12 (25 ml) column pre-equilibrated with buffer A and 1 ml fractions were collected. **A:** ○—○, A_{280nm} values of the eluted fractions; ◆—◆, endogenous kinase activity of the fraction; ◇—◇, kinase activity in the presence of GST-p25^{nck5a}. Eluates were analyzed as described in Fig. 2. Gels were immunoblotted with antibodies against Cdk5 (DC-17) (**B**) and nck5a (C-19) (**C**).

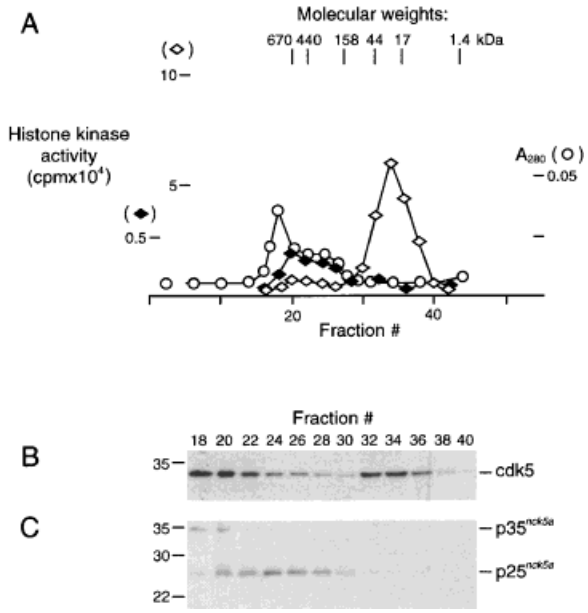

Fig. 4. The endogenous peak of Cdk5/p25^{nck5a} kinase activity from pooled Mono-S fraction III eluted at a molecular weight of ~400 kDa from a Superose 12 column. Fractions 23–31 from the Mono-S column were pooled and concentrated using Centricon-10. 2.2 mg total protein in 0.3 ml volume was injected at 0.5 ml/min into a Pharmacia Superose-12 (25 ml) column pre-equilibrated with buffer A and 1 ml fractions were collected. **A:** ○—○, A_{280nm} values of the eluted fractions; ◆—◆, endogenous kinase activity of the fraction; ◇—◇, kinase activity in the presence of GST-p25^{nck5a}. Eluted proteins were analyzed on 12.5% SDS-PAGE and immunoblotted with antibodies against Cdk5 (DC-17) (**B**) and nck5a (C-19) (**C**).

(Fig. 4A). Prolonged exposure of the immunoblot was necessary to detect nck5a from fraction III (Fig. 4C) whereas in fraction I, prolonged exposure also revealed a minimal amount of p35^{nck5a} in the high molecular weight fractions (data not shown). Therefore, our results demonstrate that Cdk5 associates with its activator protein, p25^{nck5a}, to form 60-, 200-, and 400-kDa complexes. Although some nck5a was detected in higher molecular weight fractions (>400 kDa; Fig. 2C, 3C, and 4C), it is interesting that Cdk5 appeared to be rather abundant in these fractions (Figs. 2B, 3B, and 4B). Thus, it appears that a pool of Cdk5 that is free of nck5a exists in >400-kDa macromolecular complexes.

Coprecipitation of Amphiphysin and Synapsin I With Cdk5 Complexes

There is increasing evidence that Cdk5/p25^{nck5a} is involved in cellular processes other than the cell cycle. The kinase has been particularly associated with neuronal differentiation [Nikolic et al., 1996; Nikolic et al., 1998], but it appears that Cdk5/p25^{nck5a} is also important in differentiated cell functions such as neuronal secretory exocytosis [Fletcher et al., 1999]. Because exocytosis occurs in tandem with endocytosis, it would be interesting to know whether Cdk5 is involved in regulating the latter process as well. To explore the potential interaction of Cdk5/p25^{nck5a} with proteins involved in endocytosis and exocytosis, we first examined the high molecular weight Cdk5 complexes from the gel filtration column for association with amphiphysin and synapsin I, proteins that are suggested to regulate endocytosis and exocytosis, respectively.

Initial characterization of the Cdk5/p25^{nck5a}-containing complexes from the gel filtration column was performed by immunoprecipitation using an nck5a antibody (Fig. 5). By immunoblot analysis, we found coimmunoprecipitation of Cdk5 (Fig. 5A) with p25^{nck5a} (Fig. 5B) in the 200-, 400-, and >400-kDa complexes. We also found

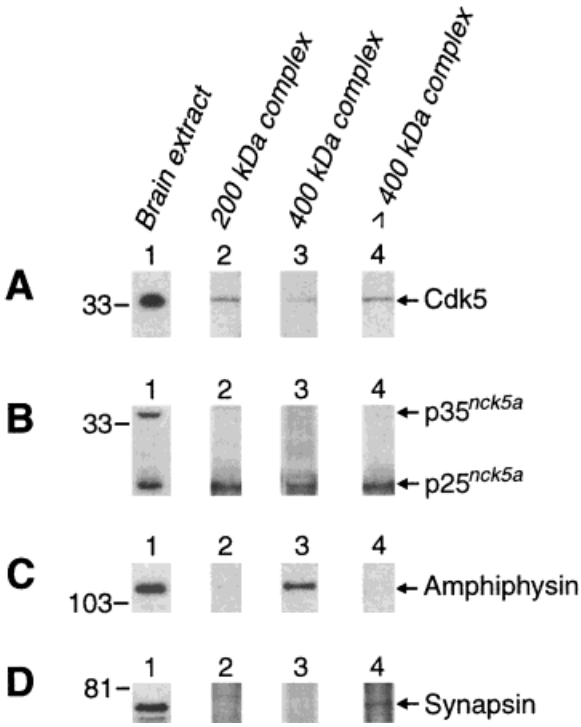

Fig. 5. Amphiphysin and synapsin I coimmunoprecipitate with Cdk5-associated p25^{nck5a}. Using an nck5a-specific antibody (C-19), nck5a was immunoprecipitated from 200 μg each of the macromolecular gel filtration eluates containing Cdk5 and p25^{nck5a}. Immunoprecipitated samples were run in a 12.5% SDS-PAGE and immunoblotted with antibodies against **A:** Cdk5 (DC-17), **B:** nck5a (C-19), **C:** amphiphysin, and **D:** synapsin I.

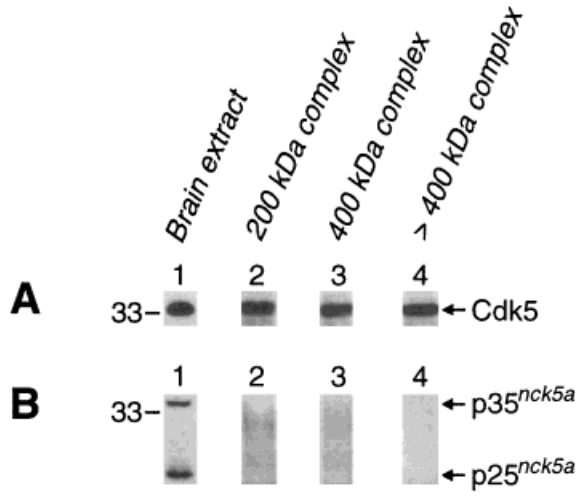

Fig. 6. An nck5a-free Cdk5 complex exists in bovine brain. Unbound proteins (supernatants) from the nck5a immunoprecipitates (Fig. 5) were subjected to another immunoprecipitation using a Cdk5-specific antibody (C-8). Immunoprecipitated samples were run in a 12.5% SDS-PAGE and immunoblotted with antibodies against **A:** Cdk5 (DC-17) and **B:** nck5a (C-19).

that amphiphysin coimmunoprecipitated with p25^{nck5a} in the 400-kDa complex (Fig. 5C, lane 3). In contrast, synapsin I coimmunoprecipitated with p25^{nck5a} in the >400 kDa complex (Fig. 5D, lane 4). Further analysis of the macromolecular Cdk5/p25^{nck5a}-containing complexes was carried out by a second immunoprecipitation step of the supernatants (unbound proteins) from the nck5a immunoprecipitates using a Cdk5 antibody. This was done to examine any remaining Cdk5 that did not coimmunoprecipitate with p25^{nck5a}. Immunoblotting analysis showed large amounts of Cdk5 (Fig. 6A) but no nck5a (Fig. 6B) remaining in the supernatants of the 200-, 400-, and >400-kDa complexes after nck5a immunoprecipitation. This indicates that most of the Cdk5 in the high molecular weight structures is not associated with nck5a. Neither amphiphysin nor synapsin I coimmunoprecipitated with the nck5a-free Cdk5 complexes (data not shown). Although it is possible that amphiphysin and synapsin I are not directly bound to Cdk5 and/or nck5a, and that bridging molecules account for their association with Cdk5/p25^{nck5a}, their association with the kinase in large multimeric complexes or macromolecular aggregates suggests a function for Cdk5/p25^{nck5a} in the neuroterminal.

Amphiphysin Acts as a Substrate for Cdk5/p25^{nck5a} In Vitro

Association of synapsin I with Cdk5/p25^{nck5a} was not surprising because it has been reported that synapsin I can act as a substrate for Cdk5 in vitro [Matsubara et al., 1996]. However, it is interesting that association was found only in the >400-kDa Cdk5 complex. In this study, we found that amphiphysin immunoprecipitated from bovine brain extract (S_{120}) and then dephosphorylated, can be rephosphorylated by bacterially expressed GST-Cdk5/GST-p25^{nck5a} (Fig. 7A, lane 6). However, we tried to further investigate the significance of amphiphysin coimmunoprecipitation with active Cdk5-associated p25^{nck5a} from the 400-kDa complex isolated by gel filtration chromatography. Since dephosphorylation results in an inactive enzyme (data not shown), two sets of nck5a immunoprecipitates were prepared from the complex. One was subjected to dephosphorylation (to dephosphorylate nck5a-associated amphiphysin) and the other left untreated (to maintain an active enzyme). These

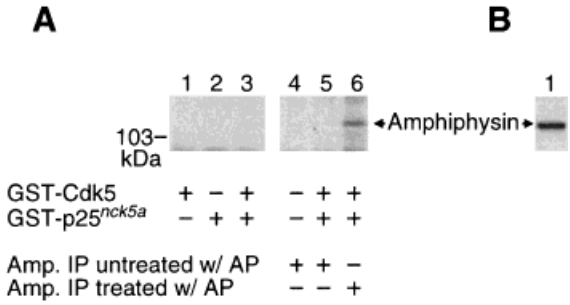

Fig. 7. Amphiphysin is phosphorylated by GST-Cdk5/GST-p25^{nck5a}. **A:** Amphiphysin was immunoprecipitated from 250 μg bovine brain extract (S_{120}) and examined for phosphorylation by GST-Cdk5/GST-p25^{nck5a} (see Materials and Methods); in lane 5, the amphiphysin immunoprecipitate (Amp IP) was not dephosphorylated whereas in lane 6, the sample was subjected to dephosphorylation (treated with alkaline phosphatase, AP, as described in Materials and Methods) prior to phosphorylation assay. **B:** Immunoblot of bovine brain extract (S_{120}) using an antibody against amphiphysin. The sample was run in the same gel as A.

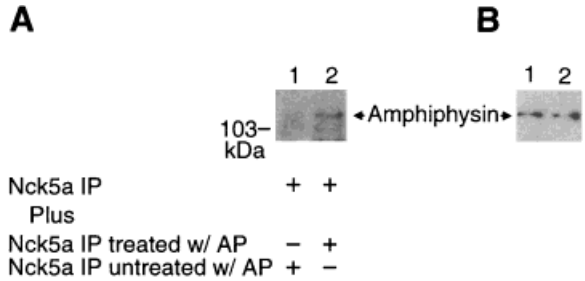

Fig. 8. Amphiphysin and Cdk5/p25^{nck5a} in the 400-kDa gel filtration eluate form a functional complex. **A:** Nck5a immunoprecipitates (IP) were prepared from the 400-kDa Cdk5- and p25^{nck5a}-containing complex. A sample of the immunoprecipitate (from 200 μg gel filtration eluate) was mixed with an equivalent amount of another nck5a immunoprecipitate that was subjected to dephosphorylation (lane 2) In lane 1, neither one of the nck5a immunoprecipitates was treated with alkaline phosphatase (AP). The mixtures were then incubated for 30 min in a kinase assay buffer containing γ-^{32}P[ATP] and analyzed for amphiphysin phosphorylation as described in Materials and Methods. **B:** Immunoblot of the membrane in A using a monoclonal antibody against amphiphysin.

samples were mixed and incubated in the presence of γ-^{32}P[ATP], and autoradiography showed phosphorylation at a molecular weight corresponding to amphiphysin (Fig. 8A, lane 2). Immunoblot analysis of the same membrane revealed the identity of the band as amphiphysin (Fig. 8B). These findings strongly suggest a functional association between Cdk5/p25^{nck5a} and amphiphysin in the complex, and it is likely that amphiphysin acts as a substrate for the kinase in the intact cell.

DISCUSSION

Control of the eukaryotic cell cycle by Cdks depends on several factors, such as the formation of Cdk-cyclin complexes, the phosphorylation state of Cdk subunits, and association with low molecular weight regulatory subunits [Morgan, 1995]. Although the function of Cdk5/p25^{nck5a}, a neuronal homologue of Cdc2, is not linked to cell-cycle regulation, as with other Cdks it has been shown to exist within macromolecular complexes [Lee et al., 1996; Lee and Johnston, 1997]. In these studies however, the kinase displayed either minimal or undetectable activity. Thus, the existence of Cdk5 in macromolecular complexes suggests that its regulation in vivo is highly dependent on its association with other cellular proteins. To further characterize Cdk5/p25^{nck5a} in bovine brain, we examined the kinase's activity and purification patterns by ion exchange and gel filtration chromatographic procedures in combination with immunologic assays.

Bovine brain extract directly loaded into a Mono-S column and analyzed for Cdk5/p25^{nck5a} histone H1 kinase activity revealed an expected peak of endogenous kinase activity in fractions containing the most p25^{nck5a}, the truncated form of p35^{nck5a}. However, an enhancement of kinase activity upon addition of exogenous GST-p25^{nck5a} indicates the presence of free Cdk5 monomers as well. The presence of activatable Cdk5 throughout the NaCl-elution gradient also agrees with our previous data suggesting that the majority of Cdk5 in bovine brain exists in an inactive form [Lee et al., 1996].

Separation of Mono-S column eluates in a Superose-12 gel filtration column, in the presence of 150 mM NaCl, showed expected peaks of kinase activity at approximately 30–40 kDa in the presence of GST-p25^{nck5a}, corresponding to fractions containing the highest concentrations of activatable free Cdk5 monomers. Endogenous peaks of kinase activity at fractions containing the highest levels of p25^{nck5a} were also expected. However, p25^{nck5a} from Mono-S fractions I, II, and III is most abundant at apparent molecular sizes of 60, 200, and 400 kDa, respectively. This is interesting because, using different purification schemes, we have previously described inactive [Lee et al., 1996] or minimally active [Lee and Johnston, 1997]

macromolecular Cdk5 structures, and our current studies suggest the existence of additional forms of Cdk5 complexes (200 and 400 kDa) in bovine brain that have moderate kinase activity.

The observation that, in >400-kDa fractions, Cdk5 levels are much greater than those of nck5a suggests that a major portion of Cdk5 in these fractions is not associated with nck5a. As with monomeric Cdk5, a population of Cdk5 in the high molecular weight complexes may serve as a reservoir for the enzyme. Apparently, Cdk5 by itself can associate with other proteins to form an inactive macromolecular complex. However, $p25^{nck5a}$ that elutes in fractions >400 kDa, as well as those that elute at 200 and 400 kDa, most likely associate with Cdk5 to form a functional Cdk5/$p25^{nck5a}$ complex similar to what we described previously [Lee and Johnston, 1997]. The differential elution of Cdk5/$p25^{nck5a}$ complexes from the gel filtration column and apparent variations in kinase activity indicate that other factors are involved in the regulation of the kinase.

The existence of macromolecular complexes of Cdk5/$p25^{nck5a}$ in bovine brain further led us to assume that the kinase associates with diverse substrates to form distinct multimeric complexes. Indeed, we found that Cdk5-associated $p25^{nck5a}$ immunoprecipitate associates with amphiphysin in a 400-kDa complex, and with synapsin I in a >400-kDa complex. Association of amphiphysin and synapsin I with nck5a-associated Cdk5 appear to be relevant, as these proteins were not associated with nck5a-free Cdk5. The existence of the latter was confirmed after subjecting supernatants from nck5a immunoprecipitates to a second immunoprecipitation step (using a Cdk5 antibody) that showed abundant Cdk5 but no nck5a in Western blots. Coimmunoprecipitation of amphiphysin and synapsin I with Cdk5/$p25^{nck5a}$, however, does not indicate direct binding and it is possible that bridging molecules account for the association. Nevertheless, their association with Cdk5/$p25^{nck5a}$ in large complexes may suggest a role for the kinase in regulating their neuronal function. Indeed, it is possible that the formation of Cdk5 complexes assists in the axonal transport of amphiphysin and synapsin I. Thus, synapsin I, which is found in outgrowing fibers and growth cones during neuronal development [Bergmann et al., 1991; Ovtscharoff et al., 1993], becomes clustered in terminals during synaptogenesis. Similarly, amphiphysin becomes localized to the nerve terminal matrix [Bauerfeind et al., 1997]. However, the engagement of Cdk5 in macromolecular assemblies may also assist in its targeted distribution to distal sites where its function is required. Indeed, consistent with our finding that synapsin I coimmunoprecipitates with Cdk5-associated $p25^{nck5a}$, Cdk5 has been shown to also bind to Munc-18 and syntaxin IA [Halachmi and Lev, 1996; Shuang et al., 1998], two other components of a network of proteins involved in neuronal secretory exocytosis. Recently, Cdk5 has been suggested to regulate neuronal secretion through phosphorylation of Munc-18 [Fletcher et al., 1999]. On the other hand, our finding that Cdk5/$p25^{nck5a}$ uses amphiphysin as a substrate in a multimeric complex may also suggest a regulatory role for the kinase in the control of endocytosis by amphiphysin. Because synapsin I can be phosphorylated by Cdk5/$p25^{nck5a}$ in vitro [Matsubara et al., 1996] as well, it is conceivable that Cdk5/$p25^{nck5a}$ possesses a role in the exocytosis-endocytosis cycle in vivo. Although further studies are necessary to confirm this possibility, our studies conform with previous reports that implicate Cdk5 in the regulation of neuronal secretory exocytosis [Halachmi and Lev, 1996; Shuang et al., 1998; Fletcher et al., 1999].

In summary, we provide evidence for the existence of (1) a moderately active 400-kDa Cdk5/$p25^{nck5a}$ complex associated with amphiphysin; (2) a modestly active >400-kDa Cdk5/$p25^{nck5a}$ complex associated with synapsin I; and (3) an inactive nck5a-free Cdk5 complex (200 to >400 kDa). We also demonstrate here that amphiphysin forms a functional complex with Cdk5/$p25^{nck5a}$, and we speculate that the kinase is involved in endocytosis at the synaptic terminal.

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