Association of Neurofilament Proteins with Neuronal Cdk5 Activator*

(Received for publication, September 15, 1997, and in revised form, October 31, 1997)

Zhong Qi‡§, Damu Tang§, Xiaodong Zhu‡, Donald J. Fujita‡¶, and Jerry H. Wang‡**

From the ‡Department of Medical Biochemistry, University of Calgary, Calgary, Alberta T2N 4N1, Canada and
§Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong

Cdk5 exists in brain extracts in multiple forms, one of which is a macromolecular protein complex comprising Cdk5, neuron-specific Cdk5 activator p35\textsuperscript{Nck5a} and other protein components (Lee, K.-Y., Rosales, J. L., Tong, D., and Wang, J. H. (1996) J. Biol. Chem. 271, 1538–1543). The yeast two-hybrid system was employed to identify p35\textsuperscript{Nck5a}-interacting proteins from a human brain cDNA library. One of the isolated clones encodes a fragment of glial fibrillary acidic protein, which is a glial-specific protein. Sequence alignment revealed significant homology between the p35\textsuperscript{Nck5a}-binding fragment of glial fibrillary acidic protein and corresponding regions in neurofilaments. The association between p35\textsuperscript{Nck5a} and neurofilament medium molecular weight subunit (NF-M) was confirmed by both the yeast two-hybrid assay and direct binding of the bacteria-expressed proteins. The p35\textsuperscript{Nck5a} binding site on NF-M was mapped to a carboxyl-terminal region of the rod domain, in close proximity to the putative Cdk5 phosphorylation sites in NF-M. A region immediately amino-terminal to the kinase-activating domain in p35\textsuperscript{Nck5a} is required for its binding with NF-M. In in vitro binding assays, NF-M binds both monomeric p35\textsuperscript{Nck5a} and the Cdk5/p35\textsuperscript{Nck5a} complex. The binding of NF-M has no effect on the kinase activity of Cdk5/p35\textsuperscript{Nck5a}.

Neuronal Cdc2-like kinase (Nckl)\textsuperscript{1} was originally purified from bovine brains on the basis of its phosphorylating activity toward serine or threonine within the sequence motif Ser/Thr-Pro-X-Lys/Arg (X for any amino acid) (1). In an independent study, Nckl was also isolated as a tau protein kinase since it catalyzed in vitro phosphorylation of tau, which is a brain microtubule-associated protein (2, 3). Purified Nckl is a heterodimer of Cdk5 and a 25-kDa regulatory subunit (p25), which is derived proteolytically from a brain and neuron-specific 35-kDa protein (p35) (4–6). In view of its specific Cdk5 activating activity, p25/p35 is called neuronal Cdk5 activator (Nck5a). In addition, mammalian brains also contain a neuron-specific 39-kDa isoform of Nck5a, called neuronal Cdk5 activator isoform (Nck5ai) (7). Nck5a and Nck5ai display a high degree of protein sequence identity, but share little sequence homology with members of the cyclin family, thus suggesting that they represent a novel family of Cdk-activating proteins.

Along with its unique structure, Nck5a is endowed with unique regulatory properties distinct from those of cyclins. While the activation of Cdc2 by cyclin depends on phosphorylation of Cdc2 by a Cdk-activating kinase at a specific threonine residue (Thr-161), the activation of Cdk5 by Nck5a is independent of Cdk5 phosphorylation (8–11). Also, several Cdc2-like kinases have been shown to be inactivated readily upon tyrosine phosphorylation by a Wee1 kinase (8, 9). However, Cdk5/Nck5a is only sluggishly phosphorylated and inactivated by Wee1 (12, 13). In addition, Cdk-cyclin complexes are susceptible to inhibitory activities of specific inhibitory proteins, such as p21\textsuperscript{cip1/Waf1} and p27\textsuperscript{kip1} (8, 9, 14). In contrast, the kinase activity of Nckl is resistant to these proteins (15, 16).

Developmental, cell biological, and transgenic mouse studies have implicated Nckl as an important regulator in development of the central nervous system in mammals and in normal functions of terminally differentiated neurons (5–7, 17–23). The apparent involvement of Nckl in neuronal differentiation and brain functions may be attributed to the roles of this enzyme in neurocytoskeletal dynamics. Among in vitro Nckl substrates are neuron-specific cytoskeletal proteins such as tau and neurofilaments (3, 24–27). In Alzheimer patients, tau from neurofilibrillary tangles is hyperphosphorylated on many proline-directed Ser/Thr sites (28). A number of studies have suggested that Nckl is a major kinase involved in catalyzing the proline-directed tau phosphorylation (2, 29). Mammalian neurofilaments consist of three protein components, neurofilament high (NF-H), medium (NF-M), and low (NF-L) molecular weight subunits, which are assembled into neurofilaments and transported down axons as discrete cytological structures (30–32). During this transport, NF-H and NF-M are heavily phosphorylated on their tail domains at many proline-directed Ser/Thr sites (32). In Alzheimer patients, tau from neurofilibrillary tangles is hyperphosphorylated on many proline-directed Ser/Thr sites (32). Cdk5 has been suggested to participate in this axonal transport-dependent phosphorylation of the neurofilament proteins (24–27). This suggestion is supported by the observation that, in cultured neuronal cells, both Cdk5 and Nck5a are present throughout the axons where neurofilaments represent the bulk of the cytoskeletal proteins (18). In the present study, we show that Nck5a and Nckl associate specifically with the neurofilament proteins, an observation that may be the molecular basis of the colocalization and possible co-transportation of Nck5a and neurofilaments in the axons. Our characterization of the interaction between Nck5a and neuro-
filaments is compatible with the notion that, in addition to kinase activation, Nck5a functions in anchoring Cdk5 to its kinase substrates.

**EXPERIMENTAL PROCEDURES**

**Library Screening with the Yeast Two-hybrid System**—The bovine p35\textsubscript{5-255}-coding sequence was amplified by PCR with the primers of NCK-1 (5'-CCAGCAGTGGGACCTCGTCGTCG-3') and NCK-2 (5'-CTGGGATCTTGAGCGTTCGCGGACG-3'). The PCR fragment was inserted into the GAL4 DNA binding domain plasmid p528 at NcoI and BamHI, resulting in a bait plasmid p528\textsubscript{nck5a/pQE12}. The prey is a human brain cDNA library in the GL4C transcription activation domain plasmid pACT2 (CLONTech Laboratories, Inc.). The bait and prey were sequentially transformed into yeast CG1945 (MATa, ura3-52, his3-200, lys2-801, trp1-901, ade2-101, leu2-3, 112, gal4-542, gal80-538, lys2::GAL1-HIS3, cy2, URA3::GAL1 17-mer-ATG) and pACT2-1-2. The cotransformants harboring two interacting proteins were selected on a synthetic dextrose medium lacking leucine, tryptophan, and histidine, and transformants harboring two interacting proteins were selected on a synthetic dextrose medium lacking leucine, tryptophan, and histidine, and buffer B (10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM dithiothreitol, and 0.2 mM PMSF). The incubation was continued with shaking for 1 h at 4 °C. The Sepharose beads were then collected and washed extensively with the washing buffer. Bound proteins were dissolved in SDS-PAGE sample buffer and subjected to SDS-PAGE. Alternatively, the bound proteins were eluted with GSH buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, and 20 mM GSH), and subjected to protein phosphorylation reactions.

**Protein Phosphorylation Reactions**—The Cdk5 kinase activity was measured by phosphorylation of the histone H1 peptide HS(9–18) as described previously (10). Phosphorylation of NF-M fragments was carried out at 30 °C in a mixture containing 20 mM MOPS at pH 7.5, 5 mM MgCl\textsubscript{2}, 100 μM γ\textsuperscript{32}P-ATP (5000 dpm/pmol), 1 mM dithiothreitol, and indicated protein samples. The reactions were terminated by addition of SDS-PAGE sample buffer. The samples were boiled for 5 min before loading on gels. Protein phosphorylation was visualized by autoradiography.

**RESULTS**

In a previous study, we showed that Nck5a existed in bovine brain extracts either as a 25-kDa fragment in a heterodimeric complex of Cdk5/p35\textsubscript{5-255}, or as the full-length protein (p35\textsubscript{5-255}) in a macromolecular complex of over 600 kDa, which also contained Cdk5 as a constituent protein (38). This observation has led to the suggestion that p35\textsubscript{5-255} has the ability to undergo high affinity association with certain cellular proteins. The yeast two-hybrid system was therefore employed to screen a human brain cDNA library to identify the putative p35\textsubscript{5-255}-binding proteins. About 3.5 × 10\textsuperscript{3} cotransformants were analyzed further. A total of 108 positive clones were isolated and introduced into another yeast strain to test binding specificities. 14 clones from the primary isolates displayed specific interaction with the original bait p35\textsubscript{5-255}/pAS2. Upon sequence analysis, these 14 clones were revealed to originate from seven different cDNA sequences. One of the isolated sequences, which represents three individual clones, encodes a carboxyl-terminal fragment of glial fibrillary acidic protein (GFAP).

Since Nck5a is a neuron-specific protein, whereas the expression of GFAP is restricted to glial cells, it is unlikely that these two proteins associate in brains under normal physiological conditions. However, the association of Nck5a with GFAP may reflect the ability of Nck5a to undergo association with certain neuronal proteins containing protein sequences similar to that of the Nck5a-binding region of GFAP. GFAP is a member of the intermediate filament family (30). All intermediate filaments share the basic architecture of a conserved central rod domain flanked by variable head and tail domains (30). The isolated Nck5a-binding fragment consists of a carboxyl-terminal portion of the rod domain and the entire tail region of GFAP. Among the intermediate filament family members are the neuron-specific intermediate filaments, NF-L, NF-M and NF-H (30–32). NF-M and NF-H have been suggested as in vivo substrates of Cdk5 (3, 24–27). Sequence alignment displays high degrees of protein sequence identity between the Nck5a-binding region of GFAP and the corresponding rod regions in neurofilaments (Fig. 1), suggesting that Nck5a may bind neurofilaments in cells.
To test whether neurofilaments are indeed capable of association with Nck5a, a fragment of NF-M, NF-M346–467, corresponding to the Nck5a-binding fragment of GFAP, was engineered into the plasmid pACT2 for the two-hybrid assay. As shown in Fig. 2, the reporter activity was detected when NF-M346–467/pACT2 was cotransformed with p35nck5a/pAS2 into yeast cells, whereas cotransformation of a control plasmid Nck5a and p25Figs. 2 and 3 show that NF-M interacts with both full-length their abilities to bind NF-M in the yeast two-hybrid system. To determine whether the interaction between NF-M and Nck5a, this protein-protein interaction was carried out and characterized in vitro using bacterially expressed proteins. GST-fusion proteins of two NF-M carboxyl-terminal fragments: NF-M346–915 and NF-M411–915 were expressed and in isolated from bacteria (Figs. 5, A and B). The results from the yeast two-hybrid assay suggested that NF-M346–915 contains the Nck5a-binding site, whereas NF-M411–915 does not. To demonstrate protein associations, the GST-NF-M proteins were incubated with (His)6-tagged p35nck5a and then affinity-purified using GSH beads. The precipitates were then analyzed on an immunoblot using an anti-Nck5a antibody to determine whether Nck5a had co-precipitated with the NF-M fragments. Fig. 5C shows that the bacteria-expressed and affinity-purified p35nck5a-His contains intact p35nck5a and two partially proteolyzed fragments showing immunoreactive protein bands of approximately 30 and 28 kDa. The p35nck5a-His proteins were present in the precipitate of GST-NF-M346–915 but not in the precipitates of GST and GST-NF-M411–915 (Fig. 5C). It supports the results from the yeast two-hybrid analysis suggesting that Nck5a undergoes specific association with NF-M and that the Nck5a-binding site is localized at the carboxyl-terminal region of the NF-M rod domain.

To further characterize the interaction between NF-M and Nck5a, the interaction was examined with the heterodimer of Cdk5/p25nck5a, which is the active form of Nck5 in existing in bovine brain extracts. Nck5 was prepared by reconstitution from bacterially expressed Cdk5 and p25nck5a as described previously (10). Samples of Nck5 were incubated with GST-NF-M346–915, GST-NF-M411–915, and GST separately. The GST fusion proteins were then affinity-purified using GSH beads. After being washed extensively, the precipitates were subjected to immunoblot analysis using antibodies against Cdk5 and Nck5a. As shown in Fig. 6, both Cdk5 and Nck5a could be detected in the precipitated sample containing GST-NF-M346–915 but not in those of GST-NF-M411–915 and GST. The binding of Nck5 and NF-M346–915 is essentially quantitative under the experimental conditions. The results indicate that NF-M binds both Nck5a monomer and the Cdk5/Nck5a heterodimer, and the same site in NF-M may be involved in the binding of the two protein species.

To further address the question of potential physiological significance of the interaction between NF-M and Nck5a, we examined any effect on the Cdk5-activating activity of Nck5a by interaction with NF-M. In one experiment, the activation of Cdk5 by a suboptimal amount of p25nck5a was determined in the presence of NF-M346–915. It was found that NF-M346–915

**Fig. 1.** Sequence alignment of the GFAP Nck5a-binding fragment and corresponding regions in neurofilaments. The amino acid sequences are human GFAP and neurofilament proteins. Vertical lines and dots indicate identical and homologous amino acid residues, respectively.
over a large concentration range, could neither enhance nor impede the Cdk5-activating activity of Nck5a (Fig. 7A). All three proteins used in the experiment of Fig. 7A were bacterially expressed GST fusion proteins. However, similar results were obtained if the proteins were pretreated by thrombin to remove the GST moiety (data not shown). To ensure that the kinase activity measured was indeed that of NF-M-bound Nclk, samples of reconstituted Cdk5/p25nck5a were incubated with varying amounts of GST-NF-M346–915. GST-NF-M346–915 along with its bound-Nclk was then affinity-precipitated using GSH beads and the Cdk5 kinase activity in the precipitates was then assayed. As shown in Fig. 7B, the Cdk5 kinase activity could be recovered in the precipitates in a NF-M 346–915 concentration-dependent manner. The kinase in the precipitate could also phosphorylate NF-M as shown in Fig. 7C. In a separate experiment, the Cdk5-catalyzed phosphorylation of NF-M346–915 was compared with that of NF-M411–915, and they were about the same (data not shown). NF-M346–915 is capable of binding to Nck5a, but NF-M411–915 is not. Thus, under various conditions, NF-M-bound Nclk and free Nclk appeared to have the same kinase activity.

**DISCUSSION**

It has become increasingly clear that protein-protein interactions play key roles in cellular regulatory mechanisms. The present study shows that neurofilament NF-M undergoes a specific association with Nck5a. The region of NF-M involved in

---

**Fig. 2. Interaction of Nck5a with NF-M fragments in the two-hybrid system.** The recombinant pACT2 plasmids were introduced into yeast Y190 with p35nck5a/pAS2 or lamin C/pAS. Cotransformants were selected on a synthetic dextrose medium without leucine and tryptophan. The colonies were then patched on the medium without leucine, tryptophan, and histidine, but containing 15 mM 3-amino-1,2,4-triazole. The cloned NF-M fragments are schematically shown beside the yeast growth pattern.

**Fig. 3. The two-hybrid assay of interaction between NF-M and Nck5a or Nck5ai.** The pACT2 constructs containing NF-M346–467 or NF-M346–413 were introduced into yeast Y190 with the pAS2 constructs of p351–98, p25nck5a (p3529–307), and p39nck5ai individually. After colonies appeared on synthetic dextrose plates lacking leucine and tryptophan, the cotransformants were patched on the medium without leucine, tryptophan and histidine, but containing 15 mM 3-amino-1,2,4-triazole.

**Fig. 4. Association of NF-M with Nck5a fragments in the two-hybrid system.** NF-M346–467/pACT2 and NF-M346–413/pACT2 were transformed into yeast Y190 with the pAS2 constructs containing various truncated fragments of Nck5a. Cotransformants were selected on synthetic dextrose plates lacking leucine and tryptophan. After colonies appeared, β-galactosidase activity was tested by a filter lift assay using X-gal. Plus (+) means that the colonies turned blue, and minus (−) means that the colonies remained white in the assay of β-galactosidase activity.
Association of NF-M and Nck5a

The Nck5a binding is conserved in NF-H and NF-L, suggesting that these neurofilament proteins are also capable of associating with Nck5a. Several lines of evidence implicate that NF-M and NF-H are in vivo substrates of Nck5. For example, NF-M and NF-H are heavily phosphorylated in neurons on their carboxyl-terminal regions at proline-directed Ser/Thr residues. Many of these in vivo phosphorylation sites can be phosphorylated in vitro by reconstituted reactions containing purified and dephosphorylated neurofilament proteins and Nck5 (3, 24, 25).

Cotransfection of SW13cl2Vim cells, which are lacking cytoplasmic intermediate filaments, with Cdk5, Nck5a, and NF-H generated heavily phosphorylated NF-H in the cells, whereas transfection of NF-H with Cdk5, Nck5a, and a dominant negative mutant of Cdk5 failed to phosphorylate NF-H (25). Glycogen synthase kinase 3β is another proline-directed Ser/Thr protein kinase that has been suggested to catalyze tau phosphorylation in brains (40). When NF-H was introduced into SW13cl2Vim cells with glycogen synthase kinase 3β, there was no significant extent of NF-H phosphorylation observed (25). The interaction between Nck5a and the NF subunits suggests that Nck5a, in addition to being an activator of Cdk5, serves as an anchoring protein to localize Cdk5 to its protein substrates. This may provide a molecular mechanism for the colocalization of neurofilaments and Nck5.

While the use of anchoring proteins to localize an enzyme to its specific site of action may no longer be considered as a novel observation, the highly asymmetrical morphology of neurons and the unique distribution and movement of neurofilaments in neurons have rendered the need for such an anchoring function in the neurofilament phosphorylation system especially obvious and important. Neurofilament proteins are synthesized in the neuronal cell body and assembled at the entrance of the axon. Immediately after the assembly, neurofilaments are transported toward the distal region of the axon along with other cytoskeletal proteins as a discrete structure. It is during this axonal transport, that the carboxyl-terminal tails of NF-H and NF-M become phosphorylated. Both Cdk5 and Nck5a are present in and throughout the axon in cultured mammalian embryonic neurons (18). The interaction between Nck5a and neurofilaments appears to be the molecular basis of the axonal localization of Nck5a and Cdk5. Furthermore, the association of Nck5 to neurofilaments suggests that Nck5 may be transported together with neurofilaments in the axon.

Several features of the interaction between Nck5a and neurofilaments are compatible with such a suggestion. The binding of NF-M to Nck5a is localized proximal to the amino-terminal junction of the Cdk5-activating domain of Nck5a. As a result, the binding of NF-M has no effect on the Cdk5-activating activity of Nck5a. In addition, the Nck5a-binding site on NF-M borders on the amino terminus of the tail region where the putative Cdk5 phosphorylation sites are located. It seems to be strategically localized to maximize the Cdk5 activity as a neurofilament kinase. Indeed, the phosphorylation of NF-M by the carboxyl-terminal region at proline-directed Ser/Thr residues is another potential site of action for Cdk5, as the phosphorylation at this site may alter the structure or stability of the neurofilament protein, thereby affecting its function in the axon.
While significant advances have been made in elucidation of the general regulatory properties of the Cdk family, the regulation of Cdk5 appears to be governed by distinct mechanisms which are poorly understood. Cdk5 activation by Nck5a does not require Cdk-activating kinase and Cdk5 is not phosphorylated in highly active Nck5a (10). Also, Cdk5/Nck5a shows significant resistance to the Weel kinase and p21^{cip1/WAF1} and p27^{kip1}, which are common Cdk inhibitory kinase and inhibitory proteins, respectively (12, 13, 15, 16). On the other hand, phosphorylation of the neurofilament proteins during axonal transport is temporally regulated, since the extent of the protein phosphorylation increases gradually during the transport. Knowledge of how the neurofilament kinase is regulated in this process awaits elucidation of the regulatory properties of Cdk5 and Nck5a. Perhaps some of the other Nck5a-binding proteins identified by the two-hybrid screening procedure are factors that can regulate Nck5a. Most of the Cdk5 protein in bovine brain extracts exists in an inactive state either as monomeric Cdk5 or as a macromolecular complex that also contains the full-length Nck5a (38). Circumstantial evidence in the literature points to the existence in mammalian brains of an inhibitory protein factor(s) and an inhibitory protein kinase(s) for Cdk5 (38, 41).

Protein-protein interactions detected in the yeast two-hybrid system do not necessarily mean that the interactions occur in vivo. However, even a nonphysiological interaction may provide insight into physiologically relevant reactions. The initial interaction detected in this study was between Nck5a and a glial-specific protein GFAP, and neurofilaments were not in the isolated seven sequences encoding p35^{nck5a}-binding proteins. The distinct localizations of GFAP and Nck5a in brains have ruled out this protein-protein association as being physiologically significant. However, this interaction has led to the identification of closely related NF-M as a Nck5a-binding protein. The interaction between Nck5a and neurofilaments has obvious physiological significance. Elucidation of the molecular basis of this protein-protein interaction may shed important light on the mechanism of Nck action and function in neurons.

Acknowledgments—We thank Dr. C. J. Brandl (University of Western Ontario) for his valuable advice and help with the yeast two-hybrid system. We are also grateful to Dr. M. Treuner for discussion throughout the work and reading the manuscript, and F. Hwang for technical assistance.

REFERENCES


FIG. 7. Effect of the NF-M binding on the kinase activity of Cdk5/Nck5a. A, effect of GST-NF-M^{346–915} on Cdk5 activation by p25^{nck5a}. 0.6 µg of GST-Cdk5 was mixed with 0.1 µg of GST-p25^{nck5a} and different amounts of GST-NF-M^{346–915} as indicated. After incubation at 30 °C for 30 min, the Cdk5 kinase activity was measured by phosphorylation of the HS(9–18) peptide at 30 °C for 20 min. B, the kinase activity of NF-M-bound Cdk5/p25^{nck5a}. 7 µl of Cdk5/p25^{nck5a} were incubated with indicated amounts of GST-NF-M^{346–915} at 30 °C for 30 min. The GST fusion proteins were retrieved with GSH-Sepharose 4B, and then released from the beads with the GSH buffer (see “Experimental Procedures”). The Cdk5/p25^{nck5a} activity co-precipitated with GST-NF-M^{346–915} was measured by phosphorylation of the HS(9–18) peptide at 30 °C for 20 min. C, phosphorylation of NF-M^{346–915} by associated Cdk5/p25^{nck5a}. 10 µg of GST-NF-M^{346–915} were incubated without (lane 1) or with (lane 2) 7 µl of Cdk5/p25^{nck5a}. The proteins were then retrieved using the GSH beads and then released with the GSH buffer. The samples were subjected to phosphorylation reactions at 30 °C for 30 min. The reaction was terminated by addition of SDS-PAGE sample buffer. The samples were resolved by SDS-PAGE. Protein phosphorylation was visualized by autoradiography.