Phosphorylation of the High Molecular Weight Neurofilament Protein (NF-H) by Cdk5 and p35*

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The high molecular weight neurofilament protein (NF-H) is highly phosphorylated in the axon. The phosphorylation sites have been identified as KSP (Lys-Ser-Pro) repeats in the tail domain of NF-H. These KSP sequences are present more than 50 times in the NF-H tail, and most of these sites are normally phosphorylated in vivo. These KSP sites can be further divided into two separate consensus sequences, KSPXK and KSXY (where Y is not K). The extensive phosphorylation of NF-H has been proposed to play a critical role in the determination of axonal diameter. Recent studies have shown that Cdk5, a kinase related to the cell cycle-dependent kinase Cdc2, is expressed in the brain and associates with the cytoskeleton. In vitro phosphorylation studies have shown that Cdk5 in conjunction with its activator, p35, is able to phosphorylate histone H1, dephosphorylated NF-H, as well as a synthetic peptide with the repetitive KSP motif. We have cloned the cDNAs for rat Cdk5 and p35 by reverse transcription-polymerase chain reaction and cDNA library screening and studied the phosphorylation of NF-H both in vivo and in vitro. By transient transfection assays, we have shown that NF-H can only be extensively phosphorylated in the presence of both Cdk5 and p35. This phosphorylation can be inhibited by a Cdk5-dominant negative mutant, an observation which further supports that Cdk5 is a kinase that is able to phosphorylate NF-H. By immunoprecipitating Cdk5 and p35 from the transfected cells, we have been able to show that the KSPXK repeats are the preferred phosphorylation sites for Cdk5, while the KSXY repeats are not directly phosphorylated by Cdk5 and p35.

Neurofilaments are 8–10 nm fibrous structures present in all vertebrate axons. In mammals, neurofilaments consist of three protein subunits, known as high (NF-H), middle (NF-M), and low (NF-L) molecular weight neurofilament proteins (1–4). These three proteins belong to the intermediate filament protein family and are classified along with α-internexin as type IV intermediate filaments. Like all other intermediate filament proteins, neurofilament proteins contain an amino-terminal head domain, a central α-helical domain of ~310 amino acids, and a carboxyl-terminal tail domain of variable length. The tail domains increase in length with increasing size of the neurofilament proteins and are extensively phosphorylated in NF-M and NF-H (5, 6). The consensus sequence for phosphorylation has been identified as Lys-Ser-Pro (KSP) (7–9), which can be further divided into two separate consensus sequences, KSPXK and KSXY (where Y is not K). There are about 50 potential phosphorylation sites in the rat, mouse, and human NF-H carboxyl-terminal tail domains (10), although the relative numbers of KSPXK and KSXY repeats differ markedly among different species. Phosphate determinations have shown that most of these sites are phosphorylated in vivo (8, 11, 12). Rat NF-M contains only 5 KSP sites (13), and human NF-M has 12 KSP sites (14). Antibodies have been raised which can distinguish phosphorylated and nonphosphorylated KSP epitopes (15). Several studies have shown that after extensive dephosphorylation by alkaline phosphatase, NF-H migrates more rapidly on SDS-PAGE (16). This characteristic mobility shift upon phosphorylation of NF-H and the antibodies specific for the phosphorylated and nonphosphorylated KSP epitopes are therefore useful tools for studying its phosphorylation.

The functional role of neurofilament phosphorylation is still not completely clear. Early studies indicated that NF-H might function as a cross-linking protein whose phosphorylation was thought to be important for the formation of cross-linking bridges between neurofilaments (17, 18). However, a later study showed that nonphosphorylated NF-H was also situated between intermediate filaments (19). More recently it has been suggested that the phosphate groups of NF-H may result in electrostatic repulsion, which in turn could increase axonal diameter (20, 21). Phosphorylation of NF-H has also been correlated with its ability to interact with microtubules (22).

The kinase(s) which phosphorylate the KSP sequences on NF-H are still not completely identified. A number of different protein kinases, including casein kinase (23), cyclic AMP-dependent protein kinase (24), Ca2+-calmodulin-dependent protein kinase (25), and protein kinase C (26) are able to phosphorylate the neurofilament proteins. However, NF-M is a better substrate than NF-H for all these protein kinases, and the characteristic mobility shift on SDS-PAGE due to phosphorylation of NF-H is not observed. Other studies have revealed protein kinases which are associated with neurofilaments, including a protein kinase activity from bovine spinal cord neurofilament-enriched preparations (27) and a 115-kDa neurofilament-associated kinase isolated by affinity chromatography using bacterially produced NF-H (28). These protein kinases...
also only phosphorylate NF-H in a limited manner and there is no evidence that they phosphorylate NF-H at the KSP sites.

Recent results have pointed to the family of cyclin-dependent protein kinases (Cdks) as candidate kinases for the KSP sites on NF-H. The cell cycle kinase Cdk2 was shown to phosphorylate neurofilaments (29, 30) and cause the characteristic gel mobility shift of NF-H upon phosphorylation (30). However, these results do not have much physiological relevance, since it is known that Cdk2 kinase is absent in terminally differentiated neurons. By using the polymerase chain reaction (PCR) with degenerative oligonucleotide primers corresponding to conserved regions of the Cdk5, a family of novel Cdk2-related cDNA clones have been isolated (31). Among them, PSSALRE is expressed in neuronal cell lines and brain, as well as other cell lines and organs (31). This same kinase was also separately identified by another laboratory, shown to be able to bind cyclin D1 (32) and renamed as Cdk5 (cyclin-dependent kinase 5). A number of recent studies have shown that Cdk5 is associated with the cytoskeleton and able to phosphorylate NF-H in vitro. (33–35). An additional protein with a molecular mass of 23–25 kDa was observed in most of the Cdk5 preparations. cDNA cloning showed that this 23–25-kDa protein is a degradation product of a larger protein, p35, which acts as a neurally-specific regulatory subunit of Cdk5 (36, 37). p35 associates physically with and activates Cdk5 kinase. Although it has no homology with cyclins, it serves a similar function in modulating the activity of Cdk5.

In this study, we have cloned the cDNAs for both Cdk5 and p35 from rat brain and conducted NF-H phosphorylation studies by transient transfections and immunoprecipitation/kinase assays. We have demonstrated that NF-H can be phosphorylated only in the presence of both Cdk5 and p35. Furthermore, this phosphorylation is specific for the KSP sites.
and the supernatants were mixed with 1 μg of antibody for 2 h at 4°C. 20 μl of Protein G Plus-Agarose (Santa Cruz Biotech) was added, and the mixtures were subsequently incubated for 2 h at 4 °C. The immune complexes were pelleted by centrifugation at 14,000 × g and washed with cold lysis buffer four times. The kinase assays were initiated by mixing kinase buffer containing 50 mM Hepes, pH 7.4, 5 mM MgCl₂, 5 mM MnCl₂, 1 mM dithiothreitol, 1 mM EDTA, 100 mM NaCl, 1 μCi of [γ-³²P]ATP (Amersham), and 2 mg of substrate with the final immune complex pellet. The reaction mixtures were then incubated in a 30°C water bath for 30 min and centrifuged at 14,000 × g for 10 min. The supernatants were saved and the pellets were washed 3 times. An equal amount of 2 × sample buffer (43) were added to the supernatants and pellets, and the samples were boiled for 10 min before SDS-PAGE.

RESULTS

Cloning of Rat Cdk5, p35, and GSK3β cDNAs—In order to study Cdk5 and its activator, p35, we first isolated their cDNAs by RT-PCR. To obtain the Cdk5 cDNA, reverse transcription was carried out using rat brain mRNA as template, and the resulting RT-PCR product was amplified by two primers complementary to the 5’ and 3’ ends of the published Cdk5 cDNA sequence (45). This PCR product was subcloned into the pGEM-5 vector, its sequence was determined and confirmed to be identical with the Cdk5 sequence from GenBankTM

The full-length rat p35 cDNA was obtained by first isolating a cDNA corresponding to the partial sequence of bovine p35 (p23) by RT-PCR using bovine brain mRNA as template (46) and screening a rat brain cDNA library using the bovine p23 cDNA as a probe. The resulting p35 cDNA was subcloned into pGEM-5 and sequenced. The deduced amino acid sequence of rat p35 is nearly identical with those of human and bovine p35 (Fig. 1). There are only four amino acid differences between rat and human p35 (36) or between rat and bovine p35 (37).

The Cdk5 dominant-negative (Cdk5dn) construct was generated by PCR and subcloned into the expression vectors described under “Materials and Methods.” This dominant-negative Cdk5dn contains a point mutation resulting in a single amino acid residue change (Asp144 → Asn) in the kinase domain of Cdk5. This mutant form has previously been shown to result in an inactive kinase, which competes with the activator protein p35 and thereby inhibits Cdk5 kinase activity (40).

A full-length cDNA for glycogen synthase kinase 3β (GSK3β) was also generated by RT-PCR using primers based on the published sequence (61). The resulting GSK3β cDNA was subcloned into pGEM-5 and sequenced. The sequence was found to be identical with the GSK3β sequence obtained from GenBankTM.

NF-H, “KSPXY,” and “KSPXK” Constructs—Rat NF-H con-
Amino acid sequence of constructs containing the NF-H KSPXY and KSPXX repeats. The KSPXY and KSPXX repeats are clustered in the tail domain of NF-H. A, the sequence for the "KSPXY" protein corresponds to amino acids 508–763 of the published rat NF-H sequence (10). The resulting "KSPXY" protein contains 41 KSP repeats and no KSPXX sequences. Note that this protein starts in the middle of the only KSPXX sequence in the rat NF-H tail located before this cluster of KSPXY repeats. B, the "KSPXX" protein corresponds to amino acids 769–1072 of rat NF-H. This construct has 7 KSP repeats (and 2 KSPXY repeats) clustered in the tail domain of NF-H.

Phosphorylation of NF-H by Cdk5 and p35

Cdk5 is expressed in a number of cell lines and tissues, but its activity has been shown to be present only in brain (36, 37). Consistent with these reports, our Northern blot analysis showed that the SW13d2Vim− cell line does not have any p35 mRNA (data not shown). These results indicate that the SW13d2Vim− cell line is a good system to study the phosphorylation of NF-H by Cdk5 and its activator, p35.

For every transient transfection experiment, 20 μg of each plasmid was used, and the resulting cell lysates were analyzed by Western blots using antibodies SM136 and SM132. SM136 is specific for the phosphorylated KSP epitope on NF-H, while SM132 recognizes this epitope in the nonphosphorylated form (15). Purified NF-H from rat spinal cord shows an apparent molecular mass of approximately 200 kDa on SDS-PAGE. After alkaline phosphatase treatment, the dephosphorylated NF-H migrates faster on SDS-PAGE (5, 49). We used this mobility shift on SDS-PAGE and the immunoreactivity of NF-H with the twophosphorylation state-dependent antibodies as the criteria to determine whether or not the transfected NF-H was phosphorylated.

As shown in Fig. 3, when NF-H is transfected by itself (lane 2) or co-transfected with Cdk5 (lane 3) into SW13d2Vim− cells, the exogenously expressed NF-H protein is detected only by SM132 and does not show the mobility shift characteristic of the phosphorylated NF-H, indicating the absence of any significant amount of NF-H phosphorylation (Fig. 3, A and B). However, when both Cdk5 and p35 are co-transfected along with NF-H (Fig. 3, lane 6), the expressed NF-H shows an apparent molecular mass of 200 kDa, comparable to that of the phosphorylated NF-H. Furthermore, the protein is recognized...
Protein kinase Cdk5 activated by the transfected p35 can extensively phosphorylate NF-H in cultured non-neuronal cells. In order to show the levels of Cdk5 and Cdk5dn in the transfected cells, the same Western blots were immunostained with the Cdk5 antibody CD17 (Fig. 3C). The endogenous Cdk5 can be observed along with the transfected Cdk5 or Cdk5dn. The mutant can be distinguished readily from the endogenous Cdk5, because its mobility is different from the endogenous Cdk5 due to the presence of a hemagglutinin (HA) tag (Fig. 3C).

From the intensity of the Cdk5 band, it is obvious that the level of the exogenous Cdk5 is much higher than the endogenous Cdk5.

Like Cdk5, GSK3β has been shown to phosphorylate SP sites on the microtubule-associated protein, τ, and to associate with the cytoskeleton. Recent transfection studies have shown that overexpression of GSK3β and τ in non-neuronal cells resulted in τ phosphorylation on SP sites, which have been associated with the abnormal phosphorylation of τ in Alzheimer’s disease. To determine if NF-H could serve as a substrate for GSK3β, we co-transfected GSK3β and NF-H in the SW13 cells (Fig. 3, lane 8). The results show that no significant amount of SM136-reactive NF-H can be observed in the cells transfected with GSK3β (Fig. 3A) and there is also no apparent shift observed with SM132 (Fig. 3B). These experiments indicate that NF-H phosphorylation is not induced to a significant extent by the overexpression of GSK3β.

Immunoprecipitation experiments show that Cdk5 is able to phosphorylate only the KSPXK sequence on NF-H—it has been noted that NF-H has two different consensus phosphorylation KSP sequences, KSPXX and KSPXY. In an effort to determine which one of these sequences is the phosphorylation site for Cdk5 and to show that the results in Fig. 3 are not due to another kinase activated by Cdk5/p35, we performed immunoprecipitation experiments followed by kinase assays. Immunoprecipitations were conducted by using the Cdk5 polyclonal antibody C-8 on protein extracts from cells, which had been transiently transfected with various combinations of Cdk5, p35, and Cdk5dn constructs. After the immunoprecipitated Cdk5 complexes were immobilized on Protein G beads, kinase buffers containing the different substrates, histone H1, NF-H, “KSPXK,” and “KSPXK” proteins were mixed with the beads. The mixtures were incubated in the presence of [γ-32P]ATP and separated on SDS-PAGE, and the gel was subsequently exposed to x-ray film.

Purified, bacterially expressed NF-H, “KSPXK,” and “KSPXK” proteins were not phosphorylated as determined by Western blot analysis (data not shown). Histone H1 was used as a positive control in these experiments, since it is a common kinase substrate for most Cdks and has previously been used for Cdk5 in vitro assays. Immunoprecipitates of the Cdk5 and p35 co-transfections yielded the most dramatic phosphorylation of histone H1, NF-H, and the “KSPXK” proteins (Fig. 4, A, B, and C, lane 5). There was no phosphorylation of the “KSPXK” protein by Cdk5 and p35 under the same conditions used for the other substrates (Fig. 4D). Cdk5 or Cdk5dn alone could not phosphorylate any of the substrates (Fig. 4, lanes 2 and 3). A long exposure of the autoradiograms showed that some phosphate incorporation on histone H1, NF-H, and “KSPXK” protein occurred in the immunoprecipitation kinase reaction from the p35-transfected cells (Fig. 4, lane 4). Thus, from the in vitro kinase assay results, we conclude that Cdk5 in conjunction with p35 is able to phosphorylate NF-H, and its phosphorylation site is on the KSPXK sequence in the NF-H tail. Furthermore, these results are consistent with the in vivo phosphorylation data obtained from the transfection experiments (Fig. 3).

DISCUSSION

Neurofilament phosphorylation has been postulated to play an important role in maintaining axonal caliber (1). The highly phosphorylated KSP XL sequence in the NF-H tail appears to be critical in maintaining the integrity and stability of the nerve fiber (1). A number of other phosphorylation sites, including some SP sites, have also been shown to play important roles in axon caliber (1). The results presented here indicate that Cdk5 and p35 are capable of phosphorylating the NF-H tail, and that the phosphorylation of some of these sites is significant enough to have an observable effect on the stability of the NF-H scaffold.
phosphorylated carboxyl-terminal tail domains of NF-H and NF-M are unique among intermediate filaments and are presumed to mediate neuron-specific functions, including maintaining the shape of the axon, which presumably requires a specialized cytoskeleton. The kinase(s) responsible for the phosphorylation of the NF-H tail domain have not been definitively identified. Several lines of evidence have pointed to Cdk5 as a candidate kinase for the phosphorylation of NF-H. Cdk5 is a kinase which was originally identified by homology to the cyclin-dependent kinase, Cdc2 (31). Several other reports identified a protein kinase activity in the central nervous system, which was co-isolated with the cytoskeleton and upon further characterization turned out to be Cdk5 (45, 50, 51). In a number of these studies, NF-H or a synthetic peptide containing the KSP repeats was used as the substrate for these kinase assays. In addition, the microtubule-associated protein, τ, was shown to be a substrate for Cdk5 (52). It was noted from early studies that Cdk5 and its mRNA are expressed in a number of cell lines and tissues. However, immunoprecipitation experiments showed that Cdk5 kinase activity was only detected in brain (48) suggesting the presence of a brain-specific activator. Two different activator proteins have been proposed for Cdk5. One of these, p35, is only expressed in brain (36, 37), and phosphorylation of histone H1 by Cdk5 is observed when p35 and Cdk5 are co-transfected into non-neuronal cells. In vitro kinase assays using bacterially produced Cdk5 and p35 also showed that p35 is able to activate Cdk5 to phosphorylate histone H1 (36). A second putative activator, p67, has been identified in purified kinase preparations containing Cdk5 and shown to activate bacterially expressed Cdk5 in in vitro kinase experiments (53). p67 is identical to nsec-1 (rbsec1 or munc-18) and plays a role in exocytosis (54), but has not been reported to be a kinase activator. In this report, we have focused our attention on the first of these activators, p35.

In order to show that Cdk5 is present in the presence of p35 is able to phosphorylate NF-H in vivo, we performed transient transfections with various combinations of the Cdk5, p35, and NF-H expression constructs into the SW13 cell line. We have previously shown that NF-H is not significantly phosphorylated when transfected in fibroblasts. In the present study, we have also determined that in SW13 cells, NF-H remains predominantly in the nonphosphorylated state (Fig. 3B). Although Cdk5 is expressed in untransfected SW13 cells, the endogenous Cdk5 does not show any kinase activity as determined by the lack of phosphorylation of NF-H or histone H1, a commonly used substrate for cyclin-dependent kinases (Fig. 4A). By Northern blot analysis, we have determined that p35 mRNA is not expressed in SW13 cells, a result consistent with the observed lack of kinase activity from the endogenous Cdk5.

Several lines of evidence presented in this study show that Cdk5, in conjunction with p35, is capable of phosphorylating NF-H in vivo. Co-transfections of Cdk5, p35, and NF-H resulted in highly phosphorylated NF-H as shown by the significant mobility shift of NF-H on SDS-PAGE (Fig. 3A). In addition to the mobility shift, NF-H, which has been co-transfected with Cdk5 and p35 is readily recognized by an antibody specific for the phosphorylated form of NF-H, but not by an antibody to the nonphosphorylated form of NF-H. Since it is possible that the phosphorylation of NF-H is not directly by Cdk5, but rather by a kinase, which is in turn activated by Cdk5, we performed the immunoprecipitation experiments. These experiments show that immunoprecipitated Cdk5 can phosphorylate NF-H provided p35 is present (Fig. 4). These experiments also show that of the two possible consensus sequences for NF-H phosphorylation, Cdk5 is able to phosphorylate only one sequence (KSP) and not the other (KSPX). Another line of evidence for the identification of NF-H as a substrate for Cdk5 comes from the experiment with the dominant-negative Cdk5dn, which can compete with Cdk5 and reduce its kinase activity on NF-H.

The data presented in this paper, as well as data from others using synthetic peptides, have demonstrated that the KSPX repeats on NF-H are the preferred sites of phosphorylation by Cdk5. The lack of reactivity of SM132 against exogenously expressed NF-H from cells co-transfected with Cdk5 and p35 is therefore somewhat surprising, since the KSPX motif accounts for only ~20% of the KSP sites of rat NF-H and might indicate that the SM132 antibody is specific for this motif in the nonphosphorylated form. Alternatively, additional phosphorylation of NF-H on the KSPXY sites by Cdk5 (or by a different kinase) could occur only after prior phosphorylation of the KSPX sites by Cdk5. It is therefore interesting to note that recent studies have shown that in vitro phosphorylation of recombinant τ by Cdk5 and by glycogen synthase kinase 3β (GSK3β) resulted in the phosphorylation of some of the same sites as τ from Alzheimer’s disease-paired helical filaments (57, 58). This abnormal phosphorylation of τ has also been observed in cultured cells in which GSK3β and τ were overexpressed by transient transfections (59). In vitro experiments have shown that τ becomes a better substrate for GSK3β after it is first phosphorylated by Cdk5 (60). In our experiments, we could not detect any significant level of phosphorylation of NF-H by transient co-transfection with GSK3β (Fig. 3). However, it is possible that in the cells co-transfected with NF-H, Cdk5, and p35 constructs, further phosphorylation of NF-H by the endogenous GSK3β occurred after the KSPX sequences were phosphorylated by Cdk5/p35. It is also interesting to note that although τ is a substrate of Cdk5 it contains a KSPXY sequence, but does not have a KSPX sequence.

Even though Cdk5 is related to the cyclin-dependent kinase family, it has several unique features, such as its expression in terminally differentiated neurons and the fact that its activator, p35, has nearly no homology to cyclins. We have observed no differences in the ability of rat p35 and bovine p23 (which corresponds to the carboxyl-terminal portion of p35) to activate Cdk5 in the co-transfection experiments. These results are consistent with the report showing kinase activity from the bacterially produced Cdk5 and truncated p35 (53) and support the notion that the carboxyl-terminal portion of p35 is sufficient to activate Cdk5. What role, if any, the amino-terminal part of p35 plays in regulating the Cdk5 activation remains an open question. Recently, a p35 isofrom, which is able to activate Cdk5 in vitro, has been isolated from human brain (56). It will be interesting to see whether p35 belongs to a larger protein family and whether Cdk5 or other related kinases can be activated by different members of this family. The restricted expression of p35 to the central nervous system also raises the possibility that in the peripheral nervous system, other protein kinases and/or activators are present which phosphorylate NF-H.

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