Brain Proline-directed Protein Kinase Is a Neurofilament Kinase Which Displays High Sequence Homology to p34*cdck2*

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The carboxyl-terminal regions of neurofilament high (NF-H) and middle (NF-M) molecular weight proteins have been suggested to be phosphorylated in vivo by a p34*cdck2-like protein kinase, on the basis of the in vivo phosphorylation site motif and in vitro phosphorylation of the proteins by p34*cdck2 kinase (Hisanaga, S.I., Kusubata, M., Okumura, E. and Kishimoto, T. (1991) J. Biol. Chem. 266, 21798-21803). A novel proline-directed protein kinase previously identified and purified from bovine brain has been found in this study to phosphorylate NF-H and NF-M at sites identical to those phosphorylated by HeLa cell p34*cdck2 kinase. The proline-directed kinase is composed of a 33-kDa and a 25-kDa subunit. The 33-kDa kinase subunit was partially sequenced, and degenerate oligonucleotide primers corresponding to the amino acid sequence information were used to clone the subunit by polymerase chain reaction (PCR). Two overlapping PCR products comprised a complete open reading frame of 292 amino acids. The sequence contains all features of a protein kinase, suggesting that the 33-kDa peptide represents the catalytic subunit of the kinase. The 33-kDa subunit shows high and approximately equal homology to human p34*cdck2 and human cdk2, with about 56% and 59% amino acid identity, respectively. These results suggest that the brain kinase represents a new category of the cdc2 family, and that some members of the cdc2 kinase family may have major functions unrelated to cell cycle control.

Mammalian neurofilaments are a class of intermediate filaments which are composed mainly of three protein subunits known as high (NF-H), middle (NF-M), and low molecular weight (NF-L) neurofilament proteins. Each of these proteins is organized into three domains, the amino-terminal globulin domain, an α-helical central core, and a carboxy-terminal extension. The long carboxy-terminal extensions of NF-H and NF-M contain repeating units of amino acid sequence and are phosphorylated in vivo (2-4). Neurofilament proteins purified from spinal cord exist mainly in the highly phosphorylated forms. The phosphorylated NF-H and NF-M undergo changes in SDS-PAGE mobility (5) and in their interaction with microtubules (6) upon dephosphorylation by alkaline phosphatase.

Although the importance of the carboxy-terminal phosphorylation of NF-M and NF-H in neurofilament transport and interaction is generally recognized, the protein kinases responsible for the in vivo phosphorylation is not known. Many of the phosphorylation sites of the proteins have been determined to have the motif KSP (7, 8). This suggests that a member of the proline-directed protein kinase family may be the in vivo protein kinase protein. Kinases of this family have a common minimal recognition site specificity of -X-S/P-T-P-X- (9). Among the members of this family is the cell cycle regulatory kinase, p34*cdck2. Recently, p34*cdck2 has been shown to phosphorylate the dephosphorylated NF-H and NF-M, to result in the restoration of the SDS-PAGE mobility and microtubule interactions, which are characteristics of the in vivo phosphorylated NF-H and NF-M (10).

The cdc2 gene of fission yeast (or its equivalent cdc28 in budding yeast) controls cell progression both in G1/S and G2/M transitions (11). The activity of its protein product p34*cdck2 depends on the association with cyclins which are a family of regulatory proteins involved in the stage-specific cell cycle control (12-14). In higher eukaryotes, there are more than one p34*cdck2-like proteomes. Recent studies suggest the existence of a subgroup of S-phase kinases, known as cyclin-dependent kinase 2 (cdk2; also, Eg1, cdc2b (Refs. 15-17)) and another subgroup of M-phase kinases, the p34*cdck2 kinase originally identified. The cDNAs from both subgroups are capable of rescuing a cdc2 yeast mutant suggesting that they both play regulatory functions in the cell division cycle.

Although the studies of p34*cdck2-like kinases have emphasized the roles of these kinases in cell cycle control, the observation that the enzymes phosphorylate neuronal specific proteins suggests additional roles of the kinases. Inconsistent results have been obtained in the attempts of demonstrating p34*cdck2 in adult mammalian brains by immunological criterion (18, 19). Developmental studies have shown that cdc2 mRNA, although present at a high level in proliferating neuronal precursor cells, is not detected in terminally differentiated neurons (20). Recently, we identified and purified a novel proline-directed protein kinase from bovine brain, which is composed of two subunits, a 33- and a 25-kDa subunit (21). The present report describes two main observations about...
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Preparation of Dephosphorylated Neurofilament Proteins—Neurofilaments were prepared from frozen bovine spinal cord tissue following the protocol described by Hisanaga and Hirokawa (6). Spinal cord tissue (15 g) was homogenized in 15 ml of buffer A (0.1 M PIPES, 1 mM EGTA, 1 mM MgCl₂, 0.5 mM DTG, 10 μg/ml leupeptin, 0.5 mM PMPSF, pH 6.8), and the crude homogenate was centrifuged at 55,000 × g for 50 min. The supernatant was concentrated to 15 mg/ml protein, and 800 μl of the concentrated extract was mixed with 60 units of Escherichia coli alkaline phosphatase (Sigma). The sample was dialyzed against 350 ml of buffer B (50 mM Tris-HCl, 150 mM NaCl, 2 mM MgCl₂, 0.5 mM EGTA, 0.1 mM DTG, 0.1 mM leupeptin, and 0.1 mM PMPSF, pH 8.5) at 35 °C overnight. The resultant neurofilament proteins were then subjected to SDS-PAGE mobility shift (6). The dephosphorylated neurofilament proteins were then subjected to two-dimensional PAGE, and the protein that migrated the same distance as neurofilament proteins were excised. The dephosphorylated neurofilament proteins were eluted at the void volume. A control sample was treated similarly, with alkaline phosphatase.

Dephosphorylation of Neurofilaments—5 μg of the peak fraction of the neurofilament proteins from the Sepharose CL-4B column was incubated with 100 μM γ32P[ATP] (11,000 dpm/pmol), 100 μM PIPES, 1 mM MgCl₂, 1 mM EGTA (pH 6.8) for 2 h at 30 °C, with either the brain proline-directed protein kinase or p34cdc2 kinase from mitotic Hela cells. The amount of kinase activity used was in the range of 350-500 pmol/min histone H peptide phosphorylation (21). The proline-directed kinase and p34cdc2 kinase were purified by SDS-PAGE and stained with Ponceau S (1% Ponceau S, 1% acetic acid). Bands corresponding to neurofilament proteins were excised and digested with calf intestine phosphatase (500 units/ml) in 100 mM NaCO₃, pH 7.3, overnight at 37 °C. After 4 h, a second aliquot of trypsin was added and the reaction allowed to proceed overnight. The supernatant was collected and lyophilized. The phosphopeptides were taken up in 20 μl of H₂O and subjected to two-dimensional TLE and TLC analysis. Electrophoresis on 1-2 μl of the sample was carried out at 340 V for 3 h in water-acetic acid/pyridine (89:10:1) at a pH of 3.5. The plates were dried and chromatography was carried out in the second dimension in n-butanol:pyridine:acetic acid:water (40:50:10:49) for 5-6 h. Phosphopeptides were identified by autoradiography.

Cloning—Tryptic peptide fragments of the purified 33-kDa subunit of the bovine brain proline-directed protein kinase were purified by HPLC and three peptides selected for sequencing. The tryptic digestion, HPLC purification of the peptides, and microsequencing of the selected peptides, were carried out by staff at the Harvard Microsequencing Facility. Degenerate oligonucleotide primers derived from the tryptic peptide sequence were synthesized for PCR at the Oligonucleotide Synthesis Facility at the University of California (DykyPyPMY, primer A; DykyPyMPYM, primer B; EQWPMAT, primer C). First strand cDNA was synthesized from poly(A) RNA prepared from bovine brain, using one of the three different oligo(dT) primer anchors: T₅, T₁₀, or K₁₀. 3'-RACE of template DNA using primer C and primer anchor T₁₀ was carried out for 40 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. This amplified DNA was reamplified using primer A or B, together with anchor T₁₀ for 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. A single 3'-RACE fragment (BT) was obtained and directly sequenced by PCR. Second strand cDNA was synthesized from first strand cDNA, which was 5'-polyadenylated, using terminal transferase. This was primed with oligonucleotide primer anchor T₁₀ or T₀, and RNA was digested by alkali and removed by Centricon 10 filtration. 5'-RACE was carried out using anchor T₁₀ or T₀, together with a specific primer (primer D), corresponding to nucleotides 841–860 within the 3'-RACE fragment, for 30 cycles of 1 min at 94 °C, 1 min at 65 °C, and 1 min at 72 °C. The specific 5'-RACE fragment (T,D) was identified by Southern hybridization. The 3'-RACE fragment internally labeled using random hexamer primers, and cloned into pBluescript KS for sequencing. The sequence of the complete coding region was obtained by sequencing at least three individual clones. Nucleotides 513–570 were sequenced directly by PCR, using a primer corresponding to nucleotides 654–651. Nucleotides 1–42 in the 5'-untranslated region are represented by the sequence of only one clone.

RESULTS AND DISCUSSION

Comparison of Neurofilament Protein Phosphorylation by p34cdc2 Kinase and Bovine Brain Proline-directed Protein Kinase—Neurofilament proteins isolated from mammalian spinal cord exist mainly in phosphorylated forms which can be converted to the dephosphorylated forms by E. coli alkaline phosphatase. Although the phosphorylated neurofilament proteins are poor substrates of p34cdc2 kinase, dephosphorylated NF-H and NF-M, but not NF-L, are readily phosphorylated by p34cdc2 (10). Since bovine brain proline-directed protein kinase displayed similar substrate specificity as p34cdc2 kinase toward a number of synthetic peptides and H₁ histone (21), we have examined the possibility that the brain protein kinase may substitute for p34cdc2 in the in vitro phosphorylation of neurofilament proteins. The results indicated that the two protein kinases shared similar characteristics in the neurofilament phosphorylation under various conditions. Both kinases catalyzed the phosphorylation of dephosphorylated NF-M and NF-H, but not NF-L, and both showed little activity toward the phosphorylated forms of the neurofilament proteins.

To define more precisely the similarity of the two protein kinases in their activity toward neurofilament proteins, the sites of NF-H and NF-M phosphorylated by the two kinases were examined. Aliquots of purified and dephosphorylated neurofilament proteins were subjected to phosphorylation by these two protein kinases in the presence of [γ32P]ATP. Tryptic phosphopeptides obtained from the phosphorylated NF-H and NF-M were then analyzed by peptide mapping. Fig. 1 compares the autoradiograms of the two-dimensional TLE/TLC tryptic peptide maps of NF-H and NF-M phosphorylated by the two kinases. For both NF-H and NF-M, the phosphorylated proteins obtained using either of the two kinases have essentially indistinguishable tryptic phosphopeptide maps. The result strongly suggests that the brain proline-directed kinase and p34cdc2 kinase have identical site specificities in their phosphorylation of the dephosphorylated NF-H and NF-M.

Cloning and Sequencing of the 33-kDa Subunit—The brain proline-directed protein kinase was purified to near-homogeneity from bovine whole brain as previously described (21). The subunits of the proteins were separated by SDS-PAGE and transblotted onto nitrocellulose membrane. The 33-kDa subunit of the kinase was subjected to microsequencing at the Harvard Microsequencing Facility. Three tryptic peptides, designated peptides 1, 2, and 3, were selected for sequencing; their amino acid sequences are shown in Fig. 1. These peptides contain sufficient homology to specific sequences of human p34cdc2 to enable the localization of peptide 1 to a region proximal to the amino terminus and peptides 2 and 3 to...
amplified from double-stranded cDNA which was anchored at the 5' end. This fragment was cloned for sequencing, the sequence of the coding region represented by that of at least three individual clones. The 3' end of this PCR product (T3D) contained the exact overlapping nucleotide sequence of the 3'-RACE fragment (BT).

The 5'- and 3'-RACE PCR products comprise an open reading frame of 292 amino acids (876 nucleotides) (Fig. 2). The sequence of all three tryptic peptides is encoded within this contiguous open reading frame, confirming that this sequence represents the 33-kDa subunit of the brain protein kinase. As this subunit is highly homologous to ~34'~''~ and its sequence contains all the hallmarks of a protein kinase sequence (23), it is suggested to be the catalytic subunit of the enzyme. The criteria for assigning the reported sequence to be full-length are: (i) identification of an initiator methionine residue preceded by a stop codon in the same reading frame (this site is equally good as a translation initiation in almost all cdc2 homologs); (ii) identification of a single open-reading frame following the start of translation which predicts a protein related to ~34'~'' at the amino acid level; (iii) the location of a stop codon in the same reading frame allowing translation of a product of 292 amino acids, which closely corresponds to the observed molecular weight of the brain kinase catalytic subunit. During the preparation of this manuscript, a paper by Meyerson et al. (24) describing the cloning of human proline-directed kinase cDNA and the deduced amino acid sequence of peptide 1.

A degenerate oligonucleotide primer (primer C) corresponding to amino acid sequence of peptide 2 was prepared and used for PCR amplification of reverse-transcribed poly(A)+ bovine brain mRNA which was anchored at the 3' end. The PCR products were found to contain several DNA bands. This material was then re-amplified by PCR using one of the two degenerate primers, primer A or B, corresponding to the 5' end of this PCR product (T3D) was sequenced and contained the exact overlapping nucleotide sequence of the 33-kDa subunit of the brain protein kinase cDNA and the deduced amino acid sequence. Shaded region represents the amino acid sequence of the tryptic peptides, determined by chemical protein sequencing. Bars indicate regions to which oligonucleotide primers (a-d) were made for PCR. Primers a, b, and c were used for 3'-RACE; primer d was used for 5'-RACE.

![Fig. 2. Nucleotide sequence of the 33-kDa subunit of the brain proline-directed protein kinase cDNA and the deduced amino acid sequence.](image)

**Fig. 1. Comparison of NF-H and NF-M sites phosphorylated by ~34'~'' or the brain kinase in the presence of [Y-~'P]

ATP for 1 hr.** The phosphorylated NF-H and NF-M were isolated, trypsin-digested and the resulting phosphopeptide analyzed by two-dimensional TLE/TLC and autoradiography as described in detail under "Materials and Methods." A, phosphopeptides of NF-H phosphorylated by ~34'~''; B, phosphopeptides of NF-H phosphorylated by the brain kinase; C, mixture of phosphopeptides of A and B; D, phosphopeptides of NF-M phosphorylated by ~34'~''; E, phosphopeptides of NF-M phosphorylated by the brain kinase; F, mixture of phosphopeptides from D and E.

regions carboxyl-terminal of the protein (Fig. 1).

A degenerate oligonucleotide primer (primer C) corresponding to amino acid sequence of peptide 2 was prepared and used for PCR amplification of reverse-transcribed poly(A)+ bovine brain mRNA which was anchored at the 3' end. The PCR products were found to contain several DNA bands. This material was then re-amplified by PCR using one of the two degenerate primers, primer A or B, corresponding to the amino acid sequence of peptide 3. A single fragment of approximately 450 base pairs was obtained with either primer, designated AT1 or BT1, respectively. The 3'-RACE fragment obtained by using primer B (BT1) was sequenced and shown to contain an open reading frame, predicting a translation product homologous to carboxy-terminal region of human p34'~''~. The 7 amino acids coded by the 5' end of this fragment matched exactly the corresponding amino acid sequence of these residues in peptide 3. Thus, this PCR fragment appears to represent the cDNA for the carboxy-terminal fragment of the 33-kDa subunit of the brain kinase. To deduce the sequence of the rest of the molecule, a primer (primer D) corresponding to nucleotide sequence of a selected region of the 3'-RACE PCR fragment was used for 5'-RACE, and resulted in a 950-base pair PCR product (T3D), being amplified from double-stranded cDNA which was anchored at the 5' end. This fragment was cloned for sequencing, the sequence of the coding region represented by that of at least three individual clones. The 3' end of this PCR product (T3D) contained the exact overlapping nucleotide sequence of the 3'-RACE fragment (BT1).

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of 11 human cdc2 kinase-related genes appeared. These genes were cloned by using degenerate oligonucleotides corresponding to conserved regions of the cdc2 gene via PCR, followed by screening cDNA libraries using the PCR fragments. Seven of the 11 genes are novel, among these is the human homolog (PSSALRE) of the bovine brain proline-directed protein kinase gene cloned in the present study. The deduced amino acid sequence of the human homolog differs from that of the bovine enzyme in only 2 residues.

Sequence Comparison with p34<sup>cdc2</sup>-related Protein Kinases—We have previously used antibodies against synthetic peptides corresponding to an amino terminus, a carboxyl terminus, and the highly conserved PSTAIRE regions of human p34<sup>cdc2</sup> sequence to examine structural similarity of the bovine brain proline-directed protein kinase and p34<sup>cdc2</sup> kinase (21). Of the three antibodies, the amino-terminal antibody and PSTAIRE antibody reacted with the brain protein kinase, the former antibody displaying a much more intense immunostain on Western blot. The carboxyl-terminal antibody showed no activity toward the enzyme (21). This result, along with the substrate specificity characterization has led us to suggest that bovine brain protein kinase and human p34<sup>cdc2</sup> are distinct but related protein kinases. As shown in Fig. 3, the amino acid sequences of the two protein kinases in the amino-terminal and PSTAIRE regions display considerable homology, whereas the sequences of the carboxyl-terminal regions are totally divergent, consistent with the observed differential immunoreactivities of the two kinases toward the three antibodies.

A large number of p34<sup>cdc2</sup>-related protein kinases have been cloned and sequenced. These protein kinases may be divided into two main categories: 1) the authentic homologs of p34<sup>cdc2</sup> kinase from different organisms, and 2) other cdc2-like kinases which are present in addition to cdc2, such as the cyclin-dependent kinase 2 (cdk 2), which appears to be important in G1/S transition (16, 25, 26). Fig. 3 shows the amino acid sequence alignment of the bovine brain kinase with 9 members of the cdc2 kinase-related protein kinase family. The brain kinase cannot be placed in either of the two categories of the cdc2 kinase family. It shows a high and approximately similar degree of sequence homology to both human cdc2 kinase and human cdk2 with amino acid identity of 58 and 59% respectively. In general, amino acid sequences which are highly conserved among the nine members of p34<sup>cdc2</sup> kinase family are also conserved in the brain kinase. p34<sup>cdc2</sup> kinase is known to be regulated by phosphorylation and dephosphorylation mechanisms; the phosphorylation sites have been determined for vertebrate p34<sup>cdc2</sup> to be Thr-14, Tyr-15, Thr-161, and Ser-277 (27). Essentially all these sites are conserved in the brain kinase, except for Thr-161, which in the brain kinase is a serine residue, a conservative substitution.

The observation suggests that, like p34<sup>cdc2</sup>, the brain kinase is regulated by similar protein phosphorylation mechanisms. The conservation of this regulatory property of the brain kinase is in agreement with our previous observation that bovine spleen p56<sup>ncd</sup>, which is capable of phosphorylating p34<sup>cdc2</sup> on Tyr-15, can similarly phosphorylate the purified bovine brain kinase (21). Although the brain proline-directed protein kinase shows sequence conservation to other members of cdc2 kinase family, there are notable exceptions at specific regions of the protein. For example, amino acid sequence corresponding to human p34<sup>cdc2</sup> residues 42–53:EGPSTAIRES is totally conserved among all nine members of the cdc2 kinase family, but contains three amino acid substitutions in the brain kinase sequence (Fig. 3). Also, a sequence corresponding to human p34<sup>cdc2</sup> kinase residues 206–212:GDSFEDIQ is completely conserved in members of the cdc2 kinase family, whereas the brain kinase sequence has four amino acid substitutions.

Biochemical characterization of the brain proline-directed kinase has indicated that the enzyme is distinct from other cdc2-related kinases in its regulation by specific regulatory proteins (21). In addition to specific phosphorylation reactions, the H1-histone kinase activity of p34<sup>cdc2</sup> is dependent upon its specific interaction with cyclin. In contrast, the brain kinase does not appear to be associated with any of the known cyclins, and is highly active toward H1 histone (21). The purified brain enzyme does, however, contain a 25-kDa subunit in addition to the 33-kDa catalytic subunit. The 25-kDa subunit has been subjected to microsequencing, and three tryptic peptides have been sequenced. The results indicate that it is not a previously described cyclin. In addition, the brain kinase is distinct from p34<sup>cdc2</sup> in that it does not display...
specific interaction with p13\textsuperscript{Cyc} (21). p13\textsuperscript{Cyc} is a small regulatory protein that undergoes specific association with p34\textsuperscript{Cdc2}, but its regulatory function is not well defined.

The structural bases of interactions of p34\textsuperscript{Cdc2} kinase with cyclin and p13\textsuperscript{Cyc} have been investigated by site-directed mutagenesis with emphasis on the phosphorylation sites and charge clusters of the enzyme molecule (28). The results suggest that the interactions of the enzyme with these regulatory proteins involve amino acid residues from different regions of the enzyme. Interestingly, residues of p34\textsuperscript{Cdc2} kinase important for these protein associations are also conserved in the brain enzyme. For example, three charge clusters are important for the p34\textsuperscript{Cdc2} kinase association with p13\textsuperscript{Cyc}. They are the clusters of Lys-20, Arg-22, His-23, and Lys-24; Lys-56, Asp-57, Arg-59, and His-60; and Arg-215 and Arg-218 (29). These results suggest that kinases of this family may have been focusing mainly on the roles of these kinases in the control of cell division. On the other hand, ~34\textsuperscript{Cdc2} antibody-reactive proteins with M\textsubscript{r} similar to p34\textsuperscript{Cdc2} have been detected in differentiated tissue such as brain (18) and platelets (30), and neuronal specific proteins such as neurofilament proteins are the clusters of Lys-20, Arg-22, His-23, and Lys-24; Lys-56, Asp-57, Arg-59, and His-60; and Arg-215 and Arg-218 (29). Essentially all these residues are conserved in the brain kinase except histidine 23, which is substituted by asparagine, and arginine 59, which is conservatively substituted by lysine. Thus, sequence comparison of the brain kinase and p34\textsuperscript{Cdc2} kinase has failed to shed light on the molecular basis of the differential interactions of the two kinases with p13\textsuperscript{Cyc} and cyclins.

In conclusion, the studies of ~34\textsuperscript{Cdc2} and related kinases have been focusing mainly on the roles of these kinases in the control of cell division. On the other hand, p34\textsuperscript{Cdc2} antibody-reactive proteins with M\textsubscript{r} similar to p34\textsuperscript{Cdc2} have been detected in differentiated tissue such as brain (18) and platelets (30), and neuronal specific proteins such as neurofilament proteins have been phosphorylated by p34\textsuperscript{Cdc2}-like kinases. These results suggest that kinases of this family may have functions other than cell division cycle control. Results from the present and our previous studies (21), as well as from the study of Meyerson \textit{et al.} (24), suggest the existence of a new category of p34\textsuperscript{Cdc2}-like protein kinases whose primary functions are other than the control of cell cycle. Thus, the cDNA of the human homolog of bovine brain proline-directed protein kinase is not capable of rescuing a cdc28 fission yeast mutant (24), and the brain protein kinase that represents the predominant p34\textsuperscript{Cdc2}-like kinase activity in bovine brain (21) is capable of phosphorylating the neurofilament proteins at identical sites as p34\textsuperscript{Cdc2} kinase.

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