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Resolution and purification of a neurofilament-specific kinase

(cytoskeleton/neuronal maturation/phosphorylation/Alzheimer disease)

BARBARA A. WIBLE, KIRK E. SMITH, AND KIMON J. ANGELIDES*

Department of Physiology and Molecular Biophysics and Program in Neuroscience, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030

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ABSTRACT **Both *in vivo* and *in vitro*, neurofilaments (NFs) are among the most highly phosphorylated proteins known. The majority of the NF phosphorylation sites reside on the carboxyl-terminal tails of the proteins. We have isolated and characterized an effector-independent neurofilament-specific protein kinase from bovine spinal cord that is associated with the NF complex and exhibits a marked substrate specificity for NF-H, the largest subunit of the NF triplet. This kinase activity emerges from a NF-conjugated affinity column coincident with a 67-kDa doublet on NaDodSO$_4$/polyacrylamide gels and has a purity of >90%. The purified enzyme exclusively phosphorylates NF-H tails and is dependent on prior phosphorylation of this molecule. The enzyme is also not autophosphorylated. While the molecular properties and substrate specificities of the NF kinase distinguish it from cAMP-dependent protein kinase, protein kinase C, Ca^{2+}/calmodulin kinase, and casein kinases I and II, it exhibits certain properties similar to, but different from, the growth-associated histone H1 kinase. The molecular properties and specific sequence requirements of the NF kinase suggest that this enzyme could play a pivotal role in the phosphorylation of NFs in normal and pathological states such as Alzheimer disease, where NFs are hyperphosphorylated.**

Nerve cell intermediate filaments, or neurofilaments (NFs), are composed of three proteins of low (66 kDa; NF-L), medium (130 kDa; NF-M), and high (180 kDa; NF-H) molecular masses (1, 2). While the three NF subunits share a highly conserved α-helical rod region with other intermediate filament proteins, NF-H and NF-M differ in that they each possess a highly acidic carboxyl-terminal tail domain. NF-M and NF-H are also highly phosphorylated [i.e., estimates of 25 and 53 mol of phosphate per mol of bovine NF-M and NF-H, respectively (3)], with the majority of phosphoserine located on the tail in the sequence Lys-Ser-Pro-(Val or Ala) (4, 5). While the rod appears to be important in the assembly of NF-L to form the filament core, NF-M and NF-H associate on the periphery, possibly forming cross-links between NFs and/or cytoskeletal proteins.

Several recent findings underscore the importance of phosphorylation for NF function. First, NF phosphorylation dramatically alters the physical and chemical behavior and the antigenicity of the proteins (6, 7). Second, phosphorylated NFs are preferentially localized in the axon, compared with the cell body or dendrites, except in Alzheimer-disease brain tissue, in which NFs are hyperphosphorylated and concentrated in the cell body (8).

To more fully understand the involvement of the phosphorylation of NFs in neuronal function, we have isolated and characterized a protein kinase that catalyzes NF phosphorylation. This enzyme shows a marked preference for NF-H and has the same domain specificity that is found *in vivo*.

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MATERIALS AND METHODS

Chemicals. The following protease inhibitors at 1 μg/ml were included in all preparative buffers: pepstatin, leupeptin, N^2-(*p*-tosyl)-L-lysine chloromethyl ketone, and L-1-tosylamido-2-phenylethyl chloromethyl ketone; and 0.2 mM phenylmethylsulfonyl fluoride was also added.

Neurofilaments and Purified Subunits. NF-enriched fractions and purified subunits (NF-H, NF-M, and NF-L) were prepared from fresh bovine spinal cord as described (2).

NF-enriched cytoskeletal proteins and resolved NF subunits were dephosphorylated by incubation with 2–20 units of *Escherichia coli* alkaline phosphatase per mg of NF protein for various times ranging from 5 min to 18 hr (6). Dephosphorylated subunits were brought to 50 mM EDTA, boiled, and dialyzed against 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes; pH 6.5) prior to assay.

Phosphorylated NF-H was digested with chymotrypsin at an enzyme/NF-H ratio of 1:400 (wt/wt) for 15 min at 22°C (9). Under these conditions NF-H is cleaved into two fragments, a 40-kDa rod and a 160-kDa tail.

Microtubules (MT) and microtubule-associated proteins (MAPs) were prepared by using temperature-dependent cycles of assembly/disassembly (10).

NF Kinase Assays. Prior to their use as phosphate acceptors, NF proteins or MT/MAPs were boiled for 5 min to destroy endogenous protein kinase activity. NF proteins (1 mg/ml), NF subunits (0.2 mg/ml), or MT/MAPs (1 mg/ml) were incubated in 50 mM Mes, pH 6.5/10 mM $MgCl_2$/20 nM ATP at 22°C for 5 min. Assays were performed with high-specific-activity [^{32}P]ATP (8.8×10^6 cpm/pmol). Even though assays were conducted at [ATP] $<< K_m$, initial velocity conditions were maintained. Reactions were terminated after 5 min by the addition of an equal volume of electrophoresis buffer [50 mM Tris, pH 6.8/1% (wt/vol) 2-mercaptoethanol/1% (wt/vol) NaDodSO$_4$/15% (wt/vol) glycerol]. Samples were electrophoresed on 7.5% (wt/vol) polyacrylamide/NaDodSO$_4$ gels (13), stained with fast green, dried, and autoradiographed.

During the purification, the specific activity of the enzyme was monitored by assaying 1 μg of purified NF-H under the same conditions described above. After staining with fast green to localize NF-H on gels, the band was excised and the ^{32}P incorporated was measured by scintillation counting.

Extraction. NF-enriched cytoskeletal preparations were homogenized (4 mg/ml) in 0.8 M KCl/10 mM $MgCl_2$/2 mM EGTA/1 mM EDTA and stirred for 4 hr at 4°C. The enzyme activity was harvested from this extract by centrifugation for 1 hr at 155,000 $\times$ *g*. The kinase-containing supernatant was dialyzed against 50 mM Mes, pH 6.5/1 mM $MgCl_2$/1 mM dithiothreitol at 4°C and clarified by centrifugation at 10,000 $\times$ *g* for 10 min.

Abbreviations: MT, microtubule; MAP, microtubule-associated protein; NF, neurofilament; NF-L, NF-M, and NF-H, low molecular mass (66 kDa), medium molecular mass (130 kDa), and high molecular mass (180 kDa) NF.

*To whom reprint requests should be addressed.

Ion-Exchange Fast Protein Liquid Chromatography (FPLC). Between 4 and 16 mg of protein from the extract was applied to an LKB HPLC/FPLC system with a DEAE-5PW FPLC column that had been equilibrated with 50 mM Mes/1 mM $MgCl_2$/1 mM dithiothreitol (column buffer). After protein from the flow-through was collected, a linear gradient from 0 to 0.5 M NaCl (50 ml) was run. Fractions (0.5 ml) were collected, dialyzed to remove the NaCl, and tested for substrate specificity by using NFs, NF subunits, or MT/MAPs.

Cibacron Blue-Agarose Chromatography. Flow-through fractions were pooled and applied to a 6-ml column of Cibacron Blue-3GA agarose equilibrated in column buffer. Nucleotide binding proteins were eluted with column buffer containing 5 mM ATP and 5 mM $MgCl_2$. The remaining protein was eluted with a 50-ml linear NaCl gradient (0–1.0 M) in column buffer. Peak fractions were pooled and dialyzed against column buffer prior to enzyme assays.

NF-Conjugated Affinity Column. For the last step, a NF-conjugated affinity column was prepared by coupling 2 mg of electrophoretically pure NFs to 500 mg of cyanogen bromide-activated Sepharose 4B. Pooled fractions eluted from the Cibacron Blue-agarose column that included ATP were applied to a 3-ml NF-coupled affinity column equilibrated in column buffer. Elution of specifically bound proteins was achieved with a gradient of NaCl in column buffer. Fractions (1.0 ml) were collected, dialyzed, and then assayed with NFs and MT/MAPs.

Analytical Methods. Protein determinations were performed by the method of Bradford (11) or by fluorescamine (12).

RESULTS

Extraction of NF Kinase. Several phosphoprotein kinase activities were released from NF-enriched material upon treatment with high ionic strength buffers. Both NFs and MT/MAPs were phosphorylated by this extract (Fig. 1*B*). MT/MAP phosphorylation was stimulated by cAMP and largely inhibited by the cAMP-dependent protein kinase inhibitor, while NF phosphorylation was unaffected (data not shown). Although all three NF subunits were phosphorylated, NF-M was the most highly phosphorylated as seen in *in vitro* phosphorylation of NF-enriched fractions (Fig. 1*B*).

Because NF-H was the most heavily phosphorylated of the three NF proteins *in vivo*, we chose to concentrate on a NF-H-specific phosphorylation activity and to resolve this activity by ion-exchange and affinity chromatography.

Ion-Exchange FPLC. The extract was dialyzed against column buffer and chromatographed on an LKB DEAE-5PW FPLC column. Of the protein applied, 20–30% did not bind to the column, while the remainder was eluted with a linear 0–0.5 M NaCl gradient (Fig. 1*A*). Four major peaks of kinase activity with different substrate specificities were resolved (Fig. 1*B*). While fractions from the column flow-through were able to phosphorylate NFs as well as MT/MAPs, densitometry of the autoradiogram of NF phosphorylation suggests an enrichment of NF-H phosphorylation relative to NF-M and NF-L when compared with the activity of the initial extract. Furthermore, electrophoretically pure NF-H was also an excellent substrate for this activity, and a chymotryptic digest of phosphorylated NF-H localized the phosphorylation to the large tail domain (Fig. 1*B*). With elution of bound protein by NaCl, the phosphorylation profile changed to one that in general had specificity for NF-M and MT/MAPs. Because the flow-through fractions showed a modest increase in the phosphorylation of NF-H tails relative to the extract and the DEAE-bound fractions, we chose to focus on the kinase activity in the flow-through.

Cibacron Blue Affinity Chromatography. The pooled flow-through fractions from the FPLC DEAE column were further purified by chromatography on Cibacron Blue-agarose. Two nucleotide binding protein peaks constituting <5% of the total protein were eluted with 5 mM ATP and 5 mM $MgCl_2$ (Fig. 2). A striking enrichment of the phosphorylation of NF-H over NF-M was observed in these fractions (Fig. 2 *Inset*), with no distinguishable difference in the activity or the sites of phosphorylation between the two peaks. Further-

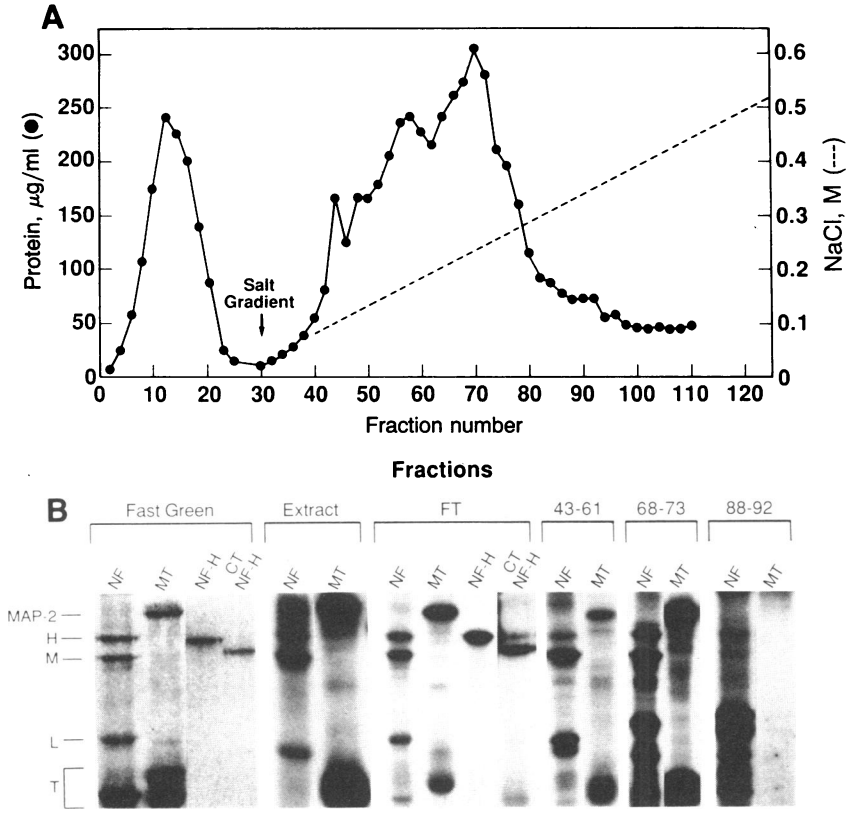

FIG. 1. Ion-exchange chromatography of soluble kinase extract on a DEAE-5PW FPLC column. (*A*) Protein elution profile. The extract (7 mg of protein) was loaded onto a DEAE-5PW column (LKB). The flow-through was collected, and bound protein was eluted with a 50-ml linear gradient of NaCl (0 to 0.5 M). The protein content of the eluted material was determined by the method of Bradford (11). (*B*) Phosphorylation of NFs and MT/MAPs by kinase extract and DEAE column fractions. The lanes labeled "Fast Green" show the polypeptide composition of the substrates NF (H, M, and L), NF-H, and MT [MAP-2 and T (α and β tubulin)]. The large tail fragment of NF-H generated by chymotryptic (14) cleavage is also shown (CT NF-H). The remaining lanes are autoradiograms of column fraction phosphorylations: extract, flow-through (FT), and DEAE-bound fractions 43–61, 68–73, and 88–92. Aliquots (15 μl) of each fraction were incubated with [^{32}P]ATP under standard phosphorylation conditions with either NFs or MT/MAPs as the substrate, followed by electrophoresis on 7.5% $NaDodSO_4$/polyacrylamide gels and autoradiography.

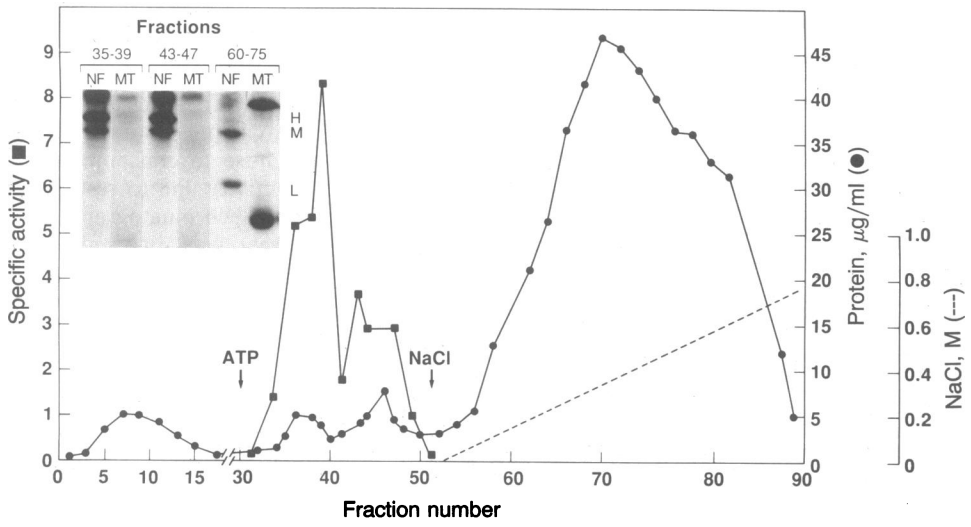

FIG. 2. Cibacron Blue-agarose chromatography of HPLC DEAE column flow-through fractions. Flow-through fractions from the DEAE column were pooled (1.3 mg) and applied to a 6-ml column of Cibacron Blue-agarose. Protein in the fractions eluted with ATP was measured by the fluorescamine assay, whereas the Bradford method was used to measure protein eluted within the 0–1.0 NaCl gradient. The specific activity of the fractions eluted with ATP is expressed as pmol of ^{32}P incorporated into NF-H per min per mg of protein. (*Inset*) Autoradiograms of NF and MT phosphorylation by ATP-eluted fractions 35–39 and 43–47 and NaCl-eluted fractions 60–75.

more there was no phosphorylation of MT/MAPs. Within the protein peak eluted with NaCl is a peak of phosphorylation activity that showed a substrate preference for NF-M as well as an ability to phosphorylate MT/MAPs.

Affinity Chromatography. The peaks from the Cibacron Blue-agarose column, with ATP bound, were pooled and applied to a column to which pure NF triplet proteins were covalently coupled. Two protein peaks with virtually identical NF kinase specific activities and substrate specificities for NF-H bound to the column and were eluted at 0.3–0.6 M NaCl and 0.9–1.0 M NaCl (Fig. 3). The two protein peaks eluted from the Cibacron Blue-agarose and NF-coupled affinity columns appear to be isozymes with virtually indistinguishable molecular and catalytic properties and reflect differences in either residual bound nucleotide or in the nucleotide binding affinity of the two forms.

Table 1 summarizes the purification of the NF kinase. The specific activities of each fraction are expressed in terms of the phosphorylation of NF-H. From the extract to the NF-coupled affinity column-purified material, an enrichment of about 130-fold is seen. This relatively modest purification must be considered only a very minimum estimate, however, because in the extract and anion-exchange fractions, several kinases other than the NF kinase could phosphorylate NF-H

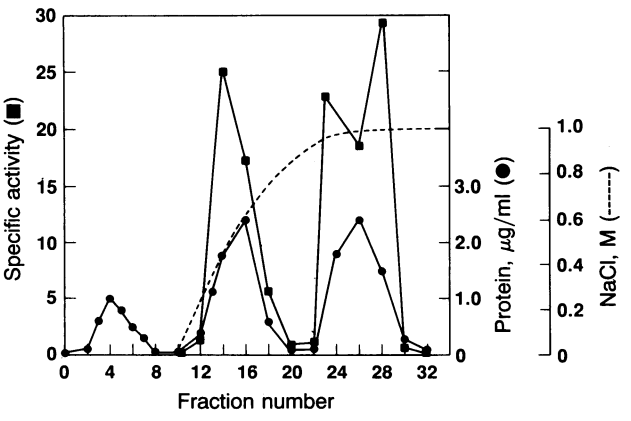

FIG. 3. Affinity chromatography of Cibacron Blue-agarose-purified NF kinase. NF kinase fractions eluted from the Cibacron Blue-agarose column with ATP and $MgCl_2$ were pooled (40 μg) and loaded onto a 3-ml NF-coupled affinity column. Bound protein was eluted with 1.0 M NaCl in column buffer. A short NaCl gradient was achieved as the column became equilibrated in NaCl buffer. Protein was determined by the fluorescamine assay. Specific activity of the bound protein is expressed as pmol of ^{32}P incorporated into NF-H per min per mg of protein.

and thus contribute to the ^{32}P incorporated into NF-H. The apparent specific activities are also lower because 20 nM ^{32}P-ATP was used. Because [ATP] << K_m, the data are expressed as V_{max}/K_m. In the most pure preparations, a V_{max}/K_m ratio of 1.4×10^{-3} liter·min^{-1}·mg^{-1} was obtained.

Examination of the protein composition at each step of the purification by $NaDodSO_4$/PAGE and silver staining showed that the NF kinase activity from Cibacron Blue-agarose and NF-coupled affinity columns correlated with the enrichment of a 67-kDa protein doublet that constituted >90% of the protein in the final fractions from the affinity column (Fig. 4). Although the possibility exists that a minor protein band with protein kinase activity escaped detection by silver staining, even labeling with ^{125}I-labeled Bolton–Hunter reagent did not reveal additional proteins. The minor polypeptide at 55 kDa may be a contaminant or a proteolytic product of the 67-kDa doublet and was often present in variable amounts relative to the major proteins.

Specificity and Properties of NF Kinase. The enzyme shows a distinct preference for the NF-H component of the NF triplet (Fig. 5, lanes 1–4). Although the enzyme catalyzed the incorporation of moderate levels of ^{32}P into NF-M when assembled into NFs (lane 2), it did not phosphorylate isolated NF-M (lane 10) or NF-L in either state (lane 2 for NF-L in NFs). Furthermore, the purified enzyme did not phosphorylate MT/MAPs (lane 12) or myosin light chains (lane 16). In addition, neither casein nor phosvitin served as substrates for the kinase. However, histone H1 could be weakly phosphorylated (lane 13).

Since NF-H in our assays was already partially phosphorylated, potential NF kinase phosphorylation sites may have been masked. Therefore, we tested whether we could reveal additional phosphorylation sites by using enzymatically de-

Table 1. Purification of NF kinase activity

Purification step	Protein, mg	Specific activity*	Fold purification
Extract	6.88	0.17 ± 0.05	1
DEAE flow-through	1.28	0.51 ± 0.17	3
Cibacron Blue-agarose, ATP eluant	0.04	4.81 ± 1.75	28
NF-conjugated affinity column	0.02	22.6 ± 4.4	133

*Measured as pmol of ^{32}P incorporated in NF-H per min per mg of protein. Kinase assays were carried out at 20 nM ATP. Thus, [ATP] << K_m, so values for V_{max} and K_m could not be determined. A V_{max}/K_m ratio was determined from the velocity (specific activity) measurement at an ATP concentration of 20 nM.

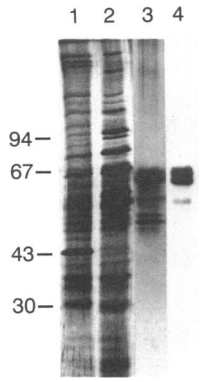

FIG. 4. $NaDodSO_4$/PAGE of fractions from NF kinase purification. Aliquots of fractions from each stage of the purification were electrophoresed on a 7.5–12% gradient polyacrylamide gel containing $NaDodSO_4$ and were silver-stained to visualize the proteins. Lanes: 1, extract (2 μg); 2, DEAE flow-through (2 μg); 3, Cibacron Blue-agarose, ATP eluant (0.5 μg); 4, NF-coupled affinity column, NaCl eluant (0.3 μg). Sizes are shown in kDa.

phosphorylated NF-H. Lane 7 in Fig. 5 shows the electrophoretic mobility change that accompanies dephosphorylation of NF-H (6). To our surprise, we were unable to demonstrate any appreciable phosphorylation of exhaustively dephosphorylated NF-H (lane 8), although there was a striking increase in ^{32}P incorporation into partially dephosphorylated protein (5 min) that decreased dramatically upon further dephosphorylation (Fig. 5, lane 6).

The substrate specificity was not due to the selective enrichment of an NF-M- or NF-L-specific phosphatase. When NFs were labeled with ^{32}P by the extract and incubated with NF kinase active fractions, no ^{32}P was released.

The NF kinase requires Mg^{2+} for activation, with optimal activity between 5 mM and 10 mM. Mn^{2+} (5 mM) was only 10% as effective as Mg^{2+} and Ca^{2+} and Zn^{2+} were totally ineffective. NF kinase activity was optimal in the pH range 6–8 and was inhibited by increasing ionic strength (100 mM NaCl).

It has been shown previously that NF phosphorylation is not affected by cAMP, cGMP, cAMP-dependent protein kinase inhibitor, Ca^{2+}/calmodulin, Ca^{2+}/phospholipid, or phorbol

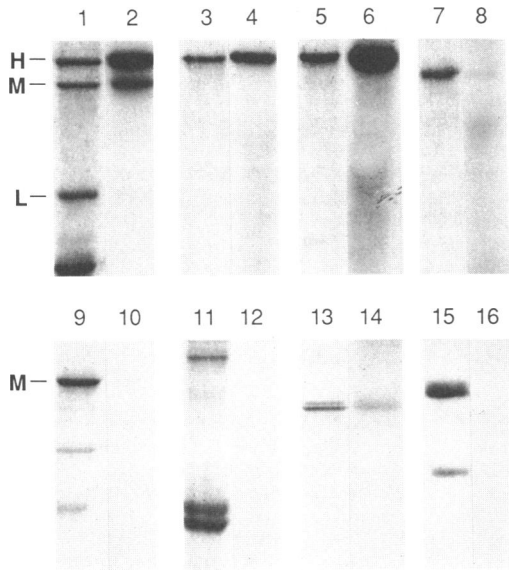

FIG. 5. Substrate specificity of purified NF kinase. Various substrates were incubated with 15 μl of purified NF kinase in the standard reaction mixture with [γ-^{32}P]ATP. Protein stain (odd-numbered lanes), autoradiogram (even-numbered lanes). Lanes: 1 and 2, NF-enriched cytoskeletal pellet (5 μg); 3 and 4, NF-H (1 μg); 5 and 6, NF-H partially dephosphorylated (5 min) (1 μg); 7 and 8, NF-H extensively dephosphorylated (18 hr) (1 μg); lanes 9 and 10, NF-M (1 μg); 11 and 12, MT/MAPs (5 μg); 13 and 14, histone H1 (1 μg); and 15 and 16, myosin light chains (6 μg). Samples were subjected to $NaDodSO_4$/PAGE (lanes 1–12, 7.5% acrylamide; lanes 13–14, 15% acrylamide; lanes 15–16, 12% acrylamide).

esters. These properties were preserved with the purified enzyme. Furthermore, the NF kinase could not utilize GTP.

Finally, when the purified NF kinase was incubated with [γ-^{32}P]ATP, no phosphate was incorporated into any protein, suggesting that the enzyme is not autophosphorylated.

DISCUSSION

We have described the purification and characterization of a NF-specific protein kinase from bovine spinal cord. The enzyme is solubilized from NF-enriched fractions and purified by sequential chromatography on DEAE, Cibacron Blue-agarose, and NF-conjugated affinity columns. The purified enzyme phosphorylates NFs with a substrate specificity restricted primarily to the NF-H component of the NF triplet and does not phosphorylate MT/MAPs, casein, phosvitin, or myosin light chains. Phosphorylation by the kinase is on the tail domain of NF-H, which contains the repeated sequence Lys-Ser-Pro, which is the major locus of *in vivo* phosphorylation (4, 5, 15). The enzyme appears to be tightly associated with the NF complex because high ionic strength buffers are required to remove the enzyme from its association with NFs. The enzyme activity coincides with a 67-kDa doublet, which, in the purest preparations, constitutes >90% of the protein. Whether the two proteins are NF kinase isozymes with identical catalytic properties or result from proteolysis of the larger protein is unknown. The latter seems unlikely because a 1:1 ratio is maintained between the two throughout the purification and in several preparations. In addition, it is possible that one polypeptide is a catalytic subunit, while the other is a modulatory subunit or a subunit that links the enzyme to the NF complex.

Both the specificity and properties of the NF kinase isolated here distinguish it from previously described effector-dependent or -independent kinases. Several protein kinases can phosphorylate NFs, including cAMP-dependent protein kinase and Ca^{2+}/calmodulin-dependent protein kinase (16, 17), but NF-M is the preferred substrate for these enzymes. A previously described NF-associated kinase (14) differs from the NF kinase that we have purified because it shows a much broader substrate specificity and does not phosphorylate NFs in the *in vivo* pattern. In contrast, the NF kinase that we have isolated shows a strong preference for NF-H, unlike any NF-associated enzyme activity previously reported, and has an activity that results in the characteristic *in vivo* NF phosphorylation pattern.

When we compared the catalytic and molecular properties of our enzyme with other effector-independent protein kinases, we were surprised to find similarities between the NF kinase and the growth-associated histone H1 kinase. Both enzymes have a molecular weight of 67 kDa and are not autophosphorylated (18, 19). Independent of these observations, the repeated sequence, Lys-Ser-Pro-(Val or Ala), was reported as the major *in vivo* NF phosphorylation site (4, 5, 15), which we noted is remarkably similar to the histone H1 sequence Lys-(Ser or Thr)-Pro-Lys, specifically phosphorylated by the growth-associated histone H1 kinase (20). The occurrence of such a sequence is infrequent. Although the NF kinase weakly phosphorylates histone H1, presumably on this sequence, other structural features of the NFs contribute to their phosphorylation by the NF kinase (see below). Certain molecular and catalytic properties also distinguish the two enzymes. For example, the NF kinase is more sensitive to ionic strength and migrates in a different position on DEAE resins from that of the histone H1 kinase. Furthermore, the growth-associated histone H1 kinase is bound to chromatin, in contrast to the cytoskeletal association of the NF kinase. It is highly unlikely that our activity arises from histone H1 kinase contamination because nuclei are removed early in our preparations. Also, the V_{max}/K_m

ratio of 1.4×10^{-3} liter·min^{-1}·mg^{-1} is 100-fold higher than the value for the growth-associated histone H1 kinase ($1.5–6.2 \times 10^{-5}$ liter·min^{-1}·mg^{-1}). However, the NF kinase has an activity in the same range as other protein kinases, being 1/10th to 1/50th as active as brain Ca^{2+}/calmodulin kinase phosphorylating tubulin (21), phosphorylase kinase (20), or gizzard myosin light chain kinase (20).

Immunological studies have suggested differences in either the primary sequence or secondary structure between bovine NF-M and NF-H at or near the major phosphorylation sites (5). In the absence of primary sequence data on the bovine subunits, the inability of the NF kinase to phosphorylate NF-M to the same extent as NF-H could be due to fewer phosphorylation sites or minor sequence or conformational differences at these sites. In the case of human NF subunits, NF-H possesses >40 Lys-Ser-Pro repeats, whereas NF-M has only 15 such sites (15, 22). In contrast, rat NF-H and NF-M both contain the same number of Lys-Ser-Pro sites (5), with variability of the flanking amino acids responsible for the differential phosphorylation of the two proteins (23, 24).

At least for NF-H, conformation appears to be important for phosphorylation. Since the native, phosphorylated form of NF-H, both in the assembled form or as a free soluble protein, is an excellent substrate, we were surprised to find that the enzyme does not phosphorylate exhaustively dephosphorylated NF-H or a 26-residue synthetic peptide that we had synthesized—Lys-Ser-Pro-Val-Pro-Lys-Ser-Pro-Val-Glu-Glu-Lys-Gly-Lys-Ser-Pro-Val-Pro-Lys-Ser-Pro-Val-Glu-Glu-Lys-Gly, derived from the human NF-H and NF-M sequences (15, 22)—but avidly phosphorylates partially dephosphorylated NF-H. In fact, had we used dephosphorylated NFs or NF-H or the synthetic peptide solely as substrates, we would not have detected this activity. These observations suggest the intriguing possibility that a defined and regulated secondary structure of NF-H is required for recognition and catalysis by the NF kinase. Phosphoserines at other sites contributed by other kinases may be required to adopt a NF-H structure that allows binding of the enzyme or phosphoryl transfer. The notion of sequential phosphorylation of NF-H by several kinases including the NF kinase is consistent with recent observations of the existence of a spectrum of partially phosphorylated forms of NF-H and NF-M in cells (25), which are detected in the axon at specific times during neuronal maturation.

The phosphorylation of a variety of proteins including vimentin (26), desmin (27), the nuclear lamins (28), and myosin heavy chain (29) has been shown to affect their assembly and/or relationship with other cellular components. While evidence is accumulating to suggest that NFs play a major role in maintaining axonal shape and volume, how the incorporation of massive amounts of phosphate into NFs alters their functional properties remains a mystery. The heavily phosphorylated tail domains of NF-H and NF-M are thought to be involved in direct NF–NF contact or interaction with other cytoskeletal proteins. During axonal maturation, NF phosphorylation increases as axonal caliber increases and stable NF networks are laid down (30). Interestingly, as others have suggested, NF transport along the axon involves interactions with other axonal structures, and, as NFs are progressively posttranslationally modified as they traverse the axon, the character of these interactions could be modified by phosphorylation leading to a change in NF transport and their eventual incorporation into a stable NF network contributing to radial axonal growth (31).

As NFs are phosphorylated by several different protein kinases including the NF kinase, the role each enzyme assumes in the sequential phosphorylation of NFs and its effects on NF structure and function remain to be elucidated. The fact that the NF kinase described here preferentially phosphorylates NF-H, the most highly phosphorylated of the NF subunits, would suggest that it plays a significant role *in vivo*, perhaps as a terminal step in differentiation. In addition, both τ protein and NF-H contain the sequence Lys-Ser-Pro (5, 15) and are recognized by antibodies to either the phosphorylated epitopes of NFs or the neurofibrillary tangles of Alzheimer disease (32–35). Further characterization of this enzyme especially with regard to its topological relationship with the NF complex and its cellular distribution should provide more insights into the physiological consequences of NF phosphorylation in both normal and pathological states.

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