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Characterization of Neurofilament-Associated Protein Kinase Activities from Bovine Spinal Cord

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SUMMARY

1. A neurofilament-enriched preparation from bovine spinal cord contains endogenous protein kinases that phosphorylate high, middle, and low molecular weight neurofilament subunits (NF-H, NF-M, and NF-L), as well as certain other endogenous and exogenous substrates.

2. Most of this associated kinase activity can be separated from the neurofilament subunits and the bulk of the protein by extraction of the neurofilament preparation with 0.8 *M* KCl. Assays using specific exogenous substrates, activators, and inhibitors for known kinases reveal significant levels of Ca^{2+}-calmodulin-dependent, cyclic nucleotide-dependent, Ca^{2+}-phosphatidylserine diglyceride-dependent, and regulator-independent kinase activities in the high-salt extract.

3. Fractionation of the salt extract on a gel filtration column resolves a regulator-independent kinase activity identified by its ability to phosphorylate purified NF-M. This preparation can phosphorylate all three neurofilament proteins either in purified form or in the assembled form, as well as α-casein. Only the regulator-independent kinase activity in this fraction is responsible for the phosphorylation of neurofilament proteins.

4. While this partially purified kinase activity does not show a strong substrate specificity between the three neurofilament subunits, the phosphorylation pattern it produces upon incubation with salt-extracted neurofilaments is

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similar to the regulator-independent phosphorylation pattern found in the original neurofilament preparation and, thus, represents a useful starting point for the further purification of this neurofilament-associated kinase activity.

INTRODUCTION

Mammalian neurofilaments (NFs) are composed of three subunits usually designated high, medium, and low molecular weight NF proteins or NF-H, NF-M, and NF-L. All three NF subunits, isolated from various sources, are found to be phosphorylated and may contain about 1–3, 6–11, and 13–22 mol of phosphate per mol of NF-L, NF-M, and NF-H, respectively (Julien and Mushynski, 1982; Carden *et al.*, 1985; Georges *et al.*, 1986; Geisler *et al.*, 1987). The phosphorylation state of NF-H and NF-M subunits varies according to their subcellular location. The heavily phosphorylated forms are predominant in the axon, whereas NF-H and NF-M found in the perikaryon and dendrites either are not phosphorylated or contain relatively few phosphate groups (Lee *et al.*, 1987; Oblinger, 1987; Glicksman *et al.*, 1987).

The identities of protein kinases responsible for the phosphorylation of NF subunits in the intact tissue are yet unclear. Neurofilament preparations from different mammalian species were shown to contain endogenous kinase activities that can phosphorylate all three subunits in the absence of activators (Runge *et al.*, 1981; Julien *et al.*, 1983; Caputo *et al.*, 1989). Such regulator-independent kinase activity capable of phosphorylating NFs has also been described in NF-enriched preparations from squid and myxicola axoplasm (Pant *et al.*, 1986; Schecket and Lasek, 1982). The kinase activities associated with NF preparations from vertebrate and invertebrate species appear to have certain common characteristics. For example, incubation of NF preparations with ATP results in the phosphorylation of all NF subunits present; heparin, at concentrations known to inhibit casein kinase II, has little or no effect on their phosphorylation; casein and certain histones serve as substrates for the kinase activity (Julien *et al.*, 1983; Pant *et al.*, 1986; Shecket and Lasek, 1982). In addition to the regulator-independent kinase activity, several regulator-dependent kinases have been reported to phosphorylate NF proteins *in vitro*, including cyclic AMP-dependent protein kinase, Ca^{2+}-calmodulin-dependent protein kinase and protein kinase C (Julien *et al.*, 1983; Sihag *et al.*, 1988; Sihag and Nixon, 1989; Vallano *et al.*, 1985).

It is probable that the NF-associated regulator-independent kinase activity responsible for the endogenous phosphorylation of NF subunits in NF preparations is composed of more than one type of kinase. In fact, up to now, two apparently unique kinases associated with NF preparations from the mammalian tissue have been described. One of these, partially purified and characterized by Toru-Delbauffe and Pierre (1983) and Toru-Delbauffe *et al.* (1986), was found to phosphorylate all three subunits but appears to have a higher specificity for NF-H. More recently, Wible *et al.* (1989) reported the isolation of yet another kinase which shows a marked preference for NF-H as a substrate, which phosphorylates NF-M only in its assembled form and which does not phosphorylate NF-L.

Since in the above two studies the reported specificities of the isolated kinases did not reflect the pattern of phosphorylation found in the original NF-enriched preparation, we reexamined this preparation for a kinase activity that would do so. Our results indicate that the bovine NF preparation contains Ca^{2+}- and cyclic nucleotide-regulated kinase activities, as well as activator-independent kinase activity. Furthermore, we report the resolution of an activator-independent kinase fraction which can phosphorylate all three NF proteins in purified and associated forms. When a NF preparation devoid of kinase activity was incubated with the fraction, the pattern of phosphorylation was found to be similar to that of the endogenous phosphorylation in the intact NF preparation.

MATERIALS AND METHODS

Materials

Calmodulin, cyclic AMP, cyclic GMP, ATP, alkaline phosphatase (from *Escherichia coli,* type III-S), 1.2-dioleoly-*sn*-glycerol (diglyceride), phosphatidylserine, histone type III-S, α_s-casein, phosvitin, and heparin were purchased from Sigma. Staurosporin was purchased from Kyowa, and W7 from Seikagoku Kogyo. Peptide substrates and inhibitors for different kinases including Leu-Arg-Arg-Ala-Ser-Leu-Gly (kemptide), Arg-Lys-Arg-Ser-Lys-Glu (substrate for cyclic GMP-dependent protein kinase), Pro-Leu-Arg-Arg-Thr-Leu-Ser-Val-Ala-Ala-NH_2 (calmodulin-dependent protein kinase substrate analogue), Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NH_2 (Wiptide; cyclic AMP-dependent protein kinase inhibitor), and Arg-Lys-Arg-Ala-Arg-Lys-Glu (cyclic GMP-dependent protein kinase inhibitor) were from Peninsula Laboratories. [γ-^{32}P]ATP tetra(triethylammonium) salt was purchased from New England Nuclear.

Neurofilament Preparation

A neurofilament-enriched preparation was obtained from bovine spinal cords by the method of Carden *et al.* (1985) with certain modifications. Following removal of the dural sheath and gray matter, spinal cords were cut transversely into thin segments and placed into 100 m*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, and 10 m*M* sodium phosphate, pH 7.0. After washing two or three times with the same solution and decanting, the tissue was resuspended in an equal volume of 1 m*M* EDTA, 1 m*M* EGTA, 10 m*M* sodium phosphate, pH 7.0. The suspension was made 100 m*M* in NaCl by the addition of 5 *M* salt solution and homogenized using a Waring Blendor. The homogenate was centrifuged at 10,000*g* for 30 min. The supernatants were collected. The pellets were rehomogenized in the 100 m*M* NaCl solution and centrifuged at 10,000*g* for 60 min. The supernatants from the first and second centrifugation steps were stored at −70°C in 20% (v/v) glycerol until further processing. To obtain a NF-enriched fraction, the supernatants

were homogenized in 0.85 *M* sucrose, 1% Triton X-100, 1 m*M* dithiothreitol, and centrifuged 110,000*g* × 18 hr. At the end of the centrifugation the myelin layer on the top and the supernatant were discarded. NF-rich pellets were homogenized in 50% glycerol, 10% ethylene glycol, 1 m*M* EGTA, 0.5 m*M* EDTA, 1.5 m*M* dithiothreitol, 50 m*M* Hepes–Tris, pH 7.0, and stored at −20°C.

Extraction of NF Preparation with 0.8 *M* KCl

NF preparation in 50% glycerol (6–7 mg protein ml^{-1}) was mixed with an equal volume of extraction buffer to make up final concentrations of 0.8 *M* KCl, 10 m*M* $MgCl_2$, 2 m*M* EDTA, 1 m*M* EDTA, 0.05 mg ml^{-1} leupeptin, 10 m*M* sodium phosphate, pH 7.0. The homogenate was shaken overnight at 4°C, then centrifuged at 150,000*g* for 60 min. The pellets were resuspended in 10 m*M* sodium phosphate, pH 7.0, and recentrifuged as above. The washed pellets were stored at −20°C until further use. The supernatants from the first centrifugation step were recentrifuged (150,000*g* × 150 min) in order to sediment trace amounts of particulate material. This "high-salt extract" was stored at −20°C until further use.

Fractionation of High-Salt Extract by Gel Filtration Chromatography

Gel filtration chromatography was chosen as a first step in the resolution of proteins from the high-salt extract, with the hope of minimizing loss of kinase activity during fractionation. The medium employed, Ultragel AcA44, is composed of a polyacrylamide and agarose gel matrix and is reported to be suitable for the fractionation of proteins of 10–130 kDa, a molecular weight range that covers many known protein kinases.

The kinase-rich high-salt extract (10 ml, 17 mg protein) was applied to an Ultragel AcA44 column (53 × 3 cm) preequilibrated with 100 m*M* KCl, 10 m*M* $MgCl_2$, 1 m*M* EGTA, 1 m*M* DTT, and 10 m*M* Hepes–Tris, pH 7.1. The proteins were eluted at a flow rate of 0.7 ml min^{-1} and 4 ml fractions were collected. The fractions were stored in 45% glycerol and 5% ethylene glycol at −20°C.

Purification of NF Subunits

Following extraction with 0.8 *M* KCl, the NF pellets were homogenized with 6 *M* urea in buffer A [5 m*M* EDTA, 1 m*M* EGTA, 0.1% (v/v) β-mercaptoethanol, 10 m*M* sodium phosphate, pH 7.5], in a total volume of 50 ml. The homogenate was stirred at room temperature for 90 min and then centrifuged at 150,000*g* × 60 min at 20°C. The supernatant was applied onto an anion-exchange column (Whatman DE-52, 18 × 1.5 cm), preequilibrated with 8 *M* urea in buffer A. Unbound proteins were eluted with 100 ml of the same solution. Subsequently, a linear increasing-NaCl (0–250 m*M*) and decreasing-urea (8–3 *M*) gradient in buffer A was run and 12 ml fractions were collected, at a flow rate of 1 ml min^{-1}. Decreasing-urea gradient concomitant with increasing-NaCl gradient

was introduced following the observations of Tokutake (1984) on the effect of urea concentration on the resolution of NF subunits. According to the author, the separation of NF-H from glial acidic protein (a major contaminant of NF preparations) is optimal in 8 *M* urea, whereas NF-M and NF-L are optimally separated from each other at a urea concentration of 3 *M*. The introduction of a decreasing urea gradient enables a one-step purification of NF subunits from the urea extract. At the end of the run, aliquots from fractions were analyzed by polyacrylamide gel electrophoresis. The fractions containing NF subunits were pooled and dialyzed overnight against 10 m*M* sodium phosphate, pH 7.0, 0.5 m*M* dithiothreitol, and 1 m*M* EGTA. The pooled fractions were then concentrated using polyethylene glycol 20,000 as an absorbent and stored at −20°C.

Molar concentrations for bovine NF subunits were calculated using the following values for molecular weights—NF-H, 179 kDa; NF-M, 129 kDa; NF-L, 66.5 kDa—estimated by Scott *et al.* (1985), by Ferguson analysis.

Phosphorylation of NF Proteins, Kinase Assay, Polyacrylamide Gel Electrophoresis, and Autoradiography

NF preparation, subfractions from the preparation, purified NF subunits, α-casein, and phosvitin were phosphorylated in medium containing 100 m*M* NaCl or KCl, 10 m*M* $MgCl_2$, 1 m*M* EGTA, 1 m*M* dithiothreitol, 1 m*M* PMSF, and 20 m*M* Hepes–Tris, pH 7.0. Media containing 10 m*M* $MgCl_2$, 1 m*M* PMSF, and 20 m*M* Tris–HCl, pH 7.5, were employed to assay cyclic nucleotide and Ca^{2+}-dependent kinase activities with other additions as indicated in the text. The concentration of ATP was 100 μM with a specific activity of 300–750 cpm $pmol^{-1}$. Unless indicated otherwise, the incubation volume was 50 μl. The reaction was started by the addition of 5 μl of [γ-^{32}P]ATP and the tubes were incubated at 31°C for 10 min. For kinase assays, the method of Roskoski (1983) was followed. Briefly, at the end of the incubation time, 25-μl aliquots from the above reaction mixtures were spotted on squares of Whatman P81 phosphocellulose paper, which were immediately transferred into 75 m*M* phosphoric acid. The squares were washed three times with the same solution. After drying, radioactivity was measured by liquid scintillation spectrometry using Econofluor as scintillant. When the phosphorylated proteins were to be separated by PAGE following labeling with [γ-^{32}P]ATP, unless indicated otherwise, the reaction was terminated by the addition of an equal volume of electrophoresis sample buffer (4% SDS, 20% glycerol, and 0.125 *M* Tris–HCl, pH 6.8). Following heating at 80°C for 10 min, β-mercaptoethanol was added to a final concentration of 5%.

Electrophoresis was carried out essentially by the method of Laemmli (1970). Following staining (Coomassie blue R-250), destaining, and drying, ^{32}P-labeled bands were visualized by autoradiography using Kodak X-Omat AR film and kodak X-Omatic regular intensifying screens. The amount of radioactivity incorporated into electrophoretic bands was determined by cutting individual bands from gels and liquid scintillation spectrometry using Aquasol as scintillant. Protein determination was done by the methods of Peterson (1977) and Bradford (1976).

RESULTS

Extraction of NF-Associated Kinases

As a first step in the characterization of NF-associated kinase activities, we aimed to obtain a soluble kinase-rich fraction devoid of NFs. Since Toru-Delbauffe and Pierre (1983) and Wible *et al.* (1989) reported the dissociation of kinases from NFs upon treatment with salt solutions of a high ionic strength, a similar approach was adopted. Figure 1 demonstrates the protein composition and kinase activities in supernatants and pellets obtained following homogenization of the NF preparation with 0.8 *M* KCl and centrifugation. The NF subunits remain mostly insoluble and are recovered in the pellet (Fig. 1A; lane 3). The supernatant (high-salt extract) and the NF-enriched pellet do not show any significant amount of endogenous phosphorylation, except for the low molecular weight bands (Fig. 1B; 2 and 3). However, when these two fractions are

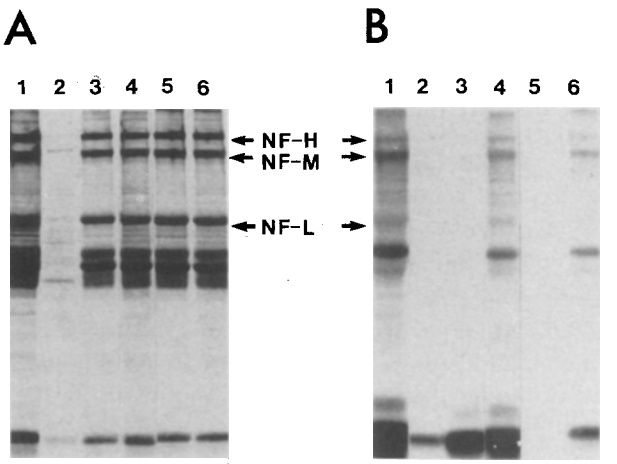

Fig. 1. Protein composition and kinase activity in supernatant and pellet following 0.8 *M* KCl extraction of the bovine NF preparation. Neurofilament preparation was homogenized in 0.8 *M* KCl and centrifuged as described under Materials and Methods. Aliquots from the original preparation and from the pellet and supernatant were incubated with [γ-^{32}P]ATP in a final volume of 200 μl, either individually or in combinations described below. The reaction was stopped by the addition of sodium deoxycholate and trichloroacetic acid, to give final concentrations of 0.012 and 6%, respectively. The precipitate was recovered by centrifugation, neutralized, and dissolved in electrophoresis sample buffer. Electrophoresis was carried out on 10% acrylamide gels. (A) Protein staining patterns; (B) autoradiographs. Lane 1, neurofilament preparation; lane 2, high-salt extract (supernatant); lane 3, pellet following salt extraction; lane 4, supernatant + pellet; lane 5, pellet incubated for 10 min in boiling water (boiled pellet); lane 6, boiled pellet + supernatant. Lane 1 corresponds to a starting amount of 50 μg protein; lanes 2–6 correspond to fractions recovered from an equivalent amount of neurofilament preparation.

coincubated, a protein phosphorylation pattern similar to that of the parent NF preparation is obtained (Fig. 1B; 1 and 4). These results suggest that a high proportion of the NF-associated kinase activity, including the activity responsible for the phosphorylation of NF subunits, is extracted by treatment of the preparation with 0.8 *M* KCl. It can be observed that the pellet also contains some endogenous kinase activity that phosphorylates the low molecular weight bands (Fig. 1B, 3). Incubation of the resuspended pellets for 10 min in boiling water inactivates the endogenous phosphorylation, while the proteins retain their capacity to be phosphorylated by the kinase activity in the high-salt extract (Fig. 1B; 3, 5, and 6). This boiled preparation was used in later experiments to test the phosphorylation of assembled NF subunits by kinase-containing fractions.

Identification of Kinase Activities in the High-Salt Extract

The high-salt extract was tested for the presence of Ca^{2+}-calmodulin-dependent, cyclic AMP-dependent, cyclic GMP-dependent kinases and of protein kinase C, as well as for its ability to phosphorylate casein. The criteria to establish the presence of regulator-dependent kinases were (1) the ability of the extract to phosphorylate specific peptides or proteins known to be good substrates for the specific kinase and (2) stimulation of phosphorylation by the activators of that kinase and inhibition of phosphorylation by specific inhibitors. The data presented in Table I indicate that the salt extract contains a significant level of Ca^{2+}-calmodulin-dependent activity, as seen by the substantial degree of phosphorylation of the specific peptide substrate in the presence of Ca^{2+} and calmodulin and the complete inhibition of the Ca^{2+}-calmodulin-stimulated phosphorylation by the calmodulin antagonist W7. The extract also contains Ca^{2+}-phosphatidylserine diglyceride- and cyclic AMP-activated protein kinases and a trace amount of cyclic GMP-dependent protein kinase activity (Table I). In addition, the extract causes substantial phosphorylation of α-casein in the absence of activators. Heparin, an inhibitor of casein kinase II [$K_i = 20\ \text{ng ml}^{-1}$ (Hathaway *et al.*, 1980)] has only a small inhibitory effect at a concentration as high as $1\ \mu\text{g ml}^{-1}$.

Resolution of a Regulator-Independent Kinase Activity by Gel Filtration

A gel filtration column (Ultragel AcA44) was employed to fractionate the kinase-rich high-salt extract. Pooled fractions from the column were assayed for their capacity to phosphorylate purified NF-M. It can be observed from Fig. 2A that the group of fractions 45–54 contains a markedly high level of kinase activity phosphorylating NF-M. Also, a smaller activity peak elutes around the void volume (Fig. 2A). When the fractions 45–54 are assayed individually, it is seen that the kinase activity elutes as a narrow peak, mainly in two fractions (Fig. 2B). These two fractions, 49 and 50, were pooled (referred to as the kinase fraction) and used in later experiments to characterize this kinase activity. When kinase assays were performed, using 2.5 μg NF-M and either kinase fraction (0.4 μg protein) or high-salt extract (5.4 μg protein) in a final volume of 50 μl, it was found that the preparation from the column constitutes a nine-fold purification of

Table I. Characterization of Protein Kinase Activities in High-Salt Extract from NF Preparation[a]

Substrate	Activators	Inhibitor	pmol P_i incorporated
Casein			9.5
Casein		Heparin	7.8
His III-S			5.0
His III-S	Ca^{2+}, PS, DG		7.9
His III-S	Ca^{2+}, PS, DG	ST	3.2
Pep sub for Ca-CaM-PK			13.6
Pep sub for Ca-CaM-PK	Ca^{2+}, CaM		150.0
Pep sub for Ca-CaM-PK	Ca^{2+}, CaM	W7	14.8
Kemptide			3.2
Kemptide	Cyclic AMP		7.0
Kemptide	Cyclic AMP	Wiptide	2.0
Pep sub for cGMP-PK			1.3
Pep sub for cGMP-PK	Cyclic GMP		2.1
Pep sub for cGMP-PK	Cyclic GMP	Pep inh	1.4

[a] Exogenous substrates were incubated with the salt extract (3.6 μg protein) in a final volume of 50 μl. Concentrations of protein and peptide substrates in incubation media were approximately equal to their reported apparent K_m values for their respective kinases as indicated below. Casein, 0.75 mg ml^{-1} (Itarte *et al.*, 1981); histone III-S, 0.6 mg ml^{-1} (O'Brian *et al.*, 1984); peptide substrate for Ca^{2+}-calmodulin-dependent protein kinase (pep sub for Ca-CaM PK), 8 μM (Pearson *et al.*, 1985); Kemptide, 15 μM (Kemp, 1976); and peptide substrate for cyclic GMP-dependent protein kinase (pep sub for cGMP PK), 22 μM (Glass and Krebs, 1982). The concentration of specific activators and inhibitors for various kinases were as follows: heparin, 1 μg ml^{-1}; $CaCl_2$, 0.5 mM when histone was used and 1 mM when peptide substrate for Ca^{2+}-calmodulin-dependent protein kinase was used; phosphatidylserine (PS), 30 μg ml^{-1}; diglyceride (DG), 3 μg ml^{-1}; staurosporine (ST), 50 nM; calmodulin (CaM), 10 μg ml^{-1}; calmodulin antagonist W7, 0.3 mM; cyclic AMP, 50 μM. Wiptide, 5 μM; peptide inhibitor for cyclic GMP-dependent protein kinase (pep inh), 200 μM. EGTA (1 mM) was included in all media that did not contain $CaCl_2$. Following incubation for 10 min at 31°C, 25-μl aliquots were blotted on phosphocellulose paper and processes as described under Materials and Methods. Values listed are the averages from two replicates.

the NF-M-phosphorylating activity relative to the high-salt extract. Calculations based on the above experiments also show that about 32% of the total NF-M-phosphorylating activity present in the high-salt extract is recovered in these two fractions from the column.

Characterization of the Kinase Fraction from the Column

Table II shows the degree of phosphorylation of different exogenous substrates following incubation with the column fraction. The conditions for the assays were the same as those described in Table I, except that the partially purified kinase activity (fractions 49–50 in Fig. 2B) was employed instead of the high-salt extract. It can be observed that, in contrast, to the parent high-salt extract, the column fraction does not cause significant phosphorylation of substrates for the Ca^{2+}- and cyclic nucleotide-dependent kinases in the presence or absence of activators. In contrast, incubation with the fraction results in substantial phosphorylation of α-casein. This phosphorylation is not inhibited by heparin at a concentration of 1 μg ml^{-1}.

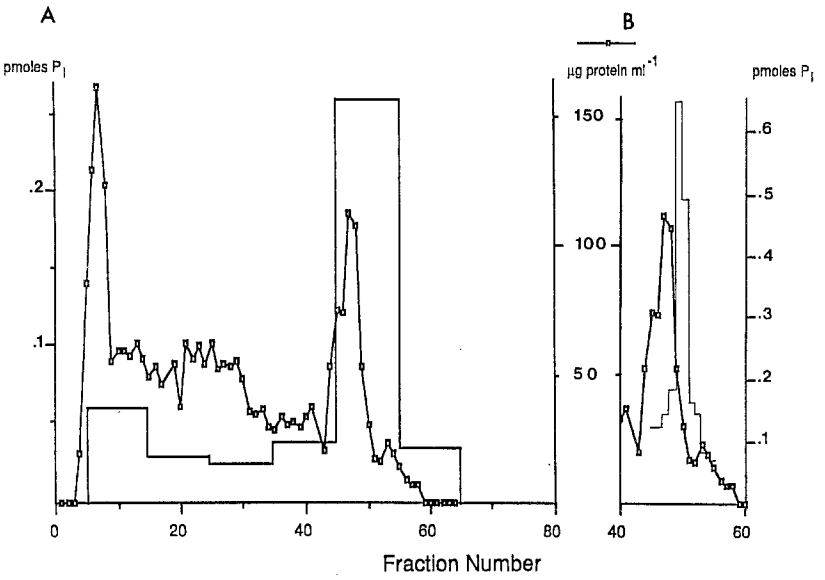

Fig. 2. Fractionation of high-salt extract on Ultragel AcA44 column: (A) Column elution profile (□) Protein content in fractions. Histogram: kinase activity in pooled fractions using NF-M as substrate. (B): High-resolution plot of kinase activity and protein profile in individual fractions 45–54, using NF-M as substrate. Kinase activity was measured as the amount of ^{32}P incorporation into 2.5 μg NF-M upon incubation with 25 μl of column fractions and [γ-^{32}P]ATP for 10 min as described under Materials and Methods. The reaction was stopped by the addition of electrophoresis sample buffer. Following electrophoresis on 10% acrylamide gels and staining, bands corresponding to NF-M were excised from the gels and the radioactivity was determined by liquid scintillation spectrometry. Note (in B) that the peak of kinase activity elutes later than the protein peak.

Table II. Characterization of Protein Kinase Activities in Fractions (49 + 50) from Gel Filtration Column[a]

Substrate	Activators	Inhibitor	pmol P_i incorporated
Casein			21.1
Casein		Heparin	20.5
His III-S			0.6
His III-S	Ca^{2+}, PS, DG		0.7
His III-S	Ca^{2+}, PS, DG	ST	0.2
Pep sub for Ca-CaM-PK			0.3
Pep sub for Ca-CaM-PK	Ca^{2+}, CaM		0.1
Pep sub for Ca-CaM-PK	Ca^{2+}, CaM	W7	0.2
Kemptide			0
kemptide	Cyclic AMP		0.1
Kemptide	Cyclic AMP	Wiptide	0.2
Pep sub for cGMP-PK			0.1
Pep sub for cGMP-PK	Cyclic GMP		0.2
Pep sub for cGMP-PK	Cyclic GMP	pep inh	0.1

[a] Exogenous substrates were incubated with the kinase preparation from the AcA44 column (0.65 μg protein) in a final volume of 50 μl. Reaction conditions, concentrations of substrates, activators, and inhibitors, and other procedural details were as described in Table I, footnote *a*.

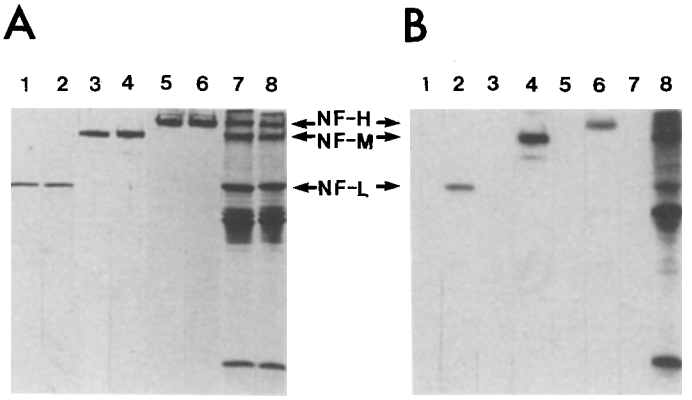

Fig. 3. Phosphorylation of NF subunits with the kinase fraction from gel filtration column. Twenty picomoles each of purified bovine NF-H, NF-M, NF-L, and aliquots from the NF pellet, boiled to inactivate endogenous kinase activities (see lane 5 in Fig. 1), were incubated with or without the kinase preparation (fractions 49 + 50; 0.4 μg protein) and [γ-^{32}P]ATP in a final volume of 50 μl for 10 min at 31°C. The reaction was stopped by the addition of electrophoresis sample buffer and the samples were run on 10% acrylamide gels. (A) Protein staining patterns; (B) autoradiographs. Lanes 1 and 2, NF-L; lanes 3 and 4, NF-M, lanes 5 and 6, NF-H; lanes 7 and 8, NF pellet. Samples represented by even numbers contain kinase preparation, whereas those represented by odd numbers do not.

In order to characterize further the regulator-independent kinase activity, casein, and phosvitin were compared for their ability to act as substrates. When 4 mg ml^{-1} casein and phosvitin were incubated with the fraction under the conditions described in Table I, the amount of phosphate incorporated into phosvitin was 20% of that incorporated into α-casein (data not shown).

Figure 3 demonstrates that the kinase fraction recovered from the column can phosphorylate NF-H and NF-L as well as NF-M, either in their purified form or in their assembled form. Also, certain other proteins present in the NF

Table III. Relative Efficiencies of Purified NF Subunits and α-Casein to Act as Substrates for Kinase Activity in Fractions (49 + 50) from Gel Filtration Column[a]

Substrate	pmol phosphate incorporated into 20 pmol of substrate	Relative incorporation
NF-H	0.20 ± 0.21	43%
NF-M	0.49 ± 0.103	100%
NF-L	0.17 ± 0.028	36%
α-Casein	0.66 ± 0.141	140%

[a] NF-H, NF-M, NF-L, and casein, each at a concentration of 0.4 μM, were incubated with [γ-^{32}P]ATP in the presence of kinase preparation from the AcA44 column (0.4 μg protein) in a final volume of 50 μl for 10 min at 31°C. ^{32}P incorporation into individual proteins was determined as described in the legend to Fig. 2. Values represent the average of four measurements ±SEM.

preparation act as substrates (Fig. 3B, lane 8). When equimolar concentrations of purified proteins are employed in the incubation medium, among the three neurofilament proteins, NF-M is the one that incorporates the highest level of phosphate (Fig. 3, Table III). Of the four protein substrates tested, the level of phosphate incorporation per mole of protein is in the following order: α-casein > NF-M > NF-H $\cong$ NF-L (Table III).

Upon incubation of NF-M with the kinase fraction up to 4 hr, a maximum incorporation of 0.36 mol of phosphate/mol of NF-M was obtained. However, it is not possible to come to a conclusion on the stochiometry of phosphorylation from the present data, since NF-M showed a steady increase in phosphorylation within the time range studied, indicating that even after 4 hr exhaustive phosphorylation was not attained (data not shown).

DISCUSSION

A major portion of NF-associated protein kinase activity can be extracted by treatment of the preparation with 0.8 *M* KCl (Fig. 1). This high-salt extract contains significant regulator-dependent and -independent kinase activities (Table I). Among the regulator-dependent kinases, Ca^{2+}-calmodulin-dependent activity appears to be most prominent. Consistent with this observation are the reports by Vallano *et al.* (1985) and Caputo *et al.* (1989) of the copurification of NFs and Ca^{2+}-calmodulin-dependent protein kinases. Taken together, these results suggest that this kinase associates with NFs. The possible physiological significance of such an association remains to be investigated.

Since the application of inhibitors of specific regulated kinases to the NF preparation does not significantly alter the pattern of the NF phosphorylation (unpublished data), we were especially interested in the regulator-independent kinase activity associated with NFs. It is probable that NF preparations contain more than one type of independent kinase. For example, the kinase purified by Wible *et al.* (1989) does not phosphorylate NF-L or purified NF-M, whereas the high-salt extract from the NF preparation does. At this point, it is interesting to note that an analysis of the published sequences of mammalian NF proteins reveals, in addition to the widely recognized KSP sequences, certain other highly conserved sequence motifs that contain serine and threonine residues (Shaw, 1989). These conserved motifs, some of which are found only in NF-M, may represent recognition sequences for other unique kinases.

In this study, we focused on the isolation of a regulator-independent kinase which could phosphorylate purified NF-M. Therefore, NF-M was employed as a substrate in the kinase assays of the AcA44 column eluants. The major peak of kinase activity that phosphorylated NF-M was further tested for the presence of regulator-dependent kinases. The data presented in Table II demonstrate that this fraction did not contain any significant Ca^{2+}- and cyclic nucleotide-dependent activities. It is known that the autophosphorylated form of Ca^{2+}-calmodulin-dependent protein kinase II, the catalytic subunit of the cyclic AMP-dependent protein kinase, and a proteolytic fragment of protein kinase C are active in the

absence of regulators. However, since this fraction did not appreciably phosphorylate any of the preferred substrates for these kinases, we concluded that the kinase fraction was also devoid of such unregulated forms of these kinases.

Among the kinase substrates tested, the partially purified fraction was able to phosphorylate casein, NF-L, NF-M, and NF-H as well as certain other proteins that are present in the NF preparation. In addition, unlike the kinase activity identified by Wible *et al.* (1989), our kinase fraction could phosphorylate NF-H following its exhaustive dephosphorylation by alkaline phosphatase (unpublished data). When equimolar quantities of purified proteins were incubated with the fraction, the degree of phosphate incorporation was in the following order: α-casein > NF-M > NF-H $\cong$ NF-L. All of the above characteristics indicate that the fraction contains a potent kinase activity which is different from that of the NF kinases described by Wible *et al.* (1989) and Toru-Delbauffe *et al.* (1986).

The NF-associated kinase activity we have partially purified cannot be identified as any known kinase. The data presented in Table II demonstrate that the proteins and peptides containing sequences specific for the Ca^{2+}-calmodulin-dependent, cyclic AMP-dependent, and cyclic GMP-dependent protein kinases do not act as substrates. Its preference for α-casein suggests that the kinase activity may be related to the family of casein kinases. However, the lack of inhibition of its activity by heparin indicates that it is not casein kinase II which is potently inhibited by this reagent. Its relationship to casein kinase I cannot be entirely ruled out at present. However, the preference of the kinase activity in the fraction for casein over phosvitin as a substrate [which is much greater than the values reported for casein kinase I (Itarte *et al.*, 1981)] would appear to argue against this possibility.

In summary, the data presented demonstrate that while the NF-enriched preparation from bovine spinal cord contains various identified regulator-dependent kinase activities, it is principally the regulator-independent kinase activity which appears to be responsible for the neurofilament phosphorylation in this preparation. We describe a partial purification of this unique kinase activity that effectively phosphorylates NF subunits. This kinase fraction is devoid of regulator-dependent kinases and should constitute an excellent starting point for the further purification of enzymes relevant to the phosphorylation of neurofilament proteins.

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