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Identification and Characterization of a Novel (115 kDa) Neurofilament-associated Kinase

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Neurofilaments (NF), the major cytoskeletal component in neuronal cells, are one of the most highly phosphorylated proteins expressed in brain. Apart from the structural role NFs play in maintaining neuronal architecture, little else is known of their function. We describe here evidence suggesting that NF may support many other proteins in the neuronal axoplasm including protein kinases. In order to isolate proteins that bind NF, we first expressed the carboxyl-terminal tail domain of the mouse high-molecular-weight NF subunit (NF-H) as a fusion protein in bacteria and then used this portion of NF-H as a ligand in affinity chromatography. A number of different proteins were isolated from mouse brain lysate that specifically bound to the NF-H column and that did not bind to a control column to which BSA was bound as a ligand. The proteins eluted from the NF-H column contained kinases able to phosphorylate NF proteins efficiently *in vitro.* We characterized these kinases further by separating proteins on denaturing polyacrylamide gels and reconstituting kinase activity *in situ.* Using this assay we identified a number of individual kinases including a 115 kDa polypeptide that showed a significant preference for NF proteins as substrate. Native NF was found to be the best substrate for the 115 kDa kinase, followed by a bacterially expressed NF-H nonfusion protein, and NF-H fusion protein. However, low-molecular-weight NF subunit (NF-L) was a poor substrate. Two different NF monoclonal antibodies, SMI31 and SMI32 (Sternberger Monoclonal Inc.), were used to demonstrate further that the 115 kDa kinase is associated with NF *in vivo.* The kinase was coimmunoprecipitated along with NF by the two NF monoclonal antibodies but appeared to be preferentially associated with phosphorylated forms of NF. We discuss here some of the novel properties of the 115 kDa NF-associated kinase we have termed NAK115 (for NF-associated kinase with a molecular weight of 115 kDa).

[Key words: neurofilaments, affinity chromatography, phosphorylation, protein kinases, immunoprecipitation]

The neuronal cytoskeleton is composed of actin filaments, intermediate filaments, and microtubules. The most abundant intermediate-filament components expressed in mature neuronal cells are neurofilaments (NF). Mammalian NF are typically composed of three subunits, NF-L (68 kDa), NF-M (160 kDa), and NF-H (200 kDa) (Hoffman and Lasek, 1975; for reviews, see Fliegner and Liem, 1991; Shaw, 1991). The three subunits differ from one another in length of their carboxyl-terminal sequences, which are modified by phosphorylation. In fact, NF-M and NF-H are among the most heavily phosphorylated proteins found in brain (for review, see Nixon and Sihag, 1991).

The major site phosphorylated in both NF-M and NF-H is the sequence Lys-Ser-Pro-Val (other repeats have either Ala/Glu/Thr/Leu in place of the Val) that is present as 5–9 and 39–52 copies, respectively, in the carboxyl-terminal regions of the molecules (see Fliegner and Liem, 1991). In fact, both molecules are phosphorylated at a molar ratio that approximates the occurrence of the repeats (Julien and Mushynski, 1982, 1983; Carden et al., 1985; Geisler et al., 1987). A number of other sequences on NF are also phosphorylated (see Xu et al., 1990, 1992; Fliegner and Liem, 1991; Nixon and Sihag, 1991). Further, there is both developmental regulation of NF expression and phosphorylation during development (Shaw and Weber, 1982; Julien et al., 1986; Carden et al., 1987; see also Fliegner and Liem, 1991; Nixon and Sihag, 1991). The consequence of phosphorylation on NF function is not known. However, in a number of neurodegenerative diseases in which neuronal structure is affected, NF phosphorylation was shown to be significantly altered (Sternberger et al., 1985; Schmidt et al., 1987; Zhang et al., 1989; Arai et al., 1990; Trojanowski et al., 1993).

There appear to be several kinases that phosphorylate NF *in vitro,* including some that copurify with NF preparations (Toru-Delbauffe and Pierre, 1983; Pant et al., 1986; Toru-Delbauffe et al., 1986; Caputo et al., 1989; Dosemeci et al., 1990; Floyd et al., 1991; Nixon and Sihag, 1991). Kinases that phosphorylate NF *in vitro* can be divided into second messenger–dependent kinases and second messenger–independent kinases. The former appear to phosphorylate the "head" domain of NF whereas the latter appear to phosphorylate the "tail" domain (for review, see Nixon and Sihag, 1991). The second messenger–dependent kinases include calcium- and calmodulin-dependent as well as cAMP- and phosphatidylserine-dependent kinases. Recently, evidence was also obtained for a role of both protein kinase A (PKA) as well as protein kinase C (PKC) in phosphorylation of NF after axotomy (Hall and Kosik, 1993).

Roder and Ingram (1991) have identified two kinases, PK36 and PK40, which phosphorylate NF-H, NF-M, and tau proteins.

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Phosphorylation of NF-H and NF-M by these kinases reduced the mobility of the NF proteins on SDS-polyacrylamide gels. PK36 and PK40 were found to be second messenger–independent kinases that did not autophosphorylate or associate with the cytoskeleton.

Another well-characterized NF kinase is an activity that had marked specificity for NF-H (Wible et al., 1989). This activity copurified with a 67 kDa doublet protein. The kinase was found to be unrelated to either PKA, PKC, Ca^{2+}/calmodulin-dependent kinase, or casein kinase I and II. It was shown to be an effector-independent kinase that did not autophosphorylate. An interesting feature of the kinase was its specificity for NF-H but not for NF-M or NF-L substrates. The specificity for NF-H was limited only for partially dephosphorylated NF-H. Thus, the kinase was unable to phosphorylate completely dephosphorylated NF-H. These results suggest that NF phosphorylation occurs in a processive manner and that multiple kinases may phosphorylate NF.

Using NF-H affinity chromatography in combination with an *in situ* gel kinase assay, we have identified a novel kinase from mouse brain extracts that appears to differ in many respects from any of the other identified NF kinases. This kinase has a molecular weight of 115 kDa, can autophosphorylate, and is found associated with NF. Furthermore, an apparently related kinase having similar properties was identified in human brain lysates, indicating that this kinase may be important in neuronal function.

Materials and Methods

Expression of NF proteins in bacteria. NF proteins were expressed in bacteria using pATH expression plasmids. Two different portions of NF-H were expressed: the tail region as a fusion protein, and almost the entire coding sequence as a nonfusion protein.

To express the fusion protein, the mouse NF-H genomic clone (Julien et al., 1988) was digested with BstY1, and the approximately 2.5 kilobase pair fragment spanning codons 458 to the stop codon, which includes almost the entire tail domain of NF-H, was ligated in frame into the BamHI site of the *trpE* expression vector pATH1 (Koerner et al., 1991). This recombinant produces a fusion polypeptide of 323 amino acids (aa) of *trpE* sequences, 11 aa of polylinker sequences, and 629 aa of NF-H sequences.

The method used to express NF-H as a nonfusion protein in bacteria has been described (Mercy et al., 1992). This clone expresses 1004 aa of rat NF-H, from codon 69 until the stop codon (see Chin and Liem, 1990; Mercy et al., 1992).

In order to express the complete NF-M polypeptide in bacteria, two DNA primers were used to amplify by the polymerase chain reaction (PCR) a specific portion of rat NF-M cDNA (a kind gift from Dr. Ronald K. H. Liem, Columbia University, NY). The forward primer began at the first base of the third codon of NF-M (5′-TACACGCTGGACTCGCT-3′) (Napolitano et al., 1987), and the reverse primer (5′-CCGCGAAGCTTGCAATGGACTCCGATC-3′) was complementary to the part of the coding strand just downstream of the stop codon. The PCR product of about 2.5 kilo-base pairs was blunt-end ligated with NruI-digested pATH10.1 plasmid DNA (Mercy et al., 1992) and recombinants containing the insert in the appropriate orientation were selected by restriction mapping. These recombinants should express the complete NF-M polypeptide as a nonfusion polypeptide.

The entire mouse NF-L polypeptide was expressed in bacteria as a nonfusion protein, as described previously (Mercy et al., 1992).

NF cDNAs under the control of the *trpE* regulatory sequences were expressed in *Escherichia coli CAG456* by addition of indole acrylic acid (IAA), essentially as described by Mercy et al. (1992).

Purification of NF proteins expressed in bacteria. Bacteria induced from a 4 liter culture were suspended in 50 ml of 25 mM Tris-HCl (pH 7.0), 5 mM EDTA, and 1 mM EGTA. Lysozyme was then added to 1 mg/ml and the suspension incubated on ice for 1 hr. Bacteria were then lysed by adding NP40 to 0.1% and urea to 6 M. The lysate was sonicated to shear DNA, which was then clarified by centrifugation at 20,000 × *g* for 20 min. The supernatant was applied to a pre-equilibrated DE52 (Whatman, Hillsboro, OR) column. The column was then washed with buffer A [6 M urea, 1 mM EGTA, 1 mM dithiothreitol (DTT), 10 mM BisTris, pH 6.5], and the proteins that bound to the column were eluted with a linear gradient of NaCl (0–0.5 M) in buffer A. This procedure was used to purify bacterially expressed NF-H fusion protein (bNF-H), the bacterially expressed NF-H nonfusion protein, and NF-L. This single DE52 chromatography procedure yielded approximately 100 mg of approximately 65% pure bNF-H protein from 4 liters of an induced bacterial culture (purity was determined by scanning the bNF-H fraction with a laser densitometer after separation by SDS-PAGE and staining with Coomassie blue; Fig. 1*C*). However, in order to purify NF-H nonfusion protein or bNF-L, additional purification was necessary.

To purify the bacterially expressed NF-H nonfusion protein, fractions from the DE52 column were dialyzed against 8 M urea and adjusted with ampholytes (pH 3–10) to 1%, and the proteins were separated by preparative isoelectric focusing using the Bio-Rad Rotofor Cell. Using this procedure, NF-H nonfusion protein fractionated free of other bacterial proteins in the pH 7.0–7.5 range of the pH 3–10 gradient that was established (see Fig. 1*E*).

bNF-L was purified by dialyzing fractions from the DE52 column against low pH buffer [150 mM NaCl, 1 mM EGTA, 1 mM DTT, and 25 mM 2-[*N*-morpholinobethanesulfonic acid (MES), pH 6.5] for 15 hr at 4°C. During this procedure bNF-L precipitated, probably because the subunits self-assembled to form filaments, which were collected by centrifugation at 100,000 × *g* for 20 min. Although some contaminating *E. coli* proteins were still present after the first centrifugation step, they were subsequently removed upon repeated disassembly (in 6 M urea) and reassembly.

The purified bacterially expressed NF proteins were dialyzed extensively against 25 mM MES, pH 6.5, 1 mM EGTA, and 1 mM EDTA prior to using the proteins for *in vitro* and *in situ* gel kinase assays.

Preparation of mouse brain extract. Approximately 25 gm of CD1 mouse brain tissue was homogenized in extraction buffer (100 mM KCl, 0.3% NP40, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM aprotonin, 1 mM leupeptin, 1 mM bactricin, and 50 mM HEPES, pH 7.5). The lysate was centrifuged at 40,000 × *g* for 30 min to remove particulate material, and the supernatant adjusted with buffer so that the final concentrations of KCl and NP40 were 50 mM and 0.15%, respectively. This extract was used to isolate NF-binding proteins by affinity chromatography.

Isolation of NF-binding proteins by affinity chromatography. Purified bacterially expressed NF-H fusion protein (10 mg) and BSA (10 mg; Sigma) were each covalently coupled to 12 ml of activated CH-Sepharose 4B (Pharmacia) according to the coupling instructions provided by the manufacturer. The Sepharose-coupled protein matrices were packed into two columns. The columns were washed with 1 M ethanolamine (pH 8.0) to inactivate remaining CH-Sepharose binding sites. Extract buffer containing 5% BSA, 50 mM KCl, and 0.1% NP40 was then circulated over both columns to block further the nonspecific protein binding sites. Mouse brain extract was then pumped over the two affinity columns overnight. The eluate from the columns was collected into a common reservoir and was circulated over both columns repeatedly (Fig. 2*A*). The columns were then washed extensively, until no trace of protein was detected in the eluate by UV absorbance at 280 nm. Proteins bound to the columns were then eluted with 10 ml portions of the following solutions made in extract buffer containing 3 mM $MgCl_2$: (1) 5 mM ATP, (2) 100 mM KCl, (3) 300 mM KCl, (4) 500 mM KCl, and (5) 1 M KCl. The fractions eluted from the columns were adjusted by addition of glycerol to 50% and stored at −85°C. Portions of the fractions were either analyzed by SDS-PAGE, immunoblotted, or assayed for kinase activity by *in vitro* and *in situ* gel kinase assays as described below.

SDS gel electrophoresis and immunoblotting. Protein samples were boiled in Laemmli loading buffer containing 2% 2-mercaptoethanol for 10 min and separated by electrophoresis through 6.5–10% SDS polyacrylamide gels (Laemmli, 1970). The fractionated proteins were either visualized by staining gels with Coomassie blue, or in some cases by silver (Morrissey, 1981). For immunoblotting, gels after electrophoresis were transferred to nitrocellulose filters by electroblotting and incubated for 17–20 hr at 4°C with appropriate antibodies in a solution containing 5% Carnation nonfat milk in TBS buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl). After incubation the filters were washed with 0.2% NP40, 0.1% milk in TBS buffer. Filters that were reacted with mouse monoclonal antibodies were further incubated for 2 hr at 25°C with rabbit

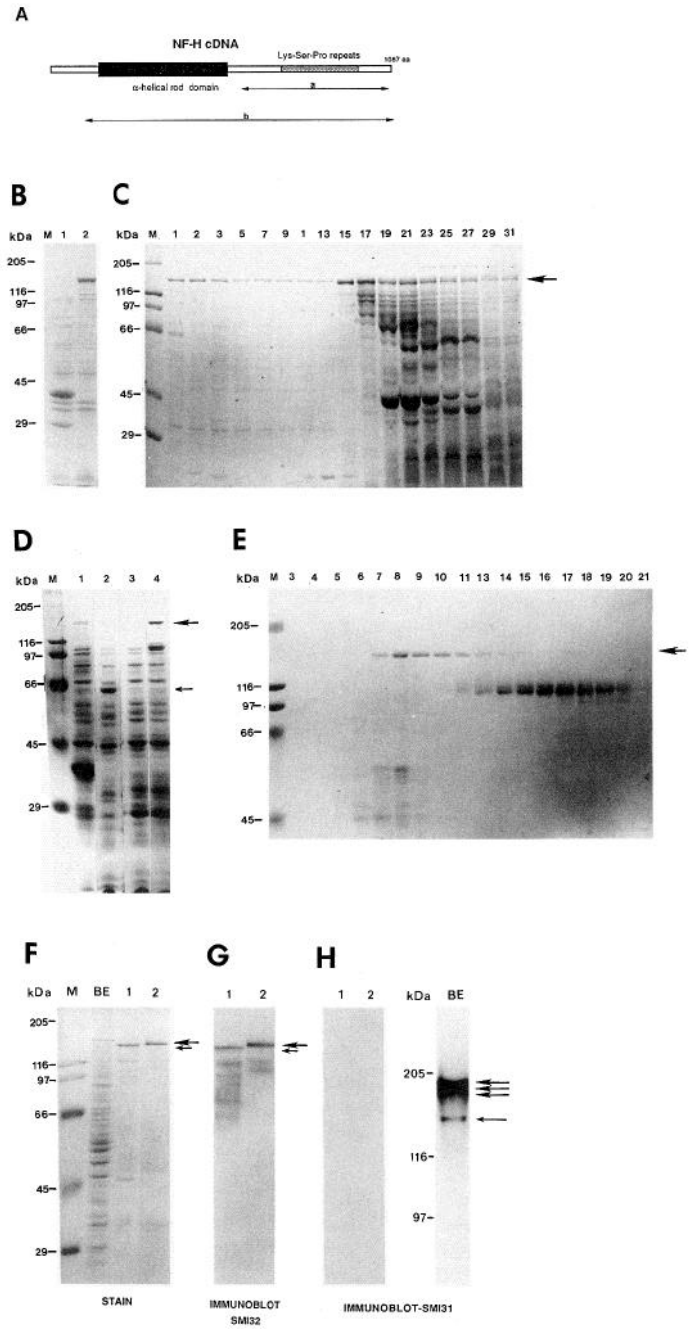


Figure 1. Structure and expression of NF genes in bacteria. *A,* Schematic structure of the mouse NF-H cDNA, indicating the two segments expressed in bacteria, as a fusion protein (*a*) and as a nonfusion protein (*b*) (see Materials and Methods). *B,* Fragment *a* was cloned in-frame with *trpE* gene in pATH1 and the resulting recombinant plasmid DNA was transformed into bacteria CAG456. Bacteria transformed with this plasmid were treated for 5 hr with IAA to induce expression of the trpE–NF-H fusion polypeptide. After induction bacterial proteins were separated by SDS-PAGE through an 8.5% gel and identified by Coomassie blue staining. *Lane M,* protein molecular weight standards; *lane 1,* bacterial proteins from a culture transformed with a control plasmid pATH 10 (induce expression of trpE, 37 kDa); *lane 2,* bacteria transformed with pATH1–NF-H express an approximately 150 kDa bNF-H fusion protein. *C,* bNF-H fusion protein was induced from a 4 liter culture and the bacterial lysate applied to a DE52 anion-exchange column. Proteins that bound to the column were eluted with a salt gradient (0–0.5 M NaCl). A 1/500 portion of the proteins eluted in alternate fractions of the column (*numbered*) was separated through an 8.5% SDS polyacrylamide gel and stained with Coomassie blue. *M,* protein molecular weight standards. The position of the NF-H fusion protein is marked with an *arrow.* Please note that *fraction 15* contained almost 100 mg of approximately 65% pure bNF-H. This fraction was used to prepare the NF-H affinity column (see Materials and Methods). *D,* Expression of nonfusion NF proteins in bacteria. NF-L, NF-M, and NF-H cDNAs were cloned into pATH expression plasmids (see Materials and Methods). After induction bacterial proteins were separated by SDS-PAGE through an 8.5% gel and stained with Coomassie blue. *Lane M,* protein molecular weight standards; *lane 1,* bacteria transformed with control pATH10 plasmid DNA induce expression of a 37 kDa trpE polypeptide; *lane 2,* bacteria transformed with pATH10.1-NF-L (1–645 aa) plasmid DNA express the complete NF-L polypeptide as a nonfusion protein of the expected size (approximately 68 kDa; marked by the *small arrow*); *lane 3,* bacteria transformed with pATH2.1-NF-M (1–845 aa) express little if any NF-M nonfusion protein; *lane 4,* bacteria transformed with pATH2.1-NF-H (69–1072 aa) express nonfusion NF-H protein of approximately 155 kDa (marked with the *large arrow*). *E,* Purification of NF-H nonfusion protein. Fractions containing NF-H nonfusion protein from the DE52 column were dissolved in 8 M urea and 1% ampholytes, pH 3–10, and subjected to isoelectric focusing using the Bio-Rad Rotofor Cell. Fractions collected after focusing were analyzed on an 8.5% SDS-PAGE gel after staining with Coomassie blue. The position of NF-H nonfusion protein is indicated with an *arrow. F–H,* The purified NF-H nonfusion and fusion proteins were separated by SDS-PAGE and immunoblotted with either phosphorylation-specific or nonphosphorylation-dependent NF antibody, SMI31 or SMI32, respectively. *Lane M,* protein molecular weight standards; *lane BE,* mouse brain extract; *lane 1,* purified bNF-H fusion protein; *lane 2,* purified NF-H nonfusion protein. *F,* Coomassie blue–stained gel. *G,* Immunoblot of proteins showing reaction of the bacterially expressed NF proteins with SMI32 antibody that recognizes nonphosphorylated NF-H protein. *H,* Immunoblot showing SMI31 antibody, which recognizes phosphorylation-dependent epitopes on NF-H, fails to recognize the bacterially expressed NF-H proteins. This antibody, however, recognizes both NF-H and NF-M proteins in mouse brain lysate (*lane BE*), both of which contain the Lys-Ser-Pro repeat sequences known to be part of the epitope recognized by this antibody. Please note that *lane BE* in *H* was fractionated on a 6.5% SDS-PAGE gel to resolve the different phosphorylated forms of NF-H that migrate as three major forms of about 190–205 kDa. The fastest-migrating form (approximately 160 kDa) that is recognized by SMI31 is probably NF-M.

anti-mouse IgG (Organon Tekinika-Cappel, Durham, NC) in TBS buffer containing 5% milk and subsequently washed as described above. All filters were finally reacted for 1–2 hr at 25°C with ^{125}I-labeled *Staphylococcus aureus* protein A in wash buffer. The filters were then washed and exposed to x-ray film (Kodak X-Omat AR film) at −85°C. Primary antibodies used for immunoblotting were SMI32 mouse monoclonal anti-nonphosphorylated neurofilaments ascites (Sternberger Monoclonals, Inc., Baltimore, MD), SMI31 mouse monoclonal anti-phosphoneurofilaments ascites (Sternberger Monoclonals), and a rabbit polyclonal anti-pan protein kinase C antibody (Upstate Biotechnology, Inc., Lake Placid, NY).

In vitro *and* in situ *kinase assays. In vitro* kinase assays were preformed in a 100 μl reaction volume in a test tube. Typically, 1–5 μl volumes of mouse brain lysate or individual affinity-purified fractions were assayed for kinases able to phosphorylate 10–20 μg of different NF proteins upon incubation in 20 mM MES (pH 6.5), 10 mM $MgCl_2$, 10 mM β-glycerophosphate, 10 mM EGTA, and 1 mM γ-^{32}P-ATP for 3–10 hr at 30°C. After the reaction, NF proteins were immunoprecipitated (see below) and separated by SDS-PAGE. Native NF were not immunoprecipitated but separated directly by SDS-PAGE. Phosphorylation of NF proteins was determined by first staining the gels with Coomassie blue to identify the NF proteins; the gels were then dried and exposed to x-ray film. Autoradiograms were correlated with the stained gels in order to determine phosphorylation of the appropriate size NF proteins. Phosphorylation was measured by scanning the radioactive protein bands with a laser phosphoimager (Molecular Dynamics Phosphoimager).

The *in situ* gel kinase assay was performed essentially as described by Geahlean et al. (1986). In this procedure, 1–30 mg of NF or casein protein was added to the resolving SDS polyacrylamide gel before polymerization. Samples that were assayed for kinase activity were boiled and separated by electrophoresis using the standard SDS-PAGE pro-

cedure (see above). After electrophoresis the gel was incubated in 6 M guanidine-HCl for 1 hr at 20°C to denature proteins. The denaturant was then removed and the proteins slowly renatured by several changes with 20 mM MES (pH 6.5) and 5 mM 2-mercaptoethanol at 4°C for at least 16 hr. After renaturation, the gel was preincubated in 20 mM MES (pH 6.5), 10 mM $MgCl_2$, 10 mM β-glycerophosphate, 10 mM EGTA, and 1 mM DTT (KA buffer) at 30°C for 30 min. The buffer was then exchanged for fresh buffer containing 250–500 μCi of radioactive γ-^{32}P-ATP (3000 Ci/mmol; Amersham Corp., Arlington Heights, IL) and incubated for 2 hr at 30°C with gentle shaking. Cold ATP was added to a final concentration of 1 mM and the incubation was continued for a further 2 hr. The gel was then washed extensively with 5% trichloroacetic acid (TCA), stained with Coomassie blue, dried, and exposed to x-ray film. The radioactivity incorporated into individual bands was measured using the laser phosphoimage scanner.

Immunoprecipitation. A 5 ml portion of Pansorbin cells (Calbiochem, La Jolla, CA) was first homogenized in 20 ml of PBSE (standard phosphate buffered saline containing 1 mM EDTA) containing 5% (w/v) BSA. The cells were then collected by centrifugation, washed twice with 20 ml of PBSE containing 0.5% NP40, and incubated with 2.0 mg of affinity-purified rabbit anti-mouse IgG (Organon Tekinika-Cappel, Durham, NC) in 10 ml of PBSE at 25°C for 30 min. The cells were washed again with PBSE containing 0.5% NP40, and finally resuspended in 5 ml of PBS and stored at −85°C. A 0.1 ml aliquot of this suspension was diluted with 1 ml of PBS and NF antibodies were then allowed to bind to the cell complex for 1.5 hr by adding appropriate volumes (1–5 μl) of SMI31 antibody, SMI32 antibody, or a rabbit polyclonal antibody that we had raised using purified bNF-H as immunogen. The cells were collected by centrifugation, washed, and then resuspended in 100 μl of PBSE containing 0.5% NP40 and incubated for 20 hr at 4°C with 5–10 μl volumes of extracts from which NF protein precipitation was sought. Immunoprecipitation of native NF was performed using 5 μl of a freshly prepared sample of mouse brain lysate (15 μg/μl). After incubation the cells were collected by centrifugation. The pellet was then washed thrice and finally resuspended in 100 μl of distilled water. Equal fractions of both the supernatant and pellet suspensions were separated by SDS-PAGE and analyzed for distribution of NF proteins by immunoblotting or for cofractionation of kinases by the *in situ* gel kinase assay.

Results

Since NF are the major cytoskeleton component in neurons we reasoned that they may act as a structural support for other functional proteins in the axon. Using affinity chromatography we demonstrate that many proteins do indeed bind to NF.

To isolate NF-binding proteins we used the affinity chromatography procedure that proved useful for isolating actin-binding proteins (Miller and Alberts, 1989; see also Miller et al., 1989) as well as microtubule-binding proteins (Kellogg et al., 1989). However, we were concerned that the procedure would yield a more complex mixture of NF-binding proteins because NF are much more irregular in structure compared to actin filaments or microtubules. We therefore concentrated on identifying proteins that bind to specific domains of NF. In this approach the tail domain of NF-H was first expressed as a recombinant protein and then used as an affinity ligand to purify NF-binding proteins.

Expression of the carboxyl-terminal domain of NF-H in bacteria

We chose to identify proteins that associate with the carboxyl-terminal domain of NF-H since this region has been implicated in cross-bridging NF and also contains the Lys-Ser-Pro repeat that is the major NF motif phosphorylated *in vivo*. Thus, the DNA fragment encoding the carboxyl-terminal sequences of mouse NF-H (see Fig. 1*A*), containing all of the Lys-Ser-Pro repeat sequences, was expressed as a *trpE*-fusion polypeptide in bacteria (Fig. 1*B*; see Materials and Methods). As expected, the fusion protein appeared to be nonphosphorylated since it reacted strongly with SMI32 (Fig. 1*G*), a monoclonal antibody that reacts with dephosphorylated NF-H sequences (see Sternberger and Sternberger, 1983; Lee et al., 1988; Chin and Liem, 1990). The inability of bacterial kinases to phosphorylate Lys-Ser-Pro sequences of NF-H was shown by absence of reactivity with SMI31 (Fig. 1*H*), an antibody that requires phosphorylation of the serine residue of the Lys-Ser-Pro motif for its recognition (Lee at al., 1988). We will henceforth refer to this fusion polypeptide as bNF-H (for bacterially expressed NF-H fusion protein).

The fusion protein was purified by DEAE ion-exchange chromatography (Fig. 1*C*). The bNF-H fusion protein was identified both on the basis of its molecular weight on SDS gels (Fig. 1*F*; ≈150 kDa) and by its recognition with the NF-H antibody, SMI32 (Fig. 1*G*).

Isolation of NF-binding proteins by affinity chromatography

To isolate NF-binding proteins we prepared an affinity column onto which approximately 10 mg of purified bNF-H protein was covalently bound (see Fig. 2*A*). A control column was prepared, onto which 10 mg of BSA was bound. Next a mouse brain extract was circulated over both columns (see Fig. 2*A* and Materials and Methods). The columns were washed until no trace of protein was detected by UV absorbance in the flow through. Proteins bound to the columns were then eluted with a series of buffers over a range of salt concentrations (see Materials and Methods).

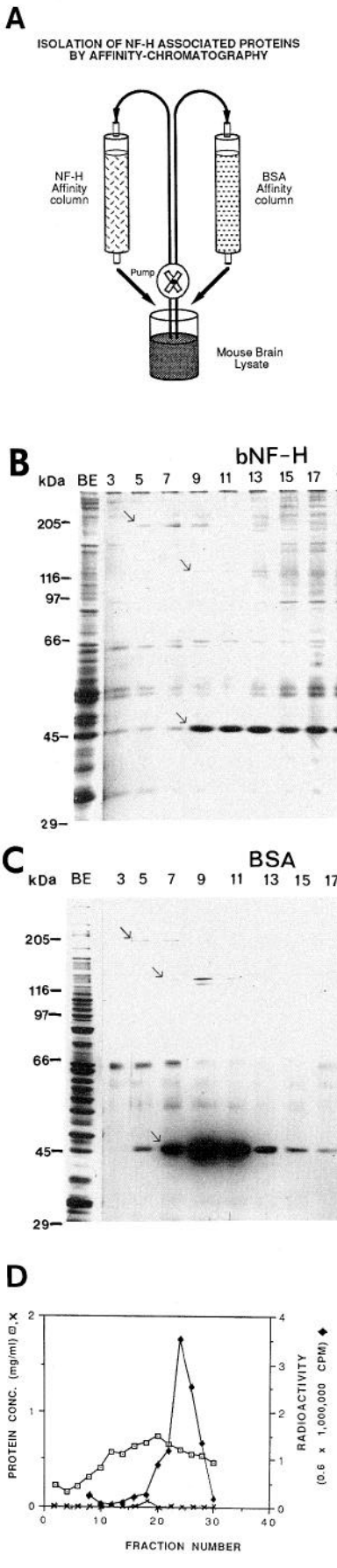
The complexity of proteins eluted was examined by silver staining following separation by electrophoresis through an 8.5% SDS polyacrylamide gel (see Fig. 2*B, C*). A significant number of proteins were eluted from the bNF-H column (see Fig. 2*B*). These proteins appeared to show specificity for binding to bNF-H since they were not eluted from the control BSA column. In fact, relatively few proteins were eluted from the BSA column (Fig. 2*D*). The latter were also eluted from the bNF-H column and thus were probably nonspecifically bound.

Proteins that specifically bound to the bNF-H column showed differences in their elution patterns, indicating a variety of binding affinities (see Fig. 2*B*). Few if any were eluted with ATP buffer (fractions 7–10). A small group was released with low salt (0.1–0.3 M; fractions 11–18) but the major group was eluted with medium salt (0.5 M; fractions 19–21). Interestingly, some proteins were eluted with high salt (>0.8 M; fractions 22–29), which has been shown to be required to remove kinases from NF (Toru-Delbauffe and Pierre, 1983; Dosemeci et al., 1990).

It was difficult to determine the exact enrichment of each eluted protein after affinity chromatography since they have yet to be purified or characterized. However, it was clear that certain proteins were enriched upon affinity chromatography compared to their relative abundance in the original mouse brain extract. For example, the approximately 90 and 58 kDa proteins (marked with arrows in Fig. 2*B*) were hardly detectable in mouse brain extracts, but were substantially enriched after affinity chromatography.

NF-binding proteins are enriched in protein kinases

Since a number of previous studies (see introductory remarks) had indicated that protein kinases copurify with NF, we investigated whether kinases were present in our NF affinity-purified fractions. We demonstrate that kinase activity was indeed present and moreover was significantly enriched upon affinity chromatography.



We used an *in vitro* assay containing bNF-H as substrate and radioactive γ-^{32}P-ATP to measure kinase activity present in crude mouse brain homogenate, the mouse brain extract loaded onto the columns (the homogenate after centrifugation at 45,000 × *g*), and alternate fractions eluted from the bNF-H column. After the reaction the products were precipitated with TCA and the radioactivity incorporated determined by scintillation counting.

The profile of ^{32}P TCA-precipitable counts indicated that kinase activity was indeed present in the NF affinity-purified fractions (Fig. 2*D*). Furthermore, the majority of this activity was present in fractions eluted with medium–high salt (>0.5 M; Fig. 2*D*). In fact, when compared with crude mouse homogenate, kinase activity was found to be enriched approximately 200-fold after affinity chromatography (compare fraction 24, 3,500,000 ^{32}P TCA precipitable cpm/μg protein, with kinase activity in crude homogenate, 17,000 cpm/μg protein).

Substrate specificity of NF-binding kinases

We next determined whether kinases recovered by affinity chromatography had any substrate preference for NF. Using a number of different substrates we demonstrated that the affinity-purified kinases are able to phosphorylate both native NF and bacterially expressed NF, although the former was phosphorylated to much higher stoichiometry.

The affinity-purified fractions were assayed for phosphorylation of purified bNF-H protein using a standard *in vitro* kinase assay (Fig. 3*A*; see Materials and Methods). Thus, kinase activity of equivalent amounts of protein, in alternate fractions eluted from the NF-H column, was compared with that found in unfractionated mouse brain extract. After the assay, bNF-H was immunoprecipitated and phosphorylation of the approximately 150 kDa bNF-H molecule was determined by phosphoimage laser densitometry of the appropriate ^{32}P radioactive bands following separation on SDS-polyacrylamide gels.

It was clear from this experiment that kinases capable of phosphorylating bNF-H were indeed recovered by affinity chromatography (see Fig. 3*A*). In fact, the peak affinity-purified fraction (fraction 23) contained as much as 18-fold more bNF-H kinase activity than unfractionated brain extract. The ability of

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Figure 2. Purification of NF-H–associated proteins by affinity chromatography. *A*, Schematic drawing of the isolation of NF-associated proteins. Purified bNF-H and BSA were each coupled to activated CH-sepharose to form two different affinity columns. Mouse brain lysate was then circulated over the columns and proteins that bound to the columns were eluted with different buffers. *B* and *C*, Mouse brain proteins that were eluted in different fractions (*numbered*) from the two affinity columns were separated by SDS-PAGE through an 8.5% gel and the gel was stained with silver. *B*, bNF-H affinity-purified proteins. *C*, BSA affinity-purified proteins. *BE*, mouse brain extract. The conditions used to elute the fractions were as follows: *fractions 3–7*, wash buffer; *fractions 7–10*, 5 mM ATP; *fractions 11–14*, 0.1 M KCl; *fractions 15–18*, 0.3 M KCl; *fractions 19–21*, 0.5 M KCl; *fractions 22–29*, 1 M KCl. Proteins that were specifically eluted from the NF-H column are marked with the *thick arrows*, and proteins that appeared to elute from both columns with the *thin arrows*. Please note that gel containing BSA eluted proteins was developed longer to stain the small quantity of proteins that bound to this column (see protein elution profile in *D*). *D*, Amount of protein (mg/ml) eluted in individual fractions from the bNF-H (□) and BSA columns (×), respectively. Equal samples of protein in alternate fractions (◆) eluted from the bNF-H column were assayed for kinase activity in an *in vitro* assay and the precipitable radioactive ^{32}P counts were determined.

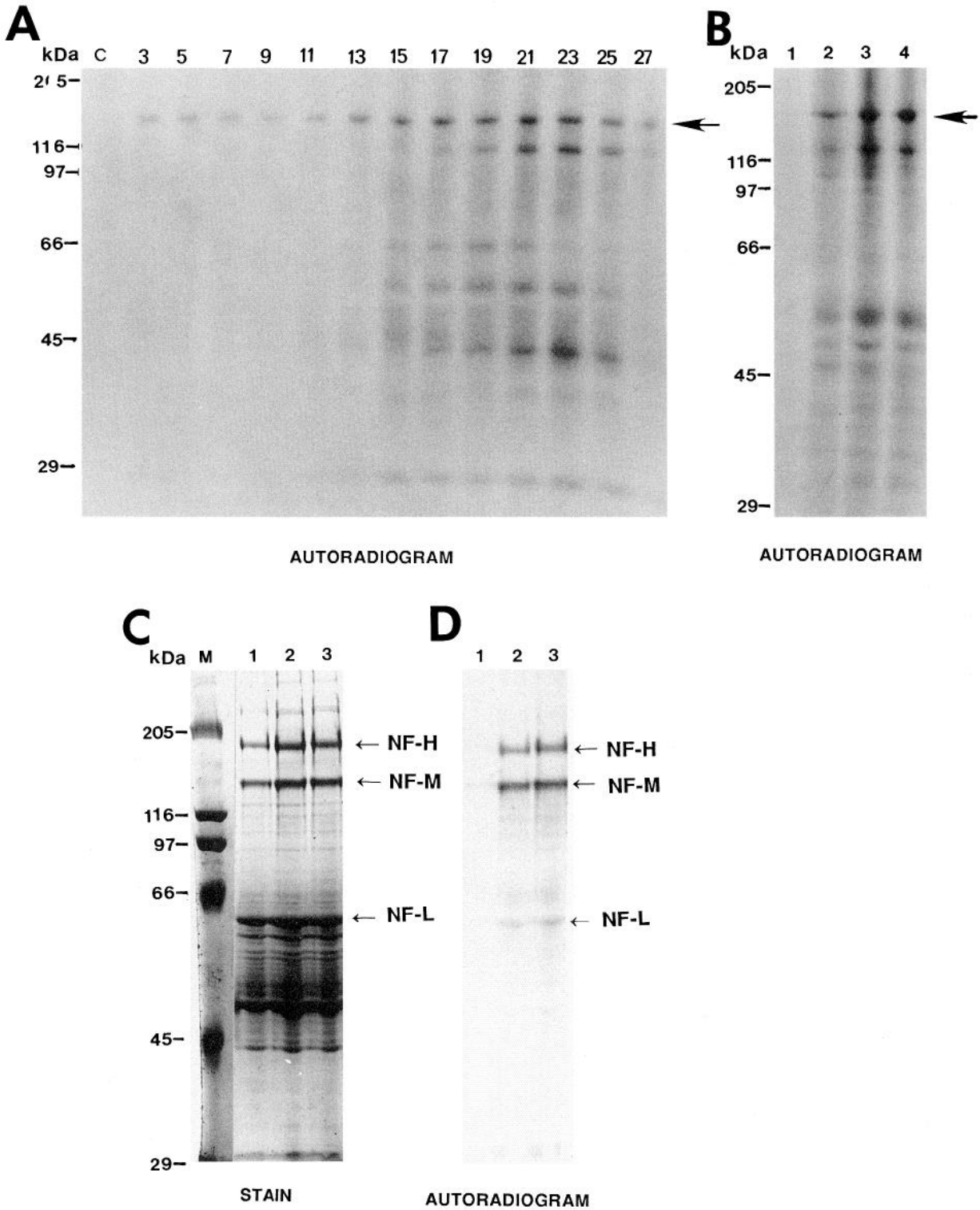

Figure 3. NF-H affinity-purified mouse brain proteins are enriched for NF kinases. Equal amounts of protein of a number of affinity-purified bNF-H fractions were assayed for phosphorylation of both native and bacterially expressed NF proteins *in vitro* (see Materials and Methods). *A,* Purified bNF-H fusion protein was used as substrate for this assay. After the assay, bNF-H was immunoprecipitated with a rabbit polyclonal antibody and the pellet fractions separated by SDS-PAGE on an 8.5% gel. The gel was stained with Coomassie blue to identify bNF-H (position is indicated with an *arrow*), dried, and exposed to x-ray film. The autoradiogram shows the pattern of bNF-H phosphorylation by selected fractions (*numbered*). *Lane C,* kinase activity present in mouse brain homogenate before affinity chromatography. *B,* This autoradiogram was prepared as in *A* except that the substrate used here was purified bacterially expressed nonfusion NF-H protein. The phosphorylation of this protein is indicated by the *arrow.* The fractions assayed for kinase activity were as follows: *lane 1,* mouse brain homogenate; *lanes 2–4,* fractions 20, 22, and 24, respectively. *C,* Native rat NF preparation was used as a substrate to assay the affinity-purified fractions for phosphorylation of individual NF subunits. After the assay the reaction mixture was separated by SDS-PAGE on an 8.5% gel. The migration of NF-H, NF-M, and NF-L (indicated) was then determined by Coomassie blue staining. *Lane M,* protein molecular weight standards; *lane 1,* incubated with total mouse brain extract; *lane 2,* incubated with fraction 22; *lane 3,* incubated with fraction 24. *D* is the autoradiogram of the gel shown in *C.*

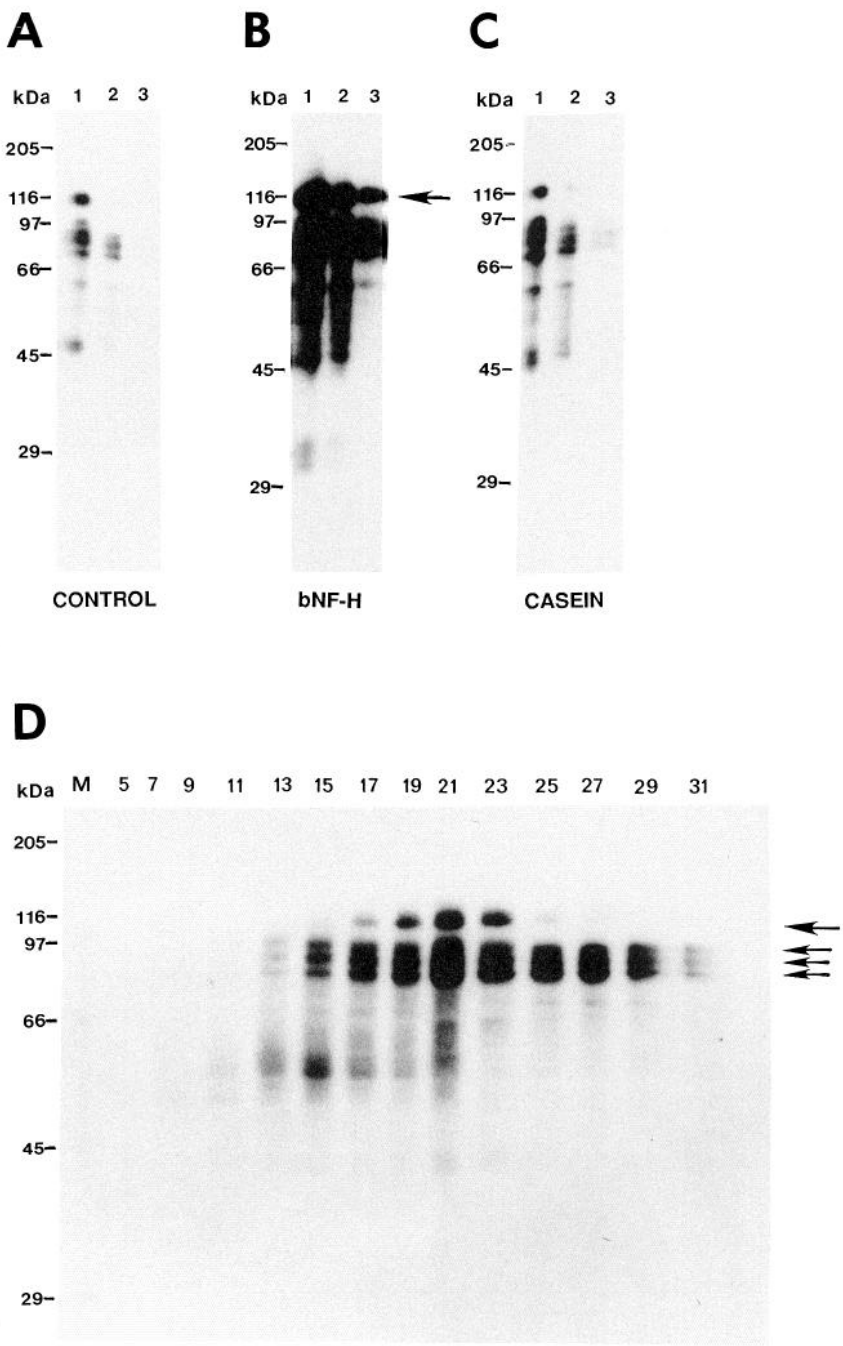

Figure 4. Identification and characterization of individual protein kinases by the *in situ* gel kinase assay. *A–C,* Equal amounts of protein of three NF-H affinity-purified fractions were each resolved by SDS-PAGE through three different 8.5% polyacrylamide gels. After electrophoresis the gels were treated in a series of solutions to renature the proteins and to identify polypeptides with kinase activity (as described in Materials and Methods). After the *in situ* kinase assay the gels were washed, stained with Coomassie blue, dried, and exposed to x-ray film. *Lanes 1–3* contain proteins from the affinity-purified fractions 20, 22, and 24, respectively. *A,* Autoradiogram of standard SDS-PAGE gel in which no additional protein was polymerized into the gel matrix. *B,* Autoradiogram of the gel in which bNF-H protein (1 mg/ml) was polymerized into the gel matrix. *C,* Autoradiogram of gel in which casein (1 mg/ml) was polymerized into the gel matrix. The *arrow* indicates a 115 kDa protein kinase, which we have termed NAK115, that showed the greatest increase in ^{32}P incorporation due to inclusion of bNF-H in the gel matrix. *D,* Profile of elution of individual kinases by NF-H affinity chromatography. Alternate fractions eluted from the bNF-H column were separated by SDS-PAGE on an 8.5% gel containing bNF-H protein (0.5 mg/ml), and kinases were identified by the *in situ* gel assay (see above). The autoradiogram shows the profile of kinases in fractions 5–31 (*numbered*) that were eluted from the NF-H column. Please note that these fractions contain four major kinases, a 115 kDa protein (*large arrow*) and three smaller proteins of approximately 95, 89, and 84 kDa (*small arrows*) that eluted differently from the column. *Lane M* contains protein molecular weight standards.

these kinases to phosphorylate bNF-H, which is not phosphorylated when expressed in bacteria (see Figs. 1*G,H*; 3*A,B*), is in contrast to the NF-H kinase isolated by Wible et al. (1989), which was unable to phosphorylate completely dephosphorylated NF-H.

Since phosphorylation of NF-H was previously shown to be affected by its prior phosphorylation state, we investigated if native NF-H would be a more effective substrate than bNF-H for phosphorylation by affinity-purified kinases. Thus, we used a purified rat spinal cord NF preparation, containing all three NF subunits, as substrate to assay the affinity-purified fractions for NF kinase activity. After phosphorylation *in vitro,* the NF subunits were separated by SDS-PAGE and identified by Coomassie blue staining (Fig. 3*C*), and their corresponding phosphorylation was determined by autoradiography (Fig. 3*D*) and phosphoimage analysis (data not shown).

It was clearly evident from this analysis that affinity-purified kinases were able to phosphorylate efficiently not only NF-H but apparently all three NF subunits. Interestingly, tubulin and microtubule-associated proteins present in this preparation were phosphorylated poorly by the kinases. Further, partial dephosphorylation of rat NF with alkaline phosphatase did not result in any detectable difference in phosphorylation of the NF-H and NF-M subunits (there was a modest increase in NF-L phosphorylation), suggesting that native NF contain many sites that are accessible to phosphorylation (data not shown). Phosphor-

ylation was accounted for entirely by the exogenously added kinases since heat-treated NF did not contain any detectable phosphorylation activity (data not shown).

Native NF-H was found to be phosphorylated approximately 35-fold more efficiently than bNF-H, suggesting that the latter may indeed be compromised in its ability to be phosphorylated by the kinases (data not shown).

In vivo, the three NF subunits NF-H, NF-M, and NF-L are phosphorylated at approximately 40, 15, and 3 sites, respectively (see introductory remarks). It was therefore of interest to compare NF-H phosphorylation with that of the other two NF subunits. For this comparison the ^{32}P counts incorporated into each protein were calculated relative to the molar concentration of each subunit in the NF preparation (the molar concentration of the three subunits in this NF preparation was 1:2:4, for NF-H:NF-M:NF-L, similar to that described by others) (Scott et al., 1985). Interestingly, NF-M was phosphorylated approximately twofold greater than NF-H. This phenomenon has also been found by others studying NF phosphorylation *in vitro* (see Toru-Delbauffe and Pierre, 1983). As expected, NF-L was phosphorylated the least, in agreement with the relatively few phosphorylation sites found on this molecule. These results provided strong evidence that the affinity-purified fractions contained kinases capable of phosphorylating NF.

Identification of novel NF-associated kinases by in situ *kinase assay*

The above experiments did not distinguish whether multiple kinases were isolated by NF affinity chromatography. In this section we describe experiments designed to resolve these kinases.

A powerful method for separating and characterizing protein kinases is an *in situ* gel kinase assay developed by Geahlen et al. (1986; see also Hutchcroft et al., 1991). This method can detect individual kinases after separation on SDS-polyacrylamide gels. Further, since it is possible to trap different protein substrates in the SDS-PAGE gel matrix (Geahlen et al., 1986; Hutchcroft et al., 1991), this method further enables detection of kinases that phosphorylate different protein substrates.

We used the *in situ* gel assay to characterize kinases present in the NF-H affinity-purified fractions. A portion of lysate of three column fractions (fractions 20, 22, and 24) were separated on three 8.5% SDS-polyacrylamide gels that differed with respect to the gel matrix used. The control gel was a standard polyacrylamide gel. The second gel contained bNF-H fusion protein polymerized in the gel matrix. The third gel contained dephosphorylated casein polymerized in the gel matrix. After electrophoresis the three gels were processed for kinase activity using γ-^{32}P-ATP as a substrate (see Materials and Methods). The autoradiograms revealed that the affinity-purified fractions contained multiple polypeptides with kinase activity (Fig. 4). At least 15 polypeptides could be detected in fraction 20 in longer exposures of the autoradiogram. The proteins are true kinases and not ATP-binding proteins by the following criteria. First, cold ATP did not compete the radioactive ^{32}P from the gels. Second, *in situ* gels incubated with α-^{32}P-ATP did not bind any detectable radioactivity (data not shown).

The number of kinases detected in the three gels was somewhat similar (compare Fig. 4*A–C*). Thus, the pattern of phosphorylated proteins in the gel to which no protein was added was weak but similar to the gels into which proteins had been polymerized. This indicates that most of the kinases were able to autophosphorylate. It is also possible, although unlikely, that phosphorylation of a comigrating protein could account for the phosphorylation seen in the control gel. However, in contrast to this low level of phosphorylation, kinase activity was increased significantly in the gel into which bNF-H fusion protein was coupled into the gel matrix (Fig. 4*B*) and to a lesser extent in the gel containing casein (Fig. 4*C*).

To quantify the increase in kinase activity, a laser phosphoimage scanner was used to measure the radioactivity of each major band in the three gels. The results revealed that a 115 kDa kinase showed the most significant difference between gels. ^{32}P incorporation in this band was increased at least 25-fold and 2-fold in the bNF-H and casein gels, respectively. This indicated the 115 kDa kinase demonstrated a greater specificity for bNF-H as substrate compared to casein. Although we cannot exclude the possibility of a specific increase in autophosphorylation upon addition of bNF-H, it seems more likely that the increase is due to direct phosphorylation of bNF-H in the gel. Moreover, other kinases had only a two- to fivefold increase in the gels, further suggesting that the 25-fold increase was specific for the 115 kDa kinase.

Using the *in situ* assay and bNF-H as substrate we next determined the elution profile of kinases from the bNF-H column. It was clear from this analysis that many kinases with different elution profiles were recovered by affinity chromatography (Fig. 4*D*). The most prominent activities detected were those associated with a 115 kDa polypeptide and three smaller polypeptides with molecular weights of approximately 95, 89, and 84 kDa (Fig. 4*D*). Interestingly, both the 115 kDa kinase and the three smaller kinases were eluted with a peak salt concentration of 0.5–0.7 M. However, in contrast to the 115 kDa kinase, the three smaller kinases were not completely eluted even with 1 M salt. This complex elution profile suggests that this latter group of kinases may have multiple and variable binding affinities.

The molecular weights of the four prominent kinases detected by the *in situ* assay are substantially different from any of the kinases thought to phosphorylate NF, casein kinase I (Floyd et al., 1991), casein kinase II (Link et al., 1992), calcium/calmodulin-dependent kinase (Vallano et al., 1986), cdc or cdk2 kinases (Hisanaga et al., 1991), erk kinases (Drews et al., 1992), the 67 kDa kinase doublet described by Wible et al., (1989), or the 36 and 40 kDa kinases described by Roder and Ingram (1991). The size and heterogeneity of the kinases were, however, similar to PKC isoforms. We thus, tested for the presence of PKC by immunoblot analysis using a polyclonal antibody specific for PKC. PKC immunoreactivity, as expected, was detected in crude mouse homogenate, but was not detected in any of the affinity-purified fractions (data not shown). These results suggest that PKC does not bind to the carboxyl-terminal sequences of NF-H. Further, the property and size of the kinases identified here appear distinct from any of the known NF kinases. For this reason we have termed these kinases NAK115 (for NF-associated kinase), NAK95, NAK89, and NAK84, respectively.

Substrate specificity of NAK115

We have begun to characterize the NF kinases identified in this study. In the previous section we demonstrated that there was a marked difference in NAK115 kinase activity depending on whether NF or casein was used as a substrate in gel assays. In this section we compared different NF substrates on NAK115 activity using the *in situ* gel kinase assay.

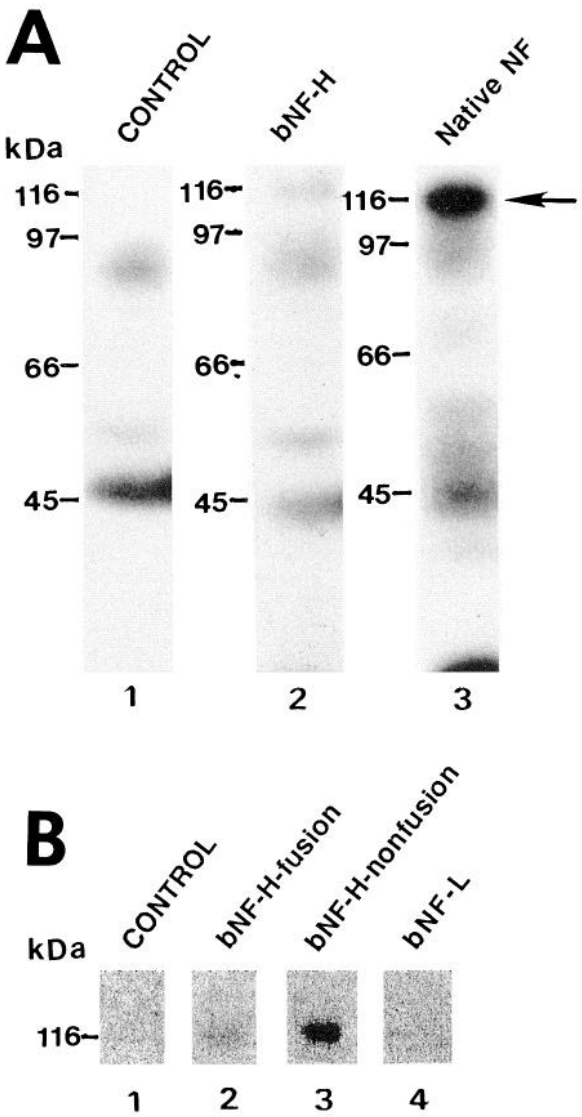

Figure 5. *In situ* gel kinase assay of different NF substrates. A number of different NF substrates were used in the *in situ* gel assay to determine the substrate preference for NAK115. A freshly prepared mouse brain extract was prepared and separated by electrophoresis through the different gels. After separation the gels were processed to determine kinase activity using the *in situ* gel assay. *A,* Autoradiogram of gels exposed for the same length of time, and treated in an identical fashion, to measure kinase activity. *Lane 1,* control gel in which no protein was embedded in the gel matrix; *lane 2,* gel in which purified bNF-H (0.5 mg/ml) was embedded in the gel matrix; *lane 3,* gel in which native rat NF preparation was embedded in the gel matrix. The *arrow* indicates the 115 kDa kinase, which showed the most significant increase in activity due to incorporation of NF substrates in the gel. *B,* Autoradiogram of the portion of the gel containing the 115 kDa kinase of an experiment in which different NF subunits were compared using this assay. *Lane 1,* control gel; *lane 2,* gel in which bNF-H fusion protein was embedded into the gel matrix; *lane 3,* gel in which purified bacterially expressed NF-H nonfusion protein was embedded in the gel matrix; *lane 4,* gel in which purified bacterially expressed NF-L was embedded in the gel matrix.

The efficiency of native NF as a substrate was first assessed. Thus, partially purified NF from rat spinal cord (Fig. 3*C* demonstrates the proteins found in this preparation) was solubilized in SDS buffer and polymerized in the polyacrylamide gel matrix. A gel similar to that used before, containing bNF-H, and a control gel containing no added protein were also prepared. A partially purified mouse brain NAK115 fraction was electrophoresed through the gels and kinase activity was determined. As had been found previously, NAK115 activity (measured as the ^{32}P incorporation of the band) in the gels varied according to the substrate immobilized in the gel matrix (Fig. 5*A*). NAK115 activity was approximately 10-fold greater in the gel containing native NF, and 3.5-fold greater in the gel containing bNF-H, than in the control gel (see Fig. 5*A*). In this experiment ^{32}P incorporation was approximately sevenfold less upon addition of bNF-H than was found previously (Fig. 4), probably due to differences in bNF-H substrates used in the gels or due to the quality of the kinase fractions assayed. Nevertheless, NAK115 showed the biggest increase of all the kinases detected in the mouse brain fraction, confirming our earlier finding that this kinase showed the greatest affinity for NF as a substrate. It should be noted, however, that differences in phosphorylation of other kinases were also found.

The reason why native NF was better than bNF-H as a substrate for NAK115 kinase may have been due to differences in the NF-H proteins of the two substrates, or due to phosphorylation of proteins in addition to NF-H (like NF-M, NF-L, tubulin, or microtubule-associated proteins) in the former. The NF-H substrate used in all the studies described so far was a fusion protein containing 37 kDa of *trpE* sequences fused with 629 residues of NF-H sequences. We were concerned that the *trpE* portion interfered in some unknown way with phosphorylation of the fusion protein. Further, it was important to eliminate the possibility of phosphorylation of bacterial sequences that can occur when using fusion proteins (see Ward and Kirschner, 1990). In addition, it was possible that phosphorylation of bNF-H was poor because we had expressed only a small portion of NF-H in bacteria.

In order to circumvent these potential problems we expressed each of the three NF-subunits in bacteria as nonfusion proteins. We used the new pATH vectors we had constructed (Mercy et al., 1992) to express the complete NF-L, the complete NF-M, and almost the entire NF-H cDNAs (from residue 69 to its stop codon, i.e., 1004 aa) as nonfusion proteins in bacteria. Both NF-L and NF-H were efficiently expressed in bacteria (Fig. 1*D,* lanes 2 and 4, respectively). However, NF-M was expressed poorly for some unknown reason (Fig. 1*D,* lane 3). Since the *in situ* gel assay requires abundant quantities of purified protein, at present we have been able to use only the former two as substrates for the *in situ* gel assay.

A newly prepared mouse brain extract was separated on four different gels that varied according to the protein polymerized into the gel matrix. After electrophoresis, the four gels were processed in an identical manner to measure gel kinase activity. The autoradiogram revealed that the greatest activity of the NAK115 kinase was seen when NF-H nonfusion protein was used as substrate (Fig. 5*B*). bNF-H, as had been found previously, was the next best substrate but NF-L was a poor substrate (see Fig. 5*B*). These data provide further support for phosphorylation of NF-H by the 115 kDa kinase.

Since the bacterially expressed NF-H nonfusion protein was a better substrate than bNF-H for NAK115 by the *in situ* gel assay, we investigated whether the two would also be phosphorylated differently *in vitro* by kinases eluted from the affinity-purified column. Thus, equivalent portions of mouse brain extract and three fractions eluted from the column were assayed

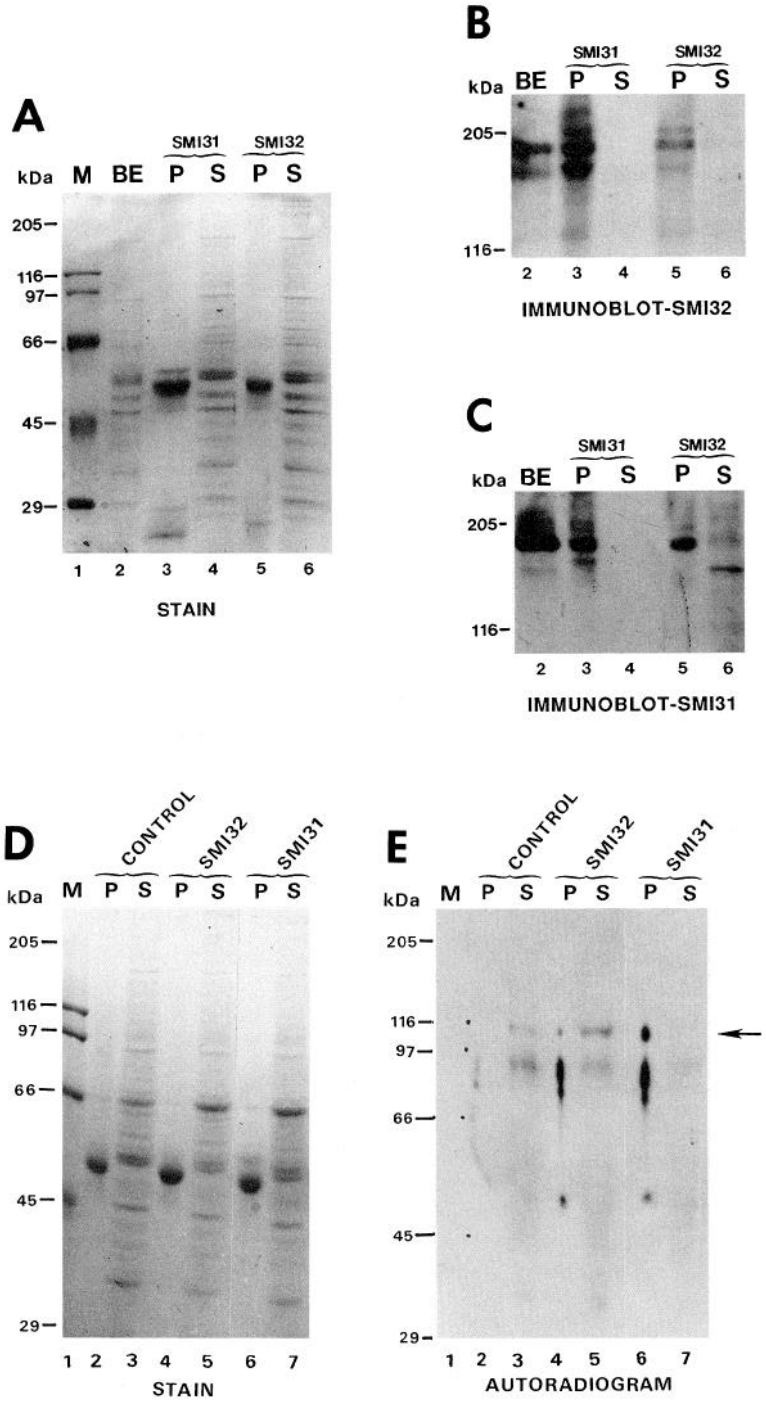

Figure 6. Demonstration that NAK115 kinase is associated with NF *in vivo.* Antibodies that were known to react with NF were used to immunoprecipitate NF proteins. The pellet and supernatant fractions were then assayed to determine if NAK115 was coimmunoprecipitated with NF. Equal portions of mouse brain lysate (75 μg) were incubated with Pansorbin cells containing either SMI31 (a monoclonal antibody that recognizes phosphorylation-dependent epitopes of NF) or SMI32 (a monoclonal antibody that recognizes dephosphorylated NF). After incubation the cells were collected by centrifugation and equal portions of both the supernatant and pellet fractions were separated by SDS-PAGE. Gels containing these fractions were either stained with Coomassie blue (*A*), immunoblotted with SMI32 (*B*), immunoblotted with SMI31 (*C*), or processed by the *in situ* gel kinase assay (*E*). *D* shows a gel equivalent to *E* but stained with Coomassie blue. *A–C, Lane M,* protein molecular weight standards; *lane 2,* brain extract (*BE*); *lanes 3* and *4,* pellet (*P*) and supernatant (*S*) fractions after immunoprecipitation with antibody SMI31; *lanes 5* and *6, P* and *S* fractions after immunoprecipitation with antibody SMI32. *D* and *E, Lane 1,* protein molecular weight standards; *lanes 2* and *3, P* and *S* fractions that had no antibody added; *lanes 4 and 5,* and *6 and 7, P* and *S* fractions after immunoprecipitation with antibody SMI32 and SMI31, respectively.

for phosphorylation of purified bNF-H and NF-H nonfusion proteins in an *in vitro* kinase reaction carried out in a test tube. After phosphorylation the proteins were immunoprecipitated and separated by SDS-PAGE, and the radioactivity incorporated into each protein was determined by autoradiography and by counting with a laser phosphoimage machine. These results indicated that the nonfusion protein was phosphorylated about twofold greater than bNF-H (data not shown). Interestingly, both substrates were phosphorylated about 7-fold and 18-fold greater by the affinity-purified kinases than crude mouse lysate, confirming NF kinases were substantially enriched after affinity chromatography (Fig. 3*B*).

NAK115 is associated with NF-H in vivo

We used an immunoprecipitation assay to determine whether NAK115 kinase is associated with NF *in vivo.* We reasoned that if these proteins were physically associated *in vivo,* then upon immunoprecipitation of NF proteins, NAK115 should be coimmunoprecipitated. We demonstrate that NAK115 is indeed associated with NF using two different antibodies to immunoprecipitate NF.

We used two monoclonal antibodies, SMI31, which recognizes only phosphorylated epitopes of NF, and SMI32, which recognizes dephosphorylated epitopes of NF, to immunopre-

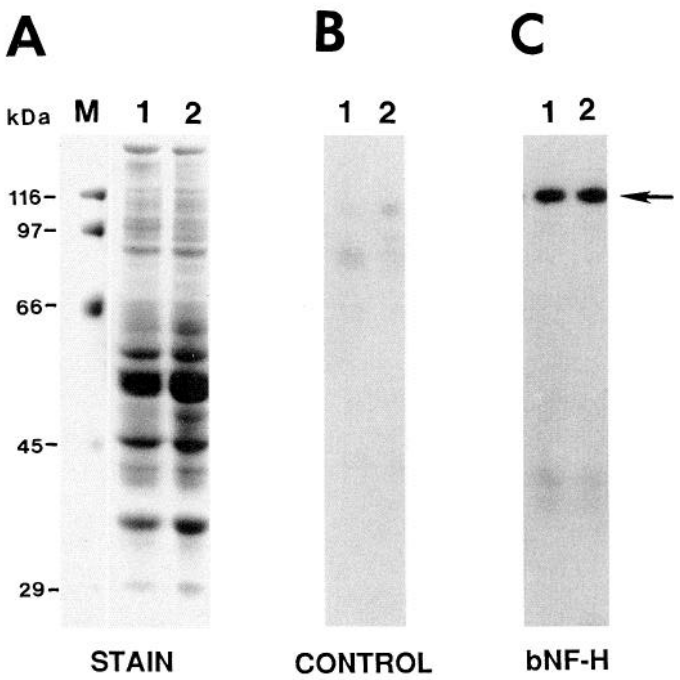

Figure 7. A kinase related to murine NAK115 is found in human brain lysates. The *in situ* gel kinase assay was used to detect kinases present in human brain. Human brain lysates from two individuals was separated through two different 8.5% SDS-polyacrylamide gels. One gel contained bNF-H (1 mg/ml) polymerized in the gel matrix and the other was the control that had no protein polymerized in the gel. After electrophoresis both gels were treated in an identical fashion, as described in Materials and Methods, to assay kinase activity *in situ*. The gels were then washed, dried, and exposed to x-ray film. *A*, Coomassie blue–stained gel. *B*, Autoradiogram of kinases detected in the control gel by the *in situ* assay. *C*, Autoradiogram showing the presence of a 115 kDa kinase (indicated with an *arrow*) in the gel containing bNF-H polymerized in its gel matrix. Both autoradiograms were exposed for 2 hr.

cipitate NF from a mouse brain lysate (see Materials and Methods). Upon immunoprecipitation equal portions of the pellet and supernatant fractions were separated on SDS-PAGE gels and analyzed for precipitation of NF by immunoblot analyses (Fig. 6*A–C*). A parallel set was analyzed for kinase activity by the *in situ* gel assay (Fig. 6*D,E*).

Immunoblot analyses revealed that, as expected, NF were present exclusively in the supernatant fraction and were not precipitated when anti-NF antibodies were not used for immunoprecipitation (Fig. 6*A–C*, lanes 2). Addition of anti-NF antibodies resulted in differential immunoprecipitation of NF When SMI31 was used almost all NF-H species detected by immunoblotting, using SMI31 or SMI32 antibodies, were immunoprecipitated since reactivity was found exclusively in the pellet fraction (Fig. 6*B,C*, lanes 3). In fact, no NF reactivity could be found in the supernatant fraction that contained the majority of the brain extract proteins (Fig. 6*A–C*, lanes 4). Similarly, when SMI32 was used all of the NFs detected by SMI32 were found in the pellet fraction (Fig. 6*B*, lane 5). However, in contrast to SMI31, which immunoprecipitated all detectable NF, SMI32 immunoprecipitated only a fraction (approximately 60%) of the total NF detectable by SMI31 (Fig. 6*C*, lanes 5, 6). Thus, the supernatant fraction contained a number of polypeptides, especially forms that migrated slower on SDS-polyacrylamide gels, that reacted with SMI31. Since SMI31 reacts with phosphorylated forms of NF-H and NF-M (see above), and SMI32 with dephosphorylated forms of these proteins, this result indicates that most if not all NF-H and NF-M proteins expressed in mouse brain are phosphorylated to some extent. Further, the results suggest that approximately 40% of NF were phosphorylated extensively, presumably the slower migrating forms, and could not be immunoprecipitated by SMI32.

Having established that it was possible to immunoprecipitate NF with SMI31 and SMI32 antibodies we next investigated whether NAK115 kinase was associated with NF that had been precipitated. We thus assayed pellet and supernatant fractions after immunoprecipitation for kinase activity using the *in situ* gel kinase assay. For this assay we included BSA as a blocking agent to prevent nonspecific binding of the kinase with the immunoprecipitated complex. The inclusion of BSA resulted in an uneven spacing of proteins in some lanes of the SDS gel but the distribution of NAK115 kinase was not obscured by this outcome (Fig. 6*E*).

As expected NAK115 kinase was present in the supernatant along with NF when anti-NF antibodies were not included during immunoprecipitation (Fig. 6*E*, lanes 2, 3). However, its distribution changed in a manner similar to that of NF when anti-NF antibodies were used. Thus, NAK115 was found exclusively in the pellet, and was almost completely immunodepleted from the supernatant when SMI31 was used (see Fig. 6*E*, lanes 6, 7). Since all of the detectable NF was immunoprecipitated by this antibody, this suggests that almost all NAK115 kinase is associated with NF *in vivo*.

In contrast to the complete immunoprecipitation of NAK115 seen with SMI31, only a fraction of this activity was immunoprecipitated by SMI32 (Fig. 6*E*, lanes 4, 5). Approximately 25% of NAK115 was precipitated by SMI32, which contrasts with the 60% fraction of NF precipitated by this antibody (compare with the immunoblot shown in Fig. 6*B,C*). We hypothesize that this difference is due to association of the kinase with predominantly phosphorylated forms of NF (see Discussion). These results, taken together, suggest that NAK115 is associated with NF *in vivo*. In contrast to the specific immunoprecipitation of the NAK115 kinase, a number of other kinases (50–95 kDa in molecular weight) were immunoprecipitated by both SMI31 as well as SMI32 antibodies (see Fig. 6*E*). Moreover, these kinases were not completely removed from the supernatant. At present it is not clear if these kinases are proteins that are precipitated nonspecifically or whether they bind NF. Evidence in support of both these possibilities was obtained. For example, these kinases precipitated even when no NF-specific antibody was used (see Fig. 6*E*, lane 2). However, a greater fraction of these kinases was immunoprecipitated upon addition of SMI31 or SMI32 antibodies (see Fig. 6*E*, lanes 4, 6). It should be possible to study the properties of these kinases once antibodies specific for the kinases are obtained.

A protein related to mouse NAK115 is found in human brain

It was of interest to know if a kinase similar to mouse NAK115 was expressed in humans since NF proteins are highly conserved across species (see Fliegner and Liem, 1991). Using the *in situ* gel assay we demonstrate that a kinase related to mouse NAK115 is present in human brain extracts.

Equivalent amounts of protein from two human brain homogenates were separated by electrophoresis through two different SDS-polyacrylamide gels. One gel contained bNF-H that had been polymerized in the gel matrix and the other was a standard gel containing no additional protein. After electrophoresis the gels were processed for protein kinase activity using the *in situ* gel assay. The autoradiogram revealed that protein

kinases were present in the human brain extracts (Fig. 7*B,C*). Interestingly, the kinase patterns were somewhat similar to that found in crude mouse brain homogenate although minor differences were noted (compare Figs. 5*A*, 7*B,C*; data not shown). More significantly, we found a kinase in human brain that was both identical in size to that of mouse NAK115 (see Fig. 7*C*, arrow) and which also incorporated more radioactive ^{32}P in the gel to which bNF-H was added as a substrate (compare Fig. 7*B,C*). However, the increase seen in ^{32}P incorporation was 14-fold, which is in contrast to the 25-fold increase seen for the mouse enzyme. This difference may be due to differences in the two kinases and could reflect a preference of the mouse enzyme for the bacterially expressed mouse bNF-H substrate.

Discussion

We describe here the identification and characterization of a novel NF-associated kinase, NAK115. NAK115 was found to bind to NF by two different criteria. First, when NFs were immunoprecipitated from mouse brain extracts, NAK115 was precipitated along with NFs. Second, NAK115 bound to NF-H *in vitro* by affinity chromatography. These findings represent the first characterization of a kinase that has been shown to associate directly with NF both *in vivo* and *in vitro*. Although the precise sites on NF that are phosphorylated by NAK115 have yet to be mapped, our demonstration that NAK115 associates with NFs should provide clues as to the role of this kinase in NF phosphorylation.

NF phosphorylation is developmentally regulated (see Fliegner and Liem, 1991). Considerable evidence exists that suggests phosphorylated NF and nonphosphorylated NF are present in spatially distinct regions in the neuron. This segregation was first found by Sternberger and Sternberger (1983), who demonstrated that the former were restricted to axons whereas the latter were predominantly localized in the cell body. The apparent segregation of NF forms that are differently phosphorylated suggests that either the kinases and phosphatases that modify these proteins are spatially segregated, or NF proteins that are phosphorylated are rapidly transported into the axons. Although the mechanism underlying this segregation is not known, a number of recent studies support a mechanism by which cell–cell interactions determine the differential segregation of NF kinases and phosphatases in neurons. In particular, a role of Schwann cells (in PNS and, presumably, oligodendrocytes in CNS) has been implicated in this interaction. Thus, in *trembler* mice, in which mutations in the PMP-22 gene result in myelin deficiency of PNS axons, both NF phosphorylation and transport are decreased (de Waegh and Brady, 1990; de Waegh et al., 1992; Suter et al., 1992a,b). Other evidence supports the notion that NF kinases or phosphatases are in fact modulated by the axolemma in contact with Schwann cells. Thus, at the nodes of Ranvier of myelinated axons, which are characterized by an absence of myelination, NF phosphorylation is reduced (Mata et al., 1992). The reduction of NF phosphorylation seen in these two examples results in a concomitant reduction in axonal caliber. This reduction in caliber is also found during axotomy when NF synthesis decreases (Hoffman et al., 1984, 1985, 1987), and is in accord with the proposal that NF are intrinsic determinants of axonal caliber (Friede and Samorajski, 1970; Friede et al., 1971).

The findings described here of an association between NAK115 and predominantly phosphorylated forms of NF suggest the NAK115 kinase may be compartmentalized in neurons. This would be in accord with the hypothesis that NF kinases are spatially distributed according to the levels of NF phosphorylation seen in neurons. We used the same two antibodies, SMI31 and SMI32, that have proved instrumental in uncovering differential NF phosphorylation to show that NAK115 was associated differently with the two NF forms immunoprecipitated by these antibodies. Interestingly, almost all of the detectable NAK115 activity was coimmunoprecipitated when we used SMI31 but only approximately 25% was coimmunoprecipitated when SMI32 was used. This difference is most easily accounted for by the association of NAK115 with predominantly highly phosphorylated forms of NF.

The epitope recognized by SMI31 and SMI32 antibodies has been shown to be the Lys-Ser-Pro sequence that is repeated many times in both NF-H and NF-M. Phosphorylation of the Ser residue of this repeat is essential for recognition by SMI31, whereas the dephosphorylated epitope is recognized by SMI32. Two interesting properties emerge from the use of these antibodies for immunoprecipitation. First, almost all of the NF expressed in brain appears to be phosphorylated at least to some degree since most of the NF detected by immunoblotting could be precipitated by SMI31. Second, a subset of this NF population appears to be partially phosphorylated, accounting for precipitation of a portion of NF by SMI32, whereas the remaining subset that was not precipitated was presumably extensively phosphorylated. The substoichiometric amount of NAK115 that was coprecipitated with NF by SMI32 suggests that NAK115 is predominantly associated with heavily phosphorylated NF forms. Furthermore, the absence of NAK115 activity in the supernatant fraction after precipitation with SMI31 suggests that most of the kinase may be exclusively associated with NF and not with other cellular proteins. A remote possibility that we cannot exclude at present is that SMI31 immunoprecipitates NAK115 directly. This appears unlikely since SMI31 does not react with a 115 kDa protein in mouse brain extracts in immunoblots (see Figs. 1*H*, 6*C*).

The region on NF to which NAK115 binds has not been established. For example, it is not known if the association we have found by immunoprecipitation is due to binding of NAK115 to individual NF subunits (NF-H or NF-M, and possibly NF-L) or binding to small NF fragments. Furthermore, our experiments do not distinguish whether NAK115 binds to NF directly or whether it is associated with proteins that bind NF. The former would appear to be the case, as NAK115 was isolated on the basis of its retention by an NF-H affinity column. Paradoxically, the bacterial NF-H coupled to the column is not phosphorylated, yet NAK115 bound to this polypeptide, contrary to its preferred association for phosphorylated NF inferred by the immunoprecipitation experiments. It is possible that NAK115 binding is complex and the two procedures enable detection of different binding properties of this protein. In this regard it would be of interest to know if NAK115 would bind more efficiently to an affinity column containing phosphorylated NF.

The *in situ* gel assay that we used to identify kinases in this study would not be expected to identify kinases that require multiple subunits for activity. However, the procedure enabled us to resolve and identify NAK115 and at least three other polypeptides (95, 89, and 84 kDa) with abundant kinase activity from the mixtures of proteins present in the affinity-purified fractions. At present we do not know if these kinases are single subunit kinases or the catalytic subunits of multiple subunit complexes. Their size and properties suggest these are novel

kinases, unrelated to PKC by immunoblotting, and are likely to be involved in NF phosphorylation.

In addition to the association of NAK115 with NF, another indicator that NAK115 is indeed involved in NF phosphorylation is the dramatic increase of incorporation of γ-^{32}P-ATP seen in *in situ* gel assays when different NF substrates are immobilized in the gel matrix. Of all the kinases detected by the *in situ* gel assay, NAK115 showed as much as a 25-fold difference in ^{32}P incorporation when NF were incorporated into the gel matrix. This increase was not due to a nonspecific effect of including any protein in the gel since incorporation of casein, which is an excellent substrate for a number of kinases that phosphorylate NF, caused only a twofold increase in activity. The most likely explanation for the dramatic increase in ^{32}P incorporation seen with NAK115 is the direct phosphorylation of NF present in the gel matrix. This increase was seen when both native and purified bacterially expressed proteins were used for the *in situ* gel assay. However, the increase was at least three-fold smaller when bacterially expressed substrates were used. This difference may in part be explained by a preference for the kinase for phosphorylated NF substrates. Indeed, Wible et al. (1989) have identified an NF-H–specific kinase activity, which copurifies as a 67 kDa doublet, that is able to phosphorylate partially phosphorylated NF-H but is unable to phosphorylate completely dephosphorylated NF-H. Interestingly, we have found a similar effect on NF-H phosphorylation when we compared the phosphorylation of native NF with that of bacterially expressed proteins, both by the *in situ* gel and *in vitro* kinase assays. Thus, when the affinity-purified fractions were assayed in a test tube for phosphorylation of bNF-H or bacterially expressed NF-H nonfusion proteins, they were both phosphorylated approximately 15—35-fold less compared with native NF-H (data not shown). Although both of the former substrates were phosphorylated less efficiently, the profile of phosphorylation paralleled that of NAK115 kinase activity (compare Figs. 3*A*, 4*D*). Therefore, using purified bacterially expressed NF proteins we have unequivocally demonstrated that the affinity-purified fractions contain kinases that are able to phosphorylate completely dephosphorylated NF proteins.

There are a number of properties that make NAK115 interesting in terms of NF kinases. It has a large molecular weight and a pI of about 4.8 (data not shown) and is able to autophosphorylate. It appears to be an effector-independent kinase since we have not observed any cofactor requirement for its activity (data not shown). Furthermore, a kinase of identical size and properties is present in human brain. This would be expected of a kinase that plays an important role in phosphorylation of highly conserved proteins. A comparison of different NF substrates reveals that NF-L is not an effective substrate but that NF-H proteins are good substrates (we have not tested NF-M as a substrate due to difficulties in expressing NF-M in bacteria). Interestingly, the nonfusion NF-H protein was a better substrate than the fusion protein. It is possible that the former is a better substrate due to lack of *trpE* sequences or the presence of additional NF-H sequences. However, it was not surprising that NF-L was a poor substrate in the gel kinase assay since *in vivo* it is phosphorylated at a molar ratio of 1:50 compared to NF-H. Additional properties should emerge as this and other kinases that phosphorylate NF are purified. In this regard the *in situ* gel assay should be useful to identify kinases that phosphorylate NF-L and NF-M.

Finally, we would like to speculate on the identity of the other proteins that were isolated by bNF-H affinity chromatography. Using this approach we have been able to show that a number of proteins including kinases bind to the carboxyl-terminal region of NF-H. Binding of proteins to this region is not surprising since the tail of NF-H forms lateral projections from the filament core and thus would be expected to interact with proteins in the axonal cytoplasm. Apart from the protein kinases that are known to phosphorylate this region of NF, the identity of other NF-binding proteins is not known. In fact, apart from this study, relatively little is known of proteins that interact with NF. The best-characterized NF-associated protein is MAP2, which is thought to facilitate binding of NF with microtubules (see Fliegner and Liem, 1991; Hirokawa, 1991). Another protein, fodrin (brain spectrin), has been shown to bind NF and may thus link NF with other neuronal cytoskeletal elements (Frappier et al., 1987). An indication that NF may provide support for other functional proteins is the finding that synapsin I is associated with NF (Goldenring et al., 1986; Steiner et al., 1987). From these studies it appears that the NF-binding proteins identified in this study could play diverse roles such as linking NF with the cytoskeleton, modifying NF, or using NF as a functional support or for their transport along the axon. We are presently preparing monoclonal antibodies against these proteins in order to characterize them further. A study of NF and their associated proteins should provide clues as to the role of these proteins in neuronal function and in diseases associated with disruption of the cytoskeleton.

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