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Two Novel Kinases Phosphorylate Tau and the KSP Site of Heavy Neurofilament Subunits in High Stoichiometric Ratios

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We have identified, purified, and characterized two neurofilament/tau kinases from bovine brain, PK36 and PK40, with apparent M_r of 36,000 and 40,000 and with novel biochemical properties. A specially designed immunoassay for phosphorylated epitopes in neurofilament (NF) proteins was used in the early stages of the purification. Neither kinase is closely associated with the cytoskeleton. Both kinases phosphorylate bovine intermediate (NF-M) and heavy (NF-H) NF subunits and also bovine tau at the expected KSP sequences, though other sites cannot be ruled out. In human paired helical filaments, tau, phosphorylated at these same KSP sites, is a major characterized constituent. Neither kinase is activated by the usual second messengers. Tau and the above NF subunits are phosphorylated in high stoichiometric ratios. In the intermediate NF subunit, all the expected sites appear to be phosphorylated, but in the heavy NF subunit only 7 out of the >40 expected sites can be phosphorylated by our kinases. We demonstrate that both kinases can induce considerable shifts of apparent M_r with SDS-PAGE for tau and, for the first time *in vitro*, also for the intermediate NF subunit.

Interestingly, PK36 and particularly PK40 are strongly inhibited by an excess of free ATP. We propose that during normal aging, and in Alzheimer's disease, age-related mitochondrial dysfunction would reduce ATP levels, which in turn might release the neurofilament/tau kinase from inhibition with consequent paired helical filament formation.

Neurofilaments (NFs), the intermediate filaments specific for neurons, are an assembly of three subunits of apparent M_r on SDS-PAGE of 68, 160, and 200 kDa, termed NF-L, NF-M and NF-H. All three subunits contain a highly conserved helical rod domain. The two heavier subunits also have extended C-terminal tail domains that are heavily phosphorylated. The cDNA-derived sequences of the two heavy NF subunits have revealed the presence of 5, 12, and >40 Lys-Ser-Pro-(Val,Ala,*X*) repeats in the C-terminal domains of rat NF-M, human NF-M, and human NF-H, respectively (Myers et al., 1987; Napolitano et al., 1987; Lees et al., 1988). These sequences very likely account for the majority of phosphorylation sites in NF-M and NF-H and also form the epitopes of several phosphoepitope-specific anti-NF monoclonal antibodies (mAbs) (Lee et al., 1988). The physiological significance of NFs and their phosphorylation is not very well understood yet (reviewed by Matus, 1988); correlative evidence suggests involvement in the regulation of axonal diameter (Hoffman et al., 1987; Pleasure et al., 1989). Electron microscopic studies in conjunction with antibody decoration (Hirokawa et al., 1984) and biochemical evidence (Minami and Sakai, 1983) favor NF-H as a component in interactions of the NF and microtubule networks. The phosphorylation status of NFs and their ability to promote tubulin polymerization are correlated *in vitro* (Minami and Sakai, 1985).

The existence of NF kinase(s) not activated by common second messengers and some of its expected properties were postulated from *in vivo* phosphorylation studies on extruded axoplasm of the giant axons of the squid (Pant et al., 1978, 1986) and of *Myxicola* (Shecket and Lasek, 1982). *In vitro* characterization of purified NF kinases has focused so far on activities that copurify with the NF cytoskeleton and can be dissociated under high salt conditions (Runge et al., 1981; Toru-Delbauffe and Pierre, 1983). There is currently no evidence of second messenger dependence of any of these activities. From a mixture of such kinases, one 67 kDa activity has been purified to apparent homogeneity (Wible et al., 1989). This kinase prefers NF-H as a substrate, but only if not completely dephosphorylated. A cAMP-dependent kinase copurifying with microtubules has been shown to phosphorylate preferentially NF-M in NF triplets (Leterrier et al., 1981). In no case are the stoichiometry or the sites of phosphorylation known, and no shift of apparent M_r of NF-M and NF-H on SDS-PAGE has been demonstrated. Such a shift is expected after incorporation of phosphate in high stoichiometric ratios into the dephosphorylated subunits. A smaller than expected gel shift associated with a heterogeneous state of KSP phosphorylation of NF-M is induced by uncharacterized kinases in mouse L-cells transfected with a human NF-M clone (Pleasure et al., 1990).

A possible pathological role of aberrant NF phosphorylation was considered when the anti-rat-NF mAb 07-5 (commercially available as SMI-34) was found to stain neurofibrillary tangles in brain tissue from Alzheimer's patients (Sternberger et al., 1985) but did not stain normal human brain tissue, except for cerebellar basket cell axons and certain motoneuron axons of patients >60 yr of age (Blanchard and Ingram, 1989). On the other hand, there is a report that the localization of the SMI-34 epitope is exclusively perikaryonal, while most other mAbs

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reacting with NF phosphoepitopes stain axons preferentially (Sternberger and Sternberger, 1983).

However, immunochemical evidence (Grundke-Iqbal et al., 1986; Kosik et al., 1986; Wood et al., 1986; Nukina et al., 1987) concerning the cross-reactivities of a series of mAbs with NFs, microtubule-associated protein tau, and the main component of tangles and paired helical filaments (PHF) point to tau as a major constituent of PHFs. This deduction is reinforced by the isolation from PHFs of tau-derived peptides (Wischik et al., 1988), while no NF-derived peptides (Kondo et al., 1988) were obtained. A number of anti-NF mAbs cross-reacting with tau, among them SMI-31 and RT97, recognize the phosphorylated KSP sequence repeat in NF proteins (Lee et al., 1988) and may therefore also bind to one or both of the same phosphorylated sequences occurring in human and bovine tau (Goedert et al., 1989; Himmler et al., 1989). PHFs react strongly with RT97, but only after prolonged treatment with SDS, suggesting the presence of this phosphorylated epitope in PHF in a nonperipheral location (Rasool and Selkoe, 1984). Recently, a peptide derived from bovine tau carrying the epitope for the Alzheimer's disease (AD)-specific mAb Alz-50 has been shown to contain the C-terminal KSPV sequence that may be at least partially phosphorylated (Uéda et al., 1990). Several other lines of evidence indicate an abnormal level or an abnormal site of phosphorylation in the C-terminal portion of the tau molecule in AD (Grundke-Iqbal et al., 1986; Kondo et al., 1988; Iqbal et al., 1989). The fact that tau and NF share phosphoepitopes, very likely the KSPV sequence, suggests the possibility that kinases acting on the multiphosphorylation repeat (MPR) of NF proteins might also phosphorylate tau and contribute to its pathological involvement in PHF formation.

We describe here the purification, identification, and characterization of two kinases from bovine brain with novel biochemical properties, which became feasible with a specially designed immunoassay (Roder and Ingram, 1990). Neither kinase is closely associated with the cytoskeleton. Both activities phosphorylate tau and the MPR sequence of NF-M and NF-H, among other sites, in a second messenger–independent fashion and in high stoichiometric ratios. Moreover, we demonstrate shifts of apparent M_r with SDS-PAGE for tau and, for the first time *in vitro,* also for NF-M.

Materials and Methods

Protein assays

Protein concentrations in column fractions were determined by a commercial Bradford assay (Pierce CB 250) with bovine serum albumin (BSA) as standard. Suspensions and solutions of purified NF were assayed with the BCA assay (Pierce Chemical Co., enhanced protocol, 60°C, 30 min) and BSA as standard. The concentration of expressed human tau protein was determined by amino acid analysis.

SDS-gel electrophoresis

SDS-PAGE was carried out on 1.5-mm gels as described previously (Laemmli, 1970). Acrylamide concentrations were 7.5% for NF proteins, 10% for tau proteins, and 12% for kinase fractions. Silver staining of gels was performed with the Bio-Rad kit based on the method of Merril et al. (1984).

Nondenaturing gel electrophoresis

Resolving gel, stacking gel, and buffers were as for SDS-PAGE, but SDS was omitted and 5 mM Mg-ATP and 1 mM dithiothreitol (DTT) were added; 7.5% gels were run at 4°C. For preparative purposes, samples were loaded into 50-mm-wide slots. PK36 and PK40 bands after preparative runs were localized by loading test slices from a side strip in SDS sample buffer after 5 min of boiling and performing analytical SDS-PAGE.

Western blots and dot blots

Previously described methods (Towbin et al., 1979) were used for electrophoretic transfer of proteins from SDS gels to nitrocellulose (0.22 μm; Schleicher and Schüll). Blots were blocked by 1 hr incubation with 3% BSA in 10 mM PBS (pH 7.2) and washed once in 0.5% Triton X-100 and 10 mM PBS. Antibodies were diluted in sterile 10 mM PBS (pH 7.2), 0.5% Triton X-100, and 10% fetal calf serum. Blots were incubated with SMI mAbs for at least 2 hr, with all other antibodies overnight. After incubations with antibodies blots were washed five times. Mouse mAbs were detected by reaction with horseradish peroxidase–linked goat anti-mouse antibody (Cappel) in 1:200 dilution and by staining with 0.05% 4-chloro-1-naphthol (Sigma) and 0.05% H_2O_2 in 50 mM Tris-buffered saline (pH 7.5) and 33% ethanol for 5–20 min. Rabbit polyclonal antibodies were detected by the Vectastain method (Vector Laboratories, Burlingame, CA): incubation for 1 hr with 1:200 biotin-conjugated goat anti-rabbit IgG diluted as above, followed by 1 hr in 1:200 avidin/biotin–peroxidase reagent.

All incubations and washes were at room temperature. Incubations were sealed in plastic bags with 50 μl of solution/cm² of membrane.

Preparation of neurofilament triplet proteins[1]

The preparation of "native" NF triplet is a modification of previously described procedures (Tokutake et al., 1983; Lee et al., 1987). A freshly obtained bovine spinal cord (100–150 gm; Arena and Sons, Hopkinton, MA) was desheathed, minced with a razor blade, and left for 2 hr in 3 liters of 10 mM Tris (pH 7.0), 50 mM NaCl, 2 mM EGTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C for swelling. The supernatant was decanted, and the swollen tissue was homogenized for 1 min in 200 ml of a similar buffer containing 150 mM NaCl (isotonic buffer) with an Ultra-Turax at 2/3 speed. After 15 min centrifugation at 12,000 × *g,* the precipitate was twice rehomogenized in 200 ml of isotonic buffer for 1 min at full speed. Supernatants of the centrifugations were combined and brought to 0.85 M in sucrose by adding solid sucrose (1 mol/liter). Centrifugation at 100,000 × *g* for 4 hr yielded about 200 mg of gelatinous precipitate, which was dissolved (aided by slow Ultra-Turax homogenization) in 100 ml of adsorption buffer: 10 mM potassium phosphate (pH 7.4), 8 M urea [deionized for 1–2 hr over mixed-bed ion exchanger AG 501-X8 (D), Bio-Rad], and 0.5% β-mercaptoethanol (β-ME). NFs were adsorbed by shaking this solution for 10 min at 4°C with HTP (40 gm dry weight; Bio-Rad), preequilibrated in adsorption buffer. The adsorbent was sedimented for 10 min at 15,000 × *g* and washed (10 min each) subsequently with 100 ml of adsorption buffer, 3 × 85 ml 130 mM KPO_4 (pH 7.0), 8 M urea, and 0.5% β-ME, and once each with 50 ml 300 mM and 250 mM KPO_4 (pH 7.0), 8 M urea, and 0.5% β-ME. The supernatants of the latter two washes contained the bulk amount of NF-L, NF-M, and NF-H and were combined for reconstitution of the NF triplet by dialysis into three changes of 1 liter of 10 mM morpholineethanesulfonic acid (MES; pH 6.8), 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM EGTA. After 30 min of incubation at 37°C and centrifugation for 6 hr at 120,000 × *g,* 40–60 mg of NF triplet proteins were obtained. The gelatinous precipitate was rehomogenized in 40% glycerol with a glass–Teflon homogenizer to form suspensions of 2.5–3 mg/ml and stored at −20°C.

In a similar way, the so-called "reconstituted" NF triplet was reconstituted from the three purified subunits (see below) after recombination of the appropriate DEAE column fractions.

We have observed a striking difference in the behavior of native and dephosphorylated native NF triplet during prolonged storage in glycerol-containing solutions at −20°C. Both preparations can be homogenized to form finely dispersed suspensions initially. Native NF triplet suspensions can be stored for at least 2 yr without apparent physical change, while the dephosphorylated native NF triplet will not resuspend after several weeks. This change is reflected by a much lower susceptibility of NF-H in stored dephosphorylated native NF triplet preparations to

[1] We used two preparations of NF triplet protein—one, called "native" NF triplet, was obtained by batch hydroxyapatite (HTP) chromatography and removing the urea used by dialysis; the other preparation was the "reconstituted" NF triplet, obtained from fast protein liquid chromatography (FPLC)-purified NF subunits, again removing urea by dialysis.

phosphorylation by our kinase(s) compared to a fresh preparation (not shown).

Separation of individual NF subunits

For separation of the individual NF subunits, a previously described procedure (Tokutake, 1984) was modified. The reconstituted NF triplet precipitate was taken up (0.5–1 ml/mg NF protein) in 10 mM sodium phosphate (pH 6.8), 6 M urea, and 0.5% β-ME (starting buffer), centrifuged at 100,000 × g for 1 hr, and loaded onto a 40 × 1.5 cm DEAE-Sephacel column (Pharmacia). NF subunits were eluted at room temperature with 600 ml of a linear gradient formed by starting buffer and 400 mM sodium phosphate (pH 6.8), 6 M urea, and 0.5% β-ME at 10–15 ml/hr. Fractions were collected (120 fractions, 5 ml each), and fractions 41–48, 71–80, and 85–94 were pooled; these contained pure NF-H, NF-M, and NF-L according to anaysis by SDS-PAGE. The three fractions were concentrated to 2–3 ml by vacuum dialysis and dialyzed into water. NF-L was obtained as a clear gelatinous precipitate after centrifugation for 1 hr at 100,000 × g; NF-M and NF-H were precipitated by ammonium sulfate. For storage at −20°C, the pure subunits were homogenized (NF-L) or dissolved (NF-M, NF-H) in 40% glycerol to form stock concentrations of about 1 mg/ml of protein.

NF subunits were also separated by FPLC on a Mono-Q 5/5 column (Karlsson et al., 1987).

Dephosphorylation of the NF triplet

Typically, 1 ml (2.5–3 mg) of NF triplet stock solution was incubated for 5 d at 37°C with 10 U (about 400 μg) of *Escherichia coli* alkaline phosphatase (type III-N) in a total volume of 2 ml containing 50 mM Tris (pH 8.5), 100 mM NaCl, 0.5 mM $MgSO_4$, 0.5 mM $ZnSO_4$, 1 mM PMSF, and 5 μg of leupeptin. The NF triplet protein was separated from the phosphatase by centrifugation for 1 hr at 100,000 × g, 4°C. The pellet was washed twice by rehomogenization in 2 ml of water. The final pellet (yield, 40–50%) was resuspended by a glass–Teflon homogenizer in 40% glycerol to form a stock solution of about 0.5 mg/ml, stored at −20°C. Dephosphorylated NF triplet tended to aggregate over several weeks of storage.

After analytical SDS-PAGE of dephosphorylation reactions, phosphatase and accompanying impurities were removed by subjecting the gel for 6 hr to a "Western blot electrophoresis" in an SDS-free buffer prior to staining. Under those conditions very little of the NF proteins was lost.

Dephosphorylation of subunits NF-M and NF-H

NF-M (0.5 gm) was dephosphorylated by incubation with 2 U (80 μg) of *E. coli* alkaline phosphatase for 5 d in a total volume of 1 ml under the same buffer conditions as used for the NF triplet. The phosphatase was removed by gel filtration of the mixture on a 50 × 1.5 cm Sephadex G200 column (50–120 μm, 10 ml/hr flow rate), equilibrated with 10 mM Bis-Tris (pH 7.0) and 100 mM NaCl. Fractions were analyzed by SDS-PAGE. NF-M–containing fractions around the exclusion volume were pooled (4 ml), dialyzed into water, concentrated in a SpeedVac, and stored at −20°C as a 0.3 mg/ml stock solution containing 40% glycerol. The yield was 270 μg (54%).

NF-H (1.05 mg) was dephosphorylated by incubation with 120 μg of calf intestinal alkaline phosphatase for 6 d at 37°C in a total volume of 1.5 ml containing 50 mM Tris (pH 8.5), 1 mM $MgSO_4$, 1 mM PMSF, and 15 μg of leupeptin. Separation from the phosphatase, concentration, and storage were as described for NF-M. The yield was 700 μg (67%).

The dephosphorylation reactions for both NF subunits were monitored by spotting 1–1.5 μg of NF protein on nitrocellulose. Blocking, staining with SMI-31 and SMI-34, and development of the blots were performed as described for Western blots (see above).

Immunodot blot assays of kinases

Assays were performed in 50 mM HEPES (pH 7.0), 2 mM $MgCl_2$, 1 mM ATP, and 2 mM DTT in a total volume of 30 μl with 5 μg of dephosphorylated native NF triplet or 1.2 μg of dephosphorylated pure subunits NF-M or NF-H as substrates together with a control assay lacking NFs. After incubation at 37°C for 18 hr, assays were diluted to 100 μl with 10 mM PBS (pH 7.2), and aliquots of 50 μl were spotted on nitrocellulose. Further processing was carried out as described for Western and dot blots (see above).

^{32}P assays of tau/NF kinases

Radioactive assays in the same buffer system as for immunoassays contained 5 μg of HTP-purified native NF triplet as substrate (3 μg of substrate proteins other than NFs) and 150–250 cpm/pmol γ-^{32}P-ATP. Incubation times were 15 min at 37°C for activities up to about 1 pmol/min/assay, since the assay responses were linear within these time intervals. Assays were stopped by cooling on ice, addition of 20 μl of 25 mM EDTA, and immediate transfer of the mixture onto glass filters (Whatman GF/A) wetted with 10% trichloroacetic acid (TCA) and 2% sodium pyrophosphate (PPA). The glass filters were washed twice for 1 hr and once for at least 3 hr in 10% TCA and 2% PPA and finally in ethanol and were air dried. Radioactivity was assessed by scintillation counting (Beckman LS 230) with 5 ml of "Liquiscint" (National Diagnostics) for 20 min. Assays were routinely carried out in triplicate except for some duplicate assays in a few explicitly mentioned cases; a control assay lacking NFs was subtracted from the mean value.

Assays to be analyzed with SDS-PAGE were stopped with an equivalent amount of sample buffer, boiled for 3 min, and run on 7.5% gels. After staining with Coomassie blue, destaining, and drying on Whatman 3MM paper, autoradiography was performed with a Du Pont Cronex screen intensifier at −70°C. For quantitative measurements, radioactive bands of individual NF subunits were cut out and placed in an Eppendorf vial immersed in 20 ml of water, and the Cerenkov radiation of the sample was counted. Counting efficiency was about 30%.

Phosphorylations with Ca^{2+}/calmodulin-dependent kinase II and protein kinase C

Incubation conditions were at 37°C in 30 μl of 50 mM HEPES (pH 7.5), 10 mM Mg^{2+}, 5 mM Ca^{2+}, 1 mM EGTA, 2 mM DTT, 1 mM ATP, and 50 μg/μl calmodulin and phosphatidylserine, respectively, and 5 μg of NF triplet protein.

Purification of the NF/tau kinases

Step I

A fresh bovine brain (350–450 gm wet weight) was cleaned from meninges and blood vessels and homogenized at 4°C in 350 ml of homogenization buffer (10 mM Bis-Tris, pH 7.0, 150 mM NaCl, 2 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 5 μg/ml leupeptin) with an Ultra-Turax or a Sorvall Omni-Mixer for 3 min. The pellet of the centrifugation at 20,000 × g for 20 min was extracted twice, each with 300 ml of homogenization buffer. The turbid supernatants were clarified by centrifugation at 100,000 × g for 4 hr. Solid ammonium sulfate (AS) was added slowly over about 4 hr while keeping the pH at 8.0–8.5 with ammonia. The precipitate obtained between 35% and 45% saturation was collected by centrifugation at 20,000 × g for 20 min, redissolved in 20 ml of 10 mM HEPES (pH 7.0), 1 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT, and dialyzed extensively against this buffer to form a "crude enzyme" stock solution of about 20 mg/ml protein, which could be stored for several weeks at 4°C with little loss of activity.

Step II

20 ml of crude enzyme were dialyzed into CM-Sepharose starting buffer (5 mM magnesium acetate, 5 mM ATP, 1 mM DTT, 10% glycerol, 0.02% sodium azide, adjusted to pH 6.0 with Bis-Tris) and loaded onto a 3 × 2.5 cm CM-Sepharose column equilibrated with starting buffer. The column was washed with 60 ml of starting buffer at about 50 ml/hr, and then the kinases were eluted in one step with 85 mM magnesium acetate, 5 mM ATP, 1 mM DTT, 10% glycerol, and 0.02% sodium azide (pH 6.0), as a fraction of 15 ml volume.

Step III

The combined fractions of the CM-Sepharose chromatography containing the bulk of the activity were dialyzed into fivefold-diluted gel filtration buffer (48 mM Bis-Tris, pH 7.0, 5 mM $MgCl_2$, 5 mM ATP, 1 mM DTT, 0.02% sodium azide), concentrated to about 3 ml in a SpeedVac, and loaded onto a 95 × 2.5 cm column of Sephadex G200 Superfine (Pharmacia). After elution of 155 ml at a flow rate of 1.5–2 ml/hr, fractions of 5 ml were collected. No significant contaminating phosphatase activity could be detected at this stage in the fractions containing PK36 and PK40 (see below).

Step IV

Method A. Gel filtration fractions containing significant amounts of PK40 (fractions 17–19) and PK36 (fractions 21–22), according to SDS-PAGE analysis, were pooled, dialyzed into Mono-Q starting buffer (20 mM Tris, pH 8.0, 20 mM $MgCl_2$, 5 mM ATP, 1 mM DTT, 0.02% sodium azide), and loaded on an HR 5/5 Mono-Q FPLC column (Pharmacia) equilibrated with starting buffer. Elution of PK40 at a flow rate of 1 ml/min started with 5 ml of starting buffer followed by a linear gradient of 7 ml up to 65 mM $MgCl_2$, 7 ml isocratic elution at 65 mM $MgCl_2$, and finally a linear gradient up to 110 mM $MgCl_2$ formed with elution buffer (20 mM Tris, pH 8.0, 110 mM $MgCl_2$, 5 mM ATP, 1 mM DTT, 0.02% sodium azide). The gradient profile for PK36 was similar, except that the isocratic step was at 60 mM $MgCl_2$. Peak fractions of the NF kinases (PK40, fractions 11–12; PK36, fractions 12–13) were pooled, dialyzed into storage buffer (20 mM Bis-Tris, pH 7.0, 2 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 0.02% sodium azide), and concentrated about 10-fold in microconcentrators (Amicon 10) for storage purposes. The enzyme is stable in the Mg-ATP–containing storage buffer. Activity was retained for several days at 4°C and after five cycles of freeze–thawing with little loss.

Method B. All gel filtration fractions containing SMI epitope reconstituting activity (fractions 14–23) were pooled, and PK40 was separated from PK36 by Mono-Q FPLC with a gradient profile similar to method A for PK40. Fractions 9–10 and 11–12 contained almost exclusively PK36 and PK40, respectively, although in much lower purity than in method A.

Step V

Elution of kinase activity from gel slices. The purity of PK40 was substantially improved by preparative gel electrophoresis. For detection of PK36 and PK40 activity, gel slices were left overnight in 30 μl of 20 mM Bis-Tris (pH 7.0), 2 mM Mg-ATP, and 1 mM DTT; aliquots of supernatants were used in standard ^{32}P and immunoassays. PK40 was eluted preparatively from gel slices of a 7.5% polyacrylamide gel in an electroeluter (model UEA, International Biotechnologies, New Haven, CT) in two consecutive 30 min runs at 120 V and 4°C into a trapping buffer consisting of 7.5 M ammonium acetate, 10 mM Mg-ATP, 2 mM DTT, and a trace of bromophenol blue. The elution buffer contained 25 mM Tris (pH 8.3), 192 mM glycine, 2 mM Mg-ATP, and 1 mM DTT. The kinase was dialyzed into a storage buffer of 20 mM Bis-Tris (pH 7.0), 2 mM Mg-ATP, and 1 mM DTT and concentrated about 10-fold in a microconcentrator (Amicon 10).

A relatively pure mix of PK36/40, as used for the identification of the 36,000 and 40,000 proteins as kinases, was obtained after pooling of gel filtration fractions 16–23 and elution from Mono-Q with an uninterrupted linear gradient of 17 ml from 20 to 110 mM $MgCl_2$.

An uncharacterized and labile activity not due to PK40 or PK36 was found in the trailing fractions 23–25 of the gel filtration. Elution from Mono-Q with a gradient profile as used for PK36 (method A) occurred in fractions 11–12 (PK36, fractions 12–13) with >90% loss of activity. No band could be assigned on a silver-stained SDS polyacrylamide gel electrophoresis, and no 36 or 40 kDa band was found.

High salt extraction of the brain cytoskeletal fraction. The pellet of the 100,000 × *g* centrifugation of the whole-brain homogenate was resuspended in 70 ml of 20 mM PBS, 0.8 M KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA, and 0.5% β-mercaptoethanol, pH 7.0 (Toru-Delbauffe and Pierre, 1983), and stirred for 4 hr at 4°C. After 1 hr centrifugation at 100,000 × *g*, the pellet was reextracted with 70 ml of fresh high-salt buffer for another 4 hr. The combined supernatants were subjected to ammonium sulfate fractionation as described for the whole-brain supernatant (see above). Precipitates were collected by centrifugation at 27,000 × *g* for 15 min, redissolved in 2 ml each of 100 mM MES, 2 mM $MgCl_2$, 1 mM EGTA, and 0.5% β-mercaptoethanol (pH 6.5), and dialyzed into this buffer.

Phosphatase assays

^{32}P-labeled NF triplet was prepared by incubation of 465 μg of dephosphorylated NF triplet under standard assay conditions (see above) with 14 units (1 unit = 1 pmol of $^{32}P-PO_4$ transferred/min) of NF kinase I activity and 275 cpm/pmol γ-^{32}P-ATP. After 18 hr at 37°C, the mixture was diluted to 1.2 ml with water and the NF triplet was recovered by centrifugation for 1 hr at 100,000 × *g*. The pellet was rehomogenized twice in 1 ml of water and resedimented to remove excess ATP as monitored by scintillation counting of the wash supernatants. The final pellet containing 2000 cpm/μg of protein was homogenized in 80 μl of 40% glycerol and stored at −20°C.

To assay for contaminating phosphatase activity in the two kinase peaks of step III, 5.6 μg of ^{32}P-NF triplet were incubated at 37°C with amounts of the two fractions as used in NF kinase assays under standard conditions (see above) for 15 min and for 18 hr. Identical control assays were performed without kinase fractions. Each assay was mixed with 20 μl of 1% BSA solution and 120 μl of 10% TCA, left for 15 min at room temperature, and centrifuged at 12,000 × *g* for 5 min. Aliquots (100 μl) of each supernatant were scintillation counted.

Preparation of tau protein

Tau protein was isolated in the course of the kinase purification procedure as a by-product from gel filtration fractions preceding the fractions containing activity. The bulk of tau eluted as a very broad peak in fractions 1–12, as detected by immunodot blotting with the mAb 5E2 (Kosik et al., 1986). From the pooled fractions, tau was obtained after $HClO_4$ treatment and AS fractionation of the supernatant as described (Uéda et al., 1990). Two tau fractions were obtained by subsequent FPLC on Mono-S with a linear gradient of 0–200 mM NaCl, 20 mM HEPES (pH 6.9), 1 mM EDTA, and 1 mM DTT (Hagestedt et al., 1989), which were distinguished only by the relative amounts of the three major isoforms resolved by SDS-PAGE. Fraction tau I (0.48 mg) was obtained from the flow-through fraction; fraction tau II (0.90 mg) eluted as a broad peak between 50 and 150 mM NaCl.

Dephosphorylation of tau protein

Tau I and tau II (165 μg total of a 1:2 mix) were incubated overnight with 4.8 μg of *E. coli* alkaline phosphatase and 6.5 μg of calf intestinal phosphatase in 0.2 ml 50 mM Tris (pH 8.5), 0.5 mM $MgSO_4$, 0.5 mM $ZnSO_4$, and 0.5 mM PMSF at 37°C. The phosphatases were quantitatively removed by precipitation with 6 μl of 70% $HClO_4$ and centrifugation for 15 min at 12,000 × *g*; a considerable amount of the tau I and tau II proteins also precipitated in this step. Dephosphorylated tau I and tau II (25 μg) were recovered from the supernatant after neutralization and dialysis into water.

Materials

E. coli alkaline phosphatase type III-N, histone type III-S (calf thymus, lysine-rich fraction), dephosphorylated α-casein (bovine), phosvitin (egg yolk), calmodulin from bovine brain, synthetic Walsh inhibitor (rabbit), cAMP and cGMP (sodium salts), adenosine 5′-triphosphate highest purity (sodium salt), and guanosine 5′-triphosphate (lithium salt) were purchased from Sigma Chemicals. Calf intestinal alkaline phosphatase (special molecular biology grade) was obtained from Boehringer Mannheim Biochemicals. γ-^{32}P-ATP (3000 Ci/mmol) and γ-^{32}P-GTP were from Du Pont–New England Nuclear. The monoclonal mouse anti-rat-NF antibodies SMI-31, SMI-33, and SMI-34 were from Sternberger-Meyer Immunochemicals, Jarretsville, MD, and polyclonal anti-tubulin was from ICN. Twice-cycled microtubules and purified tubulin from calf brain were a gift of Dr. F. Solomon (Department of Biology, MIT). L-α-Phosphatidylserine and samples of protein kinase C and calcium/calmodulin-dependent kinase II were kindly provided by Dr. A. C. Nairn (Rockefeller University). Human tau protein expressed in *E. coli* from the clone htau40 was generously provided by Dr. E.-M. Mandelkow (Max Planck Research Unit for Structural Molecular Biology, Hamburg).

Results

Preparation of dephosphorylated NF proteins for kinase immunoassays

We had to prepare NF proteins completely devoid of immunoreactivity with mAbs SMI-31 and SMI-34 in order to use an assay for kinase activity specific for these epitopes, the repeated KSP sequences (multiphosphorylation repeat [MPR]). This specificity was necessary because crude brain extracts are expected to contain a very large number of protein kinases. The

epitope of SMI-31 had been shown earlier to be the phosphorylated MPR sequence (Lee et al., 1988), while the SMI-34 phosphoepitope remained undefined. Such a preparation of dephosphorylated NF proteins would, however, be strongly reactive with the mAb SMI-33, which is specific for the nonphosphorylated KSP sequence (Lee et al., 1988).

Successful dephosphorylation of NF triplet depends on its method of preparation

We used two preparations of NF triplet protein—one, called *native* NF triplet, was obtained by batch HTP chromatography and removing the urea used by dialysis; the other preparation was the *reconstituted* NF triplet, obtained from FPLC-purified NF subunits, again removing urea by dialysis.

Dephosphorylation of native NF triplet with *E. coli* alkaline phosphatase reportedly removes >90% of all phosphate groups in NF-M and NF-H (Carden et al., 1985). In our experiments, the shift of apparent M_r on SDS-PAGE acccompanying dephosphorylation of NF-M and NF-H in the triplet proceeded very quickly and was virtually completed within minutes (Fig. 1, lanes B). However, 5 d of incubation were necessary to abolish completely the SMI-31 and SMI-34 immunoreactivity (data not shown). Before analysis, the phosphatase was removed quantitatively by repeated sedimentation of the dephosphorylated triplet.

Surprisingly, the NF-M and NF-H subunits in the NF triplet that had been reconstituted from FPLC-purified subunits reacted very much more slowly as monitored by gel shift, removal of SMI-31 reactivity, and generation of the SMI-33 epitope (Fig. 1, lanes *C*). Loss of SMI-31 reactivity was not complete even after 5 d of incubation.

The reason for this differential behavior of native and reconstituted NF triplet remains unclear. Tubulin was detected as a minor impurity in the readily reacting triplet preparation by Western blotting, but addition of tubulin or crude microtubules to the FPLC-purified NF triplet did not affect the dephosphorylation reaction.

Different phosphatases are required for dephosphorylation of purified NF-M and NF-H

FPLC-purified NF-M but not FPLC-purified NF-H could be satisfactorily dephosphorylated with *E. coli* alkaline phosphatase so as to be unreactive to SMI-31 and SMI-34 under conditions similar to those used for the NF triplet. The shift of apparent M_r on SDS-PAGE and the removal of SMI-31 and SMI-34 immunoreactivity remained incomplete even after 5 d of incubation of FPLC-purified NF-H with high concentrations of phosphatase (Fig. 2). This behavior resembled somewhat that of the NF-H subunit in the reconstituted NF triplet (Fig. 1, lanes A vs. lanes C). The immunoreactivity of purified NF-H with SMI-31/SMI-34 can only be removed with calf intestinal phosphatase after 5 d of incubation. The mobility of dephosphorylated NF-H with SDS-PAGE (Fig. 2, center panel, lane C) resembles that of native NF-M (lane A; lane B: native NF-H). NF-M can be completely dephosphorylated with both phosphatases under identical conditions (right panel).

Since the purified subunits of NF-M and NF-H are soluble and cannot be sedimented, the phosphatases had to be removed by gel filtration. Heat treatment (and freezing) of the NF was avoided because the proteins tended to aggregate.

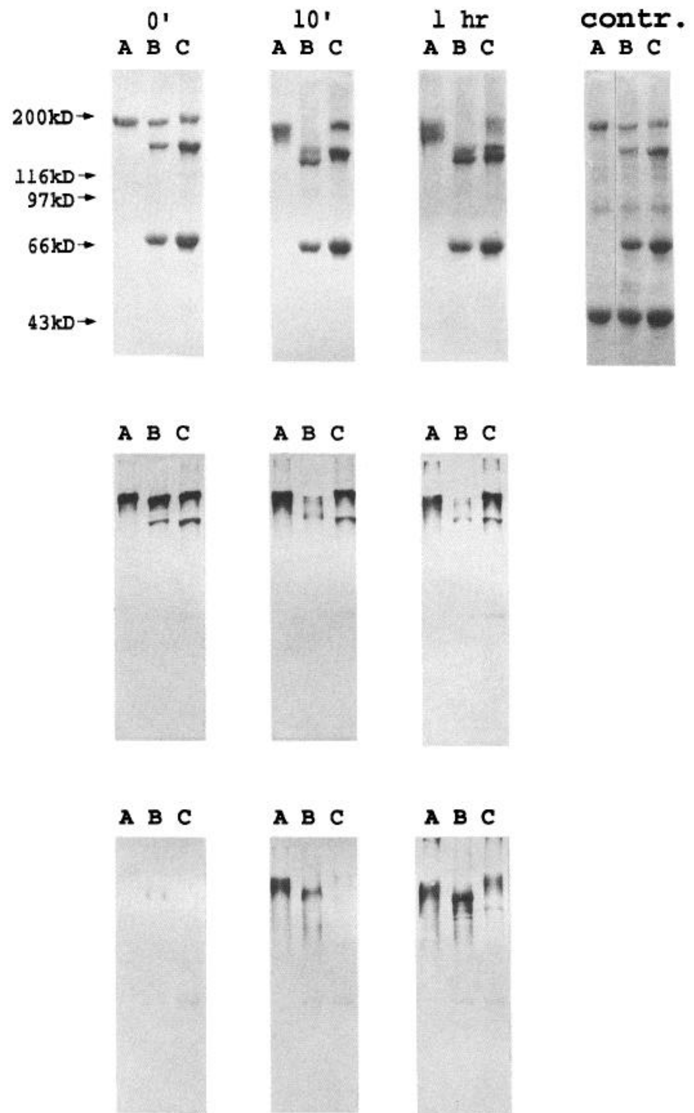

Figure 1. *Lanes: A,* purified soluble NF-H; *B,* "native" NF triplet, reconstituted after HTP chromatography; *C,* NF triplet reconstituted from FPLC-purified subunits. *Top,* 7.5% SDS-PAGE; *middle,* Western immunoblots with mAb SMI-31 (phosphorylated epitope); *bottom,* Western immunoblots with mAb SMI-33 (unphosphorylated epitope). All gels except the control were subjected to a brief electrophoretic transfer in SDS-free buffer to remove the phosphatase and accompanying impurities; NFs did not transfer under these conditions. *Control,* With the phosphatase activity inhibited by 10 mM phosphate and 5 mM EDTA, the NF proteins did not appear altered after 24 hr of incubation.

Detection of KSP-phosphorylating kinases in bovine brain by immunoassay

In initial ^{32}P assays of crude whole-brain supernatants with and without common second messengers, NF phosphorylation was relatively weak with only a slight activation in the presence of cAMP. No gel shift of the heavy NF subunits could be demonstrated even after 12 hr of incubation (not shown). Likewise, AS-concentrated fractions of supernatant and high salt cytoskeletal extract did not reveal any prominent NF-specific activity in initial rate assays.

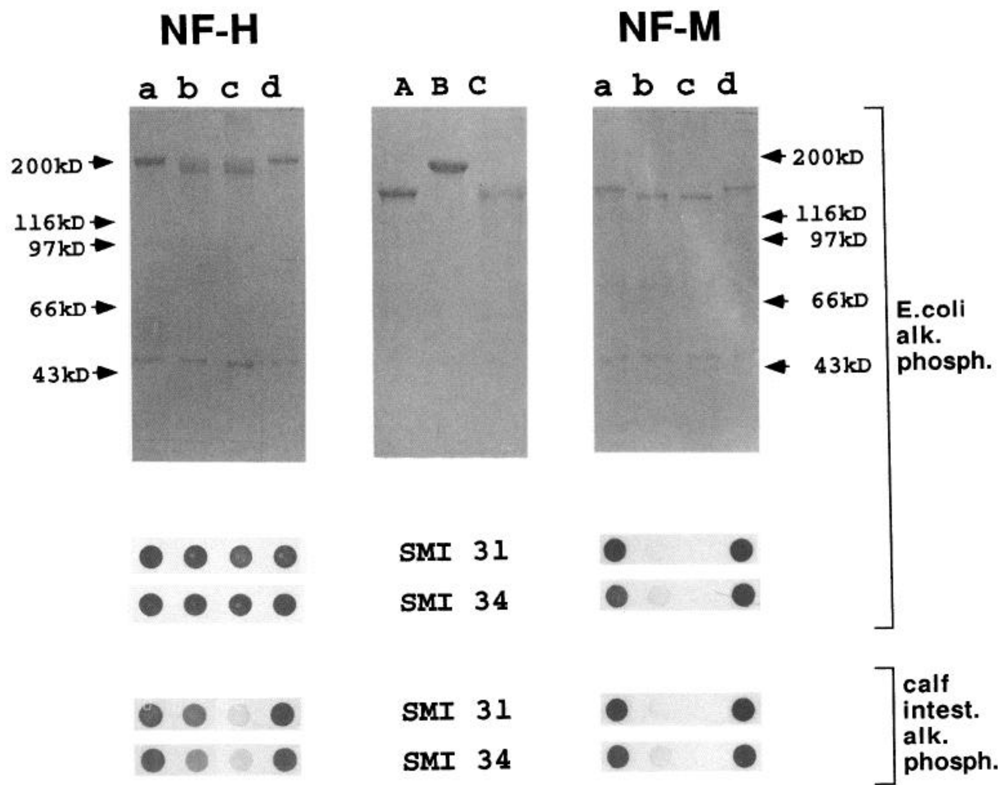

Figure 2. Dephosphorylation of NF-H and NF-M by *E. coli* alkaline phosphatase or with calf intestinal phosphatase. *Top, E. coli* alkaline phosphatase; SDS-PAGE and immunodot blots with mAbs SMI-31 and SMI-34 (phosphorylated epitope). *Lanes: a,* 1 hr; *b,* 24 hr; *c,* 5 d of incubation at 37°C; *d,* control incubation for 5 d with the phosphatase blocked by 10 mM phosphate (pH 8.5) and 5 mM EDTA. *Middle,* SDS-PAGE. *Lanes: A,* native NF-M; *B,* native NF-H; *C,* dephosphorylated NF-H (*E. coli* alkaline phosphatase). *Bottom,* Calf intestinal phosphatase; immunodot blots with mAbs SMI-31 and SMI-34. Lanes *a–d* are as above.

SMI-31 and SMI-34 immunoassays detect a NF kinase not associated with the cytoskeleton

The SMI-31 and SMI-34 epitopes could only be reconstituted after 18 hr but not after 6 hr of incubation of the dephosphorylated NF triplet with the 30–40% AS fraction of the brain supernatant. Later, a 35–45% fraction was found to be more favorable for preparative purposes. The activity was NF specific, since control immunoassays lacking dephosphorylated native NF triplet were negative, in contrast to the 40–50% fraction (Fig. 3*A*). An additional, less prominent NF-specific activity was detected in the 40–55% and 55–70% AS fractions of a cytoskeletal extract with 0.8 M KCl, where we had expected the main NF kinase activity to be, judging from reports in the literature. The soluble nature of our kinase(s) was confirmed when the activity did not cosediment under low salt conditions (10 mM HEPES buffer, pH 7) after 15 min incubation with dephosphorylated native NF triplet at 37°C or with assembled cold-solubilized microtubules according to the method of Shelanski et al. (1973) (4 M glycerol, 1 mM GTP, 37°C, 30 min), in the absence or presence of 5 mM Mg-ATP (not shown).

Optimization of the SMI-31 and SMI-34 immunoassays

The site-specific kinase immunoassays, though only semiquantitative, allowed for the estimation of some of the properties of the enzyme(s) while still in a crude form (Fig. 3*B,C*). The 18 hr incubation period was chosen for saturation assays to be consistent with the early experiments and to maximize phosphorylating activity. The pH optimum was around pH 7; the best Mg^{2+} and ATP concentrations were found to be 2 mM and 1 mM, respectively. ATP (5 mM) inhibited the activity. Mn^{2+} was about twice as effective as Mg^{2+} (not shown). GTP could not substitute for ATP. Concentrations of NaCl of >20 mM diminished the assay response, due to either inhibition or destabilization of the activity during the lengthy incubation period. This effect was attributable to ionic strength rather than specifically to Na^+ or Cl^- ions, since the same decline was seen with $(NH_4)_2SO_4$ at comparable ionic strength (not shown).

Purification of the KSP-phosphorylating kinases

To select for suitable adsorbents and elution conditions, the 35–45% AS fraction was exposed to a variety of chromatography media at 4°C. The activity as assayed with the SMI mAbs was lost in almost every case. Even largely nonionic media like agarose, and to a lesser degree Sephadex, produced substantial losses. These losses occurred also in the presence of 4 M NaCl, which by itself did not affect enzyme survival in control experiments. NaCl was added to prevent binding of the kinase or possible essential subunits to the chromatography media. However, inclusion of Mg-ATP stabilized the activity on some media but not others. In order of decreasing survival of activity, Sephadex, agarose, CM-Sepharose, and quarternary ammonium anion exchangers were found to be the only useful chromatography media in the presence of Mg-ATP. In a small-scale gel filtration of the 35–45% AS fraction on Sephadex under low salt conditions, a major activity peak of 50–60 kDa and a minor activity peak around 30–40 kDa were found both by ^{32}P assay, followed by SDS-PAGE, and by SMI immunoassays.

Chromatography on CM-Sepharose

The activity was enriched about five- to sixfold before gel filtration by chromatography of the AS fraction on CM-Sepharose at pH 6.0 with 5 mM Mg-ATP and 10% glycerol as stabilizing agents. The NF-specific kinase activity was eluted in one step

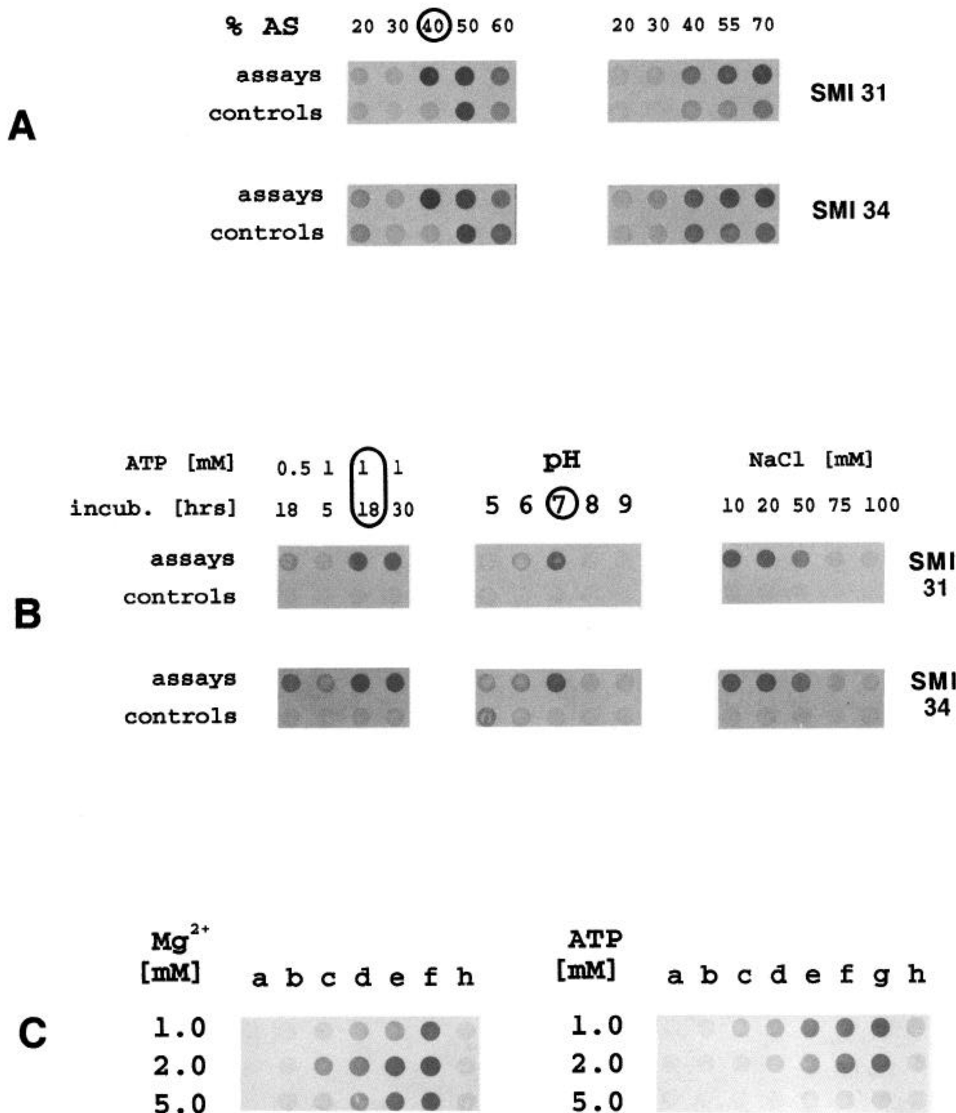

Figure 3. *A*: *Left,* Immunodot blot assays (0.5 mM Mg^{2+}, 0.5 mM ATP) of AS fractions from whole-brain supernatant. Control assays have no added NF proteins. *Right,* Imunodot blot assays of AS fractions from a 0.8 M KCl cytoskeletal extract. *B,* Time, pH, and ionic strength requirements of the SMI-31 and SMI-34 immunoassays. The assay responses were optimal at pH 7.0, low salt conditions, 1 mM ATP. All assays were performed for 18 hr with 0.5 mM Mg^{2+} when not indicated differently. *C,* Optimization of the SMI-31 immunoassay (identical results were obtained with SMI-34) with respect to Mg^{2+} (*left*; 1 mM ATP) and ATP concentrations (*right*; 2 mM Mg^{2+}) by determination of the lowest concentration of crude enzyme (40% AS cut) required for assay response. *a–g*: 0.04, 0.09, 0.13, 0.18, 0.22, 0.33, and 0.44 μg of crude enzyme protein per assay, respectively. *h,* Control assay without NFs; 0.4 μg of crude enzyme protein per assay.

by raising the Mg-acetate concentration to 85 mM. Elution with a gradient of Mg-acetate resulted only in a broader peak of activity but did not improve the enrichment. Little NF-specific activity was found in the flow-through.

Several kinase activities are resolved by gel filtration and Mono-Q FPLC

Gel filtration proved to be the most effective step in the purification scheme and was carried out in the presence of Mg-ATP on superfine grade of Sephadex. The activity as assayed by ^{32}P incorporation into dephosphorylated native NF triplet and by SMI immunoassays eluted in most cases as one very broad peak (Fig. 4), sometimes a major and a minor activity were resolved with apparent M_r of 60,000 and 40,000, respectively. No significant NF-specific phosphatase activity could be detected in the relevant kinase fractions by monitoring the liberation of phosphate under assay conditions from ^{32}P-labeled dephosphorylated native NF triplet, prepared by phosphorylation with partially purified NF kinase.

Initially, all fractions containing high amounts of SMI-31 epitope reconstituting activity were pooled (method B) and subjected to FPLC on Mono-Q with a linear gradient of $MgCl_2$. A 40 kDa band (PK40) with SDS-PAGE analysis of the fractions matched very closely the activity profile (not shown). However, subsequent detailed analysis (see below) revealed the presence of two more distinct activities in the gel filtration fractions; one of them was correlated with a 36 kDa protein (PK36), which contaminated PK40 (PK36/40; see Fig. 5*C,* lane a). Separation of PK36 and PK40 was accomplished on Mono-Q by a modified gradient profile with an isocratic component (method B). However, both kinases were obtained only in relatively low purity, and a preparative gel electrophoresis in the presence of Mg-ATP was required to yield PK40 in the highest purity (Table 1; Fig. 5*C,* lane b) achieved so far. This preparation was used to characterize PK40.

Alternatively, gel filtration fractions were pooled according to their content of the 40 and 36 kDa protein known to be associated with kinase activity (Fig. 4; method A). Subsequent elution of each kinase from Mono-Q with the modified gradient profile (Fig. 6) rendered PK40 in a purity comparing well to electrophoretically purified material (Table 1; Fig. 5*C,* lane c) and PK36 in the highest purity obtained so far (Fig. 5*C,* lane

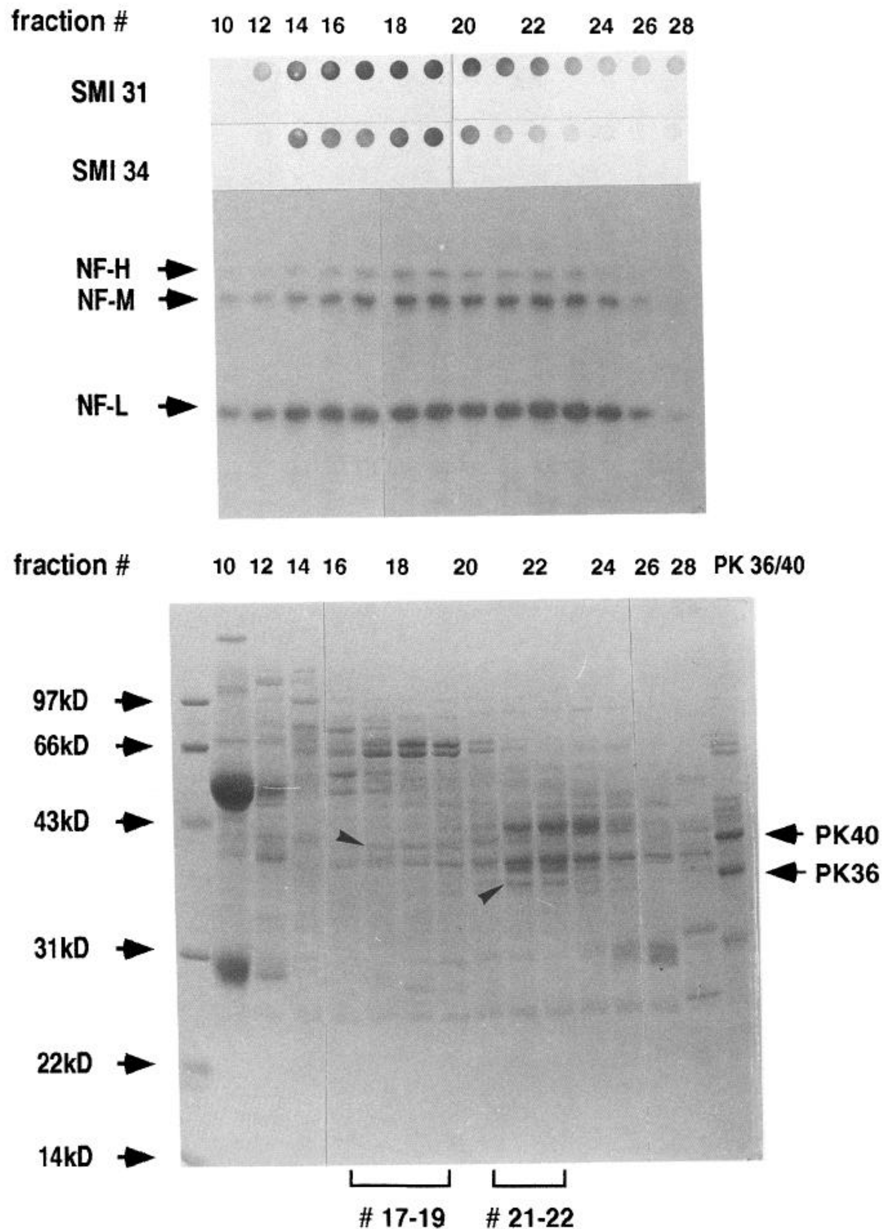

Figure 4. Analysis of gel filtration fractions 10–28 by SMI-31/SMI-34 immunoassays after 18 hr of incubation with NF triplet (*top*), by ^{32}P assays after 30 min of incubation with NF triplet (*middle*), and by 12% SDS-PAGE (*bottom*). In method A (Materials and Methods), fractions 17–19 and 21–22 were pooled according to the most prominent occurrence of PK40 and PK36 (*arrowheads*; see Fig. 5). In method B (Materials and Methods), fractions 12–22 were pooled as indicated by the SMI-31 immunodot blot assays.

d). Preparative electrophoresis of PK40 resulting from this method did not improve the purity significantly. Efforts to purify PK40 and PK36 further by FPLC on Mono-S resulted in complete loss of activity even in the presence of Mg-ATP.

A third uncharacterized kinase activity was found in gel filtration fractions after the broad activity peak and was sometimes seen as a separate peak of activity. Significant reconstitution of the SMI epitopes by this activity occurred only after concentration of these fractions (relatively weak immunoassays of fractions 23–25, Fig. 4). The activity eluted from Mono-Q at somewhat lower ionic strength than PK40 or PK36 with >90% loss of the activity loaded (not shown). No band on silver-stained SDS-PAGE of concentrated fractions could be matched to the activity elution profile of the third kinase. The activity migrated over a wide range of mobilities on a nondenaturing PAGE, suggesting that it might represent a heterogeneous mixture of degradation products but probably unrelated to PK40 or PK36 (see below). Pooling of PK40 and PK36 in the gel filtration and FPLC steps according to method A precluded contamination with this kinase.

Correlation of a 40 and a 36 kDa protein with NF kinase activity

The Mono-Q elution profile of PK36/40 (Fig. 5*C*, lane a; see Materials and Methods) strongly suggested association of a 40 kDa protein with NF kinase activity (not shown). To confirm this assignment further and to assess homogeneity of activity, PK36/40 was subjected to electrophoresis on SDS-free polyacrylamide gel in the presence of Mg-ATP to stabilize the kinase. Consecutive slices of the gel were extracted (see Materials and Methods) and analyzed for kinase activity by ^{32}P assay and immunoassay and for protein content by SDS-PAGE. The 40 kDa band correlated with activity as expected (Fig. 5*A*); how-

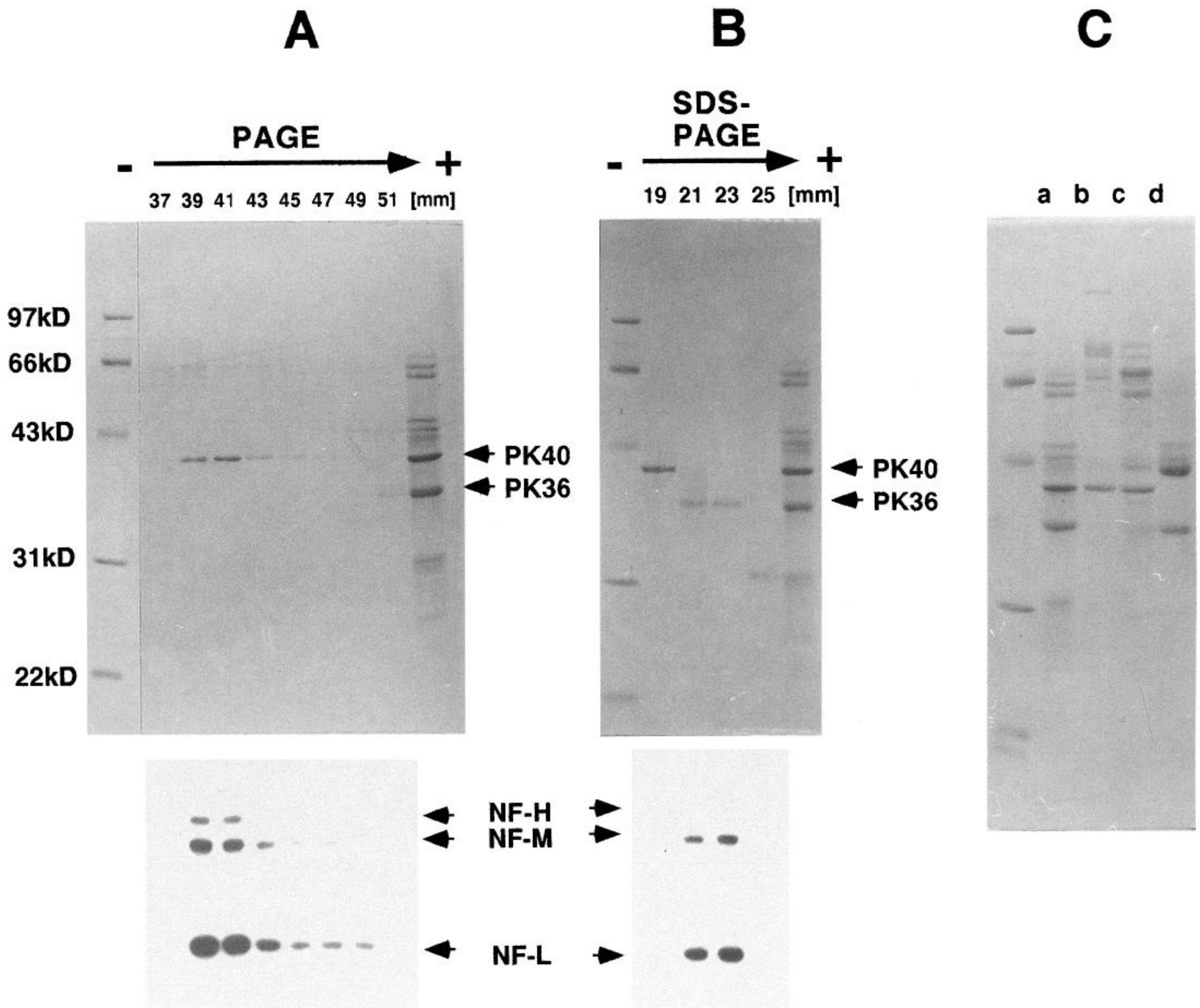

Figure 5. Identification of a 40 and a 36 kDa protein as kinases. *A,* The PK36/40 mixture (as in step IV, method B; see Materials and Methods) was separated on nondenaturing 7.5% polyacrylamide gel electrophoresis of 110 mm length containing Mg-ATP. Slices (2 mm) were each partially eluted to assay for NF kinase activity by the ^{32}P assay, followed by SDS-PAGE and radioautography. This correlated well with the 40 kDa protein content of the slices analyzed by 12% SDS polyacrylamide gel electrophoresis. A 36 kDa band was eluted in slices >49 mm from the top but showed only weak enzymatic activity, perhaps due to inefficient elution from gel slices. *B,* The PK36/40 mixture was separated on a 10% polyacrylamide gel containing SDS and Mg-ATP. Slices (2 mm) were analyzed as in *A*. Only the 36 kDa band shows activity after contact with SDS . However, 24 hr of exposure of the radioautograph was needed as opposed to only 2 hr in *A,* again pointing to a relatively low recovery of PK36 by elution of gel slices. Note that phosphorylation of NF-H is seen with PK40 but not PK36. *C,* Comparison by SDS-PAGE and staining with Coomassie blue of several NF kinase preparations. *Lane a* was obtained by pooling of PK40 and PK36 after gel filtration and elution from Mono-Q with an uninterrupted linear gradient; *lane b,* PK40 obtained by preparative gel electrophoresis (step V in method B). This preparation was used for characterization purposes. PK40 (*lane c*) and PK36 (*lane d*) were obtained after pooling of gel filtration fractions and Mono-Q elution protocol according to method A. Preparative gel electrophoresis of preparation *c* did not improve the purity beyond *b.*

ever, another activity was detected in a broad range of higher relative mobility with PAGE than PK40 (not shown).

A clearer identification of PK36 was possible when PK36/40 was separated with SDS-PAGE in the presence of Mg-ATP and gel slices were analyzed as above. In this case, only the 36 kDa protein retained NF kinase activity, while the activity of the 40 kDa protein could not be reconstituted after SDS exposure (Fig. 5*B*).

Both kinases, PK40 and PK36, also reconstituted the epitope of RT97 (data not shown), another mAb recognizing the MPR sequence (Lee et al., 1988).

N-terminal sequencing of the two proteins failed, probably

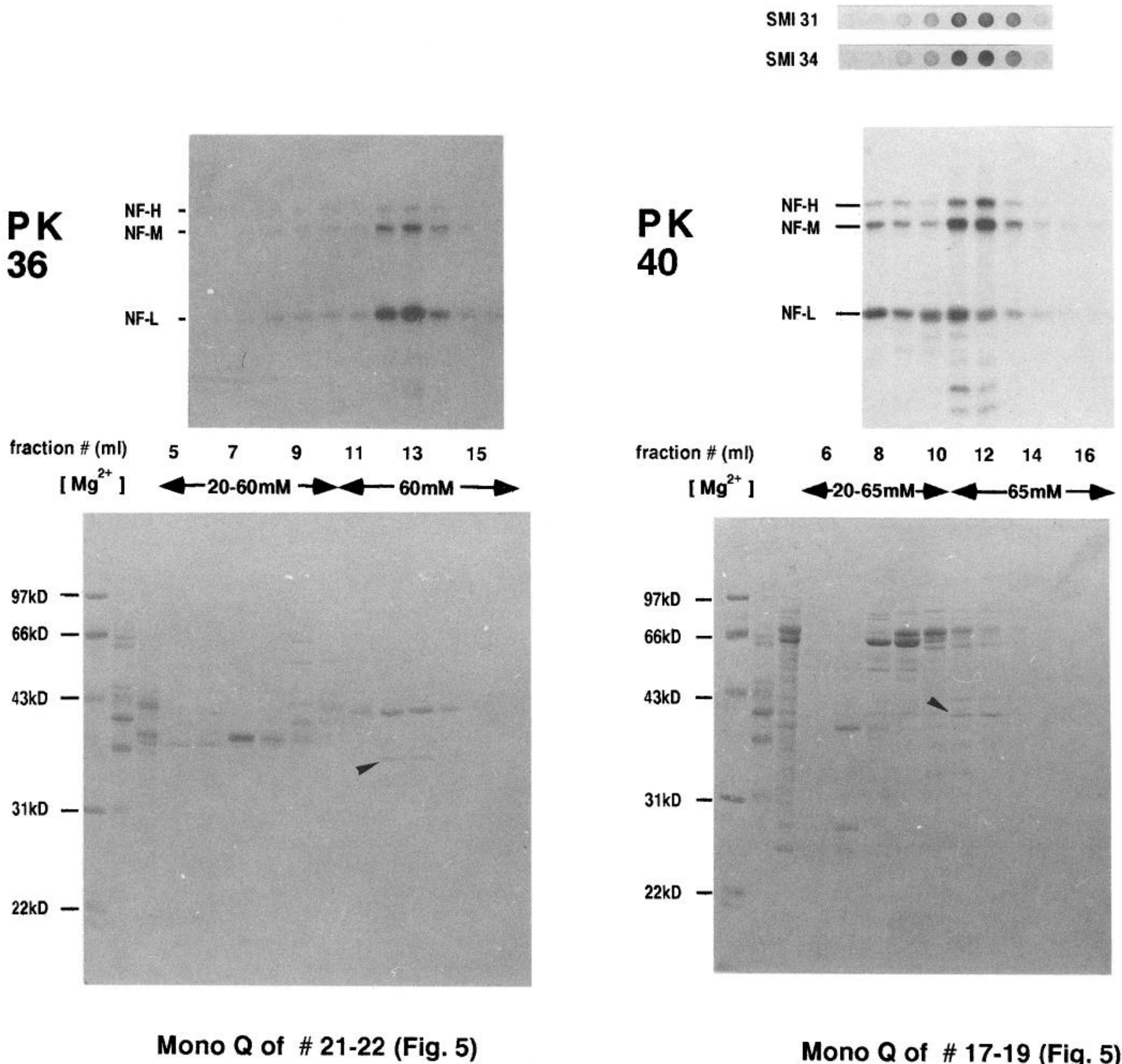

Figure 6. Elution of PK36 and PK40 from Mono-Q with a $MgCl_2$ gradient. Peaks of activity in the ^{32}P assays and immunoassays correlated well with the 36 and 40 kDa bands, respectively (*arrowheads*). The immunoassays responded only weakly to PK36. Note again the relatively prominent phosphorylation of NF-M and NF-H by PK40. The weaker activity in fractions 8–10 of the PK40 elution may be correlated with a band that has a slightly higher apparent M_r on 12% SDS-polyacrylamide gel than PK40 and was therefore not combined with PK40. Pooling of PK40 fractions 11 and 12 and PK36 fractions 12 and 13 resulted in preparations *c* and *d* in Fig. 5*C*.

as a result of blocked N-termini, which are common in mammalian proteins.

Characterization of PK40 and PK36

Substrate specificity of PK40 and PK36

Lysine-rich histone type III was found to be the most preferred substrate of both kinases and might be more favorable for assay purposes than NF. The acidic protein phosvitin and tubulin were very poor substrates.

Among neuronal proteins tested, the specificity of PK40 for dephosphorylated NF-M is most striking (Fig. 7). Other substrates are less efficient, in the following order: dephosphorylated NF-M $\gg$ tau > NF-M = NF-L > dephosphorylated NF-H > NF-H.

PK36 has a lower specific activity; its substrates form a somewhat different sequence: NF-L = tau = dephosphorylated NF-M > NF-M $\gg$ NF-H = dephosphorylated NF-H.

The third uncharacterized activity from the gel filtration (see above) phosphorylated the heavy NF subunits less well than NF-L, especially when these had been dephosphorylated, but had a significant specificity for phosvitin, suggesting that it might not be related to either PK40 or PK36 (not shown).

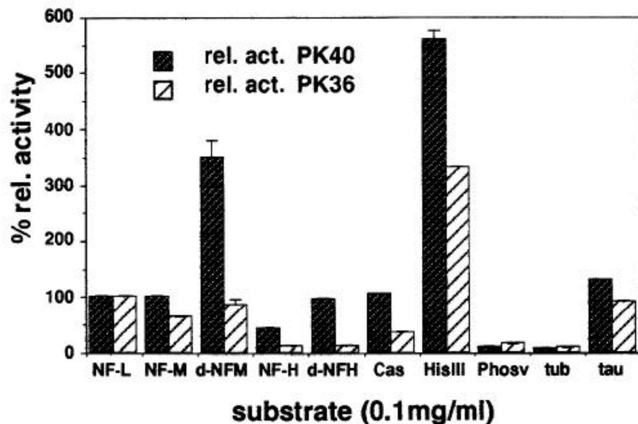

Figure 7. Specificity of the kinases PK40 and PK36 (see Fig. 5*C*, lanes b and d, and Table 1 for preparations used) for various neuronal proteins (see text) and general kinase substrates (see text) relative to NF-L. The ^{32}P assay of kinase activity was used (see Materials and Methods). The indicated values are means (±SD) of triplicate assays. Note the striking preference of PK40 for dephosphorylated NF-M and the level of tau specificity of both kinases.

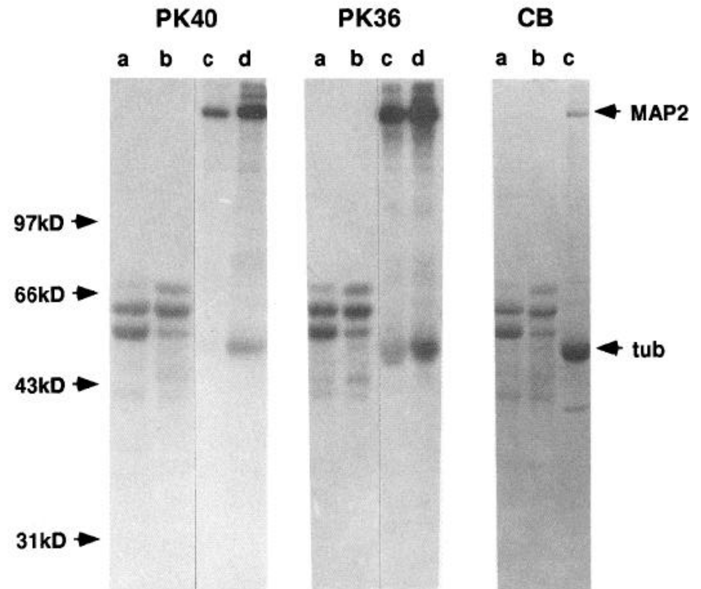

Figure 8. Comparison of MAPs on autoradiograms after phosphorylation with PK40 and PK36 or microtubule-associated kinase activity. Two distinct bovine tau fractions, tau I (*lanes a*) and tau II (*lanes b*; see Materials and Methods) were good substrates for PK40 as well as PK36. Comparison with the Coomassie blue–stained gel (*CB*) revealed no preference for any isoform of tau. MAP2 is phosphorylated by both kinases (*lanes d*) above background level (PK40, 2.5×; PK36, 1.5×; determined by Cerenkov counting). Background labeling of MAP2 is due to a second messenger–independent activity intrinsic to cycled microtubules (*lanes c*). *tub,* tubulin.

Some microtubule-associated proteins (MAPs) are also good substrates for both kinases. Two chromatographically separable tau preparations (taus I and II; see Materials and Methods) were equally well phosphorylated by PK40 and PK36. No tau isoform specificity was detectable in either case (Fig. 8, lanes a,b). MAP2 in a crude microtubule preparation was a substrate comparable to the tau proteins or better, especially for PK40 (see difference between lanes c and d, Fig. 8).

ATP dependence and inhibition of the activity of PK40 and PK36

The ATP dependence of the activities of PK40 and PK36 was determined at 2 mM Mg^{2+} and with soluble dephosphorylated NF-M as second substrate to avoid uncertainties arising from the aggregation state of NF triplet in suspension (Fig. 9). The optima were at 0.5–1 mM ATP for both kinases, which confirmed the concentration suggested earlier for the crude enzymes by the semiquantitative SMI immunoassays (see above). Apparent K_m values for ATP of both kinases were estimated from Woolf–Hanes plots (Dixon and Webb, 1979) for a range of ATP concentrations sufficiently below the onset of inhibition (Fig. 9). Three determinations of the K_m of PK40 at three different

Table 1. Enrichment of PK40 and PK36 through various chromatographic steps by alternative methods A (standard) and B

		Specific activity[a] (nmol/min/mg)		Total activity[b] (nmol/min)		Amount retrieved (mg)	
Step		PK40	PK36	PK40	PK36	PK40	PK36
I	AS fractionation	—[c]	—	—	—	290	
II	CM-Sepharose	0.55		23.6		43	
III	Sephadex G200						
	Method A	1.45	1.15	3.80	2.27	2.6	2.0
	Method B	0.26		4.5		17.5	
IV	Mono-Q FPLC						
	Method A	3.8	2.8	0.73	0.39	0.19	0.14
	Method B	0.83	0.28	0.78	0.47	0.94	1.7
V	Preparative Gel electrophoresis (method B only)	5.2	—[d]	0.67	—	0.13	—

Method A: gel filtration fractions analyzed by SDS-PAGE were pooled to contain either the 36 or the 40 kDa band (see fig. 4). Method B: all fractions containing SMI-31/SMI-34 epitope reconstituting activity were pooled after gel filtration, and PK36 and PK40 were separated in the subsequent step.

[a] nmol of $^{32}P\text{-}PO_4$ transferred/min/mg protein.

[b] nmol of $^{32}P\text{-}PO_4$ transferred/min.

[c] Not determined; NF-specific activity too low against background.

[d] Electroelution of PK36 from a preparative SDS gel was unsuccessful.

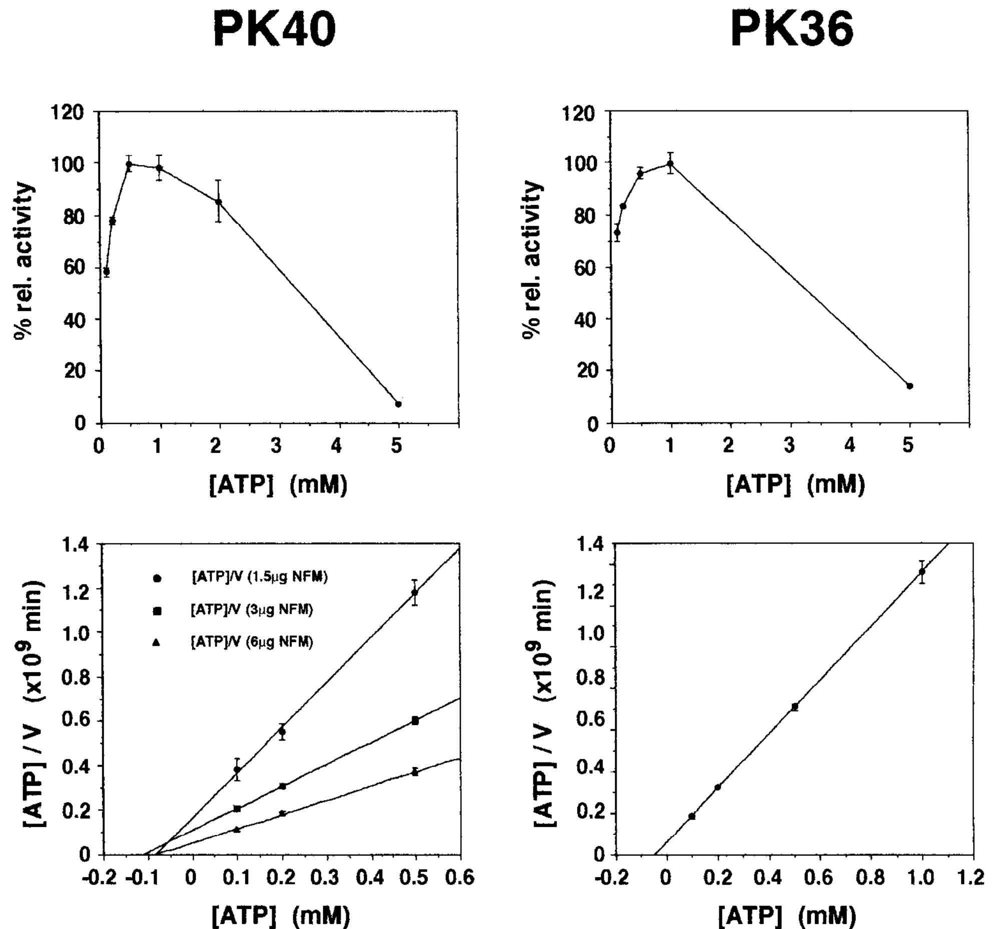

Figure 9. Top, Dependence of activity on ATP concentration at a Mg^{2+} concentration of 2 mM. *Bottom,* Hanes–Woolf plots for PK40 and PK36 with NF-M as substrate. *Error bars* are ±SD.

concentrations of the second substrate, NF-M, gave similar values (mean ± SD, 93 ± 12 μM), indicating little influence of the concentration of NF-M on ATP affinity of PK40. The apparent K_m of PK36 is approximately 50 μM.

PK36 and particularly PK40 were strongly inhibited to 14% and 7%, respectively, of the control level in the presence of 5 mM ATP amounting to 3 mM excess of free (uncomplexed) ATP over Mg^{2+}. In contrast, with excess Mg^{2+} (5 mM over 1 mM ATP) or 5 mM Mg^{2+}/5 mM ATP, little or no inhibition was observed for PK40, while the activity of PK36 was significantly reduced under these conditions (Table 2).

The activity of the kinases was also reduced to 27% (PK40) and 40% (PK36) in the presence of 150 mM NaCl. Inhibition by NaCl in this concentration range has been observed with other kinases also, among them cAMP-dependent kinase (Moll and Kaiser, 1977) and a nuclear casein kinase from HeLa cells (Friedrich and Ingram, 1989). None of the above effects could be explained by destabilization of the enzymes, since preincubations without substrate for 1 hr under inhibiting conditions, dialysis, and subsequent assay of remaining activity for 15 min under standard conditions did not reveal losses very much beyond control levels (Table 2, preincubation). Inhibition by the Walsh inhibitor was seen only for PK36, but not PK40, with an estimated IC_{50} of 50 μM. No significant activity change was seen over a wide range of concentrations of both kinases, indicating that regulatory mechanisms like homomer formation or intermolecular autophosphorylation probably do not play a role.

Certain other kinases do not phosphorylate the MPR site of NF proteins

We tested several common neuronal kinases and also a mixture of PK40 and PK36 (PK36/40) under comparable conditions for phosphorylation of the KSP sequence in dephosphorylated NF triplet and dephosphorylated NF-M, using the SMI-31 immunoassay. While calcium/calmodulin-dependent kinase II and protein kinase C phosphorylated native NF triplet relatively weakly, all NF subunits were very good substrates for the catalytic subunit of the cAMP-dependent kinase. However, reconstitution of the SMI epitopes in dephosphorylated NF triplet or purified dephosphorylated NF-M was only possible with PK36/40, even when much less activity was used than of the former kinase (Fig. 10). Incubation with the second messenger–independent kinase and the cAMP-dependent activity known to be associated with microtubules (Leterrier et al., 1981) did not increase the response in the immunoassay significantly above the level seen with microtubules alone in the absence of exogenously added NF, although all subunits were strongly ^{32}P labeled.

PK40 and PK36 induce mobility shifts of the heavy NF subunits on SDS-PAGE and incorporate phosphate in high molar ratios

To determine the maximum number of phosphates incorporated into the heavy NF subunits by PK40 and PK36, the purified activities (Fig. 5*C*, lanes b,d) were incubated in increasing

concentrations with dephosphorylated NF-M and dephosphorylated NF-H (Fig. 11). The stoichiometry of phosphorylation was determined by assuming that the correct molecular masses of NF-M and NF-H are 110 and 140 kDa, respectively, as determined by Kaufmann et al. (1984), since SDS-PAGE considerably overestimates their M_r. PK40 incorporated up to 15 phosphate groups into NF-M, which corresponds well to the number of phosphates found in isolated bovine NF-M (Wong et al., 1984) and induced a complete shift of the NF-M band on SDS-PAGE to the higher apparent M_r of native NF-M. In contrast, only a partial shift of NF-H was achieved with a maximum of seven phosphates introduced into a molecule with presumably about 40 KSP sites (Fig. 11*A*). The phosphorylation of NF-M with PK36 appeared to be saturated at 10 mol phosphate/mol NF-M with a substantial gel mobility shift; however, the NF-M band remained diffuse, possibly due to a heterogeneous phosphorylation state (Fig. 11*B*). NF-H was not phosphorylated very well by PK36 and showed virtually no gel shift, in correlation with its poor substrate properties for PK36. Both kinases reconstituted the SMI epitopes, but only weakly in the case of NF-H and PK36.

The maximal phosphorylation of NF-M was not significantly higher with a mixture of the two kinases (PK36/40), indicating that PK40 and PK36 might have a largely overlapping site specificity on NF-M. After incorporation of 7–13 phosphates, NF-M had a gel mobility comparable to native NF-M (Fig. 11*C*). The SMI immunoassay responses were correlated with the gel mobility shift but did not respond at lower levels of phosphorylation of <5 mol PO_4/mol NF-M. Interestingly, the SMI-34 immunoassay required a higher level of phosphorylation than the SMI-31 assay.

PK40 induces a substantial shift of apparent M_r of tau proteins with SDS-PAGE

Incubation of native bovine tau with a mixture of bacterial and calf intestinal alkaline phosphatases converted the pattern of three distinguishable isoforms from SDS-PAGE into a four-band pattern as expected (Lindwall and Cole, 1984), accompanied by a shift of about 15 kDa to a lower apparent M_r (Fig. 12*A*, lanes c,d). This shift could be completely reversed and the original three-band pattern restored after phosphorylation with

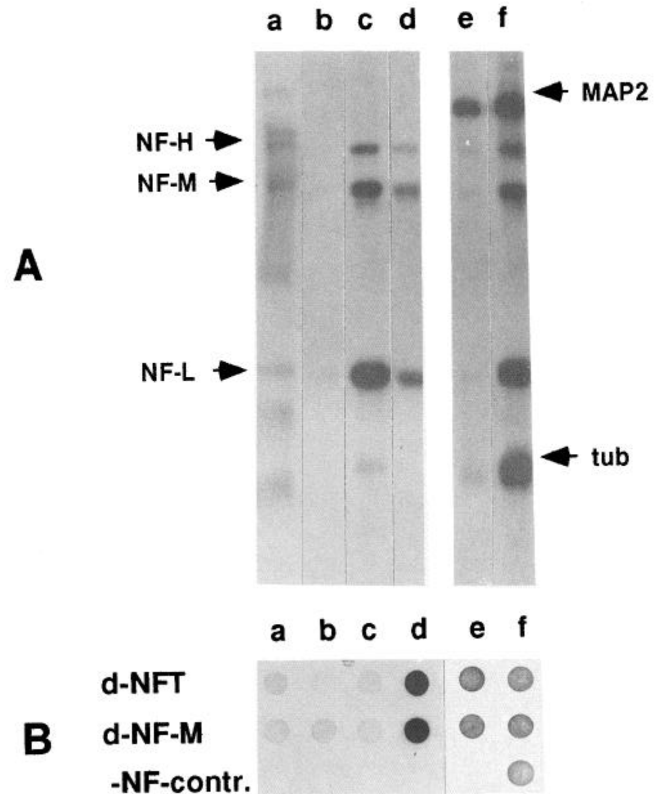

Figure 10. *A*, ^{32}P assays (*a–d*, 1 hr; *e* and *f*, 15 min) with native NF triplet. *B*, SMI-31 immunoassays after 16 hr of incubation with dephosphorylated NF triplet (*d-NFT*) and dephosphorylated NF-M (*d-NF-M*) as substrates. *a*, Calcium/calmodulin-dependent kinase II (0.6 μg); *b*, protein kinase C (0.05 μg); *c*, catalytic subunit of cAMP-dependent kinase (0.1 μg); *d*, a mix of PK40 and PK36, as used in Figure 5*C*,a (0.13 μg); *e*, second messenger–independent, microtubule-associated kinase (30 μg of twice-cycled microtubules); and *f*, cAMP-dependent, microtubule-associated kinase (30 μg of twice-cycled microtubules, activated by 50 μM cAMP). *-NF-contr.*, control without NF proteins.

PK40. The kinase also incorporated substantial amounts of phosphate into native bovine tau but induced only a small additional mobility shift. Preparations of bovine tau were previously found to contain five isoforms distinguishable with one-

Table 2. Effect of sodium chloride, excess magnesium and ATP, and the Walsh inhibitor on the relative activity (%) and stability of PK40 and PK36

	Control: 2 mM Mg^{2+} 1 mM ATP	2 mM Mg^{2+} 5 mM ATP	5 mM MG^{2+} 1 mM ATP	5 mM Mg^{2+} 5 mM ATP	NaCl 150 mM	Walsh inhibitor (μM) 4.5	15	45
PK40[b]	100[c] ± 6.9	7.2 ± 0.6	107 ± 3.5	78 ± 7.1	27 ± 1.8	102 ± 5.3	107 ± 3.7	101 ± 1.1
PK36[b]	100 ± 1.1	14 ± 0.5	40 ± 0.2	38 ± 2.2	40 ± 0.8	103 ± 0.5	87 ± 0.6	58 ± 3.3

	1 hr preincubation[a] with: 2 mM Mg^{2+} 1 mM ATP	2 mM Mg^{2+} 5 mM ATP	NaCl 150 mM
PK40[b]	42 ± 0.2	42 ± 0.3	69 ± 2.1
PK36[b]	68 ± 3.1	33 ± 1.1	62 ± 4.3

The values represent the mean of three assays (±SD); except for the Walsh inhibitor assays, which were carried out in duplicate.

[a] Assays were preincubated in the absence of substrate at 37°C, followed by microdialysis at 4°C into storage buffer, and assayed under control conditions for survival of activity: 15 min assay with 2 mM Mg^{2+} and 1 mM ATP.

[b] Preparations of PK40: see Table 1, Method B, Step V, and Figure 5*C*, lane b; preparations of PK36: see Table 1, Method A, Step IV and Figure 5*C*, lane d.

[c] All values represent relative activities in percentage of the control.

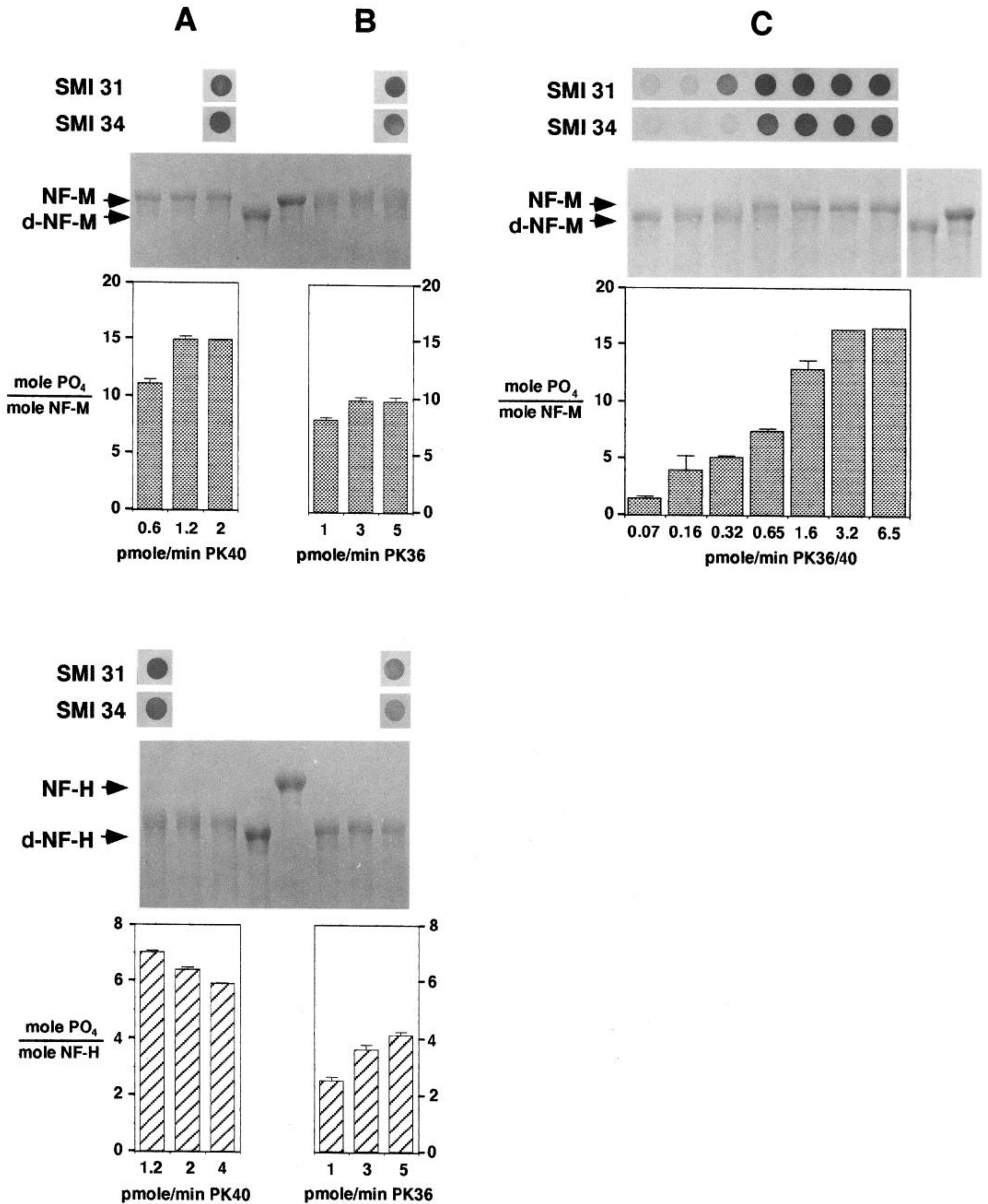

Figure 11. Saturation phosphorylation of dephosphorylated NF-M (*top*) and NF-H (*bottom*) by PK40 (*A*), PK36 (*B*), and PK36/40 (*C*) (as used in Fig. 5*C*, a), by increasing amounts of enzyme activity in 18 hr assays monitored by ^{32}P incorporation, gel mobility shift on 7.5% SDS polyacrylamide gels, and SMI-31/34 immunoassays. PK40 can induce a complete shift of dephosphorylated NF-M (*d-NF-M*) but only a partial shift of NF-H; the *lanes between A* and *B* and the *last two lanes in C* indicate the mobilities of the dephosphorylated and native NF subunits, respectively. *Error bars* are mean values ±SD.

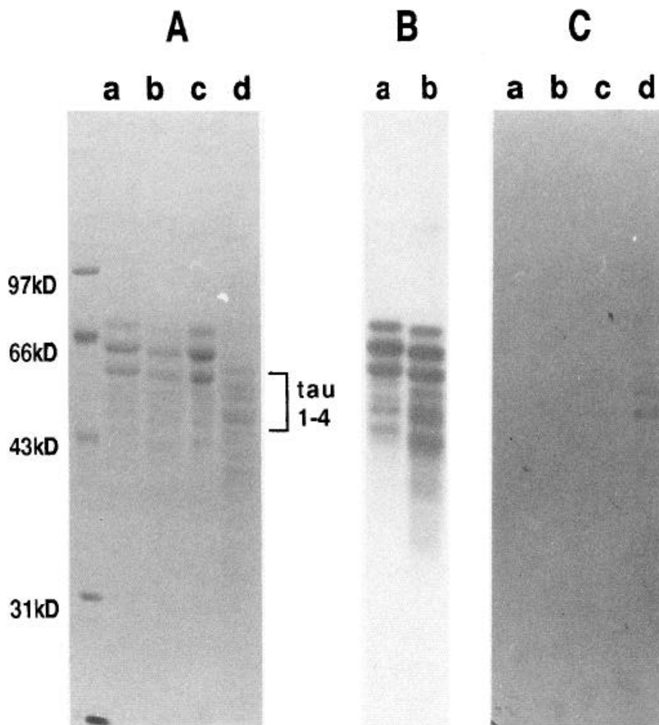

Figure 12. Saturation phosphorylation of native and dephosphorylated tau protein by PK40 monitored by SDS-PAGE, followed by Coomassie blue staining (*A*), autoradiography (*B*), and Western blotting with SMI-33 (*C*). Dephosphorylation of native bovine tau (*lanes c*) resulted in four bands, *tau 1–4* (*lane d*), of lower apparent M_r as expected. The original M_r could be completely restored by PK40 phosphorylation (*lane b*). Saturation phosphorylation of native tau resulted in only a small additional gel shift (*lane a* vs. *b* and *c*). The fact that less than 10% of the ^{32}P-labeled tau protein was recovered on the nitrocellulose blot may account for the weak staining of the blot (*C*).

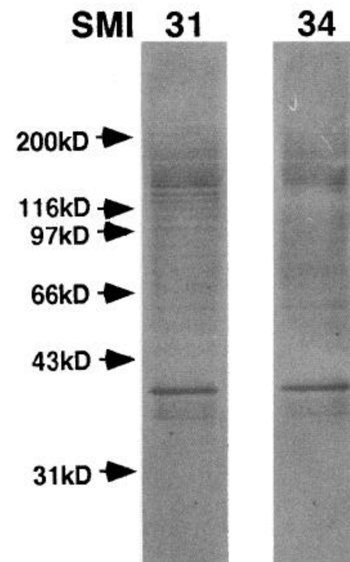

Figure 13. Comparison of peptide maps of NF-H after protease V8 digestion by Western blotting with SMI-31 and SMI-34. Pure NF-H (12 μg) was partially digested with 0.25 μg of V8 protease in ammonium bicarbonate buffer at pH 7.8 for 1 hr at 37°C. The peptide mixture was separated on a 10–20% SDS gradient gel, and Western blots were prepared.

dimensional SDS-PAGE (Butler and Shelanski, 1986). The fact that we obtained fewer apparent isoforms that did not exhibit any further substantial shift on phosphorylation might be due to selective enrichment of isoforms or a higher degree of phosphorylation in our preparation. We isolated tau as a side-product in the course of the kinase purification after prolonged exposure to the crude enzymes in Mg/ATP buffers at 4°C.

Only the dephosphorylated tau, and not the native or the rephosphorylated protein, reacted with SMI-33 (Fig. 12*C*) as a probe for the unphosphorylated KSP sequence (Lee et al., 1988) that occurs twice in the sequence of all bovine tau isoforms (Himmler et al., 1989). Conversely, the SMI-34 epitope is only found in the phosphorylated or rephosphorylated tau species (data not shown). This fact together with the ability of PK40 to phosphorylate the KSP site in NF suggests that one or both of the KSP sites of tau may also be target sequences for PK40.

Similar results were obtained with a pure human tau isoform expressed in *E. coli* from the clone Htau 40 (Goedert et al., 1989). Under saturating conditions, PK40 incorporated up to 14 phosphates into the 42 kDa tau isoform (H. Roder and E.-M. Mandelkow, unpublished observations). These experiments were carried out under conditions similar to those of Figure 11. This may not be an unreasonably high number, although lower phosphate content was found in tau previously (Lindwall and Cole, 1984). The phosphate content of isolated tau may not correspond to the *in vivo* situation. For example, in the case of MAP2, 8–50 mol of phosphate/molecule were found, depending upon the speed of preparation (A. Matus, personal communication). PK36 induced an incomplete mobility shift in tau protein, as in the case of NF-M.

The relationship between the SMI-31 and SMI-34 epitopes

In almost all aspects of the characterization of the kinases PK40 and PK36, the SMI-31 immunoassays closely paralleled the SMI-34 assays, indicating a close relationship. This was further substantiated by probing a peptide map of native NF-H after digestion with V8 protease with both mAbs on Western blots. The similarity of the staining patterns suggests that the two epitopes are probably not qualitatively different (Fig. 13). The two mAbs more likely require different degrees of phosphorylation for binding to MPR sites in the heavy NF subunits. The differential binding of SMI-31 and SMI-34 to NF-M at intermediate degrees of phosphorylation by PK40 (Fig. 11*C*) supports this notion; it seems to be the only significant difference between the two mAbs observed so far.

Discussion

PK40 and PK36 are distinguished from other kinases

The physiological relevance of NF and tau phosphorylation by common kinases, such as cAMP-dependent kinase (Leterrier et al., 1981) or protein kinase C (Sihag et al., 1988) remains in doubt. With a novel immunoassay, we have identified two new kinases that are capable of phosphorylating the MPR sequences of the heavy NF subunits in high stoichiometric ratios and of inducing, by phosphorylation, complete or partial shifts on SDS-PAGE of apparent M_r of dephosphorylated NF-M and NF-H and of tau proteins (as observed *in vivo,* Lindwall and Cole, 1984; Nixon et al., 1987; Pleasure et al., 1990). This property, together with the behavior during purification, apparent molecular mass on SDS-PAGE, the independence from common second messengers, and other enzymatic properties like sub-

strate specificity, distinguishes PK40 and PK36 from other well-known kinases. We suggest that they are novel enzymes by biochemical criteria. In addition to phosphorylating KSP sites, PK40 is further distinguished from the 40 kDa catalytic subunit of protein kinase A by lack of inhibition with the Walsh inhibitor.

The PK40 and PK36 kinases were isolated as catalytic proteins. They do not appear to be closely associated with the cytoskeleton, nor do they display obvious regulatory properties such as subunit association, homomer formation, or autophosphorylation, as seen in other common kinases. However, we cannot exclude the possibility that regulatory domains were lost by proteolysis during processing or that regulatory phosphorylation had occurred during the lengthy purification in Mg/ATP-containing buffers. A dissociating subunit could have been lost during the AS fractionation but probably not afterward, since the M_r of the kinase activities in the crude AS fraction, as determined by low-salt gel filtration, resembled closely the apparent M_r of the purified enzymes.

The apparent K_m values for ATP of PK40 (~100 μM) and PK36 (~50 μM) are significantly higher than for most other kinases (2–20 μM) and reflect a requirement for relatively high ATP concentrations. Interestingly, both kinases, but especially PK40, are strongly inhibited when ATP is in relatively small excess over Mg^{2+}. A similar regulatory phenomenon is known for phosphofructokinase, which is much more strongly inhibited by free ATP in the low-millimolar range than by the Mg-ATP complex (Lowry and Passonneau, 1966). A physiological consequence of this behavior is the "Pasteur effect"—a drastic increase of 1,6-fructose-bisphosphate levels under hypoxic conditions, for example, in stroke (Lowry and Passonneau, 1962), due to release of phosphofructokinase inhibition with decreasing ATP concentration.

While PK40 and PK36 share some properties, PK36 does not show such a clear preference for dephosphorylated NF-M as PK40 and has only a marginal specificity for NF-H, supporting an *in vivo* relevance in NF phosphorylation more for PK40 than PK36. The Walsh inhibitor, which does not affect PK40, lowers the activity of PK36 in a dose-dependent fashion, although only at relatively high concentrations. Thus, the active sites of the two kinases may be quite unrelated to each other and to the catalytic site of protein kinase A.

Significance of NF phosphorylation

Little information is available concerning stoichiometry and sites of phosphorylation for other kinases phosphorylating NF subunits. Protein kinase C has been shown to phosphorylate several sites in NF, among them a site in NF-L that is phosphorylated *in vivo* (Sihag et al., 1988), but the specificity of this kinase for NF protein was not very high. In NFs prepared without solubilization in urea, only NF-M of the three subunits was reported to be phosphorylated by microtubule-associated kinase (Leterrier et al., 1981).

The fact that PK36 and PK40 can phosphorylate only a subset of KSP sites in NF-M and NF-H, respectively, may indicate that not all KSP sites in the heavy NF subunits are conformationally and perhaps also functionally equivalent. Likewise, the MPR sequences in NF-M and NF-H are obviously different and may serve different purposes, since PK40 apparently can saturate this site in NF-M but not NF-H and since different phosphatases have to be used for complete dephosphorylation of each isolated subunit. In this respect, it is interesting to note that the 67 kDa NF-associated kinase isolated by Wible et al. (1989) accepts only partially phosphorylated NF-H as substrate. This suggests that NF-H may be primed by another kinase, perhaps PK40, for further phosphorylation by NF-associated kinases of the Wible type. *In vivo,* multiple phosphorylated NF-H variants distinguished by apparent M_r were found to be differentially distributed along axons of retinal cell neurons, with generally increasing levels of phosphorylation toward the distal end (Lewis and Nixon, 1988). However, caution has to be exercised in the comparison of *in vivo* behavior of NF with results obtained *in vitro* with isolated NF subunits, since their substrate properties may be different from the assembled NF complex *in vivo* and may even depend on subtle differences in the nature of the assembly, as exemplified by our dephosphorylation studies. Different roles in the cytoskeletal network for NF-M and NF-H are suggested by ultrastructural and *in vitro* studies (Minami and Sakai, 1983; Hirokawa et al., 1984) where positive evidence for a cross-linking function in the NF and microtubule networks was found only for the C-terminal domain of NF-H but not NF-M. There is no clear evidence of a specific function for NF-M to date.

Our observation that dephosphorylated NF triplet in contrast to the original native triplet does not form a stable suspension *in vitro* (see Materials and Methods) may point to a function of NF proteins associated with NF phosphorylation, not necessarily of the MPR sequence, in stabilizing a gel formed by NF proteins, as suggested earlier (Hoffman et al., 1987).

Possible pathological and cell biological significance of PK40 and PK36

One of our immunoassays was designed to detect specifically a kinase that establishes the epitope of the mAb SMI-34, a marker of AD (Sternberger et al., 1985) and aging (Blanchard and Ingram, 1989) in the human brain. Our results indicate that the SMI-34 epitope is closely related to the phosphorylated MPR sequence of NF and probably to the KSP site(s) of tau rather than constituting a unique "pathological" epitope. However, at least in the case of NF-M, a relatively high saturation of the MPR sequence with phosphates seemed to be necessary for SMI-34 binding. Thus, the presence of the epitope in rat brains but not normal human brains (Sternberger et al., 1985; Blanchard and Ingram, 1989) might be due to different threshold levels of phosphorylation required for SMI-34 binding that are exceeded in human brains only in a pathological condition like AD or in the process of aging. This hypothesis would invoke a hyperphosphorylation of cytoskeletal proteins rather than the formation of new abnormal epitopes.

One particularly interesting mechanism involves the upregulation of PK40 (and PK36 to a lesser extent) due to degenerative or pathological metabolic insufficiency. PK40/PK36 activity, normally confined primarily to the cell processes where most of the KSP phosphorylation occurs (Sternberger and Sternberger, 1983; Lee et al., 1987), might be released from inhibition and might then spread into the perikaryon when ATP levels decrease due to age or degenerative disease (Bennett et al., 1990). Increased formation of the KSP phosphoepitopes of RT97 (Rasool and Selkoe, 1984) and of SMI-34 (Sternberger et al., 1985), as found in PHFs/tangles, would follow and lead to extensive tangle formation in neuronal cell bodies (Roher et al., 1988).

Recent reports by Blass and his colleagues (Baker et al., 1988; Blass et al., 1990) support the possibility that the regulatory properties of PK40(36) might indeed be of relevance in neu-

rodegenerative diseases like AD. Uncoupling of oxidative phosphorylation in fibroblasts under culture conditions that favor neuronal differentiation and the consequent decrease of the ATP concentration induces the appearance of PHF and Alz-50 epitopes. In agreement with these observations and with our findings, decreased levels of ATP are to be expected in AD neurons and, perhaps to a less severe degree, in normally aged neurons. Oxidative metabolism was found to be decreased in aged neurons as well as in fresh brain tissue of AD patients and was linked to ACh deficiency (Gibson and Peterson, 1981; Sims et al., 1987). Mitochondria from AD brains appear to be partially uncoupled (Sims et al., 1987). Furthermore, a selective deficiency in the respiratory enzyme cytochrome oxidase has been reported in blood platelets of AD patients (Parker et al., 1990).

The phosphorylation of tau protein by PK40 and PK36 is particularly interesting in view of the prominent involvement of tau in the formation of PHFs (Goedert et al., 1989). A possible effect of inappropriate tau(NF) protein phosphorylation in the perikaryon via the above mechanism could be interference with normal folding pathways (Urry et al., 1989) leading to extremely insoluble aggregates. Alternatively, aluminum might become chelated by phosphate groups in tangle-bearing neurons (Garruto et al., 1984) and thus cross-link hyperphosphorylated tau protein. *In vitro,* aluminum can precipitate phosphorylated, but not dephosphorylated, NF proteins (Nixon et al., 1990).

Several reports indicate the presence of abnormal tau-derived phosphoepitopes in PHF (Grundke-Iqbal et al., 1986; Ishiguro et al., 1988; Kondo et al., 1988). A recent study (Bancher et al., 1991) concludes that abnormal phosphorylation of tau occurs prior to its incorporation into PHF in the nerve cell body. Two classes of tau phosphorylation have been distinguished previously, according to whether the electrophoretic mobility of tau is altered (mode I) or not (mode II) (Lindwall and Cole, 1984). Immunochemical studies suggest that PHF in brains of AD patients contain only mode I phosphorylated tau proteins (Grundke-Iqbal et al., 1986; Ihara et al., 1986). Calcium/calmodulin-dependent kinase II has recently been shown to induce an apparent M_r shift of tau of about 4 kDa after phosphorylation of a single site (not KSP) in the C-terminal domain of tau (Baudier and Cole, 1987; Steiner et al., 1990). A tubulin-dependent kinase copurifying with microtubules also phosphorylates tau in mode I and forms a PHF-specific phosphoepitope (Ishiguro et al., 1988). PK40 and perhaps PK36 deserve equal attention as potential tau kinases since both perform mode I phosphorylation on tau and are capable of forming the tangle/PHF-associated epitopes of the mAbs SMI-34 and RT97.

Of particular interest is the recent demonstration that a form of tau can be extracted from PHFs that retains the ability to form PHF-like structures and differs from normal human tau only by phosphorylation (Lee et al., 1991). In the abnormal form, the KSP sites are phosphorylated, as suggested earlier by Rasool and Selkoe (1984). The isoform pattern of this human isolate and the magnitude of the gel shift on a 10% SDS polyacrilamide gel are very similar to changes that our PK40 induced with our dephosphorylated bovine tau preparations.

The phosphorylation of the KSP sites in the heavy NF subunits and in tau protein by a single kinase and the ability of PK40 to shift completely the apparent M_r of both NF-M and tau on SDS-PAGE to the values found for the proteins, as isolated by us, support the notion that NF-M and tau may share a common epitope that may be of significance for determining the conformation of these proteins. It is also striking that PK36 can induce only a partial shift in *both* NF-M and tau. Perhaps the phosphorylation of tau and NF-M, and to a lesser degree of NF-H, serves a general common purpose in the cell biology of neurons, and perhaps the KSP sites together with an as yet unspecified sequence or conformation environment label both proteins as substrates for a kinase associated with this purpose.

A more complete analysis of the phosphorylation sites of cytoskeletal proteins and of the distribution and functional properties of the relevant kinases is required to substantiate the above hypotheses. The identification of PK40 and PK36 provides new tools to address the questions about the normal and pathological significance of the phosphorylation of cytoskeletal proteins.

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