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Properties of neurofilament protein kinase

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Neurofilament (NF) protein kinase, partially purified from NF preparations [Toru-Delbauffe & Pierre (1983) FEBS Lett. **162**, 230–234], was found to be distinct from both the casein kinase present in NFs and the cyclic AMP-dependent protein kinase which is able to phosphorylate NFs. NF-kinase phosphorylated the three NF protein components. The amount of phosphate incorporated per molecule was higher for NF 200 than for NF 145 and NF 68. Other proteins present in the NF preparations were also used as NF-kinase substrates. Two of them might correspond to the myelin basic proteins with M_r values of 18000 and 21000. Four other substrates in the NF preparation were not identified (respective M_r values 53000, 55000, 65000 and > 300000). NF kinase also phosphorylated two additional brain-cell cytoskeletal elements: GFAp and vimentin. Casein, histones and phosvitin, currently used as substrates for protein kinase assays, were very poor phosphate acceptors. Half-maximal NF-kinase activity was obtained at an NF protein concentration of about 0.25 mg/ml in heated, salt-washed, NF preparations. The specific activity was about 5 pmol of ^{32}P incorporated/min per μg of NF kinase preparation protein. ATP was a phospho-group donor (K_m 8×10^{-5} M), but GTP was not. NF-kinase activity remained stable at 65 °C for more than 1 h. The enzyme was not degraded by storage at −20 °C for several months in a buffer containing 50% (w/v) sucrose. Maximal activity was obtained with 5 mM-Mg^{2+} (Mg^{2+} could be replaced by Co^{2+}); Zn^{2+} and Cu^{2+} inhibited the reaction. NF-kinase was not dependent on cyclic AMP, cyclic GMP, Ca^{2+} or Ca^{2+} plus dioleoylglycerol and phosphatidylserine.

INTRODUCTION

Neurofilaments (NFs), which are intermediate filaments specifically present in neurons, can be phosphorylated *in vivo* and *in vitro* [for a review, see Lazarides (1982)]. NF phosphorylation is probably catalysed, at least in part, by protein kinases present in NF preparations (Lazarides, 1982; Nestler & Greengard, 1983). These enzymes are known to phosphorylate the three protein components of NFs (NF 200, NF 145 and NF 68) (Lazarides, 1982; Nestler & Greengard, 1983), but no effectors have yet been found for them.

Toru-Delbauffe & Pierre (1983) reported previously that the protein kinases present in NF preparations could be separated into two activities: one, identified as casein kinase I (CK_I), which did not phosphorylate NF preparations, and another, which did.

In the present paper we report the properties of the latter enzyme, which we named 'NF-protein kinase' or 'NF-kinase', and compare it with CK_I and cyclic AMP-dependent protein kinase.

MATERIALS AND METHODS

Materials

Cyclic AMP, cyclic GMP, ATP, GTP, leupeptin, Tos-Arg-OMe, PMSF, dioleoylglycerol ('diolein') phosphatidylserine, phosvitin, histones, cyclic AMP-dependent protein kinase and calf intestinal alkaline phosphatase (type VII) were purchased from Sigma; [γ^{-32}P]ATP and [γ^{-32}P]GTP were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; P11 phosphocellulose was from Whatman; acrylamide and bisacrylamide were from Eastman Kodak; antibody against GFAp was from Sanbio (Holland) and ^{125}I-labelled antibody (sheep anti-mouse IgG) was from The Radiochemical Centre.

Neurofilament preparation

The procedure described in Toru-Delbauffe & Pierre (1983) was employed with a single modification. All the buffers used contained leupeptin (2 mg/ml), Tos-Arg-OMe (0.5 mg/ml) and 2 mM-PMSF.

Isolation of the astrocyte cytoskeletons

Cytoskeletons were isolated by the method of Chiu & Norton (1982), from astrocyte-enriched secondary cell cultures, grown as previously described (Lennon *et al.*, 1983).

Microtubule-associated protein (MAP) preparation

Brain microtubules were purified by a slightly modified temperature-dependent assembly–disassembly procedure (Shelanski *et al.*, 1973).

Large amounts of MAPs were obtained by thermodenaturation of the purified microtubules in the presence of high salt concentrations (Fellous *et al.*, 1977).

Extraction of protein kinase activities from NF preparations

The procedure was described in Toru-Delbauffe & Pierre (1983).

Phosphocellulose chromatography

Extract containing NF-kinase was applied to a phosphocellulose column (0.9 cm × 5 cm) pre-equili-

Abbreviations used: NF(s), neurofilament(s); MAP, microtubule-associated protein; GFAp, glial fibrillary acidic protein; CK_I, casein kinase I; Tos-Arg-OMe, tosyl-L-arginine methyl ester; PMSF, phenylmethanesulphonyl fluoride; Tos-Phe-CH_2Cl, *N*-tosyl-L-phenylalanylchloromethane ('TPCK'); Tos-Lys-CH_2Cl, tosyl-L-lysylchloromethane ('TLCK').

brated with buffer (50 mM-Tris/HCl/2 mM-$MgCl_2$/150 mM-KCl, pH 7.1). The column was washed with the same buffer and the protein kinase activities were eluted with a linear gradient of KCl (150–800 mM). Fractions were assayed for NF kinase activity (see below) and for casein activity (Toru-Delbauffe & Pierre, 1983). The eluted NF-kinase was concentrated by dialysis against 50 mM-Tris/HCl buffer (pH 7.1)/2 mM-$MgCl_2$/50 mM-KCl/50% (w/v) sucrose.

Protein kinase assay (standard conditions)

The salt-washed NFs (2 mg of protein/ml) to be used as substrate were preincubated for 5 min at 50 °C just before protein kinase assay (see the Results section).

Assays were performed in 50 mM-Mes and 5 mM-$MgCl_2$, pH 6.5, containing 0.5 mg of proteins (NFs, or other proteins when indicated)/ml, 50–100 μM-[γ-^{32}P]-ATP or [γ-^{32}P]GTP (1–2 μCi/assay) and portions of enzymic fraction, making a total volume of 120 μl. The reaction was initiated by the addition of the nucleotide. Incubation proceeded at 30 °C for 3 min. When the incubation time was longer than 3 min, proteinase inhibitors (4 μg of leupeptin/ml, 1 μg of trypsin inhibitor/ml, 1 mM-benzamidine, 0.4 μg of antipain/ml, 10 mM-PMSF, 0.1 mM-Tos-Phe-CH_2Cl and 0.01 mM-Tos-Lys-CH_2Cl were added. Incorporation of radioactivity was monitored by spotting 50 μl aliquots of reaction mixture on to squares of Whatman 3 mm filter paper. The filters were then washed and counted for radioactivity as previously described (Toru-Delbauffe & Pierre, 1983).

NF-kinase was also assayed in 1 mM-potassium phosphate buffer, pH 7, containing 50 mM-glycerophosphate, 0.3 mM-EGTA and 5 mM-magnesium acetate, but its activity was lower under these conditions.

Polyacrylamide-gel electrophoresis, autoradiography and immunoblotting

NFs (or other proteins) were phosphorylated as described above by using 5 μCi of [γ-^{32}P]ATP. Where indicated, NFs were pretreated with alkaline phosphatase as described by Julien & Mushynski (1982). Incubation was terminated by the addition of concentrated sample buffer. SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970) with the acrylamide concentrations indicated in the Figures. Each sample loaded on the gels contained 10–30 μg of protein. The gels were stained with Coomassie Blue and dried. Gel autoradiography was carried out on Kodak X-Omat R film with an intensifying screen.

After SDS/polyacrylamide-gel electrophoresis, proteins were electrophoretically transferred to nitrocellulose sheets for immunoblotting (Towbin *et al.*, 1979). The sheets were then incubated with a monoclonal antibody against human GFAp (GFAp-6F2; 1:50 dilution); the second ^{125}I-labelled antibody was sheep anti-mouse IgG. The nitrocellulose sheets were washed as described by Towbin *et al.* (1979) and autoradiographed.

Determination of phosphate incorporation

Electrophoregrams of ^{32}P-labelled NF preparations were scanned after staining. The amount of protein in each band was estimated by comparison with serum albumin and phosphorylase subunit markers. The incorporated radioactive phosphate was measured by cutting and counting the bands from the dried, stained, polyacrylamide gel. The corresponding number of mol of phosphate was calculated from the specific radioactivity of ATP.

RESULTS AND DISCUSSION

The NF-kinase purified by phosphocellulose chromatography (Toru-Delbauffe & Pierre, 1983) showed one major polypeptide with an M_r of 40000 and one minor band (M_r > 200000) on SDS/polyacrylamide-gel electrophoresis (Fig. 1). However, it was not possible to ascertain which one of these polypeptides corresponds to the NF kinase and which is a contaminant. The NF-kinase preparation did not autophosphorylate (Fig. 1), and its specific activity was generally about 5 pmol of ^{32}P/min per μg of protein with NF preparations as substrate.

Attempts to further purify the NF kinase on other resins (DEAE-cellulose, CM-Sephadex, hydroxyapatite...) after phosphocellulose chromatography have failed. The enzyme activity was lost during subsequent purification steps.

NF-kinase could be stored at −20 °C for several months in a buffer containing 50 mM-Tris/HCl/2 mM-$MgCl_2$/50 mM-KCl/50% (w/v) sucrose, pH 7.4, with no loss of activity.

Heating the partially purified enzyme for 5 min at temperatures between 40 and 85 °C did not modify its activity. NF-kinase is fairly stable on heating, since it can be incubated at 65 °C for 1 h without any loss of activity. This property can be used to destroy other enzymic activities which might be present in the preparations.

The measurement of NF-kinase activity was improved by incubating the salt-washed NF preparations used as substrate at 50 °C for 5 min before assay. This treatment decreased residual kinase activity by 80% without impairing the phosphorylation of the NF proteins by solubilized enzyme (Table 1). The SDS/polyacrylamide-gel-electrophoretic staining patterns of preparations heated at 50 °C were no different from those of unheated NFs (Fig. 1). The same polypeptides were phosphorylated both before and after heating at 50 °C for 5 min (Fig. 1). Incubation at 55 °C completely inactivated residual kinase activity, but also suppressed the capacity of NFs to be phosphorylated.

It thus appears that the NF-kinase activity which remains associated with the NFs is much more thermosensitive than is solubilized NF-kinase. There are two possible explanations for this difference. First, they may be two distinct forms of NF-kinase, one of which is thermolabile and is strongly bound to NF preparation and another which is soluble and heat-stable. Second there is a single NF-kinase whose activity is modified by its environment. At this time we are unable to choose between these hypotheses.

In order to proceed with NF-kinase characterization we tested its activity with different substrates and effectors. Fig. 2 shows the NF-kinase activity observed in the presence of Mg^{2+} (0.1–10 mM). Activity was optimal at 5 mM. As shown in Table 2, Mg^{2+} was fully replaced by Co^{2+}. Ca^{2+} could not replace Mg^{2+}, but did not inhibit NF-kinase when Mg^{2+} was present. Other bivalent cations, such as Zn^{2+} and Cu^{2+}, inhibited NF-kinase activity in the presence of Mg^{2+}.

NF-kinase was not activated by cyclic AMP, cyclic GMP or Ca^{2+}, or by Ca^{2+} plus phosphatidylserine and dioleoylglycerol. These compounds were examined at concentrations that have been shown to be effective with

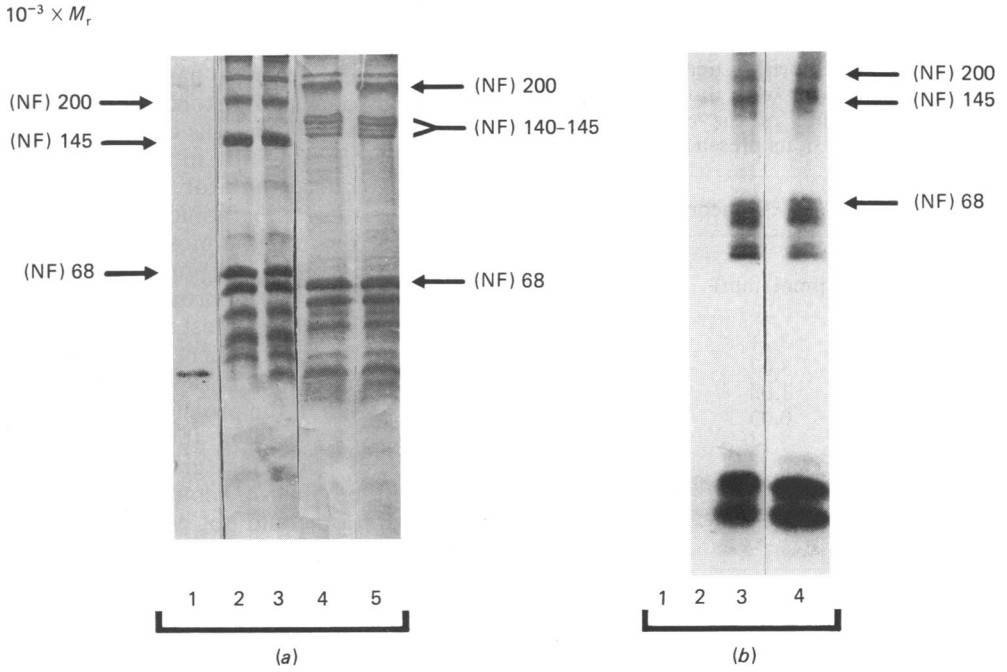

Fig. 1. Effect of temperature on the protein composition and phosphorylation of salt-washed NFs

(*a*) Salt-washed NFs were incubated in 10 mM-sodium phosphate (pH 6.5)/0.85 M-sucrose for 5 min at 50 °C. Control preparations were not heated. They were analysed by SDS/polyacrylamide-gel electrophoresis on 8.5% -acrylamide/0.23% -bisacrylamide (2,3) or 15% -acrylamide/0.1% -bisacrylamide (4,5) gels and stained with Coomassie Blue. NF kinase also was analysed on 8.5% -acrylamide/0.23% -bisacrylamide gels (1). (*b*) Salt-washed NFs were preincubated in 10 mM-sodium phosphate (pH 6.5)/0.85 M-sucrose for 5 min at 50 °C (2,3) or left unheated (4). After this preincubation, NFs were incubated with [γ-^{32}P]ATP under standard conditions in the presence (3) or the absence (2,4) of NF-kinase. NF kinase alone was also incubated with [γ-^{32}P]ATP under standard conditions (1). After this the samples were analysed on 15% acrylamide/0.1% bisacrylamide gels, which were then stained and autoradiographed.

Table 1. Thermal inactivation of residual NF-kinase activity in NFs after salt extraction

Salt washed NFs were heated in 10 mM-sodium phosphate (pH 6.5)/0.8 M-sucrose for 5 min at the indicated temperature. They were then used as substrates in NF-kinase assays. ^{32}P incorporation was measured under standard conditions.

NF heating conditions (°C)	^{32}P incorporation into NFs (c.p.m.)		Ratio $\frac{+}{-}$
	− NF kinase	+ NF kinase	
None	8090	11552	1.43
40	4741	6947	1.47
45	2022	8088	4.00
50	1336	6013	4.50
55	0	126	-

their respective protein kinase. The failure of NF-kinase to respond to cyclic nucleotides was also observed in autophosphorylation studies of NF preparations (Eagles & Gilbert, 1979; Runge *et al.*, 1981*b*; Julien *et al.*, 1983; Shecket & Lasek, 1982).

The phosphate donors and phosphate acceptors currently used for protein kinase assays were also tested. NF-kinase used ATP as a phosphate donor, exhibiting a K_m value of about 8×10^{-5} M. GTP, however, was not a phosphate donor for this enzyme (Table 3). Fig. 3 shows

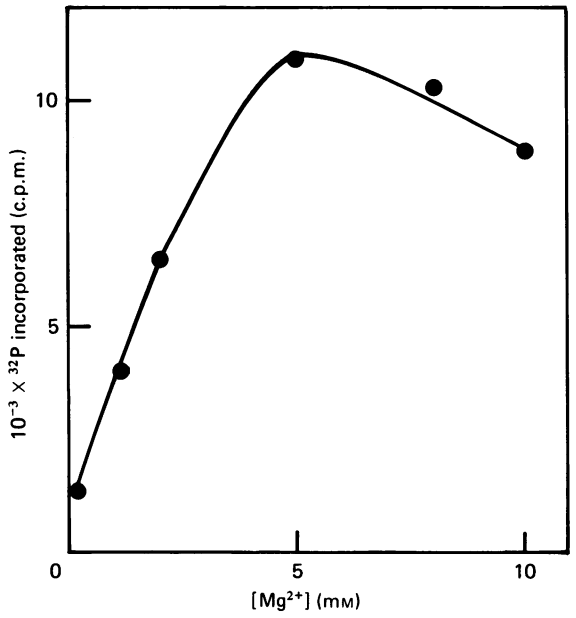

Fig. 2. Dependence of NF-kinase activity on Mg^{2+} concentration

^{32}P incorporation into NFs was measured under standard conditions, in the absence or presence of NF-kinase at various Mg^{2+} concentrations. NF-kinase activity was calculated by subtracting the values obtained in the absence of the enzyme from those measured in its presence.

Table 2. Cation specificity of NF kinase

Protein kinase assays were performed under standard conditions, except that the cations were those indicated. Values measured in the absence of the enzyme were subtracted from those obtained in its presence.

Bivalent cation	^{32}P incorporated (pmol/min)	^{32}P incorporated [% of control (5 mM-Mg^{2+})]
Mg^{2+} (5 mM)	3.21	100
Co^{2+} (5 mM)	3.07	96
Mn^{2+} (5 mM)	1.08	30
Ca^{2+} (5 mM)	0.03	0
Mg^{2+} (5 mM)+Ca^{2+} (5 mM)	3.14	98
Mg^{2+} (5 mM)+Zn^{2+} (2 mM)	0.85	26
Mg^{2+} (5 mM)+Cu^{2+} (2 mM)	0.02	0

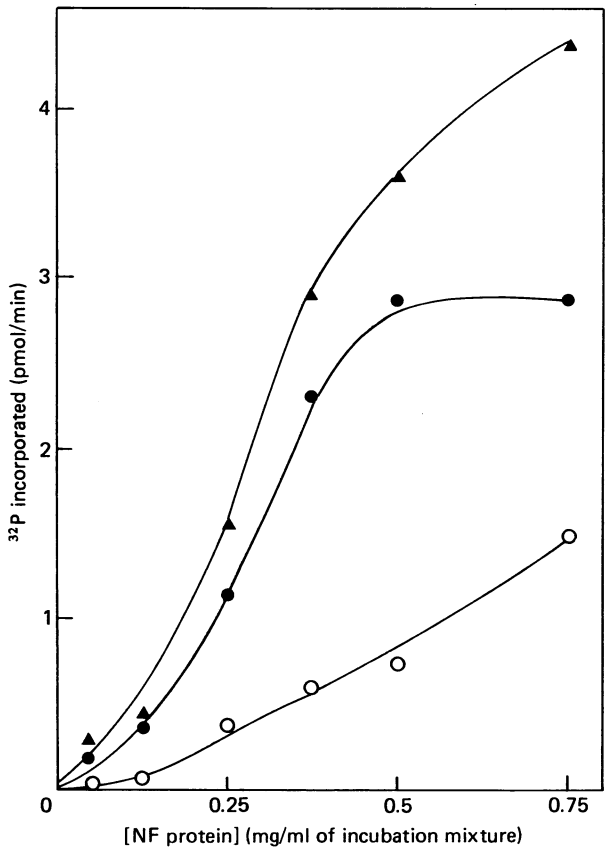

Fig. 3. Dependence of NF-kinase activity on the NF concentration

^{32}P incorporation was measured under standard conditions in the absence (○) or presence (▲) of NF-kinase, at various concentrations of salt-washed and heated NFs. NF-kinase activity (●) was calculated by subtracting the value measured in the absence of the enzyme from those measured in its presence.

the activity of NF-kinase at various NF concentrations. Half-maximal activity was obtained at about 0.25 mg of protein/ml. Its specific activity was 10 pmol of phosphate incorporated/min per μg of NF-kinase preparation protein. Of the proteins currently used as phosphate acceptors for protein kinase assays, NF-kinase did not effectively phosphorylate either acidic proteins such as phosvitin and casein, or basic proteins such as protamine, whole histones and histone subfractions (Table 3).

The three NF proteins components (NF 200, NF 145 and NF 68) present in NF preparations were all phosphate acceptors for NF-kinase (Figs. 1 and 4). Though autoradiographic patterns showed that the NF 145 band was the most ^{32}P-labelled, the number of phosphate groups incorporated *in vitro* by the NF-kinase per molecule of NF protein was 2–3 times higher in NF 200, the protein most phosphorylated *in vivo* (Jones & Williams, 1982; Julien & Mushynski, 1982), than in NF 145, and 10–20 times higher in NF 200 than in NF 68 (Table 4). Of all the proteins present in NF-preparation, NF 200 was the one that incorporated the greatest number of phosphate groups per molecule of protein (except the 65000-M_r component, for which phosphate incorporation could not be determined). The amount of phosphate incorporated by the NF-kinase into these proteins appears low when compared with the total phosphate contents of these proteins (Jones & Williams, 1982; Julien & Mushynski, 1982). It is possible that the amount of NF-kinase available in our experiments did not permit phosphorylation of all the potential sites in the incubation time (up to 2 h) during which we could prevent proteolysis with a mixture of proteinase inhibitors. It is also likely that the NF proteins may be phosphorylated by different protein kinases *in vivo* on

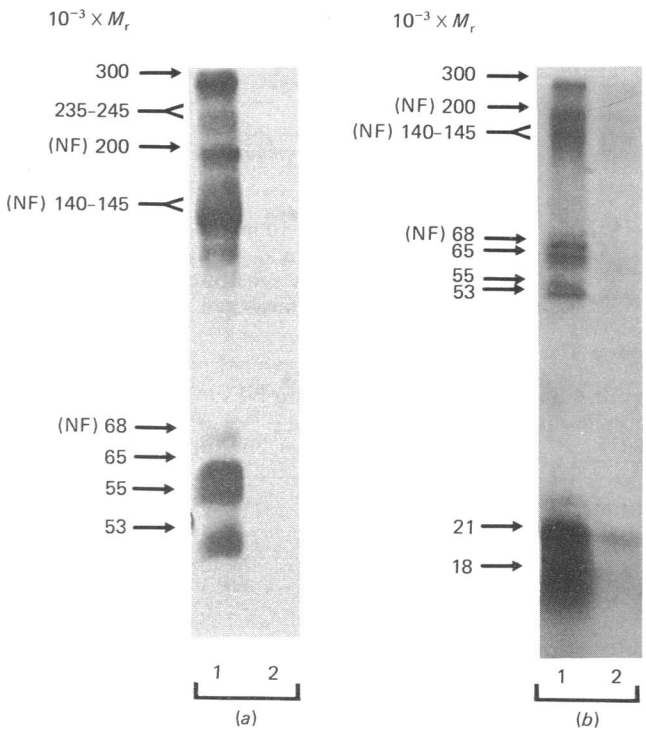

Fig. 4. Identification of the neurofilament proteins phosphorylated by NF-kinase

Heated salt-washed NFs were incubated with [γ-^{32}P]ATP in the presence (1) or absence (2) of NF-kinase and analysed on 8.5%-acrylamide/0.23%-bisacrylamide (*a*) or 15%-acrylamide/0.1%-bisacrylamide (b) gels. Electrophoresis was stopped when the dye marker reached the end of the gel. The gels were autoradiographed.

Table 3. Comparative substrate specificity of NF-kinase and other protein kinases

Protein kinase assays were performed under standard conditions, except that the phosphate acceptor, phosphate donor and protein kinases were those indicated. The concentrations of all the substrates in the reaction mixture was 0.5 mg/ml. Abbreviation used cAMP-PK, cyclic AMP-dependent protein kinase.

Phosphate acceptor	^{32}P incorporated (pmol/min)		
	NF-kinase	cAMP-PK	Casein kinase I
Donor ATP			
Whole histone	0.17		0.09 (8%)
Lysine-rich histone (III Sigma)	0.2		
Histone			
H1	0.18		
H2A	0.06		
H2B	0.03 (0.8%)	1 (100%)	
H3	0.06		
Protamine sulphate	0.12		
Casein	0.1		1.2 (100%)
Phosvitin	0.07		
NF	3.85 (100%)	0.3 (30%)	0.08 (6.5%)
Donor GTP			
NF	0.35 (9%)		

Table 4. Stoichiometry of phosphorylation of neurofilament proteins by NF-kinase

NF preparations were phosphorylated by NF-kinase under standard conditions for 2 h. The ratio '^{32}P (mol/mol of protein)' was determined as described in the Materials and methods section.

M_r	^{32}P (mol/mol of protein)
>300000	0.10
200000 (NF 200)	0.50
150000 (NF 150)	0.19
68000 (NF 68)	0.04
65000	NCBS*
55000	0.16
53000	0.06
21000	0.09
18000	0.28

* No Coomassie Blue Staining.

different sites, since it has been shown that a cyclic AMP-dependent protein kinase (Leterrier *et al.*, 1981) and a calmodulin-dependent protein kinase (Lou Vallano *et al.*, 1985) can also phosphorylate NF proteins *in vitro*. Thus, despite the presence of several phosphorylation sites on NF proteins, only few of them might be available to NF kinase. Another possibility is that the sites recognized by the NF-kinase had already been phosphorylated *in vivo*. This possibility was tested by treating NFs with alkaline phosphatase as described by Julien & Mushynski (1982) before incubation with NF-kinase. No additional incorporation was observed (results not shown).

Analysis on an SDS/15%-(w/v)-polyacrylamide gel, indicated that the NF 145 appeared to be composed of four forms, with M_r values ranging from 140000 to 145000 (Fig. 1*a*). Goldstein *et al.* (1983) and Nixon *et al.* (1982) have previously shown the great microheterogeneity of both the NF 145 and NF 200 subunits. The latter authors reported that the above polypeptides in the 140000–145000-M_r range included at least three components, which were differently distributed along the axon. A large autoradiographic band seemed to spread over the four Coomassie Blue-stained components (Figs. 1 and 4), but we could not determine whether all four of the components had incorporated ^{32}P.

Several other polypeptides presents in the NF preparations were also phosphorylated to various extents by NF-kinase (Table 4; Fig. 4, arrows). They included: (i) a protein of $M_r > 300000$ that was also previously observed in NF preparations (Burridge *et al.*, 1982) and whose the electrophoretic migration did not correspond to that of MAP_2 (results not shown); (ii) a weakly labelled protein, probably fodrin, which migrated as doublet of M_r 235000–245000 and was identified by an antibody directed against spectrin (Burridge *et al.*, 1982; present results not shown); (iii) a highly phosphorylated component (M_r 65000); the absence of Coomassie Blue staining at the level of this component prevented calculation of the number of phosphates group incorporated; Liem *et al.* (1984) claimed that an antiserum directed against polypeptide with an M_r of 66000 isolated from NFs recognized one τ protein; however, according to our results, the polypeptide whose M_r was 65000 did not co-migrate on SDS/polyacrylamide gel with any of the τ components (results not shown); (iv) two polypeptides with M_r values of 55000 and 53000; the 55000-M_r polypeptide incorporates as much phosphate as does NF 150 (Table 4); polypeptides in the 40000–65000-M_r range which are co-purified with NFs may exist *in vivo* according to Carden & Eagles (1983); however, Autilio-Gambetti *et al.* (1981) and Chin *et al.* (1983) believe that they might be proteolytic products of the NF 145 and NF 200 proteins; these suggestions were confirmed by Brown *et al.* (1983), who showed that two polypeptides with M_r 65000 and 53000 were recognized

by anti-(NF 140–145) and anti-(NF 68) sera; (v) two low-M_r polypeptides; from their M_r values of 21000 and 18000 they may be tentatively identified as two of the four basic myelin proteins in rat brain (Kelly & Luttges 1976; Brown *et al.*, 1980); the number of phosphates groups incorporated into the 18000-M_r component was between the values determined for NF 200 and NF 150 (Table 4).

These observations illustrate the specificity of NF-kinase and distinguish it from casein kinase and from cyclic AMP-dependent protein kinase. Indeed, as indicated above, casein and histones are poor substrates for the NF-kinase and there is no known activator for this enzyme. The casein kinase associated with NF preparations does not phosphorylate NF proteins (Table 3 and Fig. 5) and the cyclic AMP-dependent protein kinase, which also phosphorylates the triplet proteins and several polypeptides in NF preparations (Table 3, Fig. 5), preferentially uses histones as substrates. Furthermore, the best substrate of the cyclic AMP-dependent protein kinase was NF 145 (Leterrier *et al.*, 1981; Zimmerman & Schlaepfer 1985). The other polypeptides in NF preparations that were phosphorylated by NF-kinase were bad substrates for the cyclic AMP-dependent protein kinase, particularly the 65000-M_r protein. Conversely, at least two components of the NF preparations (Fig. 5) that were phosphorylated by the cyclic AMP-dependent protein kinase were not used as substrates by NF-kinase.

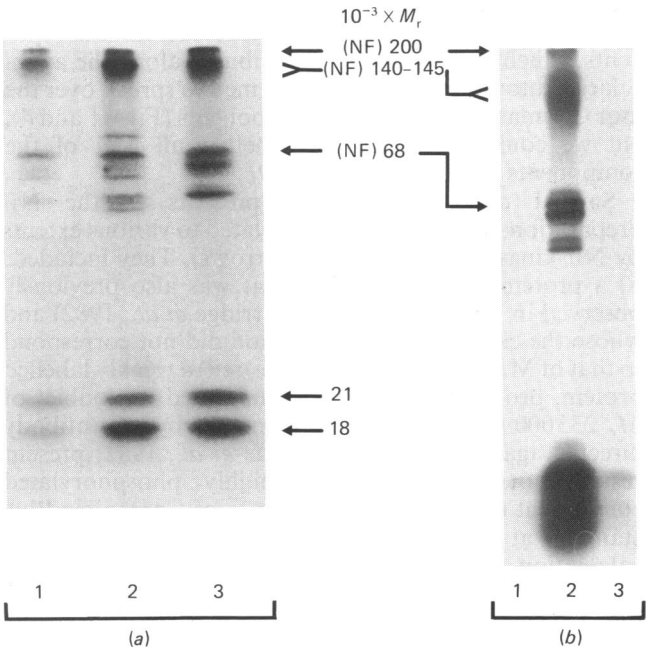

Fig. 5. Phosphorylation of NFs by various protein kinases

Heated salt-washed NFs were incubated and analysed as described in the legend to Fig. 4(*b*). (*a*) Comparison of NF-kinase and cyclic AMP-dependent protein kinase (both enzymes incorporated 3 pmol of phosphate/min into NFs under standard conditions). 1, without protein kinase; 2, cyclic AMP-dependent protein kinase; 3, NF-kinase. (*b*) Comparison of NF-kinase (same activity as in *a*) and casein kinase I (the enzyme incorporated 1.5 pmol of phosphate/min into casein under standard conditions). Casein kinase I was prepared as described previously (Toru-Delbauffe & Pierre, 1983). 1, without protein kinase; 2, NF-kinase; 3, casein kinase I.

The ability of NF-kinase to phosphorylate the cytoskeleton of cultured astrocytes, which contain GFAp and vimentin (Chiu *et al.*, 1981), was examined, since several authors have described structural analogies between the various intermediate-filament proteins (Geisler *et al.*, 1982; Geisler, 1983; Steinert *et al.*, 1983). The 51000-M_r polypeptide identified as GFAp by using a monoclonal antibody (Fig. 6) was phosphorylated by NF-kinase, as were the 57000-M_r polypeptide, vimentin, and other unidentified proteins.

It has also been suggested that microtubules might be associated with NFs in neurons (Runge *et al.*, 1981*a*; Hirokawa, 1982; Leterrier *et al.*, 1982; Minami *et al.*, 1982; Nagele & Roisen, 1982; Pachter *et al.*, 1984; Aamodt & Williams, 1984). We therefore attempted to phosphorylate the MAPs by NF-kinase. Preliminary observations indicated that the NF-kinase preparations could phosphorylate MAP_2 and τ_1 but, in contrast with the phosphorylation of NF proteins, they were no longer phosphorylated by NF-kinase which had been heat-treated (as described above).

The role of NF phosphorylation remains unknown. It might be involved in regulating NF-subunit assembly or it might mediate interactions between NFs and other cell organelles. The functions of NFs will probably have to be clarified before the biological role of this phosphoryla-

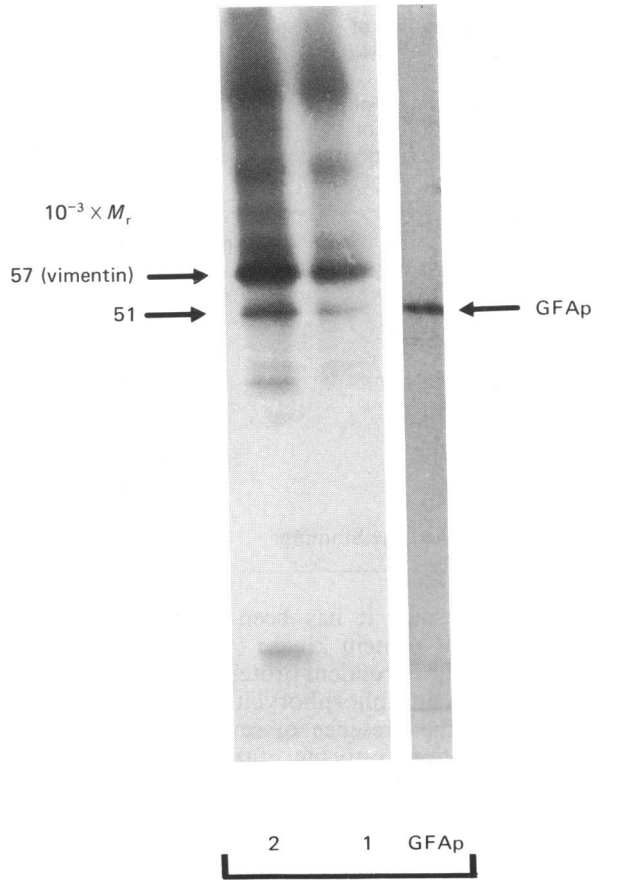

Fig. 6. Cytoskeletal brain proteins phosphorylated by NF-kinase

Astrocyte cytoskeletons (1 mg/ml) were incubated with [γ-^{32}P]ATP in the absence (1) or presence (2) of NF-kinase and analysed on 15%-acrylamide/0.1%-bisacrylamide gels and autoradiographed. GFAp was detected by a monoclonal antibody.

tion can be understood. Although we have not identified any effector for NF-kinase, one possible approach to the identification of the mechanisms regulating the activity of this enzyme might be to examine the effect of extracellular signals, possibly hormonal, on NF phosphorylation in cell cultures.

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