# A rat brain protein kinase phosphorylating specifically neurofilaments

D. Toru-Delbauffe and M. Pierre

*Unité de Recherche sur la Glande Thyroide et la Régulation Hormonale, INSERM U.96, 78 rue du Général Leclerc, 94270 Bicetre, France*

Received 10 August 1983; revised version received 23 August 1983

Protein kinase activities associated with neurofilament (cAMP, cGMP, $Ca^{2+}$-independent) were almost completely extracted by 0.8 M KCl. Two activities were separated by either sucrose gradient ultracentrifugation of phosphocellulose chromatography. One of them phosphorylates specifically neurofilament proteins and preferentially the triplet (200 kDa, 145 kDa and 68 kDa) but neither casein nor type $II_A$ histone. The second activity was identified as casein kinase I and does not catalyze the phosphorylation of neurofilament protein.

*Neurofilament* *Rat brain* *Protein kinase* *Casein kinase*

## 1. INTRODUCTION

Neurofilaments represent a class of intermediate filaments specifically present in neurons. They are essentially constituted, in the case of mammals, by a polypeptide triplet of 200 kDa, 145 kDa and 68 kDa [1–3]. The protein subunits of neurofilaments, like those of several other classes of intermediate filaments, display the ability to be phosphorylated [3]. This property has been exhibited in vivo and in vitro in numerous animal species [4–10]. Protein kinase activities are associated with the neurofilament preparations and are very likely responsible for their phosphorylation [5,6,10–12]. At present, the isolation and identification of these protein kinase(s) have not been accomplished.

Here, we report the isolation of protein kinase from the neurofilament preparation. It phosphorylates, very specifically, the constituent proteins of these cytoskeletal elements. A casein kinase I was also solubilized from the same preparation. It does not phosphorylate subunits constituting the neurofilament triplet, nor the proteins with which they are associated.

## 2. MATERIALS AND METHODS

### 2.1. *Neurofilament preparation*

A neurofilament enriched fraction was prepared after ablation of the cerebellum from the brain of 250–280 g Wistar rats by a slightly modified version of the procedure in [13]. A typical preparation was performed with 50 rats. A 4 g sample of brain underwent a gentle homogenization in 60 ml 10 mM sodium phosphate buffer (pH 6.5) containing 100 mM NaCl, 1 mM EDTA and 0.85 M sucrose in a glass–teflon homogenizer. The homogenate was centrifuged in a Sorvall SS 34 rotor at 27000 × *g* for 15 min. After collection of the floating pad of myelinated axons, the flotation was repeated 3 times. The myelinated axons were strongly homogenized in 1% (w/v) Triton X-100, 10 mM sodium phosphate buffer (pH 6.5). The solution was stirred overnight at 4°C to remove myelin and then centrifuged through a layer of 0.85 M sucrose in 10 mM sodium phosphate (pH 6.5) at 120000 × *g* for 1 h. The final pellet, which represented the neurofilament preparation, was stored at −80°C until utilization.



*Published by Elsevier Science Publishers B.V.*

2.2. *Extraction of protein kinase activities from neurofilament preparation*

The pellet of neurofilaments was rehomogenized with a small volume (4–5 ml) of modified Dahl buffer [14] (phosphate-buffered saline plus 0.8 M KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA, 0.5 mg/ml tosyl-arginine–methylester–HCl, 0.15 mM phenylmethylsulphonyl fluoride, pH 7.1). Protein kinases were extracted from the neurofilament preparation with stirring over 4 h. The suspension was centrifuged for 1 h at 150000 × *g*. The supernatant was dialyzed overnight against buffer 'A' (50 mM Tris–HCl (pH 7.1), 2 mM $MgCl_2$ and 150 mM KCl). The pellet was washed overnight by stirring in the modified Dahl buffer and it was centrifuged at 150000 × *g* for 1 h. The final pellet was strongly rehomogenized in 0.85 M sucrose in 10 mM (pH 6.5) sodium phosphate. It was then utilized as a substrate for protein kinase assays.

2.3. *Partial purification of protein kinase from neurofilament preparation*

2.3.1. Analysis of extracted protein kinases on sucrose gradient ultracentrifugation

A linear sucrose gradient of 5–20% was used. A 1 ml extract containing the protein kinase activity was layered on the top of the gradient. Centrifugation was performed at 4°C in a Beckman SW 40 rotor at 38000 rev./min for 14 h; 300 μl fractions were collected and assayed for protein kinase activity on the casein, histones and washed neurofilaments.

2.3.2. Chromatography of extracted protein kinases

Extract containing protein kinase activity (5 ml) was applied to a phosphocellulose column (0.9 × 5 cm) pre-equilibrated with buffer A. The column was washed with the same medium and then the protein kinase activity was eluted with a linear gradient of KCl (150–800 mM) in buffer A. Fractions of 1.7 ml were collected and assayed for protein kinase activity on the casein and on the washed neurofilaments.

2.4. *Protein kinase assays*

Phosphorylation was done in 50 mM 2-(*N*-morpholino)ethane–sulfonic acid, 10 mM $MgCl_2$ (pH 6.5) containing 0.5 mg protein/ml of washed neurofilaments or 2 mg/ml of type $II_A$ histone or 1.5 mg/ml of casein, 5 μM [$\gamma$-$^{32}$P]ATP (1 μCi/assay) and aliquots of enzymatic fraction to assay. The reaction was initiated by the addition of ATP. Incubation was at 30°C for 5 min in 200 μl final vol. Measurements were made by removing 50 μl aliquots and placing them on squares of Whatman 3MM filter paper. The filters were then washed as in [15].

2.5. *Polyacrylamide gel electrophoresis*

Neurofilaments were phosphorylated as above using 5 μCi [$\gamma$-$^{32}$P]ATP/assay. Incubation was terminated by addition of 0.5 vol. concentrated sample buffer [12]. SDS–polyacrylamide gel electrophoresis was following [16] on 8.5% polyacrylamide slab gels. Each sample loaded on the gel contained 30 μg neurofilament proteins. The gels were then stained with Coomassie blue and dried. Autoradiography of gels was carried out by using Kodak X-Omat R film.

## 3. RESULTS

To solubilize the protein kinases responsible for the phosphorylation of neurofilament proteins, some crude preparations of these cytoskeletal elements were treated with buffers containing variable saline concentrations. KCl was retained at 0.8 M for experimentation. Under these conditions of salinity, 85–90% of the protein kinase activity associated with the neurofilament preparations was extracted (fig.1). This enzymatic activity

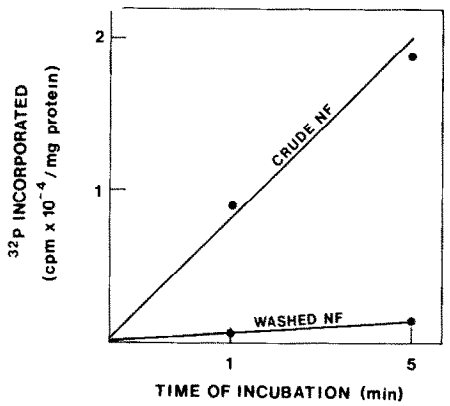


Fig.1. Autophosphorylation of crude and washed (0.8 M KCl) neurofilaments (details in section 2).

phosphorylated the casein and the proteins from the neurofilament preparation which had undergone extensive preliminary washing with 0.8 M KCl. In contrast, no phosphorylation was exhibited on type $II_A$ histone. Electrophoresis by SDS–polyacrylamide gel of proteins from the washed neurofilament preparation revealed (fig.4 (2,4)) that the protein triplet (200 kDa, 145 kDa and 68 kDa) still remained the major component of the neurofilament, even when these were treated extensively with 0.8 M KCl. Other proteins were co-purified with the neurofilament preparation.

The analysis of protein kinase activity by sucrose gradient ultracentrifugation (fig.2) revealed the presence of one activity, sedimenting at 3–3.2 S, which catalyzed strongly the phosphorylation of casein while catalyzing only weakly that of type $II_A$ histones. A second protein kinase activity, with a sedimentation coefficient of 1.8–2 S, was found. It catalyzes the phosphorylation of proteins from the washed neurofilament preparation but it does not phosphorylate either casein or histones. It was followed by a minor component, incompletely separated, that sedimented at 3 S like the peak of casein kinase activity, and by some minor peaks of higher sedimentation coefficients. These protein kinase activities phosphorylate only the neurofilament proteins.

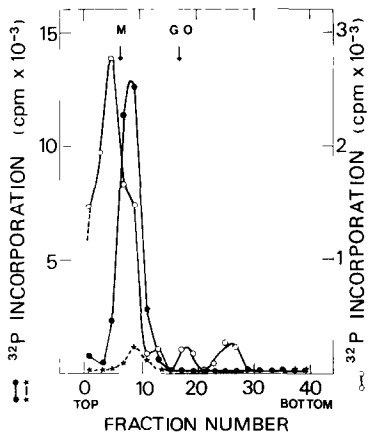


Fig.2. Sucrose gradient ultracentrifugation of protein kinases extracted from neurofilament preparations. Protein kinase activity was measured as in section 2; using salt-washed neurofilaments (○—○), casein (●—●) or $II_A$ histones (*—*) as substrate: M, myoglobin (2 S); G.O., glucose oxidase (7.9 S).

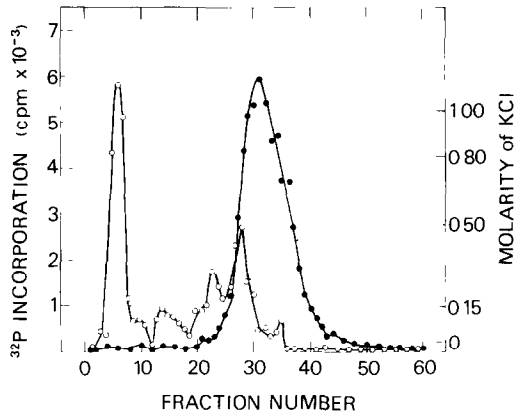


Fig.3. Phosphocellulose chromatography of protein kinases extracted from neurofilaments preparations. Protein kinase activity was assayed using salt-washed neurofilaments (○—○), and casein (●—●) as substrate.

Chromatography on phosphocellulose of the enzyme extract recovered from the neurofilament preparation allowed a total separation between the two principal activities revealed by sucrose-gradient ultracentrifugation (fig.3). Indeed, a protein kinase activity not retained on the column catalyzed exclusively the phosphorylation of proteins from the washed neurofilament preparation. No casein kinase activity was displayed at this level. This first peak was followed by several minor peaks which also phosphorylated the proteins of the neurofilament preparation. Some of them were not retained and others were eluted between 0.15 M and 0.22 M KCl. Casein kinase activity was, however, adsorbed on the phosphocellulose and was eluted by 0.35 M KCl.

We characterized, by electrophoresis and autoradiography, the proteins of the neurofilaments which were phosphorylated by the two principal protein kinases obtained by chromatography on phosphocellulose (fig.4). Neither of the two enzymatic fractions used displayed proteolytic activity with regard to the proteins from the neurofilament preparation (fig.4 (1–3)). It can be noted that the protein kinase activity which catalyzes the phosphorylation of neurofilament proteins exhibited only one band which did not correspond to the protein triplet (fig.4 (5)). Fig.4 (7,9) represent the incorporation of $^{32}$P catalyzed by residual protein kinase activity. Its proportion was estimated at

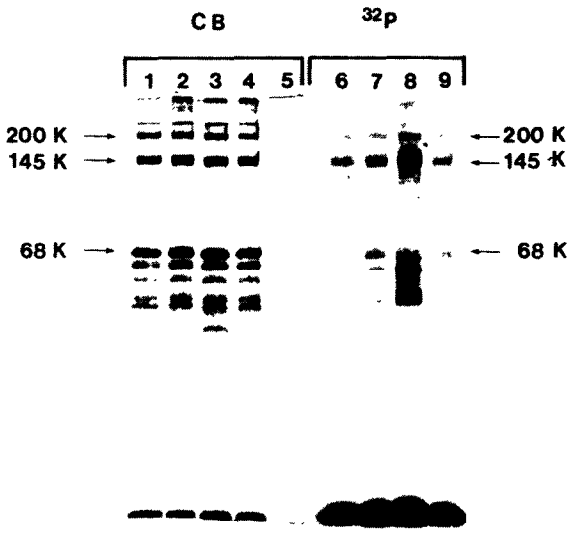


Fig.4. Phosphorylation of neurofilaments by the two principal protein kinases obtained from phosphocellulose chromatography. The Coomassie blue-stained gel (CB) shows proteins of neurofilaments incubated with [$\gamma$-$^{32}$P]ATP and: (1) with $CK_I$; (2,4) without addition; (3) with specific neurofilament protein kinase; (5) only fractions containing specific neurofilament protein kinase. An autoradiograph $^{32}$P of the same gel is shown alongside; (6–9) correspond to (1–4) (details in section 2).

about 10–15% of protein kinase activity prior to the washing of the neurofilaments (fig.1). It phosphorylates the triplet proteins, one protein >200 kDa and at least two proteins <68 kDa. Fig.4 (8) shows that the first peak of phosphocellulose phosphorylates the same proteins. In this case, a 3-fold increase in the phosphorylation of the proteins phosphorylated by residual activity, notably those of the triplet proteins, can be observed. In contrast, casein kinase activity (fig.4 (6)) increased neither the phosphorylation of the protein triplet nor that of the other proteins associated with the neurofilaments visible on the acrylamide gels of 8.5%. In all cases, small polypeptides migrating to the front were phosphorylated.

## 4. DISCUSSION

Our work shows that protein kinase activity associated with neurofilaments can only be solubilized following treatment by a solution of high ionic strength. In fact, 0.8 M KCl must be used to extract 85–90% of the total protein kinase activity associated with neurofilaments. That may explain the lack of protein kinase activity observed in the neurofilament preparation [7] (i.e., preparation involving treatment of the neurofilaments with 0.8 M KCl).

After 3 washings of neurofilament preparation with 0.5 M NaCl, 79% of protein kinase activity remained associated with the neurofilaments [6]. The use of a solution of high ionic strength to extract this enzymatic activity indicates that it is strongly associated with these cytoskeletal elements.

The purification of protein kinase activity by sucrose-gradient ultracentrifugation and especially by chromatography on phosphocellulose permitted us to establish that at least 2 distinct activities were associated with the neurofilament preparation. One of them can be characterized as casein kinase I based on its substrate specificity, on its sedimentation coefficient and its elution molarity by chromatography on phosphocellulose. This confirms [12] where protein kinase associated with neurofilaments was reported as a casein kinase which could not be $CK_{II}$. We have also shown that this casein kinase activity is not responsible for the phosphorylation of neurofilament proteins. Its rôle remains to be clarified.

The other protein kinase activity catalyzes the incorporation of phosphate into the 3 principal protein subunits of the neurofilaments (200 kDa, 145 kDa and 68 kDa) (fig.4). This activity phosphorylates other proteins co-purified with the triplet, including perhaps vimentin. This protein kinase whose activity is independent of cAMP, cGMP and $Ca^{2+}$ (not shown), acts very specifically on the neurofilament proteins, and in particular on the triplet, since neither casein nor type $II_A$ histones were a phosphate acceptor under our standard conditions.

Phosphorylation of neurofilament proteins, and in particular, those of the 200 kDa protein projection [17] may be able to mediate interactions between neurofilaments and cellular organelles, notably other cytoskeletal elements. This specific neurofilament protein kinase could modulate such interaction. For example, it could be involved in the formation, dependent upon ATP, of a complex

containing neurofilaments and microtubules [7,18,19]. However, there is no evidence that phosphorylation plays a rôle in the regulation of neurofilament assembly but this possibility should not be excluded.

Purification of this protein kinase is underway to permit a better characterization of its properties. It will then be possible to examine, for example, if it is capable of recognizing all of the phosphorylated sites in vivo on the triplet and if it is able to phosphorylate other cytoskeletal proteins. It will also be possible to investigate the presence of this protein kinase in other cellular compartments and, as a longrange objective, see if its activity may be modulated by various stimuli.

## ACKNOWLEDGEMENTS

We thank Mrs Claude Sais, Annick Guedec and Mr Mostefa Bahloul for the preparation of this manuscript.

## REFERENCES

[1] Lasek, R.J. and Hoffman, P.N. (1976) in: Cell Motility (Goldman, R. et al. eds) Microtubules and Related Proteins, vol.3, Cold Spring Harbor Laboratory, New York.
[2] Shelanski, M.L. and Liem, R.K.H. (1979) J. Neurochem. 33, 5–13.
[3] Lazarides, E. (1980) Nature 283, 249–256.
[4] Pant, H.C., Shecket, G., Gainer, H. and Lasek, R.J. (1978) J. Cell Biol. 78, 23–27.
[5] Julien, J.P. and Mushynski, W.E. (1981) J. Neurochem. 37, 1579–1585.
[6] Runge, M.S., El-Maghrabi, M.R., Claus, T.H., Pilkis, S.J. and Williams, R.C. (1981) Biochemistry 20, 175–180.
[7] Leterrier, J.F., Liem, R.K.H. and Shelanski, M.L. (1981) J. Cell. Biol. 90, 755–760.
[8] Eagles, P.A.M., Gilbert, D.S. and Maggs, A. (1981) Biochem. J. 199, 101–111.
[9] Shecket, G. and Lasek, R.S. (1982) J. Biol. Chem. 257, 4788–4795.
[10] Jones, S.M. and Williams, R.C. (1982) J. Biol. Chem. 257, 9902–9905.
[11] Eagles, P.A.M. and Gilbert, D.S. (1979) J. Physiol. Lond. 287, 10.
[12] Julien, J.P., Smoluk, D.G. and Mushynski, W.E. (1983) Biochim. Biophys. Acta 755, 25–31.
[13] Julien, J.P. and Mushynski, W.E. (1982) J. Biol. Chem. 257, 10467–10470.
[14] Dahl, D., Rueger, D.C., Bignami, A., Weber, K. and Osborn, M. (1981) Eur. J. Cell. Biol. 24, 191–196.
[15] Delbauffe, D., Ohayon, R. and Pavlovic-Hournac, M. (1979) Mol. Cell. Endocrinol. 14, 141–155.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Julien, J.P. and Mushynski, W.E. (1983) J. Biol. Chem. 258, 4019–4025.
[18] Runge, M.S., Laue, T.M., Yphantis, D.A., Lifsics, M.R., Saito, A., Altin, M., Reinke, K. and Williams, R.C. (1981) Proc. Natl. Acad. Sci. USA 78, 1431–1435.
[19] Leterrier, J.F., Liem, R.K.H. and Shelanski, M.L. (1982) J. Biol. Chem. 95, 982–986.