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Phosphorylation of neurofilament proteins by protein kinase C

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The low molecular mass (70 kDa) subunit of neurofilaments (NF-L) contains at least three phosphorylation sites in vivo and is phosphorylated by multiple kinases in a site-specific manner [(1987) J. Neurochem. 48, S101; Sihag, R.K. and Nixon, R.A. submitted]. In this study, we observed that the three subunits of neurofilament proteins from retinal ganglion cell neurons are substrates for purified mouse brain protein kinase C. Two-dimensional α-chymotryptic phosphopeptide map analyses of the NF-L subunit demonstrated that protein kinase C phosphorylates four polypeptide sites, two of which incorporate phosphate when retinal ganglion cells are pulse-radiolabeled with [^{32}P]orthophosphate in vivo.

Cytoskeletal protein; Neurofilament protein; Protein kinase C; Neuron; Phosphoprotein; Peptide mapping

1. INTRODUCTION

The three subunits of mammalian neurofilaments, designated NF-L (70 kDa), NF-M (140–160 kDa) and NF-H (200 kDa), are extensively phosphorylated in vivo [1–5]. Post-translational modification of these polypeptides occurs at various locations within neurons and may represent a mechanism to coordinate interactions between neurofilaments and other cytoskeletal proteins [4,6]. Several protein kinases phosphorylate neurofilament proteins in vitro, including a cAMP- and calcium-independent protein kinase that co-purifies with neurofilaments [7–10], and a calcium- and calmodulin-dependent kinase associated with neurofilaments [11] or axonal cytoskeletons [10]. In addition, a cAMP-dependent protein kinase that co-purifies with brain microtubule proteins phosphorylates NF-M as well as microtubule-associated proteins and tubulin [12]. Recently we found that purified cAMP-dependent kinase and an endogenous form of this enzyme associated with isolated axonal cytoskeletons phosphorylates all three subunits of the neurofilaments and that the phosphate groups on NF-L are added selectively to a polypeptide site that is normally phosphorylated in vivo [10].

In this report, we demonstrate that protein kinase C purified from mouse brain phosphorylates the three neurofilament subunits in vitro, and that the principal sites of phosphorylation on NF-L include two of the three α-chymotryptic peptides that we observed to be phosphorylated in vivo.

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Abbreviations: protein kinase C, calcium and phosphatidylserine-dependent protein kinase; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, SDS-polyacrylamide gel; TLC, thin-layer chromatography; cAMP, adenosine 3′,5′-cyclic monophosphate; DTT, dithiothreitol

2. MATERIALS AND METHODS

10–14-week-old male or female C57BL/6J mice were killed by cervical dislocation. Optic pathways (optic nerve and optic tract) containing the axons of retinal ganglion cells were dissected from rapidly cooled brains as previously described [13] and used immediately or stored at −70°C. For in vivo phosphorylation experiments, 100 μCi of [^{32}P]orthophosphate (spec. act. 1000 Ci/mmol, New England Nuclear) was injected

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intravitreally into anesthetized mice as described previously [4], and the optic pathways were dissected 24 h after injection.

The axonal cytoskeleton fractions were prepared by minor modifications [4] of the method of Chiu and Norton [14]. Briefly, four optic pathways (8 mg wet wt) were homogenized in 1 ml of 50 mM Tris-HCl, 5 mM EGTA, 100 mM KCl, 0.1% aprotinin, 0.5 mM PMSF, 50 μg/ml leupeptin and 1% Triton X-100, pH 6.8. The axonal cytoskeleton from in vivo ^{32}P-labeled optic pathways was prepared in the presence of 50 mM NaF and 0.5 mM Na_3VO_4. The Triton X-100-insoluble fraction, after treatment with DNase and RNase, was resuspended in buffer A (20 mM Hepes, 1 mM EDTA, 10 mM $MgCl_2$, 0.2 mM DTT, 50 μg/ml leupeptin, 0.2 mM PMSF, 0.5 mM Na_3VO_4, pH 7.4). To assay phosphorylation by the endogenous Ca^{2+}- and cAMP-independent kinase associated with the cytoskeleton, the reaction (in a final volume of 200 μl) was initiated by adding 20 μM of [γ-^{32}P]ATP (50 μCi/ml). In some experiments, the activity of this kinase was inhibited by including heparin (100 μg/ml) in the assay mixtures [10]. Protein kinase C-dependent phosphorylation was similarly performed in buffer A containing 100 μg/ml phosphatidylserine, 1.1 mM $CaCl_2$ and 0.5 μg of protein kinase C which was purified to electrophoretic homogeneity from mouse brain and assayed as previously described [15]. After a 10-min incubation at 30°C, the assay mixtures were quickly cooled to 4°C and centrifuged at 10000 × g for 5 min. Sedimented cytoskeletal proteins were solubilized in Laemmli buffer containing 4% SDS.

The proteins were separated on 32-cm 5–15% linear gradient polyacrylamide gels using minor modifications [4] of the method of Laemmli [16]. The gels were stained with Coomassie brilliant blue, dried under vacuum and autoradiographed [4]. Incorporation of ^{32}P into neurofilament proteins was measured by excising protein bands identified as the neurofilament protein subunits from gels and counting these samples for Čerenkov radiation.

A modification of the method of Elder et al. [17] was used for phosphopeptide mapping analyses. The phosphoprotein bands identified as NF-L [4] were excised from SDS-PAGE gels and digested with TLCK-α-chymotrypsin (10 μg in 1.0 ml of 50 mM NH_4HCO_3 for 22 h at 37°C). The complete α-chymotryptic digests were resolved in two dimensions on 20 × 20 cm cellulose-coated TLC plates (E. Merck, Darmstadt, FRG) as described [17] except that the chromatography was conducted in butanol/acetic acid/pyridine/water (80:12:30:40, v/v). The ^{32}P-labeled peptides were located by autoradiography.

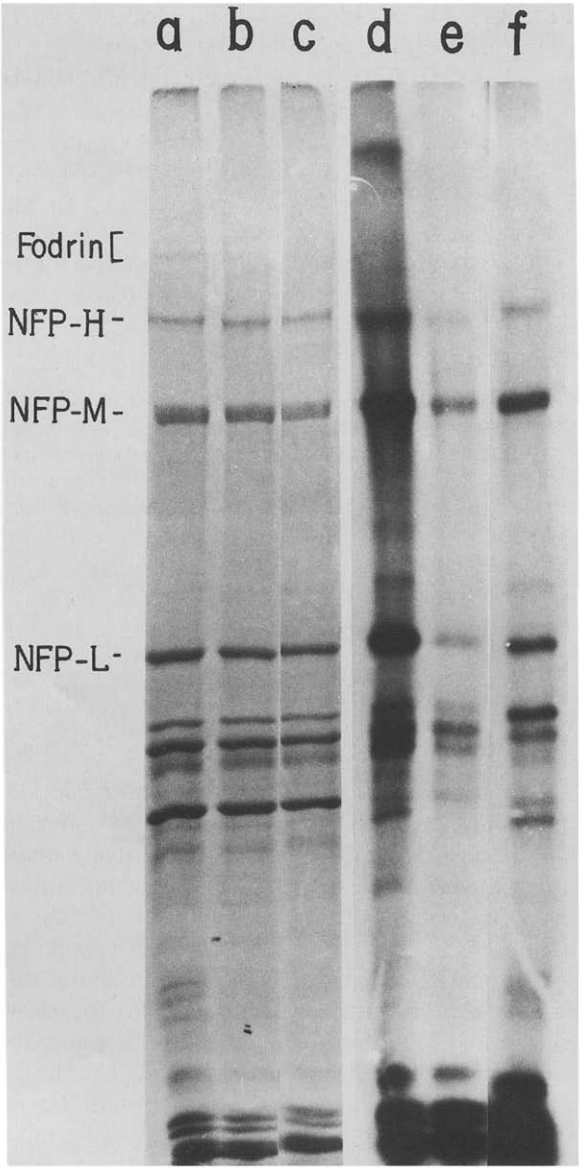

Fig.1. Phosphorylation of neurofilament subunits by protein kinase C in vitro. Triton-insoluble axonal cytoskeletons prepared from mouse optic pathway (see section 2) were incubated with [γ-^{32}P]ATP in the presence of the following additions: no addition (control) (lanes a,d); 100 μg/ml heparin (lanes b,e); 0.5 μg of protein kinase C plus 100 μg/ml heparin (lanes c,f). The proteins were separated on 5–15% gradients of SDS-PAGE and stained with Coomassie blue (lanes a–c). Phosphorylated proteins on the same gels were then visualized by autoradiography (lanes d–f, respectively). NF-L, NF-M and NF-H and other identified protein bands are labeled.

3. RESULTS

The three polypeptide subunits composing neurofilaments in retinal ganglion cell axons were good substrates for the endogenous cAMP- and calcium-independent protein kinase(s) associated with the cytoskeleton [10]. When Triton-insoluble cytoskeleton preparations were incubated with [γ-^{32}P]ATP, all three subunits were intensely labeled (fig.1) and their phosphorylation was nearly completely inhibited by heparin (100 μg/ml) (fig.1,

lanes d,e). The addition of protein kinase C and its activators, calcium and phosphatidylserine, to the axonal cytoskeleton in the presence of heparin stimulated phosphorylation of the three subunits (fig.1, lanes e,f). The incorporation of [^{32}P]phosphate into NF-L, NF-M and NF-H was 215, 210 and 160% higher, respectively, in the presence of protein kinase C.

Under in vitro conditions, kinases are capable of adding phosphate groups to sites on proteins that are not normal sites of phosphorylation in vivo [18]. Therefore, to investigate the site specificity and possible physiological relevance of neurofilament protein phosphorylation by protein kinase C, we characterized the pattern of sites on NF-L that were phosphorylated in vitro and compared it to the phosphorylation pattern when this polypeptide was isolated from retinal ganglion cells radiolabeled in vivo by injecting mice intravitreally with [^{32}P]orthophosphate [10]. The two-dimensional phosphopeptide map of NF-L, obtained after cytoskeleton preparations were incubated with [γ-^{32}P]ATP, demonstrated that the endogenous calcium- and cAMP-independent cytoskeleton-associated kinase(s) phosphorylated all three sites (L-1, L-2 and L-3) on NF-L that incorporate la-

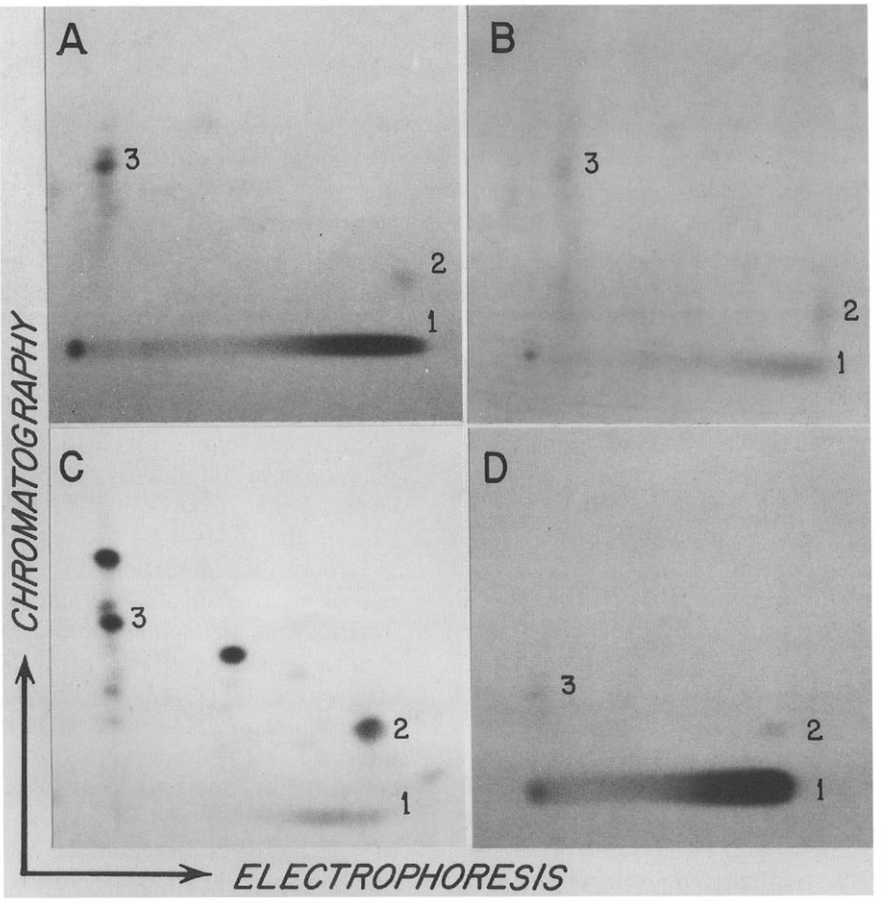

Fig.2. Autoradiograms of two-dimensional α-chymotryptic peptide maps of ^{32}P-labeled NF-L. The Triton-insoluble axonal cytoskeleton preparations were phosphorylated and separated on SDS-PAGE as described in fig.1. The band corresponding to NF-L was excised from each lane and incubated with TLCK-α-chymotrypsin. The α-chymotryptic digests were separated in two dimensions on cellulose-coated TLC plates as described in section 2. The phosphorylation conditions for the results presented here were: no addition (panel A); 100 μg/ml heparin (panel B); 0.5 μg of protein kinase C and 100 μg/ml heparin (panel C). The phosphopeptide map of NF-L phosphorylated in vivo is shown in panel D. The peptides that show incorporation of [^{32}P]phosphate in vivo are labeled 1, 2 and 3.

beled phosphate in vivo (fig.2A and D, [10] and Sihag, R.K. and Nixon, R.A., submitted). The addition of heparin to the phosphorylation assay mixture inhibited the phosphorylation of phosphopeptides L-1 and L-3 but not L-2 (fig.2A and B). When axonal cytoskeletons were incubated with protein kinase C in the presence of heparin, two-dimensional phosphopeptide map analyses showed that phosphopeptide L-2 and L-3, but not L-1, were phosphorylated by protein kinase C (fig.2B and C). Two additional peptides were labeled in vitro which are not detectably radiolabeled when neurofilament proteins in retinal ganglion cell neurons are pulse-labeled in vivo (fig.2C and D).

4. DISCUSSION

Our results provide the first evidence that neurofilament proteins are a substrate for protein kinase C in vitro. This finding adds to growing evidence that protein kinase C is capable of phosphorylating various cytoskeletal proteins, particularly those that may have associations with the plasma membrane [19–24]. Phosphorylation of NF-L by protein kinase C is interesting in this regard, since intermediate filaments in non-neural cells have been shown to be associated with the plasma membrane [25–29]. It has been suggested that phosphorylation of cellular proteins by protein kinase C may play a key role in signal transduction and the regulation of cell proliferation and differentiation [30].

Several observations suggest that the phosphorylation of NF-L by protein kinase C may be a physiologically relevant event. The two principal sites phosphorylated by protein kinase C, L-2 and L-3, correspond to two of the three α-chymotryptic peptides from NF-L that are phosphorylated when neurofilaments in retinal ganglion cell neurons are radiolabeled in vivo with [^{32}P]orthophosphate (fig.2D and [10]). Furthermore, the relative specificity of protein kinase C for these three sites on NF-L differs from that of other kinases that act on this subunit. Calcium- and cyclic nucleotide-independent protein kinases and the calcium- and calmodulin-dependent protein kinase phosphorylate site L-1 and, to a much lesser extent, L-3. cAMP-dependent protein kinase selectively phosphorylates L-2. In contrast, peptide L-3 was the preferred substrate for protein kinase C. The two sites phosphorylated by kinase C for which in vivo counterparts were not found cannot be disregarded as being physiologically irrelevant, since in vivo labeling of retinal ganglion cells reflects the phosphorylation events occurring shortly after neurofilament proteins are synthesized. Phosphorylation of additional sites, including these two, could involve a population of NF-L subunits in a restricted neuronal compartment (e.g., the perikaryon) or may occur much later as neurofilaments are transported toward distal axonal levels [4]. When neurofilaments are isolated from axons one day after retinal ganglion cells are injected intravitreally with [^{32}P]orthophosphate, sites L-2 and L-3 on NF-L are labeled less intensely than L-1 (fig.2D), which suggests either that only a subpopulation of neurofilaments contained phosphates at sites L-2 and L-3 or that [^{32}P]phosphates at these sites are turned over during the first 24 h after labeling. In support of the latter possibility is the observation that, in retinal ganglion cells, NF-L loses more than half of the initial incorporated radiolabeled phosphate groups within 3 days after isotope administration in vivo [4] and undergoes a net decrease in phosphorylation state during this time period [31]. Phosphopeptide map analyses at 1 and 4 days after isotope administration demonstrate that phosphate groups may be lost more rapidly from L-2 and L-3 than from L-1 [32]. These observations suggest that phosphate groups added to L-2 and L-3 may be important in the early fate of neurofilaments such as subunit polymerization or initial stages of axoplasmic transport.

Since newly synthesized neurofilament proteins in neuronal perikarya must assemble into neurofilaments, enter and advance along axons, integrate with other proteins into the axonal cytoskeleton, and turn over in part within nerve terminals [31,33], the differential phosphorylation of specific sites by multiple second messenger-regulated protein kinases in various domains of the neuron may partly underly the specific and diverse molecular changes necessary to coordinate these events. In this regard, it is interesting to note the different distributions of second messenger-mediated kinases in the brain and within various parts of the neuron [34–36]. The actions of these multiple kinase systems on NF-L emphasize the

potential importance of second messengers in regulating the dynamics of neurofilament structure and function.

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