Casein kinase II is responsible for phosphorylation of NF-L at Ser-473

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Received 12 April 1999; received in revised form 9 June 1999

Abstract Ser-473 is solely phosphorylated in vivo in the tail region of neurofilament L (NF-L). With peptides including the native phosphorylation site, it was not possible to locate responsible kinases. We therefore adopted full-length dephosphorylated NF-L as the substrate, and employed MALDI/TOF (matrix-assisted laser desorption and ionization/time of flight) mass spectrometry and a site-specific phosphorylation-dependent antibody recognizing Ser-473 phosphorylation. The antibody showed that casein kinase I (CK I) as well as casein kinase II (CK II) phosphorylated Ser-473 in vitro, while neither GSK-3β nor calcium/calmodulin-dependent protein kinase II did so. However, the mass spectra of the tail fragments of the phosphorylated NF-L indicated that CK II was the kinase mediating Ser-473 phosphorylation in vitro as opposed to CK I, because CK I phosphorylated another site as well as Ser-473 in vitro. The antibody also demonstrated that NF-L phosphorylated at Ser-473 was abundant in the neuronal perikarya of the rat cortex, indicating that phosphorylation of Ser-473 may take place there. This result may support the suggestion that CK II is the kinase responsible for Ser-473 phosphorylation. Despite many reports showing that CK I mediates phosphorylation of neurofilaments, CK II may phosphorylate NF-L in vivo.

Key words: Neurofilament; Neurofilament L; Casein kinase; Neuronal culture

1. Introduction

Neurofilaments, a major cytoskeletal component of neurons, are composed of three subunits, NF-L, NF-M and NF-H. NF-M and NF-H are highly phosphorylated in vivo, whereas about half of NF-L is singly phosphorylated at Ser-473 in vivo except for a small amount of NF-L phosphorylated at Ser-55 [1,2]. The phosphorylation of the tail regions of NF-M and NF-H has been investigated well, and is thought to be related to the maintenance of the axonal architecture. The phosphorylation of NF-L at Ser-473 has not been studied at all since the determination of the site. Without radio-isotopes, it would be quite difficult to monitor the extent of the phosphorylation at the native sites. Mass spectrometric analysis with MALDI/TOF (matrix-assisted laser desorption and ionization/time of flight) enables us to count the number of phosphate groups in large fragments. Otherwise, site-specific phosphorylation-dependent antibodies inform us of the phosphorylation state of specific sites, and of the distribution of phosphorylated proteins [3]. We thus combined the two methods in an attempt to determine the kinase responsible for Ser-473 phosphorylation, and investigated the distribution of NF-L phosphorylated at Ser-473 using a site-specific phosphorylation-dependent antibody. These studies may also provide hints as to the roles and functions of Ser-473 phosphorylation.

2. Materials and methods

2.1. Mass spectrometric analysis of phosphorylation of NF-L tail

NF-L was purified from bovine spinal cords as previously described [4]. NF-L, dialyzed against dialysis buffer (0.1 mM EGTA/25 mM Tris-HCl, pH 7.5), was incubated with 2.5 U bovine thrombin (Sigma)/1 mg NF-L at 37°C for 10 min [5]. The digested samples dissolved in 8 M urea were subjected to reversed-phase HPLC on a cosmosil column C4-AR (diameter 0.46×25 cm) using a 20–40% linear acetonitrile (AcN) gradient in 0.1% aqueous trifluoroacetic acid (TFA) for 20 min.

The molecular weights of the tail fragments were measured by voyager RP/DE (Perceptive, flight path = 1.3 m). An aliquot (0.67 μl) of each of the samples was mixed with 0.33 μl of the saturated solution of α-cyano-4-cinnamic acid (αCHCA, Sigma) in 50% AcN/50% H_2O/0.1% TFA on a metal plate, and dried in air for 30 min. The mass spectra were obtained under the linear mode (accelerating voltage: 25 000 V) with delayed extraction (150 ns), and the results for 256 scans were averaged. The external calibration was done using myoglobin as the standard under the same conditions as above described.

2.2. Preparation of dephosphorylated NF-L

Purified NF-L was dialyzed against 120 mM Tris/2 mM $MgCl_2$/2 mM EGTA, pH 8.5 at 4°C overnight, and reacted with 20 U *Escherichia coli* alkaline phosphatase (Boehringer Mannheim)/1 mg NF-L in a dialysis tube by dialyzing against the same buffer at 30°C for 10 h to ensure completely dephosphorylation. The reaction was stopped by dialysis against 20 mM Bis-Tris/8 M urea, pH 6.5 at 4°C overnight. To remove the phosphatase, the sample was applied to a DEAE-Q-Sepharose column (diameter 1.0×30 cm, Pharmacia) equilibrated with the same buffer. Dephosphorylated NF-L was eluted at 0.3 M NaCl by application of a NaCl gradient (0–1 M).

2.3. Preparation of a polyclonal antibody specifically recognizing Ser-473 phosphorylation of NF-L (ab-pNFL473)

A phosphopeptide (DPPS(p)EEAEC) and a non-phosphopeptide (DPPSEEAEC) were synthesized by the Fmoc method and purified by reverse-phase HPLC. Their identities were confirmed by MALDI/TOF mass spectrometry and amino acid analysis, and the purities were over 95% on reverse-phase HPLC. They were conjugated with maleimide-activated keyhole limpet hemocyanin (Pierce) and immunized to New Zealand White rabbits [1]. Sera of high titer against the peptides were affinity-purified by the columns conjugated with the peptides using SulfoLink Coupling Gel (Pierce). Thereafter, the affinity-purified antibody against the phosphopeptide was applied to the affinity column conjugated with the non-phosphopeptide to remove any contamination of the immunoreactivity against the non-phosphopeptide, and the void fraction was used as ab-pNFL473. Meanwhile,

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0014-5793/99/$20.00
PII: S0014-5793(99)00832-7

the antibody against the non-phosphopeptide was applied to the column conjugated with the non-phosphopeptide, and the void fraction was used as ab-indNFL473. The protein amount of the purified antibody was measured by the Bradford method using normal rabbit IgG as a standard protein.

2.4. Phosphorylation of dephosphorylated NF-L by various kinases

Dephosphorylated NF-L (dpNF-L) was dialyzed against dialysis buffer at 4°C overnight. Two micrograms of dpNF-L was phosphorylated by incubation with 100 U of recombinant casein kinase I (New England BioLabs), 10 mM $MgCl_2$, 5 mM DTT, 0.2 mM ATP, and 50 mM Tris-Hcl/pH 7.5 at 30°C for 2 hours, with 100 U of recombinant casein kinase II (New England BioLabs), 50 mM KCl, 10 mM $MgCl_2$, 0.2 mM ATP, 20 mM Tris-Hcl/pH 7.5 at 30°C for 2 hours, with 100 U of recombinant GSK3-β (New England BioLabs), 50 mM KCl, 10 mM $MgCl_2$, 5 m DTT, 0.2 mM ATP, and 20 mM Tris-HCl, pH 7.5 at 30°C for 2 h, or with 100 U of recombinant calcium/calmodulin-dependent protein kinase II (CaMK II, New England BioLabs), 2.4 μM calmodulin, 2 mM $CaCl_2$, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, 0.1 mM ATP, and 20 mM Tris-HCl, pH 7.5 at 30°C for 2 h.

2.5. Dot blot analysis

An aliquot (2 μl) of the fragments diluted with TBS was placed on the nitrocellulose membrane (Nitro-plus 2000, MSI). The membranes were blocked by 5% skim milk (Difco)/TBS, and reacted with the antibodies (0.1 μg/ml). After washing twice with 0.05% Tween/1% skim milk/TBS, they were reacted with ALP-labeled anti-rabbit-IgG (Fc) (Promega). The optical densities of the spots on the developed membranes were measured by Image Master (Pharmacia) with subtraction of the background. The intensity of immunoreactivity was expressed in $OD \times mm^2$, which was the product of the optical density and the area of a spot.

2.6. Western blot analysis

Samples were subjected to SDS-PAGE, and transferred onto the membrane. The membranes were stained as above described. In the case of cell lysate, cells grown on dishes were directly dissolved in 10 M urea solution and sonicated. The lysates were centrifuged at $100\,000 \times g$ for 30 min, and the supernatants were used for samples.

3. Results

3.1. Mass spectrometric measurement of phosphorylation of NF-L tail region

The tail fragment of bovine NF-L (438–554) was obtained by thrombin digestion [5,6]. No signal was obtained using sinapinic acid as a matrix for the usual MALDI/TOF measurement of high molecular weights. However, the mass spectra of the fragments were obtained using αCHCA as the matrix. The tail fragment prior to dephosphorylation showed two major peaks, corresponding to the non-phosphorylated (calculated: 12956.1) and the singly phosphorylated form (calculated: 13036.0), indicating that part of NF-L was singly phosphorylated in the tail region (Fig. 1A) [6].

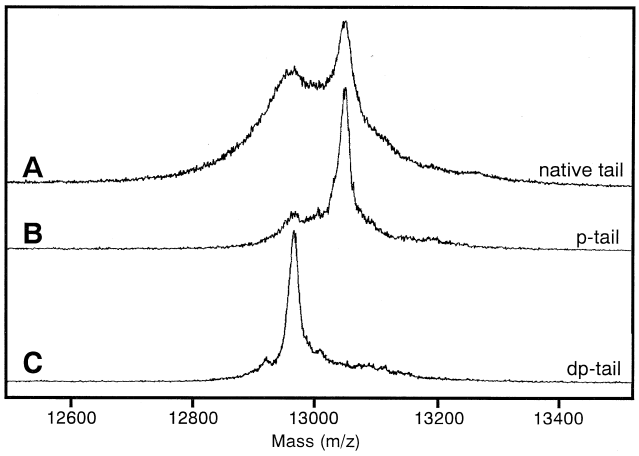

Fig. 1. Mass spectra of native (A), phosphorylated (B) and dephosphorylated (C) tail fragments of NF-L obtained by MALDI/TOF.

3.2. Specificity of antibody ab-pNFL473

Phosphorylated and non-phosphorylated tail fragments of NF-L were prepared in order to examine whether the antibodies were able to distinguish between the phosphorylated and the non-phosphorylated NF-L. The retention time of the phosphorylated tail fragment (p-tail) was a little shorter than that of the non-phosphorylated one (dp-tail). The p-tail and dp-tail were almost completely separated by three times recolumning with a gently sloped AcN gradient. The separation was confirmed by MALDI/TOF mass-spectrometry (Fig. 1B,C). In the dot blot analysis of the p-tail and dp-tail, ab-pNFL473 recognized the p-tail at 100 times higher intensity than the dp-tail (Fig. 2A). Considering that the fraction of dp-tail contained only a small amount of p-tail (Fig. 1C), ab-pNFL473 was able to recognize the phosphorylated form of NF-L with high specificity. However, ab-indNFL473 failed to distinguish between them, and recognized both of them to

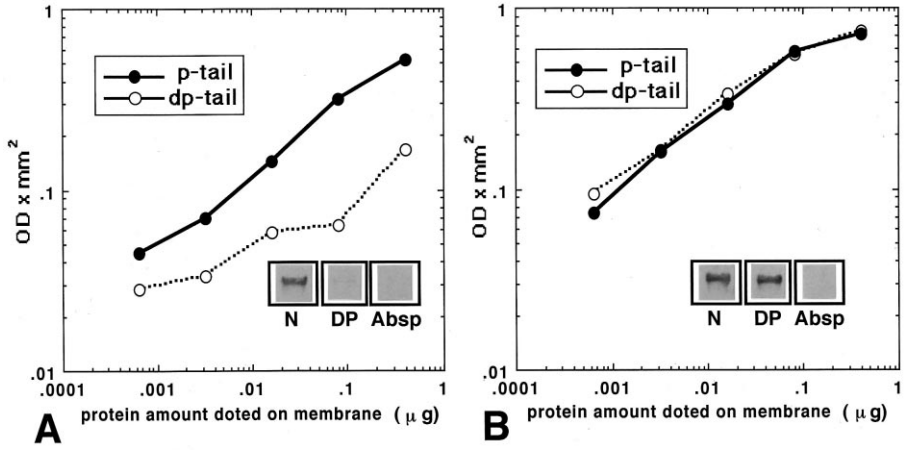

Fig. 2. Specificity of ab-pNFL473 (A) and ab-indNFL473 (B) evaluated by dot blot analysis of phosphorylated and non-phosphorylated tail fragments of NF-L (p-tail and dp-tail), and by Western blot analysis of native and dephosphorylated NF-L (N and DP in each graph, Absp: absorbed by the immunized peptides).

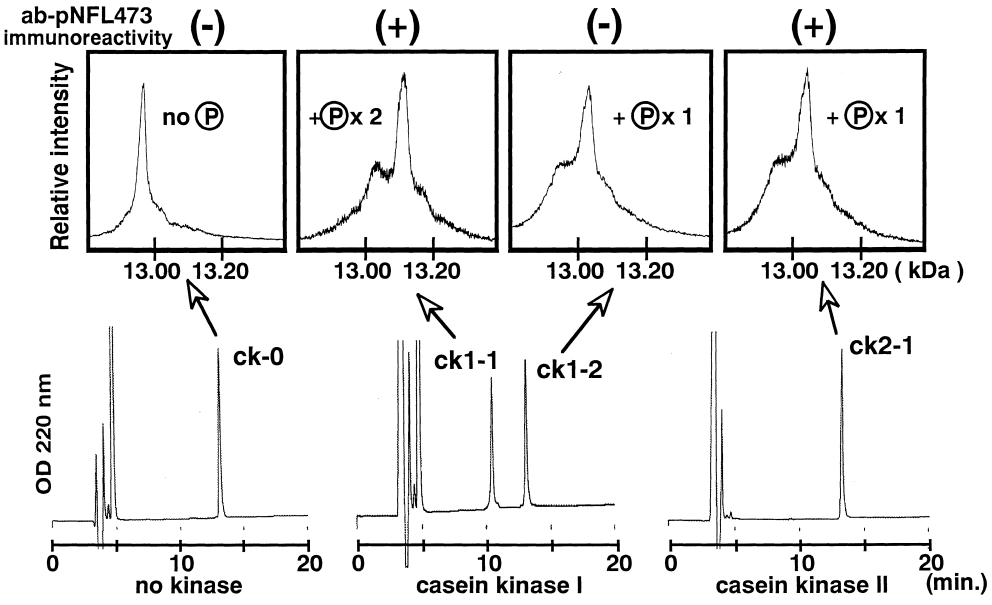

Fig. 3. Diagram of the exploration of the kinase responsible for Ser-473 phosphorylation in the casein kinases. The figure shows the immunoreactivity of ab-pNFL473, the mass spectra and the retention times of the peaks, which were observed by application of the control or phosphorylated samples to a column.

almost equal extents (Fig. 2B), although ab-indNFL473 was absorbed by the column conjugated with the phosphopeptide. This may be because ab-indNFL473 recognizes the conformation of NF-L around Ser-473. Western blot analysis showed compatible results to the above dot blot analysis (Fig. 2A,B).

3.3. Exploration of the kinase responsible for NF-L Ser-473

The kinase responsible for Ser-473 phosphorylation was explored by using the phospho-specific antibody. The peptide, DDSPEAEEEC, was used as the substrate of the kinases examined here, however, none of them phosphorylated the peptide (data not shown). Full-length dephosphorylated NF-L, free of the treated phosphatase, was then used as the substrate. Phosphorylated samples were digested by thrombin as described above. Digested samples dissolved in 8 M urea were subjected to HPLC, and the peaks were analyzed by mass spectrometry and dot blot.

The digested sample of NF-L phosphorylated by CK I showed two peaks, whereas those of NF-L phosphorylated by CK II, GSK-3 and CaMK II showed only one peak (Fig. 3). The molecular weight of the first peak (ck1-1) of NF-L phosphorylated by CK I, which was positively recognized by ab-pNFL473, showed that two phosphate groups were added to the tail fragment. On the other hand, the molecular weight of the second peak (ck1-2), which was not recognized by ab-pNFL473, showed that one phosphate group was added. These results demonstrated that Ser-473 was the secondary phosphorylation site of CK I in the tail region. As the native phosphorylation site is solely Ser-473 in the tail region, it was concluded that CK I was not responsible for Ser-473 phosphorylation in vivo. The major molecular weight of the peak (ck2-1) of NF-L phosphorylated by CK II, which was recognized by ab-pNFL473, showed that one phosphate group was added. This well matched the fact that the native phosphorylated site of NF-L is single in the tail region. Otherwise, the molecular weights of the peaks of NF-L phosphorylated by GSK-3 or CaMK II showed that no phosphate group was added, indicating that GSK-3 and CaMK II were not to involved in the phosphorylation of the tail region of NF-L. Of all the kinases examined here, CK II was the most likely kinase for mediation of Ser-473 phosphorylation.

3.4. Distribution of NF-L phosphorylated at Ser-473

Distribution of NF-L phosphorylated at Ser-473 was examined in the frozen sections of rat cortex in order to investigate the location where Ser-473 is phosphorylated (Fig. 4). The

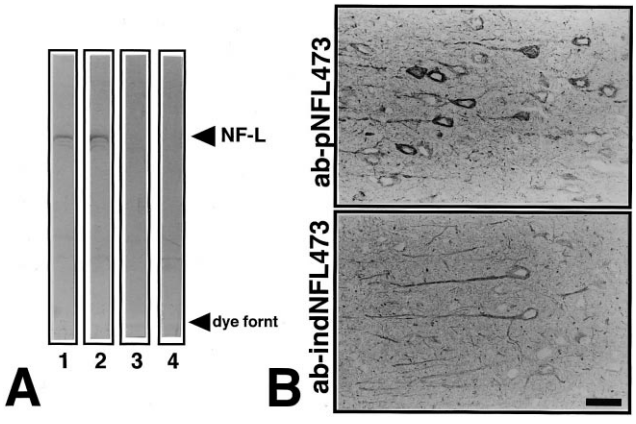

Fig. 4. Immunohistochemical study of the distribution of phosphorylated NF-L using ab-pNFL473 and ab-indNFL473. A: Specificity of ab-pNFL473 and ab-indNFL473 in the homogenate of the rat cortex: ab-pNFL473 (lane 1); ab-indNFL473 (lane 2); ab-pNFL473 absorbed by the phosphopeptide (lane 3); ab-indNFL473 absorbed by the non-phosphopeptide (lane 4). 20 μg protein of the homogenate was applied to each lane. B: Immunohistochemistry of the rat cortex. Bar represents 20 μm. 10 μm thick sections of freshly frozen samples of the rat cortex were fixed in 100% methanol. The sections were incubated overnight at 4°C with the antibodies (0.1 μg/ml) in TBS containing 10% normal goat serum and 0.05% Triton X-100, and developed by Vectastain Elite kit (Vector Lab) and diaminobenzidine (Dojin).

perikarya of the pyramidal neurons were strongly stained by ab-pNFL473, whereas ab-indNFL473 stained the entire neurons at almost the same intensity. These results indicate that phosphorylation of Ser-473 may take place in the neuronal perikarya.

4. Discussion

Exploration of a possible kinase responsible for Ser-473 phosphorylation was performed by means of a phospho-specific antibody and mass spectrometry. Usually, candidate kinases are estimated by using peptides including a phosphorylation site, however, the kinases examined here could not phosphorylate the peptide (DDSPEAEEEC). Full-length dephosphorylated NF-L was therefore used for the substrate. The tail fragments of NF-L, which had been incubated with kinases, were obtained by reverse-phased HPLC, and analyzed by the phospho-specific antibody and mass-spectrometry. These methods informed us of a possible kinase.

Although protein kinase A has been reported to phosphorylate the tail region of NF-L, the phosphorylation sites is not Ser-473 [7]. Protein kinase C is reported not to phosphorylate the tail region of NF-L [8]. GSK-3, one of the proline-directed kinase, did not phosphorylate it; nor did CaMK II, which includes the consensus sequence Ser-X-Glu/Asp [9]. Although CK I actually phosphorylated Ser-473, this was a secondary site. Considering that the tail fragment was singly phosphorylated in vivo, it was concluded that CK I was not the responsible kinase. As the sequence around Ser-473 showed the Ser-X-Glu motif, CK II was estimated to be the candidate kinase [10,11]. However, the peptide including the motif was not phosphorylated by CK II. When full-length dephosphorylated NF-L was used as the substrate, Ser-473 was solely phosphorylated in the tail region. Thus, the in vivo responsible kinase for Ser-473 phosphorylation was estimated to be CK II.

Observation of the distribution of the NF-L phosphorylated at Ser-473 indicated that the kinase phosphorylating Ser-473 may be located in the neuronal perikarya. The same findings were obtained in the rat cortical primary culture (data not shown). These observations match with the fact that CK II is rich in neurons and located in the neuronal perikarya [12–14].

Casein kinases have been investigated as the kinase phosphorylating neurofilaments in vivo. CK II is not reported to be a candidate, but CK I is, because CK I phosphorylates neurofilaments and is rich in the axon-derived neurofilament-rich fraction [15–17]. In vitro, NF-M is a good substrate for CK I [18–20]. However, CK II actually phosphorylates neurofilaments to a relatively lower extent than CK I [15,16]. In our experiment, CK I phosphorylated NF-L to a higher extent than CK II, but did so at a different location to the native site, Ser-473. Without the mass spectrometric measurement, we could not reject the possibility that CK I would be a more likely candidate than CK II, due to the higher efficacy of phosphorylation.

Only the combination of MALDI/TOF mass spectrometry and the site-specific phosphorylation-dependent antibody enabled us to determine the responsible kinase for Ser-473 phosphorylation, demonstrating that it may be CK II. Without these procedures, it would be much more difficult to make the same conclusion. CK II, a multi-functional second messenger-independent kinase, is reported to be involved in neuritogenesis and in phosphorylation of MAP1B [21–24]. In the early stage of primary neuronal culture, the immunoreactivity of ab-pNFL473 was also observed (data not shown). Phosphorylation of NF-L Ser-473 might be also involved in the neuritogenesis, or it might only represent the results of the CK II activation in the neuronal perikarya when the neuritogenesis occurs.

Moreover, ab-pNF473, the site-specific phosphorylation-dependent antibody, may also be useful in investigating the pathological changes of NF-L and the CK II activity in neurodegenerative diseases.

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