Vol. 196, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1993 Pages 115-123

PHOSPHORYLATION OF A 62 KD PORCINE α-INTERNEXIN, A NEWLY IDENTIFIED INTERMEDIATE FILAMENT PROTEIN

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Received August 19, 1993

Summary: A 62 kd protein was purified from the Triton-insoluble fraction of porcine brain white matter. This protein formed 10nm filaments, in vitro. The phosphorylation of the 62 kd protein by cAMP-dependent protein kinase caused electrophoretic mobility to shift to 66 kd on SDS-PAGE and a complete loss of the filament forming ability ensued. Amino acid sequences of four peptide fragments obtained from the 62 kd protein by lysylendopeptidase were identical with that of a 66 kd rat brain α-internexin. Amino acid analyses of the phosphopeptide fragment derived from phosphorylated porcine α-internexin revealed that the phosphorylation sites by cAMP-dependent protein kinase located in the amino-terminal head domain of this protein. These results strongly suggest that α-internexin polymerizes into 10nm filaments in vitro and that phosphorylation of the amino-terminal domain of α-internexin controls its polymerizability.

Intermediate filaments(IFs) are major components of the cytoskeleton of eukaryotic cells and appear to play a significant role maintaining organization of the cytoplasmic space (1). The subunit structure defines five major classes of IFs that can be distinguished

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Abbreviations: IF, intermediate filament; NF, neurofilament; GFAP, glial fibrillary acidic protein; HPLC, high performance liquid chromatography; 2ME, 2-mercaptoethanol; EGTA, ethylene glycol bis(β-aminoethyl ether) N, N, N', N'-tetraacetic acid; PMSF, phenyl-methylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

0006-291X/93 $4.00

both biochemically and immunologically : vimentin filaments in mesenchymal cells, desmin filaments in muscle cells, glial filaments in glial cells, neurofilaments in neurons and keratin filaments in epithelial cells (1-3). The purified constituted proteins form 10nm filaments in vitro and site / domain specific phosphorylation controls their polymerizability (4-15).

The 66 kd rat brain cytoskeletal protein α-internexin,has been classified as an IF-associated protein but not as an IF protein, since α-internexin was seen to bind to IF proteins yet did not polymerize into 10nm filaments, under conditions that reassemble the neurofilament proteins and GFAP (16). However, it has recently been reported that the predicted amino acid sequence of a cDNA encoding α-internexin bears all the characteristics of an IF protein sequence (17).

We report here evidence that α-internexin purified from porcine brain does polymerize into 10nm filaments in vitro, and that phosphorylation of the amino-terminal domain of this protein induces disassembly of the filament structure as well as other IF proteins.

MATERIALS AND METHODS

Purification of the 62 kd cytoskeletal protein (α-internexin): Porcine brain white matter (100 g) was used as starting material, and a fraction enriched in intermediate filaments (IFs) was obtained by the method of Zackroff and Goldman (18). The 62 kd protein was solubilized by 36 h extraction of IF preparation with 20 mM Tris-HCl, pH 7.5, containing 50 mM 2-ME, 2 mM EGTA, 2 mM PMSF, and 8 M urea at 4 °C. The homogenate was centrifuged for 120 min at 100,000 x g. The supernatant (576 mg) was applied to a DE52 cellulose column (ϕ2.5 x 20 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, containing 50mM 2-ME, 2mM EGTA, 1 mM PMSF, and 7 M urea (Buffer A). The column was washed with 150 ml of the same solution. The 62 kd protein was eluted from the column by application of a 1000 ml linear concentration gradient of NaCl (0.0 - 0.25 M) in Buffer A at a flow rate of 25 ml/h. The 62 kd protein was eluted as a peak between 0.023 and 0.03 mM NaCl. The DEAE fraction was dialyzed against 20 mM formate buffer, pH 4.0, containing 50 mM 2-ME, 2 mM EGTA, 1 mM PMSF, and 7 M urea (Buffer B), and the dialyzed fraction (29.4 mg) was applied to a Mono S column (ϕ0.5 x 5 cm) previously equilibrated with Buffer B. The column was washed with 5 ml of the same buffer. The 62 kd protein was eluted from the column by application of a 40 ml linear concentration gradient of NaCl (0.0 - 0.5 M) in Buffer B at a flow rate of 30 ml/h. The 62 kd purified protein (6.38 mg)eluted as a sharp

peak between 0.22 - 0.24 mM NaCl. Removal of the urea by dialysis for 24 - 36 h yielded a protein that was soluble in 5mM Tris-HCl, pH 8.8 . These samples were incubated with 25mM imidazole, pH 6.75, containing 1mM $MgCl_2$ at 37 °C for 1h. Polymerization competence was analyzed by electron microscopy and by centrifugation (15,000 x g for 30 min. at 30 °C).

Phosphorylation of the 62 kd α-internexin: The reconstituted porcine brain α-internexin IF (0.2 mg/ml) was phosphorylated by incubation with 3 μg/ml of the catalytic subunit of cAMP-dependent protein kinase, 0.1 mM [γ-^{32}P] ATP, 1mM $MgCl_2$, 25 mM Imidazole-HCl, pH 6.75 at 25 °C.

Fragmentation of Native and Deiminated α-internexin: 0.5 mg of native and phosphorylated α-internexin was digested with 0.8 μg of lysylendopeptidase in 70 μl of 50 mM Tris-HCl, pH 9.0, containing 4 M urea at 30 °C for 16 h. Peptides thus obtained were separated by HPLC on a C_{18} reverse-phase column (φ 0.46 x 15 cm) equilibrated with 12 % (v/v) acetonitrile containing 0.1 % trifluoroacetic acid. Elution was carried out with a 60 min linear gradient of acetonitrile concentration from 12 to 48 % (v/v) at a flow rate of 0.8 ml/min.

Amino Acid Analysis: Purified phosphopeptides (0.5 - 1 nmol) were subjected to 6N HCl hydrolysis in vacuo at 110 °C for 24 h. Amino acid analysis was performed by reverse-phase HPLC of phenylthiocarbamoyl derivatives(19), using the Waters Pico-Tag system.

Sequence Analysis: An aliquot of the purified fragments (1 - 3nmol) dissolved in 0.1% trifluoroacetic acid was analyzed with an ABI 470A gas-phase sequencer equipped with an ABI 120A on-line PTH amino acid analyzer, using 03R PTH program.

RESULTS AND DISCUSSION

Electrophoretic profiles of the crude IF fraction and the purified 62 kd cytoskeletal protein from porcine brain white matter are shown in Fig.1A. The DE52 and Mono S columns separated the 62 kd cytoskeletal protein from other proteins such as GFAP and neurofilament triplet protein. The purified 62 kd protein reassembled into very long 10nm filaments in vitro (Fig. 1B). On Western blots containing crude IF proteins from porcine brain white matter, antisera raised to the purified 62 kd protein recognized only the 62 kd protein (Fig.1A). Furthermore, no reactivity was observed against purified vimentin, desmin, GFAP,NF-L, NF-M, NF-H, Keratin no.8 and no.18 (data not shown). Therefore, the 62 kd protein is not probably immunologically related to the IF proteins we tested.

To better characterize this 62 kd protein, the amino acid sequence of the peptide fragments derived from this protein were analyzed. The

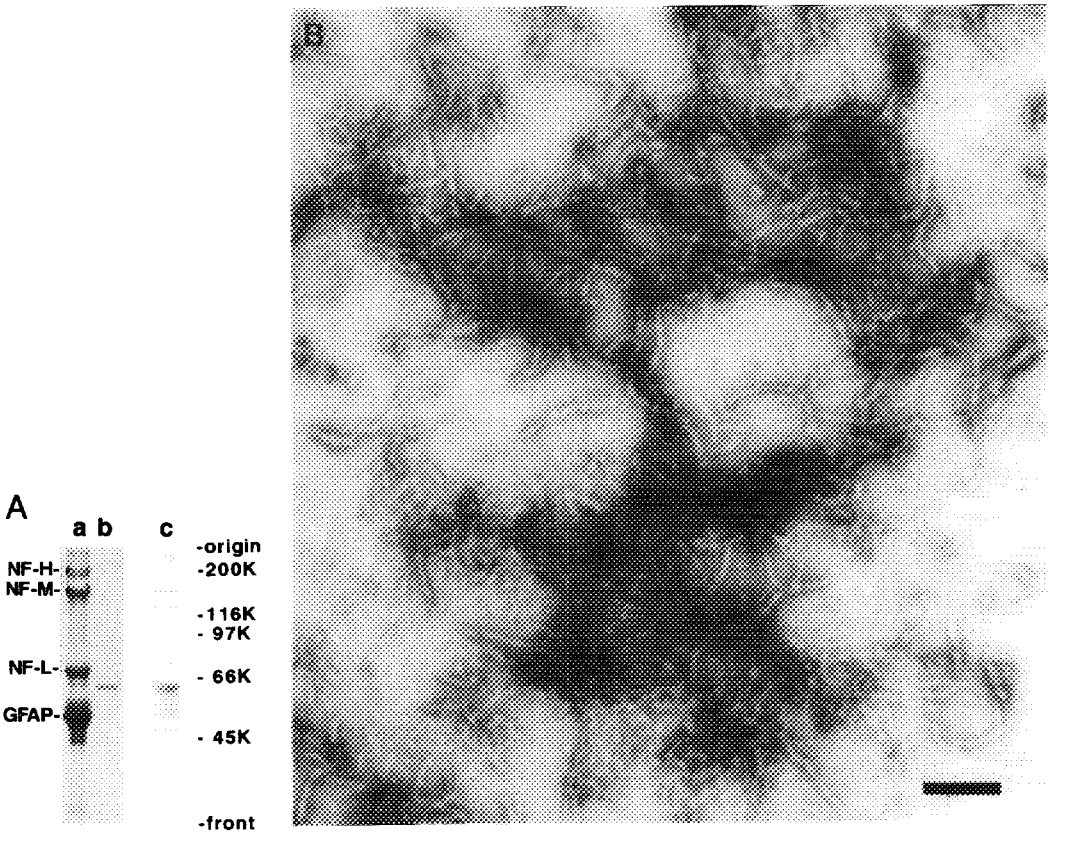
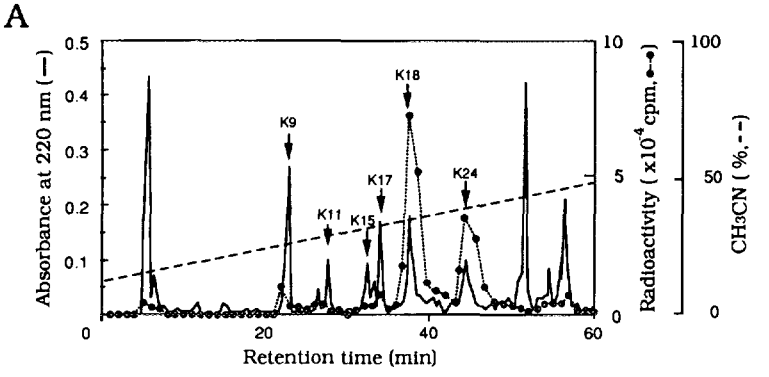


Fig. 1. A, Purification was done as described under MATERIALS and METHODS. The samples were boiled in a water bath for 1 min and subjected to SDS-polyacrylamide slab gel electrophoresis, under conditions described by Laemmli (28). The separating and stacking gel contained 8 and 3 % acrylamide, respectively. An 8M urea extract of porcine brain white matter (a) and purified 62 kd protein (b) are shown. Both lanes were stained with Coomassie blue. An 8M urea extract of porcine brain white matter was analyzed by immunoblotting using antisera for 62 kd protein (c). B, Electron microscopy of the negatively stained 62 kd protein. The sample was incubated as described under MATERIALS and METHODS. Scale bar = 200 nm, magnification x 50,000.

purified 62 kd protein was digested with lysylendopeptidase and the peptide fragments were separated by reverse-phase chromatography, the elution profiles are shown in Fig.2A. Peptides K9, K11, K15 and K17 were then applied to a gas-phase sequencer and phenylthiohydantion-amino acids were identified. As shown in Fig.2B, the amino acid sequences were identical with those of rat brain 66 kd α-internexin.



B

Porcine 62 kd protein	(peptide K9)	NLQSAEEWYK
Rat 66 kd α-internexin	(275-284)	NLQSAEEWYK
Rat NF-L	(272-281)	NMQNAEEWFK
Porcine 62 kd protein	(peptide K11)	AQQRDVDGATLARLDLEK
Rat 66 kd α-internexin	(198-215)	AQQRDVDGATLARLDLEK
Rat NF-L	(193-210)	EARKGADEAALARAELEK
Porcine 62 kd protein	(peptide K15)	MALDIEIAAYRK
Rat 66 kd α-internexin	(383-394)	MALDIEIAAYRK
Rat NF-L	(380-391)	MALDIEIAAYRK
Porcine 62 kd protein	(peptide K17)	SEMARHLREYQDLLNVK
Rat 66 kd α-internexin	(366-382)	SEMARHLREYQDLLNVK
Rat NF-L	(363-379)	SEMARYLKEYQDLLNVK

Fig. 2. The 62 kd protein or the 62 kd protein phosphorylated by the catalytic subunit of cAMP-dependent protein kinase was digested with lysylendopeptidase, as described under MATERIALS and METHODS. An aliquot of the reaction mixture was applied to HPLC on C18 reverse-phase column (ϕ 0.46 x 15 cm). A, The elution profile of the peptides. B, Amino acid sequences of four of the purified peptide fragments obtained were determined and compared with those of rat α-internexin and NF-L. Numbers in the parentheses of the rat α-internexin and NF-L indicate the amino acid positions from the amino termini.

These results indicate that α-internexin, like other IF proteins (except for NF-M and NF-H), can polymerize to 10nm filaments itself, in vitro.

Several in vitro and in vivo studies suggested that the assembly-disassembly of IFs is regulated by phosphorylation of their subunit proteins(4-15,20-27), hence we asked whether phosphorylation events

also regulate the assembly-disassembly of α-internexin. The time course of phosphorylation of α-internexin IFs by cAMP-dependent protein kinase is shown in Fig.3A . Approximately 2.6 mol of phosphate were incorporated into α-internexin present in the filaments. Interestingly, phosphorylation by cAMP-dependent protein kinase shifted the electrophoretic mobility of α-internexin upward (Fig.3Ba and 3Bb). Sedimentation analysis of the cAMP-dependent phosphorylation mixture after 60 min was a significant release of soluble α-internexin from α-internexin IFs (Fig.4A). Similar results were obtained using electron microscopy, and typical images are shown in Fig.4B .

To determine if a specific region of α-internexin was phosphorylated under conditions of disassembly, ^{32}P-labeled α-internexin was analyzed using the same procedure described for Fig.2A. As shown in Fig.2A ,use of the HPLC procedure led to separation of one major and one minor radioactive peaks (phosphopeptides K18 and K24). Since phosphopeptide K18 contained > 60% of the total radioactivity, we analyzed for this major peak. Amino acid composition

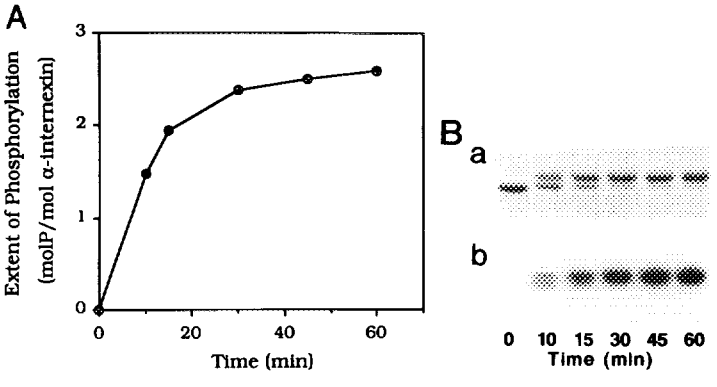


Fig. 3. A, Time course of phosphorylation of α-internexin by the catalytic subunit of cAMP-dependent protein kinase. α-internexin was phosphorylated by incubation with 3 μg/ml of the catalytic subunit of cAMP-dependent protein kinase, 0.1 mM [γ-^{32}P] ATP, 1 mM $MgCl_2$, 25mM Imidazole-HCl, pH 6.75 at 25 °C. B(a), SDS polyacrylamide electrophoresis; B(b), autoradiography.

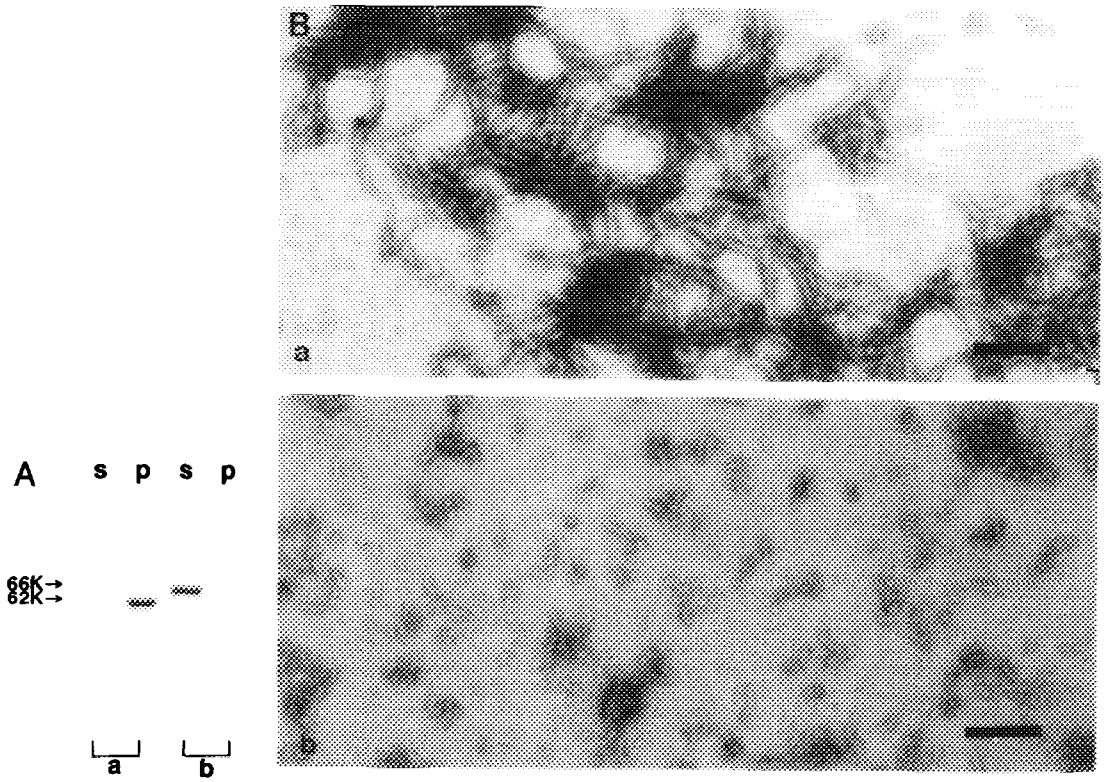


Fig. 4. α-Internexin IFs(a), and α-internexin IFs phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (b). A,SDS polyacrylamide electrophoresis.The samples were subjected to high speed centrifugation (15,000 x g) for 30 min, yielding a pellet (p) and a supernatant (s). The separating and stacking gel contained 10 and 3% acrylamide, respectively. B, Electron microscopy of negatively stained α-internexin IFs samples.Scale bars = 200 nm, magnification x 50,000.

of phosphopeptide K18 is listed in Table 1. By comparison with the sequence of rat α-internexin(17), the amino-terminal head domain is the location of the phosphopeptide along the α-internexin molecule.

We and other investigators reported that specific phosphorylation of head domain abolishes the potential of vimentin, desmin, GFAP. NF-L and keratin proteins to assemble(4-15, 20-27). In the present studies, we obtained similar evidence for α-internexin. These combined results suggest that the head domains of the IF proteins, although differing in sequence, are the common target of protein kinases which control polymerizability.

Table 1. Comparison of amino composition of rat α-internexin with the phosphopeptide K18

Residue	phosphopeptide K18 (%)	rat α-internexin (residue numbers 18-111) (%)
Ala	11.5	12.8
Val	3.9	3.2
Leu	11.8	11.7
Ile	2.7	3.2
Pro	1.7	2.1
Met	0.1	0
Phe	6.1	4.4
Gly	10.3	9.5
Ser	14.2	17.0
Thr	1.3	3.2
Cys	0.6	1.1
Tyr	2.0	2.1
Asx	9.0	8.5
Glx	12.6	8.5
Lys	3.6	3.2
Arg	8.6	9.5

Asx = Asn + Asp , Glx = Gln + Glu.

Acknowledgments: We are grateful to Drs. K. Tsujimura, T. Kiyono, T. Yano, and M. Ohara for advice throughout; A. Ishiyama for secretarial assistance. This research was supported in part by a Grant-in-aid for Scientific Research and a Grant for Cancer Research from the Ministry of Edication, Science and Culture of Japan, and special coordination funds from the Science and Technology Agency of the Government of Japan.

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