Volume 253, number 1,2, 55–58 FEB 07453 August 1989

Identification of Nsp100 as elongation factor 2 (EF-2)

Shinichi Koizumi, Alexey Ryazanov*, Tokiko Hama, Hao-Chia Chen+ and Gordon Guroff

Section on Growth Factors and +Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA and **Institute of Protein Research, Academy of Sciences of the USSR, 192292 Pushchino, Moscow Region, USSR*

Received 8 May 1989

The nerve growth factor-sensitive phosphoprotein from PC12 cells, previously designated Nsp100, has been shown to be elongation factor 2 (EF-2). The criteria used for this identification include: (i) similarity of N-terminal sequence; (ii) phosphorylation by the same kinase; (iii) ADP-ribosylation mediated by diphtheria toxin; (iv) comparable function in cell-free protein synthesis. According to these criteria, Nsp100 and EF-2 are identical and the kinase that phosphorylates Nsp100 in PC12 cells is calcium/calmodulin kinase III.

Nerve growth factor; Elongation factor 2; Ca^{2+}/calmodulin kinase III; (PC12 cell)

1. INTRODUCTION

Previous work from this laboratory has shown that the phosphorylation of a specific soluble protein in PC12 cells is decreased by treatment of the cells with nerve growth factor (NGF) [1]. This protein, designated Nsp100 (nerve growth factor-sensitive protein, 100 kDa), is phosphorylated in a cofactor-independent manner at pH 6.2 by a specific kinase; both kinase and substrate have been partially purified from PC12 cells [2]. Nsp100 and its kinase have been found in a variety of tissues [3], but the identity and function of Nsp100 has remained unknown.

The major substrate of calcium/calmodulin-dependent protein kinase III (Ca^{2+}/CaM kinase III) in several different tissues is a protein of 100 kDa [4]. Recently it was shown that the 100 kDa protein is elongation factor 2 (EF-2), and that the activity of the kinase phosphorylating it in PC12 cells is reduced by treatment of the cells with nerve growth factor.

In this report, we present evidence that the protein we have called Nsp100 is EF-2 and that the kinase we have called Nsp100 kinase is Ca^{2+}/CaM kinase III.

Correspondence address: G. Guroff, Section on Growth Factors, Building 6, Room 130, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA

Abbreviations: Nsp100, nerve growth factor-sensitive protein, 100 kDa; EF-2, elongation factor 2; NGF, nerve growth factor; Ca^{2+}/CaM kinase III, calcium/calmodulin-dependent protein kinase III; DMEM, Dulbecco's modified Eagle's medium; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol

2. MATERIALS AND METHODS

2.1. *Cell culture*

PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 7% fetal bovine serum, 7% horse serum, and 100 μg of streptomycin and 100 units of penicillin per ml (HEM Research Inc., Rockville, MD) at 37°C in a humidified atmosphere containing 6% CO_2. The cells were split in a 1:6 or 1:8 ratio each week and the medium changed once between splits.

2.2. *Preparation of Nsp100 and EF-2*

For the preparation of Nsp100 PC12 cells were collected by centrifugation, homogenized with 10 mM Tris-HCl, pH 7.4, containing 0.5 mM PMSF, 1 mM EGTA, and 1 mM DTT (buffer A). The supernatant fraction was obtained by centrifugation at 100000 × g for 60 min. Nsp100 was purified by sequential chromatography on DEAE Sephacel, Blue Sepharose, Mono Q, and Sephacryl S200. The fractions containing Nsp100 were identified by incubation with partially purified Nsp100 kinase,

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

followed by analysis of the phosphorylated products by SDS-polyacrylamide gel electrophoresis [7] and autoradiography. EF-2 was purified from rabbit reticulocytes as described by Shestakova and Ryazanov [8].

2.3. *Preparation of Nsp100 kinase*

Nsp100 kinase was purified from the supernatant fraction of PC12 cells by mono Q chromatography. The column was developed with a linear gradient of from 0 to 0.5 M NaCl in buffer A. The active fractions were eluted at about 0.45 M NaCl and were detected using Nsp100 as substrate.

2.4. *Phosphorylation of Nsp100/EF-2*

The phosphorylation of Nsp100 was done by incubating the protein in 50 mM Mes buffer, pH 6.2, containing 10 mM $MgCl_2$, 1 mM EGTA, 20 μM ATP, and 4 μCi of [γ-^{32}P]ATP. The phosphorylation of EF-2 was done by incubating the protein in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM EGTA, 10 μg/ml bovine brain calmodulin, 20 μM ATP, and 4 μCi of [γ-^{32}P]ATP. In both cases the reactions were terminated by the addition of SDS sample buffer and the samples heated in a boiling water bath for 3 min. The phosphorylated products were analyzed by SDS-polyacrylamide gel electrophoresis [7] and autoradiography.

2.5. *ADP-ribosylation*

Purified Nsp100 (3 μg) was incubated in 50 μl of 25 mM Tris-HCl, pH 8.0, containing 3 mM $MgCl_2$, 30 mM NH_4Cl, 1 mM EDTA, 5 mM DTT, 2 μg diphtheria toxin (List, Campbell, CA), and 6.8 μM [^{14}C]NAD (0.1 μCi). The incubation was carried out for 30 min at 37°C and terminated by the addition of SDS sample buffer [7]. The reaction products were analyzed on SDS-polyacrylamide gel electrophoresis followed by autoradiography.

2.6. *N-terminal sequencing*

The N-terminal sequence of Nsp100 was determined using a preparation purified as described by Togari and Guroff [2], followed by SDS-polyacrylamide gel electrophoresis. The Nsp100 band was cut from the gel, Nsp100 eluted, and the N-terminal sequence examined using an Applied Biosystems model 470A gas-phase amino acid sequencer with an on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems, Foster City, CA).

2.7. *Measurement of elongation factor activity*

Elongation factor 2-dependent polyphenylalanine synthesis was assayed in the poly(U)-directed cell-free translation system from rabbit reticulocytes according to the method of Ryazanov et al. [9].

3. RESULTS AND DISCUSSION

The sequence of the first 18 amino acid residues from the N-terminus of purified Nsp100 is: X-X-Phe-Thr-X-Asp-Gln-Ile-Arg-Ala-Ile-Met-Asp-Lys-Lys-Ala-Asn-Ile- where X indicates some uncertainty about the identity. The fifteen amino acids shown are identical with the N-terminal sequence of authentic rat EF-2, determined either by amino acid sequencing [10], or by deduction from the sequence of the cDNA [11]. The sequence is also identical to the sequence given for the 100 kDa substrate for Ca^{2+}/CaM kinase III [5].

Purified Nsp100 has the same migration on SDS-polyacrylamide gels as does EF-2 from rabbit reticulocytes (fig.1). In addition, both Nsp100 and EF-2 are phosphorylated at pH 6.2 in a cofactor-independent fashion by a fraction containing Nsp100 kinase, and both require the presence of calcium and calmodulin to be phosphorylated by the same fraction at pH 7.4 (fig.1). This experiment resolves the apparent conflict about the properties of the kinase phosphorylating Nsp100 [2], and the one phosphorylating the 100 kDa substrate from pancreas [4]. The data indicate that the requirement of Ca^{2+}/CaM kinase III for calcium and calmodulin depends on the pH at which the reaction is run.

EF-2 has been shown to be a substrate for diphtheria toxin-catalyzed ADP-ribosylation in mammalian cells [12]. Purified Nsp100 was found, also, to be ADP-ribosylated in the presence of diphtheria toxin (fig.2).

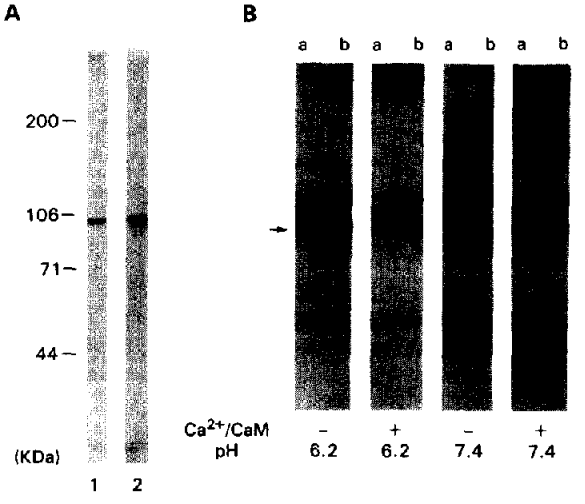

Fig.1. Comparison of the properties of Nsp100 and EF-2. (A) SDS-polyacrylamide gel electrophoresis of (1) Nsp100 from PC12 cells and (2) EF-2 from rabbit reticulocytes stained with Coomassie brilliant blue R-250. (B) Phosphorylation of (a) Nsp100 and (b) EF-2 by partially purified PC12 Nsp100 kinase at pH values 6.2 and 7.4 in the presence or absence of calcium and calmodulin. The arrow indicates the position of Nsp100.

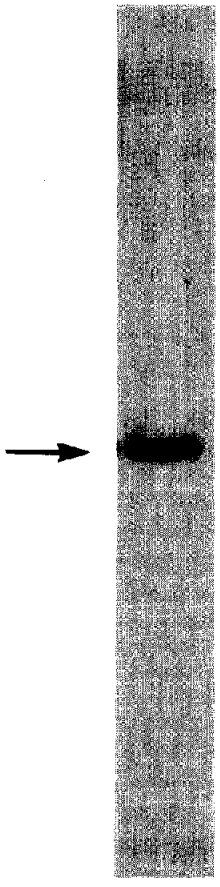

Fig.2. ADP-ribosylation of Nsp100 in the presence of diphtheria toxin. The products of the incubations were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Finally, purified Nsp100 had the same specific activity as did authentic EF-2 for the poly(U)-directed synthesis of polyphenylalanine by a cell-free preparation from rabbit reticulocytes (fig.3).

Thus, it is clear that Nsp100, EF-2, and the 100 kDa substrate for Ca^{2+}/CaM kinase III are the same protein and that Nsp100 kinase is, in fact, Ca^{2+}/CaM kinase III. The mechanism by which NGF and other agents regulate the action of Ca^{2+}/CaM kinase and, in turn, the phosphorylation of EF-2 is not yet clear. There are some reports implicating cAMP-dependent kinases in this regulation [6,13] and the involvement of protein kinase C has also been described [14]. Complete purification of Ca^{2+}/CaM kinase III has not yet been reported, so direct evidence for its phosphorylation has not yet been obtained.

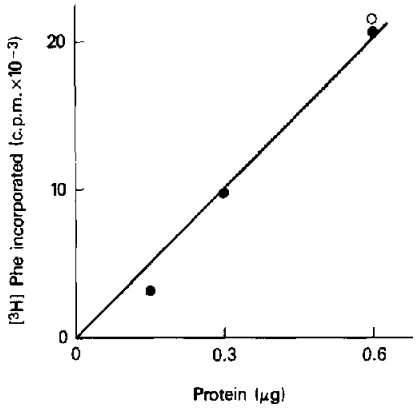

Fig.3. Poly(U)-directed polyphenylalanine synthesis in the presence of Nsp100 from PC12 cells (●) or of EF-2 from rabbit reticulocytes (○).

Definitive information on the regulation of this important enzyme must await the availability of the purified enzyme.

The finding that nerve growth factor decreases the phosphorylation, and, by implication, alters the function of Nsp100/EF-2 [1–3] provides the second example of a nerve growth factor-induced change in the protein synthetic mechanism; nerve growth factor has been shown to increase the phosphorylation of the ribosomal protein S6 [15,16]. The functional meaning of this latter change remains to be revealed, but the increasing involvement of nerve growth factor in these alterations in the components of the protein synthesis system suggests that nerve growth factor and PC12 cells could provide a very useful tool for studies on the regulation of protein synthesis.

Acknowledgement: The authors appreciate the help and advice of Dr David Neville.

REFERENCES

[1] End, D., Tolson, N., Hashimoto, S. and Guroff, G. (1980) J. Biol. Chem. 258, 6549–6555.
[2] Togari, A. and Guroff, G. (1985) J. Biol. Chem. 260, 3804–3811.
[3] Hama, T. and Guroff, G. (1985) J. Neurochem. 45, 1279–1287.
[4] Nairn, A.C., Bhagat, B. and Palfrey, H.C. (1985) Biochemistry 82, 7939–7943.
[5] Nairn, A.C. and Palfrey, H.C. (1987) J. Biol. Chem. 262, 17299–17303.

[6] Nairn, A.C., Nichols, R.A., Brady, M.J. and Palfrey, H.C. (1987) J. Biol. Chem. 262, 14265–14272.
[7] Laemmli, U.K. (1970) Nature 227, 680–685.
[8] Shestakova, E.A. and Ryazanov, A.G. (1987) Dokl. Akad. Nauk SSSR 297, 1495–1498.
[9] Ryazanov, A.G., Shestakova, E.A. and Natapov, P.G. (1988) Nature 334, 170–173.
[10] Takamatsu, K., Uchida, Y. and Okada, Y. (1986) Biochem. Biophys. Res. Commun. 134, 1015–1021.
[11] Kohno, K., Uchida, T., Ohkubo, H., Nakanishi, T., Fukui, T., Otsuka, E., Ikehara, M. and Okada, Y. (1986) Proc. Natl. Acad. Sci. USA 83, 4978–4982.
[12] Pappenheimer, A.M. (1977) Annu. Rev. Biochem. 46, 69–94.
[13] Sitikov, A.S., Simonenko, P.N., Shestakova, E.A., Ryazanov, A.G. and Ovchinikov, L.P. (1988) FEBS Lett. 228, 327–331.
[14] Hama, T., Huang, K.-P. and Guroff, G. (1986) Proc. Natl. Acad. Sci. USA 83, 2353–2357.
[15] Halegoua, S. and Patrick, J. (1980) Cell 22, 571–581.
[16] Matsuda, Y. and Guroff, G. (1987) J. Biol. Chem. 262, 2832–2844.