Vol. 198, No. 1, 1994
January 14, 1994

THE 100-kDa PROTEIN, WHOSE PHOSPHORYLATION PRECEDES THE FUSION OF CHICK EMBRYONIC MYOBLASTS, IS THE EUKARYOTIC ELONGATION FACTOR-2

Young Ju Jeon, Hye Seon Kim, Hye Sun Kim, Man-Sik Kang, Chin Ha Chung[1] and Doo Bong Ha

Department of Molecular Biology and SRC for Cell Differentiation, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea

Received November 25, 1993

SUMMARY: We have previously shown that Ca^{2+}/calmodulin-dependent phosphorylation of the 100-kDa protein dramatically increases during the early period of myoblast fusion and inhibition of the protein phosphorylation prevents the fusion. Here, we show that the protein phosphorylation occurs exclusively at Thr residue(s) and the purified 100-kDa protein can be ADP-ribosylated upon treatment with diphtheria toxin. Furthermore, the 13 N-terminal amino acid sequence of the 100-kDa protein, N-Val-Asn-Phe-Val-Asp-Gln-Ile-Arg-Ala-Ile-Met-Asp-Lys, exactly matches with that of elongation factor-2 from rat and hamster. These results indicate that the 100-kDa protein in chick embryonic myoblasts is identical to the eukaryotic elongation factor-2.

A prominent event in myogenesis is the membrane fusion of mononucleated myoblasts into multinucleated myotubes (1). A number of reports have suggested that protein phosphorylation plays an important role in mediating myoblast fusion (2,3). We have recently shown that the level of phosphorylation of the 100-kDa protein dramatically increases during early period of myoblast fusion and treatment of trifluoperazine (TFP), a calmodulin (CaM) antagonist (4,5), blocks both the protein phosphorylation and the membrane fusion (6). It has also been demonstrated that sphingosine, a potent inhibitor of protein kinase C (7), prevents phosphorylation of the 100-kDa protein and this inhibitory effect can be reversed upon treatment of CaM (8). In addition, sphingosine has been shown to also block myoblast fusion. Therefore, we have suggested that phosphorylation of the

[1]To whom correspondence should be addressed.

0006-291X/94 $5.00

100-kDa protein is mediated by a Ca^{2+}/CaM-dependent protein kinase and may provide a potential mechanism for regulating myoblast fusion (6,8). However, little is known about the biochemical feature of the 100-kDa protein and its role in myoblast fusion.

In the present study, we partially purified the 100-kDa protein from the extracts of chick embryonic breast muscle and unequivocally demonstrated that the protein is identical to the eukaryotic elongation factor-2 (EF-2).

METHODS

Purification of the 100-kDa protein: The breast muscle tissues obtained from 16-day-old chick embryos were homogenized in 50 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM EDTA and 10% (v/v) glycerol and centrifuged at 100,000 x g for 1 h. The supernatant fractions (2.5 g) were loaded on a DEAE-cellulose column (2.2 x 25 cm) equilibrated with the Tris buffer. The flow-through fractions containing the 100-kDa protein were collected and added with solid ammonium sulfate to 40-60% (w/v) saturation. The precipitated proteins were resuspended and dialyzed against the same buffer. The sample was then loaded on a hydroxylapatite column (2.2 x 12 cm) equilibrated with the Tris buffer containing 50 mM KH_2PO_4/K_2HPO_4 (pH 7.5). After washing the column, proteins were eluted by a linear gradient of 50 to 250 mM phosphate. The fractions containing the 100-kDa protein were pooled, dialyzed against the Tris buffer, and loaded on a heparin-agarose column (0.75 x 5 cm) equilibrated with the same buffer. Proteins were eluted by a linear gradient of 0 to 300 mM NaCl by collecting 0.7 ml fractions at a flow rate of 5 ml/h. The fractions containing the 100-kDa protein were kept at 4℃ for further studies.

Phosphoamino acid analysis: The partially purified 100-kDa protein was phosphorylated, electrophoresed in the presence of sodium dodecyl sulfate (SDS) (9), and transferred to polyvinylidene difluoride (PVDF) membranes. The bands corresponding to the 100-kDa protein were cut off and hydrolyzed in 6 N HCl for 1 h at 110℃ (10). The hydrolyzates were dissolved in the mixture of phosphoamino acid standards (i.e., 1 mg/ml solution of phospho-Thr, -Ser and -Tyr) and subjected to two dimensional thin-layer chromatography as described (11). The resulting plate was autoradiographed, and the standard amino acids were also visualized by staining with ninhydrin.

RESULTS AND DISCUSSION

In order to purify the 100-kDa protein, embryonic muscle tissues were dissected out from 200 chick embryos of 16-day-old. Purification of the 100-kDa protein from the tissues was carried out as described under Methods. Electrophoresis of the fractions obtained from the heparin-agarose column, which

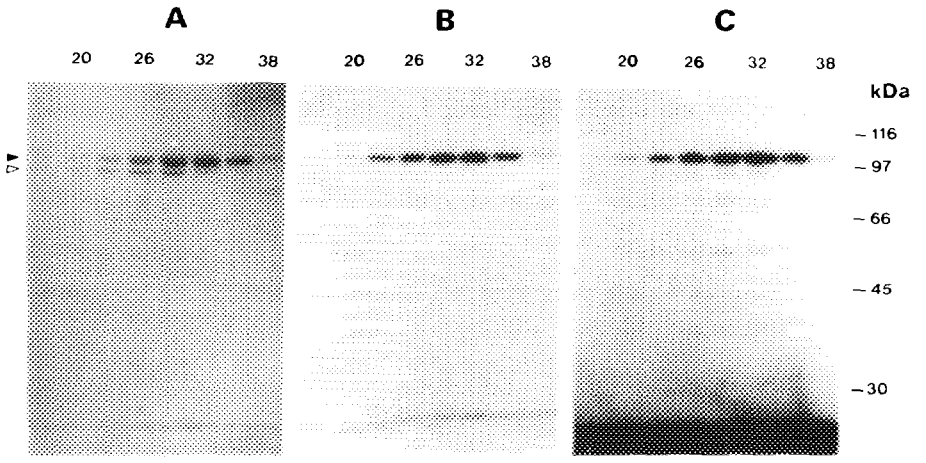

FIG. 1. Purification (A), phosphorylation (B), and ADP-ribosylation (C) of the 100-kDa protein in chick embryonic muscle. (A) Aliquots (30 μl each) of the fractions obtained from the heparin-agarose column were electrophoresed on a 10% (w/v) polyacrylamide slab gel in the presence of SDS. The gel was stained with Coomassie R-250. (B) Phosphorylation assays were performed by incubating 7 μl each of the same fractions with 1.5 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol) and the partially purified Ca^{2+}/calmodulin-dependent kinase for 10 min at room temperature. The protein kinase was obtained from the same extract used for the purification of the 100-kDa protein or from the extract of cultured myoblasts as described previously (6). (C) Aliquots (7 μl each) of the same fractions were also subjected to ADP-ribosylation by incubation with 1 μg of diphtheria toxin and 0.75 μCi of [^{32}P]NAD (800 Ci/mol) as described (14). The resulting samples were then electrophoresed as above and autoradiographed. The closed and open arrow-heads indicate the 100- and 92-kDa proteins, respectively.

was used as the final purification step, shows that the 100-kDa protein in most of the fractions were contaminated with a 92-kDa protein (Fig. 1A). Therefore, we examined whether the contaminating protein may interfere with the phosphorylation of the 100-kDa protein by the Ca^{2+}/CaM-dependent protein kinase, which was partially purified from the same tissue extract (data not shown) or from the extract of cultured myoblasts (6). Fig. 1B shows that the degree of the 100-kDa protein phosphorylation is almost proportional to the intensity of the corresponding Coomassie-stained bands of the 100-kDa protein (Fig. 1A). These results clearly show that the 92-kDa protein does not affect the phosphorylation reaction, and therefore no further attempt was made for complete purification of the 100-kDa protein.

Our previous studies (6) have suggested that the Ca^{2+}/CaM-dependent kinase from cultured myoblasts shares a number of similar properties with CaM-kinase III, including the fact that both use the 100-kDa protein as their major, if not

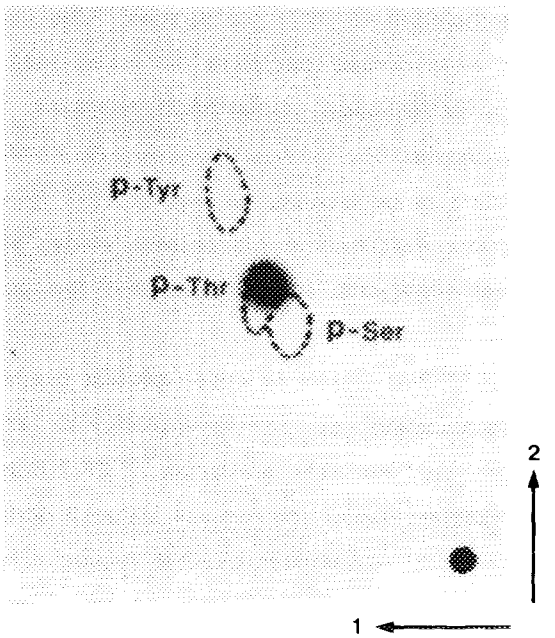

FIG. 2. Analysis of phosphoamino acids in the 100-kDa protein. Phosphoamino acids were identified as described in Methods. The dotted circles indicate the positions where the standard phosphoamino acids migrated on the thin-layer cellulose plate.

exclusive, substrate, unlike other CaM-dependent protein kinases (12,13). Because the 100-kDa protein substrate of CaM-kinase III is known to be same as the eukaryotic elongation factor-2 (EF-2) (14), we suspected if the 100-kDa protein in chick embryonic muscle may also be identical to EF-2. To test this possibility, aliquots of the same fractions from the heparin-agarose column were subjected to ADP-ribosylation. Fig. 1C shows that, upon treatment of diphtheria toxin, ADP-ribosylation of the 100-kDa protein occurred to extents proportional to the intensity of the Coomassie-stained bands (Fig. 1A). To clarify the possibility further, the 100-kDa protein from embryonic muscle was also subjected to the phosphoamino acid analysis. Fig. 2. shows that the protein is exclusively phosphorylated at its Thr residue(s). Nearly identical data were obtained when the analysis was performed with the partially purified 100-kDa protein from cultured myoblasts (data not shown) (6). These results strongly suggest that the 100-kDa protein in embryonic muscle cells and the eukaryotic EF-2 are identical proteins and that the Ca^{2+}/CaM-dependent kinase phosphorylating the 100-kDa protein may be same as CaM-kinase III (15).

To determine the N-terminal amino acid seqeunce, the partially purified 100-kDa protein obtained from heparin-agarose column was electrophoresed in the

presence of SDS and transferred to PVDF membranes. The bands corresponding to the 100-kDa protein were excised out from the membranes and subjected to Edman degradation using ProSequencer (model 6600, MilliGen). The 13 N-terminal amino acid seqeunce, N-Val-Asn-Phe-Val-Asp-Gln-Ile-Arg-Ala-Ile-Met-Asp-Lys, of the 100-kDa protein was found to be identical to that of EF-2 from rat and hamster (16). Thus, by all criteria described in the present study, it is clear that the 100-kDa protein in chick embryonic muscle cells and the eukaryotic EF-2 are identical proteins.

Phosphorylation of EF-2 is known to inhibit protein synthesis by preventing the EF-2-promoted translocation of aminoacyl-tRNA on ribosomes (17,18). Therefore, it is perplexing why the phosphorylation of EF-2 dramatically increases at the early period of myoblast fusion and remains elevated during the later period of myogenic differentiation (6), circumstances that require efficient synthesis of proteins, particularly of muscle specific proteins, such as myosin, α-actin and creatine kinase (19,20). By an analogy, EF-2 has been reported to be transiently phosphorylated in fibroblasts treated with serum growth factors (14), circumstances that also cause a stimulation of protein synthesis. However, it remains puzzling how the physiological role of EF-2 is related with myoblast fusion, particularly because inhibition of EF-2 phosphorylation impairs the myogenic process (6).

Acknowledgments: We are grateful to Dr. Young Mok Park for the analysis of the N-terminal sequence of the 100-kDa protein. This work was supported by grants from Korea Science and Engineering Foundation and the Ministry of Education.

REFERENCES

1. Bischoff, R., and Holtzer, H. (1969) J. Cell Biol. 41, 188-200.
2. Toutant, M., and Sobel, A. (1987) Dev. Biol. 124, 370-378.
3. Rapuano, M., Ross, A. F., and Prives, J. (1989) Dev. Biol. 134, 271-278.
4. Levine, R. M., and Weiss, B. (1978) Biochim. Biophys. Acta 540, 197-204.
5. Bar-Sagi, D., and Prives, J. (1983) J. Cell Biol. 97, 1375-1380.
6. Kim, H. S., Lee, I. H., Chung, C. H., Kang, M. S., and Ha, D. B. (1992) Dev. Biol. 150, 223-230.
7. Hannun, Y. A., Loomis, C. R., Merrill, A. H., and Bell, R. M. (1986) J. Biol. Chem. 261, 12604-12609.
8. Kim, H. S., Lee, I. H., Jeon, Y. J., Chung, C. H., and Ha, D. B. (1993) Exp. Cell Res. 205, 408-411.
9. Laemmli, U. K., (1970) Nature 227, 680-685.
10. Kamps, M. P. (1991) Methods Enzymol. 201, 21-27.

11. Duclos, B., Marcandier, S., and Cozzone, A.J. (1991) Methods Enzymol. 201, 10-21.
12. Nairn, A. C., Hemmings, H. C., and Greengard, P. (1985b) Annu. Rev. Biochem. 54, 931-976.
13. Hanson, P. I., and Schulman, H. (1992) Annu. Rev. Biochem. 61, 559-601.
14. Nairn, A. C. and Palfrey, H. C. (1987) J. Biol. Chem. 262, 17299-17303.
15. Nairn, A. C., Bhagat, B., and Palfrey, C. (1985a) Proc. Natl. Acad. Sci. USA 82, 7939-7943.
16. Kohno, K., Uchida, T., Ohkubo, H., Nakanishi, S., Nakanishi, T., Fukui, T., Ohtsuka, E., Ikehara, N., and Okada, Y. (1986) Proc. Natl. Acad. Sci. USA 83, 4978-4982.
17. Weissbach, H., and Ochoa, S. (1976) Annu. Rev. Biochem. 46, 191-216.
18. Ryazanov, A. G., Shestakova, E. A., and Natapov, P. G. (1988) Nature 334, 170-173.
19. Nadal-Ginard, B. (1978) Cell 15, 855-864.
20. Endo, T. and Nadal-Ginard, B. (1987) Cell 49, 515-526.