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In vivo and in vitro methylation of the elongation factor EF-Tu from *Euglena gracilis* chloroplast

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1. SUMMARY

Based on amino acid sequence similarities between the methylated elongation factor EF-Tu from *Escherichia coli* and the EF-Tu from *Euglena gracilis* chloroplast, we predicted that the latter could also be methylated in the presence of an appropriate methyltransferase. We found that, as reported for the eubacterial homologous protein, the organellar factor could be methylated in vivo and in vitro to yield monomethyllysine.

2. INTRODUCTION

The translational apparatus from chloroplasts shows many features in common with those of eubacteria (ref. 1, and references therein). Plastid ribosomes not only have a similar size and number of ribosomal proteins, but they are inhibited by several antibiotics specific against eubacterial ribosomes (summarized by Gillham, ref. 2). In addition, antibodies against the organellar ribosome showed immunological cross-reactivity with *Escherichia coli* ribosomes [3,4]. Furthermore, heterologous hybrids of *E. coli* and chloroplast 30S and 50S subunits are active in polyuridilate-directed polyphenylalanine synthesis [5,6]. The structure and organization of the ribosomal RNA genes from the chloroplasts are very similar to the eubacterial ones [1]. Moreover, translational factors from eubacteria and chloroplasts are essentially interchangeable in in vitro protein synthesizing systems [2,7].

The previous facts, and several other similarities between the plastidial and bacterial translational machineries have been considered a strong argument in favor of the independent eubacterial origin of plastids [1].

Based on DNA sequence analysis, it was recently found that the elongation factor EF-Tu from chloroplasts of *Euglena gracilis* shows 70% homology with the factor from *E. coli* [8]. The latter factor was found to be methylated on Lys 56 [9–11]. When comparing the amino acid sequence of 14 residues around the methylation site (Lys 56) in EF-Tu from *E. coli* with a similar sequence present in EF-Tu from *E. gracilis* chloroplasts (Fig. 1), one finds that 14 out of 15 amino acid residues are identical in both proteins. Stock et al. [12] have discussed criteria that could be used in

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Fig. 1. Comparison between the amino acid sequence of EF-Tu from *E. coli* containing the methylated lys-56 and the equivalent sequence present in the Ef-Tu from *E. gracilis* chloroplast. Sequence data were taken from references 10 and 8. The asterisk indicates the methylated lysine-56 residue in *E. coli*.

conjunction with primary sequence data to predict proteins that might be subject to methylation. In this case, if a methyltransferase is present in the chloroplast, the methylation of the organellar EF-Tu could be predicted. In this communication, we show that the elongation factor EF-Tu from *E. gracilis* chloroplast is in fact methylated both in vivo and in vitro.

3. MATERIALS AND METHODS

E. gracilis (var. *bacillaris*) was kindly supplied by G. Mora and J. Schiff. Cells were grown at 30°C in Hutner medium in the presence of light [13] and were harvested at the mid-logarithmic growth phase. Cell-free extracts from *E. gracilis* were obtained by ultrasonic disruption of the microorganisms. For immunoprecipitation assays [14,15], disruption of the cells was carried out in the presence of an immunoprecipitation buffer which contained 50 mM Tris-HCl, pH 7.4, 0.5 M NaCl and 1% Triton X-100. For in vitro assays, the cells were broken in the presence of 50 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$ and 5 mM 2-mercaptoethanol. Both kinds of cell homogenates were then centrifuged twice at $30000 \times g$ for 20 min at 4°C to give the S-30 cell-free extracts. Chloroplasts were isolated free of cytoplasmic components [16], and their extracts were obtained after sonic disruption of a suspension of the organelles in 50 mM Tris-HCl, pH 7.8, 50 mM NH_4Cl, 20 mM $MgCl_2$ and 5 mM 2-mercaptoethanol followed by two centrifugations at $14000 \times g$ for 20 min.

Specific antiserum against purified *E. coli* EF-Tu was raised in rabbits as before [14,15]. The chloroplast EF-Tu was isolated by immunoprecipitation of the *E. gracilis* cell-free extract with anti-EF-Tu from *E. coli* antiserum, followed by washings of the immunoprecipitates and SDS–PAGE analysis [14,15].

To determine the in vivo methylated products, *E. gracilis* cells which had reached the mid-logarithmic growth phase, were supplemented with 1 mCi of [*methyl*-^{3}H]methionine (85 Ci/mmol) to give a final concentration of 200 μCi/ml and the incubation of the cells was continued for 24 h. These cells were used for the preparation of extracts containing the radioactive methylated products. For the determination of the methylated amino acid residues present in the chloroplast EF-Tu labeled in vivo, the protein was immunoprecipitated from the radioactively labeled cell-free extracts, separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and after extracting it from the gel, the protein was hydrolyzed for 24 h in 5.7 N HCl. The radioactively labeled methylated amino acids were then separated by either high voltage electrophoresis or paper chromatography as previously described [14,15,17,18]. The in vitro methylation of Ef-Tu from chloroplasts was essentially carried out as described before [14,15,17,19], in a final volume of 100 μl containing 50 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$ and 5 mM 2-mercaptoethanol, 200 to 600 μg of *E. gracilis* cell-free extract protein or 160 μg of chloroplast extracted protein and 20 μCi of S-Ado-[*methyl*-^{3}H]methionine (85 Ci/mmol). Incubations were carried out for 25 min at 37°C and the methylated products were analyzed by SDS–PAGE, directly or after immunoprecipitation as described above.

4. RESULTS AND DISCUSSION

Fig. 2 shows the results obtained when a cell-free extract from *E. gracilis* grown in the presence of [*methyl*-^{3}H]methionine was immunoprecipitated with anti-EF-Tu from *E. coli* and the products were separated by electrophoresis in a 10% polyacrylamide–SDS gel. There were two main radioactively labeled immunoprecipitated protein

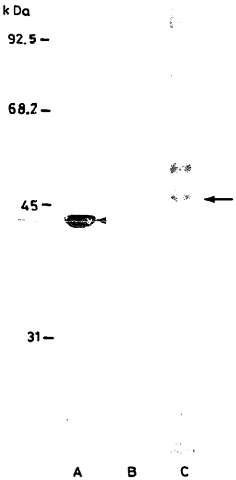

Fig. 2. Immunoprecipitation of EF-Tu from chloroplasts. After harvesting *E. gracilis* cells grown in the presence of [*methyl*-^{3}H]methionine (200 μCi/ml) a cell-free extract was prepared and it was reacted either with a preimmune antiserum (B) or with an anti-EF-Tu from *E. coli* (C). The products were then separated by 10% polyacrylamide SDS–PAGE and detected by fluorography. Chemically tritiated EF-Tu from *E. coli* [14,15] was used as standard (arrowhead in A). The arrow in C indicates the immunoprecipitated protein band taken for further analysis. Numbers at left indicate molecular mass in thousands.

bands, one of them having an apparent molecular mass of 46 000 (arrow). This value is close to the M_r of 45 011 for the chloroplast EF-Tu, estimated from the DNA sequence of the corresponding gene [8]. The second major immunoprecipitated band, with an apparent M_r of 54 000, would correspond to the EF-1a from *E. gracilis*, since there is evidence indicating a certain degree of homology between the bacterial EF-Tu and the eukaryotic one [20–22]. In addition, the size of EF-1a from different species is around 53 000 [20,23].

The protein band of 46 kDa was eluted out of the gel and then subjected to acid hydrolysis and high voltage paper electrophoresis to determine the presence of methylated amino acid residues. As shown in Fig. 3a, a radioactive peak comigrating with methylated lysine was observed. Since all forms of methylated lysine migrate together under these conditions, the different methylated in the hydrolysate were also analyzed by paper chromatography [17,18] as shown in Fig. 3b. The result clearly indicate the presence of monomethyllysine in the chloroplastic EF-Tu molecule. Therefore, it is possible that the EF-Tu from chloroplasts of *E. gracilis* is methylated in vivo in a fashion similar to that of *E. coli* EF-Tu. However, it will be necessary to confirm the site(s) of methylation within the organellar EF-Tu by sequencing the protein. Experiments in this direction are currently being done.

On the other hand, the second immunoprecipitated band was also found to be methylated, but contained trimethyllysine instead (results not shown). The latter result supports the idea that this protein may correspond to EF-1a from the protozoan, since the EF-1a from several organisms mainly contains trimethyllysine [20,22,24,25].

To confirm the methylation of the chloroplast EF-Tu and the presence of a methyltransferase we employed an in vitro methylation system in which cell-free extracts or chloroplastic extracts were incubated in the presence of S-Ado-[*methyl*-^{3}H]methionine, and the methylated products were detected either directly or after immunoprecipitation by SDS–PAGE. In general the in vitro N-methylating systems have rather low efficiencies, since the methyltransferases substrates are already methylated in the cell. The incorporation of methyl groups is greatly increased when undermethylated substrates such as those found in cells grown in the presence of ethionine are used [15]. Nevertheless, in the present studies, the different extracts prepared had comparable efficiencies which were sufficient for a qualitative estimation, as shown in Fig. 4. When an S-30 supernatant extract from mid-logarithmic phase grown *E. gracilis* was used, there was a 46 000 band (Fig. 4A, arrowhead) and other methylated products present in the extract. Some of these modified proteins could be ribo-

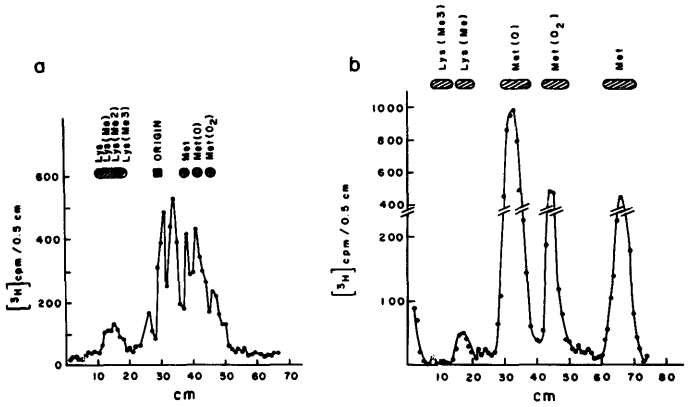

Fig. 3. Analysis of the methylated amino acids present in the in vivo methylated EF-Tu from *E. gracilis* chloroplasts. Five immunoprecipitated bands such as the one indicated by the arrow in Fig. 2C were extracted from a similar slab gel and the protein was eluted and hydrolyzed. 100 μl aliquots of the hydrolysate were mixed with 10 μg of each of the following indicated standards; Lys; monomethyllysine, Lys(Me); trimethyllysine, Lys(Me)3; Met; methionine sulfoxide, Met(O) and methionine sulfone, Met(O_2) and were separated by (a) high voltage paper electrophoresis (anode was to the right) or by (b) descending paper chromatography.

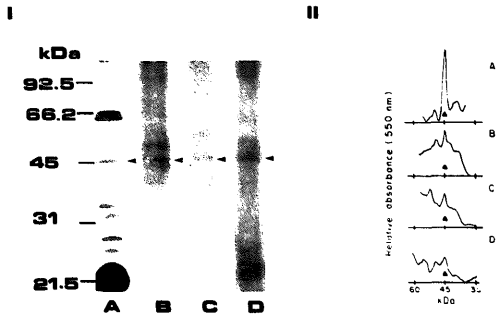

Fig. 4. In vitro methylation of chloroplast EF-Tu. (I) The *E. gracilis* in vitro methylated products obtained in the presence of S-Ado-[*methyl*-^{3}H]methionine were immunoprecipitated with anti-EF-Tu from *E. coli* (B, D) previous to 10% polyacrylamide SDS-PAGE or were directly analyzed (A, C) by electrophoresis followed by fluorography, (II) Densitometric analysis of the X-ray film in the region where chloroplast EF-Tu migrates (arrowheads). A,B, S-30 cell-free extract; C,D chloroplast extract. Numbers to the left indicate molecular mass in thousands.

somal proteins [26] or some other organellar proteins which are also known to be methylated [27]. When the methylated products were immunoprecipitated from the extract with anti-EF-Tu from *E. coli*, there was a major band with a molecular mass of 46000 (Fig. 4B). This result not only confirms the data obtained in vivo but shows the presence of a methyltransferase activity in the *E. gracilis* cell-free extracts.

Since a total cell-free extract from *E. gracilis* might contain mitochondrial or cytoplasmic methylated products, purified chloroplasts free of cytoplasmic components [16] were methylated in vitro, and again a 46000 M_r, methylated protein band was obtained (Fig. 4C). As expected, this protein was also recognized by the eubacterial anti-FF-Tu (Fig. 4D). Therefore, our results not only suggest that the chloroplast EF-Tu methyltransferase activity is present in the organelle but, as it occurs with the eubacterial EF-Tu, the chloroplast factor is methylated both in vivo and in vitro. When chloroplast extracts were immunoprecipitated with *E. coli* anti-EF-Tu serum, the presence of a higher molecular weight methylated band was also seen (Fig. 4D). At present, we have no explanation for this modified product. Since *Euglena gracilis* chloroplast EF-Tu is synthesized inside the organelle [8], it is not likely to be an EF-Tu precursor molecule.

Bacterial EF-Tu and the eukaryotic equivalent EF1-a have been described to be methylated in several species [7,9,20,21]. Previous data along with our finding suggest that modifications of the translational apparatus, such as methylation are conserved. In this regard, we previously found that methylation of the 50S ribosomal proteins was conserved within several eubacteria [17,26,28], showing a typical "eubacterial methylation pattern" which also appears to be present in the chloroplast ribosome (Sanhueza, Amaro and Jerez, unpublished results).

The apparent conservation and the ubiquitous nature of this chemical modification suggest that it may have an important biological role. In this regard, it has been recently found that the methylation of the elongation factor EF-Ru from *E. coli* affects the rate of trypsin degradation [29] and the tRNA-dependent GTP hydrolysis [11,29]. In addition, the methylated form of EF1-a from *Mucor racemosus* has also been shown to have a greatly increased activity in protein synthesis [25].

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REFERENCES

[1] Gray, M.W. and Doolittle, W.F. (1982) Microbiol. Rev. 46, 1–42.
[2] Gillham, N.W. (1978) Organelle Heredity, Raven Press, New York.
[3] Bartsch, M., Kimura, M. and Subramanian, A. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 6871–6875.
[4] Schneeman, R. and Surzycki, S. (1979) Mol. Gen. Genet. 176, 95–104.
[5] Grivell, L.A. and Walg, H.L. (1972) Biochem. Biophys. Res. Commun. 49, 1452–1458.
[6] Lee, S.G. and Evans, W.R. (1971) Science 173, 241–242.
[7] Ciferri, O. (1978) Trends Biochem. Sci 3, 256–261.
[8] Montandon, P.E. and Stutz, E. (1983) Nucleic Acids Res. 11, 5877–5892.
[9] Ames, G.F.L. and Niakido, K. (1979) J. Biol. Chem. 254, 9947–9950.
[10] Laursen, R.A., L'Italien, J.L., Nagarkatti, S. and Miller, D. (1981) J. Biol. Chem. 256, 8102–8109.
[11] Van Noort, J.M., Kraal, B., Sinjorgo, K.M.C., Persoon, N.L.M., Johanns, E.S.D. and Bosch, L. (1986) Eur. J. Biochem. 160, 557–561.
[12] Stock, A., Clarke, S., Clarke, C. and Stock, J. (1987) FEBS Lett. 220, 8–14.
[13] Schiff, J.A., Lyman, H. and Russell, G.K. (1971) Methods Enzymol. 23, 143–163.
[14] Jerez, C. and Weissbach, H. (1980) J. Biol. Chem. 255, 8706–8710.
[15] Toledo, H. and Jerez, C.A. (1985) FEBS Lett. 193, 17–21.
[16] Avadhani, N.G. and Buetow, D.E. (1972) Biochem. J. 128, 353–365.
[17] Amaro, A.M. and Jerez, C.A. (1984) J. Bacteriol. 158, 84–93.
[18] Chang, C.N. and Chang, F.N. (1975) Biochemistry 14, 468–477.
[19] Chang, C.N. and Chang, F.N. (1974) Nature 251, 731–733.
[20] Amons, R., Pluijms, W., Roolbol, K. and Moller, W. (1983) FEBS Lett. 153, 37–42.
[21] Crechet, J-B., Canceill, D., Bocchini, V. and Parmeggiani, A. (1986) Eur. J. Biochem. 161, 635–645.

[22] Van Hemert, F.J., Amons, R., Pluijms, J.M., van Ormondt, H. and Moller, W. (1984) EMBO J. 3, 1109–1113.
[23] Linz, J.E., Katayama, C. and Sypherd, P. (1986) Mol. Cell. Biol. 6, 593–600.
[24] Coppard, N.J., Clark, B.F.C. and Cramer, F. (1983) FEBS Lett. 164, 330–334.
[25] Fonzi, W.A., Katayama, C., Laethers, T and Sypherd, P.S. (1985) Mol. Cell. Biol. 5, 1100–1103.
[26] Toledo, H., Amaro, A.M., Sanhueza, S. and Jerez, C.A. (1988) Arch. Biol. Med. Exp. 21, 219–229.
[27] Black, M.T., Meyer, D., Widger, W.R. and Creamer, W.A. (1987) J. Biol. Chem. 262, 9803–9807.
[28] Mardones, E., Amaro, A.M. and Jerez, C.A. (1980) J. Bacteriol. 142, 355–358.
[29] Toledo, H. and Jerez, C.A. (1989) FEBS Lett. 252, 37–41.