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Reduced levels of ADP-ribosylatable elongation factor-2 in aged and SV40-transformed human cell cultures

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The elongation step is involved in the regulation of protein synthesis during the cell cycle, environmental stress, ageing and transformation. Using a diphtheria toxin-mediated assay for measuring the levels of ADP-ribosylatable elongation factor EF-2, we have observed an irreversible decrease of up to 64% in the amount of ADP-ribosylatable EF-2 in normal diploid human fibroblasts MRC-5 undergoing ageing in vitro. However, a similar decrease in low serum-associated G_0/G_1-arrested cells is reversible both in MRC-5 cells and in their SV40-transformed counterparts. Reduced levels of ADP-ribosylatable EF-2 could account for the slowing-down of protein synthesis during cell cycle arrest and during cellular ageing in culture.

Elongation factor-2; ADP-ribosylation; Diphtheria toxin; Protein synthesis; Ageing; Transformation

1. INTRODUCTION

A change in the rate of protein synthesis can have wide-ranging effects including those on gene expression, on cell physiology and on cell proliferation [1]. However, our understanding of the regulation of protein synthesis in eukaryotic cells is still very limited. One of the main reasons for this is the lack of detailed studies on quantitative and qualitative changes in the components of the protein synthesis machinery during various biological events, such as cell cycle, growth, development, ageing and carcinogenesis.

Although the role of the initiation step in the regulation of protein synthesis is widely recognised as crucial [1], several recent studies have indicated that the elongation step may also be important in this regard. For example, the regulation of protein synthesis at the level of elongation has been demonstrated during the cell cycle [1], heat shock [2], phorbol ester treatment [3], growth factor stimulation [4], transformation [5] and ageing [6,7]. However, changes in the amounts and activities of various elongation factors (EFs) involved in this regulation remain to be documented. Therefore, we have undertaken such studies in order to elucidate changes in EFs during serial passaging of normal and transformed human cells in culture. Previously, we have shown that the activity and amounts of active EF-1α, an enzyme which catalyses the binding of aminoacyl-tRNA to the A site of the ribosome, undergo cell cycle- and age-related changes in normal human fibroblasts and in SV40-transformed cells derived from them [7].

Here, we report changes in the amounts of ADP-ribosylatable EF-2 in serially passaged human fibroblasts undergoing the typical Hayflick aging process in culture [8], and in SV40-transformed immortal cells derived from them. EF-2 is the other elongation factor which catalyses the translocation of peptidyl-tRNA from the A site to the P site of the ribosome [1]. For this purpose, we have used the diphtheria toxin-mediated 1:1 stoichiometric ADP-ribosylation assay in cell-free extracts in which ADP-ribosylation of the diphtamide residue of EF-2 results in the complete inhibition of its activity [9]. Details regarding the sensitivity, accuracy and advantages of this assay have been published elsewhere [10–12].

2. MATERIALS AND METHODS

Normal human embryonic diploid lung fibroblasts, MRC-5, and SV40-transformed cells, MRC-5V2, derived from them were used in this study. Cells were cultured routinely in Dulbecco's modified Eagle's minimum essential medium (DMEM, Biochrom, FRG) containing 10% fetal calf serum and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin), at 37°C in an atmosphere of 5% CO_2. In vitro lifespan of MRC-5 cells was estimated by calculating the cumulative population doubling level (PDL) attained on 1:2 serial passaging, as described before [7]. In this series of experiments, MRC-5 cells reached a final PDL 49 during serial passaging, which is here considered as 100% lifespan. As described by Hayflick [8], cultures were considered to be in Phase II until slowing-down of their growth during the last few passages (Phase III) when they leave the cycle irreversibly. No such change in growth characteristics, however, is observed in serially passaged MRC-5V2 cells which continue to proliferate indefinitely without showing any signs of ageing.

For assaying EF-2 in different phases of the growth cycle, cultures were considered to be in an exponential phase of growth 20 h after

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seeding. At this stage, 80–90% of Phase II MRC-5 cells, less than 20% of Phase III MRC-5 cells, and more than 95% of MRC- 5V2 cells are capable of entering S phaser of the cell cycle as determined by [^{3}H]thymidine labelling and autoradiography [7]. To arrest the cells in G_0/G_1 phase of the cell cycle, normal medium was replaced with DMEM containing 0.2% serum for MRC-5 cells and no serum for MRC-V2 cells for 72 h. Under these conditions, more than 98% of both cell types became growth-arrested, as checked by [^{3}H]thymidine labelling and autoradiography.

Methods for both the preparation of cell-free extracts and the estimation of ADP-ribosylatable ADP-ribosylatable EF-2 have been described previously [10, 11]. Briefly, triplicate samples of about 6×10^6 cells each were sonicated in 0.5 ml buffer T (25 mM Tris/HCl pH 8, 25 mM KCl, 0.5 M NH_4Cl, 10 mM $Mg(CH_3COO)_2$, 10 mM dithiothreitol, and 3 mM benzamidine) containing 4% (w/v) activated charcoal, using a Branson Sonifier Cell Disrupter B15 in mode 4, pulse 20% for 20 seconds. The homogenate was shaken vigorously in charcoal for 5 min, in order to remove endogenous NAD. Charcoal was removed by centrifugation (15 000 × g, 20 min) and the supernatant was centrifuged again (27 000 g, 15 min) to remove cellular debris and to ensure the removal of all charcoal. All operations were performed at 0–4°C.

The diphtheria toxin-mediated EF-2 assay was performed in a final volume of 50 μl in buffer T containing 2Lf (5 μg) of diphtheria toxin (a kind gift from Dr. Iver Heron, Statens Seruminstitut, Copenhagen). Histamine was added to give a final concentration of 0.18 M, in order to inhibit the cellular ADP-ribosyltransferases, together with 1.2 mM [^{14}C]NAD. The reaction was started by adding the cell-free extract and the assay mixture was incubated for 20 min at 37°C. The reaction was stopped by adding 1 ml 10% ice-cold TCA, and the acid insoluble radioactivity was determined using a Beckmann LS7500 scintillation counter. The total amounts of soluble protein were determined by a modified Bradford method using 3% perchloric acid and BSA as the standard [13].

3. RESULTS

The method used in this study measures the total amount of ADP-ribosylatable EF-2. Previously, we have demonstrated a linear relationship between amounts of either purified EF-2 or cell-free extracts and the estimated amount of ADP-ribosylatable EF-2 [11,12]. Since the ADP-ribosylation of the diphthamide residue of EF-2 completely abolishes its activity, estimating the amount of ADP-ribosylatable EF-2 gives an indirect measure of the amount of active EF-2 in cell-free extracts.

There is a decline in the amount of ADP-ribosylatable EF-2 in serially passaged senescent MRC-5 cells (Fig. 1). Exponentially growing MRC-5 cultures of up to 75% lifespan completed contained on avarage 48.3 fmol EF-2/μg protein. Invididual values for three separate observations at each time-point in lifespan are given in Fig. 1. There was an abrupt decline in the amount of ADP-ribosylatable EF-2 within the next few passages and an average value of 18.3 fmol EF-2/μg protein was reached in Phase III senescent cultures with more than 80% lifespan completed (Fig. 1). No further change in the amount of ADP-ribosylatable EF-2 could be observed during the rest of the lifespan of MRC-5 cells in culture. In comparison, exponentially growing SV40-transformed immortal MRC-5V2 cells contained

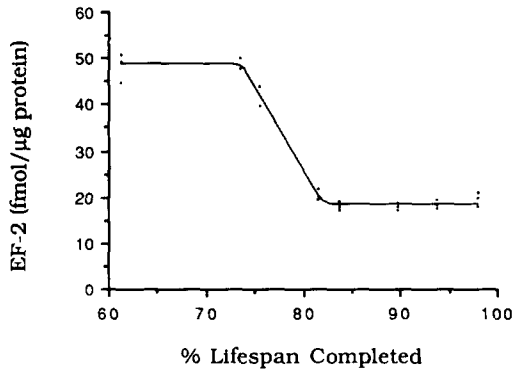

Fig. 1. Age-related decline in the amount of ADP-ribosylatable EF-2 (fmol/μg protein) in serially passaged cultures of human fibroblasts, MRC-5. Values given on each time-point are of three separate observations. 100% lifespan = PDL 49.

on average 23.8 fmol EF-2/μg protein, which did not change during serial passaging.

In order to distinguish age-related changes from those due to cell cycle, amounts of ADP-ribosylatable EF-2 during G_0/G_1-arrest and after restimulation with fresh medium containing 10% serum were also determined. For this purpose, Phase II young and Phase III senescent cultures of MRC-5 cells were compared with immortal MRC-5V2 cells, after serum starvation and restimulation. A significant reduction in the amount of ADP-ribosylatable EF-2 was observed in G_0/G_1-arrested both for Phase II MRC-5 cells (from 50.2 fmol to 28.2 fmol) and for immortal MRC-5V2 cells (from 23.8 fmol to 11.9 fmol; Fig. 2). After restimulation, this change was largely reversed within 20 h (almost 80% recovery in MRC-5 cells and 100% recovery in MRC-5V2 cells). In the case of senescent Phase III MRC-5 cells, which already have irreversibly reduced amounts of ADP-ribosylatable EF-2 due to ageing (average 18.3 fmol EF-2; respective values of three separate observations were 17.4, 18.3 and 19.4 fmol), serum starvation resulted in a further decrease of about 40% reaching an average value of 10.9 fmol EF-2/μg

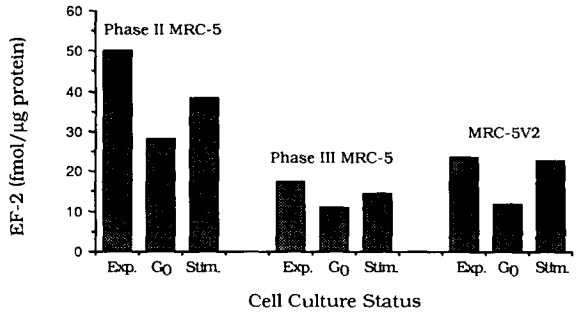

Fig. 2. Average amounts of ADP-ribosylatable EF-2 (fmol/μg protein) in exponentially growing (Exp.), in low serum-associated G_0/G_1-arrested (G_0), and in 20 h restimulated (Stim.) cultures of Phase II (72% lifespan completed), Phase III MRC-5 cells (92% lifespan completed), and SV40-transformed MRC-5V2 cells.

protein (individual values for three separate observations were 10.1, 10.9 and 11.5 fmol). However, this cell cycle-related decrease in senescent cells was reversible after restimulation with serum, although at a much slower pace (Fig. 2).

4. DISCUSSION

Cell cycle- and age-related changes in the amounts of ADP-ribosylatable EF-2 reported in this study fill an important gap in our knowledge of the regulation of protein synthesis during these processes. Our results show that the amounts of ADP-ribosylatable EF-2 are reduced significantly during cell cycle-related quiescence and during ageing, two of the conditions in which protein synthetic rates have been also observed to become low [14,15].

The age-related decline in amounts of ADP-ribosylatable EF-2 in MRC-5 cells is not attributable simple to the fact that the majority of cells in Phase III are non-dividing. This is because even though experimentally induced G_0/G_1-arrest reduces the amounts of ADP-ribosylatable EF-2, this change is reversible both in Phase II cells and in immortal cells when normal serum concentration is restored. On the other hand, Phase III MRC-5 cells with already reduced amounts of ADP-ribosylatable EF-2, show a further reduction due to low serum, which is also reversible, although at a much slower pace. Similar slow reversion of a cell cycle-related decline in senescent MRC-5 cells has been observed in the case of EF-1α [7]. This difference in the rate of serum-stimulated recovery in young and old cells is not surprising, because it is well known that there is an age-related decline in the responsiveness of old cells to treatment with serum and various growth factors [16].

Like many other cellular, biochemical and physiological changes observed in late passage cultures of diploid cells [7], the decline in the amount of ADP-ribosylatable EF-2 also became apparent only after the cells had completed about 75% of their proliferative lifespan *in vitro*. Therefore, although we were unable to obtain much younger MRC-5 cells of less than 60% lifespan, due to the limited availability of stocks, it does not affect the conclusions derived from the present studies. Thus the reduced amounts of ADP-ribosylatable EF-2 in late passage senescent MRC-5 cells are related to their intrinsic ageing process and could well be involved in determining rates of protein synthesis in old cells [6, 12, 15]. Transformed MRC-5V2 cells, however, differ from normal MRC-5 cells in respect of the amount of ADP-ribosylatable EF-2 present in them. Since our assay system estimates only the amount of ADP-ribosylatable EF-2, it is not clear whether the absolute amounts of EF-2 also vary between normal and transformed cells. Our studies are in progress to resolve these issues by using antibodies against ADP-ribosylated and unribosylated forms of EF-2. Furthermore, at present it is also not clear if the specific activities of EF-2 vary significantly between normal and transformed cells, which may account for the differences in their rates of protein synthesis [7, 10]. Our studies are in progress in this regard.

The next step in the investigation of the role of EF-2 during ageing will be determining the levels of mRNA and the total content of EF-2, using cDNA probes and antibodies, respectively. It also needs to be shown whether there is any change in the endogenously ADP-ribosylated pool of EF-2 during the cell cycle, transformation and ageing. Furthermore, reports of heterogeneity of native forms of phosphorylated and ADP-ribosylated EF-2 in rat liver [17], and of changes in the relative proportion of phosphorylated and unphosphorylated variants of EF-2 during the human cell cycle [18] open up new lines of investigation of the role of EF-2 in the regulation of protein synthesis.

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