Coordinated synthesis of the nuclear protein cyclin and DNA in serum-stimulated quiescent 3T3 cells

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Quantitative two-dimensional gel electrophoretic analysis (IEF) of the nuclear polypeptide cyclin together with autoradiographic studies have revealed a coordinate synthesis of cyclin and DNA after serum stimulation of quiescent 3T3 cells. These results strengthen the notion that cyclin may be a central component of the pathway(s) that regulate cell proliferation.

Transformation-sensitive protein *Quiescent cell* *DNA synthesis*
Two-dimensional gel electrophoresis

1. INTRODUCTION

The identification of proteins that are preferentially synthesised in proliferating cells is essential for understanding the mechanisms underlying growth regulation and cellular transformation. The transformation-sensitive nuclear protein, cyclin (M_r 36000), whose synthesis correlates directly with the proliferative state of cells has been identified in several cell types of human, mouse and hamster origin [1–5]. This protein is present in very small amounts in normal non-dividing cells or tissues, and is synthesised by normal proliferating as well as transformed cells and tumours [1,2,4–10]. The levels of cyclin fluctuate during the cell cycle with a clear increase in the S-phase [11]. It has been shown that cyclin and the proliferating cell nuclear antigen (PCNA) [11–14] are the same protein (Mathews et al., submitted).

Previous studies suggested that the activity of cyclin may be associated with events related to DNA replication [10,11]. It seemed important, therefore, to search for a possible correlation between the synthesis of this protein and DNA. For this purpose, I present here a detailed study of the synthesis of cyclin and DNA following serum stimulation of quiescent 3T3 cells.

Abbreviation: IEF, isoelectric focussing

2. MATERIALS AND METHODS

2.1. Cells

Mouse 3T3 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics (penicillin, 100 units/ml; streptomycin 50 μg/ml).

2.2. DNA synthesis assay

To prepare cells for autoradiography, approx. 3.5×10^5 cells were plated in 35-mm culture dishes containing 3 ml of medium supplemented with 10% fetal calf serum. Once the culture reached confluency the medium was replaced by DMEM containing 0.5% serum. The cells were then left for 2–3 days at 37°C before use. Cells were stimulated by adding DMEM supplemented with 20% serum and samples labeled at 2-h intervals for 2 h with 1 μCi/ml [^{3}H]thymidine. They were processed for autoradiogaphy according to standard procedures. About 500 nuclei were counted in each case. All experiments were performed in duplicate.

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2.3. Labelling of cells with [^{35}S]methionine

Cells were grown to confluency in 0.3-cm^2 microtitre plates with 0.2 ml medium supplemented with 10% fetal calf serum. The medium was then changed to DMEM containing 0.5% serum and the cells used 2–3 days later. Cells were stimulated by adding DMEM with 20% serum. Labelling of the cells was carried out for 2 h at different periods after stimulation in 100 μl of medium supplemented with 20% dialysed serum and 100 μCi [^{35}S]methionine (Amersham SJ204, England) as in [15].

2.4. Two-dimensional gel electrophoresis

Two-dimensional electrophoretic analysis was performed as in [15–17]. Briefly, the first dimension separations were performed on 230 × 1.2 mm, 4% (w/v) polyacrylamide gels containing 2% ampholytes (1.6%, pH 5–7; 0.4%, pH 3.5–10) at 1200 V for 20 h. The second dimension separates were carried out in a 15% acrylamide gel (25 × 25 cm) and run at room temperature overnight. Gels were processed for fluorography, dried and exposed at −70°C [18]. Approx. 10^6 trichloroacetic acid-precipitable cpm were routinely applied per gel. Quantitation of the radioactive cyclin was done as in [15].

One-dimensional peptide mapping was performed as in [19,20].

3. RESULTS AND DISCUSSION

A two-dimensional gel analysis of the acidic (IEF [16]) [^{35}S]methionine-labelled polypeptides of asynchronous mouse 3T3 cells is shown in fig.1. The positions of the nuclear acidic polypeptide cyclin [2], vimentin, tropomyosin polypeptide IEF 52 [1,20], total actin and α- and β-tubulins are indicated. The amount of labelled cyclin in proliferating 3T3 cells corresponds to 0.1% of the total radioactively labelled protein [7]. The identi-

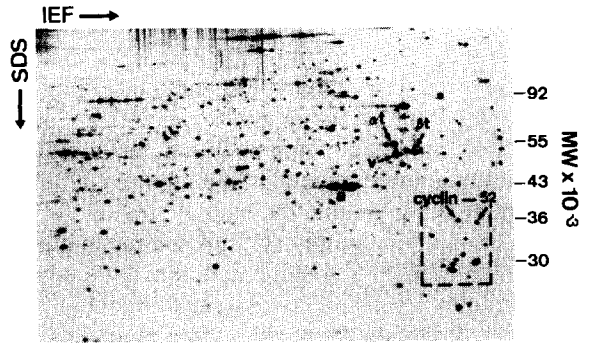

Fig.1. Two-dimensional map (IEF) of [^{35}S]methionine-labelled polypeptides from asynchronous 3T3 cells. Cells were labelled for 16 h with [^{35}S]methionine (1 mCi/ml) as in [15]. a, actin; αt, α-tubulin; βt, β-tubulin; v, vimentin. The area of interest is enclosed in a box.

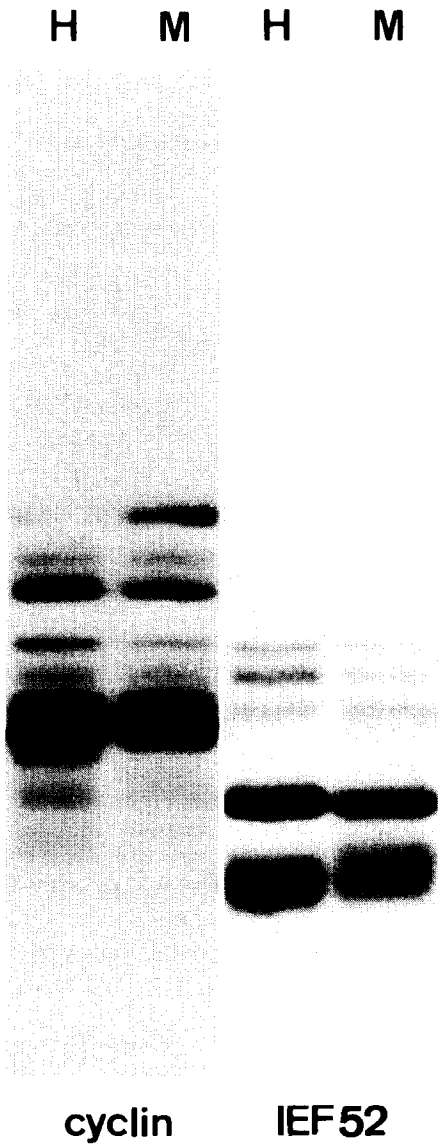

Fig.2. One-dimensional peptide map of [^{35}S]methionine-labelled cyclin and IEF 52. Polypeptides recovered from a two-dimensional gel were digested with *Staphylococcus aureus* V8 protease as in [19,20]. IEF 52 and cyclin from HeLa cells were used as standards [1,2,21]. H, human; M, mouse.

fication of cyclin in these cells was confirmed by one-dimensional peptide mapping using partial proteolytic digestion [19,20] as shown in fig.2.

The levels of cyclin in quiescent and serum-stimulated cells were determined by quantitative two-dimensional gel electrophoretic analysis of [^{35}S]methionine-labelled proteins at various times after serum stimulation. Some of the results are shown in fig.3 and the quantitative data are presented in fig.4. An increase in cyclin synthesis could be observed, only 8–10 h after serum stimulation, reaching a maximum at 14–16 h (corresponding to a 7-fold increase over the level observed in quiescent cells). After this maximum, cyclin synthesis declined and 26 h after stimulation its level was 2.5-times that of quiescent cells (not shown). Synthesis of cyclin increased again, indicating that the level of this protein does not return to that of the unstimulated quiescent cells. No changes were detected in polypeptide IEF 52. The synthesis of this polypeptide has been reported to decrease with

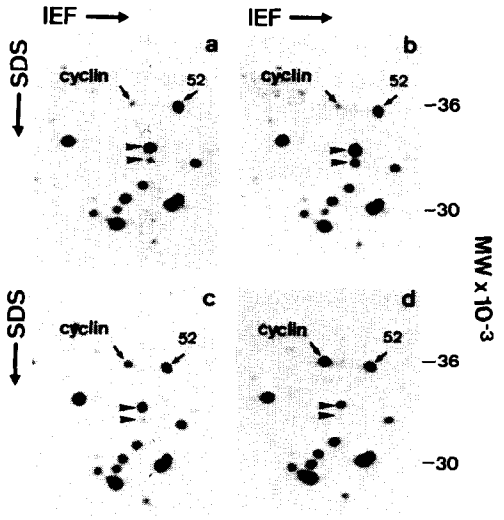

Fig.3. Two-dimensional gel electrophoresis (IEF) of [^{35}S]methionine-labelled polypeptides from serum-stimulated quiescent 3T3 cells. (a) quiescent non-stimulated cells, (b) cells after 6 h stimulation, (c) after 10 h stimulation, (d) after 14 h stimulation. Only the area of interest is shown. The arrowheads indicate two other proteins, IEF 52t and 54a (mouse numbering system [20]) whose synthesis has been shown to decrease in transformed mouse fibroblasts. However, no clear correlation could be established between the levels of these proteins and cell proliferation [2].

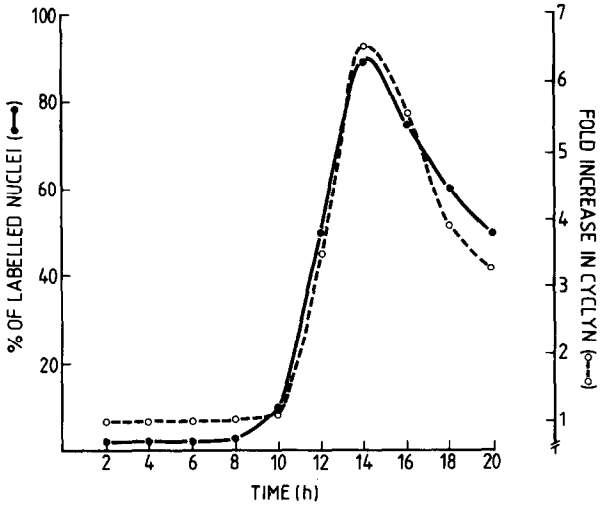

Fig.4. Synthesis of cyclin and DNA after serum stimulation of quiescent 3T3 cells. Quantitation of radioactive cyclin was performed as in [15]. The data are given as the -fold increase of cyclin compared with cyclin in non-stimulated quiescent 3T3 cells. Equal amounts of incorporated radioactivity were applied to the gels. Cells were processed for autoradiography according to standard procedures (2 weeks exposure). About 500 nuclei were counted in each case. All experiments were done in duplicate.

different degrees of transformation [1,2,5] and a possible correlation with cell proliferation has been suggested. This was not evident however in my studies.

Interestingly DNA synthesis (as determined by autoradiography) followed very closely the levels of cyclin (see fig.4), suggesting that the activity of this protein may be associated with events related to DNA replication or to DNA synthesis itself. An increase in the amount of this protein has also been observed in phytohaemagglutinin-stimulated human lymphocytes [14]. I have demonstrated a clear correlation between cyclin synthesis and the effect of epidermal growth factor on A431 cell proliferation ([17], submitted).

My results strongly support the previous suggestion that cyclin may be a central component of the pathway(s) that regulate cell proliferation [4,10]. Work is now in progress to prepare antibodies against cyclin. These should be valuable for understanding its role in cell proliferation.

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