Inhibitory diffusible factor IDF45, a G_1 phase inhibitor

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An inhibitory diffusible factor of 45 kDa (IDF45) was isolated from medium conditioned by dense cultures of 3T3 cells. The procedure involved Bio-Gel P150 chromatography and 2 reverse-phase FPLC. After the final step of purification, 60 ng/ml of IDF45 inhibited 50% of α-globulin-stimulated DNA synthesis. It was shown that IDF45 acted in the G_1 phase of the cell cycle. When added for 8 h in the G_1 phase of the cell cycle, it was able to inhibit DNA synthesis in the S phase which followed this G_1 phase. Furthermore, IDF45 inhibited the early stimulation of RNA synthesis induced by α-globulin.

Growth factor *Inhibitory diffusible factor* *G_1 phase* *(3T3 cell)* *RNA synthesis*

1. INTRODUCTION

The factors which control the growth of cells in culture are poorly understood. We have previously shown that 3T3 cell cultures diffuse in the medium inhibitory [1,2] and stimulatory [2] factors. We assumed [3] that normal cell cultures enter stationary phase when the concentration of inhibitory molecules in the medium counterbalances autocrine and exocrine stimulatory factors. Growth inhibitory factors of 45 kDa [2,4,5] have been fractionated from medium conditioned by dense cultures of 3T3 cells. From this medium a growth inhibitory molecule of 13 kDa has been recently purified [6]. Growth inhibitory factors have also been fractionated or purified from medium conditioned by different cell types [7–9]. Recently however, similarities have been shown between the inhibitory molecules diffusing from BSC_1 cells [7] and type β-TGF which may be a bifunctional regulator of cellular growth [10–12].

In this study, IDF45, the inhibitory molecule of 45 kDa diffusing from dense cultures of 3T3 cells [3,4], has been further purified by reverse-phase FPLC. We have shown that IDF45 acts in the G_1 phase of the cell cycle. The early stimulation by growth factors of RNA synthesis [13] was prevented by IDF45.

2. MATERIALS AND METHODS

2.1. *Cells*

Swiss 3T3 cells (American Type Culture Collection) were grown at 37°C in Dulbecco's modified Eagle Medium (DMEM) containing 5% newborn calf serum and antibiotics.

2.2. *Assays of IDF45 activity*

Target 3T3 cells (5×10^3) were seeded in DME medium with 5% serum in 96-well culture plates. After 1 day, the medium was discarded and replaced by serum-free medium; 2 days later, the lyophilized fractions were solubilized in the serum-free medium of the cultures and added with 75 μg α-globulin to the quiescent target cultures as described [5]. α-Globulin (Cohn Fraction IV Flucka) has properties of growth factors [14]. At this concentration, the stimulation of DNA was about 30% of the maximal stimulation induced by serum. Except when noted, DNA synthesis was determined by labelling the cells with [^{14}C]thymidine (0.05 μCi/well) between 6 and 24 h

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after the addition of fractions. The inhibitory activity of IDF45 was determined by the percent inhibition of stimulation of DNA synthesis by α-globulin. [^{14}C]Thymidine incorporation into the cells in the absence of α-globulin was between 12 and 16% of incorporation in the presence of α-globulin.

Nucleic acid synthesis was determined by labelling the cells with [^{14}C]inosine (0.25 μCi/well). [^{14}C]Inosine incorporation into nucleic acids (acid-insoluble fraction), RNA and DNA was determined as described in [2].

2.3. *Isolation of IDF45*

Serum-free medium (about 3 l) was conditioned by dense cultures of 3T3 cells and macromolecules of this medium were concentrated, solubilized in acetic acid (1 M) and acid-soluble proteins were fractionated over a 'Bio-Gel P150' column as described [2].

The lyophilized fractions containing IDF45 activity were pooled, solubilized in 0.05% TFA (trifluoroacetic acid), then adjusted to 17% acetonitrile, 0.05% TFA and fractionated by FPLC (Pharmacia). The sample was injected onto a C_8 ProRPC HR5/2 column (Pharmacia). Elution was achieved with a linear 35-min gradient of 17–47% acetonitrile, 0.05% TFA, followed by a linear 15-min gradient 47–100% acetonitrile, 0.05% TFA at a flow rate of 0.3 ml/min. An aliquot of each fraction was lyophilized and tested for IDF45 activity.

Fractions containing IDF45 activity from reverse-phase FPLC described above were adjusted to 17% acetonitrile, 0.05% TFA and rechromatographed by injection on a C_8 ProRPC HR 5/10 column (Pharmacia). The column was eluted with a linear 10-min gradient of 17–24% acetonitrile in 0.05% TFA, followed by a 40-min gradient of 24–37% acetonitrile in 0.05% TFA, at a flow rate of 0.3 ml/min. Fractions were lyophilized.

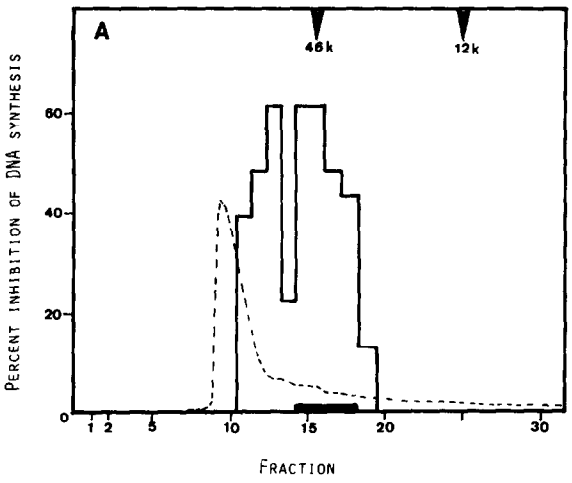
2.4. *SDS-PAGE*

Lyophilized fractions from FPLC were solubilized in an SDS sample buffer (10% glycerol, 2.3% SDS, 0.0625 M Tris-HCl, pH 6.8). SDS-polyacrylamide gel electrophoresis was performed with 10% acrylamide according to Laemmli [15]. After electrophoresis, proteins were revealed by silver staining [16]. Bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and trypsin inhibitor (20 kDa) were used as molecular mass markers.

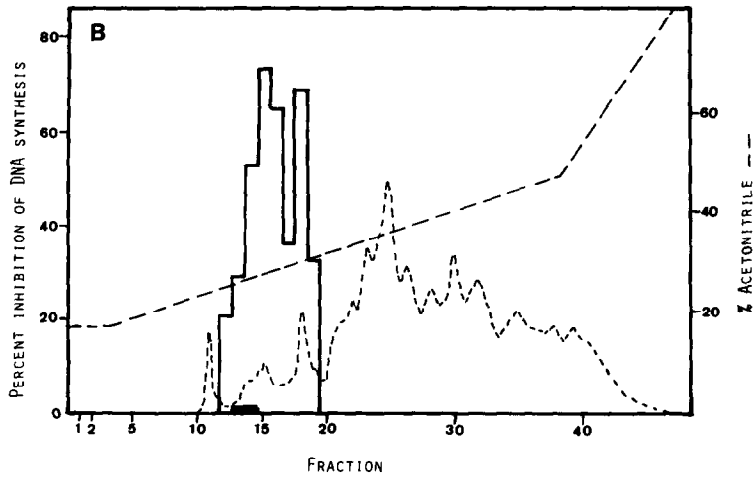
3. RESULTS AND DISCUSSION

The isolation of IDF45 is shown in fig.1. Two peaks of inhibitory activity were observed when macromolecules (soluble in 1 M acetic acid) of medium conditioned by 3T3 cells were fractionated by Bio-Gel P150 chromatography (fig.1A). One peak, which eluted like ovalbumin (fractions 15–18), was enriched in IDF45. The other peak (fractions 11–13), of larger molecular mass, was not studied further. Fractions 15–18 were pooled and used for further purification by reverse-phase FPLC. In each experiment, two peaks of inhibitory activity were observed (fig.1B). The first, peak I (fractions 13–16), eluted at about 26% acetonitrile; another, peak II (fractions 18, 19), which eluted at 29% acetonitrile, was more or less abundant depending on the experiments. SDS-PAGE of the different fractions showed that the inhibitory activity of peak I coincided with the presence of a 46-kDa protein band, whereas the inhibitory activity of peak II coincided with a 30-kDa protein band (not shown). Fractions (13,14) which eluted at 26% acetonitrile were pooled and used for further purification by another reverse-phase FPLC (fig.1C). After this final step of purification, about 60 ng/ml of IDF45 was necessary to decrease stimulation of DNA synthesis by 50% (fig.2) and two protein bands (one of 46 kDa) were observed after SDS-PAGE (fig.3). It is difficult to assess the degree of purification of IDF45, since conditioned medium contains different inhibitory molecules [2]. However, we have noted that, after the first Bio-Gel P150 chromatography, IDF45 activity is concentrated in about 10% of the total protein applied to the column. Furthermore after the two reverse-phase FPLC, the specific activity of IDF45 was multiplied by 1000 compared to its activity after Bio-Gel P150 chromatography.

We know (and have verified) that the stimulation of DNA synthesis starts between 12 and 16 h after addition of growth factors to quiescent cultures of 3T3 cells [17]. Furthermore, the presence of growth factors for 8 h in the G_1 phase



is sufficient to trigger the stimulation of DNA synthesis [17]. In experiment A (fig.4a) α-globulin was added for 8 h to the medium in the presence or absence of IDF45; then the medium was changed, replaced by DME medium without α-globulin and IDF45, and cells were labelled immediately with [^{14}C]thymidine for 16 h. The stimulation of DNA synthesis induced by the presence of α-globulin for 8 h was inhibited by IDF45. Therefore, the presence of IDF45 in the G_1 phase was sufficient to inhibit DNA synthesis in the S phase which immediately follows the G_1 phase. However, when IDF45 was in the medium for the first 8 h in the presence of α-globulin and the medium was then changed, and replaced by DME medium + α-



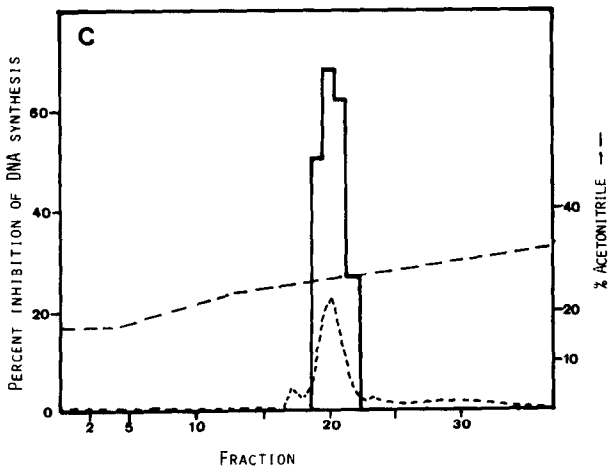


Fig.1. Isolation of IDF45. (A) About 20 mg of macromolecules concentrated from conditioned medium and solubilized in acetic acid (1 M) were chromatographed on a 'Bio-Gel P 150' column (22 × 2.5 cm) packed in 1 M acetic acid. Absorbance at 280 nm was determined (– – –). Fractions of 2.7 ml were collected, lyophilized and assayed for inhibitory activity on DNA synthesis (——). (B) The lyophilized fractions (15–18) from Bio-Gel P150 chromatography were pooled. About 600 μg solubilized in TFA (0.05%) were injected on a ProRPC-HR 5/2 column (Pharmacia) and eluted as described in section 2. An aliquot of each fraction was lyophilized and tested for IDF45 inhibition (——), A_{280} (– – –). (C) Fractions 13–14 (B) were combined, injected into a ProRPC-HR 5/10 column (Pharmacia) and eluted as described in section 2. Fractions (0.3 ml) were lyophilized and aliquots were tested for IDF45 inhibition (——), A_{280} (– – –).

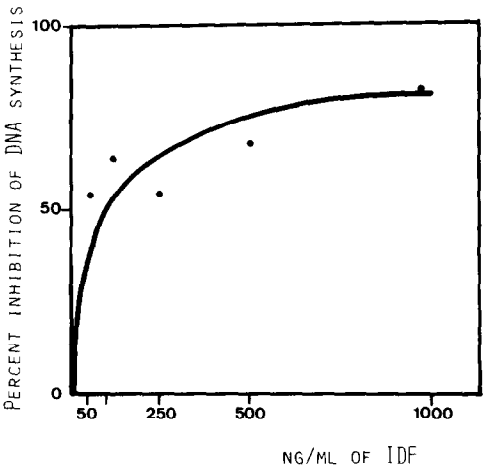


Fig.2. Dose-response curve. Inhibitory fraction 19 from the second FPLC chromatography was tested at different concentrations for the inhibitory activity of DNA synthesis.

Fig.3. SDS-PAGE of IDF45 purified by two reverse-phase FPLC.

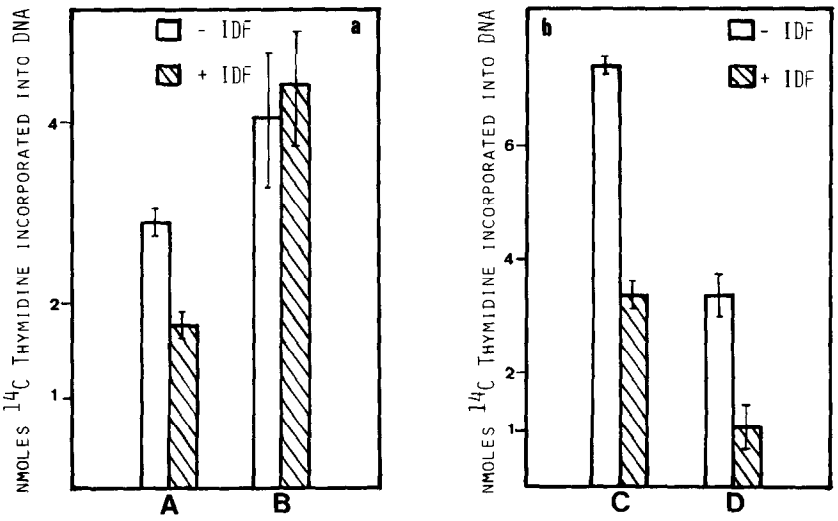


Fig.4. Inhibition in the G_1 phase of the cell cycle (typical results). In expt A (a), α-globulin was added for 8 h to the medium in the presence or absence of IDF45 (40 ng); then the medium was changed and replaced by DME medium without α-globulin and IDF45. Cells were labelled immediately for 16 h with [^{14}C]thymidine. In expt B (a), as in expt A, α-globulin and IDF45 were added for the first 8 h, after which the medium was replaced by DME medium with α-globulin, and DNA synthesis was determined between 24 and 48 h after the addition of IDF45 (16–40 h after the change in medium). In expt C, (b) IDF45 and α-globulin were added at time 0 and cells labelled with [^{14}C]thymidine, between 6 and 24 h after the addition of IDF45 and growth factors. In expt D, (b) as in expt C, IDF45 and α-globulin were added at times 0 but cells were labelled with [^{14}C]thymidine between 24 and 48 h after the addition of IDF45 and growth factors. Data are means of 3 determinations (± SD).

globulin, and DNA synthesis was determined between 24 and 48 h after addition of IDF45 (16–40 h after medium change), inhibition was not observed (expt B, fig.4a). Inhibition by IDF45 was thus reversible.

Experiment D (fig.4b) shows that the inhibitory effect of IDF45 was observed when inhibitor and α-globulin were present in the medium for 48 h and DNA synthesis determined between 24 and 48 h after their addition. These results suggest that IDF45 is different from β-TGF, which was inhibitory in the first 24 h after its addition, with α-globulin, to 3T3 cells, but was stimulatory when DNA synthesis was determined between 24 and 48 h after its addition (not shown).

The experiments (fig.5) also demonstrated that IDF45 was effective in the G_1 phase of the cell cycle, since it was able to rapidly decrease the stimulation induced by α-globulin of [^{14}C]inosine incorporation into nucleic acids. We have verified that this stimulation is not the consequence of a change in [^{14}C]inosine transport, but reflects the increase in ribosomal RNA synthesis induced by growth factors [13]. Determination of [^{14}C]inosine incorporation in DNA at different times revealed that 27 h after the addition of α-globulin, 90% of acid-insoluble ^{14}C radioactivity was incorporated into the RNA fraction, but only 10% into DNA. In the first 6 h after addition of α-globulin, more than 99% of the acid-insoluble radioactivity was incorporated into RNA. These experiments thus showed that stimulation, in the G_1 phase, of RNA synthesis was inhibited by the addition of IDF45. Since IDF45 had a rather slight effect on unstimulated cells compared to its effect on stimulated cells, we assumed that IDF45 was not a specific inhibitor of RNA synthesis, but rather inhibited one of the early events, induced by growth factor addition, which might be responsible for the stimulation of RNA synthesis. Further experiments would be necessary to determine the nature of this event.

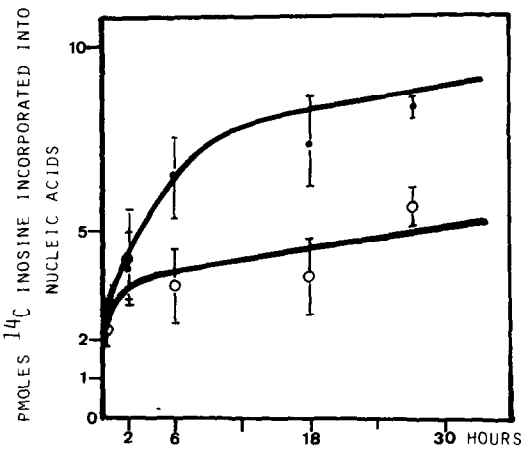


Fig.5. Early inhibition of nucleic acid synthesis (typical results). IDF45 (40 ng) and α-globulin were added at time 0 and cells were labelled with [^{14}C]inosine for 2 h from 0 to 2 h, 4 to 6 h, 16 to 18 h and 25 to 27 h after the addition of α-globulin + IDF45. [^{14}C]Inosine incorporation in acid-insoluble fraction in stimulated cells, in the absence (●—●) or presence (○—○) of IDF45. The data are the mean of 3 determinations (± SD).

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