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RAPID SELECTIVE STIMULATION BY GROWTH FACTORS OF THE INCORPORATION BY BALB/C 3T3 CELLS OF [^{35}S]METHIONINE INTO A GLYCOPROTEIN AND FIVE SUPERINDUCIBLE PROTEINS

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SUMMARY: Within four hours of adding fibroblast growth factor, epidermal growth factor, prostaglandin $F_{2\alpha}$, or serum to quiescent Balb/c 3T3 cells we observe selective increases in the incorporation of [^{35}S]methionine into six proteins; "major excreted protein" (MEP) and five "superinducible proteins" (SIPs). The mechanisms regulating the extracellular expression of MEP and the SIPs differ. 1) The levels of MEP but not SIPs are increased by NH_4Cl; and 2) Cycloheximide increase SIP and decreases MEP production. These results suggest that production of MEP and the SIPs are controlled by other proteins; MEP by a positive, and the SIPs by a negative effector.

INTRODUCTION

Little is known about the intracellular mechanism by which growth factors stimulate DNA synthesis or of the internal molecules through which the growth stimulus is imparted from the cell surface to the nucleus. It seems likely, however, that some of those internal molecules are proteins: when fresh serum is added to growth-arrested fibroblasts, total protein synthesis is rapidly increased, well before DNA synthesis starts (for references see 1). The rate at which serum-stimulated fibroblasts enter the S phase is retarded by moderately inhibiting protein synthesis (2,3). Based on data indicating that specific proteins need to be made for the rate of DNA synthesis to increase in response to growth stimulation, we began with the aim of identifying proteins that are specifically synthesized in growth-arrested, murine

ABBREVIATIONS: EGF, Epidermal growth factor; FGF, Fibroblast growth factor; MEP, "major excreted protein"; SIPs, "Superinducible proteins".

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fibroblasts (Balb/c 3T3) in response to the mitogens, FGF, EGF, and serum. We found that the most dramatic, specific increases in labeling with [^{35}S]methionine were of secreted rather than intracellular proteins. The secreted levels of these proteins begin to rise within 2 h of adding the mitogens to the growth-arrested 3T3 cells. We reported that the secreted levels of one of these proteins (called "major excreted protein" (MEP) (4); Mr 39,000) increases about 8-fold in response to FGF (5).

Here, we show that mitogens also increase the secreted levels of a set of five proteins that share the property of being "superinduced" by cycloheximide; that is, their secreted levels are increased two- to five-fold by concentrations of cycloheximide (present during induction but not present during labeling with [^{35}S]methionine) that inhibit total protein synthesis by about 85%. Cycloheximide acts in synergism with FGF. We have named these "superinducible proteins" or SIPs. They have molecular weights of 12,000 (SIP12), 24,000 (SIP24), 29,000 (SIP29), 48,000 (SIP48), and 62,000 (SIP62), and they may be the same proteins that increase intracellularly in these cells in response to stimulation by platelet-derived growth factor (6). By contrast, cycloheximide prevents FGF from raising the secreted levels of the glycoprotein, MEP.

The secreted levels of MEP and the SIPs are also regulated differently in that NH_4Cl, which inhibits lysosomal proteolysis, increases the secreted level of MEP, but not the SIPs. Finally, the ability of the growth factors to raise the secreted levels of MEP and the SIPs depends on whether the 3T3 cells are growing or quiescent and confluent. For growing 3T3 cells, the growth factors are much more effective at raising the secreted levels of MEP than they are for quiescent 3T3 cells. The opposite is true for the SIPs.

MATERIALS AND METHODS

Materials. FGF was prepared from bovine brain according to Gospodarowicz et al (7) and a preparation was received as a gift from Dr. D. Gospodarowicz (University of California in San Fransisco, CA). [^{35}S]methionine (600 - 1500 Ci/mmole) was from Amersham. All other reagents were analytical grade.

Cell Culture. Stock cultures of Balb/c 3T3-A31 cells were grown in DME, with 10% calf serum. The cultures were kept at 37°C in a humidified atmosphere of 15% CO_2 in air.

Labeling of 3T3 cells with [^{35}S]methionine. We determined the amount of [^{35}S]methionine incorporated into proteins released into the extracellular medium as follows. Cells, plated in a multiwell, plastic dish (Falcon Plastics) in DME with 10% calf serum, were grown until confluent then the medium was changed to DME with 2% calf serum to make the cells quiescent. Two to 4 days later FGF and cycloheximide were added where indicated and the cells incubated for 14 hrs at 37°C. The cells were then rinsed twice (1 ml/well) with Buffer A [.0.14 M NaCl, 6.7 mM KCl, 0.68 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.37 mM Na_2HPO_4, 25 mM Tris (pH 7.4)], and medium from parallel dishes of untreated cells was placed over the cells for 30-60 min. The medium was then removed and the cells rinsed once with Buffer A. [^{35}S]methionine labeling was done by adding DME containing 20 uM [^{35}S]methionine (50-100 uC/ml), 0.2% calf serum and FGF, but no cycloheximide (0.15 ml/1.1cm diameter well). The cells were incubated with gentle rocking at 37°C in an atmosphere of 15% CO_2 and 85% air. After 2 to 4 h, the medium was removed and spun for 20 min at 3000 X g. The supernatants were diluted with concentrated electrophoresis sample buffer, resolved by SDS polyacrylamide gel electrophoresis, the gels dried, exposed to film and the [^{35}S]methionine quantitated by densitometry as described previously (5,8).

RESULTS AND DISCUSSION

Effects of Fibroblast Growth Factor on the Incorporation of [^{35}]methionine into Proteins Secreted by Balb/c 3T3 cells.

When Balb/c 3T3 cells are incubated for various times with FGF and then labeled for 2 h with [^{35}S]methionine, the amount of [^{35}S]methionine incorporated into several secreted proteins is increased compared with other secreted proteins (Fig. 1). The estimated molecular weights of these proteins range from 12,000 to 62,000 Mr (the protein of Mr 12,000 is not shown in Fig. 1). The secreted levels of all of the induced proteins begin to increase about 2 h after FGF is added; after about 10 h, the secreted levels of these proteins decline at variable rates.

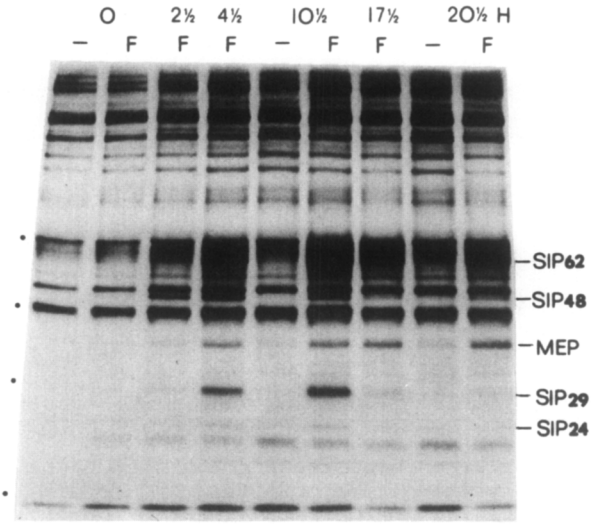

Figure 1. The Effect of FGF on the Incorporation of [^{35}S]Methionine into Proteins Secreted by Balb/c 3T3 Cells. Balb/c 3T3 cells at a density of 4.6 X 10^5 cells/well were incubated for the indicated times with FGF (100 ng/ml). The medium was then removed and at various times after adding FGF, the cells were labeled for 2 h with [^{35}S]methionine and FGF. Control wells contained no FGF. The figure shows a fluorogram of an SDS polyacrylamide gel containing the proteins secreted into the medium over the 2 h labeling period beginning at the times indicated above each channel. Medium from the same number of cells has been loaded on each channel. Shown are the proteins secreted into the medium by cells treated with (F) or without (–) FGF. The positions of MEP, and the SIPs: SIP12, SIP24, SIP29, SIP48, and SIP62 are shown. The positions of the standard proteins which were used to determine the molecular weights of the SIPs are also indicated (●). They are: bovine serum albumin (Mr 67,000), ovalbumin (Mr 45,000), carbonic anhydrase (Mr 29,000), and myoglobin (Mr 18,000).

The Effect of Cycloheximide on the Extracellular Levels of the FGF-Induced, Proteins.

To see if synthesis of proteins during the induction period influences the secreted levels of MEP or the SIPs, we inhibited protein synthesis with cycloheximide. Cycloheximide (1 ug/ml) was added with FGF and removed after 14 h (30-60 min before labeling with [^{35}S]methionine). Cycloheximide (1 ug/ml) decreases the incorporation of [^{35}S]methionine into acid-insoluble material by 85%. At this concentration, when present with FGF during the first 14 h of induction only, cycloheximide acts alone and in synergism with FGF to increase the levels of the SIPs (Table 1). When measured as a function of cycloheximide

Table 1 Effect of FGF and Cycloheximide on the Incorporation of [^{35}S]methionine into Secreted Proteins.

ADDITION	Area Under Peak			
	MEP (n=3)	SIP24 (n=6)	SIP62 (n=3)	107K (n=4)
Control	0.29	0.0	3.1 (0-8.9)	16.5
FGF, 100 ng/ml	0.77 (0.37-1.05)	13.8	26.9	19.4 (10.1-36)
Cycloheximide, 1 ug/ml	0.34 (0.33-0.37)	44.8 (10-104)	10.2 (1.5-24.6)	22.8 (10.4-49.7)
FGF and Cycloheximide	0.32 (0.19-0.45)	131.4 (43-350)	44.9 (25-70)	21.1 (8.7-49)

Quiescent 3T3 cells were incubated for fourteen hours in the presence of 1 ug/ml cycloheximide and the FGF as indicated. The cycloheximide-containing medium was then replaced with medium lacking cycloheximide. After 30-60 min the cells were labeled for 4 h with [^{35}S]methionine and the secreted proteins resolved by SDS polyacrylamide gel electrophoresis. The amount of [^{35}S]-methionine incorporated into each protein band was determined from their densities on the autoradiograms. The results have been normalized to the number of cells at the end of the experiment, and are expressed as arbitrary values that represent the relative densities of each band. The densities of the control cells varied from 1.1 to 4.1 X 10^5 cells/well. FGF increased the cell number by an average 17% and cycloheximide had no effect on cell number. The number of experiments used to obtain each set of values is indicated in parentheses below the name of the protein. The ranges for each set of values are given below the average number.

concentration (up to 3 ug/ml without FGF and up to 1 ug/ml with FGF), the amount of [35S]methionine incorporated into acid-insoluble protein is inversely proportional to the amount of secreted SIPs (data not shown). These results suggest that the levels of the SIPs are controlled by a labile protein. Several examples of superinduction by cycloheximide have been reported; e. g., in the induction of interferon (9,10) and tyrosine amino transferase (11). In each case a labile protein is proposed to regulate some aspect of gene expression.

In contrast to its effect on the induction of the SIPs, a similar incubation with cycloheximide inhibits the increase in MEP induced by FGF (Table 1). Inhibition of the FGF induction of MEP is reversible, disappearing approximately 4 h after removing

cycloheximide. These results suggest that one or more proteins need to be made for FGF to be able to increase the secreted levels of MEP.

The induction of tryptophan oxygenase (12), ovalbumin and conalbumin (13), and $\alpha_{2\mu}$-globulin (14) by steroids are also inhibited if cycloheximide or other inhibitors of protein synthesis are present during induction. The mechanism by which steroids induce these proteins is by increasing the rate of synthesis of the mRNA coding for that protein; and this increased rate of transcription induced depends on the synthesis of other proteins. The induction of MEP by FGF is inhibited if actinomycin D is present during the induction period (manuscript in preparation). This suggests that the induction of MEP by growth factors involves an increased rate of transcription. The rate of MEP mRNA synthesis is increased by the tumour promotor 12-O-tetradecanoyl phorbol 13-acetate, which in many ways mimics the effects of peptide growth factors on fibroblasts. We are now testing the hypothesis that growth factors such as FGF also increase the rate of MEP mRNA production, and whether cycloheximide, present during the induction period, inhibits that increase.

Effect of Ammonium Chloride.

The secreted levels of MEP are increased when NH_4Cl or any of several other weak bases is added to the 3T3 cells during labeling with [^{35}S]methionine (5). We have evidence that NH_4Cl inhibits degradation of MEP in the lysosomes (submitted for publication). To see if the secreted levels of the SIPs are regulated by NH_4Cl as is MEP, we tested the effect of NH_4Cl on the secreted levels of the SIPs. In contrast to its effect on the MEP, NH_4Cl does not increase the secreted levels of the SIPs, and in some experiments, the secreted levels of the SIPs are slightly

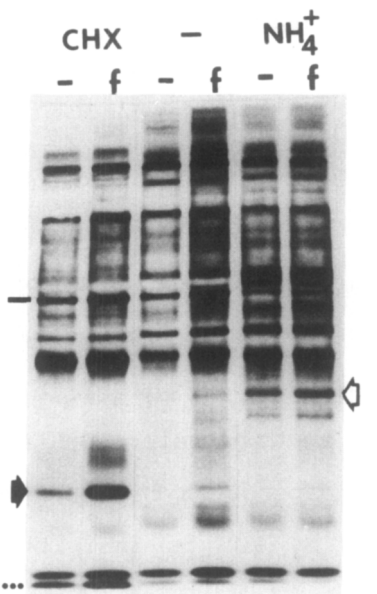

Figure 2. The Effects of Ammonium Chloride and Cycloheximide on the Secreted Levels of MEP and SIP24. Quiescent Balb/c 3T3 cells were incubated for 14 hr with (f) or without (-) FGF (100 ng/ml); and in the presence or absence of cycloheximide (1 ug/ml) (CHX). The cells were then rinsed and labeled with [^{35}S]methionine. During the four hour labeling period, FGF (f) and 10 mM NH_4Cl (NH_4^+) were present where indicated. The cell density was 1.1 X 10^5 cells/well. The positions of MEP (open arrow), SIP13 (...), SIP24 (closed arrow), and SIP62 (-) are indicated.

decreased by NH_4Cl (Fig. 2). The differential response of MEP and the SIPs to NH_4Cl and cycloheximide suggests that the secreted levels of these two types of FGF-induced proteins are regulated independently.

Effect of Other Growth Factors.

We have previously shown that several peptide mitogens for 3T3 cells increase the secreted levels of MEP (5). These other mitogens also increase the secreted levels of the SIPs (Table 2). Insulin, a poor growth factor for 3T3 cells, which does not increase the secreted levels of MEP, also does not increase the secreted levels of the SIPs. The ability to induce the SIPs is not restricted to the peptide growth factors; prostaglandin $F_{2\alpha}$, also a growth factor for 3T3 cells (15), induces SIP and MEP production. The apparent association of an increase in the levels

Table 2 Effect of Other Growth Factors on the Induction of SIPs

	Area under peak (Sq. in.)	
	SIP24	SIP62
Experiment 1:		
Control	0.10	0.09
EGF, 5 ng/ml	0.46	0.66
FGF, 100 ng/ml	0.45	0.67
Calf Serum, 10%	0.69	0.48
Insulin, 50 ng/ml	0.08	0.08
Experiment 2:		
Control	0.04	0.30
FGF, 100 ng/ml	0.62	0.70
Calf serum, 10%	0.79	0.89
Prostaglandin $F_{2\alpha}$, 100 ng/ml	0.48	0.63
Prostaglandin $F_{2\alpha}$, 300 ng/ml	0.40	1.09

Quiescent Balb/c 3T3 cells were incubated with FGF and cycloheximide, labeled with [^{35}S]methionine, and the relative amount of [^{35}S]methionine incorporated into each secreted protein was determined as described for Table 1. The results have been normalized to cell number. Each value is the average of duplicates (average standard deviation of the mean, 12%). After labeling with [^{35}S]methionine, the densities of the control cells were 2.4 X 10^5 (experiment 1) and 1.4 X 10^5 cells/well (experiment 2). The densities of cells treated with EGF, FGF, serum and insulin were 126%, 140%, 160% and 106% of the control values.

of MEP and the SIPs with the mitogenic response suggests that these secreted proteins could contribute to this response.

Effect of Growth State on Growth Factor Inducibility of Secreted Proteins.

As a final example that the secreted levels of MEP are controlled differently from those of the SIPs: FGF and EGF stimulate MEP expression much more in growing 3T3 cells than in quiescent 3T3 cells. Our results suggest that this is because MEP is degraded at a faster rate in quiescent than in growing cells. On the other hand, FGF and EGF increase the secreted levels of the SIPs much more in quiescent than in growing 3T3 cells. Similarly the inducibility of tyrosine amino transferase depends on growth state (16,17). Cycloheximide, however, appears to induce the SIPs equally well in growing and quiescent cells.

An hypothesis that we are testing now is that, associated with the mitogenic response <u>in vivo</u> is the induction of a range

of secreted proteins and glycoproteins that stimulate the growth and differentiation of the parent cell and its neighbours in a manner analogous to the intercellular communication that occurs in the immune system. Such interactions could be important in transmitting the mitogenic signal during tissue remodeling, such as occurs in wound healing or during embryonic development. We have expanded on this idea in a recent review (18).

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