INSULIN-LIKE GROWTH FACTOR: INSULIN OR SERUM INCREASE PHOSPHORYLATION OF RIBOSOMAL PROTEIN S6 DURING TRANSITION OF STATIONARY CHICK EMBRYO FIBROBLASTS INTO EARLY G_1 PHASE OF THE CELL CYCLE

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1. Introduction

Insulin-like growth factor (IGF) is a newly discovered peptide isolated from human serum [1,2]. The primary structures of two non-allelic forms of IGF (IGF-I and -II) have been determined [3,4]. The growth-promoting effects of IGF in vitro have been amply demonstrated [5,6], and its postulated physiological role as a growth hormone dependent hormone or somatomedin [7] is supported by recent findings using a radioimmunoassay in various clinical disorders [8].

We have found that one if not the major effect of physiological concentrations of IGF in stationary chick embryo fibroblasts (CEF) is on the transition from G_0 into G_1 phase of the cell cycle, an effect shared by pharmacological doses of insulin [9] and by serum [10]. The transition of cultured cells from resting to growing states is a critical step in growth control [11]. In order to delineate the triggering mechanism(s) for this response, various early changes elicited by growth stimuli have been studied [12–15]. Since rapid protein phosphorylation–dephosphorylation reactions may be common mediators of regulatory signals [16,17] we undertook a search for cellular components which are phosphorylated at the transition of cultured cells from the resting state into G_1. Here we report that within 5 min of inducing serum-deprived secondary chick embryo fibroblasts with IGF, insulin or serum to re-enter the cell cycle, there is a several-fold increase in the incorporation of radioactive phosphorus into ribosomal protein S6.

2. Materials and methods

2.1. *Tissue culture and $^{32}P_i$-labelling*

Secondary chick embryo fibroblasts were seeded at 5×10^6 cells/100 mm tissue culture plate in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% fetal calf serum, 100 μg/ml streptomycin and 100 U/ml penicillin (Gibco). After 2 days the cells were synchronized by maintaining them for 18 h in serumfree DMEM, and then incubated for 30 min with 100 μCi $^{32}P_i$/ml (Amersham) in 5 ml modified KRB phosphate-free buffer [12] in the absence or presence of IGF (100 μU/ml = 300 ng/ml), insulin (sperm whale, 24 IU/mg, 100 mU/ml = 4 μg/ml) or serum (10%, v/v). IGF was prepared as in [1].

2.2. *Separation and analysis of ribosomal subunits*

Cell lysates were made by extracting the cultures directly on the plate with hypotonic buffer containing 1% Triton X-100 and 1% Na-deoxycholate [18]. Lysates were then centrifuged through a discontinuous sucrose gradient [19], the crude ribosomal pellets resuspended in Buffer C [20], and the subunits separated on isokinetic sucrose gradients [20]. The gradients were analyzed at 270 nm and the fractions were collected. To a 200 μl aliquot from each fraction was added 20 μg BSA and 50 μl of 50% trichloroacetic acid. After 10 min on ice the samples were heated to 90°C for 10 min, filtered on Whatman GF/C glass fiber filters, dried and counted in 5 ml of Bray's solution in a liquid scintillation counter.

2.3. *One- and two-dimensional polyacrylamide gel electrophoresis (PAGE)*

Analysis of 40 S and 60 S proteins by one-dimensional electrophoresis in the presence of SDS was as in [21,22]. After electrophoresis gels were stained, destained, dried and autoradiographed. For two-dimensional polyacrylamide gel electrophoresis, 0.9 A_{260} units of ^{32}P-labelled 40 S ribosomal subunits was added to 22 A_{260} units of unlabelled 40 S ribosomal subunits from immature White Leghorn chicks [19]. The ribosomal proteins were then extracted, washed and electrophoresed as in [20,22].

3. Results

Cells arrested by serum deprivation were incubated with $^{32}P_i$ for 30 min in the absence or presence of insulin. Proteins derived from nuclear, mitochondrial, crude microsomal or cytosol fractions were then compared by one-dimensional SDS–polyacrylamide gel electrophoresis. The only notable difference between insulin-treated and control cultures was in a protein band derived from the crude microsomal fraction (not shown).

To ascertain whether this protein was of ribosomal origin, the crude microsomal pellet was separated on high salt sucrose gradients into 40 S and 60 S subunits (fig.1). When the amount of hot trichloroacetic acid-insoluble counts was plotted against A_{270}, a 30-fold increase over control cultures was found associated with the 40 S subunits derived from the insulin-treated cells. In contrast, there was no significant difference in the amount of labelling of the 60 S subunits.

Proteins from 40 S subunits were then analysed on one-dimensional SDS–polyacrylamide gel electrophoresis followed by autoradiography. Except for the labelled RNA, only one major band of ~31 000 mol. wt was detected (fig.2, slot 4). The labelled band was absent after prior treatment with pronase or alkaline phosphatase and was resistant to pancreatic ribonuclease (fig.2, slots 10–12). The rate of hydrolysis of the radioactive protein was slow at low pH and rapid at high pH, as expected for an ester bond. After hydrolysis and separation of the products

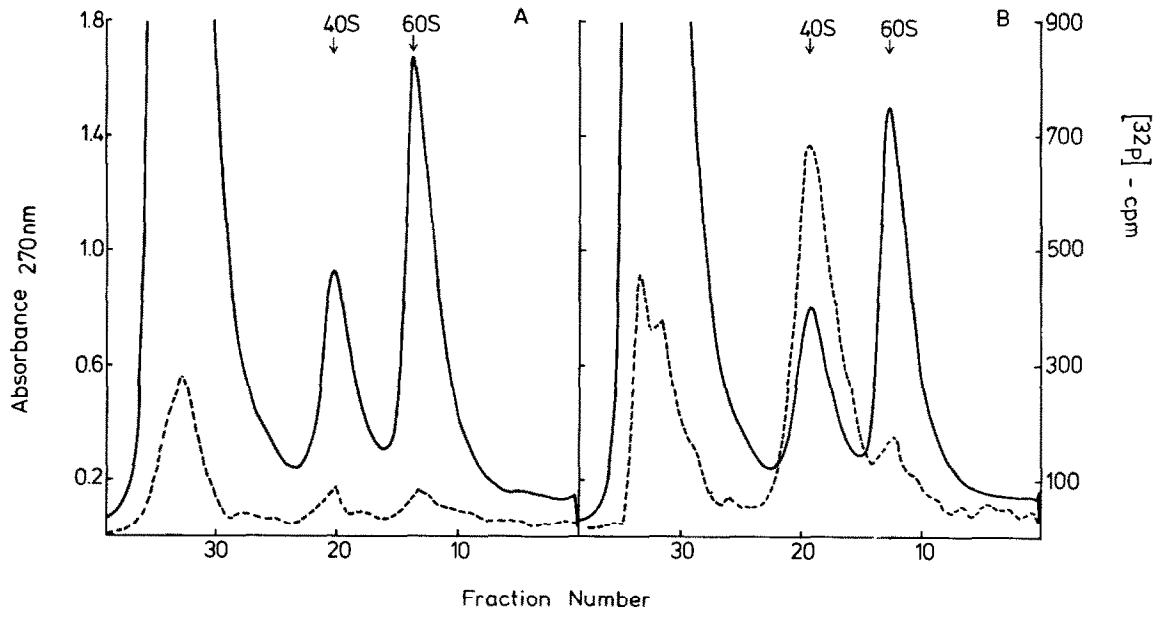

Fig.1. Sucrose gradient analysis of ribosomal subunits. (A) Resting cells. (B) 30 min after addition of insulin (100 mU/ml). Absorbance (——), cpm/fraction (– – –).

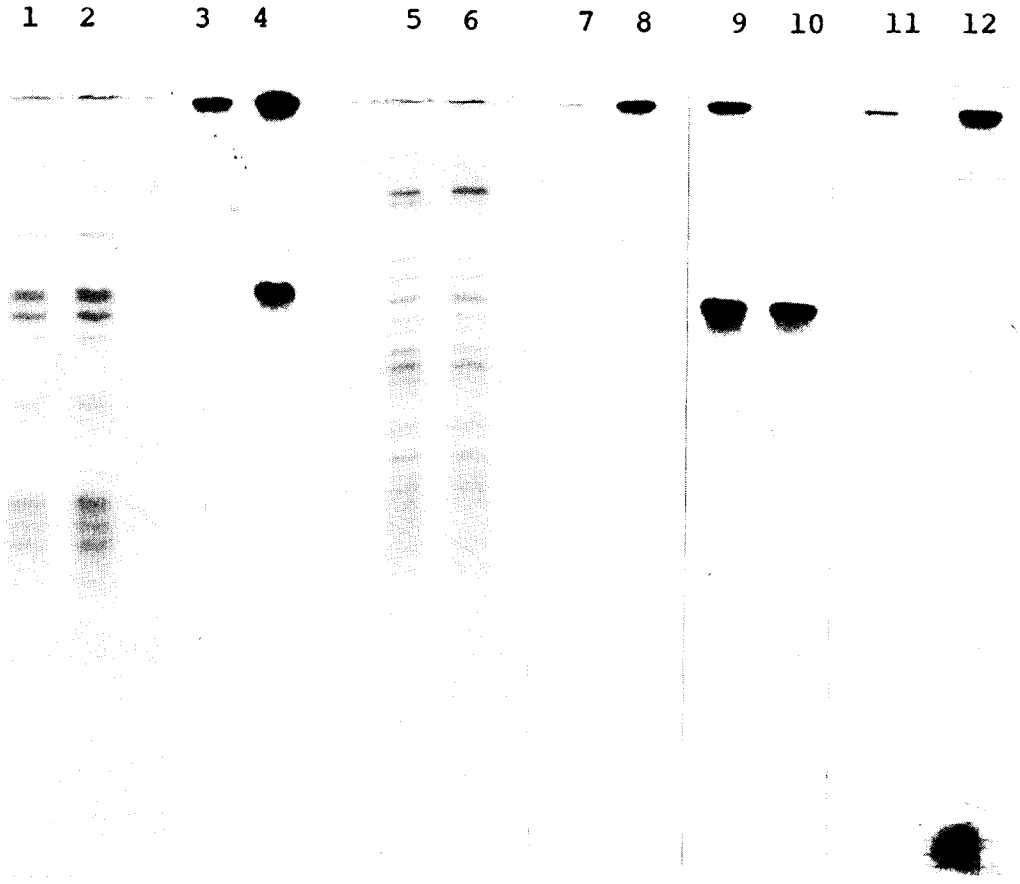

Fig.2. Polyacrylamide gel electrophoresis of 40 S and 60 S ribosomal subunits and susceptibility of 40 S ribosomal subunits to enzymatic hydrolysis. Slots (1–4) contain 40 S ribosomal subunits isolated from (1) unstimulated or (2) insulin-stimulated cultures and their corresponding autoradiograms (3,4). Slots (5–8) contain 60 S ribosomal subunits isolated from (5) unstimulated or (6) insulin-stimulated cultures and their corresponding autoradiograms (7,8). Slots (9–12) are autoradiograms of 40 S ribosomal subunits from insulin-stimulated cultures which have been incubated for 30 min at 37°C with the following enzymes: (9) control, no added enzyme; (10) pancreatic RNase, 20 μg/ml; (11) pronase, 10 μg/ml; (12) alkaline phosphatase, 1 mU/ml.

on a cation exchange column, a radioactive peak of phosphoserine was obtained (not shown).

To identify the phosphorylated protein, 40 S ribosomal subunits from chicken liver were added as carrier to a portion of radioactively labelled 40 S subunits and analysed by two-dimensional polyacrylamide gel electrophoresis as in fig.3. S6 on the autoradiogram (fig.3B) seems to consist of several spots. The most lightly labelled protein comigrated with stained chicken liver ribosomal protein S6. Other spots lay in the anodal direction of S6, the most heavily labelled being furthest from S6. If parallel cultures were induced with IGF or serum, there was a corresponding increase in the amount of S6 phosphorylation which was also demonstrated by its slower electrophoretic mobility on two-dimensional polyacrylamide gel electrophoresis (fig.3C,D) as compared to control cultures (fig.3E).

In order to investigate the extent of phosphorylation of S6 at later time points after induction, cells were exposed to $^{32}P_i$ for 30 min after various times of induction with 4 μg/ml insulin. The amount of radio-

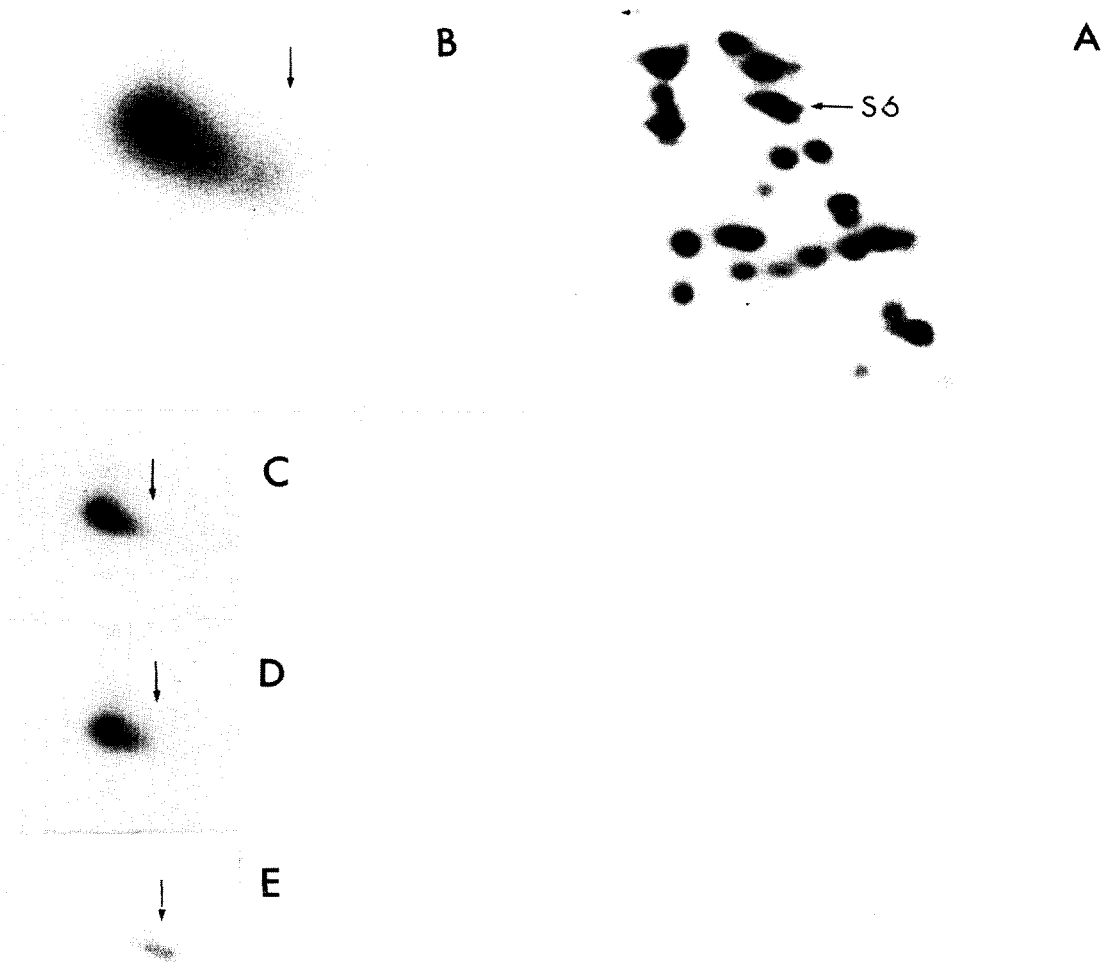

Fig.3. Identification of the phosphorylated 40 S ribosomal protein by two-dimensional polyacrylamide gel electrophoresis. (A) Stained gel of 40 S ribosomal proteins from insulin-stimulated cultures. (B–E) autoradiograms of 40 S ribosomal proteins from (B) insulin-stimulated cultures, (100 mU/ml), (C) serum-stimulated cultures, 10% (v/v), (D) IGF-stimulated cultures, 100 μU/ml, (E) unstimulated cultures. In each autoradiogram the position of the carrier-stained S6 is indicated by the arrow.

active label in S6 decreased with increasing time after induction, reaching ~1/6 at beginning of S phase of the value at 30 min after induction (not shown).

When stationary CEF were prelabelled with $^{32}P_i$ for 60 min and subsequently incubated with IGF a 3–4-fold increase in the specific radioactivity of S6 occurred after 5 min (fig.4). A further increase was seen over the next 15 min.

4. Discussion

Although there are many studies of the arrest of cell growth in culture by serum deprivation and of its reinitiation by addition of serum or purified growth factors [11] little is known about the initial events after growth stimulation leading to the complex changes described as pleiotypic response [23].

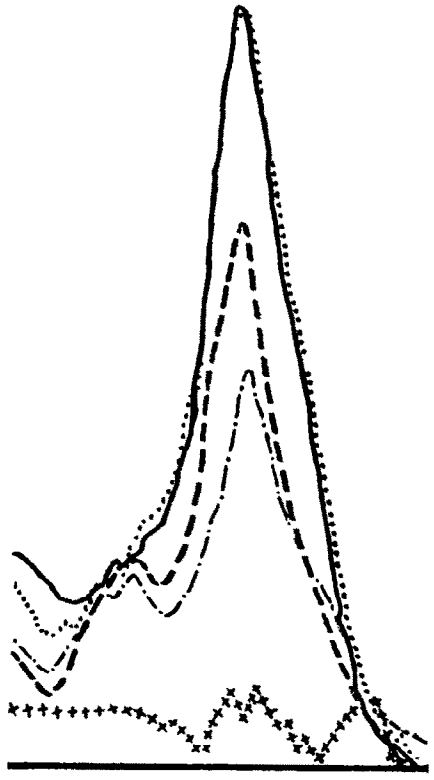

Fig.4. Densitometer tracing of autoradiogram prepared from a one-dimensional polyacrylamide gel electrophoresis. Time course of appearance of phosphorylated S6 after addition of IGF (100 μU/ml). (XXX) 0 min; (.–.–) 5 min; (– – –) 10 min; (——) 20 min; (. . .) 30 min.

Evidence is presented [24] for an increased phosphorylation of ribosomal protein S6 2 h following the transfer of growing HeLa cells to fresh serum-containing medium. Here we show that IGF, a serum-derived growth factor, increases phosphorylation of S6 considerably within 5 min.

The anodal shift of S6 on autoradiograms following induction by insulin, IGF or serum argues for a quantitative increase in the amount of S6 phosphate incorporated into S6. The results are also consistent with the findings [25] that there are several derivatives of S6 depending on the extent of S6 phosphorylation. In addition, the same shift in S6 has been observed in comparable experiments with serum-stimulated 3T3 cells labelled to equilibrium with [^{35}S]methionine (G.T. et al., in preparation). Thus, the increase in labelling cannot be simply attributed to an increase in the specific activity of the precursor pool. The time course of ^{32}P incorporation after induction and the known half life of phosphorylated ribosomal proteins [26] suggest that the phosphorylation of S6 is highest at the transition from the resting state into G_1. However, these experiments cannot rule out changes in turnover of phosphorylated S6 or in precursor pools although insulin has been shown to have no effect on phosphate uptake during the first 60 min of induction.

The extent to which ribosomal protein S6 is phosphorylated has been studied under a variety of growth conditions in vivo [25,27] and in vitro systems [28–30]. The fact that the incorporation of radioactive phosphorus is several-fold higher in our experiments than in previous experiments is most likely due to a more synchronous re-entry of cells into the cell cycle. Evidence for increased phosphorylation was also reported [31] where crude ribosomes were used as a source of both kinase and substrate. A 2-fold increase was found in the incorporation of phosphorus from ribosomes derived from serum-stimulated cultures. Phosphorylation of several HeLa cell proteins in vitro by a ribosome-associated protein kinase was shown [32]. It may be that the various stimuli mentioned all activate a specific enzymatic mechanism which in turn regulates the phosphorylation of S6. Phosphorylation of S6 was suggested [33] to be a possible means of increasing the efficiency of initiation, since they found phosphorylation of S6 to be restricted to polyribosomes in confluent BHK cells. The phosphorylation of S6 being a very early event after growth stimulation may thus be postulated to constitute a link between a growth stimulus and increased translational activity, a hypothesis presently under investigation.

The finding that IGF at physiological concentrations elicits the same effect on CEF as insulin at concentrations 4-orders of magnitude higher than those occurring in human serum is in accord with receptor binding studies [6]. These authors have shown that fibroblasts contain a high affinity IGF receptor site (app. K_d ~1 nM) for which insulin competes, but very weakly. The well-known effects of insulin on cultured cells such as stimulation of RNA, DNA and protein synthesis, on cell division

[9,17] and on ornithine decarboxylase (EC 4.1.1.17) [34] are all shared by IGF [1,8,15] and are probably mediated via the IGF receptor [8].

The effect of serum on cultured cells is certainly more complex than the effect of a single growth factor derived from serum [35]. However, the qualitatively identical effects of IGF, insulin and serum on phosphorylation of S6 suggest a common mechanism at least for this early response of fibroblasts to a growth stimulus. The rapid phosphorylation of S6 by all 3 growth stimuli argues for a specific role as an early and obligatory step in the chain of events leading from the binding to a membrane receptor of resting cells to their transition into G_1 phase. Moreover, the identification of one chemically defined component of serum as a promoter for re-entry into the cell cycle of resting cells is estimated to contribute significantly to the unraveling of the role of serum for cultured cells.

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