Control by insulin and insulin-related growth factor 1 of protein synthesis in a cell-free translational system from chick-embryo fibroblasts

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Insulin and insulin-related growth factor 1 (IGF-1) increase by 1.5–1.6-fold the rate of [³H]leucine incorporation into protein in primary monolayer cultures of chick-embryo fibroblasts (CEF); half-maximal hormone concentrations are 10 and 0.25 nM respectively. To investigate the mechanism of this effect, a rapid method is used to prepare a lysate from CEF which is active in protein synthesis. Lysate derived from cells treated for 30–150 min with insulin synthesized protein at 1.8–3.0-fold greater rate than did controls; the increased rate persisted for 20 min *in vitro*. Pactamycin (0.5 μM), an inhibitor of peptide-chain initiation, inhibited protein synthesis by 50% in lysates derived from insulin-treated and control cells. Thus insulin and IGF-1 cause an increase in the protein-synthesis rate *in vivo*, which persists in cell-free protein-synthesizing lysates of CEF.

INTRODUCTION

Insulin and other growth factors can promote the net synthesis of protein both by increasing the rate of protein synthesis (Morgan *et al.*, 1971) and by inhibiting its breakdown (Rannels *et al.*, 1975; Mortimore & Mondon, 1970). Early studies indicated that the principal effect of insulin on protein synthesis was secondary to stimulated amino acid and glucose uptake (Levine & Goldstein, 1955) or to aminoacyl-tRNA synthetase activity (Davey & Manchester, 1969). Subsequently, however, it has been shown in a variety of tissues that insulin enhances protein synthesis independently of its effects on transport of substrate (Wool, 1965) or tRNA charging (McKee *et al.*, 1978). At longer time intervals, insulin and other growth factors can influence protein synthesis by increasing specific mRNA contents (Korc *et al.*, 1981; Bolander *et al.*, 1981).

In addition to an hormonal effect on the synthesis of specific proteins, there is evidence for a rapid generalized effect of insulin and other growth factors on protein translation. Here, investigations have revealed effects on the translation efficiency of ribosomes and, at longer time intervals, on their concentration (Wool, 1975; Jefferson, 1980). Most data implicate the step of peptide-chain initiation as the locus of the effect, in that polysome profiles (Jefferson *et al.*, 1974), 40S initiation complexes (Kelley & Jefferson, 1985) and the phosphorylation of eukaryotic initiation factor 2 (Towle *et al.*, 1984) have been shown to be altered minutes after hormone addition.

To investigate the effects of insulin and IGF-1 on protein synthesis, we have used resting confluent primary cultures of CEF. Upon exposure to insulin after a period of serum deprivation, there is accelerated protein synthesis (Baseman *et al.*, 1974; Baseman & Hayes, 1975); maximal insulin concentrations raise the incorporation of [³H]leucine into total protein by

1.3–1.5-fold and that into ribosomal protein 4-fold (DePhilip *et al.*, 1979), under circumstances where amino acid transport (Vaheer *et al.*, 1973), the specific radioactivity of [³H]leucyl-tRNA (Sato *et al.*, 1981) and mRNA production (Ignotz *et al.*, 1980) are unchanged. Because insulin does not change relative or absolute transit times in CEF (Sato *et al.*, 1981), a hormonal effect on elongation or termination is unlikely, pointing to initiation as the implicated step. Direct evidence for this mechanism is seen when, immediately after insulin addition, the polysome profile of stimulated CEF is altered, with increased polysomes and decreased monosomes, under circumstances where both total cellular ribonucleoprotein and the size of the proteins translated are constant (Sato *et al.*, 1981). Finally, the large differential effect of insulin on the synthesis of total protein, as compared with ribosomal protein, and the modifications of this difference by specific translational inhibitors, are most consistent with the hypothesis that insulin changes initiation rates *in vivo* (Ignotz *et al.*, 1980).

Here the role of the IGF-1 receptor in mediating the insulin effect *in vivo* is examined, and a cell-free protein-synthesizing system derived from these cells has been prepared. Most importantly, conditions have been found which permit rapid rates of translation *in vitro* under circumstances where hormonal stimulation of synthesis is maintained. This system should greatly facilitate the search for the detailed metabolic mechanism whereby insulin and other growth factors exert translational control of protein synthesis.

MATERIALS AND METHODS

Chicken eggs (10 days incubated) were obtained from Spafas, Norwich, CT, U.S.A. IGF-1 (*N*-methionyl analogue) was from Kabi Vitrum, and crystalline pig insulin (U-100; Actrapid) was from Novo. Pactamycin

Abbreviations used: IGF-1, insulin-related growth factor; CEF, chick-embryo fibroblasts.

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was generously given by Richard L. Keene, Upjohn Co. The radiochemicals were from Dupont, puromycin was from Sigma, and the mixture of all L-amino acids was from Amersham.

Primary cultures of CEF were prepared from 10–11-day chick embryos (Amos & Moore, 1963); cells were grown in 10 ml of basal medium (Minimum Essential Medium, Gibco 410-1100, containing 4% fetal bovine serum, 100 units of penicillin/ml and 100 µg of streptomycin/ml) and reached confluence in 2–3 days.

To measure [3 H]leucine incorporation *in vivo*, 2 h before the labelling period the medium containing fetal bovine serum was removed, and confluent monolayer cultures were washed with 3 × 5 ml of basal medium without serum. Labelling with [3 H]leucine was initiated by replacement with 3 ml of basal medium supplemented with 0.5 µCi of [3 H]leucine (40–60 Ci/mmol)/ml, crystalline bovine serum albumin (0.5 mg/ml) and hormone as indicated. At the end of the labelling period, the incorporation of [3 H]leucine into acid-precipitable material was assayed (DePhilip *et al.*, 1980).

Cell-free extracts capable of protein synthesis were prepared from cell monolayers by a modification of the method of Brown *et al.* (1983). The medium was aspirated from monolayer cell cultures, and the cells were washed with 5 ml of 0.15 M-sucrose/33 mM-NH₄Cl/7 mM-KCl/4.5 mM-magnesium acetate/30 mM-Hepes, pH 7.4, at 4 °C. Cells were then permeabilized by treatment with 2 ml of the above solution supplemented with lysophosphatidylcholine (100 µg/ml) for 1 min at 4 °C. The Petri dishes were then drained upright for 1 min, and the remaining solution was aspirated. For the preparation of each lysate, approx. 10⁷ cells were scraped with a sterile policeman into 200 µl of reaction cocktail containing 100 mM-Hepes, pH 7.4, 300 mM-potassium acetate, 10 mM-phosphocreatine (dipotassium salt), 300 µM of each L-amino acid, 40 units of creatine kinase/ml, 1 mM-ATP (dipotassium salt), 1 mM-GTP (sodium salt), 10 mM-dithiothreitol, 0.1 mM-S-adenosylmethionine, 1.0 mM-magnesium acetate and 0.1 mM-spermidine. The preparation was aspirated with a 1.0 ml sterile plastic syringe, the cells were homogenized by a single passage through a 27-gauge needle, and the nuclei removed from the extract by centrifugation at 800 g for 5 min at 4 °C. The resultant extracts were entirely cell-free as judged by both optical and electron microscopy, and were used immediately for protein synthesis *in vitro* by transferring 25 µl samples to tubes containing 12.5 µCi of [3 H]leucine, which had been evaporated to dryness in an evacuated centrifuge. Both in the preparation of the reagents and in the execution of the experimental protocol, anti-RNAase precautions were adopted (Maniatis *et al.*, 1982).

Protein synthesis in the lysates was determined by the incorporation of [3 H]leucine into acid-precipitable material by the procedure of Mans & Novelli (1961); protein was measured by the method of Bradford (1976). Results are expressed as pmol of leucine incorporated/mg of protein, based on the measured specific radioactivity of [3 H]leucine in the lysate (Airhart *et al.*, 1979).

RESULTS AND DISCUSSION

When resting confluent cultures of CEF are incubated for 150 min with insulin, there is an increase in protein

Fig. 1. Dose-response relation between insulin and IGF-1 concentration and [3 H]leucine incorporation into protein in CEF

After a 2 h preincubation in basal medium without serum, the medium of resting confluent primary cultures of CEF was replaced with medium supplemented with crystalline bovine serum albumin (0.5 mg/ml), [3 H]leucine (0.5 µCi/ml) and no addition (control), insulin (●) or IGF-1 (○), and the cells were incubated for 150 min at 37 °C. Each point represents the mean ($\pm$ S.E.M., $n = 5$) of quadruplicate plates in each experiment.

synthesis as assayed by the incorporation of [3 H]leucine into acid-precipitable material (Fig. 1). Insulin maximally stimulates protein synthesis (to 150–160% of control) at 0.1 µM, with half-maximal stimulation at 0.01 µM. Because insulin at these concentrations competes with IGF-1 for binding to the IGF-1 receptor (Rechler *et al.*, 1977), the influence of IGF-1 concentration on protein synthesis was assayed. As shown in Fig. 1, the dose-response relation for IGF-1 is shifted two orders of magnitude to the left of that for insulin, with a maximal effect at 1 nM and a half-maximal effect at 0.25 nM. At maximal hormone concentrations, however, the extent of stimulation is approximately the same (150%). Since both IGF-1 and insulin receptors are tyrosine kinases and otherwise structurally homologous (Ullrich *et al.*, 1986), there may also be parallels between these receptors in the signalling pathways leading to stimulation of translation.

In order to explore the mechanism of the hormone effect on protein synthesis, a cell-free protein-translational system derived from CEF was prepared by modifications of the procedure of Brown *et al.* (1983); the most important change for CEF was the maintenance of high concentrations of all amino acids both in the culture media and subsequently in the protein-synthesis cocktail. When lysates are prepared from monolayer cultures of CEF which have been incubated for 150 min in serum-free media, they incorporate leucine at rapid rates for the first 10 min of incubation at 37 °C (Fig. 2); thereafter, the rates of incorporation slowly diminish. In contrast, lysates prepared from cells which have been treated with insulin (0.5 µM) for 150 min incorporate leucine at approximately twice the control rate for the first 5 min. This stimulated rate is maintained for longer than in the control lysate (Fig. 2), remaining linear for 10 min and then declining gradually as in the control

Fig. 2. $[^3\text{H}]$ Leucine incorporation into protein in cell-free lysates derived from CEF which had been treated with insulin or control, and its inhibition by pactamycin and puromycin

CEF cultures were either treated with insulin ($0.1 \mu\text{M}$) or not (control) for 150 min, and then lysates were prepared from both cultures by using either complete unsupplemented reaction cocktail or reaction cocktail containing pactamycin ($0.5 \mu\text{M}$) or puromycin ($600 \mu\text{M}$). Results are means $\pm$ S.E.M. ($n = 3$): x, insulin; o, insulin + pactamycin; Δ , control; $\bullet$, control + pactamycin; $\square$, insulin + puromycin.

preparation. When lysates derived from either insulin- or control-treated monolayers are incubated in the presence of pactamycin, at a concentration which specifically inhibits translational initiation ($0.5 \mu\text{M}$) (Lodish *et al.*, 1971), incorporation in both lysates is inhibited by approx. 50% (Fig. 2). Addition of puromycin ($600 \mu\text{M}$) completely inhibited the process (Fig. 2).

In the absence of translational inhibitors, initial synthesis rates of 250–500 pmol of leucine incorporated/h per mg of protein were achieved, on the basis of the reasonable assumption that in the fibroblast lysate $[^3\text{H}]$ leucyl-tRNA is in isotopic equilibrium with the free $[^3\text{H}]$ leucine, whose specific radioactivity was measured. This compares with estimated rates of proline incorporation into total protein of cultured human lung fibroblasts of 10–12 nmol/h per mg, based on the specific radioactivity of $[^3\text{H}]$ prolyl-tRNA; estimates based on the specific radioactivity of free intracellular proline were 3–5-fold lower (Hildenbran *et al.*, 1980). Previously lysates from CEF have been developed which were capable of initiation and translation of viral mRNA *in vitro*, but the actual rate of synthesis was not measured (Kerr *et al.*, 1971).

In vivo, insulin causes an alteration of the rate of protein synthesis detectable within 10 min of stimulation (DePhilip *et al.*, 1980). In order to determine the minimum period of hormonal stimulation *in vivo*

Fig. 3. Influence of duration of hormonal stimulation *in vivo* on the subsequent rates of cell-free protein synthesis in lysates of CEF

After incubation for 120 min in basal medium, CEF were incubated with or without insulin ($0.1 \mu\text{M}$) for 30, 60 or 150 min, and then lysates were prepared and assayed for $[^3\text{H}]$ leucine incorporation into protein. In all instances, values for insulin-treated CEF are greater than the corresponding controls (mean $\pm$ S.E.M., $n = 4$): o, insulin versus control, 150 min; $\square$, insulin versus control, 60 min; Δ , insulin versus control, 30 min.

required to bring about an altered rate of protein synthesis *in vitro*, parallel incubations of CEF monolayers were conducted with either insulin or control for periods of 30, 60 and 150 min. As seen in Fig. 3, the 2-fold effect of insulin was clearly evident at all times tested. At earlier times, the difference between insulin treatment and control could not be demonstrated because manipulation of the monolayers causes a rise in the rate of protein synthesis in the control lysate (results not shown).

The differential rate of $[^3\text{H}]$ leucine incorporation between lysates derived from hormone- and control-treated cells is not due to hormone-induced alterations in specific radioactivity of the labelling amino acid, since the extensive dilution of endogenous leucine by the reaction cocktail renders the specific radioactivity of $[^3\text{H}]$ leucine identical in both lysates. Similarly, it is unlikely that hormone addition causes alterations in the activity of aminoacyl-tRNA synthetases, leading to differential effects on the specific radioactivity of $[^3\text{H}]$ leucyl-tRNA in the lysate. This does not occur *in vivo* (Sato *et al.*, 1981), and is even less likely *in vitro*, owing to the large dilution with cocktail noted above; furthermore, the hormonal effect is independent of the amino acid used (results not shown). Mechanisms based on the stability or covalent structure of mRNA are also

without experimental support, since mRNAs derived from either hormone-treated or control cells translated identically in a heterologous system (Sato *et al.*, 1981). More attractive is a mechanism based on hormone-directed alterations of components of the translational machinery, leading to changes in the rates of initiation. Direct assays for initiation of endogenous message should be developed to evaluate this point quantitatively.

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