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RESEARCH COMMUNICATION

Regulation of protein synthesis in Swiss 3T3 fibroblasts

Rapid activation of the guanine-nucleotide-exchange factor by insulin and growth factors

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Insulin, whole serum, phorbol esters and epidermal growth factor each rapidly stimulate protein synthesis in serum-depleted Swiss 3T3 fibroblasts. The activation of protein synthesis by each of these agents is associated with stimulation of the activity of the guanine-nucleotide-exchange factor (GEF). This protein recycles the initiation factor eIF-2 by promoting exchange of GDP bound to eIF-2 for GTP. Activation of GEF is rapid, becoming maximal within 15 min. The degree of activation of GEF by these stimuli (to > 170 % of control for insulin, serum or epidermal growth factor; 120 % for phorbol dibutyrate) is more than enough to account for their effects on the overall rate of translation. Stimulation of protein synthesis and GEF activity occurs at low nanomolar insulin concentrations, indicating they are mediated through the insulin receptor. The best-characterized mechanism for regulating GEF activity is through changes in the phosphorylation of the smallest subunit of eIF-2 (eIF-2α); however, none of the stimuli studied altered the level of phosphorylation of eIF-2α in Swiss fibroblasts. It seems that direct regulation of GEF activity may be occurring here, and possible mechanisms for this are discussed.

INTRODUCTION

Protein synthesis is acutely regulated at the level of mRNA translation by a wide variety of stimuli in mammalian cells [1]. These include whole or dialysed serum [2–6], hormones (including insulin [3,7–9]), growth factors (e.g. [3,10,11]) and amino acids [5,12,13], all of which exert their effects specifically at the level of peptide-chain initiation, as opposed to other stages of translation. Over the past decade, several potential mechanisms have been identified through which peptide-chain initiation may be regulated (reviewed in [1] and [14]). In general they involve the modulation of the activity of translational components and, in particular, individual initiation-factor proteins, by reversible phosphorylation. For example, insulin, growth factors and serum have been reported to alter the phosphorylation of certain other initiation factors (e.g., the cap-binding protein, eIF-4E) in a variety of cell types (reviewed in [14]). The best characterized of these mechanisms concerns initiation factor eIF-2, which mediates the binding of the initiator Met-tRNA$_i$ to the ribosome. Phosphorylation of the α-subunit of this factor (eIF-2α) impairs its recycling between successive rounds of peptide-chain initiation, a process involving exchange of bound GDP for GTP which is catalysed by a further, pentameric, initiation factor, the guanine-nucleotide-exchange factor, GEF (also known as eIF-2B) ([15]; for reviews, see [1,14,16,17]). The activity of GEF may also be modulated by other mechanisms, including phosphorylation [18], and by allosteric means ([19,20]; S. Oldfield & C. G. Proud, unpublished work).

The stimulation of peptide-chain initiation by insulin and growth factors has been widely studied by a number of research groups, although the basis of its action remains unclear. Here we report that insulin rapidly increases the activity of GEF in serum-depleted fibroblasts. Other agents which stimulate peptide-chain initiation in these cells also activate GEF, and this stimulation appears to reflect alterations in the activity of GEF *per se* rather than changes in the phosphorylation state of eIF-2α.

MATERIALS AND METHODS

Chemicals and biochemicals

Chemicals and biochemicals were obtained respectively from BDH (Poole, Dorset, U.K.) and Sigma Chemical Co. (Poole, Dorset, U.K.), unless otherwise indicated. Acrylamide and methylenebisacrylamide were from Pharmacia–LKB (Milton Keynes, U.K.). [^{3}H]GDP (12.4 Ci·mmol^{-1}) was from Amersham International (Amersham, Bucks., U.K.). For protein-synthesis measurements, EXPRE^{35}S^{35}S ^{35}S-labelling mix from New England Nuclear Corp.–du Pont, Dreiech, Germany, was used. This contains cysteine and methionine, both radiolabelled. The specific radioactivity of the latter was ~ 1000 Ci·mmol^{-1}. Culture medium and foetal-calf serum were from Gibco (Paisley, Renfrewshire, Scotland, U.K.).

Protein preparations

eIF-2 was prepared from rabbit reticulocyte lysates by a modification (S. Oldfield & C. G. Proud, unpublished work; details available from C. G. P. on request) of a procedure described previously [21].

Cell culture and treatment of cells

Swiss 3T3 fibroblasts were generously given by Dr. George Thomas (Friedrich-Miescher-Institut, Basel, Switzerland) and were grown in 75 cm^2 culture flasks in a humidified atmosphere of air/CO_2 (19:1). The growth medium (Dulbecco's modified Eagle's medium) was supplemented with 10 % (v/v) foetal-calf serum, 2 mM-glutamine, 0.25 mg of streptomycin sulphate/ml,

Abbreviations used: EGF, epidermal growth factor; eIF-2, eukaryotic initiation factor-2; eIF-2α, the α-subunit of eukaryotic initiation factor-2; GEF, guanine-nucleotide-exchange factor; PDBu, phorbol dibutyrate; IGF-1, insulin-like growth factor-1.

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0.14 mg of benzylpenicillin/ml and 45 mM-$NaHCO_3$. Cells used for experiments were seeded at a density of 1.5×10^4 cells·cm^{-2} growth area into appropriately sized dishes (see below). After seeding, cells were allowed to grow in the presence of 10% serum for 3–4 days, and this was then replaced with medium containing 4% serum for a further 24 h. Cells were then treated with the agent(s) under investigation. This procedure is closely based on that described by Hesketh & Campbell [22].

Measurement of protein synthesis

For these experiments cells were grown in 35 mm-diameter Petri dishes. The radiolabelled amino acids (methionine plus cysteine) were added at the same time as the agent under investigation. After 15 min the medium was aspirated off and the cells were lysed in 0.2% (w/v) SDS. Trichloracetic acid was added to 20% (w/v), and the precipitated protein was then collected on glass-fibre (GF/C) filters (Whatman, Maidstone, Kent, U.K.) and washed with 3 × 1 ml of 10% (w/v) trichloracetic acid. After washing with 95% (v/v) ethanol, filters were dried and subjected to liquid-scintillation counting in toluene containing 0.4% (w/v) diphenyloxazole.

Assay of GEF activity

GEF was assayed by the exchange of tritiated GDP in preformed eIF-2·[^{3}H]GDP complexes for unlabelled GTP as described elsewhere [23]. For these experiments cells were grown on 100 mm-diameter dishes. After treatment with the agent under study for the indicated time, cells were washed three times with ice-cold phosphate-buffered saline [0.03% (w/v) KCl/1.0% (w/v) NaCl/0.14% (w/v) Na_2HPO_4/0.3% (w/v) KH_2PO_4, pH 7.4] and then lysed into buffer A (200 μl) comprising 20 mM-Tris/HCl, pH 7.6, 0.2 mM-EDTA, 0.2 mM-EGTA, 1 mM-dithiothreitol, 100 mM-KCl, 10% (v/v) glycerol, 1% Triton, 3 μM-microcystin LR and proteinase inhibitors [0.2 mM-benzamidine, leupeptin and pepstatin (0.4 μg·ml^{-1} each) and 0.5 mM-phenylmethanesulphonyl fluoride]. The lysate was briefly centrifuged to remove cell debris and extracts were matched for protein content (measured by the method of Bradford [24]) before assay for GEF activity. Protein contents of extracts prepared in parallel were usually within 3%, and never more than 5%, of one another. Incubations (30 °C, 20 μl) contained 10 μl of cell extract. Samples (15 μl) were removed at the indicated times and filtered through nitrocellulose to measure remaining eIF-2·[^{3}H]GDP complexes.

Isoelectric focusing and immunoblotting

Isoelectric focusing was carried out as described by Maurides *et al.* [25], modified to suit a Bio-Rad mini-gel apparatus and using a shorter electrophoresis time of 5 h. Immunoblotting was performed as described previously [21]. Samples were prepared as follows. Cells were grown on 60 mm-diameter dishes; at 15 min after treatment (e.g. with insulin) the medium was aspirated off and the cells were lysed into buffer A (see above). The lysate was briefly centrifuged to remove cell debris and then added to 50 μl of fast-flow Sepharose S equilibrated in the above buffer. The mixture was subjected to constant agitation for 2 h at 4 °C and then centrifuged. The unbound material (supernatant) was removed and the beads were washed twice in the above buffer and twice in the above buffer containing 200 mM-KCl and the eIF-2 was then eluted with 50 μl of buffer containing 400 mM-KCl. The recovery of eIF-2 after the Sepharose S step, measured by immunoblotting of the bound and unbound material, was 80–90%. A 10 μl portion was then diluted 2-fold with the original buffer before addition of the sample buffer and application to the gel.

RESULTS AND DISCUSSION

Stimulation of protein synthesis in Swiss 3T3 cells by insulin, phorbol esters and serum

In order to study the mechanism(s) by which hormones and growth factors stimulate protein synthesis in Swiss 3T3 fibroblasts, it was first necessary to establish conditions appropriate to obtaining marked and reproducible activation of translation by these agents, and considerable effort was devoted to this. Using

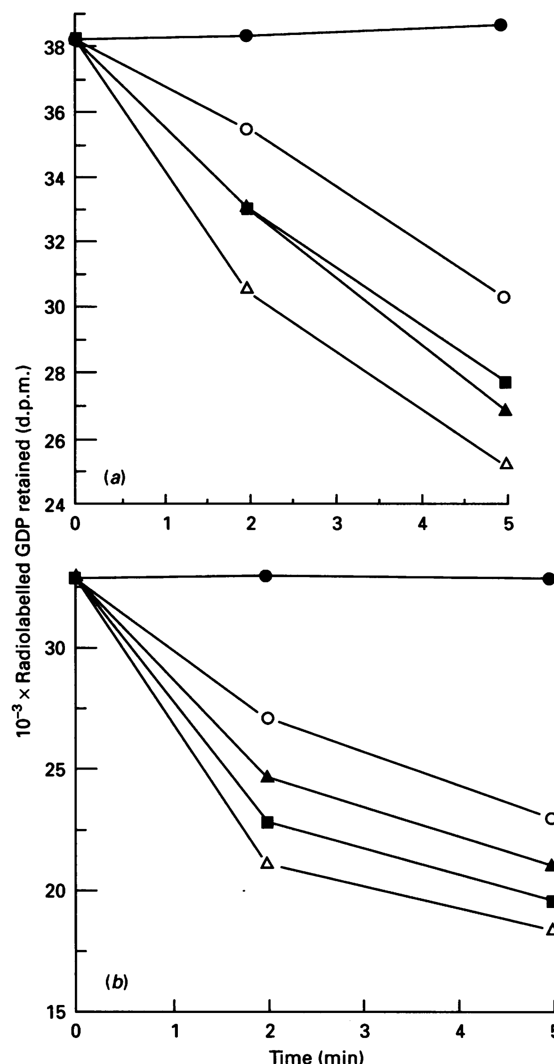

Fig. 1. Effects of insulin, serum, EGF and PDBu on GEF activity in Swiss 3T3 cells

Cells were grown in the presence of medium containing 10% (v/v) serum for 3–4 days and this was then replaced with medium containing 4% serum for a further 24 h. Cells were then treated for 15 min with the agent under investigation. GEF activity measurements were carried out as described in the Materials and methods section. (*a*) Time course of the effect of insulin on GEF activity. Cells were incubated either without added insulin (○) or for 5 (■), 15 (△) or 60 (▲) min with 20 nM-insulin before lysis. The eIF-2·[^{3}H]GDP complexes were stable in the absence of added cell extract (●). The results presented are typical of four separate experiments in which essentially the same time course was observed. (*b*) Effect of foetal-calf serum, EGF and PDBu on GEF activity. Cells were incubated without further addition (○), or with 10% (v/v) serum (△), 100 ng/ml EGF (■) or with 2 μM-PDBu (▲) before lysis. The eIF-2·[^{3}H]GDP complexes were stable in the absence of added cell extract (●). The results shown are from a typical experiment; the full data are summarized in Table 1.

Table 1. Effects of insulin, serum, EGF and PDBu on protein synthesis, GEF activity in Swiss 3T3 cells and eIF-2α phosphorylation

Cells were grown in the presence of medium containing 10% (v/v) serum for 3–4 days and then this was replaced with medium containing 4% (v/v) serum for a further 24 h. Cells were then treated for 15 min with the agent under investigation. Protein synthesis and GEF activity measurements were carried out as described in the Materials and methods section, as was the level of eIF-2α phosphorylation. Data for GEF activity are based on 2 min time points. Results for protein synthesis and GEF activity are given as percentage of control ± S.D. Data for eIF-2α phosphorylation are from a typical experiment; in four entirely separate experiments, eIF-2α phosphorylation was found to be 14 ± 0.5%. Abbreviation used: n.d., not determined.

Agent	Protein synthesis (% of control)	GEF activity (% of control)	eIF-2α phosphorylation (%)
Control	100	100	14.2
Insulin (20 nM)	134 ± 9 ($n = 21$)	198 ± 61 ($n = 12$)	14.1
Serum (10%, v/v)	161 ± 14 ($n = 8$)	182 ± 17 ($n = 3$)	14.7
EGF (100 ng/ml)	132 ± 11 ($n = 3$)	174 ± 44 ($n = 3$)	14.1
PDBu (2 μM)	121 ± 7 ($n = 8$)	122 ± 2 ($n = 3$)	13.9
IGF-1 (10 nM)	100 ± 5 ($n = 3$)	n.d.	n.d.

the procedure we have developed we found that protein synthesis was markedly stimulated by whole serum, insulin, epidermal growth factor (EGF) or phorbol dibutyrate (PDBu) (Table 1), the degree of stimulation varying from one agent to another. In all cases stimulation was rapid, the standard time used being 15 min, although stimulation was already apparent by 5 min. It is possible that we have slightly underestimated the magnitude of the observed effects on protein synthesis by commencing our measurements at zero time. The effects of insulin, serum and EGF on protein synthesis in Swiss 3T3 cells have previously been shown to be exerted principally at the level of peptide-chain initiation by Thomas and co-workers [3], although the mechanism(s) underlying these effects have remained obscure.

Effects of insulin, serum and other agents on GEF activity

To see whether the stimulation of protein synthesis by these agents was accompanied by activation of GEF, its activity was measured in extracts of control and treated cells. GEF activity was measured by the release of radiolabelled nucleotide from preformed eIF-2·[^{3}H]GDP complexes, as described by many other workers (see e.g., [5,6,26,27]). Before addition to the GEF assay incubation mixture, cell extracts were matched for protein content, so that similar amounts of total cell protein were used in each assay. The quantities of cell extract used in the incubations always gave GEF activities within the linear range of the assay (results not shown). Extracts of cells treated with insulin, EGF, serum or PDBu for 15 min showed enhanced GEF activity relative to untreated controls, as shown in Figs. 1(*a*) and 1(*b*), which depicts the results of a typical set of experiments in each case. Table 1 summarizes the results of multiple experiments for each of the four stimuli used.

In the case of insulin, activation of GEF was evident after 5 min treatment, was maximal after 15–30 min and persisted up to at least 1 h (Fig. 1*a*). A similar time course was seen with serum stimulation (results not shown). Maximal stimulation of GEF activity was obtained with 20 nM-insulin, and half-maximal with about 1 nM (Fig. 2*a*); similar values were obtained for maximal and half-maximal stimulation of protein synthesis by insulin (results not shown). For EGF the corresponding values were 100 ng·ml^{-1} (maximal) and 10 ng·ml^{-1} (half-maximal) (Fig. 2*b*). The doses of insulin required were therefore relatively low, and it is thus likely that it is operating through its own receptor, rather than, for example, through the receptor for insulin-like growth factor-1 (IGF-1) [28]. This interpretation is consistent with our observation that IGF-1 did not stimulate protein synthesis in these cells (Table 1).

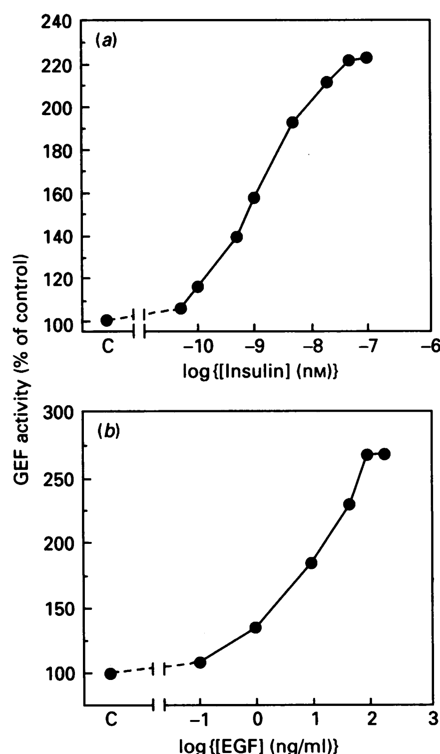

Fig. 2. Dose–response curves for the effects of insulin and EGF on GEF activity in Swiss 3T3 cells

Cells were incubated with various concentrations of either insulin (*a*) or EGF (*b*) for 15 min before lysis. Cell extracts were matched for protein content before measurements of GEF activity were carried out. In each case the data shown are typical of results from four separate experiments in which similar dose–response curves were obtained. Abbreviation: C, control.

This is the first demonstration that EGF and phorbol esters can stimulate GEF activity in intact cells, and also the first time that this has been shown for insulin in a well-defined system (i.e. cells in culture), although Kimball & Jefferson [27] and Jeffrey

et al. [29] have reported that insulin stimulates skeletal muscle GEF activity in intact experimentally diabetic rats.

Phosphorylation state of eIF-2α

The best-characterized mechanism for the regulation of GEF activity is through changes in the phosphorylation state of eIF-2α, since eIF-2 phosphorylated in its α-subunit inhibits GEF (reviewed in [14] and [16]). We therefore assessed the level of phosphorylation of eIF-2α in cells subjected to the treatments detailed above, as described in the Materials and methods section, using the widely employed technique of isoelectric focusing/immunoblotting. In four entirely separate experiments, no difference in the level of eIF-2α phosphorylation was observed when extracts from control cells or from cells treated with insulin, serum, EGF or PDBu were analysed. In a typical experiment the proportion of eIF-2α in the phosphorylated form was $14 \pm 0.3\%$ in each case (Table 1). The crucial parameter determining the sensitivity of GEF activity to changes in eIF-2α phosphorylation seems to be the ratio of eIF-2 to GEF in a particular cell type. In the small number of cases where significant changes in GEF activity have been recorded, relatively large alterations in the level of eIF-2α phosphorylation were involved (e.g. in serum-deprived Ehrlich-ascites-tumour cells eIF-2α rose from 18.5 to 44.5% [30]; in Chinese-hamster ovary cells containing a temperature-sensitive mutant leucyl-tRNA synthetase, eIF-2α phosphorylation was 5 and 17% at permissive and non-permissive temperatures respectively [31]). Thus it appears that the changes in GEF activity that we have observed cannot reasonably be explained by alterations in eIF-2α phosphorylation. However, we cannot completely discount this possibility, since the ratio of eIF-2 to GEF in Swiss 3T3 cells is not known, although it would have to be extremely high to be consistent with our data, since we have not observed any changes in eIF-2α phosphorylation using our technique which we have applied previously to demonstrate alterations in eIF-2α phosphorylation [32,33] and which should be able to detect changes of 1–2%, given that our data indicate a spread of $\pm 0.5\%$ across our experiments. The changes in rates of nucleotide exchange reported here may therefore involve alterations in the intrinsic activity of GEF itself.

Mechanism by which GEF is activated

Given that the stimuli used here failed to decrease the level of phosphorylation of eIF-2α, an alternative mechanism for the activation of GEF must presumably be operating here. Other workers have previously obtained evidence for regulation of GEF activity by mechanisms not involving changes in eIF-2α phosphorylation. Jeffrey *et al.* [29] found that GEF activity was decreased in extracts of skeletal muscle from starved or diabetic rats, but that the level of phosphorylation of eIF-2α was unaltered, indicating that GEF activity was being regulated through another mechanism. Similarly, Rowlands *et al.* [30] found that, although glutamine-deprivation of Ehrlich-ascites-tumour cells resulted in decreased GEF activity, as measured in extracts, this did not seem to be due to increased phosphorylation of eIF-2α, since it was not overcome by an excess of unphosphorylated [eIF-2·GDP], although a modest increase in eIF-2α phosphorylation was seen under these conditions. In the case of serum, however, changes in eIF-2α phosphorylation have been reported by a number of workers [6,30,34,35]. There is a single report that insulin decreases eIF-2α phosphorylation (in chondrocytes; [36]). Another consideration which suggests that alterations in the level of eIF-2α phosphorylation were unlikely to be involved in regulating GEF activity in our experiments is the fact that, under our standard conditions, a 20-fold excess of unphosphorylated eIF-2 (as the substrate eIF-2·[^{3}H]GDP) was employed when a 150-fold excess of eIF-2·[^{3}H]GDP was used, no diminution in the difference in GEF activity between extracts of cells subjected to different treatments was observed. These ratios were determined by quantitative immunoblotting of the cell extracts and the purified eIF-2 used in our experiments. A large excess of substrate should overcome the competitive inhibition of GEF due to [eIF-2·GDP] complexes containing phosphorylated eIF-2α (as discussed by Rowlands *et al.* [30], who used this as a way of distinguishing possible mechanisms for the control of GEF activity).

The observation by Dholakia & Wahba [18] that casein kinase-2 phosphorylates the largest subunit of GEF and that this is associated with activation of GEF offers a potential mechanism by which insulin, EGF and serum could activate the factor, since all three agents have been reported to stimulate casein kinase-2 in a variety of cell types [37–40]. On the other hand, the alterations in GEF activity could in principle arise from allosteric regulation by, for example, nicotinamide–adenine dinucleotides, sugar phosphates or polyamines [19–21], but we consider this unlikely, owing to the high overall degree of dilution of the samples in the assay.

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