RESEARCH COMMUNICATION

Growth hormone activates mitogen-activated protein kinase and S6 kinase and promotes intracellular tyrosine phosphorylation in 3T3-F442A preadipocytes

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Physiological concentrations of growth hormone induced a rapid and transient activation of mitogen-activated protein kinase (MAP kinase) and S6 kinase in 3T3-F442A preadipocytes. These effects were abrogated by staurosporine and in cells chronically pretreated with phorbol esters, suggesting that protein kinase C is involved in the mechanism of activation. In addition, three cytosolic proteins exhibited a growth-hormone-dependent increase in tyrosine phosphorylation.

INTRODUCTION

It is now widely recognized that growth hormone (GH) effects on cell function are not mediated solely via the promotion of insulin-like growth factor 1 (IGF-1) secretion from the liver. High-affinity receptors for GH have been identified in a variety of cell types, including hepatocytes [1], lymphocytes [2] and adipocytes [3]. One suggested action of GH is the promotion of cellular differentiation. Much of the evidence for this has been derived from studies with various preadipocyte cell lines, which appear to require GH during acquisition of the adipocyte phenotype [4,5]. However, attempts to define precise cellular actions for GH have been hampered in recent years by the relatively poor understanding of its intracellular signalling mechanisms.

Intracellular protein phosphorylation contributes greatly to the mechanism of action of many hormones. Evidence indicating that the GH receptor possesses an intrinsic or closely associated tyrosine kinase activity [6] suggests that GH may also utilize such signalling machinery. In support of this, GH has been shown to alter the phosphorylation status of several intracellular proteins in rat hepatocytes [7]. In addition, the stimulation by GH of *c-fos* gene expression in various cells [8–10] and of IGF-1 mRNA expression in rat hepatocytes [11] is thought to be mediated, at least in part, by protein kinase C (PKC).

In this study, the involvement of protein kinases in GH action was investigated further. The results demonstrate that, in 3T3-F442A preadipocytes, GH stimulates the activity of two cytosolic protein kinases which are known to be involved in the signalling mechanisms of many hormones and growth factors.

MATERIALS AND METHODS

Materials

Calf serum, epidermal growth factor (EGF) and pertussis toxin were supplied by Gibco, Paisley, Scotland, U.K. All other cell culture media and supplies were from Northumbria Biologicals Ltd. Ovine growth hormone was supplied by NIADDK, Bethesda, MD, U.S.A. 4-Nitrophenyl phosphate was purchased

from Serva, and phenyl-Sepharose was from Pharmacia. Myelin basic protein (MBP), phorbol 12-myristate 13-acetate (PMA), proteinase inhibitors and all other chemicals were obtained from Sigma. [γ - 32 P]ATP (> 3000 Ci/mol) was from Amersham. Donkey anti-sheep/goat IgG was provided by SAPU, Carlisle, Scotland, U.K.

Cell culture and treatment

Stocks of 3T3-F442A preadipocytes were generously donated by Dr. Howard Green, Harvard Medical School. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum and 2.5 mM-glutamine (standard medium), and used between passages 5 and 20. Cells were routinely incubated in standard medium without serum for 18 h prior to the addition of hormones. Down-regulation of PKC was achieved using the method of Blackshear *et al.* [12]. Briefly, cells were washed three times with DMEM and then incubated in the presence of 16 μ M-PMA for 16 h. The cells were then washed four times in DMEM and incubated for a further 2 h prior to the addition of hormones.

Cell lysis and partial purification of mitogen-activated protein kinase (MAP kinase)

Cytosolic extracts were prepared as described previously [13], except that cells were lysed at 4 °C by rapid passage through a 26 G needle attached to a 1 ml disposable syringe. This procedure caused > 95% cell breakage, as assessed by light microscopy. MAP kinase was partially purified from cytosolic extracts by batch adsorption to phenyl-Sepharose as described in detail previously [13]. Briefly, cytosolic extracts were mixed with 150 μ l of packed phenyl-Sepharose, followed by successive washing cycles of the matrix with buffers containing 10% and 35% ethylene glycol. MAP kinase was eluted from the phenyl-Sepharose in a buffer containing 60% ethylene glycol.

Protein kinase assays

MAP kinase was assayed using MBP as substrate, essentially as described previously [14]. S6 kinase was assayed [15] using, as the substrate, 40 S ribosomal subunits which were purified from commercially available brine shrimp eggs [16]. After SDS/PAGE

Abbreviations used: GH, growth hormone; IGF-1, insulin-like growth factor 1; EGF, epidermal growth factor; MBP, myelin basic protein; DMEM, Dulbecco's modification of Eagle's medium; PKC, protein kinase C; MAP kinase, mitogen-activated protein kinase; PMA, phorbol 12-myristate 13-acetate.

and autoradiography, the amount of ^{32}P incorporated into S6 was assessed using a Bio-Rad Model 620 video densitometer.

Generation of specific antibodies to phosphotyrosine

Polyclonal antibodies to phosphotyrosine were raised in sheep, using as immunogen phosphotyrosine coupled to keyhole limpet haemocyanin. Immune sera were purified by ammonium sulphate precipitation and adsorption to a column of phosphotyrosine-Sepharose. Specific antibodies were eluted with 10 mM-phenyl phosphate, and, after dialysis, were stored at -20°C . The specificity of the purified antibodies was confirmed in competitive ligand studies using enzyme-linked immunoadsorbent assay and Western blotting. At a concentration of 10 mM, phosphotyrosine and, to a lesser degree, sodium phenyl phosphate prevented a 1:100 dilution of the antibodies from recognizing phosphotyrosine-containing proteins, whereas phosphoserine and phosphothreonine were without significant effect.

Western blotting

Cytosolic extracts (see above) containing 75 μg of protein (measured using the Bradford protein assay [17]) were subjected to SDS/PAGE and immunoblotted using anti-phosphotyrosine antibodies (1:100 dilution). Reactive protein bands were visualized using horseradish peroxidase-linked donkey anti-sheep secondary antibodies.

RESULTS

In preliminary experiments, 3T3-F442A preadipocytes responded to physiological concentrations of GH with a marked (at least 3-fold) elevation in cytosolic protein kinase activity when MBP was used as substrate. Elevations in MBP-phosphorylating activity have been observed in a large number of cell types following stimulation with hormones, growth factors and other extracellular agents, and correlates with activation of MAP kinase, which utilizes MBP as an efficient and selective substrate. Fractionation of cytosolic extracts by phenyl-Sepharose chromatography has previously been shown to purify MAP kinase extensively, so that it constitutes the major protein kinase activity eluted from this matrix using buffers containing 60% ethylene glycol [13,18]. Purification of cytosolic extracts from GH-treated 3T3-F442A cells in this way did not decrease, and indeed in most cases increased, the observed stimulation of MBP-phosphorylating activity (results not shown). All subsequent data therefore represent MBP-phosphorylating activity in extracts purified by phenyl-Sepharose chromatography and are representative of MAP kinase activity present.

Activation of MAP kinase by GH was dependent upon the prior incubation period of the cells in serum-free media. Thus little or no stimulation was observed in cells serum-starved for 2 h (Table 1). Optimal responses required an overnight (18 h) preincubation in the absence of serum. In contrast, a 2 h preincubation in serum-free conditions was sufficient to render the cells maximally responsive to EGF (Table 1).

Activation of MAP kinase by GH occurred transiently (Fig. 1), with a maximal response around 10 min after the addition of hormone. Thereafter, the activation declined but remained above basal levels for at least 60 min. Separate experiments showed that MAP kinase activity remained above basal levels in cells treated for 18 h with GH (results not shown).

Activation of MAP kinase was observed at physiological concentrations of GH, with an average half-maximally effective dose of 2.95 ± 0.59 nM (mean $\pm$ S.D. from three separate experiments), and maximal effects around 10 nM. There was no significant difference in potency between ovine GH, routinely

Table 1. Comparison of effects of short- and long-term serum deprivation on subsequent stimulation of MAP kinase by GH and EGF

Cells were incubated in DMEM without serum for the indicated times prior to the addition of GH or EGF. Cytosolic extracts were then prepared and processed for MAP kinase assay as described in the Materials and methods section. Data represent the average fold stimulation of enzyme activity $\pm$ S.E.M. from 3–6 separate experiments.

Agent	Time without serum (h)...	Activation (fold)	
		2	18
GH (10 nM, 10 min)		1.37 ± 0.11	3.44 ± 1.26
EGF (10 nM, 10 min)		9.27 ± 0.33	8.00 ± 0.93

Fig. 1. Time course for activation of MAP kinase and S6 kinase by GH in 3T3-F442A preadipocytes

Cells, preincubated for 18 h in serum-free media, were treated with GH (10 nM) for the indicated times, followed by extraction and processing for assay of MAP kinase or S6 kinase as described in the Materials and methods section. MAP kinase data points (■) are averages of triplicate assay determinations for each time point. S6 kinase data (△) represent the incorporation of ^{32}P into S6 for 15 min under standard assay conditions, determined by densitometric scanning of the exposed X-ray film, and are expressed in arbitrary absorbance units. Both sets of data are from a representative experiment repeated on two further occasions.

used in these studies, and recombinant human GH (results not shown), indicating that these effects were due to GH itself and not to a contaminating ligand.

Since p42 MAP kinase has been shown previously to phosphorylate and activate the M_r -90000 S6 kinase [15], it was expected that the activation of MAP kinase by GH would be accompanied by increased S6 kinase activity. Fig. 1 shows that GH increased, in a time-dependent manner, the activity of a kinase(s) in cytosolic extracts which uses ribosomal protein S6 as substrate. The time course for activation of this S6 kinase was, as for MAP kinase, transient, with an apparent peak response at around 20 min.

A correlation between activated MAP kinase and the anti-phosphotyrosine immunoreactivity of an approx. M_r -40000 cytosolic protein has been widely observed in cells stimulated with growth factors [13,19–22]. Immunoblotting of cytosolic extracts from GH-treated cells with anti-phosphotyrosine antibodies revealed an immunoreactive protein of approx. M_r 40000 which was undetectable in control cell extracts (Fig. 2). Interestingly, GH treatment also induced an increase in the phospho-

Fig. 2. Immunoblotting of extracts from 3T3-F442A preadipocytes with anti-phosphotyrosine antibodies

Cells, preincubated in serum-free media for 18 h, were treated without (lane 1) or with (lane 2) GH (10 nM, 10 min). The cells were then lysed and equal quantities (75 µg) of protein from each sample were subjected to SDS/PAGE and Western blotting as described in detail in the Materials and methods section. Arrows indicate the three protein bands of approx. M_r 40000, 43000 and 95000 which exhibit a GH-dependent increase in phosphotyrosine content.

tyrosine content of two other cytosolic proteins, of approx. M_r 43000 and 95000 (Fig. 2).

Since PKC is thought to be involved in the transduction of some GH signals [8–11], and may also participate in stimulatory pathways leading to MAP kinase [13,20,21,23], the role of PKC in the activation of MAP kinase by GH was investigated. Chronic treatment of most isolated cells with high concentrations of tumour-promoting phorbol esters such as PMA leads to a > 95% loss of cellular PKC [12]. Pretreatment of 3T3-F442A cells in this way abolished the stimulatory effect of acute low doses (10 nM, 15 min) of PMA on MAP kinase activity (Table 2) and therefore is likely to have effectively depleted PKC to very low levels. This treatment also markedly reduced the stimulatory effect of GH on MAP kinase activity (Table 2) and on S6 kinase activity (results not shown). Pretreatment of cells with the selective PKC inhibitor staurosporine [24] also greatly reduced the ability of GH to activate MAP kinase (Table 2). In contrast, the degree of activation of MAP kinase by EGF was only marginally affected either by PMA-induced depletion of PKC or by pretreatment with staurosporine (Table 2).

Signalling by GH has also been postulated to involve a pertussis toxin-sensitive G-protein [25]. Pretreatment of 3T3-F442A cells with pertussis toxin, which abolishes the function of the G_i and G_o families of GTP-binding proteins [26], had no significant effect on the subsequent stimulation of MAP kinase by GH (results not shown). The activity of the pertussis toxin was confirmed by testing its ability to catalyse the ADP-ribosylation of an M_r 41000 protein in Rat-1 fibroblast membranes.

DISCUSSION

The findings of this study provide direct evidence that GH, at physiological concentrations, rapidly activates both MAP kinase and S6 kinase in 3T3-F442A preadipocytes. The time course for the activation of MAP kinase (Fig. 1), with maximal stimulation occurring around 10 min after addition of GH, is very similar to that observed for other growth factors [18,23,27].

MAP kinase has been shown to phosphorylate and activate the 90000- M_r form of S6 kinase in a protein kinase cascade which may be involved in the hyperphosphorylation of ribosomal protein S6 induced by many mitogenic agents [15,19]. The genetically distinct 70000- M_r S6 kinase, which also becomes activated in many growth factor-stimulated cells, is not a substrate for MAP kinase [28,29], and therefore probably lies on a separate pathway. S6 kinases of M_r 90000 are maximally activated within 10–15 min, whereas S6 kinases of M_r 70000 reach a peak of activation 30–60 min after cell treatment [19]. S6 kinase activity in 3T3-F442A cells peaked around 20 min after the addition of GH (Fig. 1), suggesting that at least part of the observed increase in S6 kinase activity in GH-treated cells was due to activation of p90 S6 kinase. However, the data do not exclude the possibility of p70 S6 kinase activation by GH, and further work will be required to demonstrate unequivocally which forms of S6 kinase are activated by GH in this system.

Since activation of MAP kinase requires its phosphorylation on tyrosine and threonine residues [14], the discovery that GH activates this kinase thereby shows that GH promotes tyrosine phosphorylation within the cell. A more direct demonstration of this was achieved by anti-phosphotyrosine immunoblotting of cell extracts, which showed that GH promoted the tyrosine phosphorylation of three cytosolic proteins (Fig. 2). The 40000- M_r species is very likely to be p42 MAP kinase itself, since a phosphotyrosine-containing protein of this size is normally detected in cells with elevated MAP kinase activity [13,19–22]. The 43000- M_r species probably represents p44 MAP kinase, a genetically close relative of p42 MAP kinase whose activation is also accompanied by tyrosine phosphorylation. Indeed, tyrosine phosphorylation of two cytosolic proteins in the M_r 40000–45000 range is a characteristic feature in many cells treated with

Table 2. Effects of staurosporine and cellular depletion of PKC on the stimulation of MAP kinase by GH and EGF

Cells were depleted of cellular PKC by pretreatment with 16 µM-PMA for 16 h [14], followed by incubation with GH or EGF. Control cells received the equivalent amount of dimethyl sulphoxide (0.1%, v/v). In separate experiments, cells were pretreated for 15 min with 500 nM-staurosporine prior to hormone treatment, with control cells receiving the equivalent amount of dimethyl sulphoxide (0.01%, v/v). After hormonal stimulation, cytosolic extracts were prepared and processed for the MAP kinase assay as described in the Materials and methods section. Data represent the average fold stimulation of enzyme activity ± S.E.M. from three separate experiments. n.d., not determined.

Agent	Pretreatment ...	Activation (fold)			
		Chronic PMA		Staurosporine	
		–	+	–	+
PMA (10 nM, 15 min)		7.64 ± 0.71	0.93 ± 0.08	n.d.	n.d.
GH (10 nM, 10 min)		4.35 ± 0.76	1.63 ± 0.17	5.72 ± 0.52	0.79 ± 0.22
EGF (10 nM, 10 min)		4.74 ± 0.15	3.85 ± 0.17	6.88 ± 0.67	5.02 ± 0.17

mitogens and other extracellular agents [19]. The identity of the 95000-*M_r* species exhibiting GH-dependent increased tyrosine phosphorylation remains unknown at present.

Tyrosine phosphorylation of MAP kinase is not believed to occur through the direct action of any receptor tyrosine kinase. It is thought instead that a complex chain of events initiated after ligand binding leads to the activation of certain factors which promote MAP kinase phosphorylation on tyrosine and threonine residues [30,31]. It is unlikely, therefore, that the GH receptor, postulated to possess tyrosine kinase activity [6], phosphorylates MAP kinase directly.

Intracellular pathways leading to MAP kinase activation have been shown previously to involve PKC. For example, phorbol esters are potent activators of MAP kinase in intact cells [13,20,23], and depletion of cellular PKC levels, by prolonged phorbol ester treatment, has been shown to abrogate the activation of MAP kinase by some, although not all, hormones [13,23]. Depletion of PKC in 3T3-F442A cells greatly reduced activation of MAP kinase by GH (Table 2), suggestive of a role for PKC in the mechanism whereby GH activates this enzyme. This idea is supported by the results obtained using the PKC inhibitor staurosporine (Table 2). This agent, though selective, is not entirely specific for PKC and has been shown to inhibit a number of other protein kinases [24]. Nevertheless, taken together, the data strongly suggest that PKC plays a role in mediating the activation of MAP kinase by GH, and support other studies suggesting that PKC is involved in GH signalling mechanisms [8–11]. The observed residual stimulation by GH after prolonged phorbol ester treatment is unlikely to have been due to incomplete removal of all cellular PKC, since cells treated in this way showed no subsequent response to acute PMA challenge (Table 2). It is more likely that there exist both PKC-dependent and -independent mechanisms for activation of MAP kinase by GH.

GH appears to differ from EGF and other agents in the period of serum deprivation required to prime cells to the stimulatory effects of the hormone on MAP kinase (Table 1, and refs. [13,14,27]). Positive effects of GH on other cellular processes such as glucose transport [32] and protein synthesis [33] also require prolonged preincubations in the absence of GH, although it is not clear whether such effects are relevant physiologically or are peculiar to cultured cell systems grown in high concentrations of animal sera. Another apparent difference between GH and EGF lies in the PKC requirement for MAP kinase activation. Whereas activation of MAP kinase and S6 kinase by GH appears to be largely dependent upon functional PKC, activation by EGF proceeds almost normally in its absence (Table 2). Such differences may reflect subtle variations in the signalling mechanisms of a mitogenic agent (EGF) and a putative differentiative agent (GH), as reported in rat PC12 cells where MAP kinase is activated by both EGF, which is mitogenic, and nerve growth factor, which promotes differentiation [21]. The fundamental question remains, however, as to why MAP kinase and S6 kinase should be activated by two agents which promote widely different cell responses. Further work investigating the activation of MAP kinase and S6 kinase isoenzymes may help to clarify this apparent anomaly.

To summarize, the results of this study show that GH activates MAP kinase and S6 kinase, and promotes the tyrosine phosphorylation of three cytosolic proteins in 3T3-F442A preadipocytes. GH thus shares features of its signal transduction mechanism with many other hormones and growth factors known to activate these enzymes in many cells. The activation of MAP kinase and S6 kinase may be mediated in part by PKC, but appears not to involve pertussis toxin-sensitive G-proteins.

Future studies are required to address the question of which members of the MAP kinase and S6 kinase families are regulated by GH, and should attempt to delineate their role in the transduction of individual signals which ultimately lead to the promotion of cell growth and differentiation in preadipocytes.

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