Requirement for phosphoinositide 3-OH kinase in growth hormone signalling to the mitogen-activated protein kinase and p70^{s6k} pathways

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Pituitary growth hormone (GH) co-ordinately stimulates three distinct signalling pathways in 3T3-F442A preadipocytes, the STAT (signal transducer and activator of transcription) pathway, the mitogen-activated protein (MAP) kinase cascade and p70^{s6k}. The mechanisms linking the GH receptor to these signals have not been fully identified. In this study we have examined the role of phosphoinositide 3-OH kinase (PI 3-kinase). Pretreatment of cells with wortmannin, a specific inhibitor of PI 3-kinase, prevented the activation of p70^{s6k} and partially inhibited the activation of p42 and p44 MAP kinases by GH. In contrast, wortmannin failed to appreciably affect the GH-stimulated tyrosyl phosphorylation of JAK-2 or STAT-1. GH transiently increased the activity of PI 3-kinase recovered in anti-phosphotyrosine immunoprecipitates. In addition, several tyrosyl-phosphorylated proteins were specifically adsorbed from lysates of cells exposed to GH by a glutathione S-transferase fusion protein containing the 85 kDa regulatory subunit of PI 3-kinase. GH also induced an increase in the PI 3-kinase activity associated with both JAK-2 and insulin receptor substrate-1 (IRS-1) immunoprecipitates. These results establish PI 3-kinase as an important mediator of GH signalling to the MAP kinase and p70^{s6k} pathways and suggest that PI 3-kinase is activated by a mechanism involving JAK-2 and IRS-1.

INTRODUCTION

Pituitary growth hormone (GH) influences the growth and metabolism of many different cell types (see [1] for review). Among the best characterized of these actions are those in adipose cells. In the preadipocyte cell line 3T3-F442A GH promotes differentiation [2], whereas in primary mature adipocytes GH exerts acute insulin-like and chronic anti-insulin metabolic effects [3]. At the intracellular level the molecular mechanisms underlying these actions have not been fully elucidated, although recently some of the early post-receptor signalling events have been characterized. Most notably GH has been shown to induce the tyrosyl phosphorylation of several intracellular proteins [4–7]. The GH receptor is a member of the cytokine receptor superfamily [8] and as such does not itself possess intrinsic tyrosine kinase activity. Instead, tyrosine phosphorylation is initiated by activation of a receptor-associated cytosolic tyrosine kinase JAK-2 [9]. JAK-2 is a member of the recently described Janus family which act as surrogate tyrosine kinases for receptors of the cytokine superfamily (see [10,11] for reviews). The *in vivo* substrates for JAK-2 have not been clearly defined although members of the STAT (**s**ignal **t**ransducer and **a**ctivator of **t**ranscription) family of cytosolic transcription factors are strong candidates. GH induces the tyrosyl phosphorylation and nuclear accumulation of STAT-1α [12], and activates c-*fos* transcription via a STAT-binding element [13].

GH also induces signals which lead to activation of mitogen-activated protein (MAP) kinase [4,14,15], p90^{rsk} [16] and p70^{s6k} [16]. Co-ordinate activation of these enzymes is believed to lead to selective changes in gene transcription and protein synthesis and is therefore likely to be crucial for cellular responses to GH. Although JAK-2 is proposed to be required for activation of MAP kinase by GH [17], the signalling events which link JAK-2 activation to MAP kinase and indeed p70^{s6k} have not been established.

The lipid kinase phosphoinositide 3-OH kinase (PI 3-kinase) is implicated in the regulation of several cellular processes including membrane trafficking, mitogenesis and glucose transport (see [18] for review). This family of proteins comprise an 85 kDa regulatory subunit (p85) and a 110 kDa catalytic subunit which exhibits both lipid and protein kinase activity. The regulatory subunit contains two *src*-homology 2 (SH2) domains which contact phosphorylated tyrosyl residues within the sequence Y-X-X-M (single-letter amino acid code) on receptors and other proteins, leading to stimulation of lipid kinase activity. There is evidence that *in vivo* PI 3-kinase phosphorylates phosphatidylinositol-4,5-bisphosphate [19,20] generating phosphatidylinositol-3,4,5-trisphosphate, a candidate second messenger which activates certain isoforms of protein kinase C (PKC) *in vitro* [21]. In this regard we have obtained evidence that implicates PKC in GH signalling to MAP kinase and p70^{s6k} [4,16]. This prompted us to examine first, whether GH is capable of activating PI 3-kinase in 3T3-F442A cells and secondly, whether PI 3-kinase is involved in the activation of the signalling pathways described above.

MATERIALS AND METHODS

Materials

Purified human GH (Lot no. AFP 9755A) was obtained from the National Hormone and Pituitary Program, Bethesda, MD,

Abbreviations used: GH, pituitary growth hormone; STAT, signal transducer and activator of transcription; PI 3-kinase, phosphoinositide 3-OH kinase; PDGF, platelet-derived growth factor; PKC, protein kinase C; GST, glutathione S-transferase; IRS-1, insulin receptor substrate-1; p85, 85 kDa regulatory subunit of PI 3-kinase; SH2, *src* homology domain 2; MAP, mitogen-activated protein.

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U.S.A. Culture media and calf serum were obtained from Life Technologies, Paisley, U.K. Wortmannin, phosphatidylinositol, phosphatidylinositol 4-phosphate and myelin basic protein were obtained from Sigma. [γ-^{32}P]ATP (> 3000 Ci/mmol) was obtained from Amersham. Silica gel 60 glass plates for TLC were obtained from Merck. Protein A–agarose and Protein G–agarose were obtained from Pierce-Warriner, Chester, U.K. Phenyl-Sepharose and glutathione-Sepharose were obtained from Pharmacia.

Cell culture

3T3-F442A cells (from Dr. Howard Green, Harvard Medical School) were grown as described previously [4]. Confluent cultures were incubated in serum-free Dulbecco's modified Eagle's medium containing 25 mM Hepes for 16–20 h before treatment with GH or other agents. Wortmannin was dissolved in DMSO at a concentration of 10 mM and stored in the dark. Immediately prior to use it was diluted into water before addition to cells.

Antibodies

Anti-phosphotyrosine (clone 4G10) and anti-JAK-2 antibodies were purchased from TCS, Botolph Claydon, Bucks., U.K. Anti-STAT-1 was purchased from Affiniti, Nottingham, U.K. Rabbit polyclonal antibodies to p70^{s6k} were raised to a synthetic N-terminal peptide from the rat sequence (amino acids 1–31, [22]). Rabbit polyclonal antibodies to the GH receptor were raised to a synthetic peptide from the mouse sequence (amino acids 425–436, [23]). Rabbit polyclonal antibodies to p44 MAP kinase (ERK1) were raised to a synthetic peptide from the rat sequence (amino acids 325–345). Polyclonal antibodies to JAK-1 and insulin receptor substrate-1 (IRS-1) and a monoclonal antibody to p42 MAP kinase (ERK2) were generous gifts from Dr. Andrew Wilks (Melbourne), Professor Ken Siddle (Cambridge) and Professor Ailsa Campbell (Glasgow), respectively.

PI 3-kinase assay

PI 3-kinase activity was measured on anti-phosphotyrosine, anti-JAK-2 or anti-IRS-1 immune complexes using slight modifications of the method described by Carter and Downes [24]. Approximately 5×10^6 cells were lysed in 0.75 ml of ice-cold Buffer A (25 mM Tris/acetate, pH 8.0, 2.5 mM EDTA, 2.5 mM EGTA, 50 mM NaCl, 50 mM NaF, 1 % Nonidet P-40, 1 mM PMSF, 1 mM sodium orthovanadate and 4 μg/ml each of pepstatin A, aprotinin and leupeptin). After 20 min the lysate was centrifuged at 12000 ***g*** in a microfuge and the resulting supernatant incubated with Protein A–agarose for 1 h. The precleared lysates were then incubated with either 2 μg of anti-phosphotyrosine (4G10), 5 μg of anti-JAK-2, 10 μl of anti-JAK-1 or 7 μl of anti-IRS-1 for 2 h. A 20 μl aliquot of a 50 % slurry of Protein A–agarose was then added and the incubation continued for 16 h. The immunoprecipitates were then washed exactly as described in [24] and assays were performed directly on the immune complex. Reactions (60 μl total volume), comprising 40 μg of phosphatidylinositol in assay buffer (20 mM Hepes, pH 7.4, 0.4 mM EGTA and 0.4 mM sodium phosphate), were initiated by adding ATP/$MgCl_2$ (final concentrations 40 μM ATP, 10 mM $MgCl_2$ and 20 μCi of [γ-^{32}P]ATP per reaction). After 20 min at 30 °C reactions were stopped by the addition of 30 μl of 4 M HCl and 130 μl of chloroform/methanol (1:1, v/v). Tubes were then vortexed and centifuged at 3000 ***g*** in a microfuge for 2 min. The lower phase was washed three times with a synthetic upper phase [24] and 20 μl of the lower phase was then applied directly to a silica gel 60 TLC plate previously treated with 1 % potassium oxalate and developed for 3–4 h using chloroform/methanol/35 % ammonia/water (49.4:38.7:7.1:4.8, by vol.).

Western blotting

Cells were lysed in Buffer B (25 mM Hepes, pH 7.5, 2.5 mM EDTA, 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 10 % glycerol, 1 % Triton X-100, 1 mM sodium orthovanadate, 0.4 mM PMSF and 2 μg/ml each of leupeptin, pepstatin A and aprotinin), clarified by centifugation (12000 ***g***, 10 min) and denatured by adding 0.25 vol. of 5 × concentrated sample buffer. Equal quantities of lysate protein (50 μg) were run on 8 or 10 % polyacrylamide gels. Following transfer to nitrocellulose, blots were probed with anti-p70^{s6k} (1:1000 dilution), anti-(MAP kinase) (1:1000) or anti-phosphotyrosine (1:4000) antibodies and immunoreactive bands detected using the enhanced chemiluminescence (ECL) (Amersham) system.

MAP kinase assays

MAP kinase was assayed following partial purification of cell lysates by batch adsorption to phenyl-Sepharose, as described previously [25]. Briefly, cell extracts were mixed with 150 μl of phenyl-Sepharose for 5 min. The Sepharose was then washed, in a step-wise fashion, with buffers containing increasing concentrations of ethylene glycol. MAP kinases, which bind tightly to this matrix [26], were eluted with buffer containing 60 % ethylene glycol and assayed using myelin basic protein as substrate.

Adsorption of proteins from cell lysates with recombinant GST–p85

Cell lysates prepared in Buffer A containing 10 % glycerol were precleared for 30 min with 50 μl of a 50 % slurry of glutathione-Sepharose. The lysates were then incubated for 1 h with 3 μg of GST–p85 precoupled to glutathione-Sepharose. The beads were then washed twice with Buffer A and once with Tris-buffered saline. Precipitated proteins were eluted by the addition of 75 μl of sample buffer followed by heating to 100 °C for 5 min. Eluted proteins were electrophoresed on 7 or 8 % polyacrylamide gels, transferred to nitrocellulose and immunoblotted with the appropriate antibody. The peptides used for competition experiments were generously provided by Dr. Phillip Hawkins (Cambridge). They were made from the region of the platelet-derived growth factor (PDGF) β receptor known to bind to the SH2 domains of PI 3-kinase [18] (amino acids 737–757). One peptide (740/751) was chemically phosphorylated on tyrosines 740 and 751 and the other non-phosphorylated as a control. The phosphorylated peptide has been shown previously to activate PI 3-kinase *in vitro* [27]. The appropriate peptide (final concentration 5 μM) was preincubated with glutathione-Sepharose beads containing prebound GST–p85. Excess peptide was removed by washing, and lysates containing 5 μM peptide were then mixed with the beads and incubated as above.

Presentation of data

Except where indicated all data shown are representative of experiments done on at least three occasions.

RESULTS

Wortmannin affects GH signalling

To examine the role of PI 3-kinase in GH action we first tested whether the highly potent and selective PI 3-kinase inhibitor wortmannin [28] affected any of the signalling pathways activated by GH in 3T3-F442A cells. Figure 1 shows that treatment of cells with wortmannin prevented, in a dose-dependent manner, the activation of $p70^{s6k}$ by GH. Activation was assessed by mobility shift due to enhanced phosphorylation of $p70^{s6k}$ which correlates with enzyme activation [16,29]. Complete inhibition occurred with 50 nM wortmannin, with a half-maximal effect around 10 nM; this is consistent with concentrations reported to inhibit PI 3-kinase *in vitro* [30,31].

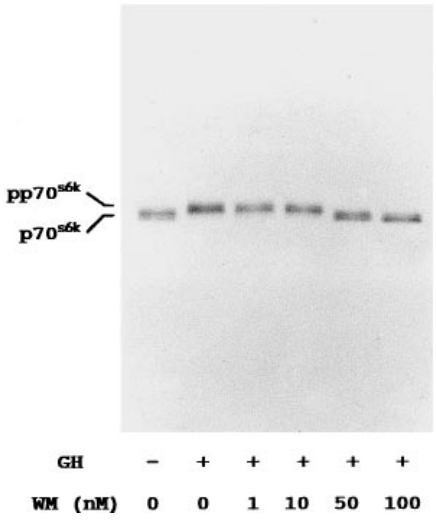

Figure 1 The effect of wortmannin on the activation of $p70^{s6k}$ by GH

Cells were pretreated with the indicated concentrations of wortmannin for 5 min prior to the addition of GH for a further 10 min. Cells were lysed and equal quantities of protein immunoblotted with anti-$p70^{s6k}$ antibodies. The positions of the hypo- (p70) and hyper- (pp70) phosphorylated forms of $p70^{s6k}$ are indicated.

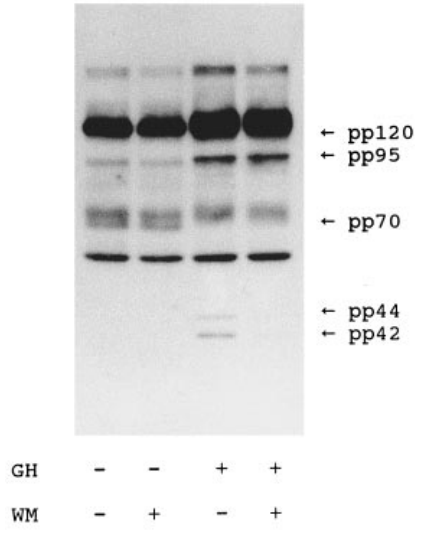

Figure 2 The effect of wortmannin on GH-induced protein-tyrosyl phosphorylation

Cells were pretreated with 100 nM wortmannin or vehicle for 5 min followed by addition of GH or vehicle for a further 10 min. Cells were lysed and equal quantities of protein immunoblotted with anti-phosphotyrosine antibodies. The apparent molecular masses (kDa) of major proteins exhibiting increased tyrosyl phosphorylation in response to GH are indicated.

Table 1 Wortmannin blocks GH but not phorbol ester activation of MAP kinase

Cells were pretreated with 100 nM wortmannin for 5 min followed by the addition of GH (10 nM) or phorbol 12-myristate 13-acetate (PMA) (10 nM) or vehicle for a further 10 min. Cells were then lysed and MAP kinase activity determined after partial purification of extracts with phenyl-Sepharose, as described in the Materials and methods section. MAP kinase activities are expressed as incorporation of ^{32}P into myelin basic protein during a 10 min assay under standard conditions [4]. Data are means ± S.E.M. from three experiments.

	10^{-3} × MAP kinase activity (c.p.m.)	
	− Wortmannin	+ Wortmannin
Basal	25.1 ± 0.7	25.4 ± 0.6
Growth hormone	87.5 ± 1.4	42.6 ± 2.1
PMA	82.0 ± 1.1	71.9 ± 3.7

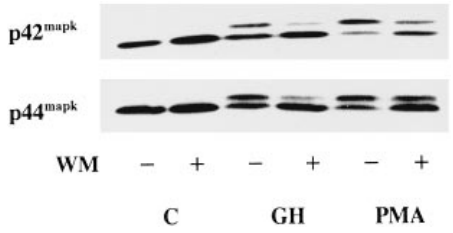

Figure 3 The effect of wortmannin on the activation of MAP kinase by GH and phorbol ester

Cells were pretreated with 100 nM wortmannin for 5 min followed by the addition of GH (10 nM) or phorbol 12-myristate 13-acetate (PMA; 10 nM) or vehicle for a further 10 min. Cells were then lysed and immunoblotted with antibodies to either p42 (ERK2) or p44 (ERK1) MAP kinase as described in the Materials and methods section.

In contrast to the above findings, wortmannin appeared to have little effect on GH's ability to promote protein-tyrosyl phosphorylation (Figure 2). Exceptions were two protein bands at 44 kDa and 42 kDa, whose tyrosyl phosphorylation was greatly decreased by wortmannin treatment. These bands were shown previously to represent the p44 and p42 forms of MAP kinase, ERK1 and ERK2 [4,14,15]. Since tyrosyl phosphorylation of MAP kinases is required for their activation [32], this result suggested that wortmannin was preventing their activation by GH. To confirm this we carried out parallel measurements of MAP kinase enzymic activity. Table 1 shows that the approximately 4-fold maximal activation of MAP kinase by GH was severely attenuated when cells were pretreated with wortmannin. Qualitatively similar effects of wortmannin were observed when MAP kinase activation was assessed by gel mobility shift (Figure 3). In contrast, stimulation of MAP kinase by phorbol ester was little affected by wortmannin (Table 1 and Figure 3), indicating that it did not interfere with the inherent ability of MAP kinase to be activated. Additional experiments revealed that wortmannin failed to affect the extent of GH-induced tyrosyl phosphorylation of JAK-2 or STAT-1, although we consistently observed a small increase in GH-induced tyrosyl phosphorylation of its receptor (results not shown).

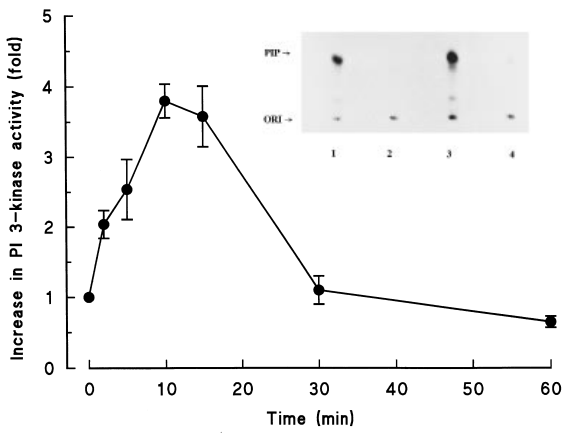

Figure 4 GH stimulates PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates

Cells were treated for the indicated times with GH followed by cell lysis and immunoprecipitation with anti-phosphotyrosine antibodies. PI 3-kinase assays were performed on the washed immune complexes as described in the Materials and methods section. Following TLC the phosphatidylinositol 3-phosphate (PIP) spot was scraped from the plate and counted for radioactivity. The data represent the mean ± S.E.M. increase in PIP formation relative to unstimulated cells and are derived from at least three independent measurements at each time point. Inset: samples from unstimulated (lanes 1 and 2) or GH-stimulated (10 min) cells (lanes 3 and 4) were assayed for PI 3-kinase in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 100 nM wortmannin. Abbreviation: ORI, Origin.

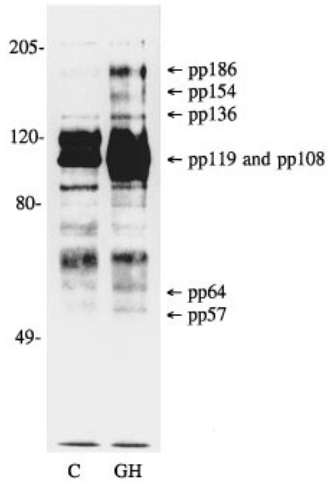

Figure 5 Specific adsorption of phosphotyrosyl proteins to GST–p85

Lysates from control (C) or GH-treated (25 nM, 5 min) cells were incubated with GST–p85 bound to glutathione-Sepharose as described in the Materials and methods section. Adsorbed proteins were eluted from the Sepharose, electrophoresed and blotted with anti-phosphotyrosine antibodies. The apparent molecular masses ($\times 10^{-3}$) of proteins whose adsorption to p85 increased in response to GH are indicated on the right and the mobilities of standard markers on the left of the Figure.

GH stimulates association of PI 3-kinase with phosphotyrosyl proteins

The preceding results provide evidence that PI 3-kinase is involved in certain aspects of GH signalling. We next attempted to demonstrate increased PI 3-kinase activity in GH-treated cells. The activation of PI 3-kinase by many growth factors involves its association with tyrosine-phosphorylated proteins through the SH2 domains of the 85 kDa regulatory subunit, p85 [18]. To test whether GH regulates PI 3-kinase in a similar fashion we measured the PI 3-kinase activity associated with anti-phosphotyrosine immunoprecipitates. Figure 4 (inset) shows that GH induced an increase in the generation of the major ^{32}P-labelled lipid product resolved by this TLC system. This material co-migrated with a monophosphorylated phosphatidylinositol standard (phosphatidylinositol 4-phosphate) which does not resolve from phosphatidylinositol 3-phosphate under these conditions [24]. The formation of phosphatidylinositol monophosphate was blocked by the inclusion of wortmannin in the assay mixture. At the concentration used (100 nM) wortmannin does not significantly inhibit mammalian PI 4-kinase [30,33], indicating that phosphorylation of phosphatidylinositol was occurring predominantly on the 3-OH and not the 4-OH position. An examination of the time course of PI 3-kinase activation by GH revealed a detectable increase by 2 min, with a maximal 3–4-fold response occurring after 10–15 min (Figure 4).

Association of p85 with phosphotyrosyl proteins

To begin the identification of tyrosyl-phosphorylated protein(s) involved in binding to PI 3-kinase we used a recombinant protein containing p85 fused to GST to probe lysates of cells stimulated with GH. The p85α isoform was used since immunoblotting experiments revealed that 3T3-F442A cells contain p85α and not p85β (results not shown). Figure 5 shows an anti-phosphotyrosine immunoblot of proteins adsorbed from cell lysates by recombinant p85. Several tyrosyl-phosphorylated proteins showed a GH-dependent increase in adsorption to p85, the most predominant of which exhibited apparent molecular masses of 119 kDa and 186 kDa. Other proteins (approx. 154, 136, 108, 64 and 57 kDa) were also consistently observed. Adsorption of these proteins to GST–p85 was prevented by the doubly tyrosyl-phosphorylated (740/751) PDGF receptor peptide but not by the equivalent non-phosphorylated peptide (results not shown).

Recently it has been shown that GH induces the tyrosyl phosphorylation of IRS-1 and its association with p85 in 3T3-F442A cells [34]. We have further observed that GH induces a time-dependent increase in PI 3-kinase activity associated with anti-IRS-1 immunoprecipitates, maximal 2.9 ± 0.4 ($n = 3$) fold activation occurring after 10 min stimulation. It seems likely therefore that the 186 kDa tyrosyl-phosphorylated protein associated with p85α (Figure 5) represents IRS-1.

Association of PI 3-kinase with JAK-2

The preceding results provide strong evidence that activation of PI 3-kinase by GH involves one or more phosphotyrosyl proteins, thus implying the involvement of a GH-stimulated tyrosine kinase. Since the only tyrosine kinase known to be activated by GH is JAK-2, we sought evidence for its involvement in PI 3-kinase activation by GH. Immunoblotting of anti-IRS-1 or anti-p85 immunoprecipitates or of recombinant p85 adsorbates failed to reveal the presence of JAK-2 protein (results not shown). In another approach we attempted to measure PI 3-kinase activity directly on anti-JAK-2 immunoprecipitates. Figure 6 shows that GH induced an increase in PI 3-kinase activity associated with anti-JAK-2 immunoprecipitates. This activity was almost completely inhibited by the presence of wortmannin in the assay and

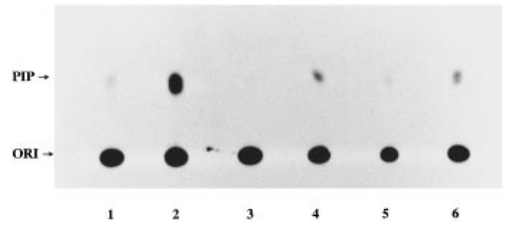

Figure 6 PI 3-kinase activity in anti-JAK immunoprecipitates

Cells were treated with GH (lanes 2, 4 and 6) or vehicle (lanes 1, 3 and 5) for 2 min then lysed and immunoprecipitated with anti-JAK-2 (lanes 1–4) or anti-JAK-1 (lanes 5 and 6) antibodies as described in the Materials and methods section. PI 3-kinase assays were performed on the washed immune complexes in the presence (lanes 3 and 4) or absence (lanes 1, 2, 5 and 6) of 100 nM wortmannin. Abbreviations: ORI, origin; PIP, phosphatidylinositol 3-phosphate.

was not precipitated with antibodies to another Janus kinase JAK-1.

DISCUSSION

GH exerts a variety of metabolic and growth-enhancing effects in different cells by mechanisms which remain to be fully characterized. We have shown previously that GH co-ordinately stimulates the MAP kinase [4] and p70^{s6k} pathways [16] in 3T3-F442A preadipocytes, where GH promotes differentiation into adipocyte-like cells. The results of the present study show, for the first time, that activation of PI 3-kinase is necessary for the full stimulation of both MAP kinase and p70^{s6k} by GH in these cells.

The activation of transcription factors by MAP kinases [35] is likely to be an important part of the mechanism by which GH promotes changes in gene expression associated with the early stages of differentiation. Indeed cellular depletion of MAP kinases blocks differentiation of the related 3T3-L1 cell line [36], as does exposure of preadipocytes to wortmannin [37,38]. Thus a signalling pathway involving the sequential activation of PI 3-kinase and MAP kinase may be critical for preadipocyte differentiation. The fact that wortmannin does not completely block GH-activated MAP kinase suggests that PI 3-kinase-independent mechanisms also exist for the activation of MAP kinase by GH. The site of PI 3-kinase involvement in MAP kinase activation cascades appears to vary depending upon both the extracellular stimulus and the cell context, with reports placing PI 3-kinase either upstream or downstream of Ras [39,40]. In the present study, the inability of wortmannin to significantly affect MAP kinase activation by the phorbol ester phorbol 12-myristate 13-acetate indicates that its cellular target lies either upstream of PKC on the pathway leading to MAP kinase or on a distinct pathway. In this context we have reported previously that PKC is required for full activation of both MAP kinase and p70^{s6k} by GH [4,16]. Furthermore, although the downstream effectors of PI 3-kinase have not been established, phosphatidylinositol-3,4,5-trisphosphate, the product of PI 3-kinase activity, has been shown to activate PKCζ [21] and other PKC isoforms [41] *in vitro*. The possible connection between PI 3-kinase and PKC in GH signalling is currently being investigated.

P70^{s6k} phosphorylates ribosomal protein S6 which results in enhanced mRNA translation and increased protein synthesis [42]. The activation of p70^{s6k} by GH is likely to be important for the process of preadipocyte differentiation in which the synthesis of more than 100 proteins is increased [43]. The mechanism of activation of p70^{s6k} is unknown but appears to involve its phosphorylation on multiple sites by more than one protein kinase [42]. Recent studies using wortmannin and other specific inhibitors have indicated that PI 3-kinase is necessary for activation of p70^{s6k} by several tyrosine kinase receptors [44,45]. Our results now show that PI 3-kinase is similarly involved in the activation of p70^{s6k} by the GH receptor, a member of the cytokine receptor superfamily. In contrast to its partial inhibitory effects on MAP kinase activation, wortmannin appears to completely inhibit the activation of p70^{s6k} by GH, indicating an absolute requirement for PI 3-kinase in the activation mechanism.

In addition to the above findings indicating an involvement of PI 3-kinase in GH signalling, we have shown, for the first time, that GH activates PI 3-kinase in 3T3-F442A cells. This increase in activity was precipitated by anti-phosphotyrosine antibodies, indicating the involvement of at least one phosphotyrosyl protein in the activation mechanism. That GH-stimulated PI 3-kinase activity was also precipitated by antibodies to IRS-1 indicates an involvement of this protein in the activation mechanism. Consistent with these findings, during the completion of these studies GH was shown to induce the tyrosyl phosphorylation of IRS-1 in 3T3-F442A cells [34].

Our experiments with the p85 fusion protein revealed that several tyrosyl-phosphorylated proteins were specifically adsorbed by p85. Although we cannot, at this stage, state which of these proteins bind directly to p85, peptide competition experiments indicated that the adsorption of all of the proteins was dependent upon interactions between tyrosyl-phosphorylated Y-X-X-M sequences and the SH2 domain of p85. GH induced an entirely different adsorption pattern of tyrosyl-phosphorylated proteins compared with insulin (N. G. Anderson and E. Kilgour, unpublished work), indicating a divergence in the mechanisms by which GH and insulin activate PI 3-kinase. Therefore although IRS-1 is known to mediate the activation of PI 3-kinase by insulin [46,47], questions remain as to whether its involvement accounts entirely for increased PI 3-kinase activity induced by GH in 3T3-F442A preadipocytes.

GH-stimulated PI 3-kinase activity was also precipitated by antibodies to JAK-2. This novel finding therefore suggests that JAK-2 participates in the mechanism of PI 3-kinase activation by GH. Since JAK-2 contains two Y-X-X-M sequences, one of which (amino acids 963–966 [48]) is conserved among other JAK kinases, it may interact with p85 directly. However, JAK-2 protein was not detectable either in p85 immunoprecipitates or adsorbed to GST–p85 and, although this could be for technical reasons, identification of the sites of phosphorylation on JAK-2 and their mutagenesis will be required to test this possibility directly. Alternatively JAK-2, which is the only tyrosine kinase known to be activated by GH, may phosphorylate a secondary protein(s) on tyrosyl residues and thereby promote its association with p85.

In conclusion we have shown that PI 3-kinase is necessary for the full activation of both MAP kinases and p70^{s6k} by GH. The activation of PI 3-kinase by GH involves the association of several tyrosyl-phosphorylated proteins with the p85 regulatory subunit of PI 3-kinase. The identification of these proteins should further elucidate the precise mechanisms leading to the activation of PI 3-kinase and hence downstream signalling pathways involved in the promotion of preadipocyte differentiation by GH.

We thank Andrew Wilks, Ken Siddle and Ailsa Campbell for antibodies and Phillip Hawkins for PDGF receptor peptides. We are very grateful to Pete Downes and Peter Thomason for advice about the PI 3-kinase assay and to Sandra Woodburn for expert technical assistance. This work was supported by a BBSRC Senior Research Fellowship to N. G. A. and by the Scottish Office Agriculture and Fisheries Department.

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Received 31 August 1995/10 November 1995; accepted 5 December 1995