RESEARCH COMMUNICATION

The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf

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Glycogen synthase kinase-3 (GSK3) is inactivated *in vitro* by p70 S6 kinase or MAP kinase-activated protein kinase-1 β (MAPKAP kinase-1 β ; also known as Rsk-2). Here we show that GSK3 isoforms are inhibited by 40% within minutes after stimulation of the rat skeletal-muscle cell line L6 with insulin-like growth factor-1 (IGF-1) or insulin. GSK3 was similarly inhibited in rabbit skeletal muscle after an intravenous injection of insulin. Inhibition resulted from increased phosphorylation of GSK3, probably at a serine/threonine residue(s), because it was reversed by incubation with protein phosphatase-2A. Rapamycin blocked the activation of p70 S6 kinase by IGF-1 in L6 cells, but had no

effect on the inhibition of GSK3 or the activation of MAPKAP kinase-1 β . In contrast, wortmannin, a potent inhibitor of PtdIns 3-kinase, prevented the inactivation of GSK3 and the activation of MAPKAP kinase-1 β and p70 S6 kinase by IGF-1 or insulin. Wortmannin also blocked the activation of p74^{raf-1}, MAP kinase kinase and p42 MAP kinase, but not the formation of GTP-Ras by IGF-1. The results suggest that the stimulation of glycogen synthase by insulin/IGF-1 in skeletal muscle involves the MAPKAP kinase-1-catalysed inhibition of GSK3, as well as the previously described activation of the glycogen-associated form of protein phosphatase-1.

INTRODUCTION

The stimulation of glycogen synthesis by insulin in skeletal muscle results from the dephosphorylation and activation of glycogen synthase [1–3]. Intravenous administration of insulin reduces the *in vivo* phosphate content by about 0.4 mol per subunit within minutes, causing a 70–100% elevation of activity when tested in the absence of its allosteric activator, glucose 6-phosphate [2]. Glycogen synthase is phosphorylated on nine serine residues *in vivo* by five or more protein kinases [4], but the phosphate released in response to insulin is mainly removed from one tryptic peptide containing three serine residues that are phosphorylated by glycogen synthase kinase-3 (GSK3) [2,5] and dephosphorylated by the glycogen-associated form of protein phosphatase-1 (PP1G) [6,7]. Thus the activation of glycogen synthase by insulin should be mediated via the activation of PP1G and/or the inhibition of GSK3.

PP1G comprises the β -isoform of PP1 complexed to a G-subunit which is responsible for its association with glycogen [6]. PP1G activity towards glycogen synthase is enhanced up to 2.4-fold by phosphorylation of the G-subunit at a serine residue (Site-1) whose level of phosphorylation increases *in vivo* in response to insulin [8]. Phosphorylation of Site-1 is catalysed by an insulin-stimulated protein kinase [8], identified as the Rsk-2

isoform of p90 ribosomal protein S6 kinase [9,10], which is activated by the p42 and p44 isoforms of mitogen-activated protein (MAP) kinase (p42/p44^{mapk}) *in vitro* [9,11]. MAP kinase is, in turn, activated by a protein kinase cascade thought to involve first the activation of the GTP-binding protein Ras and then the protein serine/threonine kinases p74^{raf-1} and MAP kinase kinase (MAPKK) (reviewed in [3]). Since Rsk-2 phosphorylates many proteins, but may not phosphorylate S6 *in vivo* [12], we prefer to use the name ‘MAP kinase-activated protein kinase-1’ (MAPKAP kinase-1) instead of p90 S6 kinase, by analogy with cyclic nucleotide-dependent and calcium/calmodulin-dependent protein kinases which are also named after their activators, and to avoid confusion with the distinct p70 S6 kinase (p70^{s6k}), which may be the real S6 kinase *in vivo* [12]. The isoforms Rsk-1 and Rsk-2 are therefore termed MAPKAP kinases 1 α and 1 β , respectively.

The α and β isoforms of GSK3 are inactivated *in vitro* by phosphorylation of a specific N-terminal serine residue (Ser-21 in GSK3 α ; Ser-9 in GSK3 β) catalysed by either MAPKAP kinase-1 β or p70^{s6k} [13,14], while protein kinase C phosphorylates and partially inhibits GSK3 β , but not GSK3 α *in vitro* [17]. p70^{s6k} is activated by insulin and other growth factors, but by a pathway distinct from that which activates MAPKAP kinase-1 β , because p70^{s6k} is not activated by p42/p44^{mapk} *in vitro* [15,16], and its

Abbreviations used: GSK3, glycogen synthase kinase-3; PP1G, glycogen-associated form of protein phosphatase 1; Rsk, ribosomal protein S6 kinase; MAP, mitogen-activated protein; MAPKK, MAP kinase kinase; MAPKAP, MAP kinase-activated protein; IGF-1, insulin-like growth factor-1; α -MEM, α -minimal essential medium; PP2A₁, protein phosphatase 2A₁; PI3K, PtdIns 3-kinase.

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activation *in vivo* is prevented by rapamycin, an immunosuppressant drug which has no effect on the activation of p42^{mapk} or MAPKAP kinase-1 [12].

Here we demonstrate that GSK3 is rapidly inhibited by a phosphorylation mechanism in the rat skeletal-muscle cell line L6 after stimulation by insulin or insulin-like growth factor-1 (IGF-1), and in rabbit skeletal muscle after injection of insulin *in vivo*. Our results suggest that MAPKAP kinase-1, rather than p70^{s6k} or protein kinase C, may be the enzyme which phosphorylates GSK3 *in vivo*.

MATERIALS AND METHODS

Materials

L6 skeletal-muscle cells were provided by Dr. A. Klip (Hospital for Sick Children, Toronto, Canada). α -Minimal essential medium (α -MEM) culture medium, foetal-calf serum, antimycotic/antibiotic solution for tissue culture, myelin basic protein and recombinant IGF-1 were obtained from GIBCO/BRL (Paisley, Renfrewshire, Scotland, U.K.) and human insulin from Novo Nordisk (Bagsvaerd, Denmark). Sterile trypsin solution and wortmannin were from Sigma Chemical Co (Poole, Dorset, U.K.) and Protein A-Sepharose from Pharmacia (Milton Keynes, U.K.). Protein phosphatase 2A₁ (PP2A₁) was isolated from rabbit skeletal muscle [18] by Dr. G. Moorhead in the Medical Research Council Protein Phosphorylation Unit, Dundee, and 1 unit of activity was that amount which catalysed the dephosphorylation of 1 μ mol of phosphorylase/min. Affinity-purified GSK3 α and GSK3 β antibodies were raised in rabbits against C-terminal peptides [19]. Polyclonal p74^{raf1} antisera (raised against the C-terminal peptide CTLTSPRLPRF by Dr. U. Rapp, NIH, Frederick, MA, U.S.A.), MAPKAP kinase-1 β (raised against the C-terminal peptide CNRNQSPVLEPVGRS by Dr. S. Pelech, Kinetek Corp., Vancouver, Canada) and p42^{mapk} (antiserum 122; Professor C. Marshall, Institute for Cancer Research, London, U.K.) [20] were generously given by the investigators indicated in parentheses. Monoclonal Ras antibody Y13259 was also provided by Professor Marshall, and affinity-purified rabbit polyclonal p70^{s6k} antibody raised against a C-terminal peptide AMIVRNAKDTAHTKAERNILEEVKHP was purchased from UBI (Lake Placid, NY, U.S.A.). Okadaic acid was provided by Dr. Y. Tsukitani (Fujisawa Pharmaceutical Co., Tokyo, Japan) and rapamycin by Dr. S. Schreiber (Department of Chemistry, Harvard University, Cambridge, MA, U.S.A.).

Cell culture and treatment with hormones and drugs

Monolayers of L6 muscle cells were grown to the stage of myotubes as described previously [21,22]. The cells were grown in six-well plates or 6 cm-diameter Petri dishes until confluent and fused. Myotubes were routinely deprived of serum for 5 h prior to analysis using serum-free α -MEM medium containing 25 mM glucose. At 1 h before assay they were incubated in 20 mM Hepes/NaOH (pH 7.4)/0.14 M NaCl/5 mM KCl/2.5 mM MgSO₄/1 mM CaCl₂/25 mM glucose (HBS buffer) to deplete them of amino acids. The cells were then stimulated with IGF-1 (30 nM) or insulin (100 nM) for the times indicated. The medium was rapidly removed by aspiration, the cells washed twice with HBS buffer, placed on ice and lysed in 0.2 ml of ice-cold Buffer A [50 mM Tris/HCl (pH 7.5, 20 °C)/1 mM EDTA/1 mM EGTA/1 % (v/v) Triton X-100/1 mM sodium orthovanadate/10 mM sodium glycerophosphate/50 mM NaF/5 mM sodium pyrophosphate/0.1 % (v/v) 2-mercaptoethanol] containing 0.27 M sucrose. The lysates were immediately frozen in liquid

nitrogen and stored at -80°C until used. Lysates were thawed just prior to use, centrifuged for 5 min at 14000 *g*, and the supernatants assayed for protein kinase activities. Wortmannin (100 nM) and rapamycin (100 nM) were added 10 min prior to stimulation with IGF-1. Wortmannin was stored at -20°C in dimethyl sulphoxide and diluted in aqueous solution immediately before use. Protein concentrations of lysates [23] ranged from 0.3 to 0.6 mg/ml.

Immunoprecipitation of protein kinases

Lysates were incubated for 2 h at 4°C on a shaking platform with Protein A-Sepharose-conjugated antibody, unless stated otherwise.

p74^{raf1}

A 0.1 ml sample of L6 lysate was incubated for 60 min with 5 μ l of Protein A-Sepharose conjugated to 2.5 μ l of p74^{raf1} antiserum. The suspension was centrifuged for 1 min at 13000 *g*, the supernatant discarded and the immunoprecipitate washed twice with 1.0 ml of Buffer A containing 0.5 M NaCl, and once with 1.0 ml of 50 mM Tris/HCl (20 °C)/0.1 mM EGTA/0.03 % (w/v) Brij-35/0.1 % (v/v) 2-mercaptoethanol.

p42^{mapk}

This was carried out as for p74^{raf1}, except that 10 μ l of L6 cell extract was incubated with 3 μ l of Protein A-Sepharose conjugated to 1.5 μ l of antiserum.

MAPKAP kinase-1 β

A 30 μ g portion of L6 lysate was incubated with 2.5 μ l of Protein A-Sepharose conjugated to 4 μ l of antiserum and the immunoprecipitates washed as described in [19].

GSK3

A 20 μ g portion of L6 lysate or 100 μ g of rabbit skeletal-muscle extract was incubated with 2.5 μ l of Protein A-Sepharose conjugated to 0.4 μ l of anti-GSK3 α serum plus 0.4 μ l of anti-GSK3 β serum and the immunoprecipitates washed as described in [19]. Where indicated, immunoprecipitated GSK3 was re-activated by incubation for 20 min at 30°C with 2 μ l of PP2A₁ (25 munits/ml) and the reaction terminated with 2 μ l of okadaic acid (12.5 μ M). Okadaic acid was added before PP2A₁, or PP2A₁ omitted, in control incubations.

Protein kinase assays

p74^{raf1} was assayed by the activation of MAPKK in a coupled assay using the MAPKK1 isoform and p42^{mapk} [24]. One unit of p74^{raf1} was that amount which increased the activity of p42^{mapk} by 1 unit/min. MAPKK was assayed using bacterially expressed p42^{mapk} [25]. Control incubations were carried out in parallel in which p42^{mapk} was omitted, and this 'reaction blank' subtracted from the value obtained in the presence p42^{mapk}. One unit of MAPKK was that amount which increased the activity of p42^{mapk} by 1 unit/min. p42^{mapk} was assayed with myelin basic protein [26] and MAPKAP kinase-1 β with a peptide related to the C-terminus of ribosomal S6 protein [Gly-245,Gly-246]S6 (218–249) [13]. GSK-3 was assayed with the peptide YRRAVPPSPSLSR-HSSPHQSEDEEE phosphorylated at its C-terminal serine either chemically or with casein kinase-2 (generously provided by Dr. O. Marin and Professor L. A. Pinna, University of Padova, Padova, Italy). Control incubations were carried out in parallel

using the dephosphorylated peptide (a gift of Dr. M. Goedert, MRC Laboratory of Molecular Biology, Cambridge, U.K.) and this 'reaction blank' subtracted from that obtained with the phosphopeptide. One unit of p42^{mapk}, MAPKAP kinase-1 β or GSK3 was that amount which catalysed the phosphorylation of 1 nmol of substrate/min. Protein kinase activities in immunoprecipitates were measured by adding the other assay components to the tubes containing the immunoprecipitated enzyme.

Activation of glycogen synthase and inhibition of GSK3 by insulin in rabbit skeletal muscle

Female New Zealand White rabbits were deprived of food for 24 h and propranolol (2 mg/kg) and insulin in 0.9% NaCl (INS) (1 unit/kg) or 0.9% NaCl (control, C) administered intravenously 15 min prior to killing the animals, and a further injection of insulin (1 unit/kg) or propranolol in 0.9% NaCl was given 7.5 min prior to a lethal dose of sodium pentobarbitone. Skeletal muscle was rapidly removed from the hind-limbs and placed in ice. The muscle was minced and homogenized at 4 °C in 2.5 vol. of 4 mM EDTA (pH 7.0)/50 mM NaF/1 mM orthovanadate/0.1% (v/v) 2-mercaptoethanol, centrifuged for 30 min at 4200 g, and the supernatant decanted, desalted and assayed for glycogen synthase activity in the absence and presence of glucose 6-phosphate as described in [2]. Both GSK3 isoforms were immunoprecipitated from the extracts and assayed as described above.

Measurement of the proportion of GTP bound to Ras in rat L6 cells

Confluent cells grown on 60 mm-diameter dishes were incubated for 3 h at 37 °C in serum-free α -MEM medium containing 25 mM glucose, then washed four times with HBS buffer and incubated for a further 4 h with HBS buffer containing 1 mCi/ml [³²P]P_i. The cells were then treated for 10 min with either 100 nM wortmannin or buffer and stimulated for 5 min with 30 nM IGF-1. The cells were lysed, and the amount of GTP and GDP bound to Ras determined as described previously [27].

RESULTS

IGF-1 or insulin stimulation of rat L6 cells and insulin stimulation of rabbit skeletal muscle inhibits GSK3 α and GSK3 β by a phosphorylation mechanism

IGF-1 stimulation of the rat skeletal-muscle cell line L6 induced a rapid decrease in GSK3 activity that was detectable after 2 min and reached a maximum (40%) inhibition after 5–10 min (Figure 1a). A similar inhibition was observed after stimulation with insulin (results not shown). In order to investigate the mechanism of inhibition, GSK3 immunoprecipitates from IGF-1 or insulin-stimulated cells were incubated with PP2A₁, a protein serine/threonine phosphatase. PP2A₁ treatment re-activated GSK3 to the level observed in unstimulated cells, while GSK3 immunoprecipitates from unstimulated cells were only activated slightly (Figure 2). Re-activation was prevented if okadaic acid (1 μ M), a specific inhibitor of PP2A₁, was added before PP2A₁ (results not shown). These experiments establish that GSK3 is partially inhibited by IGF-1 or insulin in L6 cells as a result of increased phosphorylation (probably at serine/threonine residues).

GSK3 activity was also examined in skeletal muscle *in vivo* 15 min after an intravenous injection of insulin, the time at which activation of glycogen synthase is maximal [2]. PP2A₁ activated GSK3 by 32 $\pm$ 3% [mean $\pm$ S.E.M. for four experiments (i.e. n = 4)] in immunoprecipitates from insulin-stimulated animals, but only by 12 $\pm$ 1% (n = 3) in immunoprecipitates from control animals. This indicates that insulin also decreases GSK3 activity

in skeletal muscle *in vivo* by a phosphorylation mechanism. The phosphorylation of glycogen synthase decreases its activity in the absence, but not in the presence, of the activator glucose 6-phosphate. Insulin increased the activity ratio ($\pm$ glucose 6-phosphate) of glycogen synthase from 0.22 $\pm$ 0.01 (n = 3) to 0.37 $\pm$ 0.01 (n = 4) after 15 min, similar to the increase from 0.18 to 0.35 reported in [2].

The experiments described above were carried out with a mixture of antibodies raised against GSK3 α and GSK3 β , which immunoprecipitated both isoforms. In order to study the effects of IGF-1 on each isoform, GSK3 α was first immunoprecipitated from the L6 lysates with an α -isoform-specific antibody and GSK3 β immunoprecipitated subsequently. Stimulation with IGF-1 for 10 min decreased the specific activity of GSK3 α by 36 $\pm$ 2%, and the activity of immunoprecipitated GSK3 α measured directly divided by the activity measured after incubation with PP2A₁ (as in Figure 2) was 0.57 $\pm$ 0.02 compared with 0.93 $\pm$ 0.02 for GSK3 α from unstimulated cells (n = 3). The specific activity of GSK3 β decreased by 30 $\pm$ 2% after incubation for 10 min with IGF-1, and the GSK3 β activity ratio $\pm$ PP2A₁ was 0.66 $\pm$ 0.01 compared with 0.92 $\pm$ 0.01 for GSK3 β from unstimulated cells (values are averages for two experiments). Thus both isoforms are inhibited to a similar extent by IGF-1.

Figure 1 Effect of IGF-1 on GSK3 and p70^{S6K} activity in the absence and presence of rapamycin and wortmannin

Rat L6 cells were incubated for 10 min with 100 nM rapamycin (R) or 100 nM wortmannin (W) or in the absence of either drug, then stimulated for the times indicated with 30 nM IGF-1 (I) or with buffer (control, C). (a) The cells were lysed, and both GSK3 isoforms immunoprecipitated and assayed as described in the Materials and methods section. ● Shows the specific activity of GSK3 after stimulation with IGF-1 relative to the control incubations (○). ▽ and ▼ show the effect of IGF-1 on GSK3 activity after first preincubating the cells with rapamycin and wortmannin respectively. The results are given as means $\pm$ S.E.M. for the numbers of experiments shown, and assays were performed in triplicate in each experiment. (b) Lysates were denatured in SDS, subjected to electrophoresis on a 10% polyacrylamide gel and immunoblotted with p70^{S6K} antibodies as described in [36]. The electrophoretic mobility of p70^{S6K} in unstimulated cells was identical with that observed after stimulation for 2 min with IGF-1.

Figure 2 Inhibition of GSK3 induced by IGF-1 and insulin in rat L6 cells is reversed by incubation with protein phosphatase 2A

Cells were incubated for 10 min with 100 nM rapamycin (R), 100 nM wortmannin (W) and then stimulated for 2, 5 or 10 min with 30 nM IGF-1 (I) or 100 nM insulin (INS) or in the absence of either hormone (control, C). The cells were lysed, and both GSK3 isoforms immunoprecipitated, re-activated by incubation with PP2A_i, and the reactions stopped with okadaic acid as described in the Materials and methods section. GSK3 activity is presented as S.E.M. for the numbers of different experiments indicated and expressed as the activity measured without PP2A_i treatment divided by the activity after incubation with PP2A_i. Assays were performed in triplicate in each experiment, and no re-activation occurred in control incubations where PP2A_i was omitted and where okadaic acid was added before PP2A_i.

The effect of IGF-1 on GSK3 is not mediated by p70 S6 kinase

The activation of p70^{S6K} in cells is normally monitored by the decreased electrophoretic mobility that accompanies its phosphorylation [12]. IGF-1 stimulation of L6 cells induced a striking decrease in the mobility of p70^{S6K} after 10 min (Figure 1b), which was undetectable after 2 min and barely detectable after 5 min (Figure 1b). In contrast, the inhibition of GSK3 was already pronounced after 2 min and almost maximal after 5 min (Figure 1a). Furthermore, rapamycin prevented the phosphorylation of p70^{S6K} (Figure 1b), but not the inhibition of GSK3 by IGF-1 (Figure 1a), and had no effect on the activation of MAPKAP kinase-1 β , p42^{mapk} and MAPKK by IGF-1 (Figure 3) or on the activation of MAPKAP kinase-1 β by insulin (results not shown).

Wortmannin prevents the inactivation of GSK3 and blocks the MAP kinase pathway in L6 cells

Wortmannin is a fungal metabolite which is a potent inhibitor of PtdIns 3-kinase (PI3K) [28,29]. Incubation of L6 cells with 100 nM wortmannin blocked the inhibition of GSK3 by IGF-1 (Figure 1a) and largely prevented the phosphorylation of p70^{S6K} (Figure 1b). However, it seemed unlikely that the effect of wortmannin on GSK3 inhibition was caused by inhibition of p70^{S6K}, because the blockade of p70^{S6K} activation by rapamycin had no effect on the inhibition of GSK3 by IGF-1 (Figure 1). We therefore examined the effect of wortmannin on the activation of MAPKAP kinase-1 β by IGF-1 in L6 cells and other components of the MAP kinase cascade thought to be involved in its activation. Stimulation of L6 cells for 5 min with IGF-1 increased the proportion of Ras in its GTP-bound form from 11.3 $\pm$ 1.4 % (n = 4) to 33.0 $\pm$ 2.8 % (n = 6), and incubation of the cells for

Figure 3 Activation of components of the MAP kinase cascade by IGF-1 in rat L6 cells in the presence and absence of rapamycin and wortmannin

Cells were incubated for 10 min with 100 nM rapamycin or 100 nM wortmannin or in the absence of either drug, then stimulated for the times indicated with 30 nM IGF-1 or buffer. The cells were lysed, and p74^{raf-1} (a), MAPKK (b), p42^{mapk} (c) and MAPKAP kinase-1 β (d) were assayed as described in the Materials and methods section. Symbols: ○, unstimulated cells; ●, IGF-1-stimulated cells; ▼, preincubated with rapamycin and then stimulated with IGF-1; ▽, preincubated with wortmannin and then stimulated with IGF-1. The results are presented as means $\pm$ S.E.M. for three to six different experiments.

10 min with 100 nM wortmannin did not alter the level of GTP-Ras significantly after stimulation with IGF-1 (31.1 $\pm$ 2.1 %, n = 6). In contrast, wortmannin (100 nM) inhibited the IGF-1-stimulated activation of p74^{raf-1}, MAPKK, p42^{mapk} and MAPKAP kinase-1 β by 80–90 % (Figure 3), and 50 % inhibition of p42^{mapk} occurred at 20 nM wortmannin (results not shown). Wortmannin had no direct effect on the activities of p74^{raf-1}, MAPKK, p42^{mapk}, MAPKAP kinase-1 β or p70^{S6K} *in vitro*, even when included in the assays of these enzymes at 10 μ M (results not shown).

DISCUSSION

The inhibition of GSK3 by IGF-1 or insulin in the skeletal-muscle cell line L6 and by insulin in skeletal muscle *in vivo* confirms observations made previously in Chinese-hamster ovary cells [30] and adipocytes [31], but disagrees with the findings of Yang and co-workers, who reported that insulin activates GSK3 in adipocytes [32] and in platelets [33]. The inhibition of GSK3 is mediated by increased phosphorylation, presumably at a serine or threonine residue(s), because it is reversed by incubation with PP2A_i [30,34; Figure 2], and in the following paper we show that, in A431 cells, epidermal-growth-factor-stimulated inhibition of GSK3 is accompanied by phosphorylation of a serine

residue(s) located in the tryptic peptide phosphorylated by MAPKAP kinase-1 β or p70^{s6k} *in vitro* [35]. However, the failure of rapamycin to prevent the inhibition of GSK3 (Figure 1a) excludes a role for p70^{s6k} in the inhibition of GSK3 by IGF-1, nor can protein kinase C be the relevant enzyme because it does not phosphorylate GSK3 α *in vitro* [17]. Two lines of evidence suggest that a MAPKAP kinase-1 isoform may mediate the inhibition of GSK3 by IGF-1/insulin in L6 cells and by insulin in skeletal muscle *in vivo*. Firstly, MAPKAP kinase-1 β and its presumed activator, p42/p44^{mapk}, are activated in response to IGF-1 (Figure 3) or insulin (results not shown) in L6 cells and in response to insulin in skeletal muscle *in vivo* [8,36]. Secondly, wortmannin, which prevents the inhibition of GSK3 by IGF-1 (Figure 1a) or insulin in L6 cells, strongly suppresses the activation of MAPKAP kinase-1 β by IGF-1 (Figure 3) or insulin. However, the inhibition of GSK3 is readily detectable after 2 min (Figure 1a), whereas the activation of MAPKAP kinase-1 β is relatively low at this time compared with the level attained after 10 min (Figure 3). These observations could be explained in two ways. Firstly, only a small degree of activation of MAPKAP kinase-1 may be needed for maximal inhibition of GSK3, because the potential of protein kinase cascades for amplification is enormous. Secondly, GSK3 may be inhibited by a distinct insulin/IGF-1-stimulated protein kinase whose activation is also blocked by wortmannin and which has so far escaped detection. However, we have been unable to detect any enzyme that inactivates GSK3, other than MAPKAP kinase-1 β , after Mono Q chromatography of skeletal-muscle extracts from rabbits injected with insulin (D. Cross and P. Cohen, unpublished work).

To our knowledge, a role for PI3K in the activation of MAP kinase has not been reported previously. We were therefore surprised to find that, in L6 cells, wortmannin potently inhibited the activation of the four protein kinases which form the MAP kinase cascade (Figure 3) at a concentration similar to that reported to inhibit PI3K in other cells. Wortmannin also prevents the activation of p42^{mapk} and MAPKAP kinase 1 and the inhibition of GSK3 by insulin in Chinese-hamster ovary cells [34] and Swiss 3T3 cells (Y. Saito and P. Cohen, unpublished work), although it has almost no effect on the acute activation of p42^{mapk} and p44^{mapk} by epidermal growth factor or platelet-derived growth factor in Swiss 3T3 cells or by nerve growth factor in PC12 cells (Y. Saito, Y. Doza and P. Cohen, unpublished work). If wortmannin is indeed a specific inhibitor of PI3K, then our results suggest an involvement of the putative second messenger Ptd(3,4,5)P₃ in the activation of p74^{raf-1} by insulin. Current evidence suggests that the role of Ras is to recruit Raf to the plasma membrane, where it is activated by an unknown mechanism(s) [37–39]. In L6 cells this activation mechanism may involve Ptd(3,4,5)P₃, although pure synthetic Ptd(3,4,5)P₃ (provided by Dr. R. Gigg, National Institute for Medical Research, Mill Hill, London, U.K.) did not activate c-Raf expressed in insect Sf9 cells (D. Alessi, unpublished work).

In conclusion, we suggest that the activation of glycogen synthase by insulin in skeletal muscle is mediated by the inhibition of GSK3, as well as the activation of PP1G (see the Introduction), and that phosphorylation of both interconverting enzymes is probably catalysed by MAPKAP kinase-1 β or a related isoform. An intravenous injection of insulin increases the phosphorylation of the G-subunit of PP1G at Site-1 from 0.25 to 0.5 mol/mol after 15 min [8], sufficient to increase the rate of dephosphorylation of glycogen synthase by 40%, and such a stimulation of PP1G activity in insulin-stimulated rat L6 cells has been reported [40]. This degree of activation of PP1G, coupled with a 30–40% inhibition of GSK3, is adequate to account for the 70–100%

activation of glycogen synthase by insulin measured under the same conditions [2]. Intriguingly, the stimulation of glucose transport by insulin in L6 (T. Tsakiridis, H. S. McDowell, H. S. Hundal and A. Klip, unpublished work) and other cells [28,41–43] is unaffected by rapamycin, blocked by wortmannin and (in cardiac myocytes) requires GTP-Ras [44]. Because of the need to co-ordinate glucose uptake and glycogen synthesis in skeletal muscle, it will be important to find out whether GSK3 and PP1G (which is associated with membranes as well as glycogen [45]) are also involved in the stimulation of glucose uptake by insulin.

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