RESEARCH COMMUNICATION

Wortmannin inhibits the effects of insulin and serum on the activities of glycogen synthase kinase-3 and mitogen-activated protein kinase

Gavin I. WELSH,* Emily J. FOULSTONE, Stephen W. YOUNG, Jeremy M. TAVARÉ and Christopher G. PROUD

Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K.

We have previously shown that insulin causes inactivation of glycogen synthase kinase-3 (GSK-3) in Chinese hamster ovary cells over-expressing the human insulin receptor (CHO.T cells). We now show that serum and phorbol ester also cause rapid inactivation of GSK-3, both in CHO.T cells and in the non-transfected parental cell line, CHO.K1 cells. Rapamycin was without effect on the inactivation of GSK-3 by insulin, serum or phorbol ester, indicating that the p70 S6 kinase pathway is not involved. In contrast, wortmannin, a potent inhibitor of phosphatidylinositol 3-kinase, blocked the effects of both insulin and serum on GSK-3 activity, and also substantially reduced the activation of both p90 S6 kinase (by insulin) and mitogen-activated protein (MAP) kinase (by insulin and serum). These

findings imply (i) that GSK-3 activity is regulated by a cascade involving MAP kinase and p90 S6 kinase and (ii) that wortmannin affects an early step in the MAP kinase pathway. One can infer from this that GSK-3 may be an important regulatory enzyme for the control of several biosynthetic pathways, key enzymes in which are regulated by GSK-3-mediated phosphorylation. Wortmannin had a smaller effect on the activation of MAP kinase by phorbol ester, indicating that phorbol esters may stimulate MAP kinase by a different or additional mechanism to that employed by insulin or serum. Wortmannin had very little effect on the inactivation of GSK-3 by phorbol ester: possible reasons for this are discussed.

INTRODUCTION

Glycogen synthase kinase-3 (GSK-3) was identified about 15 years ago as phosphorylating residues in glycogen synthase (GS) (Embi et al., 1980). GS catalyses the regulatory step in glycogen synthesis in animal cells and phosphorylation of these sites causes inactivation of this enzyme. These sites (termed 3a–3d) become less phosphorylated in response to insulin, which stimulates glycogen synthesis (Parker et al., 1983). Earlier work concentrated on the role of the phosphatase in bringing about decreased phosphorylation of these sites in response to insulin (Dent et al., 1990), but recent work shows that insulin causes decreased GSK-3 activity (see below). GSK-3 exists as at least two distinct isozymes (GSK-3 α and GSK-3 β) in mammalian cells (Woodgett, 1990).

Apart from GS, GSK-3 phosphorylates a number of other proteins, at least *in vitro* (Woodgett, 1991; Plyte et al., 1992). These include the proto-oncogene c-jun (Boyle et al., 1991), an inhibitor of protein phosphatase-1 (inhibitor-2) (Vandenheede et al., 1980; Hemmings et al., 1982; Vandenheede et al., 1989), ATP-citrate lyase (Hughes et al., 1992) and eukaryotic protein synthesis initiation factor-2B (eIF-2B) (Welsh and Proud, 1993). The proto-oncogene c-jun is of particular interest as it is a component of the AP-1 transcription complex which is involved in mediating nuclear processes in response to extracellular stimuli. There is now data to link phosphorylation of c-jun by GSK-3 to the control of transcription of genes which are under the control of AP-1 type promoters, i.e. overexpression of GSK-3 represses AP-1-mediated transactivation in intact cells (Nikolakaki et al., 1993; de Groot et al., 1993). Phosphorylation by GSK-3 occurs at serine residues adjacent to the DNA-binding domain of c-jun

and inhibits its activity *in vitro* (Boyle et al., 1991). Our recent data indicate that phosphorylation of eIF-2B by GSK-3 may inhibit its activity (G. I. Welsh and C. G. Proud, unpublished work). As eIF-2B mediates an essential regulatory step in peptide-chain initiation, GSK-3 may play a key role in controlling several biosynthetic pathways (glycogen synthesis, transcription and translocation) by phosphorylating and inactivating regulatory proteins in each of these processes.

We (Welsh and Proud, 1993) and others (Ramakrishna and Benjamin, 1988), using different cell-types, have shown that GSK-3 is rapidly inactivated in response to insulin, which would be consistent with a role for this kinase in controlling the anabolic processes discussed above, all of which are stimulated by insulin. Recent work from Sutherland and co-workers (Sutherland et al., 1993; Sutherland and Cohen, 1994) has indicated a potential mechanism by which the effect of insulin on GSK-3 activity may be exerted: *in vitro*, both the α and β isoforms of GSK-3 are phosphorylated and inactivated by two protein kinases known to be stimulated by insulin. They are (or are closely related to) the two protein kinases originally identified as phosphorylating ribosomal protein S6, i.e., the 90 and 70 kDa S6 kinases. The 90 kDa S6 kinase is also known as p90^{rsk} or mitogen-activated protein kinase-activated protein kinase-1 (MAPKAP-K1) and lies downstream of an increasingly well-characterized signalling pathway, the mitogen-activated protein (MAP) kinase cascade (Egan and Weinberg, 1993). MAPKAP-K1 (the name used in this paper) is activated upon phosphorylation by MAP kinase (Lavoinne et al., 1991). The 70 kDa S6 kinase (p70 S6k) is also activated by phosphorylation; the kinase(s) responsible for this has not been identified but lies on a distinct signalling pathway from the MAP kinase cascade as

Abbreviations used: p70 S6k, 70 kDa S6 kinase; CHO, Chinese hamster ovary; CK-2, casein kinase-2; eIF-2B, eukaryotic protein synthesis initiation factor-2B; GS, glycogen synthase; GSK-3, glycogen synthase kinase-3; MAP, mitogen-activated protein; MAPKAP-K1, mitogen-activated protein kinase-activated protein kinase-1; PMA, phorbol 12-myristate 13-acetate; PI, phosphatidylinositol; PKC, protein kinase C.

* To whom correspondence should be addressed.

demonstrated, for example, by the ability of the macrolide immunosuppressant rapamycin to block activation of p70 S6k without affecting the activation of MAP kinase or kinases related to MAPKAP-K1 (Price et al., 1992; Calvo et al., 1992; Kuo et al., 1992; Fingar et al., 1993).

The implication that GSK-3 may be regulated *in vivo* by phosphorylation by MAPKAP-K1 or p70 S6k raises many important questions. In this paper, we address two of them. First, since it is known that many stimuli other than insulin activate the MAP kinase cascade and/or p70 S6k (Erikson, 1991; Sturgill and Wu, 1991; Egan and Weinberg, 1993), these stimuli would also be expected to result in inactivation of GSK-3. Here we show that, in Chinese hamster ovary cells, serum and phorbol esters each cause rapid and marked inactivation of GSK-3. Second, because two distinct signalling pathways may be involved, it is important to assess the relative contributions of each to the regulation of GSK-3 activity. To address this question we have made use of two cell-permeant inhibitors. They are rapamycin, which inhibits p70 S6k activation, as discussed above, and wortmannin, which inhibits the enzyme phosphatidylinositol 3-kinase (PI 3-kinase) (Arcaro and Wymann, 1993). PI 3-kinase possesses SH2 (Src homology 2) domains through which it interacts with phosphorylated tyrosine residues (Skolnik et al., 1991; Otsu et al., 1991) and is thought to play a key role in the effects of insulin and other agents which activate membrane-associated protein tyrosine kinases (White and Kahn, 1994; Stephens et al., 1994). The data indicate that the MAP kinase pathway, rather than the one involving the p70 S6 kinase, mediates the effects of insulin and serum on GSK-3 activity in these cells. In contrast, the inactivation of GSK-3 by phorbol esters may be mediated by a different mechanism. Furthermore, we find that wortmannin blocks the activation of MAP kinase in response to insulin and serum. The implications of these findings for cellular regulation are discussed.

MATERIALS AND METHODS

Chemicals and biochemicals were obtained from BDH (Poole, Dorset, U.K.) and Sigma (Poole, Dorset, U.K.), unless otherwise indicated. Rapamycin was generously provided by Professor P. Cohen (University of Dundee, Dundee, U.K.). The GSK-3 peptide substrate (YRRAAVPPSPSLSRHSSPHQS(P)EDEEE) and the negative control peptide (YRRAAVPPSPSLSRHSSPHQAEDDEE) were synthesized by Dr. G. Bloomberg (Dept. of Biochemistry, University of Bristol, Bristol, U.K.) as outlined (Welsh et al., 1994).

The Chinese hamster ovary (CHO) cell lines CHO.K1 and CHO.T were grown and maintained as described previously (Dickens et al., 1992). Where appropriate, cells were treated with 20 nM insulin, 10% (v/v) foetal calf serum or 2 μ M phorbol 12-myristate 13-acetate (PMA) for 10 min. Where relevant, cells were pretreated with either 100 nM rapamycin for 30 min or 100 nM wortmannin for 10 min prior to addition of insulin, serum or PMA. The cells were then extracted and fractionated on a Mono-S HR5/5 column as previously described (Dickens et al., 1992; Welsh and Proud, 1993).

The ability of the fractions to phosphorylate purified rabbit reticulocyte eIF-2B and the GSK-3 peptide were assayed as described previously (Welsh and Proud, 1993) except that, because in this case the peptide was synthesized with a phosphate already in the priming site, there was no requirement to pre-phosphorylate it with casein kinase-2 (CK-2). A peptide in which the priming serine had been replaced by alanine, and which thus was not a substrate for GSK-3, was used as a negative control in all peptide assays. This is a different control peptide from the one

we used earlier (Welsh and Proud, 1993) which had a Ser in place of the Ser(P): the latter peptide is a potential substrate for CK-2 whereas the alanine peptide is not. In the case of eIF-2B, the relative levels of phosphorylation were assessed by laser densitometry of the autoradiographs using a Joyce-Loebl Chromoscan 3 scanner. All readings were within the linear range.

MAP kinase activity was assayed in anti-(p42/p44 MAP kinase) immunoprecipitates essentially as described by Young et al. (1994). However, a synthetic peptide substrate, KRELVEPLTPSGEAPNQALLR, based on the sequence around Thr-669 of the epidermal growth factor receptor was used in place of myelin basic protein to increase further the specificity of the assay for MAP kinase (Northwood et al., 1991). Protein kinase activities against the 32-mer and 8-mer S6 peptides were assayed as previously described (Dickens et al., 1992). Protein contents of the fractions were standardized by the method of Bradford (1976).

RESULTS

Effects of serum and phorbol ester on GSK-3 activity

We have previously shown that GSK-3 is inactivated in response to insulin in CHO.T cells (Welsh and Proud, 1993). The data in Figure 1 show that treatment of CHO.T cells with either serum or phorbol ester also results in inactivation of GSK-3 measured either against a specific peptide substrate (Figures 1a and 1b) or against purified eIF-2B (Figure 1c). The peaks of activity against the two substrates always exactly coincided. The results in Figure 1 show typical experiments having been performed (four for serum and five for phorbol ester). In the case of serum, the decrease in GSK-3 activity was ~70% measured against the peptide and 80–90% using eIF-2B as substrate. This is in agreement with our earlier observations that the inactivation of GSK-3 in response to insulin was apparently greater when assessed using protein substrates (eIF-2B and GS) than against the peptide substrate [(Welsh and Proud, 1993), see also (Sutherland et al., 1993; Sutherland and Cohen, 1994)]. For PMA, a fall in GSK-3 activity of 30–40% was seen using the peptide substrate and 40–50% with eIF-2B, although larger effects (up to 70% reduction in GSK-3 activity) were observed in some experiments. These data show that inactivation of GSK-3 in CHO.T cells is caused not only by insulin but by other agents which stimulate (or, by analogy with other cells, are expected to stimulate) pathways such as the MAP kinase cascade, and processes such as glycogen and protein synthesis.

The cells we used in our previous work and for the experiments shown in Figure 1 were transfected with, and express, a cDNA encoding the human insulin receptor. It was clearly possible that the effects on GSK-3 activity of insulin and the other agents might be an unusual feature of these transfected cells. We therefore examined the effects of serum and phorbol ester on the parental wild-type CHO cell line (CHO.K1 cells). The degree of inactivation of GSK-3 in the CHO.K1 cells in response to these two stimuli was essentially the same as was seen on the CHO.T cells, using both the peptide and eIF-2B to measure kinase activity (e.g. for serum, GSK-3 activity was decreased by ~75% when measured against the peptide and >80% against eIF-2B, results not shown). The effect of serum on GSK-3 activity in CHO.K1 cells is most unlikely to be due to insulin present in the serum for two reasons: first, at the concentrations found in serum (~1 nM), insulin had no effect on the activities of a range of protein kinases in these cells; and second, even at 100 nM, insulin had only small effects on them compared with the effects of much lower insulin concentrations in CHO.T cells (Dickens et al., 1992).

Figure 1 Effects of serum and phorbol ester on GSK-3 activity

Extracts were fractionated by Mono-S chromatography and assayed for GSK-3 activity using the peptide substrate [(a) serum and (b) phorbol ester] or using eIF-2B [(c) serum and phorbol ester in the same experiment] as described in the Materials and methods section. The data for the peptide substrate were corrected (by subtraction) for the incorporation into the control (alanine-containing) peptide.

The nature of the pathway leading to inactivation of GSK-3

The work of Sutherland et al. (Sutherland et al., 1993; Sutherland and Cohen, 1994) has pointed to two potential mechanisms by which insulin and other agents might inactivate GSK-3. As discussed above, purified GSK-3 is inactivated following phosphorylation by either p70 S6k or MAPKAP-K1, enzymes which are believed to lie on distinct but 'parallel' signalling pathways (Sutherland et al., 1993; Sutherland and Cohen, 1994). Rapamycin is reported to block the pathway leading to activation of p70 S6k without affecting the MAP kinase pathway (Price et al., 1992; Kuo et al., 1992; Calvo et al., 1992; Fingar et al., 1993). We therefore explored its effect on the inactivation of GSK-3 by insulin, serum or phorbol ester. As can be seen from Figure 2(a), addition of rapamycin had no effect on the inactivation of GSK-3 which occurred in response to insulin, suggesting that this effect does not involve the 70 kDa S6k pathway. The data shown in Figure 2(a) were obtained using the peptide as substrate, but the same overall result was also apparent using eIF-2B. Rapamycin also failed to counteract the inactivation of GSK-3 caused either by serum or by PMA (results not shown). In order to verify that rapamycin was indeed blocking activation of p70 S6k, we have examined this by measuring protein kinase activity against the 32-mer S6 peptide (Dickens et al., 1992) (Figure 2b). Fractions obtained from Mono-Q chromatography of cell extracts showed a broad peak of activity against this peptide, as reported earlier (Dickens et al., 1992): in samples from extracts from insulin-treated cells, activity was increased relative to the control in those fractions previously shown to contain immunoreactive p70 S6k (marked as peak III) and in the region corresponding to members of the 90 kDa S6 kinase family to which MARKAP-K1 belongs (Dickens et al., 1992) (peak I, Figure 4a). Rapamycin blocked the increased activity in peak III without altering the activity in control cells or the activity against this peptide in peaks I or II (Figure 2b). It should be noted that the same concentration of this preparation of rapamycin was shown to block the shift in electrophoretic migration associated with the activation of p70 S6k by Saito et al. (1994), using Swiss 3T3 cells. These data indicate that rapamycin blocks activation of p70 S6k in these cells (without affecting other S6 kinases) and, because rapamycin did not affect GSK-3 activity, also indicate that the effects of insulin, serum and PMA on GSK-3 activity are not mediated via the p70 S6k.

We also sought to explore the roles of other signalling pathways in the regulation of GSK-3 activity. One such potential pathway involves PI 3-kinase, as this enzyme associates through the SH2 domains of its 85 kDa α -subunit with phosphotyrosine-containing proteins such as the insulin receptor substrate IRS-1 or growth factor receptors which undergo ligand-induced tyrosine phosphorylation (Stephens et al., 1994). Wortmannin is a relatively specific inhibitor of this enzyme, showing a much lower K_i for it than for a range of other enzymes tested (Arcaro and Wymann, 1993).

In the presence of wortmannin, the effects of insulin and serum on the activity of GSK-3 were greatly diminished, and in several experiments, abolished. This was observed whether GSK-3 activity was measured against the peptide substrate (Figure 3, panel (a) for insulin; results not shown for serum) or against eIF-2B (Figures 3b–3d). However, wortmannin alone increased the measured GSK-3 activity in control cells, and close inspection of the activity profile from the Mono-S column reveals that it also appeared to cause an apparent rightward shift in the peak of GSK-3 activity, so that it eluted at higher NaCl concentrations. The inhibition of inactivation and the increase in control activity were consistently observed in a total of six separate experiments: in most cases, the shift in activity peak was also seen. The rise in

Figure 2 Effects of rapamycin on the regulation by insulin of GSK-3 and kinase activity against the 32-mer S6 peptide

(a) GSK-3 activity in fractions obtained after Mono-S chromatography was measured using the peptide substrate and the data were corrected for incorporation into the control peptide. (b) Kinase activity against the 32-mer S6 peptide was measured in fractions from Mono-Q. I, II and III refer to different portions of the overall peak of activity, as discussed in the text.

activity in the cells treated with wortmannin only and the mobility shift are consistent with the possibility that this agent causes decreased phosphorylation of GSK-3 at the inhibitory site, which would be expected to result in its later elution from Mono-S. It should be noted that in our earlier paper we showed that treatment of GSK-3 from control cells with protein phosphatase-2A resulted in an increase in activity (Welsh and Proud, 1993), all of which is consistent with the notion that GSK-3 is partially phosphorylated, and thus partially inactivated, even in the control cells. Sutherland and co-workers (Sutherland et al., 1993; Sutherland and Cohen, 1994) also showed that treatment of purified muscle GSK-3 with protein phosphatase-2A resulted in a modest rise in its activity.

In contrast to the above, wortmannin was without effect on the extent of inactivation of GSK-3 caused by phorbol ester treatment although, again, the activity of GSK-3 was increased slightly by wortmannin (Figure 3d), in this case to similar extents in the samples from control and PMA-treated cells. These findings indicate that wortmannin interferes with the signalling pathways utilized by insulin and serum, but not phorbol esters.

Figure 3 Effects of wortmannin on the inactivation of GSK-3

The activity of GSK-3 in Mono-S fractions from cells treated with insulin (a, b), serum (c) or PMA (d) were measured against the peptide substrate (a) or eIF-2B (b-d). It should be noted that the data in (a) and (b) are for representative experiments performed on separate days. (a) and (b) ■, control; ●, insulin; □, control plus wortmannin; ○, insulin plus wortmannin. (c) ■, control; ▲, serum; □, control plus wortmannin; △, serum plus wortmannin. (d) ■, control; ●, PMA; □, control plus wortmannin; ○, PMA plus wortmannin.

Effects of wortmannin on MAP kinase and p90 S6 kinase activities

The observation that wortmannin blocked the effects of insulin and serum on GSK-3 activity prompted us to examine its effect on the activity of kinases thought to lie upstream of GSK-3. Because the rapamycin data suggested that the effects of insulin and serum were not mediated by p70 S6k, we concentrated our attention on the alternative pathway, which involves enzymes of the p90 S6 kinase family such as MAPKAP-K1 (Sutherland et al., 1993; Sutherland and Cohen, 1994). Previous work on this cell line (Dickens et al., 1992) has indicated that insulin causes substantial activation of members of this family, which elute from Mono-Q at ~135–160 mM NaCl. Figure 4(a) shows such a Mono-Q profile with activity being measured against the so-called 8-mer S6 peptide (RRLSSLRA). Insulin stimulates activity against this substrate, which appears as a doublet peak, and wortmannin almost completely blocks this activation, reducing the activity of both parts of the peak.

Figure 4 Effect of wortmannin on the activation of protein kinase activity against the 8-mer S6 peptide and on MAP kinase

(a) Activity of fractions from cell extracts fractionated on Mono-Q against the 8-mer S6 peptide, RRLSSLRA. Data are shown for control and insulin-treated cells. (b) Data (means $\pm$ S.E.M.) for control (CON), insulin (INS), PMA (PMA) or foetal calf serum (SER) for the activity of MAP kinase, assayed after immunoprecipitation using anti-(MAP kinase) antibodies, against the epidermal growth factor receptor peptide. In both panels, data are shown for cells treated with or without wortmannin.

Since members of the p90 S6 kinase family are activated by the p42 and p44 MAP kinases, we then examined the effect of wortmannin on the activation of these enzymes using a peptide whose sequence is based on that of the site in the epidermal growth factor receptor which is phosphorylated by these kinases. The data in Figure 4(b) show that insulin, serum and PMA each caused substantial (5–12-fold) activation of MAP kinase measured in this assay. Wortmannin greatly diminished the activation caused by insulin or serum (to 2- and 3-fold over the control without wortmannin respectively) but only had a smaller effect on the activation by phorbol ester ($\sim 35\%$ reduction, only marginally significant ($P = 0.08$)).

DISCUSSION

The data presented here show that agents other than insulin, i.e. serum and phorbol ester, cause the inactivation of GSK-3 in the cell line used here. Since phosphorylation by GSK-3 is thought to inhibit the activities of proteins important in the regulation of

glycogen synthase (GS), protein synthesis (eIF-2B) and transcription (c-jun), this protein kinase may play a key role in the stimulation of these processes by insulin, serum and phorbol esters. The data also indicate that the effects on GSK-3 activity of the agents tested are not mediated via the p70 S6k pathway, as rapamycin did not affect the activity of GSK-3 although it did block activation of p70 S6k by insulin. The p70 S6k pathway seems therefore not to have a role in the regulation of GSK-3 in response to insulin, serum or phorbol esters in these cells, although it may clearly do so in other types of cells and under other conditions. Cohen and co-workers have recently shown that several other agents also rapidly inactivate GSK-3 in a variety of cell types, and that, in these cases too, p70 S6k seems not to be involved (Saito et al., 1994; Cross et al., 1994).

The effects of insulin and of serum, but not phorbol ester, on GSK-3 activity were almost blocked completely by application of wortmannin, an inhibitor of PI 3-kinase. Since wortmannin also blocked stimulation of MAP kinase in response to the first two agents but not the last, the data are consistent with the idea that inactivation of GSK-3 is linked to the MAP kinase pathway via MAPKAP-K1, which phosphorylates and inactivates both the α - and β -isoforms of GSK-3 (Sutherland et al., 1993; Sutherland and Cohen, 1994) *in vitro*. Consistent with this is our finding that wortmannin almost completely blocked the activation of kinase activity against the 8-mer S6 peptide, which is believed to represent members of the protein kinase family which includes MAPKAP-K1 (Dickens et al., 1992). It is perhaps surprising that, whereas insulin and serum give roughly equivalent degrees of inactivation of GSK-3, the stimulation of MAP kinase by serum is about twice as great as that caused by insulin. It seems likely that this is because the degree of activation of the MAP kinase cascade by insulin is sufficient to achieve full inactivation of GSK-3, so that serum causes no greater decrease in GSK-3 activity. A similar lack of absolute correlation has been observed by Cohen's group (Saito et al., 1994; Cross et al., 1994). The degree of amplification inherent in the MAP kinase cascade in these cells is not clear [see a discussion in (Saito et al., 1994)], and there is no reason to suppose that the degrees to which successive components in the cascade are activated will be similar. Indeed, we find that whereas wortmannin almost completely blocks the effects of insulin and serum on GSK-3 activity, it only partially blocks MAP kinase activation by these agents. A related point is that wortmannin causes a small increase in the activity of GSK-3 in control cells, perhaps by reducing the basal level of activation of the MAP kinase cascade; however, the low level of activity of MAP kinase in our control samples means that it is not possible to detect small decreases its activity in response to wortmannin treatment of the cells.

The observation that wortmannin blocks the activation of MAP kinase by insulin and serum implies a key role either for PI 3-kinase or the likely *in vivo* product of this enzyme, phosphatidylinositol 3,4,5-trisphosphate, in the MAP kinase signalling pathway. Recent data suggest that ras-GTP serves to localize raf to the plasma membrane (Stokoe et al., 1994; Leever et al., 1994) and that this involves its tight association with the cytoskeleton, where it requires a second regulatory input to achieve its activation. Given that this is apparently occurring on the membrane (Wartmann and Davis, 1994), the activatory component could be phosphatidylinositol 3,4,5-trisphosphate, which might interact with raf by binding to its cysteine-rich domain [see, for example, the work of Ghosh et al. (1994)], although other explanations could be advanced. The data presented by Cross et al. (1994) indicate that wortmannin blocks activation of several upstream components of the MAP kinase cascade, including raf but not ras, consistent with the idea that

PI 3-kinase generates an activator of raf. The finding that wortmannin inhibits the activation of MAP kinase indicates that caution must be exercised in interpreting the effects of wortmannin on other processes, such as the insulin stimulation of glucose transport, which it blocks in at least two cell lines (Kanai et al., 1993; Clarke et al., 1994).

Two aspects of the data obtained with phorbol esters deserve special comment. First, although phorbol esters and serum gave similar degrees of activation of MAP kinase, the latter had a proportionally much greater effect on GSK-3 activity. This may be because phorbol esters, acting through protein kinase C (PKC), affect GSK-3 activity via a route independent of the MAP kinase cascade. In this context, it should be noted that it has been reported that the β -isoform of GSK-3, the major form in CHO cells (Welsh and Proud, 1993), can be phosphorylated and inactivated by PKC *in vitro* (Goode et al., 1992). If this mechanism were to operate in intact cells, one might expect that phorbol esters would achieve a larger effect (acting via MAP kinase/MAPKAP-K1 and through direct effects of PKC on GSK-3) than other agents (acting via MAP kinase alone). What is not clear here, however, is whether PKC and MAPKAP-K1 phosphorylate the same sites in GSK-3 and, if not, what the relationship is between the effects of PKC-mediated and MAPKAP-K1-mediated phosphorylation on the activity of GSK-3. If the former prevented or antagonized the latter, a smaller effect could result. Further clarification requires the identification of the sites in GSK-3 β phosphorylated in intact cells in response to phorbol esters: at present, such experiments are not feasible in this laboratory.

The second point is that wortmannin had a markedly smaller effect on the activation of MAP kinase, and almost none on the inactivation of GSK-3, by phorbol ester compared with insulin or serum. This also indicates that phorbol esters activate MAP kinase largely through a route distinct from that employed by insulin and serum, which presumably feed into the MAP kinase pathway below the level at which wortmannin exerts its effect. It is not clear how phorbol esters lead to activation of MAP kinase. The available evidence points to the existence of ras-dependent and -independent pathways for the activation of the MAP kinase cascade by phorbol esters (de Vries-Smits et al., 1992; Wood et al., 1992; Thomas et al., 1992). Ras-independent pathways could feed in to the cascade at or below the level of raf, obviating the requirement for activation of PI 3-kinase. This situation is unclear, but it has been reported that in some cell-types phorbol esters may activate MAP kinase independently of raf (Chao et al., 1994).

This work was supported by Grants from the Wellcome Trust (to C.G.P.), the Medical Research Council (to J.M.T.) and the British Diabetic Association (BDA) (to C.G.P. and J.M.T.). J.M.T. is a BDA Senior Research Fellow and E.J.F. is the recipient of a postgraduate studentship from the Medical Research Council. We thank Professor Philip Cohen (University of Dundee) for invaluable discussions and for prompting us to explore the effects of wortmannin in this system, as well as the generous gift of rapamycin. We would also like to thank Dr. Graham Bloomberg (University of Bristol) for preparing synthetic peptides and Graham Rena (University of Bristol) for carrying out some of our early experiments on rapamycin.

REFERENCES

- Arcaro, A. and Wymann, M. P. (1993) *Biochem. J.* **296**, 297–301
 Boyle, W. B., Smeal, T., Defize, L. H. K., Angel, P., Woodgett, J. R., Karin, M. and Hunter, T. (1991) *Cell* **64**, 573–584
 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
 Calvo, V., Crews, C. M., Vik, T. A. and Bierer, B. E. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7571–7575
 Chao, T. S. O., Foster, D. A., Rapp, U. R. and Rosner, M. R. (1994) *J. Biol. Chem.* **269**, 7337–7341
 Clarke, J. F., Young, P. W., Yonezawa, K., Kasuga, M. and Holman, G. D. (1994) *Biochem. J.* **299**, 631–635
 Cross, D., Alessi, D., Vandenheede, J., McDowell, H. S., Hundal, H. S. and Cohen, P. (1994) *Biochem. J.* **302**, in the press
 de Groot, R. P., Auwerx, J., Bourouis, M. and Sassone-Corsi, P. (1993) *Oncogene* **8**, 841–847
 de Vries-Smits, A. M. M., Burgering, B. M. T., Leeyers, S. J., Marshall, C. J. and Bros, J. L. (1992) *Nature (London)* **357**, 602–604
 Dent, P., Lavoie, A., Nakiely, S., Caudwell, F. B., Watt, F. and Cohen, P. (1990) *Nature (London)* **348**, 302–308
 Dickens, M., Chin, J. E., Roth, R. A., Ellis, L., Denton, R. M. and Tavaré, J. M. (1992) *Biochem. J.* **287**, 201–209
 Egan, S. E. and Weinberg, R. A. (1993) *Nature (London)* **365**, 781–783
 Embi, N., Rylatt, D. B. and Cohen, P. (1980) *Eur. J. Biochem.* **107**, 519–527
 Erikson, R. L. (1991) *J. Biol. Chem.* **266**, 6007–6010
 Fingar, D. C., Hausdorff, S. F., Blenis, J. and Birnbaum, M. J. (1993) *J. Biol. Chem.* **268**, 3005–3008
 Ghosh, S., Xie, W. Q., Quest, A. F. G., Mabrouk, G. M., Strum, J. C. and Bell, R. M. (1994) *J. Biol. Chem.* **269**, 10000–10007
 Goode, N., Hughes, K., Woodgett, J. R. and Parker, P. J. (1992) *J. Biol. Chem.* **267**, 16878–16882
 Hemmings, B. A., Yellowlees, D., Kernohan, J. C. and Cohen, P. (1982) *Eur. J. Biochem.* **119**, 443–451
 Hughes, K., Ramakrishna, S., Benjamin, W. B. and Woodgett, J. R. (1992) *Biochem. J.* **288**, 309–314
 Kanai, F., Ito, K., Todaka, M., Hayashi, H., Kamohara, S., Ishii, K., Okada, T., Hazeki, O., Ui, M. and Ebina, Y. (1993) *Biochem. Biophys. Res. Commun.* **195**, 762–768
 Kuo, C. J., Chung, J., Fiorentino, D. F., Flanagan, W. M., Blenis, J. and Crabtree, G. R. (1992) *Nature (London)* **358**, 70–73
 Lavoie, A., Erikson, E., Maller, J. L., Price, D. L., Avruch, J. and Cohen, P. (1991) *Eur. J. Biochem.* **199**, 723–728
 Leeyers, S. J., Paterson, H. F. and Marshall, C. J. (1994) *Nature (London)* **369**, 411–414
 Nikolakaki, E., Coffey, P. J., Hemelsoet, R., Woodgett, J. R. and Defize, L. H. (1993) *Oncogene* **8**, 833–840
 Northwood, I. C., Gonzalez, F. A., Wartmann, M., Raden, D. L. and Davies, R. J. (1991) *J. Biol. Chem.* **266**, 15266–15276
 Otsu, M., Hiles, I. D., Fry, M. J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N., Smith, A. D., Morgan, S. J., Courtneidge, S. A., Parker, P. J. and Waterfield, M. D. (1991) *Cell* **65**, 91–104
 Parker, P. J., Caudwell, F. B. and Cohen, P. (1983) *Eur. J. Biochem.* **130**, 227–234
 Plyte, S. E., Hughes, K., Nikolakaki, E., Pulverer, B. J. and Woodgett, J. R. (1992) *Biochim. Biophys. Acta* **1114**, 147–162
 Price, D. J., Grove, J. R., Calvo, V., Avruch, J. and Bierer, B. E. (1992) *Science* **257**, 973–977
 Ramakrishna, S. and Benjamin, W. B. (1988) *J. Biol. Chem.* **263**, 16878–16882
 Saito, Y., Vandenheede, J. and Cohen, P. (1994) *Biochem. J.* **302**, in the press
 Skolnik, E. Y., Margolis, B., Mohammadi, M., Lowenstein, E., Fischer, R., Drepps, A., Ullrich, A. and Schlessinger, J. (1991) *Cell* **65**, 83–90
 Stephens, L. R., Jackson, T. R. and Hawkins, P. T. (1994) *Biochim. Biophys. Acta* **1179**, 27–75
 Stokoe, D., Macdonald, S. G., Cadwallader, K., Symons, M. and Hancock, J. F. (1994) *Science* **264**, 1463–1467
 Sturgill, T. W. and Wu, J. (1991) *Biochim. Biophys. Acta* **1092**, 350–357
 Sutherland, C., Leighton, I. A. and Cohen, P. (1993) *Biochem. J.* **296**, 15–19
 Sutherland, C. and Cohen, P. (1994) *FEBS Lett.* **338**, 37–42
 Thomas, S. M., DeMarco, M., D'Arcangelo, D. G., Halegoua, S. and Brugge, J. S. (1992) *Cell* **68**, 1031–1040
 Vandenheede, J. R., Yang, S.-D., Goris, J. and Merlevede, W. (1980) *J. Biol. Chem.* **255**, 11768–11774
 Vandenheede, J. R., Agostinis, P., Staquet, S. and Van Lint, J. (1989) *Adv. Protein Phosphatases* **5**, 19–36
 Wartmann, M. and Davis, R. J. (1994) *J. Biol. Chem.* **269**, 6695–6701
 Welsh, G. I., Price, N. T., Bladergroen, B. A., Bloomberg, G. and Proud, C. G. (1994) *Biochem. Biophys. Res. Commun.* **201**, 1279–1288
 Welsh, G. I. and Proud, C. G. (1993) *Biochem. J.* **294**, 625–629
 White, M. F. and Kahn, C. R. (1994) *J. Biol. Chem.* **269**, 1–4
 Wood, K. W., Sarnecki, C., Roberts, T. M. and Blenis, J. (1992) *Cell* **68**, 1041–1050
 Woodgett, J. R. (1990) *EMBO J.* **9**, 2431–2438
 Woodgett, J. R. (1991) *Trends Biochem. Sci.* **16**, 177–181
 Young, S. W., Dickens, M. and Tavaré, J. M. (1994) *FEBS Lett.* **338**, 212–216