RESEARCH COMMUNICATION

The mechanism by which epidermal growth factor inhibits glycogen synthase kinase 3 in A431 cells

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Glycogen synthase kinase 3 (GSK3) was inhibited by 50% within 5 min when A431 cells were stimulated with epidermal growth factor (EGF). The inhibition was unaffected by rapamycin at concentrations which blocked the activation of p70 S6 kinase, and reversed by incubation with protein phosphatase-1. EGF stimulation of A431 cells inhibited GSK3 α and GSK3 β to a similar extent, and inhibition was accompanied by phosphoryl-

ation of the tryptic peptides containing the serine residues phosphorylated *in vitro* by p70 S6 kinase or MAP kinase-activated protein (MAPKAP) kinase-1 β (also termed Rsk-2). These results demonstrate that EGF inhibits GSK3 by inducing phosphorylation of a serine residue and that GSK3 is not phosphorylated *in vivo* by either p70 S6 kinase or protein kinase C.

INTRODUCTION

Glycogen synthase kinase 3 (GSK3) is a protein kinase originally identified as an inactivator of glycogen synthase in skeletal muscle [1], but subsequently shown to phosphorylate many other proteins *in vitro* (reviewed in [2]). In addition, *Drosophila* mutants lacking GSK3 activity are defective in several developmental processes, such as larval neurogenesis [3,4], suggesting an involvement of GSK3 in numerous cellular functions.

For many years GSK3 was thought to be a constitutively active enzyme, but recent reports have revealed that its activity decreases within minutes in response to insulin *in vivo*. Insulin stimulation of rat adipocytes decreased (by 50%) the activity of an ATP-citrate lyase kinase [5] later reported to be the α -isoform of GSK3 (GSK3 α) [6], while insulin stimulation of Chinese-hamster ovary cells that overexpress the insulin receptor decreased (by up to 70%) the activity of an eIF2B kinase which was identified as the β -isoform of GSK3 (GSK3 β) [7].

The first evidence that GSK3 activity might be regulated by phosphorylation was obtained by Goode et al. [8], who reported that GSK3 β is phosphorylated by protein kinase C (PKC) decreasing its activity by about 50%. However, these investigators reported that GSK3 α was not phosphorylated or inhibited by PKC. More recently, we found that GSK3 α and GSK3 β are both phosphorylated and inhibited 80–95% *in vitro* by two protein kinases that are activated in response to insulin and other growth factors [9,10]. One of these was the β -isoform of MAP kinase-activated protein (MAPKAP) kinase-1 (also known as p90 ribosomal protein S6 kinase-2 or Rsk2), while the other was the distinct p70 ribosomal S6 kinase (p70^{S6K}). These protein kinases phosphorylate the same residue in GSK3 (Ser-21 in GSK3 α , Ser-9 in GSK3 β), which lie in Arg-Pro/Ala-Arg-Thr-Ser/Thr-Ser(P)-Phe-Ala-Glu sequences [9,10] that are also present in GSK3 from *Drosophila* [11]. However, GSK3 is also phosphorylated *in vivo* at a tyrosine (Tyr-279 in GSK3 α , Tyr-216 in GSK3 β) located eight residues N-terminal to the Ala-Pro-Glu

motif between subdomains VII and VIII [12]. This tyrosine residue appears to be phosphorylated stoichiometrically in GSK3 purified from rabbit skeletal muscle and to be important for GSK3 activity [12,13].

Here we show that epidermal growth factor (EGF) stimulation of A431 cells inhibits both GSK3 isoforms via phosphorylation of Ser-21 (GSK3 α) and Ser-9 (GSK3 β) and that neither p70^{S6K} nor PKC is responsible for the phosphorylation and inhibition of GSK3 in these cells.

MATERIALS AND METHODS

Materials

EGF was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.), Dulbecco's Modified Eagles (DME) medium from GIBCO-BRL (Paisley, Renfrewshire, Scotland, U.K.), trypsin [treated with tosylphenylalanylchloromethane ('TPCK')] from Worthington (Freehold, NJ, U.S.A.), [γ -³²P]ATP and [³²P]P_i from Amersham International (Amersham, Bucks., U.K.) and microcystin-LR from Calbiochem (Nottingham, U.K.). Antibodies were raised in rabbits against the α - and β -isoforms of GSK3 [14]. The catalytic subunit of protein phosphatase-2A from bovine cardiac muscle (Dr. R. MacKintosh) and bacterially expressed protein phosphatase 1 γ [15] (Dr. D. Alessi) were purified by the Dundee-based investigators indicated in parentheses. Rapamycin was provided by Dr. S. Schreiber (Department of Chemistry, Harvard University, Cambridge, MA, U.S.A.), and okadaic acid was a gift from Dr. Y. Tsukitani (Fujisawa Pharmaceutical Co., Tokyo, Japan). The sources of other materials are given in the preceding paper [16].

Cell culture

Human A431 cells were cultured in DME medium supplemented with 10% (v/v) foetal-calf serum (FCS). Before experimentation A431 cells were incubated in Krebs–Ringer–Hepes (KRH) buffer

Abbreviations used: GSK3, glycogen synthase kinase 3; EGF, epidermal growth factor; MAPKAP, MAP (mitogen-activated protein) kinase-activated protein; PKC, protein kinase C; DME, Dulbecco's modified Eagle's; FCS foetal-calf serum; TFA, trifluoroacetic acid.

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for 2.5 h. The cells were then incubated with growth factors and drugs and lysed in 20 mM Tris/acetate (pH 7.0)/0.27 M sucrose/1 mM EDTA/1 mM EGTA/1 mM sodium orthovanadate/10 mM sodium glycerophosphate/50 mM NaF/5 mM sodium pyrophosphate/1% (v/v) Triton X-100/1 mM benzamide/0.1 mM phenylmethanesulphonyl fluoride/0.1% (v/v) 2-mercaptoethanol (Buffer A).

Immunoprecipitation and assay of GSK3

Protein A-Sepharose (50 mg) was suspended in 1 ml of PBS, and a 25 μ l aliquot was incubated for 2 h at 4 °C with 0.5 μ l of anti-GSK3 serum. Cell lysates (30–60 μ g of protein, measured as described by Bradford [17]) were added to the Protein A-Sepharose-immobilized antibodies and incubated for 2 h at 4 °C. The immunoprecipitated GSK3 was washed essentially as described in [14] and assayed with glycogen synthase peptide-2 as described in [9,10], except that the incubations contained 0.01% (v/v) 2-mercaptoethanol, 0.03% (w/v) Brij 35 and microcystin-LR (20 nM). Microcystin was omitted during immunoprecipitation if the immunoprecipitates were to be incubated with protein phosphatases.

³²P-labelling of A431 cells and digestion of immunoprecipitated GSK3 with trypsin

These experiments were usually carried out with four 9 cm-diameter dishes of cells. Each dish of cells was washed twice with KRH buffer, then incubated for 150 min at 37 °C with 4.5 ml of KRH buffer containing 2.25 mCi of [³²P]P_i and stimulated with EGF (100 ng/ml) for 5 min. The buffer was removed, the cells washed twice with ice-cold KRH buffer, lysed with 0.4 ml of ice-cold Buffer A and centrifuged for 10 min at 13000 g. The lysates were incubated for 2 h at 4 °C with a control rabbit IgG immobilized on Protein A-Sepharose and, after centrifugation, the supernatants were removed and incubated for 2 h at 4 °C with anti-GSK3 α serum immobilized on Protein A-Sepharose. After centrifugation, the supernatant was removed and incubated for 2 h at 4 °C with anti-GSK3 β serum immobilized on Protein A-Sepharose. After centrifugation, the supernatant was discarded and the GSK3 α and GSK3 β immunoprecipitates each washed four times with 50 mM Tris/HCl (pH 7.0)/0.5 M NaCl/1 mM EDTA/1% Triton X-100, then denatured in 1% (w/v) SDS, heated for 5 min at 100 °C and subjected to electrophoresis on SDS/10%-polyacrylamide gels. The proteins were transferred to a nitrocellulose membrane, autoradiographed, and the membrane pieces containing each GSK3 isoform were excised, incubated for 30 min at 37 °C in 0.5% polyvinylpyrrolidone 40/0.1 M acetic acid, and then washed five times with water and twice with 50 mM Tris/HCl, pH 8.0. The membranes containing ³²P-labelled GSK3 α or GSK3 β (3000–10000 c.p.m. from four dishes of cells) were suspended in 0.04 ml of 50 mM Tris/HCl, pH 8.0, and digested for 30–45 min with 0.04–0.08 mg of trypsin. This treatment released > 90% of the ³²P radioactivity from the membranes, and the tryptic phosphopeptides were dried and incubated for 60 min at 0 °C with 0.1 ml of performic acid. Water (1.0 ml) was added and the solution frozen and freeze-dried. The pellet was dissolved in 0.1% trifluoroacetic acid (TFA) and applied to a small C18 column (SEP-PAK cartridge; Waters, Milford, MA, U.S.A.). After washing the column with 0.1% TFA, peptides were eluted with 0.1% TFA containing 50% acetonitrile, freeze-dried and subjected to isoelectric focusing as described in [18].

Chromatography of cell extracts on Mono Q and Mono S

Extracts (3–5 mg of protein) were applied to a 5 cm $\times$ 0.5 cm column of Mono Q equilibrated with 50 mM Tris/HCl (pH 7.3)/2 mM EDTA/2 mM EGTA/0.3 mM sodium orthovanadate/5% (v/v) glycerol/0.03% (w/v) Brij 35/0.1% (v/v) 2-mercaptoethanol/1 mM benzamide/0.1 mM phenylmethanesulphonyl fluoride. The flowthrough fractions were applied to 5 cm $\times$ 0.5 cm columns of Mono S equilibrated in 20 mM sodium glycerophosphate (pH 7.0)/1.5 mM EGTA/0.03% (w/v) Brij 35/5% (v/v) glycerol. After washing with 10 ml of equilibration buffer, the columns were developed with 40 ml linear salt gradients to 0.5 M NaCl in equilibration buffer.

RESULTS

Lack of effect of rapamycin on the inhibition of GSK3 by growth factors

Stimulation of A431 cells with EGF (Figure 1a) decreased the activity of GSK3 by about 50%. Pronounced inhibition was observed after 2 min, with maximal effects after 5–15 min. Rapamycin is an immunosuppressant which prevents the activation of p70^{S6K} by growth factors without affecting the activation of p42/p44^{mapk} or MAPKAP kinase-1 [19]. Pre-incubation of A431 cells for 10 min with 100 nM rapamycin had no effect on the inhibition of GSK3 by EGF (Figure 1a), although the expected blockade of p70^{S6K} phosphorylation was observed (results not shown).

Inhibition of GSK3 is reversed by incubation with protein serine/threonine phosphatases

The inhibition of GSK3 by EGF in A431 cells was reversed by

Figure 1 EGF induces the inhibition of GSK3 in A431 cells

Cells were stimulated with 100 ng/ml EGF for the times indicated, lysed, and both GSK3 isoforms immunoprecipitated and assayed as described in the Materials and methods section. (a) Cells were incubated for 10 min in the absence (●, -R) or presence (○, +R) of 100 nM rapamycin before the addition of EGF. The results are presented as means $\pm$ S.E.M. for the numbers of experiments indicated. The specific activity of GSK3 α + GSK3 β in the lysates from unstimulated cells (average for two experiments) was 0.091 ± 0.013 unit/mg. (b) Experiments were carried out in the absence of rapamycin as in (a), except that the GSK3 immunoprecipitates were incubated for 10 min with 200 units/ml PP1 γ (○) or 1 μ M microcystin-LR (MC) plus 200 units/ml PP1 γ (●) prior to assay. One unit of PP1 γ was that amount which catalysed the dephosphorylation of 1 μ mol of glycogen phosphorylase/min [22]. The Figure shows the results of two separate experiments. There was no activation of GSK3 by PP1 in unstimulated cells.

Figure 2 EGF induces the inhibition of both GSK3 isoforms in A431 cells

Cells were stimulated for 5 min with 100 ng/ml EGF and 4 mg of cell lysate chromatographed on Mono S as described in the Materials and methods section. The flow rate was 1 ml/min, and fractions (1 ml each) were collected and in (a) assayed for GSK3 activity with the synthetic peptide substrate phosphorylated (●, ○) or not phosphorylated (▼) at its most C-terminal serine residue. ○ Shows the results for EGF-stimulated cells and ▼, ● those for unstimulated cells. The positions of GSK3α (α) and GSK3β (β) are marked, and the NaCl gradient is shown by the diagonal line. (b and c) Aliquots of fractions 37–41 from (a) were immunoblotted with mixed antibodies to GSK3α and GSK3β as in [14]. (b) shows the immunoblot from unstimulated cells, and (c) shows the immunoblot from EGF-stimulated cells.

preincubating GSK3 immunoprecipitates with either protein phosphatase-1 (Figure 1b) or protein phosphatase 2A (results not shown). Re-activation did not occur in the presence of microcystin-LR (Figure 1b), which is a potent inhibitor of these enzymes. Protein phosphatases 1 and 2A had no effect on GSK3 immunoprecipitated from unstimulated cells (Figure 1b), indicating that GSK3 is fully active under these conditions.

Growth factors inhibit GSK3α and GSK3β

The experiments described above were carried out with mixed antibodies that immunoprecipitate both GSK3α and GSK3β (see the Materials and methods section). In order to examine the effects of growth factors on the individual isoforms, lysates from EGF-stimulated A431 cells were chromatographed on Mono S to resolve GSK3α and GSK3β [14]. These experiments demonstrated that EGF stimulation reduced the activity of both isoforms by about 50% (Figure 2). Consistent with phosphorylation being the mechanism of inhibition, EGF stimulation caused the inactive form of GSK3β to be eluted from Mono S at a slightly lower salt concentration, so that it now overlapped

Figure 3 Effect of EGF on the phosphorylation of GSK3 isoforms in A431 cells

(a) Cells were incubated with [32 P]P_i and then stimulated with EGF or with buffer (C, control) and lysed as described in the Materials and methods section. GSK3α (apparent molecular mass 51 kDa) and GSK3β (47 kDa) were immunoprecipitated sequentially from the lysates, subjected to SDS/PAGE (10% gels) and autoradiographed. (b) 32 P-labelled GSK3α and GSK3β from (a) were digested with trypsin and the tryptic phosphopeptides subjected to isoelectric focusing and autoradiography as described in the Materials and methods section. Lanes 1 and 4 show tryptic phosphopeptides 19–38 (GSK3α) and 7–28 (GSK3β) respectively, obtained by *in vitro* phosphorylation of each purified GSK3 isoform with MAPKAP kinase-1β as described in [9,10]. Lanes 2 and 5 show the GSK3 tryptic phosphopeptides A and B obtained from EGF-stimulated cells, and lanes 3 and 6 the tryptic phosphopeptides from unstimulated cells. The pI values of the two markers, Patent Blue (2.4) and azurin (5.7), are also shown.

Figure 4 Inhibition of glycogen synthase kinase-3 by okadaic acid in A431 cells is reversed by protein phosphatase-1

Cells were incubated with either 100 nM okadaic acid (○, ▽) or 400 nM okadaic acid (●, ▼). The cells were lysed at the times indicated, and both GSK3 isoforms immunoprecipitated and assayed for GSK3 activity after incubation for 10 min with either 200 munits/ml PP1γ (▽, ▼) or 200 munits/ml PP1γ plus 1 μM microcystin-LR (MC) (○, ●). Activities are expressed relative to that measured in GSK3 immunoprecipitates from cells not treated with okadaic acid. Similar results were obtained in three different experiments.

with the active form of GSK3α (Figure 2c). Consequently, treatment of fraction 38 with PP1 produced a very large increase in GSK3 activity, and incubation with fraction 41 hardly any (results not shown).

Identification of the residues on GSK3 α and GSK3 β phosphorylated *in vivo*

In order to prove that growth factors stimulate the phosphorylation of GSK3 and to identify the site(s) of phosphorylation, GSK3 α and GSK3 β were immunoprecipitated sequentially from ^{32}P -labelled A431 cells. These experiments demonstrated that GSK3 is phosphorylated in unstimulated cells and that phosphorylation increases after stimulation for 5 min with EGF (Figure 3a). EGF increased the ^{32}P -labelling of GSK3 α by $52 \pm 10\%$, and GSK3 β by $63 \pm 17\%$ in two separate experiments. Digestion of ^{32}P -labelled GSK3 α with trypsin followed by isoelectric focusing revealed two major ^{32}P -labelled tryptic peptides A and B (Figure 3b). The ^{32}P -labelling of tryptic peptide A increased in response to EGF, but the labelling of tryptic phosphopeptide B did not change significantly. Tryptic phosphopeptide A co-migrated in isoelectric-focusing experiments (Figure 3b) or during chromatography on a C18 column (results not shown) with the tryptic phosphopeptide from GSK3 α comprising residues 19–38 (Figure 3b), which is phosphorylated at Ser-21 by MAPKAP kinase-1 β or p70^{s6K}. Tryptic phosphopeptide A only contained phosphoserine, while tryptic phosphopeptide B contained equal amounts of phosphoserine and phosphotyrosine (results not shown). Similar results were obtained with GSK3 β . Tryptic digestion of ^{32}P -labelled GSK3 β also yielded two tryptic phosphopeptides, A and B. Phosphopeptide A showed a striking increase in response to EGF, whereas phosphopeptide B (which contained similar amounts of phosphoserine and phosphotyrosine) did not (Figure 3b). Phosphopeptide A co-migrated with the tryptic phosphopeptide from GSK3 β comprising residues 7–28, which is phosphorylated at Ser-9 by MAPKAP kinase-1 β or p70^{s6K} (Figure 3b).

Okadaic acid inactivates GSK3 by serine/threonine phosphorylation

Yang and co-workers recently reported that incubation of A431 cells with okadaic acid inhibited GSK3 by promoting dephosphorylation of the tyrosine residue [20]. Since okadaic acid is a potent inhibitor of PP1 and PP2A, they suggested that this compound may have promoted serine/threonine phosphorylation and activation of a protein tyrosine phosphatase which dephosphorylates and inactivates GSK3. We confirmed their observation that GSK3 is almost completely inactivated by incubation of A431 cells for 2 h with 400 nM okadaic acid, but inhibition was completely reversed by PP1 (Figure 4), demonstrating that it resulted from increased phosphorylation of a serine/threonine residue(s) and not decreased phosphorylation of the tyrosine residue.

DISCUSSION

The results presented in this and the two accompanying papers [16,21] demonstrate that GSK3 is rapidly inhibited in a number of mammalian cells, not just by insulin, but by other growth factors which signal via receptor protein tyrosine kinases. The effect is mediated by increased phosphorylation of a serine(s) located in the tryptic peptide containing the residue (Ser-21 in GSK3 α , Ser-9 in GSK3 β) phosphorylated by p70^{s6K} and MAPKAP kinase-1 β *in vitro* (Figure 3). p70^{s6K} and MAPKAP kinase-1 are both activated > 10-fold within 5 min of stimulating A431 cells with EGF (results not shown). However, the failure of rapamycin to affect the inhibition of GSK3 ([16,21]; Figure 1)

excludes a role for p70^{s6K}, and the inhibition of GSK3 α by growth factors ([16]; Figure 2) appears to eliminate the involvement of PKC, which phosphorylates GSK3 β but not GSK3 α [8]. Further work is needed to establish whether the EGF-stimulated inactivation of GSK3 results from phosphorylation by MAPKAP kinase-1 or another protein kinase that has so far escaped detection.

GSK3 is phosphorylated at a tyrosine (Tyr-279 in GSK3 α , Tyr-216 in GSK3 β) [12], and dephosphorylation of this residue decreases GSK3 activity *in vitro* [12,13]. However, the inhibitory effects of growth factors on GSK3 activity in L6, Chinese-hamster ovary and A431 cells cannot be mediated by tyrosine dephosphorylation for two reasons. Firstly, inhibition is reversed by protein serine/threonine phosphatases ([7,16,21]; the present paper). Secondly, no decrease in the ^{32}P -labelling of the phosphotyrosine-containing peptide occurs in EGF-stimulated A431 cells (Figure 3). The inactivation of GSK3 in okadaic acid-stimulated A431 cells also results from increased serine/threonine phosphorylation of GSK3 and not from decreased tyrosine phosphorylation [20], since it is reversed by incubation with PP1 (Figure 4). Incomplete immunoprecipitation of GSK3 from the lysates of okadaic acid-stimulated cells may explain the results of Yang and co-workers [20], since inspection of their data showed a decrease in the level of phosphoserine as well as phosphotyrosine.

The phosphotyrosine-containing tryptic peptide B (Figure 3) contains similar amounts of phosphoserine, suggesting that a serine residue(s) in these peptides (Ser-278 or Ser-282 in GSK3 α , Ser-215 or Ser-219 in GSK3 β) are phosphorylated *in vivo*. Bacterially expressed GSK3 β phosphorylates itself at tyrosine and serine residues [13]; thus the phosphate located at Tyr-216/Tyr-279 may be introduced by GSK3 itself. It would be interesting to know whether Ser-215/Ser-278 and Ser-219/Ser-282 are also sites of autophosphorylation.

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