Role of protein kinase B and the MAP kinase cascade in mediating the EGF-dependent inhibition of glycogen synthase kinase 3 in Swiss 3T3 cells

Morag Shaw, Philip Cohen*

MRC Protein Phosphorylation Unit, Department of Biochemistry, MSI/WTB Complex, Dow Street, University of Dundee, Dundee DD1 5EH, UK

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Abstract  Epidermal growth factor (EGF), insulin-like growth factor 1 (IGF1) and phorbol myristate acetate (PMA) induce the inhibition of glycogen synthase kinase 3 (GSK3) by stimulating the phosphorylation of an N-terminal serine. Here, we show that protein kinase B (PKB) plays a key role in mediating EGF-induced inhibition of GSK3ɛ and that the classical MAP kinase (MAPK) cascade has two functions in this process. Firstly, it makes a transient contribution to EGF-induced inhibition of GSK3ɛ. Secondly, it shortens the duration of PKB activation and GSK3ɛ inhibition. In contrast, PKB alone mediates the IGF1-induced inhibition of GSK3ɛ, while the MAPK cascade mediates the inhibition of GSK3ɛ by PMA.

Key words: Protein kinase B; Epidermal growth factor; Insulin; MAP kinase; Glycogen synthase kinase 3

1. Introduction

Glycogen synthase kinase 3 (GSK3) is a protein kinase that is thought to play multiple roles in cell regulation. For example, it phosphorylates and inhibits both glycogen synthase [1] and protein synthesis initiation factor eIF2B [2], which are likely to underlie, at least in part, the insulin-induced stimulation of glycogen synthesis [3–5] and protein synthesis [6]. Inhibition of GSK3 may also underlie the insulin or insulin-like growth factor 1 (IGF1)-induced dephosphorylation of the microtubule-associated protein Tau in cultured hippocampal neurones [7]. The inhibition of GSK3 has been suggested to underlie the phosphor ester-dependent dephosphorylation of the transcription factor c-Jun, at sites that lie proximal to the DNA binding domain [8], and to play a role in growth factor-induced protection against apoptosis [9].

The inhibition of GSK3 by insulin [10], growth factors [11] and phorbol myristate acetate (PMA) [12] results from the phosphorylation of GSK3 at a N-terminal serine residue (Ser-21 in GSK3ɛ, Ser-9 in GSK3β) and three protein kinases (protein kinase B (PKB) [10], MAP kinase (MAPK)-activated protein kinase 1 (MAPKAP-K1), also called p90rsk) and p70 S6 kinase (p70 S6K) [13,14], that become activated in response to one or more of these signals, phosphorylate these residues in vitro. The insulin- or IGF1-induced inhibition of GSK3 is prevented by inhibitors of phosphatidylinositol (Pi) 3-kinase (wortmannin or LY294002) that block the activation of PKB (also called c-Akt), but not by inhibitors of the activation of p70 S6K (rapamycin) or MAPKAP-K1 (PD98059) [10]. Moreover, cell transfection experiments using a dominant negative mutant of PKB support the view that PKB is likely to mediate the inhibition of GSK3 by insulin [15].

The inhibition of GSK3 by epidermal growth factor (EGF) is unaffected by rapamycin [16] and evidence has been presented that this effect is mediated by MAPKAP-K1. Thus, GSK3ɛ becomes phosphorylated at Ser-9 when co-transfected with MAPKAP-K1 [12] and the EGF-induced inhibition of GSK3 is largely suppressed in a cell line overexpressing a catalytically inactive mutant of MAPKAP kinase 1 (MKK1, also called MEK) [17], an enzyme that lies ‘upstream’ of MAPKAP-K1 in the classical MAPK cascade. In addition, the EGF-induced inhibition of GSK3 is enhanced in cells expressing a MKK1 mutant which displays some constitutive activity [17]. Moreover, in cells overexpressing MAPKAP-K1, the phosphorylation of GSK3β at Ser-9 is stimulated by PMA, which is known to activate the MAPK cascade, but not the activation of PKB [18].

The acute regulation of GSK3 activity by EGF and PMA was studied before the advent of inhibitors that are capable of preventing activation of the MAPK cascade. The advantage of using such small cell permeant protein kinase inhibitors (reviewed in [19]) is that they can be used to study the roles of protein kinases without the need for overexpression of active or inactive forms of protein kinases, which can lead to erroneous conclusions. We have therefore used two structurally distinct inhibitors of the MAPK cascade, namely PD98059 (which prevents the activation of MKK1 [20] and U0126 (which inhibits MKK1 [21]), in conjunction with two PI 3-kinase inhibitors (wortmannin and LY294002) and rapamycin, to identify the signalling pathways that mediate the EGF-induced inhibition of GSK3. In contrast to earlier reports, we find that a PI 3-kinase-dependent pathway, probably mediated by PKB, plays a major role in this process and that the MAPK cascade plays two distinct roles. Firstly, it contributes, albeit very transiently, to the inhibition of GSK3. Secondly, it controls the duration of inhibition of GSK3 by PKB.

2. Materials and methods

2.1. Materials

IGF1 and EGF were purchased from Gibco BRL (Paisley, UK),...
PMA and wortmannin from Sigma Chemical (Poole, UK), rapamycin, LY294002, PD98059 and U0126 from Calbiochem (Nottingham, UK) and protein G-Sepharose from Pharmacia (Milton Keynes, UK). Immunoprecipitating antibodies specific for PKB and MAPKAP-K1 were raised in sheep and affinity-purified on CH-Sepharose columns to which the appropriate peptide antigen was coupled covalently. Further antibodies were raised in sheep that recognise GSK3β phosphorylated at Ser-21 and that recognise dephosphorylated and phosphorylated GSK3 equally well. These antibodies were raised against the peptides RARTSS*FAEPG (residues 16-26 of GSK3β, where * denotes a phosphorylated residue) and QAP-DATPTLTNSS (residues 471-483 of human GSK3β) and affinity-purified as described above. All four antibodies can be purchased from UBI (Lake Placid, NY, USA).

2.2. Cell culture, stimulation and cell lysis
Mouse Swiss 3T3 fibroblasts were cultured to confluence and incubated for 16 h in Dulbecco's modified Eagles medium from which foetal calf serum was omitted. Cells were stimulated for the times indicated with IGF1, EGF or PMA with or without prior treatment for 10 min with wortmannin (100 nM), LY294002 (100 μM) or rapamycin (100 nM), or for 1 h with PD98059 (50 μM) or U0126 (10 μM). In experiments where cells were pretreated with wortmannin and either PD98059 or U0126, wortmannin was added for the last 10 min of the pre-incubation only. The cells were lysed in 1.0 ml of 50 mM Tris-HCl (pH 7.5), 1% (w/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 μM microcystin-LR, 0.1% (by volume) 2-mercaptoethanol, 1 mM benzamidine and 0.2 mM phenylmethylsulphonyl fluoride, frozen immediately in liquid nitrogen and stored at −80°C. Protein concentrations were determined according to Bradford using bovine serum albumin as a standard.

2.3. Immunoprecipitation and assay of PKB and MAPKAP-K1
PKB and MAPKAP-K1 were immunoprecipitated from 100 and 50 μg of cell lysate, respectively, and assayed using the peptide GRPRTSSFAEG, termed Crosstide [10,21,25]. One unit of activity (U) was defined as that amount which catalysed the phosphorylation of 1 nmol substrate in 1 min.

2.4. Immunoblotting of GSK3β
Cell lysates (40 μg) were denatured in 1% (by mass) sodium dodecyl sulfate (SDS), electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and immunoblotted with appropriate antibodies. Detection was performed using the enhanced chemiluminescence reagent (Amersham).

3. Results
3.1. IGF1- and EGF-dependent activation of PKB and the MAPK cascade in Swiss 3T3 cells
In order to study the potential roles of PKB and MAPKAP-K1 in the IGF1- and EGF-induced regulation of GSK3 activity, we first examined the time course of activation of these protein kinases after stimulation with each agonist. The activation of PKB and MAPKAP-K1 was measured after their immunoprecipitation from the cell lysates. In Swiss 3T3
cells, we found that PKBα and PKBγ were both activated by IGF1. The activation of both isozymes occurred with a half-time of about 1 min and was maximal after 5 min. There was no activation of PKBβ (data not shown). Since PKBγ accounted for almost 80% of the total PKB activity in cell lysates, the activity of this isoform was measured in all subsequent experiments. The MAPKAP-K1 antibody used in our experiments immunoprecipitates both MAPKAP-K1 isoforms (MAPKAP-K1α/RSK1 and MAPKAP-K1β/RSK2).

IGF1 triggered a strong and sustained activation of PKBγ, whereas activation by EGF was weaker and very transient (Fig. 1A). As expected from studies in other cells, the activation of PKBγ by either agonist was abolished by pretreatment of the cells with the PI 3-kinase inhibitors wortmannin or LY294002 (data not shown). In contrast, both IGF1 and EGF triggered a transient activation of MAPKAP-K1, a downstream component of the classical MAPK cascade, although activation induced by IGF1 was much weaker (Fig. 1B). As expected, the activation of MAPKAP-K1 by either EGF (Fig. 1B) or IGF1 (data not shown) was abolished by U0126 (Fig. 1B) or PD98059 (data not shown), the specific inhibitors of the MAPK cascade. The immunosuppressant drug rapamycin, an inhibitor of mTOR that prevents the IGF- or EGF-induced activation of p70 S6K, had no effect on the activation of PKB by either agonist (data not shown).

3.2. EGF- and IGF1-induced phosphorylation of GSK3α at Ser-21

IGF1 has been shown previously that GSK3α and GSK3β are completely inactivated by the phosphorylation of Ser-21 and Ser-9, respectively [13,14], and that IGF1-induced inhibition in cells is accompanied by the phosphorylation of these and no other sites [10]. Moreover, IGF1 is unable to induce any inhibition of a GSK3β mutant in which Ser-9 has been mutated to Ala [26]. It is therefore possible to assess the inhibition of GSK3 simply by immunoblotting cell lysates with a phospho-specific antibody that recognises the Ser-21-phosphorylated form of GSK3α.

Previous work has shown that IGF1 [27] and EGF [11] both induce 50% inhibition of GSK3, which is maximal after

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Fig. 3. Effect of specific kinase inhibitors on the IGF1- and EGF-induced phosphorylation of GSK3α at Ser-21 in Swiss 3T3 cells. Cells were stimulated for 5 min with 100 ng/ml IGF1 or for 2 or 5 min with 100 ng/ml EGF and the lysates were immunoblotted with antibodies that recognise the Ser-21-phosphorylated enzyme (pGSK3α) or with antibodies that do not distinguish between the phosphorylated and dephosphorylated forms of the enzyme (GSK3). Prior to stimulation with agonist, the cells were pretreated with wortmannin, LY294002, PD98059, U0126 or rapamycin as described in Section 2. Similar results were obtained in three separate experiments.

Fig. 4. Effect of combining inhibitors of PI 3-kinase and the MAPK cascade on the EGF-induced phosphorylation of GSK3α at Ser-21 in Swiss 3T3 cells. Cells were stimulated for the times indicated with 100 ng/ml EGF and the lysates immunoblotted with antibodies that recognise the Ser-21-phosphorylated enzyme (pGSK3α) or with antibodies that do not distinguish between the phosphorylated and dephosphorylated forms of the enzyme (GSK3). Prior to stimulation with agonist, the cells were pretreated as described in Section 2 with wortmannin or PD98059/U0126 or with combinations of these inhibitors as indicated. Similar results were obtained in three separate experiments.
about 5 min. In the present study, the phosphorylation of GSK3α at Ser-21 induced by either agonist was found to be nearly maximal after 2 min. However, while Ser-21 phosphorylation stimulated by IGF1 was sustained for at least 30 min, EGF-stimulated phosphorylation was transient and declined to near basal levels after 15 min (Fig. 2).

The time course of Ser-21 phosphorylation in response to EGF or IGF1 (Fig. 2) correlated with the activation of PKB rather than with the activation of MAPKAP-K1 (Fig. 1). Consistent with an important role for PKB, the IGF1-induced phosphorylation of GSK3α at Ser-21 after 5 min (or at any other time point) was prevented by prior treatment of the cells with wortmannin or LY294002, but not by PD98059 or U0126 (Fig. 3). The EGF-induced phosphorylation of Ser-21 after 2 min was also prevented by wortmannin or LY294002 and unaffected by PD98059 or U0126 (Fig. 3B). However, surprisingly, the EGF-induced phosphorylation of GSK3 at Ser-21 was hardly affected by inhibitors of PI 3-kinase or of the MAPK cascade after 5 min stimulation (Fig. 3). Statistical analysis of the immunoblotting data from three different experiments revealed that wortmannin inhibited EGF-induced inhibition of GSK3 by only 36 ± 2% after 5 min, while LY294002, PD98059 and U0126 had no statistically significant effect at this time point.

One possible reason for the very weak effect of PI 3-kinase inhibitors or MAPK cascade inhibitors on the EGF-induced phosphorylation of GSK3α at Ser-21 after 5 min was that activation of either pathway was sufficient for near maximal phosphorylation of Ser-21 at this time. If this was the case, then, blockade of both pathways would be required to suppress the effect of EGF. This explanation appears to be correct, because the combination of wortmannin and PD98059 or wortmannin plus U0126 completely prevented the EGF-induced phosphorylation of Ser-21 after 5 min (Fig. 4B).

Even more unexpectedly, the phosphorylation of Ser-21 was largely inhibited by wortmannin alone after stimulation with EGF for 10 or 15 min and pre-incubation with PD98059 or U0126 actually enhanced the EGF-induced phosphorylation of Ser-21 after 15 min (Fig. 4). After 15 min, the enhancement of GSK3α phosphorylation at Ser-21 was 299 ± 13% and 373 ± 15% in the presence of PD98059 and U0126, respectively (± S.E.M. for three experiments). These observations led us to re-examine the EGF-induced activation of PKB in the presence of PD98059 or U0126. These experiments revealed that, in the presence of MAPK cascade inhibitors, the activation of PKB by EGF was not nearly so transient and remained at a much higher level after 15 min (Fig. 5). These unexpected observations are considered further under Section 4.

**3.3. Effect of PMA on the phosphorylation of GSK3**

As observed in other cells, PMA induced the activation of MAPKAP-K1 (but not PKB) and this was strongly, but not...
completely, suppressed by pretreatment of the cells with PD98059 or U0126 (Fig. 6A). PMA also induced the phosphorylation of GSK3α at Ser-21 and this was partially suppressed by pretreatment with PD98059 or U0126 (Fig. 6B). Pretreatment with PD98059 plus wortmannin, or U0126 plus wortmannin, did not produce any further reduction in the PMA-induced phosphorylation of Ser-21 (Fig. 6C).

4. Discussion

The results described in this paper confirm that IGF1 triggers the phosphorylation and inhibition of GSK3 via a PI 3-kinase-dependent pathway that is unaffected by inhibitors of the MAPK cascade or rapamycin and which is presumably mediated by PKB. However, our results indicate that the signalling pathways that are rate-limiting for the EGF-induced phosphorylation of GSK3α at Ser-21 vary with the time of stimulation. A PI 3-kinase-dependent pathway (presumably mediated by PKB) is also responsible for the phosphorylation of GSK3α at Ser-21 after stimulation for 2 min. However, after 5 min, a MAPK-dependent pathway (presumably mediated by MAPKAP-K1), as well as the PI 3-kinase-dependent pathway, is also capable of phosphorylating Ser-21 maximally. Thus, after 5 min, it is necessary to block both pathways in order to observe significant inhibition of EGF-induced Ser-21 phosphorylation.

These observations are reminiscent of the tumour necrosis factor-induced phosphorylation of the transcription factor CREB at Ser-133 in HeLa cells. After 5 min stimulation, Ser-133 phosphorylation can be prevented by an inhibitor of stress-activated protein kinase 2/p38 (SAPK2/p38) but, after 15 min, CREB phosphorylation can only be blocked by inhibiting the classical MAPK cascade and SAPK2/p38 [28]. These observations highlight the need to use combinations of small cell permeant inhibitors in order to deduce which signalling pathways are rate-limiting in any particular situation.

A surprising observation was that the phosphorylation of GSK3α at Ser-21 was again largely blocked by inhibitors of PI 3-kinase alone, after stimulation with EGF for 10 min, even though the activation of MAPKAP-K1 was still quite high and the activation of PKB was declining (Fig. 1). These observations highlight the need to use combinations of small cell permeant inhibitors in order to block both pathways (presumably mediated by PKB) and to observe significant inhibition of EGF-induced Ser-21 phosphorylation.

In summary, our results demonstrate that PKB plays a major role in the regulation of GSK3 activity by EGF and that the classical MAPK cascade plays two roles in this process. Firstly, it contributes to the EGF-induced inhibition of GSK3 after 5 min. Secondly, it controls the inactivation of PKB and time of re-activation of GSK3 thereafter.

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References