Further evidence that the tyrosine phosphorylation of glycogen synthase kinase-3 (GSK3) in mammalian cells is an autophosphorylation event

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Phosphorylation of the endogenous GSK3α (glycogen synthase kinase-3α) at Tyr^{279} and GSK3β at Tyr^{216} was suppressed in HEK-293 or SH-SY5Y cells by incubation with pharmacological inhibitors of GSK3, but not by an Src-family inhibitor, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), or a general protein tyrosine kinase inhibitor (genistein). GSK3β transfected into HEK-293 cells or *Escherichia coli* became phosphorylated at Tyr^{216}, but catalytically inactive mutants did not. GSK3β expressed in insect Sf21 cells or *E. coli* was extensively phosphorylated at Tyr^{216}, but the few molecules lacking phosphate at this position could autophosphorylate at Tyr^{216} *in vitro* after incubation with MgATP. The rate of autophosphorylation was unaffected by dilution and was suppressed by the GSK3 inhibitor kenpaullone. Wild-type GSK3β was unable to catalyse the tyrosine phosphorylation of catalytically inactive GSK3β lacking phosphate at Tyr^{216}. Our results indicate that the tyrosine phosphorylation of GSK3 is an intramolecular autophosphorylation event in the cells that we have studied and that this modification enhances the stability of the enzyme.

Key words: glycogen synthase kinase-3 (GSK3), indirubin, kenpaullone, phosphotyrosine, tyrosine kinase.

INTRODUCTION

The protein kinase GSK3 (glycogen synthase kinase-3) has been studied intensively in recent years, because it is a central player in several different signal transduction pathways in mammalian cells. A variety of agonists inhibit GSK3 by triggering its phosphorylation at an N-terminal serine residue (Ser^{21} in GSK3α and Ser^{9} in the GSK3β isoform), and this can be catalysed by several different protein kinases that are switched on in response to signals that activate PI3K (phosphoinositide 3-kinase) or the classical MAPK (mitogen-activated protein kinase) cascade, or elevate cAMP. GSK3 also becomes inhibited by a different mechanism in response to secreted glycoproteins, termed Wnts, a pathway that is critical for the specification of cell fate during embryonic development (reviewed in [1,2]).

An intriguing feature of GSK3 is the presence of a phosphotyrosine residue in the 'activation loop' of this enzyme (Tyr^{279} in GSK3α and Tyr^{216} in GSK3β), a position equivalent to that of the phosphotyrosine residue that is critical for the activity of MAPK [3]. The tyrosine residue in GSK3 is important for activity, because its mutation to phenylalanine or its dephosphorylation decreases activity [3,4]. However, the mechanism by which the tyrosine residue becomes phosphorylated in cells and whether it plays a regulatory, as opposed to a purely structural role, has been the subject of much debate.

It was initially suggested that the tyrosine phosphorylation of GSK3 was catalysed by another protein kinase, because no phosphate was incorporated into tyrosine when GSK3 isolated from mammalian cells was incubated with MgATP [3]. However, it was reported subsequently that bacterially expressed GSK3β contained some phosphotyrosine, as judged by immunoblotting with a phosphotyrosine-specific antibody, and that, upon incubation with MgATP, further phosphate was incorporated into tyrosine, as well as serine and threonine, residues. [4]. The location of the phosphotyrosine residue(s) in bacterially expressed GSK3 was not determined, but these experiments suggested that the tyrosine phosphorylation of GSK3 might be an autophosphorylation event catalysed by GSK3 itself. On the other hand, genetic evidence supports the view that zaphod kinase-1 (ZAK1) catalyses the tyrosine phosphorylation of GSK3 in *Dictyostelium discoideum* in response to elevated levels of extracellular cAMP [5]. However, human homologues of ZAK1 do not appear to exist. Thus it is not clear how the findings in *Dictyostelium* relate to mammals. Nevertheless, other mammalian tyrosine kinases, such as Pyk2 and the Src-related kinase Fyn, have been reported to phosphorylate GSK3 at tyrosine residue(s) *in vitro* [6,7].

In the present paper, we have re-investigated the mechanism by which the tyrosine phosphorylation of GSK3 takes place in cells.

EXPERIMENTAL

Materials

Mouse monoclonal antibodies that recognize GSK3α phosphorylated at Tyr^{279}, as well as GSK3β phosphorylated at Tyr^{216}, were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.) and Transduction Laboratories (Lexington, KY, U.S.A.). The less-sensitive Upstate Biotechnology phospho-specific antibody was used to detect the tyrosine phosphorylation of recombinant or transfected GSK3, whereas the more-sensitive antibody from Transduction Laboratories was used to detect phosphorylation of the endogenous GSK3 isoforms in cell extracts. A mouse monoclonal antibody that recognizes the phosphorylated and unphosphorylated forms of GSK3β equally well was purchased from Transduction Laboratories and an antibody (available from Upstate Biotechnology) that recognizes all forms of GSK3α was raised in sheep. These two antibodies were mixed together in order to detect GSK3α and GSK3β simultaneously in cell

Abbreviations used: GSK3, glycogen synthase kinase-3; GST, glutathione S-transferase; NGF, nerve growth factor; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine; PTP, protein tyrosine phosphatase.

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extracts. Kenpaullone, indirubin, purvalanol, genistein and PP2 {4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine} were purchased from Calbiochem (Beeston, Notts., U.K.) and anisomycin and cycloheximide were from Sigma (Poole, Dorset, U.K.).

Site-directed mutagenesis and measurement of GSK3 activity using the standard synthetic peptide substrate YRRAAVPPSPSLSRHSSPHQpSEDEEE (where pS is phosphoserine) was carried out as described previously [8]. GSK3β was expressed as a His_6-tagged protein in Sf21 cells, or as a glutathione S-transferase (GST)-fusion protein in HEK-293 cells, and was purified as described previously [8]. GSK3β expressed in Sf21 cells was > 95 % pure as judged by SDS/PAGE and had a specific activity of 280 units/mg. GST–GSK3β expressed in *Escherichia coli* was also purified on glutathione–Sepharose. The full-length enzyme comprised 24 % of the protein, as judged by densitometric analysis of SDS/polyacrylamide gels that had been stained with colloidal Coomassie Blue. After correction for purity, the specific activity was 150 units/mg. All preparations of GSK3 were stored unfrozen at − 20 °C in 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.1 % (v/v) 2-mercaptoethanol, 150 mM NaCl, 0.03 % (w/v) Brij-35, 50 % (v/v) glycerol, 1 mM benzamidine and 0.2 mM PMSF.

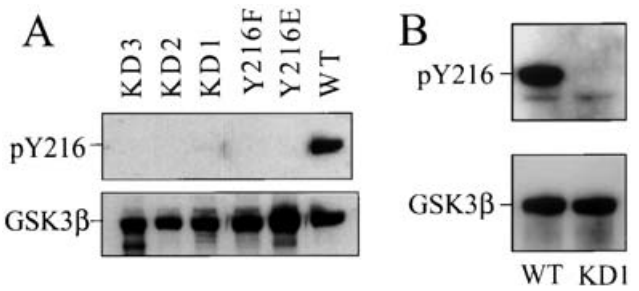

Figure 1 Wild-type (WT) GSK3β, but not catalytically inactive mutants, are phosphorylated at Tyr^{216} in cells

(**A**) Various mutants of GSK3β were expressed in HEK-293 cells, purified by affinity chromatography on glutathione–Sepharose, separated by SDS/PAGE and immunoblotted with an antibody that recognizes GSK3 phosphorylated at Tyr^{216}, and with an antibody that recognizes the unphosphorylated and phosphorylated forms of GSK3 equally well. The catalytically inactive mutants are KD1 (GSK3β[K85A]), KD2 (GSK3β[K85R,K86R]) and KD3 (GSK3β[K85M,K86A]). The two mutants in which Tyr^{216} was changed to either Phe or Glu (Y216F and Y216E respectively) were included as controls. (**B**) Same as (**A**), except that WT GSK3β and a catalytically inactive mutant were expressed in *E. coli*.

RESULTS

Effects of mutation and pharmacological inhibitors on the tyrosine phosphorylation of GSK3

To investigate the mechanism of Tyr^{216} phosphorylation in cells, several catalytically inactive mutants of GSK3β were generated in which interaction with ATP is abolished, namely GSK3β[K85A] ($Lys^{85} \rightarrow$ Ala mutation), GSK3β[K85R,K86R] ($Lys^{85} \rightarrow$ Arg and $Lys^{86} \rightarrow$ Arg mutations) and GSK3β[K85M,K86A] ($Lys^{85} \rightarrow$ Met and $Lys^{86} \rightarrow$ Ala mutations). Vectors expressing GST fusions of the wild-type and mutant enzymes were transfected into HEK-293 cells and, following purification on glutathione–Sepharose, the status of GSK3β phosphorylation at Tyr^{216} was assessed by immunoblotting. Additional mutants in which Tyr^{216} was changed to either Glu (GSK3β[Y216E]) or Phe (GSK3β[Y216F]) were included as controls. The phospho-specific antibody that recognizes phosphorylated Tyr^{216} did not recognize either GSK3β[Y216F] or GSK3β[Y216E], confirming the specificity of the antibodies (Figure 1A). None of the catalytically inactive mutants were phosphorylated at Tyr^{216}, in contrast with wild-type GSK3. Similar results were obtained when GSK3β was expressed in *E. coli*; the wild-type enzyme was phosphorylated at Tyr^{216}, but a catalytically inactive mutant was not (Figure 1B).

We next examined the effects of two structurally unrelated pharmacological inhibitors of GSK3, Kenpaullone and indirubin, on the tyrosine phosphorylation of GSK3 in HEK-293 cells [9,10]. These experiments showed that phosphorylation of the endogenous GSK3α on Tyr^{279} was greatly decreased after prolonged incubation with either compound, although the amount of GSK3α protein remained constant (Figure 2A). Phosphorylation of the endogenous GSK3β also decreased, although less markedly. Kenpaullone also induced the dephosphorylation of both GSK3 isoforms in SH-SY5Y cells and PC12 cells (results not shown).

The slow dephosphorylation of GSK3 isoforms in cells treated with GSK3 inhibitors (Figure 2A) raised the possibility that the observed decrease did not result from dephosphorylation, but from newly synthesized unphosphorylated GSK3 replacing the phosphorylated GSK3 that was already present before the addition of the drugs. To examine this possibility, we studied the half life of GSK3 isoforms by incubating cells in the presence of the protein synthesis inhibitors cycloheximide or anisomycin. These experiments failed to show any decrease in the level of either GSK3 isoform up to 12 h (Figure 2B), a time at which extensive loss of phosphate from Tyr^{279}/Tyr^{216} had taken place in cells incubated with GSK3 inhibitors (Figure 2A). The presence of cycloheximide or anisomycin did not affect the rate at which phosphate was lost from Tyr^{279}/Tyr^{216} after the addition of Kenpaullone or indirubin (results not shown). In contrast, c-myc, which is known to have a very short half life, had completely disappeared 4 h after the addition of either protein synthesis inhibitor, as expected (results not shown). These experiments demonstrate that GSK3 isoforms do indeed undergo a slow dephosphorylation at Tyr^{279}/Tyr^{216} after incubation with GSK3 inhibitors.

Kenpaullone and indirubin have been reported to inhibit cyclin-dependent protein kinases, as well as GSK3 [10]. In order to examine the specificity of the effect, we therefore performed control experiments using purvalanol, an inhibitor of cyclin-dependent protein kinases that does not inhibit GSK3 [11]. Incubation with purvalanol did not decrease the tyrosine phosphorylation of GSK3 (Figure 2C). The Src-family tyrosine kinase inhibitor, PP2 [12], or genistein, a general protein tyrosine kinase inhibitor, also had no effect on the tyrosine phosphorylation of GSK3 isoforms in HEK-293 cells (Figure 2D). The concentration of PP2 used in these experiments (10 μM) is two orders of magnitude greater than that needed to completely suppress the phosphorylation of DAPP1 (dual adaptor for phosphotyrosine and 3-phosphoinositides-1), a validated substrate of src-related kinases [13]. At the concentrations used, neither PP2 [11] nor 0.1 mM genistein (results not shown) inhibit GSK3.

If the tyrosine phosphorylation of GSK3 is an autophosphorylation event, then the absence of tyrosine phosphorylation in the catalytically inactive mutants suggested that phosphorylation at Tyr^{216} was likely to be an intramolecular process; otherwise one might have expected the catalytically inactive phosphotyrosine-deficient mutants to have been phosphorylated by the endogenous wild-type GSK3 in the transfected cells (Figure 1). To investigate this further, we purified the phosphotyrosine-deficient catalytically inactive mutants of GST–GSK3β from HEK-293 cells and incubated them with MgATP and active wild-type His_6-tagged GSK3β isolated from Sf21 insect cells. These experiments

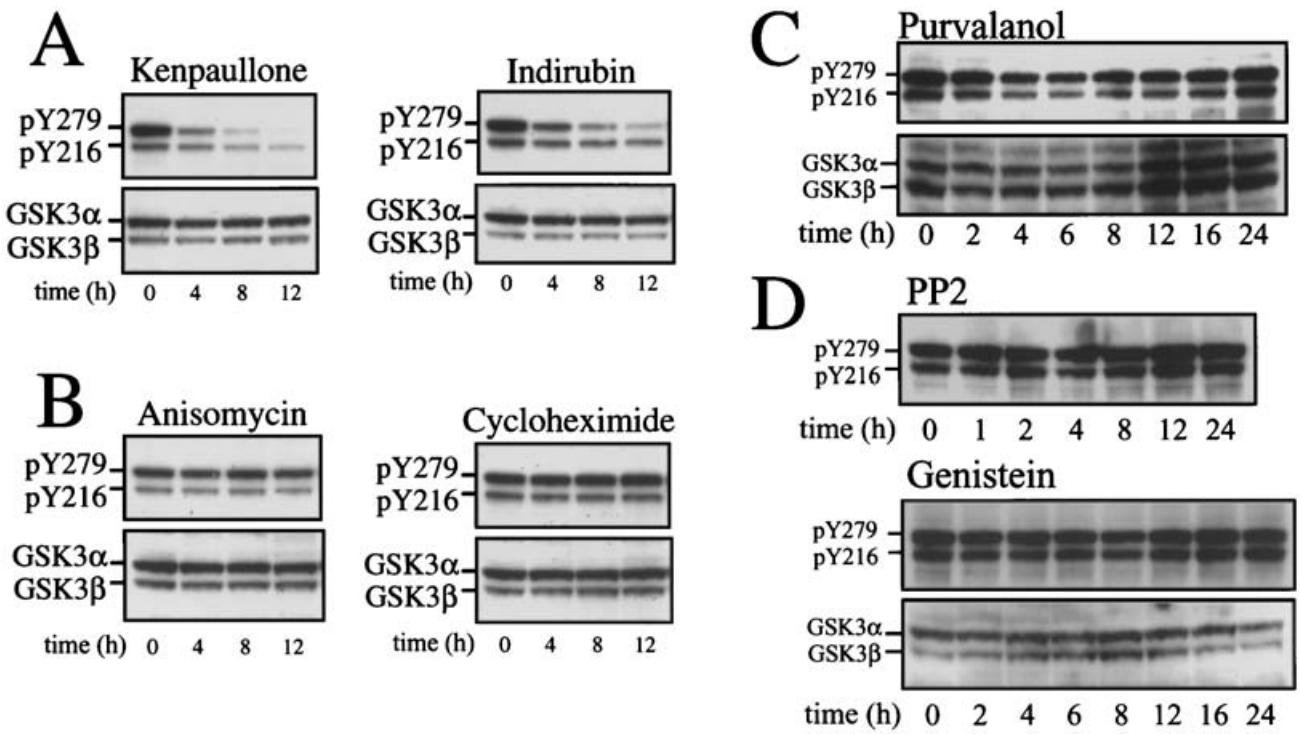

Figure 2 Effect of inhibitors on the tyrosine phosphorylation of GSK3

(**A**) HEK-293 cells were treated with Kenpaullone or indirubin (both at 10 μM). At the times indicated, the cells were lysed, subjected to SDS/PAGE, transferred on to PVDF membranes and immunoblotted with the antibodies that recognize GSK3α phosphorylated at Tyr^{279} (pY279) and GSK3β phosphorylated at Tyr^{216} (pY216), and with the antibodies that recognize unphosphorylated and phosphorylated GSK3α and GSK3β equally well (GSK3α, GSK3β). (**B**) Same as (**A**), except that the HEK-293 cells were incubated with the protein synthesis inhibitors anisomycin or cycloheximide (both at 10 μg/ml) for the times indicated. (**C**) Same as (**A**), except that the HEK-293 cells were incubated with purvalanol (10 μM) for the times indicated. (**D**) Same as (**A**), except that the HEK-293 cells were incubated with the Src-family inhibitor PP2 (10 μM) or genistein (0.1 mM) for the times indicated.

confirmed that the active His$_6$-tagged-GSK3β was phosphorylated at Tyr^{216}, but was unable to phosphorylate the catalytically inactive mutants at Tyr^{216} (Figure 3A).

Autophosphorylation of GSK3β at Tyr^{216} *in vitro*

It has been reported that bacterially expressed GSK3β undergoes autophosphorylation at tyrosine, as well as serine and threonine, residues upon incubation with MgATP, but the site(s) of phosphorylation was (were) not determined [4]. We confirmed these results using GSK3β expressed in either *E. coli* (results not shown) or Sf21 insect cells (Figure 4A). Kenpaullone (20 μM) strongly suppressed the autophosphorylation of GSK3β at Tyr^{216} *in vitro* whether GSK3 was expressed in Sf21 cells (Figure 4A) or in *E. coli* (results not shown), suggesting that it was catalysed by GSK3, and not by a contaminating kinase. The proportion of phosphate attached to Tyr^{216} was approx. 10% of that present in phosphothreonine and phosphoserine together, and the amount of phosphate incorporated into tyrosine never exceeded 0.05 mol/mol of GSK3β, even after prolonged incubation of the Sf21-expressed or *E. coli*-expressed GSK3β with MgATP or MnATP (results not shown).

If the autophosphorylation of GSK3 is an intramolecular process, then the rate of tyrosine phosphorylation should be independent of enzyme concentration. We therefore incubated 5 μg of GSK3β with Mg[γ-^{32}P]ATP in a total volume of either 25 or 250 μl. After 5 min at 30 °C, the GSK3β was precipitated with trichloroacetic acid and was subjected to phosphoamino acid analysis. This experiment showed that the incorporation of phosphate into tyrosine, threonine or serine residues per mol of GSK3β was similar at both dilutions (Figure 3B).

To establish whether the radiolabelled phosphate incorporated into GSK3β upon incubation with MgATP was associated with Tyr^{216} or another tyrosine residue(s), we digested the autophosphorylated enzyme with trypsin. The tryptic phosphopeptides were resolved by chromatography on a C_{18} column (Figure 4B) and each ^{32}P-labelled peptide was subjected to phosphoamino acid analysis. These experiments revealed that phosphotyrosine was present only in the minor phosphopeptide T2 (Figure 4C), whereas all the other peptides contained phosphothreonine or phosphoserine (results not shown). Peptide T2 was subjected to MS, which revealed the presence of a peptide with a m/z of 1361.6, corresponding to that of the tryptic peptide expected to contain Tyr^{216} (GEPNVSYICSR) plus one phosphate. To establish that the phosphotyrosine was really present at Tyr^{216}, the ^{32}P-labelled phosphopeptide was subjected to solid-phase sequencing. This experiment revealed that ^{32}P radioactivity was released from the peptide after the seventh cycle of Edman degradation (Figure 4D), corresponding to the position of Tyr^{216}.

To try and incorporate more radiolabelled phosphate into Tyr^{216}, we first incubated GSK3β (0.2 mg/ml) with high concentrations of protein tyrosine phosphatase 1B (PTP1B; 0.2 mg/ml) to dephosphorylate Tyr^{216}. An 8 h incubation at ambient temperature (21 °C) was found to be necessary to achieve nearly complete dephosphorylation of Tyr^{216} under these conditions and this was accompanied by an almost complete loss of activity towards the standard peptide substrate (results not shown). However, when the PTP1B-treated GSK3β was incubated with MgATP, and in the presence of sodium vanadate to inhibit the phosphatase, the ability

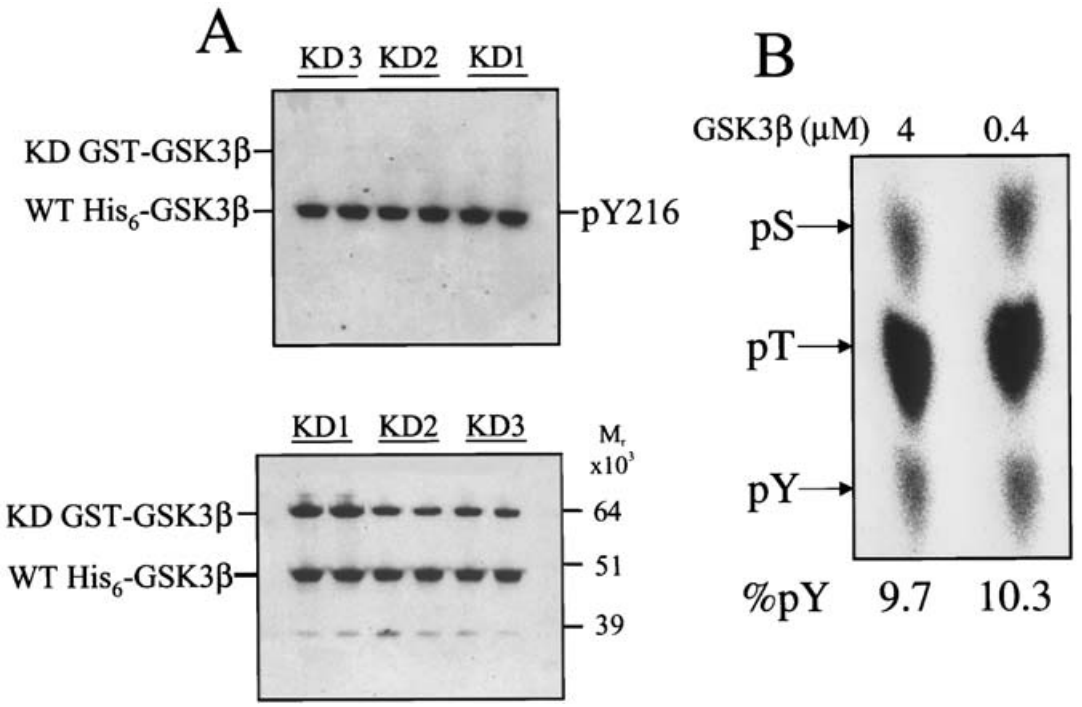

Figure 3 Wild-type (WT) GSK3β expressed in Sf21 insect cells does not phosphorylate a catalytically inactive mutant of GSK3β at Tyr^{216}

(**A**) The three catalytically inactive mutants of GST–GSK3 {KD1 (GSK3β[K85A]), KD2 (GSK3β[K85R,K86R]) and KD3 (GSK3β[K85M,K86A])} that had been purified from HEK-293 cell extracts as in Figure 1, were incubated for 1 h with 10 mM magnesium acetate and 0.1 mM ATP with active WT His_6–GSK3β purified from Sf21 insect cells. The reaction mixture was then subjected to SDS/PAGE and immunoblotted with an antibody that recognizes GSK3β phosphorylated at Tyr^{216} (upper panel) or with an antibody that recognizes the unphosphorylated and phosphorylated forms of GSK3β equally well (lower panel). (**B**) GSK3β (5 μg) purified from Sf21 insect cells was incubated for 5 min at 30 °C with Mg[γ-^{32}P]ATP (1000 c.p.m./pmol) in a total volume of either 25 μl (4 μM GSK3β) or 250 μl (0.4 μM GSK3β) of 50 mM Tris/HCl, pH 7.5, 0.03 % (w/v) Brij-35, 0.1 % (v/v) 2-mercaptoethanol and 1 mM sodium orthovanadate. The GSK3β was precipitated with 20 % (w/v) trichloroacetic acid, denatured in SDS, subjected to SDS/PAGE, then transferred on to a PVDF membrane. The bands corresponding to ^{32}P-labelled GSK3β were excised, then subjected to partial acid hydrolysis followed by electrophoresis on thin-layer cellulose to separate phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) [17]. The percentage of ^{32}P radioactivity present as phosphotyrosine relative to the total amount of ^{32}P-labelled phospho-amino acids was quantified by phosphoimager analysis (% pY).

of GSK3β to autophosphorylate at Tyr^{216} was not enhanced, but was actually abolished (results not shown), nor was there any reactivation. This unexpected finding was explained when we incubated GSK3β in the absence of PTP1B and in the presence of sodium vanadate. Under these conditions, GSK3β lost only 30–40 % of its activity after 8 h at ambient temperature, and the level of phosphorylation of Tyr^{216} remained stable as judged by immunoblotting (Figure 5, upper panel). However, the ability of GSK3β to autophosphorylate at Tyr^{216} was lost (Figure 5, lower panel). Similar results were obtained in a number of experiments. These results, which imply that molecules of GSK3β lacking phosphate at Tyr^{216} are unstable at ambient temperature, are considered further in the Discussion section.

Effects of staurosporine and NGF (nerve growth factor) withdrawal on the tyrosine phosphorylation of GSK3

It has been reported that the induction of apoptosis in SH-SY5Y cells, triggered by the addition of low concentrations of staurosporine (a non-specific protein kinase inhibitor) to the culture medium, stimulates the tyrosine phosphorylation of GSK3 [14]. Similarly, it has been reported that the withdrawal of NGF from PC12 cells triggers the tyrosine phosphorylation of GSK3 [14]. We repeated both of these experiments but, in our hands, there was no increase in the tyrosine phosphorylation of either GSK3 isoform under either of these conditions (Figures 6A and 6B).

DISCUSSION

Previous work has led to conflicting conclusions as to whether the tyrosine phosphorylation of GSK3 is catalysed by GSK3 itself or by a distinct tyrosine kinase (see the Introduction). Although it is difficult to completely exclude the existence of a protein kinase distinct from GSK3 that targets Tyr^{279} of GSK3α and Tyr^{216} of GSK3β, we present several new lines of evidence in the present paper that indicate that phosphorylation of these residues is an intramolecular autophosphorylation event in the cells that we have studied. First, we have found that Tyr^{216} is not phosphorylated when catalytically inactive mutants of GSK3β are expressed in either HEK-293 cells (Figure 1A) or *E. coli* (Figure 1B), in contrast with the wild-type enzyme. Moreover, both GSK3 isoforms become dephosphorylated if cells are incubated with cell-permeant inhibitors of GSK3 (Figure 2A). Both of these experiments strongly suggest that the phosphorylation of Tyr^{279}/Tyr^{216} is catalysed by GSK3 itself, but are not definitive because it could be argued that the mutation of GSK3 or the binding of small molecule inhibitors alters the conformation of GSK3 in such a way as to prevent another protein tyrosine kinase from phosphorylating Tyr^{279}/Tyr^{216}. It is even conceivable that GSK3 could be required to activate a protein tyrosine kinase that phosphorylates Tyr^{279}/Tyr^{216}. However, in the present study, we clearly demonstrate that GSK3β is capable of catalysing the autophosphorylation of Tyr^{216} *in vitro*. Therefore the simplest and most likely interpretation of the data is that the phosphorylation of Tyr^{279}/Tyr^{216} in cells is also catalysed by GSK3.

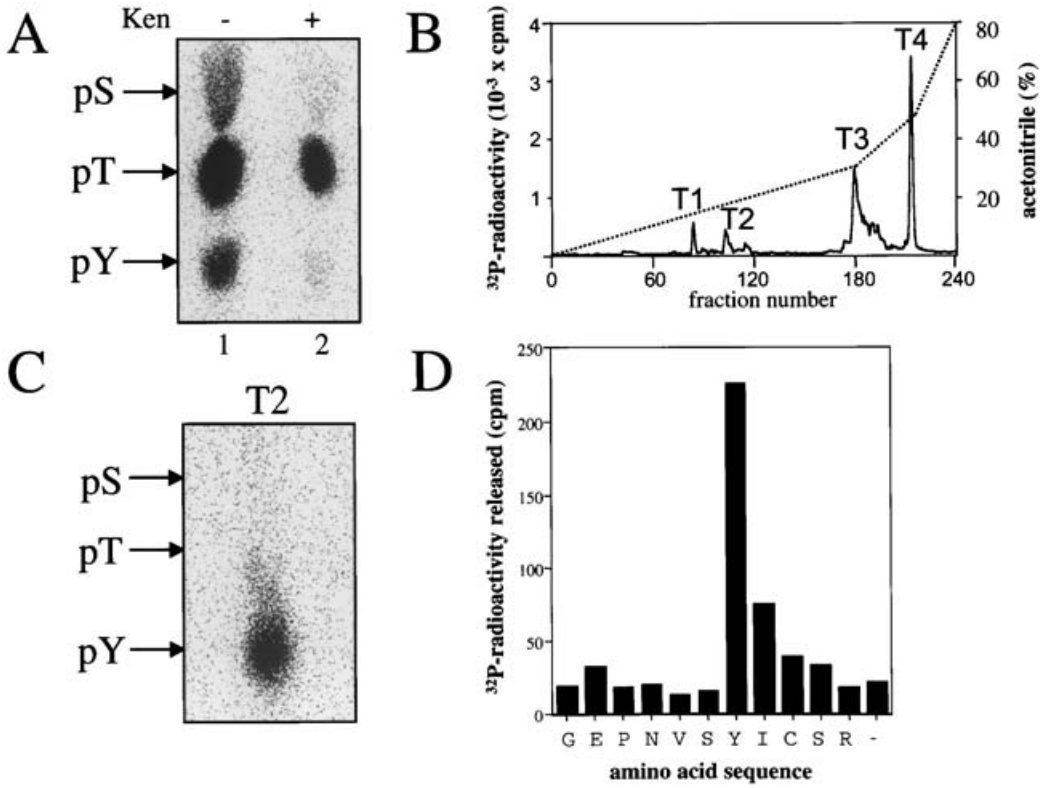

Figure 4 GSK3β autophosphorylates at Tyr^{216}

(**A**) GSK3β (0.4 μM) purified from Sf21 insect cells was incubated for 5 min at 30 °C with Mg[γ-^{32}P]ATP (7000 c.p.m./pmol) in the absence (lane 1) or presence (lane 2) of 20 μM Kenpaullone (Ken). Following SDS/PAGE and transfer on to PVDF membranes, the bands corresponding to ^{32}P-labelled GSK3β were excised and subjected to phospho-amino acid analysis (see legend to Figure 3B). (**B**) Same as (**A**), except that the concentration of GSK3 was 4 μM and autophosphorylation was carried out for 30 min. After SDS/PAGE, the gel piece containing ^{32}P-labelled GSK3β was excised, digested with trypsin and chromatographed on a Vydac C_{18} column, equilibrated in 0.1 % (v/v) trifluoroacetic acid. The column was developed with an acetonitrile gradient (broken line) in 0.1 % trifluoroacetic acid and fractions of 0.1 ml were collected. The ^{32}P radioactivity is shown by the full line. (**C**) The tryptic phosphopeptide T2 from (**B**) was subjected to phospho-amino acid analysis as in (**A**). (**D**) The tryptic phosphopeptide T2 from (**B**) was subjected to solid-phase sequencing to identify the cycle of Edman degradation at which the ^{32}P radioactivity was released from the peptide [18].

GSK3β expressed in Sf21 insect cells (Figure 4) or *E. coli* (Figure 1B) was already highly phosphorylated at Tyr^{216}, as judged by the high specific activity of these preparations, and because incubation with MgATP, which led to the incorporation of 0.03–0.05 mol of phosphate/mol of GSK3β into Tyr^{216} did not lead to any observable increase in the total amount of phosphate attached to Tyr^{216}, as judged by immunoblotting with the phosphospecific antibody (Figure 5). However, the few molecules of GSK3 in the preparation that were not phosphorylated at Tyr^{216} did become phosphorylated at this residue upon incubation with MgATP, and this was suppressed by pharmacological inhibition of GSK3 (Figures 3B, 4A and 5). It could be argued that even GSK3 purified to near homogeneity from Sf21 cells is contaminated with traces of another insect cell protein tyrosine kinase that can phosphorylate Tyr^{216}, but not if Kenpaullone is bound to GSK3 (Figure 3). However, it seems inconceivable that such an enzyme exists in *E. coli* and co-purifies with GSK3, since these bacteria do not normally express GSK3 and are not actually thought to encode any protein tyrosine kinases. Moreover, if the tyrosine phosphorylation of GSK3β was catalysed by another protein kinase, the rate of phosphorylation of Tyr^{216} should decrease upon dilution, which did not occur (Figure 3B). This finding, together with the observation that wild-type GSK3β cannot tyrosine phosphorylate catalytically inactive mutants of GSK3β that lack phosphate at Tyr^{216} (Figure 3), indicates that the phosphorylation of Tyr^{216} *in vitro* is an intramolecular autophosphorylation. Our findings also demonstrate that the phosphorylation of Tyr^{216} is not required for GSK3β to become catalytically active, at least not when it is phosphorylating itself at Tyr^{216}.

To try to incorporate more phosphate at Tyr^{216}, we first removed the phosphate already present at Tyr^{216} by prolonged incubation of GSK3β with PTP-1B, but, to our surprise, this incubation actually destroyed the ability of GSK3β to autophosphorylate at Tyr^{216}. Further experiments revealed that it was the incubation at ambient temperature, and not the dephosphorylation of Tyr^{216}, that was responsible for this phenomenon, because the same result was obtained if PTP1B was omitted (Figure 5). These observations imply that molecules of GSK3β lacking phosphate at Tyr^{216} rapidly lose the ability to autophosphorylate at Tyr^{216} when incubated at ambient temperature *in vitro*, suggesting a role for this modification in stabilizing the enzyme. In contrast, the molecules of GSK3β phosphorylated at Tyr^{216} were much more stable at ambient temperature, losing only 30–40 % of their activity towards the standard synthetic peptide substrate after 8 h. These molecules also retained the ability to autophosphorylate at serine and threonine residues (Figure 5).

In the mammalian cells that we have studied, it appears that GSK3 isoforms are highly, if not fully, phosphorylated at Tyr^{279}/Tyr^{216}. This is indicated both by the report that GSK3 purified from mammalian cells does not autophosphorylate on

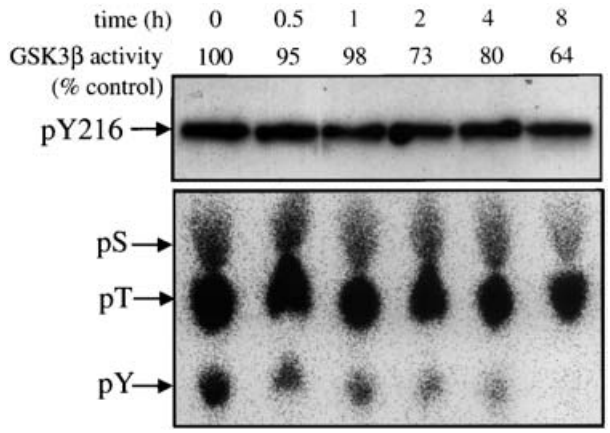

Figure 5 GSK3β loses the ability to autophosphorylate at Tyr^{216} after prolonged incubation

Six aliquots of GSK3β purified from Sf21 insect cells [4 μM in 50 mM Tris/HCl, pH 7.5, 0.03 % (w/v) Brij-35, 0.1 % (v/v) 2-mercaptoethanol, 1 mM sodium orthovanadate] were incubated at ambient temperature for one of the times indicated in a total volume of 25 μl. At each time point, 2.5 μl was removed and diluted 20-fold at 0 °C in the same buffer. The diluted enzyme was used to assay activity towards the standard peptide substrate (see the Experimental section) and to examine the extent of phosphorylation of Tyr^{216} (pY216) by immunoblotting. Activity is presented as a percentage of that obtained for GSK3β not incubated at ambient temperature ($t = 0$). The remaining 22.5 μl of each reaction was then incubated for 5 min at 30 °C with 2.5 μl of 100 mM magnesium acetate, 1.0 mM [γ-^{32}P]ATP (4500 c.p.m. per pmol). The reaction was terminated by the addition of SDS and, following SDS/PAGE and transfer on to PVDF membranes, the bands corresponding to ^{32}P-labelled GSK3β were excised and subjected to phospho-amino acid analysis (see the legend to Figure 3B).

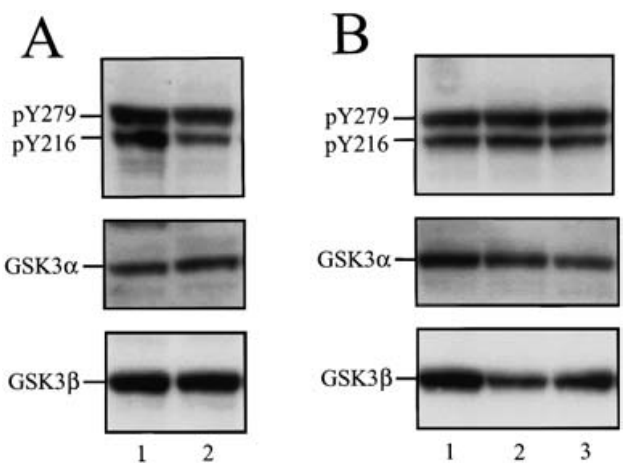

Figure 6 Effect of agonists on the tyrosine phosphorylation of GSK3

(**A**) SH-SY5Y cells in Dulbecco's modified Eagle's medium (DMEM) were treated for 2 h without (lane 1) or with (lane 2) 0.1 μM staurosporine. The cells were lysed, subjected to SDS/PAGE and immunoblotted with antibodies that recognize GSK3α phosphorylated on Tyr^{279} (pY279) as well as GSK3β phosphorylated at Tyr^{216} (pY216), and with antibodies that recognize unphosphorylated and phosphorylated forms of GSK3α or GSK3β equally well (GSK3α, GSK3β). (**B**) PC12 cells were incubated for 4 days in RPMI 1640 medium containing 1 % (v/v) foetal bovine serum without (lane 1) or with (lane 2) 50 ng/ml NGF. The NGF was then removed and cells were incubated for 2 h in RPMI 1640 medium plus 1 % (v/v) foetal bovine serum (lane 3). The cells were lysed, subjected to SDS/PAGE and immunoblotted as in (**A**).

tyrosine residues [3], and because incubation of mammalian cells for up to 8 h with 1 mM sodium orthovanadate, a general inhibitor of PTPases, does not increase the level of phosphorylation of Tyr^{279}/Tyr^{216} (P. Cohen and A. Cole, unpublished work). The near stoichiometric phosphorylation of GSK3 at Tyr^{279}/Tyr^{216} *in vivo* may be a consequence of the very low rate at which these residues are dephosphorylated in cells (Figure 2A) and their immediate rephosphorylation by GSK3, preventing significant accumulation of the unstable molecules that are devoid of phosphate at Tyr^{279}/Tyr^{216}.

Although we cannot exclude the possibility that Tyr^{279}/Tyr^{216} become dephosphorylated in mammalian cells that we have not studied and/or in response to agonists we have not examined, we have been unable to confirm any of the reports in the literature that the tyrosine phosphorylation of GSK3 changes dynamically in response to extracellular signals. For example, the phosphorylation of GSK3β at Tyr^{216} was reported to decrease when cells were exposed to insulin [15]. However, we were unable to detect any change in the level of phosphorylation of Tyr^{216} when HEK-293 cells were stimulated with insulin or IGF-1 (insulin-like growth factor-1) [16]. More recently, insulin was reported to stimulate a transient phosphorylation of GSK3β at Tyr^{216} in SH-SY5Y cells, and phosphorylation was accompanied by enhanced association of GSK3β with the tyrosine kinase Fyn [6]. We repeated these experiments, but were again unable to show a significant increase in Tyr^{216} phosphorylation under these conditions (results not shown). Moreover, PP2, a potent inhibitor of Src-family protein kinases, did not decrease the tyrosine phosphorylation of GSK3 in HEK-293 cells (Figure 2B) or SH-SY5Y cells (results not shown).

It has also been reported that the level of phosphorylation of GSK3β at Tyr^{216} increases in neuronal PC12 cells or SH-SY5Y neuroblastoma cells in response to apoptotic stimuli, such as the removal of NGF from the medium or exposure to staurosporine [14]. However, we were unable to detect any alteration in the tyrosine phosphorylation of GSK3α or GSK3β under these conditions either (Figures 6A and 6B).

We have examined a number of commercially available phosphospecific antibodies reported to recognize GSK3 isoforms phosphorylated at Tyr^{279} and Tyr^{216}, including those used in the studies cited above. However, in our hands, only the antibody from Transduction Laboratories was sufficiently specific and sensitive to unambiguously detect the endogenous tyrosine-phosphorylated GSK3 isoforms. Other commercially available antibodies recognized numerous bands in the cell lysates, some of which are likely to be other tyrosine-phosphorylated proteins. It is possible that one or more of these proteins has been mistakenly assumed to be a GSK3 isoform in some previous studies, leading to erroneous conclusions being drawn.

We thank Jim Woodgett for providing the original human GSK3β expression plasmid, Mark Peggie for making the bacterial expression vector encoding GSK3β, David Barford for providing the PTP1B, Ricardo Biondi for helpful discussions, and the Protein Production Team of the Division of Signal Transduction Therapy at Dundee coordinated by Dr Hilary Mclauchlan and Dr James Hastie for the GSK3β expressed in Sf21 cells and *E. coli*. We acknowledge the help of our DNA sequencing service co-ordinated by Dr Nick Helps and thank the U.K. Medical Research Council, The Royal Society, Diabetes U.K., AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, Merck and Co, Merck KGaA and Pfizer for financial support.

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Received 18 August 2003/9 October 2003; accepted 22 October 2003
Published as BJ Immediate Publication 22 October 2003, DOI 10.1042/BJ20031259