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Tyrosine dephosphorylation of glycogen synthase kinase-3 is involved in its extracellular signal-dependent inactivation

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Abstract **We examined whether extracellular signals regulate glycogen synthase kinase-3 (GSK-3) activity through tyrosine dephosphorylation of GSK-3. In resting Chinese hamster ovary cells overexpressing the human insulin receptor (CHO-IR cells), GSK-3 was tyrosine-phosphorylated and active. Insulin and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) induced inactivation and tyrosine dephosphorylation of GSK-3. It is known that Ser-9 of GSK-3β is phosphorylated in response to insulin and that the phosphorylation of this amino acid residue causes inactivation of GSK-3β. However, the ectopically expressed GSK-3β$^{\Delta 9}$, in which the N-terminal nine amino acids of GSK-3β were deleted, was still inactivated and tyrosine-dephosphorylated in response to insulin. Protein phosphatase 2A treatment partially reversed insulin-induced GSK-3β inactivation, but did not change GSK-3β$^{\Delta 9}$ inactivation. In CHO-IR cells where protein kinase C was down-regulated, TPA neither inactivated nor tyrosine-dephosphorylated GSK-3. However, insulin inactivated and tyrosine-dephosphorylated GSK-3, although to a lesser degree than in the control cells. These results suggest that in addition to serine phosphorylation, tyrosine dephosphorylation of GSK-3 is also important for the regulation of GSK-3 activity in response to extracellular signals and that insulin regulates GSK-3 activity through both protein kinase C-dependent as well as protein kinase C-independent pathways.**

Key words: GSK-3; Insulin; Tyrosine dephosphorylation; Protein kinase C

1. Introduction

GSK-3 has originally been characterized as a serine/threonine kinase that phosphorylates and inactivates glycogen synthase [1,2] and subsequently demonstrated to be identical to protein kinase FA that activates ATP-Mg-dependent type-1 protein phosphatase [2–4]. GSK-3 is now implicated in the regulation of several physiological responses in mammalian cells by phosphorylating many substrates including neuronal cell adhesion molecule [5], neurofilament [6], synapsin I [7], and tau [8], and transcription factors such as AP-1 [9,10] and cyclic AMP response element binding protein [11]. The cDNAs of GSK-3α and GSK-3β have been isolated and they encode protein kinases with molecular masses of 51 and 47 kDa, respectively [12]. Mammalian GSK-3β is structurally and functionally homologous to the *Drosophila zeste-white3/shaggy* gene product [13]. The *shaggy* gene product has been found to be required at several developmental stages during fruitfly embryogenesis for correct embryogenic segmentation [14,15]. In *Saccharomyces cerevisiae*, the *MCK1* and *MDS1* encode serine/threonine kinases which are homologous to mammalian GSK-3 and *Drosophila shaggy*. The *MCK1* and *MDS1* gene products play a role in the chromosomal segregation processes [16]. In *Schizosaccharomyces pombe*, the *skp1+* gene product is a homolog of GSK-3 and regulates cytokinesis [17]. *Xenopus* GSK has been shown to regulate ventral differentiation during early *Xenopus* development [18]. Furthermore, a *Dictyostelium* homolog (gskA) of GSK-3 has been found to be not essential for cell growth or the early stages of development but important for cellular differentiation [19]. Thus, GSK-3 is highly conserved through evolution. Therefore, it is possible that GSK-3 plays a fundamental role in cellular responses.

Evidence has been accumulated on how GSK-3 activity is regulated in response to extracellular signals [20–26]. GSK-3 is directly phosphorylated and inactivated by 90 kDa ribosomal protein S6 kinase (p90^{rsk}, also known as MAPKAP kinase-1) which is activated by MAPK [27]. TPA enhances the phosphorylation and inhibition of the ectopically expressed GSK-3β when coexpressed with p90^{rsk} in HeLa S3 cells [28]. EGF-induced inhibition of GSK-3 is suppressed by expression of a dominant negative mutant of MAPK kinase in NIH3T3 cells [25]. These results indicate that p90^{rsk} mediates inactivation of GSK-3 by extracellular signals which activate the MAPK pathway [29]. The phosphorylation site of GSK-3 by p90^{rsk} has been identified as Ser-21 in GSK-3α and Ser-9 in GSK-3β [27]. It has recently been found that protein kinase B (PKB, also known as Akt/RAC), which is activated by PI 3-kinase [30], phosphorylates the same serine residue of GSK-3 and inactivates GSK-3 [31]. It has also been reported that protein kinase C directly phosphorylates and inactivates GSK-3, although the phosphorylation site of GSK-3 by protein kinase C has not yet been identified [32]. These results clearly indicate that serine phosphorylation of GSK-3 induced by p90^{rsk}, PKB, or protein kinase C is important for the regulation of GSK-3 activity.

It has been found that GSK-3 is highly phosphorylated on tyrosine in vivo and that tyrosine dephosphorylation of GSK-3 by tyrosine phosphatase reduces its kinase activity in vitro [33,34]. Furthermore, it has been reported that okadaic acid and TPA induce tyrosine dephosphorylation and concurrent inactivation of GSK-3 in A431 cells [24,35]. Therefore, tyrosine phosphorylation and dephosphorylation of GSK-3 could be important for regulating its protein kinase activity. How-

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Abbreviations: GSK-3, glycogen synthase kinase-3; EGF, epidermal growth factor; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; CHO-IR cells, Chinese hamster ovary cells overexpressing the human insulin receptor; p90^{rsk}, 90 kDa ribosomal protein S6 kinase; MAPKAP, mitogen-activated protein kinase-activated protein; MAPK, mitogen-activated protein kinase; PKB, protein kinase B; PI, phosphatidylinositol; PP2A, protein phosphatase 2A; HA, hemagglutinin; PDBu, phorbol 12,13-dibutyrate

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ever, it is not clear whether extracellular signals regulate the phosphorylation state on tyrosine of GSK-3. In this paper we show that insulin and EGF induce tyrosine dephosphorylation and inactivation of GSK-3 in CHO-IR and COS cells and that GSK-3 activity is regulated by tyrosine dephosphorylation in addition to serine phosphorylation in response to extracellular signals.

2. Materials and methods

2.1. Materials and chemicals

CHO-IR cells were kindly provided by Dr. Y. Ebina (Tokushima University, Tokushima, Japan). PP2A was kindly supplied from Drs. M. Takeda and H. Usui (Hiroshima University School of Medicine, Hiroshima, Japan) [36]. Mammalian expression vector pCGN and the mouse anti-influenza virus HA1 monoclonal antibody 12CA5 [37] were provided by Dr. Q. Hu (University of California, San Francisco, CA, USA). pL1-3hyg was provided by Drs. A. Klippel (Chiron Corp., Emeryville, CA, USA). pBSSK/GSK-3β was kindly supplied by Dr. J. Woodgett (New Princess Margaret Hospital, Toronto, Canada). The GSK-3 peptide substrate (GSK peptide 1, YRRAAVPPSPSLSRHSSPHQS(P)EDEEE) and the negative control peptide (GSK peptide 2, YRRAAVPPSPSLSRHSSPHQSEDEEE) were synthesized by Dr. C.W. Turck (University of California, San Francisco, CA, USA). To make pCGN/GSK-3β and pGEX2T/GSK-3β, pBSSK/GSK-3β was digested with *Bcl*I and *Eco*RI. The fragment was blunted with *Klenow* and inserted in the *Sma*I cut pCGN and *Sma*I cut pGEX2T to generate pCGN/GSK-3β and pGEX2T/GSK-3β, respectively. GSK-3$\beta^{\Delta 9}$, in which the N-terminal nine amino acids of GSK-3β were deleted, was synthesized by polymerase chain reaction. To prepare pCGN/GSK-3$\beta^{\Delta 9}$ and pGEX2T/GSK-3$\beta^{\Delta 9}$, the fragments encoding GSK-3$\beta^{\Delta 9}$ with *Xba*I and *Sma*I sites and with *Eco*RV and *Eco*RI sites were synthesized. The former fragment was digested with *Xba*I and *Sma*I and inserted in the *Xba*I and *Sma*I cut pCGN to generate pCGN/GSK-3$\beta^{\Delta 9}$. The latter fragment was digested with *Eco*RV and *Eco*RI and inserted in the *Sma*I and *Eco*RI cut pGEX2T to generate pGEX2T/GSK-3$\beta^{\Delta 9}$. GST-fused to GSK-3β (GST-GSK-3β) and GST-GSK-3$\beta^{\Delta 9}$ were purified from *E. coli* in accordance with the manufacturer's instructions (Pharmacia Biotech, Uppsala, Sweden). COS cells were obtained from the American Type Culture Collection. Other materials and chemicals were from commercial sources.

2.2. Cell culture and fractionation of the cell extracts on Mono Q and Mono S column chromatography

CHO-IR and COS cells were cultured at 37°C in F-12 containing 10% fetal calf serum and Dulbecco's modified Eagle's medium containing 10% calf serum, respectively. After confluent cells (10-cm diameter dish) were deprived of serum for 24 h, CHO-IR and COS cells were washed with PBS and then stimulated for 5 min in serum-free medium with 500 nM TPA, 100 nM insulin, and 100 ng/ml EGF. The cells were then washed with PBS, suspended in 1 ml of buffer A (100 mM Tris-HCl, pH 7.5; 2 mM EGTA, 100 mM KCl, 25 mM NaF, 0.1% Triton X, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin), and sonicated for 5 s ten times at 4°C. To this homogenate, 1 ml of buffer B (50 mM β-glycerophosphate, pH 7.5; 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin) was added. The mixture was centrifuged at 10 000×*g* for 15 min at 4°C. The supernatant (3 mg of protein) was applied to Mono Q HR5/5 and Mono S HR5/5 columns connected in series, which were equilibrated with buffer B [26]. After the columns had been washed with 10 ml of buffer B and separated, the Mono S column was washed with 5 ml of buffer B containing 70 mM NaCl, and then the elution was performed with 3 ml of buffer B containing 0.3 M NaCl in a stepwise manner (3.5 μg of protein). The GSK-3 activity of an aliquot (10 μl) of the eluates was assayed. To measure the protein level and phosphotyrosine content of GSK-3, aliquots of the eluates were probed with the anti-GSK-3β and anti-phosphotyrosine antibodies.

2.3. GSK-3 assay

The kinase activity of GSK-3 was assayed as described [20]. The samples were incubated with 50 μM GSK peptide 1 or GSK peptide 2 in 30 μl of reaction mixture (20 mM Tris-HCl, pH 7.5; 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM [γ-^{32}P]ATP (spec. act. 500–1000 cpm/pmol)) for 20 min at 30°C. The reaction mixture was then spotted on phosphocellulose filters (Whatman P81). The filters were immersed in 150 mM phosphoric acid and then rinsed three times with fresh phosphoric acid. One unit of GSK-3 was defined as the amount which catalyzed the transfer of 1 pmol of phosphate into 1500 pmol of GSK peptide 1 for 20 min. For measuring HA-GSK-3β and HA-GSK-3$\beta^{\Delta 9}$ activities, the extracts (0.5 mg of protein) of CHO-IR cells expressing HA-GSK-3β or HA-GSK-3$\beta^{\Delta 9}$ were immunoprecipitated with the anti-HA antibody as described [38,39]. The GSK-3 activity of the precipitates was assayed as described above. All readings were within the linear ranges.

2.4. Transfection

Hygromycin-resistant cell transfectants were propagated in medium containing 200 μg/ml hygromycin B. Transfection of CHO-IR cells with pCGN/GSK-3β or pCGN/GSK-3$\beta^{\Delta 9}$ and pL1-3hyg was carried out by using Lipofectamine in accordance with the manufacturer's instructions (Life Technologies, Inc., Gaithersburg, MD, USA). The cell lines expressing HA-GSK-3β or HA-GSK-3$\beta^{\Delta 9}$ were established by selecting for resistance against hygromycin B. Colonies of the cells resistant to hygromycin B were selected at 14–20 days after transfection. Subsequently, the cells expressing HA-GSK-3β or HA-GSK-3$\beta^{\Delta 9}$ among the hygromycin B-resistant cells were further selected by immunoblot analysis using the anti-HA antibody.

2.5. PP2A treatment of HA-GSK-3β and HA-GSK-3$\beta^{\Delta 9}$

The precipitated HA-GSK-3β and HA-GSK-3$\beta^{\Delta 9}$ were incubated with PP2A (50 μU) in the presence or absence of 2 μM okadaic acid in 50 μl of reaction mixture (50 mM Tris-HCl, pH 7.5; 0.5 mM dithiothreitol, 0.05% Triton X) for 30 min at 30°C. After incubation, okadaic acid was added and the precipitated enzymes were washed with 20 mM Tris-HCl (pH 7.5) twice. The precipitates were used for GSK-3 assay.

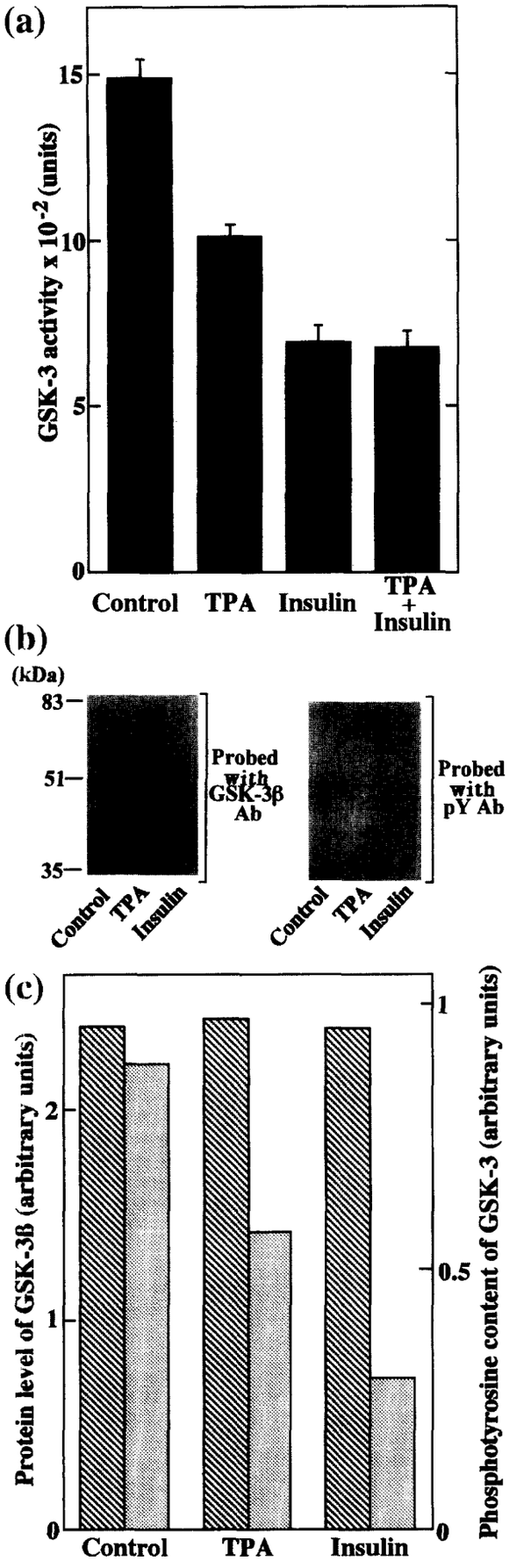
2.6. Other assays

Prolonged treatment of CHO-IR cells with PDBu was performed as described [40]. The kinase activity of protein kinase C was assayed according to [40]. Protein concentrations were determined with bovine serum albumin as a standard [41]. The protein level and phosphotyrosine content of GSK-3 were quantified using a CCD image sensor and ATTO Densitograph Ver 2.0 (ATTO Corp., Tokyo, Japan).

3. Results and discussion

3.1. Inactivation and tyrosine dephosphorylation of GSK-3 induced by extracellular signals

It has been reported that insulin inactivates GSK-3 in CHO-IR cells and L6 myoblasts [20,22,23,26]. First we examined whether insulin regulates tyrosine dephosphorylation of GSK-3 as well as GSK-3 activity in CHO-IR cells. The kinase activity against GSK peptide 1 appeared in the eluates from the Mono S column by 0.3 M NaCl (Fig. 1a). However, this kinase was not active against GSK peptide 2 (data not shown). These results indicated that this kinase activity was GSK-3. When CHO-IR cells were stimulated with TPA and insulin, GSK-3 activities were decreased (Fig. 1a). GSK-3 activities in control, TPA-stimulated, and insulin-stimulated CHO-IR cells were calculated to be 1490, 990, and 730 units, respectively. The decreases in GSK-3 activity upon stimulation with TPA and insulin were about 30 and 50%, respectively. When CHO-IR cells were stimulated with both TPA and insulin, GSK-3 activity was decreased to a level which was inhibited by insulin alone (Fig. 1a). These results were consistent with previous observations [20,22,23,26]. Immunoblot analyses of the eluates with the anti-GSK-3β and anti-phosphotyrosine antibodies were performed and then the protein level and phosphotyrosine content of GSK-3 were quan-



tified using the CCD image sensor. When the protein level and phosphotyrosine content of known amounts of GST-GSK-3β purified from *E. coli* were determined by this method, the densities of the bands on the nitrocellulose sheets were proportional to the amounts (2.5–50 ng) of GST-GSK-3β (data not shown). The protein levels of GSK-3β in control, TPA-stimulated, and insulin-stimulated CHO-IR cells were similar (Fig. 1b,c), while the stimulation of CHO-IR cells with TPA and insulin resulted in 40 and 70% reduction of the phosphotyrosine content of GSK-3 (Fig. 1b,c). The stimulation of CHO-IR cells with both TPA and insulin resulted in the same reduction of phosphotyrosine content as that caused by insulin alone (data not shown). When COS cells were stimulated with TPA and EGF, GSK-3 activities were decreased by about 25 and 50%, respectively (Fig. 2a). Approx. 35 and 65% of the phosphotyrosine content of GSK-3 were reduced in TPA- and EGF-stimulated COS cells compared to control cells under the conditions that the protein levels of GSK-3β in control, EGF-stimulated, and TPA-stimulated COS cells were similar (Fig. 2b,c). Taken together, these results indicate that extracellular signals such as insulin and EGF induce tyrosine dephosphorylation and inactivation of GSK-3.

GSK-3 is phosphorylated on a unique tyrosine residue and active in resting cells [33]. The conservation of the region of protein encompassing this residue implies a critical role for this covalent modification [33]. GSK-3 produced in *E. coli* is also tyrosine-phosphorylated and a kinase-negative mutant of GSK-3 is not tyrosine-phosphorylated [34]. Therefore, tyrosine phosphorylation of GSK-3 should be due to autophosphorylation and not to a tyrosine kinase which phosphorylates GSK-3. Tyr-216 is a phosphorylation site of GSK-3β and the substitution of Phe for Tyr-216 impairs its kinase activity [33]. These observations suggest that tyrosine dephosphorylation regulates GSK-3 activity in intact cells. It has been shown that TPA and okadaic acid induce tyrosine dephosphorylation of GSK-3 and concurrently inactivate it in A431 cells [24,35]. However, neither TPA nor okadaic acid is a physiological agonist. We have for the first time demonstrated that extracellular signals such as insulin and EGF tyrosine-dephosphorylate and inactivate GSK-3 in CHO-IR and COS cells. The degree of tyrosine dephosphorylation of GSK-3 was more than that of its inactivation when the cells were treated with insulin, EGF, and TPA. Although we do not know the exact reasons for these observations, one possibility is that when GSK-3 is inactivated, an unknown mech-

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Fig. 1. Inactivation and tyrosine dephosphorylation of GSK-3 induced by TPA and insulin in CHO-IR cells. (a) GSK-3 activity. After CHO-IR cells were left untreated (control) or stimulated with TPA, insulin, or both TPA and insulin, the cell extracts were subjected to Mono Q and Mono S column chromatography. GSK-3 activities of the eluates were assayed against GSK peptide 1. The results shown are expressed as means ± S.E.M. of three independent experiments. (b) Immunoblot analysis of phosphotyrosine of GSK-3. Aliquots of the eluates prepared in (a) were probed with the anti-GSK-3β and anti-phosphotyrosine antibodies. pY, phosphotyrosine; Ab, antibody. The results shown are representative of three independent experiments. (c) Densitometric quantification of the protein level and phosphotyrosine content of GSK-3. The protein level (hatched bars) and phosphotyrosine content (stippled bars) of GSK-3 in (b) were quantified using the CCD image sensor (ATTO Densitograph). The results shown are representative of three independent experiments.

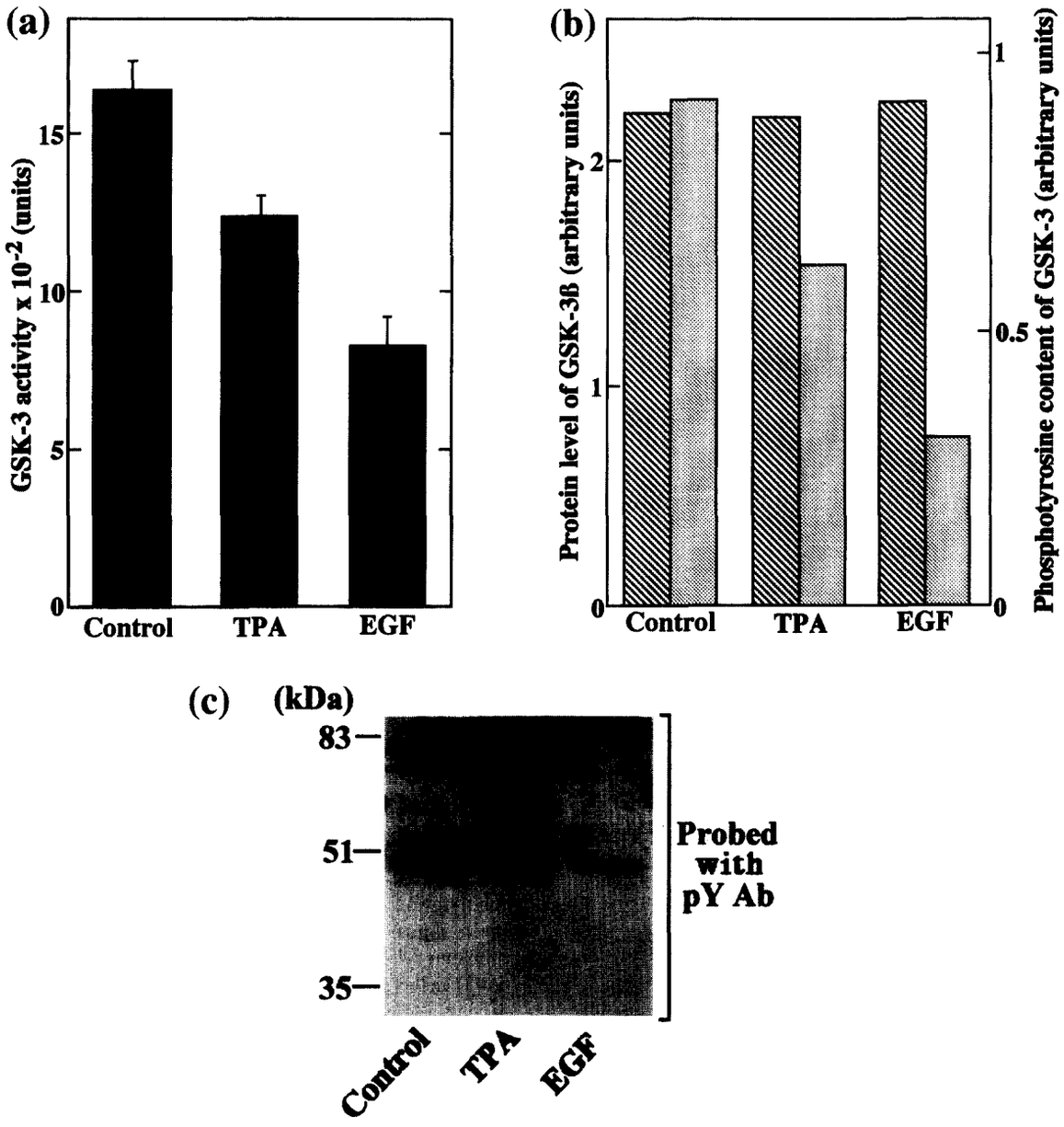
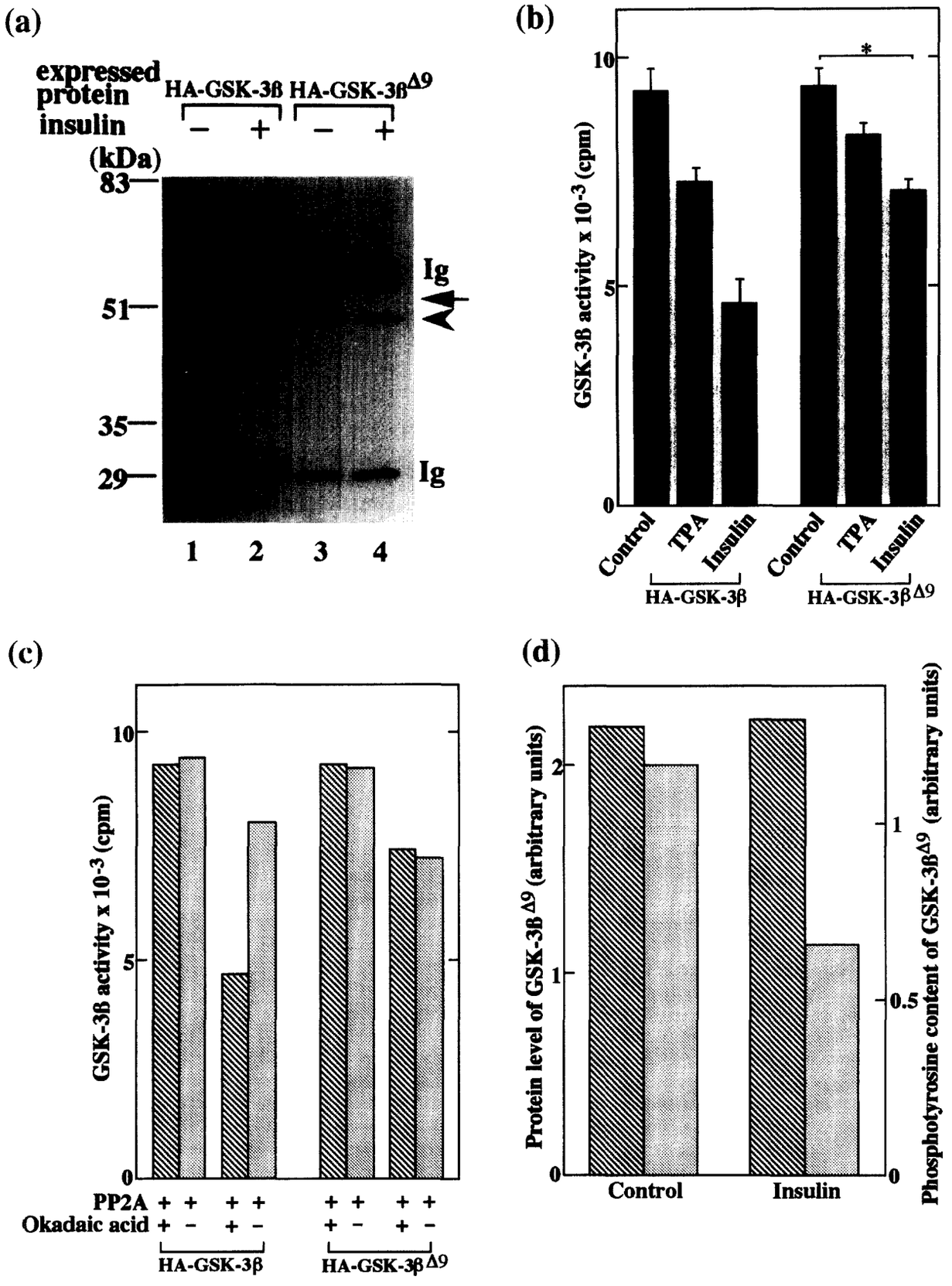


Fig. 2. Inactivation and tyrosine dephosphorylation of GSK-3 induced by TPA and EGF in COS cells. (a) GSK-3 activity. GSK-3 activities of the eluates from Mono S column chromatography of COS cells left untreated (control) or stimulated with TPA or EGF were assayed against GSK peptide 1. The results shown are expressed as means ± S.E.M. of three independent experiments. (b) Densitometric quantification of the protein level and phosphotyrosine content of GSK-3. Aliquots of the eluates prepared in (a) were probed with the anti-GSK-3β and anti-phosphotyrosine antibodies, and then the protein level (hatched bars) and phosphotyrosine content (stippled bars) of GSK-3 were quantified using the CCD image sensor. The results shown are representative of three independent experiments. (c) Immunoblot analysis of phosphotyrosine of GSK-3. Aliquots of the eluates prepared in (a) were probed with the anti-phosphotyrosine antibody. pY, phosphotyrosine; Ab, antibody. The results shown are representative of three independent experiments.

Fig. 3. Inactivation and tyrosine dephosphorylation of GSK-3β$^{\Delta 9}$ induced by insulin. (a) Protein levels of HA-GSK-3β and HA-GSK-3β$^{\Delta 9}$. After CHO-IR cells expressing HA-GSK-3β (lanes 1,2) or HA-GSK-3β$^{\Delta 9}$ (lanes 3,4) were left untreated (lanes 1,3) or stimulated with insulin (lanes 2,4), the cell extracts were immunoprecipitated with the anti-HA antibody. The precipitates were probed with the anti-GSK-3β antibody. The arrow and arrowhead indicate the positions of HA-GSK-3β and HA-GSK-3β$^{\Delta 9}$, respectively. Ig, immunoglobulin. The results shown are representative of three independent experiments. (b) Inactivation of HA-GSK-3β and HA-GSK-3β$^{\Delta 9}$ by insulin and TPA. After CHO-IR cells expressing HA-GSK-3β or HA-GSK-3β$^{\Delta 9}$ were left untreated (control) or stimulated with TPA or insulin, the cell extracts were immunoprecipitated with the anti-HA antibody. The precipitates were assayed for GSK-3 activity. The results shown are expressed as means ± S.E.M. of eight independent experiments. *Differences significant at least $P < 0.05$. (c) PP2A treatment. The precipitated HA-GSK-3β and HA-GSK-3β$^{\Delta 9}$ prepared in (a) were treated with PP2A in the presence (hatched bars) or absence (stippled bars) of okadaic acid. After this treatment, GSK-3 activities were measured. (d) Tyrosine dephosphorylation of GSK-3β$^{\Delta 9}$ in response to insulin. The precipitated HA-GSK-3β$^{\Delta 9}$ prepared in (a) were probed with the anti-GSK-3β and anti-phosphotyrosine antibodies. The protein level (hatched bars) and phosphotyrosine content (stippled bars) of HA-GSK-3β$^{\Delta 9}$ were quantified using CCD image sensor. The results shown are representative of three independent experiments.


(a)
expressed protein
insulin
HA-GSK-3ß
HA-GSK-3ß$^{\Delta 9}$
(kDa)
83
51
35
29
Ig
Ig
1 2 3 4
(b)
GSK-3ß activity x 10^{-3} (cpm)
10
5
0
*
Control
TPA
Insulin
Control
TPA
Insulin
HA-GSK-3β
HA-GSK-3β$^{\Delta 9}$
(c)
GSK-3ß activity x 10^{-3} (cpm)
10
5
0
PP2A + + + + + + + +
Okadaic acid + − + − + − + −
HA-GSK-3β
HA-GSK-3β$^{\Delta 9}$
(d)
Protein level of GSK-3ß$^{\Delta 9}$ (arbitrary units)
2
1
0
Phosphotyrosine content of GSK-3ß$^{\Delta 9}$ (arbitrary units)
1
0.5
0
Control
Insulin

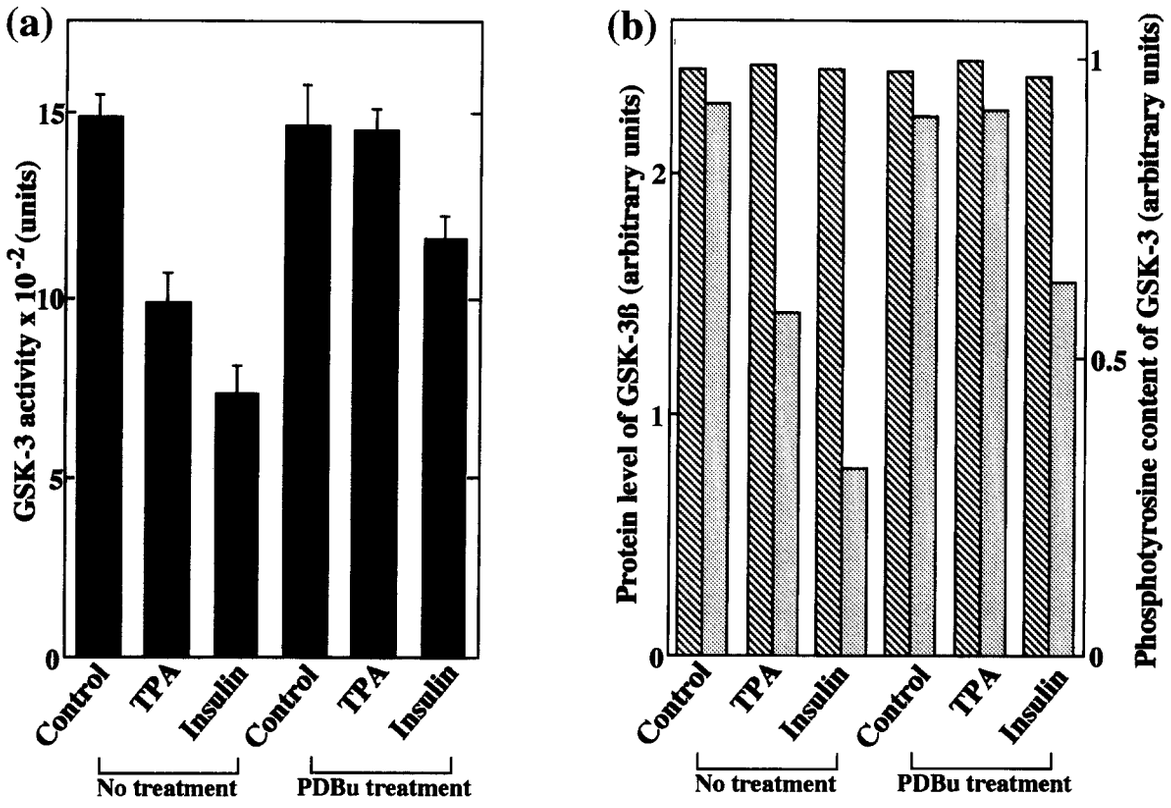


Fig. 4. Involvement of protein kinase C in insulin-dependent inactivation and tyrosine dephosphorylation of GSK-3. (a) Effect of down-regulation of protein kinase C on insulin-dependent inactivation of GSK-3. After CHO-IR cells were treated with or without PDBu for 24 h, GSK-3 activities of the eluates from Mono S column chromatography of the cells left untreated (control) or stimulated with TPA or insulin were assayed. The results shown are expressed as means ± S.E.M. of three independent experiments. (b) Effect of down-regulation of protein kinase C on insulin-dependent tyrosine dephosphorylation of GSK-3. Aliquots of the eluates prepared in (a) were probed with the anti-GSK-3β and anti-phosphotyrosine antibodies, and then the protein level (hatched bars) and phosphotyrosine content (stippled bars) of GSK-3 of the cells left untreated (control) or stimulated with TPA or insulin were quantified using the CCD image sensor. The results shown are representative of three independent experiments.

anism might work to prevent the inhibition of GSK-3 activity without affecting tyrosine dephosphorylation.

3.2. *Inactivation and tyrosine dephosphorylation of GSK-3β$^{\Delta 9}$ induced by insulin*

It is known that insulin and EGF activate p90^{rsk} and PKB and that these kinases phosphorylate Ser-21 and Ser-9 of GSK-3α and GSK-3β, respectively, and inactivate GSK-3 [20,21,23,25,27,31]. Our results shown in Figs. 1 and 2 suggest that tyrosine dephosphorylation of GSK-3 may be involved in extracellular signal-dependent inactivation of GSK-3. However, we cannot exclude the possibility that extracellular signals activate p90^{rsk} and PKB and induce the phosphorylation of GSK-3 thereby inactivating and tyrosine-dephosphorylating GSK-3. Therefore, we synthesized GSK-3β$^{\Delta 9}$, in which the N-terminal nine amino acids were deleted. When GST-GSK-3β and GST-GSK-3β$^{\Delta 9}$ purified from *E. coli* were assayed against GSK peptide 1, both proteins had the same activity (data not shown). CHO-IR cells expressing wild-type GSK-3β and GSK-3β$^{\Delta 9}$ were made. These GSK-3βs were tagged with the HA epitope at the N-terminus. After these cells were stimulated with insulin, HA-GSK-3β and HA-GSK-3β$^{\Delta 9}$ were immunoprecipitated with the anti-HA antibody. Almost equal amounts of HA-GSK-3β and HA-GSK-3β$^{\Delta 9}$ were precipitated as assessed by immunoblot analysis using the anti-GSK-3β antibody (Fig. 3a). The basal activities of HA-GSK-3β and HA-GSK-3β$^{\Delta 9}$ were almost the same. Insulin decreased the activities of HA-GSK-3β and HA-GSK-3β$^{\Delta 9}$ by 40 and 20%, respectively (Fig. 3b). TPA also decreased the activities of HA-GSK-3β and HA-GSK-3β$^{\Delta 9}$ by 20 and 10%, respectively (Fig. 3b). Although the decrease in HA-GSK-3β$^{\Delta 9}$ activity by insulin and TPA appeared to be small, these results were reproducible and statistically significant. The decrease in GSK-3β activity induced by insulin was partially restored by PP2A treatment, while that of GSK-3β$^{\Delta 9}$ activity induced by insulin was not changed (Fig. 3c). Furthermore, insulin decreased the phosphotyrosine content of HA-GSK-3β$^{\Delta 9}$ by 40% (Fig. 3d). These results clearly demonstrate that inactivation and tyrosine dephosphorylation of GSK-3 induced by insulin occurs without phosphorylation of Ser-9. Therefore, it is possible that not only serine phosphorylation but also tyrosine dephosphorylation of GSK-3 is important for the extracellular signal-dependent regulation of GSK-3 activity.

3.3. *Involvement of protein kinase C in insulin-induced inactivation and tyrosine dephosphorylation of GSK-3*

The activation of protein kinase C by insulin has been implicated in various cell functions such as the stimulation of glucose transport in BC3H1 myocytes and rat soleus muscle

[42], c-*fos* expression in neurons [43], and the activation of amino acid transport in hepatocytes [44]. However, it has been reported that protein kinase C is not required in several insulin-dependent actions [44,45]. We examined whether protein kinase C is involved in insulin-induced inactivation and tyrosine dephosphorylation of GSK-3. For this experiment, CHO-IR cells were treated with 800 nM PDBu for 24 h. After this treatment, the protein kinase C activity activated by phosphatidylserine, TPA, and Ca^{2+} was reduced to less than 5% of that of the control cells (data not shown). These results clearly indicated that protein kinase C was markedly down-regulated and reduced by prolonged treatment with PDBu. The basal activity of GSK-3 and the protein level of GSK-3β of CHO-IR cells treated with PDBu were almost similar with those of the control cells (Fig. 4a,b). In CHO-IR cells where protein kinase C activity was markedly decreased, TPA-dependent GSK-3 inactivation was completely abolished (Fig. 4a) and TPA did not induce tyrosine dephosphorylation of GSK-3 (Fig. 4b). However, insulin still inactivated and tyrosine-dephosphorylated GSK-3 in CHO-IR cells where protein kinase C was down-regulated, although the degree was attenuated compared to control cells (Fig. 4a,b). These results suggest that insulin regulates GSK-3 activity and its tyrosine dephosphorylation through both protein kinase C-dependent as well as protein kinase C-independent pathways.

Although we do not know how protein kinase C induces tyrosine dephosphorylation of GSK-3, it is intriguing to speculate that protein kinase C activates a tyrosine phosphatase for GSK-3. Two mechanisms of the inhibitory action of protein kinase C for GSK-3 have been reported. The first is that protein kinase C directly phosphorylates and inactivates GSK-3 [32], the second being that protein kinase C regulates GSK-3 through MAPKAP kinase-1 by activating the MAPK pathway [28]. It is possible that several pathways through protein kinase C activated by extracellular signals regulate GSK-3. Our results also suggest that there is a protein kinase C independent pathway which is involved in the regulatory mechanism of GSK-3 by insulin. These results are consistent with the observations that inactivation and tyrosine dephosphorylation of GSK-3 induced by insulin is more significant than those induced by TPA. Recently, it has been shown that insulin and platelet-derived growth factor activate PI 3-kinase, that PI-3-phosphate, a product of PI 3-kinase, activates PKB, and that PKB phosphorylates and inactivates GSK-3 [30,31]. This pathway could be one of the protein kinase C-independent pathways in insulin-induced inactivation of GSK-3. The mechanisms that induce tyrosine dephosphorylation of GSK-3 independently of protein kinase C in response to insulin remain to be clarified.

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