**Essential Role for Protein Kinase B (PKB) in Insulin-induced Glycogen Synthase Kinase 3 Inactivation**

**CHARACTERIZATION OF DOMINANT-NEGATIVE MUTANT OF PKB**

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Pascale C. van Weeren‡, Kim M. T. de Bruyn‡, Alida M. M. de Vries-Smits‡, Johan van Lint§, and Boudewijn M. Th. Burgering¶

From the ‡Laboratory for Physiological Chemistry, Utrecht University, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands, and §Afdeling Biochemie, Faculteit Geneeskunde, Katholieke Universiteit te Leuven, Camphuis Gasthuisberg, 3000 Leuven, Belgium

Activation of phosphatidylinositol 3'-OH kinase (PI 3-kinase) is implicated in mediating a variety of growth factor-induced responses, among which are the inactivation of glycogen synthase kinase-3 (GSK-3) and the activation of the serine/threonine protein kinase B (PKB). GSK-3 inactivation occurs through phosphorylation of Ser-9, and several kinases, such as protein kinase C, mitogen-activated protein kinase-activated protein kinase 1 (MAPKAPK-1), p70S6 kinase, and also PKB have been shown to phosphorylate this site in vitro. In the light of the many candidates to mediate insulin-induced GSK-3 inactivation we have investigated the role of PKB by constructing a PKB mutant that exhibits dominant-negative function (inhibition of growth factor-induced activation of PKB at expression levels similar to wild-type PKB), as currently no such mutant has been reported. We observed that the PKB mutant (PKB-CAAX) acts as an efficient inhibitor of PKB activation and also of insulin-induced GSK-3 regulation. Furthermore, it is shown that PKB and GSK-3 co-immunoprecipitate, indicating a direct interaction between GSK-3 and PKB. An additional functional consequence of this interaction is implied by the observation that the oncogenic form of PKB, gagPKB induces a cellular relocation of GSK-3 from the cytosolic to the membrane fraction. Our results demonstrate that PKB activation is both necessary and sufficient for insulin-induced GSK-3 inactivation and establish a linear pathway from insulin receptor to GSK-3. Regulation of GSK-3 by PKB is likely through direct interaction, as both proteins co-immunoprecipitate. This interaction also resulted in a translocation of GSK-3 to the membrane in cells expressing transforming gagPKB.

Activation of membrane-bound receptors of both the serpine and tyrosine kinase classes often results in the activation of the lipid kinase PI3-kinase, of which several different isoforms have been described (1). Activation of PI 3-kinase results in the formation of 3'-phosphorylated phosphatidylinositol (PI-3P) lipids (i.e., PI 3,4-P₂ and PI 3,4,5-P₃). These lipids were long suspected to perform a second messenger function. However, until recently the nature of the PI-3P binding proteins and their role in cellular signaling remained unsolved. It is now clear that one of the downstream effectors of PI-3P lipid signaling is the serine/threonine protein kinase PKB (2, 3), also known as c-Akt (4, 5). Adjacent to its catalytic domain, PKB contains a PH domain responsible for binding PI-3P lipids (6, 7). Although there is still debate about the precise role and nature of PI-3P lipids in the activation of PKB it is clear that in vivo for most growth factor-signaling pathways PI-3P lipid formation is both necessary and sufficient for PKB activation. Additionally, activation of PI 3-kinase has been implicated in a number of biological responses such as activation of p70S6 kinase, insulin-induced glucose transport, apoptosis protection, activation of PKCζ, activation of the small GTPase Rac, formation of Rac-dependent lamellipodia, and inactivation of GSK-3 (1).

Initially GSK-3 was shown to phosphorylate sites in glycogen synthase, which are specifically dephosphorylated when insulin promotes synthase activation leading to glycogen synthesis. A more general role for GSK-3 both in regulating cellular metabolism as well as differentiation processes is suggested by the growing number of identified GSK-3 substrates such as c-Jun (8), neurofilaments (9), tau (10), and adenosomatous polyposis coli protein (11). In addition, Zeste-White 3, the Drosophila homolog of GSK-3, has been genetically positioned within the wingless signaling pathway involved in the regulation of various differentiation processes. Within this pathway, ZW-3/GSK-3 is thought to control the transcriptional function of armadillo (β-catenin) (12).

Two highly homologous GSK-3 isozymes, GSK-3α and GSK-3β have been identified. How GSK-3 activity is controlled and whether both isozymes are controlled identically is still unclear. Following treatment of cells with insulin, GSK-3 becomes partially inactivated. In vitro phosphorylation of Ser-9 (GSK-3β) results in partial inactivation of the kinase (13), and in vivo, this site is phosphorylated after insulin treatment (14). Although this strongly suggests that GSK-3 activity is regulated through Ser-9 phosphorylation, other reports have shown that active GSK-3, isolated from resting cells is phosphorylated on tyrosine and that tyrosine dephosphorylation results in GSK-3 inactivation as well (15). Several signaling pathways have been proposed to regulate Ser-9 phosphorylation, including growth factor-induced PKC activation (16), MAPKAPK-1 (p90Rsk) activation, p70S6 kinase (13), and PKB (17). What signaling pathway leads to tyrosine dephosphorylation of GSK-3 is unknown.

Because insulin-induced GSK-3 inactivation is sensitive to
PI 3-kinase inhibitors (wortmannin) and not an inhibitor of growth factor-induced p70S6K kinase activation (rapamycin), PKB is a prime candidate for regulation of GSK-3 in vivo (17). Indeed, wild-type PKB, and not kinase-dead PKB isolated from vanadate-treated cells causes inactivation of GSK-3 in vitro (17). Although this indicates that PKB activation may be sufficient, these data do not support an essential role for PKB.

To study a possible involvement of PKB in GSK-3 regulation we therefore first tested several PKB mutants for their inhibitory effect on PKB and describe here that PKB tagged at the C terminus with the CAAX motif of Ki-Ras acts as an efficient inhibitor of insulin-induced PKB activation. In contrast, we do not observe any clear effect of either kinase-dead or PH domain constructs on PKB activation. Furthermore, with the PKB-CAAX we show that for insulin-induced GSK-3 inactivation, PKB activation is both sufficient and essential. In addition, we show that in vivo PKB not only regulates GSK-3 activity, but also appears to directly interact with GSK-3, as both proteins can be co-immunoprecipitated. This interaction may underlie the observation that in gapPKB-expressing cells, GSK-3 relocates from the cytosolic to the membrane fraction.

EXPERIMENTAL PROCEDURES

Materials—Wortmannin was purchased from Sigma and used at a concentration of 50 nM. Baculovirus cloning and expression system (Baculo-gold, FASTBAC) was purchased from Life Technologies, Inc.

Plasmids—The plasmids HA-PKB, HA-PKB kinase-defective, gagPKB, and gag have been described previously (3). HA-GSK-3 and HA-GSK-3 kinase-defective were kindly provided by Paul Coffey (Academic Hospital (AZU), Utrecht, The Netherlands). CAAX-p110 and PLAP-CAAX have also been described previously (27) and were generously provided by Marcus Thelen. PKB-CAAX was constructed as follows: In HA-PKB a Nael site at amino acid position 478 was created by in vitro mutagenesis. This amino acid change had no effect on HA-PKB activity either basal or insulin-induced. Next the CAAX-box of Ki-Ras was isolated by polymerase chain reaction from the plasmid RAFF-CAAX. The polymerase chain reaction product was digested with HpaI/EcoRI and ligated to Nael/EcoRI-digested HA-PKB resulting in HA-PKB-CAAX. To create untagged PKB-CAAX the Smal/BglII fragment of pSOS5-PKB was replaced by the Smal/BglII fragment of HA-PKB-CAAX. HA-ΔPH-PKB-CAAX was generated in a similar way: in vitro mutagenesis of Nael site was created into HA-PKB at amino acid position 7. The PH domain was then partially deleted by digesting HA-PKB with NruI/SacII, blunting the SacII site and religation. The CAAX domain was then added by the same domain swap as described above. ΔPKB-CAAX was created by digesting HA-PKB-CAAX with HindIII/BglII, blunting and ligating the HindIII/BglII fragment into pSOS digested with BamHI, and blunted. All clones were checked by partial sequencing.

Cell Culture and Cell Lines—A14 cells (3) were routinely grown in Dulbecco’s modified Eagle’s medium supplemented with 8% fetal calf serum and 0.05% glutamine. For growth factor treatment cells were seeded at approximately 50% density on day 0; on day 1 the medium of the cells was replaced with medium containing 0% fetal calf serum, and on day 2 cells were treated with insulin (1 μg/ml).

GSK-3 Assay—Cells were lysed in lysis buffer (50 mM Tris (pH 7.5), 200 mM NaCl, 1% Triton X-100, 10 mM EDTA, 1 mM Na3VO4, 50 mM phosphoric acid. After drying the samples were counted. The amount of immunocomplexed HA-GSK-3 was determined by removing the remainder of the kinase reaction and adding 25 μl of 1× Laemmli sample buffer to the beads. Expression was then analyzed by immunoblotting.

PKB Kinase Assay—The PKB kinase assay was essentially as described previously (3), except for including a wash with LiCl buffer as for the GSK-3 assay described above and using histone H2B instead of myelin basic protein as a substrate.

MAP Kinase Assay—MAP kinase assay was done as described previously (3). Cell Fractionation—A14 cells transfected with gagPKB or HA-PKB-CAAX were lysed in lysis buffer (20 mM HEPES (pH 7.4), 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 0.1 μM aprotinin) by 30 strokes with a Dounce homogenizer on ice. Nuclear components and unlysed cells were removed by centrifugation for 10 min at 14,000 rpm. Next the supernatant was centrifuged at 100,000 x g at 4 °C for 90 min, and the supernatant was collected (cytoplasmic fraction). The pellet (particulate fraction) was washed once with lysis buffer, and the pellet was extracted for 30 min at 4 °C with buffer containing 1% Triton X-100, 50 mM HEPES (pH 7.4), 50 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 0.1 μM aprotinin. Extracted particulate fraction was centrifuged (14,000 rpm for 5 min at 4 °C) to remove any remaining insoluble material. 5× Laemmli sample buffer was added (final concentration of 1× sample buffer), and cytoplasmic and particulate fractions were analyzed by SDS-PAGE and immunoblotting.

Baculovirus PKB—For use and generation baculovirus PKB the baculo-gold system was used (Life Technologies, Inc.) according to the manufacturer’s procedures. His-tagged-PKB was isolated by a procedure identical to the regular immunoprecipitation protocol modified to equip larger amounts and using Ni2+ agarose beads instead of protein A-Sepharose.

RESULTS

Membrane Targeting of PKB by the Ki-Ras CAAX Motif Results in an Inactive Kinase—We have previously shown that PKB fused to the Moloney murine leukemia virus Gag protein displays increased ligand-independent kinase activity (3). This Gag protein contains a myristoylation signal that can confer membrane localization to the fusion protein (18). Membrane targeting of PKB by myristylated sequences derived from src-like kinases (src, yes) also results in ligand-independent activation of PKB (19). With these types of mutants, it has been shown that activation of PKB is sufficient to activate p70S6K (3), stimulate glucose uptake (19), and to provide apoptosis protection (20, 21). Fusion of the membrane-targeting signal of Ki-Ras, the so-called CAAX motif, has been shown to create active versions of a variety of different signaling molecules (e.g. Raf-1 (22), mSos (23), RIF (24)) some of which are kinases.

To analyze whether the CAAX motif fused to PKB also results in an active ligand-independent kinase we constructed HA-PKB-CAAX, PKB tagged at its N terminus by the HA-peptide recognized by the 12CA5 monoclonal antibody, fused to the C-terminal end to the CAAX motif of Ki-Ras. First, we determined the subcellular localization of HA-PKB-CAAX by fractionation of A14 cells (3) transiently expressing HA-PKB-CAAX. As expected, most of the HA-PKB-CAAX was localized to the membrane fraction, and this was at least comparable to the relative amount of gagPKB found in the membrane fraction (Fig. 1A). Next, the kinase activity was determined by an immunocomplex kinase assay (Fig. 1B). Activity of HA-PKB-CAAX in the absence of ligand was clearly not increased and comparable to the activity of unstimulated wild-type HA-PKB. More surprisingly, the activity of HA-PKB-CAAX did not increase following treatment with insulin and displayed activity similar to kinase-dead PKB. GagPKB in contrast displayed a clear increase in ligand-independent kinase activity, which could be induced further by insulin treatment, albeit to a smaller extent than wild-type PKB. In this respect gagPKB is characterized as a Dominant-Negative PKB.
similar to Raf1-CAAX (22). Raf1-CAAX is constitutively active, but this activity can also be increased further by growth factor treatment.

In HA-PKB-CAAX the kinase domain is positioned between two potential membrane localization signals, the PH domain and the CAAX motif, whereas in gagPKB the myristoylation signal is in tandem with the PH domain. Therefore, in HA-PKB-CAAX, conformational restrictions might prevent interaction with upstream kinase(s) required for PKB activation. We reasoned that if so, mutation of the PH domain in HA-PKB-CAAX either by deleting or changing arginine 28 to cysteine, a mutation similar to the Btk mutation implicated in X-linked agammaglobulinemia, could restore kinase activity. However, this appeared not to be the case (Fig. 2B).

**PKB-CAAX Acts As a Dominant-Negative PKB**—The observation that HA-PKB-CAAX is a functionally inactive kinase prompted us to test whether HA-PKB-CAAX might act as a dominant-negative PKB mutant. To test this possibility we constructed PKB-CAAX lacking the HA-tag. PKB-CAAX was then transiently co-expressed with HA-PKB, and subsequently its effect on HA-PKB activity was measured (Fig. 2A). At a DNA ratio of 10:1 (PKB-CAAX:HA-PKB) there was complete inhibition of insulin-induced HA-PKB activity. Half-maximal inhibition was already observed at a DNA ratio of 1:1 (Fig. 2B) indicating that PKB-CAAX efficiently inhibited HA-PKB activation by insulin. Others have reported inhibition of PI3-kinase-dependent signaling upon expression of either kinase-dead PKB (e.g. phosphorylation of Bcl-2-associating death protein) (25) or expression of the PH domain only (e.g. anoikis) (21). To test whether these constructs were as efficient as PKB-CAAX in inhibiting PKB activation by insulin, untagged versions of these same constructs as well as mutant versions of the PH domain (not shown) were co-expressed with HA-PKB at a DNA ratio of 10:1 (inhibitor, HA-PKB). From this comparison (Fig. 2A) it is clear that in A14 cells, using insulin as inducer, only PKB-CAAX clearly inhibits HA-PKB activation. To determine what region of PKB is critical in conferring the dominant-negative action of PKB-CAAX we constructed a PKB-CAAX lacking most of the PH domain. This construct, ΔPH-PKB-CAAX, did not display any inhibiting activity, indicating a critical role for the PH domain.

To see whether PKB-CAAX inhibited signaling toward other kinases, we tested the effect of PKB-CAAX on insulin-induced HA-MAP kinase activity as well (Fig. 2C). At the same DNA ratio as used for inhibiting HA-PKB activation we did not observe any clear effect on HA-MAP kinase activation indicat-
PI 3-kinase and PKB Are Essential for Insulin-induced GSK-3 Inhibition—Previously it has been shown that GSK-3 is phosphorylated and inactivated by PKB in vitro (17). Although suggestive, at present no data support a role for PKB in the regulation of GSK-3 in vivo. Therefore, we decided to test the PKB-CAAX mutant for its effect on GSK-3 regulation. However, first we determined whether in A14 cells the kinetics of insulin-induced GSK-3 inactivation followed those of PI 3-kinase and PKB activation and whether in this system GSK-3 is regulated by a PI 3-kinase dependent mechanism.

Following treatment of A14 cells with insulin, PI 3-kinase activation, PKB activation, and GSK-3 inactivation all displayed similar kinetics (Fig. 3A). Activation was rapid with a peak approximately 2 min after PI 3-kinase and PKB and maximal inactivation of GSK-3 at approximately 5 min. This was followed by a return to near basal levels at approximately 30–40 min. Second, we tested whether in A14 cells insulin regulates GSK-3 through phosphorylation of Ser-9. This appeared indeed to be the case as in vitro kinase activity of HA-GSK-3 was transiently expressed in A14 cells was unaffected by insulin treatment (Fig. 3B, 3rd and 4th bar). Third, we tested the effect of PI 3-kinase (in)activation on GSK-3 regulation (Fig. 3B). Treatment of HA-GSK-3-expressing A14 cells with wortmannin prior to insulin treatment resulted in near complete inhibition of insulin-induced decrease in GSK-3 activity, indicating that PI 3-kinase is indeed essential for this process. This was further corroborated by co-expression of Δp85, a dominant-negative PI-3 kinase (26), which also resulted in a loss of insulin-induced GSK-3 inhibition. In A14 cells PI 3-kinase activity appeared not only necessary but also sufficient for inhibition of GSK-3 activity. Co-expression of a constitutively active PI 3-kinase (p110CAAX) resulted in a ligand-independent inhibition of HA-GSK-3 activity as compared with co-transfection of a control (PLAP-CAAX) (27). However, in this type of assay (co-transfection) inhibition of HA-GSK-3 activity by active PI 3-kinase was always less than inhibition by insulin.

As GSK-3 regulation was completely dependent on PI 3-kinase we next tested the involvement of PKB. The same set of experiments was performed using PKB-CAAX to inhibit endogenous PKB activation and gagPKB as constitutively active PKB (Fig. 3C). Co-expression of PKB-CAAX, but not of ΔPH-PKB-CAAX, resulted in a loss of insulin-induced inhibition of HA-GSK-3, and the effect of PKB-CAAX on HA-GSK-3 was similar to the effect of PI 3-kinase inhibition. Co-expression of gagPKB, but not gag alone, resulted in ligand-independent
inhibition of HA-GSK-3. However, as observed for p110CAAX, inhibition of HA-GSK-3 activity by gagPKB was always less than observed following insulin treatment.

These results clearly indicate that PI 3-kinase and PKB are both necessary and sufficient for insulin-induced GSK-3 regulation. However, as all results were derived from one time point we also tested whether the inhibition by PKB-CAA did not shift insulin-dependent regulation to later time points. This appeared not to be the case as within the normal time-course of GSK-3 regulation by insulin no inhibition of GSK-3 was observed in the presence of PKB-CAA (Fig. 3D). Although we did not observe any clear effect of either kinase-dead PKB or the PH domain itself on PKB activation, we also tested the effect of the kinase-dead PKB on HA-GSK-3 regulation by insulin. As indicated by the effects on PKB regulation no clear effect of this mutant on GSK-3 regulation was observed.

**Direct Interaction between PKB and GSK-3 in Vivo**—The results with PKB-CAA indicate that PKB is indeed essential for regulation of GSK-3 by insulin. However, other regulatory mechanism have been proposed for GSK-3 regulation. For example PKC may act in between PKB and GSK-3 as GSK-3 is a substrate for PKC (16), and PKB has been shown to interact with PKC (28). To analyze whether PKB regulation of GSK-3 might be direct, we tested whether PKB and GSK-3 co-immunoprecipitated. To this end HA-GSK-3 and HA-PKB were transiently expressed and precipitated with antisera specific to PKB and GSK-3 respectively. These results clearly indicate that PI 3-kinase and PKB are both necessary and sufficient for insulin-induced GSK-3 regulation. However, as all results were derived from one time point we also tested whether the inhibition by PKB-CAA did not shift insulin-dependent regulation to later time points. This appeared not to be the case as within the normal time-course of GSK-3 regulation by insulin no inhibition of GSK-3 was observed in the presence of PKB-CAA (Fig. 3D). Although we did not observe any clear effect of either kinase-dead PKB or the PH domain itself on PKB activation, we also tested the effect of the kinase-dead PKB on HA-GSK-3 regulation by insulin. As indicated by the effects on PKB regulation no clear effect of this mutant on GSK-3 regulation was observed.

**Characterization of a Dominant-Negative PKB**

**Fig. 3.** The PI-3 kinase/PKB pathway is necessary and sufficient for GSK-3 inactivation by insulin in A14 cells. A, kinetics of PI 3-kinase, PKB, and GSK-3 regulation by insulin. A14 cells were stimulated at the indicated time points and lysed. Following immunoprecipitation with antiphosphotyrosine (PI 3-kinase), anti-PKB, and anti-GSK-3b, kinase activities were determined as described (3, 38) using phosphatidylinositol (PI 3-kinase), Histone 2B (PKB), and P-GS1 peptide (GSK-3) as substrates. Time points for PI 3-kinase and PKB represent single measurements, whereas the time points for GSK-3 represent the average of a measurement performed in triplicate. Similar results were obtained in two independent experiments. B, regulation of GSK-3 is PI 3-kinase dependent. A14 cells were transfected with the indicated constructs, HA-GSK-3 (1 μg/dish), HA-GSK-3S9A (1 μg), or with HA-GSK-3 (1 μg/dish) in combination with Δp85 (8 μg), p110CAAX (200 ng), and kinase-dead p110CAAX (PLAP-CAAX) (200 ng) as indicated. Cells were either left unstimulated (−) or stimulated with insulin (1 μg/ml) for 7 min. Wortmannin pretreatment was for 10 min (50 nM). Cells were lysed and GSK-3 activity was determined as described. 100% activity equals 125,000 cpm, and each bar represents the average of a measurement performed in triplicate (±S.D). Following the GSK-3 assay, the reaction mix was taken off, and beads were taken up in 1× sample buffer. GSK-3 expression was checked by SDS-PAGE and immunoblotting. Similar results were obtained in two independent experiments performed in triplicate. C, regulation of GSK-3 is PKB dependent. A14 cells were transfected with HA-GSK-3 (1 μg/dish) in combination with the indicated constructs, PKB-CAAX (5 μg), ΔPH-PKB-CAAX (5 μg), gagPKB (100 ng), and gag (100 ng). Cells were either left unstimulated (−) or stimulated (+) with insulin (1 μg/ml) for 7 min. Cells were lysed, and GSK-3 activity was determined as described. 100% activity equals 125,000 cpm, and each bar represents the average of a measurement performed in triplicate (±S.D). Following the GSK-3 assay, beads were drained of reaction mix, and beads were taken up in 1× sample buffer. GSK-3 expression was checked by SDS-PAGE and immunoblotting. Results shown represent an example of an experiment representative for five independent experiments (PKB-CAAX) or three independent experiments (rest) all performed in triplicate. D, inhibition of PKB does not result in a delay of GSK-3 inhibition. A14 cells were transfected with HA-GSK-3 (1 μg/dish) in combination with PKB-CAAX (5 μg), PKBdead (10 μg), or pSG5 (10 μg). At the various time points following insulin treatment GSK-3 activity was determined as described. Each time point represents the average of a measurement performed in triplicate.
PKB and GSK-3, respectively. Co-immunoprecipitation of PKB and GSK-3 was detected by immunoblotting with the 12CA5 antibody (Fig. 4A) both in the PKB and the GSK-3 precipitations. We also analyzed whether GSK-3 immunoprecipitated from A14 cells was a substrate for active PKB. Incubation of GSK-3 immunoprecipitates with active PKB isolated from SF-9 cells infected with His-tagged PKB baculovirus, in the presence of [γ-32P]ATP. Following incubation, phosphorylated proteins were resolved by SDS-PAGE and Western blotting. After exposure to film the blot was probed with anti-GSK-3 to reveal the position of GSK-3 indicated in the figure. C, expression of gagPKB induces GSK-3 translocation. HA-GSK-3 and gagPKB were co-expressed in A14 cells. After cellular fractionation as described under “Experimental Procedures,” the presence of HA-GSK-3 was analyzed by Western blotting.

**DISCUSSION**

Activation of PI 3-kinase regulates a number of cellular responses including the activation of PKB and the inactivation of GSK-3. To enable analysis of PKB involvement in PI 3-kinase-mediated signaling in general and GSK-3 in particular, we have constructed a PKB mutant, PKB-CAAAX, and demonstrated that this mutant acted as an efficient inhibitor (i.e. at levels of only moderate overexpression) of insulin-induced PKB activation. This mutant was used to explore the role of PKB in GSK-3 inactivation. From the results it is apparent that GSK-3 is regulated by a signaling pathway consisting of insulin receptor/PI 3-kinase/PKB/GSK-3.

Previously we described activation of p70S6k kinase by active gagPKB, but we were unable to demonstrate clear inhibition of p70S6k kinase activation by co-expression of kinase-dead PKB. Here we show that kinase-dead PKB co-expression does not affect insulin-induced PKB activation. PKB-CAAAX encodes a catalytically inactive PKB as well. The reason for this is not completely understood. PKB activation requires both PI-3P lipid binding (6, 7) and phosphorylation (3). Phosphorylation occurs at threonine 308 and serine 473 (29). In PKB-CAAAX, the CAAX motif is located almost directly next to serine 473. It is therefore conceivable that C-terminal membrane targeting structurally interferes with serine 473 phosphorylation, thereby inhibiting PKB activation. Yet, this cannot be the only reason since mutating serine 473 to alanine only partially inhibits the activation of PKB (29). The residual activation of the S473A mutant is likely to result from threonine 308 phosphorylation as this mutant still shows a mobility shift on SDS-PAGE following insulin treatment (data not shown). We never observed a similar mobility shift for PKB-CAAAX after insulin treatment, suggesting that in addition to the loss of serine 473 phosphorylation, threonine 308 phosphorylation is inhibited as well.

The PH domain is critical for PKB-CAAAX to act as dominant-negative. The PH domain has been shown to bind PI-3P lipids, this suggests that PKB-CAAAX inhibits activation by sequestering the PI-3P lipid(s) involved in PKB activation. Apparently the efficiency of inhibition is greatly improved by localizing the PH domain directly to the membrane. Expression of the PH domain by itself should also result in inhibition of PKB activation, in contrast to a previous report showing that in vitro addition of the PH domain to full-length PKB resulted in activation of PKB (30). However, at the levels of overexpression employed here we do not observe a significant effect of the isolated PH domain on PKB activation.

Both the PH domain and kinase-dead PKB have been used in other studies to suggest the involvement of PKB in mediating PI 3-kinase-dependent signaling. In our model system, we observe no effect with these mutants either on PKB activation or GSK-3 regulation. The reason for this apparent discrepancy is not clear to us.

The results presented here clearly indicate in A14 cells a signaling pathway consisting of insulin receptor/PI 3-kinase/PKB/GSK-3. However, further studies will be needed to see whether in other cell types and with other stimuli this same pathway is operational. Previously, other regulatory signals for GSK-3 regulation have been put forward such as PKC (16), Ras through MAPKAPK-1 (p90Rsk) (31), and tyrosine phosphorylation (15). In A14 cells we have never obtained any evidence that insulin treatment activates PKC (32), but it is clear that for other stimuli that can activate both PI 3-kinase and PKC, PKC activation may represent a redundant pathway in GSK-3 regulation (33). In vitro phosphorylation of GSK-3 by MAPKAPK-1

(p90Rsk) results in partial inactivation of GSK-3 (13, 31) suggesting that activation of the Ras/MAP kinase cascade and subsequent MAPKAPK-1 activation could regulate GSK-3 inactivation. Expression of dominant-negative and constitutively active MAP kinase and extracellular signal-regulated kinase indeed result in attenuation of EGF-induced GSK-3 regulation (31). In contrast however, insulin-induced GSK-3 regulation in myoblasts is not affected by inhibition of MAP kinase activation by the MEK inhibitor PD98059 (34). In addition it has been shown in Chinese hamster ovary cells, that inhibition of insulin-induced Ras/MAP kinase activation by expression of SH2-containing protein tyrosine phosphatase-2 also does not interfere with insulin-induced GSK-3 inactivation (35). Although this apparently excludes a role for the MAP kinase pathway in mediating insulin-induced GSK-3 regulation, a role for Ras is still feasible, especially since Ras has been implicated to activate PI 3-kinase directly (36). PI 3-kinase activation by Ras and PI 3-kinase activation by p85 binding to tyrosine-phosphorylated proteins converge at the level of PKB. The interplay between these two regulatory mechanisms of PI 3-kinase activation is still not completely clear, but depending on the relative importance of Ras in the activation of PI 3-kinase by a ligand (37), an effect of Ras inhibition on GSK-3 regulation may be anticipated.

The interaction of PKB and GSK-3 is probably direct as both proteins co-immunoprecipitate. A consequence of this interaction is the Ser-9 phosphorylation of GSK-3. However, our results show that yet another mode of regulation is possible. In gagPKB expressing cells, constitutively active membrane bound PKB induces a translocation of GSK-3 from cytosol to membrane. Since gagPKB represents the transforming gene of v-Akt, inducing T-cell lymphomas, and we do not observe a similar translocation following insulin treatment, it will be of interest to see whether this translocation of GSK-3 to the membrane fraction is functional in the induction of transformation by gagPKB and its ability to confer protection against apoptosis.

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