MOLECULAR AND CELLULAR BIOLOGY, Sept. 2002, p. 6247–6260 Vol. 22, No. 17
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.17.6247–6260.2002

Multiple Phosphoinositide 3-Kinase-Dependent Steps in Activation of Protein Kinase B

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Received 5 February 2002/Returned for modification 26 March 2002/Accepted 4 June 2002

The protein kinase B (PKB)/Akt family of serine kinases is rapidly activated following agonist-induced stimulation of phosphoinositide 3-kinase (PI3K). To probe the molecular events important for the activation process, we employed two distinct models of posttranslational inducible activation and membrane recruitment. PKB induction requires phosphorylation of two critical residues, threonine 308 in the activation loop and serine 473 near the carboxyl terminus. Membrane localization of PKB was found to be a primary determinant of serine 473 phosphorylation. PI3K activity was equally important for promoting phosphorylation of serine 473, but this was separable from membrane localization. PDK1 phosphorylation of threonine 308 was primarily dependent upon prior serine 473 phosphorylation and, to a lesser extent, localization to the plasma membrane. Mutation of serine 473 to alanine or aspartic acid modulated the degree of threonine 308 phosphorylation in both models, while a point mutation in the substrate-binding region of PDK1 (L155E) rendered PDK1 incapable of phosphorylating PKB. Together, these results suggest a mechanism in which 3′ phosphoinositide lipid-dependent translocation of PKB to the plasma membrane promotes serine 473 phosphorylation, which is, in turn, necessary for PDK1-mediated phosphorylation of threonine 308 and, consequentially, full PKB activation.

Protein kinase B (PKB), also termed Akt, has been the subject of intense study due to its role in transducing signals from phosphoinositide 3-kinase (PI3K) that regulate cell survival and intermediary metabolism. Several protooncogene products modulate the activation of PI3K and, as a consequence, PKB has been shown to play roles in many of the cellular functions that are altered during oncogenesis and other diseases (reviewed in reference 12). Interference with PKB activation may therefore have therapeutic value.

Activation of PKB entails a complex series of events involving additional proteins. First, the PI3K-generated lipid products $PI(3,4,5)P_3$ and $PI(3,4)P_2$ recruit PKB to the plasma membrane through their affinity for the PH domain of PKB (14, 20, 21). Once membrane proximal, at least two residues of PKB are rapidly phosphorylated, including threonine 308 (T308) and serine 473 (S473) (1). T308 lies within the kinase T loop, and its phosphorylation is presumed to generate a conformational change that permits access to the substrates, analogous to T-loop phosphorylation in other protein kinases. In the case of PKB, this reaction is catalyzed by another 3′ phosphoinositide-regulated kinase termed PDK1 (2, 33). S473 is located within a hydrophobic region close to the carboxyl terminus of PKB and is also phosphorylated during activation (1), but the mechanism of its phosphorylation and the role it serves in activating PKB are incompletely understood.

Several lines of evidence suggest that S473 is autophosphorylated. For example, catalytically inactive mutants of PKB do not undergo S473 phosphorylation (34). There is also evidence for an autonomous S473 kinase. For example, in PDK1-deficient cells, PKB is largely inactive, yet phosphorylation of S473 remains responsive to insulin stimulation (37). Certain drugs that inhibit PDK1 activity prevent T308 phosphorylation and, consequently, PKB activation yet do not impact S473 phosphorylation (23). Despite the evidence for a distinct PKB kinase, the search for a "PDK2" enzyme has proven difficult, although several kinases have been shown to phosphorylate PKB at S473 in vitro, including ILK, MAPKAPK2, and RSK (18; discussed in reference 35) and a recently described kinase, NEK6 (8).

Examination of PKB activation is complicated by several factors. First, expression of membrane-targeted PKB leads to chronic high-level activation. This is best illustrated with the viral form of PKB (v-Akt), which is myristoylated due to fusion with a viral Gag protein (9). This modification renders the Gag-PKB chimera constitutively phosphorylated on both of its activating residues, T308 and S473, precluding analysis of the activation process (1, 5). Secondly, natural PKB alleles are under the control of PI3K lipid products and therefore depend upon PI3K activation to coordinate localization with the plasma membrane (5, 17, 29, 36; reviewed in reference 31). These conditions make it difficult to distinguish the events necessary for PKB localization to the plasma membrane from those necessary for phosphorylation of T308 and S473. Finally, studies examining PKB activation in vitro have the complications of both lipid dependency and omission of other, as-yet-unidentified factors (such as binding proteins), which may be important.

In this study, we have used two models to control the process of PKB activation. The first model was initially described by Roth and colleagues (25), in which the PH domain of PKB has been replaced with a myristoylation signal (Fig. 1). This pro-

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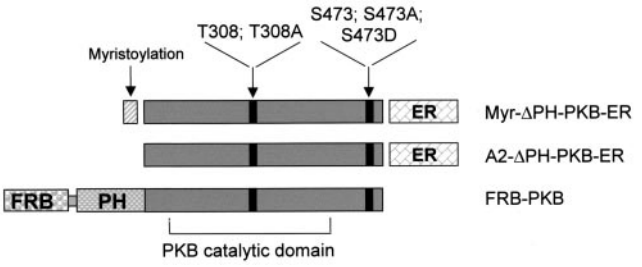


FIG. 1. Schematic representation of PKB fusion proteins used in this study. Each allele harbored either a wild-type residue occupying the relative position of T308 and S473 in PKBα or an aspartic acid or alanine residue.

motes association of PKB with membranes independently of PI3K activation. The PKB is fused to the hormone-binding domain of the estrogen receptor (ER) to generate ΔPH-PKB-ER. Activation of ΔPH-PKB-ER is under the control of an estrogen analogue, 4-hydroxytamoxifen (4-OHT). Thus, ΔPH-PKB-ER behaves like the viral form of PKB (v-Akt) except that its activation is conditional on the presence 4-OHT. This tool has been used to identify and validate downstream targets of PKB and serves as a close approximation of normally activated PKB (7, 24, 25, 27, 32), but it has not yet been utilized as a tool for probing PKB activation.

The second PKB model used here is novel and based on drug-inducible heterodimerization between drug binding fragments of FK506 binding protein (FKBP) and FRAP/mTor (FRB) (Fig. 1) (3, 16). Dimerization between FKBP12 and FRB is induced by rapamycin, or a related, nonimmunosuppressive analogue (AP21967). The FKBP12 fragment harbors a point mutation that prevents it from binding with endogenous FRAP/mTOR and thus does not perturb this pathway. In our model, we coexpressed a myristoylated FKBP12 fragment with an FRB-PKB chimera. Addition of AP21967 to cells expressing both proteins induced rapid translocation of PKB to the plasma membrane, thus mimicking the effect of PI3K-induced translocation. Unlike the first model (ΔPH-PKB-ER), the PKB-FRB fusion protein retains an intact PH domain allowing assessment of nonlipid binding roles of the PH domain in the activation process. Using these models, we have examined the intradependency between membrane-localized and cytosolic PKB on phosphorylation of T308 and S473.

MATERIALS AND METHODS

Cell culture. Human epithelial kidney (HEK) 293 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagles medium (DMEM) medium supplemented with 10% fetal calf serum and antibiotics at 37°C, 5% CO_2, and humidity.

Reagents. Antibodies used were anti-PKB/AKT, anti-phospho-specific T308 PKB/AKT, anti-phospho-specific S473 PKB/AKT (all from Cell Signaling Technology), and anti-PDK1 (Transduction Labs) antibodies. Recombinant SGK S422D was obtained from Upstate Biotechnology. 4-hydroxytamoxiphen (4-OHT) and LY-294002 were obtained from Sigma-Aldrich. The FKBP-FRB heterodimerizer AP21967 was supplied by Ariad Pharmaceuticals.

Expression vectors. cDNAs encoding myristoylated-ΔPH-PKB-ER and A2-ΔPH-PKB-ER were kindly provided by Richard Roth (25). The ARGENT Regulated Heterodimerization Kit from Ariad Pharmaceuticals was comprised of pC_4M-F2E, which encodes myristoylated 2x-FKBP12 and pC_4-R_HE encoding hemagglutinin (HA)-tagged FRB downstream of a cytomegalovirus promoter. The open reading frame of full-length, bovine PKB was inserted into the pC_4-R_HE vector in frame with FRB to produce HA-FRB-PKB and was confirmed by sequencing.

Site-directed mutagenesis. Various site mutants of ΔPH-PKB-ER, FRB-PKB, and PDK1 were generated using the Quickchange kit (Stratagene). Mutations were sequence verified.

cDNA transfections. HEK 293 cells plated onto 35-mm-diameter dishes at 80% confluency were transfected with 100 to 500 ng of cDNA using Lipofectamine (Gibco-BRL) following the manufacturers' protocol. The following day, the transfection medium was removed and replaced with complete DMEM. At 18 h prior to treatments, cells were washed and serum starved in DMEM without fetal calf serum.

Confocal microscopy. HEK 293 cells were plated on glass coverslips and transiently transfected with 1 μg of each plasmid. Cells were fixed and stained as previously described (26). Briefly, cells were incubated with primary antibodies for 2 h, washed, and then incubated with secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) and rhodamine-phalloidin (100 ng/ml) (Sigma) for an hour. Coverslips were mounted onto slides with Fluoromount-G (Southern Biotechnologies, Inc.). Immunofluorescent stained cells were visualized using a Zeiss LSM510 confocal microscope, and images were captured using LSM Software 2.3.

Cell lysis and immunoblotting. Cells were lysed in 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 25 mM NaCl, 25 mM β-glycerophosphate, 5 mM EDTA, 1 μg of Microcystin LR/ml, and protease inhibitors. Portions of the lysates were boiled with sodium dodecyl sulfate (SDS)-containing sample buffer and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, blocked in 5% skim milk for 30 min, and probed with the appropriate antibody overnight at 4°C. Secondary decoration with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies was performed at room temperature for 1 h. Proteins were visualized using ECL according to the manufacturers' protocol (Amersham).

Membrane preparations. Transfected cells were resuspended in ice-cold hypotonic lysis buffer (25 mM Tris [pH 7.4], 5 mM EDTA, 1 mM dithiothreitol [DTT], and protease inhibitors) and disrupted by five passages through a 27-gauge needle. Large cell debris was pelleted by centrifugation at 2,000 × *g* for 5 min at 4°C. Supernatants were then centrifuged at 100,000 × *g* for 20 min at 4°C. The resulting supernatant (cytosol) was removed, and the pellet was resuspended in lysis buffer containing 1% Triton X-100. The lysate was again centrifuged at 100,000 × *g* for 20 min at 4°C, and the supernatants were collected as the membrane fraction.

Kinase assays. Reserved lysates were mixed for 2 h with anti-HA (for ΔPH-PKB-ER) or anti-myc (for myc-tagged PDK1) antibody at 4°C. Antibody-protein complexes were captured with protein G-coupled Sepharose beads for 30 min and washed three times with lysis buffer. Beads were then washed twice with kinase buffer (20 mM HEPES [pH 7.4], 10 mM $MnCl_2$) and resuspended in kinase buffer containing 200 μM cold ATP and 1 μCi of [γ-^{32}P]ATP/sample as well as either 250 nM Crosstide peptide (for PKB assays) or 250 ng of recombinant SGK (S422D for PDK1 assays). Samples were incubated at 30°C for 15 min. For the assay of PKB, samples were spotted onto P81 chromatography paper and washed five times with 0.1% phosphoric acid, and radioactivity was detected using a liquid scintillation counter. For PDK1 assays, the reactions were terminated with the addition of 2× SDS sample buffer and boiling at 95°C for 3 min. ^{32}P-labeled SGK was resolved by SDS-PAGE and quantitated by phosphorimaging.

RESULTS

Treatment of myr-ΔPH-PKB-ER transfected HEK 293 cells with 4-OHT resulted in ligand-dependent activation within 15 min (Fig. 2). Treatment of the cells with IGF-1 did not stimulate myr-ΔPH-PKB-ER, as expected, as activity requires tamoxifen-dependent dissociation of heat shock proteins from the fusion protein (25). Comparison of myristoylated ΔPH-PKB-ER (myr-ΔPH-PKB-ER) with non-myristoylated ΔPH-PKB-ER (A2-ΔPH-PKB-ER) revealed only the former to be catalytically activated in response to 4-OHT (Fig. 2). Myr-ΔPH-PKB-ER activation was sensitive to PI3K inhibition by LY-294002 (Fig. 2), consistent with the original description of myr-ΔPH-PKB-ER (25) and suggesting that membrane local-

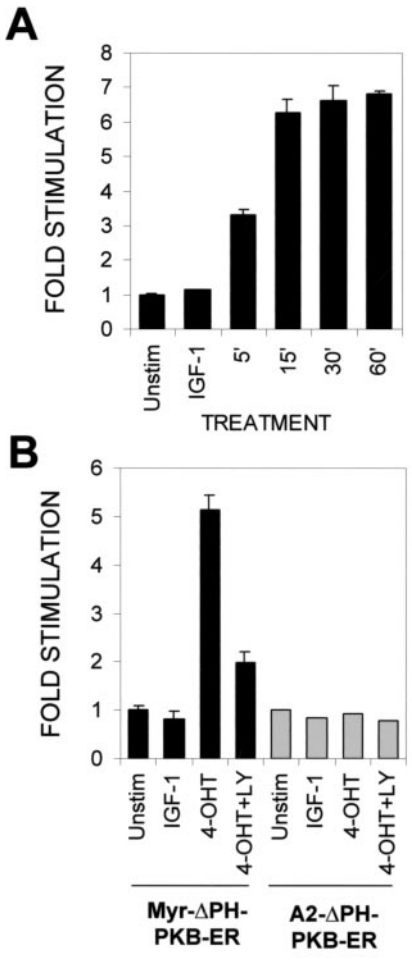


FIG. 2. Activation of myristoylated ΔPH-PKB-ER. (A) Myr-ΔPH-PKB-ER (200 ng) was expressed in HEK 293 cells for 30 h, followed by serum starvation for 18 h. Cells were treated with 1 μM 4-OHT for the indicated times and lysed in detergent-containing buffer. Myr-ΔPH-PKB-ER was immunoprecipitated with 1 μg of anti-HA antibody, and activity was measured in vitro as described in Materials and Methods. (B) Myr-ΔPH-PKB-ER (200 ng) or A2-ΔPH-PKB-ER (200 ng) was expressed in HEK 293 cells for 30 h, followed by serum starvation for 18 h. Cells were pretreated with LY-294002 (25 μM) where indicated for 15 min, followed by treatment with 4-OHT (1 μM) for 15 min. Cell were lysed and in vitro kinase assays were performed as described above.

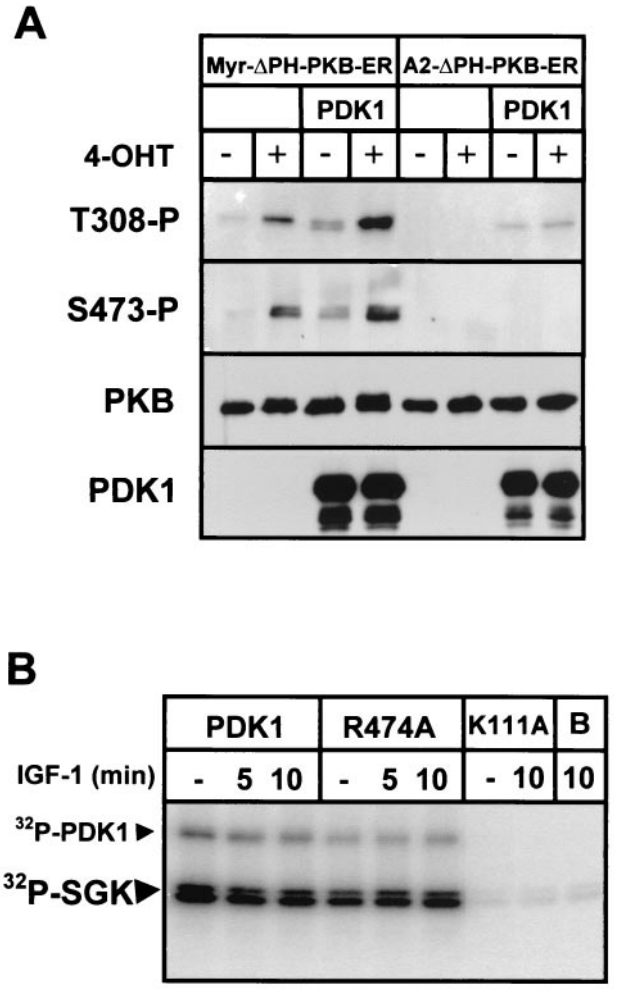


FIG. 3. Phosphorylation of T308 and S473 of myr-ΔPH-PKB-ER but not A2-ΔPH-PKB-ER. (A) HEK 293 cells were transfected with myr-ΔPH-PKB-ER or A2-ΔPH-PKB-ER (200 ng) or together with myc-PDK-1 (200 ng) where indicated. After 30 h, cells were serum starved for 18 h and then treated where indicated with 4-OHT (1 μM) for 15 min. Cells were lysed, and proteins were separated by SDS-PAGE followed by Western blotting with phospho-specific antibodies raised against PKB T308 or S473. Lysates were also blotted with anti-myc antibody (to detect PDK-1) and anti-HA antibody (to detect total ΔPH-PKB-ER). (B) Wild-type, R474A, and K111A PDK1 or an empty vector blank (lane B) were transfected into HEK 293 cells for 30 h, followed by serum starvation for 18 h. Cells were stimulated with IGF-1 for various times where indicated, and PDK1 was immunoprecipitated with anti-myc 9E10 antibody. PDK1 activity was measured in vitro using recombinant S422D SGK as substrate. After 15 min at 30°C, the reaction was terminated and ^{32}P-labeled SGK was resolved by SDS-PAGE and visualized by autoradiography.

ization cooperates with a PI3K signal to drive ΔPH-PKB-ER activation.

These data raised the issue of why only the myristoylated ΔPH-PKB-ER was phosphorylated by the endogenous PDK1 and not the cytosolic ΔPH-PKB-ER. A central tenet of PKB activation suggests that the PH domain of PKB restricts access to PDK1. Activation requires lipid binding of both PKB and PDK1, providing a condition whereby PDK1 can access T308 (35). From our results, it is obvious that this constraint is not a factor with ΔPH-PKB-ER, as it lacks a PH domain. To force phosphorylation of T308, we coexpressed PDK1 with myr-ΔPH-PKB-ER. Figure 3 shows a significant increase in the phosphorylation of T308 in the cotransfected cells compared to cells expressing only myr-ΔPH-PKB-ER, which was residually phosphorylated by endogenous PDK1. In both cases, T308 phosphorylation was conditional upon the presence of 4-OHT and occurred within 5 min of ligand addition. Consistent with the activity measurements, phosphorylation of T308 and S473 only occurred with membrane-localized myr-ΔPH-PKB-ER, as A2-ΔPH-PKB-ER was almost completely insensitive to overexpressed PDK1. We confirmed the activity of PDK1 by performing in vitro kinases assays; both the wild type and R474A mutant (Fig. 4) had similar, agonist-independent activities

(Fig. 3). Together, these results suggested that a secondary event(s) occurring at the plasma membrane but independent of the PH domain of PKB is necessary for PDK1 phosphorylation of ΔPH-PKB-ER, possibly a conformational change in PKB and/or a change in PDK1 activity (28).

To address these two possibilities, we determined whether PI3K plays a role in the phosphorylation of either T308 or S473 of membrane-localized ΔPH-PKB-ER. It is widely accepted that at least one of the roles for PI3K in PKB activation is membrane recruitment (5, 6, 17, 19, 29, 36). Using the ΔPH-PKB-ER model, we were able to address phosphorylation independently of this function. We therefore expressed myr-ΔPH-PKB-ER alone or with various forms of PDK1: wild type, myristoylated, or PH domain point mutant R474A (Fig. 4). R474A PDK1 does not interact with PI3K-derived lipid products and, consequently, resides entirely in the cytosol (4). To monitor the effect of inhibition of PI3K, we employed the specific inhibitor LY-294002. Addition of LY-294002 blocked T308 and S473 phosphorylation of myr-ΔPH-PKB-ER (Fig. 4). It also suppressed the phosphorylation of myr-ΔPH-PKB-ER coexpressed with wild-type PDK1, indicating that an increased stoichiometric ratio of PDK1 to ΔPH-PKB-ER is incapable of overriding PI3K dependence. However, coexpression of myristoylated PDK1 with myr-ΔPH-PKB-ER resulted in a high level of T308 phosphorylation that appeared to be insensitive to inhibition of PI3K (Fig. 4). Thus, under conditions in which PDK1 and PKB are colocalized at the plasma membrane, PDK1 activity towards myr-ΔPH-PKB-ER was not reduced by the presence of PI3K inhibitors. In contrast, S473 phosphorylation was LY-294002 sensitive under all conditions, regardless of PDK1 coexpression, membrane localization, or the extent of T308 phosphorylation. Finally, the R474A mutant of PDK1 phosphorylated ΔPH-PKB-ER in a manner similar to wild-type PDK1, in that PI3K inhibitors reduced the phosphorylation of both T308 and S473. Since subcellular localization of ΔPH-PKB-ER and R474A PDK1 is unlikely to be under PI3K control, this result suggests the existence of a third, PI3K-dependent event that promotes R474A PDK1 phosphorylation of ΔPH-PKB-ER at T308.

Kinase assays were also performed, and they demonstrated that the degree of T308 phosphorylation under each of these conditions strongly correlated with activity (Fig. 4). Thus, coexpression of myristoylated PDK1 with ΔPH-PKB-ER resulted in a high level of catalytic activity that was largely independent of PI3K. We also confirmed the cellular locations of myr-ΔPH-PKB-ER and A2-ΔPH-PKB-ER. Confocal microscopy placed myr-ΔPH-PKB-ER at the plasma membrane, while A2-ΔPH-PKB-ER was largely cytosolic (Fig. 4). Likewise, confocal microscopy and subcellular fractionation confirmed that wild-type PDK1 was diffusely localized within the cytosol, while myr-PDK1 was localized primarily at the plasma membrane (Fig. 4).

As shown above, the phosphorylation of ΔPH-PKB-ER by PDK1 appeared to be dependent on membrane localization in a manner that is independent of phospholipid binding of either PKB or PDK1. Thus, membrane localization primes PKB for PDK1 phosphorylation. S473 phosphorylation also appeared to be highly dependent upon subcellular localization, as phosphorylation of this residue did not occur in ΔPH-PKB-ER whether in the absence or presence of PDK1 expression (Fig.

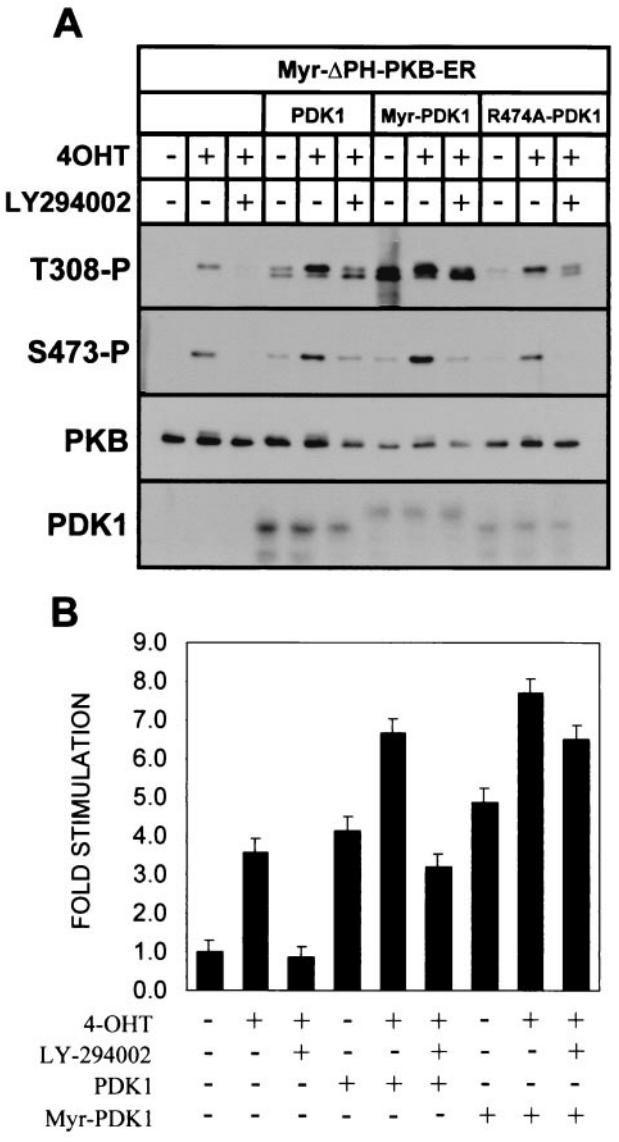
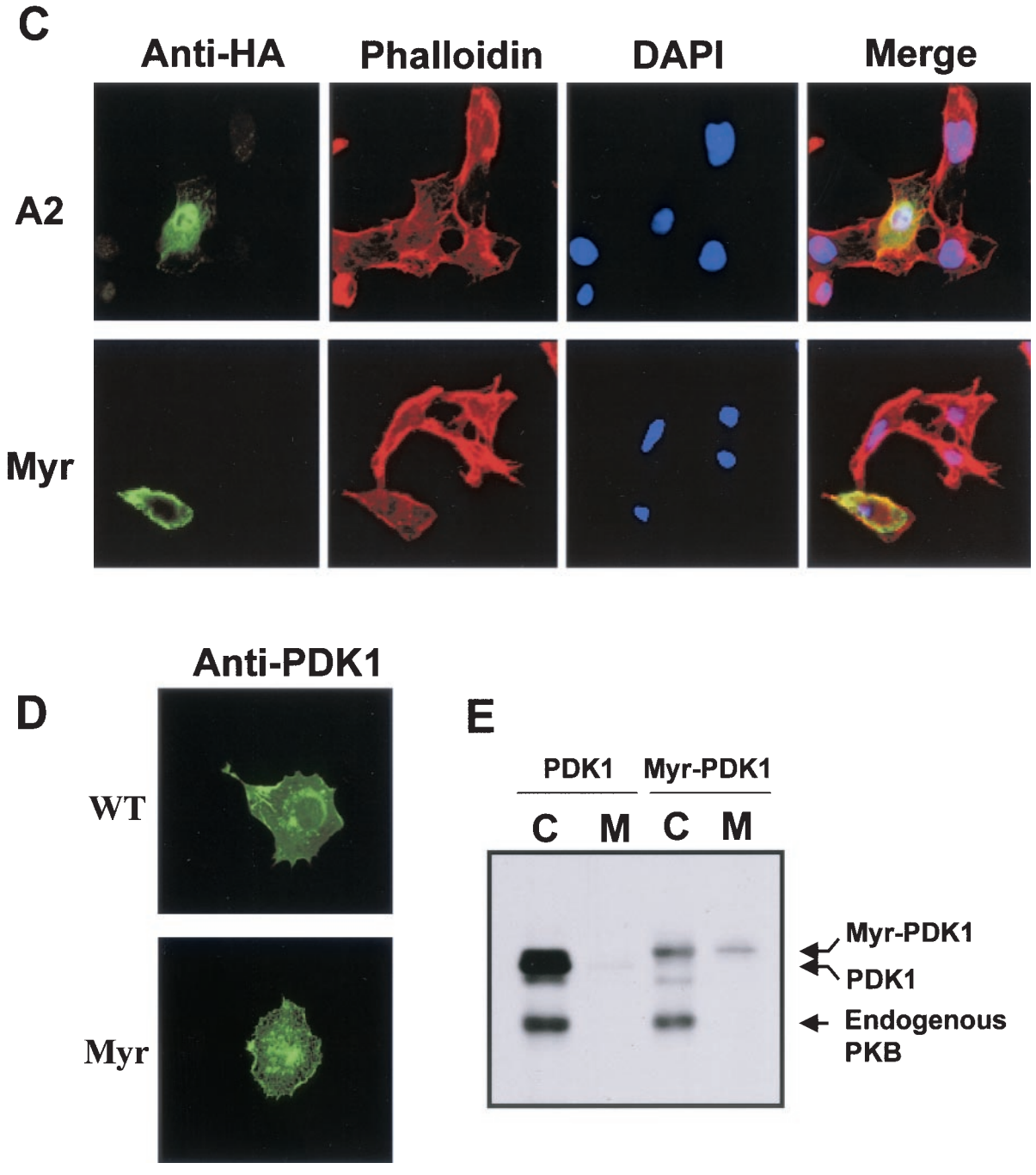


FIG. 4. (A) PI3K activity is necessary for S473 phosphorylation of myr-ΔPH-PKB-ER. HEK 293 cells were cotransfected with myr-ΔPH-PKB-ER (200 ng) and either empty vector or wild-type myc-PDK-1, myristoylated PDK-1, or myc-R474A-PDK-1 (all at 200 ng) in the wells indicated. Following 30 h to allow expression, cells were serum starved for 18 h and then treated with LY-294002 (25 μM) for 15 min. Cells were then treated with 4-OHT (1 μM) for an additional 15 min, and cells were lysed in ice-cold Triton X-100-containing buffer. Protein lysates were separated by SDS-PAGE and transferred to PVDF membranes, and PKB T308 and S473 phosphorylation was detected as described for Fig. 3. Lysates were also probed with antibodies to detect total myr-ΔPH-PKB-ER and PDK-1. (B) The catalytic activity of myr-ΔPH-PKB-ER was measured in an in vitro kinase assay following coexpression with empty vector, wild-type PDK1, or myr-PDK1 as described in Materials and Methods. Data are the averages of quadruplicate determinations from two separate experiments, with error bars representing the standard error of the mean. (C) HEK 293 cells were cultured onto glass coverslips and transfected with 1 μg of myr-ΔPH-PKB-ER or A2-ΔPH-PKB-ER. After 24 h, the cells were fixed in 3% formaldehyde and stained with anti-HA antibody, phalloidin, and DAPI (4′,6′-diamidino-2-phenylindole) as described in Materials and Methods. Cells were visualized by confocal microscopy. (D) HEK 293 cells plated on glass coverslips were transfected with 1 μg of PDK1 or 1 μg of Myr-PDK1. After 24 h, the cells were fixed and stained with anti-PDK1 antibody and visualized by confocal microscopy. (E) HEK



293 cells were trasfected with the wild type or Myr-PDK1 (200 ng). After 30 h, cells were serum starved for 18 h and then resuspended in hypotonic lysis buffer. The cytosol (C) and membrane (M) fractions were prepared as described in Materials and Methods. Samples from each were fractionated by SDS-PAGE and immunoblotted simultaneously with anti-PDK1 and anti-PKB antibodies. The myristoylated PDK1 appears at a higher molecular weight than wild-type PDK1 due to hyperphosphorylation (15) (data not shown).

3). We therefore speculated that S473 might play a regulatory role in phosphorylation of T308. To test for potential phosphorylation site interdependency, we mutated T308 or S473 to alanine. As a comparitor for the alanine mutations, K179 was mutated to glutamine to produce a catalytically inactive form of ΔPH-PKB-ER.

T308A and S473A mutants of myr-ΔPH-PKB-ER were expressed in 293 cells and treated with 4-OHT for 20 min. T308 and S473 phosphorylation demonstrated strong mutual interdependence such that the T308A mutant failed to demonstrate S473 phosphorylation, while the S473A mutation greatly reduced T308 phosphorylation (Fig. 5). The reduction in S473 phosphorylation of the T308A mutant was not unexpected, since it has been shown previously that PKB activity is neces-

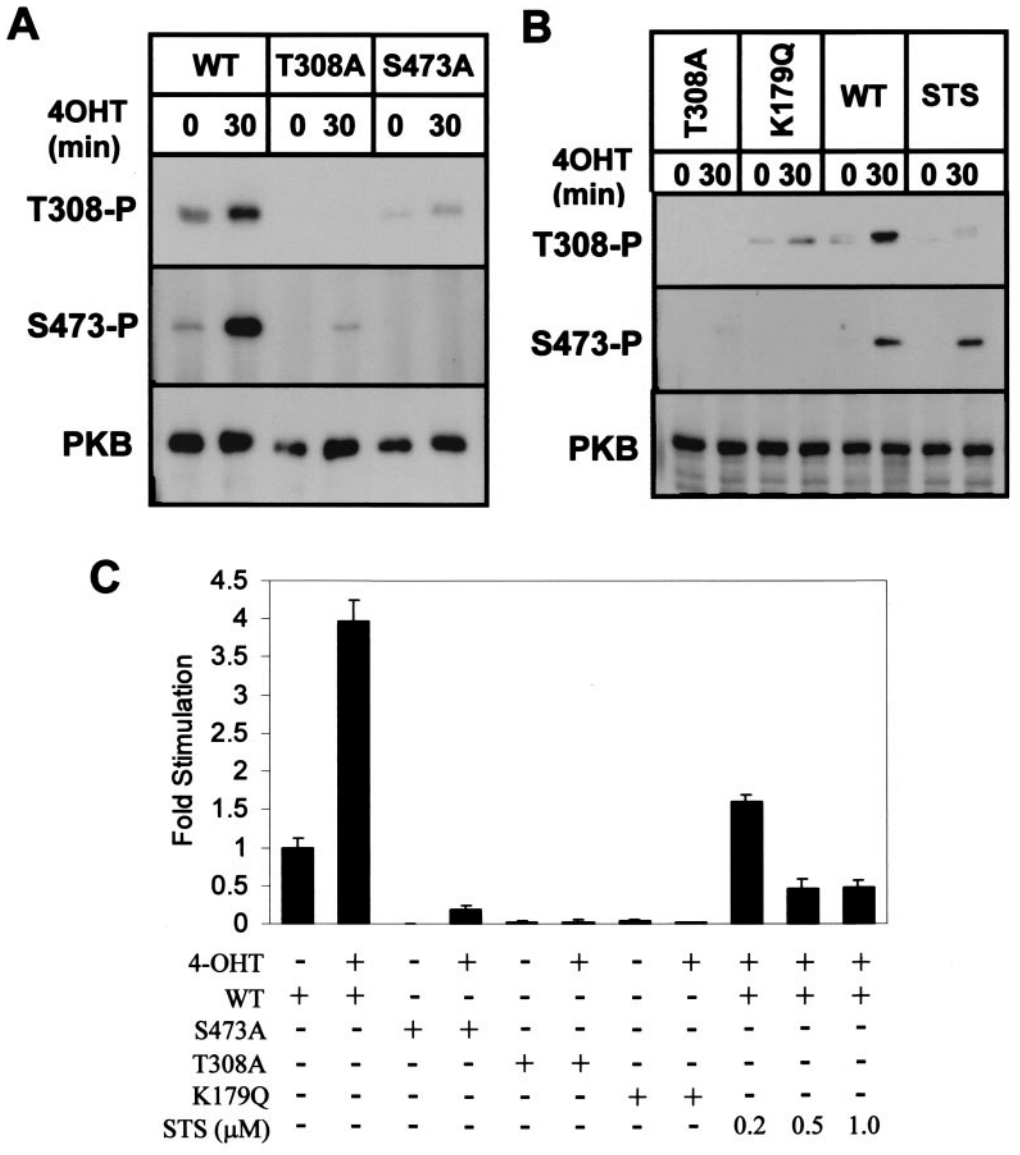


FIG. 5. T308 and S473 phosphorylations of myr-ΔPH-PKB-ER are coupled. (A) Wild-type, T308A, or S473A mutant forms of myr-ΔPH-PKB-ER were expressed in 293 cells for 30 h, followed by serum starvation for 18 h. Cells were treated with 4-OHT (1 μM) for 30 min and then lysed in ice-cold Triton X-100-containing buffer. Immunoblotting was performed to detect phosphorylated T308 or S473. (B) HEK 293 cells were transfected with wild-type, T308A, or K179Q myr-ΔPH-PKB-ER as described above. Some cells expressing wild-type myr-ΔPH-PKB-ER were pretreated with 1 μM staurosporin for 30 min prior to treatment for 30 min with 1 μM 4-OHT. Phosphorylated T308 and S473 were detected by immunoblotting. In both panels, total PKB was detected with anti-HA antibody. (C) Cell were prepared as described for panels A and B, and the catalytic activity of the K179Q, S473A, and T308A mutants of myr-ΔPH-PKB-ER were measured in an in vitro kinase assay as described in Materials and Methods. The activation of wild-type (WT) myr-ΔPH-PKB-ER was also determined after a 15-min treatment with various concentrations of staurosporin (STS).

sary for S473 phosphorylation (34) and supports a role for autophosphorylation as a contributory mechanism for S473 regulation.

In contrast, the absence of T308 phosphorylation of the S473A mutant was surprising, and it suggested that either an intact or phosphorylated serine at position 473 is required for efficient phosphorylation of T308 by PDK1. To further examine this effect, the phosphorylation of kinase-dead, myr-ΔPH-PKB-ER (K179Q mutation) was compared to myr-ΔPH-PKB-ER T308A, as well as "wild-type" myr-ΔPH-PKB-ER. Transfected cells were also treated with the PDK1 selective inhibitor staurosporin (Fig. 5). As before, the T308A mutant suppressed phosphorylation of S473. Kinase-dead myr-ΔPH-PKB-ER also displayed very little S473 phosphorylation, as expected. However, this protein also displayed significantly reduced phosphorylation of T308. This result parallels that of the S473A mutant, again suggesting that phosphorylation of S473 is important for T308 phosphorylation. Surprisingly, staurosporin treatment greatly reduced T308 phosphorylation without much effect on S473 phosphorylation. This finding

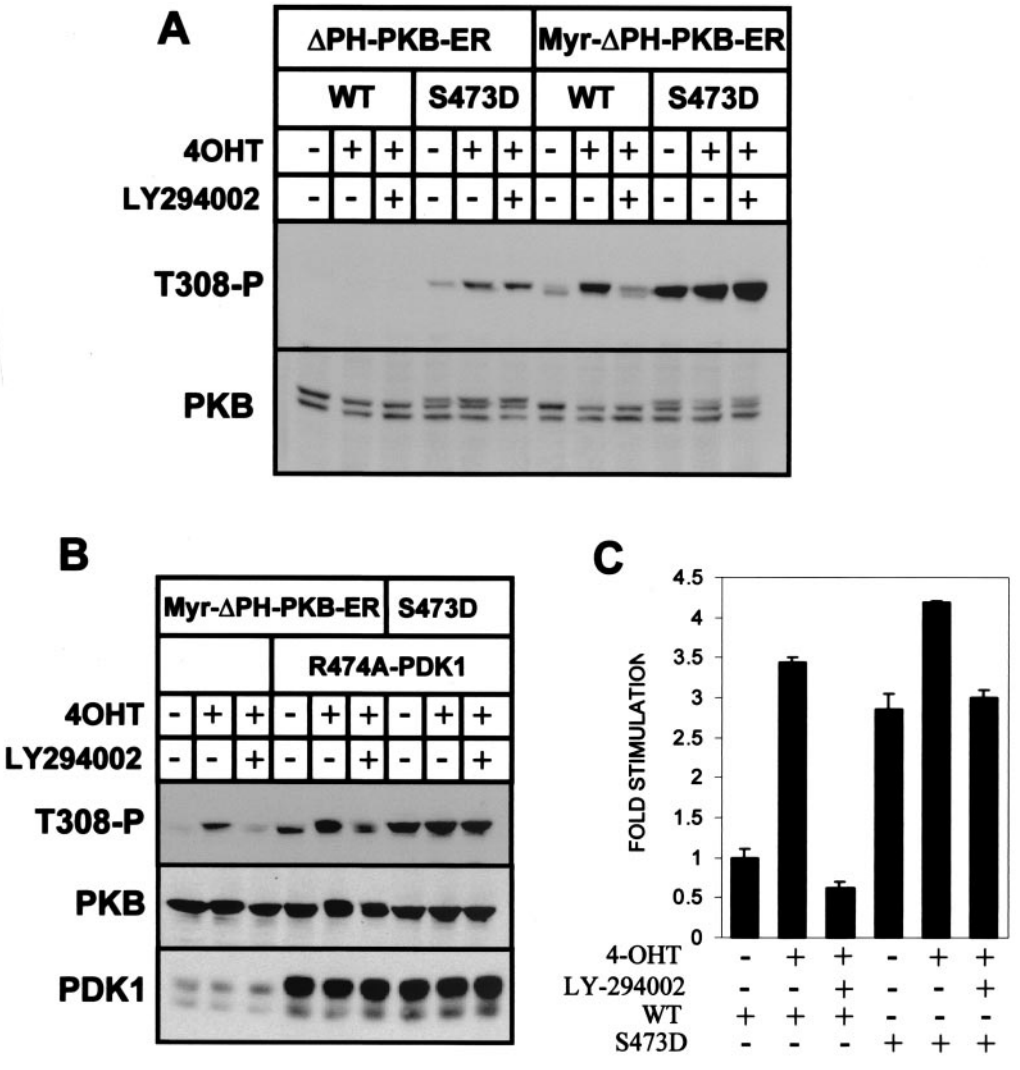


FIG. 6. S473 regulates T308 phosphorylation. (A) A2-ΔPH-PKB-ER or myr−ΔPH-PKB-ER containing either an intact S473 residue or an S473A point mutation were expressed in 293 cells for 30 h, followed by serum starvation for 18 h. Cells were treated with LY-294002 (25 μM) for 15 min where indicated, followed by treatment with 4-OHT (1 μM) for 30 min where indicated. Cellular lysates were fractionated by SDS-PAGE, and T308 phosphorylation was detected by immunoblotting. Total PKB was evaluated using a non-phosphorylation-specific PKB antibody. (B) Wild-type (WT) or S473D myr-ΔPH-PKB-ER was coexpressed with R474A-PDK1 (200 ng of plasmid for each) in 293 cells for 30 h, followed by serum starvation for 18 h. Cells were treated with LY-294002 (25 μM) for 15 min, followed by treatment with 4-OHT (1 μM) for 30 min where indicated. T308 phosphorylation, total PKB, and total PDK1 were detected as described above. (C) Wild-type (WT) or S473D myr-ΔPH-PKB-ER (200 ng) was expressed in 293 cells for 30 h, followed by serum starvation for 18 h. Cells were treated with LY-294002 (25 μM) for 15 min, followed by treatment with 4-OHT (1 μM) for 30 min, where indicated. ΔPH-PKB-ER activity was measured in an in vitro kinase assay as described in Materials and Methods. Data are the averages of duplicate determinations, with error bars representing the range.

contradicts the autophosphorylation model supported by the T308A and K179Q derived data (see below). We also measured the catalytic activity of ΔPH-PKB-ER under each of these conditions. Both the S473A and T308A mutants were resistant to activation, with T308A exhibiting a more severe effect, being comparable to kinase-dead, K179Q ΔPH-PKB-ER. Staurosporin also greatly reduced the activation of ΔPH-PKB-ER by tamoxifen, but even at the maximal dose of 1 μM it was unable to reduce the catalytic activity to that of kinase-dead ΔPH-PKB-ER.

These data suggest that S473 functions as a molecular switch to regulate the phosphorylation of T308. To test this idea, we mutagenized the S473 residue to aspartic acid to partially mimic the effect of a negative charge introduced as a result of phosphorylation. Previously, the double aspartic acid mutant of PKB was shown to be constitutively active and independent of PI3K activity, suggesting that S473D can act as a phosphomimetic (1). We expressed either membrane-localized or cytosolic S473D ΔPH-PKB-ER and examined the extent of T308 phosphorylation in response to 4-OHT (Fig. 6). The S473D mutation significantly elevated the level of T308 phosphorylation of both cytosolic ΔPH-PKB-ER and membrane-localized myr-ΔPH-PKB-ER (Fig. 6). However, the extent of T308 phosphorylation was much greater when the ΔPH-PKB-ER molecule was membrane localized. Thus, S473 phosphorylation and plasma membrane localization are the principal determinants

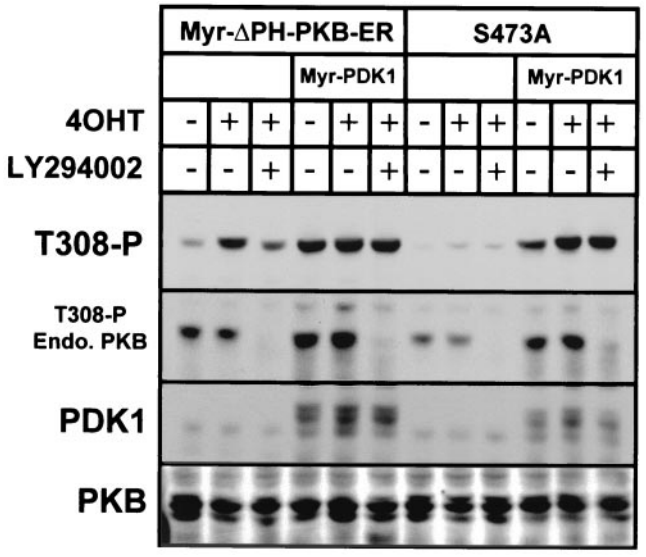


FIG. 7. Colocalizing PDK1 and PKB removes the requirement for S473 phosphorylation. Wild-type (WT) or S473A myr-ΔPH-PKB-ER was coexpressed with myristoylated PDK1 (200 ng of plasmid for each) in 293 cells for 30 h and then placed in serum-free medium for 18 h. Cells were treated with LY-294002 (25 μM) for 15 min prior to treatment with 4-OHT (1 μM) for 30 min, where indicated. Phosphorylated T308, total PDK1, and PKB were detected by immunoblotting.

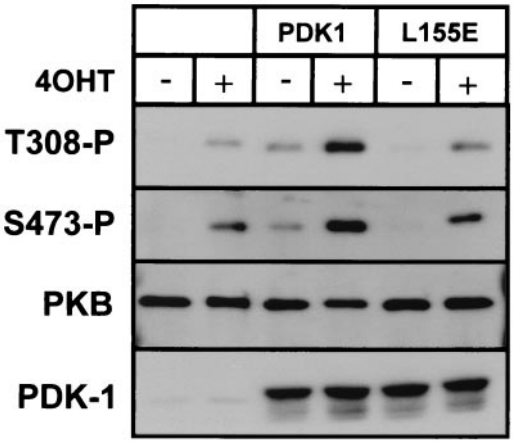


FIG. 8. Myr-ΔPH-PKB-ER is not phosphorylated by L155E PDK1. Myr-ΔPH-PKB-ER was coexpressed with either empty vector (pcDNA3), wild-type PDK1, or L155E PDK1 (200 ng for all plasmids). Cells were treated with 4-OHT (1 μM) for 30 min. Cell lysates were fractionated by SDS-PAGE and transferred to PVDF membranes, and both T308 and S473 phosphorylations were detected with phospho-specific antibodies.

for T308 phosphorylation. Also, with both cytosolic and myristoylated ΔPH-PKB-ER S473D mutants, T308 phosphorylation was insensitive to PI3K inhibition (see below). This effect was illustrated more vividly upon coexpression of PH domain mutant of PDK1 with myr-ΔPH-PKB-ER S473D (Fig. 6). Despite the high level of T308 phosphorylation promoted by coexpression of R474A PDK1, inhibition of PI3K resulted in a significant reduction in phosphorylation of T308 for the wild-type myr-ΔPH-PKB-ER. By contrast, T308 phosphorylation was insensitive to PI3K inhibition in the case of the myr-ΔPH-PKB-ER S473D mutant. As an aside, we employed R474A PDK-1 in these experiments to exclude the possibility that PI3K inhibition prevented sufficient membrane association of PDK1 to allow T308 phosphorylation.

Kinase activity measurements were also performed. Expression of S473D myr-ΔPH-PKB-ER resulted in a high level of activity compared to the wild-type protein, in agreement with the level of T308 phosphorylation, and this was only partially sensitive to PI3K inhibitors (Fig. 6). Thus, PDK1 phosphorylation of T308 and resulting kinase activity is optimal when PKB is localized to the plasma membrane and when an acidic (or phosphorylated) residue occupies the position at 473.

We next wished to address the earlier finding that myristoylated PDK1 phosphorylated myr-ΔPH-PKB-ER in the presence of PI3K inhibitors (Fig. 4). We reasoned that S473 phosphorylation is less important to T308 phosphorylation under conditions when myristoylated PDK1 is coexpressed. To test this, we expressed myr-ΔPH-PKB-ER or myr-ΔPH-PKB-ER S473A together with myristoylated PDK1 and monitored T308 phosphorylation (Fig. 7). As expected, wild-type myr-ΔPH-PKB-ER was sensitive to PI3K inhibition when phosphorylated by endogenous PDK1. Coexpression of myristoylated PDK1 generated a high degree of T308 phosphorylation, independent of PI3K, consistent with the data in Fig. 4. The myr-ΔPH-PKB-ER S473A mutant was not phosphorylated by endogenous PDK1; however, when myr-ΔPH-PKB-ER S473A was coexpressed with myristoylated PDK1, a high degree of phospho-T308 was observed, similar to wild-type myr-ΔPH-PKB-ER. Thus, S473 phosphorylation is rate limiting for ΔPH-PKB-ER phosphorylation by endogenous PDK1, but not when exogenous, myristoylated PDK1 is coexpressed.

These results support a process whereby the hydrophobic motif surrounding S473 plays a regulatory role in PDK1 phosphorylation of T308. An emerging concept for other AGC kinases, including p70S6K, SGK, and RSK, is that this hydrophobic motif serves as a docking site for PDK1 (11, 22). To validate our model that PDK1 must interact with ΔPH-PKB-ER in order to phosphorylate T308, we asked whether a change in the substrate-binding region of PDK1 would influence its ability to phosphorylate ΔPH-PKB-ER. Mutation of PDK1 at residue L155 (to glutamate) disrupts interaction of PDK1 with other AGC kinases, including p70S6K and SGK (10, 11). Coexpression of wild-type PDK1 and ΔPH-PKB-ER led to significantly elevated T308 phosphorylation, while PDK1 L155E was ineffective and resulted only in T308 phosphorylation levels that were similar to expression of ΔPH-PKB-ER alone (Fig. 8). Thus, the hydrophobic pocket of PDK1 that surrounds L155E appears essential for the phosphorylation of ΔPH-PKB-ER.

Our results with ΔPH-PKB-ER support the following mechanism of activation: localizing PKB to the plasma membrane promotes S473 phosphorylation, which is sensitive to PI3K inhibitors. Phosphorylation of S473 facilitates the ability of PDK1 to phosphorylate T308, leading to the full activation of PKB. In addition, PDK1 activity towards T308 is much greater when both are colocalized to the plasma membrane. This is evidenced by the fact that myr-ΔPH-PKB-ER-S473D is phos-

phorylated to a much greater extent on T308 than the nonmyristoylated S473D form.

We wished to confirm these observations by using a different inducible model of PKB regulation. To this end, we fused the FKBP-binding region of FRAP/mTOR (termed FRB) to the amino terminus of full-length PKB. When coexpressed with myristoylated FKBP, heterodimerization of PKB with the myristoylated FKBP was achieved by the addition of a small molecule drug, a "rapalog" of the immunosuppressant compound rapamycin, AP21967. Since FKBP is myristoylated, this serves to translocate cytosolic FRB-PKB to the plasma membrane. Immunofluorescence microscopy revealed that, prior to treatment with AP21967, FRB-PKB was diffusely present in the cytoplasm as well as the nucleus (Fig. 9). Within 30 min of AP21967 treatment, the cytosolic component of FRB-PKB was replaced by strong plasma membrane staining. Staining with anti-phospho-S473 antibody revealed the same pattern, indicating that the FRB-PKB present at the plasma membrane was undergoing S473 phosphorylation. The fraction of FRB-PKB found to be nuclear and positive for phospho-S473 antibody likely represents FRB-PKB that has become activated in the time during the transient transfection leading up to the experiment. Nucleus-free, detergent-soluble lysates from cells treated with AP29167 for 15 and 30 min also showed that phosphorylation occurred at both T308 and S473, and this was concurrent with increased kinase activity (Fig. 9).

The model of activation suggested by the ΔPH-PKB-ER data predicates FRB-PKB phosphorylation on T308 and S473 as PI3K inhibitor sensitive. Consistent with the previous data, treatment of the FRB-PKB/myr-FKBP cells with LY-294002 prior to addition of AP21967 blocked both phosphorylation events (Fig. 9). Thus, translocation of PKB to the plasma membrane is insufficient in promoting either S473 or T308 phosphorylation in the absence of PI3K activity. This result is similar to that observed with the myristoylated ΔPH-PKB-ER model (Fig. 4).

To confirm that S473 plays a role in directing T308 phosphorylation (as it does with the ΔPH-PKB-ER system), the wild type and S473A and S473D point mutants of FRB-PKB were expressed in 293 cells in the presence of myr-FKBP and treated with AP21967. The S473A mutant was largely refractory to T308 phosphorylation, while the S473D mutant was similar to wild-type FRB-PKB (Fig. 10). Kinase activity measurement demonstrated that the S473A FRB-PKB mutant had very low activity, compared with S473D, which had both high basal and AP21967-stimulated activity. Thus, mutating S473 to alanine reduces PDK1-dependent phosphorylation of T308 and reduces activity while substituting an acidic residue at this position mitigates this loss, in agreement with the results derived from ΔPH-PKB-ER.

We next examined the dependence of T308 phosphorylation on FRB-PKB phosphorylation at S473. We showed with the ΔPH-PKB-ER model that mutation of T308 to alanine produces a catalytically inactive kinase, and similar to the K179Q mutant, this prevents S473 phosphorylation. In agreement with these observations, mutation of T308 to alanine in FRB-PKB prevented translocation-induced phosphorylation of S473 (Fig. 10). Note that the kinase activity of the T308A FRB-PKB was essentially the same as that recovered from the empty vector control.

A remaining question was whether staurosporin could reduce T308 phosphorylation and activity of FRB-PKB as it did with ΔPH-PKB-ER and if this had any effect on S473 phosphorylation. Membrane translocation of FRB-PKB by AP21967 led to increased kinase activity and phosphorylation of S473 and T308 (Fig. 11). Pretreatment with staurosporin blocked the activation of FRB-PKB and phosphorylation on T308. However, there was little reduction, if any, in the phosphorylation of S473 (Fig. 11). This result agrees with our earlier results with ΔPH-PKB-ER. We rationalized that ΔPH-PKB-ER still maintained S473 phosphorylation because the activity of ΔPH-PKB-ER after staurosporin treatment was still significant compared to that of the kinase-dead mutant. This was also the case with FRB-PKB, since staurosporin only blocked AP21967-induced activity but did not reduce its basal activity. This was different from T308A FRB-PKB, which was much less active (Fig. 10).

The experiments with staurosporin and FRB-PKB were performed in HEK293 cells. To confirm that this was not a cell type-specific event, we performed the same experiment in HeLa-transfected cells. AP21967 treatment led to rapid phosphorylation of both S473 and T308 in transiently transfected HeLa cells. Pretreatment with LY-294002 attenuated the AP21967-induced phosphorylation of both sites. Significantly, staurosporin pretreatment only blocked the induction of T308 phosphorylation and had no effect on S473 phosphorylation (Fig. 11).

DISCUSSION

The results presented here represent the first report of S473-dependent PDK1 phosphorylation of T308 in vivo. The data are derived from two inducible models, one lacking the PH domain (ΔPH-PKB-ER) and a second retaining this phospholipid-binding moiety (FRB-PKB). Using these models as "inducible substrates," we monitored the rates of T308 and S473 phosphorylation after selective targeting to either the plasma membrane or the cell cytosol. Coexpression of localization mutants of PDK1 with phosphorylation site mutants of PKB allowed assessment of the relevant factors for functional interaction between PKB and PDK1 in vivo.

From this analysis, we deduce that PDK1-catalyzed phosphorylation of T308 is dependent on two criteria, the first and foremost requirement being S473 phosphorylation. A mutant of ΔPH-PKB-ER harboring an alanine at position 473 is not phosphorylated at T308, even when directed to the plasma membrane. Conversely, mutation of S473 to an acidic residue promotes PI3K-independent T308 phosphorylation, although membrane localization is still important (further discussed below). Thus, S473 and the hydrophobic motif in which it is situated may act as a docking site for PDK1 similar in mechanism to that which may occur with RSK (22) and other AGC kinases (11; see below). Further support for this model is provided by analysis of the PDK1 L155E mutant that cannot dock with other AGC kinases (10, 11). This mutant protein was similarly ineffective in promoting T308 phosphorylation of membrane-localized ΔPH-PKB-ER. Thus, the overall control of PKB activation is achieved through regulation of S473 phosphorylation and consequential phosphorylation of T308 (Fig. 12). This is in agreement with a recent study examining a

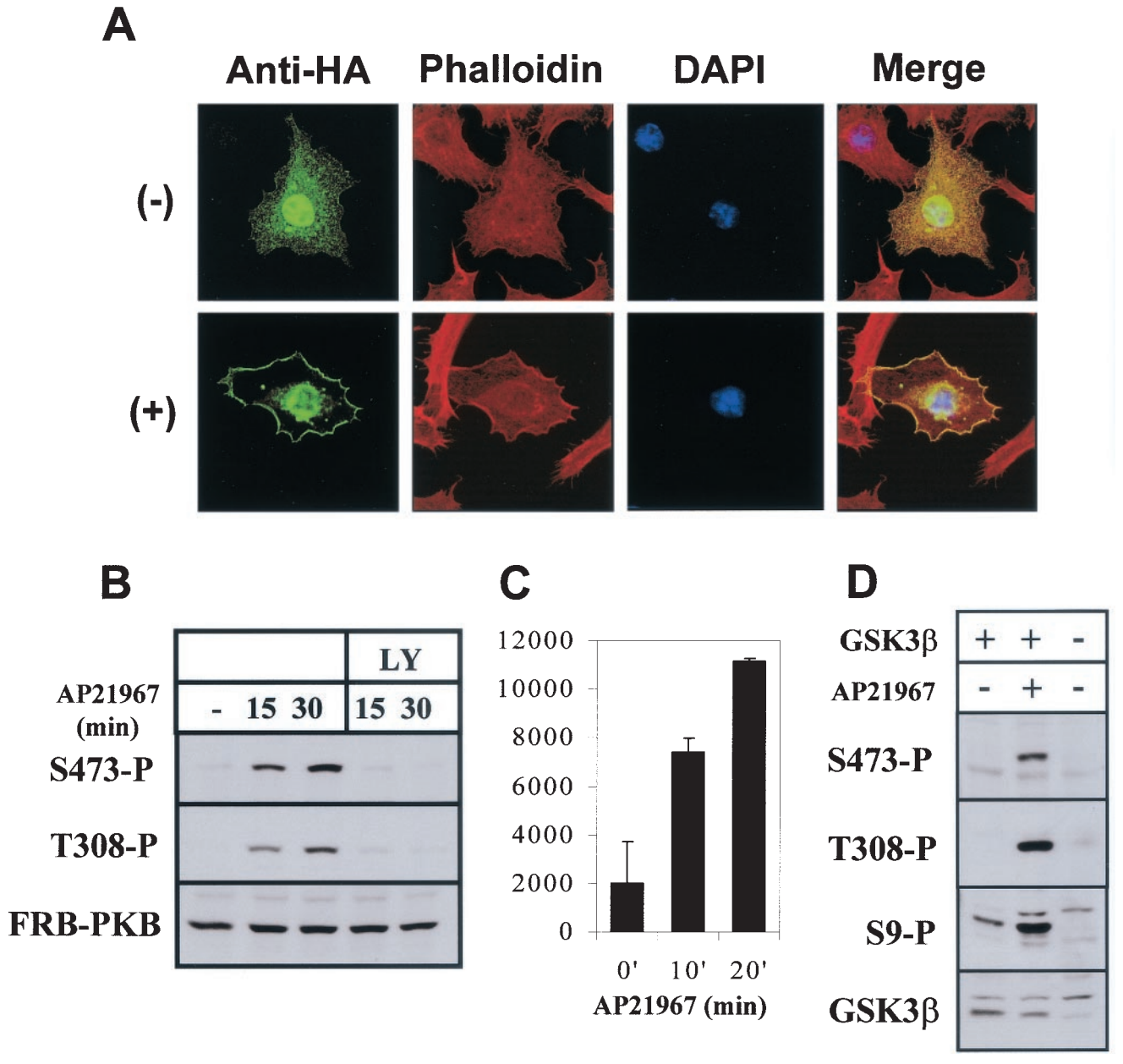


FIG. 9. Translocation of FRB-PKB to membranes leads to PI3K-dependent phosphorylation of S473 and T308 and elevated activity. (A) HEK 293 cells were plated onto glass coverslips and transfected with 300 ng of FRB-PKB and 600 ng of myr-FKBP. After 18 h, the cells were starved of serum for a further 8 h, followed by treatment with AP21967 (200 nM) for 30 min (+) or no treatment (−). Cells were fixed and stained with anti-PKB antibody, anti-phospho-S473 antibody, phalloidin (to visualize polymerized actin), and DAPI where indicated and as described in Materials and Methods, and confocal images were visualized. (B) HEK 293 cells were transiently transfected with myr-FKPB12 (400 ng), FRB-PKB, and GSK-3β (100 ng) where indicated. After 30 h, the cells were starved of serum for 18 h and then treated with LY-294002 (25 μM) for 15 min, followed by AP21967 (200 nM) for the times indicated. Phosphorylation of T308 and S473 and total FRB-PKB were detected by immunoblotting. (C) Activity of FRB-PKB following treatment of cells with AP21967 was determined in an in vitro kinase assay as described in Materials and Methods. The *y* axis represents the total count per minute of ^{32}P-labeled Crosstide. (D) The in vivo kinase activity of FRB-PKB was verified by cotransfection with GSK-3β and monitoring S9 phosphorylation by using a phospho-specific antibody following a 30-min treatment with AP21967.

spliced variant of PKBγ, which lacks the extreme carboxyl terminus, including the analogous phosphorylation site to S473 in PKBα, namely S472, and surrounding residues. This protein is poorly activated by insulin or pervanadate (13).

The second determinant for T308 phosphorylation and PKB activation is plasma membrane localization. Cytosolic ΔPH-PKB-ER and FRB-PKB are each poor substrates for PDK1, even when the latter is overexpressed. When cytoplasmic, both the activation loop site T308 and the hydrophobic motif S473 residues of these PKB molecules were not phosphorylated. It

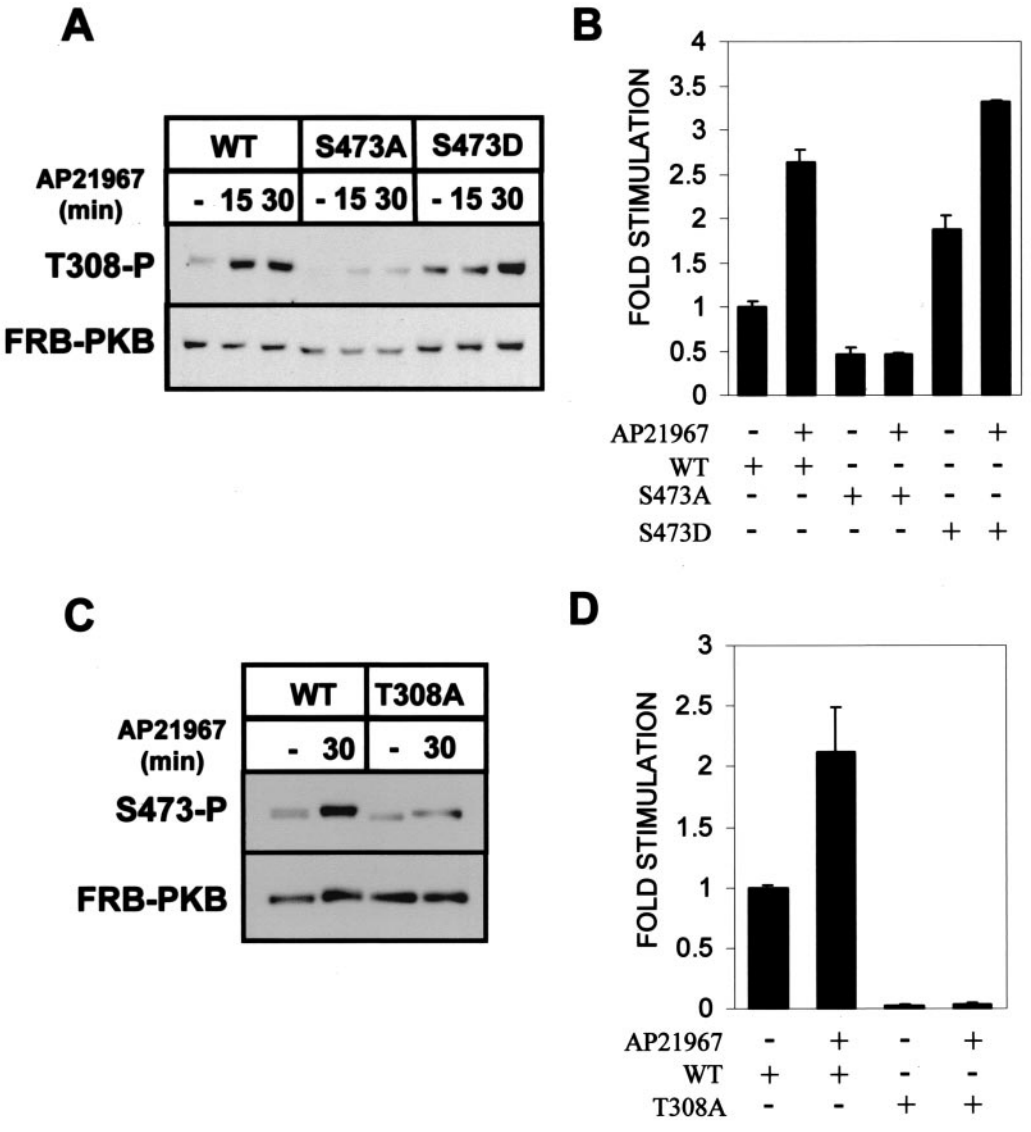


FIG. 10. Site-specific phosphorylation of FRB-PKB regulates kinase activity. (A) FRB-PKB, FRB-PKB S473A, and FRB-PKB S473D (200 ng) were cotransfected with myr-FKBP (400 ng). After 30 h, the cells were starved of serum for 18 h and then treated with 200 nM AP21967 for 15 or 30 min where indicated. Phosphorylation of T308 and total FRB-PKB were detected by immunoblotting. (B) The activities of FRB-PKB, FRB-PKB S473A, and FRB-PKB S473D were determined in an in vitro kinase assay following a 30-min treatment with AP21967. Values are the averages of quadruplicate determinations, with error bars representing the standard error of the mean. (C and D) Cells were transfected with FRB-PKB T308A and treated with AP29167 for 30 min. Phosphorylation of S473 and kinase activity were measured as done for panels A and B.

has been previously suggested that the PH domain may sterically interfere with access to the activation phosphorylation sites and that lipid binding relieves the effect. In the PKB-ER model, the PH domain was deleted from PKB. Since phosphorylation/activation was rescued upon myristoylation of PKB, we favor a simplified mechanism for the PH domain where it acts to collate the relevant molecules into the same subcellular vicinity with PI3K-dependent timing.

These results contrast with previous experiments using ΔPH-PKB. In these studies (6, 19), coexpression of ΔPH-PKB and PDK1 led to high-level phosphorylation of ΔPH-PKB on both T308 and S473. We considered that the difference in our studies might be due to the regulatory nature of ΔPH-PKB-ER. During transient expression of ΔPH-PKB, the molecule is allowed hours to accumulate phosphorylation, particularly on S473, as it interacts passively with the plasma membrane and with the overexpressed PDK1. T308 phosphorylation would closely follow. In our study, ΔPH-PKB-ER was available for phosphorylation at S473 only upon addition of tamoxifen. Thus, using a short time period (i.e., 30 min), we observed a dramatic difference between the rates of T308 phosphorylation of cytosolic and membrane-localized ΔPH-PKB-ER when coexpressed with PDK1.

Based on these observations, the lack of T308 phosphorylation of cytosolic PKB is likely a result of insufficient S473 phosphorylation. There appear to be other restrictive factors,

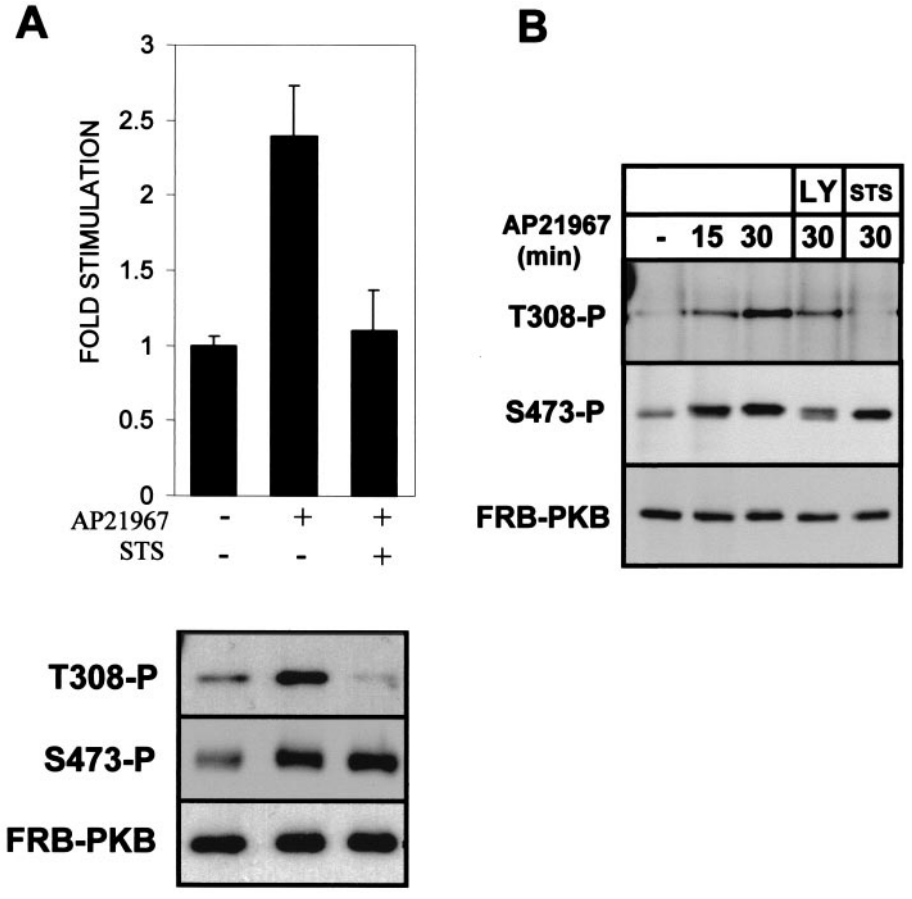


FIG. 11. Effect of staurosporin on FRB-PKB phosphorylation and kinase activity. (A) HEK 293 cells were cotransfected with myr-FKBP (400 ng) and FRB-PKB (200 ng). After 30 h, the cells were starved of serum for 18 h and then treated with 1 μM staurosporin where indicated for 15 min, followed by 200 nM AP21967 for 30 min. Kinase activity was measured, and reserved portions of the cell lysates were immunoblotted with anti-T308 antibody, anti-S473 antibody, and total PKB. (B) HeLa cells transfected with myr-FKBP and FRB-PKB were treated with LY-294002 (25 μM) for 15 min or staurosporin (1 μM) where indicated, followed by 200 nM AP21967 for various times where indicated. Lysates were immunoblotted for T308 and S473 phosphorylation and for total FRB-PKB.

however, illustrated by the finding that the S473-to-aspartic acid mutants of ΔPH-PKB-ER or FRB-PKB are not fully phosphorylated by PDK1 unless directed to the membrane. This could be a result of the concentrating effect of moving PKB and PDK1 from the cytosol to the plasma membrane and/or it may reflect an increase in PDK1 specific activity (28).

PI3K itself plays a central role in orchestrating the activation of PKB. As discussed above, PKB needs to be recruited to the plasma membrane by PI3K-generated lipids (5, 17, 29, 36); otherwise, the protein resides in the cytosol in a similar, unphosphorylated state as ΔPH-PKB-ER. Once at the plasma membrane, a PI3K-sensitive activity regulates S473 phosphorylation. We observe this experimentally, since neither myristoylated ΔPH-PKB-ER nor membrane-localized FRB-PKB is phosphorylated at S473 in the presence of PI3K inhibitors. This event appears to be independent of T308 phosphorylation such that even when T308 phosphorylation and catalytic activity are driven to high levels by coexpression of myristoylated PDK1, S473 phosphorylation is lost in the presence of LY-294002. This striking result is similar to findings of Andjelkovic and coworkers (6), who observed a comparable phenomenon during translocation of C1-ΔPH-PKB to the plasma membrane in response to phorbol esters. The Andjelkovic et al. study (6) was the first to use a translocation-inducible PKB allele to study PKB activation. The authors suggested that PI3K inactivates an S473-specific phosphatase. This scenario is consistent with our data, although we cannot exclude PI3K regulation of a distinct S473 kinase. For example, SHIP-generated

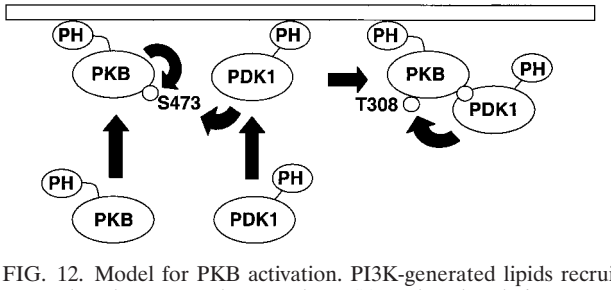


FIG. 12. Model for PKB activation. PI3K-generated lipids recruit PKB to the plasma membrane, where S473 phosphorylation occurs through autophosphorylation or an exogenous kinase. This primes PKB for PDK1 phosphorylation of T308, fully activating PKB.

$PI(3,4)P_2$ may specifically activate an S473 kinase, since it has recently been demonstrated that this lipid species is directly coupled to S473 phosphorylation in SHIP-deficient murine mast cells (30). Distinction between these possibilities is contingent upon isolation of the relevant S473 interacting proteins and understanding their mechanism of regulation by PI3K.

The possible existence of an S473 kinase is contentious. While it has been argued that S473 of PKB is targeted by an exogenous kinase (37), there is evidence supporting PKB-mediated autophosphorylation (34). Our data are consistent with autophosphorylation in that either kinase-dead or T308A mutants of ΔPH-PKB-ER exhibit much-reduced S473 phosphorylation. The same was true for FRB-PKB—the T308A mutant of FRB-PKB harbored very small amounts of S473 phosphorylation, even when the molecule was directed to the plasma membrane. A major counterargument for autophosphorylation is derived from experiments with the serine/threonine kinase inhibitor staurosporin. This compound inhibits PDK1 (among other kinases) and blocks T308 phosphorylation. We (Fig. 5) and others (6, 23) have demonstrated that this compound has little effect on S473 phosphorylation. However, we also show that membrane localization of PKB is the primary determinant for S473 phosphorylation. Thus, it may be that very little PKB activity is necessary to accumulate S473 phosphorylation when PKB is bound to the plasma membrane. Therefore, given enough time and membrane localization, S473 phosphorylation accumulates. This might explain why staurosporin-mediated inhibition of PKB leads to only a mild reduction of S473 phosphorylation, since the kinase-dead or T308A mutants of PKB represent more extreme inhibitory cases (having zero catalytic activity) and thus display a much greater impact on S473 phosphorylation. In agreement, activity measurements show that both the T308A and K179Q mutants of myr-ΔPH-PKB-ER have significantly lower activities (and are essentially catalytically inert) than cells treated with maximal inhibitory concentrations of staurosporin, in which myr-ΔPH-PKB-ER still retained 40% of basal kinase activity. This might be a consequence of residual T308 phosphorylation after staurosporin treatment, or it may reflect that an intact threonine at amino acid 308 is permissive for some activity compared with alanine substitution at the same position.

The same effect of staurosporin on PKB phosphorylation was observed with the FRB-PKB model. In this case, translocation of FRB-PKB to membrane-localized FKBP led to S473 and T308 phosphorylation. Prior treatment with staurosporin blocked T308 phosphorylation but not S473 phosphorylation. Kinase activity measurements again indicated that the residual activity of staurosporin-treated FRB-PKB was significantly higher than T308A FRB-PKB, in which no S473 phosphorylation was observed. Thus, the two distinct activation models we tested both suggest that only a small fraction of PKB activity is required for PKB phosphorylation on S473 in conjunction with translocation to the plasma membrane.

Recently, Biondi and colleagues characterized the roles of the hydrophobic motifs of S6K and SGK in binding PDK1 and phosphorylation of the activation loop (11). However, they were unable to demonstrate a similar mechanism for PKB. Using in vitro experiments, this group showed that ΔPH-PKB was dependent upon the PIF-binding pocket of PDK1 and that a peptide mimicking the PIF, PIFtide, can block the phosphorylation of ΔPH-PKB by wild-type PDK1. However, this was not the case for full-length PKB (i.e., PKB containing its normal amino-terminal PH domain). They suggested that full-length PKB was inherently different in its mode of regulation from SGK and the other AGC kinases and that the PH domain plays a role in PDK1 phosphorylation of PKB. However, as we show here (Fig. 7), overexpression of membrane-localized PDK1 can override the requirement for S473 phosphorylation, and these conditions may be similar to the in vitro experiments of Biondi and coworkers (11).

In summary, our in vivo models suggest an ordered series of events that are necessary to generate fully active PKB, as depicted in Fig. 12. First, PI3K activity draws PKB and PDK1 to the plasma membrane. There, a PI3K-dependent event is necessary to allow S473 phosphorylation to accumulate and is conditional on PKB being catalytically active. Then, this phosphorylated motif promotes the activity of PDK1 towards T308, which fully activates the kinase. This strict control on location and activation probably reflects the dire consequences of chronically activated PKB in disease.

ACKNOWLEDGMENTS

M.P.S. is supported by a Canadian Institutes of Heath Research (CIHR) postdoctoral fellowship. This work was supported by grants to J.R.W. from the CIHR and National Cancer Institute of Canada. J.R.W. acknowledges Senior Scientist support from CIHR and an International Scholarship from the Howard Hughes Medical Institute.

We gratefully thank Richard Roth for the original PKB-ER constructs. We are indebted to Jing Jin, Lisa Kockeritz, and Mike Parsons for reagents and advice.

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