

The Heat Shock Protein 90 Inhibitor Geldanamycin and the ErbB Inhibitor ZD1839 Promote Rapid PP1 Phosphatase-Dependent Inactivation of AKT in ErbB2 Overexpressing Breast Cancer Cells

Wanping Xu,¹ Xitong Yuan,¹ Yun Jin Jung,¹ Yongping Yang,² Andrea Basso,³ Neal Rosen,³ Eun Joo Chung,⁴ Jane Trepel,⁴ and Len Neckers¹

¹Cell and Cancer Biology Branch and ⁴Medical Oncology Clinical Research Unit, Center for Cancer Research, National Cancer Institute, Rockville, Maryland; ²Division of Chronic Liver Disease, Beijing 302 Hospital, Beijing, China; and ³Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York

ABSTRACT

AKT, a serine/threonine kinase that promotes cell survival, can be activated by overexpression of the receptor tyrosine kinase ErbB2. Conversely, down-regulation of ErbB2 inhibits AKT activation. Here, we identify PP1 as a serine/threonine phosphatase that associates with and dephosphorylates AKT in breast cancer cells, and we show that ErbB2 inhibits PP1-dependent dephosphorylation of AKT. Inhibition of ErbB2 by either the HSP (heat shock protein) 90 inhibitor geldanamycin or the ErbB inhibitor ZD1839 in SKBR3 cells, a human breast cancer cell line overexpressing ErbB2 protein, induces a rapid and dramatic decrease in AKT activity. Decreased AKT activity occurs many hours before the HSP90-dependent decline of AKT protein but is correlated with loss of AKT phosphorylation. Decreased AKT phosphorylation is not due to blockade of AKT activation or to preferential HSP90-mediated degradation of phosphorylated AKT. Instead, it is caused by increased AKT dephosphorylation. Sensitivity to a panel of phosphatase inhibitors suggests involvement of the phosphatase PP1 in this process. *In vitro* phosphatase assay (using PP1 immunoprecipitated from COS7 cells transiently transfected with the wild-type protein, as well as purified PP1) confirmed that AKT is a substrate of PP1. Furthermore, endogenous PP1 and AKT associate with each other in SKBR3. However, the phosphatase is phosphorylated and its activity is suppressed (determined by *in vitro* assay). In contrast, ErbB2 inhibition abrogates PP1 phosphorylation and restores its activity (measured by its ability to dephosphorylate AKT *in vitro*). Finally, transient overexpression of constitutively active PP1 in SKBR3 cells promotes marked dephosphorylation of endogenous AKT protein. These data indicate that ErbB2 acts to preserve the phosphorylation, and hence to prolong the activation, of AKT kinase by repressing the activity of the phosphatase PP1. ErbB2 thus functions to regulate AKT kinase by simultaneously promoting its activation while inhibiting its inactivation.

INTRODUCTION

AKT kinase is a major downstream target of receptor tyrosine kinases that signal via PI3k⁵ (1). AKT mediates a wide variety of biological responses to EGF, insulin, and other growth factors (2) and plays a critical role in controlling the balance between survival and apoptosis (1, 3). Activated AKT phosphorylates and inactivates multiple substrates, including caspase 9 (4), Bad (5), inhibitor of nuclear factor- κ B kinase (6), and the Forkhead transcription factors (7). Because of its antiapoptotic activity, this kinase has been identified as a molecular target for cancer treatment.

The AKT protein contains a kinase domain with specificity for Ser or Thr residues (8). NH₂-terminal to the kinase domain is a pleckstrin-homology domain, which mediates lipid-protein and/or protein-pro-

tein interactions (9, 10), whereas the COOH-terminus of AKT contains a hydrophobic and proline-rich domain. Activation of AKT involves both conformational change and phosphorylation of the protein and is usually preceded by up-regulation of receptor kinases induced by extracellular stimuli. For example, activation of ErbB2/ErbB3 heterodimers by HRG promotes association and activation of PI3k (11, 12). Activated PI3k, in turn, recruits AKT to the plasma membrane where it is phosphorylated and activated by PDK-1 (1, 13–15).

Although the mechanism of AKT activation is well documented, the reverse process of inactivation is not as well studied. Inactivation, or dephosphorylation, of AKT should be regulated by Ser/Thr phosphatases. Ser/Thr phosphatases are usually classified as type 1 (PP1) or type 2 (PP2), depending on their substrate specificity and sensitivity to inhibitors (16). In several fibroblast, smooth muscle, and osteosarcoma model systems, PP2A has been identified as a mediator of AKT dephosphorylation (17–19). However, identity of the phosphatase or phosphatases implicated in AKT dephosphorylation in epithelial malignancies is less clear.

PP1 is a major eukaryotic Ser/Thr phosphatase that regulates a broad range of cellular processes, including cell cycle progression, transcription, protein synthesis, muscle contraction, carbohydrate metabolism, and neuronal signaling (20–22). More recently, PP1 was shown to be involved in the regulation of apoptosis by affecting the phosphorylation of the Bad protein (23, 24). The catalytic subunit of PP1 exists in the cell in heteromeric complexes with a variety of regulatory subunits such as inhibitor-1, its homologue DARPP-32, and inhibitor-2 (25). The activity of PP1 is also regulated by phosphorylation of its catalytic subunit. Upon phosphorylation of Thr³²⁰, the COOH terminus of PP1 folds back to mask its catalytic center (26, 27). Mutation of Thr³²⁰ to alanine confers PP1 resistance to inhibitory phosphorylation and makes PP1 constitutively active (28). However, the regulation of PP1 phosphorylation, especially in the cytoplasm, is largely unknown.

AKT associates with the molecular chaperone HSP90, and the stability of the AKT protein depends on this association (29, 30). Disruption of HSP90 function by the antibiotic GA causes proteasome-dependent AKT degradation (30, 31). However, before its effects on AKT stabilization, GA may also negatively affect AKT kinase activity, especially in cells that express high levels of ErbB2 (30), another HSP90 client (32). Overexpression of ErbB2 in various epithelial cancers has been associated with aberrant activation of AKT (33–36).

In this article, we show that, independent of its effect on AKT activation, ErbB2 also retards AKT inactivation by promoting inhibition of the phosphatase PP1. Phosphorylated (and thus inactive) PP1 is coprecipitated with AKT in SKBR3 cells. ErbB2 inhibition does not alter this association but does promote PP1-mediated dephosphorylation of AKT. Increased PP1 activity is a result of its derepression caused by dephosphorylation of the PP1 catalytic subunit. These data suggest that in addition to promoting AKT activation, ErbB2 insures

Received 6/7/03; revised 9/5/03; accepted 9/11/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Len Neckers, National Cancer Institute, Cell and Cancer Biology Branch, 9610 Medical Center Drive, Suite 300, Rockville, MD 20850. Phone: (301) 496-5899; Fax: (301) 402-4422; E-mail: len@helix.nih.gov.

⁵The abbreviations used are: PI3k, phosphatidylinositol-3 kinase; HRG, heregulin; HSP90, heat shock protein 90; EGF, epidermal growth factor; GA, geldanamycin; GSK-3, glycogen synthase kinase-3; OA, okadaic acid; PKA, protein kinase A.

prolonged AKT activity by repressing the activity of PP1. Our findings also identify PP1 as a second Ser/Thr phosphatase (in addition to PP2A) that associates with and can dephosphorylate AKT.

MATERIALS AND METHODS

Cell Lines, Antibodies, and Reagents. SKBR3 cells were purchased from American Type Culture Collection and maintained at 37°C, 5% CO₂ in DMEM containing 1 mM sodium pyruvate and 10% fetal bovine serum. Rabbit anti-AKT and anti-phospho-AKT (Ser⁴⁷³), mouse monoclonal anti-phospho-AKT (Ser⁴⁷³) 4E2, phospho-(Ser/Thr) kinase substrate antibody sampler kit, anti-phospho-PP1 (Thr³²⁰) antibody, and AKT kinase kit were purchased from Cell Signaling Technology. Rabbit anti-PP1 (FL-18), mouse anti-PP1 (E-9), goat anti-PP1 α , and anti-PP1 γ were from Santa Cruz Biotechnology. Agarose beads immobilized with mouse anti-AKT antibody, activated AKT protein, and purified PP1 and inhibitor-1 proteins were bought from Upstate Biotechnology. Calyculin A and Microcystin-LR were bought from Calbiochem. OA was obtained from Sigma. Tautomycin was purchased from Biomol Research Laboratories. HRG β 1 and mouse monoclonal anti-phospho-ErbB2 (Ab-18) were bought from NeoMarkers. Mouse monoclonal anti-ErbB2 (Ab-3) and monoclonal anti-tubulin (Ab-1) were purchased from Oncogene Research Products.

Preparation of PP1 Constructs. The PP1 gene was cloned from SKBR3 cells. Briefly, total mRNA was extracted from SKBR3 cells by using the Trizol Reagent (Invitrogen), cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen), and the PP1 gene was amplified by PCR with the 5'-end primer GGAAGCTGGGCAAGGAGCTGCTGGCTGGA and the 3'-end primer GGCTCGAGTTTCTGGCTTGGCGGAATTGCGG. The PP1 cDNA fragment was inserted into the pcDNA3.1 vector (Invitrogen) and tagged at the COOH-terminal end with Myc and polyhistidine. Constitutively active PP1 was made by mutating Thr³²⁰ to alanine, using the QuickChange point mutation kit from Stratagene Inc.

Immunofluorescence Assay. To detect total and phosphorylated AKT, transiently transfected SKBR3 cells grown in chamber slides were washed with 1 \times PBS, immediately fixed with 3.7% formaldehyde in PBS for 10 min at room temperature, rinsed with PBS, and permeabilized with 0.1% Triton X-100 in Tris-buffered saline (pH 7.4; TBS/Triton) for 10 min at room temperature. Nonspecific binding sites were then blocked by incubating the cover slips with 3% BSA in TBS/Triton for 1 h at room temperature before processing for immunofluorescence labeling. The cells were incubated overnight at 4°C with polyclonal rabbit antiphospho-AKT or anti-AKT antibodies (1:200), and monoclonal anti-c-Myc antibody (1:200) diluted in TBS/Triton containing 3% BSA. Cells were washed with TBS/Triton three times for 10 min each at room temperature and incubated with fluorescently labeled secondary antibodies (1:200 Cy3-conjugated antirabbit IgG, 1:200 FITC-conjugated antimouse IgG; Jackson Immunoresearch Laboratories) for 1 h at room temperature. After staining, the slides were washed with TBS/Triton, rinsed quickly with water, air-dried, and coverslipped using SlowFade solution (Molecular Probes). Fluorescence was visualized using a Zeiss Axioskop microscope with a \times 63 objective and images were captured using an Optronics charge-coupled device (CCD) camera and OpenLab software (Improvision).

In Vitro Kinase Assay. The kinase activity of immunoprecipitated AKT protein from GA-treated or untreated SKBR3 cells was determined by using the AKT kinase kit from Cell Signaling Technology. Briefly, SKBR3 cells in a 10-cm plate, treated with 1 μ M GA or the same volume of vehicle (DMSO) for 1 h, were washed with cold PBS and lysed with 1 ml of lysis buffer (Cell Signaling Technology). AKT protein was immunoprecipitated and its kinase activity was assayed by using GSK-3 as the substrate, according to the manufacturer's protocol, with the exception that the reaction tubes contained either 10 μ M GA or the same volume of DMSO. To assay the kinase activity of AKT on the PP1 protein, 0.4 μ g of activated AKT protein (1 μ l) were mixed with 1 unit of purified PP1 protein (10 μ l) in 40 μ l of kinase buffer [25 mM Tris (pH 7.5), 5 mM β -glycerolphosphate, 2 mM DTT, 0.1 mM Na₃VO₄, 10 mM MgCl₂, and 0.2 mM ATP] and incubated at 30°C for 1 h. The reaction was stopped by adding in 12.5 μ l of 5 \times SDS-sample buffer and heating at 100°C for 5 min.

Western Blotting and Immunoprecipitation. Western blotting was performed as described previously (32). For immunoprecipitation of PP1 protein,

GA-treated or untreated SKBR3 cells were washed once with cold PBS and then lysed with TNE SV buffer [50 mM Tris (pH 7.5), 1% NP40, 1 mM EDTA, 100 mM sodium chloride, and 2 mM sodium orthovanadate], containing 10 mM sodium fluoride, 2 mM β -glycerol phosphate, and Complete proteinase inhibitors (Roche Diagnostics). Cell lysates were centrifuged at 13,000 rpm for 15 min, and supernatant was collected. Protein concentration was determined using the BCA method (Pierce). Cell lysates were precleared by mixing with 15 μ l of recombinant protein G-agarose beads (Invitrogen) and rotating at 4°C for 45 min. The agarose beads were pelleted by brief centrifugation, and supernatant was collected. Cleared cell lysate was then rotated with goat anti-PP1 α and anti-PP1 γ antibodies at 4°C for 2 h, followed by the addition of 10 μ l of protein G-agarose beads and rotation of another 2 h. The beads were washed four times with TNE SV buffer, precipitated proteins were dissolved in reducing SDS-sample buffer (32), and analyzed by Western blotting.

In Vitro Phosphatase Assay. The activity of immunoprecipitated PP1 was determined by using phosphorylated AKT proteins as the substrate. Specifically, COS7 cells were transfected with plasmid expressing the PP1_MycHis protein. Twenty-four h after transfection, cells were lysed with TNE SV buffer. To immunoprecipitate the PP1 protein, 1 mg of cell lysate was mixed with 2 μ g of mouse anti-Myc antibody, incubated at 4°C for 2 h, followed by the addition of 10 μ l of protein G-agarose beads and rotation at 4°C for 1 h. Pelleted beads were washed twice with TNE SV buffer and twice with PP1 buffer [50 mM Tris (pH 7.0), 0.1 mM EDTA, and 1 mM MnCl₂]. Forty μ l of PP1 buffer was added to each tube, containing 5 mM DTT, 100 ng of phosphorylated AKT protein (Upstate Biotechnology), with/without 20 μ M GA or 2 μ M inhibitor-1 (Upstate Biotechnology). In some experiments, 0.1 unit/tube purified PP1 replaced immunoprecipitated PP1. In these situations, 50 ng of phosphorylated AKT protein was used as substrate. Tubes were incubated at 30°C for 45 min, with occasional vortexing. The reaction was stopped by addition of 10 μ l of 5 \times SDS sample buffer and heating at 100°C for 5 min. Reaction products were separated on 4–20% gradient SDS-PAGE and transferred to a nitrocellulose membrane. Phosphorylation of the AKT protein was detected by Western blotting with mouse monoclonal antiphospho-AKT (Ser⁴⁷³) antibody (Cell Signaling Technology). The amount of AKT protein loaded/lane was determined with sheep anti-AKT antibody (Upstate Biotechnology). Finally, immunoprecipitation of PP1 protein was confirmed by probing the blot with mouse monoclonal anti-PP1 antibody (Santa Cruz Biotechnology).

RESULTS

AKT Kinase Activity Is Rapidly Inhibited by GA Treatment in SKBR3 Cells. Stability of the AKT protein requires its association with the molecular chaperone HSP90 (29). Disruption of HSP90 function by GA causes proteasome-dependent AKT degradation (30, 31). However, to study GA's effects on AKT kinase activity, we treated SKBR3 cells briefly with GA or its vehicle DMSO, immunoprecipitated the AKT protein from equivalent amounts of cell lysate, added the AKT substrate GSK-3, and incubated the mixture at 30°C for 30 min. The reaction was stopped by addition of SDS-sample buffer, and the phosphorylation of GSK-3 was detected with antiphospho-GSK-3 antibody. Our data indicate that a brief (1 h) exposure of SKBR3 cells to GA caused a dramatic decrease in AKT kinase activity (>90% when compared with DMSO control; Fig. 1A). However, GA had no effect on AKT kinase when added in excess (10 μ M) to the *in vitro* assay, indicating that GA did not inhibit AKT kinase directly (Fig. 1A, compare Lane 2 with Lane 4).

GA Induces a Decrease in AKT Phosphorylation before AKT Degradation. Although AKT protein level is markedly decreased by prolonged exposure to GA (31), expression of AKT protein in SKBR3 cells is only moderately affected after 1 h exposure to GA (1 μ M; Fig. 1B, bottom panel). AKT is a phospho-protein, and its kinase activity is regulated by phosphorylation. Phosphorylation of Ser⁴⁷³ and Thr³⁰⁸ is essential for kinase activation (1). Because GA-induced AKT protein degradation failed to explain the rapid loss of kinase activity, we examined the phosphorylation state of AKT. We treated SKBR3

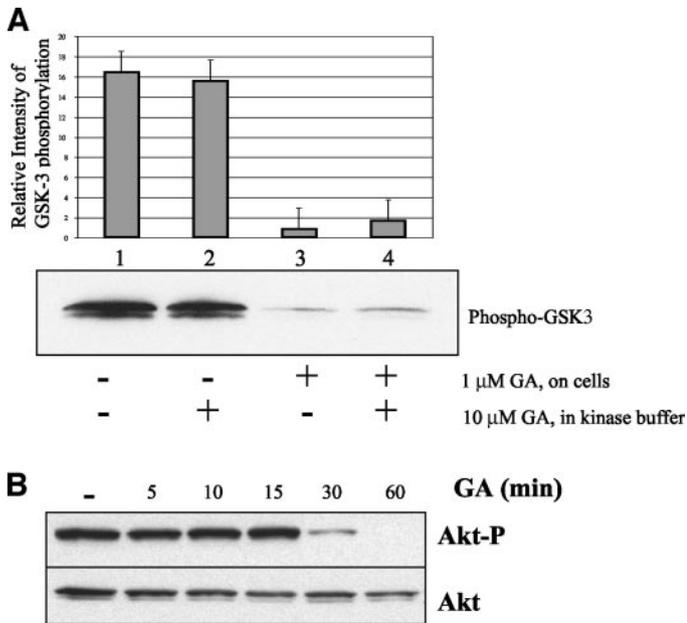


Fig. 1. GA inhibits AKT kinase activity by inducing a decrease in AKT phosphorylation level. **A**, GA inhibits AKT kinase activity indirectly. SKBR3 cells were treated with 1 μM GA or the same volume of DMSO for 1 h. Cells were lysed with lysis buffer (Cell Signaling Technology). AKT proteins were immunoprecipitated by using beads immobilized with mouse anti-AKT monoclonal antibody. AKT kinase activity was determined by detecting its ability to phosphorylate the substrate protein GSK-3. The reaction buffer contained 10 μM GA or the same amount of the vehicle DMSO. The phosphorylation of GSK-3 protein was detected by Western blotting with antiphospho-GSK-3 antibody. The density of each band was quantified using NIH Image software and plotted with Microsoft Excel. **B**, GA-induced decrease in AKT phosphorylation occurs before the decline in the AKT protein level. SKBR3 cells were treated with 1 μM GA for indicated times. AKT phosphorylation levels in total cell lysate were detected with rabbit antiphospho-AKT-(Ser⁴⁷³) antibody, and the total AKT protein levels were determined by reprobating the same blot with mouse anti-AKT antibody.

cells with 1 μM GA for increasing times and detected AKT phosphorylation by Western blotting with antiphospho-AKT antibody. The results show that GA induced a dramatic and rapid decline in AKT phosphorylation within 30 min (Fig. 1B, top panel). Importantly, the loss of phosphorylated AKT correlated temporally with the observed decrease in AKT kinase activity in that AKT phosphorylation was nearly undetectable by 1 h after GA treatment. These data support the hypothesis that the GA-induced loss of AKT kinase activity is caused by a drug-stimulated decline in its phosphorylation level.

GA Does Not Block AKT Activation. Next, we investigated the mechanism by which GA rapidly reduced the level of phosphorylated AKT. We first determined whether phospho-AKT was preferentially degraded in response to GA. Previous experiments have demonstrated that proteasome inhibitors can block GA-induced protein degradation (37, 38). We treated SKBR3 cells with 1 μM PS-341 for 1 h before the addition of GA. However, we found that PS-341 did not affect the decrease in AKT phosphorylation caused by GA (data not shown). GA treatment in the presence of proteasome inhibitors can cause insolubility of HSP90 client proteins, including AKT (31, 39). To rule out the possibility that phosphorylated AKT protein was diverted to a detergent-insoluble fraction, we collected the TNESV-insoluble pellets and solubilized them with boiling SDS-sample buffer. The presence of phospho-AKT was detected by Western blotting. We found no increase of phosphorylated AKT protein in the insoluble fraction of GA/PS-341-treated cells (data not shown). Taken together, these data indicated that GA did not promote preferential degradation of phosphorylated AKT.

Another possible explanation for the GA-induced decrease in AKT phosphorylation is that the drug blocks AKT activation. To investigate

this possibility, we tested whether AKT could be activated by growth factor in the presence of GA. We cultured SKBR3 cells in serum-free medium for 24 h, then pretreated the cells with either GA, Ly294002 (a specific PI3k inhibitor), or the vehicle DMSO. After 1 h, cells were either washed with PBS or kept in the presence of drugs and treated with 1 nM HRG (an ErbB3 ligand) for different times. Phosphorylation of AKT protein in total cell lysate was detected by Western blotting. AKT maintained a low basal phosphorylation level in serum-free medium (Fig. 2A, Lane 1), and this was additionally decreased by GA (Fig. 2A, Lane 6). HRG caused a dramatic increase in AKT phosphorylation, and there was no significant effect of brief treatment with GA or Ly294002 (Fig. 2A, compare Lanes 2–4 with Lanes 7–9 and Lanes 12–14). As expected, the continued presence of Ly294002 completely blocked AKT phosphorylation induced by HRG, confirming the importance of PI3k in mediating this response (Fig. 2A, compare Lane 14 with Lane 15). In contrast, continued presence of GA in the medium did not prevent HRG-dependent AKT activation (Fig. 2A, compare Lane 10 with Lanes 7, 8, and 9).

To ensure that we did not use a HRG concentration that was high enough to overcome a partial GA block of activation, we titrated the ligand. SKBR3 cells were pretreated with 1 μM GA for 1 h, followed by 10 min stimulation with decreasing concentrations of HRG. Confirming and extending the results of Fig. 2A, GA caused a decrease in basal AKT phosphorylation (Fig. 2B, compare Lane 7 with Lane 1), but the elevation of AKT phosphorylation in response to HRG was comparable with that seen in untreated cells (Fig. 2B, graph). These data indicate that brief exposure to GA does not block AKT activation by extracellular signals.

GA-Induced AKT Dephosphorylation Is Prevented by Inhibition of the Ser/Thr Phosphatase PP1. The steady-state AKT phosphorylation level is the outcome of a balance of activities of those kinases that phosphorylate AKT and the phosphatases that dephosphorylate it. Because GA did not block stimulus-induced AKT phosphorylation, and phosphorylated AKT was not selectively degraded, we speculated that the drug-induced decrease in AKT phosphorylation was caused by increased phosphatase activity toward AKT. To test this hypothesis, we examined whether various phosphatase inhibitors could block the loss of phosphorylated AKT induced by GA. We treated SKBR3 cells with different phosphatase inhibitors before addition of GA, and AKT phosphorylation was monitored by Western blotting. Microcystin-LR (5 nM), a preferential inhibitor of PP2A at this concentration, did not reverse GA-stimulated AKT dephosphorylation, although at this concentration Microcystin-LR significantly inhibited general phosphatase activity measured in the cell extract (data not shown). In contrast, calyculin A, an inhibitor of both PP1 and PP2A, blocked GA-induced AKT dephosphorylation completely in these cells (Fig. 2C).

To further support a functional role for PP1 in GA-stimulated AKT dephosphorylation, we used tautomycin, a preferential inhibitor of PP1 (18). Pretreatment of SKBR3 cells with tautomycin completely abolished GA-induced AKT dephosphorylation (Fig. 2D), supporting involvement of PP1 in this process in SKBR3 cells. This was additionally verified by using the unique activity profile of OA. OA inhibits PP2A at low concentrations ($\text{IC}_{50} < 0.1$ nM), but it inhibits both PP2A and PP1 at higher concentrations ($\text{IC}_{50} = 150$ nM). We pretreated SKBR3 cells with increasing OA concentrations for 4 h, followed by treatment with GA for 1 h. As is shown in Fig. 2E, OA did not affect AKT dephosphorylation at 10 or 30 nM but blocked ~60% of the GA effect at 100 nM and completely blocked it at 500 nM and 1 μM . Importantly, even 5 nM OA significantly inhibited general phosphatase activity measured in the cell extract (data not shown). This activity profile supports a role for PP1, but not PP2A, in mediating GA-induced AKT dephosphorylation in these cells. Taken to-

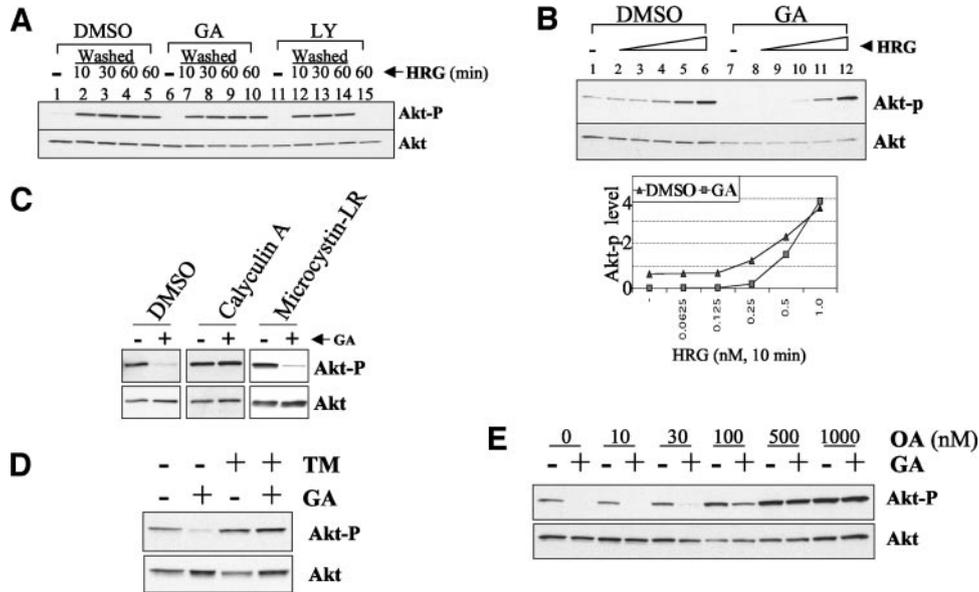


Fig. 2. GA-induced AKT dephosphorylation is because of the activity of the phosphatase PP1 but not a blockage of AKT activation. **A**, GA does not prevent HRG-induced AKT phosphorylation. 1 μ M GA (Lanes 6–10), 50 μ M Ly294002 (Lanes 11–15), or the same volume of DMSO (Lanes 1–5) were added to SKBR3 cells, which had been serum-starved for 24 h. One h later, cells were either washed with PBS (Lanes 2–4, 7–9, and 12–14) or left alone (Lanes 5, 10, and 15) and then treated with 1 nM HRG for the indicated times. Cells were lysed with TNESV buffer containing 10 mM NaF and protease inhibitors. Cell lysates were separated by 4–20% gradient SDS-PAGE and transferred to nitrocellulose membranes. Duplicate blots were probed with either rabbit anti-AKT antibody or rabbit antiphospho-AKT antibody. **B**, GA does not prevent the responsiveness of SKBR3 cells to HRG. Serum-starved SKBR3 cells were treated with 1 μ M GA or DMSO for 1 h and then stimulated with serially diluted HRG for 10 min at 37°C. Cell lysates were analyzed as described above. The density of each band was quantified and plotted as described in Fig. 1. **C**, the PP1/PP2A inhibitor calyculin A, but not the PP2A inhibitor Microcystin-LR, blocks GA-induced AKT dephosphorylation. SKBR3 cells were grown to 70% confluence and serum starved for 24 h. Cells were pretreated with 8 nM calyculin A for 4 h or 5 nM Microcystin-LR for 6 h, followed by the treatment of 1 μ M GA for another hour. Control cells were treated with the same volume of the vehicle DMSO. Cell lysis and Western blotting were as above. **D**, the PP1 inhibitor tautomycin blocks GA-induced AKT dephosphorylation. SKBR3 cells, untreated or pretreated with 10 μ M tautomycin for 6 h, were exposed to 1 μ M GA for 1 h. Cells were lysed and analyzed as above. **E**, GA-induced AKT dephosphorylation is blocked by OA at high concentration. SKBR3 cells were first treated with different concentrations of OA for 4 h. Cells were then exposed to 1 μ M GA for an additional hour. Cell lysis and AKT analysis were as above.

gether, these results indicate that GA promotes AKT dephosphorylation in SKBR3 cells by activating the phosphatase PP1.

GA Promotes Dephosphorylation of PP1. PP1 is an abundant cellular protein that is ubiquitously expressed. However, its activity is tightly regulated (21). One of the regulatory mechanisms involves phosphorylation of its catalytic subunit. Phosphorylation in its COOH terminus inhibits PP1 activity (40). Because GA did not affect the expression level of the PP1 protein in SKBR3 cells (data not shown), we wondered whether GA might activate or derepress the phosphatase by reversing PP1 phosphorylation. To test this possibility, we examined the phosphorylation status of the PP1 in GA-treated or untreated SKBR3 cells. We immunoprecipitated PP1 protein and detected its phosphorylation state with different antibodies specific for phosphorylated Tyr, Ser, or Thr. No Tyr phosphorylation of PP1 was found in either GA-treated or untreated cells (data not shown). Furthermore, based on antibody reactivity, PP1 was not phosphorylated on Ser or Thr residues by kinases of the ATM, protein kinase C, or PDK-1 families. Interestingly, PP1 in untreated cells, but not in GA-treated cells, was recognized by antibodies specific for serine or threonine residues phosphorylated by AKT and/or PKA (Fig. 3A). However, the PKA inhibitor H-89 (41) was unable to promote AKT dephosphorylation (data not shown), raising the intriguing possibility that AKT itself mediates PP1 phosphorylation. This hypothesis is supported by two pieces of evidence. First, in an *in vitro* kinase assay purified, active AKT was able to phosphorylate purified, active PP1 on residue T320 (Fig. 3B), the residue in which phosphorylation results in PP1 inactivation (26, 27). Second, the two proteins can be shown to physically interact in SKBR3 cells, and this association remains unaffected by GA (Fig. 3C).

PP1 Dephosphorylates AKT *in Vitro* and *in Vivo*. GA treatment caused a decline in PP1 phosphorylation (Fig. 3A), suggesting a derepression of its phosphatase activity. Indeed, this was confirmed

by an *in vitro* phosphatase assay in which we found, using phospho-AKT as substrate, that endogenous PP1 immunoprecipitated from GA-treated SKBR3 cells was more enzymatically active than when immunoprecipitated from untreated cells (Fig. 3E). The PP1-dependent nature of the phosphatase activity in the immunoprecipitates was confirmed by inclusion of inhibitor-1 protein, a specific PP1 inhibitor (21). However, GA did not affect PP1 activity directly because inclusion of GA in the phosphatase assay did not affect AKT dephosphorylation (data not shown). These results indicate that AKT is a substrate for PP1 and that GA reverses a phosphorylation-dependent repression of PP1 in SKBR3 cells. The ability of PP1 to dephosphorylate AKT protein was additionally verified *in vitro* using ectopically expressed PP1 isolated from COS7 cells (Fig. 3F). Finally, in an *in vitro* phosphatase assay, purified PP1 promoted significant dephosphorylation of purified AKT (Fig. 3D). This activity was blocked by inclusion of 1 μ M OA (an inhibitory concentration for PP1) but not by inclusion of 5 nM OA (an inhibitory concentration for PP2A but not for PP1). Lastly, to demonstrate that nonphosphorylatable PP1 could mediate AKT dephosphorylation in SKBR3 cells, we ectopically expressed a constitutively active, nonphosphorylatable PP1 construct [PP1(T320A)_MycHis; Ref. 28] in SKBR3 cells, and we observed a marked decrease in AKT phosphorylation *in vivo*, as observed by immunofluorescence assay (Fig. 4). Taken together, these data demonstrate that active PP1 can promote AKT dephosphorylation both *in vivo* and *in vitro*.

Specific Inhibition of ErbB2 Promotes AKT Dephosphorylation. In attempting to identify GA-sensitive upstream components that may promote phosphorylation-dependent PP1 inhibition, we focused our attention on ErbB2. In initial experiments we observed that GA-induced ErbB2 dephosphorylation was noticeable within 5 min and was complete within 30 min, thus preceding the inactivation of AKT (Fig. 5A, top panel). Loss of phospho-ErbB2 occurred before

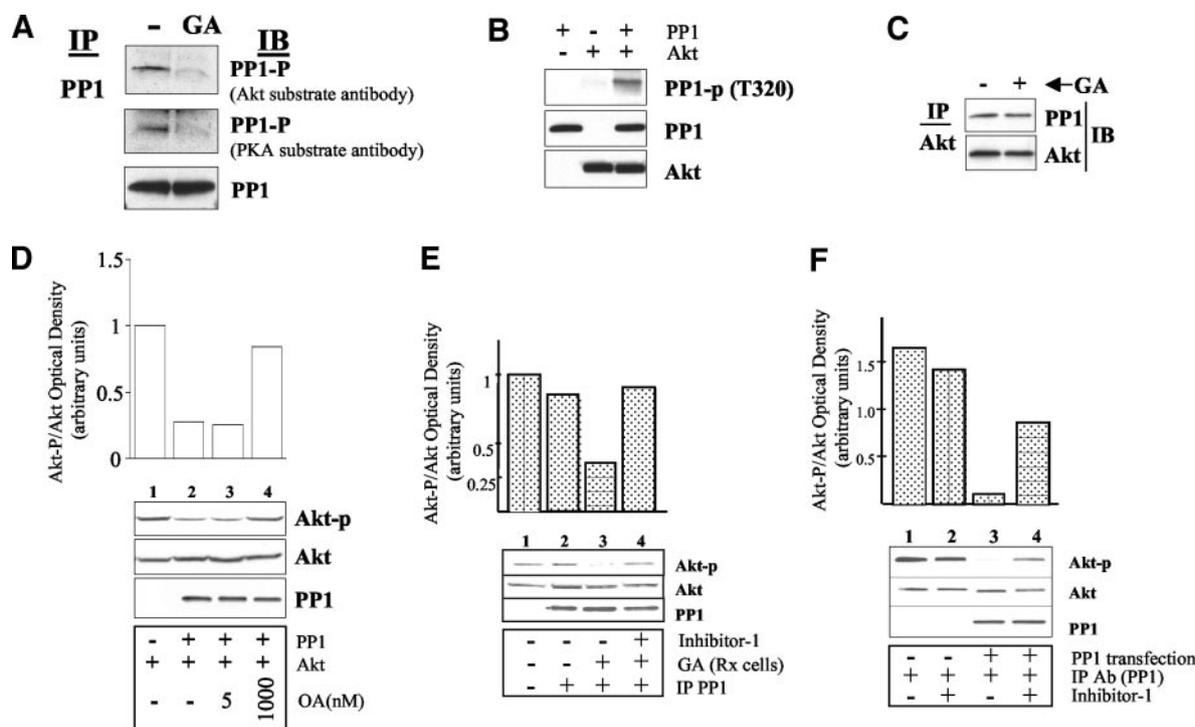


Fig. 3. PP1 mediates GA-induced AKT dephosphorylation. **A**, GA induces dephosphorylation of PP1. SKBR3 cells were either treated with $1 \mu\text{M}$ GA or its vehicle DMSO for 1 h. PP1 was immunoprecipitated with goat anti-PP1 α and anti-PP1 γ antibodies. Phosphorylation of PP1 was detected using a panel of antibodies specific for phosphorylated Ser and/or Thr in different molecular contexts so that potential phosphorylation by Ser-Thr kinases AKT, PKA, protein kinase C, PDK1, ATM/ATR, or mitogen-activated protein kinase/cyclin-dependent kinase could be evaluated. Loading of PP1 was monitored with mouse anti-PP1 monoclonal antibody. **B**, AKT phosphorylates the PP1 protein on Thr³²⁰ in an *in vitro* kinase assay. Activated AKT protein was mixed with purified PP1 protein in kinase buffer, and the reaction was carried out at 30°C for 1 h. The phosphorylation of PP1 protein at Thr³²⁰ and the protein levels of PP1 and AKT were detected by Western blotting with specific antibodies. **C**, the PP1 protein associates with the AKT protein kinase in SKBR3 cells. Lysates of SKBR3 cells treated with $1 \mu\text{M}$ GA or DMSO for 1 h were precleared with protein G-agarose beads. AKT protein was immunoprecipitated with immobilized mouse anti-AKT antibody. Immunoprecipitates were dissolved in SDS-sample buffer and separated by SDS-PAGE. The blot was first probed with mouse anti-PP1 antibody and then with rabbit anti-AKT antibody. **D**, purified PP1 phosphatase directly dephosphorylates the AKT protein in an *in vitro* phosphatase assay. Purified PP1 protein (0.1 unit) was mixed with 50 ng of phosphorylated AKT protein in phosphatase buffer in the presence or absence of 5 or 1000 nM OA and incubated at 30°C for 45 min. Change in AKT phosphorylation level and the protein levels of AKT and PP1 were detected by Western blotting. The density of each band was determined using NIH Image software, and AKT phosphorylation levels were normalized to total AKT protein levels (see graph). **E**, GA treatment of SKBR3 cells increases the activity of PP1 toward AKT. SKBR3 cells were untreated or treated with $1 \mu\text{M}$ GA for 1 h. Immunoprecipitated PP1 was mixed with phosphorylated AKT protein with (Lane 4) or without (Lanes 2 and 3) $2 \mu\text{M}$ inhibitor-1 protein. Lane 1 contained protein G-agarose beads without PP1. After incubation, AKT phosphorylation and protein level were determined by Western blotting with specific antibodies. PP1 protein was detected with mouse anti-PP1 antibody. Protein band density was graphed the same as in **D**. **F**, PP1 immunoprecipitated from COS7 cells dephosphorylates AKT protein *in vitro*. COS7 cells were transfected with MycHis-tagged wild-type PP1 (Lanes 3 and 4) or empty vector (Lanes 1 and 2). PP1 protein was immunoprecipitated with Myc antibody. *In vitro* phosphatase assay and data analysis were as above.

loss of ErbB2 protein (Fig. 5A, bottom panel). If active (*e.g.*, phosphorylated) ErbB2 were necessary to maintain AKT phosphorylation, then prevention of ErbB2 dephosphorylation should also make phospho-AKT resistant to GA. To test this hypothesis, we pretreated SKBR3 cells for 4 h with the specific tyrosine phosphatase inhibitor bpv(phen) and then exposed the cells to GA for an additional hour (Fig. 5B). Indeed, retarding ErbB2 dephosphorylation with bpv(phen) imparted GA resistance to phospho-AKT.

Because GA inhibits ErbB2 indirectly, by interfering in its association with HSP90 (32), it is possible that another HSP90 client protein may be conferring protection on phospho-AKT. To provide additional evidence that ErbB2 is involved, we used the specific ErbB inhibitor ZD1839, which was shown to efficiently inactivate ErbB2 in SKBR3 cells because steady-state ErbB2 activity in these cells is maintained primarily by ErbB1-dependent transphosphorylation (42, 43). ZD1839 treatment decreased phosphorylation of ErbB2, as well as phosphorylation of AKT and the endogenous AKT substrate Gsk-3 (Fig. 5C). Moreover, bpv(phen) blocked ZD1839-induced ErbB2 dephosphorylation and restored AKT phosphorylation (Fig. 5D). Furthermore, as with GA, ZD1839 did not prevent HRG-elicited stimulation of AKT at a concentration that effectively induced AKT dephosphorylation (Fig. 5E). Lastly, ZD1839-stimulated AKT dephosphorylation was prevented by OA at a concentration inhibitory for PP1 (Fig. 5F).

Taken together, these data strongly implicate ErbB2 as a key element in PP1-mediated AKT dephosphorylation.

DISCUSSION

Overexpression of ErbB2 leads to constitutive activation of AKT in breast cancer cells. The ability of ErbB2/ErbB3 heterodimers to promote PI3k-dependent AKT phosphorylation has been well documented (11, 12). The data we present in this report identifies a novel activity of ErbB2 in breast cancer cells, namely to prevent PP1-mediated dephosphorylation of AKT, thus prolonging the activated state of the kinase. In addition, we demonstrate that PP1 associates with AKT in SKBR3 cells and is able to dephosphorylate the kinase both *in vitro* and *in vivo*.

Both AKT and ErbB2 are HSP90 client proteins and thus both kinases are destabilized by the HSP90 inhibitor GA (31, 32). Basso *et al.* (30) recently reported that, whereas phosphorylated AKT (*e.g.*, the activated kinase) levels decline in concert with total AKT protein after GA treatment of breast cancer cells that do not overexpress ErbB2, in ErbB2-overexpressing breast cancer cells, phosphorylated AKT and apparent AKT activity decline much more rapidly than does total AKT protein level in response to GA. In the present study, we have confirmed that loss of phosphorylated AKT and AKT activity, as determined by *in vitro* kinase assay, occurs very rapidly after exposure

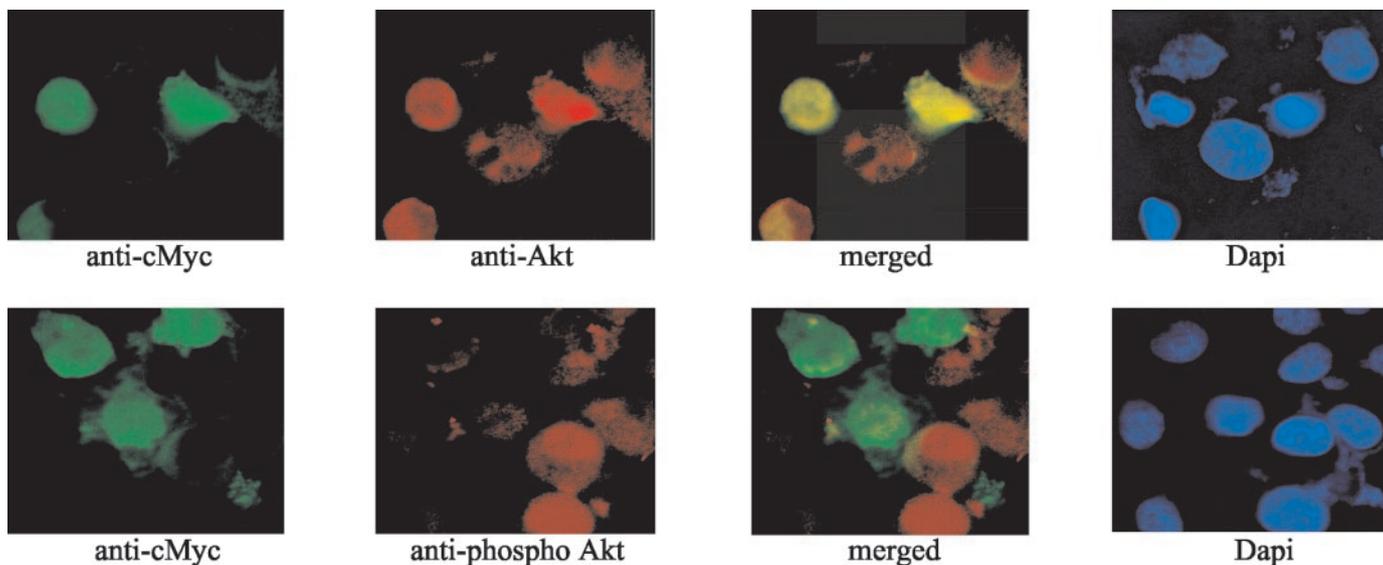


Fig. 4. PP1 dephosphorylates AKT *in vivo*. SKBR3 cells were transfected with the constitutively active PP1(T320A)_{MycHis}. Twenty-four h after transfection, total and phosphorylated AKT (red) were analyzed by fluorescence microscopy. Cells were stained with antibody to c-Myc to identify transfected cells (green), with the nuclear stain 4',6-diamidino-2-phenylindole to identify all cells (blue). The presence of either AKT or phosphorylated AKT in cells expressing transfected PP1 is depicted by a yellow color in the merged panels.

of ErbB2-overexpressing SKBR3 cells to GA. Inhibition was clearly noticeable within 15 min of drug addition and complete by 30 min. Inhibition of AKT activity was indirect because addition of GA in excess to the *in vitro* assay had no effect. However, loss of phosphorylated AKT was not a result of preferential degradation by the proteasome because proteasome inhibition failed to prevent its occurrence.

Importantly, our data reveal that AKT phosphorylation can be restimulated in GA-pretreated cells, even in the continued presence of GA, by the ErbB2/ErbB3 ligand HRG, with a similar sensitivity profile as that of untreated cells. That this reactivation of AKT in the presence of GA requires PI3k is shown by the fact that it was completely blocked by the presence of the PI3k inhibitor LY294002.

Because GA did not prevent HRG-stimulated AKT activation, we evaluated other possible mechanisms by which it might promote rapid inactivation of AKT. In our screen of various phosphatase inhibitors, we found that inhibition of PP1, but not PP2A, was able to block GA-induced AKT dephosphorylation. Thus, calyculin A, an equipotent PP1/PP2A inhibitor, was able to antagonize the effects of GA, whereas OA at low concentrations (<100 nM, PP2A preferring) was not. In contrast, OA at higher concentrations (>100 nM, PP1/PP2A preferring) and tautomycin (PP1 preferring at the concentration used) stabilized phosphorylated AKT in the presence of GA. Sato *et al.* (29) previously reported that OA (500 nM) enhanced steady-state AKT phosphorylation in 293 cells and concluded that PP2A was responsible for dephosphorylating AKT in these cells. However, 500 nM OA inhibits PP1 as well as PP2A, and Sato *et al.* (29) did not explore the potency of other phosphatase inhibitors in their study. Although several other investigators have implicated PP2A in regulating AKT phosphorylation state in cells of nonepithelial origin (17–19), the phosphatase or phosphatases involved in modulating AKT phosphorylation in epithelial neoplasms remain(s) in question.

To confirm a role for PP1 in mediating GA-stimulated AKT dephosphorylation in SKBR3 cells, we successfully coprecipitated the phosphatase with AKT, but we found that GA did not enhance this association. However, the PP1 coprecipitating with AKT from untreated SKBR3 cells was phosphorylated, whereas in GA-treated cells it was not. Because phosphorylation of PP1 inactivates the phosphatase, it is likely that in SKBR3 cells, the PP1 associated with AKT is

inactive and that its activity is restored after GA treatment. This hypothesis was supported by the finding that in an *in vitro* phosphatase assay using phospho-AKT as substrate, PP1 immunoprecipitated from GA-treated SKBR3 cells was more active than was the phosphatase immunoprecipitated from untreated cells. In addition, we demonstrated that wild-type PP1 immunoprecipitated from transiently transfected COS7 cells is able to dephosphorylate purified phospho-AKT in an *in vitro* phosphatase assay. We confirmed that the observed phosphatase activity toward phospho-AKT was due to immunoprecipitated PP1 by demonstrating reversal of the activity by inclusion in the assay of purified inhibitor-1 protein, a highly specific PP1 inhibitor. Finally, we demonstrated, using a cell-free, *in vitro* phosphatase assay, that purified PP1 was able to dephosphorylate purified AKT.

We reasoned that if the endogenous PP1 protein in SKBR3 cells were inhibited because of its phosphorylation, then constitutively active (*e.g.*, nonphosphorylatable) PP1 should be able to dephosphorylate AKT in these cells. To test this hypothesis, we cloned the endogenous *PP1* gene from SKBR3, mutated Thr³²⁰ to alanine to create the constitutively active phosphatase (28), and we reintroduced this construct into SKBR3 cells by transient transfection. Using immunofluorescence to monitor the phosphorylation state of AKT in transfected SKBR3 cells, we clearly demonstrated the disappearance of phospho-AKT without effect on total AKT levels in those cells expressing constitutively active PP1.

Phosphorylated PP1, isolated from SKBR3 cells, was recognized by antibodies specific for phosphorylated substrates of both AKT and PKA. However, a PKA inhibitor was unable to promote AKT dephosphorylation (data not shown), raising the intriguing possibility that AKT itself mediates PP1 phosphorylation in SKBR3 cells. To test whether AKT can phosphorylate PP1, we used an *in vitro* kinase assay to demonstrate that PP1 is indeed a substrate of AKT. Furthermore, AKT was able to phosphorylate PP1 on the very residue whose phosphorylation renders PP1 inactive. Because both PP1 and AKT can be found in association in SKBR3 cells, the factor or factors that give the advantage to AKT and allow it to inhibit associated PP1 instead of itself being inhibited remain to be identified, but a role for ErbB2 is suggested (see below).

Basso *et al.* (30) have shown that the effect of GA on AKT activity, as opposed to AKT stability, is seen most dramatically in breast

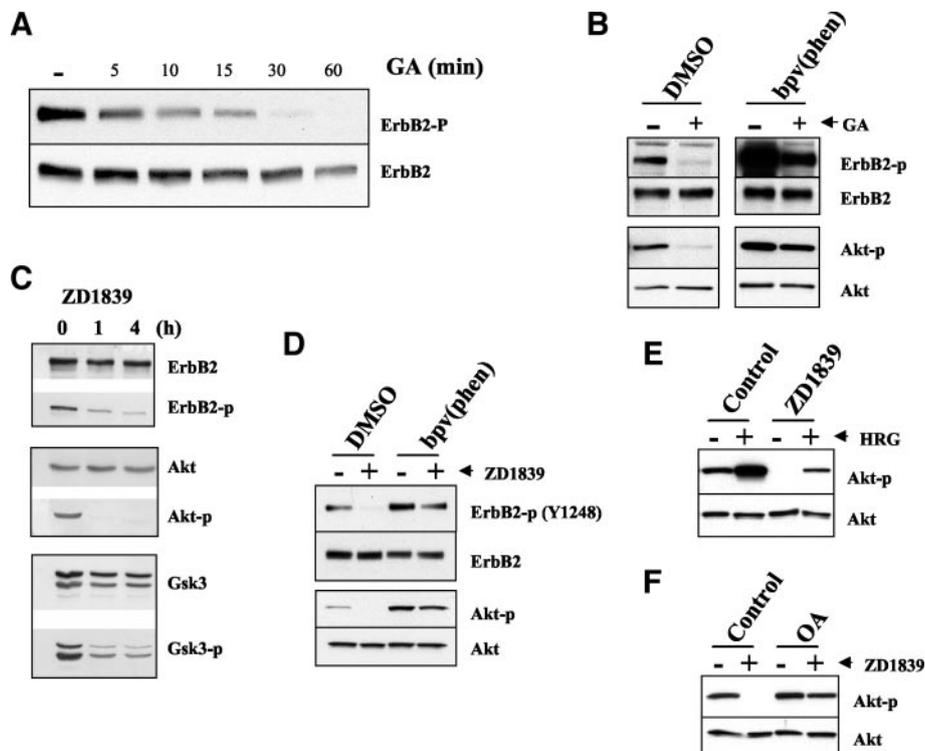


Fig. 5. Specific inhibition of ErbB2 results in AKT dephosphorylation. **A**, GA induces dephosphorylation of ErbB2 before that of AKT. SKBR3 cells were treated with 1 μ M GA for indicated times. Phosphorylation (Y-1248) and protein levels of ErbB2 in cell lysate were detected by Western blotting with specific antibodies. **B**, the Tyr phosphatase inhibitor bpv(phen) inhibits GA-induced AKT dephosphorylation. SKBR3 cells were first treated with 10 μ M bpv(phen) or the vehicle DMSO for 4 h, followed by an additional 1 h of treatment with 1 μ M GA. Cell lysates were separated by SDS-PAGE and transferred to duplicate nitrocellulose membranes. One membrane was probed for the levels of total ErbB2 and AKT and the other for the levels of the phosphorylated forms of the two kinases. **C**, the ErbB inhibitor ZD1839 promotes AKT dephosphorylation in SKBR3 cells. SKBR3 cells were treated with ZD1839 (1 μ M) for 1 and 4 h, and total and phosphorylated forms of ErbB2, AKT, and the AKT substrate protein Gsk-3 were monitored as above. **D**, Bpv(phen) inhibits ZD1839-induced AKT dephosphorylation. SKBR3 cells were pretreated with 10 μ M bpv(phen) or the same amount of DMSO for 4 h, 0.5 μ M ZD1839 was added, and the cells were incubated for an additional 40 min. The phosphorylation states and total protein levels of ErbB2 and AKT were analyzed as above. **E**, ZD1839 does not block AKT activation by HRG. SKBR3 cells were serum starved for 24 h, pretreated with 0.5 μ M ZD1839 for 1 h, and then stimulated with 1 nM HRG for 15 min. Phosphorylation and protein levels of AKT protein were detected as above. **F**, ZD1839-induced AKT dephosphorylation is inhibited by OA. SKBR3 cells were first treated with 0.5 μ M OA for 4 h and then treated with 0.5 μ M ZD1839 for an additional hour. AKT phosphorylation and protein levels were detected as above.

cancer cells that overexpress ErbB2, suggesting an important role for ErbB2 in this phenomenon. Indeed, early in the course of these experiments, we noticed that GA rapidly promoted ErbB2 dephosphorylation (within 5–10 min), even faster than the drug-affected phospho-AKT. To determine whether ErbB2 inactivation may play a role in restoring the sensitivity of AKT to PP1, we blocked GA-stimulated ErbB2 dephosphorylation with the Tyr phosphatase inhibitor bpv(phen) and found that phospho-AKT was also stabilized.

Although, taken together, these data implicate ErbB2 as a key element in preventing AKT dephosphorylation, GA inhibits ErbB2 indirectly, and its effects on other HSP90 client proteins might also contribute to rapid inhibition of AKT activity. Therefore, to additionally support a role for ErbB2, we determined whether the EGF receptor inhibitor ZD1839 could mimic the effects of GA. In cells that express high levels of both EGF receptor and ErbB2 such as SKBR3, activated ErbB2 is maintained by EGF receptor-dependent *trans*-phosphorylation, and ZD1839 therefore effectively inactivates ErbB2 (42, 43). In our model, ZD1839 promoted rapid loss of both phosphorylated ErbB2 and phosphorylated AKT and resulted in rapid inhibition of endogenous AKT activity. Similar results have been obtained in a second ErbB2 overexpressing breast cancer cell line, BT-474.⁵ Furthermore, protection of phospho-ErbB2 species with bpv(phen) abrogated the ability of ZD1839 to stimulate dephosphorylation of AKT. Although these findings do not prove a role for ErbB2 in inhibiting PP1 activity in SKBR3 cells, they are consistent with such a hypothesis.

In summary, our data demonstrate that PP1 associates with AKT in SKBR3 cells but that it is inactive (and phosphorylated). Inhibition of ErbB2 by either GA or ZD1839 resulted in rapid dephosphorylation of AKT, which could be mimicked by transfecting cells with a constitutively active, nonphosphorylatable form of PP1. Thus, although the signaling pathway linking activated ErbB2 to phosphorylation and inactivation of PP1 remains to be identified, our findings describe a novel mechanism by which ErbB2 prolongs AKT activity, and they identify the Ser-Thr phosphatase PP1 as a key regulator of AKT in these cells.

REFERENCES

- Datta, S. R., Brunet, A., and Greenberg, M. E. Cellular survival: a play in three Acts. *Genes Dev.*, 13: 2905–2927, 1999.
- Galetic, I., Andjelkovic, M., Meier, R., Brodbeck, D., Park, J., and Hemmings, B. A. Mechanism of protein kinase B activation by insulin/insulin-like growth factor-1 revealed by specific inhibitors of phosphoinositide 3-kinase—significance for diabetes and cancer. *Pharmacol. Ther.*, 82: 409–425, 1999.
- Franke, T. F., Kaplan, D. R., and Cantley, L. C. PI3K: downstream AKTion blocks apoptosis. *Cell*, 88: 435–437, 1997.
- Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. Regulation of cell death protease caspase-9 by phosphorylation. *Science (Wash. DC)*, 282: 1318–1321, 1998.
- del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science (Wash. DC)*, 278: 687–689, 1997.
- Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. NF- κ B activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature (Lond.)*, 401: 82–85, 1999.
- Biggs, W. H., III, Meisenhelder, J., Hunter, T., Cavenee, W. K., and Arden, K. C. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proc. Natl. Acad. Sci. USA*, 96: 7421–7426, 1999.

⁵ N. Rosen, A. Basso and D. Solit, unpublished observations.

8. Coffey, P. J., and Woodgett, J. R. Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. *Eur. J. Biochem.*, *201*: 475–481, 1991.
9. Datta, K., Franke, T. F., Chan, T. O., Makris, A., Yang, S. I., Kaplan, D. R., Morrison, D. K., Golemis, E. A., and Tsichlis, P. N. AH/PH domain-mediated interaction between Akt molecules and its potential role in Akt regulation. *Mol. Cell. Biol.*, *15*: 2304–2310, 1995.
10. Musacchio, A., Gibson, T., Rice, P., Thompson, J., and Saraste, M. The PH domain: a common piece in the structural patchwork of signalling proteins. *Trends Biochem. Sci.*, *18*: 343–348, 1993.
11. Yarden, Y., and Sliwkowski, M. X. Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell. Biol.*, *2*: 127–137, 2001.
12. Vivanco, I., and Sawyers, C. L. The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat. Rev. Cancer*, *2*: 489–501, 2002.
13. Bellacosa, A., Chan, T. O., Ahmed, N. N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J., and Tsichlis, P. Akt activation by growth factors is a multiple-step process: the role of the PH domain. *Oncogene*, *17*: 313–325, 1998.
14. Sable, C. L., Filippa, N., Filloux, C., Hemmings, B. A., and Van Obberghen, E. Involvement of the pleckstrin homology domain in the insulin-stimulated activation of protein kinase B. *J. Biol. Chem.*, *273*: 29600–29606, 1998.
15. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B α . *Curr. Biol.*, *7*: 261–269, 1997.
16. Wera, S., and Hemmings, B. A. Serine/threonine protein phosphatases. *Biochem. J.*, *311*: 17–29, 1995.
17. Ivaska, J., Nissinen, L., Immonen, N., Eriksson, J. E., Kahari, V. M., and Heino, J. Integrin $\alpha 2 \beta 1$ promotes activation of protein phosphatase 2A and dephosphorylation of Akt and glycogen synthase kinase 3 β . *Mol. Cell. Biol.*, *22*: 1352–1359, 2002.
18. Resjö, S., Goransson, O., Harndahl, L., Zolnierowicz, S., Manganiello, V., and Degerman, E. Protein phosphatase 2A is the main phosphatase involved in the regulation of protein kinase B in rat adipocytes. *Cell. Signal.*, *14*: 231–238, 2002.
19. Yellaturu, C. R., Bhanoori, M., Neeli, I., and Rao, G. N. *N*-Ethylmaleimide inhibits platelet-derived growth factor BB-stimulated Akt phosphorylation via activation of protein phosphatase 2A. *J. Biol. Chem.*, *277*: 40148–40155, 2002.
20. Shenolikar, S., and Nairn, A. C. Protein phosphatases: recent progress. *Adv. Second Messenger Phosphoprotein Res.*, *23*: 1–121, 1991.
21. Aggen, J. B., Nairn, A. C., and Chamberlin, R. Regulation of protein phosphatase-1. *Chem. Biol.*, *7*: R13–R23, 2000.
22. Cheng, A., Dean, N. M., and Honkanen, R. E. Serine/threonine protein phosphatase type 1 $\gamma 1$ is required for the completion of cytokinesis in human A549 lung carcinoma cells. *J. Biol. Chem.*, *275*: 1846–1854, 2000.
23. Ayllon, V., Martinez, A. C., Garcia, A., Cayla, X., and Rebollo, A. Protein phosphatase 1 α is a Ras-activated Bad phosphatase that regulates interleukin-2 deprivation-induced apoptosis. *EMBO J.*, *19*: 2237–2246, 2000.
24. Ayllon, V., Cayla, X., Garcia, A., Roncal, F., Fernandez, R., Albar, J. P., Martinez, C., and Rebollo, A. Bcl-2 targets protein phosphatase 1 α to Bad. *J. Immunol.*, *166*: 7345–7352, 2001.
25. Huang, H. B., Horiuchi, A., Watanabe, T., Shih, S. R., Tsay, H. J., Li, H. C., Greengard, P., and Nairn, A. C. Characterization of the inhibition of protein phosphatase-1 by DARPP-32 and inhibitor-2. *J. Biol. Chem.*, *274*: 7870–7878, 1999.
26. Goldberg, J., Huang, H. B., Kwon, Y. G., Greengard, P., Nairn, A. C., and Kuriyan, J. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. *Nature (Lond.)*, *376*: 745–753, 1995.
27. Eglloff, M. P., Cohen, P. T., Reinemer, P., and Barford, D. Crystal structure of the catalytic subunit of human protein phosphatase 1 and its complex with tungstate. *J. Mol. Biol.*, *254*: 942–959, 1995.
28. Berndt, N., Dohadwala, M., and Liu, C. W. Constitutively active protein phosphatase 1 α causes Rb-dependent G₁ arrest in human cancer cells. *Curr. Biol.*, *7*: 375–386, 1997.
29. Sato, S., Fujita, N., and Tsuruo, T. Modulation of Akt kinase activity by binding to Hsp90. *Proc. Natl. Acad. Sci. USA*, *97*: 10832–10837, 2000.
30. Basso, A. D., Solit, D. B., Munster, P. N., and Rosen, N. Ansamycin antibiotics inhibit Akt activation and cyclin D expression in breast cancer cells that overexpress HER2. *Oncogene*, *21*: 1159–1166, 2002.
31. Basso, A. D., Solit, D. B., Chiosis, G., Giri, B., Tsichlis, P., and Rosen, N. Akt forms an intracellular complex with Hsp90 and Cdc37 and is destabilized by inhibitors of Hsp90 function. *J. Biol. Chem.*, *277*: 39858–39866, 2002.
32. Xu, W., Mimnaugh, E., Rosser, M. F., Nicchitta, C., Marcu, M., Yarden, Y., and Neckers, L. Sensitivity of mature ErbB2 to geldanamycin is conferred by its kinase domain and is mediated by the chaperone protein Hsp90. *J. Biol. Chem.*, *276*: 3702–3708, 2001.
33. Zhou, B. P., Hu, M. C.-T., Miller, S. A., Yu, Z., Xia, W., Lin, S.-Y., and Hung, M. C. HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF- κ B pathway. *J. Biol. Chem.*, *275*: 8027–8031, 2000.
34. Zhou, B. P., Liao, Y., Xia, W., Zou, Y., Spohn, B., and Hung, M. C. HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. *Nat. Cell Biol.*, *3*: 973–982, 2001.
35. Perez-Tenorio, G., and Stal, O. Activation of AKT/PKB in breast cancer predicts a worse outcome among endocrine treated patients. *Br. J. Cancer*, *86*: 540–545, 2002.
36. Stal, O., Perez-Tenorio, G., Akerberg, L., Olsson, B., Nordenskjold, B., Skoog, L., and Rutqvist, L. E. Akt kinases in breast cancer and the results of adjuvant therapy. *Breast Cancer Res.*, *5*: R37–R44, 2003.
37. Schneider, C., Sepp-Lorenzino, L., Nimmesgern, E., Ouerfelli, O., Danishefsky, S., Rosen, N., and Hartl, F. U. Pharmacologic shifting of a balance between protein refolding and degradation mediated by Hsp90. *Proc. Natl. Acad. Sci. USA*, *93*: 14536–14541, 1996.
38. Mimnaugh, E. G., Chavany, C., and Neckers, L. Polyubiquitination and proteasomal degradation of the p185c-erbB-2 receptor protein-tyrosine kinase induced by geldanamycin. *J. Biol. Chem.*, *271*: 22796–22801, 1996.
39. Schulte, T. W., An, W. G., and Neckers, L. M. Geldanamycin-induced destabilization of Raf-1 involves the proteasome. *Biochem. Biophys. Res. Commun.*, *239*: 655–659, 1997.
40. Liu, C. W., Wang, R. H., Dohadwala, M., Schonthal, A. H., Villa-Moruzzi, E., and Berndt, N. Inhibitory phosphorylation of PPI α catalytic subunit during the G₁/S transition. *J. Biol. Chem.*, *274*: 29470–29475, 1999.
41. Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T., and Hidaka, H. Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase. *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *J. Biol. Chem.*, *265*: 5267–5272, 1990.
42. Moasser, M. M., Basso, A., Averbuch, S. D., and Rosen, N. The tyrosine kinase inhibitor ZD1839 (“Iressa”) inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells. *Cancer Res.*, *61*: 7184–7188, 2001.
43. Moulder, S. L., Yakes, F. M., Muthuswamy, S. K., Bianco, R., Simpson, J. F., and Arteaga, C. L. Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells *in vitro* and *in vivo*. *Cancer Res.*, *61*: 8887–8895, 2001.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

The Heat Shock Protein 90 Inhibitor Geldanamycin and the ErbB Inhibitor ZD1839 Promote Rapid PP1 Phosphatase-Dependent Inactivation of AKT in ErbB2 Overexpressing Breast Cancer Cells

Wanping Xu, Xitong Yuan, Yun Jin Jung, et al.

Cancer Res 2003;63:7777-7784.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/63/22/7777>

Cited articles This article cites 42 articles, 22 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/63/22/7777.full#ref-list-1>

Citing articles This article has been cited by 26 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/63/22/7777.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cancerres.aacrjournals.org/content/63/22/7777>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.