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Stabilization of Phosphatidylinositol 4-Kinase Type IIβ by Interaction with Hsp90*

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Mammalian cells express two isoforms of type II phosphatidylinositol 4-kinase: PI4KIIα and PI4KIIβ. PI4KIIα exists almost exclusively as a constitutively active integral membrane protein because of its palmitoylation (Barylko, B., Gerber, S. H., Binns, D. D., Grichine, N., Khvotchev, M., Südhof, T. C., and Albanesi, J. P. (2001) *J. Biol. Chem.* 276, 7705–7708). In contrast, PI4KIIβ is distributed almost evenly between membranes and cytosol. Whereas the palmitoylated membrane-bound pool is catalytically active, the cytosolic kinase is inactive (Wei, Y. J., Sun, H. Q., Yamamoto, M., Wlodarski, P., Kunii, K., Martinez, M., Barylko, B., Albanesi, J. P., and Yin, H. L. (2002) *J. Biol. Chem.* 277, 46586–46593; Jung, G., Wang, J., Wlodarski, P., Barylko, B., Binns, D. D., Shu, H., Yin, H. L., and Albanesi, J. P. (2008) *Biochem. J.* 409, 501–509). In this study, we identify the molecular chaperone Hsp90 as a binding partner of PI4KIIβ, but not of PI4KIIα. Geldanamycin (GA), a specific Hsp90 inhibitor, disrupts the Hsp90-PI4KIIβ interaction and destabilizes PI4KIIβ, reducing its half-life by 40% and increasing its susceptibility to ubiquitylation and proteasomal degradation. Cytosolic PI4KIIβ is much more sensitive to GA treatment than is the integrally membrane-associated species. Exposure to GA induces a partial redistribution of PI4KIIβ from the cytosol to membranes and, with brief GA treatments, a corresponding increase in cellular phosphatidylinositol 4-kinase activity. Stimuli such as PDGF receptor activation that also induce recruitment of the kinase to membranes disrupt the Hsp90-PI4KIIβ interaction to a similar extent as GA treatment. These results support a model wherein Hsp90 interacts predominantly with the cytosolic, inactive pool of PI4KIIβ, shielding it from proteolytic degradation but also sequestering it to the cytosol until an extracellular stimulus triggers its translocation to the Golgi or plasma membrane and subsequent activation.

Phosphoinositides are essential regulators of fundamental cellular processes, including signal transduction, membrane traffic, cytoskeletal dynamics, and ion transport (reviewed in Refs. 1–6). Phosphatidylinositol 4-kinases (PI4Ks) initiate the canonical phosphoinositide biosynthetic pathway by phosphorylating the D-4 hydroxyl of the inositol head group of PtdIns. The product of this reaction, PtdIns 4-phosphate (PtdIns4P),[2] serves not only as a major precursor in the synthesis of more highly phosphorylated phosphoinositides, including PtdIns 4,5-bisphosphate and PtdIns 3,4,5-trisphosphate, but also has itself been shown to be a regulator of membrane trafficking (7, 8). Two types of PI4K (PI4KII and PI4KIII) have been identified in eukaryotes. The type III kinases, which are expressed from yeast to mammals, are further subdivided into α (~230 kDa) and β (~100 kDa) isoforms. Although mammals also express two type II kinases (α and β, both ~55 kDa), *Saccharomyces cerevisiae* have only one PI4KII ortholog, known as Lsb6p (9, 10).

PI4KIIα and β have conserved catalytic domains but diverse N-terminal regions extending approximately from residues 1 to 90. Unlike PI4KIIIs, which are almost entirely cytosolic, PI4KIIs can associate integrally with membranes by virtue of palmitoylation of multiple cysteines within their catalytic domains (11, 12). The palmitoylation motif, CCPCC, is present in both PI4KIIα and β. However, whereas >90% of PI4KIIα is palmitoylated and exists in cells as an active, integrally membrane-bound species, PI4KIIβ is divided almost evenly between cytosolic and membrane-bound pools (13, 14). Moreover, almost half of membrane-bound PI4KIIβ is only peripherally associated with membranes, extractable by sodium carbonate at pH 11 in the absence of detergent. Therefore, it appears that only ~25–30% of PI4KIIβ is normally palmitoylated in cells. Because palmitoylation is essential for catalytic activity (14), 70–75% of this isoform may be inactive under resting conditions. Although a small portion (~7%) of PI4KIIβ is recruited to membranes in response to growth factor receptor activation (13), conditions have not yet been found that result in a major redistribution of the kinase from cytosol to membranes. We reported that the different membrane binding properties and palmitoylation states of PI4KIIα and β are not due to their highly diverse N-terminal regions but instead to relatively slight differences in their C-terminal 160 residues (14).

PI4KIIα has been implicated in generating PtdIns4P pools that regulate membrane trafficking from the trans-Golgi network (15–17) and in late stages of endocytosis (17). Although no specific function has been ascribed to PI4KIIβ, its partial redistribution to the plasma membrane in response to growth

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[2] The abbreviations used are: PtdIns, phosphatidylinositol; PtdIns4P, PtdIns 4-phosphate; GA, geldanamycin; PI4K, phosphatidylinositol 4-kinase.

factor receptor activation (13) and tyrosine phosphorylation in response to activation of the T-cell receptor (18) suggest that it may have a role in cellular signaling. To understand how extracellular stimuli recruit PI4KIIβ to membranes, where it can be palmitoylated and activated, we sought to understand the basis for its distribution between membranes and cytosol. To this end, we attempted to identify binding partners that (*a*) selectively bind to PI4KIIβ over PI4KIIα and (*b*) could sequester PI4KIIβ to the cytosol. The data presented below demonstrate that the molecular chaperone Hsp90 fulfills these criteria. They further demonstrate that the interaction with Hsp90 is required to stabilize the cytosolic pool of PI4KIIβ and that the PI4KIIβ-Hsp90 interaction is disrupted by growth factor receptor activation, resulting in a partial redistribution of the kinase to membranes.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—Anti-PI4KIIβ and anti-PI4KIIα antibodies were generated as described previously (13, 19). Monoclonal anti-Myc antibody 9E10 was obtained from the National Cell Culture Center (Minneapolis, MN). Hsp90 antibody (H-114) was from Santa Cruz Biotechnology (Santa Cruz, CA). Hsp70 antibody was purchased from Cell Signaling (Danvers, MA). Primers were obtained from Integrated DNA Technologies (Coralville, IA). Cloning reagents and reagents for mutagenesis were from Invitrogen. Triton X-100 and reagents for electrophoresis and immunoblotting were from Bio-Rad. Geldanamycin (GA) was obtained from InvivoGen (San Diego, CA). Other reagents, including ATP, buffers, protease inhibitors, and MG132 (proteasome inhibitor), were from Sigma.

Cell Culture and Transfection—HEK293, COS, and HeLa cells were maintained in DMEM supplemented with 10% FBS and antibiotics. The cells were replated 1 day prior to transfection. For transient expression of proteins, the cells were transfected for 20 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

cDNA Constructs and Generation of Mutants—Rat PI4KIIα (gi:16758553) and human PI4KIIβ (gi:20159767) cDNAs were cloned in pCMV5-Myc vectors as described previously (11, 13). All truncated and swapped constructs were generated as described previously (14). Cat α_{92-431} was generated as described in Ref. 19. PI4KIIβ_{1-434} and PI4KIIβ_{1-340} were subcloned with primers to delete C termini, 5′-CCTTACTCAGGCATTGAGAGACTGATCTAGAGC-3′ and 5′-GAATTCCTTATTAAAATAGCTGCATGATCTAGAGC-3′, respectively. To delete residues 312–336 of PI4KIIβ (PI4KIIβ-Δ312–336), two separate PCRs were performed using primers A (5′-CCATCGATATGGAGGATCCCTCCGAGCCCGACCGG-3′) and B (5′-CCATTATCAATTGCAGCTATGTATCTGACTAACCAATTATC-3′) as well as primers C (5′-GATAATTGGTTAGTCAGATACATAGCTGCAATTGATAATGG-3′) and D (5′-TGCACTGCAGCTACCAGGAGGAAAAAAATGGCTTCCTGC-3′). The fragments were combined and overlap-extended using primers A and D. The final PCR product was subcloned into pCMV5-Myc vector using ClaI and PstI. PI4KIIα (ΔCCPCC) was generated as described (11). Hsp90α was amplified from mouse cDNA library with primers KpnI: 5′-CGGGGTACCCATGCCTGAGGAAACCCAGACCCAAGACC-3′ and XbaI: 5′-GCTCTAGATTAGTCTACTTCTTCCATGCGTGATGTGTCG-3′ and cloned into p3XFLAG-CMV-7.1 expression vector (Sigma).

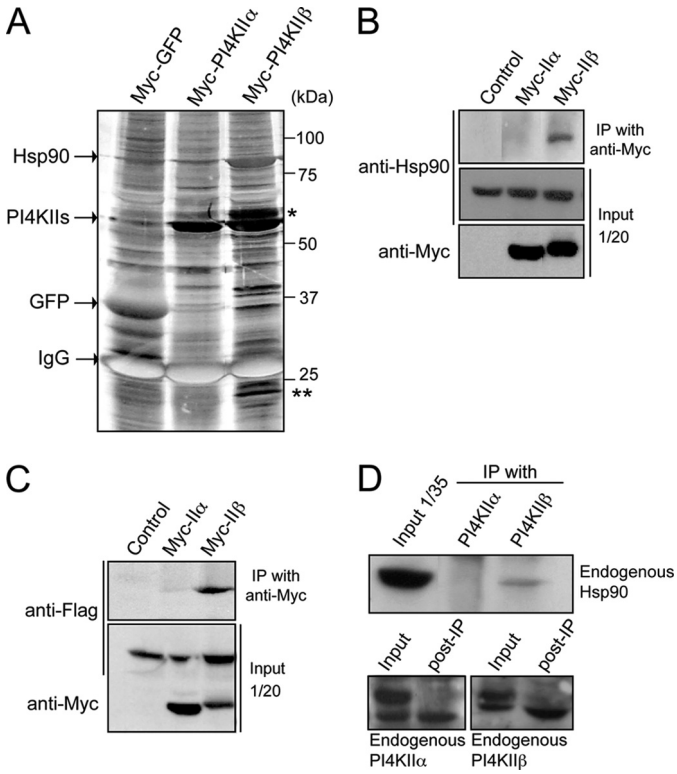


FIGURE 1. **Hsp90 co-immunoprecipitates with PI4KIIβ.** *A*, silver-stained gel showing increased intensity of a 90-kDa band in precipitates of Myc-PI4KIIβ compared with those of Myc-PI4KIIα or an irrelevant protein, Myc-GFP. Myc-tagged proteins were expressed in HEK 293 cells, which were then lysed and subjected to immunoprecipitation with anti-Myc antibodies. The 90-kDa band was extracted and identified as Hsp90 by mass spectrometry. The *asterisk* designates phospho-PI4KIIβ, and the *double asterisks* designate peroxiredoxin I, another PI4KIIβ-binding protein identified in this study. Other unique bands in the precipitates were identified by mass spectrometry as fragments of the full-length kinases. *B* and *C*, specific association between endogenous and exogenous Hsp90s and PI4KIIβ. Myc-PI4KIIα or Myc-PI4KIIβ was expressed in HeLa cells without (*B*) or with (*C*) FLAG-Hsp90α. The cell lysates were immunoprecipitated (*IP*) with anti-Myc antibody and blotted with anti-Hsp90 or anti-FLAG to show the specific association of Hsp90 with PI4KIIβ but not PI4KIIα. *D*, specific association between endogenous Hsp90 and endogenous PI4KIIβ. HeLa cell extracts prepared as above were used to immunoprecipitate endogenous PI4KIIα or PI4KIIβ with their corresponding antibodies. The *upper panel* shows an immunoblot of the electrophoresed precipitates with anti-Hsp90 antibody. The *lower panels* show depletion of the kinases from supernatants following immunoprecipitation. Because of co-migration of antibody heavy chains with the kinases on SDS gels, the amounts of precipitated PI4KIIα and PI4KIIβ could not be determined. The cross-reactive but not precipitated lower bands that appear on occasion (see also Fig. 8*B*) have not been identified. The data shown in this figure are representative of experiments performed two to four times.

In-gel Digestion and Protein Identification Using Mass Spectrometry—Silver-stained protein bands were excised from one-dimensional SDS gels and cut further into small cubes (~1 × 1 mm). After in-gel reduction, alkylation, and destaining, the proteins were digested overnight with trypsin (12 ng/μl). The tryptic peptides were extracted from gels dried in a vacuum centrifuge and analyzed by electrospray liquid chromatography-tandem mass spectrometry using a nanoscale C18 column coupled in-line with an ion trap mass spectrometer (LCQ Deca, Thermo Finnigan). The instrument was set in a data-dependent acquisition mode, cycling between one full MS scan and

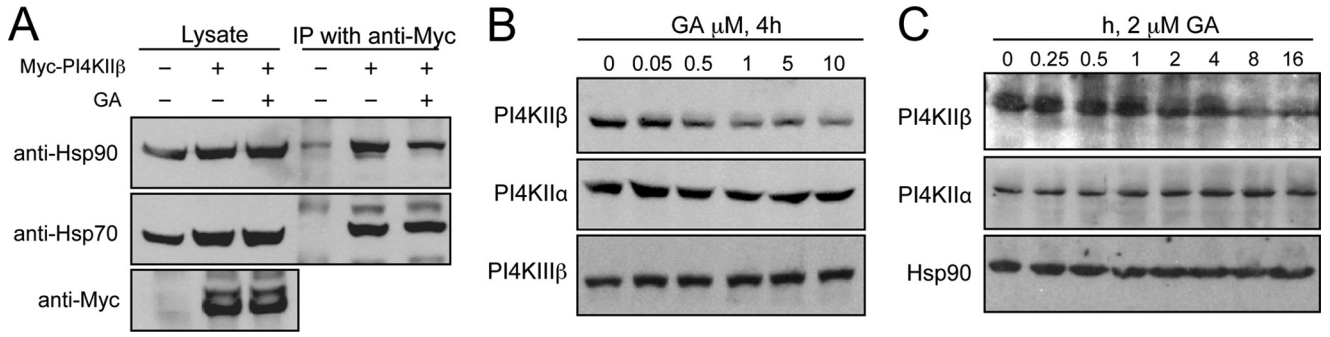


FIGURE 2. **Effects of the Hsp90 inhibitor, GA, on stability of PI4KIIβ.** *A*, disruption of the association between PI4KIIβ and Hsp90 by GA. Myc-PI4KIIβ was expressed and immunoprecipitated (*IP*) with anti-Myc antibodies from cells treated with 2 μM GA (or left untreated) for 2 h. Precipitates were electrophoresed and blotted with antibodies against Hsp90 and Hsp70. *B*, dose-dependent loss of endogenous PI4KIIβ in response to GA treatment. HeLa cells were treated for 4 h with various doses of GA prior to isolation and electrophoresis of post-nuclear supernatants. The gels were blotted with antibodies against PI4KIIβ, PI4KIIα, and PI4KIIIβ to estimate changes in kinase levels in response to GA treatment. *C*, time-dependent loss of PI4KIIβ in response to GA treatment. HeLa cells were exposed to 2 μM GA for various times and prepared as in *B*. The data in this figure are representative of at least three separate experiments.

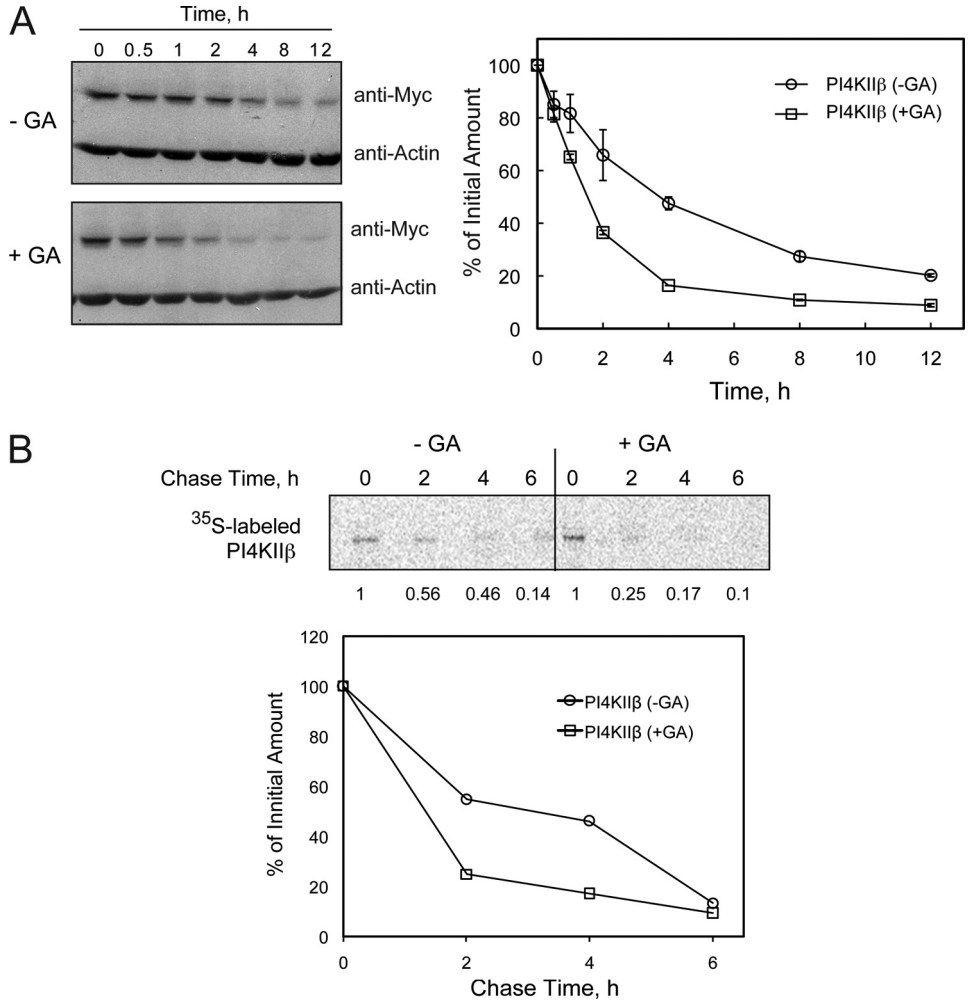


FIGURE 3. **Effects of GA on the half-life of PI4KIIβ.** *A*, cells expressing Myc-PI4KIIβ were treated with cycloheximide alone or cycloheximide together with GA (2 μM). At the indicated time points, the cells were lysed, and the lysates were electrophoresed and blotted with anti-Myc antibodies to detect kinase and with anti-actin antibodies for normalization. The graph shows the amount of PI4KIIβ remaining after cycloheximide treatment as a percentage of its initial amount, normalized to the amount of actin in each lane. The data represent the averages of two experiments, one measured in duplicate and the other measured in triplicate. *B*, pulse-chase method for determining the effect of GA on the half-life of PI4KIIβ. The cells expressing Myc-PI4KIIβ were treated with or without 2 μM GA for 2 h, starved with methionine/cysteine-free DMEM for 1 h, labeled with [^{35}S]methionine/cysteine for 1 h, then chased with unlabeled methionine/cysteine for 2, 4, or 6 h, and lysed. PI4KIIβ was immunoprecipitated with anti-Myc antibody, electrophoresed, and analyzed by fluorography. The graph displays quantification of results of a single pulse-chase experiment.

MS/MS scans of the three most abundant ions. The MS and MS/MS data were used to search the nonredundant NCBI protein database using the MASCOT search engine.

Immunoprecipitation and Immunoblotting—Untransfected cells or cells transfected for 20 h were washed with PBS and lysed in Nonidet P-40 lysis buffer consisting of 20 mM Tris-HCl,

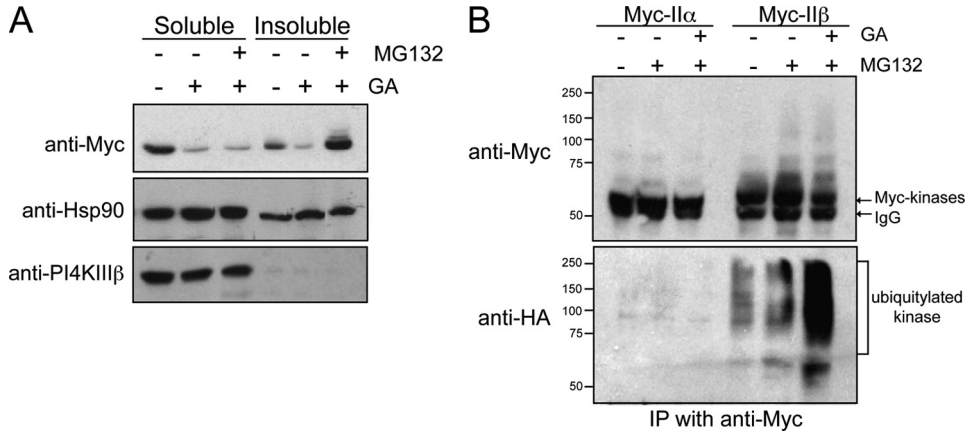


FIGURE 4. **Proteasome-dependent destabilization of PI4KIIβ.** *A*, effect of MG132, a proteasome inhibitor, on PI4KIIβ stability. The cells expressing Myc-PI4KIIβ were incubated with 1 μM GA with or without 10 μM MG132. Soluble fractions were prepared in 1% Nonidet P-40 lysis buffer. Insoluble fractions (pellets) were mixed with SDS sample buffer followed by electrophoresis and immunoblotting. Hsp90 and cytosolic PI4KIIIβ are shown as controls. *B*, ubiquitylation of PI4KIIβ. Myc-PI4KIIα or Myc-PI4KIIβ was co-expressed with HA-tagged ubiquitin in HeLa cells. Prior to scraping in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.05% SDS, 2 mM EDTA, 0.2 mM PMSF, and protease and phosphatase inhibitors), the cells were incubated with 2 μM GA and 10 μM MG132 for 1.5 h. Ubiquitylated kinases, detected by anti-HA antibody, appear as smeared bands. *IP*, immunoprecipitation.

150 mM NaCl, 1 mM EDTA, 30 mM NaF, 2 mM $Na_4P_2O_3$, 1 mM Na_2VO_4, and 0.1% Nonidet P-40. Cell lysates were obtained after centrifugation at 1000 × *g* for 5 min and were precleared of nonspecifically binding proteins by incubation with rec-Protein G-Sepharose 4B conjugate (Zymed Laboratories Inc.) for 30 min. Myc-tagged proteins were immunoprecipitated by 4 h of incubation with anti-Myc antibody that had been cross-linked to protein G-Sepharose using dimethyl pimelimidate (Pierce). The immunoprecipitates were washed three times with Nonidet P-40 lysis buffer. The samples were eluted with SDS sample buffer and loaded on SDS-polyacrylamide gels. To quantify PI4KII levels in various fractions, the samples were electrophoresed and immunoblotted with anti-Myc, anti-PI4KIIα, or anti-PI4KIIβ antibodies. To estimate relative amounts of proteins, the immunoblots were incubated with ^{125}I-labeled secondary antibody, scanned by phosphorimaging (Fujifilm BAS 1500), and quantified using the Multi-Gauge V2.3 program (Fuji Photo Film Co.). Alternatively, immunoblotted bands were quantified by densitometry using the Multi-Gauge V2.3 program (Fuji Photo Film Co.).

Preparation of Cytosol and Membranes—Transfected or untransfected cells were washed with PBS and scraped in a solution containing 0.25 M sucrose, 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, 1 mM PMSF, a protease inhibitor mixture (10 μg/ml each of N^{α}-*p*-tosyl-L-lysine chloromethyl ester, N^{α}-*p*-tosyl-L-arginine methyl ester, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, leupeptin, and pepstatin A) and phosphatase inhibitors (50 mM NaF, 50 mM $Na_4P_2O_3$, 1 mM Na_2VO_4). The cells were lysed by two freeze-thaw cycles and passage through a 27.5-gauge needle. The lysates were then centrifuged at 1,000 × *g* for 5 min to obtain post-nuclear supernatants. The post-nuclear supernatant were then centrifuged at 200,000 × *g* for 15 min to separate cytosol from membranes. The resulting membrane pellets were homogenized sequentially, first in 0.1 M Na_2CO_3 (pH 11) to extract tightly bound peripheral proteins and then in a solution containing 1% Triton X-100 (v/v), 20 mM Tris-HCl (pH 7.5), 1 mM EDTA to extract integral proteins.

Analysis of [^{32}P]P_i Incorporation into Lipids—For radiolabeling, transfected COS cells were incubated in phosphate-free medium containing 5% dialyzed FBS and 25 μCi/ml [^{32}P]P_i (PerkinElmer Life Sciences) for 4 h. After three brief washes with PBS, labeled cells were scraped into tubes containing methanol:HCl (10:1), and the lipids were extracted with chloroform. The organic phase was collected and washed with an equal volume of methanol and HCl (1:1). Aliquots were spotted onto TLC plates and separated in a solvent system consisting of *n*-propyl alcohol/H_2O/NH_4OH (65:20:15). Radioactive PtdIns4P spots were scanned using FLA-5100 (Fuji Photo Film Co.), and radioactivity of bands was quantified using the Multi-Gauge V2.3 program.

Geldanamycin Treatment of Cells—GA was solubilized in Me_2SO and added to cell culture medium to the final concentrations indicated in the text and figure legends. Equal volumes of Me_2SO without GA were used in controls. The cells were treated for 1–24 h as indicated.

Immunofluorescence Microscopy—To visualize the redistribution of endogenous PI4KIIβ, HeLa cells were grown on coverslips for 16 h. After treatment with 2 μM GA for 2 h, the cells were washed three times with ice-cold PBS, fixed with 3.7% formaldehyde in PBS for 10 min at room temperature, and permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. After two washes with PBS and blocking in solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20, and 1% BSA, the cells were incubated with anti-PI4KIIβ antibody followed by rhodamine-labeled secondary antibody (Molecular Probes). Fluorescence microscopy was performed with a Zeiss LSM 510 confocal microscope with 63× oil immersion lens.

Half-life Measurement—With the cycloheximide method, the cells expressing Myc-PI4KIIβ were treated for various times with 0.2 mg/ml cycloheximide, in the presence or absence of 2 μM GA. Lysates prepared from these cells were electrophoresed and blotted with anti-Myc antibodies to detect kinase and with anti-actin antibodies for normalization. Relative amounts of

kinase and actin were estimated using ^{125}I-labeled secondary antibodies. With the pulse-chase method, COS cells were transfected with Myc-PI4KIIβ, and after 20 h they were treated with or without 2 μM GA for 2 h. After starving with methionine/cysteine-free DMEM for 1 h, the cells were labeled with [^{35}S]methionine/cystine for 1 h and then chased with unlabeled methionine/cysteine for 2, 4, or 6 h. The cells were lysed and immunoprecipitated with anti-Myc antibody. The precipitated samples were separated by 10% SDS-PAGE and analyzed by fluorography. The results were quantified using ImageQuant software.

Other Procedures—SDS-polyacrylamide gel electrophoresis and immunoblot analysis were carried out by the methods of Laemmli (20) and Towbin *et al.* (21), respectively. Protein concentrations were determined using the modified Lowry method (22) according to Peterson (23) with BSA as a standard.

RESULTS

Identification of Hsp90 as a Binding Partner of PI4KIIβ—To understand the basis for the distinct subcellular distributions of PI4KIIα and β, we sought to identify proteins that preferentially interact with one isoform over another. Myc-tagged versions of each kinase were expressed in HEK293 cells and immunoprecipitated using anti-Myc antibodies. One electrophoretic band of 90 kDa was consistently enriched in immunoprecipitates of PI4KIIβ compared with those of PI4KIIα or of an irrelevant expressed protein, GFP (Fig. 1*A*). This band was extracted from gels and subsequently identified as Hsp90 by mass spectrometry. Apart from several other bands that proved to be fragments of PI4KIIβ, the only other band that selectively co-immunoprecipitated with PI4KIIβ was identified by mass spectrometry as peroxiredoxin I (Fig. 1*A*, **). The potential significance of this interaction is currently being investigated.

The selective binding of Hsp90 to PI4KIIβ was confirmed by immunoprecipitating PI4KIIα or PI4KIIβ and blotting the electrophoresed precipitates with an anti-Hsp90 antibody (Fig. 1, *B* and *D*). The results show that Hsp90 co-immunoprecipitates with Myc-PI4KIIβ (Fig. 1*B*) as well as with the endogenous kinase (Fig. 1*D*) but not with PI4KIIα. Selectivity was also demonstrated by co-precipitation of expressed FLAG-tagged Hsp90 with Myc-PI4KIIβ but not with Myc-PI4KIIα (Fig. 1*C*).

Inhibition of the PI4KIIβ-Hsp90 Interaction by Geldanamycin Results in Destabilization of PI4KIIβ—To determine the significance of Hsp90 binding to PI4KIIβ, we disrupted the interaction using GA, an agent that binds directly to the ATP-binding site of Hsp90 and blocks its association with many client proteins (24, 25). Treatment of cells with 2 μM GA for 2 h significantly reduced the amount of Hsp90 that co-immunoprecipitates with Myc-PI4KIIβ, whereas similar amounts of Hsp70 were present in immunoprecipitates from GA-treated and untreated cells (Fig. 2*A*). Hsp70 serves as general chaperone for newly synthesized proteins engaged in co-translational folding and for unfolded proteins. It is also a component of the Hsp90 chaperone complex, participating in the Hsp90 assisted folding cycle (reviewed in Refs. 26 and 27), but GA apparently does not disrupt the association of Hsp70 and its substrates (28).

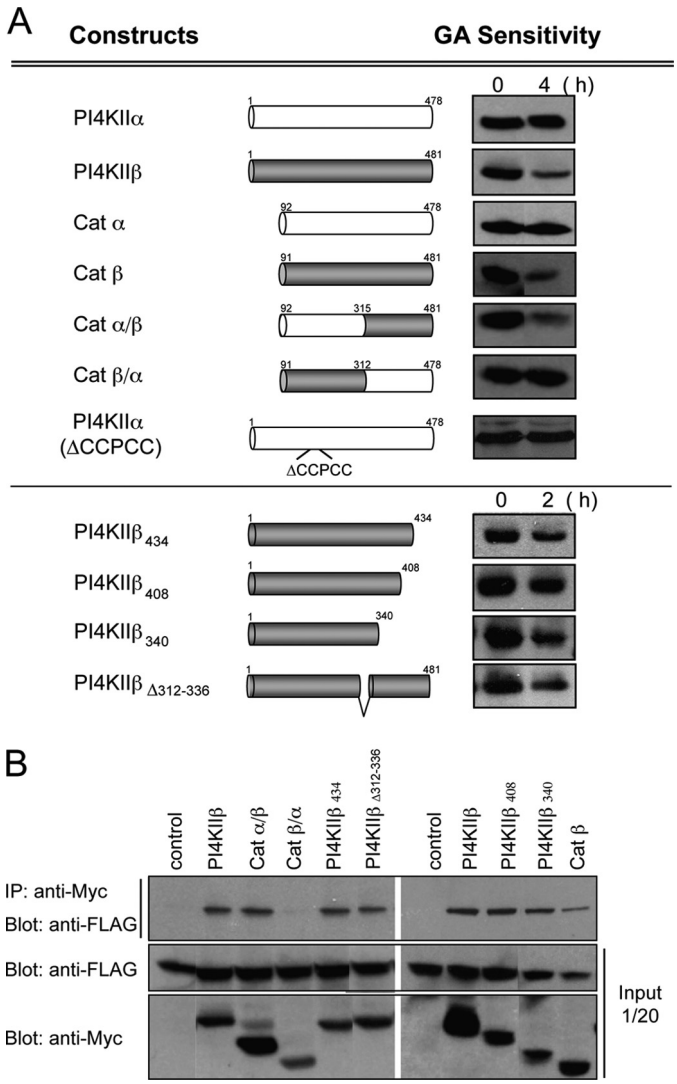
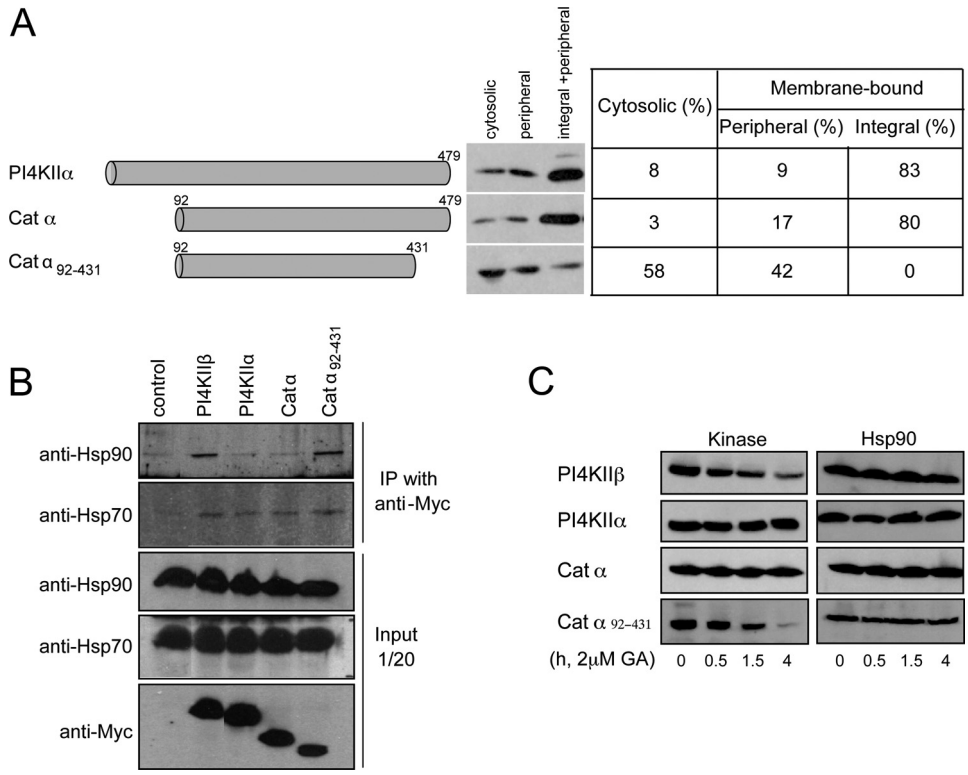


FIGURE 5. **GA sensitivity and Hsp90 binding of various PI4KII constructs.** *A*, the designated Myc-tagged constructs were expressed in cells that were treated with 2 μM GA for 4 or 2 h. The amount of each construct was detected by immunoblotting cell lysates with anti-Myc antibodies. *B*, the Myc-tagged constructs and FLAG-Hsp90 were expressed and immunoprecipitated with anti-Myc-antibodies. The binding of Hsp90 was shown by immunoblotting with anti-FLAG antibodies. The controls were anti-Myc immunoprecipitates from FLAG-Hsp90 transfected cells. *IP*, immunoprecipitation.

In many cases, interfering with the interaction of Hsp90 with its client proteins reduces the stability of those clients. To test whether this also applies to PI4KIIβ, we analyzed the effect of GA treatment on the cellular levels of PI4KIIβ in a dose-dependent (Fig. 2*B*) and time-dependent manner (Fig. 2*C*). After cell exposure to GA, we observed reduction of cellular levels of endogenous PI4KIIβ but not of endogenous PI4KIIα. PI4KIIβ levels were reduced approximately by half following 4 h of treatment with 1 μM GA (Fig. 2*B*). Reduction of PI4KIIβ was evident even after a 1-h exposure to 2 μM GA (Fig. 2*C*). We also tested the sensitivity of PI4KIIIβ to GA, because Flanagan and Thorner (29) reported that Hsp90 co-purifies with the yeast PI4KIIIβ ortholog, Pik1p. Indeed, based on this report, PI4KIIIβ has been classified in the literature as a binding protein of Hsp90 (30, 31), although the function of this interaction



Cytosolic (%)	Membrane-bound	
	Peripheral (%)	Integral (%)
8	9	83
3	17	80
58	42	0

FIGURE 6. **Hsp90 binding and GA sensitivity of a cytosolic fragment of PI4KIIα.** *A*, distribution of full-length PI4KIIα, its full-length catalytic domain (Cat α), and a truncated catalytic domain (Cat α_{92-431}) among cytosolic, peripheral membrane, and integral membrane pools. The fractions were prepared and analyzed as described under "Experimental Procedures." *B*, binding of Hsp90 to PI4KIIβ, PI4KIIα, and PI4KIIα fragments. All of the constructs were expressed as Myc-tagged proteins in HeLa cells and immunoprecipitated (*IP*) with anti-Myc antibodies. Co-precipitating Hsp90 and Hsp70 were detected by immunoblotting. *C*, effect of GA on the stability of PI4KIIβ, PI4KIIα, and PI4KIIα fragments. The cells expressing the Myc-tagged constructs were treated with 2 μM GA for the designated times, and their quantities in cell lysates were estimated by immunoblotting with anti-Myc antibodies. Anti-Hsp90 immunoblots (*right panels*) were used as loading controls.

was not investigated. Our results indicate that PI4KIIIβ is resistant to GA treatment (Figs. 2*B* and 4*A*).

Consistent with the above results, GA was found to shorten the half-life of PI4KIIβ (Fig. 3*A*). HeLa cells expressing Myc-PI4KIIβ were treated with the protein synthesis inhibitor cycloheximide in the presence or absence of 2 μM GA. At various times thereafter, the kinase levels were estimated by immunoblotting. The half-life of PI4KIIβ was reduced from 4 to 1.5 h by GA treatment. We verified that GA also reduced the half-life of Myc-PI4KIIβ, from 3 to 1.5 h, using the ^{35}S-labeling pulse-chase procedure (Fig. 3*B*). This is the first reported measurement of the half-life of a type II kinase, and it demonstrates that, like many other signaling proteins, it turns over relatively rapidly.

Hsp90 Protects PI4KIIβ from Degradation by the Proteasome—GA-mediated reductions in the cellular levels of Hsp90 client proteins are likely due to their enhanced ubiquitylation and proteasomal degradation (32–35). To check whether Hsp90 protects PI4KIIβ from proteasomal degradation, we examined the effect of treating cells expressing Myc-PI4KIIβ with both GA and the proteasome inhibitor, MG132. In the absence of these agents, the majority of Myc-PI4KIIβ was present in the soluble fraction of cells homogenized in buffer containing 1% Nonidet P-40. The remainder, presumably including aggregated kinase, was recovered in low speed pellets (Fig. 4*A*). GA treatment for 24 h reduced the amount of PI4KIIβ recovered either in the soluble or insoluble fractions. However, nearly the entire initial pool of kinase distributed to the pellet when cells were simultaneously treated with GA and MG132, indicating that PI4KIIβ is protected from proteasomal degradation but accumulates in an aggregated state. In contrast, the amounts of PI4KIIIβ (or of Hsp90) were not influenced by either GA or MG132. It was previously reported that PDK1, Raf1, and Src also accumulate in the insoluble fraction upon treatment of cells with both GA and MG132 (34, 36, 37).

We next verified that dissociation of the PI4KIIβ-Hsp90 complex by GA enhanced ubiquitylation of the kinase. The cells were co-transfected with HA-ubiquitin and Myc-PI4KIIβ or Myc-PI4KIIα to allow detection of ubiquitylated kinase with anti-HA antibodies. In the absence of GA, inhibition of the proteasome with MG132 did not appreciably increase the amount of ubiquitylated kinases detected in anti-Myc immunoprecipitates (Fig. 4*B*). However, co-treatment with GA for 1.5 h resulted in a significant accumulation of ubiquitylated PI4KIIβ, whereas the ubiquitylation of PI4KIIα was unaffected by either MG132 alone or in combination with GA.

Hsp90 Selectively Stabilizes the Cytosolic Pool of PI4KIIβ—To explain why Hsp90 binds preferentially to PI4KIIβ over PI4KIIα, we analyzed the interactions using a series of truncated and hybrid kinases. Deletion of the highly diverse N-terminal segments (~residues 1–90, constructs designated as Cat α and Cat β) had essentially no effect on Hsp90 binding or GA sensitivity (Figs. 5 and 6*B*). Therefore, as shown for a number of protein kinases (38), Hsp90 apparently interacts with the cata-

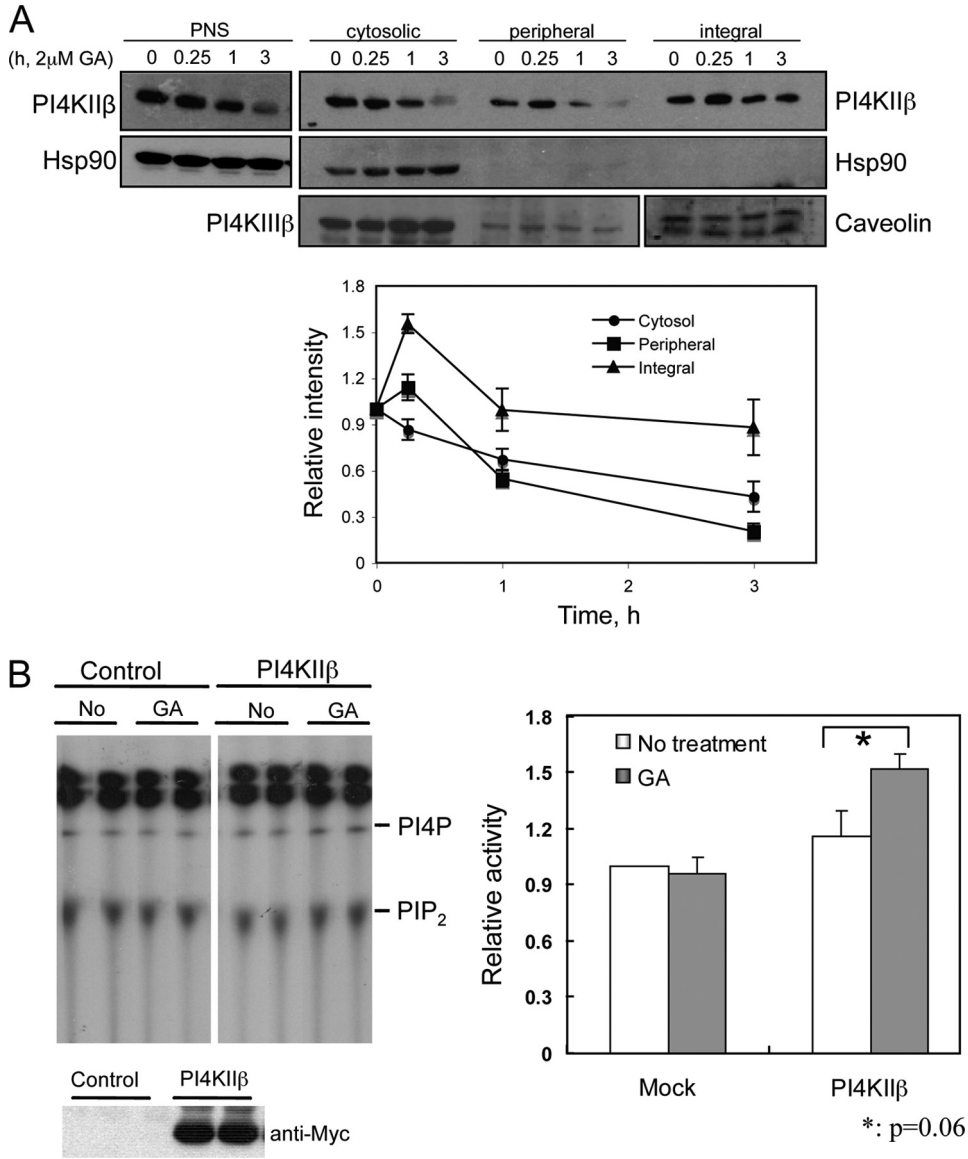


FIGURE 7. **Redistribution of Myc-PI4KIIβ to membranes in response to GA treatment.** *A*, effect of GA on the distribution of PI4KIIβ among cytosolic, peripheral membrane, and integral membrane pools. Cytosolic and membrane fractions were recovered from the supernatants and pellets, respectively, following centrifugation of post-nuclear supernatant (*PNS*) at 200,000 × *g* for 15 min. Peripheral membrane proteins were those released from membranes upon treatment with 0.1 M Na_2CO_3 (pH 11), and integral membrane proteins were those that were then solubilized by 1% Triton X-100. Cells transfected with Myc-PI4KIIβ were treated with 2 μM GA for the designated times and fractionated as described under "Experimental Procedures." The lysates were immunoblotted to estimate the relative amounts of PI4KIIβ in each fraction, as shown in the graph. Hsp90, PI4KIIIβ, and caveolin were blotted as loading controls. The graph represents the means ± S.D. from three independent experiments. *B*, PtdIns4P production in cells treated briefly with GA. Cells transfected with vector (mock) or Myc-PI4KIIβ were labeled with [^{32}P]P_i for 4 h and incubated with GA for 15 min. The cells were then scraped into methanol:HCl (10:1) to extract lipids, which were then quantified as described under "Experimental Procedures." Phosphorylated lipids were resolved by TLC. The graph shows the relative amounts of radioactive phosphate incorporated into PtdIns4P. The averages of three independent triplicate experiments are shown. The *error bars* designate the standard deviations.

lytic domain (residues 91–481) of PI4KIIβ. We next asked whether a particular region of the PI4KIIβ catalytic domain determines its GA sensitivity. The catalytic domains of the type II kinases can be divided into two segments: an N-terminal segment (residues ~90–314) having 86% sequence similarity and a C-terminal segment (residues ~315–481) having 77% sequence similarity. We generated two hybrids, Cat α/β (residues 92–314 of PI4KIIα fused to residues 312–481 of PI4KIIβ) and Cat β/α (residues 91–311 of PI4KIIβ fused to residues 315–478 of PI4KIIα). Cat α/β, like full-length PI4KIIβ, bound to Hsp90 and was destabilized by GA. Cat β/α, like PI4KIIα, displayed neither property (Fig. 5). Interestingly, Cat α/β and Cat β/α behave similarly to PI4KIIβ and PI4KIIα also in terms of their solubilities: Cat α/β is >80% cytosolic, whereas Cat β/α is >80% integrally membrane-bound (14). The above results raise two possibilities: (*a*) the C-terminal segment of PI4KIIβ contains a binding site for Hsp90 that is absent from the corresponding region of PI4KIIα and (*b*) Hsp90 binds preferentially to PI4KIIβ because a substantial portion of PI4KIIβ is cytosolic because of its C-terminal segment. To address the first possibility, we analyzed a series of truncated mutants of PI4KIIβ lacking the C-terminal 47, 74, or 141 residues. All three of these

mutants retained their ability to bind to Hsp90 and were sensitive to GA (Fig. 5). In addition, deletion of the region that is most diverse between the catalytic domains of PI4KIIα and β, residues 312–336, had no effect on Hsp90 binding or GA sensitivity. These results support the second possibility that PI4KIIβ requires stabilization by Hsp90 because of its weaker binding to membranes than PI4KIIα, a property that is conferred by its C-terminal 160 residues.

This conclusion was further supported by analysis of a truncated form of PI4KIIα, termed Cat α_{91-431}, which lacks its C-terminal 47 residues and consequently distributes predominantly to the cytosol (Fig. 6*A*). This mutant interacted with Hsp90 (Fig. 6*B*) and was susceptible to proteolytic degradation in the presence of GA (Fig. 6*C*), demonstrating that PI4KIIα contains an Hsp90-binding site that is normally occluded because of its tight association with membranes. Presumably, this tight membrane association also protects PI4KIIα from proteolytic degradation, although palmitoylation is apparently not required for stabilization, because an unpalmitoylated deletion mutant, PI4KIIα-ΔCCPCC, was not proteolytically degraded upon GA treatment (Fig. 5).

In summary, both PI4KIIα and β contain an Hsp90 interaction site, which most likely resides in the N-terminal lobe of the catalytic domains. In this respect, PI4KIIs are similar to other Hsp90-binding protein kinases, wherein the N-lobe cores have been shown to be essential for association with Hsp90 (38). PI4KIIα apparently does not require stabilization by Hsp90 binding because it associates strongly with membranes, even in the absence of palmitoylation. In contrast, PI4KIIβ must bind to Hsp90 and is highly sensitive to its release, perhaps because a substantial portion of this isoform is cytosolic. Apparently, the inactive, cytosolic pool of PI4KIIβ is very unstable unless it associates with its chaperone.

Dissociation of PI4KIIβ from Hsp90 by Exposure to GA Results in Transient Translocation to Membranes and Increased Kinase Activity—Because Hsp90 binds preferentially to soluble PI4KII molecules, it is also possible that Hsp90 sequesters PI4KIIβ to the cytosol. If so, GA treatment may promote redistribution of PI4KIIβ from cytosol to membranes. To detect this redistribution, it was necessary to subject cells to GA treatments shorter than 4 h, before the competing processes of ubiquitylation and proteolysis obscured the analysis. Cells expressing Myc-PI4KIIβ were treated with 2 μM GA for 0, 0.25, 1, or 3 h prior to fractionation into cytosolic, peripheral membrane, and integral membrane pools. The longer incubations (1 and 3 h) caused a decrease in PI4KIIβ levels from all three pools. However, the integral membrane-bound pool was much more stable, declining by only ~20% after a 3-h GA treatment *versus* 60–70% for the cytosolic and peripherally membrane-bound pools (Fig. 7*A*). This result further supports the view that tight membrane binding stabilizes the type II kinases despite the absence of Hsp90 binding. Exposure of cells to GA for only 15 min was sufficient to disrupt the Hsp90-PI4KIIβ interaction but was too brief to elicit a detectable loss of PI4KIIβ. Interestingly, this short GA treatment induces a partial translocation of the kinase from cytosol to membranes, increasing its proportion in peripheral and integral pools by 1.2- and 1.5-fold, respectively (Fig. 7*A*). The increase in the amount of integral

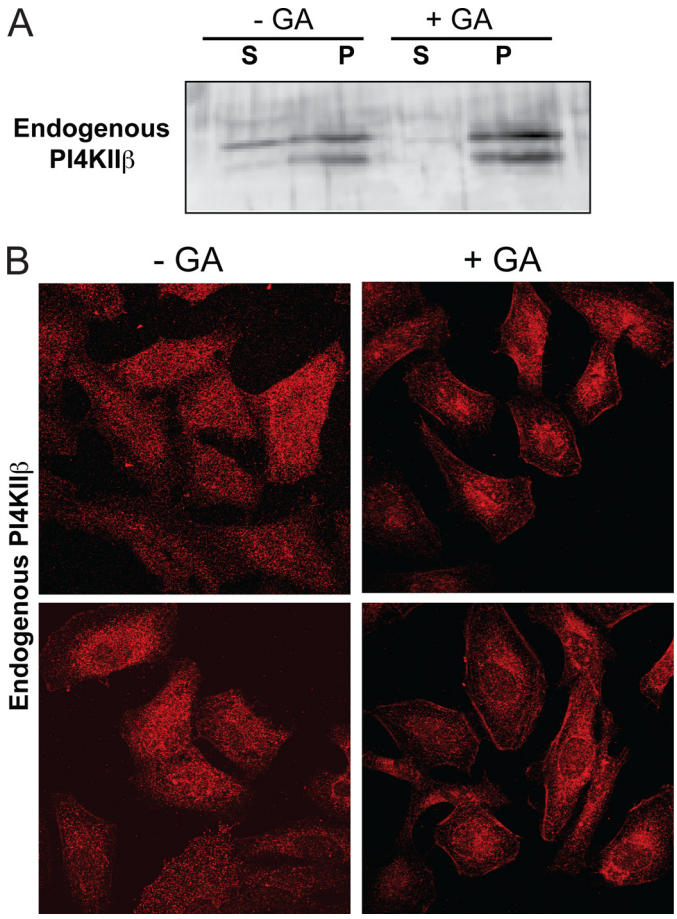


FIGURE 8. **Redistribution of endogenous PI4KIIβ to membranes in response to GA treatment.** *A*, cytosol (*S*) and membrane (*P*) fractions were prepared from untransfected HeLa cells, left untreated or treated for 2 h with 2 μM GA, as described in Fig. 7. The samples were electrophoresed, blotted, and stained with anti-PI4KIIβ antibodies (13). *B*, immunofluorescence localization of endogenous PI4KIIβ in control and GA-treated cells. HeLa cells were left untreated (*left panels*) or treated with 2 μM GA for 2 h (*right panels*). Fixed and permeabilized cells were stained with anti-PI4KIIβ primary antibodies and rhodamine-labeled secondary antibodies. Similar results were obtained in three separate experiments.

PI4KIIβ observed in GA-treated cells is particularly important, because only this species is palmitoylated and catalytically active (14). Indeed, 15-min GA treatment resulted in a 1.5-fold elevation in $[^{32}P]P_i$ incorporation into cellular PtdIns4P (Fig. 7*B*), corresponding closely to the increase in PI4KIIβ that is integrally associated with membranes.

To show that endogenous PI4KIIβ also responds to GA treatment by relocalizing to membranes, we electrophoresed membrane and cytosol fractions from COS cells that were treated or untreated for 2 h with 2 μM GA and blotted the gels with anti-PI4KIIβ antibodies. The endogenous kinase was nearly entirely depleted from cytosol of GA-treated cells (Fig. 8*A*). The GA-dependent redistribution of endogenous PI4KIIβ to membranes was confirmed by immunofluorescence microscopy (Fig. 8*B*). Interestingly, the increase in abundance of the kinase on the plasma membrane after GA treatment also occurs when cells are treated with growth factors (13). Therefore, we next asked whether activation of growth factor receptors disrupt the Hsp90-PI4KIIβ interaction. To address this question, COS cells were co-transfected with Myc-PI4KIIβ and FLAG-Hsp90, and

their co-immunoprecipitation was measured in response to treatment with EGF (50 ng/ml) or PDGF (100 ng/ml). Under both conditions, the PI4KIIβ-Hs90 complex was disrupted to a similar extent as was caused by a 15-min treatment with GA (Fig. 9).

DISCUSSION

Of the four mammalian PtdIns 4-kinase isoforms, PI4KIIβ is by far the least understood in terms of function or regulation. Unlike the other three isoforms, PI4KIIβ is almost evenly distributed between membranes and cytosol. The two type III kinases are almost entirely cytosolic (10), and the other type II isoform, PI4KIIα, is almost entirely membrane-bound (11).

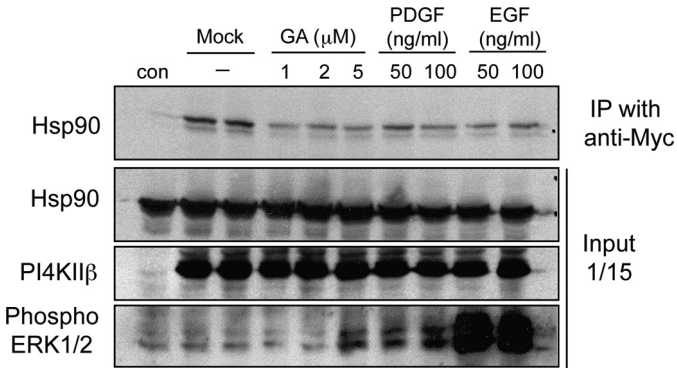


FIGURE 9. **EGF and PDGF disrupt the interaction between Hsp90 and PI4KIIβ.** COS cells were transfected with FLAG-Hsp90 alone or together with Myc-PI4KIIβ and starved for 8 h to achieve quiescence. The cells were then treated with GA, PDGF, or EGF at different concentrations for 15 min. Me_2SO was added as a mock treatment. The cell lysates were prepared in 1% Nonidet P-40 buffer, immunoprecipitated (*IP*) with anti-Myc antibodies, and blotted with anti-Hsp90 antibody. The inputs were immunoblotted with anti-Hsp90, anti-Myc, and anti-phospho-ERK1/2 antibodies (to demonstrate effectiveness of growth factor treatments). Note the slight increase in ERK1/2 phosphorylation at 5 μM GA, as reported (41, 42). These data are representative of three independent experiments. *con*, control.

Because cytosolic PI4KIIβ is catalytically inactive, its redistribution to membranes is likely to represent a major mechanism of regulation. Indeed, a portion of PI4KIIβ is recruited to membranes in response to stimuli, such as growth factor receptor activation (13). In an effort to explain the subcellular distribution of PI4KIIβ, we sought to identify binding partners that could either recruit the kinase to membranes or sequester it in the cytosol. Our results revealed that the molecular chaperone Hsp90 binds preferentially to cytosolic (and weakly membrane-bound) PI4KIIβ, inhibits its association with membranes, and protects it from proteolytic degradation. Moreover, the interaction between PI4KIIβ and Hsp90 was weakened upon treatment of cells with EGF or PDGF, suggesting that Hsp90 is a key regulator of stimulus-dependent PtdIns4P production. Based on our data, we propose a model for the life cycle of PI4KIIβ shown in Fig. 10. According to this model, cytosolic PI4KIIβ associates with Hsp90 for stabilization. An extracellular signal disrupts the interaction, allowing the free kinase to translocate to the membrane, where it may undergo palmitoylation and activation. Short exposure to GA may mimic the effect of growth factors. In contrast to PI4KIIβ, we suggest that PI4KIIα rapidly associates with Golgi membranes after its synthesis on cytosolic ribosomes and then is stably palmitoylated and, hence, constitutively active.

Hsp90 is essential for the maturation, stability, and translocation of a defined set of so-called "client" proteins (reviewed in Refs. 26, 31, 39, and 40). Currently there are more than 100 known Hsp90 clients, and many of those, including steroid hormone receptors, transcription factors, and protein kinases, participate in signal transduction pathways. To our knowledge, this is the first example of a lipid kinase as an Hsp90 client, although PI3K activity is indirectly regulated by Hsp90 (36). The chaperone function of Hsp90 is ATP-driven and involves the coordinated assistance of a variety of co-chaperones, as well

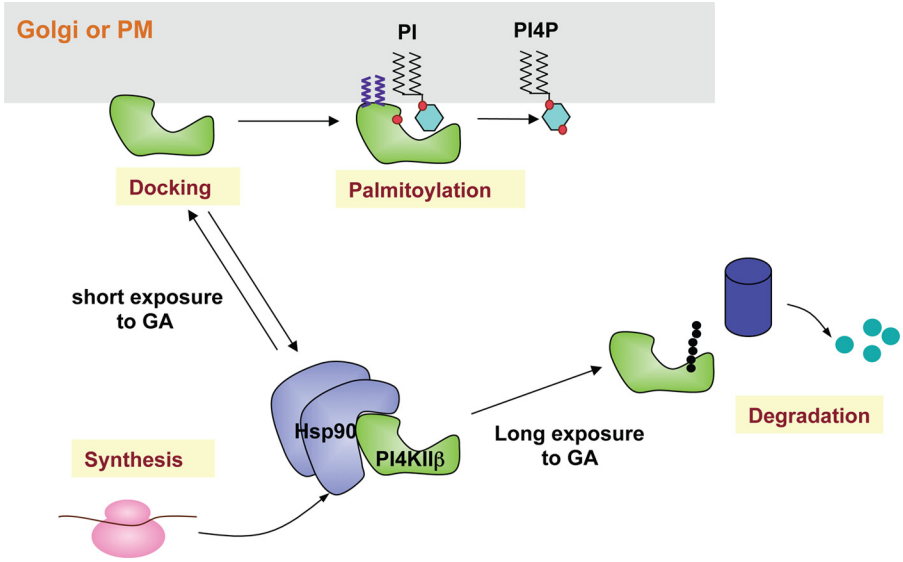


FIGURE 10. **Proposed life cycle of PI4KIIβ.** Hsp90 complex binds to cytosolic PI4KIIβ, either newly synthesized or dissociated from membranes. In response to stimuli, PI4KIIβ is released from the Hsp90 complex and then docks at the membrane through an as yet unidentified docking protein. Acyltransferases on the membrane palmitoylate peripherally bound PI4KIIβ leading to integral membrane binding and expression of catalytic activity. A portion of membrane-bound PI4KIIβ, particularly that which is peripherally associated, recycles to the cytosol, and is recaptured by Hsp90. GA disrupts the interaction between Hsp90 and PI4KIIβ. Short exposure to GA mimics the effect of other stimuli, whereas longer exposure to GA gives rise to proteasome-dependent degradation of PI4KIIβ.

as of Hsp70 (26, 27). Specific inhibitors of the Hsp90 ATPase reaction, such as GA, have been used to disrupt interactions between Hsp90 and its client proteins in cells and to establish the functional significance of these interactions (24). As shown in this study for PI4KIIβ, GA treatment has often resulted in enhanced proteolytic degradation and reduction in cellular levels of Hsp90 clients (reviewed in Ref. 41).

Based on our observations, we hypothesize that growth factor-dependent stimulation of PI4KIIβ is tightly and obligatorily linked to its release from Hsp90. There are other examples in the literature of Hsp90 serving as an inhibitor of kinase activity. For example, protein kinase R is activated by dsRNA, which triggers its dissociation from Hsp90 (42). Also, Src is transiently activated upon its release from Hsp90 (43). Thus, it appears that in some cases, interaction with Hsp90 can be inhibitory in the short term, although protective in the long term. At present, we have no information regarding the downstream signaling event(s) that might trigger growth factor-dependent dissociation of PI4KIIβ from Hsp90. The most obvious possibility, stimulus-dependent phosphorylation of PI4KIIβ, has not yet been examined, although it is interesting that this modification occurs only in the membrane-associated species (14). Moreover, because there is evidence that Hsp90 kinase interactions can be disrupted by phosphorylation (44), this direction will be pursued in future studies.

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