

Hsp90 increases LIM kinase activity by promoting its homo-dimerization

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SPECIFIC AIMS

THE ORIGINAL AIM of this study was to elucidate the mechanism that controls the concentration of LIM kinase 1 (LIMK1) in cells. Computer searches indicated that the kinase domain of LIMK1 contains Hsp90 recognition sequences similar to those found in ErbB2, an Hsp90 client protein. We, therefore, studied the effects of Hsp90 inhibition on the concentration of LIMK1 protein.

PRINCIPAL FINDINGS

1. LIMK1 stability is regulated by transphosphorylation

LIMK1 is a very stable protein with a half-life of ~20 h. In contrast, the half-life of two dominant-negative counterparts, which lack kinase activity (kinase dead) and therefore cannot be transphosphorylated, is only ~4 h. More importantly, the half-life of endogenous LIMK1 proteins that coimmunoprecipitated with the overexpressed wild-type or kinase-dead LIMK1 was similar to that of the overexpressed proteins (20 and 6 h, respectively). While overexpressed LIMK1 can transphosphorylate endogenous LIMK1, kinase-dead LIMK1 cannot transphosphorylate the endogenous protein, which results in unphosphorylated endogenous LIMK1 with a shorter half-life of 6 h. These results are consistent with the possibility that phosphorylation protects LIMK1 from degradation.

2. Hsp90 protects LIMK from degradation

Computer searches of protein databases revealed that LIMK1 contains a short amino acid sequence within its kinase domain similar to that of the ErbB-2 sequence responsible for its binding to Hsp90 (**Fig. 1A**). We, therefore, examined the effects of Hsp90 on the stability of these proteins. Treatment of 293T cells with the

well-characterized antagonists of Hsp90, 17-AAG, and Radicicol resulted in a dramatic reduction in the concentration of LIMK1 and LIMK2, with only 10% of the protein remaining after 24 h incubation (**Fig. 1B–D**). The decrease in LIMK levels was associated with reduced levels of p-cofilin, as incubation of cells with 17-AAG or Radicicol decreased the levels of endogenous p-cofilin, the main LIMK1 substrate, by 60 and 80%, respectively (**Fig. 1E**). This finding indicated that, in the absence of active Hsp90, LIMK proteins undergo degradation.

3. Identification of the amino acid-mediating LIMK1-Hsp90 association

To identify the amino acid within the kinase domains of LIMK1 and LIMK2 that is responsible for the association with Hsp90, we have chosen the proline-to-glutamic acid mutation because, in the case of EGFR/HER2, the GVGSPYVS sequence (alphaC-beta4 loop) of ErbB-2/HER2 determines Hsp90 binding. Based on analogies to other kinases, we proposed that replacing the proline of LIMK with an acidic residue would inhibit Hsp90 binding. Indeed the P394E mutation affected the interaction between LIMK1 and Hsp90, which resulted in significant reduction in the amount of LIMK1-P394E that was associated with Hsp90 compared with that of the wild-type protein (**Fig. 2A**). Moreover, addition of 17-AAG reduced Hsp90 association with wt-LIMK1 but had no further effect on its weak association with LIMK1-P394E or on its concentration in the cells (**Fig. 2A–C**). In vitro kinase assays showed that the level of LIMK-P394E phosphorylation

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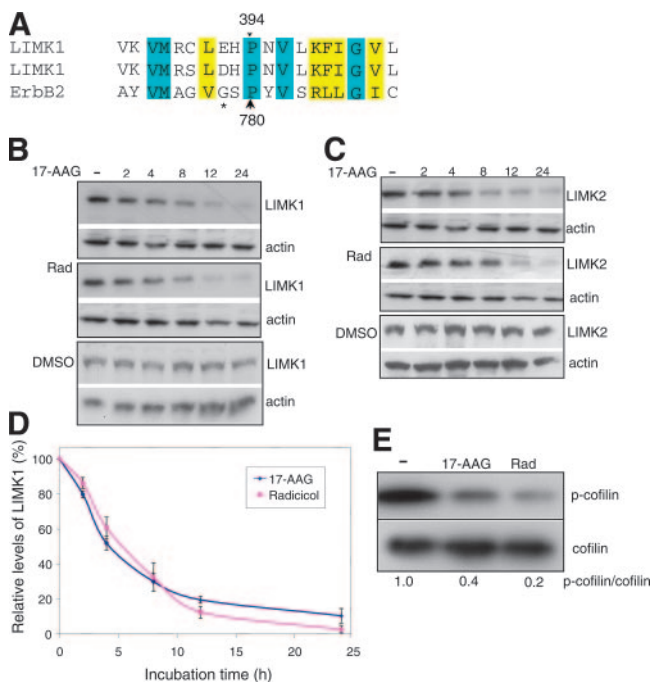


Figure 1. A) Sequence comparison of the ErbB-2 region responsible for binding to Hsp90 and the corresponding region in LIMK1 and LIMK2. Identical (cyan shade) or conservatively substituted residues (yellow shade) are shown boxed. The asterisk denotes the glycine residue implicated in interaction of ErbB-2 with Hsp90. The numbers represent the position of the proline residue in LIMK1 and ErbB-2. B–E) Inhibition of Hsp90 enhances the degradation of LIMK1 and LIMK2 and down-regulates p-cofilin levels. 293T cell lysates incubated with 1 μ M 17-AAG or 1 μ M Radicicol (Rad) for 2 to 24 h were subjected to immunoblotting with anti-actin rat anti-LIMK1 (B) or anti-LIMK2 (C) mAbs and rabbit antiactin Abs for loading control. D) Summary of the data presented in (B) and (C). E) Cell lysates of 293T incubated for 24 h with 1 μ M 17-AAG or Radicicol were subjected to Western blotting and probing with anti-phospho-cofilin (p-cofilin), and re-probing with anti-cofilin Abs. The numbers below represent the relative amount of p-cofilin after treatment with the inhibitors where its level in nontreated cells is set to 1.0.

was reduced by 70% compared with that of wt-LIMK1. While 17-AAG inhibited LIMK1 phosphorylation by 60%, it had only a slight effect (10%) on the level of transphosphorylation of the P394E mutant. Expression of LIMK1-P394E in 293T cells resulted in 25% reduction in the level of endogenous phospho-cofilin in these cells, indicating that the mutation influenced the level of LIMK1 transphosphorylation and its activity. Covalent cross-linking experiments revealed that wt-LIMK1 could form homodimers while the mutant was not able to dimerize. LIMK1 homodimerization was dependent on the presence of Hsp90, as no dimers were formed on addition of 17-AAG. Comparison of the half-lives of F-LIMK1 and the P394E mutant demonstrated that the half-life of LIMK1 was \sim 12 h, while that of LIMK1-P394E was only \sim 3 h. The half-life of the P394E mutant was similar to the half-life of the kinase-dead LIMK1-D460E.

CONCLUSION AND SIGNIFICANCE

LIMK1 is a very stable protein with a half-life of \sim 16–20 h. We have demonstrated previously that LIMK1 levels and its phosphorylation are markedly elevated in metastatic breast cancer cell lines compared with nonmetastatic and normal cells. The extended half-life of LIMK1 is not due to the stability of its mRNA because no significant differences were observed in the levels of LIMK1 mRNA in these cell lines. In addition, studies

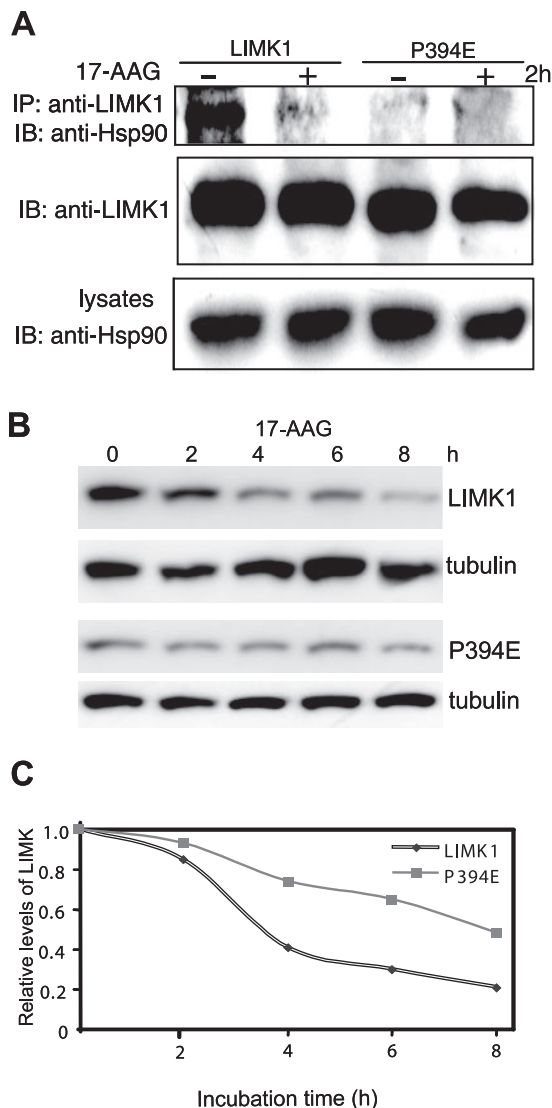


Figure 2. Proline 394 within the kinase domain of LIMK1 confers recognition by Hsp90 and sensitivity to 17-AAG. A) Lysates of 293T cells expressing Flag-LIMK1 (LIMK1) or F-LIMK1-P394E (P394E) were incubated in the absence or presence of 1 μ M 17-AAG for 2 h and subjected to immunoprecipitation with anti-LIMK1 monoclonal antibody followed by Western blotting and probing with anti-Hsp90 and anti-LIMK1 mAbs. B) 293T cells expressing wt-LIMK1 or LIMK1-P394E were incubated in the absence or the presence of 1 μ M 17-AAG for the period indicated (in hours). Cell lysates were subjected to Western blotting and probing with anti-Flag and anti-tubulin Abs. C) Summary of the data presented in (B). The experiments presented in (A) and (B) were performed twice and three times, respectively.

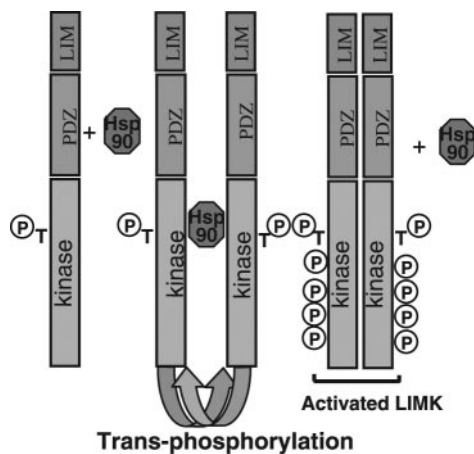


Figure 3. A proposed model depicting the role of Hsp90 in regulating the activity of LIMK1 and LIMK2. Hsp90 binding to a motif in the kinase domain of LIMK promotes a complex between two LIMK molecules facilitating transphosphorylation and stabilization of LIMK. After transphosphorylation, the complex between LIMK and Hsp90 is disrupted allowing LIMK interaction with its substrate cofilin.

using microarrays also did not reveal significant differences in the concentration of LIMK1 mRNA between invasive and noninvasive breast cancer cell-lines. Together these findings suggest that the concentration of LIMK1 protein is regulated by posttranslational modification. In this study we confirmed this hypothesis and furthermore have provided data to support that LIMK1 dimer formation and transphosphorylation are responsible for the stability of LIMK1 protein.

The data obtained in this study identified two new members in addition to the long list of Hsp90 client proteins. Binding of Hsp90 to the LIMK proteins protects them from degradation, as after 24 h incubation with Hsp90 inhibitors only less than 10% of these proteins can be detected. This corresponds to a half-life of ~4 h, which is similar to that of the kinase-dead LIMK1 and unphosphorylated endogenous LIMK1 proteins. These findings strongly suggest that Hsp90 promotes dimer formation and transphosphorylation of LIMK1. Indeed, we have shown here that Hsp90 binds to LIMK1, leading to the formation of LIMK1 homodimers. We have previously demonstrated that deletion mutants lacking the LIM and PDZ domains of LIMK1 have increased kinase activity. This was attributed to findings that the LIM and PDZ domains interact with the kinase domain and thereby inhibit its

association and activation by ROCK and PAK. We propose that the binding of Hsp90 to the kinase domain of LIMK1 enables the opening up of the molecule and promotion of homodimers formation.

Although the recognition motif of LIMK is similar to that of ErbB-2, the role that Hsp90 binding plays is different between these two protein kinases. While binding of Hsp90 to the kinase domain of ErbB-2 restrains its catalytic activity and prevents heterodimer formation with other ErbB proteins, Hsp90 binding to the kinase domain of LIMK1 results in homodimer formation enabling LIMK1 transphosphorylation and its activation. Our cross-linking experiments with over-expressed LIMK1 demonstrated the formation of LIMK1 homodimers of 140 kDa only in the absence of 17-AAG. In contrast, LIMK1-P394E does not form homodimers in the absence or presence of the Hsp90 inhibitors. This suggests that the interaction between these two proteins depends on the Hsp90 binding motif in the kinase domain of LIMK.

Recently it was revealed that the interaction between Hsp90 and ErbB-2 is mediated by a loop in the N lobe of the kinase domain of ErbB-2. Mutations in this loop that disrupt the positively charged patch and the hydrophobic strip abolish the association between these two molecules. Indeed, modeling of the kinase domain of LIMK1 demonstrated the existence of a similar loop also in LIMK1 and other EGFR family members. Moreover, comparison of the sequence within this loop demonstrated that more than half the residues are identical or conservatively substituted between LIMK1 and ErbB-2. Interestingly, three amino acids conserved between LIMK and ErbB-2 are also shared between ErbB-2 and ErbB-1 and are not involved in the association between ErbB-2 and Hsp90. In contrast, mutation of P394, a residue conserved among LIMK1, ErbB-2, and ErbB-1 to the negatively charged glutamic acid, greatly reduced the association between LIMK1 and Hsp90. It remains to reveal whether changes in the charge or/and the backbone conformation, or the introduction of a longer side chain in this position of this region affect the interaction between these two molecules.

Given the fact that Hsp90 inhibitors are known to be potent anticancer drugs and are currently in clinical trials, this study postulates a novel anticancer mechanism of Hsp90 inhibitors via their perturbation of LIMK family function, molecules that have previously been implicated in tumor growth, invasion, and metastasis. FJ

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ABSTRACT LIM kinase 1 (LIMK1) is a serine protein kinase that regulates the actin cytoskeleton by phosphorylation and inactivation of actin depolymerizing factor cofilin. LIMK1 activity is regulated by the Rho-GTPases via their serine/threonine kinase effectors Rho-kinase and p21-activated kinases 1 and 4 that phosphorylate LIMK1 on threonine 508 in its activation loop. The purpose of this study was to elucidate the pathway leading to the stability of LIMK1, a protein with a half-life of ~20 h. Because the half-life of kinase-dead LIMK1 is only 4 h, it is suggestive that trans- or auto-phosphorylation is responsible for the stabilization of LIMK1. Using known Hsp90 inhibitors, we have shown that the half-life of LIMK1 in cells depends on the presence of active Hsp90. Furthermore, endogenous LIMK1 coimmunoprecipitated with endogenous Hsp90 and this interaction promoted LIMK1 homodimer formation as seen by cross-linking experiments. Hsp90 binds LIMK1 via a recognition sequence within the LIMK1 kinase domain, homologous to that of ErbB-2. Mutation of a proline residue within this sequence to glutamic acid reduces its interaction with Hsp90, inhibits homodimer formation, and reduces its half-life to 4 h. These findings implicate Hsp90 in the stabilization of LIMK1 by promoting homodimer formation and transphosphorylation. Li, R., Soosairajah, J., Harari, D., Citri, A., Price, J., Ng, H. L., Morton, C. J., Parker, M. W., Yarden, Y., Bernard, O. Hsp90 increases LIM kinase activity by promoting its homo-dimerization. *FASEB J.* 20, E417–E425 (2006)

Key Words: Rho-GTPases • actin dynamics • half-life • trans-phosphorylation

THE LIM KINASE (LIMK) family of serine protein kinases includes two members, LIMK1 and LIMK2, which have identical genomic structure and share more than 50% homology (1, 2). LIMK1 regulates actin dynamics by phosphorylation and inactivation of the actin-depolymerizing factors (ADF)/cofilin, resulting in the stabilization of actin filaments (3, 4). The activity of LIMK1 is regulated by the members of the Rho-

GTPases via their effector serine/threonine kinases: Rho kinase (ROCK) and the p21 activated kinases (PAK), PAK1, and PAK4 (5–7). These kinases activate LIMK1 by phosphorylation of threonine 508, located in the activation loop of the kinase domain (8). Overexpressed and endogenous LIMK1 can form homodimers and heterodimers with LIMK2 (our unpublished observations). Homodimer formation results in further activation of LIMK by transphosphorylation. The level of LIMK1 phosphorylation positively correlates with its activity (our unpublished observations). Indeed, we have found very high levels of phosphorylated LIMK1 in metastatic breast and prostate cancer cell lines (9). These cell lines have increased LIMK1 activity as judged by increased levels of endogenous phospho-cofilin, the main substrate of LIMK. It is estimated that LIMK1 contains at least 5 phospho-amino acids in its kinase domain, which are mainly phospho-serines (10). We have recently demonstrated that slingshot (SSH) phosphatase, originally identified as cofilin phosphatase, is also a LIMK1 phosphatase. SSH dephosphorylates LIMK1 on Thr 508 as well as on the transphosphorylated residues, resulting in down-regulation of LIMK1 activity (11). The exact role of the transphosphorylated form of LIMK1 is not yet known.

The heat shock protein 90 (Hsp90) is a molecular chaperone that is ubiquitously expressed and is responsible for the biogenesis, regulation, and functionality of more than 100 proteins (client proteins), most of which are involved in signal transduction (12). Hsp90 regulates the activity of its client proteins by the formation of complexes and by its ATPase activity (13, 14). For example, it was recently demonstrated that Hsp90 regulates the activity of the tyrosine kinase receptor ErbB-2 by binding to a specific loop within its kinase domain, thereby restricting heterodimer formation

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with ErbB-1 or ErbB-3 (15, 16). The motif within a loop in the ErbB-2 kinase domain that mediates its association with Hsp90 has been recently identified, and a point mutation within this loop disrupts Hsp90 association (16).

Here we demonstrate that transphosphorylation of LIMK1 increases its stability. We further show that endogenous LIMK1 associates with Hsp90 *in vitro* and results in homodimer formation of LIMK1 leading to the transphosphorylation and increased activity and stability of LIMK1.

MATERIALS AND METHODS

Reagents and antibodies

17-AAG and Radicicol were dissolved in DMSO (1 mM) and were used at a concentration of 1 μ M. 17-AAG was kindly provided by Dr. E. Sausville (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD, USA) and Radicicol was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rat anti-LIMK1 and anti-LIMK2 mAbs were generated as described previously (17). Rat anti-Hsp90 monoclonal antibody (mAb) (SPA-835) was obtained from Stressgen Biotechnologies (Ann Arbor, MI, USA). Rabbit anti-phospho-cofilin antibodies were a gift from James R. Bamburg (Colorado State University); rabbit anti-cofilin antibodies were obtained from Cytoskeleton; and Flag-M2 mAb was from Sigma.

Plasmid constructs

pEF-BOS-Flag-LIMK1 (F-LIMK1) and the LIMK1 domains were described previously (18). Glutathione (GSH) S-transferase (GST)-LIMK1-D460E was constructed by subcloning a 2.2 kb *Bgl*II/*Xba* fragment from pEF-BOS-Flag-LIMK1-D460E (3) into the pEBG vector. LIMK1-P394E mutation was generated by polymerase chain reaction, and the mutation was confirmed by sequencing.

Cell culture, transfection, immunoprecipitation, and immunoblot analyses

All cell lines were cultured in DME medium supplemented with 10% fetal calf serum. 293T cells were transfected using FuGENE 6 (Roche Diagnostics, Indianapolis, Indiana, USA). After 48 h, cell lysates were prepared as described previously (18). For LIMK1 and Hsp90 immunoprecipitation, lysates were incubated with the mAbs and protein G-agarose beads. Western blotting was done as described (11).

Cross-linking and siRNA transfections

Cross-linking experiments were performed by addition of *bis* (sulfosuccinimidyl) suberate (BS⁻³) (2 mM) to the lysis buffer for 2 h on ice before addition of Tris-HCl (50 mM) for an additional 15 min. Hsp90 siRNA oligonucleotide (5'ACAA-GAAGAAGAAGAAGAA3') was from Dharmacon (Chicago, IL, USA). 293T cells were plated in 6-well plates at 60% confluency 24 h before transfection. Opti-MEM (250 μ l) containing 250 pmol siRNA was mixed with 250 μ l Opti-MEM containing 5 μ l of lipofectamine 2000 (Invitrogen) and incubated at RT for 20 min. This siRNA mixture was added to the cells containing 2 ml Dulbecco's modified Eagle's me-

dium. After 48 h, cells were prepared for the cross-linking experiments.

In vitro kinase assays

The *in vitro* kinase assay was performed as described previously (3). After SDS-PAGE and Western blotting, phosphorylated proteins were detected and quantitated using a PhosphorImager (400 series; Molecular Dynamics). The level of the loaded proteins was determined by immunoblotting and densitometry analysis.

Metabolic labeling with [³⁵S]-methionine

293T cells were transfected with GST-LIMK1 or LIMK1-D460E, and 48 h later they were washed twice with methionine-free DME medium (Thermo Trace Ltd.) containing 1% FCS or 10% dialyzed FCS. Following washes, the cells were incubated with the same medium containing 0.1 mCi/mL [³⁵S]-protein labeling mixture (NEN/PE) for 1 h. The cells were then washed twice with normal culture medium (DME/10% FCS) and incubated in this medium for various time periods before harvest. The labeled overexpressed proteins were purified with glutathione Sepharose beads and analyzed by SDS-PAGE and Western transfer followed by exposure to PhosphorImager.

LIM kinase modeling

The modeling of the kinase domain of LIMK1 was based on a crystal structure of human tyrosine kinase protein c-Src in complex with AMP-PNP (PDB code: 2SRC). The modeling process was performed by COMPOSER as contained in Sybyl7.1 (Tripos Inc.) followed by energy minimization in Sybyl7.1. The quality of the model was evaluated by Verify3D (19) and shown to be of good quality. There were only two small regions where the modeling was uncertain: residues 365–378 and 562–581. The sequences used for building the model of the LIMK1 kinase domain were combined with the sequences of the epidermal growth factor (EGF) receptor tyrosine kinase domains to produce a more extensive sequence alignment by ClustalX (data not shown) (20).

RESULTS

LIMK1 stability is regulated by transphosphorylation

LIMK1 protein is highly expressed and highly phosphorylated in metastatic breast cancer cells (9), however, the levels of its mRNA are similar in all cell lines examined (data not shown), suggesting that phosphorylation protects it from degradation. We, therefore, compared the half-life of LIMK1 with two dominant-negative counterparts that lack kinase activity and therefore could not be transphosphorylated. LIMK1-s is a splice variant that lacks 20 amino acids within the kinase domain, and LIMK1-D460E lacks kinase activity due to a point mutation in its kinase domain (3). This point mutation replaces an aspartic acid, conserved in all protein kinases and essential for their kinase activity, with glutamic acid that has no effect on the structure of the protein. 293T cells expressing GST-LIMK1, GST-LIMK1-s, or GST-LIMK1-D460E were metabolically la-

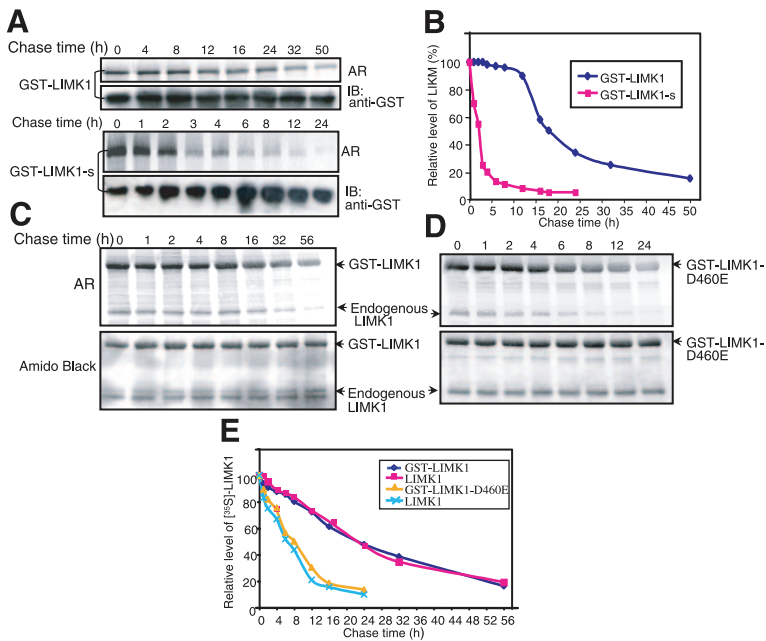


Figure 1. The half-life of LIMK1 is regulated by transphosphorylation. *A*) GST-LIMK1, or GST-LIMK1-s constructs were transfected into 293T cells. The cells were pulse-labeled with [³⁵S]-methionine and chased for the time indicated (in hours). The GST-tagged proteins were purified with glutathione Sepharose beads followed by immunoblotting. The membrane was exposed to PhosphorImager [autoradiogram (AR)] followed by probing with anti-glutathione S-transferase antibodies. *B*) Summary of the data presented in (*A*). *C, D*) 293T cells expressing GST-LIMK1 (*C*) or GST-LIMK1-D460E (*D*) were pulse-labeled with [³⁵S]-methionine, the LIMK1 proteins were immunoprecipitated with rat anti-LIMK1 mAb and subjected to Western blotting, exposure to PhosphorImager, and staining with Amido Black to visualize the immunoprecipitated proteins. *E*) Summary of the data presented in (*C*) and (*D*). Note the two bands corresponding to endogenous LIMK1 (*C*, bottom panel). The higher band corresponds to phospho-LIMK1 and is of the same size as the endogenous [³⁵S]-LIMK1 (*C*, top panel). These experiments were performed twice with similar results.

beled for 1 h with [³⁵S]-methionine followed by washing with normal medium and culturing for up to 56 h. The purified proteins were subjected to immunoblotting and exposure to PhosphorImager. The results show that GST-LIMK1 is extremely stable with a half-life greater than 20 h (Fig. 1A, top panel, and B). In contrast, the half-life of LIMK1-s and LIMK1-D460E proteins, lacking kinase activity, was only ~4 and 6 h, respectively (Fig. 1A, bottom panel and Fig. 1D, respectively). Because the half-life of the unphosphorylated LIMK1 proteins is much shorter than that of wt-LIMK1, these results are consistent with the possibility that phosphorylation protects LIMK1 from degradation.

To measure the half-life of endogenous LIMK1, we coimmunoprecipitated the overexpressed GST-LIMK1 or GST-LIMK1-D460E proteins together with endogenous LIMK1 with rat anti-LIMK1 mAb. While the half-life of the endogenous LIMK1 protein is similar to that of the coexpressed GST-LIMK1 (Fig. 1C and E), the half-life of endogenous LIMK1 in 293T cells expressing GST-LIMK1-D460E is similar to that of the overexpressed protein (~6 h) (Fig. 1D and E). GST-LIMK1 can transphosphorylate endogenous LIMK1 (see the top band of endogenous LIMK1 at the lower panel of Fig. 1C), while GST-LIMK1-D460E has no kinase activity and therefore cannot transphosphorylate endogenous LIMK1 resulting in unphosphorylated LIMK1 with a shorter half-life.

Hsp90 protects LIMK from degradation

It has been recently demonstrated that a short amino acid sequence within the kinase domain of ErbB-2 is responsible for its binding to Hsp90 (15, 16). Computer searches of protein databases revealed that a similar sequence exists in the kinase domain of LIMK1 and LIMK2 (Fig. 2A). We therefore examined the

effects of Hsp90 on the stability of these proteins. Treatment of 293T cells with the well-characterized antagonists of Hsp90, 17-AAG, and Radicolol, resulted in a dramatic reduction in the level of LIMK1 and LIMK2, with only 10% of the protein remaining after 24 h incubation (Fig. 2B–D). The decrease in LIMK levels was associated with reduced levels of p-cofilin, as incubation of cells with 17-AAG or Radicolol decreased the levels of endogenous p-cofilin, the main LIMK1 substrate, by 60 and 80%, respectively (Fig. 2E), indicating that in the absence of active Hsp90, LIMK proteins undergo degradation.

Hsp90 interacts with LIMK and promotes its homodimerization

To demonstrate that Hsp90 interacts with LIMK1 and promotes its homodimerization, we performed coimmunoprecipitation assays with endogenous LIMK1 and Hsp90 proteins using MDA-MB-231 cell lysates. Hsp90 was present in the immunoprecipitated LIMK1 complex. Likewise, LIMK1 coimmunoprecipitated with Hsp90, indicating that endogenous LIMK1 associates with endogenous Hsp90 in these cells (Fig. 3A). This association was greatly reduced after incubation with 17-AAG and Radicolol (Fig. 3B). The interaction between LIMK1 and Hsp90 was mediated via the kinase domain of LIMK1 (Fig. 3C).

To show that the association between endogenous LIMK1 and Hsp90 proteins results in LIMK1 homodimerization, we performed covalent cross-linking experiments, in the presence or absence of the Hsp90 antagonist 17-AAG as well as in the presence of Hsp90 siRNA. The formation of LIMK1 or LIMK2 homodimers was dependent on their association with Hsp90 because, in the presence of 17-AAG, the levels of LIMK1 or LIMK2 dimers were greatly reduced (Fig. 4A,

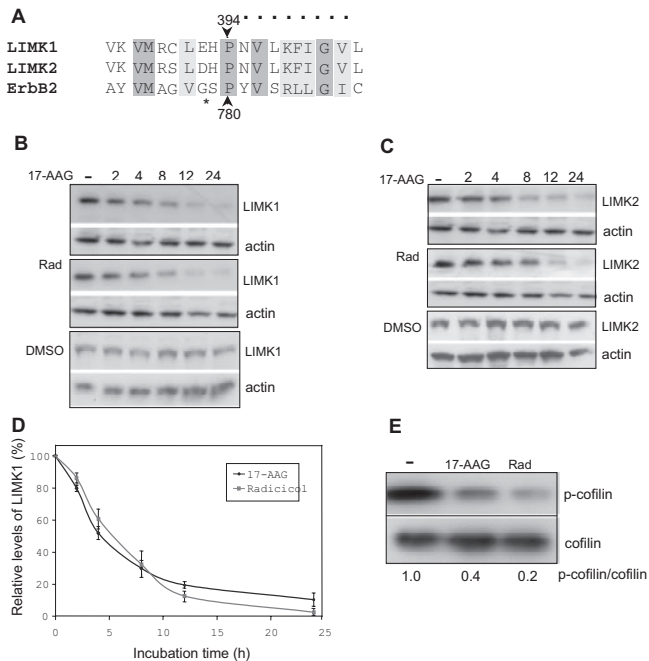


Figure 2. A) Sequence comparison of the ErbB-2 region responsible for binding to Hsp90 and the corresponding region in LIMK1 and LIMK2. Identical (cyan shade) or conservatively substituted residues (yellow shade) are shown. The asterisk denotes the glycine residue implicated in interaction of ErbB-2 with Hsp90. The numbers represent the position of the proline residue in LIMK1 and ErbB-2. B-E) Inhibition of Hsp90 enhances the degradation of LIMK1 and LIMK2 and down-regulates p-cofilin levels. B, C) 293T cell lysates incubated with 1 μ M 17-AAG or 1 μ M Radicicol (Rad) for 2 to 24 h were subjected to immunoblotting with rat anti-LIMK1 (B) or anti-LIMK2 (C) mAbs and rabbit anti-actin Abs for loading control. D) Summary of the data presented in (B). E) Cell lysates of 293T incubated for 24 h with 1 μ M 17-AAG or Radicicol were subjected to Western blotting and probing with anti-phospho-cofilin (p-cofilin), and reprobing with anticofilin Abs. The numbers below represent the relative amount of p-cofilin after treatment with the inhibitors where its level in nontreated cells is set to 1.0. These experiments were performed twice with similar results.

left and right panels, respectively). Furthermore, transfection of Hsp90 siRNA that resulted in down-regulation of the level of Hsp90 monomers (Fig. 4B, middle panel) had a great effect on the formation of LIMK1 dimers, as no dimers were observed in the presence of Hsp90 siRNA (Fig. 4B, top panel). High MW LIMK1 complexes were also evident after cross-linking (Fig. 4B, top panel), indicating that LIMK1 forms complex with other proteins. Indeed, we and others have previously demonstrated that LIMK1 interacts with Bone Morphogenetic Protein Receptor (21), slingshot phosphatase (11) and 14-3-3 ζ (22, 23). The overexpressed F-LIMK1 also forms homodimers in the presence of the covalent cross-linker BS⁻³, but no dimerization takes place in the presence of 17-AAG (Fig. 7C, first 2 lanes). These results demonstrate that Hsp90 interacts with both LIMK1 and LIMK2 to facilitate the formation of homodimers.

Identification of the amino acid mediating LIMK1-Hsp90 association

We have constructed a model of the LIMK1 kinase domain using c-Src tyrosine kinase, as described in the

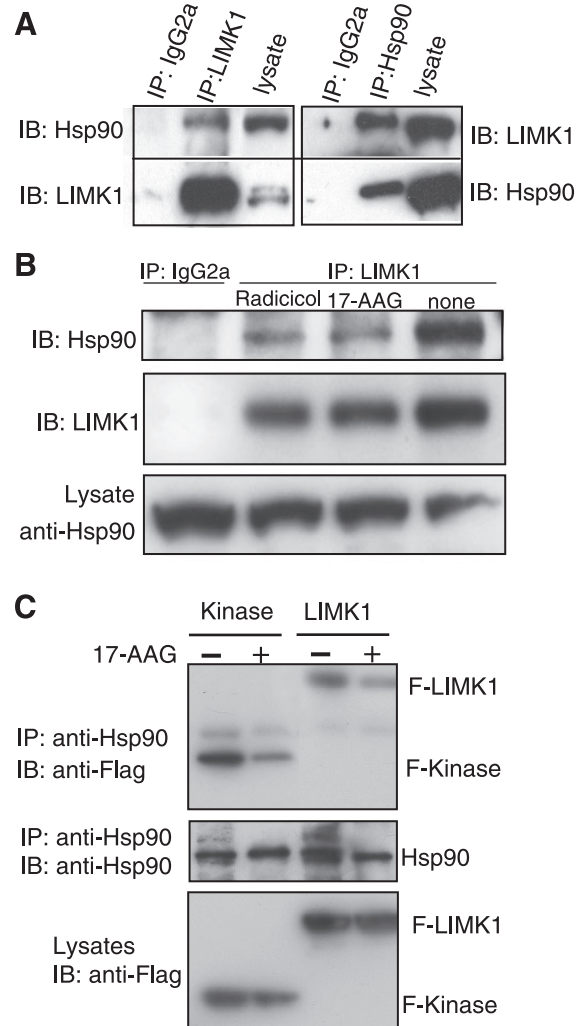


Figure 3. Endogenous LIMK1 interacts with endogenous Hsp90. A) LIMK1 or Hsp90 were immunoprecipitated from MDA-MB-231 cells with anti-LIMK1 (left panel) or anti-Hsp90 mAbs (right panel) followed by Western blotting and probing with anti-LIMK1 and anti-Hsp90 mAbs. B) The Hsp90 inhibitors inhibit the interaction between endogenous LIMK1 and Hsp90. LIMK1 was immunoprecipitated from lysates of MDA-MB-231 cells treated with 1 μ M 17-AAG or Radicicol for 2 h and subjected to immunoblotting. The filter was probed with anti-Hsp90 and anti-LIMK1 mAbs. C) Hsp90 interacts with the kinase domains of LIMK1. 293T cells were transfected with Flag-tagged full-length LIMK1 (LIMK1), and the kinase domain (kinase). After 48 h, cells were treated with 1 μ M 17-AAG for 2 h and lysates were immunoprecipitated with anti-Hsp90 mAb and subjected to immunoblotting. The filters were probed with anti-Flag mAb to show the Flag-tagged LIMK1 proteins that interact with Hsp90 (top panel) and the adequate expression of these proteins in the lysate (bottom panel) as well as with anti-Hsp90 mAb as immunoprecipitation control (middle panel). The experiments presented in (A) and (B) were performed twice while those in (C) were performed three times with similar results.

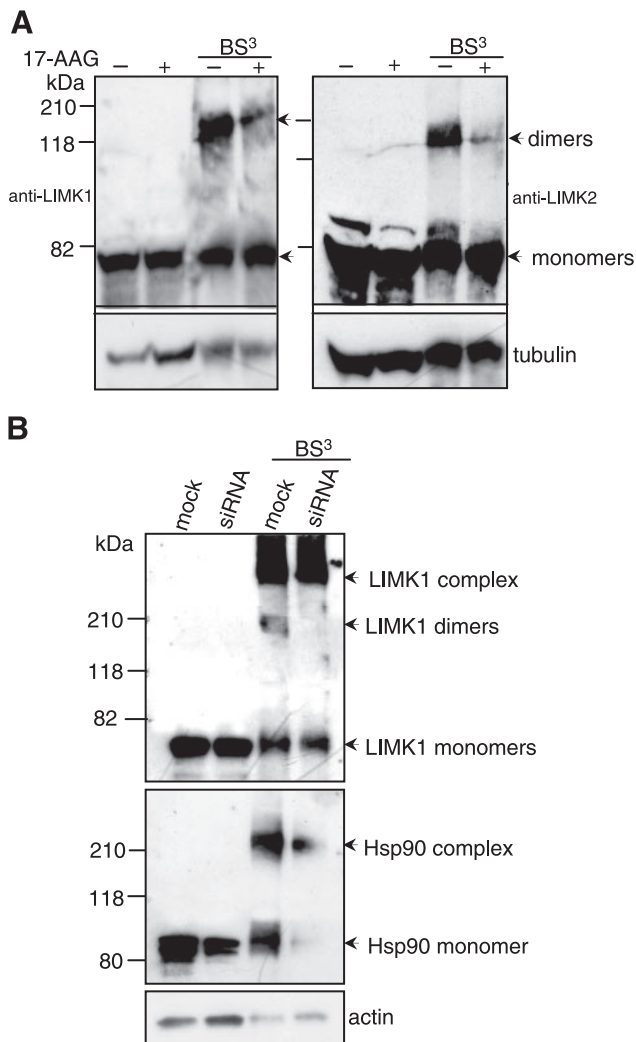


Figure 4. Hsp90 mediates dimer formation of endogenous LIMK1 and LIMK2. *A*) 293T cells incubated with or without 1 μ M 17-AAG for 4 h were washed and lysed in buffer containing BS³ followed by Western blotting and probing with anti-LIMK1 (*left panel*) or anti-LIMK2 (*right panel*) and with anti-tubulin Abs (bottom panels). *B*) Cell lysates of 293T cells transfected, with or without (mock) Hsp90 siRNA for 48 h, were subjected to cross-linking and Western blotting. The filter was probed with anti-LIMK1 (*top panel*), anti-Hsp90 (*middle panel*), and anti-actin (*bottom panel*) antibodies. These experiments were performed three times.

Experimental Procedures. As no experimental structure is available for the kinase domain of ErbB-2, we have used available structures from other members of the EGF receptor tyrosine kinase family as a basis for structural comparison. A number of such structures were superimposed on the LIMK1 model and its template structure, c-Src. Despite the low sequence identity across the family (data not shown), the conformation of the LIMK1 Hsp90 binding motif is well conserved (**Fig. 5**). Thus recognition of LIMK1 by Hsp90 is likely to be encoded by the region of primary structure shown in Fig. 2A. More than half the residues in the loop are identical or are conservatively substituted between LIMK and ErbB-2. In contrast, there is no sequence conservation in this region across the other members of the EGFR family, except with one residue (Phe 394 in LIMK1) showing some conservation.

Having identified, within the sequence of the kinase domain of LIMK1, an amino acid sequence similar to that responsible for the binding of ErbB-2 to Hsp90 (alphaC-beta4 loop) (Fig. 2A), we attempted to identify the amino acid within the kinase domains of LIMK1 and LIMK2 that is responsible for the association with Hsp90. We have chosen the proline to glutamic acid mutation because, in the case of EGFR/HER2, the GVGSPYVS sequence (alphaC-beta4 loop) of ErbB-2/HER2 determines Hsp90 binding. Based on analogies to other kinases we proposed that replacing the proline of LIMK with an acidic residue would inhibit Hsp90 binding.

We first tested whether the P394E mutation affected the interaction between LIMK1 and Hsp90 and found significant reduction in the amount of LIMK1-P394E that was associated with Hsp90 in comparison with that of the wild-type (wt) protein (**Fig. 6A**). While addition of 17-AAG reduced Hsp90 association with wt-LIMK1, it had no further effect on its weak association with LIMK1-P394E (Fig. 6A, *right lanes*).

Next, we examined the effect of 17-AAG on the stability of LIMK-P394E. While after 4 h incubation with the inhibitor the concentration of F-LIMK1 was reduced by 75%, the concentration of F-LIMK-P394E was reduced only by 20%. After 8 h incubation with the inhibitor, the concentration of LIMK1 was reduced by 80% while that of the mutant was reduced only by 50% (Fig. 6B and C).

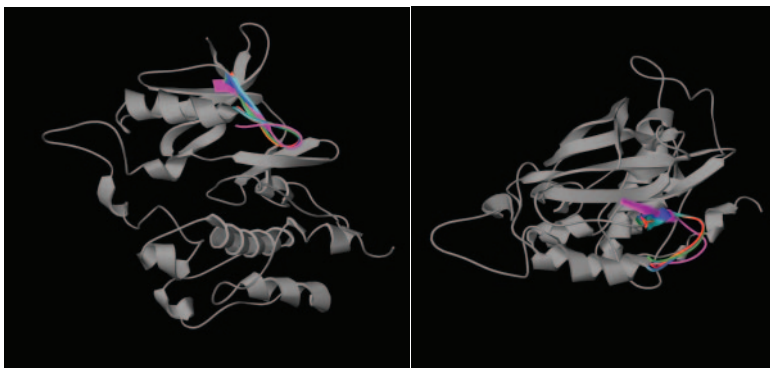


Figure 5. A model of the kinase domain of LIMK1 in gray ribbon representation shown in two different orientations. The structures of four different kinase domains from the EGFR family were superimposed on the model of LIMK1. The superposition against the LIMK1 Hsp90 binding loop is highlighted by different colors. The color code is as followed: LIMK1: cyan, 2SRC (c-Src): blue, 1XKK: (EGFR kinase domain complexed with a Quinazoline inhibitor GW572016): green, 1R1W (kinase domain of hepatocyte growth factor receptor): red and 1UU9 (Pdk1 kinase domain in complex with Bim-3): pink.

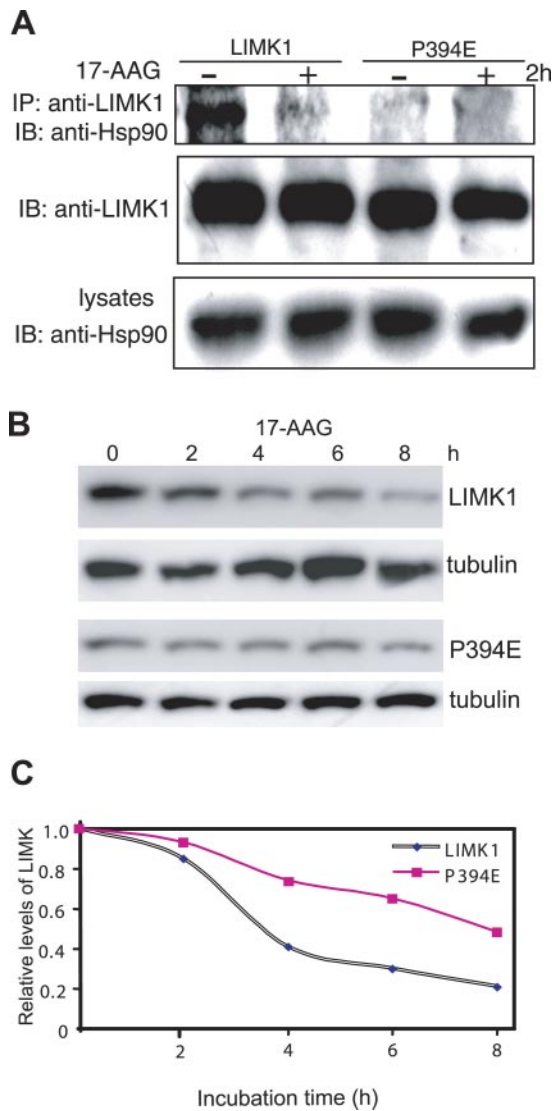


Figure 6. Proline 394 within the kinase domain of LIMK1 confers recognition by Hsp90 and sensitivity to 17-AAG. *A*) Lysates of 293T cells expressing Flag-LIMK1 (LIMK1) or F-LIMK1-P394E (P394E) were incubated in the absence or presence of 1 μ M 17-AAG for 2 h and subjected immunoprecipitation with anti-LIMK1 mAb followed by Western blotting and probing with anti-Hsp90 and anti-LIMK1 mAbs. *B*) 293T cells expressing wt-LIMK1 or LIMK1-P394E were incubated in the absence or the presence of 1 μ M 17-AAG for the period indicated (in hours). Cell lysates were subjected to Western blotting and probing with anti-Flag and anti-tubulin Abs. *C*) Summary of the data presented in *B*). The experiments presented in *A*) and *B*) were performed twice and three times, respectively.

To test whether the mutation affected the transphosphorylation of LIMK1 we performed in vitro kinase assay in the presence or absence of 17-AAG. We found that the level of LIMK-P394E phosphorylation was reduced by 70% in comparison with that of wt-LIMK1 (Fig. 7A). While 17-AAG inhibited LIMK1 phosphorylation by 60%, it had only a slight effect (10%) on the level of transphosphorylation of the P394E mutant (Fig. 7A). In addition, expression of LIMK1-P394E in 293T cells resulted in 25% reduction in the level of endoge-

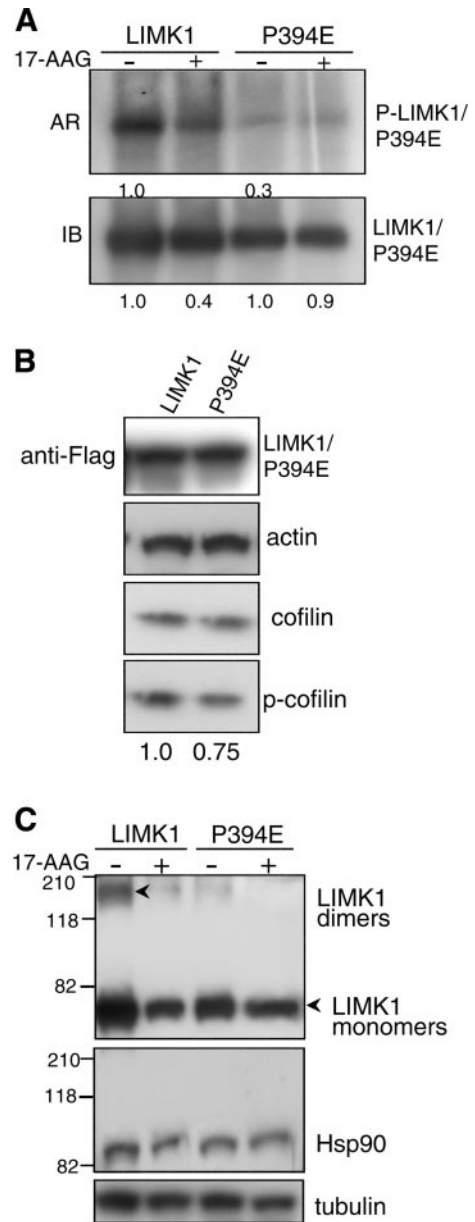


Figure 7. The concentration of LIMK1-P394E protein and its phosphorylation are not affected by 17-AAG. *A*) Overexpressed F-LIMK1 and F-LIMK1-P394E incubated in the absence or presence of 17-AAG for 4 h were immunoprecipitated from 293T cells with anti-Flag mAb followed by in vitro kinase assay Western blotting. The membrane was exposed to PhosphorImager (AR) followed by probing with anti-Flag Abs. The numbers below the top panel represent the relative radioactivity of the LIMK1 and LIMK1-P394E proteins, while those below the bottom panel represent the relative concentration of the LIMK1 proteins in the absence or presence of the inhibitor, where the concentration of the LIMK1 proteins in the absence of 17-AAG is set to 1. *B*) Western blot analysis of cell lysates of 293T cells overexpressing F-LIMK1 or F-LIMK1-P394E probed with anti-Flag, anti-actin, anti-p-cofilin, and anticofilin Abs. The numbers below represent the ratio between p-cofilin and cofilin where that of WT LIMK1 is set to 1. *C*) LIMK1 P394E cannot form homodimers. 293T cells overexpressing LIMK1 or LIMK1 P394E were treated with 1 μ M 17-AAG for 4 h followed by lysis in buffer containing BS³. The lysates were subjected to Western blot analysis and probing with anti-Flag (*top panel*), anti-Hsp90 (*middle panel*), and antitubulin (*bottom panel*) antibodies. These experiments were performed three times with similar results.

nous phospho-cofilin in these cells (Fig. 7B), indicating that the mutation influenced the level of LIMK1 transphosphorylation and its activity.

To strengthen the notion that the reduced phosphorylation and kinase activity of the P394E mutant is due to its inability to form dimers, we tested, by covalent cross-linking, its capacity to form homodimers. While overexpressed wt-LIMK1 could form homodimers, the mutant was not able to dimerize. LIMK1 homodimerization was dependent on the presence of Hsp90, as no dimers were formed in the presence of 17-AAG (Fig. 7C).

Finally, we compared the half-lives of F-LIMK1 and the P394E mutant. As expected, the half-life of LIMK1 was ~12 h (Fig. 8A and C) while that of LIMK1-P394E was only ~3 h (Fig. 8B and C). The half-life of the P394E mutant was similar to the half-life of the kinase dead LIMK1-D460E.

Together, these results demonstrate that P394 is responsible, in part, for the association between LIMK1 and Hsp90 and that mutation of this proline to glutamic acid inhibits the formation of LIMK1 homodimers, resulting in reduced transphosphorylation and the activity of LIMK1 as well as its half-life.

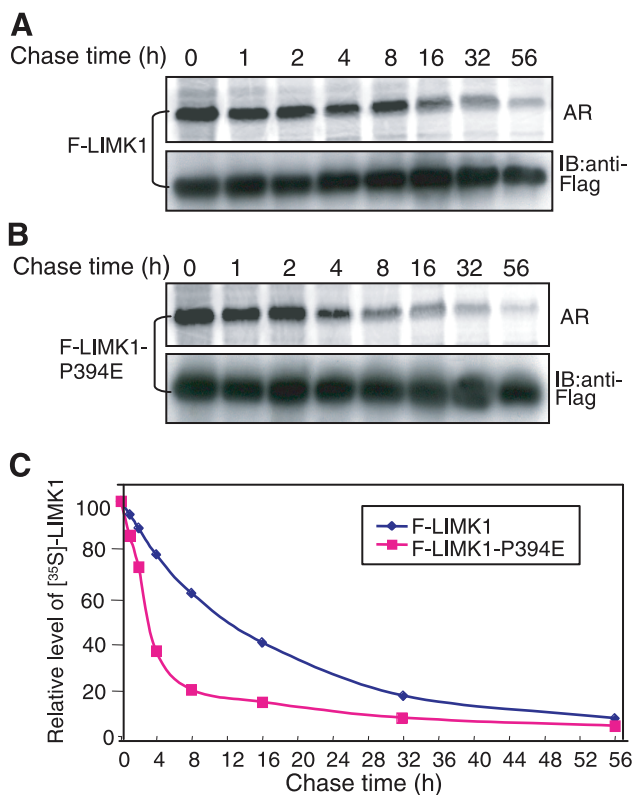


Figure 8. The half-life of LIMK1-P394E is greatly reduced. A, B) 293T cells expressing F-LIMK1 (A) or F-LIMK1-P394E (B) were pulse-labeled with [³⁵S]-methionine, and the labeled proteins were immunoprecipitated with anti-Flag mAb followed by immunoblotting, exposure of the filter to Phosphor-Imager and probing with anti-Flag mAb. C) Summary of the data presented in (A) and (B). These experiments were performed twice with similar results.

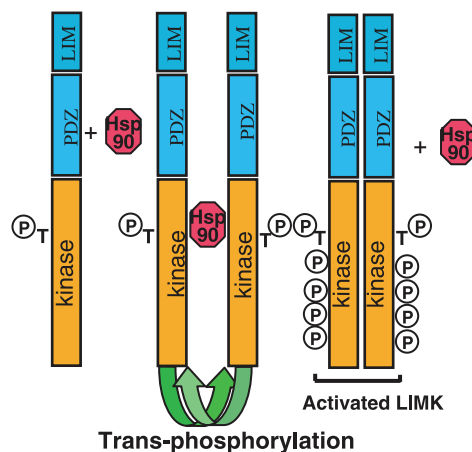


Figure 9. A proposed model depicting the role of Hsp90 in regulating the activity of LIMK1 and LIMK2. Hsp90 binding to a motif in the kinase domain of LIMK1 promotes a complex between two LIMK molecules facilitating transphosphorylation and stabilization of LIMK. After transphosphorylation, the complex between LIMK and Hsp90 is disrupted, allowing LIMK interaction with its substrate cofilin.

DISCUSSION

LIMK1 is a very stable protein with a half-life of ~16–20 h. We have previously demonstrated that LIMK1 levels and its phosphorylation are markedly elevated in metastatic breast cancer cell lines in comparison with nonmetastatic and normal cells. The extended half-life of LIMK1 is not due to the stability of its mRNA because no differences were observed in the levels of LIMK1 mRNA in these cell lines. In addition, studies using microarrays did not reveal significant differences in the level of LIMK1 mRNA between invasive and noninvasive breast cancer cell-lines (24). Together these findings suggest that the concentration of LIMK1 protein is regulated by posttranslational modification. In this study we confirmed this hypothesis and furthermore have provided data to support that LIMK1 dimer formation and transphosphorylation are responsible for the stability of LIMK1 protein.

The data obtained in this study identified two new members in addition to the long list of Hsp90 client proteins. Binding of Hsp90 to the LIMK proteins protects them from degradation as after 24 h incubation with Hsp90 inhibitors only less than 10% of these proteins can be detected (Fig. 2). This corresponds to a half-life of ~4 h, which is similar to that of the DN-LIMK1 and unphosphorylated endogenous LIMK1 proteins (Fig. 1). These findings strongly suggest that Hsp90 promotes dimer formation and transphosphorylation of LIMK1. Indeed, we have shown here that Hsp90 binds to LIMK1 leading to the formation of LIMK1 homodimers (Fig. 4). We have previously demonstrated that deletion mutants lacking the LIM and PDZ domains of LIMK1 have increased kinase activity (3). This was attributed to findings that the LIM and PDZ domains interact with

the kinase domain (25) and thereby inhibit its association and activation by ROCK and PAK (21). We propose that the binding of Hsp90 to the kinase domain of LIMK1 enables the opening up of the molecule and promotion of homodimers formation.

Although the recognition motif of LIMK is similar to that of ErbB-2, the role that Hsp90 binding plays is different between these two protein kinases. While binding of Hsp90 to the kinase domain of ErbB-2 restrains its catalytic activity by preventing heterodimer formation with other ErbB proteins, Hsp90 binding to the kinase domain of LIMK1 results in homodimer formation enabling LIMK1 transphosphorylation and its activation. Our cross-linking experiments with overexpressed LIMK1 demonstrated the formation of LIMK1 homodimers of 140 kDa only in the absence of 17-AAG. In contrast, LIMK1-P394E does not form homodimers in the absence or presence of the Hsp90 inhibitors (Fig. 7C). This suggests that the interaction between these two proteins depends on the Hsp90 binding motif in the kinase domain of LIMK.

It was recently demonstrated that ErbB2, but not ErbB1, is an Hsp90 client protein. Therefore, while binding of ErbB2 to Hsp90 promotes its homo-dimerization, in turn, it inhibits hetero-dimerization with ErbB1. As ErbB2 homo-dimers have lower kinase activity than ErbB2-ErbB1 hetero-dimers, ErbB2 homo-dimerization results in its restrained activity (26). It is well established that Hsp90 forms homodimers (27), we therefore propose that LIMK homo-dimerization requires Hsp90 dimers where each Hsp90 monomer binds to the recognition site in the kinase domain of LIMK1 enabling the formation of a quaternary complex followed by transphosphorylation and increased protein stability.

Although it appears that the role of Hsp90 in the regulation of ErbB2 is different from its role in the regulation of LIMK activity, the overall outcome of their interaction is very similar as inhibition of Hsp90 induces rapid and marked degradation of these proteins the cell. In addition to its ability to promote or restrain homo- and hetero-dimerization, respectively, Hsp90 can also promote the maturation of nascent client proteins into an active and stable conformation as well as maintain the mature form of the client protein as was demonstrated for ErbB2 [reviewed in (26)].

Recently it was revealed that the interaction between Hsp90 and ErbB-2 is mediated by a loop in the N lobe of the kinase domain of ErbB-2 (15, 16). Mutations in this loop that disrupt the positively charged patch and the hydrophobic strip abolish the association between these two molecules. Indeed, modeling of the kinase domain of LIMK1 demonstrated the existence of a similar loop also in LIMK1 and other EGFR family members. Moreover, comparison of the sequence within this loop demonstrated that more than half the residues are identical or conservatively substituted between LIMK1 and

ErbB-2. Interestingly, three amino acids conserved between LIMK and ErbB-2 are also shared between ErbB-2 and ErbB-1 and are not involved in the association between ErbB-2 and Hsp90. In contrast, mutation of P394, a residue conserved among LIMK1, ErbB-2, and ErbB-1, to the negatively charged glutamic acid greatly reduced the association between LIMK1 and Hsp90, suggesting that changes in the charge or/and the backbone conformation, or the introduction of a longer side chain in this position of this region, affect the interaction between these two molecules.

Given the fact that Hsp90 inhibitors are known to be potent anticancer drugs that are currently in clinical trials, this study postulates a novel anticancer mechanism of Hsp90 inhibitors via their perturbation of LIMK family function, molecules that have previously been implicated in tumor growth, invasion, and metastasis (9, 28, 29).

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