# Role of Acetylation and Extracellular Location of Heat Shock Protein 90α in Tumor Cell Invasion

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## Abstract

**Heat shock protein (hsp) 90 is an ATP-dependent molecular chaperone that maintains the active conformation of client oncoproteins in cancer cells. An isoform, hsp90α, promotes extracellular maturation of matrix metalloproteinase (MMP)-2, involved in tumor invasion and metastasis. Knockdown of histone deacetylase (HDAC) 6, which deacetylates lysine residues in hsp90, induces reversible hyperacetylation and attenuates ATP binding and chaperone function of hsp90. Here, using mass spectrometry, we identified seven lysine residues in hsp90α that are hyperacetylated after treatment of eukaryotic cells with a pan-HDAC inhibitor that also inhibits HDAC6. Depending on the specific lysine residue in the middle domain involved, although acetylation affects ATP, cochaperone, and client protein binding to hsp90α, acetylation of all seven lysines increased the binding of hsp90α to 17-allyl-amino-demethoxy geldanamycin. Notably, after treatment with the pan-HDAC inhibitor panobinostat (LBH589), the extracellular hsp90α was hyperacetylated and it bound to MMP-2, which was associated with increased *in vitro* tumor cell invasiveness. Treatment with antiacetylated hsp90α antibody inhibited *in vitro* invasion by tumor cells. Thus, reversible hyperacetylation modulates the intracellular and extracellular chaperone function of hsp90, and targeting extracellular hyperacetylated hsp90α may undermine tumor invasion and metastasis.** [Cancer Res 2008;68(12):4833–42]

## Introduction

Heat shock protein 90 (hsp90) is a constitutively and ubiquitously expressed, ATP-dependent molecular chaperone (1). It exerts an essential role in proper folding and in maintaining the active conformation, intracellular disposition, and proteolytic turnover of a large number of the progrowth and prosurvival substrate client oncoproteins in cancer cells (1). Therefore, hsp90 has emerged as a promising target in cancer therapy (2). Activation of client proteins by hsp90-based chaperone machine involves an ordered association with several cochaperones, e.g., p23, cdc37, and Aha-1, linked to the ATPase cycle of hsp90, which may also direct client protein specificity (3–5). Hsp90 exists predominantly as a homodimer, with transient association between $NH_2$-terminal domains, thus functioning as a dimeric "molecular clamp" (6). Each hsp90 monomer is modular with three well-defined domains. These include the $NH_2$-terminal nucleotide-binding domain, a middle domain (MD) that completes the ATPase site and binds to client proteins, as well as the COOH-terminal dimerization domain (7, 8). ATP binding and hydrolysis triggers conformational change in the hsp90 homodimer, which is crucial for its binding to the cochaperones, as well as for its interaction with various client oncoproteins in the cancer cells (1, 9, 10). The hsp90 chaperone cycle includes (*a*) an open, apo, nucleotide-free conformation in which each of the three domains in each monomer presents hydrophobic surface to the large interdomain cleft, a conformation most optimal for client protein binding; (*b*) an ATP-bound intermediate state; and (*c*) a closed ADP-bound state (8). There are two isoforms of hsp90, i.e., hsp90α and hsp90β, which are encoded by two separate genes (11, 12). Only hsp90α has been described to be extracellular, where it serves as a molecular chaperone and activates matrix metalloproteinase (MMP)-2 (11, 12).

In addition to cochaperone association as well as ATP binding and hydrolysis, posttranslational modifications such as hyperphosphorylation (13–15), S-nitrosylation, and reversible hyperacetylation have also been shown to regulate the chaperone function of hsp90 (16–18). Several serine-threonine phosphorylation sites have been identified in hsp90. Although hyperphosphorylation negatively regulates hsp90 chaperone function, the role of site-specific phosphorylation in modulating hsp90 function has yet to be fully elucidated. Lysine (K) acetylation is a reversible modification mediated by opposing actions of acetyltransferases (HAT) and deacetylases (HDAC) in which an acetyl group is covalently linked to lysine residues of target proteins (19). After treatment with a variety of pan-histone deacetylase inhibitors (HDI), including the hydroxamic acid analogues vorinostat, LAQ824, and panobinostat (LBH589), or after siRNA-mediated knockdown of HDAC6, reversible hyperacetylation of hsp90 has been documented (17, 18). Overall, hyperacetylation of hsp90 was shown to inhibit the ATP, cochaperone p23, and client protein binding to hsp90, directing the client proteins to polyubiquitylation and proteasomal degradation (18). In a recent report, Scroggins and colleagues (20) identified the K294 in the MD of hsp90α as a discrete acetylation site. They also determined that the acetylation status of K294 is a strong determinant of client protein and cochaperone binding to hsp90α. Although they noted that hsp90 is acetylated at more than one site, identification of these sites or their functional significance was not determined (20). In the present study, we determined the identity and functional significance of the domain-specific seven-lysine residues that are hyperacetylated, after treatment with pan-HDAC inhibitors that also inhibit HDAC6. Remarkably, hyperacetylated hsp90α was extracellular and acted as a chaperone for MMP-2, which promoted *in vitro* invasion by breast cancer cells. Our findings also show that treatment with antiacetyl lysine-69 hsp90α antibody markedly inhibits the invasiveness of breast cancer cells.

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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## Materials and Methods

**Cell lines, antibodies, and plasmids**. HEK293T, MDA-MB-468, MDA-MB-231, and T47D cells were all purchased from American Tissue Culture Collection. HEK293T and MDA-MB-468 cells were maintained in DMEM; T47D and MDA-MB-231 cells were maintained in RPMI containing 10% fetal bovine serum (FBS). The following antibodies used were purchased from commercial sources: anti-CHIP (Abcam); anti-hsp40 (SPA-450; StressGen); anti-hsp90α (SPA-840; StressGen); anti-hsp90α (polyclonal; GeneTex); anti-p23 (Alexis Biochemicals), anti-acetyl-lysine (monoclonal), and anti-AKT (Cell Signaling); anti-Acetyl lysine (polyclonal; Upstate-Millipore); anti-HA.11 (monoclonal; Covance), anti-cRaf, and anti-MMP2 (Santa Cruz Biotech.); and anti-FLAG (M2 monoclonal and F polyclonal), ANTI-FLAG M2 agarose, anti–β-actin (Sigma), and agarose conjugates (Upstate). Plasmids expressing FLAG (F)-hsp90 and HA-p300 have previously been described (21, 22). Hsp90 mutants were generated by site-directed mutagenesis using the QuikChange kit from Stratagene (23). All mutants were verified by sequencing at the DNA Core Laboratory of Medical College of Georgia. The sequence of mutagenesis primers is provided in Table 1.

**Acetylated-K69 hsp90α antibody**. Affinity-purified polyclonal antibody against Ac-K69-hsp90α was generated by Alpha Diagnostic based on the synthetic 12 amino acid peptide flanking K69 (acetylated) ETLTDPSKLDSGK. Affinity-purified antibody was checked by performing an ELISA, using free peptide containing acetylated lysine. The antibody specifically recognized acetylated peptide but not nonacetylated peptide dotted on nitrocellulose membrane (data not shown). The antibody also recognized increase in hsp90α acetylation after HDAC inhibitor treatment and untreated acetylated hsp90α (see Fig. 6*B*).

**Transfections, immunoprecipitations, and immunoblots**. After culture in the plates for 24 h, cells were transfected by Lipofectamine Plus following the protocol provided by Invitrogen. Transfected cells were cultured in full medium containing drugs dissolved in DMSO or vehicle for 24 h. Cellular extracts were prepared by directly adding lysis buffer [25 mmol/L Tris-phosphate (pH 7.8), 2 mmol/L DTT, 2 mmol/L 1,2-diaminocyclohexane-*N*‘,*N*’,*N*‘,*N*’-tetraacetic acid, 10% glycerol, and 0.2% Triton X-100] to the cells on ice. For immunoprecipitations (IP), $2 \times 10^6$ cells in 100-mm dishes were transfected and/or treated as described above. Cellular extracts were prepared and IP was performed as described (24). For immunoblotting, cellular extracts or immunoprecipitates were separated on SDS-PAGE, transferred to a nitrocellulose membrane, probed with antibodies, and visualized with enhanced chemiluminescence, as described (24).

**Purification of acetylated hsp90α.** Using anti-FLAG M2 affinity beads, F-tagged hsp90α protein was affinity captured from F-hsp90α–transfected HEK 293 cells that had been treated with 100 nmol/L LBH589 (Novartis Pharmaceuticals, Inc.). This was followed by IP of acetylated F-hsp90α using acetyl lysine agarose beads. The immunoprecipitated proteins were resolved by 8% SDS-PAGE gel and visualized using Coomassie Blue stain.

**Mass spectrometric determination of hsp90α acetylation sites**. For comprehensive detection of the acetylation sites, mass spectrometry (MS) in combination with MS/MS was used (25). First, gel slices from SDS-PAGE separation of cell lysates were subjected to in-gel tryptic digestion to create peptides whose mass can be searched against public data bases. For peptide detection, we used an Applied Biosystems 4700 Proteomics Analyzer with Mascot (Matrix Science) protein search engine. The sample was loaded using α-cyano-4-hydroxycinnamic acid into the instrument according to manufacturer's instructions and run in a data-dependent MS plus MS/MS mode. This allows for the documentation of the peptides creating a protein fingerprint for protein identification as well as documentation of the peptides for further analysis through postsource fragmentation. Once the peptides were detected, the instrument was told to go back to each sample and fragment the individual top 20 peptides creating fragments of different lengths that can be reassembled by the computer to predict, in combination with the identification from the fingerprint data, the sequence of the peptide. The sequence data, in turn, was used to bolster the protein identification call. The program allows for certain user input modifications that will allow for missed cleavages as a result of enzyme inefficiency as well as blocked sites. The software also allows for input of potential modifications that might add additional mass to a peptide, allowing the search engine to call the peptide in either its modified or unmodified state and with or without missed cleavages resulting from any modifications or enzyme inefficiencies. All of this information was amassed, and the call for identification and of the potential modification at a certain site was statistically calculated, and the highest probability calls are reported.

**Table 1.** Sequence of mutagenesis primers

| Mutation site | Sequence of primer |
|---|---|
| K69Q | 5-GAA AGC TTG ACA GAT CCC AGT CAA TTA GAC TCT GGG A |
| K69R | 5-GAA AGC TTG ACA GAT CCC AGT AGA TTA GAC TCT GGG A |
| K100Q | 5-GAT ACT GGA ATT GGA ATG ACC CAG GCT GAC TTG ATC |
| K100R | 5-GAT ACT GGA ATT GGA ATG ACC AGG GCT GAC TTG ATC |
| K292Q | 5-TCG ATC AAG AAG AGC TCA ACC AAA CAA AGC CCA TCT G |
| K292R | 5-TCG ATC AAG AAG AGC TCA ACA GAA CAA AGC CCA TCT G |
| K327Q | 5-TGG GAA GAT CAC TTG GCA GTG CAG CAT TTT TCA GTT G |
| K327R | 5-TGG GAA GAT CAC TTG GCA GTG AGG CAT TTT TCA GTT G |
| K478Q | 5-GTG ATG AGA TGG TTT CTC TCC AGG ACT ACT GCA CCA G |
| K478R | 5-GTG ATG AGA TGG TTT CTC TCA GGG ACT ACT GCA CCA G |
| K546Q | 5-GAA GAC TTT AGT GTC AGT CAC CCA AGA AGG CCT GGA ACT |
| K546R | 5-GAA GAC TTT AGT GTC AGT CAC CAG AGA AGG CCT GGA ACT |
| K558 | 5-TCC AGA GGA TGA AGA AGA GCA AAA GAA GCA GGA AG |
| K558R | 5-TCC AGA GGA TGA AGA AGA GAG AAA GAA GCA GGA AG |

**ATP-sepharose binding assay**. Hsp90α in 200 μg of cell lysates was affinity precipitated using KinaseBind γ-phosphate–linked ATP resin (Innova Biosciences) at 4°C for 4 h. After washing three or four times with the lysis buffer, the resin was pelleted and SDS-PAGE analysis was performed (18).

**Biotinylated-geldanamycin binding assay.** Biotinylated-geldanamycin (B-GA) binding to hsp90 was assessed as described previously (26). Briefly, cell lysates were incubated with or without 17-allyl-amino-demethoxy geldanamycin (17-AAG; Developmental Therapeutics Branch of Cancer Therapy Evaluation Program/National Cancer Institute/NIH) for 1 h at 4°C, and then incubated with B-GA to displace 17-AAG from hsp90 for another 1 h. GM-bound hsp90 was captured by biotin-GA linked to streptavidin Mutein Matrix (Roche Diagnostics) for 1 h at 4°C. The unbound supernatant was removed and the beads were washed thrice with lysis buffer. The precipitates were immunoblotted for hsp90.

**Isolation of hsp90α on cell surface.** MDA-MB-231 cells transfected with hsp90α expression constructs cultured in RPMI 1640 (without serum) were harvested by centrifugation (1,000 *g*, 10 min at 4°C) and washed twice with ice-cold PBS. Proteins on the cell surface were labeled following the protocol supplied by the manufacturer (PIERCE). Briefly, cells were resuspended in EZ-Link Sulfo-NHS-SS-Biotin (0.25 mg/mL) at 4°C for 30 min. The biotinylation reaction was terminated by addition of Quenching Solution. After biotinylation, the cells were washed thrice with TBS, pelleted by centrifugation, and solubilized in Lysis buffer. The cell lysates were

further disrupted by brief sonication. Solubilized biotinylated membrane proteins from same amount of cell lysates were captured using Immobilized NeutrAvidin Gel, extensively washed with Wash Buffer, and resuspended in 2× SDS sample buffer before immunoblot analysis.

***In vitro* invasion assays.** *In vitro* invasion assay were performed, as previously described (12, 27) by using the Cultrex Cell Invasion Assay kit (RandD Systems). In brief, serum-starved cells in 50 μL serum-free medium with or without antibody or IgG were placed in the top chamber and allowed to invade for 24 hour. The lower chambers (assay chamber) were filled with 10% FBS medium. After incubation, migrated cells on the upper chamber of the membrane were dissociated with cell dissociation solution containing Calcein AM at 37°C for 1 h and read the bottom plate at 485 nm excitation and 520 nm emission.

***In vitro* protein acetylation assay.** Protein acetylation assays were performed as described previously (28). Briefly, reactions (30 μL) were carried out at 30°C for 1 h with M2 beads bound F-hsp90α (25 μL M2 beads mixed with 10 μL of *in vitro*–translated F-hsp90α and washed thrice with HAT buffer) and 50 ng of p300 protein (Upstate) in HAT buffer [50 mmol/L Tris-HCl (pH 8.0), 10% glycerol, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 0.1 mmol/L EDTA, and 50 nmol/L acetyl-CoA (Sigma)]. After washing thrice with HAT buffer, the samples were then subjected to Western blot analysis with anti–acetyl-lysine antibody.

**Confocal microscopy.** MDA-MB-231 cells were cultured in a chamber slide in RPMI with 10% FBS or under serum-free conditions with or without 40 nmol/L LBH589 for 16 h and stained with anti-acetyl (Ac)-K69 antibody. Briefly, after 16 h incubation, cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. After this, the slides were blocked with 3% bovine serum albumin for 30 min and incubated with primary antibody at a dilution of 1:100 in blocking buffer for 2 h. After three washes with PBS, the slides were incubated in Alexa Fluor 488 anti-rabbit secondary antibody (Molecular probes; Invitrogen) for 1 h at 1:3,000 dilution. After three washes with PBS, the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) using Vectashield mountant with DAPI and imaged using Zeiss LSM510 confocal microscope.

## Results

**Hyperacetylation of hsp90α involves p300 as the acetyltransferase.** We first confirmed that hsp90 is acetylated both *in vitro* and *in vivo*, as well as determined the HAT responsible for inducing hyperacetylation of hsp90α. In HEK293 cells with ectopic expression of F-hsp90α, treatment with the pan-HDAC inhibitors LBH589 (Fig. 1*A*) or vorinostat (Fig. 1*B*) resulted in hyperacetylation of F-hsp90α. To determine whether p300 acts as the HAT for hsp90α, after incubation with p300 and acetyl-CoA, *in vitro*–translated hsp90α was analyzed for its acetylation status by Western analysis with anti-AcK antibody. As shown in Fig. 1*C*, p300 was necessary for the acetylation of hsp90α in the *in vitro* assay. Immunoprecipitated F-hsp90 from LBH589-treated cells was used as the positive control. In HEK293 cells, it was determined that p300 can be coimmunoprecipitated with hsp90α (Supplementary Fig. S1*A*). Coincubation with p300 was discovered to dose-dependently stimulate the acetylation of hsp90α (Fig. 1*D*). This was not seen with PCAF (data not shown). However, knockdown of p300 using siRNA only partially decreased LBH589-induced acetylation of hsp90 (Supplementary Fig. S1*B*). Therefore, p300 seemed not to be the sole but one of the HATs both for the *in vitro* and *in vivo* acetylation of hsp90α.

Identity and functional significance of lysine residues in hsp90α hyperacetylated by pan-HDAC inhibitors. Next, we determined the identity of the acetylated lysine residues in hsp90α induced by

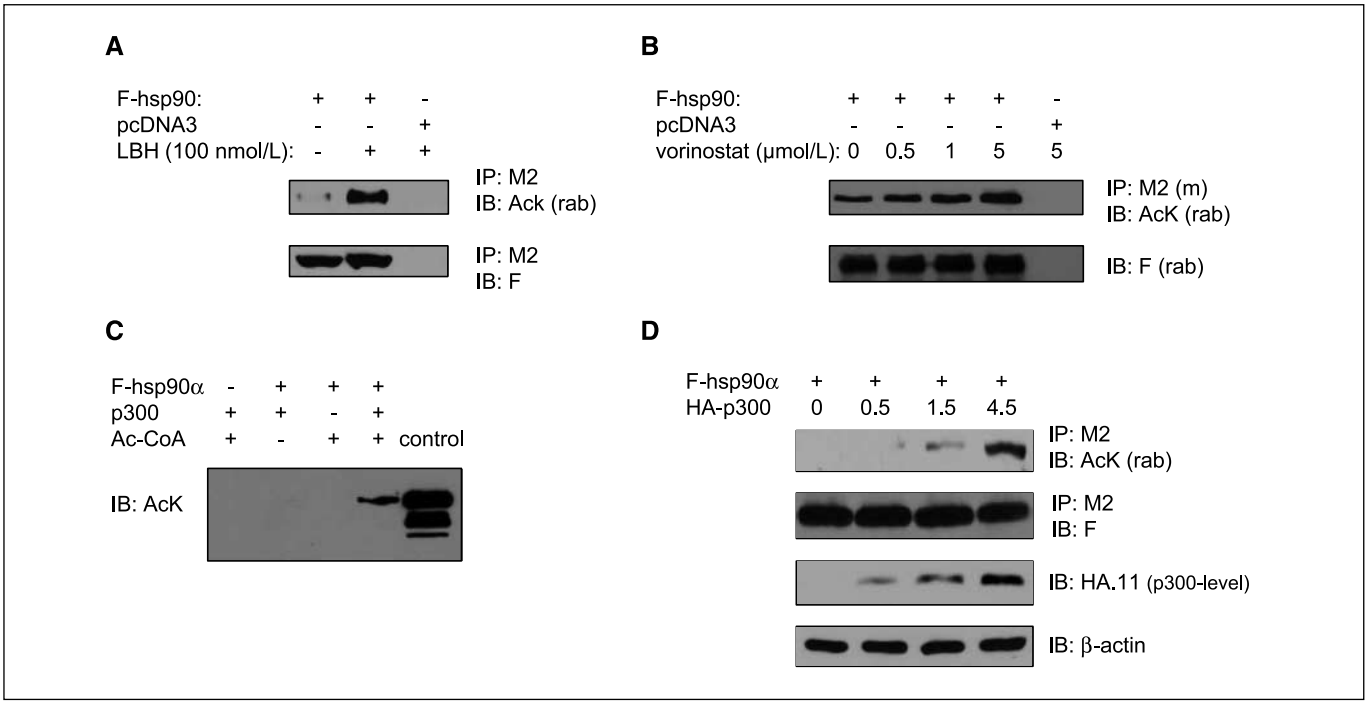


**Figure 1.** Treatment with pan-HDAC inhibitor induces acetylation of hsp90α, and p300 is the HAT for hsp90α. *A* and *B,* HEK293 cells were transfected with either F-tagged hsp90α (3 μg) or empty vector pcDNA3 and treated with the indicated concentration of LBH589 (*LBH*; *A*) or vorinostat (*B*) for 24 h. IP with anti-F (*M2*) antibody–conjugated beads were immunoblotted (*IB*) with either anti-rabbit acetyl lysine (*AcK*) or anti-rabbit FLAG (*F*) antibody. *C,* p300 acts as a histone acetyl-transferase for hsp90α *in vitro*. The *in vitro*–translated hsp90α was incubated with the combination of recombinant p300 and Ac-CoA followed by immunoblotting with anti-AcK antibody. The lane labeled "control" contained LBH589-induced acetylated F-hsp90α. *D,* p300 promotes acetylation of hsp90 in a dose-dependent manner. HEK293 cells were cotransfected with F-hsp90α and the indicated amount of HA-tagged p300 (μg). Immunoprecipitates with anti-M2 antibody were immunoblotted with either anti-AcK or anti-F antibody. Immunoblots with anti-HA.11 or β-actin antibody served as loading control for p300 levels.

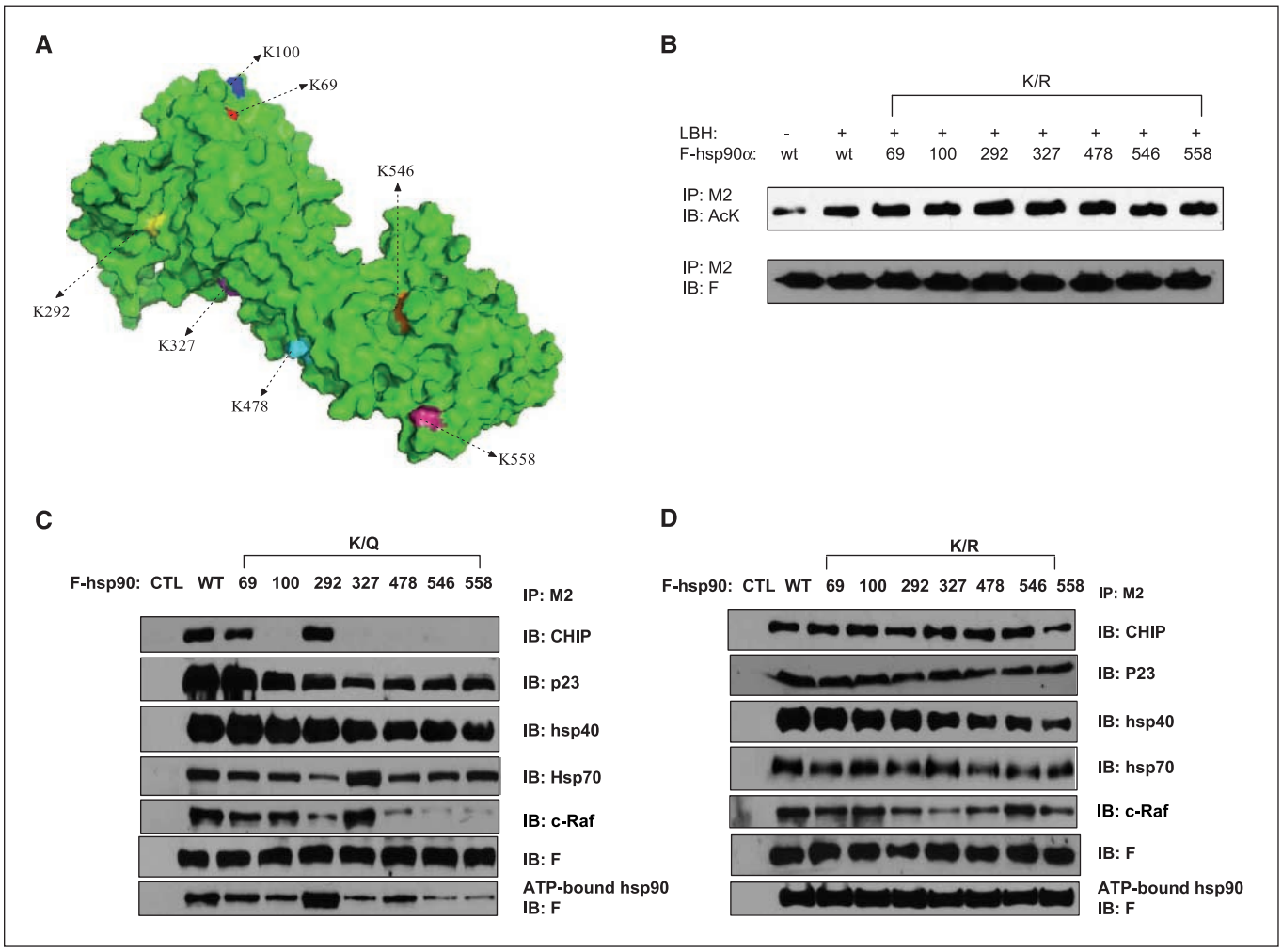


**Figure 2.** The individual K/R or K/Q substitutions do not affect the overall acetylation level of hsp90 but affect the ability of hsp90α to bind ATP, cochaperones, and client proteins. *A,* the space-filling molecular structure model of hsp90. The seven lysine residues are shown in different colors. *B,* transfectants of F-hsp90α, with or without K/R substitutions, were treated with or without 100 nmol/L of LBH589. After this, immunoprecipitates with anti-F antibody were immunoblotted with either anti-AcK or anti-F antibody. *C* and *D,* transfectants of F-hsp90α with K/Q (*C*) but not K/R substitutions (*D*) affect ATP binding of hsp90α. Precipitates from the mixture of cell lysates containing hsp90α and ATP-sepharose were analyzed with anti-F antibody. Also, after transfections of F-hsp90α, with or without K/Q or K/R substitutions, immunoprecipitates with anti-F antibody were immunoblotted with anti-CHIP, anti-p23, anti-hsp40, anti-hsp70, anti–c-Raf, or anti-F antibody. *WT,* wild-type; *CTL,* control.

HDAC inhibitor LBH589. HEK293 cells transfected with F-hsp90α were treated with 100 nmol/L LBH589, and the acetylated F-hsp90α was affinity immunopurified using anti-FLAG–conjugated M2 agarose, followed by agarose beads bearing immobilized anti-AcK antibody. The enriched acetylated hsp90α was analyzed by nano high performance liquid chromatography/MS/MS in a mass spectrometer. Seven acetylated lysine residues were identified in hsp90α: K69, K100, K292, K327, K478, K546, and K558. Figure 2*A* shows a three dimensional space-filling molecular structural model of hsp90α, which shows that all of the identified lysine residues that are acetylated reside on the surface and, thus, accessible for modification.

To assess the effect of acetylation at the various lysine residues on hsp90α function, point mutations were introduced to create acetylation-deficient (lysine to arginine, K/R) and acetylation-mimetic (lysine to glutamine, K/Q) mutants on the (F)-tagged hsp90α. First, we determined whether any of the K/R point mutations affects the overall hyperacetylation of F-hsp90α induced by either the cotransfected p300 or by treatment with LBH589. None of the individual K/R mutants showed any change in the hyperacetylation induced by either the cotransfected p300 (Supplementary Fig. S2) or by treatment with LBH589 (Fig. 2*B*). These mutants were also analyzed for their ability to bind ATP, as well as for binding to cochaperones, client proteins, and the biotinylated B-GA (26). Although none of the K/R mutants compromised the ATP binding of F-hsp90α (Fig. 2*D*), all but one of the acetylation-mimetic mutants (K/Q) showed decreased binding to ATP (Fig. 2*C*). The exception was the K292Q mutant, which showed increased binding to ATP (Fig. 2*C*). The significance of this is unclear, although K292 is in the hinge region at the beginning of the MD of hsp90α, a region that is well-conserved from yeast to human hsp90 (20, 29). Increased acetylation of hsp90 by a pan-HDAC inhibitor or HDAC6 siRNA had been shown to inhibit the binding of hsp90 with cochaperones, e.g., p23, and client proteins (18). Here, we found that acetylation-mimetic mutants (K/Q) of the lysine residues in the MD, i.e., K100, K292, K327, K478, K546, and K558, displayed decreased binding with the cochaperones p23 and to a lesser extent hsp40 (Fig. 2*C*). Although binding of

K/Q mutants at K69, K100, K327, K478, K546, and K558 to CHIP was decreased, binding of K/Q mutant at K292 was not affected (Fig. 2*C*). Additionally, binding of K/R mutants was similar to the binding of wild-type hsp90α to CHIP (Fig. 2*D*). Acetylation-mimetic mutants of hsp90α also showed disrupted binding to hsp70 and with its client protein c-Raf, except with the K327Q mutant (Fig. 2*C*). Binding of hsp70 and c-Raf to K/R mutants seemed to be not significantly altered (Fig. 2*D*). In contrast, notably, the acetylation-mimetic K/Q mutants showed increased binding to B-GA (Fig. 3*A*). This is consistent with the observation that acetylation of the endogenous hsp90α due to treatment with LBH589 was associated with increase in B-GA binding of hsp90α in MDA-MB-468 cells (Fig. 3*B*). LBH589-induced hsp90 acetylation in MDA-MB-468 cells also promoted 17-AAG binding to the acetylated endogenous hsp90 because B-GA binding to hsp90 was reduced by 17-AAG treatment more in those cells exposed to LBH589 compared with those that were unexposed (Fig. 3*C* and *D, top*). Also, after treatment with LBH589, each K/R mutant of hsp90α showed increased binding to B-GA, which could not be displaced by cotreatment with 17-AAG, suggesting decreased binding of K/R mutants to 17-AAG (Fig. 3*D*).

**Role of lysine hyperacetylation in extracellular location of hsp90α.** Previous reports have shown that the inducible isoform hsp90α, but not hsp90β, can be secreted and found on the surface of cancer cells, although it lacks the classic signal sequence (12, 30). Serum starvation, hypoxia, high concentration of glucose, as well as oxidative stress have all been shown to promote the export and extracellular location of hsp90α (30–32). Consistent with these reports, our findings show that serum starvation of breast cancer T47D cells promoted secretion and extracellular localization of the endogenous hsp90α (Fig. 4*A*), as well as of the ectopically expressed F-hsp90α (Fig. 4*B*). Notably, under both circumstances,

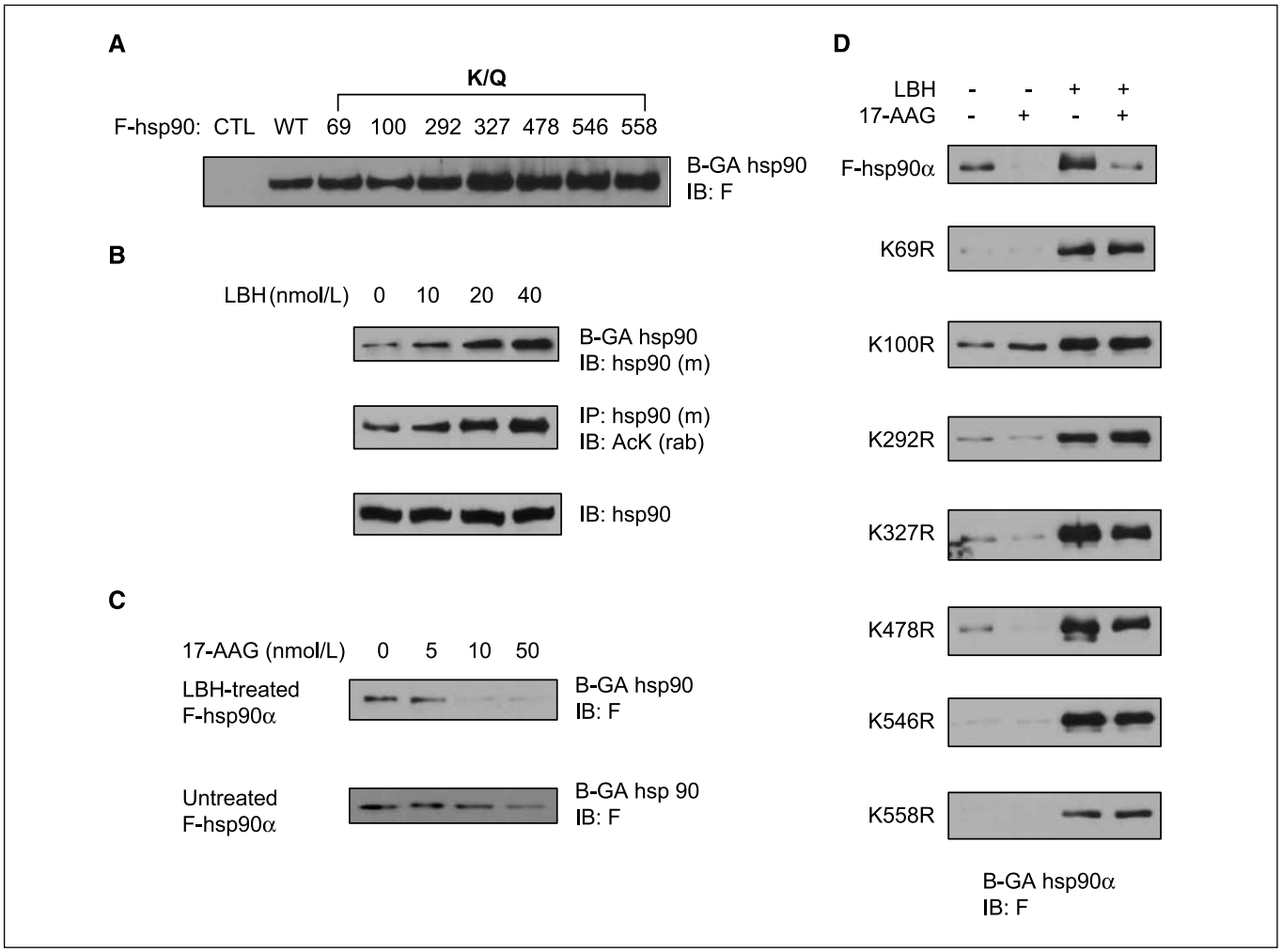


**Figure 3.** LBH589-induced acetylation increases B-GA and 17-AAG binding to hsp90α. *A,* individual K/Q substitutions increase hsp90α binding to B-GA. Cell lysates from Fig. 2*D* were also incubated with B-GA, followed by streptavidin-coated agarose beads, and eluted proteins were analyzed with anti-F antibody. *B,* LBH589 increases hsp90 binding to GA and induces hsp90 acetylation dose-dependently. MB-468 cells cultured in DMEM containing 10% FBS were treated with the indicated concentration of LBH589 for 16 h. After this, cell lysates were incubated with B-GA, followed by streptavidin-coated agarose beads, and eluted proteins were analyzed with anti-hsp90α antibody. Acetylation and expression level of endogenous hsp90 were detected with anti-AcK and anti-hsp90 antibody, respectively. *C,* LBH589 treatment preferentially increases hsp90α binding to 17-AAG. MB-468 cells ectopically expressing F-hsp90α were treated with 100 nmol/L of LBH589 for 16 h. After this, equal amount of cell lysates were incubated with the indicated doses of 17-AAG for 30 min at 4°C, followed by incubation with B-GA and streptavidin-coated agarose beads. Precipitates from streptavidin-coated beads were analyzed with anti-F antibody. *D,* individual K/R substitution disrupted the affinity of LBH589-treated hsp90α for 17-AAG. After treatment with either vehicle or 100 nmol/L of LBH589, cell lysates from HEK293 cells expressing F-hsp90 or K/R substitutions were incubated with vehicle or 50 nmol/L of 17-AAG followed by incubation with B-GA and streptavidin-coated agarose beads. Precipitates from streptavidin-coated beads were analyzed with anti-F antibody.

hsp90α was acetylated (Fig. 4*A* and *B*). Notably, treatment with LBH589, in a dose-dependent manner, also promoted and increased the export and extracellular localization of hyperacetylated hsp90α from T47D cells into the culture medium (Supplementary Fig. S3*A*). These data indicate that hyperacetylation stimulates the extracellular export of hsp90α. To further verify this, we determined the export and extracellular location of acetylation mimetic (K/Q) or acetylation-resistant (K/R) mutants transfected into T47D cells that were cultured under serum-free condition. As shown, K69Q, K100Q, and K558Q substitutions promoted the export and extracellular location of hsp90α, whereas K/R substitutions at the same residues markedly reduced the export and extracellular location of hsp90α (Fig. 4*C*). Cotreatment of cells transfected with K/R mutants with LBH589 increased the export and extracellular location of K/R mutants of hsp90α to a variable extent, with K100R and K558R mutants showing less export than the other mutants (Supplementary Fig. S3*B*). Immunoblot analysis of the solubilized biotinylated membrane proteins also showed that acetylation-mimetic mutants K69Q, K100Q, K327Q, and K558Q exist more on the cell surface compared with the wild-type hsp90 (Fig. 4*D*).

**Extracellular hyperacetylated hsp90α binds MMP-2 and promotes tumor cell invasion.** In cancer cells, extracellular hsp90α was shown to act as a chaperone and assist in the maturation of the MMP-2 to its active form (12). Consistent with this, our findings show that K/Q mutants expressed in MB-231 cells, especially K69Q, K100Q, and K558Q, which are preferentially extracellular under serum-free culture conditions, bind MMP-2 in

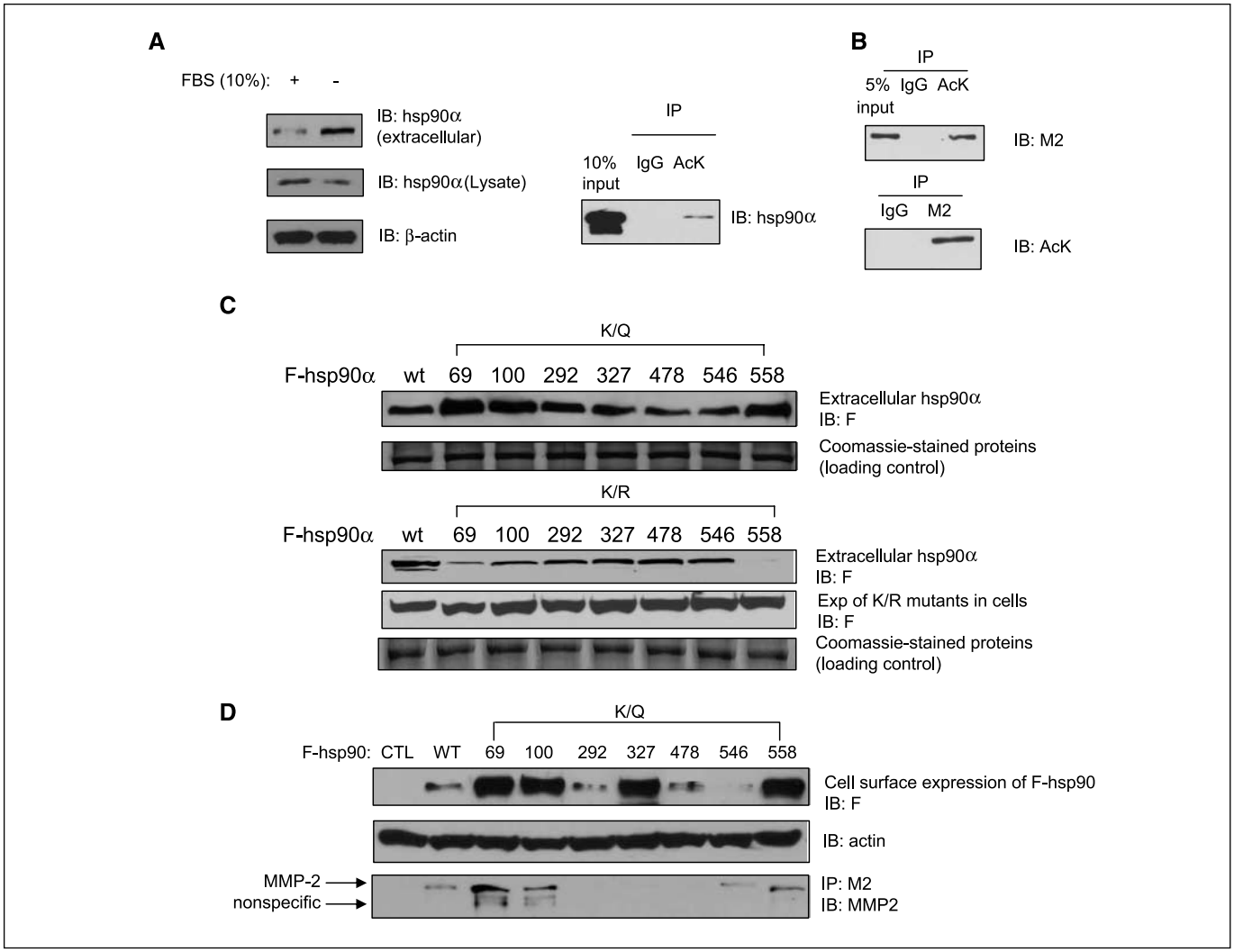


**Figure 4.** Acetylation-dependent extracellular localization of hsp90α. *A,* serum starvation of T47D breast cancer cells promotes extracellular localization of hsp90α. Concentrated extracellular medium or cell lysates from T47D cells, which were either serum starved or cultured in 10% FBS, were immunoblotted with anti-hsp90α antibody. β-actin served as a loading control. *A, right* and *B*, under starvation, both endogenous (*A, right*) and exogenous hsp90α (*B*) are secreted from T47D cells in the acetylated form. Extracellular hsp90α was immunoprecipitated with anti-AcK antibody and immunoblotted with either anti-hsp90α or M2 antibody. *C,* in serum-starved T47D cells, K/R substitutions at K69, K100, and K558 decrease, whereas K/Q substitutions increase the level of extracellular hsp90α. Supernatants of serum-starved T47D cells transfected with the indicated F-tagged hsp90α mutant constructs were concentrated and immunoblotted with anti-F antibody. Coomassie-stained nonspecific proteins served as the loading control. *D,* K/Q substitution affects hsp90 expression on cell surface. MB-231 cells transfected with the indicated constructs were cultured under serum-free condition for 24 h and followed by the labeling of surface protein with biotinylation. Biotinylated hsp90 on cell surface was detected with anti-F antibody. Biotinylated actin on cell surface served as loading control. Supernatants of serum-starved MB-231 cells transfected with the indicated F-tagged K/Q hsp90α mutant constructs were also concentrated and immunoprecipitates with anti-F antibody–conjugated beads were immunoblotted with anti–MMP-2 antibody.

the extracellular medium (Fig. 4*D*). Overall, these data indicate that acetylation promotes not only the extracellular location of hsp90α but also facilitates its chaperone association with MMP-2. Next, we determined whether following treatment with sublethal concentrations of pan-HDAC-inhibitor–increased extracellular levels of acetylated hsp90α increases invasiveness of cancer cells. Figure 5*A* shows that treatment with LBH589 dose dependently increased Matrigel invasion by MB-231 cells. This was associated with increased extracellular binding of acetylated hsp90α with MMP-2 (Fig. 5*B*). Similar increase in the invasiveness of MB-468 cells was observed after treatment with LBH589 or SAHA (Fig. 5*C*). We next determined whether increased extracellular location of K69Q, K100Q, or K558Q mutant promotes *in vitro* invasiveness of breast cancer cells. For this, we created stable transfectants of MB-468 cells expressing the wild-type hsp90α, or the K/Q or K/R mutants of hsp90α at the residues K69, K100, and K558, and their *in vitro* invasiveness in the Matrigel-based assay was evaluated. As compared with the MB-468 cells expressing the wild-type hsp90α, stable transfectants of MB-468 cells with K/Q but not K/R mutants at K69, K100, and K558 showed increased *in vitro* invasiveness (Fig. 5*D*).

**Treatment with anti-AcK hsp90α antibody inhibits *in vitro* invasion by breast cancer cells.** The findings above raised the possibility that treatment with an antibody to acetylated (Ac) hsp90α would inhibit binding of hsp90α to MMP-2 that is involved in tumor invasiveness. Therefore, the anti–Ac-hsp90α would thereby inhibit invasiveness of breast cancer cells. To verify this, first we compared the extracellular location of the ectopically expressed K69Q mutant versus the wild-type hsp90α in the breast cancer MB-231 cells. As shown, compared with the location of the wild-type hsp90α, which is mostly intracellular (extracellular to intracellular ratio of 13.1), relatively more of the K69Q mutant hsp90α was extracellular (extracellular to intracellular ratio of 1.9; Fig. 6*A*). Next, a polyclonal antibody was generated against the acetylated K69-containing peptide of hsp90α (anti–Ac-K69). The specificity of the antibody was confirmed by determining its ability to detect the increase in the acetylation of the ecotopically expressed (Fig. 6*B, top*) or endogenous hsp90α (Fig. 6*B, bottom*), after treatment with the HDAC inhibitor LBH589. As shown, after treatment of cells with LBH589, the anti–Ac-K69 hsp90α antibody recognized the increase in the acetylated hsp90α (Fig. 6*B*). Importantly, the anti–Ac-K69 hsp90α antibody selectively recognized acetylated hsp90α (Supplementary Fig. S4*A*). In contrast, the commercially available polyclonal anti-hsp90α antibody nonspecifically recognized both the acetylated and unacetylated hsp90α, without showing specific increase in the epitope detection after treatment of the cells with serum-starved condition or with LBH589 (Supplementary Fig. S4*B*). Therefore, this raised the possibility that the anti–Ac-K69 antibody might be more selective in attenuating the role of Ac-hsp90α in the invasiveness of cancer cells. Figure 6*C* shows that, compared with the control IgG–treated or untreated MB-231 cells, serum-starved MB-231 cells showed surface location of the acetylated hsp90α when stained with the anti–Ac-K69 antibody (Fig. 6*C*). Additionally, treatment of serum-starved MB-231 cells with LBH589 led to increased levels of the membrane-associated acetylated hsp90α, as detected by the anti–Ac-K69 antibody. Next, we compared the effect of anti-hsp90α with anti–Ac-K69 hsp90α antibody on the *in vitro* invasiveness of MB-231 breast cancer cells. Figure 6*D* clearly shows that, although the control IgG had no significant effect and the commercially available polyclonal anti-hsp90α antibody only modestly inhibited the *in vitro* invasiveness of MB-231 cells, treatment with the anti–Ac-K69 hsp90α markedly inhibited the Matrigel invasion by MB-231 cells (Fig. 6*D*). Thus, acetylation of K69 in hsp90α may play an important role in extracellular location and chaperone association of hsp90α with MMP-2. Additionally, exposure to anti–Ac-K69 hsp90α antibody could inhibit invasion of breast cancer cells.

## Discussion

Recently, the hsp90 ortholog in the yeast was shown to physically or genetically interact with at least 10% of the yeast proteome (33). By analogy, hsp90 is increasingly recognized as a chaperone and master regulator for the key cell signaling networks or cellular transcription in human cells, especially in the transformed cells (34). Hyperacetylation of hsp90 resulting from inhibition of HDAC6 has been shown to inhibit the ATP, cochaperone p23, and client protein binding of hsp90. This directs the client proteins, including several oncoprotein signaling kinases and nuclear hormone receptors to polyubiquitylation and proteasomal degradation (17, 18, 35–37). Although it is likely that not all of the known hsp90 client proteins would be uniformly affected (38), posttranslational modifications of hsp90 such as phosphorylation and acetylation influence the chaperone function of hsp90, thereby affecting the signaling networks that govern the malignant phenotype and response to therapy of cancer cells (39). By using mass spectrometry on hsp90α isolated from HDAC inhibitor–treated cells, we could document hyperacetylation of surface-residing seven-lysine residues in the $NH_2$ terminus and the MD of hsp90α. Unlike a previous report, in our study, under the experimental conditions we used for mass spectrometry studies, K294 was not found to be acetylated in hsp90α (20). Most likely this is so because different tryptic digestion protocols and different HDAC inhibitors were used in the two studies. Therefore, there may have been preservation and detection of K294 acetylation in the previous report and not of the other acetylated lysine residues identified in our study (20). In the previous report, authors had conjectured that lysine residues other than K294 may also be involved in the acetylation/deacetylation modification (20). Although other HATs may be involved, in the present studies, we also determined that p300 is one of the HATs involved in acetylating hsp90α.

In our studies, all but one of the acetylation-mimetic mutants showed decreased binding to ATP. The exception was the K292Q mutant, which showed increased binding to ATP. Most K/Q mutants of the lysine residues in the MD, i.e., K292, K478, K546, and K558, displayed decreased binding to p23, hsp40, hsp70, as well as with the client protein c-Raf. Binding to CHIP, which is a COOH-terminal–interacting protein of hsp70 (40), was also disrupted. Conversely, the K/Q mutants showed increased binding to B-GA. This suggests that charge neutralization at the lysine residues in the MD of hsp90α decreases the ATP and cochaperone binding but increases GA binding. Consistent with this, treatment with LBH589, which dose dependently induced hyperacetylation of hsp90, also increased the B-GA binding to hsp90α in a dose-dependent manner. In a previous report, we showed that cotreatment with a pan-HDAC inhibitor enhances antileukemia effects of 17-AAG with greater depletion of hsp90 client proteins in the leukemia cells (41). This strongly suggested that hyperacetylation of hsp90 could augment 17-AAG binding to hsp90 and inhibition of its chaperone function, resulting in superior antileukemia activity of the combination of HDAC inhibitor and 17-AAG. Indeed, in

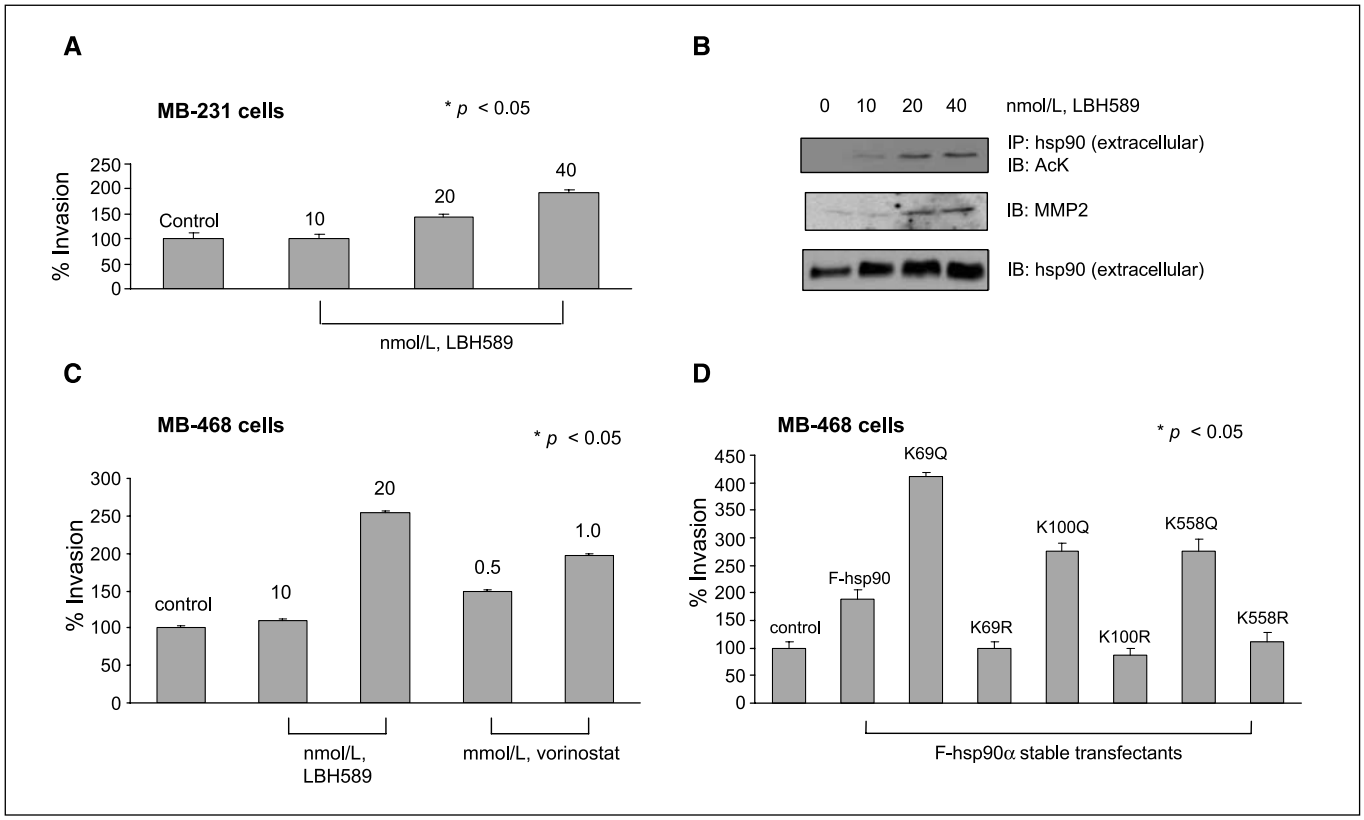


**Figure 5.** Acetylation of hsp90α promotes *in vitro* invasion by breast cancer cells. *A* and *B,* LBH589 promotes *in vitro* invasion of MB-231 cells, associated with extracellular location and binding of acetylated hsp90α to MMP-2. *A,* MB-231 cells were treated with the indicated concentrations of LBH589 for 16 h. After this, an aliquot of the cells was used for determining *in vitro* Matrigel invasion (see text for details). *Columns,* average results of three independent experiments; *bars,* SD. *, $P < 0.05$. *B,* alternatively, supernatants of serum-starved LBH589-treated MDA-MB-231 cells were concentrated and immunoprecipitates with anti-hsp90α antibody were immunoblotted with anti-AcK, anti–MMP-2, or hsp90α antibody. *C,* HDAC inhibitors promote *in vitro* invasion by MB-468 breast cancer cells. Serum-starved MB-468 cells were used for determining *in vitro* Matrigel invasion in the indicated concentrations of LBH589 or vorinostat and then evaluated for *in vitro* invasion. *Columns,* average results of three independent experiments; *bars,* SD. *, $P < 0.05$. *D,* stable transfection of K/Q- but not K/R-substituted mutants promote *in vitro* invasion by MB-468 cells. Serum-starved MB-468 cells expressing either F-hsp90α, or K/Q- or K/R-substituted mutants were used for determining *in vitro* Matrigel invasion. *Columns,* average results of three independent experiments; *bars,* SD. *, $P < 0.05$.

LBH589-treated cells, 17-AAG was able to more efficiently displace B-GA, indicating increased affinity of the hyperacetylated hsp90α for binding to 17-AAG. However, for unclear reasons, this was not observed in LBH589-treated K/R mutants of hsp90α. Overall, these observations create a rationale to determine the *in vivo* activity of the combination of HA-HDI and 17-AAG against human leukemia cells.

Recently, hsp90 was shown to be present on the surface of metastatic melanoma cells (42). It is the inducible isoform hsp90α, not hsp90β, which is secreted and present on the surface of cancer cells (12). Serum starvation of breast cancer cells promoted secretion and extracellular localization of the endogenous or ectopically expressed hsp90α, which was notably hyperacetylated under these circumstances. LBH589 treatment also promoted the export and extracellular localization of hyperacetylated hsp90α into the culture medium. Although it lacks the classic signal sequence, the hyperacetylated hsp90α is extracellular. Our data also show that K69Q, K100Q, and K558Q substitutions promote the export and extracellular location of hsp90α, whereas K/R substitutions at the same residues markedly reduce the export and extracellular location of hsp90α. To a variable extent, treatment with LBH589 was also able to induce the export and extracellular location of the K/R mutants of hsp90α. Thus, acetylation status of hsp90α at specific sites may influence the extracellular localization of hsp90α. Although hsp70, p23, cdc37, and Hip (hsp70 interacting protein) have also been detected in the extracellular environment of tumor cells, their precise function in this location has not been elucidated (30, 43). Whether ATP binding and hydrolysis is required for the extracellular chaperone function of hsp90α remains to be determined.

Extracellular hsp90α acts as a chaperone and assists in the maturation of the MMP-2 to its active form (12). Our data also show that acetylation promotes not only the extracellular location of hsp90α but also facilitates its chaperone association with MMP-2. Acetylation-mimetic mutants K69Q, K100Q, and K558Q exist more on the cell surface compared with the wild-type hsp90α. Therefore, HDAC inhibitor mediated hyperacetylation of the endogenous hsp90α at these residues most likely promotes binding and maturation of MMP-2. This is supported by the observation that K/Q mutants, especially at K69, K100, and K558 residues, bind MMP-2 in the extracellular medium. Eustace and colleagues (12) had clearly documented that the presence of hsp90α on the cell surface was critical for the *in vitro* invasiveness of HT-1080 fibrosarcoma cells (13–30). We further elucidate that sublethal concentrations of LBH589, which was associated with increased extracellular binding of acetylated hsp90α with MMP-2, increased invasiveness of breast cancer cells. Increased extracellular localization and chaperone association of K69Q, K100Q, and K558Q with

MMP-2 also promoted *in vitro* invasiveness by breast cancer cells. Previous studies have shown that treatment with extracellularly restricted GA beads, designed to inhibit extracellular chaperone function of hsp90α, decreased active MMP-2 levels and inhibited invasiveness of cancer cells by 80% (12). Here, we generated a novel antibody against the acetylated K69 containing hsp90α peptide. This anti–Ac-K69 hsp90α antibody specifically recognizes the acetylated and not the unacetylated endogenous hsp90α on the cell surface. Exposure to this antibody markedly inhibited the *in vitro* Matrigel invasion by breast cancer cells, and this effect was superior to the inhibition observed with the commercially available anti-hsp90α antibody. This evidence supports that acetylation of K69 plays an important role in extracellular chaperone association of hsp90α with MMP-2, which may regulate invasion by breast cancer cells.

Serum starvation, hypoxia, high concentrations of glucose, and oxidative stress have all been shown to promote the export and extracellular location of hsp90α (13, 16, 32). Under cellular stress, the intracellular signaling events result in phosphorylation or hyperacetylation of hsp90α. Under serum- and nutrient-depleted, hypoxia-stressed environment, in the established primaries, hsp90α is likely to be hyperacetylated and extracellular. Boyault and colleagues (44) have recently shown that cellular stress and increased levels of misfolded polyubiquitylated proteins in cancer cells triggers the dissociation of a repressive HDAC6/heat shock factor 1 (HSF1)/hsp90 complex and a subsequent HSF1 activation, which induces the expression of major cellular chaperones. Dissociation of the complex also induces hsp90α acetylation, which would export hsp90α to the cell surface. Extracellularly, it would bind and promote the maturation of MMP-2, thereby



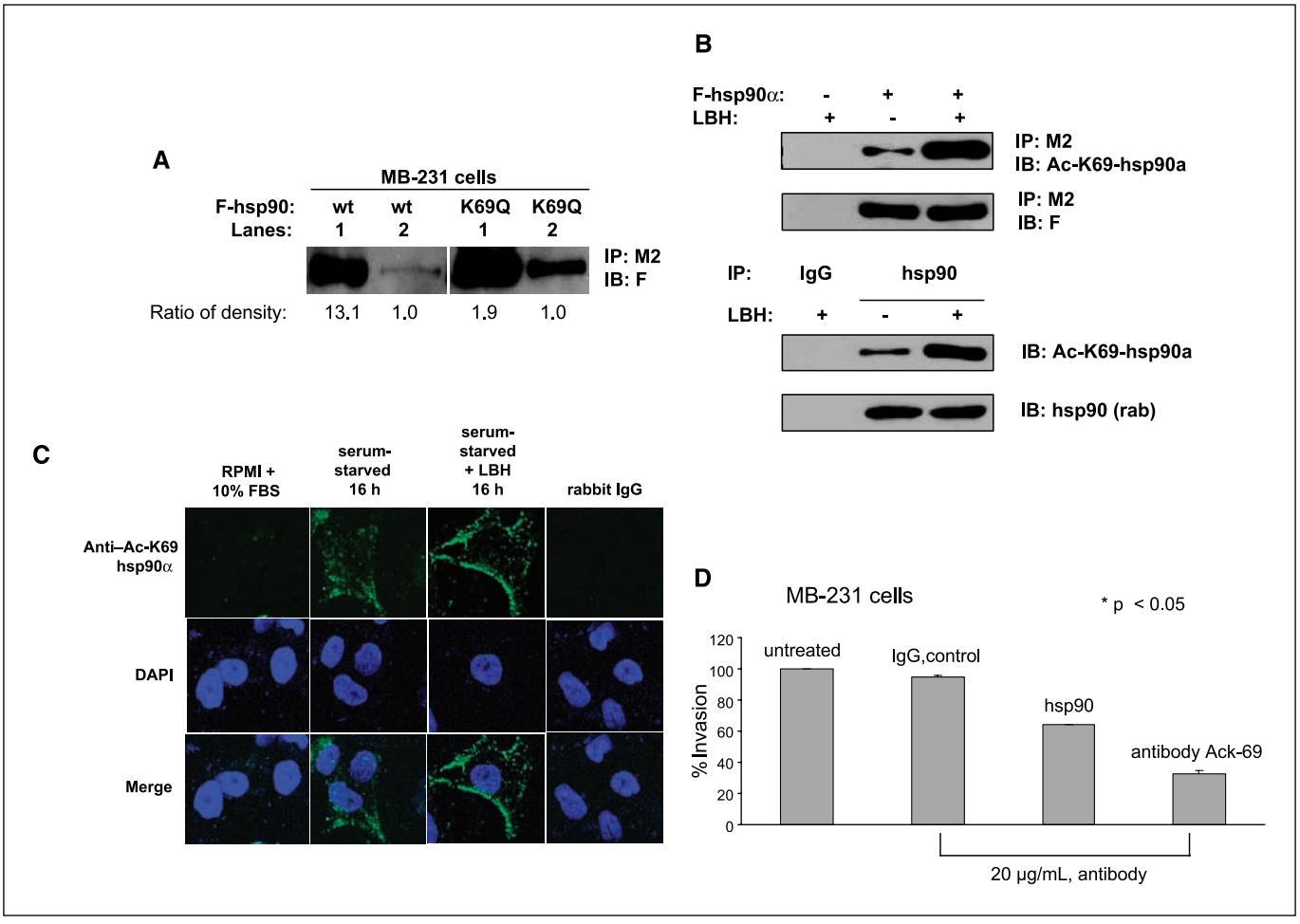


**Figure 6.** Anti–Ac-K69 antibody inhibits hsp90α-dependent *in vitro* invasion by breast cancer cells. *A*, K/Q substitution at K69 promotes extracellular location of hsp90α. MB-231 cells transfected with either F-hsp90 or K69Q mutant were cultured under serum-free condition for 24 h. Total extracellular and intracellular hsp90α were immunoprecipitated with anti–M2-conjugated beads and immunoblotted with anti-F antibody. The intensity of the bands was quantified using ImageQuant 5.2 software, and the ratio for intracellular to extracellular hsp90 is shown below the panel. *Lane 1,* intracellular; and *lane 2,* extracellular. *B,* anti–Ac-K69 hsp90α antibody specifically recognizes acetylated form of both exogenous and endogenous hsp90α expressed in MB-231 cells. MB-231 cells were transfected with F-hsp90α followed by the treatment with LBH589. Immunoprecipitates of F-hsp90α with anti–M2-conjugated beads were immunoblotted with anti–Ac-K69-hsp90α antibody for the acetylation status and anti-F antibody for F-hsp90α expression. Cells transfected with empty vector followed the treatment with LBH589 served as control for specificity. Acetylation of endogenous hsp90α induced by LBH589 was also detected with anti–Ac-K69-hsp90α antibody (*bottom*). Immunoprecipitates of endogenous hsp90α with anti-hsp90α antibody from cell lysates of MB-231 cells treated with or without LBH589 were immunoblotted with either anti–Ac-K69-hsp90α or anti-hsp90α (rabbit) antibody. IgG served as the control for specificity of the immunoprecipitates. *C,* LBH589 induces surface localization of acetylated hsp90α in MB-231 cells. Serum-starved MB-231 cells were treated with 40 nmol/L LBH589 for 16 h, followed by staining with anti-AcK69 hsp90α antibody and confocal microscopy. Cells cultured in RPMI with 10% FBS and cells stained with rabbit IgG served as controls. *D,* inhibition of *in vitro* invasion by MB-231 cells by anti-AcK69 hsp90α antibody. Serum-starved MB-231 cells treated with 20 μg/mL anti-hsp90α or anti–Ac-K69 hsp90α antibody were used for determining *in vitro* Matrigel invasion. Untreated cells, or cells treated with IgG were used as controls. *Columns,* average results of three independent experiments; *bars,* SD. *, $P < 0.05$.

facilitating invasion and metastasis. Parenthetically, activation of HSF1 and heat shock response by proteotoxic stress was identified as a potent modifier of tumorigenesis and required for tumor initiation and maintenance in cancer cells, highlighting a non-oncogene addictive stress phenotype of cancer cells (45, 46). A recent report showed that in a multivariate analysis, the level of hsp90 expression was an independent prognostic factor of survival in patients with breast cancer (47). Our findings raise the possibility that, due to intratumoral stress in the primary breast cancers, increased expression, hyperacetylation, and extracellular location of hsp90α promotes MMP maturation, increased tumor invasion, and metastasis. This may be responsible for the overall negative effect of high hsp90α expression on the survival in breast cancer patients. Therefore, perhaps it is the expression of acetylated hsp90α, which is the important determinant of metastases and overall prognosis in breast cancer. As its corollary, it would be important to determine whether the combination of LBH589 and anti-AcK69 hsp90α antibody will inhibit *in vivo* invasion and metastasis by breast cancer cells.

## Disclosure of Potential Conflicts of Interest

K. Bhalla: commercial research grant, Novartis. P. Atadja: employee, Novartis.

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