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Identification of residues on Hsp70 and Hsp90 ubiquitinated by the co-chaperone CHIP

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Abstract

Molecular chaperones Hsp70 and Hsp90 are in part responsible for maintaining the viability of cells by facilitating the folding and maturation process of many essential client proteins. The ubiquitin ligase C-terminus of Hsc70 interacting protein (CHIP) has been shown *in vitro* and *in vivo* to associate with Hsp70 and Hsp90 and ubiquitinate them; thus targeting them to the proteasome for degradation. Here, we study one facet of this CHIP-mediated turnover by determining the lysine residues on human Hsp70 and Hsp90 ubiquitinated by CHIP. We performed *in vitro* ubiquitination reactions of the chaperones using purified components and analyzed the samples by tandem mass spectrometry to identify modified lysine residues. Six such ubiquitination sites were identified on Hsp70 (K325, K451, K524, K526, K559, K561) and thirteen ubiquitinated lysine residues were found on Hsp90 (K107, K204, K219, K275, K284, K347, K399, K477, K481, K538, K550, K607, K623). We mapped the ubiquitination sites on homology models of almost full-length human Hsp70 and Hsp90, which were found to cluster in certain regions of the structures. Furthermore, we determined that CHIP forms poly-ubiquitin chains on Hsp70 and Hsp90 linked via K6, K11, K48, and K63. These findings clarify the mode of ubiquitination of Hsp70 and Hsp90 by CHIP which ultimately leads to their degradation.

Keywords

ubiquitin; protein turnover; tandem mass spectrometry; molecular chaperone; C-terminus of Hsc70 interacting protein

Introduction

Protein turnover is an essential cellular process needed for health and survival. It is a way to ensure proper function of proteins, regulate cellular mechanisms and prevent the accumulation of the aberrant proteins. Here, we focus on the turnover of the mammalian molecular chaperones Hsp70 and Hsp90. Many proteins are dependent upon these

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chaperones for proper folding and maturation. Numerous kinases and transcription factors, for example, are 'clients' of Hsp70 and Hsp90.¹

Hsp70 is a monomer with two domains: an N-terminal ATPase domain and a C-terminal client protein binding domain.² Hsp90 functions as a dimer and contains three domains: an N-terminal ATPase domain, a middle domain, and a C-terminal dimerization domain.³ Through their C-terminal sequences, Hsp70 and Hsp90 bind the tetratricopeptide repeat 1 (TPR1) and TPR2A domains of Hsp70/Hsp90 organizing protein (HOP), respectively.⁴ An unfolded client protein first associates with Hsp70, where it is partially folded, and is then passed to Hsp90, where its maturation is completed.⁵ During the folding process, both chaperones undergo conformational changes which are coupled to their ATPase activities.

Hsp70 and Hsp90 turnover is dependent upon the ubiquitin-proteasome system. In this pathway, ubiquitin ligases attach the C-terminus of a ubiquitin molecule to typically a lysine side chain on a target protein via an isopeptide bond.⁶ In many cases a poly-ubiquitin chain, in which the C-terminus of one ubiquitin is linked to a lysine on another ubiquitin, is attached to a target protein. It is known that a target protein modified by a chain of four or more ubiquitin molecules linked via K48 is recognized by the proteasome and subsequently degraded.⁷ It has recently been demonstrated that poly-ubiquitin chains linked via other lysine residues may also target the modified protein for degradation.⁸

CHIP is the ubiquitin ligase which ubiquitinates Hsp70 and Hsp90. This enzyme contains an N-terminal TPR domain, a central α -helical domain, and a C-terminal U-box ubiquitin ligase domain.^{9,10} By associating, via its TPR domain, with the C-terminal sequences of Hsp70 and Hsp90, CHIP has been proposed to ubiquitinate the chaperones' client proteins, and target them to the proteasome for degradation.¹¹ CHIP is thus the quality control regulator of the folding pathway. We and others have previously shown that Hsp70 and Hsp90 are also ubiquitinated by CHIP *in vitro*.^{12–14} In addition, CHIP-mediated turnover of Hsp70 and Hsp90 has been studied extensively *in vivo*.¹⁵ Over-expression of CHIP leads to a decrease in Hsp70 levels; conversely an increase in Hsp70 was noted in the absence of CHIP. Furthermore, addition of exogenous CHIP to CHIP knock-out cells greatly decreased the half-life of both Hsp70 and Hsp90. Our previous data suggest that the cellular concentration of CHIP is fine-tuned relative to the concentrations of the chaperones, so that there is a constant, low level of CHIP-dependent Hsp70 and Hsp90 ubiquitination and consequent proteasome-mediated degradation.¹²

Here, we further investigate the mechanism of Hsp70 and Hsp90 degradation by identifying the lysine residues on the chaperones ubiquitinated by CHIP and mapping these residues on homology models of Hsp70 and Hsp90 to find any structural regions where the ubiquitination sites cluster. We also determine that CHIP forms poly-ubiquitin chains on Hsp70 and Hsp90 and identify the types of ubiquitin-ubiquitin linkages in these chains.

Results

We performed CHIP mediated *in vitro* ubiquitination of Hsp70 and Hsp90 using purified components. UbcH5c was used as the ubiquitin conjugating enzyme because it has previously been shown to function with CHIP *in vitro*.¹⁴ Ubiquitination of Hsp70 and Hsp90 can be detected by the formation of higher molecular weight species which are evident following electrophoresis on SDS poly-acrylamide gels (Fig. 1a). To ensure that these bands predominantly correspond to ubiquitinated Hsp70 or Hsp90 and not free poly-ubiquitin chains, we performed ubiquitination assays in the absence of Hsp70 or Hsp90 (Fig. 1b). Without the chaperones, we do not observe a significant amount of other protein bands in the region of the gel where ubiquitinated Hsp70 or Hsp90 would resolve. CHIP has

previously been shown to auto-ubiquitinate itself.¹⁶ The CHIP-ubiquitin conjugates can be detected in different areas of the gels. Bands on the gel in Fig. 1a that correspond to unmodified and modified Hsp70 and Hsp90, after 30 or 45 minutes of ubiquitination, were cut out of the Coomassie-stained poly-acrylamide gels, digested with trypsin, and analyzed by liquid chromatography - tandem mass spectrometry (LC-MS/MS). Trypsin digestion of a ubiquitinated protein leaves the C-terminus of ubiquitin, Gly-Gly, attached to the ubiquitinated lysine residue. We searched the mass spectrometry data for such modification of Hsp70 and Hsp90 lysines.

Hsp70 lysine residues ubiquitinated by CHIP

Identified Hsp70 peptides in the two ubiquitinated Hsp70 samples, which have a Mascot software ion score 40 or greater, cover ~ 64 % of the protein sequence. We did not observe any ubiquitination in the unmodified Hsp70 sample. In the ubiquitinated Hsp70 samples, together we found six modified Hsp70 lysine residues: K325, K451, K524, K526, K559, K561 (Fig. S1). The MS/MS spectra of two Hsp70 peptides which include the identified ubiquitination sites K325 and K524 are depicted in Fig. 2a and 2b, respectively. The MS/MS spectra of Hsp70 peptides which contain the remaining ubiquitination sites are shown in Figs. S4 – S7.

To learn more about ubiquitination of Hsp70 by CHIP, we mapped the ubiquitinated lysine residues on the structure of Hsp70. Because a crystal structure of human Hsp70 with both domains present is not available, we built a homology model of almost full-length Hsp70 (the 30 C-terminal residues are missing) based on *Bos taurus* Hsc70¹⁷ and *Escherichia coli* DnaK¹⁸ crystal structures. Ubiquitinated lysine residues are indicated on this Hsp70 homology model (Fig. 2c). All six lysines are solvent accessible. Four out of the six identified residues are located near the interface between the ATPase domain and the client protein binding domain: K524, K526, K325, and K451. K559 and K561 are located further away on the α -helical portion of the client protein binding domain. This α -helical segment is commonly referred to as the 'lid'.² The β -sheet subdomain of the client protein binding domain contains the client protein binding site. When ADP is bound to the ATPase domain, the lid lies close to the client protein (without interacting with it) and the β -subdomain, thus enclosing the client protein.¹⁸ The homology model is of this 'closed' conformation. When the ATPase domain is bound to ATP, the lid is believed to be further away from the β -subdomain in an extended conformation, exposing the client protein binding site. Because the ubiquitination reaction is ATP dependent, we expect Hsp70 to predominantly be in this alternate 'open' conformation when being ubiquitinated by CHIP in our assays. We expect K559 and K561 therefore to be further away from the β -subdomain than they are on the model in Fig. 2c.

Hsp90 lysine residues ubiquitinated by CHIP

Identified Hsp90 peptides in the two ubiquitinated Hsp90 samples, which have a Mascot software ion score 40 or greater, cover ~ 64 % of the protein sequence. We did not observe any ubiquitination in the unmodified Hsp90 sample. In the ubiquitinated Hsp90 samples, together we found thirteen modified Hsp90 lysine residues: K107, K204, K219, K275, K284, K347, K399, K477, K481, K538, K550, K607, K623 (Fig. S2). The MS/MS spectra of two Hsp90 peptides which include the identified ubiquitination sites K284 and K481 are depicted in Fig. 3a and 3b, respectively. The MS/MS spectra of Hsp90 peptides which contain the remaining ubiquitination sites are shown in Figs. S8 – S17. Hsp90 peptides 224EISDDEAEEEEKGEKEEEDKDDEEKPK249 and 250IEDVGSDEEDDSGKDKKK267 contain a ubiquitinated lysine residue as well, although we were unable to determine the exact ubiquitination site based on the MS/MS spectra.

As for Hsp70, a crystal structure of full-length human Hsp90 is not available. We built a homology model of human Hsp90 based on the crystal structure of almost full-length *Saccharomyces cerevisiae* Hsp90.¹⁹ Ubiquitinated lysine residues are indicated on this Hsp90 homology model (Fig. 3c). All thirteen lysines are solvent accessible. Six ubiquitinated lysine residues (K477, K481, K538, K550, K607, K623) cluster at the C-terminal end of Hsp90: on the C-terminal dimerization domain or on the section of the middle domain close to the C-terminal domain. The other seven ubiquitinated lysine residues (K107, K204, K219, K275, K284, K347, K399) are located either on the N-terminal ATPase domain or on the middle domain in proximity to the ATPase domain. When ATP is bound to the ATPase domain, Hsp90 is believed to be in a closed conformation where both the C-terminal dimerization domain as well as the N-terminal ATPase domain interact with the identical domain of the other monomer.^{3,20} Hsp90 in this state is a tightly twisted dimer with two dimerization interfaces. In the absence of a bound nucleotide, Hsp90 is in an open or extended conformation in which the C-terminal dimerization domain provides the only dimerization interface. *S. cerevisiae* Hsp90 crystal structure, and hence our homology model of human Hsp90, depicts the chaperone in the closed conformation. Recent single-particle electron microscopy experiments however show that without co-chaperones human Hsp90 bound to ATP is predominantly in the open conformation with a small fraction being in the closed conformation.²¹ In our ubiquitination assays, we therefore expect the ATP bound Hsp90 to mainly be in the open conformation. CHIP is bound to the C-terminus of Hsp90 when ubiquitinating the chaperone. The open Hsp90 conformation is probably allowing the lysine residues in the more distant N-terminal ATPase domain to be brought into closer proximity with CHIP for subsequent modification by ubiquitin.

Ubiquitin chains on Hsp70 and Hsp90

Peptides corresponding to ~ 95% of the ubiquitin sequence were identified in the mass spectrometry experiments. Certain ubiquitin residues were found to be modified by another ubiquitin molecule in all the samples of ubiquitinated protein. This signifies the presence of not only mono-ubiquitination but also poly-ubiquitin chains attached to Hsp70 and Hsp90. Our LC/MS-MS data indicate that K6, K11, K48 and K63 linked poly-ubiquitin chains are conjugated to both Hsp70 and Hsp90 (Fig. S3). The MS/MS spectra of two ubiquitin peptides which include the identified ubiquitination sites K63 and K48 are depicted in Fig. 4a and 4b, respectively. The MS/MS spectra of ubiquitin peptides which contain the remaining ubiquitination sites are shown in Figs. S18 and S19.

Poly-ubiquitin chains linked via K48 are known to target modified proteins to the proteasome for degradation.⁷ Recently K6 and K11 ubiquitin linkages have also been shown to possibly function in the ubiquitin-proteasome pathway.⁸ Observing K6, K11, and K48 poly-ubiquitin chains on Hsp70 and Hsp90 thus correlates well with the *in vivo* data¹⁵ in which ubiquitination of the chaperones by CHIP leads to their degradation. Modification of a target protein does not always involve degradation, however. K63 poly-ubiquitination has been implicated in signal transduction and DNA repair⁶, although more recent studies suggest a role for this type of ubiquitin linkage in proteasome degradation as well.^{22,23} Ubiquitination of Hsp70 or Hsp90 by CHIP has not been associated with a cellular process other than degradation. The function of K63 poly-ubiquitin chains linked to Hsp70 and Hsp90 requires further elucidation.

Discussion

In summary, we have identified not only the specific lysine residues on Hsp70 and Hsp90 ubiquitinated by CHIP, but also the structural regions of the chaperones where these ubiquitination sites cluster. It is possible that CHIP also ubiquitinates other Hsp70 and

Hsp90 residues not identified by these experiments as Hsp70 peptides which include 27 out of the total 50 lysine residues and Hsp90 peptides which include 32 out of the total 75 lysine residues were not sequenced. For example, there are lysine residues not shown to be ubiquitinated in close proximity to the modified lysines. It is unclear whether these unmodified residues are not ubiquitinated by CHIP or whether ubiquitination of these lysine residues was not detected in the experiments. However, the observations that only 6 of the 23 sequenced lysine residues on Hsp70 and only 13 of the 43 sequenced lysine residues on Hsp90 were identified as the ubiquitination sites and that they cluster in certain regions of the chaperones' structures, suggest that we have identified the lysine residues likely to be ubiquitinated by CHIP *in vivo*. We further established that CHIP attaches poly-ubiquitin chains to Hsp70 and Hsp90 and determined the types of ubiquitin linkages present in these chains.

We searched the literature to determine whether such observed clusters of ubiquitination sites on structures of substrates are common. Even though many lysine residues modified by ubiquitin have been identified on various substrates, their locations on structures were not analyzed in detail. There is experimental evidence, however, that more than one lysine residue can be ubiquitinated by the cognate ubiquitin ligase and that the ubiquitination sites at least sometimes cluster to one region or a domain of the protein.^{24–26} Because of the geometric constraints of the ubiquitin conjugating enzyme – ubiquitin ligase – substrate complex, we expect only a subset of all lysine residues located in certain regions of the substrate to be capable of accepting the ubiquitin molecule.

Acetylation of Hsp90 at K286 (isoform β) has been shown to affect the function of the chaperone.²⁷ It was of interest to us to examine whether this lysine residue is also ubiquitinated by CHIP, as ubiquitination would prevent acetylation and thus the subsequent effect on Hsp90 function. K286 was not found to be ubiquitinated in our experiments. Other acetylation sites on Hsp90 α have also been identified previously.²⁸ Three such lysine residues shown to be acetylated, K284, K538, and K550 in Hsp90 β , were found to be ubiquitinated by CHIP as well. The role of this likely competition between lysine acetylation and ubiquitination requires further investigation.

Materials and Methods

DNA constructs

Full-length human CHIP was cloned as a BamHI/NcoI restriction fragment from pcDNA3-CHIP (a gift from Cam Patterson)¹¹ into pET11a (Stratagene) which had been modified to include a TEV cleavable His₆-tag between the promoter and cloning cassette to create pET11a-His₆-TEV-hCHIP. Full-length human His₆-Hsp90 β in pET-14b²⁹ and human His₆-Hsp70 in pET28 were gifts from Sophie Jackson. Full-length human Ubch5c in pET28a (without a tag) was a gift from Rachel Klevit.³⁰

Recombinant protein purification

All recombinant proteins were expressed in BL21(DE3) *E. coli* cells induced with 0.8 mM IPTG. CHIP, Hsp70, and Hsp90 were purified using Ni-NTA agarose (Qiagen) according to manufacturer's instructions. They were further purified over Superdex S200 16/60 gel filtration column (Amersham) in 50 mM Tris pH 7.4, 10 mM NaCl, 5 mM β -mercaptoethanol (β ME) buffer. Proteins were used without removing the His₆ tags.

Ubch5c purification was performed as described previously³⁰ with slight modifications. Briefly, Ubch5c in cell lysate was loaded onto UNO S1 ion exchange column (Bio-Rad) equilibrated in 30 mM MES pH 6, 1 mM EDTA, 2 mM β ME and eluted using 0–1 M NaCl gradient. Further purification was achieved using a Superdex S75 16/60 gel filtration column

(Amersham), equilibrated in 25 mM Na phosphate pH 7, 150 mM NaCl, 1 mM β ME. Final protein stocks were dialyzed into the same buffer but with lower β ME concentration of 0.1 mM. Protein concentrations were determined using absorbance at 280 nm.

In vitro ubiquitination assay and trypsin digest

For Hsp70 and Hsp90 ubiquitination assays, 0.091 μ M human ubiquitin activating enzyme (UBE1) (BostonBiochem), 20 μ M UbcH5c, 20 μ M CHIP, and either 20 μ M Hsp70 or 20 μ M Hsp90 were combined in 50 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 150 mM KCl, 10 mM ATP, 0.1 mM β ME buffer. For ubiquitination reactions in the absence of Hsp70 or Hsp90, 0.091 μ M human UBE1, 20 μ M UbcH5c, 20 μ M CHIP, and 20 μ M GST were combined in the above buffer. All reactions were pre-incubated at 30°C for ~10 minutes, initiated by the addition of 0.5 mM ubiquitin (Sigma), and kept at 30°C for the duration of the reaction. 10 μ l aliquots were quenched with SDS sample buffer at various time points after initiation of the reactions and resolved on Coomassie-stained SDS-polyacrylamide gels. Protein bands were excised out of the gel and in-gel trypsin (Promega Corp.) digest was performed as described previously³¹ with a slight modification: iodoacetamide was replaced with chloroacetamide because iodoacetamide was shown to cause a ubiquitination-like artifact.³²

LC-MS/MS

LC-MS/MS experiments were performed on LTQ Orbitrap which was equipped with a Waters nanoAcquity UPLC system, and used a Waters Symmetry® C18 180 μ m $\times$ 20mm trap column and a 1.7 μ m, 75 μ m $\times$ 250 mm nanoAcquity™ UPLC™ column (35°C) for peptide separation. Trapping was done at 15 μ l/min, 99% Buffer A (100% water, 0.1% formic acid) for 1 min. Peptide separation was performed at 300 nl/min with Buffer A: 100% water, 0.1% formic acid and Buffer B: 100% CH₃CN, 0.075% formic acid. A linear gradient (51 minutes) was run with 5% buffer B at initial conditions, 50% B at 50 minutes, and 85% B at 51 minutes. MS was acquired in the Orbitrap using 1 microscan, and a maximum inject time of 900 followed by four data dependant MS/MS acquisitions in the ion trap. Neutral loss scans (MS3) were also obtained for 98.0, 49.0, and 32.7 amu.

Database searching

All MS/MS spectra were searched in-house using the Mascot algorithm³³ (version 2.2.0) for un-interpreted MS/MS spectra after using the Mascot Distiller program to generate Mascot compatible files. The Mascot Distiller program combined sequential MS/MS scans from profile data that have the same precursor ion. A charge state of +2 and +3 were preferentially located with a signal to noise ratio of 1.2 or greater and a peak list was generated for database searching. Using the Mascot database search algorithm, a protein was identified when Mascot listed it as significant and more than 2 peptides matched the same protein. NCBI database was searched. The Mascot significance score match was based on MOWSE score. Parameters used for searching were lysine ubiquitination, partial methionine oxidation, and carboxamidomethylated cysteine with a peptide tolerance of +20ppm, MS/MS fragment tolerance of +0.6 Da, and peptide charges of +2 or +3. Normal and decoy database searches were run.

Homology modeling human Hsp70

The CLUSTAL W³⁴ multiple sequence alignment program at the Network Protein Sequence Analysis website³⁵ was used to create an alignment between human Hsp70, *B. taurus* Hsc70, and *E. coli* DnaK sequences. SwissModel in Swiss PDB viewer³⁶ was used to build a homology model of human Hsp70 N-terminal half (residues 1–536) based on the crystal structure of *B. taurus* Hsc70, residues 1–536 (1yuw)¹⁷ and the sequence alignment. A

homology model of human Hsp70 C-terminal half (residues 392–611) was then built based on the crystal structure of *E. coli* DnaK client protein binding domain (1dkz)¹⁸ and the multiple sequence alignment. The N-terminal and C-terminal models of human Hsp70 were superimposed in Swiss PDB viewer via the shared residues 392–536. RMSD of 0.85 Å was obtained. The final model of human Hsp70 (1–611) was created by combining residues 1–521 of the N-terminal model and residues 522–611 of the C-terminal model.

Homology modeling human Hsp90

The CLUSTAL W³⁴ multiple sequence alignment program at the Network Protein Sequence Analysis website³⁵ was used to create an alignment between human and *S. cerevisiae* Hsp90 sequences. SwissModel in Swiss PDB viewer³⁶ was used to build a homology model of human Hsp90 (residues 11–689) based on the crystal structure of *S. cerevisiae* Hsp90 (2cg9)¹⁹ with the co-crystallized Sba1 deleted. Each monomer of human Hsp90 was built independently of each other. They were then superimposed onto *S. cerevisiae* Hsp90 dimer and the coordinates for each human monomer were combined to create a dimer. Residues 224–274 are missing in the model of human Hsp90, because the corresponding residues are also missing in the crystal structure of *S. cerevisiae* Hsp90. Shorter segments of Hsp90 (10–14 residues) which are missing in the crystal structure, were modeled by the SwissModel software. Figures depicting the ribbon diagrams of Hsp70 and Hsp90 homology models were prepared using Swiss PDB viewer and POV-Ray (Persistence of Vision Raytracer Pty. Ltd.).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used

CHIP	C-terminus of Hsc70 interacting protein
HOP	Hsp70/Hsp90 organizing protein
TPR	tetratricopeptide repeat domain
LC-MS/MS	liquid chromatography – tandem mass spectrometry

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Fig. 1.

In vitro ubiquitination of Hsp70 and Hsp90 by CHIP. Coomassie-stained SDS-polyacrylamide gel analysis of ubiquitination reactions (a) in the presence of Hsp70 or Hsp90 and (b) in the absence of the chaperones at various times after initiation. Protein bands corresponding to unmodified Hsp70, Hsp90, CHIP, and the various ubiquitin conjugates as well as the molecular weight of the marker bands (M lane) are indicated. Trypsin digest and subsequent LC-MS/MS analysis was performed on the protein bands delineated by the red boxes in (a). The section of the gel in (b) where we would expect modified Hsp70 and Hsp90 to resolve is highlighted with a dashed box and does not include a significant amount of protein bands. Section of the gel shown in (b) zooms in on the region of interest.

Fig. 2. Hsp70 lysine residues ubiquitinated by CHIP. (a and b) MS/MS spectra of representative Hsp70 peptides (a) $^{325}\text{DAKLDKAQIHDLVLVGGSTR}_{342}$ and (b) $^{518}\text{MVQEAKEYKADEVQR}_{533}$ both containing a Gly-Gly modified (ubiquitinated) lysine residue. Modified lysine residues are labeled with $^{\text{GG}}$ and correspond to (a) K325 and (b) K524 in Hsp70. Fractionation of the peptides into b and y ions is indicated and the corresponding peaks are labeled on the spectra. Peptide fragment ions which have +2 charge or lost a water molecule are indicated with $^{+2}$ and *, respectively. The y-axis, relative abundance, was normalized to the most abundant identified peptide fragment. The Mascot software ion scores of these peptides are 88 and 82, respectively. (c) Ribbon diagram of Hsp70 homology model. Six Hsp70 lysine residues found to be ubiquitinated by CHIP are shown as ball-and-stick representations, colored in red and labeled. Hsp70 domains are labeled.

Fig. 3.

Hsp90 lysine residues ubiquitinated by CHIP. (a and b) MS/MS spectra of representative Hsp90 peptides (a) ${}^{276}\text{YIDQEELNKTkPIWTR}_{291}$ and (b) ${}^{477}\text{MKETQKSIYYITGESK}_{491}$ both containing a Gly-Gly modified (ubiquitinated) lysine residue. Modified lysine residues are labeled with GG and correspond to (a) K284 and (b) K481 in Hsp90. Fractionation of the peptides into b and y ions is indicated and the corresponding peaks are labeled on the spectra. Peptide fragment ions which have +2 charge, lost a water molecule, or lost an NH_3 group are indicated with $^{+2}$, $*$, and $\#$, respectively. The y-axis, relative abundance, was normalized to the most abundant identified peptide fragment. The Mascot software ion scores of these peptides are 60 and 72, respectively. (c) Ribbon diagram of Hsp90 dimer homology model with monomers colored differently. Thirteen Hsp90 lysine residues found to be ubiquitinated by CHIP are shown as ball-and-stick representations, colored in red and labeled on one monomer. Hsp90 domains are labeled.