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High-Throughput Targeted Quantitative Analysis of the Interaction between HSP90 and Kinases

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Abstract

Kinases, which function in numerous cell signaling processes, are among the best characterized groups of client proteins for the 90-kDa heat shock protein (HSP90), a molecular chaperone that suppresses the aggregation and maintains the proper folding of its substrate proteins (i.e., clients). No high-throughput proteomic method, however, has been developed for the characterizations of the interactions between HSP90 and the human kinome. Herein, by employing a parallel-reaction monitoring (PRM)-based targeted proteomic method, we found that 99 out of the 249 detected kinase proteins display diminished expression in cultured human cells upon treatment with ganetespib, a small-molecule HSP90 inhibitor. PRM analysis of kinase proteins in the affinity pull-down samples showed that 86 out of the 120 detected kinases are enriched from the CRISPR-engineered cells where a tandem affinity tag was conjugated with the C-terminus of endogenous HSP90β protein over the parental cells. Together, our results from the two complementary quantitative proteomic experiments offer systematic characterizations about the HSP90–kinase interactions at the entire proteome scale and reveal extensive interactions between HSP90 and kinase proteins in human cells.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.anal-chem.9b03320.

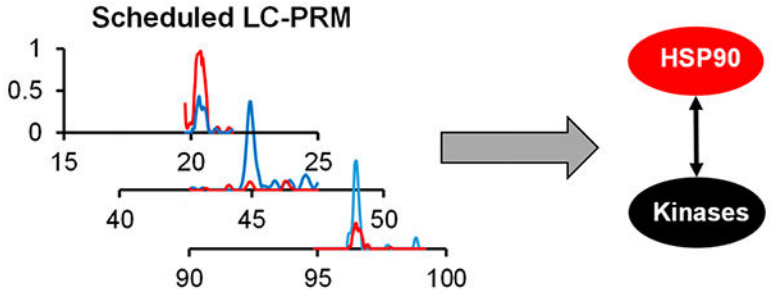
Detailed experimental procedures and additional characterization data (PDF)

Table S1, relative expression levels of kinase proteins in HEK293T cells upon a 24-h treatment with 100 nM ganetespib or mock treatment with DMSO; results were obtained from LC–MS/MS analysis in the PRM mode (XLSX)

Table S2, kinases enriched from affinity pull-down from lysates of Flag-HSP90 expressing HEK293T cells relative to wild-type HEK293T cells; results were obtained from LC–MS/MS analysis in the PRM mode (XLSX)

The authors declare no competing financial interest.

The raw files for LC-PRM analyses of kinases were deposited into PeptideAtlas with the identifier number of PASS01357 (http://www.peptideatlas.org/PASS/PASS01357).



As a molecular chaperone, HSP90 facilitates the proper folding of client proteins to maintain homeostasis of the proteome.[1] Kinases, which catalyze the phosphorylation of biological molecules[2] and play crucial roles in cell signaling and in regulation of cell proliferation and metabolism,[3] are among the best characterized groups of client proteins for HSP90.[4] Thus, it is important to investigate comprehensively the interactions between HSP90 and kinases. These interactions were previously studied with luminescence-based mammalian interactome (LUMIER) assay or affinity pull-down followed by Western blot analyses.[4] However, LUMIER assay requires ectopic expression of kinases, which may not faithfully reflect the behaviors of endogenous kinases. Additionally, Western blot analysis is time-consuming and has low throughput.

The targeted proteomic method, which utilizes multiple-reaction monitoring (MRM) or parallel-reaction monitoring (PRM), affords much better sensitivity and reproducibility than shotgun proteomic analysis in the data-dependent acquisition mode.[8,9] Moreover, PRM, owing to the use of a high-resolution mass analyzer for MS/MS acquisition, is advantageous over MRM in the specific and accurate identification/quantification of analytes in complex sample matrixes.[8,9] Hence, PRM has recently become a widely used bioanalytical method.[10–12]

We characterized comprehensively the interactions between HSP90 and the human kinome by employing a recently published scheduled LC-PRM-based targeted proteomic method[13–16] in combination with stable isotope labeling by amino acids in cell culture (SILAC).[17] In this experiment, a Q Exactive Plus mass spectrometer was set up to collect the tandem mass spectra (MS/MS) for the precursor ions from a limited number of peptides in each 8 min retention time (RT) window.[12,18,19] We first assessed the differential expression of kinases in cultured human cells upon treatment with ganetespib (Figure 1a). In this vein, ganetespib is one of the most widely used small-molecule inhibitors for HSP90.[5] It binds to the ATP-binding pocket located in the N-terminal portion of HSP90 and compromises its capability in maintaining the proper folding of client proteins,[5,7] thereby resulting in the degradation of client proteins through the ubiquitin-proteasome pathway.[5,7] Ganetespib has also been exploited in the preclinical stage for treating various human diseases.[5,6]

Our LC-PRM analysis results showed that 99 out of the 249 quantified kinases were down-regulated by at least 1.5-fold in HEK293T cells upon ganetespib exposure (Figure S1 and Table S1). It is worth noting that treatment with ganetespib did not affect the level of expression of HSP90 protein (Figure S2a). In addition, we monitored, by employing real-

time quantitative PCR analysis, the mRNA expression levels of nine randomly selected kinase genes whose protein products are diminished in HEK293T cells upon ganetespib treatment, and it turned out that only NEK1 displayed a statistically significant decrease in the mRNA expression level (Figure S2b). Therefore, the decreases in expression of most of the 99 kinases are unlikely attributable to alterations in mRNA expression levels of these kinase genes, and most of the down-regulated kinases are considered candidate client proteins for HSP90.

To further assess the interactions between kinases and HSP90, we employed a previously generated CRISPR cell line where a tandem affinity tag (3× Flag, 2× Strept) was conjugated to the C-terminus of endogenous HSP90β protein in HEK293T cells.[20] With affinity purification using anti-Flag M2 beads followed by tryptic digestion and LC-PRM analysis (Figure 1b), we were able to quantify 120 kinases. Strikingly, 86 of them were enriched by at least 1.5-fold from the lysate of the Flag-HSP90β cells relative to the lysate from parental HEK293T cells (Figure S3 and Table S2). In this vein, the smaller number of kinase proteins detected in the pull-down experiments relative to the aforementioned inhibitor experiments could be attributed to the relatively weak interactions between HSP90 and kinases, which may not sustain the washing conditions employed in the in-vitro pull-down experiment.

It is worth noting that the same retention time and dot product (dotp) values[21] of at least 0.7 were found for all PRM transitions (4–6) (Figure S4), thereby providing accurate peptide identification and quantification. Furthermore, the results obtained from forward and reverse SILAC labeling experiments are consistent (Figure 1c,d, Table S1, and Table S2). In this context, it is worth noting that a small number of kinases were quantified only in the forward or reverse SILAC experiments. This is not surprising in light of the fact that some kinase proteins are not expressed at high levels, and the kinase peptides are present in complicated sample matrixes (e.g., the tryptic digestion mixture of whole-cell protein lysate). Nevertheless, the average relative standard deviations (RSD) among the different quantified peptides from the same kinases were 11.5% (Table S1). This observation, together with the consistent results obtained from the forward and reverse SILAC experiments, supports the robustness of PRM-based method for kinase protein quantification.

Among the 98 commonly quantified kinases in ganetespib treatment and anti-Flag pull down experiments (Figure 2a), 29 were both enriched in HSP90β-tagged cells and down-regulated upon treatment with ganetespib (Figure 2b,c and Table S3). These 29 kinases are considered strong candidate client proteins of HSP90, where 8 out of the 29 kinases were previously shown by the LUMIER assay to be HSP90 clients.[4] Importantly, the remaining 21 kinases could be novel client kinases for HSP90 (Table S3). In this vein, MAP4K4 and ARAF were previously identified as strong clients for HSP90 (Table S3),[4] and the interaction between HSP90 and ARAF was validated previously.[20] We also confirmed these interactions by using immunoprecipitation followed by Western blot analyses (Figures 3 and 4). Furthermore, our Western blot analyses revealed the binding between HSP90 and LATS1 discovered in this study and confirmed it as a novel client kinase for HSP90 (Figure 4).

In summary, we employed, for the first time, a PRM-based targeted proteomic approach, together with CRISPR genome editing technology, to fulfill high-throughput analysis of the

interactions between HSP90 and kinase proteins. We found that the expression levels of approximately 40% of the 249 detected kinases were diminished in human cells upon treatment with ganetespib. By using a CRISPR cell line with a tandem affinity tag being integrated to the C-terminus of endogenous HSP90β, we found that more than 70% of the 120 quantified kinases could be enriched by affinity pull-down of HSP90 from the Flag-HSP90β-engineered cells over the parental cells, supporting the interactions between kinases and HSP90. The results led to the discovery of novel kinase proteins as clients for HSP90. Together, this represents the first comprehensive quantitative proteomic characterizations about the interactions between HSP90 and the human kinome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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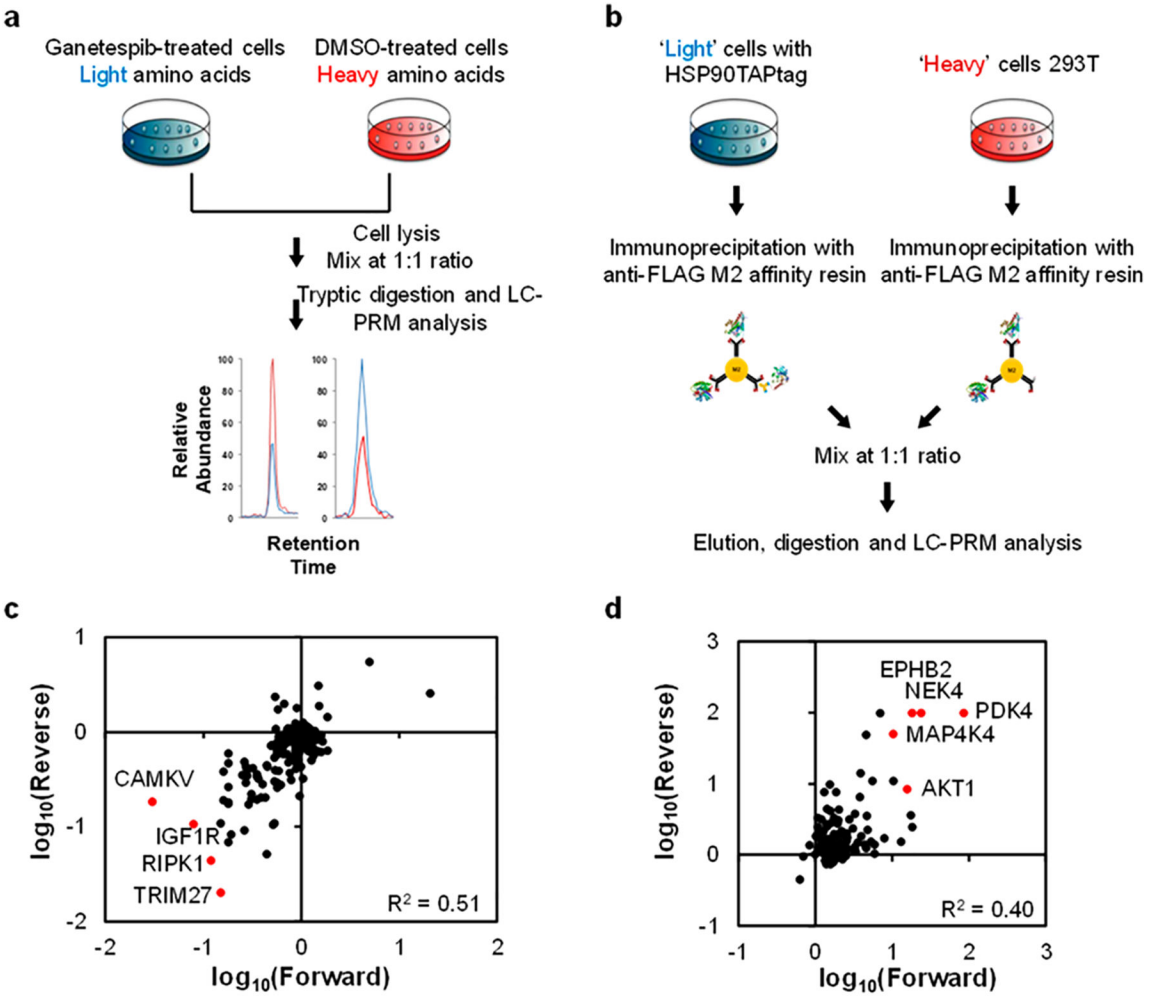

Figure 1.

PRM-based targeted proteomic approach for examining the interaction between HSP90 and the human kinome. (a) Experimental approach, involving the use of forward SILAC labeling together with the PRM-based targeted proteomic analysis, for monitoring the changes in expression of kinase proteins in human cells upon treatment with ganetespib. (b) Experimental strategy, involving the combination of forward SILAC labeling with LC-PRM-based targeted proteomic analysis, for the identification of cellular proteins that interact with HSP90β. (c,d) Scatter plots displaying the correlation between the ratios obtained from forward and reverse SILAC labeling experiments with ganetespib treatment (c) or with anti-Flag pull-down of HSP90β and its interaction proteins (d).

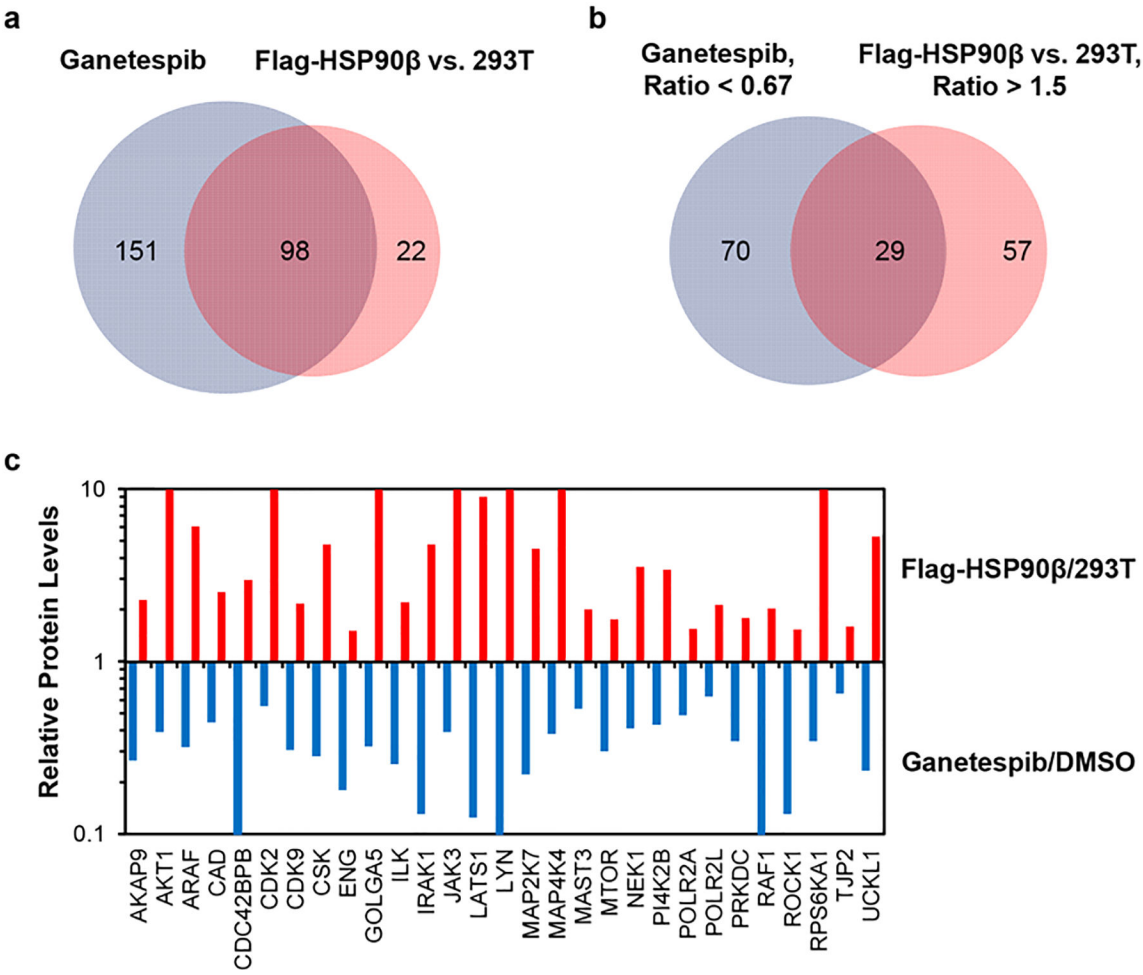

Figure 2.

Kinases that interact with HSP90. Venn diagrams depicting the number of kinases that are commonly quantified (a) or capable of binding with HSP90β (b), i.e., those kinases that could be enriched from affinity pull-down from Flag-HSP90β cells over parental HEK293T cells, and those that could be down-regulated upon HSP90 inhibitor treatment. (c) Bar graph showing the kinases that are both enriched from affinity pull-down from Flag-HSP90β cells and down-regulated upon ganetespib treatment.

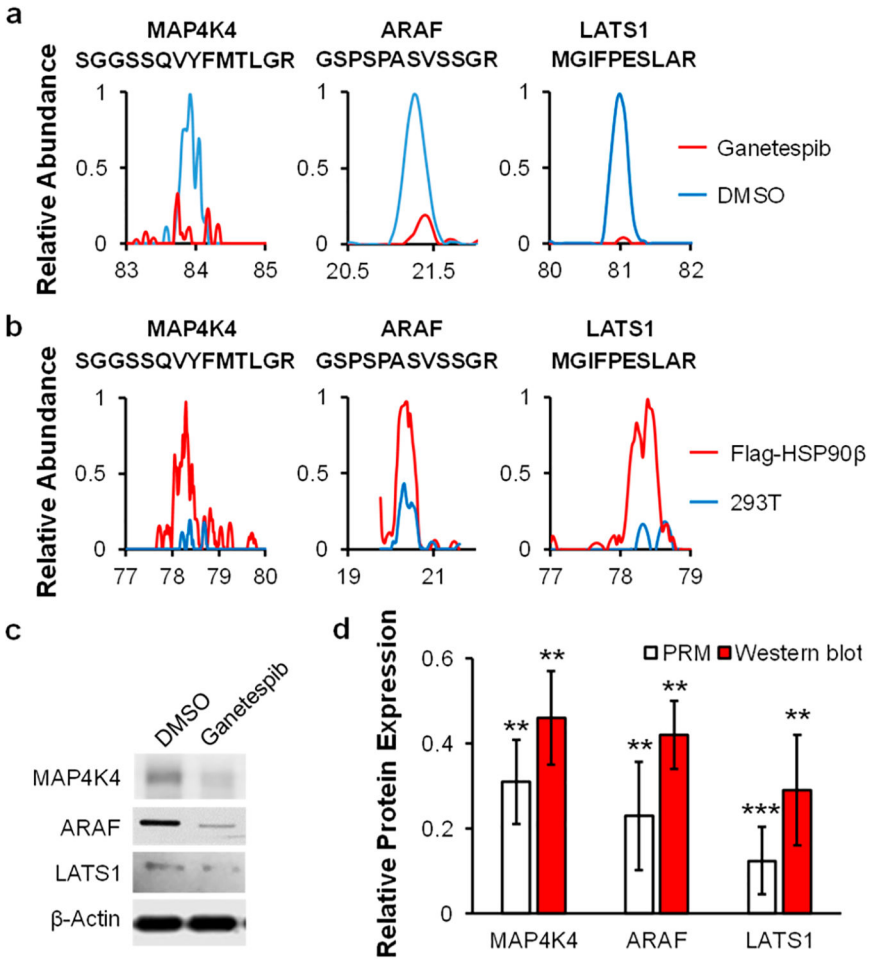

Figure 3.

Interactions between HSP90 and ARAF/MAP4K4/LATS1. Representative PRM traces showing the relative quantification results of ARAF, MAP4K4, and LATS1 from the HEK293T cells with or without ganetespib treatment (a) or from the anti-Flag pull-down mixture in HEK293T cells with or without the integration of a tandem affinity tag to the C-terminus of the HSP90β protein (b). (c) Western blot for the validation of the expression levels of ARAF, MAP4K4, and LATS1 in HEK293T cells with and without ganetespib treatment. (d) Quantitative comparisons of the ratios obtained from PRM (n = 2, one forward and one reverse SILAC labelings) and Western blot analyses (n = 3) for ARAF and MAP4K4 in HEK293T cells with vs without ganetespib treatment. Error bars represent standard deviation. The p values referred to comparison between mock treatment (with DMSO) and a 24-h treatment with 100 nM ganetespib and were calculated using unpaired, two-tailed Student's t-test: **, $0.001 \leq p < 0.01$; ***, $p < 0.001$.

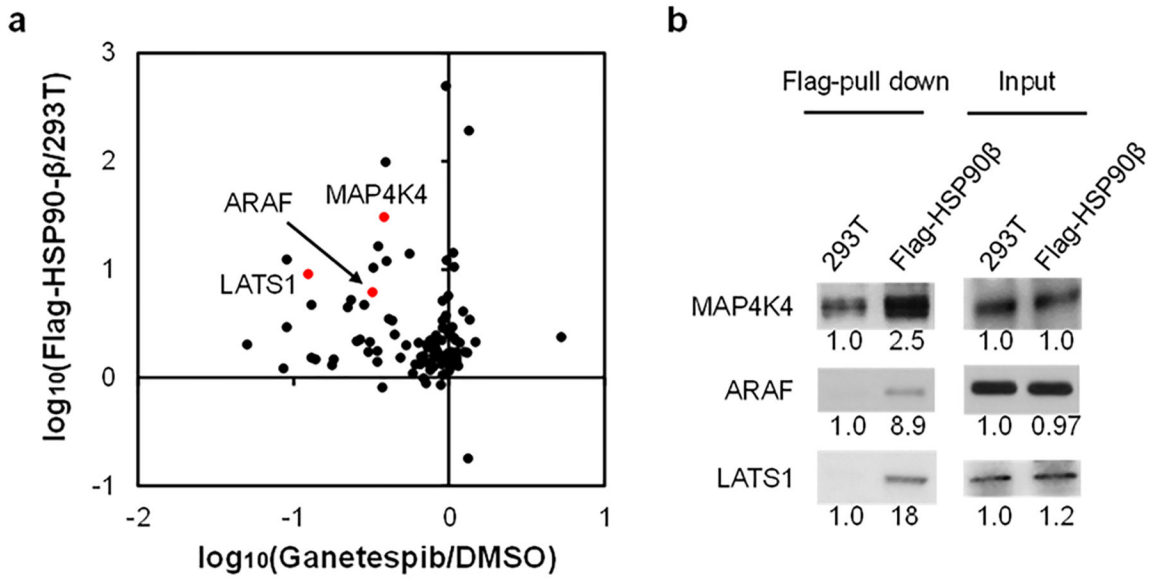

Figure 4.

Interactions between HSP90 and kinases: (a) correlation between the ratios obtained from affinity pull-down and ganetespib treatment and (b) immunoprecipitation followed by Western blot analysis for validating the interactions between HSP90β and kinases.