Chapter 16

Detecting Posttranslational Modifications of Hsp90

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Abstract

The molecular chaperone Heat Shock Protein 90 (Hsp90) is essential in eukaryotes. Hsp90 chaperones proteins that are important determinants of multistep carcinogenesis. The chaperone function of Hsp90 is linked to its ability to bind and hydrolyze ATP. Co-chaperones as well as posttranslational modifications (phosphorylation, SUMOylation, and ubiquitination) are important for its stability and regulation of the ATPase activity. Both mammalian and yeast cells can be used to express and purify Hsp90 and also detect its posttranslational modifications by immunoblotting.

Key words Heat shock protein 90 (Hsp90), Molecular chaperones, Posttranslational modification, Phosphorylation, SUMOylation, Ubiquitination

1 Introduction

Heat Shock Protein 90 (Hsp90) is an essential molecular chaperone in eukaryotes [1, 2]. Its cellular functions have been most clearly identified in mammalian cells, Drosophilia, and baker's yeast *Saccharomyces cerevisiae* [3]. Hsp90 creates and maintains the functional conformation of a subset of proteins [4–6]. These targets (or "clients") are key components of signal transduction pathways and numerous transcription factors. Hsp90 and a discrete set of co-chaperone proteins "hold" these clients in a state from which they can respond to activating signals [7].

Hsp90 chaperone activity depends on ATP binding and hydrolysis [8–10]. This is coupled to a conformational cycle involving the opening and closing of a dimeric "molecular clamp" via transient association of Hsp90's N-terminal domain [11, 12]. The N-domain also binds the antitumor antibiotics geldanamycin and radicicol, both Hsp90 inhibitors [13–17].

Hsp90 ATPase activity is also regulated by co-chaperones. For example, Hop^{Sti1} [18–20], $p50^{Cdc37}$ [21–23], and $p23^{Sba1}$ [24, 25] have an inhibitory effect on the ATPase cycle of Hsp90, while Aha1 [26–30] and Cpr6 [31, 32] have an activating effect.

Stuart K. Calderwood and Thomas L. Prince (eds.), *Chaperones: Methods and Protocols*, Methods in Molecular Biology, vol. 1709, https://doi.org/10.1007/978-1-4939-7477-1_16,

Hsp90 is posttranslationally modified (PTMs) by phosphorylation, acetylation, *S*-nitrosylation, ubiquitination, and SUMOylation [33–36]. These PTMs, in concert with co-chaperones, fine-tune Hsp90 chaperone function, which ultimately leads to chaperoning of kinase and non-kinase client proteins of Hsp90 [37, 38]. The most extensively studied Hsp90 PTM is phosphorylation [39–49]. Early work has shown that cells treated with the serine/threonine phosphatase inhibitor, okadaic acid, demonstrate Hsp90 hyperphosphorylation and decreased association of the chaperone with $pp60^{v\text{-}Src}$, suggesting a link between Hsp90 phosphorylation and chaperoning of its target proteins [50, 51]. Subsequent study has shown that c-Src directly phosphorylates Tyr300 of Hsp90 under basal conditions, reducing its ability to chaperone client proteins [48].

Recent work has shown that Hsp90 is also subject to SUMOylation, which is an addition of a small ubiquitin-like modifier to a lysine residue. This modification affects cellular localization or function of a protein rather than signal for its degradation like ubiquitination. SUMOylation of Lys191 in human Hsp90α (Lys178 in yeast) promotes its binding to the co-chaperone Aha1 and also increases cells' sensitivity to Hsp90 inhibitors [33].

Lack of PTM-specific antibodies has made it difficult to study PTMs of Hsp90. There is currently only one phospho-Hsp90α antibody (Cell Signaling) available for detecting the phosphorylation of Hsp90α-Thr5/7 [52]. Also *HSP90* gene knockouts are lethal in mammalian systems; therefore, any PTM Hsp90 mutant must be investigated in a background of highly expressed native mammalian Hsp90 proteins.

Simple baker's yeast, *Saccharomyces cerevisiae*, is a well-established and valuable tool for studying various aspects of conserved protein chaperone machinery. The yeast system has provided us with a powerful tool to study Hsp90 phosphorylation, since it readily allows plasmid exchange whereby any introduced Hsp90 gene—provided it is partially functional—can provide 100% of the Hsp90 of the cell (Fig. 1). Such genetic modifications are simply not achievable in cultured mammalian cells. This plasmid exchange (Fig. 1) was used to isolate temperature-sensitive (*ts*) Hsp90 mutants.

This chapter describes the isolation and identification of yeast Hsp90 phosphorylation using immunoblotting procedures. Using the yeast system it is possible to show that Hsp90 is constitutively phosphorylated on serine and threonine residues. However, Hsp90 threonine phosphorylation is lost upon either heat shock stress or treatment with the Hsp90 inhibitor GA (Fig. 2).

This chapter also describes the isolation and analysis of the human (h)Hsp90-N-domain from mammalian cells. This is achieved by introducing a PreScission protease cleavage site between the N-domain and adjacent charged linker region of

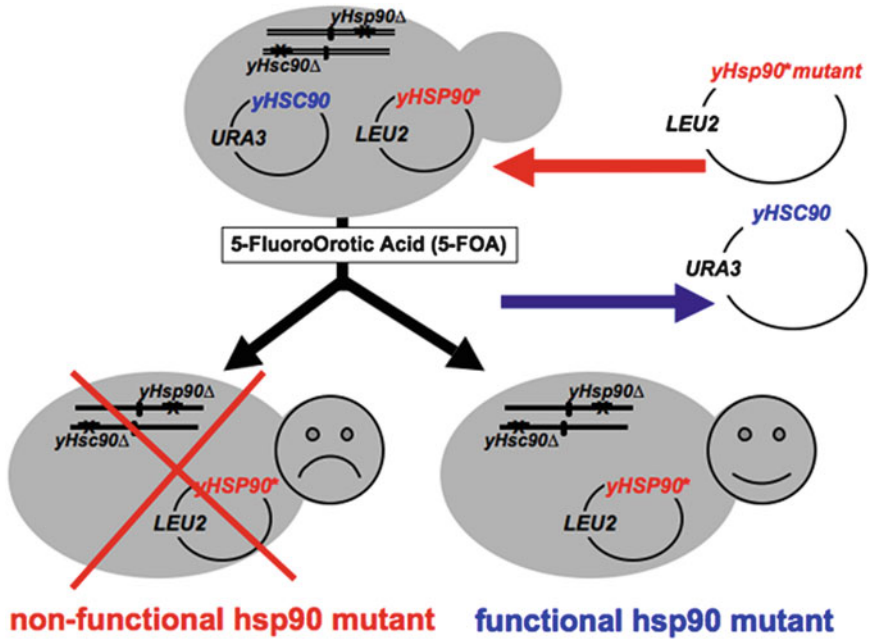

Fig. 1 With plasmid shuffling, a mutant *hsp90* gene can be made to provide all the Hsp90 of the yeast cell (yHsp90 = Hsp82 and yHsc90 = Hsc82). This involves introducing the mutation into yHsp90 on Leu2 plasmid and then introducing it into haploid yeast cells (yHsp90Δ, yHsp90Δ). Growth of these cells on 5-fluoroorotic acid (5-FOA) will "cure" the yeast cells of the wild-type yHsc90 therefore creating *hsp90* mutant

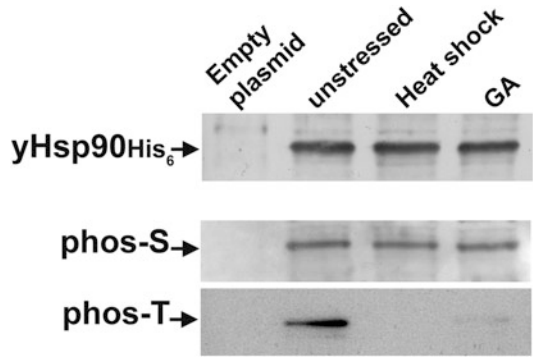

Fig. 2 Yeast Hsp90 phosphorylation on serine (phos-S) and threonine (p-T) residues. yHsp90-His_6 was purified from yeast cells that were heat shocked at 39 °C for 40 min or treated with 100 μM GA for 1 h. Wild-type cells containing the empty plasmid were used as negative control

hHsp90α, allowing isolation of the N-domain. Separation of the N-domain containing either wild-type or non-SUMOylated hHsp90α-K191R mutant from the full-length Hsp90 protein allows for better detection of SUMOylated Hsp90 by immunoblotting (Fig. 3).

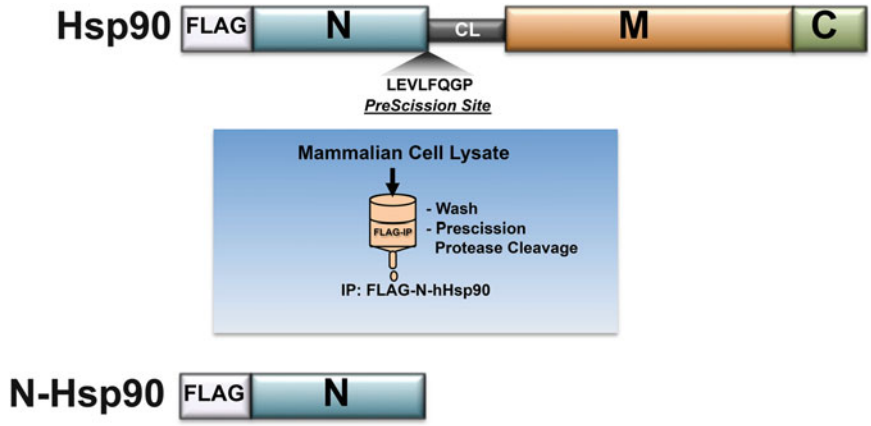

Fig. 3 Schematic representation of Hsp90-FLAG showing the amino (N-), charged linker (CL), middle (M-), and carboxy (C-) domains. Mammalian lysate with the Hsp90-FLAG will be attached to anti-FLAG agarose and Hsp90 N-domain can be isolated by PreScission protease digestion

2 Materials

1. YPD (2% (wt/vol) Bacto peptone, 1% (wt/vol) yeast extract, 2% (wt/vol) glucose, 20 mg/L adenine).
2. Yeast protein extraction buffer (yEB): 50 mM Tris–HCI, pH 6.8, 100 mM NaCl, 50 mM $MgCl_2$. One tablet of complete EDTA-free protease inhibitor cocktail (Roche) and one tablet of PhosphoSTOP (Roche) are added to 50 mL mEB.
3. 425–600 μm glass beads (acid washed) (Sigma).
4. Dulbecco's Modified Eagle's Medium—high glucose (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS).
5. Mammalian protein extraction buffer (mEB): 0.1% NP-40, 20 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 20 mM Na_2MoO_4. One tablet of complete EDTA-free protease inhibitor cocktail (Roche) and one tablet of PhosphoSTOP (Roche) are added to 50 mL mEB. (For the detection of SUMO, mEB should also contain 20 mM *N*-ethylmaleimide (NEM), *see* **Note 8**).
6. *Trans*IT-2020 transfection reagent (Mirus).
7. Bio-Rad Protein Assay Dye solution (Bio-Rad).
8. Ni-NTA agarose (Qiagen).
9. Imidazole (Sigma).
10. Anti-FLAG M2 Affinity Gel agarose (Sigma).
11. PreScission protease (GE Healthcare).

12. PreScission protease cleavage buffer: 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT (pH 7.0).
13. SDS-PAGE sample buffer (2×): 125 mM Tris–HCI pH 6.8, 20% glycerol, 2% SDS, 10% 2-mercaptoethanol, 0.01% bromophenol blue, stable at −20 °C. Aliquot and avoid freeze-thaw cycles.
14. Protran BA85, 0.45 μm Nitrocellulose membrane (Whatman).
15. Ponceau S solution (Sigma).
16. Tris-buffered saline (TBS): 150 mM NaCl, 25 mM Tris-Base. Adjust pH to 7.4 using HCI. Sterile filter and incubate at 4 °C.
17. Albumin, bovine serum (minimum purity 98%).
18. Dried skimmed milk.
19. Phospho-serine antibody (Sigma).
20. Phospho-threonine antibody (Sigma).
21. Phospho-tyrosine antibody (4G10; Millipore).
22. Acetylated lysine antibody (Cell Signaling).
23. Ubiquitin antibody (Santa Cruz).
24. SUMO-1 or SUMO-2/3 antibody (Cell Signaling).
25. 6×–His antibody (Invitrogen).
26. FLAG epitope antibody (ThermoScientific).
27. Anti-secondary mouse and/or rabbit antibody; ECL™ anti-mouse or anti-rabbit IgG, Horseradish Peroxidase-linked whole antibody (GE Healthcare).
28. ELC plus Western Blotting Detection System (GE Healthcare).
29. X-ray film, X-ray cassette, and X-ray film developing machine.

3 Methods

3.1 Extraction of Total Yeast Protein

1. Grow PP30 cells [8] expressing His_6 linked at the N domain of Hsp82 (yHsp90) on 150 mL YPD overnight a 28 °C.
2. Harvest and wash cells 2–3 times in ice-cold deionized water (dH_2O).
3. Transfer the cell pellet into a screw cap 2 mL tube.
4. Pellet the cells and remove the supernatant (*see* **Note 1**).
5. Add equal volume of cell pellets, ice-cold glass beads.
6. Add half the volume of pellet/glass beads, yEB.
7. Bead beat the cells using the mini-beadbeater (BioSpec Products, Inc.) for 30 s.
8. Incubate the cells on ice for 30 s.

9. Repeat **steps** (**7** and **8**), 10–12 times.
10. Centrifuge the tubes at (10,000 × *g*) for 10 min at 4 °C (*see* **Note 2**).
11. Transfer the supernatants into fresh 1.5 mL micro-centrifuge tubes.
12. Centrifuge the tubes at (10,000 × *g*) for 10 min at 4 °C (*see* **Note 2**).
13. Transfer the supernatants (soluble protein) into fresh 1.5 mL micro-centrifuge tubes.
14. Determine protein concentrations using Bio-Rad Protein Assay solution (Bio-Rad).
15. Transfer 40 μL of Ni-NTA Agarose slurry into a 1.5 mL micro-centrifuge tube (*see* **Note 3**).
16. Add 1.0 mL of yEB to the Ni-NTA Agarose and spin at 10,000 × *g* for 1 min at 4 °C.
17. Remove the supernatant and add 1.0 mL of yEB to the Ni-NTA Agarose (*see* **Note 4**).
18. Repeat **step** (**16**, **17**) four times.
19. Resuspend the Ni-NTA Agarose in 30 μM imidazole in yEB and incubate at 4 °C for 30 min (*see* **Note 5**).
20. Repeat **step 16–17** twice and remove the supernatant.
21. Add 1 mg of total protein to the Ni-NTA Agarose in a total volume of 600 μL.
22. Incubate the total proteins/Ni-NTA Agarose at 4 °C for 2 h (*see* **Note 6**).
23. Centrifuge the tubes at (1000 × *g*) for 1 min at 4 °C.
24. Gently remove the supernatant (*see* **Note 7**).
25. Add 1 mL of yEB to the Ni-NTA Agarose.
26. Repeat (23–25) five times.
27. Wash the Ni-NTA agarose with 30 μM imidazole in yEB.
28. Wash the Ni-NTA agarose with yEB once.
29. Centrifuge the micro-centrifuge tube at 15,000 × *g* for 1 min at 4 °C.
30. Remove as much supernatant as possible.
31. Add 40 μL of the protein sample buffer.
32. Boil the samples for 3–5 min.
33. Proceed to Subheading 3.3 for Western blotting and PTM detection.

3.2 Extraction of Total Protein from HEK293 Cells and Immunoprecipitation (IP) of hHsp90

1. Transfect HEK293 cells (~40% confluent in a 10 cm dish; growing in DMEM +10% FBS) with 2 μg hHSP90-FLAG using *Trans*IT-2020 reagent (Mirus) and incubate overnight at 37 °C, 5%CO_2.
2. Place the plates on ice and aspirate media. Wash 2× with cold PBS. Remove all remaining PBS from the plate.
3. Add 200 μL cold mEB to the plate. Scrape the cells and transfer to a 1.5 mL micro-centrifuge tube on ice (*see* **Note 8**).
4. Sonicate the lysate for 15 s. Incubate on ice for 15 s. Repeat ten times.
5. Centrifuge the tubes at (10,000 × *g*) for 8 min at 4 °C (*see* **Note 2**).
6. Transfer supernatants (soluble protein) into fresh 1.5 mL micro-centrifuge tubes.
7. Determine protein concentrations using Bio-Rad Protein Assay solution (Bio-Rad).
8. Transfer 50 μL anti-FLAG M2 Affinity Gel agarose (Sigma) into a 1.5 mL micro-centrifuge tube.
9. Add 500 μL mEB to the anti-FLAG agarose and spin at 10,000 × *g* for 1 min. Remove the supernatant (*see* **Note 4**).
10. Repeat **step 9** four times.
11. Add 1 mg of total protein to the anti-FLAG agarose in a total volume of 500 μL.
12. Incubate the total protein/anti-FLAG agarose at 4 °C for 2 h on a rotator (*see* **Note 6**).
13. Centrifuge the tubes at 1000 × *g* for 1 min.
14. Gently remove the supernatant (*see* **Note 7**).
15. Add 500 μL mEB to the anti-FLAG agarose.
16. Repeat (**steps 13–15**) five times.
17. Add 500 μL mEB to the anti-FLAG agarose.
18. Centrifuge at 15,000 × *g* for 1 min.
19. Remove as much supernatant as possible (*see* **Note 9** for optional PreScission protease cleavage, Fig. 3).
20. Add 40 μL of the protein sample buffer.
21. Boil the samples for 3–5 min.
22. Proceed to Subheading 3.3 for Western blotting and PTM detection.

3.3 Western Blotting and Detection of Hsp90 PTMs

1. Centrifuge the samples at 1000 × *g* and load the supernatant onto a 7.5% SDS-PAGE Tris-HCI gel (*see* **Note 10**).
2. Transfer the proteins from SDS-PAGE gel onto ProtranBA85, 0.45 μm nitrocellulose membrane (Whatman) (*see* **Note 11**).

3. Examine the quality and efficiency of the transfer by staining the membrane with Ponceau S solution (Sigma) for 2 min (*see* **Note 12**).
4. Wash the membrane with dH_2O.
5. Incubate the membrane in 5% milk in TBS-T for 15–20 min at room temperature.
6. Wash the membrane with 1× TBS-T for 5 min at room temperature.
7. Repeat (38) three times.
8. Incubate the membrane with primary antibody (*see* Table 1).
9. Wash the membrane three times with 1× TBS-T for 5 min at room temperature.
10. Incubate the membrane with 1:2000 dilution of secondary anti-mouse or anti-rabbit antibody in 5% milk-TBS-T for 1 h at room temperature.
11. Wash the membrane three times with 1× TBS-T for 5 min at room temperature.
12. Remove 1× TBS-T and then apply ECL plus (GE Healthcare) to nitrocellulose membrane for 2–3 min.
13. Drain nitrocellulose membrane of excess developing solution (do not let dry).

Table 1
Primary antibodies for Hsp90 post-translational modifications detection by Western blot

PTM	Antibody	Manufacturer	Dilution	Diluent	Time and temperature	Species
Phosphorylation	Phospho-serine (PSR-45)	Sigma (cat no. P5747)	1:500–1:1000	1 % BSA in TBS-T	o/n 4 °C	Mouse
	Phospho-threonine (PTR-8)	Sigma (cat no. P6623)	1:500–1:1000	1 % BSA in TBS-T	o/n 4 °C	Mouse
	Phospho-tyrosine (4G10)	Millipore (cat no. 05-321)				Mouse
Acetylation	Acetylated lysine	Cell Signaling (#9441)				Rabbit
Ubiquitination	Ubiquitin (P4D1)	Santa Cruz (sc-8017)	1:500–1:2000	5 % milk in TBS-T	o/n 4 °C	Mouse
SUMOylation	SUMO-1 (2A12)	Cell Signaling (#5718)				Mouse
	SUMO-2/3 (18H8)	Cell Signaling (#4971)				Rabbit

14. Wrap the blot in saran wrap.
15. Place the blot in the X-ray film cassette (*see* **Note 13**).
16. Expose the blots to X-ray films by placing X-ray film directly against the western blot at different lengths of time.

4 Notes

1. The cell pellet must be kept on ice.
2. At this stage, Bio-Rad Protein Assay solution (Bio-Rad) should be prepared.
3. Ni-NTA Agarose is precharged with Ni2+ ions and appears blue in color. It is provided as a 50% slurry in 30% ethanol.
4. Do not disturb the Ni-NTA or anti-FLAG Agarose pellet.
5. Imidazole at low concentrations is commonly used in the binding and wash buffer to minimize binding of unwanted host cell proteins.
6. Use Eppendorf Thermomixer R to gently mix total proteins/Ni-NTA Agarose solution.
7. Avoid disturbing the Ni-NTA or anti-FLAG Agarose.
8. For the detection of SUMO, mEB should always contain 20 mM *N*-ethylmaleimide (NEM).
9. PreScission protease cleavage: incubate hHsp90α-FLAG bound to anti-FLAG agarose with 2 units of PreScission Protease in 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT (pH 7.0) at 10 °C for 16 h.
10. Criteron precast gels from Bio-Rad are suitable for this purpose.
11. The high MW setting on the Bio-Rad Trans-Blot Turbo transfer system is suitable for this purpose.
12. Prepare 5% dry milk (LabScientific Inc.) in 1× TBS-T (0.1% Tween-20, Sigma) buffer before examining the membrane.
13. This procedure must be performed in the dark.

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