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Detecting *HSP90* Phosphorylation

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Abstract

Heat-shock protein 90 (*HSP90*) is an essential molecular chaperone in eukaryotes. It is important for chaperoning proteins that are important determinants of multistep carcinogenesis. *HSP90*'s ATPase activity is associated with its chaperone function. Co-chaperones as well as posttranslational modifications (phosphorylation, acetylation, and S-nitrosylation) are important for regulating its ATPase activity. Yeast can be used to express and purify *HSP90* and also detect its phosphorylation by pan-phosphoserine or phosphothreonine antibodies.

Keywords

HSP90; Molecular chaperones; Posttranslational modification; Phosphorylation

1. Introduction

Heat-shock protein 90 (*HSP90*) is an essential molecular chaperone in eukaryotes (1–3). Its cellular functions have been most clearly identified in mammalian cells, *Drosophila* and baker's yeast *Saccharomyces cerevisiae* (4–7). *HSP90* creates and maintains the functional conformation of a subset of proteins (8). 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.

HSP90 chaperone activity depends on ATP binding and hydrolysis (9–11) which 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 (12, 13), which also binds the antitumor antibiotics geldanamycin and radicicol (14–17).

ATPase activity of *HSP90* is also regulated by co-chaperones. For example, Hop^{Sti1} (18–21), pSO^{Cdc37} (22–25), and p23^{Sbal} (26–28) have an inhibitory effect on the ATPase cycle of *HSP90* while Aha1 (29–31) and Cpr6 (32, 33) have an activating effect.

HSP90 is a phosphoprotein (34–42). Cells treated with the serine/threonine phosphatase inhibitor, okadaic acid, demonstrate *HSP90* hyperphosphorylation and decreased association of the chaperone with pp60^{v-Src}, suggesting a link between *HSP90* phosphorylation and chaperoning of its target proteins (35, 43). Recent work has shown that c-Src directly phosphorylates Tyr-300 of *HSP90* under basal conditions, reducing its ability to chaperone client proteins (41). Therefore, *HSP90* phosphorylation provides an additional means of

fine-tuning its chaperone activity. Lack of phospho-specific antibodies has made it difficult to study *HSP90* phosphorylation in mammalian cells. Also *HSP90* gene knockouts are lethal in mammalian systems, so any mutant *HSP90* must be investigated in a background of highly expressed native mammalian *HSP90* proteins.

Simple baker's yeast, *S. 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).

2. Materials

1. YPD (2% (w/v) Bacto peptone, 1% (w/v) yeast extract, 2% (w/v) glucose, 20 mg/l adenine).
2. Yeast protein extraction buffer (EB): 50 mM Tris-HCl, pH 6.8, 100 mM NaCl, 50 mM MgCl₂. One tablet of complete EDTA-free protease inhibitor cocktail (Roche) and one tablet of PhosphoSTOP (Roche) are added to 50 ml EB.
3. Bio-Rad Protein Assay solution (Bio-Rad).
4. 425–600 µm glass beads (acid washed), (Sigma).
5. SDS-PAGE sample buffer (2×): 125 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 10% 2-mercaptoethanol, 0.01% bromophenol blue, stable at –20°C. Aliquot and avoid freezethaw cycles.
6. Ponceau S solution (Sigma).
7. Tris-buffered saline (TBS): 150 mM NaCl, 25 mM Tris base. Adjust pH to 7.4 using HCl. Sterile filter and incubate at 4°C.
8. Albumin, bovine serum (minimum purity 98%).
9. Dried skimmed milk.
10. Protran BASS, 0.45 µm Nitrocellulose membrane (Whatman).
11. ECL plus Western Blotting Detection System (GE Healthcare).
12. Ni-NTA agarose (Qiagen).
13. Imidazole (Sigma).

14. Phospho-serine QS antibody (Qiagen).
15. Phospho-threonine Q7 antibody (Qiagen).
16. Tetra-His antibody (Qiagen).
17. Anti-secondary mouse antibody; ECLTM antimouse IgG, Horseradish Peroxidase linked whole antibody (GE Healthcare).
18. X-ray film, X-ray cassette, and X-ray film-developing machine.

3. Methods

The extraction of total yeast protein:

1. Grow PP30 cells (9) expressing His₆ linked at the N-domain of Hsp82 (y*HSP90*) on 150 ml YPD overnight at 28° C.
2. Harvest and wash cells two to three times in ice-cold deionized water (dH₂O).
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, EB.
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 microcentrifuge 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 microcentrifuge tubes.
14. Protein concentrations were determined using Bio-Rad Protein Assay solution (Bio-Rad).
15. Transfer 40 µl of Ni-NTA agarose slurry into a 1.5-ml microcentrifuge tube. (see Note 3).
16. Add 1.0 ml of EB to the Ni-NTA agarose and spin at 10,000 × *g* for 1 min at 4°C.
17. Remove supernatant and add 1.0 ml of EB to the Ni-NTA agarose (see Note 4).
18. Repeat step (steps 16 and 17) four times.
19. Resuspend the Ni-NTA agarose in 30 µM imidazole in EB and incubate at 4°C for 30 min (see Note 5).
20. Repeat steps 16 and 17 twice and remove supernatant.

21. Add 1 mg of total protein to the Ni-NTA agarose in a total volume of 600 μ l.
22. Incubate the total proteins/Ni-NTA agarose at 4°C for 2 h (see Note 6).
23. Centrifuge the tubes at (1,000 $\times g$) for 1 min at 4°C.
24. Gently remove the supernatant (see Note 7).
25. Add 1 ml of EB to the Ni-NTA agarose.
26. Repeat (steps 23–25) five times.
27. Wash the Ni-NTA agarose with 30- μ l EB.
28. Wash the Ni-NTA agarose with EB once.
29. Centrifuge the microcentrifuge tube at 15,000 $\times g$ for 1 min at 4°C.
30. Remove as much supernatant as possible.
31. Add 40 μ l of the protein sample buffer.
32. Boil the samples for 3–5 min.
33. Centrifuge the samples at 1,000 $\times g$ and load the supernatant on to a 7.5% SDS-PAGE Tris-HCl gel (see Note 8).
34. Transfer the proteins from SDS-PAGE gel on to ProtranBA85, 0.45 μ m nitrocellulose membrane (Whatman).
35. Examine the quality and efficiency of the transfer by staining the membrane with Ponceau S solution (Sigma) for 2 min (see Note 9).
36. Wash the membrane with dH₂O.
37. Incubate the membrane in 5% milk-TBS-T for 15–20 min at room temperature.
38. Wash the membrane with 1 $\times$ TBS-T for 5 min at room temperature.
39. Repeat (step 38) three times.
40. Incubate the membrane with 1:500–1,000 dilution of either phospho-serine (Q5) or phospho-threonine (Q7) antibodies, (Qiagen), in 2% BSA-TBS-T overnight at 4°C (see Note 10).
41. Wash the membrane three times with 1 $\times$ TBS-T for 5 min at room temperature.
42. Incubate the membrane with 1:2,000 dilution of anti-secondary mouse antibody in 5% milk-TBS-T for 1 h (see Note 11) at room temperature.
43. Wash the membrane three times with 1 $\times$ TBS-T for 5 min at room temperature.
44. Remove 1 $\times$ TBS-T and then apply ECL plus (GE Healthcare) to nitrocellulose membrane for 2–3 min.
45. Drain nitrocellulose membrane of excess developing solution (do not let dry).
46. Wrap the blot in saran wrap.
47. Place the blot in the X-ray film cassette (see Note 12).

48. Expose the blots to X-ray films by placing X-ray film directly against the western blot for 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 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 agarose.
8. Criterion precast gels from Bio-Rad are suitable for this purpose.
9. Prepare 5% dry milk (LabScientific Inc.) in 1× TBS-T (0.1% Tween-20, Sigma) buffer before examining the membrane.
10. Phospho-antibodies stock concentration is 0.1 µg/µl.
11. 1:2,000 dilution of anti-secondary mouse antibody; ECLTM antimouse IgG horseradish peroxidase-inked whole antibody (GE Healthcare).
12. This procedure must be performed in the dark.

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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 (*yHSP90* , *yHSP90*). Growth of these cells on 5-fluoroorotic acid (5-FOA) “cures” the yeast cells of the wild-type *yHsc90*, therefore creating *HSP90* mutant.

Fig. 2.

Yeast *HSP90* phosphorylation on serine (phos-S) and threonine (phos-T) residues. *yHSP90*-His₆ 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.