

Chapter 23

Detection and Analysis of Extracellular Hsp90 (eHsp90)

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Abstract

Heat Shock Protein 90 (Hsp90) is a ubiquitous molecular chaperone that comprises about 1–3% of the total cellular protein. Over the last decade, Hsp90 has been detected and studied in the extracellular space (extracellular or eHsp90) of normal and neoplastic cells. Once outside the cell, eHsp90 has been shown to interact with extracellular client proteins and promote their stabilization and function. Cell conditioned media are routinely collected to detect and quantify eHsp90, and determine its interactions with extracellular clients. Finally, targeting specifically the eHsp90 with pharmacologic inhibitors or antibodies that are unable to cross the plasma membrane has been beneficial in inhibiting tumor cell motility and invasion.

Key words Extracellular Heat Shock Protein 90 (eHsp90), Clients, Co-chaperones

1 Introduction

The molecular chaperone Hsp90 is an essential and highly abundant protein in eukaryotes [1, 2]. The Hsp90 chaperone function is regulated by a complex interplay of mechanisms including adenosine triphosphate (ATP)-dependent Hsp90 conformational changes, posttranslational modifications, and interactions with cofactors or co-chaperones, all of which facilitate the optimal function of the chaperone and assist in folding, stabilization, and activation of “client” proteins [3, 4]. Two major isoforms of Hsp90, α and β , are constitutively expressed in the cytoplasm; however, only the Hsp90 α is induced in response to cellular stressors.

Although Hsp90 has been considered a predominant cytosolic protein, recent studies have increasingly supported early findings by Eustace et al. that cells (normal and tumor) secrete Hsp90 to the extracellular space, where it could exist as free unbound protein or bound to the cell surface [5]. Following its release, Hsp90 α can be detected on the cell surface and in the conditioned media of a variety of tumor cells including HT1080 fibrosarcoma cells and MDA-MD231 breast carcinoma cells [5–7]. Detection of

extracellular Hsp90 β has been less successful and occasionally studies have resulted in conflicting observations [5, 6, 8]. In contrast, osteosarcoma cells were shown to secrete Hsp90 β , with Hsp90 α being undetectable [9].

In cancer, increased levels of eHsp90 have been associated with migration, invasion, and metastasis of tumor cells [10, 11]. eHsp90, similarly to the intracellular form, interacts with extracellular client proteins, some found in free form, others cell surface bound [6, 12, 13]. Matrix metalloproteinases (MMP-2 and MMP-9) are secreted proteases shown to interact with eHsp90, and appear to rely on eHsp90 for their stability and activity [14, 15]. Increased expression and hyperactivity of MMPs is a hallmark of tumor development and progression since extensive, irreversible extracellular matrix proteolytic degradation provides a promigratory, proinvasive, and proangiogenic environment for tumor cells [16].

The development of small-molecule inhibitors or anti-eHsp90-specific antibodies that do not readily cross the plasma cell membrane has introduced a unique approach of targeting and inhibiting specifically the extracellular Hsp90 [17, 18]. The non-membrane permeable Hsp90 inhibitor biotinylated ganetespib (STA-12-7191) was recently shown to specifically bind to eHsp90, exhibiting low toxicity in cell viability assays and blocking wound healing and breast cancer cell migration [6]. Anti-eHsp90-specific antibodies have also been used to inhibit eHsp90 function, tumor cell invasion, and metastasis in *in vivo* human xenograft mouse models [17, 19].

This chapter describes a procedure for detecting, isolating, and characterizing cell secreted, extracellular unbound Hsp90. The protocol includes detailed steps including mammalian cell culture, protein isolation, western blot, and immunoprecipitation/co-immunoprecipitation (Fig. 1).

2 Materials

1. Protein Extraction Buffer (50 mL): 1 mL 1 M Tris pH 7.4, 50 μ L 1 M MgCl₂, 1 mL 5 M NaCl, 2 mL 500 nM sodium molybdate, 500 μ L NP40 IGEPAL (Sigma), 1 Phosphatase inhibitor tablet (Thermo Fisher), 1 Protease inhibitor tablet (Sigma), ~45 mL dH₂O.
2. 10 \times TBS (1 L): 24.2 g Trizma (Sigma), 80 g NaCl, 13 mL HCl, 1 L dH₂O.
3. 1 \times TBST (1 \times , 1 L): 900 mL dH₂O, 100 mL 10 \times TBS, 1 mL Tween 20 (working solution).

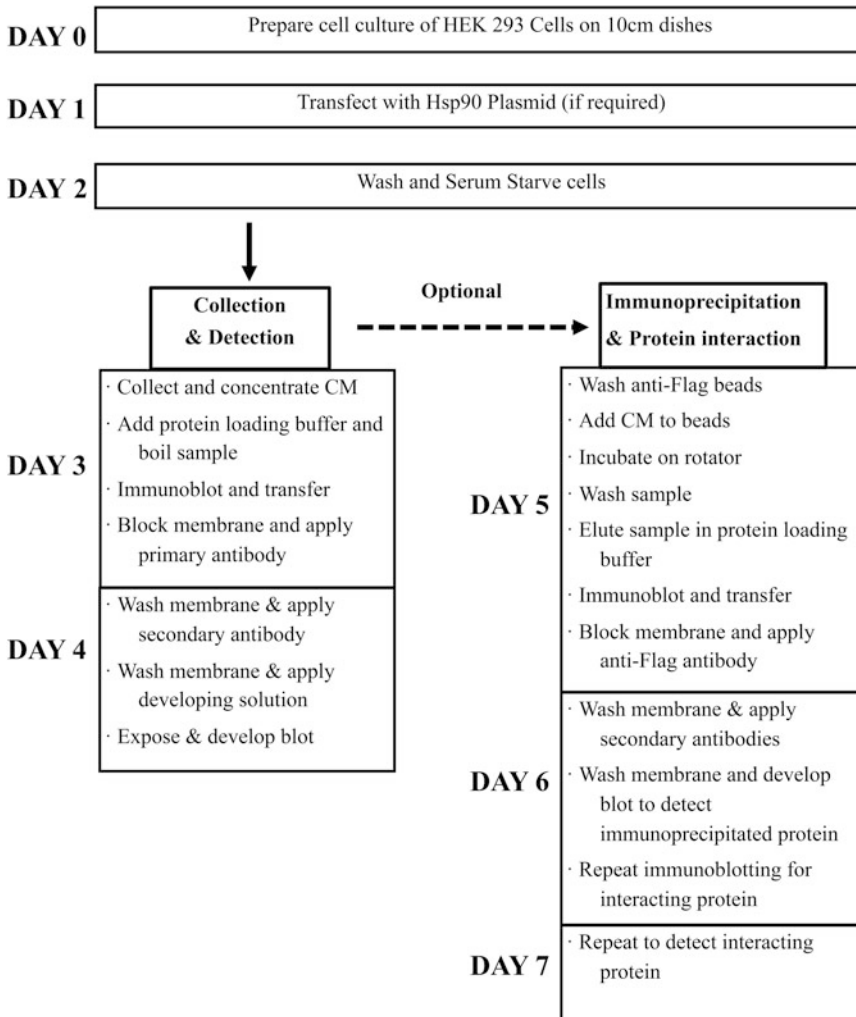


Fig. 1 Schematic representation of the experimental timeline. The experiment is completed in 4–7 days. *CM* cell-conditioned media

4. Protein Loading Buffer (5×, 15 mL): 4.7 mL 1 M Tris pH 6.8, 4.5 mL Glycerol, 1.5 g SDS, 0.75 mL 1% Bromophenol blue, 0.75 mL Beta-mercaptoethanol, ~4.3 mL dH₂O.
5. 10× Running Buffer: 121.2 g Trizma (Sigma), 576.8 g Glycine, 20 g SDS, 3.2 L dH₂O.
6. 10× Transfer Buffer: 800 mL 5× Transfer buffer (Bio-Rad), 800 mL Ethanol, 2.4 L dH₂O.
7. 4–20% Criterion™ Tris–HCl polyacrylamide gel (Bio-Rad).
8. Ponceau S solution (Sigma).
9. Dried skimmed milk.
10. Protran BA85, 0.45 μm Nitrocellulose membrane (Whatman).

11. Thermo Scientific™ Pierce™ ECL 2 Western Blotting Substrate (Thermo Scientific).
12. ECL2 or SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific).
13. Rabbit anti-Flag antibody, Cat# PA1-984B, (Thermo Scientific).
14. Rat anti-Hsp90 mAb (16F1), Cat# ADI-SPA-835F (Enzo Life Sciences).
15. Rabbit anti-MMP-2 (D8N9Y) mAb, Cat# 13132 (Cell Signaling Technology).
16. HRP-conjugated secondary antibodies (Santa Cruz, anti-rabbit Cat #sc-2004, anti-rat Cat# sc-2006).
17. X-ray film, X-ray cassette, and X-ray film developing machine.
18. Centrifugal concentrators (Amicon).
19. TransIT-2020 (Muris), transfection reagent for mammalian cells.
20. Dulbecco's Modified Eagle Medium (Sigma).
21. Fetal Bovine Serum (FBS) (Sigma).
22. Phosphate-Buffer Saline (PBS) cell culture grade (Sigma).
23. Hsp90 α or β cloned in mammalian expressing vector with Flag tag.
24. Anti-Flag agarose beads (anti-Flag M2 Affinity Gel, Cat#A2220, SIGMA).

3 Methods

3.1 Cell Culture and Immunoblotting (See Note 1)

1. Culture human embryonic kidney (HEK293) cells on a 10 cm plate at 37 °C in a humidified atmosphere of 5% CO₂, in 10% FBS/DMEM culture media for 18 h.
2. Transfect cells using 6 μ L of TransIT-2020, 600 μ L serum-free DMEM, and plasmid DNA (ranging from 1 μ g to 3 μ g) per 10 mL complete media per 10 cm plate (*see Note 2*).
3. Incubate the cells overnight at 37 °C in a humidified atmosphere of 5% CO₂.
4. Next day remove media and wash the cells gently twice with 5 mL PBS.
5. Serum starve the cells for 24 h with serum-free DMEM (*see Note 3*).
6. Next day collect CM (avoiding disruption of attached cell monolayer) (*see Note 4*).
7. Centrifuge CM at 4 °C for 5 min at a speed of $94 \times g$.

8. Collect the supernatant. Transfer CM to centrifugal concentrators.
9. Concentrate CM 10× by centrifuging at 2900 × *g* for 5–15 min depending on CM volume (*see Note 5*).
10. Add protein loading buffer to sample in a 1:1 ratio.
11. Boil samples for 5 min.
12. Briefly centrifuge sample.
13. Load samples on 4–20% Criterion™ Tris–HCl polyacrylamide gel (Bio-Rad).
14. Run the sample at 200 V until bands are clearly separated in 1× running buffer.
15. Transfer gel onto nitrocellulose membrane.
16. Briefly wash membrane with 1× TBST.
17. Block membrane in 5% milk in 1× TBST for 1 h.
18. Wash membrane three times for 5 min each in 1× TBST.
19. Rock membrane overnight at 4 °C in antibody of interest (5% Milk in 1× TBST).
20. Use rabbit anti-Flag antibody, 1:2000 in 1× TBST 5% milk, incubation for 2 h at room temperature.
21. If endogenous Hsp90 is detected, use primary anti-Hsp90 antibody at 1:2000, overnight incubation at 4 °C.
22. Rabbit anti-MMP-2 antibody, 1:2000 in 1× TBST 5% milk, incubation overnight at 4 °C.
23. Wash membrane three times for 5 min each in 1× TBST.
24. Rock membrane with secondary antibody in 5% milk in 1× TBST for 1 h at room temperature.
25. Anti-rabbit antibody 1:4000 and anti-rat antibody 1:2000, both in 1× TBST 5% milk, incubation for 1 h at room temperature.
26. Wash membrane three times for 5 min each in 1× TBST.
27. Remove 1× TBST and apply developing solution (ECL-2 or SuperSignal™ West Femto Maximum Sensitivity Substrate) to membrane for 3–4 min. 24. Remove excess of developing solution (*see Note 6*).
28. Place blot on X-ray film cassette.
29. Expose blot to X-ray films for different lengths of time.
30. Detect the Hsp90 protein at ~90 kDa.
31. Detect the MMP-2 protein at ~72 kDa (pro-MMP-2). Occasionally, a second or even a third band between 62 and 67 kDa may be present, corresponding to the intermediate and fully activated MMP-2 forms.

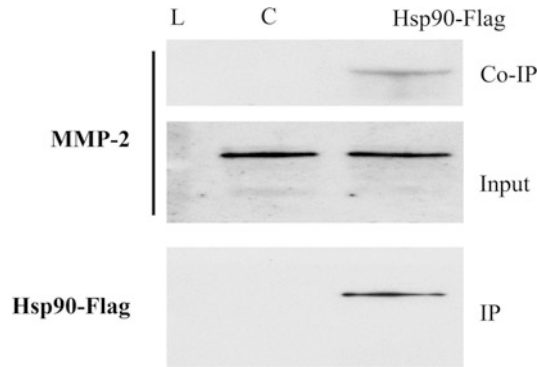


Fig. 2 Detection and immunoprecipitation of Hsp90 from the conditioned media of HEK293 cells. Hsp90-Flag was transfected and media collected for analysis. Immunoprecipitation (IP) of Hsp90 was followed by co-immunoprecipitation (co-IP) of MMP-2. Secreted MMP-2 levels are detected in the CM (input). *L* ladder, *C* control, *IP* immunoprecipitation, *co-IP* co-immunoprecipitation

3.2 Immuno-precipitation and Co-immuno-precipitation (See Note 1)

This protocol describes isolation of secreted Hsp90-Flag and determination of eHsp90-interacting proteins in co-immunoprecipitation experiments. Here, we determine interaction with extracellular protease MMP-2 (Fig. 2).

1. Following the transfection and immunoblotting protocols as described above in 3.1, Hsp90-Flag and MMP-2 expression are determined in CM.
2. Wash 50 μL /sample of anti-Flag beads with 500 μL of 0.1% protein extraction buffer for 30 s at $21,000 \times g$. Repeat this step three times and vortex samples in between washes.
3. Add adjusted volume of concentrated or non-concentrated CM to beads (based on the input levels of the interacting protein MMP-2).
4. Adjust the sample to final volume of 500 μL with 0.1% protein extraction buffer.
5. Rotate samples for 1 h at 4°C .
6. Centrifuge at 4°C for 30 s at $845 \times g$. Discard the supernatant and keep the beads pellet.
7. Wash beads twice with 500 μL of 0.1% protein extraction buffer for 30 s at $845 \times g$, and vortex in between washes.
8. Wash with 500 μL of 0.1% protein extraction buffer for 30 s at $21,000 \times g$. Remove the supernatant.
9. Elute protein from beads by adding 60–100 μL $5\times$ protein loading buffer to the beads and heat for 5 min (*see Note 7*).
10. Centrifuge at $21,000 \times g$ for 30 s. Collect the supernatant containing immunoprecipitated protein. Freeze samples at -80°C or continue with immunoblot (*see Note 8*).

11. Load supernatant samples (5 μ L) on 4–20% Criterion™ Tris–HCl polyacrylamide gel (Bio-Rad) to detect the immunoprecipitated Hsp90-Flag.
12. Run gel at 200 V until bands are clearly separated in 1 \times running buffer.
13. Transfer gel onto nitrocellulose membrane.
14. Wash membrane once with 1 \times TBST.
15. Block membrane in 5% milk in 1 \times TBST for 1 h.
16. Wash membrane three times for 5 min in 1 \times TBST.
17. Rock membrane for 1 h at room temperature with anti-flag antibody in 5% Milk in 1 \times TBST.
18. Wash membrane three times for 5 min in 1 \times TBST.
19. Incubate membrane with secondary antibody in 5% milk 1 \times TBST for 1 h at room temperature.
20. Wash membrane three times for 5 min in 1 \times TBST.
21. Remove 1 \times TBST and apply developing solution (ECL-2 or SuperSignal™ West Femto Maximum Sensitivity Substrate) to membrane for 3–4 min.
22. Remove excess developing solution.
23. Place blot on X-ray film cassette.
24. Expose blot to X-ray films for different lengths of time.
25. If inputs of Hsp90-Flag are equal proceed to co-immunoprecipitation.
26. Equalize or run equal volumes of immunoprecipitated samples in new gel and transfer as in **step 13**.
27. Wash blot three times for 5 min with 1 \times TBST.
28. Block blot for 30 min with 5% milk in 1 \times TBST.
29. Add anti-MMP-2 antibody (co-immunoprecipitated protein) and incubate overnight at 4 °C.
30. Repeat **steps 18–24**.

4 Notes

1. All the steps involving CM are performed on ice.
2. No need to transfect if endogenous, secreted Hsp90 is the protein of interest.
3. Treatment with drug or antibody should be completed following serum starvation for 24 h.

4. Cell extracts should also be collected if interested in intracellular signaling and to ensure drug/antibody did not pass through cell membrane.
5. If high levels of secreted Hsp90 are expected, no need to concentrate CM.
6. Use Femto if signal not strong enough to be seen with ECL 2.
7. Compete with FLAG peptide if heavy/light chain is close to protein of interest in immunoprecipitation (that applies to Flag-tagged proteins).
8. Store samples (CM, lysates, IPs) at -80°C if not being used right away.

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