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A proteomic snapshot of the human heat shock protein 90 interactome

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Abstract Heat shock protein 90 (Hsp90) is a molecular chaperone which modulates several signalling pathways within a cell. By applying co-immunoprecipitation with endogeneous Hsp90, we were able to identify 39 novel protein interaction partners of this chaperone in human embryonic kidney cells (HEK293). Interestingly, levels of DNA-activated protein kinase catalytic subunit, an Hsp90 interaction partner found in this study, were found to be sensitive to Hsp90 inhibitor treatment only in HeLa cells but not in HEK293 cells referring to the tumorgenicity of this chaperone.

Keywords: Hsp90; HEK293; Proteomics; Interactome; Mass spectrometry; Radicicol; DNAPK; Immunoprecipitation

1. Introduction

Heat shock protein 90 (Hsp90) is an abundant, constitutively expressed chaperone (1–2% of total cellular protein under non-stress conditions). The current opinion is that Hsp90 binds to a restricted set of substrates rather than generally to unfolded proteins. It seems to be specialised in stabilising metastable regions, such as open ligand binding pockets, by keeping them in a "holding" position, thus, preventing their collapse or aggregation [1].

Hsp90 is an ATP-dependent chaperone. Although the ATPase activity of human Hsp90 is barely detectable as compared to that of the yeast or of the bacterial homologue, it becomes stimulated by co-chaperones or substrates [2]. Binding of Hsp90 to substrates is selectively inhibited by benzoquinone ansamycins (geldanamycin and derivatives) and radicicol, which bind to the ATP-binding pocket with a much higher affinity than ATP [3].

Of the approximatively 100 client proteins of Hsp90 known so far, a considerable number are overexpressed or mutated in malignous tumours [4]. It is indicative that the first client protein of Hsp90 to be identified in vivo in vertebrates was the oncogenic viral tyrosine kinase v-Src [8]. One of the reasons for this preferential interaction is probably due to the destabilising effects of amino acid mutations. In accordance with this hypothesis, it has been recently shown that in vitro the intrinsic stability of v-Src is significantly reduced compared to the cellular counterpart c-Src [5].

Suppressing the interaction between Hsp90 and client proteins represents a promising antitumoral approach [4]. Yet, the discovery of novel Hsp90 substrates occurs rather incidentally than rationally. A global picture of Hsp90-modulated functional frameworks in vivo would greatly improve long-term challenges such as the development of more sophisticated drugs which for example might selectively suppress Hsp90 interactions situated on exclusive nodes of signal transduction pathways.

Here, we present a proteomic approach by which "all" Hsp90 clients can be identified and which can be further improved towards the detection of low-abundant as well as transient substrates of this therapeutically important chaperone.

2. Materials and methods

Unless otherwise described, reagents, chemicals and antibodies were purchased from Sigma (St. Louis, MO, USA).

2.1. Cell preparation

Human embryonic kidney cells (HEK293) and HeLa cells were grown at 37 °C and 5% CO_2 in DMEM supplemented with 10% fetal calf serum (PAA, Pasching, Austria). Confluent cells were washed once with PBS, harvested with a dish scraper, stained with trypan blue and viability was determined with a hemocytometer (Brand, Wertheim, Germany). For inhibition of Hsp90, cells were treated with 20 μM radicicol, or with DMSO as a control, respectively.

2.2. Immunoprecipitation

10^7 HEK293 cells were used for each experiment. Cells were suspended in 500 μl lysis buffer (20 mM Tris, 2.5 mM NaCl, 1% Triton X-100, pH 7.4) supplemented with protease inhibitor mix, disrupted for 20′ at 4 °C on a rotary shaker and centrifuged at 13 000 × g. The pellet was discarded and 4 μl of anti-Hsp90 antibody (clone AC68, Stressgen, San Diego, USA) were added except to the control samples. The mixture was incubated overnight at (ca. 15 h) 4 °C on a rotary shaker. A Protein-G agarose resin was then added and samples were incubated for further 3 h at 4 °C. After that, samples were centrifuged, the supernatant was removed and the protein-G agarose resin was washed 5 times with washing buffer (50 mM Tris, 150 mM NaCl, 0.1% Triton X-100, pH 7.4). Lämmli buffer was added directly to the protein-G agarose, heated at 95 °C, probes were run on a SDS–PAGE gel and finally detected by silver-staining.

2.3. In-gel protein digestion and mass spectrometry

Bands to be analysed were prepared as described in [6]. Briefly, bands of interest were excised, soaked sequentially with NH_4HCO_3 and NH_4HCO_3/acetonitrile, and dehydrated with acetonitrile (Fisher Scientific, Loughborough, UK). Each sample was then subjected to reduction of the disulfide bonds with DTT at 54 °C and to alkylation with iodacetamide. Bands were then washed again with NH_4HCO_3,

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Abbreviations: Hsp90, heat shock protein 90; HEK293, human embryonic kidney cells; DNA-PKcs, DNA-activated protein kinase catalytic subunit; co-IP, co-immunoprecipitation

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NH_4HCO_3/acetonitrile and acetonitrile, dried in a vacuum centrifuge (Eppendorf, Hamburg, Germany) and either stored at −80 °C or immediately used for proteolytic digestion. For this purpose, samples were rehydrated in NH_4HCO_3, subjected to tryptic digestion overnight and peptides were gel-extracted. Nano-HPLC–MS/MS sequencing and peptide database search were performed as previously described [6].

2.4. Detection of DNA-PKcs in cytosolic and nuclear cell lysates

For the inhibition of Hsp90, cells were treated with 20 μM radicicol, or with DMSO as a control, respectively, and incubated in serum-free medium for different periods. For the preparation of the nuclear extracts, cells were harvested as described above, resuspended in buffer containing 10 mM HEPES, 50 mM NaCl, 0.5 M sucrose, 0.1 mM EDTA and 0.5% Triton-X100, pH 7.9, incubated on ice for 5′, and centrifuged at 1000 × *g*. The cytosolic supernatant was stored separately. To obtain the nuclear extract, the pellet was resuspended in buffer containing 10 mM HEPES, 500 mM NaCl, 0.1 mM EDTA, 0.1% Igepal, pH 7.9, rotated for 15′ at 4 °C, and centrifuged at 14000 × *g* for 10′ at 4 °C.

Samples of the cytosolic and the nuclear fractions were run on a 8% SDS–PAGE gel and blotted on a Hybond nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden). Marker bands were visualised by staining with Ponceau Red. The membrane was further incubated first with anti-DNA-activated protein kinase catalytic subunit (DNA-PKcs) antibody (clone 18-2, EMD Biosciences, Darmstadt, Germany) and then with horseradish peroxidase-coupled anti-mouse-antibody. DNA-PKcs was detected by treatment with ECL solution (Amersham Pharmacia, Uppsala, Sweden) and final exposure to a Kodak X-Omat AR film for visualisation. For the control detection of vinculin, the same procedure was repeated and anti-vinculin (clone hVIN-1) was used for immunoblotting of the cytosolic fractions.

3. Results and discussion

To obtain a Hsp90/substrate-interaction map, we performed co-immunoprecipitation (co-IP) experiments in cytosolic cell lysates of confluent HEK293 cells using antibodies directed against Hsp90 as a bait. We preferred to target endogenous Hsp90 rather than to add exogenous, recombinantly expressed Hsp90, to our preparation, since endogenous conformations and post-translational modifications of Hsp90 might be required for efficient interactions with client proteins. We further used an antibody which was able to recognise both Hsp90 α and β isoforms. Due to the transient/labile nature of Hsp90-client complexes, several conditions for IP-ing these complexes were tested. For this purpose, the influence of ionic strength, pH and temperature as well as of additives like detergents and metal ions was tested. The finally optimised conditions for Hsp90 co-IP are given in Section 2.

The protein mixture which co-IPed together with Hsp90 was separated on SDS–PAGE and the detected bands were, after in-gel trypsinisation, analysed by nano-HPLC–MS/MS. Fig. 1 shows two typical silver-stained SDS–PAGE gels obtained from an Hsp90 co-IPed HEK293 lysate. After washing with 150 mM NaCl, only few bands were visible on the control lane (Fig. 1A, lane 1) indicating that background contaminations derived from unspecific binding to protein-G had been efficiently removed.

As summarised in Table 1, most of the proteins identified are metabolic enzymes (synthetases, glycolytic enzymes), ribosomal subunits, translational proteins (tRNA synthetases, initiaton/elongation factors), structural proteins and chaperones (two of the identified proteins, spot Nos. 38 and 39, have no annotated function yet). Among the chaperones identified, the interaction between Hsp90 and Hsp70 is well documented and represents one of the best investigated chaperoning cycles [1,7]. Our inability to detect further Hsp90 co-chaperones by our pull-down method may be due to the fact that the anti-Hsp90 antibody in the IP experiments displaced co-chaperones from Hsp90 by competiting for shared epitopes on the chaperone. In addition, the transient nature of the Hsp90/co-chaperone complexes combined with the low abundancy of the latter proteins rendered detection of these complexes difficult.

The interaction with glycolytic enzymes was previously unreported for Hsp90 but might be important to guarantee a higher production of ATP under stress conditions. Since Hsp90 was recently suggested to be an ATP-sensor [8], the interaction with glycolytic enzymes may also represent a feedback loop for the regulation of the ATP synthesis.

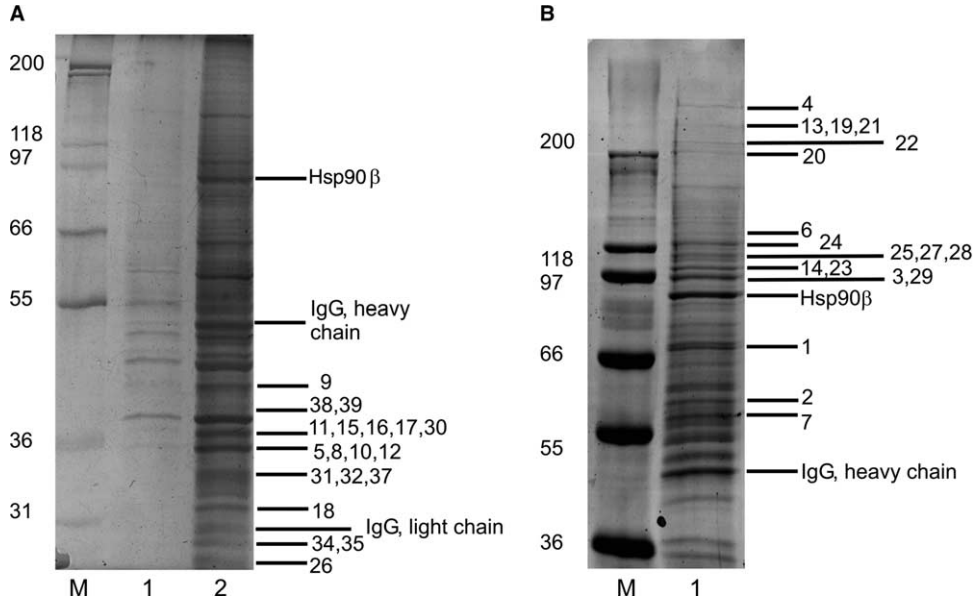

Fig. 1. (A) 10% SDS–PAGE of proteins Hsp90 co-IPed from HEK293 cell lysates. M: molecular weight marker. Lane 2: 150 mM NaCl extract. Lane 1: controls (no antibody). (B) 8% SDS–PAGE gel of proteins Hsp90 co-IPed from HEK293 cell lysates. Lane 1: 150 mM NaCl extract.