Reports

A chemical cross-linking method for the analysis of binding partners of heat shock protein-90 in intact cells

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Members of the heat shock protein-90 (Hsp90) family are key regulators of biological processes through dynamic interaction with a multitude of protein partners. However, the transient nature of these interactions hinders the identification of Hsp90 interactors. Here we show that chemical cross-linking with ethylene glycolbis (succinimidylsuccinate), but not shorter cross-linkers, generated an abundant 240-kDa heteroconjugate of the molecular chaperone Hsp90 in different cell types. The combined use of pharmacological and genetic approaches allowed the characterization of the subunit composition and subcellular compartmentalization of the multimeric protein complex, termed p240. The *in situ* formation of p240 did not require the N-terminal domain or the ATPase activity of Hsp90. Utilizing subcellular fractionation techniques and a cell-impermeant cross-linker, subpopulations of p240 were found to be present in both the plasma membrane and the mitochondria. The Hsp90-interacting proteins, including Hsp70, p60Hop and the scaffolding protein filamin A, had no role in governing the formation of p240. Therefore, chemical cross-linking combined with proteomic methods has the potential to unravel the protein components of this p240 complex and, more importantly, may provide an approach to expand the range of tools available to the study of the Hsp90 interactome.

Keywords: chaperone; heat shock protein; protein cross-linking; small interfering RNA

Members of the 90-kDa heat shock protein (Hsp90) family are molecular chaperones with an intrinsic ATPase activity that functions in the folding, maturation, and activation of large number of protein interactors known as clients (for current list of client proteins, see: <http://www.picard.ch/downloads/downloads.htm>). These client proteins have critical roles in a diverse range of biological processes, including signal transduction, cellular trafficking, metabolism, transcription, and cell growth and differentiation. Hsp90 exists in a homodimeric state in association with several co-chaperones and accessory proteins that regulate the ATPase activity of Hsp90 and ensure client protein assembly into multiprotein

complexes (1,2). Structural studies and molecular modeling methods have provided a model in which the dynamic conformational changes between the nucleotide-free and the nucleotide-bound forms of Hsp90 enable spatiotemporal recruitment of select co-chaperones while effecting transport and/or assembly of functional signaling complexes (3–5). Hsp90 has three distinct domains, termed N-terminal domain, which contains an ATP binding site, the highly charged middle domain that has high affinity for co-chaperones and client proteins, and the C-terminal domain harboring a second ATP binding site and a conserved pentapeptide sequence recognized by co-chaperones. The N-terminal nucle-

otide binding pocket has been shown to bind the ansamycin antibiotic geldanamycin, whereas the coumarin antibiotic novobiocin and some chemotherapeutic agents interact with the C-terminal ATP binding site to prevent hydrolysis of ATP. As a result, these pharmacological inhibitors disrupt the chaperone activity of Hsp90, causing ubiquitinylation and subsequent degradation of a subset of pro-oncogenic client proteins (6,7).

The study of Kang et al. (8) has shown that unlike most normal tissues, mitochondria of various tumors contain Hsp90, where it interacts with the immunophilin cyclophilin D to confer anti-apoptotic protection (9). In addition to being found intracellularly,

Method summary:

Here, we demonstrate chemical cross-linking of the 240-kDa heteroconjugate of the Hsp90 molecular chaperone using ethylene glycolbis (succinimidylsuccinate) in intact cells. Use of the cross-linking agent will enable the further study of Hsp90 function and interactions.

Hsp90 is present at the cell membrane and participates in a multitude of extra-cellular functions, from acting in skin cell motility and wound healing (10), to cancer cell invasion (11,12). Extra-cellular Hsp90 binds the surface low density lipoprotein (LDL) receptor-related protein 1/CD91, matrix metalloproteinase 2, and the extracellular domain of HER-2 (11–13).

The human Hsp90 interactome has been characterized recently using immunopurification and affinity capture with immobilized Hsp90 (14–16). Several co-chaperones and client proteins were identified; however, these experiments were not designed to study protein interactions in intact cells, as many of these interactions with Hsp90 are relatively transient due to their dependence of the dynamic ATP-dependent cycle (17,18) and cellular redox state (19,20). Here, we use a method for chemical cross-linking to evaluate the formation of hetero-conjugates of Hsp90 in intact cells. When compared with the mammalian two-hybrid system and classical proteomic approach, the protein cross-linking technique has the advantage to stabilize covalently Hsp90-interactor protein complexes, thereby minimizing the possibility of the native complexes undergoing dissociation during cell lysis and biochemical fractionation. This approach has enabled the detection of an abundant Hsp90 multimeric complex, termed p240, in various subcellular compartments, including mitochondria and the plasma membrane facing the extracellular environment.

Materials and methods

Materials

The following Hsp90 inhibitors, 17-allylamino-17-demethoxygeldanamycin (17-AAG), 17-desmethoxy-17-N,N-dimethylamino-ethylamino-geldanamycin (17-DMAG), and novobiocin, were purchased from EMD Biosciences (Gibbstown, NJ, USA). The homobifunctional protein cross-linkers dimethyl pimelimidate (DMP), disuccinimidyl suberate (DSS), ethylene glycolbis(succinimidylsuccinate) (EGS), and the water soluble ethylene glycolbis(sulfosuccinimidyl succinate) (Sulfo-EGS) were from Thermo Scientific Pierce (Rockford, IL, USA). Novex tris-glycine gels (4%–12% and 6%), iBlot apparatus, and Opti-MEM were from Invitrogen (Carlsbad, CA, USA). Non-fat dry milk and Tween-20 were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Tris-buffered saline was from ScyTek Laboratories (Logan, UT, USA).

Mouse anti-human Hsp90 α/β antibody was purchased from BD Biosciences (cat. 610419; San Jose, CA, USA); monoclonal antibodies against Hsp70 (sc32239) and GAPDH (sc32233), and polyclonal anti-I κ B α (sc371) and anti-EGFR (sc03) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-HSF1 (clone 10H8) and p60Hop (clone DS14F5) monoclonal antibodies were from Enzo Life Sciences (Farmingdale, NY, USA); mitochondrial complex V antibody was from Molecular Probes, Invitrogen (cat. 439800; Eugene, OR, USA), while Stratagene anti-FLAG M2 clone and anti-filamin A (FLNa) were from Agilent Technology (cat. 200471; Cedar Creek, TX, USA) and Fitzgerald Industries International (cat. 10-F82A; Acton, MA, USA), respectively. Protein G agarose was purchased from Millipore (Temecula, CA, USA). Enhanced chemiluminescent (ECL)

detection system, donkey anti-rabbit horseradish peroxidase-linked IgG, and sheep anti-mouse horseradish peroxidase-linked IgG were from GE Healthcare/Amersham Biosciences (Piscataway, NJ, USA). α -Minimal essential medium (α -MEM) was purchased from Invitrogen, and Ham's F12 medium was from Cellgro/Mediatech (Manassas, VA, USA). Human FLNa small interfering RNA (siRNA), Qiagen mini- and maxi-plasmid preparation kits, and Qiaquick PCR kit were from Qiagen (Valencia, CA, USA). Lipofectamine RNAiMAX reagent and Lipofectamine 2000 transfection reagent were purchased from Invitrogen.

Protein cross-linking in intact cells

The human HepG2 hepatocarcinoma and 1321N1 astrocytoma cell lines were purchased from American Type Culture Collection (ATCC; Manassas,

Figure 1. Formation of a stable 240-kDa heterocomplex of Hsp90 in intact cells. (A) HepG2 cells depleted of serum for 4 h were rinsed in PBS and then incubated in PBS with vehicle (DMSO, '-') or EGS cross-linker (+) for 30 min at room temperature. The reaction was quenched using an excess of L-glycine. Cells were lysed and processed for Western blot analysis using specific antibodies against Hsp90, Hsp70, and p60Hop. Representative immunoblots are shown where two dishes per treatment group (DMSO, lanes 1 and 2; EGS, lanes 3 and 4) were loaded onto the gel. (B) Immunoprecipitation from EGS-treated cells was carried out with anti-Hsp90 (clone F8) and control IgG, followed by detection of Hsp90 expression by Western blot analysis. (C) Serum-depleted HepG2 cells were incubated in PBS with protein cross-linkers with various spacer arm length. The positions of the molecular weight markers (in kDa) are shown on the left of immunoblots. Arrow, indicates the position of the 240-kDa heterocomplex of Hsp90 (p240). Each experiment was repeated at least twice with two dishes per group being examined each time.

Figure 2. Determination of the role of Hsp90 ATPase in EGS-mediated formation of p240. (A and B) Serum-depleted HepG2 cells were treated either with vehicle (0.1% DMSO), 17-AAG (5 μ M), or novobiocin (0.2 and 0.6 mM; Novo) for the indicated periods of time and then subjected to EGS cross-linking in PBS. (C) 1321N1 astrocytoma cells and human PBMCs were treated with DMSO or 5 μ M 17-AAG for 1 h followed by EGS-mediated cross-linking reaction. In the experiments depicted in panels (A–C), aliquots of total cell extracts were analyzed by Western blot analysis using anti-Hsp90. (D) HepG2 cells were cotransfected with the FLAG-tagged and Myc-tagged Δ NT-Hsp90 α constructs or with pcDNA alone for 48 h. Protein cross-linking reaction with EGS was then performed. Anti-Myc immunoprecipitates were subjected to Western blot analysis using FLAG antibody. These immunoprecipitation experiments were performed in the absence of a parallel control immunoprecipitation with an irrelevant antibody. Note the faster migration of the truncated Δ NT-Hsp90 α constructs. Arrow, indicates the position of p240 (A–C) or p210 (D). Each experiment was repeated at least twice with two dishes per group being examined each time.

VA, USA). Cells were maintained in α -MEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM pyruvate, 50 U/mL penicillin and 50 μ g/mL streptomycin. The human melanoma cell line lacking FLNa (M2 cells) and the isogenic cell line stably transfected with a full-length FLNa construct (A7 cells) were previously described (21). All cell lines were cultured at 37°C in a humidified incubator with 5% CO₂, and the medium was replaced every 2–3 days. Peripheral blood mononuclear cells (PBMC) collected from healthy donors (who provided informed consent) were isolated by Ficoll-Hypaque density gradient centrifugation. Cells were cultured in RPMI 1640 with 10% fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2 mM glutamine for 16 h.

Before each experiment, cells were incubated with serum-free α -MEM:Ham's F-12 (1:1) for 4 h, washed twice in phosphate-buffered saline (PBS), and then incubated either with vehicle or various

protein cross-linkers (1 mM) for 30 min at 20°C in PBS. The protein cross-linking reaction was quenched by the addition of an excess of glycine (10 mM). Cells were rinsed and immediately processed as indicated below. To assess the role of Hsp90 binding drugs, serum-starved cells were pretreated with vehicle (dimethyl sulfoxide; DMSO), 17-AAG (5 μ M), DMAG (5 μ M), or novobiocin (0.2–0.6 mM) for 1 h before EGS cross-linking reaction. Based on the nature of the experiments, the use of serum starvation prior to cross-linking reaction in PBS was to eliminate cross-reaction of the EGS with serum proteins and free amino acids present in the culture medium.

Isolation of cellular organelles

Cell mitochondria were isolated according to the manufacturer's protocol (MitoScience, Eugene, OR, USA). In brief, cells were subjected to three freeze-thaw cycles followed by a two-step homogenization procedure using Dounce homogenizer with Teflon pestle. The clarified crude homogenates were centrifuged at 12,000 $\times g$ for 10 min at 4°C, and the

pelleted mitochondria were resuspended, aliquoted, and stored at -80°C. The post-mitochondria supernatant was centrifuged at 100,000 $\times g$ for 1 h at 4°C to collect the crude membrane fraction and cytosol; both of which were aliquoted and stored at -80°C. Nuclear extracts from HepG2 cells were prepared using the NE-PER extraction kit (Thermo Scientific Pierce).

Plasmid construction

The cDNA encoding the human Hsp90 α (*HSP90A1*) with a N-terminal 94 amino acid truncation (Δ NT-Hsp90 α ; GenBank/EBI Data Bank accession no. BC023006) was purchased from ATCC. Δ NT-Hsp90 α ligated in pCMV-SPORT6 (Thermo Fisher Scientific, Open Biosystems) was subcloned in-frame into two mammalian expression vectors, pCMV-3Tag-1 and pCMV-3Tag-2 (Stratagene; vectors contain three copies of the FLAG-tag and Myc-tag, respectively), to generate the N-terminal FLAG- Δ NT-Hsp90 α and Myc- Δ NT-Hsp90 α fusion constructs. All constructs were confirmed by standard DNA sequencing.

Transient transfection experiments

HepG2 cells grown to 50% confluence on 35-mm dishes were transiently transfected with either FLAG- Δ NT-Hsp90 α , Myc- Δ NT-Hsp90 α , or empty vector plasmid with 2 μ g DNA/dish using Lipofectamine 2000 in serum-free Opti-MEM, according to the manufacturer's instructions (Invitrogen). The cells were incubated for 48 h after transfection and then serum-starved for 4 h before performing protein cross-linking reaction.

Transfection of 21-nucleotide siRNA duplexes for targeting endogenous FLNa was carried out using Lipofectamine RNAiMAX reagent and 20 nM siRNA duplex per 35-mm plate, according to the supplier's instructions (Invitrogen). Transfected HepG2 cells (250,000 cells/mL) were assayed 3 days after reverse transfection. The sequences of FLNa siRNA used were: r(GGAAGAAGAUCCAGCAGAA)dTdT (sense) and r(UUCUGCUGG-AUCUUCUUC)dAdC (anti-sense), whereas the negative control siRNA was the AllStars Neg. Control siRNA (Qiagen) that has no known target gene. These validated siRNAs have been shown to perform efficient knockdown with minimal off-target effects. Specific silencing of FLNa was confirmed by real-time PCR and immunoblotting.

Immunoprecipitation and Western blot analysis

Cells were lysed in radioimmunopre-

cipitation assay buffer (50 mM HEPES, pH 7.4, 135 mM NaCl, 1%, w/v, Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 100 mM NaF, 1 mM sodium orthovanadate, and protease inhibitor cocktail I and II; EMD Biosciences) as detailed (21). Insoluble material was removed by centrifugation (16,000× *g* at 4°C for 10 min), and the protein concentration in the soluble extracts was determined with the Pierce BCA protein assay (Thermo Scientific) using BSA as standard. For immunoprecipitation experiments, aliquots of the cell lysates were precleared with protein G agarose for 1 h at 4°C, and then incubated with 2 µg monoclonal anti-Hsp90α/β antibody (clone F8; Santa Cruz Biotechnology) for 16 h at 4°C. The immune complexes were collected by addition of protein G agarose for 90 min at 4°C, washed extensively, and eluted in 1.5× Laemmli sample buffer supplemented with 7.5% 2-mercaptoethanol. In some instances, cell lysates were incubated with anti-FLAG M2 affinity gel and anti-Myc-tag antibody. Immunoprecipitates and cell lysates were subjected to SDS-PAGE under reducing conditions and then transferred to polyvinylidene difluoride membranes using iBLOT apparatus. Details of immunoblotting are described elsewhere (21).

Results and discussion

EGS-dependent formation of a stable 240-kDa protein complex encompassing Hsp90 (p240) in intact cells

The irreversible NH₂ group-specific homobifunctional cross-linking reagent EGS was used initially to ascertain whether stable Hsp90-containing heteroprotein complexes could be generated in intact HepG2 cells. After cell lysis, cellular proteins were resolved by SDS-PAGE under reducing conditions and analyzed for Hsp90 by Western blot analysis using a monoclonal antibody that has specificity for both Hsp90α (*HSP90AA1*) and β (*HSP90AB1*) isoforms. A high molecular weight protein complex of ~240 kDa encompassing Hsp90 (p240) was detected upon EGS cross-linking (Figure 1A). Neither the co-chaperones Hsp70 and p60Hop (Figure 1) nor p23 (data not shown) were found in p240 or in any other covalent multiprotein complexes. This heteroconjugate of Hsp90 was also detected in Hsp90 immunoprecipitates from EGS-treated HepG2 cells (Figure 1B). Of significance, exposure to EGS allowed the formation of p240 in 1321N1 astrocytoma cells and human PBMCs (see Figure 2C). Finally, the potency of EGS (16.1 Å)

Figure 3. Subcellular distribution of p240. (A and B) Mitochondrial (Mito), cytosolic (cyto), and crude membrane (mem, 100,000× *g* pellet) fractions were prepared from control and EGS-treated HepG2 cells. The resulting samples were subjected to Western blot analysis using anti-Hsp90 antibody. The stripped membranes were reprobed with antibodies against complex V and IκBα (A, middle and bottom panels) or EGF receptor (B, bottom panel). The time of exposure of the immunoblot to the film was longer than usual to enable the detection of p240 in the membrane fraction of EGS-treated cells. Shorter exposures were also made to enable the detection of sharp p240 and Hsp90 protein bands in the cytoplasmic fraction of EGS-treated cells (not shown). (C) HepG2 cells were treated with EGS or its cell-impermeant analog, sulfo-EGS, followed by immunoblot analysis of total cell lysates using antibodies raised against Hsp90 (upper panel) and HSF1 (lower panel). Arrow, indicates the position of p240; the position of HSF1 trimers is also shown on the right. Each experiment was repeated at least twice with two dishes per group being examined each time.

was compared with that of DMP (9.2 Å) and DSS (11.4 Å), two irreversible NH₂ group-specific homobifunctional cross-linking reagents that have shorter spacer length. The results in Figure 1C showed that the distances between Hsp90 and interacting molecule(s) to generate p240 in situ requires a cross-linker with a 14 Å spacer.

A previous study by Rexin et al. (22) using the chemical cross-linkers DSS and EGS, demonstrated the formation of a constitutive hetero-oligomer complex of ≥300 kDa between Hsp90 and the nonactivated glucocorticoid receptor both in intact cells and in cell extracts. Similar analysis using DMP cross-linking showed the covalent association of a 59-kDa protein (p59) with Hsp90-glucocorticoid

receptor complexes in cytosolic extracts (23). Other evidences showed that DSP and EGS cross-linking in cytosolic extracts covalently linked p59 to the Hsp90-glucocorticoid receptor complexes (24). In the latter study, Hsp90 was detected in a 2:1 ratio with respect to p59, which was later determined to be Hsp56 (*FKBP4*), an immunophilin of the FK506/rapamycin binding class (25). As such, Hsp56 associates with Hsp90 in untransformed mammalian steroid receptor complexes (25) and plays an important role in basic cellular processes involving protein folding and trafficking (26).

Intracellular heat shock proteins are among the most highly expressed proteins in normal and cancerous cells,

Figure 4. Determination of the role of FLNa in the formation and cell surface distribution of p240. HepG2 cells were transfected with either negative control (ctrl) or FLNa siRNA for 72 h. (A) Western blot analysis using total cell lysates was performed with anti-FLNa antibody. (B and C) Serum-depleted cells were incubated with EGS (B) or sulfo-EGS (C), and then Hsp90 expression was detected by immunoblotting (upper panels). In all experiments, the membranes were reprobed for GAPDH as a loading control. ns, nonspecific signal; arrow, indicates the position of p240. Each experiment was repeated at least twice with two dishes per group being examined each time.

and it is likely that the Hsp90-associated partner(s) is indeed a heat shock protein. Consistent with this notion is the fact that protein-protein interactions of Hsp90 and its co-chaperones are refractory to either the pharmacological inhibition of Hsp90 ATPase function or expression of Hsp90 mutants lacking the C-terminal homodimerization domain (27,28). In contrast, pharmacological inhibition of the intrinsic ATPase activity of Hsp90 has been found to disrupt client protein interaction with Hsp90 (2). Here, we present unequivocal evidence for p240 formation existing in intact cells treated with two structurally unrelated classes of Hsp90 inhibitors. The EGS-mediated accumulation of p240 was comparable in the absence or presence of 17-AAG and novobiocin in HepG2 and 1321N1 cells and in freshly isolated PBMCs (Figure 2, A–C). To independently assess whether the N-terminal ATP binding domain of Hsp90 has any role in orchestrating the formation of p240, HepG2 cells were transfected with two distinct epitope-tagged Hsp90 α constructs that lack 94 amino acids at the N terminus (Δ NT). These constructs, termed FLAG- Δ NT and Myc- Δ NT, express truncated recombinant forms of Hsp90 α and, therefore, should give rise to a faster migrating complex when compared with the endogenous Hsp90 α protein. Indeed, when anti-Myc immunoprecipitates of EGS-treated cells were subjected to Western blot analysis

using the FLAG antibody, an ~210-kDa multiprotein complex encompassing FLAG- Δ NT and Myc- Δ NT was observed (Figure 2D, lane 4). In the absence of EGS, there was co-sedimentation of FLAG- Δ NT (~80 kDa) with Myc- Δ NT (Figure 2D, lane 3), consistent with the well-known noncovalent dimeric interaction of Hsp90 (1,2). Reciprocal immunoprecipitation was performed and gave similar results (data not shown), indicating that both the constitutive and EGS-mediated formation of Hsp90 dimers did not require the ATPase activity or binding motifs present in the N-terminal domain of Hsp90 α . Furthermore, evidence of a heteromeric complex of cross-linked Δ NT-Hsp90 α suggests that the ATPase function of Hsp90 is not necessary for the formation and stabilization of the quaternary structure of p240. Taken together, these results demonstrate that the EGS-mediated cross-linking event is specific and may provide an approach for the study of Hsp90-controlled processes that may occur in intact cells under physiological and stress-inducible conditions.

Subcellular distribution of p240 in EGS-treated cells

In addition to its cytosolic expression, Hsp90 is detected in mitochondria and on the surface of normal and tumor cell membranes (8,10–12,29). Here, mitochondrial and cytosolic fractions from control

and EGS-treated HepG2 cells were prepared and analyzed by Western blot analysis for the presence of p240 (Figure 3A). Both subcellular compartments contained monomeric Hsp90 and p240. The blots were reprobed with the mitochondrial marker, complex V, and the cytosolic marker, I κ B α , to confirm the quality of our cell fractionation. Furthermore, when crude membranes (100,000 $\times$ g pellets) from EGS-treated cells were immunoblotted with anti-Hsp90 antibody, there was clear expression of p240, although to levels that were significantly lower when compared with the cytosolic fraction (Figure 3B). As expected, the crude membrane fraction stained positively for EGF receptors (Figure 3B, lower panel).

To assess whether p240 could accumulate at the cell surface facing the extracellular milieu, cells were treated with the cell-impermeant EGS analog, sulfo-EGS (Figure 3C). The results indicate the detection of p240 in sulfo-EGS-treated cells which, when normalized to the amount of monomeric Hsp90, yielded a 10-fold lower abundance as compared with cells treated with EGS (Figure 3C, upper panel). To independently verify the presence of a subpopulation of p240 on the cell surface, we compared the effect of sulfo-EGS on the formation of p240 and HSF1 trimers in HepG2 cells. HSF1 is a cytosolic transcription factor that requires trimerization for its nuclear translocation and DNA binding properties. Consistent with our recent observation (30), constitutive formation of HSF1 trimers was observed in EGS-treated cells; in contrast, sulfo-EGS was unable to promote HSF1 oligomerization (Figure 3C, lower panel, lane 3 versus 2) while inducing the accumulation of p240.

Recent studies have demonstrated that Hsp90 gets exported at the cell surface and is secreted in conditioned media by both normal and tumor cells (reviewed in Reference 31). The reported targets for extracellular Hsp90 α include the co-chaperones Hsp70, p60Hop, p23, and others in breast cancer cells; this co-chaperone complex increases matrix metalloproteinase 2 (MMP-2) binding to Hsp90 α and assists in MMP-2 activation, thereby promoting cancer cell migration and invasion *in vitro* (32). The detection of p240 at the cell surface of EGS-treated HepG2 cells may have important biological implications, as extracellular Hsp90 has been reported to dock with membrane-bound CD91/LRP1, hyaluronan receptor CD44, and tyrosine kinase receptors (31,33), as well as acting as negative regulator for the activation of latent TGF- β 1 (34).

Role of FLNa in the formation and subcellular distribution of p240

Recent proteomic approaches have identified FLNa (also known as ABP280) as a novel interaction partner of Hsp90 (16). FLNa, a member of the nonmuscle actin binding protein family, is a widely expressed molecular scaffold protein that regulates signaling events such as subcellular localization of signaling proteins through changes in cytoskeletal actin dynamics (35). To ascertain the role of FLNa in the formation and subcellular distribution of p240, siRNA-mediated knockdown of FLNa was carried out in HepG2 cells, as evidenced by the potent and selective suppression of FLNa protein levels (Figure 4A). Under these conditions, there was no significant difference in the immunodetection (Figure 4B) and cell surface localization (Figure 4C) of p240 when cells that were transfected with either FLNa siRNA or nonsilencing control siRNA were treated with sulfo-EGS. Moreover, human M2 melanoma cells that lack FLNa also exhibited EGS-dependent accumulation of p240 (data not shown). Therefore, FLNa does not appear to be a key mediator of the recruitment, subcellular compartmentalization and/or dynamic trafficking of p240 in EGS-treated cells.

To conclude, the demonstration that p240 is stoichiometrically abundant is of significance and indicates that the interaction partner(s) is also abundantly expressed. Because of the lack of MS/proteomics data, we cannot rule out the possibility that p240 may consist of an Hsp90 dimer with aberrant electrophoretic mobility along with the existence of populations of heteromers. However, given the fact that the electrophoretic mobility of p240 is significantly slower than that of the DMS-linked Hsp90 dimers (36), it is likely that a distinct component of ~60 kDa is present in p240 in association with two Hsp90 molecules. Moreover, our observation that only EGS with its 14-Å spacer was able to stabilize covalently p240 in intact cells is in contrast with what was observed by others using cytosolic extracts and chemical cross-linkers with shorter spacer arms (22–25). The simplest interpretation for these results is that the quaternary structure of the cytosolic Hsp90 hetero-oligomeric complexes and/or availability of reactive amino groups (e.g., appropriate average molecular spacing and geometry) may differ as a consequence of cell homogenization. Nevertheless, the specificity of the cross-linking event observed here may provide an approach to expand the range of tools available to the study of the Hsp90 interactome.

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Competing interests

The authors declare no competing interests.

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