Cell surface heparan sulfate proteoglycans are involved in the binding of Hsp90α and Hsp90β to the cell plasma membrane

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Introduction

Heat shock protein 90 (Hsp90) is one of the most abundant and ubiquitously expressed proteins.1 There are 2 major cytoplasmic isoforms of Hsp90, Hsp90α (the inducible form) and Hsp90β (the constitutive form), which share a high sequence homology but show some differences in biochemical properties, expression, and function.2 Hsp90 acts inside cells as a chaperone that regulates folding, maturation, transport, and degradation of a diverse set of client proteins, in particular, signaling molecules, steroid receptors, and transcription factors.1,2 In addition, Hsp90 can be found in the extracellular space and at the cell surface membrane. Both Hsp90 cytosolic isoforms, Hsp90α and Hsp90β, were shown to be secreted by many types of normal and cancer cells and expressed at the cell surface.3,4,5 Cell stressors such as heat shock, oxidative stress and hypoxia, as well as growth factors, stimulate Hsp90 translocation to the membrane and/or secretion.6,7,8

The main function of the extracellular Hsp90 is to promote cell motility. It stimulates neurite outgrowth,9 migration processes in the developing nervous system,10 dermal fibroblast migration,3,11 promotes skin wound healing,11,12 and stimulates migratory, invasion, and metastatic activity of cancer cells.8,13-16 Extracellular Hsp90 acts as a pro-motility factor via cell surface receptors.4,17 Hsp90α was shown to bind to cell surface LRPs,18 and stimulate cell migration in an LRP1-dependent manner in normal and cancer cells.6,19 Extracellular Hsp90α plays an important role in ErbB-1 and ErbB-2 activation, downstream kinase signaling, and subsequent cell migration.16 Extracellular Hsp90α and Hsp90β can also function as chaperones for some surface-bound or secreted proteins, such as matrix metalloproteinases and extracellular matrix proteins, thus promoting the cell motility.17-23

Both Hsp90 isoforms have neither a signal peptide necessary for the secretion via the classic endoplasmic reticulum/Golgi protein secretory pathway nor a recognizable transmembrane sequence necessary for the anchoring to the cell plasma membrane. It was demonstrated that Hsp90 uses an unconventional exosomal way of secretion.3,7,14 The mechanisms of the translocation and anchoring of Hsp90 to the plasma membrane are not elucidated.

Heparan sulfate proteoglycans (HSPGs) are cell-surface and extracellular matrix glycoproteins that comprise a core protein to which heparan sulfate glycosaminoglycan chains are covalently attached.24 Surface HSPGs are involved in a large number of biological processes, including cell growth and division, cell adhesion and motility, and cell signaling.24-26 They primarily function as co-receptors for numerous surface receptors and interact with various protein ligands, enabling their more efficient interaction with respective receptors.24-26

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In this study, a line of independent evidence is presented for the first time that cell surface HSPGs are involved in the binding and anchoring of Hsp90α and Hsp90β to the cell plasma membrane.

Results and Discussion

It is known that Hsp90 binds to heparin whose chemical structure is similar to HSPG polysaccharide side chains.27 We
also observed that both isoforms of Hsp90, Hsp90α and Hsp90β, specifically bind to heparin-Sepharose (not shown). These data suggested that cell surface HSPGs may play a role in the anchoring of Hsp90 to the plasma membrane. To verify this consideration, we investigated the cell surface expression of Hsp90α and Hsp90β in human glioblastoma A-172 and fibrosarcoma HT1080 cells treated with different reagents impairing the structure of cell surface HSPGs. We revealed that Hsp90α and Hsp90β are expressed on the surface of A-172 and HT1080 human tumor cells. The contribution of cell-surface HSPGs to plasma membrane anchoring of Hsp90α and Hsp90β was estimated using different experimental approaches. First, we determined the cell-surface expression of Hsp90α and Hsp90β after the cultivation of cells in a medium supplemented with sodium chlorate, a substance known to reduce the sulfation of HSPG glycan chains during their biosynthesis. After a 24-h incubation of cells with sodium chlorate at a nontoxic concentration of 30 mM, the surface HSPG sulfation detected by heparin/heparan sulfate affinity chromatography was diminished to 22.7%, 5.0% and 7.4%, 11.4% of the respective level, respectively; for HT1080 cells, the level of cell surface Hsp90α and Hsp90β decreased to 19.1%, 13.3%, 7.9% and 23.4%, respectively (Fig. 1A-C; Fig. S1). During a 24-h sodium chlorate treatment, the amounts of total (intracellular and membrane-bound) Hsp90α and Hsp90β remained unaltered (Fig. 1D; Fig. S1), which excludes the possibility that the decrease in the amount of membrane-bound Hsp90α and Hsp90β was due to a respective decrease in the total amounts of Hsp90α and Hsp90β in cells. Then, we showed that the cleavage of sulfated glycan chains of cell surface HSPGs with a heparinase I/III blend strongly reduced the level of cell surface heparan sulfates (Fig. 2A), which was accompanied by a considerable loss of surface-associated Hsp90 isoforms. For A-172 cells, the amount of surface-bound Hsp90α and Hsp90β diminished to 33.0% ± 7.9 and 23.4% ± 6.2% of the initial level, respectively; for HT1080 cells, the level of cell surface Hsp90α and Hsp90β decreased to 22.7% ± 6.5 and 15.1% ± 5.1%, respectively (Fig. 2A-C; Fig. S2). Treatment of cells with heparinase I/III blend did not change the contents of total cellular Hsp90α and Hsp90β (Fig. 2D; Fig. S2).

The third approach involved the evaluation of the influence of heparin, a polysaccharide closely related to heparan sulfate, on the attachment of Hsp90α and Hsp90β to the cell surface. For both cell cultures, the treatment of cells at 37°C with heparin at a concentration of 50 μg/ml reduced the amount of cell-associated Hsp90α and Hsp90β by 30–35% and 70–75%, respectively (Fig. 3A, B; D; Fig. S3). At the same time, the heparin treatment of cells did not change the levels of total (intracellular and cell-associated) Hsp90α and Hsp90β in cells, as evidenced by Western blot (Fig. 3C; Fig. S3). The effect of heparin on the cell surface Hsp90 isoforms was concentration-dependent; heparin significantly decreased the levels of both Hsp90 isoforms even at a concentration of 10 μg/ml, reaching a maximum effect at concentrations of 50–100 μg/ml (Fig. 3D). Nevertheless, even at a concentration of 100 μg/ml, heparin did not completely remove surface-bound Hsp90α and Hsp90β, and a significant portion of surface-bound Hsp90 isoforms (65–70% of Hsp90α and 20–30% of Hsp90β) was insensitive to it (Fig. 3D). The effect of heparin on the surface-bound Hsp90 isoforms was time-dependent; the detachment of Hsp90 from cell surface was maximum after a 30-60-min treatment of cells (data not shown).

Some active processes that may occur during treatments of cells with heparin at 37°C, in particular, the appearance of new Hsp90s on the plasma membrane and the internalization of complexes of Hsp90 with cellular receptor(s), may complicate the interpretation of the results. To eliminate these active processes, we evaluated the surface expression of Hsp90α and Hsp90β after treatment of cells at 4°C, a temperature that inhibits the translocation of proteins across the membrane, classical and exosomal secretion, and internalization of receptor-ligand complexes. We observed that the effect of heparin was independent of temperature and was nearly the same at 37 and 4°C (Fig. 3E).

The incubation of heparin-treated cells in DMEM-FBS without heparin at 37°C resulted in a quick (15 min) restoration of cell surface expression of Hsp90α and Hsp90β up to 50% of the control level (untreated cells) followed by a slower restoration of Hsp90 expression to the control level by 6 h. The restoration of surface-bound Hsp90α and Hsp90β was not observed when the cells were incubated for 1 h at 4°C, a temperature at which proteins are not expected to be translocated across the plasma membrane (Fig. 3F), which indicates that the restoration of surface-bound Hsp90 is an active process.

Taken together, the data of 3 experimental approaches strongly indicated that cell surface HSPGs are involved in the anchoring of 2 isoforms of Hsp90, Hsp90α and Hsp90β, to the cell plasma membrane.

To support our finding, we analyzed the colocalization of cell surface HSPGs and Hsp90 isoforms. Simultaneous staining of cell surface HSPGs and Hsp90 isoforms with MAb specific to heparin/heparan sulfate and anti-Hsp90α or anti-Hsp90β antibodies revealed a colocalization of a portion of Hsp90α and Hsp90β with HSPGs on the cell surface (yellow dots in Fig. 4D). After the treatment of cells with heparin, a significant decrease in the number of yellow dots was observed, indicating the disturbance of localization of Hsp90α and Hsp90β with surface HSPGs. The results are presented for HT1080 cells (Fig. 4D); similar data were obtained for A-172 cells. The results suggest that a portion of cell surface Hsp90s is associated with surface HSPGs and provide additional support for our conclusion about the involvement of HSPGs in the anchoring of Hsp90s to the cell surface.

We also determined the influence of sodium chlorate and heparinase treatment of cells, as well as of heparin, on the direct binding of exogenous Hsp90 to the cell surface. We showed that FITC-labeled native mouse Hsp90 (a mixture of Hsp90α and Hsp90β) efficiently bound to the cell surface at 4°C of untreated cells (Fig. 4A, B, C). Unlabeled Hsp90 inhibited the binding of FITC-labeled Hsp90, suggesting the specificity of the binding of Hsp90 to cells (not...
The binding of FITC-labeled Hsp90 strongly decreased in chlorate- and heparinase-treated cells as compared to control untreated cells. Heparin at a concentration of 20 μg/ml also considerably diminished the binding of Hsp90-FITC to the surface of untreated cells (Fig. 4A, B, C). Taken together, the results suggest a role of cell-surface HSPGs in the direct binding of extracellular Hsp90 to cells.

To our knowledge, this is the first report demonstrating the role of cell surface HSPGs in the binding/anchoring of 2 Hsp90 cytosolic isoforms, Hsp90α and Hsp90β, to the cell surface. The revelation of...
heterogeneous and heterotypic modes of interaction of Hsp90α and Hsp90β with cell surface suggests that cell-surface HSPGs may serve as co-receptors in the binding of Hsp90 to the plasma membrane. It has been shown that cell-surface HSPGs and LRP1 function in a cooperative manner to mediate GRP94 cell surface binding, cellular uptake of C4b-binding protein, lipid metabolism, and internalization of apoE/ligoprotein particles, and amyloid-β uptake. Since LRP1 is considered to be one of the receptors of extracellular Hsp90, these data suggest that cell-surface HSPGs may cooperate with LRP1 in mediating the binding/anchoring of Hsp90α and Hsp90β isoforms to the cell surface.

Heparin is known to affect the migration and invasion of cells and cancer progression by inhibition of heparinase, blocking of P- and L-selectin mediated cell adhesion, and the inhibition of angiogenesis. We also observed that heparin inhibited the migration and invasion of A-172 and HT1080 cells in vitro, which correlated with the reduction of the levels of cell surface-bound Hsp90α and Hsp90β (in preparation). These results and our data presented above suggest another possible mechanism of action of heparin on tumor cells. Namely, heparin may diminish the level of cell surface-bound Hsp90 and prevent the binding of Hsp90 to the plasma cell membrane, which would inhibit the migration and invasion of tumor cells in vitro and in vivo.

The recovery of the cell-surface expression of Hsp90α and Hsp90β after heparin treatment of cells was biphasic with a relatively fast partial restoration of the level of surface Hsp90. This suggests that either Hsp90 isoforms are quickly translocated directly to the plasma membrane from cytosol by an unknown mechanism or the autocrine secreted Hsp90 isoforms are anchored to the membrane. If Hsp90 isoforms are to be secreted before being anchored to the plasma membrane, autocrine secreted Hsp90 may reach relatively high local concentrations in a close proximity to the plasma membrane, which can drastically increase the rate of the Hsp90 association with receptor(s) and enable the quick emergence of Hsp90 on the cell surface. The binding of paracrine secreted Hsp90 to adjacent or remote cells cannot be excluded, but this process needs a reasonable surface. The binding of paracrine secreted Hsp90 to the cell surface suggests that cell-surface HSPGs and Hsp90 isoforms to the cell surface.

Materials and Methods

Cell cultures

Human glioblastoma A-172 and fibrosarcoma HT1080 cells were from the Cell Culture Collection of Vertebrates (St. Petersburg, Russia). The cells were maintained in DMEM (HyClone) supplemented with 10% FBS (HyClone) (DMEM-FBS). The cells were grown till 50–60% confluence in chambered coverslips (Thermo Scientific) for confocal microscopy analysis or in culture dishes (Corning) for flow cytometry analysis. Each experiment was performed independently at least 3 times, and representative results are shown in the figures.

Antibodies and Hsp90 detection

Rabbit Hsp90α- and Hsp90β-specific antibodies directed to N-terminal fragments of human Hsp90α (PEETQTQDQPM) and Hsp90β (EEVHHGEEEEEVE) were used (USBiological H1834–55A and H1832–98H). Mouse monoclonal antibody specific to heparin/heparan sulfate (clone T320.11) was from Millipore. For confocal microscopy, the cells grown in chambered coverglasses and treated as described below were washed with cold PBS containing 0.05% Na3Na3 (PBS-Na3Na3), incubated with primary specific or control antibodies and, after washing, with Alexa 488-labeled secondary anti-rabbit IgG or anti-mouse IgG conjugates (Jacksons ImmunoResearch). For colocalization experiments, primary rabbit anti-Hsp90α or anti-Hsp90β antibodies were incubated with cells simultaneously with mouse heparin/heparan sulfate antibody. The cells were washed and incubated simultaneously with Alexa 488-labeled anti-mouse IgG and Alexa 647-labeled anti-rabbit IgG conjugates cross-adsorbed against IgG of different species; anti-mouse and anti-rabbit IgG conjugates do not cross-react with rabbit and mouse IgG, respectively. All incubations with antibodies were performed at 4°C in PBS-Na3Na3 containing 2% BSA (PBS-BSA-Na3Na3), conditions that preclude the translocation of intracellular Hsp90s to the plasma membrane and the internalization of surface Hsp90s. After washing, the cells were fixed with 0.5% formaldehyde for 15 min at 4°C and counterstained with Hoechst 33258 prior to imaging on a confocal laser-scanning microscope Leica TCS SP5 (Leica Microsystems) fitted with a 63x objective. For intracellular staining, the cells were fixed with 0% formaldehyde for 15 min at 4°C and treated with 0.1% Triton X-100 after which they were incubated with primary antibodies and Alexa 488-labeled anti-rabbit IgG conjugates, and counterstained with Hoechst 33258 prior to imaging. For flow cytometry, treated cells were detached from culture dishes by incubation for 5 min at 37°C with 0.05% Na3Na3-EDTA, washed with cold PBS-Na3Na3, incubated with primary and secondary antibodies, fixed with formaldehyde, and analyzed using an Accuri C6 flow cytometer (Becton Dickinson). Each analysis included at least 50,000 events of gated cells. The mean fluorescence intensity (MFI) was determined using the BD Accuri C6 software. To quantify the flow cytometry data, the levels of surface-bound Hsp90α and Hsp90β were expressed as the respective MFI ± SD of 3 to 6 independent replicates. The statistical significance was determined by the Student’s t-test, and p<0.05 was considered to be significant.

Treatment of cells with heparinase I/III and sodium chloride

To assess the influence of surface heparan sulfate digestion on the cell surface expression of Hsp90α and Hsp90β, A-172 and HT1080 cells were washed with DMEM and incubated for 1 h at 37°C with a heparinase I/III blend from Flavobacterium heparinum (Sigma-Aldrich) diluted in DMEM-FBS (0.03 IU/ml). Thereafter, the cells were either probed with Hsp90α- and Hsp90β-specific antibodies for confocal microscopy or were detached from the surface followed by staining with Hsp90α- and Hsp90β-specific antibodies for flow cytometry. The effect of undersulfation of HSPGs on the expression of cell-surface Hsp90α and Hsp90β was examined by growing the cells in DMEM-FBS supplemented with 30 mM sodium chloride (Sigma-Aldrich) for 24 h at 37°C. After the treatment, the
Figure 3. Treatment of A-172 and HT1080 cells with heparin results in a significant loss of membrane-bound Hsp90α and Hsp90β. Cells were treated for 1 h at 37°C (A, B, D, F) or at 37°C and 4°C (E) with heparin at concentrations of 50 μg/ml (A, B, E, F) or 0–100 μg/ml (D), stained with anti-Hsp90α and anti-Hsp90β antibodies, and analyzed by confocal microscopy (A) and flow cytometry (B, D, E, F). (A) Representative confocal microscopy images showing the surface staining with antibodies are presented. Scale bar: 20 μm. (B) Representative flow cytometry histograms for control (black lines) and heparin-treated (red lines) cells stained with Hsp90-specific antibodies, as well as cells stained with the negative control rabbit antibody (blue lines) are presented. (C) Western blot analyses of total (intracellular and cell-surface) levels of Hsp90α, Hsp90β, and β-actin (loading control) in A-172 and HT1080 cells treated with heparin at a concentration of 50 μg/ml for 1 h at 37°C. The representative results from 3 independent experiments are shown. (D, E) The flow cytometry-based quantification of membrane-bound Hsp90α and Hsp90β after treatment with heparin at different concentrations (D) and different temperatures (E). The data are presented as the MFI specific for Hsp90α and Hsp90β, expressed in percent. MFI of control cells was taken as 100%. (F) The percentage of restoration of membrane-bound Hsp90α and Hsp90β is presented. The levels of membrane-bound Hsp90α and Hsp90β were estimated by the respective specific MFI. The levels of membrane-associated Hsp90 in heparin-treated cells and control cells were taken as a 0% restoration and a 100% restoration, respectively. Each bar (D, E) and point (F) represent the mean ± SD (n = 5–6). *Statistically significantly different (P < 0.05) from untreated cells. The representative results from 3 independent experiments are shown.
Figure 4. Binding of Hsp90 to cells (A-C) and colocalization of cell surface Hsp90 with cellular HSPGs (D). (A, B) A-172 and HT1080 cells were treated with sodium chlorate or heparinase. A suspension of cells was incubated for 1 h at 4°C with Hsp90-FITC. Untreated cells were incubated with Hsp90-FITC in the presence and absence of heparin (20 μg/ml). Then, the cells were washed, fixed with formaldehyde, and analyzed by flow cytometry. Representative flow cytometry histograms for A-172 (A) and HT1080 (B) cells are presented. Red lines - chlorate- and heparinase-treated cells and cells incubated with heparin; green lines - untreated cells; black lines - control cells (autofluorescence). (C) Flow cytometry-based quantification of Hsp90 binding to cells. The data are presented as the FITC-Hsp90-specific MFI of treated and control cells, expressed in percent. MFI of control cells was taken as 100%. Each bar represents the mean ± SD (n = 4–5). *Statistically significantly different (P < 0.05) from untreated cells. (D) Live untreated cells and cells treated with heparin at a concentration of 50 μg/ml for 1 h at 37°C were stained simultaneously with antibodies specific to Hsp90α or Hsp90β (red fluorescence) and HSPGs (green fluorescence), counterstained with Hoechst 33342, and analyzed by confocal microscopy. Representative confocal microscopy images showing surface staining with antibodies are presented. Inserts are enlarged images of regions of interest. Yellow fluorescence indicates the colocalization of cell-surface Hsp90 and HSPGs. Scale bar: 20 μm.
membrane-bound Hsp90 isoforms were detected using specific antibodies as described above.

**Heparin treatment of cells and surface Hsp90 restoration experiments**

A-172 and HT1080 cells were treated for different times at 37°C or 4°C with different concentrations of heparin (Sigma or MPBiomedicals) diluted in DMEM-FBS. Then, the cells were either probed with Hsp90α- and Hsp90β-specific antibodies for confocal microscopy or were detached from culture dishes and stained in suspension with Hsp90α- and Hsp90β-specific antibodies for flow cytometry. To evaluate the restoration of membrane expression of Hsp90α and Hsp90β after heparin treatment, the cells were treated with heparin (50 μg/ml) for 1 h at 37°C, washed with DMEM and incubated for different periods of time at 37°C or 4°C in DMEM-FBS. Then, the membrane-bound Hsp90α and Hsp90β were detected using the antibodies as described above.

**Binding of Hsp90 to cells**

Native mouse Hsp90 was purified as described earlier. 42 The purity of Hsp90 was 96–97%, as indicated by SDS-PAGE. Purified Hsp90 was labeled with FITC according to a standard protocol. Cells were treated with sodium chlorate or heparinase I/III blend as described above. Treated and untreated cells were detached from the culture dishes by incubation at 37°C with 0.5% Na-EDTA, washed 2 times with ice-cold PBS-NaN₃, and incubated for 1 h at 4°C with Hsp90-FITC diluted in PBS-BSA-NaN₃. To assess the influence of heparin on Hsp90 binding, untreated cells were incubated with Hsp90-FITC in the presence and absence of heparin (20 μg/ml). Then the cells were washed, fixed with formaldehyde, and analyzed by flow cytometry. The nonspecific binding of Hsp90 to cells was determined by incubation of Hsp90-FITC with cells in the presence of a four- to eightfold excess of unlabeled Hsp90.

**Western Blot Analysis**

Cells were treated with sodium chlorate and heparinase I/III blend as described above. Cells were treated with heparin at a concentration of 50 μg/ml for 1 h at 37°C. Treated and untreated cells were lysed in PBS containing 1% Nonidet P-40, 0.5% deoxycholate, 0.3% SDS, and 1 mM phenylmethylsulfonyl fluoride. Cell samples were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked in PBS containing 0.05% Tween 20 and 2% BSA (PBS-T-BSA). The membrane was incubated with anti-Hsp90α, anti-Hsp90β, and anti-b-actin (Santa Cruz Biotechnology) antibodies in PBS-T-BSA for 2 h at room temperature, washed, and then incubated with HRP-conjugated secondary anti-rabbit IgG or anti-mouse IgG antibodies in PBS-T-BSA (AbD Serotec, STAR117P and STAR121P) for 1 h. After thorough washing, immunoreactive bands were detected using 3,3’-diaminobenzidine substrate.

**Disclosure of Potential Conflicts of Interest**

No potential conflict of interests was disclosed.

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**Supplemental Material**

Supplemental data for this article can be accessed on the publisher’s website.

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