Supplemental Information Inventory

Glucocorticoid Receptor Function Regulated by Coordinated Action of the Hsp90 and Hsp70 Chaperone Cycles

Elaine Kirschke, Devrishi Goswami, Daniel Southworth, Patrick R. Griffin, and David Agard

Supplemental Figures and Figure Legends

Figure S1, Related to Figure 2
Figures S1A, B and C validate that the F602S mutation is not changing the conclusions of this work. Figures S1C and D compliment Figure 2, correlates the nucleotide state of Hsp70 with GRLBD binding and inhibition. These were included to strengthen the argument stabilization of the ATP state of Hsp70 correlates with loss of GRLBD binding and inhibition.

Figure S2, Related to Figure 3
The limited proteolysis (Figure S3A) supports the HDX-MS results shown in Figure 3. Figures S3B shows the HDX-MS data for GRLBD alone and S3C provide the change in H/D exchange rates with Hsp70 for the individual peptides that are mapped onto the GRLBD structure in Figure 3.

Figure S3, Related to Figure4
This MBP-GRLB pull down compliments the functional ligand binding data shown in Figure 4C and 4D with protein binding data.

Figure S4, Related to Figure4
Items in this figure support the conclusions drawn from the data shown in Figure 4. The specific activity (Figure S4A) indicate that enhancement in ligand association and affinity in Figure 4E, 4G, 4H, and 4I are not the results of the chaperones activating a previously inactive fraction of the GRLBD population. Figure S4B shows that the acceleration in ligand association shown in Figure 4E and 4G is due to Hsp90. Figures S4C and S4D show that the measured increase in ligand association and dissociation reported from the data in Figure 4 are statistically significant.

Figure S5. Related to Figure 5
Figures S5A, S5B and S5C explain how the Hsp90:Hsp70:HOP:GRLBD complex used for cryoEM data collection was formed, including how the presence of GRLBD was ensured. Figures S5D and S5E are supporting data for the cryoEM reconstruction.

Figure S6. Related to Figure 5

This figure depicts the conformational rearrangements in Hsp90 (Figure S6A) and Hsp70 (Figure S6B and C) that are required to best fit the EM density reported in Figure 5.

Figure S7. Related to Figure 6
This figure provides supporting data for Figure 6. Figure S7A is the same data shown in Figure 6B, but scaled to the first 50 minutes to clearly show the different time scales in which reactivation by Bag-1 and the Hsp90 occur. Figure S7B show that HOP and p23 are important for the maintained function by Hsp90 shown in Figure 6B. Figure S7C is the same experimental data shown in Figure 6B, but also including the Hsp90 D93N mutants (excluded form Figure 6B for clarity), showing that the Hsp90 D93N is not equivalent to Hsp90 E47A, indicating that Hsp90 closer is important for maintaining GRLBD function.

Supplemental Experimental Procedures

Protein Expression and Purification
Provides in depth experimental procedures for the purification of all proteins:
- Hsp90, HOP, p23, and Hsp40 expression and purification
- Bag-1 expression
- Hsp70 expression and purification
- GRLBD expression and purification

Ligand Binding Experiments
Provides in depth experimental procedures for the ligand binding assays, with specific experimental details and data analysis used for each figure.
- GRLBD equilibrium binding, related to Figure 1
- GRLBD ligand binding with Hsp70 system, related to Figure 2
- GRLBD equilibrium binding with Hsp70/Hsp90, related to Figure 4
- GRLBD ligand binding recovery, related to Figure 6

Size Exclusion Chromatography with Multi-Angle Light Scattering (SEC-MALS)
Provides experimental procedures related to Figure S1B and S5A

Limited Proteolysis
Provides experimental procedures related to Figure S1F

MBP-GRLBD Pull Down
Provides experimental procedures related to Figure S1D and S4

Supplemental References

Glucocorticoid Receptor Function Regulated by Coordinated Action of the Hsp90 and Hsp70 Chaperone Cycles

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Supplemental Data

Figure S1. GRLBD WT and F602S comparison and Hsp70:GRLBD interaction characterization, Related to Figure 2

A) GRLBD WT binds ligand with higher affinity than GRLBD F602S. Normalized equilibrium ligand binding of 20nM F-dex to MBP-GRLBD F602S (blue) as in Figure1A and MBP-GRLBD WT (red).

B) Both GRLBD WT and F602S are monomeric. Masses were determined by analytical size exclusion chromatography (SEC) on a Shodex 803 column with an in line multi-angle light scattering (MALS) for MBP-GRLBD F602S (blue) and MBP-GRLBD WT (red). While WT elutes slightly earlier than F602S, both GRLBDs are monomeric (~75 kDa).

C) Hsp70 ATP hydrolysis is required for GRLBD ligand binding. Plotted is the equilibrium binding of F-dex to GRLBD with and without Hsp40 and Hsp70 with indicated nucleotide (±SEM). Assay conditions: 20nM F-dex, 1μM MBP-GRLBD, 2μM Hsp40, 15μM Hsp70, and 5mM $MgCl_2$/nucleotide.

D) Hsp70 ATP hydrolysis is required for GRLBD binding. MBP pull down with MBP-GRLBD visualized by coomassie stained SDS-PAGE (top), and western blot probing for Hsp40 (bottom). No interaction with Hsp70 is detected without Hsp40 (lane 1 and 2). ATP hydrolysis is required for stable Hsp70 binding (lane 3 and 4). Hsp70 binding is significantly reduced with the NEF Bag-1 (lane 5). Assay conditions: 5μM MBP-GRLBD, 2μM Hsp40, 15μM Hsp70 and Bag-1, and 5mM $MgCl_2$/nucleotide.

E) GR F602S mutation does not effect Hsp70's IC_{50}. Plotted is the equilibrium binding of F-dex to MBP-GRLBD WT (red) and F602S (blue), with Hsp40.

Inhibition for WT GRLBD fits an IC_{50} of 5.0μM, within the standard deviation of F602S (4.6±0.6 μM). Same procedure and fitting as Figure 2C with 1μM MBP-GRLBD, 2μM Hsp40, 20nM F-dex, 5mM ATP/$MgCl_2$ and 0-32μM Hsp70.

Figure S2. Partial Unfolding of GRLBD by Hsp70 detected by limited proteolysis and HDX-MS, Related to Figure 3

A) Limited proteolysis of GRLBD shows increased trypsin sensitivity with Hsp70. Western blot probed with MBP antibodies against reactions from trypsin digestion of 1μM MBP-GRLBD that were preincubated with 2μM Hsp40 and increasing concentrations of Hsp70 (0-32μM). Hsp70 promotes the production of a 52-54kDa N-terminal fragment, corresponding to cleavage on GRLBD's helix3.
B) HDX-MS data for GRLBD alone.
C) Differential HDX-MS for 10μM GRLBD ± 12μM Hsp70 and 2μM Hsp40.

Figure S3. Inhibition of Hsp90's ATP hydrolysis does not prevent binding of Hsp90 to the GR-Hsp70 complex, but prevents incorporation of p23, Related to Figure 4

A) MBP pull down with MBP-GRLBD. Coomassie stained SDS-PAGE (top), and western blot probing for HOP, p23, and Hsp40 (bottom). No/weak Hsp90 binding to GRLBD without Hsp70 (lane 1). Hsp70 binding with Hsp40 (lane 2). In addition to Hsp40 and Hsp70, there is almost no detectable Hsp90 binding without HOP and p23 (lane 3). Hsp90 binding is detected with HOP (lane 4), and p23 (lane 5). Increased Hsp90 binding is detected with both HOP and p23 (lane 6). 17AAG only inhibits p23 binding (lane 7). Hsp90 mutants, E47A (lane 8) and D93N (lane 9) similarly inhibit p23 binding. Addition of Bag-1 results in the reduction of Hsp70 and HOP binding, while increasing Hsp90 and p23 binding (lane 10). Assay conditions: 5μM MBP-GRLBD, 2μM Hsp40, and 15μM Hsp70, Hsp90, HOP, p23 and Bag-1.

Figure S4. Hsp90 Enhances Ligand Binding, Related to Figure 4

A) Specific ligand binding activity of GRLBD is unaffected by the chaperone system. 2μM F-dex, with increasing amounts of MBP-GRLBD alone (blue), and with 2μM Hsp40 and 10μM Hsp70, HOP, p23 and Hsp90 (red). The specific activity of GRLBD is 56±4% for GR alone and 59±6% with chaperones (±SEM).
B) Hsp90 accelerates association kinetics. GRLBD ligand association kinetics of 20nM F-dex (as in Figure 4D) for 1μM MBP-GRLBD alone (black square), and with 2μM Hsp40, 15μM Hsp70, HOP, and p23, and 0-8μM dimer Hsp90 (colored circles).
C) On rates for MBP-GRLBD alone and with chaperones determined from the slope of the linear fit in Figure 4G to be 0.165±0.008 for GRLBD alone and 0.304±0.072 $\mu M^{-1}min^{-1}$ with chaperones (± weighted error of slope).
D) Average off rates for MBP-GRLBD alone and with chaperones determined from 3 separate experiments as in Figure 4F to be 0.041±0.004 min^{-1} for GRLBD alone and 0.059±0.002 min^{-1} with chaperones (±SEM).

Figure S5. Purification of Hsp90:Hsp70:HOP:GRLBD Complex, Related to Figure 5

A) SEC-MALS of 10μM Hsp90, 15μM Hsp70, 15μ HOP, 2μM Hsp40 (blue) and with 20μM MBP-GRLBD (red) (Wyatt 050S5). Without GRLBD, a 300kDa complex of one HOP, one Hsp70 bound to a Hsp90 dimer is obtained. With GRLBD, a shoulder on the front of the peak with an increase in mass indicates the incorporation of MBP-GRLBD (74kDa) into the complex. Mass measurements are unreliable at low protein concentration.
B) SDS-PAGE of fractions from front shoulder in the SEC run with GRLBD in (A). Top gel shows incorporation of Hsp70, GRLBD, and HOP. Due to their comparable size, MBP-GRLBD and Hsp70 cannot be resolved by SDS-PAGE. Sample to the right of ladder are from an equivalent run with a his-tagged Hsp70, allowing the separation of Hsp70 from the MBP-GRLBD, showing the incorporation of both proteins. Bottom gel contains the same fractions as top gel, but cross-linked with 0.02% gluteraldehyde, indicating the two complexes, with and without GRLBD.

C) SEC of sample used for cryo EM data collection. Sample were incubated at 20μM Hsp90 monomer, 15μM HOP, 15μM Hsp70, 20μM MBP-GRLBD, and 3μM Hsp40 and with 400μM ATP in 30mM HEPES pH7.5, 50mM KCl, 5mM $MgCl_2$, 2mM DTT, 10% Glycerol, and 0.05% n-Octyl-β-D-Glucopyranoside (β-OG) for 1 hour at room temperature and then purified by SEC (Wyatt 050S5) in 50mM HEPES pH7.5, 50mM KCl, 5mM $MgCl_2$, 2mM DTT, 0.01% β-OG and 200μM ADP. SDS-PAGE indicates that only fractions in which complete incorporation of GRLBD were used.
D) Reference free class averages of 10,149 particles from cryo-EM data set with the number of particles per class indicated.
E) The resolution of the cryo EM reconstruction is estimated to be 38Å by the fourier shell correlation method (FSC).

Figure S6. Docking of Crystal Structures into EM density, Related to Figure 5

A) Fitting of Hsp90 into the density requires rotation of the NTD of the client bound arm. The reconstruction fit with the symmetric Hsp90 structure with the HOP induced NM rotation (left). A 23° rotation away from the center about the NM pivot point (black dot) allows the NTD to fit into the density (right).
B) EM complex fit with peptide bound structure of Hsp70 SBD (1DKX) with the α-helical lid in a bent conformation.
C) EM complex fit with a model of Hsp70 SDB with the α-helical lid in a straight conformation. The extra density between the substrate binding site and the lid is likely GRLBD.

Figure S7. HOP and p23 are required for Hsp90 to maintain full GR activity with Bag-1, Related to Figure 6

A) Ligand binding kinetics from Figure 6B scaled to the first 50 minutes. Reactivation by Hsp90, HOP and p23 (red), Bag-1 (black), and both (purple).

B) Ligand binding kinetics of 1μM MBP-GRLBD, as in Figure6A. Kinetics initiated with Bag-1 alone (black), or with Bag-1 and Hsp90 (green), Hsp90 with HOP (yellow), Hsp90 with p23 (blue), and Hsp90 with HOP and p23 (red).
C) Reactivation kinetics from Figure 6B, additionally showing the combined effect of Bag-1 and the nucleotide binding deficient mutant of Hsp90 (D93N) with p23 and HOP (grey).

Supplemental Experimental Procedures

Protein Expression and Purification

Typically, proteins were expressed and purified by the following procedure. Proteins were expressed in bacterial BL21 star (DE3) strain. Cells were grown in LB at 37°C until OD_{600} reached 0.7-0.8, and then induced with 1mM IPTG overnight at 16°C. Cells were harvested and lysed in 50mM Tris pH8, 300mM KCl, 6mM β-mercaptoethanol (βME), 0.2mM PMSF and 10mM imidazole using a EmilsiFlex-C3 (Avestin). Lysate was centrifuged and the soluble fraction was affinity purified by gravity column with Ni-NTA affinity resin (QIAGEN). The protein was eluted with 30mM Tris pH8, 50mM KCl, 6mM βME, and 300mM imidazole. Eluted protein was then loaded onto an ion exchange column, MonoQ 10/100 GL (GE Healthcare) with 30mM Tris pH8, 50mM KCl, 6mM βME and eluted with a linear gradient of 50-500mM KCl. The 6x-His tag was removed with TEV protease during the following overnight dialysis in 30mM Tris pH7.5, 150mM KCl, 6mM βME. Cleaved protein was purified by size exclusion in 30mM Tris pH7.5, 500mM KCl, 6mM βME using a Superdex S200 16/60 (GE Healthcare) or Superdex S75 16/60 (GE Healthcare). Purified protein was dialyzed overnight into storage buffer (30mM HEPES pH 7.5, 50mM KCl, 1mM DTT, and 10% Glycerol), concentrated, flash frozen, and stored in aliquots at −80°C.

Bag-1 Expression

The human gene for the Bag-1 isoform, Bag-1M (71-345), was obtained from the Protein Structure Initiative Material Repository (Cormier et al., 2010). The 26

kDa fragment corresponding to the canonical Bag-1 isoform (116-345) was cloned into a modified pET28a vector with an N-terminal TEV cleavable 6x His tag. Protein was expressed and purified as above.

Hsp70 Expression and Purification

The gene for full length human Hsp70 (GeneCopoeia) was cloned into the pET151 plasmid with the TEV cleavable 6x His-tag. Hsp70 was expressed and purified as above with the following modifications. An extra wash step with 0.1% Tween20 and 2mM ATP was added to the Ni-NTA column and the elution was loaded straight onto an ion exchange column (Q Sepharose, GE Healthcare). The first of the two Hsp70 peaks was collected and TEV cleaved overnight. The cleaved protein was purified on a Superdex S200 16/60 (GE Healthcare) and only the monomeric peak was collected. The pure protein was buffer exchanged on a HiTrap desalting column (GE Healthcare) into storage buffer with 5mM $MgCl_2$. Since the presence of residue nucleotide was detected by the ratio of 260 vs 280 nm absorption, the protein concentration was determined by Bradford assay. Investigation of the stored protein by SEC-MALS revealed the reappearance of dimers in protein stocks (data not shown). While relative amounts of dimer varied between purifications, the IC_{50}s for GRLBD inhibition was unchanged by the monomer-dimer distribution.

For cryo-EM, Hsp70 was purified from baculovirus infected Sf9 cell pellets expressing human Hsp70, produced at the Baculovirus/Monoclonal Antibody Facility at Baylor College of Medicine. Cells were lysed in 20mM Tris7.5, 20mM KCl, 1mM EDTA, and 1mM DTT and loaded onto an ion exchange HiPrep 16/60 Q FF column (GE Healthcare). Protein was eluted with a linear gradient of 20-500mM KCl. Eluted protein was affinity purified by ATP agarose (N-6 attachment) gravity column (Sigma),eluting with 20mM ATP/ $MgCl_2$. Sf9 expressed protein was then treated as the bacteria expressed Hsp70 starting with the S200 SEC column.

GRLBD Expression and Purification

A synthetic gene for human GR was codon optimized for bacterial expression (GeneArt). The LBD fragment (521-777) containing the F602S mutation was cloned into a pMAL-c2X derivative with a N-terminal TEV cleavable 6x His-MBP tag. MBP-GRLBD was expressed in BL21 star (DE3). Typically, cells were grown in 37°C for about 3 hour, and then the temperature was lowered to 16°C. When the OD_{600} reached 0.8, dexamethasone was added to a final concentration of 180μM, followed by overnight induction with 1mM IPTG. Harvested cells were lysed by an EmulsiFlex-3C (Avestin) in 50mM Tris pH8, 300mM KCl, 10% glycerol, 20mM imidazole, 50μM dexamethasone, 0.5mM TCEP, 0.04% CHAPS and protease inhibitor pills (Roche). The lysate was clarified by centrifugation and the soluble fraction batch bound to Ni-NTA affinity resin (QIAGEN). The Ni-NTA column was washed with lysis buffer supplemented with 5mM $MgCl_2$, and 2mM ATP and then eluted in 30mM Tris pH8, 50mM KCl, 0.5mM TCEP, 10% glycerol, 50μM cortisol, and 300mM imidazole. Eluted protein was loaded on a MonoQ 10/100 GL (GE Healthcare) ion exchange column and eluted with a linear gradient of 50-500mM KCl. Eluted protein was dialyzed over night against 1 liter of ligand free buffer (30mM Tris 7.5, 150mM KCl, 0.5mM TCEP and 10% glycerol). The next morning, the protein was purified by size exclusion with a Superdex S200 16/60 (GE Healthcare) in 30mM Tris pH7.5, 500mM KCl, 0.5mM TCEP, and 10% glycerol. Purified protein was dialyzed against 1 L of storage buffer (30mM HEPES pH 7.5, 100mM KCl, 4mM DTT, and 10% Glycerol) overnight, and then further against 500mL of storage buffer for 5-8 hours to ensure ligand removal. Purified protein was concentrated, flash frozen, and stored in aliquots at −80°C. For WT MBP-GRLBD, site directed mutagenesis was used to change the F602S back to wild type. WT MBP-GRLBD was expressed and purified the same but with 0.04% CHAPS in all buffers.

For untagged GRLBD, the LBD fragment (521-777) containing the F602S mutation was cloned into a pACYCDuet derivative with a N-terminal thrombin cleavable 6x His tag. Modifications to the purification included the removal of the

6x His tag with thrombin (Sigma) during the post MonoQ dialysis, and size exclusion wtih a Sephacryl S-75 column (GE Healthcare).

Ligand Binding Experiments

GR Equilibrium Binding, Related to Figure 1

For the GRLBD dilution series (Figure 1A), MBP-GRLBD was equilibrated to room temperature for 5 to 10 minutes. Ligand binding was then initiated with 20nM F-dex and the association kinetics was measured until binding reached equilibrium. Plotted equilibrium values are the average of 3 independent experiments with error bars representing the standard deviation. Equilibrium binding curves were fit with a single site binding model where Y_{max} is the maximal binding, NS is the nonspecific binding base line, and K_D is the dissociation constant:

$$Y(mP) = \frac{[GR] * (Y_{max} - NS)}{(K_D + [GR])} + NS$$

The reported K_D was determined by averaging the K_Ds obtained from the fitting of the three separate experiments (±SEM).

For ligand binding with Hsp90 (Figure 1B), MBP-GRLBD was pre-equilibrated with 5μM Hsp90 for 60-90 minutes with 5mM ATP/$MgCl_2$. Ligand binding was measured as above with the plotted values being the average of 2 independent experiments.

GRLBD Ligand Binding with Hsp70 System, Related to Figure 2

For association kinetics (Figure 2A), 1μM MBP-GRLBD was pre-equilibrated with 2μM Hsp40, 15μM Hsp70 and 5mM ATP/$MgCl_2$ for 60-70 minutes at room temperature. Reactions were equilibrated to 25°C before the kinetics was

initiated with 50nM F-Dex. Association curves fit to a single exponential binding equation were k_{obs} is the observed association rate:

$$Y(mP) = Y_{max} * \left(1 - e^{(-1-t*k_{obs})}\right) + NS$$

For the Hsp70 inhibition curve (Figure 2C), samples were prepared as in Figure 2A with varying concentration of Hsp70. Association kinetics was measured until binding reached equilibrium. Plotted equilibrium values are the average of 3 independent experiments with error bars representing the standard deviation. The IC_{50} curve fit a cooperative inhibition model were n is the Hill coefficient:

$$Y(mP) = \frac{Y_{max} - NS}{1 + (\frac{[Hsp70]}{IC_{50}})^n} + NS$$

The reported IC_{50} and n were determined by averaging the parameters obtained from the fitting of the three separate experiments (±SEM).

For dissociation kinetics (Figure 2D), the concentration of F-dex was increased to improve the signal to noise. 100nM F-dex was pre-bound to 1μM MBP-GRLBD in the presence of 2μM Hsp40 and 5mM ATP/$MgCl_2$. Association was monitored to ensure the binding had reached equilibrium. Dissociation was initiated with 100μM unlabeled dex ± 15μM Hsp70. Dissociation curves fit to a single-phase exponential decay:

$$Y(mP) = (Y_{max} - NS)e^{-t*k_{off}} + NS$$

The reported off rates (k_{off}) were determined by averaging the rates obtained from six independent experiments (±SEM).

GR Equilibrium Binding with Entire Chaperone System, Related to Figure 4

For equilibrium ligand binding in Figure 4C, proteins were pre-equilibrated together with 5mM ATP/$MgCl_2$ for 60 to 70 minutes at room temperature. Proteins and reagents were added at the following concentration: 1μM MBP-GRLBD, 2μM Hsp40, 15μM Hsp70, 15μM Hsp90, 15μM HOP, 15μM p23, and 50μM 17AAG. Ligand binding was initiated with 20nM F-dex, and association was measured until reaching saturation. Plotted equilibrium values represent the mean of 3 independent experiments, with error bars representing the standard deviation (Figure 4C).

The Hsp90 concentration dependence for GRLBD ligand binding recovery (Figure 4D), was carried out as in Figure 4C with varying concentrations of Hsp90 WT, E47A, and D93N. For Hsp90 WT, values are the mean of 3 independent experiments with error bars representing the standard deviation. Data was fit to a half maximal effective concentration equation (EC_{50}):

$$Y(mP) = \frac{Y_{max} - NS}{1 + (\frac{[Hsp90]}{EC_{50}})^n} + NS$$

GRLBD ligand association kinetics with the entire chaperone system (Figure 4E and 4G) was carried out as shown in Figure 4B. Proteins were pre-incubated at 1μM MBP-GRLBD, 2μM Hsp40, 15μM Hsp70, 10μM Hsp90, 10μM HOP, and 10μM p23 with 5mM ATP/$MgCl_2$ for 60-70 minutes at room temperature. MBP-GRLBD alone was prepared with matching volumes of chaperone storage buffer. Reactions were equilibrated to 25°C for several minutes before the kinetics was initiated with 20nM F-Dex. Association curves were fit to a single exponential to determine the observed association rate, k_{obs}:

$$Y(mP) = Y_{max} * \left(1 - e^{(-1-t*k_{obs})}\right) + NS$$

For Figure 4G, the k_{obs} obtained at varying MBP-GRLBD concentration was determined as in Figure 4E. Each point represents the average k_{obs} from 3-5

separate experiments with error bars representing the standard error of the mean. The on rate (Figure S4C) was determined from the slope of the linear fit in Figure 4G, with the error determined by the weighted errors of the individual points.

For GRLBD ligand dissociation kinetics with the entire chaperone system (Figure4F), proteins were pre-incubated at 1μM MBP-GRLBD with 2μM Hsp40, 15μM Hsp70, 10μM Hsp90, 10μM HOP, 10μM p23 with 5mM ATP/$MgCl_2$ or equivalent volume of chaperone buffer for 60 minutes at room temperature, followed by equilibration with 100nM F-dex for about 50 minutes. Ligand dissociation was initiated with 100μM unlabeled dex. Off rates were determined from the average of 3 separate experiments (±SEM) (Figure S4D).

For determination of ligand binding affinity with and without chaperones (Figure 4H), samples were prepared as for Figure 4C, with varying concentrations of MBP-GRLBD. Ligand binding was measured and fit as in Figure 1. We noted that GRLBD ligand binding behavior was affected by buffer conditions. For this reason whenever comparing GR with and without chaperones, reactions were always normalized such that without chaperones, equivalent amounts of chaperones storage buffer was added to normalize for any changes. The difference in K_D for GRLBD alone between Figure 1A and Figure 4E is therefore attributed to the difference in buffer conditions after normalizing for the significant volume of chaperone storage buffer. The K_D was determined from the average of 5 separate experiments (±SEM) (Figure 4I).

GR Ligand Binding Recovery, Related to Figure 6

For the GRLBD ligand binding recovery (Figure 6B), experiments were carried out as shown in Figure 6A. 1μM MBP-GRLBD was pre-incubated with 2μM Hsp40, 15μM Hsp70 and 5mM ATP/$MgCl_2$ for 50-60 minutes at room temperature, followed by incubation with 50nM F-dex for 45-60 minutes. Ligand

binding recovery was initiated with 15μM Bag-1 and/or 15μM Hsp90, HOP and p23.

Size Exclusion Chromatography with Multi-Angle Light Scattering (SEC-MALS)

Samples were resolved by SEC with either a Shodex 803 or a Wyatt 050S5 column on an Ettan LC (GE Healthcare). Molecular weights were determined by MALS with an in-line DAWN HELEOS MALS detector and Optilab rEX differential refractive index detectors (Wyatt Technology Corporation). Data were analyzed by the ASTRA V software package (Wyatt Technology Corporation)

Limited Proteolysis

Proteins were equilibrated at 1μM MBP-GRLBD, 2μM Hsp40, and 0.5, 1, 2, 4, 8, 16, and 32 μM Hsp70 in 30mM HEPES pH7.5, 150mM KCl, 2mM DTT, 5mM $MgCl_2$/ ATP for one hour at room temperature. Trypsin (Sigma) was added at 4ng/μL and incubated on ice for 30 minutes. Reactions were quenched with 4X SDS loading buffer. 250ng of digested reactions were separated by SDS-PAGE, followed by western transfer to nitrocellulose and probed with an MBP antibody (New England BioLabs).

MBP-GRLBD Pull Down

Proteins were incubated at 5μM MBP-GRLBD, 2μM Hsp40, 15μM Hsp70/Hsp90/HOP/p23 for 45 min at room temperature in 30mM HEPES pH7.5, 150mM KCl, 2mM DTT, 0.05% Tween20, 5mM ATP/$MgCl_2$. The ~3kDa his tag was left on Hsp70 to allow separation of Hsp70 and MBP-GRLBD by SDS-PAGE. Following incubation, 10μL amylose magnetics beads (New England BioLabs) was added per 20μL reaction and incubated on ice for one hour. Beads were then washed 3 times with the incubation buffer, before elution with 50mM maltose. Samples were separated by SDS-PAGE and visualized with Coomassie

blue stain. For enhanced detection of HOP, p23, and Hsp40, a separate SDS-PAGE was transferred to nitrocellulose and separately probed with antibodies against p23 (Santa Cruz Biotechnology), HOP (Santa Cruz Biotechnology), and Ydj1 (sigma).

Supplemental References

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