Quantitative assessment of complex formation of nuclear-receptor accessory proteins

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Like other nuclear receptors, steroid hormone receptors form large protein hetero-complexes in their inactive, ligand-friendly state. Several heat-shock proteins, immunophilins and others have been identified as members of these highly dynamic complexes. The interaction kinetics and dynamics of hsp90, hsp70, p60 (Hop), FKBP52, FKBP51, p48 (Hip) and p23 have been assessed by a biosensor approach measuring the complex formation in real time. A core chaperone complex has been reconstituted from p60, hsp90 and hsp70. p60 forms a molecular bridge between hsp90 and hsp70 with an affinity in the range of 10^5 M^{-1} . Dynamics of hsp90–p60 complex formation is modu-

lated by ATP through changes in the co-operativity of interaction. At low protein concentrations ATP stabilizes the complex. Binding of p23 to hsp90 did not change the affinity of the hsp90–p60 complex and the stabilizing effect of ATP. Saturation of the p48–hsp70 interaction could not be achieved, suggesting multiple binding sites. A picture of the protein complex, including stoichiometric coefficients, co-operativity of interaction and equilibrium-binding constants, has been formed.

Key words: co-operativity, cyclophilin, heat-shock proteins, real-time biological interaction assay, surface plasmon resonance.

INTRODUCTION

In their ligand-free state, steroid hormone receptors, such as oestrogen and progesterone receptors, form multiprotein complexes with a variety of proteins that mostly belong to the heat-shock protein and immunophilin families [1,2]. The core member of these receptor–chaperone complexes is hsp90, a protein which has been linked to a variety of different cellular events, but whose functions are yet to be defined. hsp90 is one of the most abundantly expressed proteins in the cell and plays important roles in protein folding, translocation and in cell signalling [3]. Besides steroid hormone receptors, hsp90, predominantly as a dimer [4,5], forms chaperone complexes with other transcription factors (e.g. dioxin receptor, v-erbA) [6–8], the tumour-suppressor protein p53 [9] and a number of protein kinases.

At the time when hsp90 was identified as a member of the untransformed steroid receptor complexes [10,11], it became clear that a number of other non-steroid binding proteins with different properties were present [12]. The FK506-binding protein FKBP52 [13–17] and cyclophilin-40 (CyP-40) [18,19] belong to the family of immunophilins and were identified as members of progesterone-, glucocorticoid- and oestrogen-receptor complexes. They bind immunosuppressant drugs like FK506, rapamycin or cyclosporin and possess peptidyl-prolyl-isomerase function [20–22]. Dependent on cell type and conditions, hsp70 was co-adsorbed by immunoprecipitation experiments in sub-stoichiometric levels [23–26]. Like hsp90, hsp70 is essential for protein folding in different cellular compartments or refolding after denaturation [25–31]. A number of additional members of these multiprotein complexes, such as p60 (Hop) [32], p48 [33] and p23 [34,35], have been identified. p60 interacts with both hsp70 and hsp90, forming a core unit of chaperone complexes.

p48 (Hip) binds to hsp70 and seems to promote hsp70–substrate interaction [36]. p23 binds to hsp90 in an ATP-dependent manner [34,37] and seems to play a role in signal transduction itself [38]. The current model for receptor–complex formation is the following: newly synthesized or recycled receptor is recognized by hsp70–p48 (Hip). This early complex is recognized by p60 or Hop (hsp70–hsp90-organizing protein) in context with hsp90 and p48 dissociates from hsp70. Transition-metal ions, like tungstate or molybdate, stabilize this (hsp90–p60–hsp70)–receptor complex. However, during complex maturation p60 is exchanged for FKBP52, or in certain cell types by CyP-40, and hsp70 is found in sub-stoichiometric amounts. Recent reports describe the requirement for sub-stoichiometric concentrations of a member of the hsp40 family for receptor–complex reconstitution under cell-free and defined conditions [39–41].

Most of the information on these highly dynamic chaperone–receptor complexes stems from immunoprecipitation and/or *in vitro*-reconstitution experiments using cell lysates. These methods have aided in the identification of complex members in the first place and, together with *in vitro*-reconstitution systems using cell lysates, minimal conditions for complex formation have been defined [41].

In this report, we have combined PAGE methods with a novel biosensor-based approach to study interaction of members of the chaperone–receptor complexes. Purified proteins were combined and incubated under various conditions and subsequently separated under native and denaturing conditions. Protein complexes were revealed by silver-stain and immunoblotting techniques. In the biosensor approach based on surface plasmon resonance (SPR) using the BIACORE[™] system [42–44], qualitative and quantitative aspects of interaction between members of chaperone complexes have been analysed. First, several

Abbreviations used: FKBP51 and FKBP52, FK506-binding protein; CyP-40, cyclophilin-40; bCyP-40, bovine CyP-40; SPR, surface plasmon resonance; RU, response units; GST, glutathione S-transferase; NTA, nitrilotriacetic acid; CM, carboxymethyl; EDC-NHS, *N*-ethyl-*N'*-(dimethyl-aminopropyl)carbodi-imide/*N*-hydroxysuccinimide.

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immobilization principles were tested [45] to address questions on the specificity of protein–protein interaction. Furthermore, for selected interacting partners kinetic constants have been derived based on sensorgram data employing BIAevaluation software. These results will aid in better understanding of the dynamics of chaperone–receptor complexes.

THEORY

Both specificity of interaction and affinity can be assessed in real time by biosensor systems based on SPR [42,44,46]. After sample injection, the analyte interacts with ligand immobilized to the sensor surface, which gives the association phase until a steady state is reached where association and dissociation rates are in equilibrium. As soon as the analyte injection is stopped and replaced by buffer flow, dissociation of the analyte begins. Association and dissociation phases can be employed to derive kinetic constants. Assuming pseudo-first-order kinetics:

the rate of complex formation during analyte injection is given by

$$\frac{dR}{dt} = k_a C R_{\max} - (k_a C + k_d R_t) \quad (2)$$

where dR/dt is the rate of change of the SPR signal, k_a is the association rate constant, k_d the dissociation rate constant, C is the analyte concentration, $R_{\max}$ the maximum analyte binding capacity in response units (RU), and R_t is the signal at time t . The integral form of eqn. (2), expanded by R_i , the background binding in RU,

$$R_t = \left(\frac{C k_a R_{\max} \{1 - e^{-(C k_a + k_d)t}\}}{C k_a + k_d} \right) + R_i \quad (3)$$

has been used to fit the response curve during the association phase. From these fits k_a ($M^{-1} \cdot s^{-1}$), k_d (s^{-1}) and K_A (M^{-1} ; the equilibrium-binding constant) can be estimated.

The rate of dissociation can be expressed as:

$$\frac{dR}{dt} = -k_d R_t \quad (4)$$

and the integrated form,

$$R_t = (R_0 \cdot e^{-k_d t}) + R_{(t \rightarrow \infty)} \quad (5)$$

can be used to fit response curves during the dissociation phase. Here, R_0 is the response at the beginning of the dissociation phase and $R_{(t \rightarrow \infty)}$ is the response at infinite time accounting for the background signal. The dissociation constant can be estimated from this fit. At equilibrium, association and dissociation rates are equal and the equilibrium-binding constant is given by:

$$K_A = \frac{k_a}{k_d} \quad (6)$$

According to O'Shannessy and Winzor [47], the value for k_d obtained from first-order approximation of a time span of the dissociation phase can be inserted into the fitting equation for the adsorption phase. The resulting values for k_a and $R_{\max}$ show higher confidence. Additionally, injection and analysis of different analyte concentrations can reveal deviations from first-order kinetics and limitations due to mass-transfer problems [48].

To avoid these problems, highly reliable binding constants were determined by combining data sets from the maximal response at equilibrium (R_{eq}) for each injected protein concentration. Assuming a Langmuirian adsorption characteristic [49], R_{eq} is related to the equilibrium concentration C^* by

$$R_{eq} = R_{\max} \frac{K_A C^*}{1 + K_A C^*} \quad (7)$$

C^* corresponds to the injected analyte concentration (C_0). Bound analyte is plotted against the ratio of bound (R_{eq}) and free analyte (C^*) yielding the so-called Scatchard plot. The slope of the curve gives K_A . The underlying hypothesis of this model is an interaction where the ligand-binding sites do not interfere with each other during association and dissociation phases. When co-operative interaction takes place, the relationship between the equilibrium concentration bound to the solid phase (R_{eq}) and the analyte concentration can be described by the Hill plot:

$$R_{eq} = R_{\max} \frac{K_A^n \cdot C^{*n}}{1 + K_A^n \cdot C^{*n}} \quad (8)$$

where n is the co-operativity coefficient. If $n > 1.0$, positive co-operativity is occurring. If $0 < n < 1$, negative co-operativity is observed. For positive co-operativity, the Scatchard plot results in a characteristic curvilinear shape; for negative co-operativity the curve increases rapidly with decreasing amounts of bound analyte (R_{eq}).

MATERIALS AND METHODS

Materials

All chemicals were purchased from Merck (Darmstadt, Germany) or Sigma–Aldrich (St. Louis, MO, U.S.A.). Anti-glutathione S-transferase (GST) polyclonal serum was provided by Boris Ferko (Institute for Applied Microbiology, Vienna, Austria). GST-fusion expression vectors for human hsp90 β and rabbit FKBP52, as well as rabbit anti-human hsp90 and mouse anti-rabbit FKBP52 (EC-1) antibodies, were kind gifts from Jack-Michel Renoir (CNRS, Chatenay-Malabry, France). David O. Toft (Department of Biochemistry and Molecular Biology, Mayo Graduate School, Rochester, MN, U.S.A.) kindly provided an expression vector for human p23 and mouse anti-chicken p23 antiserum (JJ3). Expression vectors for p48 and p60 were obtained from David F. Smith (Department of Pharmacology, University of Nebraska Medical Center, Omaha, NB, U.S.A.). Genomic DNA for human hsp70 was provided by Jack-Michel Renoir and cloned into a pSP72 vector (Boehringer Bioproducts, Ingelheim, Germany) by standard restriction cut-and-ligation procedures. Media components for *Escherichia coli* cultivation were from Merck (yeast extract, glucose monohydrate and sodium chloride) and bacto peptone was from Oxoid (Basingstoke, Hants, U.K.).

Vector construction and cultivation conditions

The hsp70 genomic DNA fragment was cut from a mammalian expression vector by *Bam*HI/*Bgl*II restriction digest and inserted into the multiple cloning site of a pSP72 vector (Boehringer Bioproducts). The resulting pSPhsp70 construct was transformed into electrocompetent *E. coli* TG-1 cells: positive clones were cultivated in 2 $\times$ YT (yeast extract/tryptone)/ampicillin expression medium at 30 °C.

All cDNAs were obtained in bacterial expression vectors and transformed directly into competent MC1061 or TG-1 *E. coli*

cells. Except for the pET expression vectors [His-p60, His-FKBP51 (an FK506-binding protein) and His-FKBP52] which had a kanamycin (*kan*) resistance gene, all vectors contained the ampicillin (*amp*) resistance gene. In general, 100 μ l from frozen stock cultures were used to inoculate two 250-ml shaker flasks, containing 50 ml of 2 $\times$ YT with ampicillin (100 μ g/l) or kanamycin (50 μ g/l) selection, and incubated overnight at 37 °C. Then three 1-litre flasks were inoculated with the overnight cultures to a final volume of 400 ml each and incubated at 30 °C. Recombinant protein expression was induced at A_{600} between 1 and 2 by addition of 0.5 mM isopropyl β -D-thiogalactoside for 3 h at 25 °C. Finally, the bacteria were harvested by centrifugation, washed, and the pellets were stored at -70 °C.

Protein extraction and purification

Frozen biomass was resuspended in NTEN extraction buffer 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Igepal [(octylphenoxy(polyethoxyethanol)) CA-630 supplemented with 2 mM dithiothreitol and a cocktail of protease inhibitors [PMSF, tosyl-lysylchloromethane ('TLCK') and tosyl-phenylalanylchloromethane ('TPCK'); 0.1 mM each]. For cell disruption, a combined lysozyme treatment and sonication procedure was chosen. To decrease viscosity, DNase I (10 units/ml) and RNase A (1 μ g/ml) were added to the lysis buffer. After a 30-min incubation on ice, the suspension was sonicated (three cycles of 30 s on ice) and centrifuged at 35000 *g* for 20 min. To increase solubility of recombinant hsp70, 1 M urea was added to the extraction buffer before sonication. The clear extracts were processed immediately or stored at -70 °C.

Gel filtration (Sephadex G-25 C) of clear bacterial extracts was the first step in all purification schemes. All chromatography runs were performed at 4 °C, except for the 'polishing' step (Superdex 200 prep grade) for GST-hsp90 purification.

Purification of GST-fusion proteins

GST-fusion proteins, such as hsp90, FKBP52 (hsp56, p59) and bovine CyP-40 (bCyp-40), were captured by glutathione-Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) chromatography. After gel filtration using buffer A (20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol) as running buffer, the gel filtrate was loaded on to glutathione-Sepharose that had been equilibrated previously with buffer A. Elution was performed with buffer B (20 mM GSH in buffer A, pH 7.5). GST-hsp90 glutathione eluate pools were concentrated by ultrafiltration and further separated on a Superdex 200 pg HiLoad column (Amersham Pharmacia Biotech) equilibrated with 20 mM Tris (pH 7.4) and 400 mM NaCl. FKBP52 and bCyp-40 could be expressed and purified in one step with high yields.

Purification of His-tagged proteins

After gel filtration with buffer C (50 mM sodium phosphate, pH 8.0, 300 mM NaCl), both His₆-tagged proteins used in this study were applied on to metal-chelating Sepharose fast flow (Amersham Pharmacia Biotech) loaded with Cu²⁺ and Ni²⁺. After extensive washing (buffer C, pH 6.0), bound protein was eluted with buffer D (buffer C + 300 mM imidazole, pH 8.0) in a step gradient mode. For 6 $\times$ His-p60, the 50 mM imidazole fraction was collected, concentrated by ultrafiltration (Amicon YM-10) and transferred into buffer E (10 mM sodium phosphate, pH 7.4) by repeated dilution and concentration. This preparation was loaded on to a 2-ml hydroxyapatite (Bio-Rad, Hercules,

CA, U.S.A.) column equilibrated with buffer E. Bound protein was eluted with a step gradient with buffer F (300 mM sodium phosphate, pH 7.4). The 50–250 mM sodium phosphate fractions contained 6 $\times$ His-p60, as confirmed by SDS/PAGE and Western blotting using anti-p60 (Stressgen, Victoria, BC, Canada) and anti-His-tag antibodies (Qiagen, Hilden, Germany). The first fraction was highly pure whereas purity decreased with increasing NaCl concentrations. 6 $\times$ His-p48 eluted between 10 and 50 mM imidazole, pH 8.0. Purity of 6 $\times$ His-p48 eluates from nickel-chelating Sepharose was regarded as sufficient for the following experiments.

Purification of hsp70

By conventional extraction of bacterial biomass, only a small fraction of hsp70 is soluble [50]. To increase solubility of human hsp70 expressed in *E. coli*, 1–2 M urea was added to the extraction buffer. The gel filtrate was applied on to a Q-Sepharose fast flow column equilibrated with buffer A. Fractions containing hsp70 (190–400 mM NaCl eluates) were pooled, concentrated and diafiltrated into buffer G (50 mM glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA) by ultrafiltration and loaded on to an ATP-agarose resin. The linear flow rate had to be decreased to below 60 cm/h to achieve quantitative binding. Bound hsp70 was eluted with 5 mM ATP in buffer G.

Purification of p23

Gel-filtrated bacterial extract containing p23 was loaded on to Q-Sepharose fast flow (Amersham Pharmacia Biotech) equilibrated with buffer A. Q-Sepharose fractions eluting between 235 and 440 mM NaCl were pooled, concentrated by ultrafiltration and separated on a Superdex 200 pg HiLoad (Amersham Pharmacia Biotech) column. Fractions containing p23 were pooled, concentrated and diafiltrated by ultrafiltration and loaded subsequently on to a FPLC Mono Q (Amersham Pharmacia Biotech) anion-exchange column equilibrated with buffer A. Fractions eluting between 120 and 200 mM NaCl contained p23.

SDS/PAGE and Western blotting

All purification steps were followed by SDS/PAGE using precast 4–12% or 4–20% polyacrylamide Tris/glycine gels (Novex, San Diego, CA, U.S.A.). The identity of purified proteins was confirmed by Western-blotting standard techniques using specific antibodies against the target protein and/or the respective fusion tag. In all experiments, electrophoretically separated proteins were transferred on to PVDF membranes (Immobilon P; Millipore, Bedford, MA, U.S.A.) by electrotransfer (Novex) or on to nitrocellulose membranes (Bio-Rad) by electrotransfer or overnight diffusion blotting.

Native PAGE

For native PAGE, protein preparations were transferred into 0.5 $\times$ TBE buffer (Tris/borate/EDTA; 1 $\times$ TBE = 45 mM Tris/borate/1 mM EDTA) by ultra-/dia-filtration. The respective amounts of protein were combined with 10 mM KCl, 10 mM NaMoO₄ and 10 mM ATP/Mg²⁺ or an ATP-regeneration system. The samples were adjusted to a total volume of 100 μ l and incubated for at least 90 min on ice. Samples were separated on precast polyacrylamide gels (4–20% gradient; Tris/glycine), prerun with 0.5 $\times$ TBE for 20 min, at 10 V/cm for 4 h. In some experiments, 10 μ l of an ATP-regeneration cocktail consisting of 50 mM ATP, 250 mM creatine phosphate, 20 mM magnesium

acetate and 100 units/ml creatine kinase was added. For immunological detection, proteins were transferred on to nitrocellulose membranes by semi-dry overnight diffusion blotting, incubated with specific antibodies, and colour was developed with alkaline phosphatase-conjugated secondary antibodies and Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate (NBT/BCIP).

Immobilization of proteins on CM-5 (carboxymethyl) and nitrilotriacetic acid (NTA) sensor chips

hsp90 and glutathione were immobilized directly on to carboxymethylated dextran layers of CM-5 sensor chips (BIAcore, Freiburg, Germany) by amine-coupling chemistry. His-tagged proteins were immobilized on to NTA resins of a ready-to-use sensor chip. This metal-chelating resin was preloaded with Ni^{2+} ions (1 mM NiNO_3 in 50 mM Tris, pH 7.5). For surface regeneration, 1 M NaCl was used for all applications, and additionally, EDTA and imidazole were used for NTA surface regeneration.

Interaction studies using BIAcore™

The standard running buffer HBS (Hepes-buffered saline; 10 mM Hepes, pH 7.3, 150 mM NaCl, 0.005 % Tween 20) was modified for the interaction studies described in this report by addition of 10 mM NaMoO_4 , 10 mM Mg_2Cl_2 and 10 mM KCl (HBS-interaction buffer, HBS-I). For the assessment of interaction kinetics, one reaction partner, e.g. hsp90, p60 or p48, was immobilized (ligand), and the second partner or a mixture of possible partners (analyte) was injected over this interaction surface. For detailed analysis of kinetic rate constants, different concentrations of analyte(s) and ligand were tested. Proteins not interacting with hsp90 served as negative controls for experiments with immobilized hsp90. The question of protein-complex formation was addressed by sequential injection of single proteins following a $6 \times$ His-tagged protein coupled to the NTA sensor chip. Again, samples containing 50 mM imidazole in the HBS-I served as negative controls.

Evaluation of sensorgram data

Sensorgrams of single experiments were normalized using BIA-evaluation 3.0 (BIAcore). Fitting was also performed with BIAevaluation 3.0. Dissociation rate constants were determined by fitting the slopes after sample injection (dissociation phase). The association rate constants and responses at equilibrium were obtained by fitting the association phases to the Langmuir equation. The equilibrium response (R_{eq}) was used to assess equilibrium-binding constants by Scatchard-plot analysis and first-order regression analysis using Sigma Plot 2.0 software (Jandel Scientific, Madera, CA, U.S.A.). Additionally, for hsp90–p60 interaction the concentration-dependent response after a defined dissociation phase served to calculate the maximum binding capacity and equilibrium-binding constant (K_A) by generating a binding isotherm through fitting to the Hill equation (eqn. 8). Bound analyte was quantified assuming 10000 RU for 1 ng of a 150-kDa protein and a 1-mm² sensor-chip surface with 50 % of the 100-nm dextran layer being freely accessible.

RESULTS

Accompanying proteins of ER α (oestrogen receptor α) and other nuclear receptors such as hsp90, hsp70, p60, FKBP52, FKBP51, p48, CyP-40 and p23 have been overexpressed and purified as described in the Materials and methods section. To identify or

Figure 1 Electrophoretic mobility-shift assay of hsp90–FKBP52 with Western blot of native PAGE

hsp90 and the hsp90–FKBP52 complex was detected by an anti-hsp90 antibody in the presence (+) or absence (–) of ATP. Lanes 1 and 4, hsp90; lanes 2 and 5, mixture of hsp90 and FKBP52; lanes 3 and 6, FKBP52.

verify interacting partners and estimate the sizes of protein complexes, single proteins and mixtures thereof were incubated in the absence or presence of ATP and separated electrophoretically in polyacrylamide gels under native conditions. First, the interaction of several purified proteins, namely FKBP52, p60, hsp70, bCyP-40 and p23 with hsp90 was tested. Only FKBP52 showed a marked shift with hsp90 that can be interpreted as complex formation (Figure 1). His-p60, bCyP-40 and p23 did not shift hsp90 to a detectable level under the conditions applied (results not shown). Consistently with previous reports, hsp70 did not interact with hsp90. Addition of 10 mM ATP/ Mg^{2+} / K^{+} did not alter the observed band pattern of FKBP52–hsp90 (Figure 1) and bCyP-40–hsp90 mixtures (results not shown). It seems noteworthy that hsp90 migrated homogeneously as a dimer under the experimental conditions applied.

Mixtures of three or more protein components proved to be difficult to interpret. Several emerging bands from silver-stained gels were not recognized by specific antibodies (results not shown). Definitive answers about interactions are therefore not possible.

Qualitative protein–protein-interaction studies using the BIAcore biosensor system was the next step. Five proteins, hsp90, p60 (Hop), FKBP52, FKBP51 and p48 (Hip), were immobilized on to the sensor surface via different immobilization chemistries.

hsp90 was directly immobilized to CM-5 sensor chips by EDC-NHS [*N*-ethyl-*N'*-(dimethylaminopropyl)-carbodi-imide/*N*-hydroxysuccinimide] chemistry. In parallel, glutathione was chemically modified and immobilized on to CM-5 in order to generate reversible hsp90 coupling via a GST tag. This modification was essential since direct coupling of GSH to CM-5 resulted in extremely low binding capacities for GST-tagged proteins (results not shown). Immobilization of the chemically modified glutathione resulted in binding of approximately 50 RU of hsp90 in a single-flow cell. Furthermore, an anti-GST monoclonal antibody was coupled on to CM-5 to assess interaction with GST-tagged proteins. All these approaches served to analyse protein–protein-interactions qualitatively (Table 1).

Immobilization of p60 (Hop), FKBP51, FKBP52 and p48 (Hip) via the His tag on Ni^{2+} -saturated NTA surfaces resulted in a highly improved surface concentration of the respective protein compared with hsp90. These sensor surfaces were used to approximate stoichiometric ratios between the different proteins.

hsp90 and hsp70 clearly showed interaction with immobilized p60 (Figure 2). bCyP-40 and rabbit FKBP52 were negative in the

Table 1 Summary of the interactions of the investigated proteins

Proteins were tested pairwise. k, Kinetic analysis; n.d., not determined; Yes, qualitative interaction confirmed and no interaction observed.

	hsp90	hsp70	FKBP51	FKBP52	p60 (Hop)	p48 (Hip)	bCyP-40
hsp90	—	—	—	—	—	—	—
hsp70	No	—	—	—	—	—	—
FKBP51	Yes/k	n.d.	—	—	—	—	—
FKBP52	Yes/k	Yes	n.d.	—	—	—	—
p60 (Hop)	Yes/k	Yes/k	n.d.	No	—	—	—
p48 (Hip)	Yes	Yes/k	n.d.	n.d.	n.d.	—	—
bCyP-40	Yes	n.d.	n.d.	n.d.	No	n.d.	—
p23	Yes	n.d.	n.d.	n.d.	No	n.d.	n.d.

Table 2 Summary of the equilibrium-binding constants (K_A), Hill coefficients (n) and stoichiometries of interaction estimated after saturation was achieved

—, Saturation could not be achieved under applied conditions and stoichiometry cannot be estimated; n.d., not determined.

Interacting proteins	Nucleotide	K_A (M^{-1})	n	Stoichiometry
p60–hsp90	– ATP	2.8×10^5	1.22	1:2
	+ ATP	1.0×10^5	0.64	1:2
p60–hsp90–p23	– ATP	5.0×10^5	1.29	1:1
	+ ATP	5.5×10^5	0.89	1:1
p60–hsp70	– ATP/ADP	4.1×10^4	0.52	—
	+ ADP	4.5×10^5	3.83	—
p48–hsp70	+ ATP	n.d.	n.d.	—
	– ATP/ADP	n.d.	n.d.	—
	+ ADP	n.d.	n.d.	—
	+ ATP	n.d.	n.d.	—
FKBP51–hsp90		2.7×10^5	2.25	—
FKBP52–hsp90		4.1×10^4	0.69	1:1

Figure 2 Sensorgrams from real-time BIA measurements to assess the qualitative interaction of various accompanying proteins of steroid receptors with p60 (Hop) **Figure 3** Sensorgrams from real-time BIA measurement to determine the specificity of the interaction between p60 and p23

Identical concentrations of p23 were injected on to sensor chips coated with different amounts of p60.

Figure 4 Interaction of hsp90 and p60 in the presence and absence of ATP

Experimental data from real-time BIA analysis were fitted to the Hill equation (A) and a Scatchard plot (B).

case of another recently discovered polypeptide, p23. Experiments with different p60 levels on the sensor-chip surface revealed that the interaction observed in previous experiments was non-specific. Increasing levels of coupled p60 decreased the p23 response, indicating decreasing non-specific interactions with

Figure 5 Influence of p23 on the interaction of hsp90 and p60 in the presence and absence of ATP

Experimental data from real-time BIA analysis were fitted to the Hill equation (A) and a Scatchard plot (B).

increasing p60 saturation levels (Figure 3). Those protein combinations showing significant interaction were subjected to kinetic analysis (Table 1).

Different concentrations of the respective proteins were injected sequentially in the absence or presence of nucleotides, and the slopes of association as well as the dissociation curve and bound protein at equilibrium or after a certain dissociation phase were employed to derive kinetic rate constants (k_a and k_d) and the equilibrium-binding constant (K_A). Co-operativity was assessed by plotting the ratio of bound and free protein versus bound protein. When the Scatchard plot showed a characteristic curvilinear shape the data sets were approximated by the Hill equation (eqn. 8) to estimate the Hill coefficient (n) and the correct equilibrium-binding constant (K_A).

p60 as ligand

Since p60 (Hop) plays a key role in chaperone-complex and therefore receptor-complex formation, this protein was the premier ligand for kinetic experiments. These rate constants were used to determine K_A . Comparison of the p60 amount coupled to

Figure 6 Interaction of hsp70 and p60 in the presence and absence of nucleotides (ADP or ATP)

Experimental data from the adsorption (A) and desorption (B) phases were fitted to the Hill equation. Regr., regression.

the sensor chip and the maximum hsp90 binding indicated a 1:2 stoichiometric ratio of interaction (Table 2). The K_A value for hsp90 interaction with p60 (Hop), estimated directly from sensorgrams, was between 8.5×10^5 and $3.0 \times 10^6 \text{ M}^{-1}$; the binding constant derived from fitting with the Hill equation was $3.0 \times 10^5 \text{ M}^{-1}$ (Figure 4). In presence of ATP the binding properties of hsp90 for p60 changed significantly. Highly negative cooperativity could be observed in the Scatchard plot and was confirmed by fitting with the Hill equation. A co-operativity coefficient of 0.65 was obtained, clearly indicating negative cooperativity or enhancement of hsp90 binding to p60 at low concentrations in the presence of ATP.

In another set of experiments, hsp90 was preincubated with p23 in the absence or presence of ATP, and different dilutions thereof were loaded on to the p60-coated sensor surface (Figure 5). Preincubation with p23 decreased the binding capacity of hsp90 for the p60 matrix at lower concentrations, indicated by the Hill coefficient and the shape of the Scatchard plot. Addition of ATP did not reverse this effect of p23 on hsp90. Fitting with the Hill equation equivocally resulted in equilibrium-binding constants of 5.0×10^5 and $5.5 \times 10^5 \text{ M}^{-1}$ in the absence and presence of ATP respectively.

Figure 7 Interaction of hsp90 and FKBP 52

Experimental data from real-time BIA analysis were fitted to the Hill equation (A) and a Scatchard plot (B).

Similarly, the interaction of hsp70 with p60 was investigated in a concentration range between approximately 0.1 and 10 μM in the absence and presence of ATP. k_a and k_d values, calculated from real-time measurements, indicated considerably strong interaction, with a K_A of between 1×10^6 and $9 \times 10^6 \text{ M}^{-1}$ in the absence of ADP or ATP. In this case, addition of ATP did not affect K_A .

In contrast with kinetic experiments, K_A obtained at steady state was lower. We observed a K_A of $4.0 \times 10^4 \text{ M}^{-1}$ without nucleotides, $4.5 \times 10^5 \text{ M}^{-1}$ with ADP and $5.0 \times 10^4 \text{ M}^{-1}$ with ATP. ADP changed the dynamics of interaction from negative co-operativity to a highly positive one (Figure 6).

Results with ADP found in the association and dissociation phases were not consistent (see the Discussion section).

FKBP52 and FKBP51 as ligands

In native PAGE/Western blot, hsp90 and FKBP52 showed strong interaction (Figure 1). For hsp90, dissociation rate constants (k_d) were uniformly between 1.8 and $3.2 \times 10^{-4} \text{ s}^{-1}$, whereas k_a values were difficult to assess. Below saturation levels, equilibrium-binding constants (K_A) were 5.1×10^6 – $1.3 \times 10^7 \text{ M}^{-1}$. Fitting the equilibrium data with the Hill equation, a binding constant of $4.0 \times 10^{-4} \text{ M}^{-1}$ with a negative co-operativity coefficient was obtained (Figure 7).

Figure 8 Interaction of hsp90 and FKBP 51

Experimental data from real-time BIA analysis were fitted to the Hill equation (A) and a Scatchard plot (B).

For FKBP51, K_A from real-time measurements varied from 1.3×10^6 to $6.5 \times 10^7 \text{ M}^{-1}$. A Hill fit resulted in a K_A of $2.7 \times 10^5 \text{ M}^{-1}$ with positive co-operativity. A Scatchard plot of data obtained during the dissociation phase confirmed the co-operative character of interaction. K_A values were identical in both evaluation methods (Figure 8).

p48 as ligand

Another member of steroid receptor complexes, p48 (Hip) was investigated for its interaction with hsp70 and hsp90. For hsp70, several concentrations between approximately 0.2 and 12 μM were loaded on to p48-coated sensor chips in the absence or presence of ADP or ATP. Equilibrium-binding constants for hsp70–p48 interaction estimated by real-time measurements varied widely (5.8×10^5 – $1.8 \times 10^7 \text{ M}^{-1}$). Binding sites for hsp70 on p48 in the presence of ATP or ADP could not be saturated within the investigated range. K_A was in the range of 10^{-3} to 10^{-4} M^{-1} (Figure 9).

Analysis of complex formation

Different component mixtures were analysed using p60- or FKBP52-coated biosensor surfaces. These immobilized proteins were used as anchors for the reconstitution of the protein complex. In the first case, hsp90, hsp70 and p23 were injected sequentially. The build-up of the complex was monitored in real

Figure 9 Interaction of hsp70 and p48 in the presence and absence of nucleotides (ADP or ATP)

Experimental data from the adsorption (A) and desorption (B) phases were fitted to the Hill equation.

time. After p60 was coated on to the surface, hsp90 injection led to an additional increase in the sensor signal, indicating substantial binding. Subsequent injection of p23 had no effect on the sensor signal. Injection of hsp70 again increased the response, suggesting the reconstitution of a core hsp90 complex (Figure 10).

DISCUSSION

In this study, qualitative and quantitative aspects of chaperone-complex formation have been addressed using electrophoretic methods and the BIACORE system. First, native PAGE/Western blotting was used to identify protein-protein interactions with purified components *in vitro*. This method identified hsp90 and the immunophilin FKBP52 as strong interacting partners, on both the silver-stain and the immunodetection levels, but failed to identify other interaction products. Possible reasons could be the unphysiologically high pH that has to be employed in native PAGE or masking of antibody binding sites through protein-protein interaction. To overcome these potential drawbacks, we developed a capturing step based on GST-tagged hsp90. Here, proteins were incubated under more physiological conditions and concentrated by adsorption to a glutathione-Sepharose matrix. These modifications allowed for the detection

Figure 10 Analysis of complex formation by sequential injection of purified hsp90, p23 and hsp70 on a p60-coated sensor chip

of a basic chaperone complex consisting of p23 and p60 (Hop) bound to hsp90. However, only very weak signals of p23 and p60 and no hsp70 could be detected (results not shown). Whether this was due to low reconstitution efficiency, instability of complexes under the conditions applied or low sensitivity of some antibodies employed remains to be elucidated.

Kosano et al. [41] reported that for receptor-complex reconstitution an hsp40 analogue was essential and that the yeast hsp40 homologue Ydj1 was also functional even at sub-stoichiometric amounts. We did not include hsp40 (homologues) in our studies although it seems likely that, as for yeast, the bacterial homologue co-purified with hsp70 since in SDS/PAGE the main contaminant co-migrated at a molecular mass corresponding to 40–50 kDa.

Real-time interaction studies using the BIACORE system based on SPR [42,44,46] formed the second main approach in this study. Advantages of SPR are the acquisition of kinetic information on the interaction of biomolecules in real time, the short time needed and a variety of mild immobilization methods for ligands. Drawbacks are mass-transfer limitations and possible non-specific adsorption that complicate proper interpretation of sensorgram data. To assess interactions of hsp90 with other purified members of chaperone complexes, we pursued three different immobilization strategies. Direct linking of different amounts of hsp90 to CM-5 sensor chips via EDC-NHS chemistry

Figure 11 Schematic representation of the interaction between chaperone-complex components and nuclear receptors

Arrows indicate interaction. The equilibrium-binding constants were calculated from fitting of the experimental data to the Hill equation. *, Qualitative binding detected; boxed + and - indicate positive and negative co-operativity.

resulted in very low binding capacities. Direct immobilization of GSH to CM-5 gave virtually no binding at all. Chemical modification of glutathione by addition of an ether-linker resulted in binding of 50 RU of GST-hsp90 and considerable specific interactions. Removal of by-products of the modification procedure will further improve the efficiency of this indirect linking method for GST-tagged proteins and allow for quantitative assessment of additional hsp90-co-chaperone interactions in the near future. However, interaction of p23, bCyP-40, FKBP52 and p60 with hsp90 immobilized by either strategy could be demonstrated. Not surprisingly, hsp70 did not show interaction.

Coupling of $6 \times \text{His}$ -tagged proteins to a nickel-chelated matrix sensor chip (NTA) resulted in improved interaction properties. This effect is obviously due to immobilization under physiological conditions and improved accessibility of the coupled protein. Both hsp70 and hsp90 showed specific interaction with immobilized p60 (Hop), which agrees with the current understanding that Hop plays a central role in nuclear-receptor chaperone complexes (Figure 11).

The question of specificity of interaction was addressed by immobilizing different amounts of p60. In the case of p23, for example, the degree of interaction decreased rapidly with the amount of p60 loaded. This negative correlation strongly indicates non-specificity of the observed p23 interaction with the p60-NTA sensor chip.

Kinetic rate constants can be determined directly from fitting the association and/or dissociation phase, respectively. However, binding constants derived from k_a and k_d from sensorgrams frequently deviate from constants determined by other methods. We analysed different analyte concentrations and compared the

binding constants derived directly from sensorgrams by BIA-evaluation software with constants from fits with the Hill equation and Scatchard-plot analysis.

ATP altered the dynamics of interaction between hsp90 and p60 to negative co-operativity or, in other words, increased the affinity of hsp90 at lower concentrations. This observation constitutes another proof for ATP-dependence of hsp90 function. Since p23 binds to hsp90 but not to p60, it was included in the kinetic experiments with hsp90. p23, in the absence or presence of ATP, changed the character of interaction to positive co-operativity. Maximum binding levels estimated from fitting to the Hill equation indicated that the stoichiometry of the p60-hsp90 complex was dramatically altered from 1:2 (p60/hsp90) to 1:1 in the presence of p23 and ATP.

Furthermore, we have demonstrated that hsp70 binds to p60 with significant affinity. Addition of ADP changed the dynamics of interaction from negative to highly positive co-operativity. From these results we conclude that ADP destabilizes the hsp70-p60 complexes at low protein concentrations. ATP in general lowered the affinity of hsp70 for p60.

Interaction of hsp90 with FKBP52 or FKBP51 showed different equilibrium-binding constants, of 2.7×10^3 and $4.1 \times 10^4 \text{ M}^{-1}$ respectively. Again, Scatchard plots and Hill fit indicated strong negative co-operativity of hsp90-FKBP52 interaction, whereas for the FKBP51 homologue a highly positive co-operativity coefficient was estimated. The physiological meaning of these findings remains to be clarified. Each single hsp90 molecule contains tetratricopeptide repeats needed for binding of FKBP52 and related proteins. Measuring the interaction of FKBP52 with hsp90 yielded a stoichiometry of 1:1. In a fully developed complex the binding of FKBP52 may be altered compared with the artificial situation in a two-component system, which is required to assess the binding constants.

The heat-shock-protein-interacting protein Hip (p48) was originally identified as a hsp70-binding protein and as a member of the progesterone-receptor complexes. Experiments in the presence or absence of ATP and ADP showed clearly that both nucleotides weakened the affinity of hsp70 for p48. This finding disagrees with the concept that ADP-hsp70 has high affinity for p48 [51]. We have to stress that saturation could not be observed within the investigated concentration range. This finding could mean there are multiple binding sites available. However, the underlying experimental set-up does not allow the conclusion as to which protein carries the multiple binding sites.

Reconstitution of basic chaperone complexes harbouring either p60 or FKBP52 as a bridging protein between hsp90 and hsp70 was the first step towards reconstitution of functional steroid hormone-receptor complexes anchored to sensor chips. Further efforts are currently being made to achieve this goal, which will provide multiple opportunities.

A comprehensive understanding of chaperone-complex formation, a highly dynamic process, requires models about the sequence of interaction and the equilibrium-binding constant of each step. Our results will help to provide a more comprehensive view of chaperone-complex formation as a universal concept and, in the long run, of nuclear-receptor complex formation itself.

We thank J.-M. Renoir, T. Ratajczak, D. F. Smith and D. O. Toft for providing cDNAs and antibodies. We offer special thanks to Rainer Hahn for help with glutathione modifications.

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Received 21 July 1999/5 October 1999; accepted 15 November 1999