Rabbit reticulocyte lysate contains a multiprotein chaperone system that assembles the glucocorticoid receptor (GR) into a complex with hsp90 and converts the hormone binding domain of the receptor to its high affinity steroid binding state. This system has been resolved into five proteins, with hsp90 and hsp70 being essential and Hop, hsp40, and p23 acting as co-chaperones that optimize assembly. Hop binds independently to hsp70 and hsp90 to form an hsp90-Hop-hsp70 complex that acts as a machinery to open up the GR steroid binding site. Because purified hsp90 and hsp70 are sufficient for some activation of GR steroid binding activity, some investigators have rejected any role for Hop in GR-hsp90 heterocomplex assembly. Here, we counter that impression by showing that all of the Hop in reticulocyte lysate is present in an hsp90-Hop-hsp70 complex with a stoichiometry of 2:1:1. The complex accounts for ∼30% of the hsp90 and ∼9% of the hsp70 in lysate, and upon Sephacryl S-300 chromatography the GR-hsp90 assembly activity resides in the peak containing Hop-bound hsp90. Consistent with the notion that the two essential chaperones cooperate with each other to open up the steroid binding site, we also show that purified hsp90 and hsp70 interact directly with each other to form weak hsp90-hsp70 complexes with a stoichiometry of 2:1.

Unliganded steroid receptors exist in cytosols in a heterocomplex with the ubiquitous protein chaperone hsp90 (for review, see Ref. 1). Hsp90 binds to the ligand binding domain (LBD) of the receptors (1), and the glucocorticoid receptor (GR) LBD must be bound to hsp90 for the receptor to have high affinity steroid binding activity (2, 3). The receptor-hsp90 heterocomplexes are assembled by a multiprotein chaperone system that was first studied in reticulocyte lysate (4, 5). Both biochemical data (6) and data from GR mutants (7, 8) support a model (9) in which the hydrophobic ligand binding cleft in the LBD is opened to access by steroid during heterocomplex assembly. The assembly system in reticulocyte lysate has been reconstituted (9), and a mixture of five purified proteins, hsp90, hsp70, Hop, hsp40, and p23, is now used to achieve optimal receptor-hsp90 heterocomplex assembly (10, 11).

The chaperones hsp90 and hsp70 are both essential for opening the steroid binding cleft in the GR LBD, and hsp40, Hop (hsp70/hsp90 organizing protein), and p23 act as co-chaperones to increase the rate or extent of GR-hsp90 heterocomplex assembly (12). Hop binds independently to hsp90 and hsp70 to form an hsp90-Hop-hsp70 complex (13), and assembly proceeds faster when Hop is present to bring the two essential chaperones together (12). These complexes also contain small amounts of the hsp70 co-chaperone hsp40 (10), and together they form the hsp90/hsp70-based chaperone “machinery.” The chaperone machinery can be prepared simply by mixing purified components, or it can be immunoadsorbed from reticulocyte lysate with a monoclonal antibody against Hop (10, 14). When mixed with immunoadsorbed GR, the immunoadsorbed chaperone machinery converts the GR to its steroid binding form in an ATP-dependent manner (10, 14). Once the machinery has assembled the GR-hsp90 heterocomplex, p23 binds dynamically (15) to the ATP-dependent conformation of hsp90 (16) and stabilizes its association with the receptor.

The Hop (p60) of rabbit reticulocyte lysate (17) is the homolog of a human protein cloned by Honore et al. (18) and the non-essential yeast heat shock protein Sti1 (19). Unlike hsp70 and hsp90, Hop alone does not possess any chaperone activity in protein refolding assays (20, 21). Hop contains multiple tetratricopeptide repeats (TPR), with separate TPR domains determining its binding to hsp70 and hsp90. The N-terminal TPR1 domain binds to the C terminus of hsp70, and the central TPR2 domain binds to a TPR acceptor site in the C terminus of hsp90 (13, 22–26). In addition to bringing hsp70 and hsp90 together into the machinery for opening the steroid binding cleft in the GR LBD (14), studies of purified Hop-hsp90 interaction show that Hop inhibits hsp90 ATPase activity (27, 28). Inasmuch as hsp90 ATPase activity is required to generate steroid binding activity (29) and the presence of Hop in the purified five-protein system accelerates the rate at which GR steroid binding activity is generated (12), inhibition of hsp90 ATPase activity may not be a critical component of Hop function in the activation of steroid binding sites by the hsp90-Hop-hsp70 machinery.

In 1992, we purified (by ammonium sulfate precipitation and molecular sieve chromatography) a high molecular mass complex from rabbit reticulocyte lysate that contained hsp90 and
hsp70 and had a low ability to assemble GR-hsp90 heterocomplexes with steroid binding activity (30). A factor that was separated from the complex during the ammonium sulfate step was required for efficient heterocomplex reconstitution (30), and this factor was identified as p23 (31). Subsequently, heterocomplexes containing hsp90, Hop, and hsp70 were immunoadsorbed from reticulocyte lysate with monoclonal antibodies against hsp90 (32) or Hop (14) and shown to assemble GR-hsp90 heterocomplexes with steroid binding activity. This multiprotein chaperone complex, originally called a “foldosome” (32), is now known as the hsp90/hsp70-based chaperone machinery. Yeast (Saccharomyces cerevisiae) contains similar hsp90/hsp82/Hop/Sti1/hsp70/Ssa complexes (33), and mutation of sti1 results in decreased GR activation of a reporter gene in vivo (34).

These observations suggest that the hsp90-Hop-hsp70 chaperone machinery plays a role in GR-hsp90 heterocomplex assembly under cell-free conditions by reticulocyte lysate and by yeast in vivo, but Hop is not essential in either case. Immune depletion of Hop from reticulocyte lysate, for example, reduces its ability to generate GR steroid binding activity by 50% (12), and Sti1 mutant yeast still have one-third the GR activity of wild-type yeast at high hormone concentration (34). Recently, studies utilizing purified proteins for GR-hsp90 assembly have caused some investigators to totally repudiate the notion of the hsp90/hsp70-based chaperone machinery (35). In a purified protein assembly system, GR steroid binding activity can be generated with a combination of the core chaperones hsp90 and hsp70 without Hop or hsp40 (12, 35). Also, GR-hsp90 heterocomplexes can be assembled by purified proteins in a two-step procedure that does not involve prior formation of the hsp90-Hop-hsp70 assembly machinery (36, 37). However, we have shown that the rate of GR-hsp90 heterocomplex assembly is accelerated markedly when Hop is added to a mixture of purified hsp90, hsp70, YDJ-1, and p23 (12). Because Hop binds to hsp70 and hsp90, it co-purifies with both, and fastidious purification procedures are required to assure Hop-free preprations of these chaperones (12). It is likely that Hop contaminations of the purified chaperones accounts for the difference between those who have concluded that Hop has no effect (35) and both ourselves (9, 12, 14) and the Toft laboratory (11) who have shown that the presence of Hop in a purified five-protein system optimizes receptor-hsp90 heterocomplex assembly.

Although assembly with the purified chaperones is useful for studying the mechanism by which GR-hsp90 heterocomplexes are formed and how steroid binding sites are created, the purified system does not replicate the conditions of reticulocyte lysate. The concentrations of hsp90 and hsp70 in the purified system, for example, are several times those of reticulocyte lysate. Here, we revisit the reticulocyte lysate system to determine the relative abundance of hsp90, hsp70, and Hop and the amount of each protein that is present in the chaperone machinery. We find that all of the Hop, ~30% of the hsp90, and ~9% of the hsp70 in rabbit reticulocyte lysate exist in hsp90-Hop-hsp70 heterocomplexes. When the reticulocyte lysate is separated into two pooled fractions by molecular sieving chromatography, 92% of the ability to activate GR steroid binding activity resides in the large M<sub>c</sub> peak of hsp90, which contains 92% of the Hop. A second pool of fractions containing the unbound hsp90 (38%) and hsp70 (53%) and 8% of the Hop has a low GR activating activity. This suggests that in reticulocyte lysate the Hop-containing chaperone machinery plays the major role in GR-hsp90 heterocomplex assembly, with free hsp90 and hsp70 playing a considerably lesser role. We have determined by both native gel electrophoresis and cross-linking that the chaperone machinery in reticulocyte lysate possesses an hsp90:Hop:hsp70 stoichiometry of 2:1:1. We also show by cross-linking that purified hsp90 and hsp70 bind weakly to each other in the absence of Hop, producing an hsp90-hsp70 complex with a stoichiometry of 1:1. This is consistent with the notion (37) that hsp90 and hsp70 interact directly with each other while the steroid binding cleft is being opened by either the chaperone machinery or by the purified chaperones without Hop.

**EXPERIMENTAL PROCEDURES**

**Materials**

Untreated rabbit reticulocyte lysate was purchased from Green Hector (Oregon, WI). [6,7-<sup>3</sup>H]Triamcinolone acetonide (38 Ci/mmol) and 125I-conjugated goat anti-mouse IgG were obtained from PerkinElmer Life Sciences. Sephacryl S-300 was from Amersham Pharmacia Biotech. Protein A-Sepharose, goat anti-mouse horseradish peroxidase conjugate, and molecular weight markers used for non-denaturing gels were from Sigma Chemical Co. The BuGR2 monoclonal IgG antibody against the GR and the 3G3 monoclonal IgM against hsp90 were from Affinity Bioreagents (Golden, CO). The AC88 monoclonal IgG against hsp90 and the N27F3-4 anti-72/73-kDa hsp monoclonal IgG (anti-hsp70) were from StressGen (Victoria, BC, Canada). Escherichia coli expressing YDJ-1 was a gift from Dr. Avrom Caplan (Mount Sinai School of Medicine). The DS14F5 monoclonal IgG against Hop and E. coli expressing Hop were kindly provided by Dr. David Smith (Mayo Clinic, Scottsdale, AZ). Hybridoma cells producing the FiGR monoclonal IgG against the GR were generously provided by Dr. Jack Bodwell (Dartmouth Medical School).
**Methods**

**Immunoadsorption of GR**—Mouse GR was expressed in S9 cells, and cytosol was prepared as previously described (36). Receptors were immunoadsorbed from 50-μl aliquots of S9 cytosol by rotation for 2 h at 4 °C with 14 μl of protein A-Sepharose precoupled to 7 μl of FcGR ascites suspended in 200 μl of TEG (10 mM TES, pH 7.6, 50 mM NaCl, 4 mM EDTA, 10% glycerol). Prior to incubation with reticulocyte lysate or lysate subfractions, immunoadsorbed receptors were stripped of associated hsp90 by incubating the immunopellet for an additional 2 h at 4 °C with 350 μl of 0.5 M NaCl in TEG. The pellets were then washed once with 1 ml of TEG followed by a second wash with 1 ml of Hepes buffer (10 mM Hepes, pH 7.4).

**Glucocorticoid Receptor Heterocomplex Reconstitution**—For assembly of GR-hsp90 heterocomplexes, FcGR immunopellets containing GR stripped of chaperones were incubated with 40 μl of reticulocyte lysate or with 40 μl of a lysate subtraction from Sephacryl S-300 chromatography plus 6 μg of purified p23 and 0.4 μg of purified YDJ-1. Incubation volumes were adjusted to 50 μl with HKD buffer (10 mM Hepes, 100 mM KCl, 5 mM dithiothreitol, pH 7.35) containing 20 mM sodium molybdate and 5 μl of an ATP-regenerating system (50 mM ATP, 250 mM creatine phosphate, 20 mM magnesium acetate, and 100 units/ml creatine phosphokinase). The assay mixtures were incubated for 20 min at 30 °C with suspension of the pellets by shaking the tubes every 2 min. At the end of the incubation, the pellets were washed twice with 1 ml of ice-cold TEGM buffer (TEG with 20 mM sodium molybdate) and assayed for steroid binding capacity.

**Assay of Steroid Binding Capacity**—Immune pellets to be assayed for steroid binding were incubated overnight at 4 °C in 50 μl of HEM buffer (10 mM Hepes, pH 7.35, 1 mM EDTA, 20 mM molybdate) plus 50 mM [3H]triamcinolone acetonide. Samples were then washed three times with 1 ml of TEGM and counted by liquid scintillation spectrometry. The steroid binding is expressed as counts per minute of [3H]triamcinolone acetonide bound/FcGR immunopellet prepared from 50 μl of S9 cytosol.

**Gel Electrophoresis and Western Blotting**—Immune pellets were resolved on 12%-SDS-polyacrylamide gels and transferred to Immobilon-P membranes. The membranes were probed with 0.25 μg/ml BuGR for GR or 1 μg/ml AC88 for hsp90. The immunoblots were then incubated a second time with the appropriate 125I-conjugated horse-radish peroxidase-conjugated counterantibody to visualize the immunoreactive bands. For electrophoresis under non-denaturing conditions, 10 μl of reticulocyte lysate was mixed with 50 μl of detergent-free buffer (312 mM Tris-HCl, pH 6.8, 50% glycerol, 0.05% bromphenol blue), and proteins were resolved on a 7.5% polyacrylamide gel, followed by Western blotting. The immunoblots were probed with AC88 for hsp90, 1 μg/ml N27F3-4 for hsp70, or 0.1% DS14F5 mouse ascites for Hop. Molecular weight markers for non-denaturing gels were bovine serum albumin, monomer (66,000) and dimer (132,000), and Jack bean urease, trimer (272,000) and hexamer (545,000).

**Cross-linking of Purified Proteins**—For cross-linking of purified hsp90 or hsp70, 15 μg of purified protein was incubated for 1 h at room temperature with 0.8 mM glutaraldehyde in a final volume of 50 μl adjusted with HKD buffer. The cross-linking was terminated by adding 12 μl of 0.5 M Tris, pH 8.0, and continuing incubation for 30 min at room temperature. Proteins were resolved by SDS-polyacrylamide gel electrophoresis on 5% gels followed by Western blotting.

**Relative Abundance of Proteins in Heterocomplexes and Reticulocyte Lysate**—For determining stoichiometry of hsp90/Hop-hsp70 heterocomplexes, 50-μl aliquots of reticulocyte lysate were immunoadsorbed to 18 μl of protein A-Sepharose precoupled with 0.5 μl (~7 μg) of DS14F5 antibody against Hop or non-immune mouse IgG. The samples were rotated at 4 °C for 2 h, and immunopellets were washed three times with 1 ml of TEG buffer. Relative amounts of hsp90 and hsp70 in immunoadsorbed Hop complexes were estimated by resolving the immune pellet proteins on 12% SDS-polyacrylamide gels and staining with Coomassie Blue. Ratios of hsp90 to hsp70 were determined by scanning multiple stained bands. For cross-linking, Hop immune pellets were suspended in 50 μl of HKD buffer and incubated with 0.4 mM glutaraldehyde as described above.

To determine the concentration of Hop, hsp90 and hsp70 in reticulocyte lysate, aliquots of lysate were electrophoresed on SDS-polyacrylamide gels that also contained various amounts of purified hsp90, hsp70, and Hop to provide a standard curve for each protein. ImmunobLOTS were prepared and probed with monoclonal IgGs against each protein, followed by incubation with 125I-labeled anti-IgG counterantibody. Samples and purified standards were then excised and counted to permit calculation of the concentration of each protein.

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**RESULTS AND DISCUSSION**

**Quaternary Structures of hsp90, hsp70, and Hop**—hsp90 forms homodimers, and dimerization is required for both receptor-hsp90 heterocomplex assembly in reticulocyte lysate (41) and hsp90 function in vivo (42). Bacterial DnaK (43) and its mammalian mitochondrial (44) and cytoplasmic (45) hsp70/hsc70 homologs self-associate in solution into dimers, trimers, and probably higher oligomers in a concentration-dependent equilibrium. We have examined, by both non-denaturing gel electrophoresis and cross-linking, the quaternary structure of the purified rabbit hsp90 and hsp70 used in the five-protein GR-hsp90 heterocomplex assembly system. The purified hsp90 is a mixture of monomer and dimer (Fig. 1A), and dimer can be recovered on denaturing gel electrophoresis after glutaraldehyde cross-linking (Fig. 1B). Few monomers of hsp90 are detected on non-denaturing gel electrophoresis of whole reticulocyte lysate (Fig. 1C). Thus, it appears that the hsp90 monomers are formed during purification, and the monomers account for 30–50% of each purified preparation. Our purified hsp70 is predominantly dimeric on native gel electrophoresis (Fig. 1A),
with some trimers being detectable, and the dimer can be seen on denaturing gel electrophoresis after glutaraldehyde cross-linking of the purified protein (Fig. 1B). Our purified, bacterially expressed Hop behaves as very large and diffuse aggregates on native gel electrophoresis, and we were not able to detect monomers, dimers, or discrete multimers (data not shown).

In Fig. 1C, aliquots of rabbit reticulocyte were submitted to gel electrophoresis under non-denaturing conditions and immunoblotted for hsp90, hsp70, and Hop, respectively. The hsp90 in reticulocyte lysate was detected in two major bands, with one being the homodimer (180-kDa band seen above the 132-kDa marker) and a second migrating at ~310 kDa. A portion of the hsp70 and all of the Hop in reticulocyte lysate also behave as a similar 310-kDa complex. Most of the hsp70 in reticulocyte lysate distributed on native gels as diffuse species of M_r ~ 310, and no monomers or dimers of hsp70 were detectable by immunoblotting with the N27F3-4 antibody (Fig. 1C).

The 310-kDa Complex Contains hsp90, hsp70, and Hop—To ask if hsp90, hsp70, and Hop were present in the same complex, aliquots of reticulocyte lysate were immunoadsorbed with monoclonal antibody specific for Hop (Fig. 2), and the immunoadsorbed supernatants were analyzed by native gel electrophoresis and immunoblotting. As shown in Fig. 2, immunodepletion of Hop yielded significant immunodepletion of the 310-kDa band of hsp90 and hsp70 without affecting the 180-kDa band of hsp90. Co-immunodepletion of all three proteins suggests that hsp90, hsp70, and Hop are present in the same ~310-kDa complex.

The migration of the complex at ~310 kDa under non-denaturing gel electrophoresis would be consistent with an hsp90:Hop:hsp70 stoichiometry of 2:1:1. In Fig. 3, aliquots of reticulocyte lysate were immunoadsorbed from aliquots of reticulocyte lysate (RL) as in Fig. 3A, and after washing, the immune pellets were incubated with glutaraldehyde (X-linker). The proteins in the immune pellets were electrophoresed under denaturing conditions and immunoblotted in A for hsp90 and in B for Hop or hsp70 or only with anti-IgG counterantibody (No 1° Ab). Samples in B were treated with 0.4 mM glutaraldehyde. Downward-slanting arrows point to uncross-linked hsp90 in A and Hop and hsp70 in B. NI, samples immunoadsorbed with non-immune IgG; I, samples immunoadsorbed with F5 antibody against Hop.

FIG. 3. Relative abundance of hsp90, hsp70, and Hop in Hop immune pellets and in reticulocyte lysate. A, Hop was immunoadsorbed from 50 µl of reticulocyte lysate, the immune pellet was washed three times with TEG, and the proteins in the pellet were electrophoresed under denaturing conditions and stained with Coomassie Blue. HC, immunoglobulin heavy chain. B, multiple samples prepared as in A were scanned in a densitometer to determine the relative amounts of hsp90, hsp70, and Hop. The bars present the average ratios ± S.E. from 16 samples. C, concentrations of hsp90, hsp70, and Hop were assayed in reticulocyte lysate as described under "Experimental Procedures." The data are the means ± S.E. from three experiments.

FIG. 4. Cross-linking of an hsp90-Hop-hsp70 complex. Hop was immunoadsorbed from aliquots of reticulocyte lysate (RL) as in Fig. 3A, and after washing, the immune pellets were incubated with glutaraldehyde (X-linker). The proteins in the immune pellets were electrophoresed under denaturing conditions and immunoblotted in A for hsp90 and in B for Hop or hsp70 or only with anti-IgG counterantibody (No 1° Ab). Samples in B were treated with 0.4 mM glutaraldehyde. Downward-slanting arrows point to uncross-linked hsp90 in A and Hop and hsp70 in B. NI, samples immunoadsorbed with non-immune IgG; I, samples immunoadsorbed with F5 antibody against Hop.
Thus, it appears that a significant amount of hsp70 and hsp90 are lost during washing of the Hop immune pellet. To determine the stoichiometry of hsp90:Hop:hsp70 in the complex, Hop immune pellets prepared as in Fig. 3A were cross-linked with glutaraldehyde and resolved by denaturing gel electrophoresis and immunoblotting. In Fig. 4A, the blots were probed with the AC88 monoclonal antibody specific for hsp90, and the bands were developed with radiolabeled counterantibody. As shown in lane 8 (Fig. 4A), an immune pellet that was not exposed to reticulocyte lysate but was exposed to cross-linker yielded three major bands representing cross-linked immunoglobulin chains detected with the secondary anti-IgG antibody. In lanes 4–6 of Fig. 4A, Hop immune pellets were prepared from reticulocyte lysate and treated with glutaraldehyde. These cross-linked samples reveal an hsp90-con-
The fractions eluted from the Sephacryl shown in Fig. 6, fraction A promoted assembly of GR immunoadsorbed GR that had been stripped of hsp90. As a regenerating system, and the mixtures were incubated with the Hop. Fraction B contained 38% of the hsp90, 53% of the hsp70, and 92% of the total hsp70 in reticulocyte lysate is in the hsp90/hsp70-based Chaperone Machinery. hsp90 heterocomplex assembly, both hsp90 and hsp70 are essential, but their function together is optimized by their presence in the multiprotein machinery with Hop (12). Inasmuch as Hop is widely distributed in the animal kingdom from yeast to humans (17) and Hop homologs are present in plants (47), it is reasonable to consider hsp90 as functioning together with hsp70 in the context of the multiprotein machinery where the two essential chaperones are brought into physical association by Hop (14).

Hsp90 and hsp70 Bind to Each Other Directly—Using biochemical separation procedures, we have previously shown that pure mammalian (mouse) hsp90 does not bind to purified hsp70 unless a factor from reticulocyte lysate is present to facilitate heterocomplex formation (48). Smith et al. (17) showed that hsp90 and hsp70 co-purified from reticulocyte lysate with a 60-kDa protein, which is now known to be Hop (13). In contrast to the mammalian chaperones, the purified hsp90 homolog of Neurospora crassa forms a rather tight complex with purified hsp70 that can be detected by normal biochemical separation procedures (49), and this hsp90-hsp70 complex interacts with polypeptide substrate (50). In Fig. 7, we asked if purified rabbit hsp70 and hsp70 interacted with each other. In this experiment, the proteins were mixed together in the presence or absence of glutaraldehyde, and the cross-linked products were resolved by electrophoresis under denaturing conditions. In the presence of cross-linker, a complex was recovered from the protein mixture that migrated slower than the hsp90 homodimer at a molecular mass consistent with an hsp90-hsp70 complex with a stoichiometry of 2:1.

The data of Fig. 7 suggest that mammalian hsp90 and hsp70 do have a weak ability to interact with each other. Because hsp90 and hsp70 are the only two components of the five-protein assembly system required to open the steroid binding cleft in the GR LBD, it seems likely that they may interact with each other as they modify receptor conformation. We have shown that GR-hsp90 heterocomplexes can be assembled in a two-step procedure (36). In the first step, immunoadsorbed GR is incubated with hsp70, hsp40 (YDJ-1), and an ATP-regenerating system to form a “primed” GR-hsp70 complex that can be washed free of unbound hsp70 and incubated in a second step with purified hsp90, Hop, p23, and ATP. The sequence of the two steps cannot be reversed, both steps are ATP-dependent, and steroid binding activity is generated during the second step (36). Purified hsp90 does not bind to the GR alone, but it does bind to the primed GR-hsp70 complex (36), at which time the two chaperones may directly interact with each other in opening the steroid binding cleft (37).

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Stoichiometry, Abundance, and Functional Significance of the hsp90/hsp70-based Multiprotein Chaperone Machinery in Reticulocyte Lysate
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