Heat Shock Protein 70 Is Associated in Substoichiometric Amounts with the Rat Hepatic Glucocorticoid Receptor†,‡

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ABSTRACT: The 70-kDa heat shock protein (hsp70) has been shown to be an important participant in several intracellular events, including protein folding and trafficking. Hsp70 binds to many, if not all, proteins during their translation and maintains its association with some protein complexes as a subunit. We have examined the possibility that hsp70 may be associated with one or more forms of the rat hepatic glucocorticoid receptor (GR). Unliganded GR was immunoprecipitated from cytosol with the anti-GR monoclonal antibody BUGR2 and then subjected to western blotting. Both hsp70 and the 90-kDa heat shock protein (hsp90) were found to be specifically associated with the GR. Hsp70 was also bound to the liganded unactivated and activated (transformed) forms of the GR complex, while as expected, hsp90 was absent from the activated GR. Immunoprecipitation of cytosolic hsp70 with the anti-hsp70 monoclonal antibody N27 resulted in coprecipitation of GR. The components of the immunopurified GR were also analyzed by laser densitometry after polyacrylamide gel electrophoresis and Coomassie blue staining. These experiments revealed that hsp70 is bound to the GR in an approximate 1:5 ratio. Unactivated GR complexes isolated via a ligand affinity purification scheme contained hsp90 and trace amounts of hsp70. Collectively, these experiments demonstrate that hsp70 is specifically associated with several forms of the native rat hepatic GR, although its binding is substoichiometric. This is in direct contrast to hsp90, which binds as a dimeric subunit to the heteromeric unactivated GR complex.

Once they have bound their respective hormones, all steroid receptors undergo a conformational change termed "activation" or "transformation". This activation step occurs both *in vitro* and *in vivo* [reviewed in Schmidt and Litwack (1982)] and is essential for subsequent receptor binding to specific DNA sequences called hormone response elements (HREs). Much of our understanding of the mechanisms underlying glucocorticoid receptor (GR)[1] activation stems from elucidation of the subunit structure of the unactivated receptor complex. Data from several laboratories indicate that this complex is a multimer of at least 300 kDa (Pratt et al., 1989; Denis & Gustafsson, 1989). A phospholipid referred to as modulator (Bodine & Litwack, 1990), a metal ion (Leach et al., 1982; Mesinchi et al., 1988), a non-steroid-binding 59-kDa protein (Tai et al., 1986; Rexin et al., 1991; Sanchez, 1990) recently shown to be an immunophilin (Yem et al., 1992), and a dimer of the 90-kDa heat shock protein (hsp90) (Mendel & Orti, 1988; Bresnick et al., 1990; Rexin et al., 1991) have all been reported to be associated with the unactivated GR complex. The association with hsp90 has been demonstrated with unliganded and liganded unactivated receptor complexes immunopurified from various tissue sources (Dahlman et al., 1989; Howard et al., 1990), and an identical GR–hsp90 complex has been shown to exist *in vivo* (Howard & Distelhorst, 1988). Although the functional role(s) for receptor-associated hsp90 is not clear, this heat shock protein may inhibit premature activation by masking the receptor DNA binding site (Howard et al., 1990). Other reports suggest that hsp90 may (i) anchor the receptor to actin filaments in the cytoplasm prior to activation (Miyata & Yahara, 1991), (ii) maintain the receptor in a functional, nonaggregated conformation (Pelham, 1986; Cadepond et al., 1991) or in a conformation optimal for ligand binding (Nemoto et al., 1990), or (iii) directly enhance the subsequent ability of the activated GR complex to regulate transcription of specific genes (Picard et al., 1990). Regardless of hsp90 function(s), receptor activation is accompanied by dissociation of hsp90 both *in vitro* (Mendel et al., 1986; Dahlman et al., 1989) and *in vivo* (Howard & Distelhorst, 1988).

Our laboratory has addressed the possibility that another member of the heat shock protein family could also be a subunit of one or more forms of the rat hepatic GR. More specifically, we have focused our attention on the 70-kDa heat shock protein (hsp70). Like hsp90, hsp70 is an abundant cytosolic protein even in unstressed cells (Pelham, 1986) and has been implicated in several important intracellular processes. For instance, hsp70 has been well-characterized in terms of its ability to maintain labile proteins in a functional, nonaggregated state (Beckman et al., 1990; Pelham, 1986), and in this regard it could serve a vital role as a receptor complex subunit. Since hsp70 also facilitates the transport of specific proteins across microsomal and mitochondrial membranes (Deshaies et al., 1988; Chirco et al., 1988), a GR–hsp70 interaction could also facilitate translocation of the receptor complex across the nuclear membrane. In fact, there is some correlative data to support the possibility that hsp70 might be involved in receptor transport. The rat GR contains two nuclear localization signals, one of which is composed of amino acid residues 510–517 (Picard & Yamamoto, 1987). The presence of a similar

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‡ This paper is dedicated to the memory of Dr. Byron A. Schottelius.

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[1] Abbreviations: GR, glucocorticoid receptor; hsp90, 90-kDa heat shock protein; hsp70, 70-kDa heat shock protein; triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; DEAE, diethylaminoethyl; IgG, immunoglobulin G; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; MES, 2-(*N*-morpholino)ethanesulfonic acid; PVDF, poly(vinylidene difluoride).

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sequence in several yeast proteins has been shown to promote their association with an unidentified 70-kDa protein which is required for their nuclear translocation (Silver et al., 1989). Recently, a 70-kDa protein with an identical function has been identified as hsp70 in HeLa cells (Shi & Thomas, 1992).

There are several other published studies which led us to suspect that hsp70 may in fact be a subunit of one or more forms of the rat hepatic GR. Gustafsson and colleagues demonstrated several years ago that an unidentified 72-kDa protein with unknown function binds to the purified activated form of the rat hepatic GR (Okret et al., 1984). Additionally, hsp70 has been shown to form a complex with hsp90 (Smith et al., 1992) and is associated in an ATP-dependent fashion with the unactivated and activated forms of the chicken oviduct progesterone receptor (Edwards et al., 1987; Kost et al., 1989). During the completion of our studies, Pratt and co-workers reported that the unactivated mouse GR overexpressed in Chinese hamster ovary cells is associated with hsp70, but this interaction is not evident in L cells (Sanchez et al., 1990) unless a reticulocyte lysate/ATP-regenerating system is added to immunoprecipitated GR complexes (Hutchinson et al., 1992). Gehring and colleagues showed that hsp70 binds directly to an immunomatrix during precipitation of the GR from WEHI-7 cytosol and that this was independent of receptor binding (Rexin et al., 1991). Experiments presented in this report show for the first time that hsp70 is specifically associated, albeit substoichiometrically, with the native rat hepatic GR.

MATERIALS AND METHODS

Reagents. [^{3}H]Triamcinolone acetonide (TA), specific activity 28 Ci/mmol, was purchased from Amersham. Bio-Gel A-1.5m agarose and hydroxylapatite (HTP, DNA grade) were obtained from Bio-Rad; DNA–cellulose (1.2 mg of native calf thymus DNA/mL of cellulose) was purchased from P-L Biochemicals; DEAE-cellulose (preswollen DE52) was from Whatman; 2-(*N*-morpholino)ethanesulfonic acid (MES), sodium molybdate, glycerol, Sephadex G-75, bovine serum albumin (BSA), protein A–Sepharose, and the ATP-hydrolyzing enzyme apyrase were from Sigma. Prepacked Sephadex G25 columns were purchased from Pharmacia, and deoxycorticosterone–agarose ligand affinity resin (Sterogel) was from Sterogene Biochemicals. All other chemicals were reagent-grade. Buffers utilized included buffer A (50 mM potassium phosphate, 10 mM sodium molybdate, 2 mM DTT, and 10 mM thioglycerol, pH 7.0), buffer B (10 mM potassium phosphate, 10 mM sodium molybdate, 2 mM DTT, and 10 mM thioglycerol, pH 7.0), and buffer C (10 mM MES and 0.5 mM EDTA, pH 7.0).

Antibodies. Monoclonal antibody BUGR2 (Gametchu & Harrison, 1984; Harrison & Gametchu, 1984), which recognizes an epitope in the DNA binding domain of the rat GR, was utilized for immunopurifications as well as immunoblotting. Monoclonal antibody N27 (Welch & Suhan, 1986), which recognizes an epitope in the N-terminus of hsp70 (personal communication, R. Morimoto), was utilized for immunopurifications and immunoblots. A monospecific polyclonal antibody which is directed against a sequence of 11 amino acids at the C-terminus of mouse hsp70 (Ehrhart et al., 1988) was used for immunoblotting. Since the five amino acids at the C-terminus of hsp70 and hsp90 are identical, this antibody (termed "anti-hsp70 polyclonal antibody" in this report) also cross-reacts with hsp90. Mouse IgG purified from rat serum (Sigma) was used as a control for nonspecific immunoprecipitation in several experiments. Horseradish peroxidase-conjugated second antibodies directed against rabbit and mouse IgG were also obtained from Sigma.

Preparation of Rat Hepatic GR. Rat liver cytosol, prepared in buffer A and stored under liquid N_2 as described previously (Schmidt et al., 1985), was thawed and incubated with 60 nM [^{3}H]TA at 0 °C for at least 2 h. The labeled cytosol was then subjected to gel filtration on a Sephadex G25 column preequilibrated in MES buffer in order to remove unbound [^{3}H]TA as well as the activation inhibitor, sodium molybdate. If the unactivated GR complex was to be analyzed, 10 mM molybdate was immediately readded to the eluate. In experiments that required analysis of both the unactivated and activated forms of the GR, molybdate was readded only to the sample which was to be kept unactivated. Parallel incubations of cytosol were then performed at 0 and 25 °C in order to obtain unactivated and thermally activated GR complexes, respectively. Hydroxylapatite and DNA–cellulose binding and/or DEAE-cellulose chromatography (Schmidt et al., 1985) were then performed to ensure that GR samples were either completely unactivated or activated.

Unliganded GR were prepared using the protocol described for unactivated receptors, with the exception that they were not preincubated with [^{3}H]TA. Thermal inactivation of unliganded GR was achieved by first subjecting cytosol to gel filtration on a G25 column and then heating the eluate at 25 °C for 1 h. The extent of GR inactivation was determined by comparing the subsequent specific binding of [^{3}H]TA detected in heated samples to that found in parallel samples incubated at 0 °C. Inactivated receptors routinely lost at least 70% of their original ligand binding capacity.

Immunopurification of Rat Hepatic GR. Aliquots of hepatic cytosol were incubated with a final concentration of 6.0% (v/v) BUGR2 (present in Dulbecco's Modified Eagle's medium) at 0 °C for 2.5–4 h. Parallel control incubations of cytosol containing no antibody or total mouse IgG were included for subsequent determination of nonspecific binding. The cytosol was then incubated at 4 °C for 1–1.5 h with 50 μL of protein A–Sepharose with constant mixing. The protein A–Sepharose pellet was then washed 5 times in MES buffer, pH 7.0, which contained 1.0% (w/v) sodium deoxycholate and 0.1% (v/v) Triton X-100. Sample buffer along with 5% (v/v) β-mercaptoethanol was then added to each pellet and boiled for 5 min.

Immunoblotting. The immunoblotting procedure utilized was basically that described by Burnette (1981). Polyacrylamide gels were placed against Immobilon PVDF sheets (Millipore) in a TE Series Transfor unit (Hoefer Scientific Instruments), and transfer buffer [25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol, and 0.01% (w/v) SDS, pH 8.3] was added. The Transfor unit was operated at 100 V for 2.5 h in a refrigerated cold box. After transfer, the molecular weight standards lane(s) was cut from each sheet, stained with a 0.1% Amido Black solution in 40% (v/v) methanol and 10% acetic acid, and destained with methanol. The remainder of each sheet was subsequently incubated twice for 15 min in western buffer [20 mM Tris-HCl, 150 mM NaCl, 5% (v/v) Tween 20 and 0.1% (w/v) BSA]. The sheets were then incubated in western buffer containing the appropriate dilution of antibody (1:85 for BUGR2, 1:2500 for the anti-hsp70 polyclonal antibody, or 1:1000 for N27) at 4 °C for 12–16 h or at room temperature for 2–4 h. Blots were subsequently washed twice in western buffer for 10 min at room temperature and then incubated with the appropriate horseradish peroxidase-conjugated second antibody (1:500 dilution of anti-mouse antibody for BUGR2- or N27-treated blots or 1:2000 dilution

of anti-rabbit antibody for the anti-hsp70 polyclonal) in western buffer at 4 °C for 12–16 h or at room temperature for 2–4 h. Blots were washed in western buffer, and horseradish peroxidase activity was subsequently visualized with a mixture of 30 mg of 4-chloro-1-naphthol, 30 μL of H_2O_2, and 10 mL of methanol in 50 mL of Tris-buffered saline (20 mM Tris-HCl and 200 mM NaCl, pH 7.5).

Gel Scanning. Gels stained with Coomassie blue alone or sequentially with Coomassie blue and silver nitrate were scanned with an Ultrascan XL laser densitometer (LKB Instruments). Comparison of peaks obtained was made by using the internal integration system of the densitometer.

Affinity Ligand Purification of the Unactivated GR. The ligand affinity scheme utilized for purification of the unactivated rat liver GR was a modification of the protocol developed by Grandics and colleagues (Grandics, 1981; Grandics et al., 1984). Fifty milliliters of cytosol prepared in buffer A containing 10 μg/mL each of the protease inhibitors leupeptin, pepstatin A, antipain, and soybean trypsin inhibitor was mixed by constant rotation with 8–10 mL of packed Sterogel at 0 °C for 2.5–3 h. The resin was then pelleted by centrifugation, washed at least 8 times with 15-mL portions of cold buffer A, and then subjected to four extensive washes (3–4 h each) in buffer A containing the previously described protease inhibitors. These extended washes were necessary to eliminate nonspecific copurification of hsp90 and hsp70. Receptors were then eluted from the resin by incubation with 10 mL of buffer B which contained 2 μM [^{3}H]TA as well as the protease inhibitors. The subsequent purification steps, including gel filtration on Bio-Gel A-1.5m and anion-exchange chromatography on DEAE-cellulose, were then performed as originally described (Grandics et al., 1984).

To evaluate the possibility of the copurification of unrelated proteins, mock purifications were also performed. Rat hepatic cytosol was preincubated with 20 μM unlabeled TA in order to block the subsequent binding of GR complexes to the ligand affinity resin. The purification protocol was then completed by pooling the appropriate fractions at each step based on previous purifications. Any protein(s) detected in the final eluate were defined as copurifying contaminants, and the absence of GR was confirmed by western blotting.

RESULTS

Association of Hsp70 and Hsp90 with the Immunopurified GR. The first experiment tested the possibility that hsp70 is specifically associated with the unliganded rat hepatic GR. Aliquots of rat liver cytosol were treated with the anti-receptor monoclonal antibody (BUGR2), nonspecific mouse IgG, the anti-hsp70 monoclonal (N27), or no antibody. Each sample was then incubated with protein A-Sepharose and processed for immunoblotting as described in Materials and Methods. The resulting immunoblot was then treated with antibody N27. Figure 1, lane 1, which resulted from an immunoprecipitation with N27, shows a distinct hsp70 band, along with the N27 antibody heavy and light chains (approximately 50 and 30 kDa, respectively). No hsp70 was precipitated nonspecifically by protein A–Sepharose alone (lane 2) or by total mouse IgG (lane 3). The BUGR2 immunopurification (lane 4) contains a 70-kDa band apparently identical to that found in lane 1, and also the heavy and light chains of the BUGR2 antibody. This experiment indicates that hsp70 is associated with the unliganded GR.

The experiment shown in Figure 2 was designed to confirm that hsp90 is associated with the unliganded and liganded unactivated forms of the rat hepatic GR but not with the

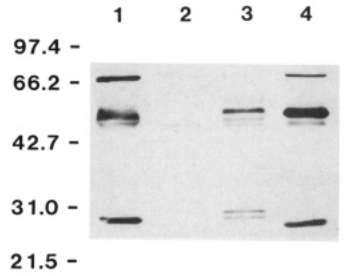

FIGURE 1: Specific association of hsp70 with the unliganded rat hepatic GR. Unliganded GR was prepared as described in Materials and Methods. Immunoprecipitations from cytosol were performed with the anti-hsp70 monoclonal N27 (lane 1), protein A alone (lane 2), mouse IgG (lane 3), or the anti-GR monoclonal BUGR2 (lane 4) and subsequently processed for immunoblotting and incubated with N27.

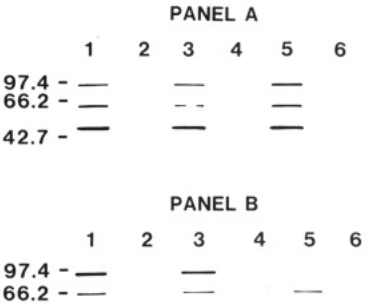

FIGURE 2: Association of hsp90 with the unliganded and liganded unactivated forms of the rat hepatic GR and its dissociation during *in vitro* activation. Unliganded and liganded GR complexes were prepared as described in Materials and Methods, and then 10 mM molybdate was added to half of the liganded sample. The unliganded and liganded, molybdate-treated samples were then incubated at 0 °C for 1 h and the liganded molybdate-free sample was incubated at 25 °C for 1 h to induce GR activation. Each sample was then immunoprecipitated with BUGR2 and immunoblotted. Panel A contains immunopurified unliganded (lane 1), liganded unactivated (lane 3), and activated (lane 5) receptors incubated with BUGR2, and panel B contains samples in the same order incubated with the anti-hsp70 polyclonal antibody, which recognizes both hsp70 and hsp90. Lanes 2, 4, and 6 contain the appropriate protein A controls for each previous lane in panels A and B.

activated form. Each of these forms of the GR was prepared as described and subsequently precipitated with BUGR2, processed for immunoblotting, and then incubated with BUGR2 (panel A) or the anti-hsp70 polyclonal antibody (panel B), which recognizes both hsp90 and hsp70. Lanes 1, 3, and 5 in panel A, which contained immunopurified unliganded, liganded unactivated, and liganded activated receptors, respectively, demonstrate the same total amount of immunoreactive GR protein either as the intact 94-kDa protein or as a proteolyzed receptor fragment at approximately 65 kDa. Nonspecific binding of GR to protein A–Sepharose was absent in each of these samples, as shown in lanes 2, 4, and 6 (controls for lanes 1, 3 and 5, respectively). Panel B, lane 1 (unliganded receptors) contains both hsp90 and hsp70 bands which were apparently equal in intensity to the hsp90 and hsp70 bands associated with the liganded unactivated receptors (lane 3). Lane 5, which contains liganded activated receptors, shows an hsp70 band but is devoid of an hsp90 band. Nonspecific binding of hsp90 and hsp70 to protein A–Sepharose in each sample was undetectable (lanes 2, 4, and 6). Thus hsp90 appears to be associated with the unliganded and liganded unactivated forms of the GR but dissociates upon thermal activation. In contrast, hsp70 appears to be associated with all three receptor forms.

Coimmunoprecipitation of GR with Anti-hsp70 Monoclonal Antibody N27. If the hsp70 coimmunoprecipitated using

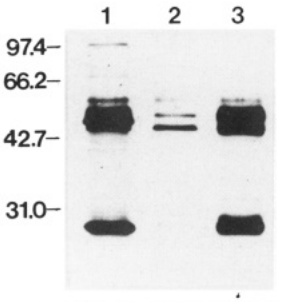

FIGURE 3: Coimmunoprecipitation of the GR with hsp70 during an immunopurification with antibody N27. Immunopurifications from rat hepatic cytosol were conducted with N27 (lane 1), protein A alone (lane 2), and mouse IgG (lane 3). After immunoblotting, each sample was incubated with BUGR2.

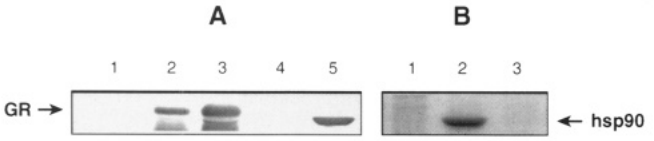

FIGURE 4: Immunoblotting and Coomassie blue staining of the GR protein. Unactivated GR complexes were immunoprecipitated with BUGR2 (panel A, lanes 3 and 5, and panel B, lane 2) as were activated complexes (panel A, lanes 2 and 4, and panel B, lane 1), and then both were subjected to SDS–PAGE followed by immunoblotting (Panel A) and Coomassie staining (Panel B). Samples in panel A, lanes 2 and 3, were incubated with BUGR2 to reveal GR protein, while samples in 4 and 5 were incubated with the anti-hsp70 polyclonal to expose hsp90. Panel A, lane 1, and panel B, lane 3, contained protein A–Sepharose controls (each was performed with the unactivated sample).

BUGR2 is truly bound to the GR, it may be possible to coprecipitate GR with an antibody directed against hsp70. The experiment shown in Figure 3 explored the possibility that N27 could coprecipitate the GR via its association with hsp70. Cytosol containing unactivated rat hepatic GR complexes was incubated with N27, while control samples were treated with total mouse IgG or with protein A–Sepharose alone. Each sample was processed for immunoblotting and incubated with BUGR2. The N27-immunopurified sample (lane 1) shows a distinct receptor band and N27 heavy and light chains, while the protein A–Sepharose (lane 2) and mouse IgG (lane 3) controls were devoid of receptor protein. Thus, it is possible to precipitate the GR with an antibody directed against hsp70, presumably due to an association between the two proteins.

Stoichiometric Analysis of the GR–Hsp70 Complex. Our strategy in determining the ratio of GR protein to hsp70 in the receptor complex was to perform GR immunoprecipitations, subject them to SDS–PAGE and Coomassie blue staining, and scan the resulting receptor and hsp70 bands with a laser densitometer. In this way, an estimate of the ratio of the GR to hsp70 could be made. Figure 4, panel B shows an SDS gel of BUGR2-precipitated activated (lane 1) and unactivated (lane 2) GR complexes and a protein A–Sepharose control for the unactivated sample (lane 3). Lane 2 contains a band at approximaely 90 kDa which could be the GR, hsp90, or both. However, lane 1 does not contain this band, suggesting that the band in lane 2 is actually hsp90 which dissociates upon activation. Panel A contains identical samples which were electrophoresed on the same SDS gel and subjected to western blotting. Lanes 2 and 4 contain activated GR complexes blotted for the GR and hsp90, while lanes 3 and 5 contain unactivated GR complexes blotted for the GR and hsp90, respectively. The protein A–Sepharose control (lane 1), which was blotted for both GR and hsp90, shows no nonspecific binding. Lane 5 contains hsp90, while lane 4 has

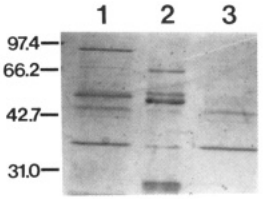

FIGURE 5: Protein staining of GR-associated hsp70 and hsp90 with Coomassie blue. Immunopurifications from rat hepatic cytosol were performed with BUGR2 (lane 1), N27 (lane 2), and protein A alone (lane 3) and then subjected to SDS–PAGE and Coomassie blue staining.

no hsp90 band due to receptor activation. Since lanes 2 and 3 both contain significant GR immunoreactivity, one must conclude that the protein band stained with Coomassie blue in panel B, lane 2 is hsp90, not the GR. Lack of GR staining by Coomassie blue is a phenomenon which has been previously noted by our laboratory as well as Pratt and colleagues (Bresnick et al., 1990). Thus, stoichiometric analysis of the association between the GR and hsp70 could not be based on scans of the Coomassie-stained GR protein itself.

In Figure 5, immunopurified unliganded GR complexes were electrophoresed on an SDS gel in parallel with immunopurified hsp70 and both were stained with Coomassie blue. Lane 1 contains immunopurified GR complexes and shows a prominent hsp90 band along with a faint hsp70 band and several other proteins, including BUGR2 heavy and light chains. Lane 2 contains immunopurified hsp70 and shows a distinct hsp70 band as well as N27 antibody heavy and light chains. The protein A–Sepharose control for both immunopurifications (lane 3) lacks hsp70 and hsp90 bands. This gel was then subjected to laser scanning and the hsp90 band was found to be present at a level which was 10-fold higher than that of hsp70. Since it is well established that the unliganded glucocorticoid receptor complex contains a dimer of hsp90 (Mendel & Orti, 1988; Bresnick et al., 1990; Rexin et al., 1991), hsp70 appears to be present in a 1:5 ratio with respect to the receptor protein. When the same gel was further stained with silver nitrate and scanned, the same 1:5 ratio was obtained (data not shown). To eliminate the possibility of dramatically different Coomassie blue staining properties of hsp70 and hsp90, known amounts of purified hsp90 and hsp70 were subjected to SDS–PAGE, stained, and scanned. Standard curves of absorbence units versus microgram quantity of protein revealed that hsp90 stains approximately 18% more intensely than hsp70 (data not shown). Thus our estimate of a 1:5 ratio of hsp70-receptor binding is not due to major differences in the staining properties of hsp90 and hsp70.

Association of Hsp90 and Hsp70 with the Ligand Affinity-Purified GR. In addition to the immunopurified GR, it was also essential to investigate the subunit structure of the GR isolated from cytosol via a purification method other than immunoprecipitation. The three-step ligand affinity scheme described by Grandics et al. (1984) for purification of the unactivated GR was chosen. Figure 6, panel A shows unactivated GR which was purified by this protocol and then subjected to immunoblotting with BUGR2. Although some of the receptor protein was intact (just below 97.4 kDa), the majority was proteolyzed to forms of approximately 66, 45, and 42 kDa in size. A sample which was mock-purified and blotted with BUGR2 is shown in panel C, lane 1, and completely lacks immunoreactive GR protein. Panel B contains a sample of purified receptor treated with the anti-hsp70 polyclonal antibody and shows a prominent hsp90 band. This lane also contained a faint hsp70 band which was not

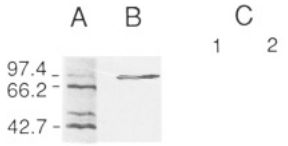

FIGURE 6: Association of hsp90 and trace amounts of hsp70 with the ligand affinity-purified GR. Two 3-mL aliquots of the purified GR were precipitated with 15% (v/v) trichloroacetic acid, dissolved in sample buffer, and subjected to immunoblotting. Mock-purified GR was also prepared and treated identically. Panel A contains purified GR immunoblotted and incubated with BUGR2, and panel B shows an immunoblot of GR incubated with the anti-hsp70 polyclonal antibody. Panel C contains immunoblotted mock-purified GR incubated with BUGR2 (lane 1) or the anti-hsp70 polyclonal antibody (lane 2).

preserved photographically. Panel C, lane 2 contains a mock-purified sample incubated with the anti-hsp70 polyclonal antibody and shows that neither heat shock protein is present nonspecifically. These blots demonstrate that although the receptor protein was partially proteolyzed, hsp90 was specifically associated with the purified unactivated GR, as was a trace amount of hsp70.

DISCUSSION

The 300-kDa unactivated GR complex minimally includes modulator (Bodine & Litwack, 1990), a 59-kDa protein (Tai et al., 1986; Rexin et al., 1991; Sanchez, 1990), an hsp90 dimer (Mendel & Orti, 1988; Bresnick et al., 1990; Rexin et al., 1991), and a 94-kDa steroid-binding subunit. In addition to these components, the unliganded, liganded unactivated, and thermally activated forms of the immunopurified rat hepatic GR were all shown in the present study to be associated with hsp70 (Figures 1 and 2). It is unlikely that hsp70 binds directly to the BUGR2 antibody, a phenomenon noted with regard to several other proteins (Hendry et al., 1993), since (i) no 70-kDa protein bound to BUGR2 in the work of Hendry and colleagues, and (ii) GR complexes immunoprecipitated with the anti-GR monoclonal antibody 250 (Okret et al., 1984) also contain hsp70 (data not shown). Importantly, the data presented here which demonstrate that hsp90 dissociates from the GR during thermal activation while hsp70 remains bound (Figure 2) argue for a direct association of hsp70 with the receptor rather than an indirect association with the GR through hsp90.

A previous observation that the GR receptor does not stain well with Coomassie blue or silver nitrate (Bresnick et al., 1990) was also noted (Figure 4), although the reason for this is unclear. Samples of immunopurified unliganded GR which were subjected to SDS–PAGE and stained with Coomassie blue revealed a distinct hsp90 band and a faint hsp70 band (Figure 5). Laser scans of this gel suggested a 1:5 ratio of hsp70 to GR. Protein staining with Coomassie blue can vary as much as 10-fold between different proteins (Read & Northcote, 1981). However, the approximate 1:5 ratio was confirmed after a series of experiments verified that hsp70 and hsp90 stain equivalently. Densitometric scans performed on immunopurified samples of liganded unactivated and activated GR complexes, as well as thermally inactivated receptors, resulted in the same ratio (data not shown). This estimated substoichiometric hsp70:GR ratio suggests that hsp70 is not a functional subunit of the GR complex but does not eliminate the possibility that limited hsp70–GR interactions may be important for receptor function(s). Paradoxically, immunoblots incubated with the anti-hsp70 polyclonal antibody, which is directed against hsp70 but also cross-reacts with hsp90, often showed hsp70 and hsp90 bands of similar intensity (Figure 2). A reasonable explanation for this apparent discrepancy is that this antibody may have a higher affinity for hsp70 than hsp90.

Hsp70 has been shown to be associated with the avian progesterone receptor, and 10 mM ATP in the presence of Mg^{2+} has been shown to stimulate hsp70 dissociation (Kost et al., 1989). However, incubation of immunopurified unactivated GR complexes with 10 or 50 mM ATP did not affect the level of hsp70 binding (data not shown). Many protein–hsp70 interactions are disrupted due to the release of ATP during tissue homogenization (Beckman et al., 1990). To test the possibility that this may also be true for hsp70–GR complexes, immunopurifications were performed in cytosol treated with the enzyme apyrase (100 μg/mL), which hydrolyzes ATP. Under these conditions, no additional hsp70 associates with the GR as compared to control samples (data not shown); thus the level of hsp70–GR binding is not artificially low due to ATP-induced hsp70 dissociation during tissue homogenization.

Hsp70 has also been shown to bind to dysfunctional proteins in order to maintain their function within the cell (Beckman et al., 1990; Pelham, 1986); accordingly, hsp70 may associate with damaged GR protein. Oxidation of GR sulfhydryls and dephosphorylation of its steroid binding domain are thought to be partially responsible for the loss of ligand binding activity during thermal inactivation (Cranberg & Ballard, 1977). However, thermal inactivation of the GR does not appear to promote additional hsp70 binding (data not shown).

Samples of unactivated GR purified from rat hepatic cytosol by the three-step ligand affinity purification scheme contain a protein of approximately 90 kDa on Coomassie blue/silver-stained SDS gels. Immunoblots of ligand affinity-purified GR reveal partially proteolyzed receptor protein, hsp90, and a trace amount of hsp70 (Figure 6). Again, due to the lack of GR staining with Coomassie blue, the 90-kDa protein seen on SDS gels was identified as hsp90. Despite the fact that the GR protein was partially proteolyzed, it remained functional in terms of its ability to undergo thermal activation and subsequently bind to both positive and negative glucocorticoid response elements (GREs) (Argentin et al., 1991; Drouin et al., 1992, 1993).

Data presented in this report demonstrate that GR immunopurified with BUGR2 as well as those obtained by ligand affinity purification contain hsp70 and hsp90. However, immunopurified receptor preparations apparently contain higher levels of hsp70 than do ligand affinity-purified receptor samples. Hsp70 could be associated with a subset of receptors which cannot bind to the ligand affinity resin. For instance, hsp70 could cover the steroid binding site on a population of GR and sterically hinder their interaction with the resin. Hsp90 is bound to the GR steroid binding domain (Pratt et al., 1988; Denis et al., 1988; Howard et al., 1990), and the hsp90–GR interaction is clearly preserved during receptor purification. However, hsp70 may be associated with a site (or sites) on the GR nearer the NH_2 terminus which is subject to proteolysis.

The potential function of the hsp70–GR interaction was not addressed in the present study, although potential functions can be inferred from other systems, such as protein trafficking (Chirco et al., 1988) and maintenance of properly folded nonaggregated protein conformations (Beckman et al., 1990; Pelham, 1986). It is also feasible that hsp70 is associated with the GR in substoichiometric amounts in order to stabilze various transition states of the GR which occur: after receptor protein translation but prior to hormone binding, after hormone

binding but prior to activation, or after activation but prior to DNA binding. Future experiments should examine the dynamics of GR–hsp70 binding and the potential involvement of hsp70 in receptor functions.

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