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Association of the Rat Liver Glucocorticoid Receptor with Hsp90 and Hsp70 Upon Whole Body Hyperthermic Stress

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The influence of whole body hyperthermic stress (41°C, 15 min) on association of the glucocorticoid receptor (GR) with heat shock proteins Hsp90 and Hsp70 was followed in rat liver cytosol during a 24 h period after the stress. Total cytosolic concentration of the GR, Hsp90 and Hsp70 and the amounts of Hsp90 and Hsp70 co-immunopurified with the GR were determined by a quantitative Western blotting using appropriate monoclonal antibodies. A significant decrease in the cytosolic GR level in response to the stress was noticed. The ratio of the amount of the GR to Hsp90 recruited by the GR was found to be unaltered by hyperthermia, in spite of the stress-induced increase in the total Hsp90 concentration in the cytosol. Hsp70 was also found in association with the GR and its 2.5-fold induction by the stress was accompanied by about 3-fold increase in its relative amount that co-immunopurified with the GR. The results suggest that heat stress influences the interaction of the GR with Hsp70 through the mechanisms controlling the untransformed rat liver GR heterocomplexes assembly process. © **1998 Published by Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Unliganded steroid hormone receptors occur both in the cytosol preparations and in intact cells as multiprotein heterocomplexes comprising heat shock proteins Hsp90 and Hsp70, an immunophilin (FKBP52/Hsp56 or CyP-40) and other, as yet unidentified, proteins such as p60, p48 and p23 (reviewed in Ref. [1]). The structure, composition, assembly and functional significance of these complexes, the isolation of which is facilitated by molybdate-stabilization are currently under intensive investigation. Intriguingly, steroid receptor complexes were shown to vary in stoichiometry and protein composition depending on cell type [2, 3], the receptor class [4] and supposedly also on momentary physiological demands and experimental conditions. Besides, it has been documented that steroid receptor complexes are dynamic structures and that the assembly process generates different transient heterocomplex states through ATP/Mg^{2+}- and K^+-dependent association/dissociation reactions of individual components [5, 6].

Some Hsps, including Hsp90 and Hsp70, serve fundamental cellular roles, chaperoning proteins during folding, functioning and intracellular trafficking and are found *in vivo* in complexes with a number of transcription factors and protein kinases. In association with steroid hormone receptors, Hsp90 plays a dual role: aids proper folding of the receptor's hormone binding domain to steroid binding conformation and represses transcriptional activity of the receptor in the absence of the hormone by maintaining it in the untransformed state. Thus, for example, in L cells, dissociation of Hsp90 induced the GR to collapse to nonsteroid-binding conformation [7], while in yeast cells transfected with the GR or ER, reduction of intracellular Hsp90 level resulted in impaired hormonal responsiveness [8]. Further evidence for the chaperoning role of Hsp90 was provided by recent studies with the drug geldanamycin, which binds to Hsp90 and disrupts its complexes with steroid receptors, causing a rapid loss of steroid binding activity and impeding hormone-dependent

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trafficking of the GR from the cytoplasm to the nucleus [9]. Dittmar and Pratt [10] have shown that GR-Hsp90 heterocomplex assembly *in vitro*, in rabbit reticulocyte lysate, requires two other components, Hsp70 and p60. The authors suggest that the three proteins, Hsp90, Hsp70 and p60, which combine spontaneously when purified, act cooperatively to form a core of a multiprotein chaperone machinery that is ubiquitously present in eukaryotic cells.

Functional properties of the glucocorticoid receptor, such as ligand binding and transactivation activity, are subject to modulation with changing physiological states, as well as under various pathophysiological and stressful conditions (reviewed in Ref. [11]). It was previously shown that heat stress leads to a rapid and reversible reduction of the GR ligand binding capacity [12–14], a slight increase in its binding affinity, and impairment of the GR–DNA complexes stability [15]. The cytoplasmic concentration of the GR protein was found to be depleted by heat stress on the account of its stimulated nuclear accumulation [14–16]. These effects of excessive heat were reported to be accompanied by an enhancement [16–18] of the receptor's transcriptional activity.

Since the glucocorticoid receptor forms stable associations with Hsp90 and Hsp70 under physiological conditions and since these interactions appear to play an important role in regulating the receptor activity, we were interested to learn whether heat shock, as a condition characterized by induction of Hsps synthesis and altered binding and transactivation activity of the GR, affects its interaction with Hsps. The results presented herein show that *in vivo* heat stress stimulates Hsp70 association with the GR, while decreasing the cytoplasmic level of the GR and increasing that of both Hsp90 and Hsp70.

MATERIALS AND METHODS

Chemicals

Monoclonal antibodies AC88 (anti-HSP90) and N27F3-4 (anti-Hsp72/Hsp73) were the products of StressGen (Victoria, British Columbia, Canada) and BuGR2 (anti-GR) of Affinity BioReagents (Neshanic Station, NJ). ^{125}I-Conjugated sheep anti-mouse IgG was obtained from ICN Pharmaceuticals (Costa Mesa, CA) and Protein A-Sepharose, nonimmune mouse IgG and horseradish peroxidase-conjugated goat anti-mouse IgG from Sigma (St. Louis, MO).

Animals and treatment

Male Wistar rats (200–250 g b.w.), reared under standard laboratory conditions (22°C, 12:12 h light/dark cycle) were used. Hyperthermic stress was achieved by exposure of the animals anesthetized with Nesdonal (4.6 mg/100 g b.w., i.p.; Specia, Paris, France) to 44°C in a ventilated and humidified chamber until rectal body temperature reached 41°C [19]. After 15 min of maintaining the rectal temperature at 41°C, the rats were transferred to room temperature and sacrificed after indicated time intervals. Regularly, body temperature returned to 37°C basal level in about 30 min after transfer to room temperature. The control (unstressed) animals were Nesdonal-anesthetized, kept at room temperature and sacrificed along with their counterparts.

Preparation of liver cytosol

The livers were perfused *in situ* with cold 0.14 M NaCl and homogenates were prepared from at least three animals, in 2 vol. (w/v) of 50 mM Tris buffer, pH 7.55 containing 0.25 M sucrose, 25 mM KCl, 10 mM $MgCl_2$ and 20 mM Na-molybdate. After centrifugation (10 min, 6000*g*, 4°C and 1 h, 105000*g*, 4°C), the upper lipid layer was aspirated and cytosols were stored in liquid nitrogen until use.

Immunoadsorption of untransformed cytosolic GR

Immobilization of BuGR2 antibodies to Protein A-Sepharose and immunoprecipitation of the untransformed GR heterocomplexes were done essentially as described by Czar *et al.* [20]. Immunoadsorbent was prepared by rotating (30 min, 4°C) 8 μl of Protein A-Sepharose pellets with 1.0 μg of BuGR2 in 300 μl of TEGM buffer (10 mM TES, pH 7.6 containing 50 mM NaCl, 4 mM EDTA, 10% glycerol and 20 mM Na-molybdate). After addition of 200 μl cytosol (4 mg protein), the rotation was continued for another 2 h at 4°C. The immune pellets were washed three times by suspension in 1.0 ml of TEGM and centrifugation. For nonimmune controls, the preimmune mouse IgG was used instead of BuGR2. The immunoadsorbed proteins were extracted by boiling in $^{2\times}$SDS-sample buffer and subjected to SDS-PAGE.

SDS-PAGE

Proteins were resolved according to Laemmli [21] on 10% SDS-polyacrylamide gels using Mini-Protean II Electrophoresis Cell (Bio-Rad Laboratories, Hercules, CA). Gels were cooled at 4°C during electrophoresis. Myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa) were simultaneously run as molecular mass references.

Immunoblotting

Western transfer of proteins from acrylamide gels to nitrocellulose membranes was performed in 25 mM Tris buffer, pH 8.3 containing 192 mM glycine and 20% (v/v) methanol, at 135 mA overnight in Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA). Unbound sites on

the membranes were blocked by 20 mM Tris buffer pH 7.4 containing 150 mM NaCl, 0.125% BSA and 0.1% Tween 20. The membranes were incubated by rocking (16 h, 4°C) in transfer buffer with 2 μg/ml BuGR2, 0.1% AC88 or 1.0 μg/ml N27F3-4 for detection of the GR, Hsp90 or Hsp70, respectively. The immunoblots were then incubated with the ^{125}I-labeled, followed by horseradish peroxidase-conjugated counter-antibodies. After autoradiography, the immunoreactive bands, visualised by peroxidase reaction, were excised out of the membranes and quantified by liquid scintillation counting as described by Meshinchi *et al.* [22].

Protein determination

Protein content in the cytosols was determined by the method of Lowry *et al.* [23] using BSA as a reference.

RESULTS

To examine the content of Hsp90 and Hsp70 within the untransformed rat liver GR heterocomplexes during a 24 h time period after exposure of the animals to 41°C *in vivo* hyperthermic stress, molybdate-stabilized cytosolic GR was immunoadsorbed to Protein A-Sepharose with monoclonal BuGR2 anti-GR antibody under mild conditions. Quantitative

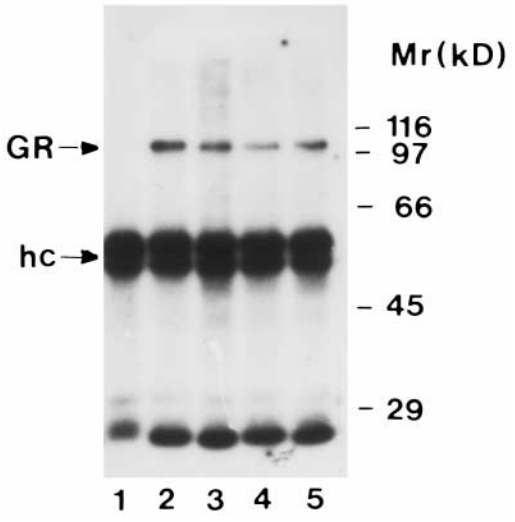

Fig. 1. The effect of 41°C whole body hyperthermic stress on the GR concentration in the rat liver cytosol. Molybdate-stabilized GR contained in 200 μl rat liver cytosols (4 mg protein) was immunoadsorbed to Protein A-Sepharose by BuGR2 anti-GR antibody. The immune pellets were washed and subjected to electrophoresis and Western blot analysis. The GR was detected with BuGR2, followed by both horseradish peroxidase-conjugated and ^{125}I-labeled counter antibodies. The representative autoradiography is shown. Lane 1: nonimmune control (nonimmune mouse IgG was used instead of BuGR2 for immunoadsorption); lane 2: unstressed control; lanes 3, 4 and 5: 2, 12 and 24 h after the stress, respectively. BuGR2 or nonimmune IgG heavy chains are designated by *hc*.

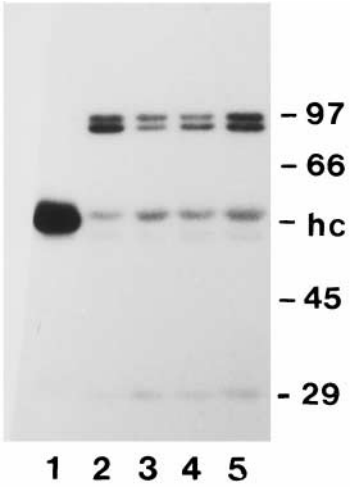

Fig. 2. The effect of 41°C whole body hyperthermic stress on Hsp90 content of the untransformed GR heterocomplexes in the liver cytosol. After immunopurification of molybdate-stabilized GR by BuGR2 antibody, electrophoretic separation and Western transfer of precipitated proteins, Hsp90 was detected by AC88 as the primary, and both peroxidase-conjugated and ^{125}I-labeled as secondary antibodies. The representative autoradiography is shown. Lane 1: nonimmune control (nonimmune mouse IgG was used instead of BuGR2 for immunopurification); lane 2: unstressed control; lanes 3, 4 and 5: 2, 12 and 24 h after the stress, respectively. BuGR2 or nonimmune IgG heavy chains are designated by *hc*.

Western blot analysis based on the use of appropriate monoclonal and both ^{125}I-labeled and peroxidase-conjugated counter antibodies, was subsequently performed to determine the amounts of immunoprecipitated GR, Hsp90 and Hsp70.

As shown in Fig. 1, the hyperthermic stress led to a decrease in the GR protein level in the liver cytosol. This decrease could be noticed throughout the examined 24 h period and reached its maximum at 12 h point, when the GR concentration made only 40% of that in the corresponding unstressed controls (Figs 4 and 7, open bars).

When anti-Hsp90 monoclonal antibody AC88 was used to detect Hsp90 co-immunopurified with the GR, two clearly resolved and approximately equally distributed isoforms of the protein (Mr 90 and 94 kDa) within untransformed GR heterocomplexes were observed (Fig. 2). For the first 12 h of recovery, the concentration of both Hsp90 isoforms in the immune pellet declined to about 40% of that before stress, while at the end of 24 h period it was again close to the basal level (Fig. 4, solid bars). Taking into account that variations observed in independent experiments were $\pm$10%, it could be concluded that heat stress-induced changes in the amount of GR-associated Hsp90 were similar to those recorded for the receptor itself, so that the GR:Hsp90 ratio remained essentially unaltered by the stress throughout the examined time interval.

Since the amount of Hsp90 associated with the GR might depend on the total cytosolic Hsp90 concen-

tration, a similar quantitative immunoblotting procedure with AC88 as a primary antibody, was performed after electrophoretical separation of 40 μg cytosol protein from the liver of unstressed and hyperthermia-exposed animals (Fig. 3). As could be expected, Hsp90 induction by whole body hyperthermic stress was noticed. A modest 15% increase in the cytosolic concentration of the protein was observed as early as 2 h after the stress. Further elevation of Hsp90 concentration to the level 1.9- and 1.3-fold higher than the basal one was detected 12 and 24 h after the stress, respectively (Fig. 4, hatched bars). Interestingly, the stress-induced increase in Hsp90 concentration in the cytosol coincided with the decrease (2 h and 12 h) or almost unchanged (24 h) amount of the protein that co-immunopurified with GR.

Determination of the GR-associated Hsp70 by N27F3-4 monoclonal antibody (Fig. 5) showed, however, that hyperthermic stress altered the GR–Hsp70 interaction in the rat liver cytosol. Namely, 2, 12 and 24 h after exposure to the stress, the amount of Hsp70 within the GR heterocomplexes was 1.7-, 1.2- and 2.2-fold higher, respectively, as compared to that determined in unstressed controls (Fig. 7, hatched bars). Together with the data on the GR level in the corresponding cytosols (Fig. 7, open bars), these results leaded to an estimation that 2, 12 and 24 h after hyperthermic stress the molar ratio of the GR to GR-associated Hsp70 rose in favour of the latter from the assumed 1:1 in unstressed controls to 1:2.1, 1:2.9 and 1:3.3, respectively. It could not be concluded, however, whether the observed changes in GR:Hsp70 ratio resulted from the stoichiometric alterations within the untransformed GR heterocomplexes, or from an increased share of the GR molecules that associate with Hsp70 in the total number of the GR molecules in the cytosol.

Total cytosolic Hsp70 concentration in the liver cytosol of unstressed and hyperthermic animals was determined from 40 μg cytosol protein using N27F3-4 monoclonal and both peroxidase-conjugated and ^{125}I-labeled counter antibodies (Fig. 6).

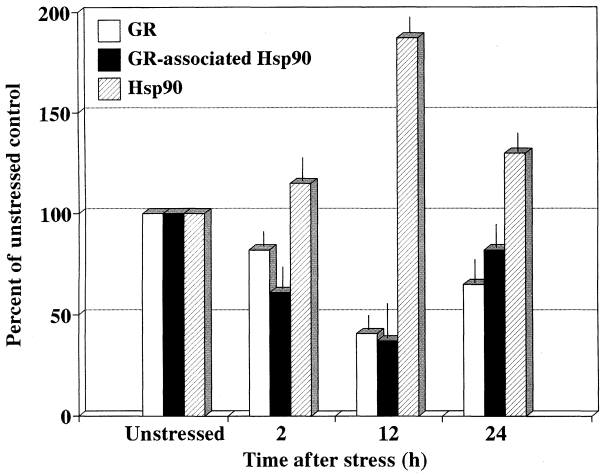

Fig. 4. Relations of the amount of GR, GR-associated Hsp90 and total cytosolic Hsp90 in the rat liver cytosol upon the hyperthermic stress. After autoradiography, the immunoreactive bands visualized by peroxidase reaction were excised from the membranes as those presented in Figs 1–3 and measured for ^{125}I radioactivity. Mean values ± SE from three independent experiments are expressed as percentage of the corresponding unstressed controls.

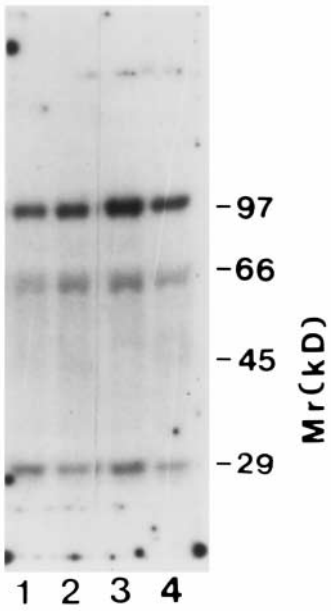

Fig. 3. Hsp90 in the liver cytosol of rats exposed to 41°C whole body hyperthermia. Cytosol proteins (40 μg) were electrophoretically separated and transferred to nitrocellulose membrane. Hsp90 was detected by incubating the membrane with AC88 anti-Hsp90 monoclonal antibody followed by ^{125}I-labeled and peroxidase-conjugated counter antibodies. The representative autoradiography is shown. Lane 1: unstressed control; lanes 2, 3 and 4: 2, 12 and 24 h after exposure to hyperthermic stress, respectively.

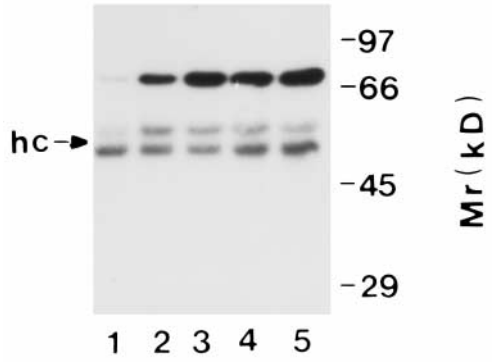

Fig. 5. The effect of 41°C whole body hyperthermic stress on Hsp70 content of the untransformed GR heterocomplexes in the liver cytosol. The procedure was the same as given for Fig. 2 except that Hsp70 was detected by N27F3-4 as the primary antibody. Lane 1: nonimmune control (nonimmune mouse IgG was used instead of BuGR2 for immunopurification); lane 2: unstressed control; lanes 3, 4 and 5: 2, 12 and 24 h after the stress, respectively. BuGR2 or nonimmune IgG heavy chains are designated by *hc*.

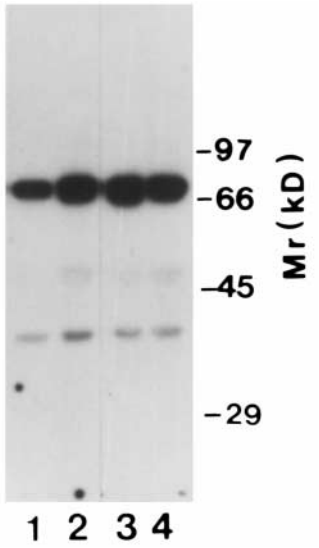

Fig. 6. Hsp70 in the liver cytosol of rats exposed to 41°C whole body hyperthermia. The procedure was the same as given for Fig. 3 except that Hsp70 was detected by N27F3-4 as the primary antibody. Lane 1: unstressed control; lanes 2, 3 and 4: 2, 12 and 24 h after exposure to hyperthermic stress, respectively.

Measurement of ^{125}I radioactivity in the immunoreactive bands revealed that hyperthermic stress led to an approximate 2.5-fold increase in the cytosolic concentration of the protein that could be seen throughout the examined recovery period (Fig. 7, stitched bars).

DISCUSSION

There is an accumulating body of evidence for the GR modifications under stress conditions. It has been reported that heat stress applied to both cell cultures [13, 14] or whole organisms [12] led to considerable loss of cytosolic glucocorticoid binding capacity that coincided with a decreased amount of the GR protein present in the cytosolic fraction and its increase in the nuclei [14–16]. Similar reduction in the steroid binding capacity was observed for the GR upon chemical stresses, such as cadmium or arsenite toxicity [24, 25], as well as for other steroid receptors after exposure to heat stress [26]. However, the extent of the reduction noticed in different studies varies considerably and the clear correlation between stress-related loss of the GR protein in the cytosol and its accumulation in the nuclei is still lacking. Therefore, other possible causes of the observed stress-induced modifications of the GR function should be considered. These include the changes in the rates of the receptor synthesis and degradation, in its covalent modifications and, as proposed in this study, in its assembly with Hsps to form hetero-oligomeric complexes of possibly altered steroid binding activity, stability and/or intracellular localization.

The results presented herein confirm our previous observation that fever-like heat stress *in vivo* induces a pronounced decrease in the rat liver GR cytosolic concentration and show that this decrease persists during the whole examined 24 h period after the treatment. Concomitantly, the GR exhibited an increased ability to associate with Hsp70 and an unaltered capacity to interact with Hsp90, although the cytosolic concentrations of both these Hsps were significantly elevated in hyperthermic animals as compared to unstressed controls.

Intensive studies on the role of Hsp90 in GR functioning, performed so far, have clearly established that GR has to bind to Hsp90 in order to be in a high-affinity steroid binding conformation [7], that dissociation of Hsp90 from unliganded receptor is followed by a simultaneous loss in cytosolic steroid binding activity [7] and that this protein is implicated in the receptor shuttling between the cytoplasm and the nucleus [9, 27]. Therefore, it is reasonable to expect that heat stress-induced elevation of Hsp90 might lead to alterations in the GR–Hsp90 association and thus, to the modulation of the GR hormone-binding and DNA-binding ability, as well as to the changes in its intracellular localization. However, our results showing that hyperthermic stress did not affect the GR:Hsp90 ratio imply that GR–Hsp90 interaction might not necessarily depend on the cellular Hsp90 level and that it could not account for the stress-induced reduction of the GR binding activity and its intracellular redistribution induced by stress. Thus, they direct the attention to Hsp70 role in the GR action and to, as yet poorly understood, mechan-

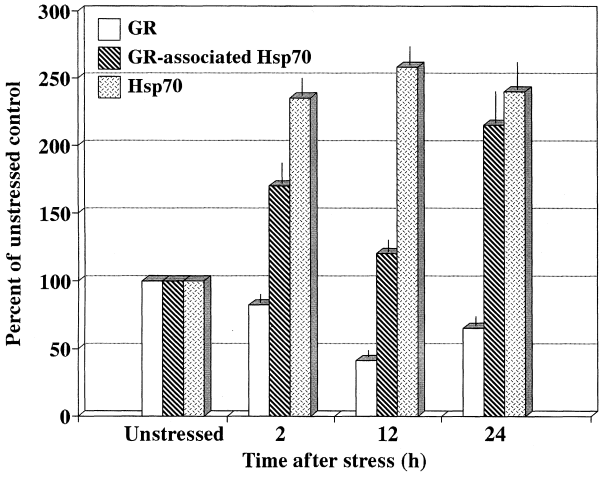

Fig. 7. Relations of the amount of GR, GR-associated Hsp70 and total cytosolic Hsp70 in the rat liver cytosol upon the hyperthermic stress. After autoradiography, the immunoreactive bands visualized by peroxidase reaction were excised from the membranes presented in Figs 1, 5 and 6 and measured for ^{125}I radioactivity. Mean values ± SE from three independent experiments are expressed as percentage of the corresponding unstressed controls.

ism(s) controlling the GR heterocomplexes assembly process.

The presence of Hsp70 within the untransformed GR heterocomplexes is still a matter of debate. Based on *in vitro* examination of the GR heterocomplexes formation mechanism in rabbit reticulocyte lysate, it was proposed that Hsp70 is required for the assembly and that, during the course of this process, a part of Hsp70 dissociates leaving the receptor bound to it in substoichiometric amounts [6, 10]. The finding that Hsp70 has not been recovered in cross-linked native receptor heterocomplexes [3, 28], however, raised reserves with respect to its involvement in the receptor action as a structural component of the untransformed receptor after the heterocomplex formation [29]. Our data presented in Fig. 5 (lane 1, nonimmune probe) clearly suggest that rat liver GR is associated with Hsp70, since only negligible binding of Hsp70 to immunoadsorbent in the absence of BuGR2 antibody has been noticed. A possible argument that Hsp70-bound GR represents a fraction of the receptors that are, or are held up, in the process of heterocomplex assembly, however, still remains. In our experiments, heat stress caused a 3-fold increase in Hsp70 binding to the GR, an alteration roughly proportional to 2.5-fold increase in its cytosolic level. It is not clear whether the observed stimulation of the GR–Hsp70 interaction reflects the alteration in the GR heterocomplexes stoichiometry, or in the number of the GR molecules capable of Hsp70 binding. In any case, a question arises whether stress-induced change in Hsp70 binding to the GR could be related to acceleration of the assembly process, or to increased accumulation of the GR in the nuclei. If no other role in the GR action beyond a transient one in the heterocomplex assembly could be ascribed to Hsp70, then an increased interaction of these two proteins observed under stress conditions might be explained solely as a mechanism aimed to provide better protection of the receptor against possibly denaturing effects of heat. On the other hand, if Hsp70 could be considered as a genuine structural component of the untransformed GR, its role might be linked to the GR intracellular transportation. Hsp70 has been shown to be required for nuclear import of a number of proteins [30], although Yang and DeFranco [31] reported that it was not the case with the GR. On the contrary, Hsp70 was found so far only in the GR heterocomplexes immunopurified from cells with the untransformed GR localized in the nuclei, but not from those in which it occurs in the cytoplasm [32], suggesting its involvement in the GR intracellular cycling between the cytoplasm and the nucleus. In line with this idea, the results on the GR–Hsp70 interaction presented here open a possibility that GR–Hsp70 association stimulated by hyperthermic stress might represent a prerequisite for the increased nuclear accumulation of the GR.

The observation that stress-induced increase in the relative abundance of the two examined Hsps in the cytosol affects only GR–Hsp70, but not GR–Hsp90 interaction imply that assembly of the untransformed GR heterocomplexes, as an orderly and functionally conserved process [5, 6, 10] does not depend solely on the cytosolic concentrations of the proteins involved. Suggesting that stress might affect the assembly process, these data point at the importance of the mechanisms that control the assembly and stability of the untransformed GR heterocomplexes modulating their biological activity in accordance with momentary physiological demands.

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