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High Levels of Non-activated Receptors in Glucocorticoid-sensitive S49wt Mouse Lymphoma Cells Incubated with Dexamethasone

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Upon agonist binding the heteromeric glucocorticoid receptor complex undergoes a conformational change (receptor activation). This event involves the dissociation of a dimer of 90 kDa heat shock proteins. Whereas receptor activation in cytosolic assays is both rapid and irreversible, less is known about the receptor activation and translocation in intact cells during challenge with an agonist. In this paper we report on the receptor status of glucocorticoid-sensitive murine S49 lymphoma cells during dexamethasone exposure. By three different assays, ligand (re)binding, nuclear translocation and hsp90 co-immunoprecipitation, it was found that the majority of the glucocorticoid receptor protein was in a non-activated conformation. Furthermore, prolonged exposure to dexamethasone did not result in increased levels of activated receptors. By assessing receptor activation in situ we found that physiological temperature was less effective in dissociating hsp90 compared to room temperature. These findings indicate that the physiological temperature negatively controls receptor activation, probably due to a thermolabile interaction between the hormone and its cognate receptor.

INTRODUCTION

Glucocorticoid hormones (GC) modulate gene transcription in a tissue-specific manner [1]. In tissues of lymphatic origin they are capable of mediating the induction of apoptosis and GC-hormones are therefore included in most protocols for treatment of leukemia and lymphoma [2, 3]. The intracellular signal transduction of the hormone is mediated by the glucocorticoid receptor (GR), a protein capable of interacting with the ligand as well as with the steroid responsive elements in the DNA [4].

Several lines of evidence have indicated that un-liganded GRs are complexed to a dimer of 90 kDa heat shock proteins (hsp90) [5–8]. Loss of these heat shock proteins results in a decrease in affinity of the receptor

for glucocorticoid by at least 100-fold [9, 10]. These observations have led to the suggestion that hsp90 maintains the receptor in a conformation capable of binding the hormone with high affinity. Both the steroid binding pocket as well as the region of interaction with hsp90 are located at the carboxyl terminal part of the protein. Truncated receptors devoid of this region are constitutively active [11, 12], underscoring another major function of hsp90 in the receptor complex: suppression of DNA binding activity. Ligand binding facilitates the dissociation of hsp90 from the complex, a process referred to as receptor activation [10]. Activated GRs are DNA-binding proteins and can be isolated from the nuclear compartment. This process of intracellular redistribution is called translocation.

Much of the assembled data on the interaction between hsp90 and the receptor stem from laboratory studies in which ligand binding and receptor activation

were assayed in cytosolic preparations at temperatures ranging from 15–25°C. In these studies receptor activation has been found to be rapid, quantitative and irreversible [13–17]. In intact, glucocorticoid responsive S49 cells assayed at physiological temperature, steroid-induced nuclear translocation of the GR ranged from 40% as measured by Distelhorst and Howard [18] to almost 100% according to Segnitz and Gehring [19]. As it is generally assumed that nuclear translocation and receptor activation are concurrent phenomena this would logically lead to a significant loss of ligand binding capacity. These results are, however, in contrast with our previous observation that no loss of specific steroid binding was observed in such cells previously exposed to dexamethasone [20]. These findings would suggest that in intact, GC-responsive cells substantial amounts of receptor were not activated and thus still complexed to hsp90.

We have therefore studied the apparent discrepancies between cytosolic and whole-cell assays by investigating the receptor status in S49 cells after various lengths of dexamethasone incubation in three different ways, specific ligand (re)binding, hsp90 association and receptor translocation. Furthermore, we have examined the effect of temperature on *in situ* activation of liganded receptors. In doing so we obtained evidence that the majority of glucocorticoid receptors in steroid-challenged S49 cells are in a non-activated conformation, even under conditions leading to maximal lysis. Finally, by assessing the receptor activation in concentrated cytosols we found that a low temperature (20°C), often used to activate cytosolic receptors, is more effective in dissociating hsp90 from the complex than the physiological temperature of 37°C.

MATERIALS AND METHODS

Cell lines and cytosol preparation

Dexamethasone-sensitive S49 mouse lymphoma cells with a doubling time of approximately 12 h were maintained as described previously [20]. Cytosols were prepared from exponentially growing cell cultures. Prior to harvesting the cells were routinely checked for viability by light microscopy. Approximately 10^9 cells were collected by gentle centrifugation ($800g$) and washed twice with cold phosphate buffered saline. After the last wash the cells were spun down at $1200g$ and the volume of cell pellet (400–600 μl) was measured. The cells were lysed by diluting the cell pellet with an equal volume of ice-cold lysis buffer. Next, the slurry was transferred to a Dounce homogenizer and the cells were disrupted by 20 strokes. The mixture was subsequently centrifuged for 1 h at 100,000g in the cold. The resulting supernatant (cytosol) was aliquoted and either stored at −70°C or directly used in the experiments. Protein concentration of the cytosols were determined using a protein assay kit (Bio-Rad, Richmond, U.S.A.).

Chemicals and antibodies

All cell culture reagents were from Gibco (Paisley, Scotland). Immobilon-P PVDF blotting membrane was from Millipore (Bedford, U.S.A.). Chemicals were of analytical grade and obtained from Boehringer Mannheim (Mannheim, Germany), except for TES (2 [tris(hydroxylmethyl)methyl-amino]-1-ethanesulfonic acid) which was from Janssen Chimica (Geel, Belgium) and acrylamide from Serva (Heidelberg, Germany). Protein A Sepharose was from Pharmacia LKB (Uppsala, Sweden). Anti-GR antibody MAI-510 (clone BuGR2) and the anti-hsp90 antibody MA3-010 (which reacts similar to the AC88 monoclonal) were from Affinity BioReagents Inc. (Neshanic Station, U.S.A.). Anti-receptor antibody GR49 was a generous gift from Dr H. M. Westphal (Marburg, Germany). Goat–anti-mouse peroxidase conjugated IgG was from Pierce (Rockford, U.S.A.) and ^{125}I-labeled sheep–anti-mouse (16 μCi/μg) and [1,2,4^{3}H]dexamethasone (37 Ci/mmol) were purchased from Amersham International (Bucks., England).

Buffers

PBS^{++}: phosphate buffered saline with 2.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$; **TBS**: Tris buffered saline; 20 mM Tris pH 7.4, 150 mM NaCl, **TEG**: 10 mM TES, pH 7.4, 4 mM EDTA, 10% (v/v) glycerol; **blocking buffer**: 0.5 mM EDTA, 0.5% (v/v) Tween-20, 0.15% (w/v) bovine serum albumin in TBS; **lysis buffer**: 10 mM Hepes, pH 7.4, 1 mM EDTA, 1 mM DTT, proteases inhibitors: PMSF, anti-trypsin; **fractionation buffer**: phosphate buffer 20 mM, pH 7.4, 250 mM sucrose, 1 mM EDTA.

[^{3}H]dexamethasone binding experiments

The number of specific dexamethasone binding sites was determined using a modified version of the whole-cell binding assay as used by Costlow *et al.* [21] and was described previously [20]. Dissociation of the ligand from the GR in intact S49 cells was accomplished by collecting the [^{3}H]dexamethasone labeled cells by centrifugation (800g) and resuspension in agonist-free growth medium at 37°C at a density of approx. 10^7 cells/ml. Rebinding with the radio-labeled steroid was done by returning the cells to the same medium as used for the initial binding experiment.

Immunoprecipitation and Western blotting

Cytosol (100 μl) was incubated with 2.5 μg of MA1-510 receptor antibody for 2 h in the cold. Subsequently the cytosol was diluted with 100 μl TEGM (TEG buffer with 20 mM Na_2MoO_4) and 30 μl Protein A Sepharose [20% (w/v)] and gently mixed for another 2 h. The sample was concentrated by centrifugation (14,000g) for 10 min and washed five times with 1 ml of cold TEGM buffer. The immunopellets were resuspended in 2 × sample buffer and boiled for

5 min. Subsequently the Protein-A-Sepharose beads were spun down and the supernatant was applied to SDS–PAGE (7.5 or 12%). After separation the proteins were transferred to PVDM membranes (2 h at 400 mA). The membranes were incubated in blocking buffer for 1 h at room temperature and subsequently incubated with the appropriate antibody dilutions (1:300 for the MA3-010 monoclonal and 1:500 for the BuGR2 clone). Primary antibody incubations were always performed overnight in the cold. Unbound antibody was removed by washing the membrane twice with cold TBS. As a second antibody, goat–anti-mouse horseradish conjugated IgG (1:3000 in blocking buffer) was used; 2–3 h at room temperature. The proteins were visualized by reacting the membranes with 5 ml 4-chloro-1-naphthol (3 mg/ml) and 100 μM H_2O_2 in 25 ml of TBS. For quantification the immunoblots were incubated with [^{125}I]RAM (sheep–anti-mouse) IgG (2 μCi in 10 ml of blocking buffer). Unbound label was removed by washing the membranes three times with cold TBS buffer. Membranes were left to dry before an autoradiogram was made or the stained bands were excised for quantification by scintillation counting.

Nuclear translocation of GRs

S49 cells are collected, washed twice, resuspended in fractionation buffer and disrupted by freeze/thawing in liquid nitrogen ($\approx 50 \times 10^6$ cells/ml). The nuclear pellet was separated from the cytoplasm by centrifugation at 14,000g in the cold for 1 h. The resulting pellet was washed twice with cold 250 mM NaCl and subsequently incubated with 500 mM NaCl for 30 min at room temperature to extract activated receptors.

The extracted proteins were precipitated with cold TCA (10% v/v) and washed twice with cold TCA (1% v/v). The pellets were boiled in sample buffer and the pH was adjusted with 5 M NaOH. Cell equivalent amounts of protein were separated on SDS–PAGE and after transfer and blotting (MoAb GR49; 1:10,000) analyzed for receptor protein.

RESULTS

Pulse–chase dexamethasone incubations in S49 cells

Glucocorticoid-sensitive S49 cells are rapidly lysed by incubation in 10^{-7} M of dexamethasone [20]. To assess the minimum time required for induction of a lytic response the cells were incubated with the steroid for various time intervals and subsequently resuspended in agonist-free growth medium. Apoptotic cells were scored microscopically after 24 h (Fig. 1). A minimal incubation time of 8–10 h was found to be required for committing the cells for lysis, during which the cells became arrested in the G_1 phase of the cell cycle as described previously [22].

Dexamethasone challenged S49 cells can be relabeled with [^{3}H]dexamethasone

The reversibility of dexamethasone-induced lysis suggested that the receptor-mediated signalling was reversible during agonist incubations up to 8 h. High affinity ligand binding capacity, indicative for non-activated receptors, was therefore assayed by measuring the dexamethasone (re)binding capacity. S49 cells were labeled at 37°C for 1 h with the tritiated ligand and stable receptor–ligand association was determined after a post-incubation dilution step (PID) at room
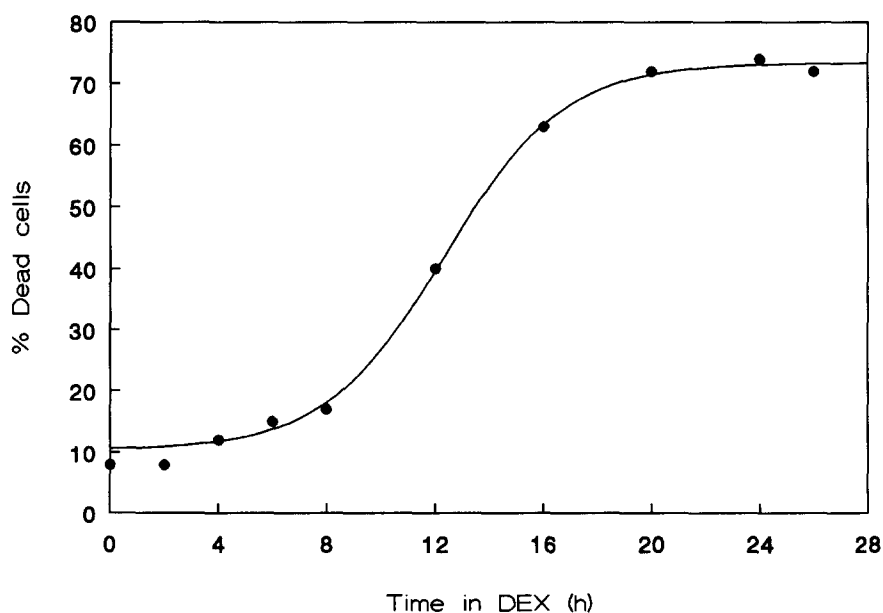

Fig. 1. Dexamethasone-induced cell death in S49 cells. S49 cells were incubated with 10^{-7} M of dexamethasone and at the indicated times collected and resuspended in agonist-free medium. The percentage of lysed cells was scored after 24 h. Presented data are the average of triplicate experiments.
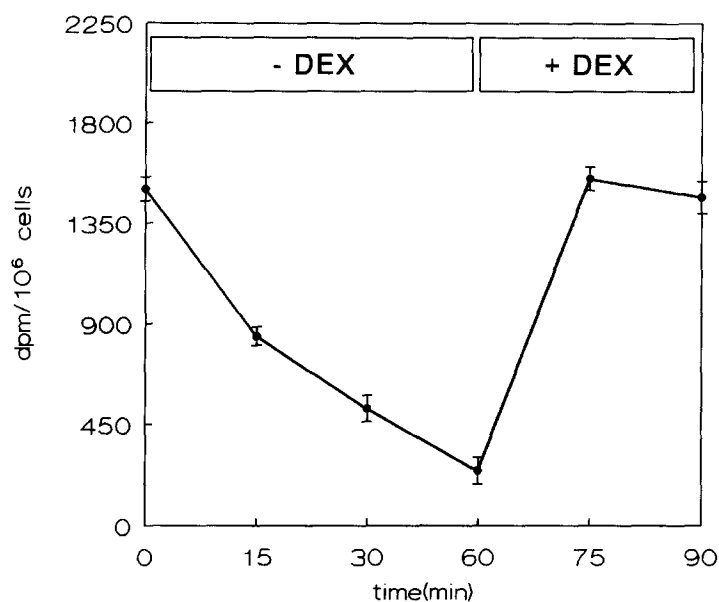
Fig. 2. Dissociation and rebinding of [³H]dexamethasone in S49 cells. S49 cells were incubated with 2 μCi/ml (0.5 × 10⁻⁸ M) [³H]dexamethasone for 1 h at 37°C (t_0 value). Subsequently the cells were transferred to agonist-free medium for 1 h and then returned to the labeling medium. At indicated times specific dexamethasone binding was determined. Data points are the mean ± SEM of three independent experiments.

temperature. If the PID was performed at 37°C a progressive loss ($t_{1/2} = 18$ min) of cell-bound dexamethasone was observed (Fig. 2), compared to stable binding of the ligand at 20°C [18]. After dissociation of the radioactive ligand at 37°C, the steroid-depleted cells were able to rebind up to control levels of ligand (Fig. 2). This response was essentially similar to that reported for different subtypes of the L1210 cell line [20]. The effect on high affinity ligand binding of longer (6 and 16 h) dexamethasone incubation was also investigated. It was found that cells under both conditions were still able to (re)bind the ligand in a high affinity manner to approx. 70% of the control value in cells pre-incubated for the 16 h with the steroid.

Cytosolic receptors and receptor-associated hsp90 can be detected in high levels in S49 cells irrespectively of dexamethasone incubation

The data in Fig. 2 suggested that the high affinity binding conformation of the receptor—indicative for hsp90 association—is present, despite prolonged incubation with the ligand. To investigate the protein composition of GRs during agonist incubation, concentrated cytosols were prepared from cells incubated with dexamethasone (10⁻⁷ M) for various times. In order to preserve receptor-associated hsp90 during preparation of the cytosols, 20 mM of Na₂MoO₄ was included in the lysis buffer. Control experiments had shown that molybdate was capable of preventing the agonist- and temperature-mediated loss of hsp90 at 20 as well as 37°C in cytosols (data not shown). Immunoaffinity

isolated receptors were detectable in the cytosolic fraction, irrespective of the length of exposure to dexamethasone [Fig. 3(A)]. Moreover, hsp90 was found to be a component of the immunoprecipitated complex. Quantification with [¹²⁵I]SAM IgG showed that both proteins were present in high levels. Indeed, results from three independent experiments showed no detectable downregulation of the cytoplasmic GR and complexed hsp90 during 2, 6 or 16 h of dexamethasone incubation [Fig. 3(B)].

GR translocation in S49 cells is rapid and reversible but not quantitative

The outcome of the above experiments is characteristic of a predominantly non-activated receptor population. To examine the translocation competence of GRs, the nuclear and cytosolic compartments from control and dexamethasone-challenged cells were assayed for receptor content. After cell fractionation, a 97 kDa, anti-receptor antibody-responsive signal was obtained from the cytoplasmic compartment in control cells only. Incubations with 10⁻⁷ M of dexamethasone for 2 h resulted in the appearance of a nuclear (250 mM NaCl resistant, 500 mM NaCl extractable) signal (Fig. 4, lane 6). It is obvious, however, that most of the receptor protein was still present in the cytosolic fraction at all conditions (Fig. 4). Limited nuclear translocation could be detected after dexamethasone incubations as brief as 5 min (data not shown), but did not increase during more prolonged incubation. Withdrawal of the steroid resulted in the disappearance of the nuclear receptor

signal within 2 h (Fig. 4, lane 7). By ^{125}I-relabeling of the bands, it was estimated that the translocated pool of receptors represented approx. 20% of the total endogenous receptor. Independent repeats, but probed with the MAI-510 antibody (BuGR2 clone) showed similar results.

Activation of GRs in concentrated cytosols is less effective at 37 than at 20°C

Efficient relabeling, persistant co-immunoprecipitation of hsp90 and limited nuclear translocation are all indicative for non-activated receptors. Therefore, the
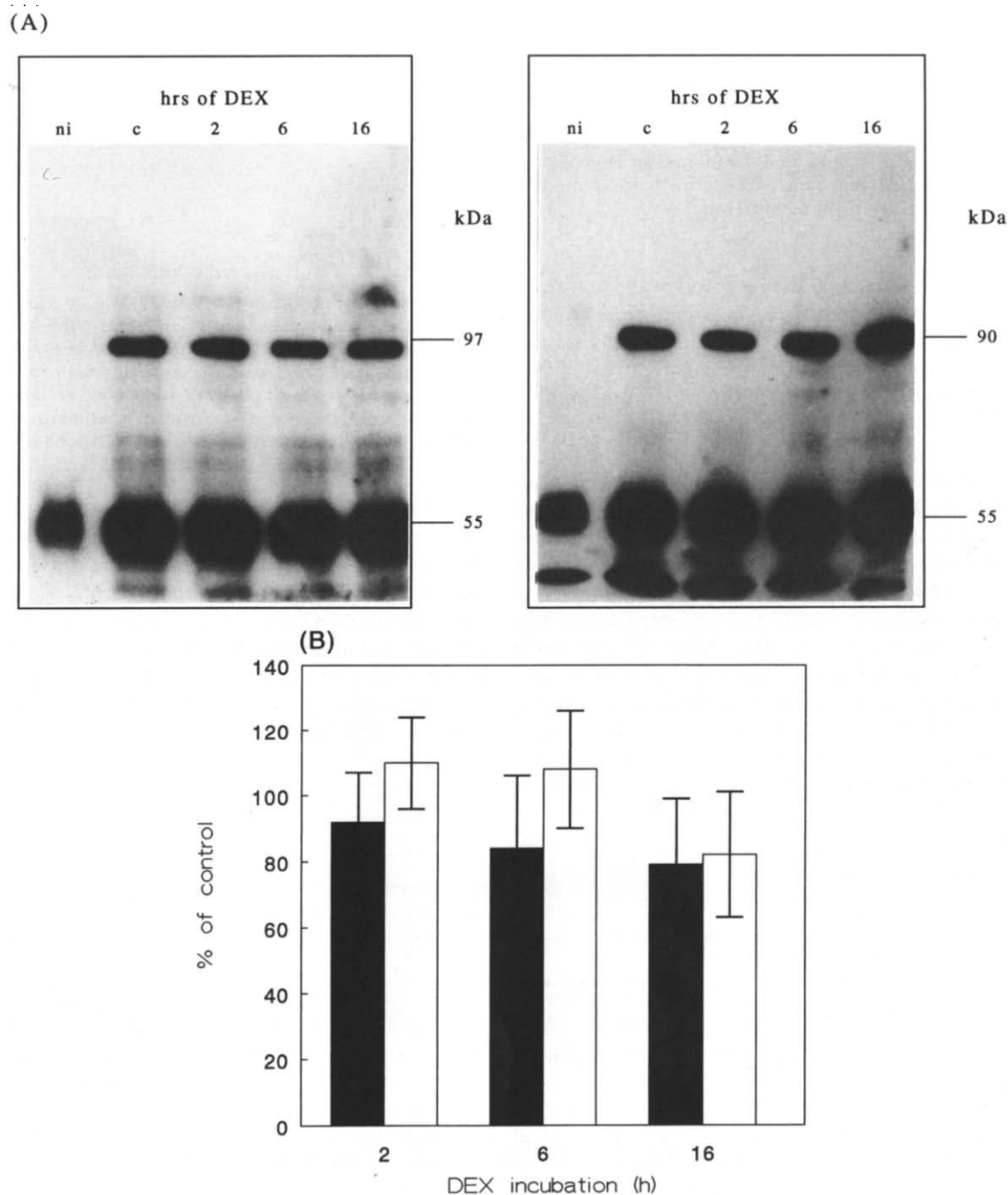

Fig. 3. The effect of 10^{-7}M dexamethasone on GR levels and receptor-associated hsp90 in S49 cells. a(A) Autoradiogram of ^{125}I-labeled Western blots of immunoprecipitated GR, probed for receptor (left panel) and receptor-associated hsp90 (right panel). S49 cells were incubated with dexamethasone and after various time intervals analyzed for GR and GR associated hsp90. Corresponding conditions are depicted above each lane. ni: non-immune serum, c: control, 2, 6 and 16 represent the incubation time in hours in 10^{-7}M dexamethasone. (B) GR levels (closed bars) and receptor-associated hsp90 (open bars) in cytosols from dexamethasone-incubated S49 cells. Result are the mean ± SEM of three independent experiments and expressed as a percentage of untreated control.
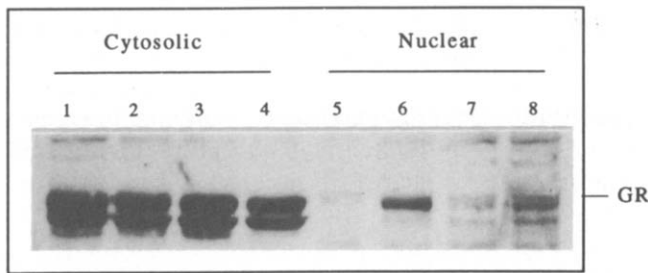
Fig. 4. Limited and reversible translocation of GRs in S49 cells. Cells were challenged with 10^{-7} M dexamethasone, lysed and cytosolic and nuclear fractions were analyzed for GR. Lanes 1–4, cytosolic fractions; lanes 5–8, nuclear fractions; lanes 1 and 5, no steroid; lanes 2 and 6, 2 h of dexamethasone; lanes 3 and 7 and 4 and 8, as in lanes 2 and 6 but post-incubated in agonist-free growth medium for 2 and 4 h, respectively.
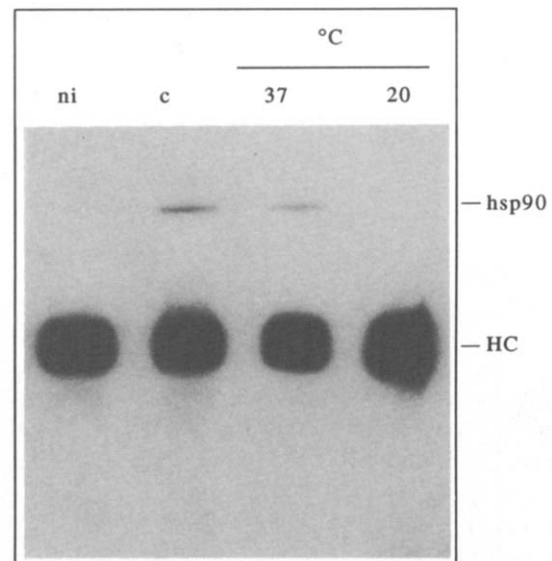

Fig. 6. Activation of pre-warmed GRs at 20°C. Dexamethasone-loaded cytosols were incubated for 1 h at 37°C. Next a sample was put on ice or further incubated for 1 h at 20°C. Both samples were analyzed by immunoprecipitation for receptor-complexed hsp90. Conditions are shown above each lane. ni, non-immune serum; c, control (no dex + MoO$_4$); 37°C, 1 h at 37°C; 20, 1 h at 37°C and 1 h at 20°C.

activational competence of the receptors was examined. To this end, concentrated cytosols were incubated in the cold with 10^{-7} M of dexamethasone and subsequently heated to either 20, 30 or 37°C. After 30, 60, 90 and 120 min, aliquots were taken and receptor activation was stopped by quickly cooling and addition of Na$_2$MoO$_4$. The samples were incubated with the MAI-510 antibody and assayed for receptor associated hsp90 by Western blotting. During the incubations at 30 and 37°C hsp90 was isolated throughout the time course (Fig. 5, middle and lower panel, lanes 3, 4 and 5). By contrast a steady decrease in receptor associated hsp90 was observed when the activation temperature was 20°C (Fig. 5, top panel, lanes 3, 4 and 5). Each temperature condition was repeated at least twice and similar observations were made.

The conditions present at 37°C did not irreversibly inhibit receptor activation capacity. This was demonstrated in Fig. 6, as receptors in dexamethasone (10^{-7} M) loaded cytosol pre-incubated at 37°C for 1 h (lane below 37°C), were still activational competent when cooled to 20°C (lane below 20°C). By Western blotting with the BuGR2 Ab it was found that only very limited receptor breakdown occurred during the course of the experiment (data not shown), confirming
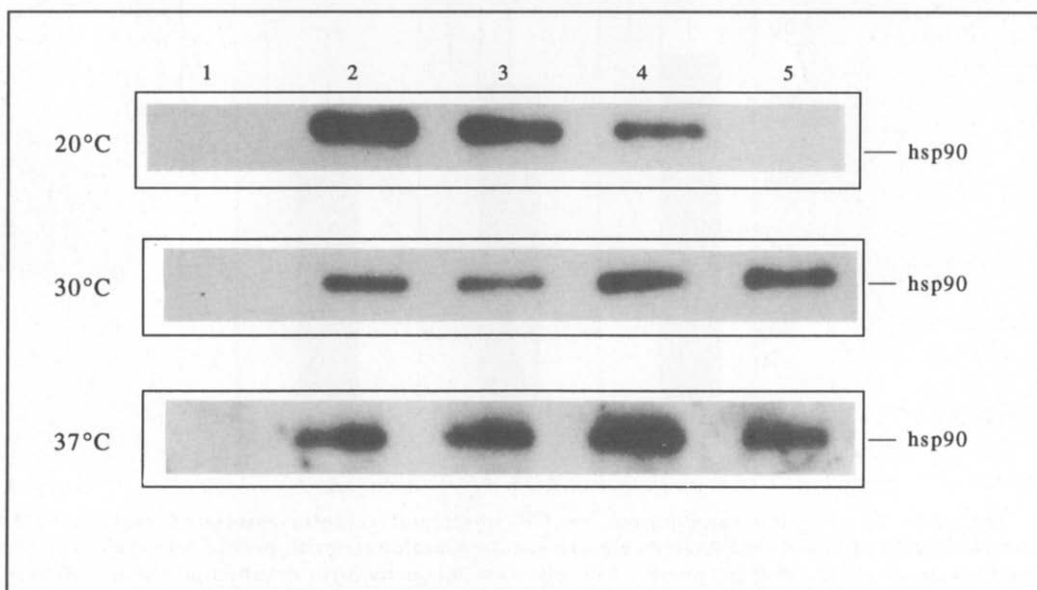

Fig. 5. *In vitro* activation of GRs at different temperatures. Cytosols were incubated overnight with 10^{-7} M dexamethasone at 4°C. Next the temperature was raised to 20, 30 or 37°C. Aliquots were taken at various time intervals, immunoprecipitated and assayed for receptor-associated hsp90 by Western blotting. Lane 1, non-immune serum; lane 2, control ($t = 0$); lane 3, 30 min; lane 4, 60 min; lane 5, 120 min.

the structural integrity of the receptor in the activation experiment.

DISCUSSION

Glucocorticoid signaling is mediated by an intracellular heteromeric receptor protein containing one steroid recipient molecule and probably a dimer of hsp90. The presence of hsp90 in the GR complex is identified with the suppression of DNA binding capacity and the maintenance of a high affinity binding pocket for its cognate ligand. Administration of glucocorticoids prones lymphoid cells for apoptosis (programmed cell death). However, the required steroid incubation time varies from <3 h for non-cycling thymocytes to 48 h for several dexamethasone-sensitive cell lines [23, 24].

Among lymphoid cell lines, S49 cells are the most sensitive, yet they require 12–16 h of steroid exposure for full lysis induction and can be successfully rescued from apoptosis by withdrawal of the agonist before the critical incubation time has been achieved (Fig. 1). We asked if the necessity for prolonged steroid incubation could be explained by an insufficiently activated receptor population. To this end, GRs, in S49 cells committed to lysis, were compared with receptors from control cells or cells from the pre-commitment phase. Receptor characteristics were analyzed in three independent ways: high affinity dexamethasone (re)binding, hsp90–receptor association and nuclear translocation. Re-labeling studies revealed that high affinity binding capacity was present in dexamethasone-incubated cells in levels comparable to those in control cells (Fig. 2). Likewise, hsp90 could co-immunoprecipitate in high levels with the GRs from dexamethasone-challenged cells (Fig. 3). Finally, dexamethasone was capable of relocating the receptor to the nucleus, but the fraction of translocated receptor at any time during dexamethasone incubation was small and the process was fully reversible (Fig. 4). Thus, limited and reversible receptor activation, sufficient to initiate a lytic programme, could be confirmed in intact S49 cells at physiological temperature in three independent experimental approaches: ligand re-binding, hsp90 co-isolation and nuclear translocation. These observations are in plain contrast with receptor activation studies in cytosols in which agonist binding caused rapid and irreversible transformation of the receptor.

Steroid-induced hsp90 dissociation from the receptor complex has been linked to the unmasking of a nuclear localization sequence and is therefore linked to receptor translocation [25]. There are conflicting reports on the degree of agonist-mediated translocation of the GR in S49 cells at physiological temperature. Segnitz and Gehring [19] have reported nearly complete depletion of cytoplasmic receptors by triamcinolone acetonide (TA). Distelhorst and Howard [18], using the same cell-type, showed that a significant amount (up to 60%) of the GR was still present in the cytosolic fraction after dexamethasone (DEX) incubations. As TA is only a slightly more potent agonist than DEX, it is unlikely that the discrepancy between these observations can be explained by the different steroids used in the experiments. Due to the different focus of these studies, further analysis of the receptor complex was only addressed to a limited extent. The use of concentrated cytosols to avoid receptor activation by dilution and the early inclusion of molybdate in our experiments has allowed the detection of limited activation and nuclear translocation of GRs in GC-sensitive S49 cells. Our results indicate that only a minor fraction of GRs is activated during steroid treatment. This finding, however, does not exclude nuclear translocation of the GR while still complexed with hsp90, as was demonstrated by hsp90 complexed, nuclear located receptors in GR.cDNA transfected CHO cells overexpressing the GR [26, 27]. Likewise, the detection of receptors complexed to hsp90 can not be taken as evidence that activation has not occurred. It is possible that nuclear receptors, devoid of hsp90, are exported to the cytoplasm where they are re-assembled with hsp90. Evidence for this cyclic receptor model has been presented previously [28, 29]. Furthermore, it appears that in S49 cells only low steady state levels of activated receptors are required, estimated to amount to approx. 20% (= about 3000 molecules per cell) of total endogenous receptors.

The finding that only a minor fraction of the total receptor population is associated with the nuclear compartment during steroid incubations raises the question what mechanisms control the receptor activation and nuclear translocation. A role for the heat shock proteins present in the GR complex has been suggested [30]. Hsp70 and 90 have been shown to be involved in intracellular and transmembrane protein trafficking [31]. More recently, the involvement of hsp56 has also been proposed by Pratt et al. [32]. Post-translational modification of the receptor has also been suggested as a regulatory element in intra-cellular receptor trafficking. Dalman et al. and others [33, 34] have reported on the steroid-dependent (hyper)phosphorylation of the rat GR. A putative phosphatase dependence of the activation/translocation process has been documented by DeFranco et al. [28] and molybdate, capable of counteracting receptor activation, is acknowledged to be a phosphatase inhibitor [35].

We have reported here on a possible role for the thermolability of the ligand–receptor complex at 37°C as a negative regulator of receptor activation. This is primarily based on the thermolabile ligand–receptor interaction at 37°C and the consequent re-binding capacity. Furthermore, in vitro receptor activation is concentrated cytosols showed that activation at physiological temperature is less effective as compared to lower temperatures, e.g. room temperature (Fig. 5).

The stability of the ligand–receptor complex is much lower at 37°C than at room temperature [20], it is possible that the half-life of the ligand–receptor association at physiological temperatures is too short for efficient transformation. The cause for this thermolabile ligand–receptor association can not be deduced from our present results. The effects could be due to (enzymic) modifications of the heteromeric complex or be a direct consequence of temperature and the structure of the steroid binding pocket.

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