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THE EFFECTS OF *ras* GENE EXPRESSION ON GLUCOCORTICOID RECEPTORS IN MOUSE FIBROBLASTS

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Summary—Analysis of induction of glutamine synthetase activity by dexamethasone showed a 2-fold increase in NIH3T3 but no change in NIH3T3 *ras* (EJ-*ras*) cells. The observed increase could be abolished by the antagonist RU486. The lack of response in *ras* transformed cells might reflect oncoprotein effects on the glucocorticoid receptor (GR). Several GR parameters were studied in order to clarify this point. Total GR level was the same for both cells; cytoplasmic receptor level however, was 3 times lower in NIH3T3 *ras* than in NIH3T3 cells. Hormone-receptor binding affinity, specificity, thermostability, sedimentation coefficient, molecular weight as well as the cytoplasmic GR transformation ratio were similar for the two cell lines. On the other hand, the fraction of the total receptor pool involved with the recycling process was approximately 20% lower in NIH3T3 *ras* than in NIH3T3 cells. After 24 h of dexamethasone treatment, no GR down regulation was observed in NIH3T3 *ras* cells, whereas normal NIH3T3 cells exhibited a decrease of GR binding capacity around 80%. Further studies are necessary to define the mechanisms underlying the association between glucocorticoid insensitivity, and modifications in the GR nuclear/cytoplasmic ratio, in the recycling GR fraction and in the down-regulation process observed in *ras* transformed cells.

INTRODUCTION

The glucocorticoid hormone plays an important role in homeostasis, growth and development by interacting with specific high affinity receptors [1]. This receptor is located in the cytoplasm of cells, associated with the heat shock protein 90 (hsp 90). Glucocorticoid hormone addition results in receptor activation, i.e. dissociation from hsp 90, translocation into the nucleus, tight binding to specific DNA sequences (hormone responsive elements) interaction with the basic transcriptional machinery and stimulation of the rate of transcription [2–4].

Some changes in the regulation of gene expression are associated with oncogenic transformation [5]. Oncoproteins may influence gene expression by interactions with DNA [6] or with specific transcription factors [7]. Alterations of inositol phospholipid metabolism [8] and bradykinin receptor levels [9] are examples of specific signal transduction pathways affected by *ras* oncogene expression.

Glucocorticoid regulation of gene expression may be altered by v-*mos* [10–13], H-*ras* [10, 12] and v-*ras* [11, 12] oncogenes. Since the p37 v-*mos*, p21 H-*ras* and v-*ras* oncoproteins are localized respectively in the cytoplasm [14] and associated to plasma membrane [15], their effects probably could not be due to a direct interaction with DNA. It was proposed that these oncoproteins mediate a glucocorticoid receptor (GR) modification [10–13].

To extend the studies about alterations on cellular glucocorticoid hormone-regulated genes, caused by *ras* oncogene expression, we analysed the dexamethasone regulation of the glutamine synthetase gene measuring its proteic product in NIH3T3 and NIH3T3 *ras* (EJ-*ras*) cells.

Glutamine synthetase (EC 6.3.1.2) induction by glucocorticoid was associated with a functional GR [16, 17] and with an increase of the enzyme mRNA [17]. Moreover the presence of GRE sequences was characterized in the corresponding gene [18].

In an attempt to associate alterations on glucocorticoid-regulated gene expression to

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possible GR abnormalities we investigated properties such as: number, affinity, specificity, thermostability, sedimentation coefficient, molecular weight, DNA binding, recycling and down-regulation in normal and *ras*-transfected NIH3T3 cells.

EXPERIMENTAL

Chemicals

[^{3}H]Dexamethasone (sp. act. 104 Ci/mmol) was obtained from Amersham International, Bucks., England and {6,7-[^{3}H]dexa-methasone-21-mesylate (49.9 Ci/mmol) from New England Nuclear, Boston, Mass, U.S.A.

RPMI 1640, Eagle's without glutamine, phenylmethylsulfonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), soybean trypsin inhibitor, pepstatin, DNA–cellulose (4 mg native DNA/g cellulose), dexamethasone, cortisol, progesterone, dihydrotestosterone and estradiol were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

RU486 (11β-dimethylaminophenyl-17β-hydroxy-17 propynyl-estra-4,9-dien-3-one) was a gift from Roussel–Uclaf, Romainville, France.

Cells

NIH3T3 and NIH3T3 *ras* cells, transfected with the EJ-*ras* oncogene (pEJ 6,6 plasmid) described by Capon *et al.* [19], were kindly supplied by Dr Bernd Groner from the Friedrich Miescher-Institut, Basel.

Cell culture

NIH3T3 and NIH3T3 *ras* cells were cultured in RPMI 1640 medium with 1.2 g/l sodium bicarbonate supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2, 95% O_2 atmosphere at 37°C. In some assays the culture medium was supplemented with 10% charcoal-treated FBS (TS), prepared by a 60 min incubation at 4°C with a dextran-charcoal pellet (2.5% Norit A, 0.25% dextran in 0.01 M Tris–HCl pH 8.0, 1/2 ml of serum). Charcoal was removed by centrifugation and serum stored at −20°C until use.

Subconfluent monolayer cells were harvested by trypsin (0.2%) treatment and neutralized by addition of RPMI plus 10% FBS. The cellular suspensions were collected by centrifugation and washed three times with phosphate buffered saline (PBS). The cellular pellets were resuspended and used for further glucocorticoid quantification and characterization.

Glutamine synthetase assay

NIH3T3 and NIH3T3 *ras* cells were cultured for 3 days in Eagle's medium without glutamine, supplemented with 10% TS with or without addition of dexamethasone, RU 486 or both. The harvested cells were suspended in 0.5–0.1 ml of ice cold PBS containing 10^{-5} M dithiothreitol (DTT). Cellular extracts were prepared as described by Barnes *et al.* [20]. Glutamyl-transferase activity of glutamine synthetase was measured spectrophotometrically (at 505 nm) according to the assay described by Barnes *et al.* [20], except that the reaction mixture contained 0.26 M L-glutamine [21]. Enzyme specific activity was expressed as nmol of γ-glutamylhydroxamate produced per min per mg protein. Protein concentration was determined as described by Lowry *et al.* [22].

Whole cell binding

Binding assays were performed using a modification [23] of the method described by Lippman and Barr [24]. Specific data were analysed by the method of Scatchard [25]. Results were expressed as fmol/10^6 cells.

Cytosol binding

NIH3T3 and NIH3T3 *ras* cells were suspended in TEDG buffer (10 mM Tris/HCl pH 8.5, 1.5 mM EDTA, 2 mM DTT, 20 mM sodium molybdate and 10% glycerol) and homogenized on ice in a polytron PT 10 ST (Brinkman). The homogenate was centrifuged for 1 h at 104,000 *g* at 4°C, and the cytosol recovered. GR binding assays were performed as previously described [26]. Data were analysed by the method of Scatchard[25] and expressed as fmol/mg protein.

Steroid-binding specificity

Cytosol preparations from NIH3T3 and NIH3T3 *ras* cells were incubated with 1.5×10^{-8} M [^{3}H]dexamethasone in the presence and absence of 100-fold molar excess of unlabeled dexamethasone, cortisol, progesterone, dihydrotestosterone and estradiol. Results are expressed as percent reduction in [^{3}H]dexamethasone binding produced by competing unlabeled steroids as compared to the reduction produced by a 100-fold molar excess of unlabeled dexamethasone.

Glucocorticoid hormone–receptor complex thermostability

Cytosols from NIH3T3 and NIH3T3 *ras* cells, prepared in TEDG without sodium

molybdate, were incubated at 4°C for 3 h with 1.5×10^{-8} M [^{3}H]dexamethasone in the absence and presence of a 200-fold excess of unlabeled dexamethasone. After separation of bound from free steroid using dextran-coated charcoal (DCC), labeled cytosol aliquots were incubated at 25°C for 15, 30 and 45 min. These aliquots were cooled at 4°C, incubated again with DCC, centrifuged and the radioactivity of supernatants determined. Percentage of GR-hormone dissociation was determined by the ratio of specific binding after and before heat treatment.

Sucrose density gradient analysis of GR

GR from NIH3T3 and NIH3T3 *ras* cytosols prepared in TEDG were analysed in sucrose density gradients as previously described for estrogen receptor by Lopes *et al.* [27]. Bovine serum albumin (BSA) was run in parallel as a molecular weight marker.

Polyacrylamide gel electrophoresis

Cytosols from NIH3T3 and NIH3T3 *ras* cells were prepared in TEDG (plus 1 mg/ml soybean trypsin inhibitor, 0.5 mM PMSF, 2 mM NEM and 2 mM pepstatin) and incubated with 1.5×10^{-7} M [^{3}H]dexamethasone 21-mesylate in the presence and absence of 3.0×10^{-5} M unlabeled dexamethasone at 4°C for 3 h. At the end of the incubation bound steroid was separated from the free by the use of DCC. Cytosol aliquots (0.1 ml) were submitted to polyacrylamide gel electrophoresis under reducing and denaturating conditions (SDS–PAGE) as described by Distelhorst *et al.* [28]. Kodak XAR-5 film (Eastman Kodak, Rochester, N.Y.) were exposed to gels for 30 days at −80°C. Molecular weight standards were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa).

DNA–cellulose binding of hormone–GR complexes

DNA–cellulose (4 mg DNA native/g cellulose) was stored at −20°C in TEN buffer (10 mM Tris/HCl pH 7.4, 1.0 mM EDTA and 150 mM NaCl) and washed with TEN without NaCl before use. Both 3 cellular lineages were incubated with [^{3}H]dexamethasone and had the cytoplasmic extract prepared as described by Holbrook *et al.* [3]. Cell-free GR transformation was accomplished by incubating the cytoplasmic extract at 25°C for 15, 30 and 45 min. After indicated times 0.15 ml aliquots from cytoplasmic extracts were incubated with a DNA-cellulose pellet (11 mg cellulose/tube) at 4°C for 45 min. The DNA–cellulose pellets were washed 3 times with TES (10 mM Tris/HCl pH 7.4, 1.0 mM EDTA and 20 mM sodium molybdate) and radioactivity determined. The percentage of transformation was calculated by the ratio between DNA–cellulose associated radioactivity and total radioactivity of each cellular lineage after heat treatment.

GR recycling

The cells used in these experiments were cultured 24 h in RPMI supplemented with 10% TS. Cells were incubated with 1.5×10^{-8} M dexamethasone in the presence and absence of unlabeled dexamethasone either at 4°C for 3 h or at 37°C for 1 h. Aliquots from the cells kept for 1 h at 37°C were washed with RPMI and submitted to further incubations at 37°C without steroid for times ranging from 1 to 3 h. After these periods, cells were re-incubated with [^{3}H]dexamethasone at 4°C for 3 h. Following different incubations, cells from all groups were washed, lysed and had the cytoplasmic and nuclear compartments prepared as described by Raaka and Samuels [29]. The receptor percentage in cytoplasm and nucleus was determined by the ratio between the receptor number in each compartment and total receptor number.

GR regulation by dexamethasone

NIH3T3 and NIH3T3 *ras* cells growing in RPMI plus 10% FBS had the serum replaced by TS (dextran-coated charcoal-treated fetal bovine serum). After 4 days in this medium, 10^{-7} M dexamethasone was added during 4 days more. Cell monolayers were then washed with PBS, had their medium changed to RPMI plus 10% TS and were further cultivated for 8 days. Before receptor quantification, cells were harvested by trypsinization, washed 3 times with 50 ml PBS, suspended in RPMI plus 10% TS and incubated at 37°C for 1 h. This procedure allowed steroid dissociation from cells treated with dexamethasone [30, 31]. GR number was determined by the whole cell method.

Statistical analysis

The non-parametric Mann–Whitney test and Student's *t*-test were used at a significance level of $P < 0.05$.

RESULTS

Glutamine synthetase activity

Glutamine synthetase activity basal levels assayed after 3 days of treatment in Eagle's medium without glutamine, supplemented with 10% TS were 28.0 ± 7.0 and 19.0 ± 8.0 nmol/min/mg protein for NIH3T3 and NIH3T3 *ras* cells respectively ($n = 4$). There were no statistical differences between both values. Treatment of the cells with 10^{-7} M or 10^{-6} M dexamethasone increased the glutamine synthetase activity by approximately 2-fold in NIH3T3 cells $P < 0.05$). In contrast, no change in enzyme activity was observed in *ras* cells. The glucocorticoid antagonist RU486 (10^{-6} M) blocked the dexamethasone-mediated increase in enzyme activity of NIH3T3 cells and had no effect in both cellular lineages by itself. The results shown in Fig. 1 are the mean ($\pm$SD) of four different experiences, each one done in triplicate.

Glucocorticoid receptor binding characteristics

Glucocorticoid receptor numbers in whole cells and in cytoplasmic compartment with respective affinities are shown on Table 1. GR studies in NIH3T3 ($n = 16$) and NIH3T3 *ras* cells ($n = 15$) revealed a similar receptor content in whole cells for both cellular lineages ($P > 0.05$). On the other hand GR number concentration in the cytoplasmic compartment was approximately 3-fold higher in NIH3T3 ($n = 14$) than in NIH3T3 *ras* cells ($n = 10$) ($P < 0.001$). Mixing cytosol from *ras* transformed cells with cytosol from NIH3T3 cells gave no evidence of the presence of excess of inhibitory factors or deficiency of positive modifiers in NIH3T3 *ras* cells (data not shown).

There were no differences in receptor affinities between NIH3T3 and NIH3T3 *ras* cells.

Specificity of glucocorticoid receptors

Specificity of cytoplasmic GR was demonstrated by measuring the ability of 1.5×10^{-6} M competing steroid analogs to displace 1.5×10^{-8} M [^{3}H]dexamethasone in the binding reaction Fig. 2 is representative of a typical experiment ($n = 3$). Dexamethasone was more effective than cortisol. Progesterone was also an effective competitor, but competition for glucocorticoid binding by high concentration of progesterone has been previously described in other cell types [16, 23]. Estradiol and dihydrotestosterone showed weak competition for GR binding. Cytoplasmic GR specificity was the same for both cellular lineages.

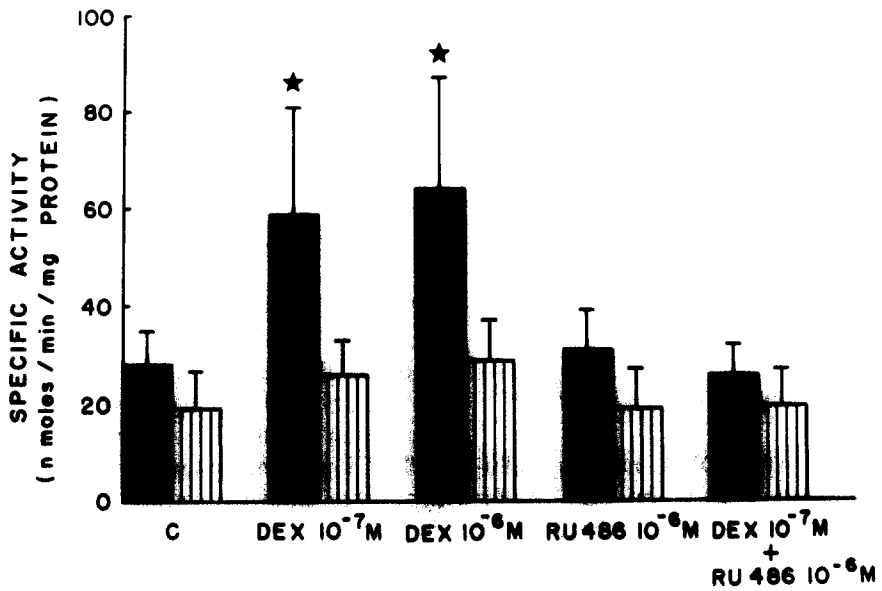

Fig. 1. Effect of dexamethasone and RU486 on glutamine synthetase activity in NIH3T3 and NIH3T3 *ras* cells. NIH3T3 (■) and NIH3T3 *ras* (▥) cells were cultured for 3 days in Eagle's medium without glutamine, supplemented with 10% TS in the absence (C) or in the presence of dexamethasone (DEX) RU486 or both, added at the concentrations shown. Glutamine synthetase was determined in the cellular extracts as described in Methods. Enzyme specific activity was expressed as nmol of γ-glutamylhydroxamate produced per min per mg protein. The height of the columns equals the mean of four different experiences, each one done in triplicate; bars, SD. *$P < 0.05$ as compared to control (C).

Table 1. Total and cytoplasmic glucocorticoid receptor number and respective affinities (K_d) in NIH3T3 and NIH3T3 *ras* cells

	Whole cell		Cytosol	
Cell	Binding capacity (fmol/10^6 cells)	($K_d \pm 10^{-9}$ M)*	Binding capacity (fmol/mg protein)	($K_d \pm 10^{-9}$ M)*
NIH3T3	159.1 ± 35.8	9.7 ± 3.3	212.7 ± 55.4†	3.0 ± 1.4
NIH3T3 *ras*	181.8 ± 81.2	10.2 ± 3.8	65.9 ± 32.1†	3.5 ± 3.0

*Mean ± SE.
†$P < 0.0001$.

Glucocorticoid hormone–receptor complex thermostability

Stability of glucocorticoid hormone–receptor complexes, assayed by submitting labeled complexes to the temperature of 25°C for various time intervals was similar for both cellular lineages (Fig. 3).

Sucrose density gradient analysis of GR

Centrifugation of cytoplasmic [^{3}H]dexamethasone–GR complexes from NIH3T3 and NIH3T3 *ras* cells in a sucrose density gradient, demonstrated specific peaks of radioactivity in the 7.4S and in the 7.3S regions for NIH3T3 and NIH3T3 *ras* cell respectively (Fig. 4).

Determination of the molecular weight of GR

Cytosols from NIH3T3 and NIH3T3 *ras* cells were incubated with [^{3}H]dexamethasone-21-mesylate and then submitted to SDS–PAGE (Fig. 5). Specific binding proteins (GR) may be distinguished from non-specific binding proteins as described by Distelhorst *et al.* [28]. The presence of a GR band corresponding to a molecular weight of 97 kDa was verified in cytoplasmic preparations from NIH3T3 and NIH3T3 *ras* cells. However, two other bands corresponding to molecular weights of 58 and 32 kDa were observed in both cellular lineages. The specific bands detected in NIH3T3 cells were more intense than those seen in NIH3T3 *ras* cells. These results agree with the ones shown in Table 1, where the cytoplasmic GR numbers in NIH3T3 cells are higher than in NIH3T3 *ras* cells. Ratios between the 97, 58 and 32 kDa band intensities were similar for both cellular lineages, suggesting a degradation of the 97 kDa GR in spite of the presence of protease inhibitors. The 58 and 32 kDa forms have not been described by Hoeck *et al.* [33] for NIH3T3 cells.

DNA binding of hormone–GR complexes

DNA–cellulose binding of cytoplasmic GR complexes was determined before and after transformation, as described in Experimental.

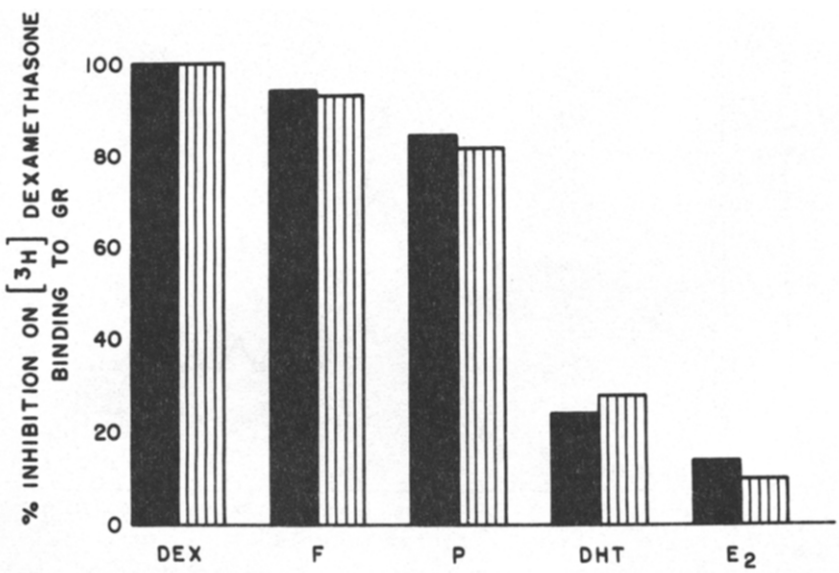

Fig. 2. Specificity of saturable [^{3}H]dexamethasone binding to GR from NIH3T3 and NIH3T3 *ras* cells. Cytosols from NIH3T3 (■) and NIH3T3 *ras* cells (▥) were incubated with 1.5×10^{-8} M [^{3}H]dexamethasone in the presence and absence of a 100-fold excess of unlabeled steroids. The reduction produced by dexamethasone (DEX) was taken as 100%. F = cortisol, P = progesterone, DHT = dihydrotestosterone, E = estradiol.

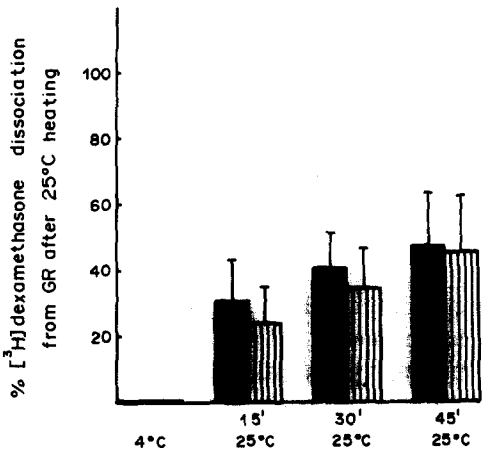

Fig. 3. Thermostability of the hormone–GR complexes. Cytosols from NIH3T3 (■) and NIH3T3 *ras* cells (▥) were incubated with [^{3}H]dexamethasone, in the presence or absence of unlabeled excess of dexamethasone, at 4°C for 3 h. Glucocorticoid hormone–receptor complexes were heated at 25°C for 15, 30 and 45 min. Percentages of complexes dissociation were determined by the ratio between specific binding before and after heat treatment. The height of the columns represents the mean for data obtained from 5 experiments; bars, SD.

Our results showed that heat treatment significantly enhanced DNA–cellulose binding activity of hormone–GR complexes in a similar extent for both cellular lineages (Fig. 6), although absolute levels of transformed GR in NIH3T3 cells were higher than in NIH3T3 *ras* cells.

GR recycling

Incubation of NIH3T3 and NIH3T3 *ras* cells with [^{3}H]dexamethasone at 4°C for 3 h and subsequent separation of the cytoplasmic and nuclear compartments showed that 64.0 ± 7.0% ($n = 4$) of the GR from NIH3T3 and 44.0 ± 7.0% ($n = 4$; $P < 0.05$) from NIH3T3 *ras* cells were in cellular cytoplasm (Fig. 7), according to our results shown in Table 1 where NIH3T3 presented a higher GR cytoplasmic concentration than NIH3T3 *ras* cells. 1 h after incubation at 37°C the cytoplasmic GR concentration decreased to 13.0 ± 11.0% for NIH3T3 and 12.0 ± 11.0% for NIH3T3 *ras* cells as compared to results obtained at 4°C. At the same time the nuclear GR concentration increased to 87.0 ± 11.0% and 86.0 ± 15.0% for NIH3T3 and NIH3T3 *ras* cells respectively. These results confirmed those obtained by DNA–cellulose binding (Fig. 6), where the cytoplasmatic GR transformation ratio was the same for both cellular lineages. The decrease in the cytosol concentration after treatment of cells with [^{3}H]dexamethasone was reversed within 3 h at 37°C after steroid withdrawal. At this time the GR number in the cytoplasmic compartment was the same as that obtained initially when both cells were incubated with [^{3}H]dexamethasone at 4°C for 3 h. A similar GR

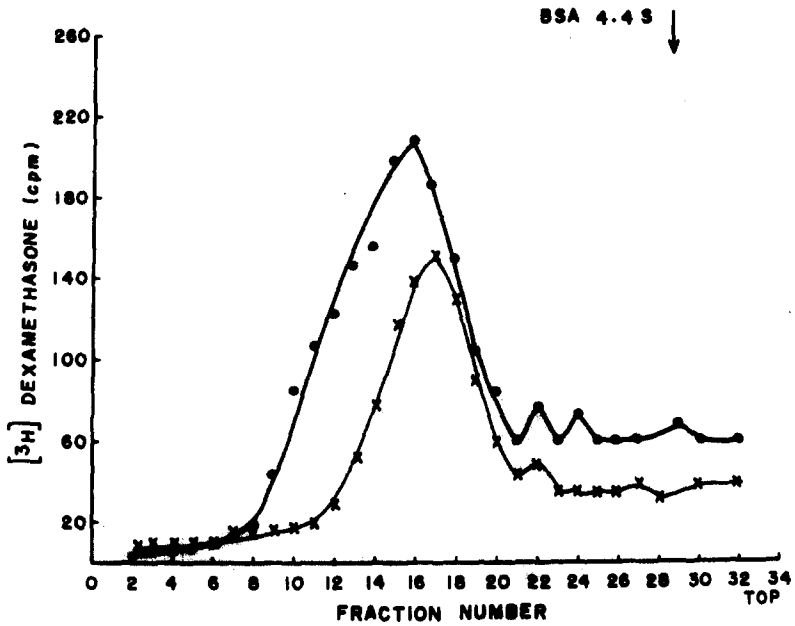

Fig. 4. Sucrose density gradient centrifugation analysis of GR. Cytosols (3 mg protein/ml) from NIH3T3 (●——●) and NIH3T3 *ras* cells (×——×) were incubated with [^{3}H]dexamethasone in the presence or absence of an unlabeled excess of dexamethasone at 4°C for 3 h; followed by DCC treatment. Aliquots were layered on 5–20% sucrose density gradients. After ultracentrifugation, gradients were fractioned and radioactivity was counted. The points represent the specific binding. Bovine serum albumin (BSA) was run in parallel as a molecular weight marker.

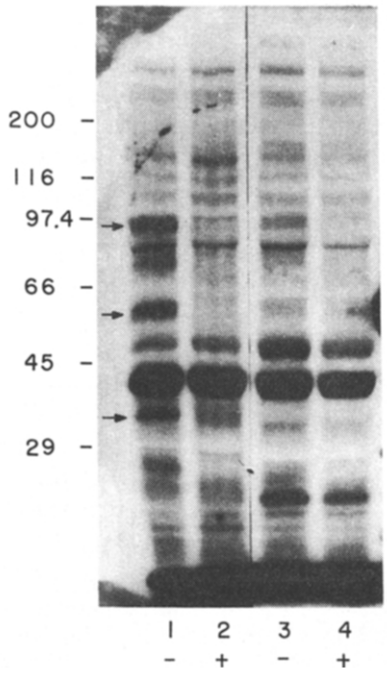

Fig. 5. Autoradiogram of affinity-labeled GRs from NIH3T3 and NIH3T3 *ras*. Cytosols aliquots from NIH3T3 (690 μg protein; lanes 1 and 2) and NIH3T3 *ras*, (870 μg protein; lanes 3 and 4) were incubated with [^{3}H]dexamethasone 21-mesylate in the absence (−) and presence (+) of unlabeled excess dexamethasone. Cytosol samples were analysed by SDS–polyacrylamide gel electrophoresis. Molecular weight (kDa) of standard proteins are indicated on the left. GR specific bands of 97, 58 and 32 kDa are indicated by arrows.

distribution for NIH3T3 has been recently reported by using an immunoblot procedure [34]. Thus, absolute number of receptors involved with the recycling process was equivalent to the initial cytoplasmic GR levels for both cells and therefore lower for NIH3T3 *ras* than for NIH3T3 cells.

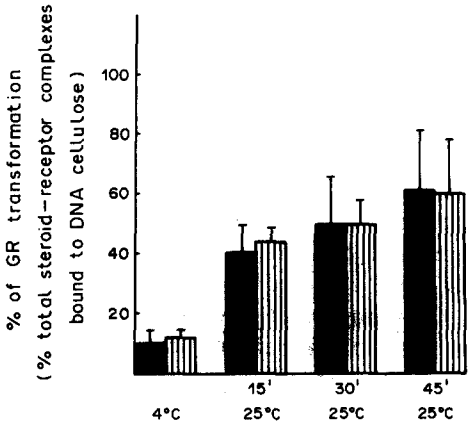

Fig. 6. Binding of [^{3}H]dexamethasone–receptor complexes to DNA NIH3T3 (■) and NIH3T3 *ras* cells (▥) were incubated with [^{3}H]dexamethasone in the presence and absence of unlabeled dexamethasone at 4°C for 3 h. Cells were lysed and cytoplasmic extracts incubated at 25°C for 15, 30 and 45 min. The amounts of transformed dexamethasone–receptor complexes were determined by a DNA–cellulose binding assay, as described in Experimental. The results presented are the mean for data obtained from 4 experiments; bars, SD.

GR regulation by dexamethasone

GR concentration was determined in NIH3T3 and NIH3T3 *ras* whole cells after different treatments. In spite of slight variations, no statistical differences in GR concentration were determined in cells maintained in TS (medium containing bovine fetal serum stripped of endogenous hormones by dextran-coated charcoal) for 7 days for both cellular lineages (Figs 8A and B). 1 day after exposure to dexamethasone (5th day), the whole cell glucocorticoid receptor content in NIH3T3 cells (Fig. 8B) decreased 60–80% ($P < 0.05$), reaching a new steady state maintained during the period of dexamethasone treatment. Upon removal of dexamethasone and incubation in hormone-free medium, the original GR numbers were totally restored after 2 days.

On the other hand, no decrease in basal GR content was observed in NIH3T3 *ras* cells after dexamethasone treatment or after hormone was withdrawn (Fig. 8A).

DISCUSSION

Our data present an increased enzyme activity in NIH3T3 cells after treatment with 10^{-7} M dexamethasone, a glucocorticoid concentration previously shown to induce glutamine synthetase in L6 cells [17]. The glucocorticoid antagonist RU486 [32] blocked dexamethasone-mediated increase in enzyme activity, suggesting a mechanism mediated via interaction of the steroid hormone with intracellular GR. On the other hand NIH3T3 *ras* cells did not show glutamine synthetase induction by 10^{-7} M or 10^{-6} M dexamethasone; this absence of inducibility by glucocorticoid might result from oncogene expression effects on the GR.

Earlier studies reported that glucocorticoid resistance might be caused either by a reduced affinity [35] or by a decrease in GR numbers [36]. Our results however showed the same GR total levels and affinities for NIH3T3 and NIH3T3 *ras* cells. Nevertheless, cytoplasmic receptor levels were 3 times lower in NIH3T3 *ras* than in NIH3T3 cells. The reduction of receptor concentration in cell cytosol was analogous to that observed by Chrousos *et al.* [35] in fibroblasts from two glucocorticoid-

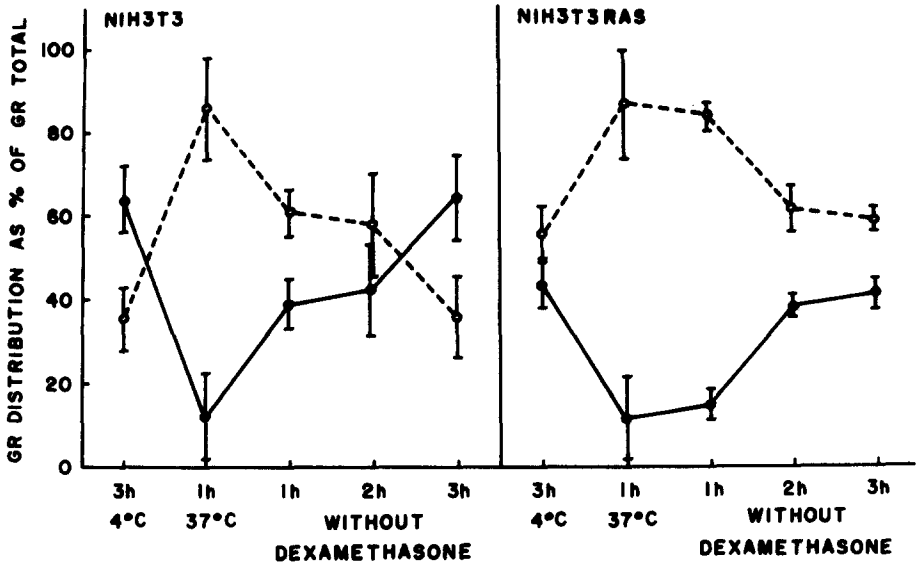

Fig. 7. GR distribution after [^{3}H]dexamethasone treatment and [^{3}H]dexamethasone withdrawal from NIH3T3 and NIH3T3 *ras* cells. NIH3T3 (left panel) and NIH3T3 *ras* (right panel) cells were maintained for 24 h before the assay in medium supplemented with 10% dextran-coated charcoal treated FBS (TS). Cells were incubated with [^{3}H]dexamethasone at 4°C for 3 h or at 37°C for 1 h. Aliquots from the last cell group were then washed and incubated at 37°C for 1, 2 and 3 h in medium without steroids. After these periods cells were re-incubated with [^{3}H]dexamethasone at 4°C for 3 h. All cellular groups were washed and lysed. Cytoplasmic (●——●) and nuclear (○- - -○) compartments were separated and GR present in each one determined as percentage of total GR. Points are the mean of 3 experiments. Bars, range.

resistant patients. These authors supposed that instability of the receptor was occurring in cytoplasmic preparations as a consequence of GR low affinity observed in those cells. In our cells, however, no differences in GR affinities and thermostability were observed; thus, the lower cytoplasmic GR in NIH3T3 *ras* cells was not a consequence of excessive hormone dissociation from the complex.

Both cellular lineages have GR with the same specificity for glucocorticoids and similar to those previously described [16, 23].

The lower cytoplasmic GR levels in NIH3T3 *ras* and the same total receptor levels for both cellular lineages suggested a preferential GR nuclear localization in transformed cells. This could be explained by the presence of a truncated 40 kDa receptor similar to that described by the S49 mutant nt^i [37]. Both cellular lineages showed however, normal 97 kDa receptors. Smaller bands probably represent proteolytic fragments. Moreover receptor sedimentation coefficients were approximately 8S which suggested a normal GR association with hsp90 [3].

Glucocorticoid resistance was described in another S49 mutant, nt$^-$ [37]. These cells presented GR–hormone complexes with low DNA binding capacity after GR thermal transformation. In spite of the lower cytoplasmic GR absolute number in NIH3T3 *ras* cells, we found that both cellular lineages have GR–hormone complexes with the same DNA binding ability (Fig. 6).

Transformed GR–hormone complexes have to bind to specific DNA sequences (GRE) to modulate gene transcription [4]. The methodology used here did not allow us to check if receptor–hormone complexes would be able to distinguish between specific and non-specific DNA sequences. Therefore, the possibility that complexes from NIH3T3 *ras* cells are unable to recognize GRE cannot be discarded.

Glucocorticoid receptor recycling was described as an important step in the appropriate physiological response to the hormone [38]. Alterations in the glucocorticoid regulation of the metallothionein gene in v-*mos* transformed cells were reported to be consequence of a modification on GR recycling [39]. Our results suggested that apparently, only the fraction of receptors found initially in the cytoplasm at 4°C seems to be involved in the recycling process. Therefore the fraction of the total receptor pool associated with this process was lower in NIH3T3 *ras* (44%) than in NIH3T3 cells (64%) (Fig. 7). Although a diminished proportion of recycling GR might alter glutamine synthetase gene regulation, this difference seems insufficient to be considered as the only cause for the observed hormone insensitivity in *ras* transformed cells.

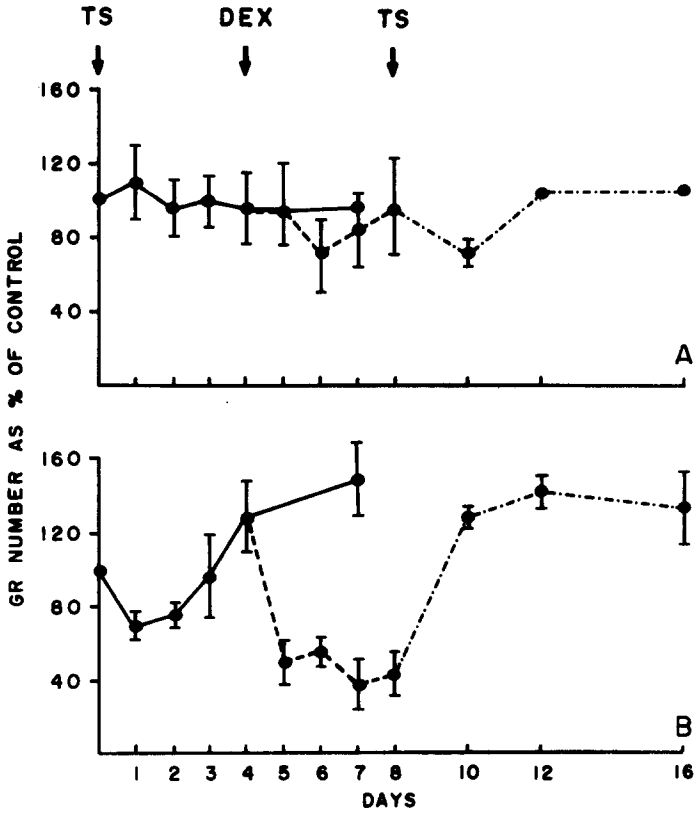

Fig. 8. Effect of dexamethasone on GR concentration in intact cells. NIH3T3 *ras* (A) and NIH3T3 (B) cell cultures were maintained in TS (medium containing bovine fetal serum treated with dextran-coated charcoal) for 4 days (●——●) and then exposed to 10^{-7} M dexamethasone (DEX) for 4 more days (●---●). Cultures were washed and fresh medium without dexamethasone (TS) was added for the next 8 days (●-•-•-•-●). Aliquots were taken to determine receptor concentration in whole cells as described in Experimental. During the period of dexamethasone addition, a parallel group of control cells were grown in TS, until day 7, (●——●). The results presented are the mean ± SD of four experiments (except for points without bars, 2 experiments). The initial GR concentration considered as 100% was 120.0 ± 18.0 fmol/10^6 cells for NIH3T3 and 140.0 ± 25.0 fmol/10^6 cells for NIH3T3 *ras* cells.

Reports from Jaggi *et al.* [11], Hamilton and DeFranco[13] and Jaggi *et al.* [12] described a transient glucocorticoid induction of MMTV [11], of metallothionein [13] and of tyrosine aminotransferase gene expression [12] in cells transformed respectively by v-*ras* and v-*mos*, v-*mos* and H-*ras* oncogenes. We are showing an absence of glutamine synthetase induction in *ras* transformed cells, which might suggest a diverse pathway in gene regulation, maybe involving different changes of the GR protein. However we did not discard the possibility of a transient enzyme induction in *ras* transformed cells that was not detectable by our assays.

Jaggi *et al.* [12] explained the transient gene regulation by a higher GR down-regulation in *ras* transformed NIH3T3 cells. Our results indicated that after 24 h of hormone treatment only 20–30% of the total GR amounts remained in normal NIH3T3 cells. Similar data were observed by Hoeck *et al.*[33] in NIH3T3 cells using an anti-GR antibody. On the other hand GR levels in NIH3T3 *ras* cells were unchanged even after 4 days of dexamethasone treatment, therefore failing to be down-regulated (Fig. 8A). The discrepancies among our results and those from Jaggi *et al.* [12] might be due to the conditions used for oncogene expression. The NIH3T3 *ras* cell line used by us had a constitutive oncogene expression while those used by Jaggi *et al.* [12] had the oncogene expressed only after exposure to 42°C.

It has been described that at least part of the GR down-regulation is conferred by GR repression of its own gene transcription [40]. One could speculate that a more complex alteration of GR, which has not been detected by our present assays hinders glucocorticoid to regulate gene induction and gene repression in NIH3T3 *ras* cells.

Hormone-dependent phosphorylation was observed in transformed glucocorticoid [33] and progesterone [41] receptors. This phosphorylation might influence the affinity of receptor for specific genes [41] or control the quality or magnitude of transcriptional activity of regulatory elements [33, 41]. The return of a binding component from the nucleus to the cytoplasmic compartment (recycling) is ATP dependent [38]. It was speculated that GR hyperphosphorylation in the nucleus might serve to destabilize its interactions with DNA and thus play some role in the cycling of receptor in the cell [42].

Jaggi *et al.* [10] and Hamilton and DeFranco [13] proposed that the V-*mos* protein could directly alter GR phosphorylation as this oncoprotein is a serine kinase [14]. However no transphosphorylating activity has been detected with any *ras* protein [15]. The *ras* transformation reported to be was associated with modified activities of phosphatidyl inositol kinase, phosphatidyl inositol 4-phosphate kinase, diacylglycerol kinase and protein kinase C [43]. An effect of the *ras* oncoprotein, on other kinase activities possibly involved with receptor phosphorylation, and thus indirectly interfering with nuclear receptor retention and receptor recycling could be assumed. Nevertheless, as receptor down-regulation is independent of receptor phosphorylation [33, 41], other GR changes might be required. More studies will be necessary to better characterize the exact GR modifications in these cells that could account for abnormal regulation of gene expression.

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