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Potential Mechanisms Underlying Autoregulation of Glucocorticoid Receptor mRNA Levels in the DHD/K12/PROb Rat Colonic Adenocarcinoma Cell Line*

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The DHD/K12/PROb rat colonic epithelial cell line, which was originally derived from a chemically induced adenocarcinoma, expresses functional glucocorticoid receptors (GR) and has been reported to be growth inhibited by glucocorticoid agonists. In the present study the potential mechanisms underlying corticosteroid-mediated autoregulation of GR mRNA levels in this colonic cell line were investigated. The GR mRNA levels in the various treatment groups were quantitated via the ribonuclease protection assay using a specific ^{32}P-cRNA probe. Time-course experiments demonstrated that in contrast to several other cell lines that are also growth inhibited by glucocorticoids, treatment of confluent monolayers of PROb cells with the pure GR agonist RU 28362 (1 μM) elicits a rapid and significant (65%) down-regulation of GR mRNA levels that is sustained for at least 36 h. This down-regulation, which is also elicited to a lesser extent by weaker GR agonists including corticosterone and aldosterone, is blocked by the GR antagonist RU 38486. The protein synthesis inhibitor cycloheximide was utilized to demonstrate that the initial phase (6 h) of agonist-mediated down-regulation occurs independently of ongoing protein synthesis, although new protein synthesis, perhaps of the GR protein itself, is required to maintain this down-regulation. Although agonist-mediated down-regulation in these cells probably occurs primarily at the level of GR gene transcription, inhibition of ongoing RNA synthesis with actinomycin D or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) demonstrated that during the initial phase (1 h) of this down-regulation, but not following maximal (18 h) down-regulation, RU 28362 treatment also significantly reduces the stability of the GR mRNA.

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INTRODUCTION

Glucocorticoid hormones influence the proliferation, differentiation and metabolism of a wide variety of target tissues and cell types. Although the effects of these steroids on the growth of colonic tumors and cell lines has not been investigated extensively, glucocorticoids have been reported to promote proliferation of chemically-induced rat colon tumors [1] and the HC845 human colon carcinoma cell line [2]. In contrast, these hormones have also been reported to inhibit the growth of several other rodent and human solid colon tumors [3] and cell lines, including the DHD/K12/PROb rat colonic adenocarcinoma cell line [4]. These glucocorticoid-mediated antiproliferative effects are mediated by intracellular glucocorticoid receptors (GR) which bind these hormones with high affinity and specificity. Once the steroid has promoted the activation/transformation of these cytoplasmic receptors (reviewed in [5]) the complexes are translocated into the nucleus where they bind to specific sequences termed hormone response elements. The consequence of this nuclear binding is either the enhancement or repression of the rate of transcription of

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Abbreviations: DMEM, Dulbecco's Minimal Essential Medium; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; GR, glucocorticoid receptor; TCA, trichloroacetic acid.

glucocorticoid-responsive genes (reviewed in reference [6]), which may include the GR gene itself.

Since the glucocorticoid sensitivity or responsiveness of target cells is directly correlated with the number of functional GR [7–9], regulation of the expression of the GR gene constitutes an important homeostatic mechanism. Numerous published studies have in fact reported that GR are autoregulated by their cognate ligand (reviewed in [10]), which suggests that the duration and extent of a glucocorticoid-mediated response may be modulated by the steroid itself. For example, Fukawa *et al.* [11] have demonstrated that homologous down-regulation of GR levels in human lymphoma cells attenuates hormonal responsiveness. Although agonist-mediated down-regulation of endogenous GR mRNA levels has been detected in a variety of target tissues [12–14] and cell types [15–17], it is clearly not common to all target tissues. For instance, glucocorticoid-mediated up-regulation of GR mRNA levels has been reported in a number of cell lines that are growth-inhibited by glucocorticoids [18–21]. Regardless of whether autoregulation is negative or positive in nature it appears to occur primarily at the level of GR gene transcription. This conclusion is based on the results of direct nuclear transcription assays [15, 16] as well as the fact that addition of the RNA synthesis inhibitor, actinomycin D, after maximal up-regulation [22] or down-regulation [14–16] has been attained, has failed to reveal any glucocorticoid-mediated alteration in GR mRNA half-life. Several laboratories have also utilized cycloheximide to evaluate the potential role of ongoing protein synthesis in glucocorticoid-mediated autoregulation and have concluded that both down-regulation [23] as well as up-regulation [22] of GR mRNA levels occur independently of protein synthesis.

In the present series of experiments we have utilized the DHD/K12/PROb rat colonic epithelial cell line to further investigate autoregulation of GR mRNA levels. This subline was isolated from a 1,2-dimethylhydrazine-induced colonic adenocarcinoma [24, 25]. These PROb cells express GR which based on several criteria [4] appear identical to GR expressed in normal rat tissues. These PROb cell GR are clearly functional, as evidenced by the fact that treatment of monolayers with glucocorticoid agonists elicits growth inhibition as well as secretion of fibonectin and an unidentified 40 kDa protein [4, 26]. These colonic epithelial cells have been utilized in this study to further evaluate potential mechanisms underlying the autoregulation of GR mRNA levels.

MATERIALS AND METHODS

Chemicals and reagents

Uridine 5′-[α-^{32}P]triphosphate ([^{32}P]-UTP) (800 Ci/mmol) and Hyperfilm-MP were purchased from Amersham, Arlington Heights, IL. RU 38486 and RU 28362 were generous gifts supplied by Roussel-Uclaf, Romainville, France. Restriction enzymes and *in vitro* transcription kits (Riboprobe II Core System) were purchased from Promega, Madison, WI. Ribonuclease protein assay reagents (RPA II Kit) and the β-actin cDNA were obtained from Ambion, Austin TX. RNAzol was purchased from Tel-Test, Inc., Friendswood, TX. Aldosterone, corticosterone, actinomycin D, DRB and other reagent grade chemicals were obtained from Sigma, St Louis, MO. Tissue culture media and antibiotics were purchased from the Diabetes and Endocrinology Research Center at The University of Iowa. Fetal calf serum and trypsin/EDTA solution were obtained from Gibco, Hazleton PA. Culture flasks and miscellaneous disposable culture supplies were obtained from Corning, Corning, NY.

Conditions of cell culture

The DHD/K12/PROb cells were routinely grown in Corning 25 cm^2 flasks in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin plus streptomycin in a humidified atmosphere at 37°C and 5% CO_2 in air. The media was replenished several times per week and the cells were subcultured every 2 weeks. The subculturing and harvesting of the cells were performed by washing monolayers three times with 0.25% trypsin/0.02% EDTA solution and subsequently incubating for 5–10 min at 37°C in a minimal volume of the same solution. Cells stored under the vapor phase of liquid nitrogen were periodically thawed to minimize any potential alterations in the properties of these cells during the course of the experiments.

Synthesis of riboprobes

The GR antisense RNA probe was synthesized using the pXGR14 transcription vector, which was a gift of Dr K. Yamamoto. This pBluescript vector contains a 1.5 kb fragment of the GR cDNA which corresponds to sequences in the amino terminal domain and in the DNA binding domain of the rat GR. In order to synthesize an appropriate probe for ribonuclease protection assays (RPA), it was first necessary to remove a *SmaI-SmaI* fragment containing sequences encoding the GR DNA binding domain. After removal of this fragment and religation of the vector, transcription of the *XhoI* linearized template with bacteriophage T7 RNA polymerase generates a 550 nucleotide probe that contains 446 nucleotides corresponding to GR cDNA sequences. The β-actin antisense RNA probe that was routinely utilized was transcribed using SP6 RNA polymerase to generate a 300 nucleotide probe containing 250 nucleotides corresponding to β-actin cDNA sequences. The antisense [^{32}P]-cRNA probes specific for either the GR or β-actin mRNA were synthesized using [^{32}P]-UTP and the Riboprobe II Core System.

Synthesis was performed according to the manufacturer's instructions and was followed by incubation with 5 units of DNAse 1 for 15 min at 37°C to digest template DNA. Following DNAse 1 digestion the riboprobes were purified on 6% polyacrylamide/urea gels and the full length probes were eluted from the gel for several hours at 37°C using probe elution buffer included in the RPA II kit.

Isolation of RNA and ribonuclease protection assays

Total cellular RNA from harvested PROb cells was isolated using the commercially available RNAzol reagent, which is a modification of the single-step procedure originally developed by Chomczynski and Sacchi [27]. The concentration of RNA in each sample was determined by spectrophotometry and the quality was verified by electrophoresis on a 1.0% agarose/formaldehyde gel and subsequent visualization with ethidium bromide staining. Ribonuclease protection assays were performed utilizing the RPA II kit, which is a modification of the solution hybridization procedure originally described by Lee and Costlow [28]. Following electrophoresis of the RNase-treated samples on 6% polyacrylamide/urea gels, the gels were exposed to Hyperfilm-MP at −70°C for various periods of time. Two separate exposures were necessary for each gel to insure that both the β-actin mRNA signal and the GR mRNA signal fell within the linear range appropriate for densitometric scanning. The resulting β-actin and GR mRNA protected fragments were quantitated by densitometric scanning (LKB Ultroscan Scanning Densitometer) of the autoradiograms (see Fig. 1 for sample autoradiogram) and the appropriate peak areas were automatically integrated. In order to perform reliable quantitation of GR mRNA levels it was important to determine in preliminary experiments the linear range of the assay, which is dependent on both the amount of total RNA in each sample as well as the linear range of the densitometric scanning. In each experiment the control treatment group (GR mRNA/β-actin mRNA) was normalized to 1.0 or 100, and the other treatment groups were expressed relative to this control group. For quantitation of GR mRNA levels all experiments were performed at least twice with duplicate flasks for each treatment group. The data from identical experiments were pooled and the total number of individual flasks per treatment group are indicated in the individual figure legends.

Western blotting

Western blotting of total (cytoplasmic plus nuclear) PROb cell GR protein was conducted using the $BuGR_2$ monoclonal antibody and the Enhanced Chemiluminescence detection system as previously described by our laboratory [14].

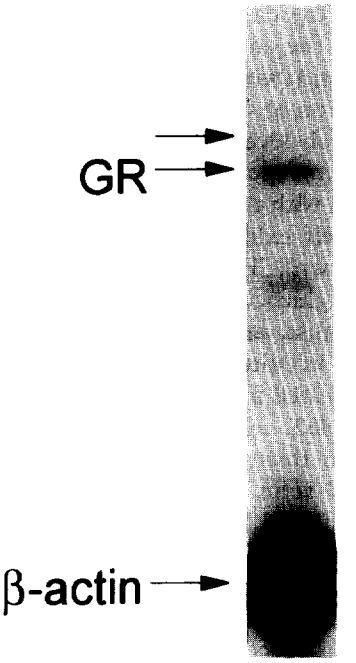

Fig. 1. Autoradiogram depicting ribonuclease protection assay for quantitation of GR mRNA levels. Quantitation of GR mRNA levels with ribonuclease protection assay was performed using an aliquot (30 μg) of PROb cell RNA. The upper arrow indicates the position of the input GR [^{32}P]-cRNA probe (550 nt), the GR arrow indicates the position of the protected GR mRNA fragment (446 nt), and the lower arrow indicates the position of the protected β-actin mRNA fragment. The β-actin mRNA level detected in each lane was used to normalize the GR mRNA level detected in the same lane. Much shorter exposures were also used to insure that the [^{32}P]β-actin mRNA fragment, which is overexposed in this autoradiogram, fell within the linear range for densitometric scanning. The faint unmarked band, which was routinely detected, may have resulted from further RNase digestion of the GR mRNA protected fragment, since the intensity of this faint band paralleled that for the protected fragment.

Statistical analysis

Experimental results are expressed as means ± standard errors. The number of culture flasks in each treatment group is indicated in the figure legend. Statistical analyses were performed using the Microsoft Excel version 4.0 software package. The Student's *t*-test (assuming equal or unequal variance as appropriate) was used to determine statistical significance between treatment groups.

RESULTS

Homologous regulation of GR mRNA levels

In order to evaluate the potential effects of a pure GR agonist on GR mRNA levels, confluent monolayers of PROb cells were treated with 1 μM RU 28362 for various lengths of time. Total RNA was subsequently isolated from the individual flasks and subjected to ribonuclease protection assays for quantitation of GR and β-actin mRNA levels. The GR mRNA/β-actin mRNA ratios from the control (time 0) flasks were set

at 100% and the treatment flasks were expressed as a percentage of the normalized control value. As is seen in Fig. 2, a rapid (within 1 h) and sustained (up to 36 h) down-regulation of GR mRNA levels to approx. 35% of the control levels was detected. This agonist-mediated down-regulation was completely blocked by co-incubation with the pure GR antagonist RU 38486, which by itself exhibited no partial agonist activity (data not shown). To evaluate the ability of the endogenous rat glucocorticoid, corticosterone, as well as the weak agonist, aldosterone, to down-regulate GR mRNA levels in cultured PROb cells, confluent monolayers were treated for 6 h with a final concentration of 1 μM RU 28362, corticosterone, or aldosterone. Following steroid treatment, RNA was isolated and GR mRNA and β-actin mRNA levels quantitated as previously described. The GR mRNA/β-actin mRNA ratio from the control flasks, which received ethanol vehicle alone, was set at 100%, and the GR mRNA/β-actin mRNA ratios from the various treatments were expressed as a percentage of the control value. As is seen in Fig. 3, although all three agonists significantly ($P < 0.05$) decreased GR mRNA levels following a 6 h treatment, RU 28362 appeared to be more effective in mediating this negative autoregulation.

Effects of cycloheximide on RU 28362-mediated homologous down-regulation of GR mRNA levels

The next series of experiments utilized the protein synthesis inhibitor cycloheximide to evaluate the necessity for ongoing protein synthesis during the initial phase of homologous down-regulation (6 h of RU 28362 treatment) as well as following maximal down-regulation (18 h of RU 28362 treatment). In preliminary studies, the concentration of cycloheximide

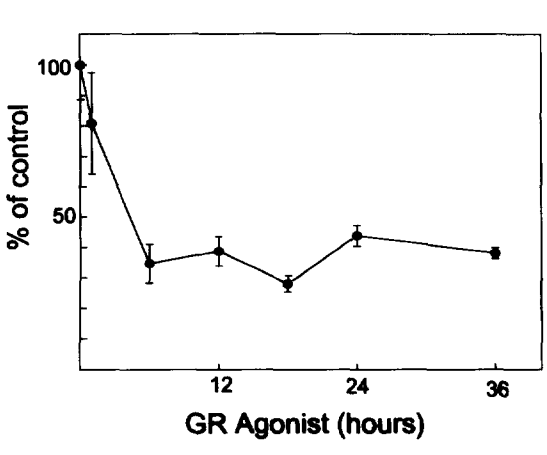

Fig. 2. Time-course for homologous down-regulation of GR mRNA levels. Monolayers of PROb cells were treated with 1 μM RU 28362 for various lengths of time. RNA from the individual flasks was then isolated and the GR and β-actin mRNA levels were subsequently quantitated via ribonuclease protection assays as described in Materials and Methods. The GR mRNA/β-actin mRNA ratios at time zero were set at 100%. The data from two experiments each performed in duplicate have been averaged and the results have been plotted plus or minus the standard error of the means.

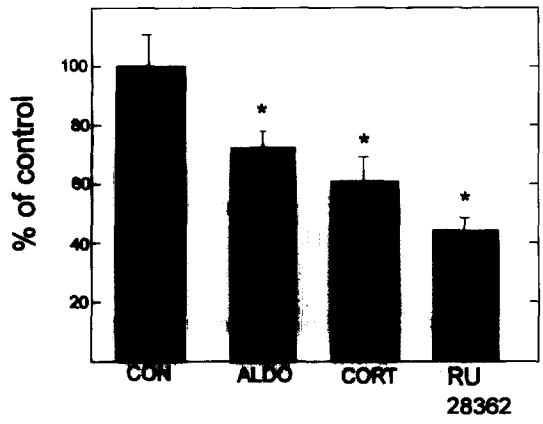

Fig. 3. Effects of aldosterone, corticosterone and RU 28362 on GR mRNA levels in cultured PROb cells. Monolayers of PROb cells were treated for 6 h with a final concentration of 1 μM steroid. RNA from the individual flasks was then isolated and the GR and β-actin mRNA levels were subsequently quantitated via the ribonuclease protection assays as described in Material and Methods. The GR mRNA/β-actin mRNA ratio in the control (minus steroid) flasks was set at 100% and the treatments are expressed relative to the control value. The average of 5 individual flasks for each treatment in a single experiment was determined and plotted plus the standard error of the mean. *Indicates a significant difference at $P < 0.05$.

(10 μg/ml) selected for utilization in these experiments was found to be effective in inhibiting >95% of [^{3}H]leucine incorporation into TCA precipitable proteins (data not shown). In the experiments designed to evaluate the role of continued protein synthesis during the initial phase of down-regulation, confluent monolayers of PROb cells were treated with cycloheximide for 12 h followed by addition of RU 28362 (1 μM) for an additional 6 h. As the data presented in Fig. 4 (Panel A, solid bars) demonstrate, this 6 h treatment with the agonist resulted in significant ($P < 0.05$) down-regulation of GR mRNA levels. Treatment with cycloheximide alone caused superinduction and increased GR mRNA levels by approx. 1.8-fold. As indicated by the data, a 12 h pretreatment with cycloheximide had no effect on the subsequent ability of the GR agonist to mediate repression of GR mRNA levels. This lack of effect is more clearly seen in Panel B (solid bars), in which the GR mRNA level detected in the cycloheximide-treated cells has been normalized to 100%. The 6 h RU 28362 treatment resulted in a 50% decrease (significant at $P < 0.05$) in GR mRNA levels in both the absence (Panel A) or presence of (Panel B) of cycloheximide. These results demonstrate that continued protein synthesis is not required for, nor does it enhance, the initial phase of homologous down-regulation of GR mRNA levels.

In experiments designed to evaluate the potential role of ongoing protein synthesis in the maintenance of homologous down-regulation of GR mRNA levels, confluent monolayers of PROb cells were treated for

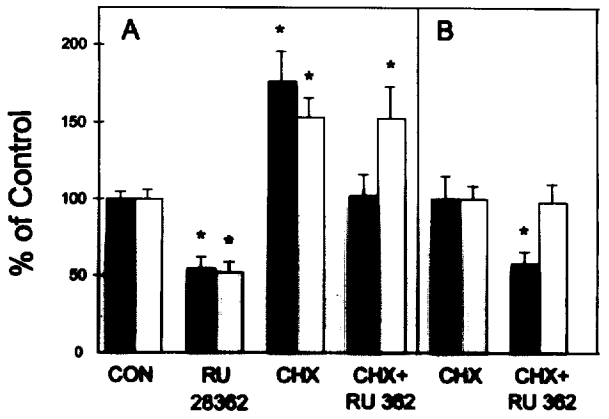

Fig. 4. The effects of protein synthesis inhibition on the initial and maintenance phases of homologous down-regulation of GR mRNA levels. Monolayers of PROb cells were incubated with 1 μM RU 28362, cycloheximide (CHX, 10 μg/ml) alone, or cycloheximide plus RU 28362 for 18 h. In the combined treatment designed to evaluate the role of ongoing protein synthesis during the initial phase of homologous down-regulation (solid bars), cells were treated with cycloheximide for 12 h prior to addition of 1 μM RU 28362 and incubation for an additional 6 h. In the combined treatment designed to evaluate the role of ongoing protein synthesis during the maintenance phase of homologous down-regulation (open bars), cells were treated with cycloheximide plus RU 28362 for 18 h. At the end of the various incubations RNA from individual flasks was isolated and the ribonuclease protection assay was subsequently performed for quantitation of GR and β-actin mRNA levels as described in Materials and Methods. The GR mRNA/β-actin mRNA ratios from the control flasks were set at 100% and the treatment values are expressed as a percentage of this normalized value. Each experiment was performed twice and the mean values of each treatment group (control, $n = 6$; RU 28362, $n = 6$; chx, $n = 8$; chx plus RU 28362, $n = 8$) plus the standard error of the means are plotted. In Panel A, the means of the cycloheximide-treated flasks are expressed relative to the non-treated controls. In Panel B, the means of the cycloheximide-treated flasks have been set at 100% and the mean of the cycloheximide plus RU 28362 treated flasks is expressed relative to this control (this normalization facilitates comparison with the effects of RU 28362 in the absence of cycloheximide shown in Panel A). *Indicates a significant difference at $P < 0.05$.

18 h with RU 28362 plus cycloheximide. As seen in Fig. 4 (Panel A, open bars) treatment with RU 28362 alone for 18 h again resulted in a 50% decrease (significant at $P < 0.05$) in GR mRNA levels. Treatment with cycloheximide alone again resulted in superinduction and increased GR mRNA levels by approx. 1.8-fold. In Panel B (open bars) the GR mRNA/β-actin mRNA ratio detected in the cycloheximide treated cells has again been normalized to 100% and the RU 28362 plus cycloheximide ratio has been expressed relative to this value. As shown by the data, co-incubation with cycloheximide abolished the response to RU 28362, which demonstrates that the continued synthesis of an unidentified protein(s) is required for the maintenance of this repression. Taken collectively, the results presented in Fig. 4 (Panels A and B) demonstrate that although ongoing protein synthesis and/or the glucocorticoid-mediated induction of a specific protein(s) is not required for the initial phase of glucocorticoid-induced repression of PROb cell GR mRNA levels, new protein synthesis is required for the continued maintenance of this negative autoregulation.

These data suggested that after an 18 h incubation of PROb cells with RU 28362 in the presence of a protein synthesis inhibitor the GR protein levels may be down-regulated below the minimum threshold level required for continued repression of GR mRNA levels. To test this hypothesis total (cytoplasmic plus nuclear) GR protein levels were quantitated via immunoblotting following incubation of monolayers for 18 h with RU 28362 (1 μM) plus or minus cycloheximide. Treatment with agonist alone resulted in significant down-regulation of GR protein levels, but relatively long exposures of the autoradiograms facilitated detection of an intact 94 kDa protein band (Fig. 5, lane 1). Based on densitometric scanning data, simultaneous treatment with the agonist plus cycloheximide resulted in an additional 50% reduction in total GR protein levels (Fig. 5, lane 2).

Effects of RU 28362 treatment on GR message stability

The next series of experiments was conducted to test the hypothesis that glucocorticoid treatment may decrease GR mRNA half-life either during the initial phase or during the maintenance phase of homologous down-regulation. In order to address this question two different RNA synthesis inhibitors (actinomycin D and DRB) were utilized. Confluent monolayers of PROb

Fig. 5. Western immunoblotting for quantitation of total (cytoplasmic plus nuclear) GR protein levels. Monolayers of PROb cells (8 flasks/treatment group) were incubated for 18 h with 1 μM RU 28362 (lane 1) or 1 μM RU 28362 plus cycloheximide (lane 2). The GR in the pooled cytosolic extracts from the two treatment groups were then immunoprecipitated with the BuGR2 monoclonal antibody and subsequently quantitated via Western immunoblotting with the same antibody as previously described [14]. The GR protein bands were then detected with the Enhanced Chemiluminescence system. The arrows indicate the intact 94 kDa GR protein bands on the resulting autoradiogram.

cells were treated with 1 μM RU 28362 for either 1 h (initial phase) or 18 h (maintenance phase) followed by addition of DRB (50 μg/ml) for an additional 2 or 4 h. This dose of DRB was determined in preliminary experiments to inhibit > 85% of the incorporation of [^{3}H]uridine into TCA precipitable RNA molecules. This protocol provided adequate time for the agonist to bind to GR and initiate down-regulation of mRNA levels. The 2 and 4 h incubation periods with DRB were chosen since the half-life for the GR mRNA in other target cells has been reported to be 3–4.5 h [15, 16]. In order to compare the extent of receptor message degradation between the steroid-treated and non-steroid-treated cells, it was necessary in these experiments to set both the control and the RU 28362 treatment values at 100%, thus canceling any decreases due to steroid treatment alone. The data presented in Fig. 6 show the relative rate of GR message degradation in the presence or absence of steroid. As is seen in panel A, following 1 h of steroid treatment the GR mRNA has an increased rate of degradation (shorter half-life) as compared to that detected in non-steroid-treated cells. Panel B demonstrates that following 18 h of steroid treatment, the GR message has the same relative rate of degradation as that detected in non-steroid-treated cells. These results obtained using DRB suggest that glucocorticoid treatment is capable of decreasing receptor mRNA stability in the initial phase of down-regulation, but not following maximal down-regulation.

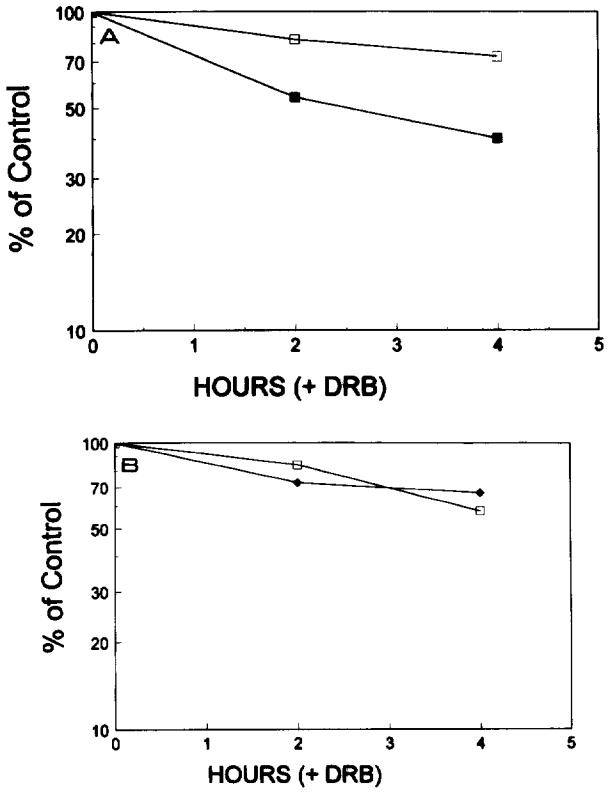

Fig. 6. Effect of RU 28362 on GR mRNA half-life as determined using the RNA synthesis inhibitor DRB. Monolayers of PROb cells were incubated for either 1 h (A) or 18 h. (B) in the absence (open squares) or presence (closed squares) of 1 μM RU 28362 prior to the addition of DRB for an additional 2 or 4 h. RNA was isolated following these incubations and the ribonuclease protection assay was subsequently performed for quantitation of GR and β-actin mRNA levels as described in the Materials and Methods. The GR mRNA/β-actin mRNA ratios from the control flasks (time 0) were set at 100%, and the other treatment values are expressed as a percentage of this value. The data presented are the means of one experiment performed in duplicate. The experiment was repeated with essentially the same results.

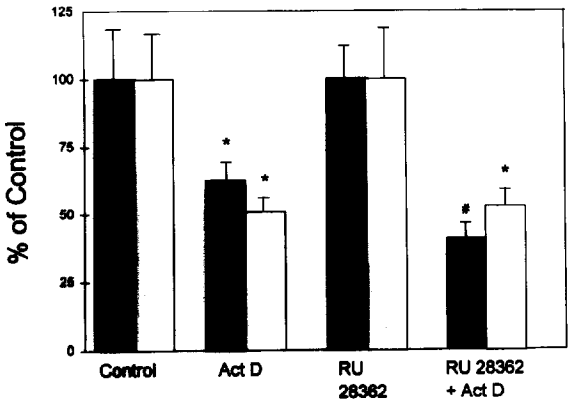

Fig. 7. The effects of RU 28362 on the half-life of GR mRNA during the initial and maintenance phases of homologous down-regulation. Monolayers of PROb cells were incubated for 1 h (initial phase; solid bars) or 18 h (maintenance phase; open bars) in the absence (control) or presence 1 μM RU 28362 prior to a 4 h incubation with actinomycin D (Act D, 10 μg/ml). RNA was isolated following the 1 or 18 h incubations and following the subsequent 4 h incubation with actinomycin D in the absence (control) or presence of RU 28362. The ribonuclease protection assay was subsequently performed for quanitation of GR and β-actin mRNA levels as described in Materials and Methods. The GR mRNA/β-actin mRNA ratios from the control flasks and RU 28362 treated flasks were set at 100%, and the other treatment values are expressed as percentages of these normalized values. The experiment was performed twice and the mean values of each treatment group (control, $n = 8$; Act D, $n = 8$; RU 28362, $n = 8$; Act D plus RU 28362, $n = 8$) plus the standard error of the means is plotted. *Indicates a significant difference ($P < 0.05$) from control value. †Indicates a significant difference ($P < 0.05$) from Act D treatment value using a one-tailed t-test.

Similar experiments were also performed utilizing either a 1 or 18 h incubation of confluent monolayers of PROb cells with RU 28362 followed by a 4 h incubation with actinomycin D (10 μg/ml). This dose of actinomycin D was found sufficient to inhibit > 95% of incorporation of [^{3}H]uridine into TCA precipitable RNA molecules in preliminary experiments. As is seen in Fig. 7 (solid bars), this 4 h incubation with actinomycin D in the absence of steroid resulted in a 40% decrease (significant at $P < 0.05$) in GR mRNA levels when compared with control cells. The GR mRNA/β-actin mRNA ratios obtained for the RU 28362-treated cells were set at 100% to facilitate comparison between

the ratios obtained for the cells treated with RU 28362 plus actinomycin D and those treated with actinomycin D alone. Actinomycin D treatment of RU 28362 pretreated cells resulted in a significant decrease (60%) in GR mRNA levels when compared to cells treated with RU 28362 alone. The difference in the GR mRNA levels dected in the absence (60% of control) or presence (40% of control) of RU 28362 is significantly different ($P < 0.05$). These data reveal that a 1 h treatment with RU 28362 decreases GR mRNA levels via a mechanism which is independent of transcriptional repression, suggesting that GR mRNA stability is significantly decreased during the initial phase of homologous down-regulation.

In Fig. 7 (open bars), the results from the experiments designed to evaluate agonist-mediated effects on GR mRNA levels during the maintenance phase (18 h) of down-regulation are also presented. These data demonstrate that actinomycin D treatment alone resulted in a 50% decrease (significant at $P < 0.05$) in GR mRNA levels. These data also reveal that in steroid-treated cells 50% of the GR mRNA is degraded during the 4 h actinomycin D treatment, which is identical to the decrease detected in the cells treated with actinomycin D alone. These actinomycin D data are consistent with the conclusion that an 18 h incubation with RU 28362 has no detectable effect on GR mRNA stability. These results are in contrast to those obtained from the 1 h steroid treatment (solid bars), which demonstrate a significant difference in the extent of receptor mRNA degradation following steroid treatment.

DISCUSSION

The first series of experiments reported herein evaluated the abilities of several agonists to mediate autoregulation of PROb cell GR mRNA levels. The data demonstrate that in this colonic cell line down-regulation of GR mRNA levels occurs as early as 1 h and is maintained for 36 h following addition of the agonist RU 28362. This agonist-mediated negative autoregulation contrasts with the positive autoregulation that has been reported in a number of other cell lines that are also growth inhibited by glucocorticoids [18, 19–21]. These observations made in other cell lines lead to the initial speculation that up-regulation of GR mRNA levels by glucocorticoids plays an important role in the ultimate growth inhibition by agonists. However, a subsequent series of experiments conducted by Denton *et al.* [22] using glucocorticoid-resistant lymphoid cells that express functional GR clearly demonstrated that this positive autoregulation is unrelated to agonist-mediated growth inhibition.

The high-affinity glucocorticoid agonist, RU 28362, was the most effective ligand with regard to eliciting down-regulation of PROb cell GR mRNA levels. The actions of this ligand could be fully blocked by the antagonist, RU 38486 (data not shown), confirming that the observed down-regulation was indeed GR-mediated. Although the endogenous rat glucocorticoid, corticosterone, as well as the weak agonist, aldosterone, were capable of down-regulating GR mRNA levels, they appeared to be slightly less effective than the synthetic agonist. These results were predicted based on the lower affinities of corticosterone and aldosterone for the GR [29, 30]. Despite the fact that 1 μM aldosterone has been reported to be incapable of eliciting another GR-mediated response in these PROb cells, more specifically the induction and secretion of an unidentified 40 kDa glycoprotein [26], this mineralocorticoid was capable of down-regulating GR mRNA levels in the present study. Aldosterone has been shown to exhibit similar glucocorticoid-like agonist activity in other systems. For example, aldosterone–GR complexes have been reported to induce tyrosine aminotransferase activity in rat hepatoma cells [31] and electrogenic sodium transport in A6 frog kidney cells [32]. Although it is interesting that GR-aldosterone complexes are capable of eliciting repression of GR mRNA levels without apparently inducing the synthesis and secretion of this this glycoprotein in PROb cells, the mechanism(s) underlying these differential responses is unclear.

The next pair of experiments examined the effects of inhibition of ongoing protein synthesis on agonist-mediated homologous down-regulation of GR mRNA levels. The first experiment addressed the potential requirement for protein synthesis at an early time point, since blocking the synthesis of a glucocorticoid-inducible protein could potentially decrease the efficacy of the agonist in mediating down-regulation. The data, however, indicate that inhibition of protein synthesis has no effect on the ability of RU 28362 to mediate the initial phase of down-regulation in PROb cells. These data thus do not support a model in which a glucocorticoid-inducible protein facilitates agonist-mediated repression of a glucocorticoid responsive gene such as the endogenous GR gene itself [33], and are in agreement with data obtained in rat hepatocytes [15]. In the second experiment the effect of inhibition of PROb cell protein synthesis was also examined following an 18 h treatment with RU28362. The data generated from this experiment revealed that ongoing protein synthesis is essential for the maintenance of homologous down-regulation in these colonic cells. The loss of agonist-mediated homologous down-regulation under these conditions is most likely due to the loss of the GR protein itself, since it has been demonstrated that the level of GR expression dictates the glucocorticoid sensitivity of a number of different cell lines [9, 11]. In addition to their ability to down-regulate GR mRNA levels, glucocorticoid agonists have also been reported to shorten the half-life of the GR protein in several cell lines [34, 35] including PROb cells [36]. Therefore when new receptor protein synthesis is inhibited in

agonist-treated PROb cells the GR levels may rapidly fall below the minimum threshold concentration required to mediate a glucocorticoid response (i.e. down-regulation). The immunoblot presented in this study demonstrates that not only does treatment with agonist result in significant down-regulation of GR protein levels, but that addition of cycloheximide results in a further reduction (50%) in detectable GR protein levels. This observation, coupled with the report that a 2-fold increase in GR levels in a rat hepatoma cell variant converts these cells from glucocorticoid-resistant to fully sensitive [37], supports our conclusion concerning a minimum threshold level of PROb cell GR protein. This dependency on ongoing protein synthesis in PROb cells contrasts with data generated using other glucocorticoid-responsive cell lines [22, 23] and may reflect differences in the extent of agonist-mediated GR protein down-regulation or in the sensitivity threshold of these other cell lines.

As previously mentioned, glucocorticoid treatment results in a reduction in the rate of transcription of the GR gene in a variety of target tissues and cell types [15, 16]. However, direct demonstration of this effect via nuclear run-on experiments has not been feasible in other cell lines due to technical difficulties that may relate to the low level of GR mRNA expression [16]. Although we have not performed nuclear run-on experiments in the present study, we believe that it is very reasonable to conclude that the observed agonist-mediated down-regulation of PROb cell GR mRNA levels involves a decreased rate of transcription of the GR gene. This conclusion would apply to the initial phase of down-regulation and clearly to the phase of maximal down-regulation, since no effect of agonist on mRNA stability was detected during the latter. In contrast to the well-documented effect of agonists on GR gene transcription, the role of potential agonist-mediated alterations in GR message stability has not been well delineated. For example, following maximal down-regulation of GR mRNA levels, no effect of agonist on message stability has been detected in rat hepatocytes [15] or human lymphocytes [16, 22]. In contrast, when GR message stability in rat AtT-20 pituitary cells has been analyzed during the initial phase of agonist-mediated homologous down-regulation, a decrease in message half-life has been detected [38]. Since these studies utilized different cell lines and protocols for inhibition of RNA transcription, their interpretation is not straightforward. Thus it was important to test the hypothesis that glucocorticoid treatment may not only inhibit PROb cell GR gene transcription but may also be capable of decreasing the half-life of GR mRNA during the initial phase of down-regulation, without necessarily altering mRNA stability after maximal down-regulation has been achieved. In the present study the results generated using two inhibitors of RNA polymerase [39, 40] demonstrate that GR mRNA stability is significantly reduced during the initial phase of homologous down-regulation. Using these inhibitors, GR message half-life was significantly reduced after 1 h of steroid treatment. These results are similar to those reported by Vedeckis and colleagues [38], who demonstrated that the absolute GR mRNA (levels not normalized) half-life in the AtT-20 pituitary cell line was reduced from 3 to 1 h by treatment with dexamethasone. In the present study the normalized GR mRNA half-life was reduced from approx. 7 h to approx. 3 h following 1 h of glucocorticoid treatment. In contrast to these results, no effect of glucocorticoid treatment on the stability of message was detected following maximal down-regulation of GR mRNA levels.

This ability of glucocorticoids to not only inhibit the transcription of a specific gene but to also decrease the stability of the mRNA transcribed from that gene is not unique to the GR gene. For instance, glucocorticoids have been shown in specific cell types to repress the transcription of a number of other genes including those encoding proopiomelanocortin [41], type I procollagen [42], collagenase [43] and interleukin 1β [44]. Although treatment with these steroids has been reported to have no effect on the stability of the mRNAs transcribed from several of these genes [42, 43], glucocorticoid hormones have been reported to decrease the stability of the interleukin-1β message [44]. Lee *et al.* [44] reported that this destabilizing effect is blocked in the presence of actinomycin D or cycloheximide, which indicates that this glucocorticoid-mediated response requires *de novo* mRNA and protein synthesis. These investigators therefore concluded that a glucocorticoid-inducible protein(s) may influence the stability of the interleukin-1β message much the same way as a nuclease or a nuclease-directing protein may destabilize histone mRNAs that contain specific target 5′-leader sequences [45].

The precise mechanism(s) underlying the glucocorticoid-induced destabilization of GR mRNA reported in this study is unknown. In contrast to the destabilizing effect of glucocorticoids on interleukin-1β mRNA, their effect on the stability of the PROb cell GR mRNA was not blocked in the presence of transcription inhibitors and hence does not appear to require *de novo* RNA synthesis. Thus other possible mechanisms, including a model proposed several years ago by Vedeckis and colleagues [38], should be considered. These investigators noted that the 3′-untranslated region of the GR mRNA contains a number of tRNA-like sequences which potentially are able to form double-stranded RNAs. Since these double-stranded structures tend to stabilize mRNA, they could potentially lend stability to the receptor mRNA molecules. Since they had previously demonstrated that activated GR complexes can bind tRNA molecules *in vitro* [46, 47], these investigators proposed a model in which activated GR could bind to these tRNA-like regions of the GR mRNA, thus preventing the formation of double-stranded

mRNAs and blocking the subsequent stabilization. Although this model was not tested in the present study, it is consistent with the observation that destabilization of GR mRNA occurs in the presence of transcription inhibitors.

In conclusion, the experiments described herein have tested several key hypotheses concerning the potential mechanism(s) underlying homologous down-regulation of GR mRNA levels in the rat PROb cell line. The data presented demonstrate that GR ligands including; the pure agonist, RU 28362, the endogenous agonist, corticosterone, and the weak agonist, aldosterone, are all capable of mediating homologous down-regulation of GR mRNA levels to variable degrees. Although ongoing protein synthesis is not necessary for the initial phase of this homologous down-regulation, it is required for the maintenance of this negative autoregulation. Additional experiments performed using two different inhibitors of RNA synthesis demonstrate that treatment of monolayers with the agonist RU 28362 is capable of decreasing the half-life of the GR message during the initial phase of down-regulation, but not following maximal down-regulation. Thus although agonist-mediated negative autoregulation of GR mRNA levels in PROb cells most likely involves a decrease in the rate of transcription of the GR gene, a reduction in the stability of the GR message also contributes to the initial phase of this form of autoregulation.

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