Synergistic Induction of Apoptosis with Glucocorticoids and 3′,5′-Cyclic Adenosine Monophosphate Reveals Agonist Activity by RU 486

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Glucocorticoids induce a programed cell death in immature murine T cells through a process that has been named apoptosis. Cyclic AMP-dependent protein kinase (PKA) activity contributes to this response but acts through an undefined mechanism. Steroid-induced cytolysis can be completely blocked by the glucocorticoid antagonist RU 486, which inhibits the transformation of the glucocorticoid receptor (GR) into a fully activated transcription factor. However, the ability of cAMP to act synergistically with steroids to cause apoptosis has revealed that a limited portion of RU 486-bound GR can translocate to the nucleus and contribute to a loss of cell viability. The combination of cAMP and RU 486 was also found to act cooperatively to regulate mRNA levels of specific genes. A 5-kilobase VL30 retroviral element transcript, which had previously been shown to be regulated synergistically by cAMP and dexamethasone, was strongly induced by the combination of RU 486 and cAMP. There was no agonist effect of RU 486 on the induction of the the VL30 5-kilobase transcript in a variant cell line with defective PKA. Thus, the ability of RU 486 to act as an agonist, in this instance, was cAMP dependent. A similar response was also seen with the chondroitin sulfate proteoglycan core protein gene. On the other hand, mouse mammary tumor virus mRNA levels, which were not affected by cAMP alone, did not respond to a combination of RU 486 and cAMP. Studies of GR function, evaluating nuclear translocation and DNA binding at a GR-specific DNA regulatory element site found no evidence for a general effect of PKA activation on these receptor functions. We propose that cAMP may contribute to the induction of apoptosis at a step beyond receptor transformation by promoting an interaction between GR and other gene-specific regulatory proteins. Moreover, this interaction is sufficient to overcome the inhibition imposed by RU 486 on the functional capacity of nuclear glucocorticoid receptors. (Molecular Endocrinology 7: 104–113, 1993)

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INTRODUCTION

The synthetic steroid RU 486 (1) has been used therapeutically as an abortifacient (2, 3) and in the laboratory as a tool to study mechanisms of steroid receptor function (4). The rationale for its use resides in RU 486's capacity to bind avidly to both progesterone and glucocorticoid receptors (GR) without promoting an effective conversion of these proteins into activated transcription factors (5, 6). The compound is particularly useful experimentally, since any steps in receptor transformation that are blocked by the drug help to define critical hormone-dependent changes in receptor activity. In this regard, RU 486 has been shown to inhibit the release of GR from cytoplasmic complexes containing heat shock proteins, thus suppressing the subsequent translocation of receptors into the nucleus (7, 8). There is accumulating evidence that, in certain instances, this block in nuclear translocation is incomplete and implies that the drug must also restrict subsequent steps in the protein's transition to a fully activated transcription factor (5, 9–11). In both intact cells and *in vitro*, RU 486 has been found to cause a significant conversion of receptors into DNA-binding proteins (10, 11). However, analysis by anion exchange chromatography has shown that, compared to agonist-bound GR, the RU 486-transformed receptors exhibited an altered elution profile (11). This behavior indicates that RU 486 was inefficient in producing changes in receptor structure that are normally seen after the GR is released from a complex containing heat shock proteins. One such change had been indicated by the finding that RU 486 failed to provoke a measurable increase in receptor phosphorylation normally seen after agonist binding (12). The existence of measurable amounts of RU 486-bound GR in the nuclei of hormone-treated cells leaves open the possibility that some of these receptors can acquire functional capacity. It is of considerable interest,

therefore, to know if and under what circumstances RU 486-transformed GRs can gain the ability to contribute to transcriptional regulation of specific genes.

Derivatives of the murine T-lymphoma cell lines S49 and WEHI-7 have been used to study the cytolytic effects of glucocorticoids and cAMP on thymus-derived cells (13–18). Recent work carried out in this laboratory has led to the hypothesis that the glucocorticoid and cAMP signaling pathways cooperate to promote a programed cell death (apoptosis) by the coregulation of lysis-related genes (18) in WEHI-7 cells. Loss of cAMP-dependent protein kinase (PKA) activity was shown to produce a reduction in the capacity of dexamethasone to induce apoptosis. Conversely, normally innocuous concentrations of $(Bu)_2$cAMP enhanced the capacity of dexamethasone to cause cell death in wild type cells. This report demonstrates a similar functional synergism between cAMP and RU 486.

RESULTS

Induction of Apoptosis

Previous studies had shown that RU 486 could completely antagonize the cytolytic response to dexamethasone in the murine T cell lymphoma line WEHI-7 (5). Moreover, RU 486 alone, at concentrations up to 1 μM, has little or no effect on the proliferation of WEHI-7 cells. Therefore, it was somewhat surprising when we found that 50 nM RU 486 could enhance the cytolytic effect of cAMP in these cells. Figure 1 illustrates the results of cell proliferation assays carried out with a

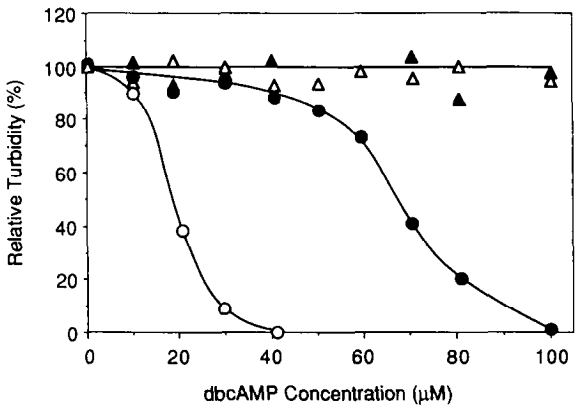

Fig. 1. Effect of RU 486 on the Induction of Apoptosis by $(Bu)_2$cAMP in WEHI-7 Cells

Cells were dispensed (10^3/ml) into medium containing the indicated concentrations of $(Bu)_2$cAMP and allowed to proliferate for 10 days. At the end of the incubation period, turbidity measurements (660 nm) were taken and expressed relative to untreated control cultures. Each *point* is the average of two determinations. The *open symbols* represent cultures that contained RU 486 (50 nM) in addition to the (Bu)2cAMP. ○ and ●, Wild type cell line W7TG; △ and ▲, CXG-56 cells containing no PKA activity.

WEHI-7 derivative, W7TG, and a cAMP-resistant ($cAMP^r$) variant, CXG-56. CXG-56 cells provide a control since they are devoid of detectable PKA activity (17). In W7TG, $(Bu)_2$cAMP alone caused a loss of proliferative capacity with an ED_{50} of approximately 70 μM. In combination with RU 486, however, the dose response to $(Bu)_2$cAMP was shifted to the left (ED_{50} of 20 μM). RU 486 had no effect on the cAMP dose response in another WEHI-7 cell line that contained no GR (not shown). In the $cAMP^r$ variant CXG-56, $(Bu)_2$cAMP, either alone or in combination with RU 486, failed to kill the cells. Seen from a slightly different perspective, these results indicate that RU 486, acting through an association with glucocorticoid receptors, displayed agonist activity when used in concert with cAMP to inhibit proliferation.

One of the hallmarks of steroid-induced apoptosis is the degradation of DNA into a characteristic population of fragments (19), reflecting a preferential nuclease attack at sites between adjacent nucleosomes. This phenomenon has provided a means to qualitatively monitor DNA degradation using agarose gel electrophoresis. Figure 2 and Table 1 present a parallel analysis of DNA fragmentation and cell viability in cells that had been treated with either dexamethasone, RU 486, a low concentration of $(Bu)_2$cAMP, or combinations thereof. After 24 h, treatment with a combination of dexamethasone and $(Bu)_2$cAMP caused nearly half of the cells to die (as measured by trypan blue exclusion) and resulted in the greatest degree of DNA digestion, as evidenced by the amount of DNA appearing in the typical nucleosome ladder pattern. DNA digestion was also obvious in cells treated with dexamethasone but not in the untreated or the RU 486- and the $(Bu)_2$cAMP-treated cells. When RU 486 was added along with dexamethasone, it prevented DNA degradation as well as loss of viability. RU 486 added in combination with $(Bu)_2$cAMP, however, caused a reduction in viability nearly equivalent to that of dexamethasone and a nucleosome ladder of DNA fragments which was not observed with either drug alone.

Regulation of mRNA Accumulation

There is considerable evidence indicating that an initiating event in apoptosis is caused by steroid-induced changes in gene expression. Thus, the results of Figs. 1 and 2 suggested an important question. Does RU 486 have the capacity to promote expression of specific genes in lymphoma cells containing activated PKA? To address this issue, RNA was analyzed from cells incubated with dexamethasone, RU 486, $(Bu)_2$cAMP, and their various combinations. Probes for mouse mammary tumor virus (MMTV) and VL30, a murine retroelement, were used. The mouse genome contains 100–200 copies of VL30 (20), and the level of its encoded 5-kilobase (kb) RNA had previously been shown to be synergistically regulated by dexamethasone and cAMP in WEHI-7 cells (21). Transcription of a 35S MMTV transcript is regulated by glucocorticoids but not cAMP,

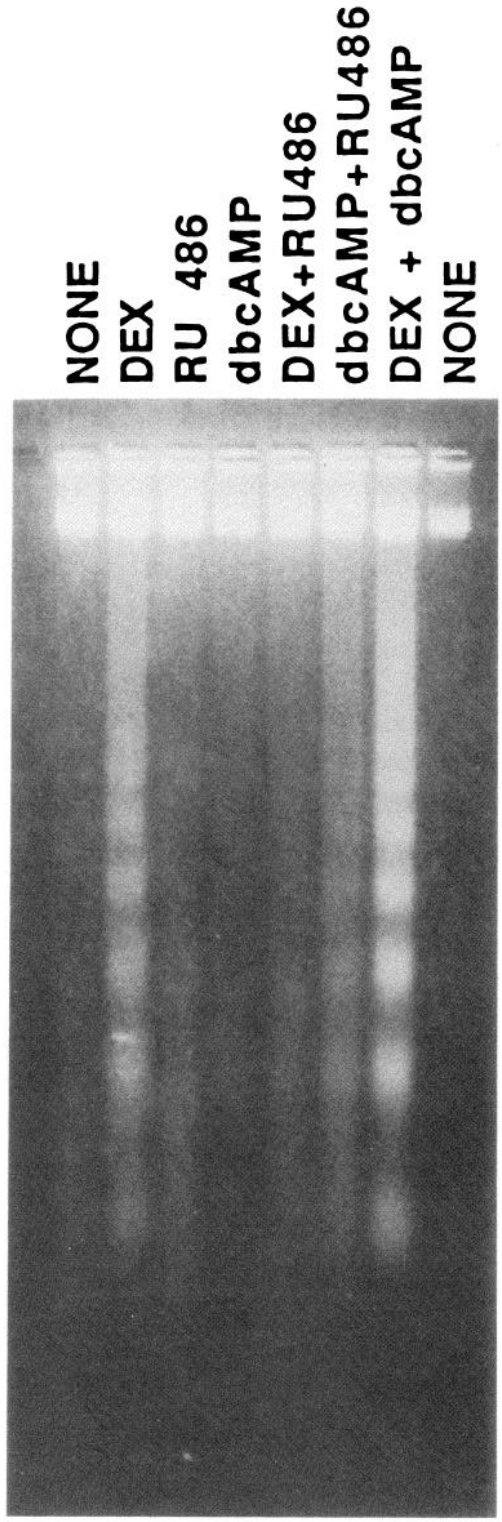

Fig. 2. Agarose Gel Analysis of DNA From the Cell Cultures Described in Table 1

DNA was isolated from the cell cultures used to generate the data shown in Table 1. The DNA samples were fractionated by electrophoresis in 1.8% agarose gels and stained with ethidium bromide.

Table 1. Effects of dex, RU 486, and $(Bu)_2$cAMP on the Viability of W7TG Cells

Treatment	Dead cells (%)	
	Exp. in Fig. 2	Average of 4 experiments
None	5	5.0 ± 0.8
Dex (50 nM)	20	26 ± 4.9
RU 486 (250 nM)	4	6.3 ± 1.7
$(Bu)_2$cAMP (30 μM)	8	8.8 ± 5.7
Dex + RU 486	5	5.3 ± 0.5
$(Bu)_2$cAMP + RU 486	18	21 ± 4.7
Dex + $(Bu)_2$cAMP	48	54 ± 5.4

W7TG cells were incubated for 24 h in the presence of the indicated compounds. At the end of the incubation period, the number of living and dead cells in the cultures was measured using trypan blue exclusion.

as will be verified below. The results presented in Fig. 3 were obtained using a WEHI-7 line (W7 MG-1) which contains glucocorticoid-inducible MMTV (22). The figure is a series of autoradiograms obtained by Northern blot analysis of RNA that was isolated from W7 MG-1 after the cells had been incubated under the conditions indicated. The *top panel* shows the results obtained using the MMTV probe. Dexamethasone induced an increased accumulation of MMTV RNA, while $(Bu)_2$cAMP and RU 486 did not. RU 486 also inhibited the effect of dexamethasone and caused no effect when added in combination with $(Bu)_2$cAMP. This is an instance of RU 486 acting as a pure antagonist. It is also important to note that the combination of $(Bu)_2$cAMP and dexamethasone was no more effective than dexamethasone alone. Thus, $(Bu)_2$cAMP did not exert a measurable influence on the steroid induction of MMTV RNA through a hypothetical effect on the overall activity of the glucocorticoid receptors or by increasing the number of GR. The *bottom panel* of Fig. 3 demonstrates that there was no effect of these agents on the constitutively expressed CHO-B gene and acts as a control that equivalent amounts of RNA were analyzed in each case.

The response of the VL30 genes in the same cells was significantly different in several ways (Fig. 3, *middle panel*). Dexamethasone and $(Bu)_2$cAMP each induced accumulation of the major (*arrow*) 5-kb mRNA (3.7-fold and 3.1-fold, respectively), and the effect of the combination of the two was more than additive (9.4-fold). RU 486 alone caused a small increase (1.9-fold) but, again, was capable of antagonizing the effect of dexamethasone. Most importantly, RU 486 and $(Bu)_2$cAMP in combination induced an accumulation of the 5-kb mRNA (4.9-fold), which was greater than that seen with either $(Bu)_2$cAMP or dexamethasone alone. Taken together, the results indicate that, while RU 486 can antagonize the effects of dexamethasone, it is also capable of exhibiting significant agonist activity, particularly when participating as a partner with cAMP to regulate VL30 RNA levels.

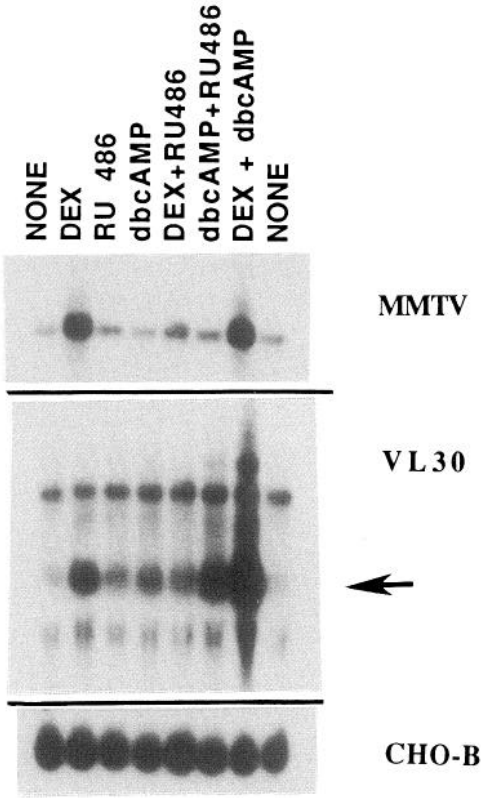

Fig. 3. Northern Blot Analysis of RNA From Lymphoma Cells Incubated with Dexamethasone, RU 486, and $(Bu)_2$cAMP

Total RNA was isolated from cell cultures (W7 MG-1 cell line) treated for 6 h with the same concentrations of drugs as used in Table 1. The RNA was fractionated by electrophoresis in a 1% agarose gel containing formaldehyde. The RNA was transferred to a nylon filter and successively hybridized with probes to MMTV, VL30, and CHO-B. The amount of hybridization, in each case, was evaluated using autoradiography.

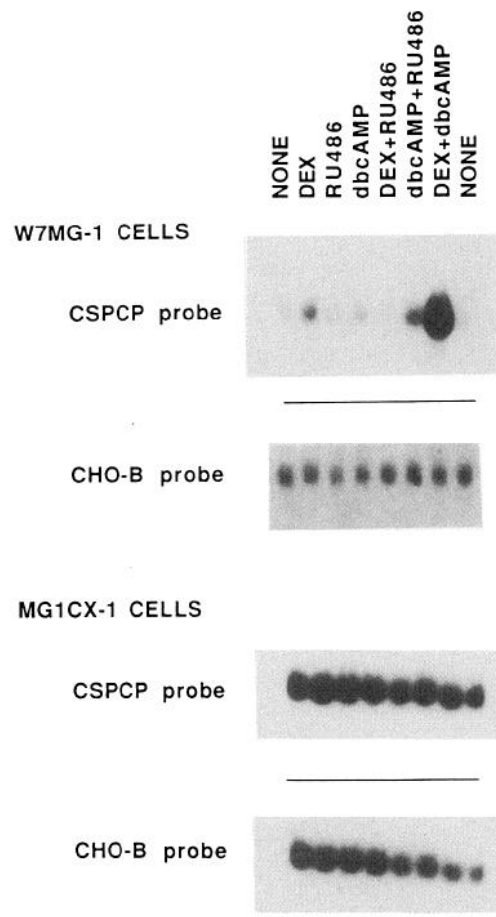

Fig. 4. Northern Blot Analysis of mRNA Levels in W7 MG-1 and MG1CX-1 Cells

Total RNA was isolated form W7 MG-1 and MG1CX-1 cells treated as in Fig. 3 and analyzed in a similar fashion. The *top panel* shows the results using a cDNA probe specific for the 1.2-kb mRNA for CSPCP and RNA expressed in W7 MG-1 cells. As in Fig. 3, a probe for the CHO-B gene was used as a control. The *bottom two panels* show the results obtained with the same two probes and RNA from MG1CX-1 cells.

Figure 4, *top panel*, demonstrates that the phenomenon of cAMP-dependent expression of agonist activity by RU 486 can be observed with genes other than VL30. Again, Northern blot analysis was used to characterize the RNA isolated from cells that had been incubated under the same spectrum of conditions employed for Fig. 3. In this instance, the RNA was hybridized with a probe for the chondroitin sulfate proteoglycan core protein (CSPCP) gene (23). This gene had also been found to exhibit a synergistic response to dexamethasone and cAMP (21). The autoradiogram confirms that synergistic behavior along with the capacity of RU 486 to cooperate with $(Bu)_2$cAMP in causing a significant (equivalent to dexamethasone) induction of this mRNA. Once again, the CHO-B probe was used to check that equivalent amounts of RNA has been loaded into each well. To verify that the effects of $(Bu)_2$cAMP on RU 486 activity were mediated by PKA, $cAMP^r$ variants containing defective PKA were isolated from W7 MG-1 cells. The cell line MG1CX-1 is typical of the variants that were obtained. It possesses a 10-fold increase (ED_{50} of 700 μM *vs.* 70 μM for the wild type) in cellular resistance to $(Bu)_2$cAMP and an *in vitro* PKA activity with a corresponding shift (to higher concentrations) in the dose response to cAMP (not shown). The *bottom two panels* in Fig. 4 are autoradiograms that resulted from hybridization of RNA from MG1CX-1. When the CSPCP probe was used, the film was exposed three times longer (3 days) than that for the W7 MG-1 RNA so that a signal from all lanes could be clearly visualized. The results show that none of the conditions produced a significant increase in CSPCP mRNA accumulation. Only dexamethasone (±cAMP) appeared to cause a slight increase in hybridization. A similar result was obtained when Northern blots containing MG1CX-1 RNA were hybridized with a VL30 probe (not shown). In that instance, dexamethasone induced a 2-fold increase in VL30 5-kb mRNA, indicating that the steroid response was not ablated in these cells. RU 486 had no effect on the expression of either gene whether it was added with cAMP or not. Thus, it

appears that for these two gene transcripts (VL30 and CSPCP), not only was the response to RU 486 PKA dependent, but to some extent, the response to dexamethasone was as well.

Evaluation of GR Activation

The results shown in Figs. 3 and 4 demonstrate that RU 486 can increase the accumulation of mRNA in a gene-specific and PKA-dependent manner. The lack of an effect on MMTV expression suggests that the result of activating PKA is not a general promotion of activity in all the glucocorticoid receptors bound with RU 486. Such would be the case, for example, if cAMP caused a complete release of RU 486-bound receptors from their cytoplasmic heat shock protein complexes or caused a more efficient recognition of GR-specific DNA regulatory elements (GREs). In order investigate these possibilities more thoroughly, the ability of RU 486 to activate receptors in response to $(Bu)_2$cAMP was measured in two ways. First, receptor nuclear translocation assays were carried out with cells that had been incubated with either RU 486 or the agonist triamcinolone acetonide (TA). The cells were also preincubated with or without $(Bu)_2$cAMP. The results of the study are shown in Table 2. The agonist TA [±$(Bu)_2$cAMP] caused approximately half of the receptors to translocate to the nucleus. Much less of the receptor bound with RU 486 was found associated with the nuclear fraction (~15%) and, as with TA, there was no change produced by $(Bu)_2$cAMP. The only consistent difference caused by $(Bu)_2$cAMP that was observed was a 50% increase in total steroid binding capacity (not shown). This effect was detected with both hormones and is likely to represent the cAMP-dependent increase in receptor synthesis due to higher GR mRNA levels (18).

Earlier indirect receptor/DNA binding studies had indicated that, compared with agonist-bound GR, RU 486-bound GR had a lower affinity for specific GRE-containing DNA fragments (5). Based on some of the results presented above, one might speculate that activation of PKA serves to increase the affinity of RU 486-bound receptor for DNA. We have investigated this issue using band shift assays to assess GR binding to GRE-containing DNA fragments. Figures 5 and 6 demonstrate the specificity of GR binding to a 39-base pair probe containing a palindromic GRE site. (Please see *Materials and Methods* for a description of the probe.) In this set of experiments, the GR had been activated with the agonist TA. Figure 5 shows that addition of GR slowed the migration of the probe into two bands. The shift of the major band was completely competed by an excess of identical unlabeled DNA fragments (GREpal). The smaller and more defined band migrating slightly faster than the major band was partially competed but, as will be shown below, does not appear to contain the GR. Two other DNA fragments which do not contain a GRE sequence (CRE and TSE) failed to compete the shift, while another GRE-containing fragment (TATGRE) also proved to be an effective compet-

Table 2. Effect of PKA Activation on Nuclear Translocation of the GR

Pretreatment	Ligand	Nuclear translocation (%)
None	TA	45 ± 4.6
None	RU 486	16 ± 3.1
$(Bu)_2$cAMP	TA	48 ± 3.2
$(Bu)_2$cAMP	RU 486	14 ± 3.2

Intact cells were incubated with labeled ligand (2×10^{-8} M) after pretreating the cultures ± $(Bu)_2$cAMP (200 μM) for 4 h. Unbound hormone was washed out and the cells lysed with 0.4% NP40 to generate nuclear and cytoplasmic samples. The values represent the means and their SDs from three separate experiments.

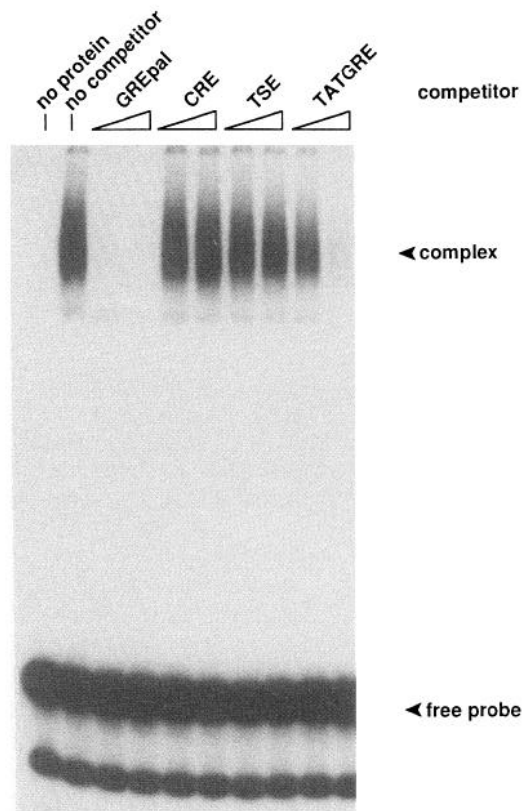

Fig. 5. Competition of GR Binding to a Specific GRE-Containing DNA Fragment

Samples containing GRs that had been activated with TA were incubated with a ^{32}P-labeled 39-base pair probe containing a palindromic GRE (GREpal) site. Competition of binding was carried out by including a 100- or 1000-fold excess of unlabeled DNA. The increasing concentrations are indicated by the height of the *triangles*. The samples were analyzed using a nondenaturing 4% acrylamide gel followed by autoradiography. The specific competitors were GREpal, which is identical to the probe, and TATGRE, a fragment from the rat TAT gene 5′-flanking region. The nonspecific competitors were sequences containing the CRE-binding protein binding site (CRE) and tissue-specific elements from the chorionic gonadotropin α-subunit gene (TSE).

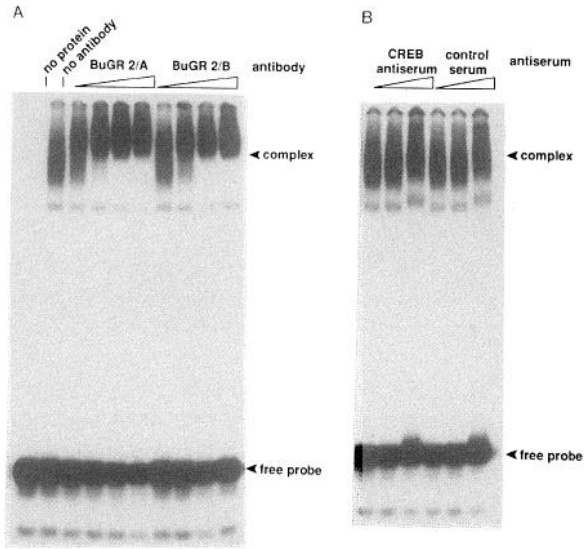

Fig. 6. Effect of Antibodies on the Migration of DNA-Protein Complexes

A, A fixed concentration (1 nM) of TA-activated GR and GREpal probe was incubated with decreasing dilutions of the BuGR-2 monoclonal antibody (1:125, 1:25, 1:5, and 1:1). Two different antibody-containing tissue culture supernatants were used for comparison. The resulting DNA/protein complexes were analyzed by electrophoresis and autoradiography as in Fig. 5. B, Control incubations were carried out as in A using a polyclonal antibody against the rat CRE-binding protein and a preimmune rabbit serum. The dilutions were 1:25, 1:5, and 1:1.

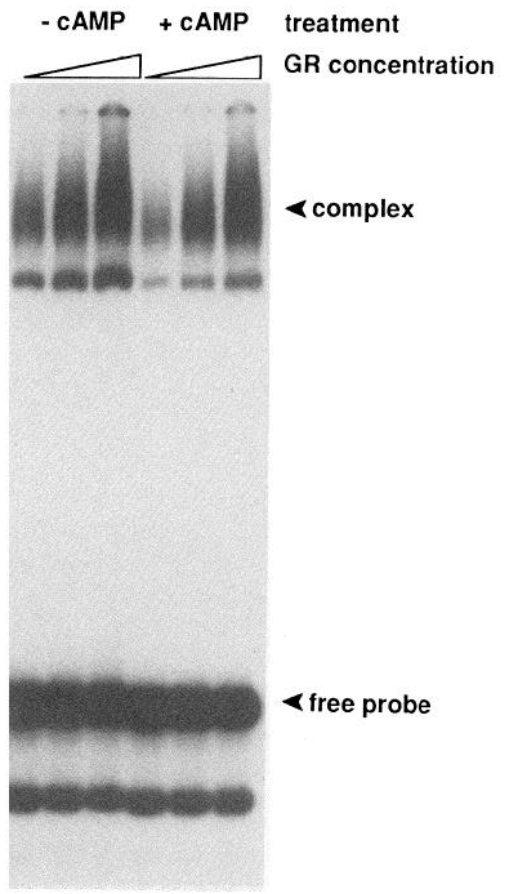

Fig. 7. Effect of PKA Activation on the Formation of DNA/Protein Complexes Containing the RU 486-Bound Receptor

W7TG cells were incubated at 37 C for 4 h with or without $(Bu)_2$cAMP (200 μM). Cytoplasmic extracts were prepared, incubated with RU 486, and the GR activated by incubating the samples at 27 C. Increasing concentrations of GR were incubated with a fixed amount of probe and the complexes evaluated as in Fig. 5. The GR concentrations were 0.25 nM, 0.5 nM, and 1 nM.

itor. Figure 6A shows the results of incubating the GR/DNA complexes with the BuGR-2 monoclonal antibody which is specific for GR. Two different antibody preparations are shown for comparison. In this instance, the migration of the major band was progressively slowed by increasing amounts of antibody. The minor band was essentially unchanged. Figure 6B shows the results obtained when two antibodies that do not recognize the GR were used. In each instance there was no significant change in the migration of the major band. Therefore, the major band represents a complex minimally composed of GR bound to the probe.

We next evaluated the ability of PKA activation to influence the capacity of RU 486-bound GR to cause a band shift. The results shown in Fig. 7 represent a comparison of extracts containing GR isolated from cells that had been preincubated with or without $(Bu)_2$cAMP. In this instance, the receptors were activated *in vitro* with RU 486. An equivalent range of GR concentrations from each sample was incubated with a constant amount of probe. There was no significant difference between the results with each of the two samples [$\pm(Bu)_2$cAMP] in regard to the amount of probe that was shifted. Moreover, the migration of the complex containing the RU 486-activated GR was identical to that seen with the agonist-activated receptor. Thus, there was no indication that prior activation of PKA (in intact cells) caused alterations in GR which promoted more efficient GRE binding by RU 486-activated receptors or that the resulting GR-DNA complexes contained a conformational difference that altered their migration. We have also looked for and not been able to detect any differences resulting from *in vitro* incubations of GR with PKA catalytic subunit and ATP (not shown).

DISCUSSION

This report documents two important aspects related to steroid-induced apoptosis in murine T-lymphoma cells. The first is that the glucocorticoid and cAMP signaling pathways cooperate synergistically to cause a loss of cell viability. The second and more novel aspect is that the glucocorticoid antagonist RU 486 has the potential to act as an agonist of apoptosis in cells with functional PKA activity. Moreover, this phenome-

non does not appear to be simply the result of a cAMP-induced change in the size or activity of the total GR population, since the induction of MMTV synthesis was unaffected by cAMP. In addition, nuclear translocation of RU 486-bound GR did not increase in response to cAMP, demonstrating that the antagonist still inhibited the efficient release of receptors from a cytoplasmic complex containing the 90-kilodalton heat shock protein. Rather, the results indicate that the effect of cAMP was to induce activity in the fraction of RU 486-bound GR that does translocate to the nucleus. In this regard, it is important to note that 8-Br-cAMP was found to promote activation of avian progesterone receptors (PRs) even in the absence of steroid (24). These observations may be analogous to ours since, unlike GR, ligand-free PRs are normally localized to the nucleus where they have the potential to directly interact with other chromatin components. In another study, RU 486 binding was not found to inhibit PR dissociation from the 8–10S complexes or the receptor's conversion into a form with higher avidity for nuclei in intact cells (25). However, RU 486-bound PRs were found to have an altered mobility on band shift assays when compared to agonist-bound receptors. These results were interpreted as reflecting steroid-dependent conformational differences in PR which could potentially influence protein-protein interactions. The effect of cAMP on RU 486-bound PR in band shift assays was not addressed.

While cAMP can contribute to the induction of apoptosis by glucocorticoids, we have not found a cAMP-dependent enhancement of the GR's capacity to bind DNA, either specifically to a GRE site or nonspecifically to DNA cellulose (not shown). Earlier studies had indicated that RU 486-bound receptors might not be able to bind GRE sites with the same affinity as agonist-bound GR (5). More recently, RU 486 was shown to produce different kinetics of specific GR/DNA binding when compared to the agonist triamcinolone acetonide (26). However, our results with band shift experiments did not detect a significant difference between the ability of TA and RU 486 to promote GR binding to a GRE site. Nor was there an increase in receptor DNA binding capacity after the cells had been preincubated with $(Bu)_2$cAMP. This suggests that the effects promoted by cAMP may occur at a distal step. One possibility is that the capacity of nuclear RU 486-bound GR to interact with other chromatin proteins is affected. The lack of a general effect was also consistent with the observation that RU 486 influenced mRNA levels in a gene-specific manner. RU 486 acted as a pure antagonist of glucocorticoid induction of MMTV mRNA expression. This was not the case for the VL30 5-kb transcript, where RU 486 alone had little agonist activity but was a far more efficient agonist in combination with cAMP. A similar result was also obtained with the chondroitin sulfate proteoglycan core protein gene. Thus, the data show that there is a subset of GR-regulated genes which have the potential to respond to RU 486. Furthermore, a requirement for the participation of additional cAMP-regulated factors is indicated. The cytolytic response to the combination of RU 486 and $(Bu)_2$cAMP supports this proposal and indicates that the gene(s) responsible for apoptosis may fall within this category (18).

There is at least one other case where RU 486 was found to possess agonist activity when used in combination with another drug. This example provides an interesting counterpoint to our work, since it involves the regulation of MMTV expression by glucocorticoids. Inhibitors of ADP ribosylation had been shown to enhance (~2-fold) the ability of dexamethasone to induce transcription of MMTV in the mouse mammary tumor cell line 34I (27, 28). RU 486 antagonized the induction of MMTV expression by dexamethasone while displaying agonist activity when used in combination with the ADP ribosylation inhibitor 3-amino benzamide (3-AB). The drug (3-AB) also caused increased nuclear translocation of the GR, but it is not clear if this was the sole basis for the effect on transcription since no other GR-regulated genes were tested.

The antiglucocorticoid dexamethasone-mesylate (dex-mes) has also demonstrated a variable capacity to act as a steroid agonist. Simons and his colleagues (29, 30) have shown that dex-mes can exhibit considerable agonist activity relative to expression of the tyrosine aminotransferase (TAT) gene. However, the compound's effect on TAT expression was cell, not cAMP, dependent. Dex-mes induced TAT expression in Fu5–5 cells but not in HTC cells, but the differential behavior of dex-mes did not reflect a property inherent to the receptor from either of the two cell lines (31). Rather, it was proposed that the agonist behavior was produced by other factors contributing to the receptor's regulation of the TAT gene in Fu5–5 cells. Interestingly, this group also reported a cell-dependent agonist effect of RU 486 with TAT. These observations are consistent with a variety of other instances where cooperative interactions between steroid receptors themselves as well as with other proteins were required to produce a full steroid response (32–37). Reduction in either component of the pair was sufficient to disrupt the regulatory synergism, and the concept of composite regulatory elements has been proposed to explain the complex forms of GR-mediated regulation that have been observed (38). Thus, it is virtually certain that protein-protein interactions play a key role in GR regulation of transcription. We believe that RU 486's agonist activity with respect to the VL30, CSPCP, MMTV, and TAT genes is also likely to reflect a manifestation of this phenomenon. In this context, it is significant that two different agents (cAMP and 3-AB) which act by producing changes in protein modification (phosphorylation and ADP ribosylation) each had the capacity to convey agonist activity to RU 486-bound receptors. In particular, our results indicate that cAMP may contribute to the induction of glucocorticoid-regulated genes, and to apoptosis, by inducing modification of proteins that cooperate with GR to activate transcription.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

WEHI-7 is a thymoma cell line obtained from a female BALB/c mouse after x-irradiation (39). W7TG is a thioguanine-resistant variant of WEHI-7. CXG-56 is a cAMP-resistant variant of W7TG which contains no detectable cAMP-dependent protein kinase activity (17). W7 MG1 is a derivative of WEHI-7 containing expressed copies of MMTV (22). The W7 MG1 cell line was generously provided by Dr. Michael Stallcup (University of Southern California, Los Angeles, CA). MG1CX-1 is a variant selected from W7 MG1 for increased resistance to $(Bu)_2$cAMP. MG1CX-1 was isolated by plating W7 MG1 cells (2×10^5 cells/ml) into multiwell dishes (1 ml/well) in medium containing 100 μM $(Bu)_2$cAMP and 75 μM methylisobutylxanthine. Resistant colonies were obtained with a frequency of approximately 5×10^{-7}. All of the cell lines were grown in Dulbecco's modified Eagle's medium as previously described (40).

Evaluation of Cellular Drug Sensitivity

The viability of the cells in culture was determined by a trypan blue exclusion assay. The capacity of cells to proliferate was determined using a turbidity assay described by Gruol and Dalton (40). Qualitative evaluation of induced DNA digestion was carried out by analyzing DNA size on 1.8% agarose gels with Tris-borate buffer. Briefly, nuclei were prepared (41) from drug-treated cells and their DNA isolated by the method of Blin and Stafford (42). Final DNA concentrations were estimated by UV absorbance at 260 nm. DNA fragments were separated on 1.8% agarose gels containing ethidium bromide (0.25 μg/ml). Each sample contained 10 μg DNA.

Northern Blot Analysis of RNA

Total cellular RNA was isolated as described by others (43). RNA (20 μg) was heated (65 C for 10 min) in a buffer containing 50% formamide and 2.2 M formaldehyde before loading on 1% agarose gels containing 2.2 M formaldehyde. After electrophoresis, RNA was transferred onto nylon filters and hybridized with specific cDNA probes. All of the probes were prepared using a multiprime labeling kit (Amersham, Arlington Heights, IL) and by following the instructions provided by the manufacturer. Probes specific for MMTV (44), VL30 (21), the chondroitin sulfate proteoglycan core protein (21), and the constitutively expressed CHO-B gene (45) were used to evaluate drug-induced synthesis of RNA. The filters were hybridized for 18 h at 42 C in a shaking water bath. The hybridization buffer contained 50% formamide and was described by Church and Gilbert (46). After hybridization, the filters were washed and the amount of ^{32}P-labeled probe bound was detected by autoradiography.

Receptor Nuclear Translocation Assays

The capacity of receptors bound with RU 486 or TA to become tightly bound to nuclei was measured as previously described (41).

Band Shift Assays

Cytoplasmic extracts containing partially purified GR were prepared as described by Gruol and Wolfe (47) with the modifications outlined below. Briefly, cell suspensions (approximately 10^9 cells) were lysed using a Dounce homogenizer in a buffer containing 10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (pH 7.1), 2.5 mM EDTA, 2 mM KCl, 1 mM dithiothreitol, and protease inhibitors (23 μg/ml leupeptin, 32 μg/ml antipain, and 33 μg/ml pepstatin). The lysates were spun for 20 min at 17,000 × g and the pellet discarded. The supernatants were subjected to further centrifugation, 60,000 × g for 30 min, and the resulting supernatants retained. The samples were brought to 30 mM in KCl and incubated with DNA cellulose to remove hormone-independent DNA-binding proteins. The DNA cellulose was pelleted by low-speed centrifugation and the resulting supernatants passed through Millex-GV (0.22 μm) filters (Millipore, Bedford, MA). The glucocorticoid receptors were then incubated with hormone and activated. Each sample was incubated with ^{3}H-labeled hormone (either TA or RU 486 at 3×10^{-8} M) for 30 min at 0 C, 30 min at 27 C, and 30 min at 0 C. After the incubation, the samples were passed through PD-10 (Pharmacia, Piscataway, NJ) sieving columns (equilibrated with 20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), 1 mM EDTA, and 30 mM KCl) to remove free hormone. The amount of labeled hormone bound was subsequently used to estimate the concentration of ligand-containing receptor. The samples were concentrated using CentriCells (Polysciences, Warrington, MA) with a 30,000-Dalton nominal molecular mass limit. The final protein concentrations ranged from 10–20 mg/ml. The samples were made 20% in glycerol and stored at −20 C until use.

The oligonucleotide used as a probe contains a palindromic GRE and has the following sequence:

Upper strand, 5′-AAGGAAGCTTCTCTGC *AGAACATGATGTT CT*AGCTAC-3′;
Lower strand, 5′-AAGGAAGCTTGTAACT *AGAACATCATGTT CT*GCAGAG-3′.

The italic portions represent the nucleotides comprising the palindromic GRE. The single-stranded oligonucleotides were purified by gel-electrophoresis through a 15% polyacrylamide gel in 50% (wt/vol) urea, eluted, and precipitated. Complementary strands were annealed in TE buffer (10 mM Tris/Cl, pH 7.4, 1 mM EDTA, pH 8.0) and then labeled using [α-^{32}P]dATP (>3000 Ci/mmol) and the Klenow fragment of DNA-polymerase I. Double-stranded radiolabeled oligonucleotides were purified on a 6% polyacrylamide gel, eluted, ethanol-precipitated, and resuspended in TE buffer. The competitor oligonucleotides represent the CRE and TSE from the human CG α-subunit gene (48) and have been described elsewhere (49). The TAT-GRE oligonucleotide is derived from the rat TAT gene (position −2510 to −2889) and has been described by Tsai *et al.* (50).

Binding reactions were performed in a final vol of 15 μl in 10 mM Tris/Cl, pH 7.5, 70 mM KCl, 6% (vol/vol) glycerol, 2% (wt/vol) Ficoll 400, 3 mg/ml BSA, and 0.07% (vol/vol) NP-40. They contained 0.1 μg poly(dI-dC)·poly(dI-dC) as nonspecific competitor DNA. Unless indicated otherwise, the final concentration of the glucocorticoid receptor was 10^{-9} M as judged by hormone binding capacity. After preincubation for 15 min at 20 C, 1 fmol probe was added, and binding was allowed to proceed for 15 min at 20 C. When unlabeled competitor oligonucleotides were added, they were included in the preincubation step. The reactions were loaded onto 4% polyacrylamide-gels (30:1 acrylamide:bis-acrylamide) in 0.25× TAE buffer, 5% (vol/vol) glycerol (1× TAE is 40 mM Tris-acetate, pH 8.0, 1 mM EDTA) which had been prerun at 8 V/cm for 30 min. After samples were loaded, the gels were run at 8 V/cm for additional 3.5 h at room temperature, dried, and autoradiographed.

Reagents

[6,7-^{3}H] RU 486 (SA, 38.4 Ci/mmol) and unlabeled RU 486 was obtained as a generous gift from Roussel-Uclaf (Romainville, France) [1,2,4-^{3}H(*N*)]TA (SA, 29 Ci/mmol) was purchased from Amersham Corp. Hybond-N filters and the multiprime cDNA labeling kit were also purchased from Amersham. [α-^{32}P]dATP was obtained from ICN (Irvine, CA). Dex, N^6, O^2 $(Bu)_2$cAMP, and 3-isobutyl-1-methyl-xanthine were obtained from Sigma (St. Louis, MO).

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