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Transformation of Glucocorticoid Receptors Bound to the Antagonist RU 486: Effects of Alkaline Phosphatase†

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ABSTRACT: RU 486 is a synthetic steroid that binds avidly to glucocorticoid receptors without promoting their transformation into activated transcription factors. A significant part of this behavior has been shown to be due to a failure of the RU 486 bound receptor to be efficiently released from a larger (sedimenting at 8-9 S) multimeric complex containing the 90-kDa heat shock protein. Our studies have found that in vitro at 15 °C the RU 486-receptor was slowly released from the 8-9S complex and converted into a DNA binding protein by a process that could be blocked by sodium fluoride. Moreover, this transition was significantly accelerated by treatment with alkaline phosphatase. High-resolution anion-exchange chromatography showed that the profile of receptor subspecies released from the 8-9S complex (in the absence of phosphatase treatment) was different for the RU 486 bound receptor when compared to the receptor occupied by the agonist triamcinolone acetonide. Production of the earliest eluting receptor form (peak A) was inhibited with RU 486. Peak A had previously been shown to be the predominant form of the receptor possessing a capacity to bind DNA. Treatment of the RU 486-receptor with alkaline phosphatase increased the formation of the peak A subspecies as well as the capacity of receptor to bind DNA-cellulose. Taken together, the results indicate that phosphorylation of the receptor or a tightly bound factor contributes to defining the capacity with which individual steroids can promote dissociation of the 8-9S complex and conversion of the glucocorticoid receptor into a DNA-binding protein.

The steroid antagonist RU 486[1] binds avidly to glucocorticoid receptors (Jung-Testas & Baulieu, 1983; Bourgeois et al., 1984) but does not provoke the response normally manifested by agonist hormones at the level of gene expression (Baulieu, 1987; Becker et al., 1986; Chasserot-Golaz & Beck, 1984). This behavior is indicative of the antagonist's failure to promote one or more critical steps in the receptor's transition into an activated transcription factor (Baulieu et al., 1989). Therefore, RU 486 has the potential of helping to define the changes in receptor structure, which play an important role in converting the protein into its activated state. For example, early characterization of the effects of RU 486 found that it caused a lower (relative to agonists) amount of glucocorticoid receptor to translocate to the nucleus in vivo (Jung-Testas & Baulieu, 1983; Bourgeois et al., 1984). More recent in vitro

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[1] Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton; PAGE, polyacrylamide gel electrophoresis; RU 486, 17β-hydroxy-11β-[4-(dimethylamino)phenyl]-17α-propynylestra-4,9-dien-3-one; TA, triamcinolone acetonide.

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biochemical studies have demonstrated that RU 486 also did not promote the efficient dissociation of receptor from a large (sedimenting at 8–9S) cytoplasmic complex containing the 90-kDa heat shock protein (Sanchez et al., 1985; Sanchez et al., 1987; Sablonniere et al., 1984; Lefebvre et al., 1988; Groyer et al., 1987). Thus, these observations support the proposal that release of receptor from the 8–9S complex in vivo is necessary for the receptor to become tightly bound to chromatin (Vedeckis, 1989b; Howard & Distelhorst, 1988).

The fact that a significant, albeit low, level of RU 486–receptors was found in nuclei indicates that the stabilization of the 8–9S complex was not complete and leaves open the possibility that other requisite changes in the monomeric receptor structure/function may also be inhibited by the antagonist ligand. Additonal studies have shown that receptor containing RU 486 bound to the glucocorticoid regulatory element DNA sequences in vitro (Willmann & Beato, 1986) but not in the intact cell (Becker et al., 1986). Furthermore, the kinetics of DNA binding in vitro have been shown to be different for the RU 486–receptor compared to agonist-bound receptor (Willmann & Beato, 1986). Chimeric glucocorticoid receptors (containing the GAL4 DNA-binding site) bound with RU 486 also blocked the action of the GAL4 transcription factor (Webster et al., 1988). The latter observation was interpreted as a competition of antagonist-containing chimeric receptors with GAL4 at the level of the chromatin binding site. Therefore, the complete basis for the inhibitory nature of RU 486 has not been fully resolved and a comparison of receptor subspecies generated during transformation has the potential to identify relevant structural differences resulting from bound agonist versus antagonist.

The dissociation of glucocorticoid receptors from the heat shock protein complex into the smaller 4S form appears to be a necessary but not a sufficient change to generate a fully activated receptor. Reports from several laboratories have shown that receptor transformation is a multistep process and that release from the 8–9S complex does not result in the complete conversion of receptor into a protein that can bind DNA (Schmidt et al., 1985; Harmon et al., 1988; Ben-Or & Chrambach, 1988; Gruol & Wolfe, 1989). Moreover, the transition may be dependent upon cellular activities that are not inherent to the receptor protein itself. One such class may be proteins that regulate receptor phosphorylation levels. The glucocorticoid receptor is a phosphoprotein (Housley & Pratt, 1983; Tienrungroj et al., 1987; Mendel et al., 1987; Smith et al., 1989; Dalman et al., 1988) whose overall phosphate content was found to increase subsequent to binding agonist hormones (Orti et al., 1989b; Hoeck et al., 1989). The dependence of this process on the prior release of receptor from the 8–9S complex has not yet been delineated. Furthermore, RU 486 failed to provoke a measurable increase in receptor phosphorylation levels, indicating that these changes might have a functional significance.

If phosphorylation plays a significant role in controlling receptor function, dephosphorylation must also participate in the process. Both Barnett et al. (1980) and Reker et al (1987) have demonstrated that alkaline phosphatase treatment accelerated the release of agonist-bound glucocorticoid receptor from the 8–9S complex. Similarly, we (Gruol & Wolfe, 1989) found that in vitro incubation with phosphatase produced an increased rate of receptor conversion into a DNA-binding protein. We also observed that treatment with this enzyme changed the DEAE-Sepharose elution profile of the subspecies within the 4S receptor population. The result was a loss of the more "acidic" subspecies. Consistent with our findings, Dalman et al. (1988) have shown that the glucocorticoid receptor was a substrate for alkaline phosphatase. We will demonstrate below that phosphatase treatment was capable of producing transformation of glucocorticoid receptors that have bound RU 486 and that the chromatographic profile of the receptor subspecies was likewise affected during the process.

Materials and Methods

Cell Culture. WEHI-7 is a thymoma cell line obtained from a female Balb/c mouse after X-irradiation (Harris et al., 1973). A derivative of WEHI-7 resistant to thioguanine (W7TG) was used for all of the experimentation except that shown in Figure 1. W7MG1 is a derivative of WEHI-7, which contains expressed copies of the mouse mammary tumor virus (Danielsen et al., 1983). The W7MG1 cell line was generously provided by Dr. Michael Stallcup. The cells were grown as previously described (Gruol & Dalton, 1984).

Preparation of Cytoplasmic Extracts. Cytoplasmic extracts were prepared as previously described (Gruol & Wolfe, 1989). Briefly, a cell pellet was suspended in a buffer containing 10 mM Pipes (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 cells were lysed using a Dounce homogenizer and the particulate matter was removed by centrifugation at 17000*g* for 20 min. The supernatant was removed and subjected to further centrifugation (60000*g* for 30 min) and the resulting cytosol passed through a Millex-GV (22 μm) filter. For the experiments described in Figures 4 and 5B, the sample was passed twice through a PD-10 sieving column that had been equilibrated with Pipes (pH 7.1), 1 mM dithiothreitol, and 2 mM KCl to remove low molecular weight molecules. This treatment resulted in an increase in total DNA-binding capacity.

Labeling of Receptors with Hormone. (1) Intact cells: The cells were concentrated to 2×10^7 cells/mL into a 1:1 mixture of culture medium and phosphate-buffered saline. Incubations with hormone were carried out in a CO_2 incubator at 37 °C. When [^{3}H]triamcinolone acetonide was used, the concentration was 2×10^{-8} M. When [^{3}H]RU 486 was used, the concentration was $(1–2) \times 10^{-8}$ M. After a 1-h incubation, the cells were washed three times in cold (0 °C) phosphate-buffered saline to remove unbound hormone.

(2) Cytoplasmic extracts: Cell extracts were prepared and incubated with hormone (3×10^{-8} M) for 30 min at 0 °C and then at 27 °C for 20 min. When no other manipulations were to be employed, the extracts were chilled to 0 °C for 30 min and passed through a PD-10 sieving column to remove free hormone. The sieving columns were preequilibrated with 10 mM Pipes (pH 7.1), 1 mM dithiothreitol, and 2 mM KCl.

Anion-Exchange Chromatography. Chromatography was carried out by using DEAE fast flow Sepharose as previously described in detail (Gruol & Wolfe, 1989).

Receptor Binding to DNA–Cellulose. DNA–cellulose was prepared as described by Litman (1968). Prior to use, it was washed five times in DNA-binding buffer [10 mM Pipes (pH 7.1) and 70 mM KCl]. Typically, 0.3 mL of sample was incubated on a rotating wheel with 25 mg of DNA–cellulose for 1 h at 4 °C in DNA-binding buffer. The DNA–cellulose was pelleted by centrifugation and washed one time in the same buffer. Both supernatants were retained and counted. The final pellet was resuspended in 95% ethanol, removed, and placed in a scintillation vial for measurement of bound hormone. Measurement of the nonspecific binding was made with samples that had been incubated with an additional excess of unlabeled triamcinolone acetonide.

Receptor Nuclear Translocation Assays. The capacity of receptors bound with RU 486 or triamcinolone acetonide to become tightly bound to nuclei was measured as previously described (Gruol et al., 1984).

Analysis of Dexamethasone Mesylate Labeled Receptors. A cytosol sample was prepared as described above and split into two equal portions and each made 50 mM in Tris/Cl (pH 8.8). One portion was used to reflect total binding of the labeled hormone; the other portion was made 10^{-5} M in unlabeled triamcinolone acetonide and reflects the nonspecific binding. [^{3}H]Dexamethasone mesylate was added to both (10^{-7} M) and the samples were incubated at 0 °C for 30 min, at 27 °C for 20 min, and again at 0 °C for 30 min. The samples were made 1 mM in dithiothreitol and passed through PD-10 sieving columns to remove unbound hormone. The samples were loaded onto DEAE-Sepharose columns and washed with 100 mL of loading buffer and then with 25 mL of buffer containing 25 mM KCl. The receptor was step eluted from the columns with buffer containing 150 mM KCl, and the eluates were concentrated by using Centricells (MNWL 30000, Polysciences, Inc.) and lyphylized after removal of the salt. The samples were rehydrated in gel-loading buffer. Receptors labeled with [^{3}H]dexamethasone mesylate were analyzed on 7.5% polyacrylamide gels prepared as described by Laemmli (1970). The electrophoresis tank buffer contained 2 mM EDTA in addition to the standard components. After electrophoresis, the gels were impregnated with Enhance (NEN), dried, and used for fluorography. ^{14}C molecular weight markers (Amersham) were used to estimate the M_r of the receptor.

Northern Blot Analysis of MMTV mRNA Levels. Total cellular RNA was isolated as described by others (Chomczynski & Sacchi, 1987). RNA (15 μg) was heated (65 °C for 10 min) in a buffer containing 50% formamide and 2.2 M formaldehyde prior to loading on 0.8% agarose gels containing 2.2 M formaldehyde. After electrophoresis, RNA was transferred onto nylon filters and hybridized with probes specific for mouse mammary tumor virus (MMTV) (Pfahl, 1982) and the constitutively expressed CHO-B gene (Harpold et al., 1979). The probes were prepared by using a multiprime labeling kit (Amersham) and by following the protocol provided by the manufacturer. The filters were hybridized for 18 h at 42 °C in a shaking water bath. The hybridization buffer contained 50% formamide, 0.6 mg/mL denatured salmon sperm DNA, 5× SSPE (150 mM NaCl, 10 mM NaH_2PO_4, 1.25 EDTA, pH 7.4), and 10× Denhardt's solution [0.2% each of BSA, poly(vinylpyrrolidone), and Ficoll]. After hybridization, the filters were washed and the amount of ^{32}P probe bound was measured by using autoradiography. The autoradiograms were scanned with an Ultroscan XL laser densitometer (LKB) and, in each case, the MTV-specific data were normalized to the corresponding value obtained with the CHO-B probe. All samples were analyzed in duplicate.

Reagents. [6,7-^{3}H]RU 486 (specific activity 47 Ci/mmol) was obtained from Roussel-Uclaf (Romainville, France). [1,2,4-^{3}H(N)]Triamcinolone acetonide (specific activity 29 Ci/mmol) was purchased from Amersham Corp. Dexamethasone mesylate (specific activity 49.9 Ci/mmol) was purchased from Du Pont NEN. DEAE fast flow Sepharose and Sephadex PD-10 (G25M) columns were obtained from Pharmacia. Alkaline phosphatase (calf intestinal, molecular biology grade) and dithiothreitol were purchased from Calbiochem. Chemicals from Sigma included leupeptin, antipain, pepstatin A, triamcinolone acetonide, and Pipes buffer. Hybond-N filters and the multiprime labeling kit were purchased from Amersham (Arlington Heights, IL). [α-^{32}P]ATP was obtained from ICN (Irvine, CA).

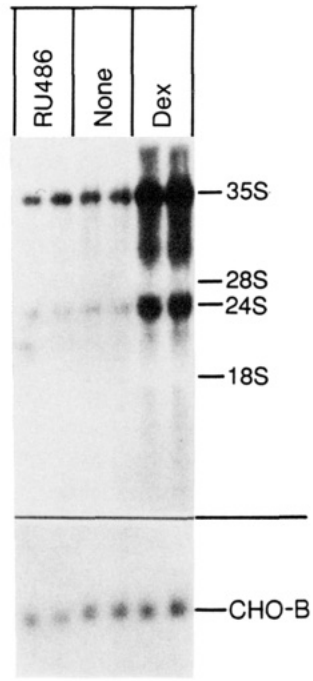

FIGURE 1: Northern blot analysis of MMTV RNA levels in W7MG-1 cells. Total cellular RNA was isolated from W7MG-1 cells that had been treated with dexamethasone or RU 486 for 6 h. Duplicate RNA samples were separated by electrophoresis using 0.8% agarose gels containing 2.2 M formaldehyde. The resulting blots were hybridized with [^{32}P]cDNA probes for mouse mammary tumor virus and the constitutively expresses CHO-B gene. Hybridization was evaluated by using autoradiography and scanning densitometry. The concentrations of steroids used were as follows: dexamethasone, 1 μM; RU 486, 0.5 μM.

RESULTS

RU 486 is a unique glucocorticoid antagonist due to its capacity to bind glucocorticoid receptors with a high affinity without producing increased rates of transcription in steroid-regulated genes. An example of this behavior is illustrated by the Northern blot analysis of RNA from W7MG-1 cells shown in Figure 1. W7MG-1 is a derivative of the WEHI-7 cell line and contains several expressed copies of the mouse mammary tumor virus (MMTV) genome (Danielson et al., 1983). Dexamethasone caused an increase in the two constitutively expressed RNA species (35S and 24S) which are typical of MTV (Hynes et al., 1981). Also induced was an MTV-specific RNA band, which migrated between the 35S and 24S species. This band is atypical of the reported MTV transcription products and may be the result of a partial deletion in one or more of the integrated viral genomes. When the quantified autoradiographic data were normalized to the constitutively expressed CHO-B RNA levels, incubation of W7MG-1 cells with dexamethasone was found to have caused a 5.82 ± 2.1-fold (n = 8) increase of MMTV mRNA levels within 6 h of hormone treatment. RU 486, on the other hand, produced no significant increase in MMTV RNA above the basal level (1.34 ± 0.46, n = 8), demonstrating its failure to activate the transcriptional capacity of the receptors. RU 486 was also found to block the effect of dexamethasone on MMTV expression as well as the steroid-mediated cytolytic response (Bourgeois et al., 1984; D. Gruol, unpublished results).

An early event in the transition of glucocorticoid receptors into active transcription factors is nuclear translocation, the

passage of receptor from the cytoplasm into the nucleus. It should be noted, for purpose of comparison, that the glucocorticoid agonist triamcinolone acetonide (TA) produces approximately 50% receptor translocation in the murine lymphoma cell line W7TG (Gruol et al., 1984, 1986). This value corresponds to approximately 14 000 receptors per nucleus (maximum). However, only 5000 receptors per nucleus bound with agonist are sufficient to elicit an effective cytolytic response in W7TG cells (Gruol et al., 1989a,b). During our present studies we found that RU 486 produced a lower level of receptor nuclear translocation (15 ± 5%, $n = 4$) than TA. This value is significant since it corresponds to 4000 receptors per nucleus when all the receptors in a cell are bound with ligand. On the other hand, incubations of intact cells with the antagonist RU 486 led to no loss in viability and, in fact, had the capacity to block the cytolytic effect of dexamethasone. Thus, the inability of RU 486 to initiate even a partial cytolytic response in W7TG cells suggests that another step, in addition to that of nuclear translocation, was also impeded by this drug. A similar conclusion was reached by Schmidt as a result of his studies with RU 486 and the steroid-sensitive human leukemic cell line CEM-C7 (Schmidt, 1986).

Recent work carried out in several laboratories has demonstrated that glucocorticoid receptor bound with RU 486 did not readily dissociate from the large (sedimenting at 8–9 S) heteromeric complex containing the 90-kDa heat shock protein (Groyer et al., 1987; Lefebvre et al., 1988). This process, which is thought to be necessary for subsequent nuclear translocation, can be monitored in vitro by using anion exchange chromatography (Parchman & Litwack, 1977; Sakaue & Thompson, 1977). Examples of this assay are illustrated by the results shown in Figure 2A, where cytosol extracts containing receptor incubated with triamcinolone acetonide or RU 486 were analyzed on DEAE-Sepharose columns. Receptors that had bound triamcinolone acetonide were completely converted to the peak I form. Receptor that had bound RU 486 exhibited a much more complex pattern with only 50% of the receptor eluting at the peak I position of the profile. Another 40% of the receptor remained as peak II, characteristic of the 8–9S receptor complex. An additional peak (approximately 5–10%) was released between peaks I and II at a position (eluting at 160 mM KCl) coincident with a very minor component of the TA–receptor profile. The multiple forms of the RU 486 receptor observed within the region of peak I are indicative of subspecies within the 4S form (Gruol & Wolfe, 1989) and will be described in more detail below.

The results shown above demonstrate that RU 486 was less effective than triamcinolone acetonide at causing in vitro conversion of receptor from the peak II form into that of peak I. Since the inhibition was not an absolute block, it suggests that a significant difference between the effectiveness of RU 486 and TA as agonists might be related to the kinetics with which each promotes transformation of the receptor. This possibility had been raised previously and was discussed by Baulieu (1987). Interestingly, Reker et al. (1987) had shown that treatment with alkaline phosphatase increased the rate of glucocorticoid receptor conversion from the 8–9S form into a smaller 5.2S species. We (Gruol & Wolfe, 1989) had reported a similar effect relative to the conversion of receptor into a DNA-binding protein. Both of these studies, however, were carried out with agonist hormones. Figure 2B shows that the incubation of a cytosolic sample with alkaline phosphatase caused a nearly complete conversion of RU 486–receptor into the peak I form. For comparison, a sample that had been incubated with RU 486 and then at high ionic strength (0.4 M KCl) is also shown. Phosphatase was much more effective in promoting the production of the peak I form of the RU 486–receptor complex than the commonly employed high-salt condition (Schmidt & Litwack, 1982). The narrow width of the large peak (I) seen with the phosphatase-treated sample reflects a loss of the more "acidic" receptor subspecies that exist in the entire peak I population. This will be illustrated more definitively below. Peak I for the KCl-treated sample remained broad and indicative of a more complex receptor subpopulation. The additional peak that eluted at 160 mM KCl was also more prominent after the high salt treatment.

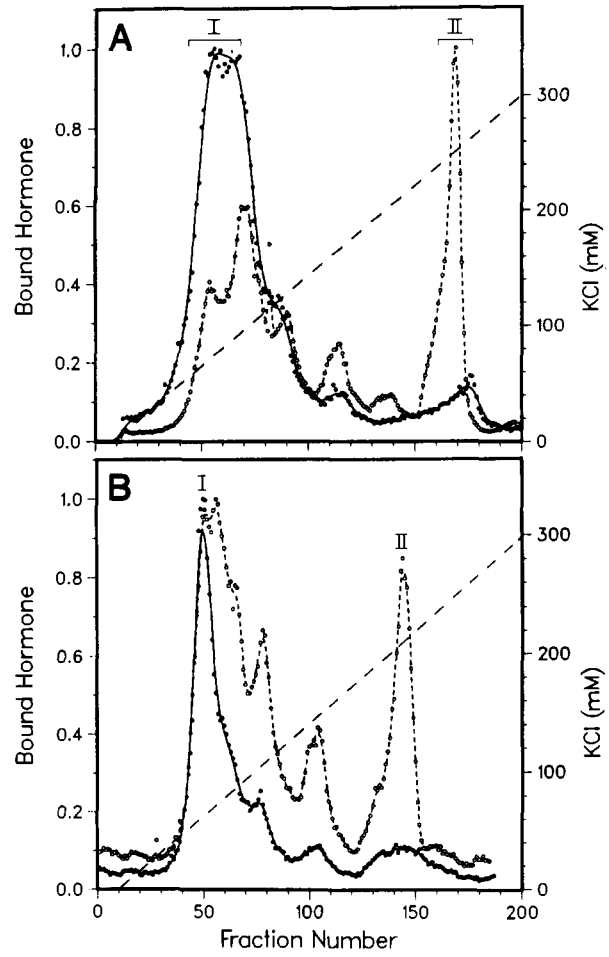

FIGURE 2: (A) DEAE-Sepharose elution profile of glucocorticoid receptors incubated with triamcinolone acetonide or RU 486. Cytosol extracts were prepared and incubated with either [^{3}H]RU 486 or triamcinolone acetonide for 30 min at 0 °C and then for 20 min at 27 °C. The samples were loaded onto separate columns of DEAE fast flow Sepharose, and the bound receptor was eluted with a linear gradient of KCl (2–300 mM KCl). Fractions, 1.5 mL, were collected and analyzed for hormone content by liquid scintillation measurements. The bound hormone is expressed normalized to the fraction with the most radioactivity for each set: triamcinolone acetonide, 2432 cpm (–●–); RU 486, 8544 cpm (--o--). (B) DEAE-Sepharose elution profile of glucocorticoid receptors incubated with RU 486 and treated with alkaline phosphatase or high salt concentrations. Cytosol extracts were prepared and incubated with RU 486 for 30 min at 0 °C and then for 20 min at 27 °C. One extract was incubated further with 50 units/mL alkaline phosphatase for 60 min at 15 °C (–●–). The other extract was maintained at 0 °C but made 0.4 M in KCl (--o--). The samples were analyzed by anion exchange chromatography as in (A). The curves were normalized to the fraction containing the most radioactivity for each set: phosphatase treated, 2382 cpm; KCl treated, 1166 cpm).

The effect of phosphatase observed above was reflected in the conversion of RU 486–receptor into a DNA-binding protein and is documented in Table I. Receptors bound with TA were found to have a DNA-binding capacity of 43%. An

Table I: Effect of Phosphatase on the Transformation of Glucocorticoid Receptors Bound with TA or RU 486[a]

conditions of steroid incubation	% bound to DNA–cellulose
Triamcinolone Acetonide	
0 °C, 1 h	9.1 ± 3.7 (n = 7)
27 °C, 20 min	42.7 ± 12.9 (n = 7)
27 °C, 20 min; 15 °C for 1 h with alkaline phosphatase	43.4 ± 11.0 (n = 4)
RU 486	
incubated at 27 °C for 20 min then	
0 °C, 1 h	24.4 ± 4.4 (n = 8)
15 °C, 1 h	29.8 ± 3.0 (n = 8)
15 °C, 1 h with alkaline phosphatase	46.4 ± 8.9 (n = 8)
15 °C, 1 h with *boiled* alkaline phosphatase	28.4 ± 1.3 (n = 4)

[a] Cytosol extracts were incubated with hormone for 30 min at 0 °C and then under the conditions listed in the table. Free hormone was removed with dextran charcoal. Specific hormone binding was determined with parallel incubations containing an excess (500-fold) of triamcinolone acetonide. The incubations with alkaline phosphatase were carried out at 50 units/mL. The samples were tested for their receptors capacity to bind DNA–cellulose during a 1-h incubation at 4 °C. Data points are the mean ± SEM. Values in parentheses represent the number of independent determinations for each measurement.

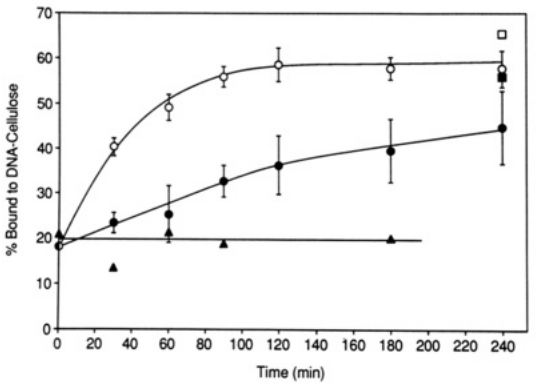

FIGURE 3: Time course of the conversion of RU 486–receptor into a DNA binding protein. Cytosol extracts were incubated with RU 486 for 30 min at 0 °C, for 20 min at 27 °C, and then at 15 °C for the time indicated. The capacity to bind DNA–cellulose was measured for each sample. During the incubations at 15 °C, the extracts contained the following additions: none, –●–; 50 units/mL alkaline phosphatase, –○–; 50 mM NaF, –▲–. The circles represent the mean ± SEM of three separate determinations and the triangles the average of two determinations. The squares represent samples that were incubated with triamcinolone acetonide as well as with (□) or without (■) alkaline phosphatase.

additional incubation with alkaline phosphatase produced no further change. Receptors bound with RU 486 had a lower DNA-binding capacity (24.4%) after an initial incubation at 27 °C, which is consistent with the incomplete conversion of peak I receptor shown in Figure 2A. However, addition of active alkaline phosphatase produced an increase in DNA binding up to 46.4%, similar to the level seen with the TA–receptor complex. This latter observation is also consistent with the increased production of peak I seen in Figure 2B. Incubation of RU 486–receptor samples for 1 h at 15 °C with or without boiled alkaline phosphatase introduced only a small increase in the DNA-binding capacity.

The effect of phosphatase treatment on the rate of transformation of RU 486–receptor is more vividly illustrated by the results shown in Figure 3. These data represent a time course of RU 486–receptor conversion to a DNA-binding protein in the presence and absence of alkaline phosphatase. After an initial incubation at 27 °C for 20 min, the samples were incubated at 15 °C (with or without phosphatase) for the times indicated. Without phosphatase, the DNA-binding

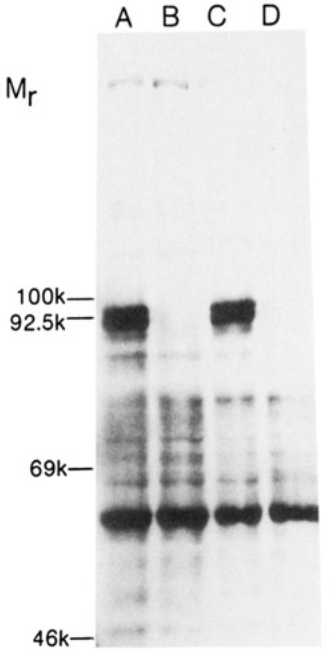

FIGURE 4: Size of the glucocorticoid receptor and alkaline phosphatase treatment. A cytosol sample was prepared and the receptors covalently labeled with [^{3}H]dexamethasone mesylate as described in the Materials and Methods section. The sample was analyzed by SDS–PAGE and autoradiography. Prior to analysis, half of the sample was incubated with alkaline phosphatase (80 units/mL) for 1 h at 15 °C (lanes C and D). The other portion was left untreated (lanes A and B). The samples in lanes B and D represent nonspecific binding, since the incubation with [^{3}H]dexamethasone mesylate contained an excess of unlabeled triamcinolone acetonide.

capacity slowly increased over a period of at least 4 h. Addition of phosphatase accelerated the process to the extent that the final level of DNA binding approached that found with triamcinolone acetonide and was completed within 2 h. It should be noted that for this experiment, the sample preparation was slightly modified to include removal of low molecular weight components as described in the Materials and Methods section. This change produced a greater final DNA-binding capacity (approximately 60% compared with 40–50%).

The work of Reker et al. (1987) had also indicated that an endogenous cytoplasmic activity could contribute to the receptor transformation process. Since these authors had reported that sodium fluoride could inhibit the putative endogenous activity, this salt was tested to see if it would inhibit the slow conversion of RU 486–receptor seen at 15 °C. The results, which are also shown in Figure 3, indicate that 50 mM NaF completely arrested the increase in DNA-binding capacity. Since this compound is not an effective inhibitor of alkaline phosphatase activity, it was not tested in this context. In contrast to the results of Reker and colleagues, however, we found little effect of sodium fluoride on the transformation of receptor promoted by triamcinolone acetonide under these conditions (data not shown). Thus, the effect of NaF may be confined only to a set of circumscribed conditions that dictate very low rates of receptor transformation.

Figure 4 demonstrates that treatment with phosphatase did not cause a detectable change in the size of the receptor protein. In this experiment, the receptors in a cytosol extract were covalently labeled with [^{3}H]dexamethasone mesylate (with or without unlabeled hormone as competitor) and analyzed by polyacrylamide gel electrophoresis (Eisen et al., 1981). The sample was divided into two sets and one set was incubated with alkaline phosphatase prior to analysis. The other re-

mained untreated. Both sets of samples contained ligand-specific bands corresponding to proteins with molecular weights of 93K and 91K. A similar receptor doublet had been observed by others using Western blotting (Northrop et al., 1985). Incubation with alkaline phosphatase did not produce a change in the receptor doublet bands or evidence of the specific proteolysis products (ranging from 54 to 46 kDa) of the mouse glucocorticoid receptor that have been reported by others (Middlebrook & Aronow, 1977; Vedeckis, 1983a). In addition, we have used sieving chromatography with Sephacryl S-300 under elevated salt concentrations (300 mM KCl) and found no evidence that phosphatase treatment caused the production of a receptor form smaller than the intact 6-nm particle (data not shown).

We had previously shown that the peak I form of the glucocorticoid receptor is a mixed population of receptor subspecies that can be resolved by anion-exchange chromatography (Gruol & Wolfe, 1989). A typical profile generated by receptor bound with TA is shown in Figure 5A (filled circles) and represents resolution of receptor subspecies contained within peak I. The profile of receptor subspecies generated by incubating receptor with RU 486 is also shown. The two patterns are distinctly different. RU 486 resulted in a greater relative amount of receptor released as the more "acidic" subspecies (eluting at higher ionic strength), peaks C–E. This difference is significant since continued incubation of TA–receptor produced a progressive loss of the peaks C–E (Gruol et al., 1988). This is exemplified in Figure 5B, which shows another comparison of the patterns obtained from incubations with TA and RU 486. In this instance, an initial incubation at 27 °C for 20 min was followed by a second incubation at 15 °C for 2 h. The profile obtained with TA shows a nearly complete loss of peaks C–E with a concomitant relative increase in peak A. The profile of the RU 486–receptor exhibited a smaller relative decline in peaks C–E, along with a significant continued inhibition in the formation of peak A. Since peaks C–E have been shown to be converted into peaks A and B (Gruol & Wolfe, 1989), the results confirm that this process (production of peak A) is also impeded for the RU 486–receptor.

We had also shown earlier that the peak A subspecies is the predominant DNA-binding form of the receptor (Gruol & Wolfe, 1989; Gruol et al., 1989c) and is, therefore, diagnostic of the conversion of receptor into a DNA-binding protein. The reduced production of peak A (relative to the TA–receptor) seen with RU 486 in Figure 5A,B correlates well with the lower capacity of the RU 486–receptor to bind DNA. Since alkaline phosphatase was found to accelerate receptor transformation, its effect on the DEAE elution profile was also studied. The results are shown in Figure 5C as a comparison between incubations with active and boiled enzyme. Treatment with active enzyme produced a large increase in the relative amount of peak A such that most (51.4%) of the receptor was observed in this form. A measurement of DNA-binding capacity prior to chromatography found 47% of the receptor capable of binding DNA after phosphatase treatment compared with 27% before incubation with enzyme. Moreover, the large change in the DEAE elution profile was not due to the selective loss of bound steroid in the more "acidic" forms since the overall RU 486 steroid-binding capacity did not change as a result of enzyme treatment. Additional experiments involving more extensive enzyme treatment found that more than 80% of the RU 486–receptor could be converted into the peak A form (data not shown). A second sample that had been incubated with boiled alkaline phosphatase is also

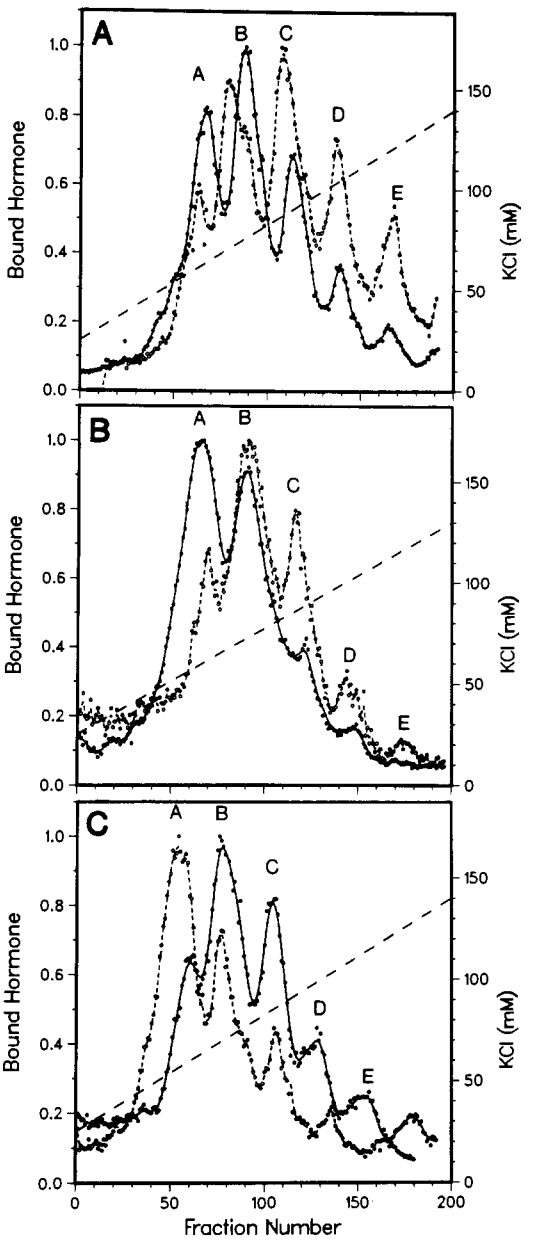

FIGURE 5: (A) High-resolution DEAE-Sepharose elution profile of peak I receptors containing bound triamcinolone acetonide or RU 486. Cytosol samples were incubated with tritiated triamcinolone acetonide or RU 486 for 30 min at 0 °C and then at 27 °C for 20 min. After removing bound hormone, the samples were loaded onto separate DEAE-Sepharose columns and the receptor eluted with linear gradients of KCl confined to the peak I region (25–140 mM). The bound hormone is expressed normalized to the fraction containing the most radioactivity: triamcinolone acetonide, 4442 cpm (–●–); RU 486, 1142 cpm (--o--). (B) DEAE-Sepharose elution profile of peak I receptors after extended incubations with triamcinolone acetonide or RU 486. Cytosol extracts were prepared and passed through PD10 columns to increase their overall DNA binding capacity. The samples were incubated with hormone for 30 min at 0 °C, for 20 min at 27 °C, and finally for 2 h at 15 °C. The receptor is described in (A). The bound hormone is expressed normalized to the fraction containing the most radioactivity: triamcinolone acetonide, 2346 cpm (–●–); RU 486, 514 cpm (--o--). (C) DEAE-Sepharose elution profile of RU 486–receptors treated with alkaline phosphatase. Cytosol extracts were incubated with RU 486 for 30 min at 0 °C, for 20 min at 27 °C, and then for 60 min at 15 °C. Alkaline phosphatase (50 units/mL) was added to the samples prior to the incubation at 15 °C. One sample received enzyme that had been treated by boiling to destroy its activity. The receptor subspecies were separated by using DEAE-Sepharose chromatography as described in (A). The bound hormone is expressed normalized to the fraction containing the most radioactivity: treated with active phosphatase, 946 cpm (--o--); treated with boiled phosphatase, 1190 cpm (–●–).

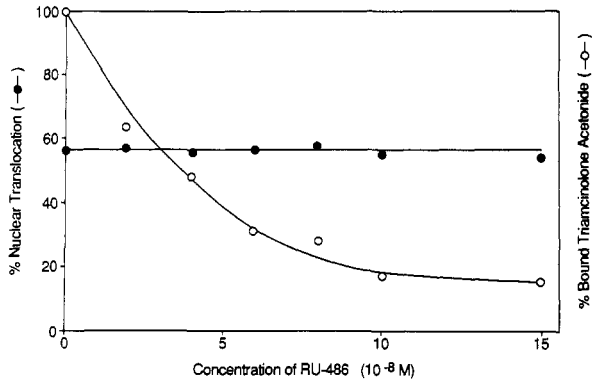

FIGURE 6: Measurement of the effect of RU 486 on the nuclear translocation of receptors containing bound triamcinolone acetonide. W7TG cells were incubated with a fixed concentration of [^{3}H]TA (1.2×10^{-8} M) and increasing concentrations of unlabeled RU 486 for 1 h at 37 °C. The percent of TA–receptor bound to nuclei (—●—) and the total amount of TA bound to the cells (—○—) were measured. The hormone binding values are expressed relative to the samples that did not contain any RU 486. Each point is the average of duplicate measurements.

shown for comparison in Figure 5C. As in Figure 5A,B, the pattern shows a consistent inhibition in the production of the peak A subspecies, thus indicating that active enzyme was necessary to effect the change.

The glucocorticoid receptor is thought to bind to its DNA regulatory elements (GRE's) as a dimer (Tsai et al., 1988; Wrange et al., 1989). This observation raises the possibility that glucocorticoid receptors, like estrogen receptors (Gordon & Notides, 1986), interact with each other prior to binding DNA. If this is the case, RU 486 containing receptors could potentially affect the function of receptors containing bound agonist. Figure 6 shows the results of an experiment that addressed this issue relative to nuclear translocation. W7TG cells were incubated with a fixed concentration of labeled triamcinolone acetonide and increasing concentrations of unlabeled RU 486. The amount of bound TA was progressively competed by the RU 486. When a maximum of 1.5×10^{-7} M RU 486 was used isn the incubation, only 13% of the receptors contained bound TA. This corresponds to a value of 6.7 (87%/13%) for the RU 486–/TA–receptor ratio. Measurements of nuclear translocation were made for each concentration of RU 486 employed. The fraction of receptor found tightly bound to nuclei did not change regardless of the differing ratios (lowest, 0.59; highest, 6.7) of RU 486–/TA–receptor. Thus, in regard to nuclear translocation, receptors containing bound agonist functioned independently of receptors bound with antagonist RU 486. The results also indicate that RU 486 did not act indirectly by inhibiting a transformation-promoting cytoplasmic factor. In this regard, in vitro experiments failed to find an effect of RU 486 on cytoplasmic phosphatase activity or on the ability of receptors prebound with TA (at 0 °C) to be converted to DNA-binding proteins (data not shown).

Discussion

The facilitated conversion of glucocorticoid receptors into functioning transcription factors is thought to require structural changes that not only expose a DNA-binding site but also allow for altered interactions with other cytoplasmic as well as chromatin proteins (Baulieu et al., 1989; Sablonniere et al., 1984; Strahle et al., 1988; Schule et al., 1988a,b). The exact role of the steroid molecule is only partially understood. The hormone binding portion of the receptor resides in the carboxy-terminal third of the molecule (Giguere et al., 1986; Rusconi & Yamamoto, 1987; Danielsen et al., 1986) and has been shown to act as a repressor of receptor function (Godowski et al., 1987; Danielsen et al., 1987). Removal of the steroid-binding domain produces a constitutively active receptor. Moreover, this portion of the receptor can be transposed to the amino terminus and still retain its steroid-dependent repressive capacity (Picard et al., 1988). These and other observations (Pratt et al., 1988; Denis et al., 1988a) have led to the proposal that a structural component of the steroid-binding domain is responsible for stabilizing the receptor's association with the 90-kDa heat shock protein. In this type of model, the binding of steroid produces conformational changes in the receptor that lead to its release from the heat shock protein complex. Hormone antagonists such as RU 486 would be viewed as deficient in this capacity (Baulieu et al., 1989).

Conversion of receptor from the 8–9S form into the smaller 4S species is a temperature-dependent process (Schmidt & Litwack, 1982; Denis et al., 1988b), which may involve activities not inherent to the receptor protein itself. Support for this view comes from work carried out in several laboratories. Schmidt et al. (1985) found that purified 8–9S receptor complex required a heat-sensitive cytoplasmic activity to affect receptor transformation. On the other hand, Harmon et al. (1988) have demonstrated the existence of a heat-stable macromolecular factor, which acted to convert monomeric receptors into DNA-binding proteins. These activities may also be related to the 70-kDa protein reported by Tymoczko et al. (1988), which had the capacity to enhance the conversion of receptor into a DNA-binding protein. Thus, the observed dependence of transformation on temperature could reflect modification of either the receptor itself or another component contained within the 8–9S heat shock protein complex.

Several studies have demonstrated that the rate of receptor transformation can be accelerated by alkaline phosphatase treatment (Barnett et al., 1980; Reker et al., 1987; Gruol & Wolfe, 1989). Since the glucocorticoid receptor has also been shown to be a substrate for alkaline phosphatase (Dalman et al., 1988), the results support a model where removal of phosphate destabilizes the receptor's association with the 90-kDa heat shock protein. Our results indicate that glucocorticoid receptors bound with RU 486 had the capacity to undergo a slow in vitro conversion to the DNA-binding state at 15 °C. This transition was inhibited by sodium fluoride, possibly reflecting inhibition of an endogenous activating mechanism, although we have not observed an equivalent effect of fluoride on receptor bound with triamcinolone acetonide. The RU 486–receptor was similar to the TA–receptor, however, in that its transformation could also be accelerated by alkaline phosphatase treatment. Thus, added phosphatase can overcome the constraints on receptor transformation imposed upon the system by temperature or ligand.

The peak I form of the glucocorticoid receptor is composed of a set of receptor subspecies (peaks A–E) that can be resolved by anion-exchange chromatography. All of the peak A receptor formed during in vitro incubations with triamcinolone acetonide (without alkaline phosphatase) has the capacity to bind to DNA–cellulose (Gruol & Wolfe, 1989). A comparison of the receptor elution profiles from incubations with TA or RU 486 indicated that the formation of peak A was inhibited for the RU 486–receptor. Treatment of RU 486–receptor samples with phosphatase resulted in the enhanced conversion of receptor from the other peaks (C–E) into that of peak A. Thus, phosphatase not only promoted a release of receptor from the 8–9S complex but altered the overall charge dis-

tribution associated with the receptor as well. These results verify that the receptor protein (or a tightly bound factor) could be a substrate for phosphatase within, of after its release from, the 8–9S complex.

Neither Tienrungroj et al. (1987) nor Orti et al. (1989a) were able to detect a change in receptor phosphate levels as a result of an in vitro transformation with agonist hormone. Thus, the effects of alkaline phosphatase that we have observed may represent a general destabilization of the 8–9S complex and reflect an important role for phosphate groups in determining the overall stability of interations between receptors and heat shock proteins. Dephosphorylation, on the other hand, need not normally contribute to the transition of receptor into a DNA-binding protein. However, phosphorylation would still have considerable significance in this regard if, as has been suggested (Bresnick et al., 1989), a receptor/heat shock protein interaction is necessary to confer steroid binding capacity to the receptor. This is particulary true since earlier work had shown that alkaline phosphatase treatment caused a loss of hormone binding capacity in ligand-free receptors (Nielsen et al., 1977). Destabilization of the 8–9S complex by removing phosphate from either the receptor or the heat shock proteins would then explain the loss of hormone binding function. Conversely, binding of agonist hormones normally causes conformational changes in the receptor that destabilize protein–protein interactions, which may involve phosphate groups. Antagonists such as RU 486 appear to produce the requisite conformational changes at a lower rate, which can be accelerated in vitro by phosphatase treatment. Thus, this model suggests that the state of phosphorylation of the glucocorticoid receptor has a potential to influence the relative agonist/antagonist quality of a variety of steroids that exhibit partial agonist activity.

Experiments carried out with intact cells have indicated that after binding hormone phosphate levels were increased for both the nuclear receptor and the receptor remaining in the cytoplasm (Orti et al., 1989b). This phenomenon requires, however, that phosphate be removed at some stage since the receptor is thought to recycle between transformed and untransformed states (Munck & Holbrook, 1984; Raaka and Samuels, 1983). It is also important to keep in mind that, in the intact cell, phosphate may be removed from one set of sites and added to another with little change in the overall phosphate levels. Whatever the case, our results show that the profile of receptor subspecies released from the 8–9S complex after binding RU 486 is different than that seen with triamcinolone acetonide. If this distinction is untimately reflected in a receptor function such as chromatin binding or transcriptional activation, it would indicate that regulation of receptor structure/function can be inhibited at more than one level by the antagonist RU 486.

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