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Comparison of DNA Binding Properties of Activated, Covalent and Noncovalent Glucocorticoid Receptor–Steroid Complexes from HTC Cells†

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ABSTRACT: Several differences in the interaction with DNA of noncovalent vs. covalent glucocorticoid receptor–steroid complexes are described. HTC cell glucocorticoid receptors were incubated under cell-free conditions with the potent reversible glucocorticoid dexamethasone and with the irreversible antiglucocorticoid dexamethasone 21-mesylate to yield noncovalent and covalent complexes, respectively. Using DNA immobilized on cellulose, we found that the noncovalent dexamethasone complexes were activated (by dilution in pH 8.8 buffer at 0 °C) to a DNA binding species 2-fold faster than were covalent dexamethasone 21-mesylate labeled complexes. The affinity of activated, noncovalent dexamethasone complexes for DNA in an equilibrium binding assay was 2-fold higher than that of the activated, covalent dexamethasone 21-mesylate complexes. This conclusion was supported by the observations in a DNA–cellulose pellet assay that covalent receptor–steroid complex binding to DNA was disrupted by lower NaCl concentrations than was noncovalent complex binding. The same studies of DNA binding at various NaCl concentrations failed to provide evidence that glucocorticoid receptor–steroid complex binding to DNA is a multistep process. These quantitative distinctions in the DNA binding properties of covalent and noncovalent receptor–steroid complexes represent the first physicochemical differences between the complexes of antiglucocorticoid and glucocorticoid steroids and may partially account for their divergent biological properties.

Antiglucocorticoids which bind to glucocorticoid receptors are potentially useful probes of the mechanism of action of glucocorticoid steroids. Unfortunately this line of research has been severely limited by the facts that most antiglucocorticoids (1) have a low affinity for, and a rapid rate of dissociation from, the receptor protein and (2) interact with other receptor proteins (Samuels & Tomkins, 1970; Raynaud et al., 1980; Svec & Rudis, 1982). We therefore have been

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preparing derivatives of glucocorticoid steroids in an effort to obtain receptor-specific, high-affinity antiglucocorticoids which would not readily dissociate from the glucocorticoid receptor (Simons et al., 1980a,b; Simons & Thompson, 1981; Simons, 1982; Lamontagne et al., 1984).

The most useful antiglucocorticoid that we have so far developed is dexamethasone 21-mesylate which forms a covalent receptor–steroid complex (Simons & Thompson, 1981; Eisen et al., 1981; Simons, 1982) and which is an irreversible antagonist in HTC cells (Simons & Thompson, 1981; Simons, 1981). Dexamethasone 21-mesylate does display some agonist activity, the amount of which varies with different systems (Mercier et al., 1983) as has been seen with the antiestrogen tamoxifen (Mester et al., 1981; Martin, 1981). Nevertheless, dexamethasone 21-mesylate has proved to be a useful probe of glucocorticoid hormone action. An examination of the effects of modifications of steroid structure yielded the conclusion that no one portion of the steroid molecule determines the biological activity of glucocorticoid steroids (Lamontagne et al., 1984). Dexamethasone 21-mesylate was found to form a covalent receptor–steroid complex in whole cells (Simons et al., 1983), thus indicating that not all antiglucocorticoids must rapidly dissociate from the receptor (Raynaud et al., 1980). Finally, covalent receptor–dexamethasone 21-mesylate complexes could be activated in cell-free systems to a DNA binding complex with the same efficiency as could noncovalent receptor–dexamethasone complexes (Simons et al., 1983). Therefore, the antiglucocorticoid activity of dexamethasone 21-mesylate is not due to an inability of the covalent complexes to undergo activation (Samuels & Tomkins, 1970). However, while the apparent affinity of activated complexes for DNA was the same for both covalent and noncovalent complexes, the whole cell nuclear binding properties of the two complexes were not the same (Simons et al., 1983). This observation, plus recent results by others (Hunziker, et al., 1983; Svec & Williams, 1983), suggests that "activation" may be a multistep process. Since acquisition of DNA binding properties could be an early event in whole cell activation, we have reexamined the DNA binding of covalent and noncovalent receptor–steroid complexes for differences in complex binding to DNA which could contribute to the altered nuclear binding properties of the covalent complexes and to the whole cell antiglucocorticoid activity of dexamethasone 21-mesylate. These studies with dexamethasone 21-mesylate have uncovered several differences in the interactions of covalent and noncovalent receptor–steroid complexes with DNA. The rate of activation to a DNA binding complex is about 2-fold faster for the noncovalent complexes. The affinity of complex binding to DNA is 2-fold higher for the noncovalent complexes. Finally, the DNA binding of noncovalent complexes is less sensitive to increasing concentrations of NaCl.

Materials and Methods

Materials. [^{3}H]Dexamethasone (46.0 Ci/mmol) was purchased from Amersham; [^{3}H]dexamethasone 21-mesylate (46.0 or 48.9 Ci/mmol) was obtained from New England Nuclear. Hydrofluor was supplied by National Diagnostics. Nonradioactive dexamethasone was obtained from Sigma Chemical Co. Fetal bovine and newborn bovine sera were purchased from KC Biological, Inc. Swim's S77 medium without antibiotics was custom formulated by GIBCO Laboratories. TAPS[1] and Tricine were purchased from Calbiochem-Behring Corp. Tris was obtained from Research Plus Laboratories. DNA–cellulose (native calf thymus DNA; 1.2 or 2 mg of DNA/mL of cellulose) was prepared by P-L Biochemicals and stored at −20 °C.

Buffers and Solutions. $TAPS_0$ buffer, which consisted of 25 mM TAPS, 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA), and 10% glycerol, was adjusted to pH 8.8 or 9.5 at 0 °C on a Beckman 3500 pH meter with an Altex combination electrode calibrated with Beckman pH 7 and 10 standards at 0 °C. TENN buffer, composed of 10 mM Tris, 1 mM EDTA, 150 mM NaCl, and 0.02% NaN_3, and TE buffer, composed of 10 mM Tris and 1 mM EDTA, were adjusted to pH 7.4 at room temperature. SDS (2×) sample buffer contained 0.6 M Tris (pH 8.85 at room temperature), 2% sodium dodecyl sulfate (SDS), 0.2 M dithiothreitol, 20% glycerol, and bromphenol blue.

DNA–cellulose was suspended in TENN buffer and centrifuged for 45 s at 500*g* in a Sorvall GLC-4 centrifuge. The supernatant, containing fines and any dissociated DNA, was removed by aspiration; fresh TENN was added such that the DNA–cellulose pellet comprised one-third of the final total volume. This DNA–cellulose solution was stored in 5–10-mL aliquots at −20 °C.

Cell Culture and Preparation of Cytosol. HTC cells were grown in spinner or monolayer culture in Swim's S77 medium supplemented with 5% fetal bovine serum, 5% newborn bovine serum, and 0.03% glutamine as described by Thompson (1979). Cytosol (~20 mg of protein/mL) was prepared from washed, frozen cell pellets by freeze–thaw lysis and stored in liquid nitrogen as described elsewhere (Reichman et al., 1984). Specific, cell-free glucocorticoid binding activity was assayed as previously described (Simons et al., 1979). Briefly, cytosol was incubated in pH 8.8 $TAPS_0$ buffer at 0 °C for 2.5 h with 5×10^{-8} M [^{3}H]dexamethasone in the absence or presence of 600-fold excess [^{1}H]dexamethasone. Free steroid was then removed by the addition of 0.1 volume of a 10% dextran-coated charcoal solution followed by centrifugation (2300*g* for 10 min); bound steroid was quantitated by scintillation counting of an aliquot of the supernatant with Hydrofluor in a Beckman LS 7800 or LS 133 scintillation counter (counting efficiency = 40–50%). Specifically bound steroid, the difference between the amounts of [^{3}H]dexamethasone bound in the presence and absence of [^{1}H]dexamethasone, ranged from 0.8 to 2.0 pmol/mg of cytosolic protein.

Labeling and Activation of Cytosol. As has been previously described (Simons et al., 1983), cytosol was treated for 2.5 h at 0 °C in 0.67 volume of $TAPS_0$ buffer, pH 9.5, with $(1.5–2.0) \times 10^{-7}$ M [^{3}H]dexamethasone 21-mesylate in the absence or presence of 80-fold excess [^{1}H]dexamethasone or with 5×10^{-8} M [^{3}H]dexamethasone in the absence or presence of 600-fold excess [^{1}H]dexamethasone. This mixture was then diluted with an equal volume of $TAPS_0$, pH 8.8. β-Mercaptoethanol was added to a final concentration of 50 mM to prevent further covalent labeling by [^{3}H]dexamethasone 21-mesylate; an 80-fold excess of [^{1}H]dexamethasone was added to cytosols labeled just with [^{3}H]-dexamethasone 21-mesylate in order to block the noncovalent binding of the reaction product of free [^{3}H]dexamethasone 21-mesylate and β-mercaptoethanol. The diluted cytosol was incubated either at 20 °C for 30 min to activate receptor–steroid complexes or at 0 °C for 30 min to consume the unreacted [^{3}H]dexamethasone 21-mesylate without effecting activation.

The efficiency of the covalent labeling procedure was assessed by quantification of the specifically labeled M_r 98 000 protein observed after SDS–polyacrylamide gel electrophoresis

[1] Abbreviations: TAPS, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)aminomethane; HTC, hepatoma tissue culture.

as described by Simons et al. (1983). Samples to be analyzed were treated with an equal volume of 2× SDS sample buffer, heated at 100 °C for 5 min, and electrophoresed on 10.8% polyacrylamide gels at 30 mA/gel (20 mA/gel until the sample entered the running gel). The gels were then fixed and stained with Coomassie blue, sliced, digested in 30% hydrogen peroxide, and counted in Hydrofluor at 33% counting efficiency on a Beckman LS-7800 liquid scintillation counter. In those experiments employing 1.5×10^{-7} M [^{3}H]dexamethasone 21-mesylate, the amount of covalently labeled 98K receptor equalled 77 ± 16% (SD; $n = 16$) of the total receptor, which was measured by the specific charcoal-resistant binding of 5×10^{-8} M [^{3}H]dexamethasone in a parallel assay solution. Incubation with 2×10^{-7} M [^{3}H]dexamethasone 21-mesylate gave a labeling efficiency of 98K receptor equal to 98 ● 11% (SD; $n = 8$).

DNA–Cellulose Binding Assay. The binding of receptor–steroid complexes to DNA–cellulose was measured with the following modifications of an assay described previously (Simons, 1977). Aliquots of DNA–cellulose slurry were added to 4.5–mL conical polystyrene centrifuge tubes and washed with 1.5–2.0 mL of 0 °C $TAPS_0$ buffer, pH 8.8. After centrifugation (1250*g* for 2 min at 0 °C), the supernatant was removed by aspiration. The final pellet volume was one-third that of the initial aliquot. $TAPS_0$ buffer, pH 8.8 (150 μL), and labeled cytosol containing activated complexes (250 μL) were added to the washed DNA–cellulose pellets. Tubes were vortexed to resuspend DNA–cellulose and then incubated on a Bellco roller drum apparatus at 13 rpm at 4 °C. Cytosol labeled with [^{3}H]dexamethasone was incubated with DNA–cellulose for 0.5–1.25 h; cytosol labeled with [^{3}H]dexamethasone 21-mesylate was incubated with DNA–cellulose for 1–1.75 h. Cytosol labeled with either steroid in the presence of excess [^{1}H]dexamethasone was incubated from 1.5 to 3 h. Receptor–steroid complex binding to DNA–cellulose was maximal and stable over these time intervals (see Results). Binding was terminated at equilibrium by centrifugation (1250*g* for 2 min at 0 °C), and the supernatant was removed by aspiration. A single wash of the DNA–cellulose pellets dramatically reduced the nonspecific binding but had little effect on the observed specific binding; a second wash caused little further change (data not shown) and was omitted. Thus, the pellets were routinely washed with 1.0 mL of $TAPS_0$ buffer, pH 8.8, centrifuged as described above, aspirated to complete dryness with a flame-tapered Pasteur pipet, and incubated for at least 1 h at room temperature with 0.2 mL of $TAPS_0$ buffer, pH 8.8, before being transferred to a scintillation vial with two 0.1-mL H_2O washes and counted in 10 mL of Hydrofluor. Duplicates generally varied by less than 5%. Specific binding of [^{3}H]steroid–receptor complexes to DNA–cellulose was calculated as the difference between binding of cytosol labeled in the absence (total binding) and presence (nonspecific binding) of excess [^{1}H]dexamethasone. Nonspecific binding was usually less than 7%, or 25%, of the total binding in [^{3}H]dexamethasone, or [^{3}H]dexamethasone 21-mesylate, labeled cytosols, respectively.

Measurement of DNA and Protein Concentration. DNA concentration was determined spectrophotometrically; the OD_{260}/OD_{280} ratio was between 1.8 and 2, and 1 OD_{260} unit was equal to 50 μg of DNA/mL. Protein concentration was measured by the method of Lowry et al. (1951), with bovine serum albumin as the standard.

Results

Binding of Receptor–Steroid Complexes to DNA–Cellulose in Suspension. The binding of receptor–^{3}H steroid complexes to cellulose has commonly been used as a measure of the nonspecific binding of complexes to DNA–cellulose (LeFevre et al., 1979; Schmidt et al., 1981; Norris & Kohler, 1983). Because so much more protein sticks to DNA–cellulose than to cellulose under our binding conditions (Figure 1), we chose to use the binding to DNA–cellulose of receptors labeled with ^{3}H + excess ^{1}H steroid as a measure of nonreceptor binding to DNA. This was particularly important in the case of [^{3}H]dexamethasone 21-mesylate labeled receptor solutions where proteins other than the receptor are covalently labeled in various amounts (Simons & Thompson, 1981; Eisen et al., 1981; Reichman et al., 1984).

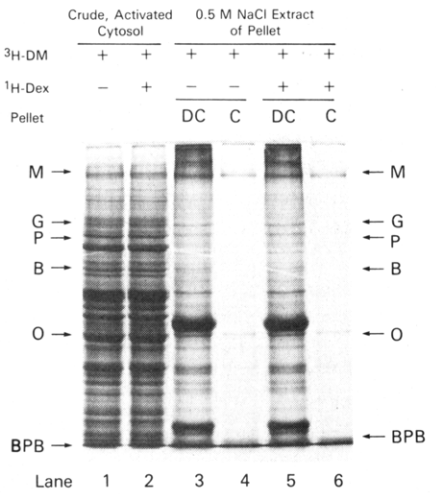

FIGURE 1: Coomassie Blue stained SDS–polyacrylamide slab gel of [^{3}H]dexamethasone 21-mesylate ± [^{1}H]dexamethasone labeled cytosol bound to DNA–cellulose and to cellulose. Crude HTC cell receptors were labeled with 1.6×10^{-7} M [^{3}H]dexamethasone 21-mesylate ● 1.6×10^{-5} M [^{1}H]dexamethasone and then activated as described under Materials and Methods. Aliquots of the activated solutions were diluted 1:1 with 2× SDS sample buffer for subsequent analysis on gels. Four washed pellets (20 μL each) of DNA–cellulose, or of cellulose, in 300 μL of pH 8.8 $TAPS_0$ were each incubated with 500 μL of the above activated solutions for 2.5 h/0 °C and then washed with pH 8.8 $TAPS_0$ as described under Materials and Methods. Each group of four pellets was combined and extracted (2 × 0.5 mL) with pH 8.8 TAPS containing 0.5 M NaCl. The protein in the combined two salt extracts was precipitated with acid (Cl_3CCOOH), dissolved in a minimum of 1 N NaOH, and diluted with 2× SDS sample buffer. SDS–polyacrylamide slab gels (9%) were then run of the activated cytosols (lanes 1 and 2), of the Cl_3CCOOH-precipitated 0.5 M NaCl extracts of [^{3}H]dexamethasone 21-mesylate labeled cytosol that bound to DNA–cellulose (DC) and cellulose (C) (lanes 3 and 4), and of the Cl_3CCOOH-precipitated 0.5 M NaCl extracts of [^{3}H]dexamethasone 21-mesylate + [^{1}H]dexamethasone labeled cytosol that bound to DNA–cellulose (DC) and cellulose (C) (lanes 5 and 6). The positions of the molecular weight markers myosin (M), β-galactosidase (G), phosphorylase *b* (P), bovine serum albumin (B), ovalbumin (O), and bromphenol blue (BPB) are indicated by the arrows.

The abundance of dexamethasone 21-mesylate labeled, nonreceptor species in crude cytosol (Figure 2A) necessitated determining what species are detected by our DNA–cellulose binding assay. DNA–cellulose pellets were incubated with crude cytosol that had been treated with [^{3}H]dexamethasone 21-mesylate ± 100-fold excess [^{1}H]dexamethasone. The pellets were extracted with $TAPS_{500}$ buffer (=$TAPS_0$ + 0.5 M NaCl; 2 × 125 μL) to yield ≥86% of the radioactivity bound to DNA–cellulose. Greater than 99% of the extracted, specifically bound [^{3}H]dexamethasone 21-mesylate was acid precipitable (data not shown). Greater than 80% of the extracted specific binding was present in the 98K holoreceptor (Figure 2B); the remaining specific binding was found in what appear to be proteolytic fragments of the 98K receptor (Simons et al., 1983; Reichman et al., 1984). The lower yield of

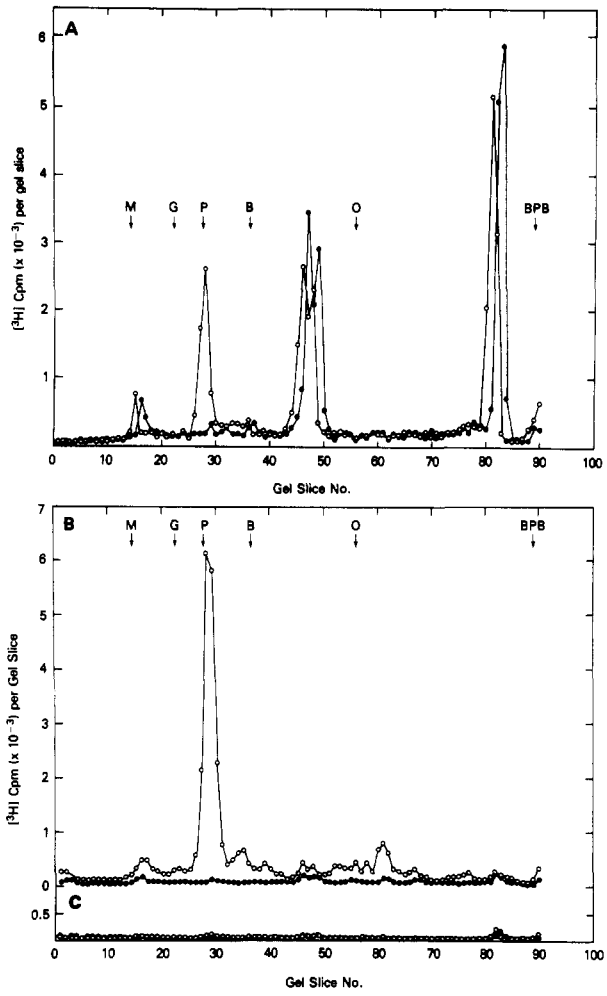

FIGURE 2: Binding of [^{3}H]dexamethasone 21-mesylate labeled cytosol to DNA–cellulose and to cellulose. The lanes of the gel in Figure 1 were sliced and counted [(○) cytosol labeled with [^{3}H]dexamethasone 21-mesylate; (●) cytosol labeled with [^{3}H]dexamethasone 21-mesylate + [^{1}H]dexamethasone] as follows: (A) activated cytosol (lanes 1 and 2 of Figure 1), (B) 0.5 M NaCl extracts of DNA–cellulose pellets (lanes 3 and 5 of Figure 1), and (C) 0.5 M NaCl extracts of cellulose pellets (lanes 4 and 6 of Figure 1) that had been incubated with [^{3}H]dexamethasone 21-mesylate ± [^{1}H]dexamethasone labeled cytosol. The arrows indicate the positions of the molecular weight markers.

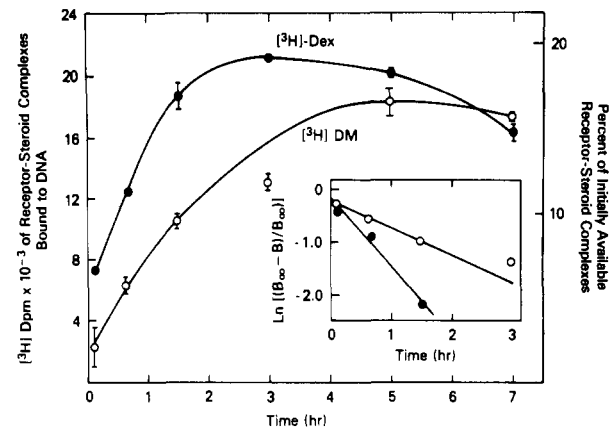

FIGURE 3: Rate of activation at 0 °C of covalent and noncovalent complexes to a DNA-binding complex upon dilution in pH 8.8 buffer. Unactivated receptor–steroid complexes (250 μL; labeled with 5.3 × 10^{-8} M [^{3}H]dexamethasone ± 3.0 × 10^{-5} M [^{1}H]dexamethasone or with 2.0 × 10^{-7} M [^{3}H]dexamethasone 21-mesylate ± 1.6 × 10^{-5} M [^{1}H]dexamethasone) were prepared at a final pH of 8.7 at 0 °C, incubated at 4° C in duplicate tubes containing 16.7 μL of DNA–cellulose pellet in 150 μL of pH 8.8 $TAPS_0$ buffer for the indicated times, and processed as described under Materials and Methods. The specific DNA binding of [^{3}H]dexamethasone (●) and [^{3}H]dexamethasone 21-mesylate (○) labeled receptors was plotted as a function of the time of incubation with DNA–cellulose. Because a limiting amount of DNA–cellulose was used in these assays, the maximal amount of DNA-bound complexes represents only about half of those complexes that could bind to DNA (see Figure 4). In order to determine the rate of complex activation, the maximum observed specific DNA binding for the covalent and noncovalent complexes was set equal to B_∞. The specific DNA binding at other times was used to calculate ln $[(B_\infty - B)/B_\infty]$, which was plotted vs. time (see insert). The best-fit lines for the data were generally determined by linear least-squares analysis.

nonspecifically bound, covalently labeled species from cellulose pellets (Figure 2C) was expected from the above results and further supports our decision to use DNA–cellulose binding of receptors labeled with ^{3}H ± excess ^{1}H steroid to assess the level of nonspecific binding to DNA.

Changes in the amount of DNA associated with cellulose can lead to artifactual results during the assay (LeFevre et al., 1979). The crude HTC cell receptor preparations contain little, if any, endogenous nuclease activity, as shown by a lack of increase of acid-soluble, ^{14}C-labeled, nick-translated MMTV–LTR DNA after a 3.8-h incubation with [^{1}H]dexamethasone bound receptors under the conditions of the assay (data not shown). The binding of both covalent and noncovalent complexes to DNA–cellulose was fast (>80% of the maximal binding was seen after 15 min at 0 °C). The level of bound complex was stable for the duration of the assays (1–4 h). This stability of binding is a further indication that there is no nuclease digestion of cellulose-bound DNA; it also demonstrates that the dissociation of DNA from the cellulose during the course of this assay was negligible [cf. LeFevre et al. (1979)]. With longer incubation times, we sometimes did see a decrease in complex binding to DNA–cellulose which may reflect a slow dissociation of DNA. However, this decrease may also be related to an occassionally observed, time-dependent loss of steroid-bound receptors (data not shown).

Rate of Activation of Covalent and Noncovalent Receptor–Steroid Complexes. In $TAPS_0$ buffer with 20 mM Na_2MoO_4 (pH 8.8), unactivated complexes do not bind to DNA–cellulose (Reichman et al., 1984) while activated complexes bind rapidly (see above). Thus, the rate of activation of receptor–steroid complexes can be approximated by following the kinetics of DNA binding for initially unactivated complexes. Both covalent and noncovalent complexes were found to undergo activation at 0 °C with reasonably rapid rates upon dilution in high pH (i.e., 8.8) buffer (Figure 3). In both cases the amount of DNA binding by the spontaneously activated complexes was about equal to that seen with conventional, heat-activated complexes (data not shown). Linear semilog plots of the kinetics of activation (see insert of Figure 3) indicate that, for both complexes, the rate of activation was first order and that the noncovalent receptor–steroid complexes undergo spontaneous activation at a rate that is 2.6 times faster than that of the covalent complexes [$T_{1/2}$ for activation = 0.49 ± 0.05 h (n = 3) for noncovalent complexes; $T_{1/2}$ = 1.32 ± 0.37 h (n = 3) for covalent complexes]. In a preliminary experiment, the rate of spontaneous activation of noncovalent receptor–dexamethasone complexes at a lower pH (i.e., 8.2 vs. 8.8) was significantly slower ($T_{1/2}$ = 0.77 h). These results confirm and extend earlier studies that demonstrated first-order kinetics of activation for noncovalent glucocorticoid receptor–steroid complexes (Atger & Milgrom, 1976; Munck & Holbrook, 1984).

Effect of NaCl on Binding of Activated Complexes to DNA. If receptor–steroid complex binding to DNA were a reversible,

Table I: Effect of NaCl on Covalent and Noncovalent Receptor–Steroid Complex Binding to DNA[a]

receptors labeled with	value of $NaCl_{50}$ (mM) for salt added during DNA binding	after DNA binding at equilibrium
[^{3}H]dexamethasone	62.4 ± 7.8 (n = 4)	68.8 ± 3.6 (n = 5)
[^{3}H]dexamethasone 21-mesylate	44.6 ± 8.5 (n = 5)	53.9 ± 8.7 (n = 6)

[a] Activated, ^{3}H-labeled cytosol (250 μL) was incubated with $TAPS_0$, pH 8.8 (150 μL), and DNA–cellulose (16.7 μL of DNA–cellulose pellet) at 4 °C. If NaCl was present during binding, the NaCl concentration of the $TAPS_0$ buffer was varied such that the final concentration of the assay mixture (400 μL) was 0, 20, 40, 60, 90, or 120 mM NaCl. Cytosol labeled with dexamethasone or dexamethasone 21-mesylate was incubated for 0.75 h or from 1 to 1.25 h, respectively. If NaCl was added after equilibrium binding (approximately 0.75-h incubation), however, the initial TAPS buffer contained no added NaCl. $TAPS_0$ buffer (32.5 μL) containing 13.3× the desired final concentration of NaCl was added at 0.75 h. Samples containing cytosol labeled with dexamethasone or dexamethasone 21-mesylate were then incubated for a total of 1.25 h or from 1.5 to 1.75 h, respectively. Samples were processed as described under Materials and Methods with the following modification: pellets were washed with TAPS buffers whose ionic strengths were comparable to those of the final assay mixtures. The NaCl concentration required for half-maximal inhibition of control binding was interpolated from graphs where specific binding, expressed as a percentage of control (specific binding at no added NaCl), is plotted as a function of NaCl concentration. It is important to note that the cytosol preparations do contribute to the final ionic strength of the assay mixture. With a Radiometer (Model CDM2e) conductivity meter, we have determined that the salt concentration of cytosol diluted to its assay strength is comparable to 15.9 ± 3.0 (SD; n = 5) mM NaCl. The table values are based solely on added NaCl.

single-step reaction, then the effect of added NaCl during complex association with, and dissociation from, DNA–cellulose should generate identical curves of DNA bound complexes vs. NaCl concentration. Quantitatively, the concentrations of added NaCl required to reduce the binding in $TAPS_0$ buffer by 50% (defined as "$NaCl_{50}$") should be identical, whether the NaCl is added during the DNA binding reaction or after the binding has reached equilibrium, and would be related to the strength of the complex–DNA interaction. Conversely, unequal $NaCl_{50}$ values would indicate the presence of an irreversible reaction and possibly a multistep reaction, similar to that seen in the formation of RNA polymerase–DNA transcription initiation complexes (Chamberlin, 1974). Prior to conducting these experiments with receptor–steroid complexes, we determined that added NaCl did not change the rate of complex binding to DNA, that the level of DNA-bound complexes was stable for 1–4 h with added NaCl, and that addition of NaCl to solutions of complexes prebound to DNA produced new equilibrium binding levels within 40 min (data not shown). Interestingly, the nonspecific binding was relatively insensitive to changes in NaCl concentration.

The $NaCl_{50}$ values for covalent dexamethasone 21-mesylate complex binding to DNA, or for dissociation of these complexes from DNA, were lower than the $NaCl_{50}$ values for the noncovalent dexamethasone complexes (Table I). These differences are not large; but, in six paired experiments (three for binding and three for dissociation), the $NaCl_{50}$ value for the covalent complexes was always lower than that of the noncovalent complexes. It should also be noted that, for each type of receptor–steroid complex, the $NaCl_{50}$ values for complex binding and dissociation were essentially the same.

The DNA binding of activated complexes in TAPS buffer was more sensitive to added NaCl than was the binding of activated complexes in Tricine buffer at the same pH. While 27–49% of the activated complexes in Tricine buffer plus 75 mM NaCl bound to DNA–cellulose columns (Simons et al., 1983), only 18% of the activated complexes in TAPS buffer plus 50 mM NaCl bound to columns (data not shown). This behavior may be due to the larger amount of proteolyzed receptor fragments in the Tricine buffer cytosol preparations (Simons et al., 1983; Reichman et al., 1984) which could have a higher affinity for DNA than the holoreceptors (Wrange & Gustafsson, 1978).

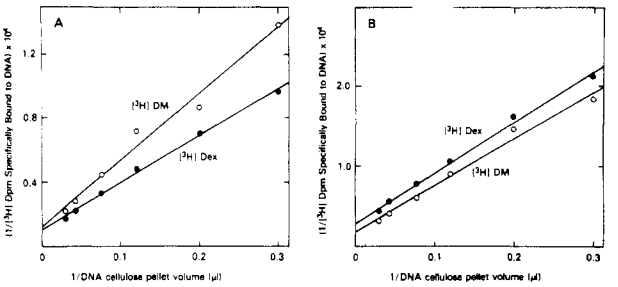

FIGURE 4: Determination of affinity of covalent and noncovalent receptor–steroid complex binding to DNA. Activated cytosol (250 μL; labeled with 5.0×10^{-8} M [^{3}H]dexamethasone ± 3.0×10^{-5} M [^{1}H]dexamethasone or with 15×10^{-8} M [^{3}H]dexamethasone 21-mesylate ± 1.2×10^{-5} M [^{1}H]dexamethasone) was prepared as described under Materials and Methods and incubated with various amounts of DNA–cellulose (pellet = 3.3, 5, 8.3, 13.3, 23.3, and 33.3 μL) in pH 8.8 $TAPS_0$ at 4 °C for 0.75–2.25 h. Specific DNA binding was determined as described under Materials and Methods, expressed as the reciprocal, and plotted vs. 1/(DNA–cellulose pellet volume). The K_d for receptor–steroid complex binding to DNA was obtained from these double-reciprocal plots as the quotient of (slope)/(y intercept). Different preparations of crude receptors gave double-reciprocal plots for DNA binding of covalent [^{3}H]dexamethasone 21-mesylate (○) or noncovalent [^{3}H]dexamethasone- (●) labeled complexes that were either convergent (A) or parallel (B).

Affinity of Covalent and Noncovalent Receptor–Steroid Complex Binding to DNA. The above $NaCl_{50}$ data suggest that the covalent receptor–steroid complexes have a lower affinity for DNA than do the noncovalent complexes. We had previously concluded, on the basis of salt elution profiles of DNA–cellulose bound complexes, that both complexes had identical affinities for DNA (Simons et al., 1983). This question was therefore reexamined by means of double-reciprocal plots of equilibrium binding of a constant amount of receptor–steroid complexes to increasing amounts of DNA (Simons et al., 1976; Simons, 1977). With this equilibrium assay, the affinity for DNA of noncovalent dexamethasone-bound complexes was found to be 1.8 times greater than that of the covalent dexamethasone 21-mesylate labeled complexes.[2] These double-reciprocal graphs always displayed either convergent (Figure 4A) or parallel (Figure 4B) data. The graphs with convergent data indicated that equal amounts of covalent and noncovalent complexes can be activated to DNA-binding species, as was previously described (Simons et al., 1983). In the experiments yielding graphs with parallel data, and thus unequal amounts of activated complexes, it appears that this difference in quantity of activated complexes is due to an increase in the percentage of covalent complexes that were activated (data not shown). This behavior (convergent or parallel double-reciprocal plots) was specific for a given preparation of crude receptors and may eventually provide

[2] The double-reciprocal plots give values for receptor–steroid complex affinity for DNA that have the units of "DNA–cellulose pellet volume (in μL)"; the ratio of the K_d values for covalent and noncovalent complex binding to DNA is unitless. In six paired experiments, K_d^{DM} = 38.2 ± 17.4 and K_d^{Dex} = 21.7 ± 5.3. These values were significantly different at $P < 0.05$ (t test). The average of the six paired ratios of K_d^{DM}/K_d^{Dex} = 1.80 ± 0.75.

interesting clues regarding the hitherto unknown mechanism of activation.

Discussion

We have described three physicochemical differences in cell-free systems between the covalent receptor–steroid complexes of the irreversible antiglucocorticoid dexamethasone 21-mesylate and the noncovalent receptor–steroid complexes of the potent synthetic glucocorticoid dexamethasone: (1) the covalent complexes are spontaneously activated to a DNA-binding complex at a 50% slower rate than that seen for the noncovalent complexes, (2) DNA binding of the covalent complexes is inhibited by lower concentrations of NaCl than that for the noncovalent complexes, and (3) DNA binding affinity of the covalent complexes is about half that of the noncovalent complexes. As far as we are aware, this is the first time that physicochemical differences have been noted for receptor–antiglucocorticoid steroid complexes. The prior inability to observe such differences was almost certainly due to the rapid rates of receptor–steroid dissociation that are associated with all of the previously available antiglucocorticoids (Samuels & Tomkins, 1970; Raynaud et al., 1980; Svec & Rudis, 1982). The use of an antiglucocorticoid which forms a covalent receptor–steroid complex (Simons & Thompson, 1981; Eisen et al., 1981; Simons, 1982; Simons et al., 1983) obviates this problem.

These experiments were conducted with crude cytosol in order to retain cytoplasmic factors which may participate in receptor–steroid complex activation and binding to DNA. The differences between covalent and noncovalent receptor–steroid complexes do not appear to be due to indirect effects of the alkylating steroid dexamethasone 21-mesylate on nonreceptor molecules because (1) very low concentrations of dexamethasone 21-mesylate were used (i.e., $\leq 2 \times 10^{-7}$ M), (2) the labeling of nonreceptor species was not inhibited by excess dexamethasone (see Figure 2A), indicating that these species must have low steroid affinity and are present in large excess, and (3) the rate of activation of both covalent and noncovalent complexes is consistent with a first-order or pseudo-first-order process, which would rule out the involvement of other species that are not present in large excess.

The rates of activation of covalent and noncovalent complexes seen here were considerably faster than those observed elsewhere (Higgins et al., 1973; Milgrom, 1981; Schmidt & Litwack, 1982; Norris & Kohler, 1983; Kumar & Dickerman, 1983; Vedeckis, 1983a). Several experimental differences could account for the present behavior. We have conducted our experiments at a higher pH, which is known to accelerate activation (Bailly et al., 1978; Schmidt & Litwack, 1982). Our assay solutions contained high concentrations of thiols (i.e., 31 mM β-mercaptoethanol) which can increase the amount of activation (Vedeckis, 1983b). The assay system contained DNA which, by virtue of high local concentrations of ionic strength, could cause activation (Milgrom, 1981). Finally, the use of TAPS buffer appeared to faciliate activation since much more binding to DNA–cellulose columns was seen for "unactivated" complexes in $TAPS_0$ buffer than for "unactivated" complexes in $Tricine_0$ buffer (Simons et al., 1983; data not shown).

The salt sensitivity (and affinity) of receptor–steroid complex binding to DNA is often assessed by means of salt elution profiles of complexes bound to DNA–cellulose columns (Yamamoto et al., 1974; Gehring & Hotz, 1983; Okret et al., 1983; Simons et al., 1983). We have found that receptor–steroid complex binding to DNA–cellulose pellets is much more sensitive to added NaCl than is complex binding to DNA–cellulose columns. Thus, receptor–dexamethasone complex binding to pellets was inhibited by 50% at ~85 mM NaCl (includes 16 mM NaCl from cytosol) while 120 mM NaCl was required to half-maximally elute the same complexes from a DNA–cellulose column (data not shown). This higher value of NaCl for column elution is probably due to weak DNA binding of the complexes in the increasing NaCl gradient which artifactually retards the elution of complexes from the column. It would appear that the equilibrium pellet binding assay that we have used thus affords a more accurate assessment of the effect of NaCl on glucocorticoid complex binding to DNA. These results are very similar to those obtained by Rousseau et al. (1975) in which 50 mM NaCl was found to decrease complex binding by 50% to the DNA appearing in the void volume of Sepharose 2B columns.

In equilibrium binding studies to DNA–cellulose or to free DNA, estrogen receptor complexes with the antiestrogen 4-hydroxytamoxifen exhibited a 2-fold lower affinity for DNA than did complexes with the agonist estradiol (Evans et al., 1982). This result is identical with what we have found with the antiglucocorticoid vs. glucocorticoid complexes.[2] We cannot yet say whether all antagonist complexes will have 2-fold lower affinity for DNA than the agonist complexes, but the results with 4-hydroxytamoxifen indicate that our present observations are not unique for an alkylating steroid which yields a covalent receptor–steroid complex.

The differences described above for the covalent dexamethasone 21-mesylate vs. the noncovalent dexamethasone labeled receptors appear insufficient to account for the major differences in biological activity (Simons & Thompson, 1981; Simons, 1982) and in nuclear localization of receptors (Simons et al., 1983) that are observed with the antagonist dexamethasone 21-mesylate and the agonist dexamethasone. Some of the other factors that could contribute to the observed biological responses are receptor–steroid complex interactions with cytoplasmic components not found in the cytosol preparations, with chromosomal proteins, and with specific sequences of DNA. The following paper (Miller et al., 1984) describes our studies of receptor–steroid complex binding to portions of the MMTV genome that have been found to contain the glucocorticoid regulatory site in whole cells (Lee et al., 1981; Huang et al., 1981).

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