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Glucocorticoid Receptor Binding to Calf Thymus DNA. 2. Role of a DNA-Binding Activity Factor in Receptor Heterogeneity and a Multistep Mechanism of Receptor Activation

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ABSTRACT: In the preceding paper [Cavanaugh, A. H., & Simons, S. S., Jr. (1990) *Biochemistry* (preceding paper in this issue)], we characterized an apparently identical factor in the cytosol and the nuclear extract of HTC cells that is required for the DNA binding of approximately 43% of the activated receptor–glucocorticoid complexes. In the present study, both those activated complexes that are influenced by this factor and the role of this factor in the process of activation are examined. We find that sodium arsenite inhibits only the DNA binding of those complexes that require factor. Conversely, methyl methanethiolsulfonate inhibits the DNA binding of only those complexes that are independent of factor. These results provide direct chemical evidence for two populations of activated complexes. Double-reciprocal plots revealed that the increase in DNA binding with endogenous factor occurred by recruiting new complexes for DNA binding as opposed to increasing the binding affinity of existing complexes. These results further suggest that factor associates only with the receptor–steroid complex and does not additionally interact with DNA. A saturable association of factor with complexes was indicated since the amount of available factor in cytosolic solutions decreased after activation of the complexes. Sodium molybdate is known to inhibit the activation of HTC cell receptor–steroid complexes. When factor was added to complexes that had been subjected to activating conditions in the presence of the inhibitor sodium molybdate, no increased DNA binding was observed. In contrast, the addition of factor to Sephadex G-50 chromatographed, activated complexes did stimulate DNA binding, even in the presence of molybdate. Collectively, these data establish the presence of two populations of activated receptor–steroid complexes. Activation of one population of complexes was shown to involve an additional step that is not seen for the other population. This additional step is characterized by an insensitivity to molybdate and a requirement for our recently characterized DNA-binding activity factor.

Activation of receptor–steroid complexes, whereby the initially formed complexes attain a high affinity for DNA, is still a poorly understood process (Baxter et al., 1972; Kalimi et al., 1975; Milgrom, 1981). Progress has been impeded both by the lack of a rigorous definition of activation and by the apparent involvement of several nonreceptor components, only some of which have been studied in any detail (Schmidt & Litwack, 1982; Harmon et al., 1984, 1988; Schmidt et al., 1985; Tai et al., 1986; Webb et al., 1986; Pratt, 1987; Bodine & Litwack, 1988a,b; Mechinchi et al., 1988). One major advance has been the discovery that sodium molybdate can block activation [Leach et al., 1979; for review see Dahmer et al. (1984)]. In the preceding paper in this issue (Cavanaugh & Simons, 1990), we described and characterized a macromolecular factor that is required for the maximal binding of glucocorticoid receptors to DNA. This factor was first identified in the cytosol of HTC cells by the observation that Sephadex G-25 or G-50 gel filtration chromatography decreased the ability of activated receptor–[^{3}H]dexamethasone (Dex)[1] complexes to bind to calf thymus DNA–cellulose. This decrease could be reversed by the addition of cytosolic components. This decreased binding was not observed when the same chromatographed complexes were tested for their ability to bind to isolated nuclei. We subsequently found that salt extracts of crude nuclei contained 5–10 times more of the apparently identical DNA-binding activity factor. Hence the removal of cytosolic factor during the chromatography of activated complexes caused a decrease in the binding to DNA but not to nuclei since the nuclear binding assay supplied an

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[1] Abbreviations: Dex or dexamethasone, 9α-fluoro-16α-methyl-11β,17,21-trihydroxypregna-1,4-diene-3,20-dione; TAPS, 3-{[tris(hydroxymethyl)methyl]amino}propanesulfonic acid; MMTS, methyl methanethiolsulfonate.

excess of the same endogenous factor. This result typifies the problems inherent in activation studies. While the binding of complexes to both nuclei and especially DNA has been used to quantitate the extent of activation, the two methods do not always give the same results.

Physicochemical analysis revealed that our factor was precipitable with $(NH_4)_2SO_4$, was stable to heat (100 °C for 10 min) as well as several freeze–thaw cycles, was resistant to digestion with nucleases and several proteases, and possessed a molecular weight between 700 and 3000 [preceding paper (Cavanaugh & Simons, 1990)]. Taking advantage of these properties, we examined the role of this factor in the process of activation. Chemical inhibitors of DNA binding were used to establish the existence of two subpopulations of activated complexes, only one of which interacted with the DNA-binding activity factor. Our data also provide direct evidence that the activation of one subpopulation of glucocorticoid complexes proceeds via at least a two-step mechanism. These steps can be separated by their sensitivities to Na_2MoO_4, to our newly described factor, and to the chemical inhibitors of DNA binding.

Experimental Procedures[2]

The sources of most reagents, and the preparation of all buffers, are described in the preceding paper in this issue. Sodium molybdate and sodium arsenite were purchased from Baker. Methyl methanethiolsulfonate (MMTS) was obtained from Aldrich Chemical Co.

Preparation of cytosol from HTC cells to provide glucocorticoid receptors as well as the conditions for [^{3}H]Dex binding to receptor, for preparation of nuclear extract factor, and for preparation of factor-depleted cytosol by chromatography through Sephadex G-50 are described in the preceding paper in this issue.

Receptor–steroid complex binding to calf thymus DNA–cellulose and DE-52 cellulose (DEAE) was quantitated by the minicolumn technique of Holbrook et al. (1983) incorporating the modifications of the preceding paper. Determination of the K_d for receptor–steroid complex binding to DNA was carried out with a DNA–cellulose pellet assay exactly as described by Simons and Miller (1984). All other experimental details are given in the legends.

The computer analysis of the effect of activation on the concentration of free factor in cytosol, as seen by a shift in the titration curves of Figures 3 and 4, was conducted in collaboration with Dr. Harry Saroff (NIH). For these studies, we assumed the model (see also Figure 5):

$$\text{receptor–steroid complex (RS)} + \text{factor (F)} \overset{K_1}{\rightleftharpoons} \text{RS·F}$$

and

$$\text{RS·F} + \text{DNA} \overset{K_2}{\rightleftharpoons} \text{RS·F·DNA}$$

The other variables were the concentration of binding sites for RS·F on DNA and the concentration of F. The concentration of RS (=the total amount of steroid-bound receptor that could be induced to bind to DNA after the addition of F) was set at 0.2 nM, which was the value obtained in the experiment of Figure 3. Using the equations for the equilibrium association constant, we derived the equation for $(RS{\cdot}F{\cdot}DNA)_i/(RS)_{total}$ in terms of the other parameters, except that F was always expressed in terms of the total added F. The curves best fitting the observed data [after replotting the data of Figure 3 as $(RS{\cdot}F{\cdot}DNA)_i/(RS)_{total}$] were determined manually by successive approximations using reasonable values for the various parameters.

[2] Unless otherwise indicated, all manipulations were performed at 0 °C.

Table I: Effect of Sephadex G-50 Chromatography, Time, and Addition of Nuclear Extract on DNA Binding of Activated and Factor-Depleted Cytosol[a]

receptors	nuclear extract factor	% of complexes bound to DNA after 1 h/0 °C	% of complexes bound to DNA after 4 h/0 °C
activated cytosol	–	72	79
activated cytosol	+		82
unactivated cytosol	–	19	50
unactivated cytosol	+		47
unactivated cytosol through Sephadex G-50	–		48
unactivated cytosol through Sephadex G-50	+		85

[a] HTC cytosol [60% (v/v) in TAPS buffer; pH at 0 °C ~8.8] was incubated with [^{3}H]Dex for 2.5 h. The solution was diluted 1:2 with TAPS buffer and an aliquot kept at 0 °C to yield unactivated cytosol, while activated cytosol was prepared by warming the complexes at 25 °C for 30 min. An aliquot (500 μL) of unactivated cytosol was loaded onto a 9-mL Sephadex G-50 column, and receptor–[^{3}H]Dex complexes were eluted in the void volume. Aliquots (40 μL) of receptor preparations were mixed with 60 μL of nuclear extract factor (which had been desalted by Sephadex G-10 chromatography), the volume was brought to 120 μL with TAPS buffer, and receptor binding to the DNA of DNA/DEAE minicolumns was assayed as described previously [preceding paper (Cavanaugh & Simons, 1990)]. The percentage of receptor bound to DNA is expressed as (dpm of complexes bound to DNA/dpm of complexes bound to DNA + DEAE) × 100. In some experiments, samples were assayed for DNA binding ability after 1-h incubation. In other experiments with added factor, all samples were assayed after a total of ~4 h.

Results

Activation of Glucocorticoid Receptor–Steroid Complexes by Sephadex G-50 Chromatography. Gel filtration chromatography is often used to activate receptor–glucocorticoid complexes (Higgins et al., 1973; Ali & Vedeckis, 1987; Bodine & Litwack, 1988b). This was confirmed under our conditions for Sephadex G-25 [preceding paper in this issue (Cavanaugh & Simons, 1990)] and G-50 chromatography (data not shown), but the extent of activation was always less than that achieved by heat and dilution (preceding paper). We suspected that Sephadex chromatography not only caused activation but also removed the DNA-binding activity factor to give overall, submaximal DNA binding (preceding paper and data not shown). The data of Table I support this hypothesis by showing that Sephadex chromatography of unactivated cytosol yields complexes for which the DNA binding is increased by the addition of factor. In most of the following experiments, we have used extracts of crude HTC nuclei as our source of factor.

Considerable activation of unactivated complexes occurred spontaneously over 4 h at 0 °C (Table I). Such slow activation has been previously documented (Simons & Miller, 1984). Hence, the extent of activation caused by Sephadex G-50 chromatography after 4 h is much less than when the unactivated complexes are assayed sooner (compare with Table II of preceding paper). There can be no doubt, however, that factor stimulates the DNA binding of unactivated complexes only after they have been chromatographed (Table I).

Effect of Factor on the Affinity of Activated Complexes for DNA. The addition of factor could increase the amount of Sephadex G-50 chromatographed complexes that bind to DNA either by increasing the affinity of complexes for DNA

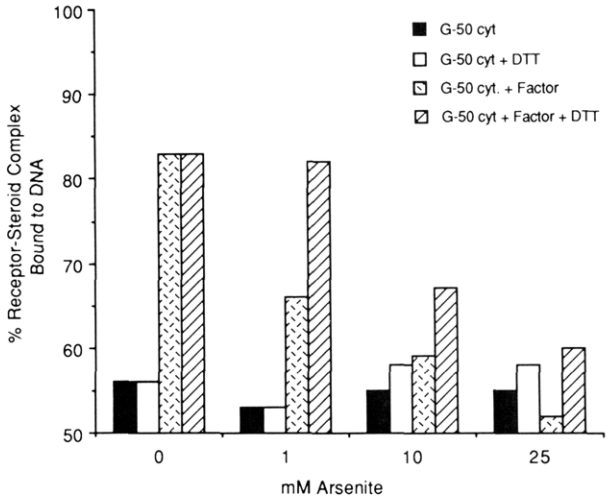

FIGURE 1: Inhibition of activated complex binding to DNA by sodium arsenite. [^{3}H]Dex-bound receptors were activated by heat and dilution and depleted of factor by chromatography on Sephadex G-50 as described previously (preceding paper in this issue). Aliquots (40 μL) of G-50 cytosol were mixed with $NaAsO_2$ at the concentrations indicated from a 10× stock and incubated for 30 min. The DNA-binding activity factor was supplied by a nuclear extract of HTC cells which had been desalted on Sephadex G-10, heat treated, and fractionated on a Centricon C-3 microconcentrator (see previous paper). Factor or TAPS buffer (80 μL) was added to the incubations. Where indicated, DTT was added to a final concentration of 1 mM from a 100× stock. The mixtures were incubated an additional 20 min and loaded onto DNA/DEAE minicolumns. The percentage of receptor–steroid complexes that bound to DNA was determined as in Table I. Similar results have been obtained in at least one additional experiment.

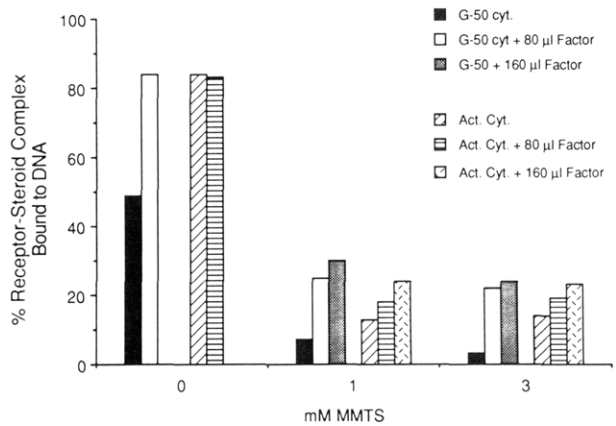

FIGURE 2: Inhibition of activated complex binding to DNA by MMTS. [^{3}H]Dex-bound receptors were activated by heating and dilution, and a 500-μL aliquot was chromatographed on Sephadex G-50. Aliquots (40 μL) of either activated cytosol or G-50 cytosol were mixed with MMTS at the final concentrations indicated, by appropriate additions from a 100× stock, and incubated for 30 min. Factor was supplied from a nuclear extract of HTC cells treated as described in Figure 1. Aliquots of factor or TAPS buffer (80 or 160 μL) were added, and the mixture was incubated for an additional 20 min before being assayed on DNA/DEAE minicolumns. Nonspecific binding to cellulose was determined from the binding to DNA–cellulose/cellulose and cellulose/cellulose columns and was subtracted from each value. The percent of receptor–steroid complexes that bound to DNA was then determined as in Table I. Similar results have been obtained in at least one additional experiment.

that could already bind or by enabling the DNA binding of a previously nonbinding population of complexes. A double-reciprocal plot of dpm of ^{3}H-complexes bound to DNA vs amount of DNA for activated [^{3}H]Dex complexes, before and after depletion of the endogenous factor by Sephadex G-50 chromatography, yields a unitless parameter that is proportional to the K_d (Simons, 1977; Simons & Miller, 1984). The ratio of these K_d values (K_d = [slope][y intercept] for the linear plots generated by least-squares fit) for complexes ± factor respectively was 1.29 ± 0.38 (SD, n = 4, data not shown). Clearly, the presence of the factor does not give rise to receptor–steroid complexes with an altered affinity for DNA. Instead, the factor appears to recruit complexes that were unable to bind to DNA as a result of the Sephadex G-50 chromatography.

We have previously shown that the application of Sephadex G-50 chromatographed complexes to DNA–cellulose columns gave flow-through fractions that were greatly enriched in those complexes that require factor for DNA binding (see Figure 2 in preceding paper). These results, plus the inability of factor to alter the affinity of complexes for DNA, indicate that the factor interacts with a distinct subpopulation of complexes to cause DNA binding. In an effort to obtain more definitive evidence for these subpopulations, we looked at the ability of known inhibitors of DNA binding to block the DNA binding of activated complexes ± factor.

Effect of Sodium Arsenite on Activated Complexes. We have recently found that sodium arsenite is a partial inhibitor of activated complex binding to DNA (Simons et al., 1990). The percentage of DNA binding that is inhibited by arsenite was very similar to the percentage of activated complexes that require factor in order to bind to DNA. It therefore appeared possible that only the factor-dependent DNA binding is blocked by arsenite. In order to investigate this possibility, [^{3}H]Dex-bound receptors were depleted of factor by gel filtration chromatography and incubated with arsenite before the DNA binding was quantitated. As seen in Figure 1, concentrations of up to 25 mM sodium arsenite have no effect on the DNA binding of those complexes that have been depleted of factor. However, increasing concentrations of arsenite progressively eliminate the ability of the factor to reconstitute the DNA binding of chromatographed receptors. Arsenite is known to specifically react with vicinal dithiols[3] (Jocelyn, 1972). Therefore, it was not surprising that the effects of 1 mM arsenite could be reversed by the addition of 1 mM DTT. It should be noted that 1 mM DTT had no effect on the DNA binding of chromatographed complexes (Figure 1). Concentrations of DTT higher than 1 mM gave a precipitate in the presence of the arsenite. Hence, complete reversal of the inhibition by ≥10 mM arsenite with higher concentrations of DTT was not attempted.

Effect of MMTS on the DNA Binding of Activated Complexes. Several investigators have reported that the thiol reagent MMTS also blocks the DNA binding of activated complexes (Bodwell et al., 1984; Tienrungroj et al., 1987; Miller & Simons, 1988). However, this blockage is not complete. The residual DNA binding (Miller & Simons, 1988) appeared to be of the same magnitude as the amount of DNA binding that requires factor. This was investigated further by incubating activated cytosol and factor-depleted cytosol with increasing concentrations of MMTS ± factor. The presence of 1 mM MMTS reduced the DNA binding of activated complexes to less than 20% (Figure 2). However, increasing the concentration to 3 mM had no further effect [see also Miller and Simons (1988)]. While factor activity is blocked by sodium arsenite (Figure 1), the ability of factor to stimulate DNA binding is not blocked by MMTS (Figure 2). MMTS appears, however, to attenuate the efficacy of the factor in that more factor caused further DNA binding of the chromatographed complexes. This is consistent with the observation that MMTS treatment of activated cytosol, which contains low levels of factor, caused a greater inhibition of DNA binding than would be expected. However, the predicted levels

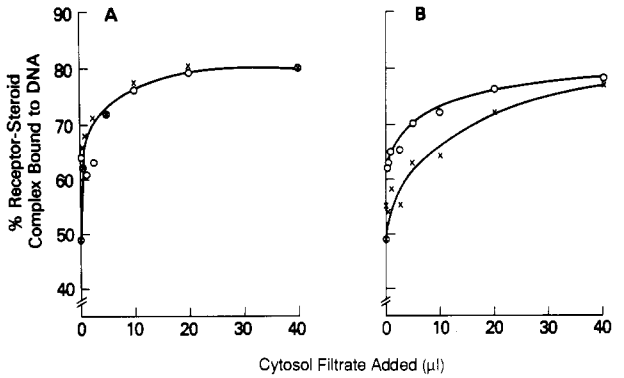

FIGURE 3: Titration of DNA-binding activity factor in steroid-free, unactivated, and activated cytosols. HTC cytosol [60% (v/v) in TAPS buffer] was incubated with buffer ±2.5 × 10^{-5} M [^{1}H]Dex for 2.5 h. Samples of steroid-free and unactivated cytosols also contained 20 mM Na_2MoO_4 throughout the entire experiment. All cytosols were then diluted 1:2 (v/v) with TAPS buffer and either warmed at 25 °C for 30 min (activated) or held on ice (steroid-free and unactivated) before being filtered through Centricon C-10 microconcentrators (1 h/2000*g*) to separate factor (in filtrate) from receptor (in retentate). Aliquots of the filtrate were added to 40 μL of factor-depleted, activated receptor–[^{3}H]Dex complexes, and the volume was brought to 120 μL with TAPS buffer. After 20 min, the DNA binding was assayed on DNA/DEAE minicolumns. The percentage of complexes that bound to DNA was determined as in Table I. Panels A and B represent two different experiments. Panel A: Factor from unactivated cytosol containing steroid-free receptors (○) or receptors bound with [^{1}H]Dex (×). Panel B: Factor from cytosol containing unactivated (○) or activated (×) receptor–[^{1}H]Dex complexes.

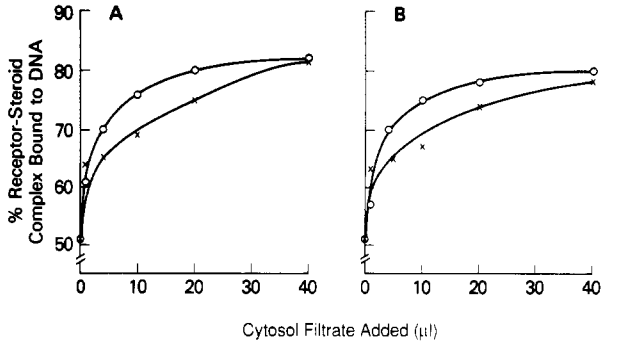

FIGURE 4: Determination of relative concentrations of uncomplexed, low molecular weight factor in unactivated and activated cytosols. Activated and unactivated glucocorticoid receptors were prepared as described in Figure 3. Panel A: Activated and unactivated cytosol preparations were loaded onto individual Centricon C-10 microconcentrators and centrifuged at 2000*g* for 1 h. Aliquots of the cytosol filtrate were mixed with 40 μL of factor-depleted cytosol and brought up to 120 μL with TAPS buffer. The mixture was incubated for 20 min and assayed for DNA binding as in Figure 3: (○) filtrate of unactivated cytosol; (×) filtrate of activated cytosol. Panel B: Five hundred microliter aliquots of C-10 filtrates, prepared as in panel A, were loaded onto individual C-3 microconcentrators and centrifuged at 5500*g* for 1 h. Aliquots of these C-3 filtrates were assayed for DNA binding as described above: (○) filtrate of unactivated cytosol; (×) filtrate of activated cytosol.

can be approximated simply by adding more factor (Figure 2). These results were independent of the order of addition; treatment of chromatographed complexes with MMTS before or after the addition of factor did not block the factor-induced increase in DNA binding (data not shown). Thus two different forms of activated complexes exist which can be distinguished by their ability to bind to DNA in the presence of sodium arsenite or MMTS and by their requirement of factor for DNA binding.

Since the factor does not bind to DNA (see Figure 5 of preceding paper), it seemed reasonable that the factor causes increased complex binding to DNA by some sort of association with the receptor molecule. We therefore inquired whether that subpopulation of complexes which requires factor is always associated with factor or whether factor becomes associated with the receptor at some point after the binding of steroid.

Amount of Factor in Cytosol Containing Steroid-Free Receptors and Unactivated and Activated Complexes. Titration curves quantitating the amount of free, nonmacromolecularly bound factor in steroid-free cytosol and in steroid-bound unactivated and activated cytosols would show a difference if the factor associated preferentially with one species of receptor. In each case, Centricon C-10 microconcentrators were used to separate the free factor from receptors and possible macromolecularly bound factor. Due to its small molecular weight (MW = 700–3000, see preceding paper), the factor can be recovered in the Centricon C-10 filtrates with no alteration in factor concentration. Aliquots of Centricon C-10 filtrates were than added to factor-depleted cytosol, and the mixture was assayed on the DNA/DEAE minicolumns. The quantity of assayable factor in cytosol solutions of steroid-free and steroid-bound receptors was the same (Figure 3A). However, there were marked differences in the amount of titratable factor in activated and unactivated cytosols (Figure 3B). Reconstitution of DNA binding of factor-depleted activated complexes required 3–4 times more filtrate from activated cytosols than from unactivated cytosols. Since the only difference between the filtrates of these cytosols was a 30-min incubation at 20 °C, it is reasonable to conclude that the activation process involves an association between receptor and factor, resulting in a decrease of endogenous factor that is free in solution.

We have found that cytosol actually contains two factors (M_r = 700–3000 and 3000–10 000) which are separable by filtration on a Centricon C-3 microconcentrator (preceding paper). The more abundant, smaller factor is identical with the nuclear factor. In order to determine which cytosolic factor was reduced in concentration, the level of factor in Centricon C-10 (Figure 4A) and C-3 (Figure 4B) filtrates of unactivated and activated complexes was assayed. The concentration differences in assayable factor in activated and unactivated cytosols were maintained after the Centricon C-3 filtration step (Figure 4). This argues that the small molecular weight cytosolic factor does indeed associate with the receptor as a consequence of activation. The minimal differences between the data obtained with C-10 and C-3 filtrates (panels A and B of Figure 4) further support our previous conclusion that the larger molecular weight DNA-binding activity factor is only a minor component in HTC cell cytosol (preceding paper).

Any differences in the titration curves of Figures 3B and 4 would be observable only if the amount of factor in unactivated cytosol was slightly higher than the amount of factor that associates with those receptors which require the factor for DNA binding. This conclusion was supported by computer modeling studies (data not shown).

Effect of Additional Factor on DNA Binding of Unactivated and Activated Receptor–Steroid Complexes. While the above experiments show that there is an excess of factor in both unactivated and activated cytosol solutions, our inability to achieve more than ~80% DNA binding (Figure 2 and preceding paper) could result from the concentration of factor in various cytosol solutions being below the equilibrium dissociation constant for factor plus receptor. This may be particularly true for steroid-free receptors and unactivated complexes. We therefore inquired whether additional factor (from nuclear extracts) would have any effect on the DNA-binding capacity of unactivated and activated complexes. However, increasing the amount of available factor by 9–18-

Table II: Effect of Na_2MoO_4 on Reconstitution of Glucocorticoid Receptor–Steroid Complex Binding to DNA[a]

receptor source	nuclear extract factor	% of complexes bound to DNA
Part A		
unactivated cytosol at 0 °C	–	46
unactivated cytosol + 20 mM Na_2MoO_4 at 0 °C	–	15
unactivated cytosol + 20 mM Na_2MoO_4 at 0 °C	+	16
unactivated cytosol + 20 mM Na_2MoO_4 + 25 °C/30 min	–	26
unactivated cytosol + 20 mM Na_2MoO_4 + 25 °C/30 min	+	24
unactivated cytosol + 20 mM Na_2MoO_4 through G-50	–	52
unactivated cytosol + 20 mM Na_2MoO_4 through G-50	+	63
unactivated cytosol + 20 mM Na_2MoO_4 through G-50 + 25 °C/30 min	–	67
unactivated cytosol + 20 mM Na_2MoO_4 through G-50 + 25 °C/30 min	+	90
Part B		
activated cytosol	–	79
activated cytosol through G-50	–	48
activated cytosol through G-50	+	85
activated cytosol through G-50 + 20 mM Na_2MoO_4	+	85

[a] Part A: HTC cytosol was incubated with [^{3}H]Dex and kept on ice to yield unactivated cytosol. Alternative treatments (as indicated) included the addition of 20 mM Na_2MoO_4 at various points and subjecting the complexes to the activating conditions of heat and dilution or gel chromatography. Aliquots of nuclear extract factor (60 μL) that had been desalted by chromatography on Sephadex G-10 were incubated with 40 μL of the various receptor preparations as indicated, brought to a final volume of 120 μL with TAPS buffer, and assayed for DNA binding as in Table I. Part B: HTC cytosol was activated by heat and dilution with TAPS buffer. A 500-μL aliquot was chromatographed on Sephadex G-50 to yield factor-depleted cytosol, which was treated with buffer ± Na_2MoO_4. Nuclear extract factor (60 μL) that had been desalted by chromatography on Sephadex G-10 was then added to 40-μL aliquots of the G-50 cytosol, and complex binding to DNA was quantitated as above.

fold had little, if any, effect on the amount of activated complexes that could bind to DNA (Table I and Figure 2). Thus activated complexes are already maximally associated with factor. Similarly, a 9–18-fold increase in factor was unable to stimulate the DNA binding of unactivated complexes, even when some activation had already occurred during the extended storage of unactivated complexes at 0 °C (Table I).

Effect of Sodium Molybdate on the Ability of Factor to Stimulate Receptor–Steroid Complex Binding to DNA. Since there is increased association of factor with activated complexes and activation is inhibited by sodium molybdate (Dahmer et al., 1984), it was of interest to determine whether sodium molybdate could block the association of factor and the resulting increase in DNA binding of factor-depleted receptor preparations. When [^{3}H]Dex-bound receptors were incubated in the presence of 20 mM sodium molybdate, only 15% of the total receptors bound to DNA–cellulose (Table IIA). The addition of factor to molybdate-containing complexes resulted in no increase in the ability of complexes to bind to DNA. When unactivated complexes were warmed for 30 min at 25 °C in the presence of sodium molybdate, some activation occurred but less than the time-dependent activation at 0 °C (Table IIA). More importantly, the addition of factor to these warmed complexes containing sodium molybdate had no effect on the total amount of DNA binding.

While Sephadex G-50 chromatography of unactivated complexes yields a preparation that shows increased DNA binding upon the addition of factor (Table I), the same factor-induced increase in DNA binding is almost entirely prevented if molybdate was initially present (Table IIA). The maximum factor-dependent DNA binding is seen only if the molybdate-containing complexes that were subsequently gel chromatographed are then heated before factor is added (Table IIA). This argues that activation by gel chromatography, or by heating, of those complexes that require factor can be blocked by sodium molybdate. In contrast, molybdate does not block the activation by gel chromatography of those complexes that do not require factor in order to bind to DNA.

Similar experiments with complexes that had been previously activated by heat and dilution and then chromatographed revealed that full reconstitution of the DNA binding occurred even in the presence of sodium molybdate (Table IIB). Thus the maximal stimulation of factor-depleted receptor–steroid complex binding to DNA by the addition of factor occurs only after the complexes have been subjected to activating conditions. However, the effect of factor itself cannot be blocked by sodium molybdate.

Discussion

In the preceding paper in this issue, we described and characterized a small macromolecular factor (MW 700–3000) which is required for the maximal binding of glucocorticoid receptor–steroid complexes to DNA. We have not yet purified this factor to homogeneity. Preliminary results with computer modeling suggest that this factor is present at low concentrations in cytosol ($\leq 10^{-7}$ M) so that considerable purification will be required. However, our current experiments with crude and partially purified factor preparations have succeeded in elucidating how this newly described factor works. In addition, we have demonstrated functional heterogeneity in glucocorticoid complexes.

There is ample evidence for charge heterogeneity in the wild-type glucocorticoid receptor (Housley & Pratt, 1983; Cidlowski & Richon, 1984; Smith et al., 1986). However, as far as we are aware, our current results provide the first evidence for functional heterogeneity among activated receptor–glucocorticoid complexes. This heterogeneity was initially implied by our discovery that a cytosolic factor was required for the DNA binding of 43 ± 12% (SD, n = 266) of the activated complexes (preceding paper; see also Tables I and II). The subpopulation of complexes that required factor for DNA binding (i.e., factor-dependent complexes) could be separated from the other, factor-independent complexes by Sephadex G-50 chromatography followed by DNA–cellulose chromatography (Figure 2 in preceding paper). These two populations were further distinguished in the present study by their responses to two different inhibitors of DNA binding—arsenite and MMTS. Arsenite, which selectively reacts with vicinal dithiols (Jocylen, 1972), blocked the DNA binding of factor-dependent complexes and had no effect on factor-independent complexes (Figure 1). We suspect that arsenite acts by preventing the DNA binding of factor-associated complexes since arsenite is equally effective when added to solutions of activated complexes which already contain the factor.[3] In contrast, the thiol reagent MMTS (Simons, 1987) has little effect on the DNA binding of factor-dependent complexes but completely prevented the binding of factor-independent complexes (Figure 2). This nicely explains why even ≥10 mM MMTS was previously found not to eliminate all of the DNA binding of activated complexes (Bodwell et al.,

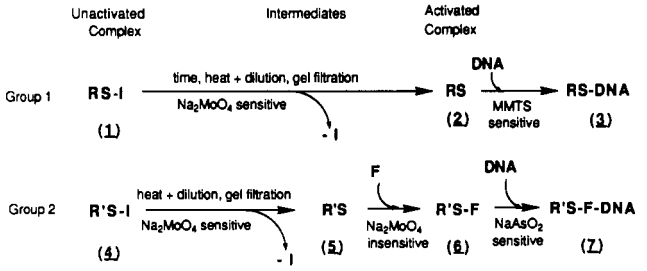

FIGURE 5: Proposed model for mechanism of glucocorticoid receptor activation involving different populations of receptor. In the schematic, S = steroid, R = glucocorticoid receptor, I = activation inhibitor component, and F = DNA-binding activity factor characterized in this study. Conversion of **1** to **2**, and **4** to **5** (but not **5** to **6**), is inhibited by Na_2MoO_4.

1984; Tienrungroj et al., 1987; Miller & Simons, 1988). MMTS does reduce the potency of the factor (Figure 2) but does not block the ability of factor to act. Thus arsenite and MMTS are the first reagents that are able to chemically detect two different populations of activated complexes, only one of which requires factor for DNA binding.

The factor does not bind to DNA (preceding paper). Instead, the factor appears to preferentially associate with activated complexes (Figures 3 and 4) to cause an increased amount of complex binding to DNA. The association of factor with activated complexes must be rapidly reversible since the factor can be removed by Sephadex G-50 chromatography. Nevertheless, the association is sufficiently strong that all of those complexes in activated cytosols that can bind factor are already bound. Thus added factor does not increase the amount of activated complexes that will bind to DNA (Table I).

Activation is conventionally defined as a process that converts receptor–steroid complexes (or steroid-free receptors) from a non-DNA-binding form to a DNA-binding form. The mechanism of activation is still unclear, but it is generally considered to involve a single step, such as the dissociation of heat shock protein 90 (hsp90) (Pratt, 1987). Nevertheless, evidence has been accumulating that activation may be a two-step process (Schmidt et al., 1985; Gottlieb et al., 1987; Colvard & Wilson, 1987; Ben-Or & Chrambach, 1988; Harmon et al., 1988, Gruol & Wolfe, 1989). Our data provide four lines of evidence that activation of receptor–glucocorticoid complexes proceeds via two *different* pathways, with the activation of factor-dependent complexes involving one more step than the activation of factor-independent complexes (Figure 5). First, we have demonstrated the existence of two separable populations of activated complexes, but only one population (**5**) requires the presence of our low molecular weight factor for DNA binding. Since activation is defined as conferring the ability to bind to DNA, this is prima facie evidence that two different pathways of activation exist. Factor cannot convert unactivated complexes to the DNA binding form (Table I), can be separated from the unactivated complexes by Sephadex G-50 chromatography (Table I and preceding paper), and becomes associated with activated complexes (Figures 3 and 4). We therefore propose that activation of the factor-dependent complexes (**4**) occurs via two steps (Figure 5): the first step can be achieved by gel filtration or heat and dilution, is blocked by molybdate (Table II), and may involve the removal of either the inhibitor of activation (I) described by others (Schmidt et al., 1985; Bodine & Litwack, 1988a; Meshinchi et al., 1988), hsp90, or even part of the receptor itself (Godowski et al., 1987), to give **5**. The second step requires the association of our low molecular weight factor to give **6**. A logical consequence of these data is that the dissociation of inhibitory molecules such as hsp90 from unactivated complexes may be necessary (Denis et al., 1988), but it is not sufficient for the DNA binding of receptor–glucocorticoid complexes. Furthermore, the common observation that not all dissociated 4–5S complexes can bind to DNA [e.g., Tienrungroj et al. (1987) and Recker et al. (1987)] may result from the absence of our factor. Second, only the activation of factor-independent complexes (**1**) can be accomplished by time at 0 °C or by gel chromatography when molybdate is present. In the absence of molybdate, gel chromatography or heat and dilution afford activation of factor-independent (**1**) and factor-dependent (**4**) complexes (Tables I and II). Third, while the activation of both populations of unactivated complexes can be blocked by molybdate (i.e., **1** to **2** and **4** to **5**), the ability of factor to complete the activation process for gel chromatographed, factor-dependent complexes (i.e., **5** to **6**) cannot be blocked by molybdate (Table II). Thus activation of factor-dependent complexes (**4**) consists of a molybdate-sensitive step which precedes a molybdate-insensitive step. Fourth, the two pathways are specifically and differentially affected by the thiol-specific reagents arsenite and MMTS (Figures 1 and 2). Collectively, we feel that these data provide convincing evidence that at least two populations of receptor–glucocorticoid complexes exist and that activation of one population requires a second step not seen for the other population. Our results therefore differ from the published reports of activation being a two-step process in that we find a two-step process with only a subpopulation, as opposed to all, of the complexes. However, our results are not incompatible with these published reports since we appear to be examining a different step.

In conclusion, we have discovered and characterized a previously unknown, low molecular weight factor that is involved in a multistep activation process of one of at least two populations of glucocorticoid receptors. This factor is not required for the nuclear binding of either population of complexes, presumably because nuclei contain high concentrations of this factor. In this respect, it will be interesting to see whether both populations interact with a recently described 48K protein that functions as a dimer to increase binding of activated glucocorticoid complexes to nuclei but not to DNA (Okamoto et al., 1988, 1989). The existence of heterogeneous populations of glucocorticoid receptor, each displaying slightly different mechanisms of activation, also affords new opportunities at the molecular level for the modulation and selective control of gene expression by glucocorticoids.

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