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THE HEAT-STABLE CYTOSOLIC FACTOR THAT PROMOTES GLUCOCORTICOID RECEPTOR BINDING TO DNA IS NEITHER THIOREDOXIN NOR RIBONUCLEASE

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Summary—Treatment of rat liver cytosol containing temperature-transformed [^{3}H]dexamethasone-bound receptors at 0°C with the sulfhydryl modifying reagent methyl methanethiosulfonate (MMTS) inhibits the DNA-binding activity of the receptor, and DNA-binding activity is restored after addition of dithiothreitol (DTT). However, transformed receptors that are treated with MMTS and then separated from low M_r components of cytosol by passage through a column of Sephadex G-50 have very little DNA-binding activity when DTT is added to regenerate sulfhydryl moities. The receptors will bind to DNA if whole liver cytosol or boiled liver cytosol is added in addition to DTT. The effect of boiled cytosol is mimicked by purified rat thioredoxin or bovine RNase A in a manner that does not reflect the reducing activity of the former or the catalytic activity of the latter. This suggests that the reported ability of each of these heat-stable peptides to stimulate DNA binding by glucocorticoid receptors is not a biologically relevant action. We suggest that stimulation of DNA binding of partially purified receptors by boiled cytosol does not constitute a reconstitution of a complete cytosolic system in which the dissociated receptor must associate with a specific heat-stable accessory protein required for DNA binding, as has been suggested in the "two-step" model of receptor transformation recently proposed by Schmidt *et al.* (Schmidt T. J., Miller-Diener, A., Webb M. L. and Litwack G. (1985) *J. biol. Chem.* **260,** 16255–16262).

INTRODUCTION

Sulfhydryl groups are important for permitting several actions of steroid receptors. For example, SH-groups are required for glucocorticoid receptors in cytosol preparations to bind steroid [1], and subsequently, for transformed§ glucocorticoid–receptor complexes to bind to DNA [2]. Bodwell *et al.*[3] have provided good evidence that the sulfhydryl groups required for DNA binding by the transformed receptor are different from those required for steroid binding. In addition, there is evidence that sulfur moieties must be in a reduced form for progesterone [4], androgen [5] and glucocorticoid¶ [6] receptors to undergo transformation. Our laboratory has been interested in defining the endogenous heat-stable cytosolic factors that are responsible for converting glucocorticoid receptors from a non-steroid-binding to a steroid-binding form [7, 8]. We have published evidence that glucocorticoid receptors in rat liver cytosol are maintained in a reduced, steroid-binding state by a NADPH-dependent, thioredoxin-mediated thiol–disulfide interchange system [9, 10].

Recently, in pursuing studies on receptor transformation to the DNA-binding state, we have considered a second possible interaction of thioredoxin with the glucocorticoid receptor [11]. We began by asking whether an enzyme was involved in temperature-mediated transformation of the glucocorticoid–receptor complex. Rat liver cytosol containing untransformed steroid-bound receptor was passed through a column of Bio-Gel ASM (in molybdate-free buffer) to separate the receptor from smaller cytosolic components that might be required for transformation. The fractions containing the steroid–receptor complex were combined and rapidly concentrated back to the original sample volume by filtration on an Amicon filter. The steroid–receptor complex in this Bio-Gel peak had little DNA binding activity, and in contrast to untreated cytosol, when the peak material was heated at 25°C, there was little or no increase in DNA binding [11].

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§It should be noted that we will use the term "transformation" to describe the process whereby the steroid-bound receptor is converted to a form that binds to nuclei, DNA-cellulose, etc. We have used the term "activation" to describe the process whereby the oxidized receptor is converted from a nonbinding form to a form that binds steroids.

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Abbreviations: MMTS, methyl methanethiosulfonate; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; DTT, dithiothreitol.

As the Bio-Gel-separated complex did not show increased binding when it was heated, it seemed possible that it had been separated from a smaller cytosolic component (or components) required for subsequent temperature-mediated transformation. Accordingly, we added either whole cytosol (containing inactivated receptors) or boiled cytosol to the Bio-Gel-separated glucocorticoid–receptor complex and found that both cytosol preparations permitted increased binding of receptors to DNA-cellulose [11]. Not only was the boiled cytosol effective at increasing the DNA binding, but it was effective at 0°C. These observations suggested that a heat-stable component of cytosol was promoting DNA binding in a non-enzymatic manner. The activity in boiled cytosol behaved like thioredoxin in that it was smaller than the excluded material on Sephadex G-50 chromatography ($M_r < 30{,}000$), but most of it did not pass through an Amicon YM10 filter ($M_r > 10{,}000$). Therefore, we added purified rat liver thioredoxin to the Bio-Gel-separated receptors and found that it was as active as boiled cytosol in promoting DNA binding [11].

Taken together, these observations led us to speculate that thioredoxin might be involved in the binding of transformed receptor to DNA [11]. Thioredoxin could act either by reducing the sulfur moieties required for DNA binding, or possibly by binding to the receptor itself and producing a conformational change leading to an increased affinity for DNA. Thioredoxin is known, for example, to bind tightly to bacteriophage T7 DNA polymerase and through a conformational rather than a reductive action it permits the enzyme to function on double-stranded nucleic acids [12, 13]. Huber *et al.*[13] have speculated that thioredoxin binds to the enzyme near the binding crevice for double-stranded DNA and locks the duplex DNA into position. In this paper, we examine the effects of thioredoxin in promoting DNA binding by transformed glucocorticoid receptors whose DNA binding activity has been reversibly inactivated with a sulfhydryl modifying agent. We find that neither the effect of thioredoxin nor the similar effect produced by RNase in this system reflects a biologically relevant action of these heat-stable peptides.

EXPERIMENTAL

Materials

[6,7-^{3}H]Dexamethasone (45.8 Ci/mmol) was obtained from New England Nuclear, Boston, MA. Insulin isolated from bovine pancreas, dithiothreitol, nonradioactive dexamethasone, methyl methanethiosulfonate, ribonuclease A (EC 3.1.27.5) from bovine pancreas (Type XII-A), ribonuclease S-protein from bovine pancreas (Grade XII-PE) were obtained from J. T. Baker Chemical Co., Phillipsburg, New Jersey.

Cell source and fractionation

Liver was obtained from 100–200 g male Sprague–Dawley rats which had been adrenalectomized and maintained on 9% saline for 1 day, prior to sacrifice. Livers were removed immediately upon death, placed in ice-cold Earle's balanced salt solution, and homogenized in a Waring blender in 1.5 vol of 10 mM Hepes buffer, pH 7.35, per gram of wet weight, followed by Dounce homogenization. The tissue homogenate was centrifuged for 20 min at 27,000 *g* and the resulting supernatant was centrifuged at 100,000 *g* for 2 h. The 100,000 *g* supernatant (referred to as cytosol) was used as the source of receptor, and was stored at −70°C until used.

Incubation conditions and steroid binding assay

Incubations containing cytosol and other additions were prepared as noted in the figure and table legends. Most additions were made from stock solutions concentrated at least 10-fold. The cytosol was preincubated at 0°C with 50 mM [^{3}H]dexamethasone, with or without a 1000-fold excess of nonradioactive dexamethasone, for at least 3 h to form bound receptor and was then subjected to the various experimental conditions. The steroid–receptor complexes were first transformed to the DNA-binding state by incubating the cytosol for 45 min at 25°C. Cytosol containing transformed [^{3}H]dexamethasone–receptor complexes was then incubated for 1 h on ice with 3 or 3.5 mM MMTS to inactivate DNA-binding capacity. To reverse the MMTS inhibition of DNA binding in whole cytosol, DTT (20 mM) was added to the indicated samples and steroid binding and DNA binding were assayed.

Transformed steroid-receptor complexes that had been treated with MMTS in whole cytosol were separated from free MMTS and small M_r($< 30{,}000$) components of cytosol by passage through a column (2.5 × 83 cm) of Sephadex G-50, eluting with 10 mM Hepes buffer, pH 7.35. The column eluate was monitored at 280 nm, and the fractions containing the macromolecular material (void volume peak) were pooled and concentrated to original volume by filtration on an Amicon YM10 filter which excludes molecules of M_r greater than approx 10,000. This preparation was then incubated on ice for 1 hour with DTT and other additions (as indicated in the figure and table legends) to permit reactivation of DNA-binding capacity prior to assay of steroid binding and binding to DNA-cellulose.

Assay of steroid binding and binding to DNA-cellulose

Steroid binding was assayed by the charcoal absorption method. Aliquots of each incubation mixture (0.1 ml), preincubated with steroid as described above, were incubated with 0.15 ml of a suspension of dextran-coated charcoal (1% charcoal (w/v) and 0.2% dextran (w/v) in 10 mM Hepes, pH 7.35, at 4°C) for 10 min at 0°C. After centrifugation at 12,000 *g* for 4 min, 0.1 ml of the supernatant was assayed for radioactivity in 5 ml of scintillation fluid. The specific binding represents the average of dupli-

cate assays made in the absence of competing dexamethasone minus the nonspecific binding value and is expressed in cpm/0.2 ml of original undiluted cytosol. In samples of MMTS-treated cytosol that were passed through Sephadex G-50, all of the bound steroid recovered in the macromolecular peak was considered to represent steroid–receptor complex. The assumption is valid, as a control experiment in which a nonspecifically bound sample was passed through the column revealed no binding in the macromolecular peak.

To assay DNA binding, duplicate 0.1 ml aliquots of incubation mix were incubated for 45 min with 0.1 ml of a suspension of DNA-cellulose under constant stirring in an ice bath. The DNA-cellulose-bound material was washed and assayed for radioactivity as described previously [8]. DNA-cellulose was used as a 12.5%(v/v) suspension of DNA-cellulose (0.36 mg of native DNA/ml of cellulose).

Preparation of the endogenous activating factor

The endogenous receptor activating factor (partially purified thioredoxin), which is called EAF in the legends to the tables and figures, was prepared from the 27,000 *g* supernatant of rat liver essentially as previously described [9]. Briefly, the 27,000 *g* supernatant fraction from rat liver was placed into a boiling water bath for 2 min and the denatured protein was removed by centrifuging at 27,000 *g* for 5 min. One hundred ml of the boiled supernatant were lyophilized to dryness, redissolved in 10 ml of 0.1 mM Hepes buffer, pH 7.35, and desalted on a Sephadex G-50 column (2.5 × 67 cm). All of the fractions obtained prior to the salt peak were pooled, lyophilized, dissolved in 4 ml of 0.1 mM Hepes buffer, pH 7.35, and incubated with 10 mM DTT for 30 min at 20°C to reduce all thioredoxin in the preparation. This material was applied to another Sephadex G-50 column (2.5 × 80 cm) and eluted with 0.1 mM Hepes buffer, pH 7.35. The eluent was monitored at 280 nm and aliquots from every other fraction were assayed for thioredoxin activity by the insulin disulfide reduction method described below. The thioredoxin-containing fractions, which are eluted in the same fractions as the endogenous receptor activating activity [9], were pooled, lyophilized to dryness and stored at −20°C until used.

Thioredoxin assay

Thioredoxin was assayed by the insulin disulfide reduction method of Holmgren[14]. An aliquot of each test sample was incubated in a final volume of 0.6 ml in a 1 ml cuvette at room temperature with 500 μl of bovine insulin (1 mg/ml), 8 μl of 50 mM dithiothreitol, and the appropriate volume of 100 mM phosphate buffer, pH 7.0, containing 2-mM EDTA. Measurements were performed at A_{650} every 5–10 min. Rates of insulin reduction were expressed as the change in turbidity with time observed within a linear range between 0.2 and 1.0 A_{650}. The non-enzymatic reduction of insulin by dithiothreitol was recorded in a control cuvette without thioredoxin and this value (0.008–0.01 $A_{650nm} \times min^{-1}$) was subtracted from that recorded with each test sample to yield the rate of insulin reduction due to thioredoxin. A standard curve generated from purified rat liver thioredoxin was used to determine the thioredoxin concentration of the EAF preparation.

Purified thioredoxin and thioredoxin antiserum

Rat liver thioredoxin was purified to 80% of homogeneity at the Karolinska laboratory by the method of Holmgren and Luthman[15]. The purified thioredoxin (45 μm) was sent to the United States in the oxidized form in 50 mM Tris–HCl buffer, pH 7.5, 8 mM EDTA. Antiserum against purified rat liver thioredoxin was prepared in rabbits as described previously [10]. The gamma-globulin fraction of pre-immune and antithioredoxin serum was prepared as described [10], precipitated to 90% of saturation with solid ammonium sulfate and the suspensions sent by airmail from Stockholm to Ann Arbor. On arrival, the precipitate was collected by centrifugation, dissolved in 10 mM Hepes buffer, p 7.35, and dialyzed against Hepes buffer overnight. After dialysis, the sera were returned to original volume with buffer and stored in 0.2 ml aliquots at −20°C until used.

RESULTS

Reversal of MMTS inhibition of DNA binding

Methyl methanethiosulfonate (MMTS) reacts specifically with protein thiols to derivatize them by adding a thiomethyl group through the formation of a mixed disulfide. Bodwell *et al.*[2] have shown that 0.25 mM MMTS is required to produce half-maximal inhibition of DNA binding by transformed glucocorticoid receptors in rat thymocyte cytosol. Tienrungroj *et al.** have found that a 10-fold higher concentration of MMTS is required to inhibit DNA binding by transformed receptors in rat liver cytosol, which has a higher concentration of glutathione available to react with the reagent [9]. The inhibition of DNA binding caused by 3 mM MMTS in whole rat liver cytosol is shown at the top of Table 1. At this concentration of MMTS, the steroid–receptor complex is not disrupted but DNA binding is inhibited and the inhibition can be reversed by adding 10 mM dithiothreitol (DTT) to the cytosol.

After derivatization with MMTS, sylfyhdryl groups can be regenerated by reagents, like DTT, which promote thiol-disulfide interchange. If regeneration of receptor sulfhydryl groups is the only thing that is required to permit the MMTS-treated, transformed receptor to bind to DNA, then MMTS-treated

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Table 1. Small, heat-stable components of cytosol are required in addition to DTT to reverse MMTS inhibition of DNA binding

Condition	Expt	Specific binding	Binding to DNA-cellulose	% of total complexes bound to DNA-cellulose
		cpm/0.2 ml		
Transformed cytosol alone	1	29,600	8760	30
	2	20,400	9270	45
plus MMTS	1	33,100	4960	15
	2	17,700	4400	25
plus MMTS then DTT	1	32,200	8120	25
	2	20,100	7940	40
MMTS-treated cytosol filtered through Sephadex G-50				
no additions	1	20,000	1240	6
	2	10,100	866	9
plus DTT	1	21,700	2270	10
	2	11,900	1850	16
plus whole cytosol	1	20,800	2500	12
plus whole cytosol and DTT	1	21,800	4590	21
plus boiled cytosol	1	21,100	2220	11
	2	12,000	2530	21
plus boiled cytosol and DTT	1	20,400	4250	21
	2	11,600	4360	38

Rat liver cytosol containing [^{3}H]dexamethasone-bound, temperature-transformed receptors was incubated for 1 h on ice with 3 mM MMTS to reduce the DNA-binding activity of the receptor. A portion of MMTS-treated cytosol was then maintained at 0°C while the remainder was filtered through a column of Sephadex G-50 to separate the steroid–receptor complex from unreacted MMTS and from small components of cytosol. The fractions containing the material in the Sephadex void volume peak were concentrated back to original volume by Amicon filtration. Additions of whole rat liver cytosol or boiled rat liver cytosol (each at 40% of final incubation volume) or DTT (10 mM) were made as indicated and both steroid binding and binding to DNA-cellulose were assayed 1 h later. Receptors in whole cytosol were inactivated by incubation at 37°C prior to addition. MMTS treated cytosol that was not filtered through Sephadex G-50 was maintained at 0°C and DTT was added to restore DNA-binding activity 5 h later, which is the time required for the rest of the material to be run through the Sephadex and Amicon procedures. The results of two experiments are presented. The DNA-binding capacity of the untransformed, 0°C control cytosol was 1820 cpm/0.2 ml in experiment 1 and 900 cpm/0.2 ml in experiment 2.

receptors that have been separated from unreacted MMTS and small M_r components of cytosol should be returned to the DNA binding state by exposing them to DTT alone. In the experiments shown in Table 1, temperature-transformed receptors were treated with MMTS and the cytosol was then filtered through a column of Sephadex G-50 to separate MMTS-treated receptor from any unreacted MMTS and small M_r cytosol components ($<30,000$). This procedure takes several hours and is accompanied by a loss of 20–50% of the steroid binding. Transformed receptors treated in this manner have a very low DNA binding activity that is only partially restored by DTT. Readdition of cytosol components in the

Table 2. Reversal of MMTS inhibition of DNA binding by DTT and the endogenous receptor activating factor or purified thioredoxin

Condition	Specific binding	Binding to DNA-cellulose	% of total complexes bound to DNA-cellulose
	cpm/0.2 ml		
Transformed cytosol alone	31,400	12,800	41
plus MMTS	40,500	6430	16
plus MMTS then DTT	44,200	14,000	32
MMTS-treated cytosol filtered through Sephadex G-50			
plus buffer alone	29,600	1610	5
plus DTT	29,600	2490	8
plus boiled cytosol	32,900	7760	24
plus boiled cytosol and DTT	31,600	10,200	32
plus EAF	32,300	6890	21
plus EAF and DTT	30,900	10,500	34
plus thioredoxin	31,300	3960	13
plus thioredoxin and DTT	29,000	10,100	35

Rat liver cytosol containing [^{3}H]dexamethasone-bound, temperature-transformed receptors was incubated for 1 h on ice with 3.5 mM MMTS and filtered through Sephadex G-50 as described in the Legend to Table 1. Additions of boiled rat liver cytosol (at 40% of final incubation volume), endogenous activating factor (EAF, 40% by volume), or purified thioredoxin (4.5 μmol), or each of these preincubated for 1 h at room temperature with 20 mM DTT were made as indicated, and the mixtures were incubated on ice for 1 h prior to assay of steroid binding and binding to DNA-cellulose. The concentration of DTT in the incubation with the receptor was 10 mM. MMTS-treated cytosol that was not filtered through Sephadex was reactivated by addition of DTT after 1 h.

form of whole cytosol or boiled cytosol yields a modest increase in DNA binding. The presence of both boiled cytosol and DTT yields more DNA binding than expected from an addition of the two effects; although the percent of total steroid–receptor complexes bound to DNA-cellulose is not as high as that in the original temperature-transformed cytosol before MMTS treatment. This observation suggested to us that the MMTS-derivatized receptor may have been separated from a heat-stable component of whole cytosol that promotes receptor binding to DNA.

In the light of our previous results with Bio-Gel-separated receptors [11], we considered the possibility that thioredoxin might act like boiled cytosol to permit DNA binding. As thioredoxin is a thiol-containing, heat-stable protein of M_r 12,000, it would be inactivated by MMTS and separated from the receptor during the Sephadex G-50 procedure employed in the experiments of Table 1. Accordingly, in the experiment of Table 2 a preparation of partially purified rat liver thioredoxin or a preparation of highly purified thioredoxin was added back to MMTS-treated receptors that were passed through Sephadex G-50. The partially purified thioredoxin was originally called endogenous activating factor (EAF) on the basis of its ability to activate the oxidized glucocorticoid receptor to its steroid binding form [9] and we will refer to it here as EAF. As shown in Table 2, both EAF and rat liver thioredoxin purified to 80% of homogeneity were as effective as boiled cytosol in permitting Sephadex-separated, MMTS-treated, transformed receptors to bind to DNA in the presence of DTT. As shown in Fig. 1, the EAF preparation is maximally effective when it is present in the system at a concentration of about 1 micromolar based on its thioredoxin activity as assayed by the insulin disulfide reduction method. The concentration of thioredoxin in the boiled rat liver cytosol is 3–4 micromolar [9] and its is being added to the Sephadex-separated receptor system at 40% of the assay volume, yielding a final concentration of 1–1.5 micromolar thioredoxin. Thus, the EAF preparation promotes receptor binding to DNA at concentrations of thioredoxin activity that exist in rat liver cytosol.

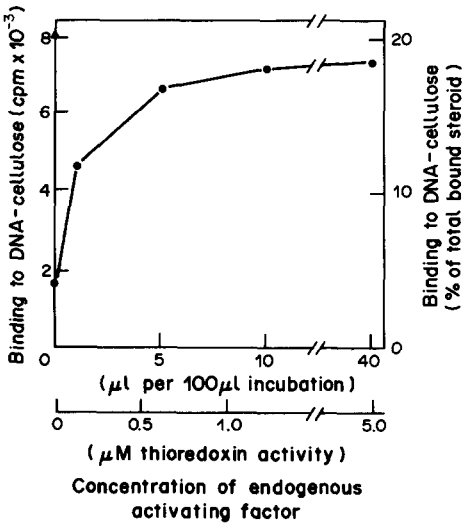

Fig. 1. Effect of a partially purified preparation of thioredoxin (EAF) on DNA-cellulose binding activity of transformed receptors that have been treated with MMTS and passed through Sephadex G-50. Rat liver cytosol containing [^{3}H]dexamethasone-bound transformed receptors was treated with 3.5 mM MMTS and filtered through Sephadex G-50 as described in the legend to Table 1. The Sephadex-filtered receptor preparation was divided into replicate aliquots which were incubated for 1 h on ice with 10 mM DTT in the presence of boiled cytosol (▲), which was present at 40% of the final incubation volume, or with various amounts of the endogenous activating factor, EAF (●). The EAF was prepared from boiled rat liver cytosol by Sephadex chromatography as described under Experimental. In this experiment boiled cytosol and the EAF were preincubated at 25°C for 30 min with 10 mM DTT and cooled to 4°C prior to addition to the Sephadex-filtered receptor preparation. Binding to DNA-cellulose is presented both as cpm/0.2 ml of undiluted cytosol and as the percent of total steroid–receptor complexes (assayed by charcoal assay) bound to DNA. The concentration of EAF is expressed both as the number of microliters of EAF added to the incubation and as the final concentration of thioredoxin activity, which was assayed in the EAF preparation by insulin disulfide reduction method using purified rat liver thioredoxin as standard.

The stimulation of DNA binding by thioredoxin is not inhibited by thioredoxin antiserum

If stimulation of DNA binding by Sephadex-separated, MMTS-treated receptors is specifically permitted by thioredoxin, then one might expect the effect to be blocked by thioredoxin antiserum. A rabbit antiserum against rat thioredoxin has been prepared, and as shown in Fig. 2, when the antiserum is present at high concentration in the insulin

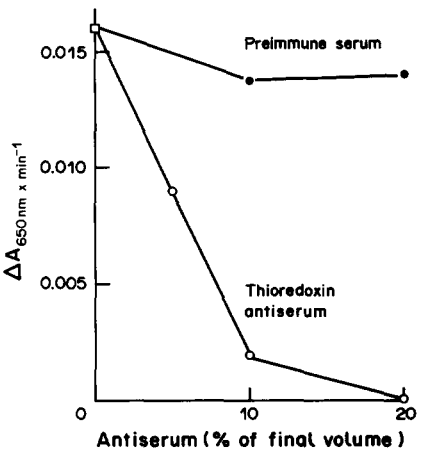

Fig. 2. Effect of thioredoxin antiserum on the reduction of insulin by a reducing system composed of dithiothreitol and EAF. The rate of insulin reduction by EAF and DTT in the presence of buffer (□), preimmune control serum (●), or thioredoxin antiserum (○) was determined as described under 'Experimental'. EAF was present at 7.5% of final incubation volume (45 μl in a total assay volume of 600 μl).

Table 3. Failure of thioredoxin antiserum to affect reversal of MMTS inhibition by DTT and partially purified thioredoxin (EAF)

Condition	Binding to DNA-cellulose	% of total complexes bound to DNA-cellulose
	cpm/0.2 ml	
MMTS-treated cytosol filtered through Sephadex G-50		
plus Buffer	2040	5
plus DTT	1900	4
plus EAF	3750	9
plus DTT + EAF	9490	24
plus DTT + EAF + preimmune serum	8720	24
plus DTT + EAF + immune serum	9960	27

Rat liver cytosol containing [^{3}H]dexamethasone-bound, temperature-transformed receptors was incubated for 1 h with 3.5 mM MMTS and filtered through Sephadex G-50. Additions of DTT (10 mM, endogenous activating factor (EAF, 40% by volume), or EAF preincubated with preimmune or immune serum were made as indicated. The mixtures were incubated on ice for 1 h, and steroid binding and binding to DNA-cellulose were assayed. EAF was preincubated with 35% immune or preimmune serum at 25°C for 30 min, DTT was added and the preincubation was continued for an additional hour on ice prior to adding the mixture to Sephadex G-50 filtered cytosol. The concentration of antiserum in the final incubation mix containing the receptor was 17% by volume.

disulfide reduction assay, there is an immune-specific inhibition of the protein reducing activity of EAF. As shown in Table 3, however, the presence of a high concentration of antiserum did not affect stimulation of receptor binding to DNA caused by the combination of EAF and DTT. As the same concentration of antiserum was shown to inhibit the reducing function of the partially purified thioredoxin in Fig. 2, it is clear that the reducing function of thioredoxin is not required for the effect on receptor binding to DNA.

RNase also stimulates DNA binding

Grandics *et al.*[16] have reported that rat liver glucocorticoid–receptor complexes which have been purified approx 4000-fold in the untransformed form by affinity chromatography undergo little conversion to the DNA-binding state when they are heated, but DNA binding can be conferred on the purified complexes in a reconstituted system in which crude cytosol has been added. The active component in rat liver cytosol was found to be a trypsin-sensitive, heat-stable (90°C for 30 min) factor that is excluded from Sephadex G-25 [17]. The factor promotes DNA binding by purified receptors that have been thermally transformed and it is active at 0°C. This led Schmidt *et al.*[17] to propose that transformation is a "two-step" process, with the first step being a temperature-mediated dissociation of the receptor and the second being an interaction of the dissociated receptor with a heat-stable cytoplasmic protein that enhances its ability to bind to DNA-cellulose.

Schmidt *et al.*[18] have recently shown that RNase

Table 4. Effect of RNase A, S-protein and S-peptide on receptor binding to DNA

Condition	Specific binding	Binding to DNA-cellulose	% of total complexes bound to DNA-cellulose
		cpm/0.2 ml	
Transformed cytosol alone	57,900	20,400	35
plus MMTS	55,100	7340	13
plus MMTS then DTT	58,000	13,400	23
MMTS-treated cytosol filtered through Sephadex G-50			
plus buffer alone	43,900	2450	6
plus DTT	45,200	3730	8
plus boiled cytosol	47,200	9210	20
plus boiled cytosol and DTT	40,000	11,900	30
plus EAF	51,900	9470	18
plus EAF and DTT	46,000	10,600	23
plus RNase	45,800	10,100	22
plus RNase and DTT	44,100	12,300	28
plus S-protein	46,600	5200	11
plus S-protein and DTT	45,800	8000	17
plus S-peptide	45,700	1770	4
plus S-peptide and DTT	45.600	2810	6

Rat liver cytosol containing temperature-transformed [^{3}H]dexamethasone-bound receptors was incubated for 1 h on ice with 3.5 mM MMTS and filtered through Sephadex G.50. Additions of endogenous activating factor (EAF, 40% by volume, final protein concentration 0.68 mg/ml), RNase A (0.68 mg/ml), an equimolar amount of RNase S-protein, or S-peptide, or each of these preincubated for 1 h at room temperature with 20 mM DTT were made as indicated, and the mixtures were incubated on ice for 1 h prior to assay of steroid binding and binding to DNA-cellulose. The concentration of DTT in the incubation with the receptor is 10 mM. MMTS-treated cytosol that was not filtered through Sephadex was reactivated by addition of DTT after 1 h.

Table 5. Pretreatment of EAF, RNase or S-protein with *N*-ethylmaleimide does not affect their ability to promote receptor binding to DNA

Condition	Binding to DNA-cellulose	% of total complexes bound to DNA-cellulose
	cpm/0.2 ml	
Transformed cytosol alone	13,700	31
plus MMTS	7590	17
Plus MMTS then DTT	14,400	33
MMTS-treated cytosol filtered through Sephadex G-50		
plus buffer alone	4220	18
plus DTT	5590	24
plus NEM and DTT	4210	18
plus EAF	8100	34
plus EAF and DTT	10,900	46
plus NEM-treated EAF and DTT	9150	39
plus RNase	9610	41
plus RNase and DTT	12,200	51
plus NEM-treated RNase and DTT	13,700	58
plus S-protein	7670	32
plus S-protein and DTT	11,100	47
plus NEM-treated S-protein and DTT	12,700	54

Sephadex-filtered, MMTS-treated, transformed steroid–receptor complexes were incubated for 1 h on ice with the indicated additions and steroid binding and binding to DNA-cellulose were assayed as in previous experiments. For NEM-treatment, EAF (final concentration in assay 0.68 mg protein/ml), RNase (final concentration 0.68 mg/ml), and S-protein (at equimolar concentration to RNase) were preincubated at room temperature for 15 min with 1 mM DTT, then 30 min with 3 mM NEM, and then 10 mM DTT was added to inactivate unreacted NEM. NEM-treated or non-NEM-treated preparations were added to the Sephadex-filtered, MMTS-treated cytosol as indicated.

A mimics the effect of the endogenous heat-stable protein in permitting DNA binding. RNase S-protein, a catalytically inactive fragment of RNase A that lacks a 20 amino acid segment (RNase S-peptide) at the amino terminal [19], was found to be as effective as intact RNase in their system. As shown in Table 4, both intact pancreatic RNase and RNase S-protein promote DNA binding by Sephadex-separated, MMTS-treated, transformed receptors. An equimolar concentration of the 20 amino acid S-peptide cleavage product does not promote DNA binding.

As the intact RNase (M_r 13,700) and RNase S-protein (M_r 11,200) are similar to thioredoxin in both size and heat stability, it is possible that both preparations could be contaminated with thioredoxin. We have assayed both the RNase and the S-protein preparations by the insulin disulfide reduction method, however, and find no thioredoxin activity, even when RNase is present in the assay at a concentration that is 10-fold higher than that employed in the experiment of Table 4. As shown in Table 5, neither the activity of the RNase preparations nor the activity of the thioredoxin preparation in promoting DNA binding is affected by treatment with *N*-ethylmaleimide. This again suggests that stimulation of receptor binding to DNA is a nonspecific effect that is unrelated to the presence of thiol moieties in either the thioredoxin or RNase preparations.

DISCUSSION

Two laboratories have reported that a heat stable component of rat liver cytosol promotes DNA binding by glucocorticoid receptors that were partially purified or extensively purified without being submitted initially to thermal transformation [11, 17]. As the effect of boiled cytosol in stimulating DNA binding can be replaced by partially purified or highly purified thioredoxin at concentrations that exist in the rat liver cytosol preparation (ref. 11, Table 2, Fig. 1), we have considered the possibility that thioredoxin might play a direct role in facilitating binding of the receptor to DNA. In the case of phage T7 DNA polymerase, it has been shown that thioredoxin acts as an accessory protein that endows the polymerase with the ability to function on double-stranded nucleic acids [13]. Although modification of the SH-groups in thioredoxin by *N*-ethylmaleimide destroys its activity on the phage polymerase, no thiol–disulfide exchange appears to take place after thioredoxin binds to the enzyme [13]. It is tempting to consider that an analogous interaction might be occurring between the glucocorticoid receptor and thioredoxin to affect the DNA binding activity of the receptor. Several observations, however, argue against any biochemically meaningful interaction between thioredoxin and the receptor: (1) the effect of partially purified thioredoxin in promoting DNA binding by MMTS-treated receptors in the presence of DTT is not affected by antithioredoxin serum (Table 3); (2) RNase A and RNase S-protein produce a similar increase in binding (Table 4); (3) treatment with *N*-ethylmaleimide does not affect the stimulation of DNA binding caused by either the thioredoxin or RNase preparations (Table 5).

It seems rather clear that there is also no biochemically meaningful interaction between RNase and the glucocorticoid receptor in promoting DNA binding.

This is inferred from the fact that the catalytically inactive RNase S-protein promotes DNA binding by receptors, both in our system (Tables 4 and 5) and in the purified receptor system of Schmidt *et al.*[18]. At least, it can be said that the effect of the RNase preparation in our system is unrelated to the presumed catalytic effects of RNase on the size and DNA-binding activity of glucocorticoid receptors that have been reported in rat liver cytosol and other systems [20, 21].

Neither our observations nor the more recent observations of Schmidt *et al.*[18] support the "two step" model [17] of transformation in which a dissociated receptor must associate with a heat-stable cytoplasmic peptide in order to bind to DNA-cellulose. It is entirely possible however that the receptor must associate with a metal in order to bind to DNA. It is known from studies of primary structure that the DNA binding domains of glucocorticoid and other steroid receptors [22, 23] contain cysteine-rich motifs that by analogy with other proteins may constitute metal binding domains [24]. Similar motifs in the *Xenopus* transcription factor TF111A are thought to bind zinc which is required for DNA binding by that protein [25]. It is possible that the presence of such a trace metal could explain the effects of boiled cytosol and the partially purified thioredoxin and RNase preparations on DNA binding observed in our experiments and in the experiments published by Schmidt *et al.*[17, 18]. Schmidt *et al.*[17] have inactivated the DNA-binding factor activity in liver cytosol by trypsin digestion. In contrast, we have not been able to affect the activity of either boiled cytosol or the partially purified thioredoxin preparation by incubation with trypsin or chymotrypsin. This argues against a heat-stable protein factor being required for DNA binding and would be consistent with a metal effect.

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