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Effects of Bovine Pancreatic Ribonuclease A, S Protein, and S Peptide on Activation of Purified Rat Hepatic Glucocorticoid–Receptor Complexes†

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Received April 15, 1986; Revised Manuscript Received June 16, 1986

ABSTRACT: Bovine pancreatic ribonuclease (RNase) A and S protein (enzymatically inactive proteolytic fragment of RNase A which contains RNA binding site) stimulate the activation, as evidenced by increasing DNA–cellulose binding, of highly purified rat hepatic glucocorticoid–receptor complexes. These effects are dose dependent with maximal stimulation of DNA–cellulose binding being detected at approximately 500 μg (50 units of RNase A/mL). RNase A and S protein do not enhance DNA–cellulose binding via their ability to interact directly with DNA or to increase nonspecific binding of receptors to cellulose. Neither S peptide (enzymatically inactive proteolytic fragment which lacks RNA binding site) nor cytochrome *c*, a nonspecific basic DNA binding protein, mimics these effects. RNase A and S protein do not stimulate the conformational change which is associated with activation and is reflected in a shift in the elution profile of receptor complexes from DEAE-cellulose. In contrast, these two proteins interact with previously heat-activated receptor complexes to further enhance their DNA–cellulose binding capacity and thus mimic the effects of an endogenous heat-stable cytoplasmic protein(s) which also function(s) during step 2 of in vitro activation [Schmidt, T. J., Miller-Diener, A., Webb, M. L., & Litwack, G. (1985) *J. Biol. Chem. 260*, 16255–16262]. Preadsorption of RNase A and S protein to an RNase affinity resin containing an inhibitory RNA analogue, or trypsin digestion of the RNA binding site within S protein, eliminates the subsequent ability of these two proteins to stimulate DNA–cellulose binding of the purified receptors. These data indicate that the effects of RNase A on activation do not require RNA hydrolysis but do require an intact RNA binding site. This conclusion is consistent with numerous observations which suggest that a small RNA molecule(s) may be an integral component of the glucocorticoid receptor and may influence activation.

Once glucocorticoid molecules bind with high affinity and specificity to target cell intracellular receptors, these steroid–receptor complexes must undergo "activation" or "transformation" before they can bind to nuclear acceptor sites and ultimately modulate gene expression. Activation is a temperature-dependent process which is thought to involve a conformational change in the glucocorticoid–receptor complex resulting in the exposure of positively charged amino acid residues on the surface of the protein (Milgrom et al., 1973; DiSorbo et al., 1980) and hence an increased affinity for nuclei and polyanions such as DNA and DNA–cellulose (Milgrom et al., 1973; Baxter et al., 1972; Kalimi et al., 1975; LeFevre et al., 1979). This conformational change is also reflected in an altered elution profile of the activated glucocorticoid–receptor complexes from anion-exchange resins such as DEAE-cellulose (Sakaue & Thompson, 1977). Despite the fact that activation occurs in vivo under physiological conditions and appears to be rate limiting for nuclear binding (Munck & Foley, 1979; Markovic & Litwack, 1980; Miyabe & Harrison, 1983), its underlying biochemical mechanism has not been elucidated. Although numerous theories have been proposed [reviewed by Schmidt & Litwack (1982)], recent studies have implicated subunit dissociation as a mechanism of glucocorticoid–receptor activation (Vedeckis, 1983; Raaka & Samuels, 1983). However, reconstitution experiments with purified unactivated glucocorticoid–receptor complexes have also demonstrated that maximal DNA–cellulose binding of thermally activated complexes requires the additional involvement of an endogenous heat-stable cytoplasmic protein(s) (Schmidt et al., 1985).

Just as the precise mechanism of activation requires further clarification, the exact molecular composition of the unactivated glucocorticoid receptor itself has not been ascertained. Numerous studies have suggested a homotetramer–homodimer–monomer model for the unactivated receptor (Raaka & Samuels, 1983; Vedeckis, 1983; Holbrook et al., 1983;

† At The University of Iowa, this work was supported by a Helmar Maage Memorial Grant for Cancer Research from the American Cancer Society (BC 464) and by Research Grant 1 R01 AM 34490-01 PC from the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health. T.J.S. is a Scholar of the Leukemia Society of America, Inc. At the Temple University School of Medicine, this work was supported by Research Grants AM-13531 and AM-32870 from the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health.
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0006-2960/86/0425-5955$01.50/0

Sherman et al., 1983), while others have suggested that nonhormone binding proteins may also be components of the oligomeric forms (Grandics et al., 1984b; Gehring & Arndt, 1985; Joab et al., 1985). It has also been speculated that RNA may play a role in the structure and function of many steroid receptors including those for glucocorticoids (Liao et al., 1980; Chong & Lippman, 1982; Hutchens et al., 1982; Rossini & Barbiroli, 1983; Tymoczko & Phillips, 1983; Tymoczko et al., 1984; Economidis & Rousseau, 1985), progestins (Liao et al., 1980), androgens (Liao, 1980; Haase et al., 1983), estrogens (Liao et al., 1980; Liang & Liao, 1974; Chong & Lippman, 1982; Thomas & Kiang, 1985), and vitamin D (Franceschi & DeLuca, 1979; Franceschi et al., 1983; Franceschi, 1984). In many of these studies, crude cytosolic receptors were incubated with exogenous ribonuclease, and subsequent alterations in the physicochemical properties of the receptor complexes were detected. Because ribonuclease converted these receptor complexes to more slowly sedimenting species and enhanced their DNA–cellulose binding capacity, it has been speculated that ribonuclease may actually play a role in receptor activation. The obvious assumption which has been made in these studies is that ribonuclease enhances activation via its ability to interact directly with the unactivated receptors and hydrolyze RNA which may be associated with these complexes. Obviously, however, it has not been possible to completely rule out the possibility that in these experiments cytoplasmic RNA may have nonspecifically or artifactually associated with the crude cytosolic receptors.

In the present study, we have investigated the direct effects of bovine pancreatic ribonuclease A (RNase A) on the activation of rat hepatic glucocorticoid–receptor complexes purified to near homogeneity by a three-step procedure which includes affinity chromatography (Grandics et al., 1984b). In addition, we have tested the effects of S protein and S peptide, two RNase proteolytic fragments which lack enzymatic activity. The data presented demonstrate that both RNase A and S protein, but not S peptide, stimulate the DNA–cellulose binding capacity of thermally activated receptor complexes in a dose-dependent manner. The effectiveness of S protein in these experiments implies that although an intact RNA binding site is required, actual hydrolysis of an RNA molecule(s), which presumably form(s) an integral component of the purified unactivated complexes, may not be required.

Materials and Methods

Chemicals. [1,2,4(N)-^{3}H]Triamcinolone acetonide ([^{3}H]TA; 30 Ci/mmol) was purchased from Amersham. Bio-Gel A-1.5m agarose and hydroxylapatite (HTP, DNA grade) were obtained from Bio-Rad; DNA–cellulose (1.2 mg of native calf thymus DNA/mL of cellulose) was from P-L Biochemicals, Inc.; DEAE-cellulose (preswollen DE52) was from Whatman; 2-(*N*-morpholino)ethanesulfonic acid (MES), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), sodium molybdate, glycerol, trypsin, soybean trypsin inhibitor, nonradioactive TA, Sephadex G-75, bovine serum albumin (BSA), bovine pancreatic ribonuclease A (RNase A; 100 Kunitz units/mg of solid), S protein (1.6 Kunitz units/mg of solid), S peptide (<0.1 Kunitz unit/mg of solid), and cytochrome *c* were from Sigma; all other reagent-grade chemicals were from Fisher. The Sterogel A affinity resin (deoxycorticosterone–agarose) was purchased from Sterogene Biochemicals. The RNase A affinity resin [5′-(4′aminophenylphosphoryl)uridine–2′(3′)-phosphate–Sepharose 4B] was purchased from Miles Scientific.

Buffers. Buffers used were buffer A (50 mM potassium phosphate, 10 mM sodium molybdate, and 10 mM thioglycerol, pH 7.0 at 4 °C), buffer B [10 mM potassium phosphate, 10 mM sodium molybdate, 10 mM thioglycerol, 2 mM dithiothreitol (DTT), and 10% (v/v) glycerol, pH 7.0 at 4 °C], and buffer C [10 mM MES, 0.5 mM ethylenediaminetetraacetic acid (EDTA), and 0.5 mM dithiothreitol, pH 6.5 at 4 °C]. The RNase A and S protein stocks were prepared in buffer C at 1 mg/mL, and the RNase A stock was routinely heated at 90 °C for 30 min to inactivate any potential protease and/or DNase activities.

Preparation of Cytosol. Adrenalectomized male Sprague Dawley rats (175–200 g) were purchased from Hormone Assay Laboratories, Inc., and were used 5–8 days following surgery. Following anesthetization with ether, rat livers were perfused in situ via the portal vein with cold 0.9% NaCl and then with buffer A containing 2 mM DTT. The excised and minced livers were weighed and then homogenized in 1 volume of ice-cold buffer A (2 mM DTT) with a Brinkmann Polytron (PT-10-35) homogenizer. After the crude homogenate was centrifuged at 4000*g* for 15 min and the upper lipid layer was aspirated, the supernatant was centrifuged at 105000*g* for 1 h to obtain the cytosol. All procedures were performed at 0–4 °C, and the final cytosol was stored under liquid nitrogen until further use.

Purification of Unactivated Glucocorticoid Receptors. Unactivated hepatic glucocorticoid–receptor complexes were purified to near homogeneity by using minor modifications of a published scheme which includes affinity chromatography with a deoxycorticosterone-derivatized agarose, gel filtration on Bio-Gel A-1.5m agarose, and DEAE-cellulose chromatography (Grandics et al., 1984b; Schmidt et al., 1985). Following its elution from DEAE-cellulose at approximately 250 mM potassium phoshate, the pooled unactivated [^{3}H]-TA–receptor peak was eluted with buffer C from a Sephadex G-75 column (50-mL bed volume; inner diameter 2.8 cm) to remove the high salt. The fractions containing the unactivated [^{3}H]TA–receptor complexes were again pooled and adjusted to 10% glycerol (v/v) and 10 mg of BSA/mL. Addition of the carrier protein (BSA) to the purified receptor does not affect subsequent thermal activation but does reduce subsequent heat-induced [^{3}H]TA dissociation (Schmidt et al., 1985). One-milliliter aliquots were then stored under liquid nitrogen for subsequent activation experiments. After the aliquots were thawed, but prior to 1:1 dilution (v/v) with buffer C and subsequent thermal activation, the purified complexes were adjusted to 10 mM DTT.

Assay of [^{3}H]TA–Receptor Complexes. Receptor-bound [^{3}H]TA was determined by using the hydroxylapatite batch assay originally described for the crude estrogen receptor (Erdos et al., 1970) and adopted for the purified glucocorticoid receptor (Schmidt et al., 1985). Briefly, triplicate aliquots (50 μL) of the purified receptor were incubated with 400 μL of a hydroxylapatite slurry [10% (w/v) in 50 mM potassium phosphate and 10 mM MoO_4, pH 7.0 at 4 °C] for a minimum of 15 min at 0–4 °C. The pellets were then washed with 2 mL of 50 mM potassium phosphate (10 mM MoO_4, pH 7.0 at 4 °C) and centrifuged. After the supernatants were aspirated, the pellets were resuspended in 5 mL of Ready-Solv liquid scintillation fluid (Beckman), quantiatively transferred to vials, and assayed for bound radioactivity in a Beckman liquid scintillation spectrometer with an average counting efficiency for tritium of 45%.

DNA–Cellulose Binding Assay. The binding of purified [^{3}H]TA–receptor complexes to DNA–cellulose was determined by the procedure of Kalimi et al. (1975) with slight modification. Briefly, 100-μL aliquots of purified receptor were

Table I: Effect of RNase A, S Protein, and S Peptide on DNA–Cellulose Binding Capacity of Purified Thermally Activated [^{3}H]TA–Receptor Complexes[a]

treatment	total binding of [^{3}H]TA (dpm/100 μL)	DNA–cellulose binding	
		dpm/100 μL	%
(1) unactivated control (0 °C, 30 min)	6160	1040	16.9
(2) thermal activation (25 °C, 30 min)	5390	1670	31.0
(3) thermal activation plus RNase A (500 μg/mL)	4870	3740	76.8
(4) thermal activation plus S protein (500 μg/mL)	4730	2870	60.7
(5) thermal activation plus S peptide (500 μg/mL)	3920	940	24.0
(6) thermal activation plus equimolar mixture of S protein plus S peptide	4530	2680	59.2
(7) thermal activation plus cytochrome *c* (500 μg/mL)	5310	1360	25.6
(8) DNA–cellulose preincubated with RNase A	5260	1700	32.3
(9) DNA–cellulose preincubated with S protein	5220	1730	33.1
(10) DNA–cellulose preincubated with cytochrome *c*	5380	1800	33.5

[a] Aliquots of purified unactivated [^{3}H]TA–receptor complexes were mixed 1:1 (v/v) with either buffer C alone or buffer C containing the various proteins as indicated. The mixtures were then thermally activated at 25 °C for 30 min. The hydroxylapatite and DNA–cellulose binding assays were then performed as described under Materials and Methods, and the DNA–cellulose binding was expressed as a percentage of the total receptor-bound [^{3}H]TA. All dpm values represent the means of triplicate determinations (coefficients of variation <5.0%).

added in triplicate to 50 μL of packed DNA–cellulose and were then incubated at 0–4 °C for a minimum of 45 min. After being washed once with 2 mL of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.0 at 0–4 °C), the pellets were quantitatively transferred to scintillation vials and assayed for bound radioactivity as described.

Results

Effects of RNase A, S Protein, and S Peptide on DNA–Cellulose Binding of Purified Receptors. As seen in Table I, thermal activation (25 °C for 30 min) of purified unactivated [^{3}H]TA–receptor complexes resulted in approximately a 2-fold increase in subsequent DNA–cellulose binding (treatment 2 vs. treatment 1) as previously reported (Schmidt et al., 1985). When added prior to thermal activation at a concentration of 500 μg/mL, both RNase A (treatment 3) and S protein (treatment 4) [a large RNase A proteolytic fragment containing amino acids 21–124 which is devoid of ribonuclease activity (Richards & Wyckoff, 1971; Richards & Vithayathil, 1959)] enhanced the subsequent DNA–cellulose binding of the purified [^{3}H]TA–receptor complexes an additional 2.5- and 2.0-fold, respectively, when compared to the heated control. In contrast, S peptide (treatment 5), a small RNase proteolytic fragment containing amino acids 1–20, failed to enhance the DNA–cellulose binding of the purified complexes. A mixture of S protein and S peptide (treatment 6) [mixed at an equimolar ratio which has been shown to restore full ribonuclease activity due to a noncovalent association between the two polypeptides (Dasher, 1967; Richards & Vithayathil, 1959)] resulted in a 2-fold increase in DNA–celulose binding similar to that detected when S protein alone was added (treatment 4). These data suggest that the S peptide does not interfere with the stimulatory activity of the S protein nor does restoration of enzymatic activity appear to enhance the subsequent stimulation of DNA–cellulose binding. The failure of cytochrome *c*, a low molecular weight basic protein, to enhance DNA–cellulose binding of the purified complexes (treatment 7) suggests that the stimulatory effects of RNase A and S protein cannot be a consequence of their net charge or size.

To rule out the possibility that RNase A and S protein may enhance the DNA–cellulose binding of the purified [^{3}H]TA–receptor complexes via their ability to interact directly with DNA, DNA–cellulose pellets were preincubated with each protein (treatment 8 and 9). After being washed with TE buffer to remove free proteins, the DNA–cellulose pellets were incubated with thermally activated [^{3}H]TA–receptor complexes. The subsequent DNA–cellulose binding was identical

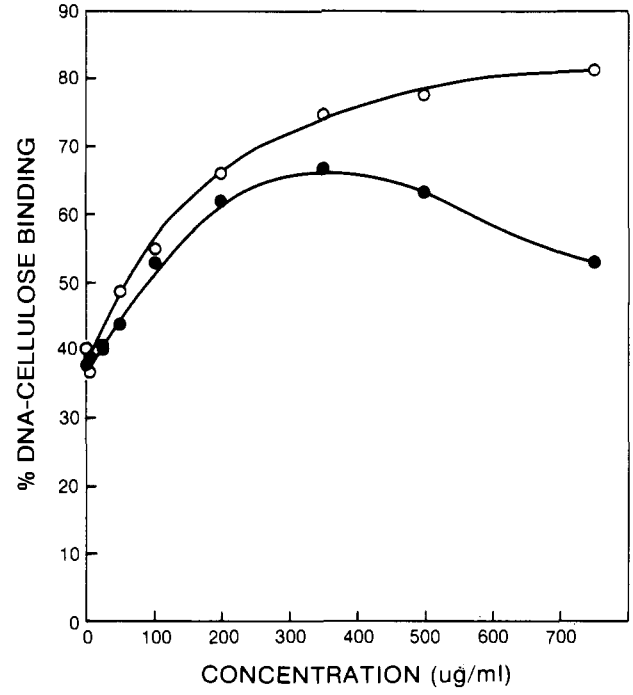

FIGURE 1: Dose–response relationship for the effects of exogenous RNase A and S protein on DNA–cellulose binding of purified [^{3}H]TA–receptor complexes. Aliquots of purified unactivated [^{3}H]TA–receptor complexes were mixed 1:1 (v/v) with either RNase A (○) or S protein (●) at the final concentrations indicated and were then incubated at 25 °C for 30 min. The mixtures were then adjusted to 10 mM MoO_4, and the hydroxylapatite and DNA–cellulose assays were performed as described under Materials and Methods. The DNA–cellulose binding was then expressed as a percentage of the total receptor-bound [^{3}H]TA.

with that detected after incubating thermally activated complexes with nonpretreated DNA–cellulose (treatment 2). Similarly, pretreatment of DNA–cellulose with cytochrome *c*, another nonspectific DNA binding protein, had no effect on the subsequent binding of thermally activated [^{3}H]TA–receptor complexes (treatment 10). Taken collectively, these data suggest that both RNase A and S protein enhance DNA–cellulose binding via their ability to interact directly with the purified [^{3}H]TA–receptor complexes.

Dose–Response Relationships for the Effects of RNase A and S Protein. The effects of increasing concentrations of RNase A and S protein on the DNA–cellulose binding of the thermally activated purified [^{3}H]TA–receptor complexes are depicted in Figure 1. This dose–response curve demonstrates that as the concentrations of these two proteins were increased

Table II: Effect of Preincubation with RNase Affinity Resin on Subsequent Ability of RNase A and S Protein To Stimulate DNA–Cellulose Binding of Purified Thermally Activated [^{3}H]TA–Receptor Complexes[a]

treatment	total binding of [^{3}H]TA (dpm/100 μL)	DNA–cellulose binding dpm/100 μL	DNA–cellulose binding %
(1) unactivated control (0 °C, 30 min)	10400	2060	19.8
(2) thermal activation (25 °C, 30 min)	9190	2850	31.0
(3) thermal activation plus RNase A (500 μg/mL)	8960	6910	77.1
(4) thermal activation plus S protein (500 μg/mL)	8870	5610	63.2
(5) thermal activation plus RNase A pretreated (batchwise) twice with 0.5 mL of packed RNase affinity resin	8830	2750	31.1
(6) thermal activation plus S protein pretreated (batchwise) twice with 0.5 mL of packed RNase affinity resin	9020	2720	30.2

[a] Aliquots of purified unactivated [^{3}H]TA–receptor complexes were mixed 1:1 (v/v) with either buffer C alone or buffer C containing either RNase A or S protein as indicated. The mixtures were then thermally activated at 25 °C for 30 min. The hydroxylapatite and DNA–cellulose binding assays were then performed as described under Materials and Methods, and the DNA–cellulose binding was expressed as a percentage of the total receptor-bound [^{3}H]TA. All dpm values represent the means of triplicate determinations (coefficients of variation <5.0%).

Table III: Effects of Trypsin Preincubation on Ability of RNase A and S Protein To Subsequently Stimulate DNA–Cellulose Binding of Purified Thermally Activated [^{3}H]TA–Receptor Complexes[a]

treatment	total binding of [^{3}H]TA (dpm/100 μL)	DNA–cellulose binding dpm/100 μL	DNA–cellulose binding %
Experiment A			
(1) unactivated control (0 °C, 30 min)	11000	2190	20.0
(2) thermal activation (25 °C, 30 min)	8950	2970	33.2
(3) thermal activation plus RNase A (500 μg/mL)	9630	7210	74.9
(4) thermal activation plus RNase A (500 μg/mL) preincubated (37 °C, 30 min) with trypsin (10 μg/mL) followed by a 10-fold excess of soybean trypsin inhibitor (37 °C, 30 min)	10400	8100	77.9
Experiment B			
(1) unactivated control (0 °C, 30 min)	23800	5080	21.3
(2) thermal activation (25 °C, 30 min)	20600	6640	32.2
(3) thermal activation plus S protein (500 μg/mL)	20100	12400	61.7
(4) thermal activation plus S protein (500 μg/mL) preincubated (37 °C, 30 min) with trypsin (10 μg/mL) followed by a 10-fold excess of soybean trypsin inhibitor (37 °C, 30 min)	19300	6620	34.3

[a] Aliquots of purified unactivated [^{3}H]TA–receptor complexes were mixed 1:1 (v/v) with either buffer C alone or buffer C containing either RNase A or S protein as indicated. The mixtures were then thermally activated at 25 °C for 30 min. The hydroxylapatite and DNA–cellulose binding assays were then performed as described under Materials and Methods, and the DNA–cellulose binding was expressed as a percentage of the total receptor-bound [^{3}H]TA. All dpm values represent the means of triplicate determinations (coefficients of variation <5.0%).

from final concentrations of 5–750 μg/mL, DNA–cellulose binding was concomitantly enhanced. Maximal stimulation of DNA–cellulose binding by RNase A and S protein was detected at a final concentration of approximately 500 μg/mL, and therefore we continued to use this concentration in all subsequent experiments. RNase A appeared to be slightly more effective than S protein in enhancing DNA–cellulose binding over the entire range of concentrations tested. At a concentration of 500 μg/mL, RNase A and S protein enhanced DNA–cellulose binding 2.0- and 1.7-fold, respectively, when compared to the appropriate heated controls. Unlike RNAse A, S protein appeared to lose some of its stimulatory activity at the highest concentration tested (750 μg/mL).

Effects of Preadsorption to RNase Affinity Resin and Trypsin Digestion on Subsequent Stimulatory Effects of RNase A and S Protein. Although the ability of S protein to enhance DNA–cellulose binding of the thermally activated purified [^{3}H]TA–receptor complexes demonstrates that ribonuclease activity is not required to elicit this effect, the ineffectiveness of S peptide (Table I, treatment 5) suggests that an intact substrate (RNA) binding site may be required. To test this hypothesis, two independent approaches were pursued. First, both RNase A and S protein were preincubated with an affinity resin [5′-(4-aminophenylphosphoryl)uridine–2′(3′)-phosphate–Sepharose 4B] which contains an inhibitory RNA analogoue and was initially developed by Wilchek and Gorecki (1969) for the purification of ribonuclease. The data presented in Table II indicate that preadsorption of both RNase A (treatment 5) and S protein (treatment 6) to this affinity resin precludes their subsequent ability to enhance the DNA–cellulose binding of the thermally activated, purified [^{3}H]TA–receptor complexes (treatments 3 and 4). These results are consistent with those of Wilchek and Gorecki (1969), who reported that S protein was bound strongly to this affinity resin, as well as those of Bernfield (1965), who concluded that the 20 amino-terminal residues (S peptide) are not required for substrate binding to S protein.

Second, in order to further test the hypothesis that an intact RNA binding site is required in this system, both RNase A (Table III, experiment A) and S protein (Table III, experiment B) were preincubated with trypsin (10 μg/mL), which was subsequently inhibited with a 10-fold excess of soybean trypsin inhibitor, prior to their incubation with the purified [^{3}H]TA–receptor complexes at 25 °C for 30 min. As seen in Table III (treatment 3 vs. treatment 4), preincubation of RNase A with trypsin failed to prevent its subsequent ability to stimulate DNA–cellulose binding. This result is consistent with the known resistance of the intact enzyme at neutral pH and room temperature to trypsin digestion (Richards & Wyckoff, 1971). In contrast, preincubation of S protein with trypsin totally abolished its subsequent ability to stimulate DNA–cellulose binding (Table III, treatment 3 vs. treatment 4). This result, which is consistent with the reported sensitivity of S protein to trypsin digestion (Allende & Richards, 1962), suggests that an intact RNA binding site is necessary in order for this enzymatically inactive fragment to elicit an effect on the

Table IV: Effects of RNase A and S Protein at 0–4 °C in the Presence of Molybdate on DNA–Cellulose Binding of Previously Activated Purified [^{3}H]TA–Receptor Complexes[a]

treatment	total binding of [^{3}H]TA (dpm/100 μL)	DNA–cellulose binding dpm/100 μL	DNA–cellulose binding %
(1) unactivated control (0 °C, 30 min), then incubated at 0 °C for 30 min plus 10 mM MoO_4	5640	1060	18.8
(2) activated control (25 °C, 30 min), then incubated at 0 °C for 30 min plus 10 mM MoO_4	4620	1110	24.0
(3) activated (25 °C, 30 min), then incubated with RNase A (500 μg/mL) at 0 °C for 30 min plus 10 mM MoO_4	4430	3200	72.2
(4) activated (25 °C, 30 min), then incubated with S protein (500 μg/mL) at 0 °C for 30 min plus 10 mM MoO_4	4020	2220	55.2

[a] Aliquots of purified unactivated [^{3}H]TA–receptor complexes were first incubated at 0 °C for 30 min (treatment 1) or thermally activated at 25 °C for 30 min (treatments 2–4). The preincubated [^{3}H]TA–receptor complexes were then adjusted to 20 mM MoO_4 and were then mixed 1:1 (v/v) with buffer C (adjusted to pH 7.0 at 4 °C and 1 mg of BSA/mL) alone or buffer C containing either RNase A (treatment 3) or S protein (treatment 4). After an additional incubation at 0 °C for 30 min, the hydroxylapatite and DNA–cellulose assays were performed as described under Materials and Methods, and the DNA–cellulose binding was expressed as a percentage of the total receptor-bound [^{3}H]TA. All dpm values represent the means of triplicate determinations (coefficients of variation <5.0%).

thermally activated, purified [^{3}H]TA–receptor complexes.

Effects of RNase A and S Protein on Thermally Activated, Purified [^{3}H]TA–Receptor Complexes at Low Temperature in the Presence of Molybdate. The results of preliminary experiments (data not shown) indicated that RNase A could enhance the DNA–cellulose binding of previously activated (25 °C for 30 min) purified [^{3}H]TA–receptor complexes when incubated at 0–4 °C in the presence of 10 mM MoO_4. Similar experiments were repeated in order to test whether the effect of S protein, like that of RNase A, is temperature independent. Purified [^{3}H]TA–receptor complexes were first thermally activated (25 °C for 30 min) and were then adjusted to 10 mM MoO_4 [blocks any subsequent activation as reflected by a shift in the elution profile from DEAE-cellulose (Schmidt et al., 1985)]. The activated complexes were then incubated for an additional 30 min at 0–4 °C with buffer C, RNase A, or S protein (final concentrations of 500 μg/mL). The results of such of two-step experiment are summarized in Table IV. The data demonstrate that both RNase A (treatment 3) and S protein (treatment 4) are capable of interacting with previously thermally activated, purified [^{3}H]TA–receptor complexes and enhancing their subsequent DNA–cellulose binding capacity. This apparent lack of temperature dependency is obviously consistent with a nonenzymatic mechanism of action of both RNase A and S protein. If the purified unactivated [^{3}H]TA–receptor complexes eluted from DEAE-cellulose were pooled, adjusted to 20 mM MoO_4, and incubated at 0 °C for 30 min with either RNase A or S protein (final concentration of 10 mM MoO_4), no subsequent increase in DNA–cellulose binding capacity was detected (data not shown). These data suggest that both RNase A and S protein potentiate the DNA–cellulose binding of only thermally activated [^{3}H]TA–receptor complexes.

Discussion

The data presented in this report demonstrate that both exogenous bovine pancreatic RNase A and S protein, a proteolytic fragment of the enzyme which lacks enzymatic activity but possesses an RNA binding site, enhance the DNA–cellulose binding capacity of purified thermally activated rat hepatic glucocorticoid–receptor complexes in a dose-dependent manner (Figure 1). The similarity of the dose–response curves for these two polypeptides clearly indicates that the effectiveness of S protein is not a consequence of its residual ribonuclease activity (<2% of intact enzyme). The observation that an equimolar mixture of S protein plus S peptide, which results in restoration of full enzymatic activity via a noncovalent interaction, was no more effective than S protein alone (Table I) also supports this conclusion. The stimulatory effects of RNase A and S protein appear to be relatively specific since they cannot be mimicked by cytochrome *c*, another protein of similar molecular weight and net charge (Table I). The stimulatory effects also appear to result from a direct interaction of both RNase A and S proteion with the receptor complexes rather than with DNA–cellulose (Table I). Although ribonuclease hydrolytic activity does not appear to be required for stimulatory activity, both preadsorption to an RNase affinity resin (Table II) and trypsin preincubation (Table III) suggest that an intact RNA binding site is required. The observations that the stimulatory effect of RNase A on previously thermally activated complexes is not temperature dependent (data not shown) and that relatively high concentrations (approximately 500 μg/mL) of both RNase A and S protein are required for maximal stimulation of DNA–cellulose binding (Figure 1) are also consistent with a nonenzymatic mechanism. It is important to note that thioredoxin has also been reported to enhance the DNA–cellulose binding capacity of partially purified rat hepatic glucocorticoid receptors (Grippo et al., 1986). However, when the RNase A and S protein utilized in the present study were assayed for thioredoxin activity by using the insulin disulfide reduction assay, they were found to be devoid of contaminating activity (Dr. W. B. Pratt, personal communication).

Their effectiveness at 0–4 °C in the presence of 10 mM MoO_4 (Table IV) suggests that both RNase A and S protein enhance the DNA–cellulose binding of thermally activated glucocorticoid–receptor complexes during the recently proposed step 2 (temperature independent, MoO_4 insensitive) of in vitro activation (Schmidt et al., 1985). In a similar manner, we have shown that an endogenous heat-stable (90 °C for 30 min) cytoplasmic protein(s) also stimulate(s) the DNA–cellulose binding capacity of thermally activated complexes during step 2 (Schmidt et al., 1985). In light of the fact that this (these) endogenous stimulatory protein(s) and exogenous RNase A are both stable at elevated temperature and that 90 °C treated cytosol contains significant ribonuclease activity (data not shown), it is tempting to speculate that the endogenous stimulatory protein may in fact be hepatic ribonuclease. However, several observations argue against this identity. First, exogenous RNase A is trypsin resistant (Table III; Richards & Wyckoff, 1971) while the endogenous stimulatory protein(s) is (are) trypsin sensitive (Schmidt et al., 1975). Second, preliminary experiments demonstrate that the cytoplasmic, heat-stable stimulatory protein(s) is (are) not adsorbed by the RNase affinity resin utilized in the present study. Finally, the high concentration (500 μg/mL) of exogenous ribonuclease required for maximal stimulation of DNA–cellulose binding in the present study obviously exceeds the endogenous con-

centration of the enzyme. It should be noted, however, that limited studies from other laboratories have suggested that endogenous ribonuclease may be involved in activation of crude glucocorticoid–receptor complexes. Tymoczko and Phillips (1983) reported that a placental ribonuclease inhibitor could block the increase in crude glucocorticoid–receptor complex binding to DNA caused by exogenous RNase A, but not by heating. However, Rossini (1985) reported that the same inhibitor could also prevent the heat-induced activation of crude dexamethasone–receptor complexes in the absence of exogenous RNase. Although these latter results might suggest a role of endogenous RNase in activation, an indirect effect of the placental inhibitor unrelated to its ability to inhibit RNA hydrolysis could not be totally excluded. Further experiments aimed at identifying the endogenous heat-stable protein(s) and defining the role, if any, of endogenous ribonuclease in mediating activation are currently in progress in our laboratory.

The results presented in this report indicate that the ability of both RNase A and S protein to stimulate DNA–cellulose binding requires the presence of RNA associated with the purified receptors. Although RNA hydrolysis does not appear to be required, the data presented do not allow us to distinguish between the possibility that both RNase A and S protein enhance DNA–cellulose binding by facilitating the removal of unhydrolyzed RNA from the purified receptor complexes or by forming ternary complexes with the RNA which remains bound to the receptors. Obviously, both of these possibilities require that RNA be associated with the purified thermally activated complexes. Another possible mechanism which we have considered is that RNA molecules associated with the purified unactivated receptor complexes dissociate from the receptors during thermal activation and subsequently compete with DNA for binding of activated complexes. If this were the case, addition of RNA binding proteins, such as RNase A or S protein, could effectively remove this free RNA and hence enhance DNA–cellulose binding. This mechanism seems unlikely for several reasons. First, it is difficult to conceive of there being sufficient RNA released during activation to effectively compete with the large quantity of DNA bound to cellulose. Second, we have thermally activated purified receptor complexes and subsequently subjected them to gel filtration on Sephadex G-75, which would effectively separate these complexes from any relatively small RNA molecules released during the earlier activation step. However, the fact that both RNase A and S protein significantly enhanced the DNA–cellulose binding capacity of the filtered receptors (data not shown) suggests that the RNA molecules with which these two proteins interact are still associated with the activated receptors.

Numerous lines of experimental evidence clearly suggest that RNA is a component of the purified unactivated receptor complexes. Grandics et al. (1984a) tested the possibility that rat hepatic glucocorticoid receptors become phosphorylated in vivo by injecting [^{32}P]orthophosphate into adrenalectomized rats and subsequently purifying the unactivated glucocorticoid–receptor complexes by the same three-step procedure employed in the present study. Their results demonstrated not only that the receptor (M_r 90 000) incorporated ^{32}P but also that a lower molecular weight component also became heavily phosphorylated. This component (M_r 24 000) did not stain with Coomassie Blue, and the authors speculated that it may in fact be a polynucleotide. In a related study, Housley and Pratt (1984) demonstrated that when mouse L cells were incubated in the presence of [^{32}P]orthophosphate, several labeled components, including the receptor protein itself, could be detected after purification by the same scheme. The ^{32}P detected at a heavily phosphorylated M_r 21 000 position could be eliminated by treatment with exogenous ribonuclease, which again suggests that an RNA species copurifies with unactivated receptor complexes.

Clearly RNA may associate artifactually with proteins in vitro (Girard & Baltimore, 1966; Baltimore & Huang, 1970) and, more specifically, with steroid receptors. However, although it has been suggested that following activation the estrogen receptor might interact with nonspecific RNAs to produce heterogeneity (Skipper et al., 1985), this situation appears to be less likely when the starting preparation, as in the present study, is the purified unactivated receptor complex. To copurify with the unactivated glucocorticoid receptor (without actually being complexed with the receptor), the RNA would have to bind to the affinity resin, be subsequently eluted with [^{3}H]TA, and coelute with the large (M_r 303 000) unactivated receptor complexes from the Bio-Gel column. M. Webb and colleagues have recently performed experiments (unpublished data) which suggest that RNA is associated with the partially purified unactivated form of the receptor up to the point of anion-exchange chromatography. At this point, the RNA (some of which coelutes from DEAE–cellulose with the unactivated complexes) can be largely separated from the receptor protein but can be subsequently eluted from DEAE-cellulose at approximately 0.6 M KCl. When this eluted material, which incorporates ^{32}P in vivo and stains with ethidium bromide at approximately 100–110 nucleotides, is added back to the purified unactivated receptor complexes, it blocks the RNase A stimulation of DNA–cellulose binding of thermally activated complexes reported in the present study. Likewise, Kovacic-Milivojevic et al. (1985) have reported that after elution of receptor complexes from DEAE-cellulose, further elution with 0.5 M KCl yields a fraction which contains RNA. When added back to the 3.8–4.2S receptor monomer, the sedimentation coefficient is increased to 5.2 S on molybdate-containing sucrose gradients. Again, these data suggested that a low molecular weight RNA may be an integral component of the receptor.

Although one could argue that RNA artifactually binds to the unactivated receptors during preparation of the cytosol, recent studies suggest that RNA may be a component of the receptor in vivo. Economidis and Rousseau (1985) covalently labeled the receptors in cultured rat hepatoma cells with [^{3}H]dexamethasone mesylate, and the putative RNA–receptor complexes were subsequently stabilized by either cell-free cross-linking using formaldehyde or irradiation of intact cells incubated with [^{14}C]uridine. Subsequent analyses by CsCl gradients and polyacrylamide gel electrophoresis demonstrated that the glucocorticoid receptor behaves as a ribonucleoprotein after cross-linking in vitro and in vivo. Obviously, the precise role, if any, that this RNA plays in terms of regulating the structure and/or function of the glucocorticoid receptor is unknown. Several interesting and sometimes contrasting possibilities which have been suggested include the following: maintaining the receptor in the unactivated state by masking its DNA binding site; facilitation of receptor translocation from the cytoplasm across the nuclear membrane (Economidis & Rousseau, 1985) in the same way that the RNA component of the "signal recognition particle" may participate in the injection of secretory proteins into the endoplasmic reticulum (Walter et al., 1984); facilitation of mRNA transport from the nucleus out into the cytoplasm (Knowler, 1985); stabilization of hormonally induced mRNA; aiding in recognition of specific gene sequences, perhaps via homology to acceptor

sites, and hence increasing the efficiency of transcription (Kovačič-Milivojević et al., 1985); or, finally, by acting as an "off" signal to terminate the enhanced transcription of specific genes by activated receptor complexes. The availability of highly purified unactivated glucocorticoid–receptor complexes and their associated RNA(s) should facilitate direct testing of some of these hypotheses.

Added in Proof

Since submission of the manuscript, we have demonstrated that RNase A and S protein also stimulate activation of receptors purified from hepatic cytosol prepared using an isotonic homogenization buffer. These data suggest that any potential association of RNA with the purified receptor complexes cannot simply result from the use of a hypotonic homogenization buffer.

Acknowledgments

We thank Connie Spurgeon and Thomas Chambers for technical assistance. We also thank Dr. Carol Barnett for helpful comments and suggestions during the preparation of the manuscript and Carol Johnson for secretarial assistance.

Registry No. Ribonuclease A, 9001-99-4.

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