Assessment of estrogen receptor-histone interactions

(affinity chromatography/DNA-cellulose/protein-protein interactions/chromosomal proteins/steroid hormone action)

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ABSTRACT Several different *in vitro* binding assays show that the estrogen receptor from rabbit uterus interacts selectively with purified histones from calf thymus. The estrogen receptor binds strongly to histones H2B and H2A, moderately to histones H3 and H4, and poorly to histone H1. In the presence of histones H2B or H2A, the position at which the estrogen receptor focuses in an isoelectric gradient is shifted to a more basic zone. Kinetic experiments show that, if histone H2B is bound to a DNA, the estrogen receptor dissociates more slowly from that DNA. The portion of the estrogen receptor molecule required for binding to histone H2B is relatively stable to tryptic digestion; in contrast, the portion of the receptor molecule responsible for DNA binding is promptly lost during limited tryptic digestion. These *in vitro* findings suggest that the mechanism by which the estrogen receptor selectively alters gene expression may involve specific contacts with histone molecules.

Steroid hormone receptors are regulatory proteins that can migrate from cytoplasm to nucleus and somehow alter the expression of specific genes (1-3). Although the precise nature of the interaction between steroid receptors and the genome remains unclear, many studies suggest that DNA and chromosomal proteins play an important role in the binding of the receptor complex to the nucleus (2-11). In particular, steroid hormone receptors can bind to isolated DNA *in vitro* (3-9). Yet, *in vivo*, virtually all the DNA in the eukaryotic nucleus is folded by histones into nucleosomes (12-14). Very little is known about how the receptor might recognize histone-DNA complexes (8-9). Such recognition could occur in at least two general, non-exclusive ways. First, the receptor might recognize the presence (or absence) of critical histone molecules directly via a histone-receptor interaction. Alternatively, the receptor might not make direct contact with the histones, but the histones could induce changes in the secondary and tertiary structure of DNA and these changes could then serve as partial determinants of recognition. The limited interaction of steroids with histones (15, 16) could be attributed, at least in part, to trace amounts of steroid hormone receptors "contaminating" the histone fractions (17, 18). Subsequent studies have shown that steroid hormone receptors can bind to histones and protamines *in vitro* (19-23), but these studies have often implied that the receptor-histone interactions were nonspecific.

To explore the possibility of direct and specific receptor-histone interactions, we have used the estrogen receptor from rabbit uterus to address the following questions: (i) Does the estrogen receptor bind to histones? If so, do preferential interactions occur with certain histones? (ii) Do histones affect the physicochemical properties of the estrogen receptor? (iii) Do histones influence the binding of the estrogen receptor to DNA? (iv) Where on the estrogen receptor is the histone recognition site?

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Is it associated with the DNA-binding site or with a topographically distinct domain of the receptor?

MATERIALS AND METHODS

Chemicals. [³H]Estradiol (58 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels) was obtained from Amersham. Diethylstilbestrol was purchased from Sigma. DNA-cellulose (400 μg/ml) was prepared by the method of Alberts and Herrick (24) using calf thymus DNA from P-L Biochemicals and Cellex grade cellulose from Bio-Rad Laboratories. Norit A charcoal was from Amend Drug and Chemical (Irvington, NJ), and Dextran T-70 was from Pharmacia. All other chemicals were of reagent grade.

Histone Purification. Histones were extracted from fresh calf thymus glands (25, 26) and purified by chromatographic procedures (27-29). Histone H1, prepared by method 1 of Johns (25), was purified by chromatography on a Bio-Rex 70 column (27). Histone H3 was purified from Johns' fraction f3 (25) by affinity chromatography on a mercurial Sepharose column (28). Histone H4 was obtained by chromatography of Johns' fraction f2a + f3 on a Bio-Gel P-60 column (29). The same gel filtration procedure was used to purify histone H2A from fraction f2a2 (26) and to purify histone H2B from fraction f2b (prepared by method 1 of Johns) or from fractions f1 and f2b (prepared by method 2 of Johns) (25). After chromatography, the histones were dialyzed against 2% acetic acid, lyophilized, and stored at -20°C. The purity of each histone was evaluated by electrophoresis on long acid urea gels (30, 31) and on NaDodSO₄ slab gels (27, 32). Histones H1, H2B, H3, and H4 were >99% pure; histone H2A was >95% pure.

Histone H1 and bovine serum albumin were coupled to activated Sepharose 4B by a standard procedure (33). To efficiently couple histones H2A, H2B, H3, or H4 to activated Sepharose, salts and buffer were omitted from the coupling solution (34). The purified histone was dissolved in distilled water, and the pH was adjusted to 9.0-9.5 with dilute NaOH; this solution was added directly to water-washed activated Sepharose. The pH of the slurry was monitored frequently and adjusted as necessary.

Preparation of Estrogen Receptor. The procedure of Feldman *et al.* (35) was used to prepare ³H-labeled estrogen receptor from rabbit uterus. Minced uteri were homogenized in 2 vol of cold buffer A (20 mM Tris-HCl, pH 7.4/1 mM EDTA/30 mM KCl/1 mM 2-mercaptoethanol). Centrifugation (190,000 × g, 30 min) of this homogenate yielded a clear supernatant that was then incubated with [³H]estradiol for 1 hr at 0°C. The solution was then adjusted to 25% saturated ammonium sulfate and centrifuged (10,000 × g, 20 min). The resulting pellet was dissolved in buffer A and used directly for the DNA-binding experiments. Protein concentrations were determined by the method of Lowry *et al.* (36).

DNA-Binding Assay. Binding of ³H-labeled estrogen receptor to DNA-cellulose was carried out as described (6). The DNA-cellulose suspension was rewashed on the day of use,

suspended in buffer B [buffer A/0.2% bovine serum albumin/10% glycerol (vol/vol)], adjusted to contain 10 μg of DNA/0.1 ml of suspension and pipetted into small test tubes in 0.1-ml aliquots. An equivalent volume of unsubstituted cellulose was used as a control. Aliquots (0.3 ml) of labeled estrogen receptor were added to the DNA-cellulose and the unsubstituted cellulose suspensions and incubated at 20°C for 30 min; the incubation was carried out in a water bath with vigorous gyratory shaking. The mixture was then chilled by addition of 2.0 ml of cold buffer C (buffer B containing 0.5 μM unlabeled estradiol) and centrifuged ($2500 \times g$, 5 min). The supernatants were removed by aspiration, and the DNA-cellulose pellet was washed with 2.0 ml of ethanol at 37°C for 15 min with constant shaking. After centrifugation, an aliquot of the ethanol solution was assayed for radioactivity. The specific binding of labeled estrogen receptor to DNA-cellulose was determined by subtracting the radioactivity bound to unsubstituted cellulose from that bound to DNA-cellulose. Estradiol binding to receptor was determined by a conventional dextran-coated charcoal technique with minor variations (37).

Histone-Sephadex Binding Assays. ^3H -Labeled estrogen receptor was prepared from rabbit uteri as described except that the ammonium sulfate pellet was dissolved in buffer A/0.15 M KCl. Various amounts (10^3 – 10^4 cpm) of this receptor preparation were added to 0.25 ml of a suspension of histone-Sephadex containing ≈ 50 μg of a purified calf thymus histone (covalently linked to Sephadex), and the mixture was incubated with gentle shaking at 4°C for 90 min in a final volume of 0.60 ml. In the competition experiments, the soluble histone and the Sephadex-immobilized histone were mixed thoroughly before the labeled receptor was added. At the end of the incubation period, the mixture was diluted with 0.75 ml of cold buffer A and centrifuged ($1000 \times g$, 3 min). The pellet was washed with three 0.8-ml portions of buffer A, centrifuged as above, and the final pellet was extracted with ethanol and assayed for radioactivity by liquid scintillation counting.

RESULTS

Receptor Binding to Histone-Sephadex. When increasing concentrations of ^3H -labeled estrogen receptor from rabbit uteri were incubated at 4°C for 90 min with a fixed amount of histone H2B-Sephadex, increasing amounts of the labeled steroid bound to the matrix (Fig. 1). No substantial binding was

FIG. 1. Binding of estrogen receptor complex to histone H2B-Sephadex. Various amounts of labeled receptor were incubated with either histone H2B-Sephadex (○) or bovine serum albumin-Sephadex (□) at 4°C for 1.5 hr. Labeled receptor was denatured by heating at 60°C for 20 min, and the denatured receptor was incubated with histone H2B-Sephadex (▲). Each point represents the average of three determinations.

observed with either bovine serum albumin-Sephadex or Sephadex alone. The difference in radioactivity associated with histone-Sephadex and blank Sephadex was attributed to specific histone binding. The estrogen receptor-histone interaction is reversible; >85% of the bound [^3H]estradiol can be eluted with 0.8 M KCl. Also, the extracted radioactivity (at least 70%) was in macromolecular form; i.e., it was excluded by Sephadex G-25 (data not shown). If the receptor interacts specifically with histone H2B, soluble histone should compete with histone-Sephadex for receptor binding. When increasing amounts of soluble histone H2B were added to a fixed amount of histone-Sephadex and mixed with labeled receptor, the soluble histone competed as expected and decreased the binding of labeled receptor to histone-Sephadex (Fig. 2). To determine whether the receptor can discriminate among different histones, we also examined histones H3, H4, and H1 for their ability to compete with the Sephadex-immobilized H2B for receptor binding. The different histones have quite different abilities to bind to receptor as measured by competition. H2B was an effective competitor, H3 and H4 were less so, and H1, even at a high concentration, was a poor competitor. These results are consistent with the view that the estrogen receptor binds specifically and preferentially to histone H2B. The ability of soluble histone H2A to compete with Sephadex-immobilized H2B was similar to that of soluble H2B (data not shown).

Competition experiments of this type must be interpreted with caution because the added soluble histones could reduce receptor binding to the histone-Sephadex by two distinct mechanisms: (i) a true competition between soluble histone and

FIG. 2. Competition of soluble histone and Sephadex-immobilized histone for ^3H -labeled estrogen receptor. Various amounts of soluble histone H1 (●), H3 (□), H4 (○), or H2B (▲) were mixed with histone H2B-Sephadex (containing 50 μg of coupled H2B) in 250 μl of buffer A, and 200 μl of partially purified ^3H -labeled receptor was added. The mixture was incubated for 2 hr at 4°C with gentle shaking. The samples were then centrifuged, and the histone-bound radioactivity in the pellet was determined. Results are expressed as percent of receptor bound to H2B-Sephadex in the absence of soluble histones.

Sepharese-immobilized histone for the receptor and (ii) histone-histone interactions between the two forms of histone that might effectively block the site on histone-Sepharese to which receptor binds (13, 14). For example, if the receptor were to bind to a domain of histone H2B situated at the H2B-H4 interface, then soluble histone H4 could reduce the binding of estrogen receptor to histone H2B-Sepharese by blocking this site. These considerations led us to test the capacity of the receptor to bind to histone H4 directly. Indeed, estrogen receptor did bind to histone H4-Sepharese and this interaction was disrupted by salt (Fig. 3). As lower salt concentrations are required to release the receptor from H4-Sepharese than from H2B-Sepharese, the receptor-H4 interaction is apparently weaker than the receptor-H2B interaction.

Separation of DNA, Histone, and Steroid Binding Sites of the Estrogen Receptor. Limited proteolysis (7) and photooxidation (35) experiments have shown that the DNA-binding domain of the estrogen receptor is physically separate from the estrogen-binding site. We have used mild tryptic digestion to see whether the histone-binding domain and the DNA-binding domain of the receptor display similar sensitivities to proteolysis. The receptor was incubated with trypsin and, at various times, aliquots were removed to measure DNA, histone, and estrogen binding (by DNA-cellulose, histone-Sepharese, and dextran-coated charcoal assays, respectively). In general agreement with previous observations (7), the DNA-binding activity was reduced by 75% in the first 10 min (Fig. 4). In marked contrast, the histone-binding and estrogen-binding activities were almost fully retained. These data indicate that the histone-binding and the DNA-binding sites are on different parts of the receptor molecule.

To examine the effect of histone binding on the charge properties of the receptor molecule, samples of receptor were ana-

FIG. 3. Salt-dependent release of ^3H -labeled estrogen receptor from Sepharose-immobilized histones H2B (○) and H4 (▲). Labeled receptor was allowed to bind to histone H2B- or H4-Sepharese as described in the legend to Fig. 1. The Sepharose beads were then centrifuged, washed with buffer A and suspended in 0.75 ml of buffer A containing various concentrations of KCl. After shaking for 30 min at 4°C , the samples were washed, centrifuged, and assayed for radioactivity.

FIG. 4. Effect of trypsin on the capacity of estrogen receptor to bind DNA, histone H2B, and estradiol. Ammonium sulfate-fractionated receptor (1.5–2 mg of protein/ml) was incubated with trypsin (5 $\mu\text{g}/\text{mg}$ of protein) at 10°C . At the indicated times, aliquots were removed, soybean trypsin inhibitor (2 $\mu\text{g}/\mu\text{g}$ of trypsin) was added, and the binding of the receptor to DNA (●) was assayed by incubating ^3H -labeled receptor with DNA-cellulose ($\approx 10 \mu\text{g}$ of DNA in 200 μl of buffer A) for 30 min at 22°C with shaking; the DNA-bound labeled receptor was assayed as described. The binding of labeled receptor to histone H2B-Sepharese (▲) was measured as in Fig. 1. The binding of [^3H]estradiol (□) to the receptor was measured by the dextran-coated charcoal technique (36).

lyzed by isoelectric focusing in the presence and absence of histones H2A or H2B (Fig. 5). In the absence of histone, the receptor was resolved into two broad bands with isoelectric focusing points at pH 5.7 and 6.2, as expected. In the presence of histones H2A or H2B, however, a large proportion of the receptor focused at a more basic pH.

Histone Stabilizes Estrogen Receptor-DNA Interactions. To determine whether the presence of histones on DNA modulates the binding of estrogen receptor to DNA, we compared the

FIG. 5. Isoelectric focusing of ^3H -labeled estrogen receptor in the presence and absence of histone H2B. Labeled receptor (10–20 μl of a 15 mg/ml solution prepared directly from cytosol) was electrofocused in polyacrylamide 2% (wt/vol) Ampholines, pH 3.5–9.5 (LKB-Produkter AB, Bromma, Sweden) at 700–1000 V. The approach to equilibrium was monitored by observing the focusing of hemoglobin, myoglobin, and ferritin markers in adjacent gel lanes. After the run (1–1.5 hr), the pH (x) along the 9-cm gel was determined with a surface electrode, and longitudinal strips of gel (corresponding to applied samples) were cut into 1-cm fractions, which were subdivided into two or three segments for assay by liquid scintillation counting. The arrow marks the point at which the sample was applied in the presence (▲) or absence (○) of 10 μl of purified calf thymus histone H2B (50 $\mu\text{g}/\text{ml}$).

rates at which the receptor dissociates from naked DNA-cellulose and from DNA-cellulose to which histones had been added (without reconstitution). The dissociation is first order for at least 30 min (Fig. 6). Furthermore, the receptor dissociates more slowly from H2B-DNA-cellulose than from naked DNA-cellulose. Histone H2B is more effective in stabilizing the estrogen receptor-DNA interaction than equivalent amounts of histone H4 (see Fig. 6) or histone H3 (data not shown).

DISCUSSION

Whereas previous reports on the binding of estrogen receptor to histones (or protamines) implied that these interactions were nonspecific (19–23), our data demonstrate that estrogen receptor discriminates among mammalian histones. It has a high affinity for histones H2B and H2A, a moderate affinity for histones H4 and H3, but only a weak to negligible affinity for histone H1. Thus, estrogen receptor has binding sites not only for estrogen and DNA but also for histones. The extent to which it binds to histones in the intact nucleus and the functional significance of this binding remain to be established.

In addition, our trypsin digestion experiments indicate that the histone-binding portion of the receptor can be distinguished from that portion of the receptor required for DNA-binding. There is no direct evidence for the existence *in vivo* of a receptor form similar to that generated *in vitro* by mild proteolysis (38). Nonetheless, such a trypsin-treated receptor, which can bind to histones but not to DNA, may prove useful in assessing how receptor-DNA and receptor-histone interactions contribute to the binding of receptor to chromatin.

In agreement with the work of Thanki *et al.* (39), our kinetic data suggest that histones, especially H2B, stabilize the binding

of estrogen receptor to DNA. In light of our other data, it seems likely that this stabilization is caused, at least in part, by a direct protein-protein interaction between the estrogen receptor and the histone. It is also possible that the conformation of histone-bound DNA may be more favorable than that of naked DNA for receptor binding. In similar fashion, when histone binds to the receptor, it may promote an analogous allosteric transition so that the resulting conformation of the receptor may have an enhanced affinity for DNA.

The capacity of estrogen receptor to interact with histones may be related to one or more of the intracellular functions of the receptor. For example, histones might act as "escort proteins" that modulate the translocation of receptor into the nucleus. The DNA-binding and the histone-binding sites, situated on different surfaces of the receptor, might provide the receptor with the necessary leverage to convert selected nucleosomes from an inactive nontranscribable configuration to an active transcribable one (40, 41). If the segments of histone recognized by the receptor are exposed and accessible on only some nucleosomes, these histone segments might provide additional specificity determinants that facilitate the putative search of the receptor for estrogen-sensitive genes. Finally, receptor-histone interactions might be partly responsible for that portion of the nuclear receptor that is resistant to salt extraction (42).

Our studies and those of Thanki *et al.* (39) on the estrogen receptor, together with recent work on the thyroid hormone receptor (43) and the glucocorticoid receptor (44, 45), suggest that histones influence the activity of several hormone receptors. Thus, some eukaryotic regulatory proteins that alter gene expression may do so, in part, by interacting with histones, the structural proteins around which genes are packaged.

FIG. 6. Rate of dissociation of ^3H -labeled estrogen receptor from histone-DNA-cellulose. DNA-cellulose was preincubated in the absence (○) or presence of histone H2B (□) or H4 (▲) ($2\text{ }\mu\text{g}$ of histone/ μg of DNA) for 30 min at 4°C and washed with buffer. Labeled receptor was allowed to bind to the resulting histone-DNA-cellulose (or to control DNA-cellulose), and the rate of dissociation of the labeled receptor was measured as described (6). At time zero, soluble calf thymus DNA (20-fold excess) was added to initiate the dissociation. Aliquots were taken at the indicated times to determine radioactivity remaining in the DNA-cellulose pellet. Data are presented as the fraction of radioactivity associated with DNA-cellulose before addition of the soluble DNA (cpm/cpm₀).

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