Intermolecular Engagement of Estrogen Receptors Indicated by the Formation of a High Molecular Weight Complex during Activation†

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ABSTRACT: After exposure to ligand at 0–4 °C, estrogen receptors from mouse uteri characteristically eluted between thyroglobulin (M_r 669 000) and ferritin (M_r 443 000) during size-exclusion HPLC. However, when preparations were warmed with ligand under mild activating conditions, most or all of the receptor was observed as a much larger complex, which eluted between dextran blue 2000 and thyroglobulin. Formation of the large complex required ligand, was inhibited by molybdate, and occurred even in 0.4 M KCl. Slower ligand dissociation characterized the large complex, indicating that activated receptors were included preferentially. This large complex did not form when charged cytosols were aged, concentrated, or precipitated, indicting that formation was not the result of random aggregation. After exposure to conditions commonly used for activation (25 °C, 60 min), most receptor existed as a very large, monodisperse complex of finite size, predicting an ordered structure for these large complexes that should be useful for defining the types of proteins which can interact with estrogen receptors. Formation of the large complex was not impeded or disrupted by EDTA, RNase, DNase I, thiourea, or mercaptoethanol; however, the capacity to form this large complex was not demonstrated by preparations that had been exposed to trypsin or by the small receptor forms obtained after salt extraction. Proteolytic sensitivity and lack of sensitivity to RNase or DNase indicate that interactions between receptors and other proteins are involved in peak A formation. Since the formation of this large complex was observed in preparations obtained from CF-1 mouse uteri, pituitaries, and hypothalami, nude mouse uteri, and MCF-7 human breast cancer cells, this capacity appears well distributed across biological sources. Multiple characterizations described here indicate that the formation of this large complex is distinct from the broader consideration termed receptor aggregation. Since the formation of this large complex conforms with receptor activation, this complex is a direct indication that through activation estrogen receptors acquire the capacity to engage in intermolecular associations with other proteins. Since this type of structural engagement could provide a means through which estrogen receptors unite regulatory elements for the initiation of the complex series of events associated with hormone action, it may be helpful for identifying these types of individual regulatory components.

Normal tissues, as well as tumors, can respond to estrogens after specific hormone binding proteins interact with chromatin and initiate events that control the rates of cellular transcription, translation, and replication (Jensen & DeSombre, 1972, 1973; O'Malley & Means, 1974; Yamamoto & Alberts, 1976; Katzenellenbogen, 1980). Coordinated with the initiation of these events is the involvement of receptors in stable interactions within chromatin, often referred to as receptor "fixation" in chromatin (Sheridan et al., 1979; Martin & Sheridan, 1982; Molinari et al., 1985). This process of fixation can be considered to be composed of at least two processes involving (1) attachments that are highly stable and (2) associations between receptors and other components in a physical alliance that mediate hormone action. In cell-free systems, receptor "activation" may be related to in situ receptor fixation within chromatin because activated receptors demonstrate a high affinity for nuclei (Jensen et al., 1968; Shyamala & Gorski, 1969), chromatin (McGuire et al., 1972; Chamness et al., 1974), DNA–cellulose (Yamamoto, 1974), and ATP–Sepharose (Miller & Toft, 1978). Additional characteristics of receptor activation include slower rates of ligand dissociation (Weichman & Notides, 1977; deBoer & Notides, 1981), transitions involving positive cooperativity (Notides et al., 1981; Sassan & Notides, 1983), and conversion of the salt-transformed 4S–5S cytoplasmic receptor to a 5S–7S form (Notides & Nielsen, 1974, 1975; Notides et al., 1975). Receptor activation can be inhibited by sodium molybdate (Nishigori & Toft, 1980; Shyamala & Leonard, 1980; Mauck et al., 1982; Muller et al., 1982). Several structural forms of steroid receptors have been identified including the mero-receptor (2S–3S, M_r[1] 20 000–25 000), receptors with fragmented structure (3S–4S, M_r 40 000–70 000), and the intact receptor monomer (4S–5S, M_r 90 000–110 000) (Sherman & Stevens, 1984). When warmed in the presence of 0.4 M KCl, the receptor monomer converts to the activated 5S–7S component

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[1] Abbreviations: BSA, bovine serum albumin; DES, diethylstilbestrol (α,α'-diethyl-4,4'-stilbenediol); DME, Dulbecco's modified Eagle's medium; 17β-estradiol, estra-1,3,5(10)-triene-3,17β-diol; EDTA, ethylenediaminetetraacetic acid; M_r, molecular weight; PALEP, PMSF, aprotinin, leupeptin, EDTA, and pepstatin; P_{100} buffer, KH_2PO_4/K_2HPO_4 (100 mM, pH 7.0); PEG, poly(ethylene glycol); PMSF, phenylmethanesulfonyl fluoride; SEHPLC, size-exclusion high-performance liquid chromatography; tamoxifen aziridine, (*Z*)-1-[4-[2-(*N*-aziridinyl)ethoxy]phenyl]-1,2-diphenylbut-1-ene; V_e, elution volume.

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which may represent dimerizations, as well as associations with nonreceptor proteins (Notides, 1978; Sherman & Stevens, 1984). Receptors extracted from the nucleus with 0.4 M KCl also sediment predominately as a 5S–7S component (Giannopoulos & Gorski, 1971; Traish et al., 1978, 1979; Clark & Peck, 1979) indicating that nuclear receptors can either dimerize or associate with other proteins. Large cytosol receptor complexes (9S–10S; M_r 320000–350000) have been observed in hypotonic buffers containing 10–20 mM molybdate or leupeptin, a protease inhibitor (Sherman & Stevens, 1984). These large complexes have been reported within intact cells (Raak et al., 1985), and although it has been suggested that they represent receptor tetramers (Sherman & Stevens, 1984), it is also likely that they contain a heat-shock protein (M_r 90000) that does not bind hormone (Dougherty & Toft, 1982; Puri et al., 1982; Housley et al., 1983; Mendel et al., 1986).

Since steroid receptors interact in vitro with specific DNA target sequences irrespective of the presence of ligand (including antagonist) or of conditions permissive for activation (Joab et al., 1984; Willman & Beato, 1986; Bailly et al., 1986; Green & Chambon, 1986), it has been suggested that interactions between receptors and other proteins are required for action in vivo. We set out to determine if, after activation, interactions between estrogen receptors and other proteins could be identified chromatographically. Since under conditions which promote receptor activation estrogen receptors may have a tendency to aggregate (Yamamoto & Alberts, 1974, 1978; Nishizawa et al., 1981; Skipper et al., 1985a,b), our efforts included comparisons between interactions involving receptors and the characteristics of aggregation. Even though a number of investigators have suggested the possibility that receptor aggregation may reflect events that are biologically significant (Yamamoto & Alberts, 1974, 1976; Sherman & Stevens, 1974; Moncharmont et al., 1982), this phenomenon has not been studied directly or extensively. Under conditions generally used to study receptor activation, receptor aggregation has been thought to have only a minor occurrence, so that most receptors exist after activation as the 9S–10S form in low salt or as the 5S–7S form in 0.4 M KCl. Using chromatographic supports capable of resolving very large molecular species, we found to our surprise that, after exposure to mild activating conditions, most estrogen receptors became involved in the formation of a monodisperse complex of large finite size. Since this formation concurred with conditions that activate receptors, formed to a major extent even when 0.4 KCl was present, and differed substantially from the characteristics of receptor aggregation, these efforts identify a novel process which previously has not been recognized. Importantly, these observations indicate that receptors can acquire the ability to engage in interactions with a limited number of proteins through activation. Because proteins which interact with receptors have the potential to modulate hormone action, we have attempted to better define this process of engagement.

Experimental Procedures

Materials. 17β-[2,4,6,7-$^3H_4$]Estradiol (95–115 Ci/mmol), [*ring*-^{3}H]tamoxifen aziridine (25.5 Ci/mmol), and 16α-[^{125}I]iodoestradiol (200 Ci/mmol) were purchased from Amersham Corp. Alcohol dehydrogenase, β-amylase, BSA, carbonic anhydrase, dextran blue 2000, DES, DNase I (deoxyribonucleate 5′-oligonucleotide hydrolase, EC 3.1.21.1), 17β-estradiol, ferritin, γ-globulin, ovalbumin, PEG 3350, RNases A (ribonucleate 3′-pyrimidino-oligonucleotide-hydrolase, EC 3.1.27.5), T1 (ribonucleate 3′-guanylo-oligonucleotidohydrolase, EC 3.1.27.3), T2 (ribonucleate 3′-oligonucleotidohydrolase, EC 3.1.27.1), and S (protease-modified RNase A), thyroglobulin, trypsin (type XI), and trypsin inhibitor (type I-S) were obtained from Sigma Chemical Co. Aprotinin, Na_2EDTA, leupeptin, pepstatin, and PMSF were obtained from Boehringer Mannheim Biochemicals. Other chemicals were sodium molybdate (reagent grade, Mallinkrodt), Tris (ultrapure grade, Schwarz/Mann, Inc.), and dimethylformamide (Burdick & Jackson Chemicals). Centricon-10 and -30 ultramembrane microconcentrators were obtained from Amicon. Female mice were used between 20–23 days of age ([NSA] CF-1/HSD strain, Harlan Sprague–Dawley, Indianapolis, IN). MCF-7 human breast cancer cells were provided by Diane Bronzert and Marc Lippman at NIH.

Preparative Procedures. Mice were sacrificed by cervical dislocation and decapitation. Tissues were collected and transferred to Hank's balanced salt solution (Ca^{2+} and Mg^{2+} free) on ice. Homogenization was in P_{100} buffer (five uteri/mL) unless stated otherwise. The inclusion of PMSF (0.2 mM), aprotinin (0.5 μg/mL), leupeptin (0.5 μg/mL), EDTA (1 mM), and pepstatin (0.07 μg/mL) as multiple protease inhibitors is designated by +PALEP. Preparation of cytosols and nuclei and quantitation of estrogen receptor specific binding were as described previously (Pavlik et al., 1982a,b, 1985a,b, 1986). When [^{3}H]estradiol (10 nM) or [^{125}I]iodoestradiol (5 nM) was used, radioinert DES (2 μM) was employed as the competitor to assess nonspecific binding, while radioinert 17β-estradiol (2 μM) was employed when [^{3}H]tamoxifen aziridine was used (10 nM). For receptor concentration experiments, cytosol was charged with [^{3}H]-estradiol ± DES and subjected to ultramembrane concentration with Amicon Centron-10 (10-kDa cutoff) or Centron-30 (30-kDa cutoff). Starting volumes (2 mL) were reduced to 100 μL and held at this concentration for 2 h at 0–4 °C. Concentrates were then diluted to their original volume with P_{100} buffer before SEHPLC analysis. For receptor precipitation experiments, charged and uncharged cytosols were exposed to PEG 3350 at a final concentration of 30% (w/v) at 0–4 °C overnight. After centrifugation (3000*g*, 30 min), the precipitated receptor-containing pellet was allowed to stand for 2 h at 0–4 °C. The pelleted material was allowed to dissolve in fresh P_{100} buffer at 0–4 °C for 1–5 h. Precipitated, uncharged cytosol was redissolved and then charged. On preparations obtained by SEHPLC, determinations of the rate of ligand dissociation were performed at 0–4 °C (for 60 h) or at 20 °C (for 3 h) after resaturation of receptor preparations with 10 nM [^{3}H]estradiol (±2 μM DES) and use of 4 μM DES to prevent rebinding of ligand as described previously (Pavlik et al., 1985a, 1986). When ligand dissociation and chromatographic behavior were simultaneously analyzed, preparations were charged under conditions favoring formation of peak A or maintenance of peak B and followed at 0–4 °C for 360 min. The presence of two slopes in the dissociation curves always correlated with two peaks of specific binding in SEHPLC analyses. Each dissociation time point was determined as specific binding [i.e., the difference between mean total binding (n = 4) and mean nonspecific binding (n = 4)]. Free ligand was removed by exposure to dextran-coated charcoal (Pavlik et al., 1985), and excess DES (4 μM) was added to prevent ligand rebinding. At each time point, cytosols in P_{100} buffer were exposed to dextran-coated charcoal (15 min, 0–4 °C), while preparations in P_{100} containing KCl were subjected to adsorption to hydroxylapatite (Pavlik et al,. 1982a,b). Linear regression analysis was performed on the log values for specific binding for fast- and slow-dissociating components. Significance was based on the t distribution with

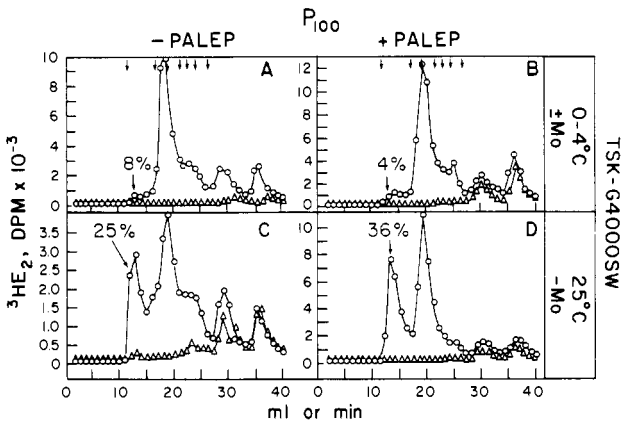

FIGURE 1: Formation of a large molecular complex when cytosol is warmed. Cytosol was prepared in the absence [(A–C) –PALEP] and presence [(B–D) +PALEP] of multiple protease inhibitors. Preparations were charged for 2 h at either 0–4 °C (A and B) or 25 °C (C and D). Total (circles) and nonspecific (triangles) binding profiles are shown. Arrows mark the elution of (left to right) blue dextran 2000, thyroglobulin, ferritin, γ-globulin, BSA, ovalbumin, and molybdate. Percentages indicated estimate the amount of specific binding activity associated with the peak A region.

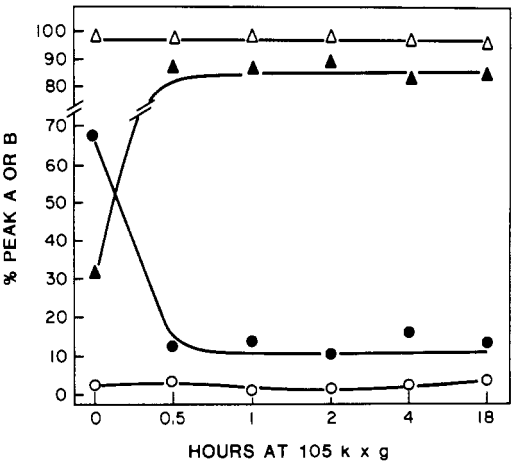

FIGURE 2: Sedimentation of peak A receptor complexes during high-speed centrifugation. High-speed cytosols (105000g, 45 min, 0–4 °C) were charged for 2 h with [^{3}H]estradiol ± DES at 0–4 °C in the presence of 50 mM Na_2MoO_4 (open symbols) to define peak B receptor complexes or at 37 °C (without Na_2MoO_4, solid symbols) to define peak A receptor complexes. Preparations were then centrifuged for the times indicated and analyzed by SEHPLC in order to determine the chromatographic size of receptor that resisted sedimentation. Activity associated with peak A receptor complexes is represented by circles, while triangles represent peak B receptor complexes.

the t value = $t_{m+n-2} = (\bar{x}_B - \bar{x}_A)/S(\bar{x}_B - \bar{x}_A)$, where m = number of determinations for peak A, n = number of determinations for peak B, $S(\bar{x}_B - \bar{x}_A)^2 = S^2(1/m + 1/n)$, and S^2 = pooled estimate of variance = $[(m - 1)S^2(A) + (n - 1)S^2(B)]/(m + n - 2)$.

Size-Exclusion High-Performance Liquid Chromatography. All procedures have been previously described in detail (Pavlik et al., 1982a,b; Nelson et al., 1984). Buffers were filtered, and low protein binding filters (Millex GV, Millipore Corp.) were used to prepare cytosols for analysis (Pavlik et al., 1985b). Isocratic elution was performed with flow-metered pumps (Beckman Models 110 and 112; ISCO Model 2300), using Spherogel TSK-G4000SW (7.5 × 600 mm) exclusion columns that were fitted with a guard column (Spherogel-TSK precolumn, 7.5 × 100 mm). A 250-μL sample loop was used for injection. Elution was with P_{100} buffer containing 7.5% dimethylformamide. The HPLC systems were maintained at 0–5 °C in a refrigerated chromatography cabinet. Elutions of specific receptor sites are referenced to the elutions of standard proteins. These referenced elutions are designated by their hydrodynamic property (hdp) and are expressed relative to the marker proteins eluting before and after any specific binding peak; hdp = leading marker/trailing marker (i.e., hdp = ferritin/γ-globulin) (Pavlik et al., 1985a).

Results

Formation of Large Estrogen Receptor Containing Complexes Chromatographically Identified as Peak A. The murine estrogen receptor complex prepared in hypotonic buffers and charged under nonactivating conditions (0–4 °C, 2 h) chromatographed between thyroglobulin (M_r 669 000) and ferritin (M_r 443 000) in the presence and absence of multiple protease inhibitors (Figure 1A,B), as previously reported (Pavlik et al., 1985a; Atrache et al., 1985). In contrast, when cytosol was charged at 25 °C for 2 h, receptor eluted as a much larger complex, between dextran blue 2000 (M_r 2 000 000) and thyroglobulin (M_r 669 000), (Figure 1C,D). The larger, early-eluting receptor complex has been designated as peak A (hdp = dextran blue 2000/thyroglobulin; V_e = 11–15 mL) and the later-eluting receptor complex as peak B (hdp = thyroglobulin/ferritin; V_e = 16–23 mL). Only minor receptor activity eluted in the peak A region in preparations charged at 0–4 °C, even in the absence of molybdate (Figure 1). Since the formation of peak A receptor complexes has been observed on Superose 6B columns as well, these identifications are not due to some column-related anomaly, but are true indications of complex size. Moreover, these large receptor complexes occurred only after warming and could be eliminated by high-speed centrifugation (Figure 2, solid circles). Consequently, rapid sedimentation indicates that these receptor complexes are quite large and that they are not the exclusive result of alterations in molecular symmetry that appear chromatographically as changes in Stokes radii.

In order to determine the extent of peak A formation, warming-time analyses revealed that most receptor was included in peak A (Figure 3A,B). When protease inhibitors were present, the rate of peak A formation occurred more extensively at 37 °C (Figure 3B) than at 25 °C (Figure 3A), while protease inhibitors also provided a dramatic stabilization of estrogen receptors at this higher temperature (Figure 3D).

In order to investigate the influence of ligand, preparations were warmed (5–60 min, 25 °C) before and after exposure to ligand. In the absence of ligand, very little peak A formation occurred (Figure 4, solid profile); however, considerable peak A formation occurred after warming in the presence of radioligand (Figure 4, dot-dashed profile). Exposing receptor to ligand under activating conditions resulted in ~25% more peak A formation than when the same preparations were precharged (0–4 °C, 3 h) before being activated (data not shown). Thus, peak A formation depends upon the presence of ligand and can be influenced by ligand binding that occurs prior to activation.

Because receptor activation can be inhibited by molybdate, the influence of molybdate on peak A formation was examined. Molybdate inhibited peak A formation (Figure 5). This inhibition occurred similarly when cytosol was diluted over a 100-fold range (Figure 5 inset, top) with dilution per se having no apparent influence on peak A formation (Figure 5 inset, bottom).

Formation of Peak A Complexes in Hypertonic KCl. Since the phenomenon known as aggregation has been reported to be inhibited by moderate salt concentration (Yamamoto & Alberts, 1974; Sherman & Stevens, 1984), it is important to

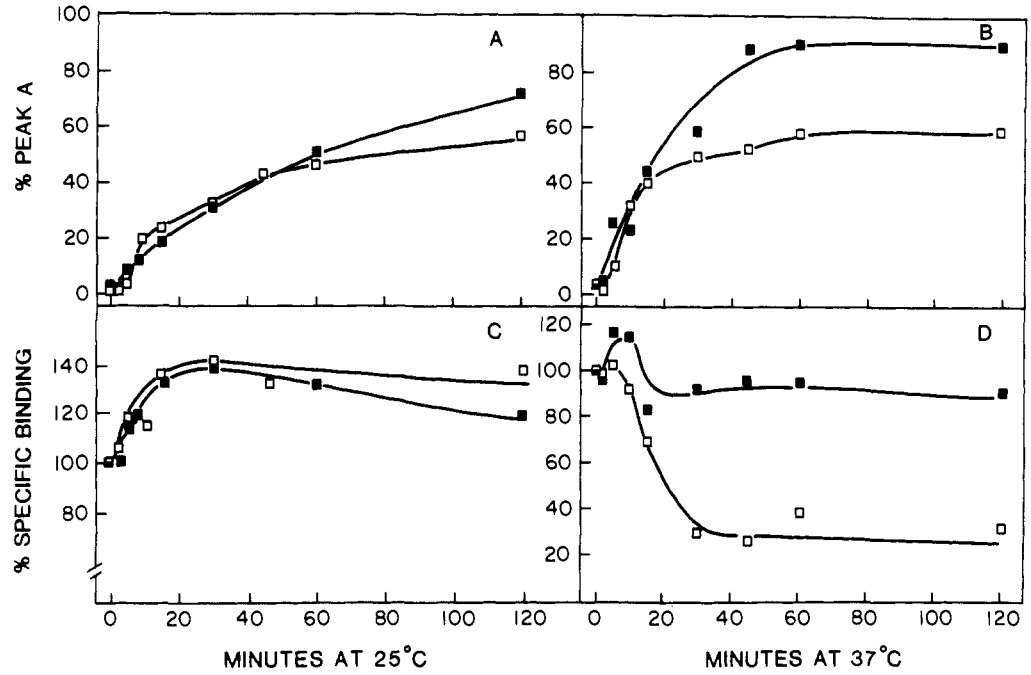

FIGURE 3: Formation and stability of peak A complexes. Cytosol, prepared with (solid symbols) or without (open symbols) multiple protease inhibitors, was exposed to ligand ([^{3}H]E_2 ± DES) and warmed at 25 °C (A and C) or 37 °C (B and D) for the times indicated. Receptor stability is expressed as percent specific binding (C and D) and is normalized relative to initial activity present in preparations charged for 2 h at 0–4 °C before warming. Percent peak A activity was based on specific binding.

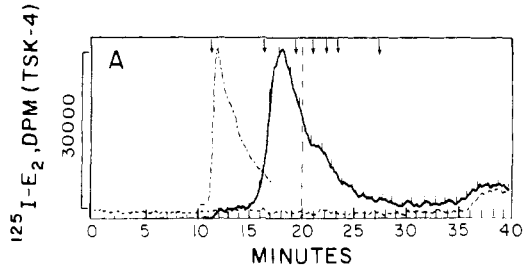

FIGURE 4: Dependence upon ligand for peak A formation. Cytosols were held at 25 °C for up to 60 min, cooled, and then charged with [^{125}I]iodoestradiol ± DES for 3 h at 0–4 °C (solid line). Radioactivity was monitored with an in-line γ-radioisotope detector (Beckman Model 170). Cytosol charged with [^{125}I]iodoestradiol for 1 h at 25 °C is shown by the dot-dash total binding profile. Nonspecific binding activity is indicated by the dashed line. Vertical arrows indicate the elution of (left to right) dextran blue 2000, thyroglobulin, ferritin, γ-globulin, β-amylase, alcohol dehydrogenase, and molybdate.

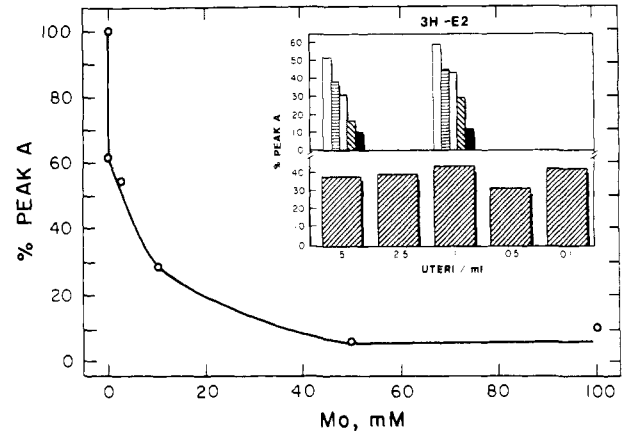

FIGURE 5: Sensitivity of peak A formation to molybdate. Cytosol was exposed to [^{3}H]estradiol ± DES (2 h, 25 °C) in the presence of Na_2MoO_4 at the concentrations indicated. Inset (bottom): cytosol was diluted as indicated (five uteri/mL ~ 2 mg of protein/mL) and charged (2 h, 25 °C), in the absence of Na_2MoO_4 at the concentrations shown. Profiles of Na_2MoO_4 inhibition (top inset) were determined at 5 (left profile) and 0.1 (right profile) uteri/mL with 0 (open), 0.5 (horizontal lines), 1 (shaded), 5 (diagonal lines), or 10 (solid) mM Na_2MoO_4. Specific binding activity was used to determine percent peak A activity.

examine the effect of 0.4 M KCl on peak A formation. First, peak A receptor complexes, formed under hypotonic conditions, were not disrupted either when salt was added or when 0.4 M KCl was present in the SEHPLC elution buffer (Figure 6 inset). Second, peak A complexes formed in the presence of 0.4 M KCl as the major chromatographic form and did not disperse even when salt was present in the elution buffer (Figure 6). Third, the presence of salt facilitated more peak A formation at lower temperatures (<30 °C), while slightly less peak A formation occurred in the presence of salt at temperatures of ≥30 °C, (Figure 7). Less complete formation of peak A at higher temperatures was probably the result of thermal instability since, in contrast to hypotonic preparations, protease inhibitors failed to stabilize specific binding when preparations containing 0.4 M KCl were warmed to temperatures of ≥30 °C (data not shown).

Formation of Peak A Complexes under a Variety of Conditions. Receptor preparations were subjected to a wide variety of conditions under which the capacity for peak A formation was examined, as summarized in Table I. Peak A receptor complexes formed equally well in P_{100} buffer and Tris (10 mM)–EDTA (1 mM) buffer (pH 7.4), with and without multiple protease inhibitors. Peak A formation was not impeded when either RNase, DNase, thiourea, or mercaptoethanol was present in the reaction, nor were preformed peak A complexes (25 °C, 120 min, in the presence of ligand) disrupted by introduction of these agents. Ultrafiltration to remove proteins smaller than 30 kDa also did not interfere with the capacity for peak A formation. When prepared in the presence of 0.4 M KCl, mouse uterine estrogen receptors chromatographed as smaller, later-eluting complexes (hdp = ovalbumin/molybdate), which upon warming were converted to peak A complexes. Formation of peak A complexes was not observed in the presence of molybdate (10 mM), after receptors were exposed to trypsin or after receptors were extracted from chromatin. Since the capacity for peak A for-

Table I: Peak A Formation under Different Conditions[a]

receptor preparation	hdp, TSK-G4000SW (without warming)	% peak A (after warming) <10%	11–20%	21–49%	>50%
CF-1 mouse uterine cytosol					
P_{100} or Tris–EDTA	thyroglobulin/ferritin				+
+PALEP	thyroglobulin/ferritin				+
+molybdate	thyroglobulin/ferritin	+			
+RNase[b]	thyroglobulin/ferritin				+
+DNase I[c]	thyroglobulin/ferritin				+
+thiourea[d]	thyroglobulin/ferritin				+
+mercaptoethanol[e]	thyroglobulin/ferritin				+
+ultrafiltration, >10 kDa[f]	thyroglobulin/ferritin				+
+ultrafiltration, >30 kDa	thyroglobulin/ferritin				+
+0.4 M KCl	ovalbumin/molybdate				+
+trypsinization[g]	ovalbumin/molybdate		+		
+fresh cytosol[h]	ovalbumin/molybdate		+		
CF-1 mouse nuclear extract					
0.4 M KCl extract[i]	ovalbumin/molybdate	+			
+DES cytosol[j]	ovalbumin/molybdate		+		
CF-1 mouse pituitary cytosol					
P_{100}[k]	thyroglobulin/ferritin				+
CF-1 mouse hypothalamus cytosol					
P_{100}	thyroglobulin/ferritin				+
nude mouse uterine cytosol					
P_{100}	thyroglobulin/ferritin				+
MCF-7 human tumor cell cytosol					
P_{100}[l]	thyroglobulin/ferritin				+

[a] Preparations (five uteri/mL) were chromatographed before and after warming (25 °C, 120 min) in the presence of ligand, and peak A formation was quantitated. [b] RNases examined: A, T1, T2, and S (100–500 units/mL). [c] DNase I was present at 500 units/mL. [d] Thiourea at 100–500 μg/mL. [e] Mercaptoethanol at 1%. [f] Ultrafiltration was performed on Centron-10 and -30. [g] After warming in the presence of ligand, preparations were exposed to trypsin (145 μg/mL, 0–4 °C, 1 h), followed by the addition of soybean trypsin inhibitor (2 μg/μg of trypsin). [h] Preparations charged at 0–4 °C were exposed to trypsin, as above, and were combined (1:1) with nontrypsinized cytosol (10 uteri/mL) that had been charged with 100 nM DES prior to warming. [i] Washed nuclei were extracted with P_{100} containing 0.4 M KCl (pH 7.4) at 0–4 °C (1 h). [j] Extracts were combined with an equal volume of cytosol that had been charged with DES at 0–4 °C. [k] Preparations (90 pituitaries or hypothalami/mL of P_{100} buffer) were treated identically as uterine cytosols. [l] Cells were transferred to steroid-free HL-1 medium (Ventex Labs) for 24–48 h before harvesting. Cells from 70 flasks (150 cm^2) produced a packed volume of 5 mL and were homogenized in 3.5 mL of P_{100} + PALEP.

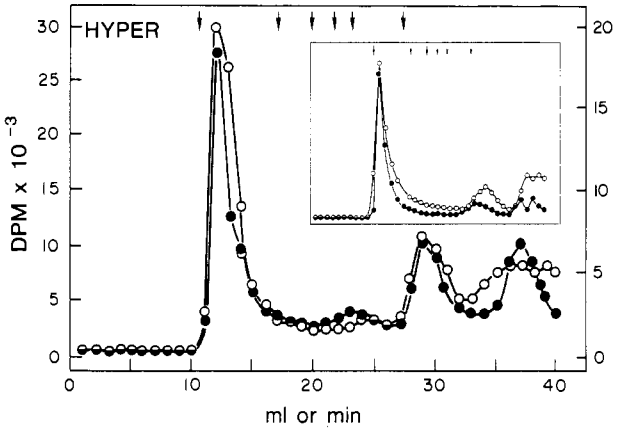

FIGURE 6: Formation and stability of peak A estrogen receptor complexes during SEHPLC in low- and high-salt buffers. Cytosols were prepared in P_{100} buffer (inset) or P_{100} buffer containing 0.4 M KCl and were charged with [^{3}H]estradiol ± DES (120 min 25 °C) in order to generate peak A. Cytosols were eluted either with P_{100} + 7.5% DMF (open circles) or with P_{100} + 0.4 M KCl + 7.5% DMF (solid circles). Vertical arrows mark the elution of (left to right) blue dextran 2000, thyroglobulin, ferritin, γ-globulin, BSA, and molybdate.

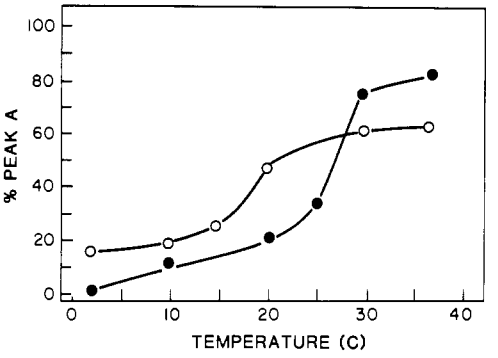

FIGURE 7: Formation of peak A in hypotonic and hypertonic environments. Cytosols were prepared in P_{100} buffer + PALEP (solid symbols) or P_{100} buffer containing 0.4 M KCl + PALEP (open symbols) and were exposed to [^{3}H]E_2 ± DES for 2 h at the temperatures indicated.

mation was not restored when fresh cytosol was added to trypsin-exposed receptors or to nuclear receptors, loss of the capacity to form peak A complexes appears to involve receptors per se rather than other factors in the preparation. Finally, since peak A formation occurred in cytosols prepared from CF-1 mouse pituitary and hypothalamus, nude mouse uteri, and human breast tumor cells (Table I), the capacity to form peak A complexes is present in preparations obtained from widespread sources. Taken together, these observations demonstrate that peak A formation does occur in a variety of preparations from varied sources, but appears to be lost after exposure to trypsin or extraction of nuclear receptors.

Peak A Complexes Do Not Form When Cytosols Are Aged, Are Concentrated, or Are Precipitated. Because aggregation may occur in older animals (Rochefort & Baulieu, 1972), when preparations are aged, (Sica et al., 1976), or when cytosol is concentrated or precipitated, peak A formation was examined with these factors in mind. No appreciable difference was observed in the capacity for peak A formation between adult ovariectomized mice and immature mice (data not shown). Moreover, when uterine cytosol was aged for up to 10 days at 0–4 °C in the presence of 10 nM [^{3}H]estradiol ± 2 μM DES, no spontaneous formation of peak A complexes was observed either in the presence or in the absence of molybdate (Figure 8). It is interesting that in these preparations a shift to smaller sized receptor complexes occurred after ~4 days, probably due to endogenous proteolysis or to receptor subunit

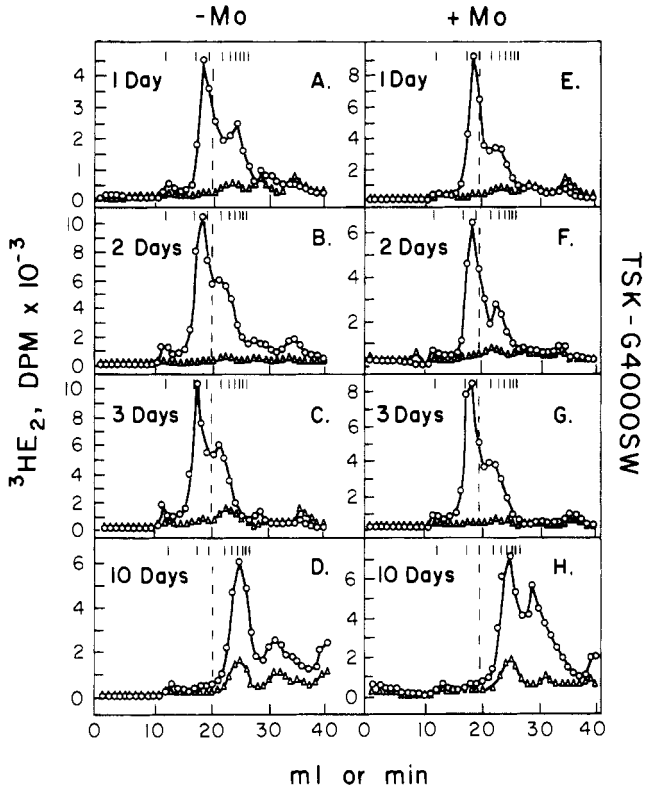

FIGURE 8: Lack of peak A formation during prolonged exposure to radioligand at 0–4 °C. Cytosols were prepared in the absence (A–D) or presence (E–H) of 25 mM Na_2MoO_4 and charged for up to 10 days at 0–4 °C in the presence of [^{3}H]estradiol ± DES. SEHPLC analyses were performed at 8 h and 1, 2, 3, 4, 7, 8, and 10 days with selected results shown. Vertical markings in the top panels identify the elution of (left to right) dextran blue 2000, thyroglobulin, ferritin, γ-globulin, BSA, ovalbumin, trypsin, and trypsin inhibitor. The broken vertical line references fraction 20. Total (circles) and nonspecific (triangles) binding profiles are shown.

dissociation. Concentration of charged cytosol by 20-fold also did not result in peak A formation in preparations held at 0–4 °C (Figure 9A,B). Similarly, no peak A formation occurred due to precipitation with PEG, with either charged (Figure 9C,D) or uncharged preparations (Figure 9E), even though receptor that was redissolved after precipitation was capable of peak A formation during warming in the presence of ligand (Figure 9F). Note that large early-eluting molecules can be observed if tamoxifen aziridine is employed (Figure 9D). These molecules are antiestrogen binding sites, which have been described previously (Kon, 1983) and should not be mistaken for peak A estrogen receptors. These observations indicate that peak A formation is not merely a consequence of random collisions involving estrogen receptors and components of the cytosol, since concentration and precipitation, which increase the opportunity for random interactions between the various components of the cytosol, do not result in peak A formation.

Peak A Receptor Complexes and Ligand Dissociation Kinetics. Since the conditions which activate receptors promote slower ligand dissociation, the kinetics of ligand dissociation were examined in order to determine if peak A complexes, formed under activating conditions, contained receptors characterized by slower ligand dissociation. Thus, if lower dissociation rates were found to characterize peak A complexes, it would indicate that activated receptors become engaged in peak A formation. Conversely, any equivalence in rates of dissociation would support the notion that peak A formation might trap or aggregate both activated and nonactivated receptors with equal facility. After chromatography on TSK-G4000SW columns, pooled peak A preparations demonstrated slower ligand dissociation at 20 °C [(55 ± 21) × 10^{-6} s^{-1}] than pooled preparations from peak B [(220 ± 19) × 10^{-6} s^{-1}]. However, since considerable time elapsed while multiple SEHPLC injections, separation, pooling, and recharging of peak A and B preparations were performed, it was possible that endogenous proteases in the peak B preparation might be responsible for the difference in observed ligand dissociation kinetics. Consequently, simultaneous analyses of chromatographic behavior and ligand dissociation were performed in order to eliminate any effects on receptors that might result during extended preparation. Preparations were exposed to conditions that promoted peak A formation to extents varying from total to partial, when peak B complexes were also present. Kinetic studies of ligand dissociation showed that ligand dissociation was always slower for peak A complexes.

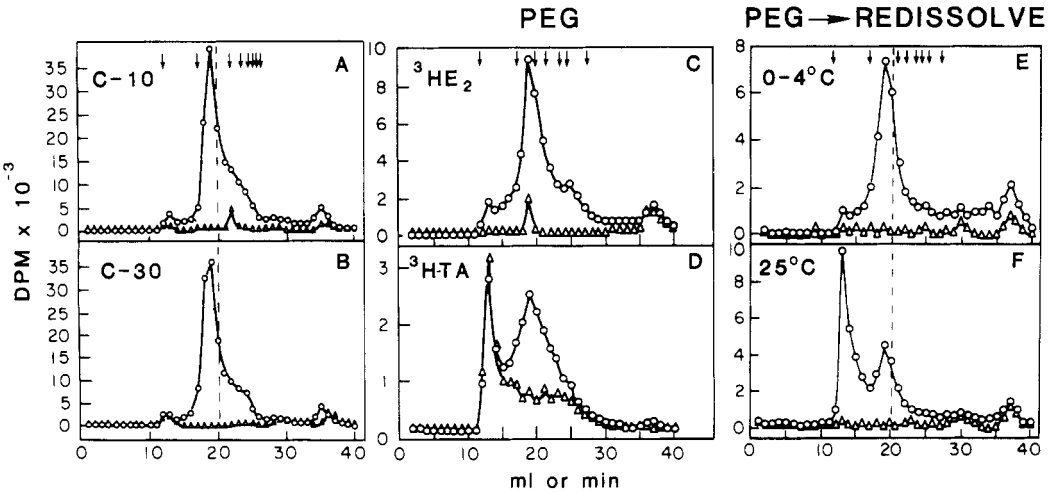

FIGURE 9: Lack of peak A formation during concentration and precipitation. Cytosol was charged with [^{3}H]estradiol ± DES and subjected to ultramembrane concentration [(A) Amicon Centron-10 or (B) Amicon Centron-30]. Preparations, charged with [^{3}H]estradiol ± DES (C) or [^{3}H]tamoxifen aziridine ± E_2 (D) for 2 h at 0–4 °C, were precipitated with PEG 3350. The precipitated material was dissolved in fresh P_{100} buffer at 0–4 °C for 1–5 h before injection. Uncharged cytosol was precipitated and allowed to stand for 2 h at 0–4 °C. After being redissolved, cytosol was charged for 2 h at 0–4 °C (E) or 25 °C (F) with [^{3}H]estradiol ± DES. Total (circles) and nonspecific binding (triangles) profiles are shown with the dashed vertical line identifying the twentieth fraction. Calibration standards were as indicated in Figure 4 (panels A, B, E, and F) and as follows for panels C and D (left to right): dextran blue 2000, thyroglobulin, ferritin, γ-globulin, BSA, ovalbumin, and molybdate.

Table II: Peak A Formation and Receptor Aggregation[a]

aggregation	peak A formation
occurs at 0–4 °C at low ionic strength[b–d]	not observed
occurs more in ovariectomized adults[e]	equivalence observed
occurs as cytosols age[e]	not observed
occurs when receptors are concentrated[b]	not observed
occurs when receptors are precipitated[b]	not observed
prevented by 0.4 M KCl[b–d]	occurs in 0.4 M KCl
disrupted or prevented by RNase[b,d]	RNase has no effect
prevented by thiol-reducing mercaptoethanol	mercaptoethanol has no effect
inhibited by molybdate at 0–4 °C[b]	not observed at 0–4 °C
inhibited by molybdate at 25 °C[b]	*observed or confirmed*
polydisperse[f]	monodisperse
elutes in G-200 V_o[f]	elutes after V_o
sediments during high-speed centrifugation[c]	*observed or confirmed*

[a] Characteristics of aggregation collected from the literature as indicated. Italics identify characteristics shared between peak A receptor complexes and receptor aggregates. Polydisperse is here taken to mean multiple or broad profiles of molecules larger than native receptor, while monodisperse refers to a single narrow profile. [b] Jensen et al., 1971. [c] Yamamoto & Alberts, 1974. [d] Sherman & Stevens, 1984. [e] Rochefort & Baulieu, 1972; Sica et al., 1976. [f] Nishizawa et al., 1981.

When two slopes were present in the dissociation analysis, both peak A and peak B complexes were always present in the SEHPLC determinations. Under these circumstances, the slower rate of dissociation was always presumed to represent peak A complexes; however, because this assumption could bias the overall interpretation, analyses were also made where only preparations showing monophasic dissociation were included. Using this approach and segregating monophasic dissociation data in brackets, the mean dissociation rate (±SEM) from peak A was $(3.99 \pm 0.499) \times 10^{-6}$ s^{-1}, $n = 14$, 6.86_{max}, 0.90_{min} [$(4.32 \pm 0.43) \times 10^{-6}$ s^{-1}, $n = 7$, 6.01_{max}, 3.2_{min}], while the dissociation rate from peak B was $(53.5 \pm 14.1) \times 10^{-6}$ s^{-1}, $n = 15$, 221_{max}, 4.85_{min} [$(56.1 \pm 10.9) \times 10^{-6}$ s^{-1} $n = 8$, 110_{max}, 4.8_{min}]. Thus, the differences in the rates of dissociation between peak A and peak B complexes were significantly different ($p > 0.005$) when all values were used, as well as when only monophasic values were included, so that ligand dissociation from peak A complexes was 13 times slower overall. These observations show that slower rates of ligand dissociation are associated with peak A receptor complexes and, consequently, indicate that activated receptors preferentially engage in the formation of peak A complexes.

Discussion

The observations reported here lead to two significant conclusions. First, under conditions which promote activation, a major fraction of estrogen receptors becomes involved in the formation of large complexes that are here designated as peak A complexes. Since peak A formation is ligand-dependent, inhibited by molybdate, and includes receptors demonstrating slower ligand dissociation, activated estrogen receptors are involved in these large complexes. Thus, through activation, estrogen receptors acquire the capacity to engage in quite stable intermolecular interactions. Importantly, this capacity includes the acquisition of superior binding by peak A receptor complexes to ATP–Sepharose and DNA–cellulose (unpublished results). Second, peak A formation is distinct from the broader consideration termed *receptor aggregation*. As shown in Table II, only two characteristics appear shared between peak A complexes and receptor aggregates. It is significant that peak A formation occurred even in the presence of 0.4 M KCl, which prevents aggregates from forming. Peak A formation involves interactions between receptors and other proteins since neither RNase nor DNase prevented or disrupted peak A formation and since proteolysis prevented peak A formation. A consideration of steroid receptor aggregates is relevant to the observations reported here. Some investigators have referred to the 9S–10S estrogen receptor (i.e., peak B complexes; hdp = thyroglobulin/ferritin) as the aggregated receptor state and to 4S–5S receptors as deaggregated (Muller et al., 1983), so that receptor forms larger than the 4S–5S receptor monomer become an indication of aggregation. In general, interactions involving estrogen receptors have been reported to be sensitive to temperature (Sherman & Stevens, 1984; Monocharmont et al., 1982), salt (Sherman & Stevens, 1984; Notides, 1978), chaotrophic agents (Auricchio et al., 1978b; Sica et al., 1976), EDTA (Sica, 1976), heparin (Shyamala, 1971; Chamness & McGuire, 1972; Auricchio et al., 1978a), and pyridoxal 5-phosphate (Seeley et al., 1984), to be insensitive to modifications of arginyl residues (Muller et al., 1983), and to be increased when preparations have been aged (Sica et al., 1976). Because peak A complexes eluted as molecules that were larger than even 9S–10S receptor complexes, it is likely that peak A formation has been overlooked in numerous investigations, probably because of tube-bottom sedimentation and exclusion in column void volumes. While peak A complexes can easily be sedimented by centrifugation, they also will chromatograph in the internal volume if columns with a high enough fractionation range are used. Thus, on TSK-G4000SW or Superose 6B columns, peak A complexes eluted after dextran blue 2000 in a monodisperse fashion, which indicates finite or defined size. On columns with a shorter fractionation range (Sephadex G-200, for example), peak A complexes will coelute with estrogen receptors near the void volume and will not be noticed as distinct from peak B (9S–10S) receptors. Our observations are supported by recent reports that warming rat uterine estrogen receptor preparations produced large receptor forms, referred to as aggregates because they were excluded from Sephacryl S-300 and failed to enter polyacrylamide gels (Skipper et al., 1985a,b). However, chromatographic behavior shows that peak A formation is limited to a large, fixed molecular size and indicates that a defined stoichiometry exists within peak A complexes. This anticipation of ordered structure is noteworthy, providing a means for defining proteins which can interact with receptors to modulate hormone action.

Peak A formation was not observed when opportunities for interaction between cytosol components were increased by concentration or precipitation, so that the formation of this large complex does not appear to result from generalized aggregation. It is important to realize that PEG was used for precipitation because this agent, in contrast to salt precipitation, does little to change the distribution of bound water but is effective primarily by influencing overall molecular charge (Hansen & Gorski, 1985). As a consequence, in the studies performed here, changes in molecular conformation induced by the distribution of bound water have been minimized.

It is noteworthy that exposure to trypsin dramatically reduced the capacity for peak A formation because trypsin-treated estrogen receptors have been incapable of demonstrating the properties of activated receptors (Andre & Rochefort, 1973; Weichman & Notides, 1977). Taken together, these comparisons support the concept that peak A formation is an event coordinated with the conditions that promote in vitro receptor activation. Notably, this coordination

is maintained even under conditions of elevated salt concentration. Peak A formation cannot be explained on the basis of interactions between protease inhibitors and estrogen receptors (Puca et al., 1986) because this large complex forms readily even when protease inhibitors are absent. The important implication of these observations is that, as part of receptor activation, receptors acquire the ability to interact with other cellular proteins, demonstrated here by the majority of receptors that engage in the formation of a large molecular entity. Consequently, it is possible that during activation estrogen receptors can acquire certain properties from protein components which are united in the peak A receptor complex.

In a more global perspective, these observations are related to our proposal that ligand can mediate transitions in receptor structure (Pavlik et al., 1985a). A straightforward interpretation of these data is that favorable thermal conditions perturb the structure of ligand-occupied receptors so that the ensuing molecular orientations become translated to the receptor's exterior and thereby convey the capacity for stabilized attachments between receptors and other proteins. This perturbation does not occur with unoccupied receptors. We speculate that the ultimate relevancy of the capability to engage in stabilized interactions would occur within the nucleus as a prelude to the initiation of hormone-mediated responses. Such a concept would provide an explanation of how receptors become fixed within chromatin through attachment to other proteins. This suggestion is supported by our identificaton of large nuclear receptor complexes in vivo which chromatograph indistinguishably from the peak A complexes that form in cytosol under cell-free conditions (Baranowska-Kortylewicz et al., 1987). Thus, evidence of receptor engagement within chromatin in the form of peak A complexes has been obtained. It should be noted that these large nuclear receptor complexes have been obtained in 0.4 M KCl extracts only when protease inhibitors were employed. After KCl extraction in the absence of protease inhibitors, we suggest that a salt-induced proteolysis of chromatin–receptor interaction sites yields receptor complexes which are smaller than peak A. Thus, these smaller receptor complexes may not demonstrate the function domains that are included in receptor structure or those acquired through interactions with other proteins. This speculation is further supported by the dearth of reports demonstrating that functional properties are associated with extracted nuclear receptors. In this regard, the large peak A complexes that can be extracted from the nucleus or formed under cell-free conditions can more completely present a unit with which regulatory functions can be associated. In light of the growing body of evidence that estrogen receptors have a predominately nuclear localization (Welshons et al., 1984, 1985; Gravanis & Gurpide, 1986; King & Greene, 1984; McClellan et al., 1984; Gasc et al., 1984), the formation of peak A complexes demonstrates that receptors, which become redistributed from the nuclear fraction to the cytosol during preparation, still can demonstrate the potential for engaging in stabilized interactions with other components. Because smaller extracted nuclear estrogen receptors were obtained in the present effort without the use of protease inhibitors, we believe that they represent proteolyzed receptor forms which, like receptors exposed to trypsin, have lost the ability to form peak A complexes. Since cytosol, which had been precharged (0–4 °C, 120 min) before warming for peak A formation, demonstrated less ability to form peak A than receptors warmed in the presence of ligand, the capacity for peak A formation may decline after ligand is bound. In addition, since the very large peak A nuclear receptor complexes were not released by the conditions used here for salt extraction, it appears that considerable nuclear receptor activity remains fixed within chromatin and demonstrates the stability of interactions between nuclear receptors and other components in vivo.

Ultimately the significance of peak A complexes must be addressed. If the stabilized interactions involved in peak A formation can be moderated biologically and also can be governed by specificity or selectivity, it would be appropriate to propose that significance can be established through regulatory elements which become engaged by receptors in a unit capable of initiating the complex series of events associated with hormone action. Additional experimentation will be needed to explore these possibilities.

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