Androgen Receptor Binding to Nuclear Matrix in Vitro and Its Inhibition by 8S Androgen Receptor Promoting Factor†

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ABSTRACT: The partially purified 4.5S [^{3}H]dihydrotestosterone receptor binds to nuclear matrix isolated from rat Dunning prostate tumor with properties similar to those reported for androgen receptor binding in intact nuclei [Colvard, D. S., & Wilson, E. M. (1984) *Biochemistry* (preceding paper in this issue)] in that it requires Zn^{2+} and mercaptoethanol, is saturable, and is temperature dependent and of high affinity ($K_a \sim 10^{13}$ M^{-1}). On a milligrams of DNA equivalent basis, the extent of matrix binding of androgen receptor (700 fmol of receptor bound/mg of matrix protein) is similar to that of intact nuclei, corresponding to ~1400 sites/nucleus. Association rate constants (k_a) for 4.5S androgen receptor binding to matrix at 0, 15, and 25 °C are 2.7×10^5, 1.2×10^6, and 2.4×10^6 M^{-1} min^{-1}, respectively, indicating an energy of activation of 15 kcal/mol. Up to 50% of matrix-bound receptor is extractable in buffer containing 3 mM ethylenediaminetetraacetic acid plus either 0.4 M KCl or 5 mM pyridoxal 5′-phosphate. A protein fraction designated 8S androgen receptor promoting factor that promotes conversion of the 4.5S androgen receptor to 8 S [Colvard, D. S., & Wilson, E. M. (1981) *Endocrinology* (*Baltimore*) *109*, 496–504] has been further purified and found to inhibit the binding of the 4.5S androgen receptor to isolated nuclei and nuclear matrix in a concentration-dependent manner. The results support the hypothesis that the 8S steroid receptor is a complex of the activated 4.5S androgen receptor with a non-steroid binding protein that renders the receptor incapable of binding in nuclei.

It is becoming increasingly apparent that the nuclear matrix is a key site in the regulation of gene activity. All rapidly labeled RNA is associated with nuclear matrix (Miller et al., 1978; Herman et al., 1978), including heteronuclear (Faiferman & Pogo, 1975; van Eekelen & van Venrooij, 1981), preribosomal, and ribosomal RNA (Herlan et al., 1979). Genes that are transcriptionally active appear to be associated with nuclear matrix (Robinson et al., 1982; Nelkin et al., 1980; Pardoll & Vogelstein, 1980). For example, the ovalbumin gene has been found associated with matrix in chick oviduct cells, but not in chick liver cells, while the β-globin gene, a transcriptionally inactive gene in the oviduct, was not enriched in the matrix (Robinson et al., 1982).

Since steroid hormones and their receptors are thought to activate specific genes, it is conceivable that matrix association of receptors could facilitate this process. Steroid binding studies using exchange-labeling techniques have revealed that isolated nuclear matrix contains steroid receptors (Barrack & Coffey, 1980). These binding sites are generally found to be resistant to extraction with high ionic strength buffers, suggesting to some that they may be important in gene activation (Barrack & Coffey, 1982). In this paper, we present evidence that the in vitro binding of the 4.5S [^{3}H]dihydrotestosterone receptor to nuclear matrix is temperature dependent, saturable, and of high affinity.

An additional objective of this work was to determine whether a protein fraction, designated 8S androgen receptor promoting factor, would alter the ability of the 4.5S androgen receptor to bind to nuclear matrix. We have shown previously (Colvard & Wilson, 1981) that the 8S androgen receptor promoting factor promotes the conversion of the partially purified 4.5S androgen receptor to 8 S on low-salt sucrose gradients. Using conditions found to optimize androgen receptor binding to nuclei (Colvard & Wilson, 1984) and matrix in vitro, we have found that the 8S-promoting factor inhibits receptor association with intact nuclei and nuclear matrix, supporting the hypothesis that 8S steroid receptors are incapable of binding to nuclei.

Materials and Methods

Materials

[1,2,4,5,6,7-^{3}H]Dihydrotestosterone (120 Ci/mmol) was purchased from New England Nuclear; RNase A was from Worthington Biochemicals; diisopropyl fluorophosphate was from Calbiochem; pyridoxal 5′-phosphate was from Aldrich Chemical Co.; 2-mercaptoethanol was from Eastman; Trizma base [tris(hydoxymethyl)aminomethane (Tris)[1] buffer] and phenylmethanesulfonyl fluoride were from Sigma; DEAE-Sepharose, CM-Sepharose, and ATP–Sepharose were from Pharmacia Fine Chemicals; hydroxylapatite was from Bio-Rad; phosphocellulose P11 was from Whatman; reagent-grade chemicals and Scintiverse were from Fisher Scientific Co.; chelating agarose was from Bethesda Research Labs; Triton X-100 was from Research Products International.

Methods

Preparation of the 4.5S [^{3}H]Dihydrotestosterone-Labeled Receptor. Dunning rat prostate tumors (R3327H), propagated by subcutaneous implantation in Copenhagen-Fischer rats, were obtained from the Papanicolaou Cancer Research Institute, Inc., Miami, FL, and were the source of androgen receptor and nuclear matrix. Tumor-bearing rats were castrated through an abdominal incision under ether anesthesia and decapitated 18 h later. Blood was collected by exsanguination and allowed to clot for 1 h at room temperature

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[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; TE, 1 mM EDTA–50 mM Tris, pH 7.5; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; CM-Sepharose, carboxymethyl-Sepharose; 8S-PF, 8S androgen receptor promoting factor; SDS, sodium dodecyl sulfate.

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followed by overnight at 4 °C. Serum obtained by centrifugation was stored at −20 °C. Serum was the starting material for isolation of the 8S androgen receptor promoting factor.

All of the following procedures were carried out at 4 °C unless indicated otherwise. The 4.5S [^{3}H]dihydrotestosterone receptor was partially purified on phosphocellulose from the cytosol fraction of the Dunning prostate tumor as previously described (Colvard & Wilson, 1981). Tumor cytosol was prepared in 10% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, 2 mM diisopropyl fluorophosphate, and 50 mM Tris, pH 7.5, by centrifuging the homogenate at 100000g for 75 min. Cytosol was labeled with 15 nM [^{3}H]dihydrotestosterone in the presence of 10 mM 2-mercaptoethanol for 18 h at 0 °C and fractionated by phosphocellulose chromatography. Following washing with 0.14 M KCl in 5 mM 2-mercaptoethanol–50 mM Tris, pH 7.5, the [^{3}H]dihydrotestosterone-labeled androgen receptor was eluted in 0.3 M KCl, 5 mM 2-mercaptoethanol, 15% glycerol, and 50 mM Tris, pH 7.5. The pooled peak of radioactivity was analyzed by sucrose gradient centrifugation and found to be 4.5 S in buffer containing 0.025 or 0.15 KCl due to its separation from the 8S androgen receptor promoting factor, as previously described (Colvard & Wilson, 1981). Glycerol was added to the receptor fractions to a final concentration of 30%. The enriched receptor fraction was stored in small aliquots at −70 °C prior to its use. Phosphocellulose chromatography results in a 40–50-fold purification of receptor (780–970 fmol/mg of protein).

Isolation of Nuclei and Nuclear Matrix. The Dunning rat prostate tumor (R3327H), liver, and ventral prostate were taken from Copenhagen-Fischer rats that were castrated 18 h previously. Nuclei were isolated by sedimentation through 1.9 M sucrose as previously described (Spelsberg et al., 1971; Colvard & Wilson, 1984). Protein/DNA weight ratios of nuclei, which ranged from 2.5 to 3.0, were determined by measuring protein (Lowry et al., 1951) and DNA (Burton, 1956) concentrations. Nuclear matrix was isolated from 2 to 4 mL of nuclei (5 mg of DNA/mL) as previously described (Berezney & Coffey, 1977; Ross et al., 1982). Nuclei were sedimented at 2000g_{max} for 10 min and suspended at 4 °C in 10 mL of buffer A [0.1 mM phenylmethanesulfonyl fluoride (PMSF), 0.2 mM $MgCl_2$, and 10 mM Tris, pH 7.5] plus 0.35 M NaCl. All suspensions were done in a Dounce homogenizer followed by sedimentation at 2000g for 10 min. A 10 mM PMSF stock solution in absolute ethanol was prepared fresh. The 0.35 M NaCl wash step was repeated and the pellet dispersed in buffer A (15–20 mL) to a concentration of ~1 mg of DNA/mL. DNase I was activated by diluting 0.2 mL of 2 mg/mL pancreatic DNase I (stored at −20 °C) into 1.8 mL of buffer B (0.1 mM PMSF, 5 mM $MgCl_2$, and 10 mM Tris, pH 7.5) plus 92 μg/mL bovine serum albumin (37 μL of a 5 mg/mL solution) followed by incubation for 1–2 h at 0 °C. Activated DNase I was added (0.1 mL/mL of nuclei) to a final concentration of 20 μg of DNase I/mL. The mixture was incubated for 30–60 min at 34 °C with occasional shaking, cooled to 0 °C, and sedimented. The nuclear residue was resuspended in buffer A containing 2 M NaCl and sedimented at 2000g_{max}, and then this procedure was repeated. The pellet was resuspended in buffer B plus 1% Triton X-100, sedimented, and washed twice in buffer B. Total protein (Lowry et al., 1951), DNA (Burton, 1956), and RNA (Ceriotti, 1955) were determined prior to the DNase I step and in the final matrix preparation. Approximately 15% of total nuclear protein was recovered in the matrix fraction of the prostate tumor with the loss of 93% of the RNA and >99% of the DNA.

Nuclear Matrix in Vitro Binding Assay. The nuclear matrix–androgen receptor binding assay was carried out essentially as described previously for nuclei–androgen receptor binding (Colvard & Wilson, 1984) except the binding buffer did not contain sucrose.

Partial Purification of 8S Androgen Receptor Promoting Factor. Rat serum was used as starting material for 8S-promoting factor purification. Blood was collected from adult male, tumor-bearing Copenhagen-Fischer rats that were decapitated 18 h after castration. Serum was obtained following blood clot formation for 1 h at room temperature followed by 18 h at 4 °C. The 20–40% saturated $(NH_4)_2SO_4$ pellet of 70–100 mL of serum was resuspended in 30–40 mL of 50 mM Tris, pH 7.5, and 1 mM EDTA (TE) and dialyzed overnight at 4 °C against TE. The sample was applied to a DEAE-Sepharose column (2.6 × 14 cm) equilibrated in TE, and after the column was washed with TE, the sample eluted with 0.09 M KCl–TE followed by 0.2 M KCl–TE. Aliquots of all column fractions were dialyzed overnight at 4 °C against 5 mM KCl–5 mM Tris, pH 7.5, and lyophilized. The residue was resuspended in a small volume of distilled H_2O and assayed for 8S-promoting factor activity as described below.

Following chromatography on DEAE-Sepharose, purification of 8S-promoting factor proceeded as indicated in the figure legends, either by chromatography on an anion-exchange column, such as carboxymethyl-Sepharose (CM-Sepharose) or phosphocellulose, or by chromatography directly onto hydroxylapatite. Prior to anion-exchange chromatography, the 0.2 M KCl fraction from DEAE-Sepharose containing 8S-promoting factor activity was dialyzed against a large volume of TE and applied to a column of CM-Sepharose (2.6 × 14 cm) or phosphocellulose (2.5 × 4 cm) equilibrated in TE. The CM-Sepharose column was sequentially eluted in 0.06 M KCl followed by 0.18 M KCl. Phosphocellulose was eluted with 0.08 and 0.2 M KCl. The 8S-promoting factor activity was detectable in the second eluate from either column.

Prior to chromatography on hydroxylapatite, samples containing 8S-promoting factor activity were dialyzed against 4 L of 30 mM KH_2PO_4, pH 7.0, in TE overnight at 4 °C. The hydroxylapatite column (2.6 × 4 cm) was equilibrated and washed with 30 mM KH_2PO_4, pH 7.0, and then sequentially eluted with 0.08 and 0.25 M KH_2PO_4, pH 7.0. The latter fraction containing 8S-factor activity was dialyzed against 4 L of TE and applied to an ATP–Sepharose column (1.6 × 8 cm) equilibrated in TE. ATP–Sepharose was prepared as previously described (Moudgil & Toft, 1975). Following sample application and washing of the column, the ATP–Sepharose column was sequentially eluted with 0.05, 0.1, and 0.2 M KCl. The 8S-promoting factor activity eluted at 0.1 M KCl.

Assay for 8S Androgen Receptor Promoting Factor. The presence of 8S-promoting activity was determined in sucrose gradients containing 0.025 M KCl as previously described (Colvard & Wilson, 1981).

Results

The Dunning prostate tumor, a well-differentiated androgen-dependent adenocarcinoma, was used as the source of the [^{3}H]dihydrotestosterone-labeled 4.5S androgen receptor and for isolation of nuclei and nuclear matrix. This tumor androgen receptor has been extensively characterized and found to be more stable than that of the normal rat prostate, due to a low level of endogenous proteases in tumor cytosol (Wilson & French, 1979). In electron micrographs, nuclei of the prostate tumor display a prominant nucleolus, which remains evident in the nuclear matrix fraction (Figure 1). The nuclear

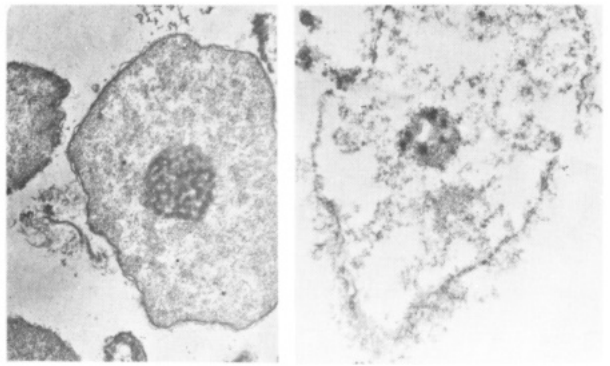

FIGURE 1: Electron micrographs of nuclei and nuclear matrix isolated from the Dunning rat prostate tumor. Nuclei and nuclear matrix were isolated as described under Methods, fixed with 2.5% glutaraldehyde–0.1 M phosphate buffer, pH 7.2, and postfixed in 2% osmium tetraoxide in 0.1 M phosphate buffer, pH 7.2, as previously described (Kierszenbaum & Tres, 1974). Thin sections were stained with uranyl acetate followed by lead citrate.

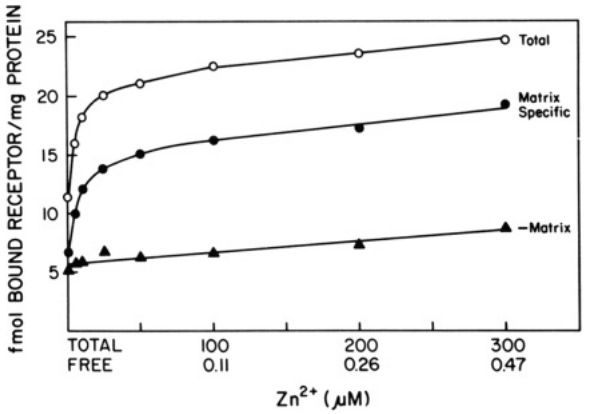

FIGURE 2: Androgen receptor binding to nuclear matrix at increasing Zn^{2+} concentrations. The 4.5S [^{3}H]dihydrotestosterone-labeled androgen receptor partially purified on phosphocellulose was incubated in the presence (○) or absence (▲) of nuclear matrix for 1 h at 25 °C in 0.3 mL containing 13 fmol of [^{3}H]dihydrotestosterone-labeled receptor, 250 μg of matrix protein, 0.15 M KCl, 3 mM $MgCl_2$, 5 mM mercaptoethanol, and 50 mM Tris, pH 7.5, plus increasing concentrations of $ZnCl_2$. Receptor binding was measured by ethanol extraction of pellets as described under Methods. The difference in radioactivity extracted in the presence (○) and absence (▲) of matrix represents "matrix-specific" binding of receptor (●) at increasing concentrations of Zn^{2+}. Total added and estimated free Zn^{2+} concentrations are indicated. Free Zn^{2+} concentration was calculated as previously described (Colvard & Wilson, 1984).

matrix, isolated by using standard techniques (Berezney & Coffey, 1977; Ross et al., 1982), accounts for ~15% of the total nuclear protein, with removal of >99% of the DNA. Although DNA was not detectable in the matrix preparation using the Burton spectrophotometric assay (Burton, 1956), we cannot rule out the presence of a select population of DNA in association with the matrix. Nuclear matrix was not routinely treated with RNase A, although partial hydrolysis of the RNA by treatment with RNase A (37 °C for 40 min) did not appear to alter receptor binding to nuclear matrix.

Requirements for Matrix Binding of Receptor. We have found previously (Colvard & Wilson, 1984) that mercaptoethanol (5 mM) and salt (0.15 M KCl) are required in an in vitro binding reaction carried out in the presence of low levels of Zn^{2+}. Under these conditions, the addition of $ZnCl_2$ results in a concentration-dependent increase in binding of the 4.5S [^{3}H]dihydrotestosterone receptor to nuclear matrix (Figure 2). Sedimentation of receptor in the absence of matrix is only

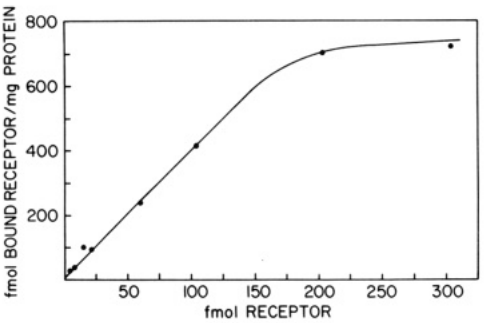

FIGURE 3: Saturation of nuclear matrix binding sites with the androgen receptor. Increasing amounts of the partially purified 4.5S [^{3}H]-dihydrotestosterone receptor were incubated with nuclear matrix (48 μg of total matrix protein) in 0.3 mL of 0.15 M KCl, 300 μM $ZnCl_2$, 5 mM mercaptoethanol, and 50 mM Tris, pH 7.5, for 30 min at 25 °C. Following sedimentation at 2600*g* for 10 min, pellets were washed in the above buffer, sedimented, and extracted in 0.3 mL of ethanol. Parallel incubations were carried out in the absence of matrix to assess nonspecific sedimentation of the receptor. Specific binding was calculated as the difference in binding in the presence and absence of matrix, as shown in Figure 2.

slightly Zn^{2+} concentration dependent and represents ~20% of total matrix binding. Matrix-specific binding is represented by the difference in the amount of receptor sedimented in the presence and absence of matrix. Analysis of the receptor fraction prior to and following incubation with nuclear matrix using either the Lowry protein assay (Lowry, 1951) or SDS–polyacrylamide gel electrophoresis (Laemmli, 1970; Wilson & French, 1980) revealed no change in the total protein concentration or pattern on gels. These results suggest that the receptor is preferentially binding to the matrix under our assay conditions. Half-maximal binding occurs at 20 μM total Zn^{2+}. We have shown previously that mercaptoethanol displays cooperative binding to Zn^{2+} in a ratio of three mercaptoethanol molecules per Zn^{2+} atom (Colvard & Wilson, 1984). The zinc–mercaptoethanol binding constants predict that 20 μM total Zn^{2+} corresponds to ~20 nM free Zn^{2+}, a value which would be considered within a physiological range.

Various divalent cations in addition to Zn^{2+} were tested for their effectiveness in potentiating receptor binding to matrix, including Ni^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, and Mn^{2+}. The cation specificity for potentiation of receptor binding to matrix was nearly identical with that found for receptor binding to nuclei (Colvard & Wilson, 1984) in that Zn^{2+} was most effective followed by Ni^{2+}. Other cations were essentially without effect (data not shown).

The matrix binding of receptor was saturable at 700 fmol of receptor/mg of matrix protein (Figure 3). The addition of ovalbumin to maintain constant protein levels did not influence receptor binding or saturation. An apparent binding constant of ~10^{13} M^{-1} (K_a) was estimated. If it is assumed that there are 12 pg of DNA/rat prostate cell nucleus (Bruchovsky et al., 1975) and that matrix accounts for 10–15% of nuclear protein, then there are approximately 1400 receptor binding sites/nucleus. The similarity in the number of sites for nuclei (Colvard & Wilson, 1984) and nuclear matrix suggests that matrix binding of receptor accounts for the majority of nuclear binding sites under our in vitro assay conditions.

The temperature dependence of matrix binding shown in Figure 4 indicates that while binding occurs at 0 °C, it is quite slow, requiring about 19 h to reach the level observed at 25 °C after 2 h. Binding is enhanced at 15 and 25 °C, although the receptor steroid binding site becomes inactivated at elevated temperature for prolonged periods (Figure 4A). Association rate constants calculated as described in the legend of Figure

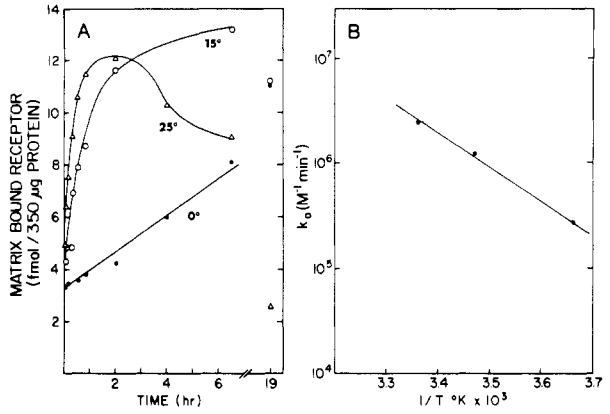

FIGURE 4: Temperature dependence (A) and an Arrhenius plot (B) of androgen receptor binding to nuclear matrix. (A) The 4.5S [^{3}H]dihydrotestosterone receptor (21.3 fmol) was incubated with nuclear matrix (350 μg of protein) at 0 (●), 15 (○), and 25 °C (△) for increasing times up to 19 h and assayed for binding as described under Methods. Maximal binding achieved at 15 °C for 6 h represented 61% of the total added receptor. (B) Arrhenius plot of temperature-dependent androgen receptor binding to nuclear matrix. Association rate constants (k_a's) were calculated by using the equation $[tk_a(b-a)]/2.303 = \log(a/b) + \log[(b-x)/(a-x)]$ where a is the maximal number of matrix receptor binding sites (817 fmol/mL), b is the total amount of [^{3}H]dihydrotestosterone receptor added to the binding reaction (71 fmol/mL), and x is the amount of matrix-bound receptor (in femtomoles per milliliter) at a given time t (in minutes). The activation energy (15 kcal/mol) was calculated from the slope of the Arrhenius plot: $\Delta H = (\log k_1/\log k_2)[2.303R/(T_1 - T_2)]$ where $R = 1.987$ cal mol^{-1} deg^{-1}.

4 are 2.7 × 10^5 M^{-1} min^{-1} at 0 °C, 1.2 × 10^6 M^{-1} min^{-1} at 15 °C, and 2.4 × 10^6 M^{-1} min^{-1} at 25 °C. The energy of activation determined from an Arrhenius plot (Figure 4B) is 15 kcal/mol.

Effects of High Salt Concentrations or Pyridoxal 5′-Phosphate on Receptor–Matrix Binding. The addition of KCl up to 0.4 M to the binding reaction reduced receptor binding to matrix by only 15%, which 5 or 10 mM pyridoxal 5′-phosphate reduced binding by 50% (not shown). Following the binding reaction, either of the above reagents in the presence of 3 mM EDTA was effective in releasing approximately half of the bound receptor. These results resemble those required for extraction of receptor from intact nuclei (Colvard & Wilson, 1984) and suggest that the high-affinity binding of receptor to nuclear matrix involves hydrophobic interactions, which are in part disrupted by EDTA, an effective chelating agent of Zn^{2+}. Pyridoxal 5′-phosphate appears to disrupt steroid receptor–nuclear interactions, probably through the formation of a Schiff base with lysine group(s) as proposed for the progesterone receptor (Nishigori et al., 1978).

Tissue Specificity. The data of Figure 3 demonstrate that nuclear matrix isolated from the prostate tumor contains a limited number of high-affinity binding sites for the 4.5S androgen receptor (approximately 1400 sites/nucleus). Figure 5 shows the effect of incubating increasing amounts of the 4.5S [^{3}H]dihydrotestosterone receptor with a constant amount of nuclear matrix (120 μg of protein) isolated from the Dunning prostate tumor, ventral prostate, and liver taken from Copenhagen-Fischer rats 18 h after castration. In all three tissues, saturation of the binding sites was evident. When expressed relative to milligrams of DNA equivalents, the Dunning tumor contained more sites for the receptor, followed by the prostate, with liver showing the lowest number of sites. As summarized in Table I, ventral prostate nuclear matrix contained 410 sites/mg of DNA equiv, 29% of the sites of prostate tumor nuclear matrix. Liver nuclear matrix contained only 14% of the binding sites of the prostate tumor. The lower number

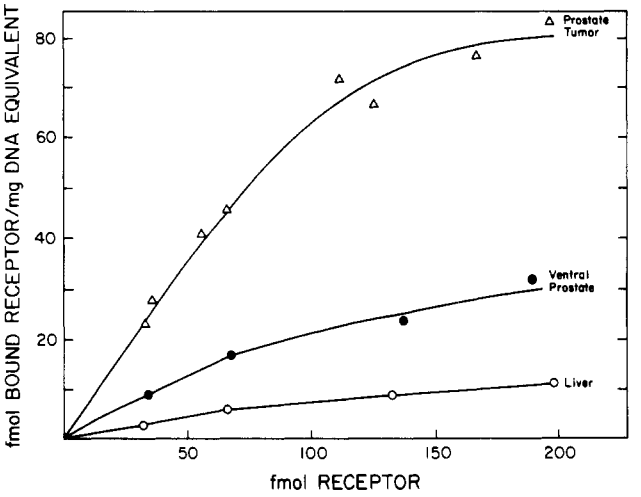

FIGURE 5: Tissue specificity of androgen receptor binding to nuclear matrix. Increasing amounts of the phosphocellulose-purified 4.5S [^{3}H]dihydrotestosterone receptor were incubated with 120 μg of nuclear matrix protein isolated from the Dunning prostate tumor (△; 0.47 mg of protein/mg of DNA equiv), the ventral prostate (●; 0.11 mg of protein/mg of DNA equiv), and the liver (○; 0.05 mg of protein/mg of DNA equiv) as described under Methods except that the total volume was 0.7 mL.

Table I: Androgen Receptor Binding to Nuclear Matrix of Different Tissues

tissue	no. of binding sites[a] fmol/mg of DNA equiv	sites/nucleus
Dunning prostate tumor	110	800
ventral prostate	52	410
liver	22	170

[a] Binding sites were estimated by plotting the data of Figure 5 in the form of Scatchard plots (1949).

Table II: Partial Purification of 8S-Promoting Factor (8S-PF) from Rat Serum

	total protein (mg)	8S-PF activity[a] (units/mg of protein)
serum of mature male rat	5000	0.5
40% saturated $(NH_4)_2SO_4$	2500	0.7
DEAE-Sepharose (eluted at 0.12 M KCl)	600	2.5
CM-Sepharose (eluted at 0.15 M KCl)	200	5.0
hydroxylapatite (eluted at 0.25 M phosphate)	70	20
ATP-Sepharose (eluted at 0.1 M KCl)	12	50

[a] One unit is the amount of total protein needed to reconstitute approximately 60 fmol of 4.5S [^{3}H]dihydrotestosterone-labeled androgen receptor to the 8S form.

of binding sites in liver was consistent with its lower degree of androgen responsiveness. It is conceivable the higher number of sites in the prostate tumor may reflect an alteration related to its neoplastic state.

The tissue-specific differences noted above, which appear to parallel androgen sensitivity of the organ, were similar when the binding was expressed per milligram of matrix protein (data not shown), even though in the prostate tumor, a 2–3-fold greater portion of total nuclear protein was recovered in the nuclear matrix fraction (16%) compared to the ventral prostate or liver (6–7%).

Inhibition by the 8S Androgen Receptor Promoting Factor. The 8S androgen receptor promoting factor, when added to the partially purified 4.5S [^{3}H]dihydrotestosterone receptor, results in a shift in sedimentation to an 8S form (Colvard & Wilson, 1981). Partial purification using standard chroma-

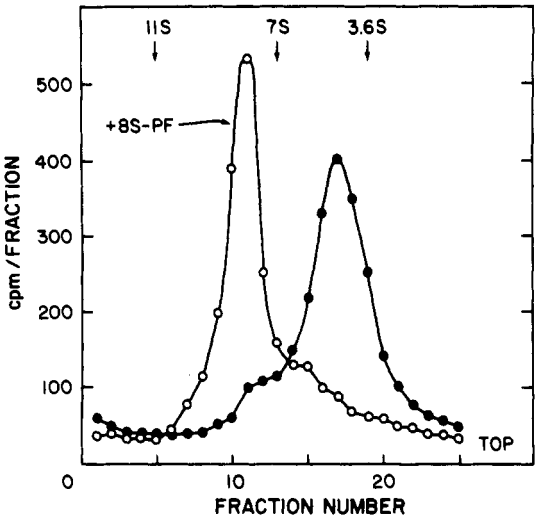

FIGURE 6: Sucrose gradient centrifugation of the 4.5S [^{3}H]dihydrotestosterone receptor in the presence or absence of 8S androgen receptor promoting factor. The 4.5S [^{3}H]dihydrotestosterone receptor was obtained by chromatography on phosphocellulose as described under Methods. The 8S-promoting factor (8S-PF) was partially purified as described under Methods by fractionation of adult male rat serum with $(NH_4)_2SO_4$, followed by sequential chromatography on DEAE-Sepharose, hydroxylapatite, and ATP–Sepharose. Receptor fractions (62 fmol each) were dialyzed with (○) or without (●) the 8S-promoting factor fraction (62 μg of protein eluted from ATP–Sepharose at 0.1 M KCl) against 250 mL of 25 mM KCl, 10% glycerol, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 50 mM Tris, pH 7.5, for 2 h at 4 °C. Aliquots (0.3 mL) were layered onto 2–20% sucrose gradients prepared in the same buffer and analyzed as described under Methods.

tographic procedures (see Methods) results in a 100-fold purification of the 8S factor from serum (Table II); following chromatography on ATP–Sepharose, the 8S-promoting factor retains its ability to shift the 4.5S receptor to the 8S form (Figure 6). The 8S-promoting factor in serum of the adult rat likely originates from androgen target tissues, since we have demonstrated its presence in androgen-responsive organs of the immature male rat but its absence in the serum of these animals (Colvard & Wilson, 1981). Previous estimates indicate that the 8S-promoting factor is a protein of ~170000 molecular weight that does not bind ^{3}H-labeled androgens.

The partially purified 8S-promoting factor was tested for its ability to alter Zn^{2+}-potentiated binding of the 4.5S [^{3}H]dihydrotestosterone receptor to isolated nuclei and to nuclear matrix. As shown in Figure 7, addition of the partially purified 8S-promoting factor (130 μg of protein) to the nuclear binding assay, carried out at increasing amounts of DNA up to 360 μg, showed a striking inhibition. Similarly, at increasing concentrations of Zn^{2+}, the 8S factor inhibited receptor association with nuclear matrix (Figure 8) or nuclei (data not shown). Inhibition of receptor binding to nuclei or nuclear matrix was dependent on the concentration of the 8S-promoting factor. From 40 to 100 μg of partially purified 8S factor was required to inhibit nuclear binding by 50%. The amount of 8S-promoting factor required to inhibit nuclear binding by 50% was approximately half the minimal amount required to shift an equivalent amount of the 4.5S receptor to 8 S on low-salt sucrose gradients. Therefore, it appears that the 8S-promoting factor, in addition to promoting formation of the 8S complex, causes inhibition of the receptor–nuclear matrix interaction.

A question that arises is whether one protein has both 8S reconstituting activity and inhibitory activity, or if two proteins of differing activities have been copurified. The 8S receptor is stable in buffer containing 0.025 M KCl, yet it dissociates

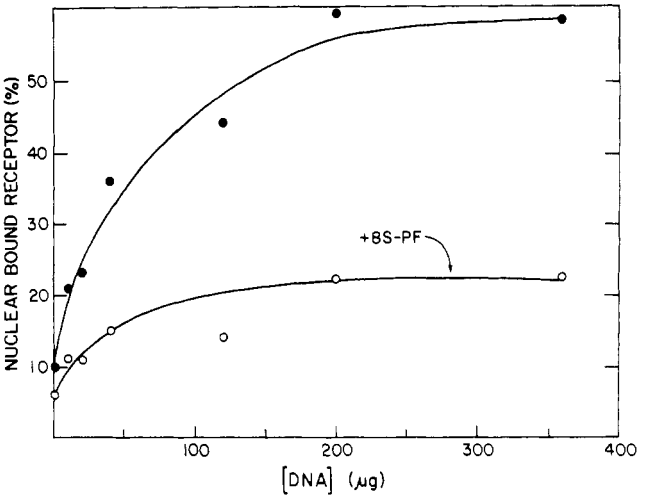

FIGURE 7: Inhibition of Zn^{2+}-dependent 4.5S androgen receptor binding to nuclei by the 8S androgen receptor promoting factor at increasing concentrations of nuclei. Increasing amounts of nuclei from the Dunning prostate tumor were incubated under standard conditions with 33 fmol in 0.3 mL of [^{3}H]dihydrotestosterone-labeled 4.5S androgen receptor in the presence (○) or absence (●) of 130 μg of partially purified 8S androgen receptor promoting factor. The 8S-promoting factor (8S-PF) was isolated as described under Methods by $(NH_4)_2SO_4$ fractionation, followed by sequential chromatography on DEAE-Sepharose, hydroxylapatite, and phosphocellulose. The 8S-promoting factor fraction was added at the beginning of the 30-min incubation at 25 °C. Radioactivity in nuclear pellets was extracted and is expressed as the percent of total radioactivity added in the form of the 4.5S [^{3}H]dihydrotestosterone receptor.

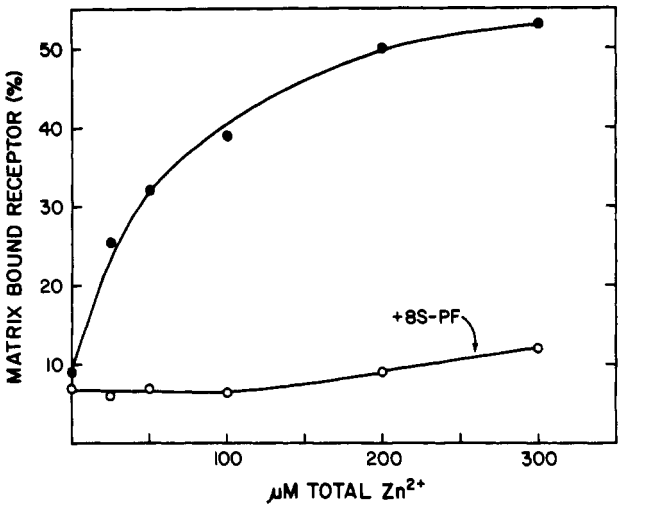

FIGURE 8: Inhibition of binding of the 4.5S androgen receptor to nuclear matrix by the 8S androgen receptor promoting factor at increasing concentrations of Zn^{2+}. The 4.5S [^{3}H]dihydrotestosterone receptor (33 fmol in 0.3 mL) was incubated with 250 μg of nuclear matrix protein from the Dunning prostate tumor for 1 h at 25 °C in the presence (○) or absence (●) of the partially purified 8S androgen receptor promoting factor (460 μg of total protein) at increasing concentrations of $ZnCl_2$. The 8S-promoting factor (8S-PF) was partially purified as described under Methods by fractionation with $(NH_4)_2SO_4$ and chromatography on DEAE-Sepharose (eluted at 0.22 M KCl) followed by hydroxylapatite (eluted at 90 mM phosphate buffer). Binding is expressed as the percent of the total added radioactivity that was extracted with ethanol.

to the 4.5S form during centrifugation for 21 h through sucrose gradients containing 0.15 M KCl. At the same time, the nuclear inhibitory activity of the 8S-promoting factor is detected in an assay buffer containing 0.15 M KCl. An explanation for this apparent discrepancy is that dilution effects and the long centrifugation time required during sucrose gradient analysis may drive the equilibrium toward dissociation of the

8S complex. In agreement with this, dilution of the receptor–nuclear binding assay by 2–4-fold or decreasing the concentration of the 8S-promoting factor resulted in loss of its inhibitory activity. Dilution effects have been shown previously to activate the progesterone receptor (Buller et al., 1975) and may explain the instability of the 8S complex in 0.15 M KCl on sucrose gradients.

The use of chromatofocusing columns, gel filtration, sucrose gradient centrifugation, and zinc chelating columns, in addition to those outlined in Table II, has not separated 8S reconstituting activity from inhibiting activity of nuclear binding. The addition of column fractions which lacked 8S reconstituting activity showed no inhibition of nuclear binding. Various purified proteins such as ovalbumin, bovine γ-globulin, or myoglobin did not alter nuclear binding of the receptor. Heat inactivation of 8S-promoting factor (60 °C for 30 min), assayed by loss of its ability to shift the 4.5S receptor to 8 S, likewise destroyed the inhibiting activity of receptor–nuclear binding. However, since the 8S-promoting factor has not been purified to homogeneity, we cannot rule out the possibility that the 8S factor fraction is composed of two distinct components, one that reconstitutes the 8S receptor form and one that inhibits nuclear binding of the 4.5S receptor. That both activities are inherent in one protein is in agreement with the hypothesis that 8S steroid receptors are incapable of binding in nuclei and represent the nontransformed receptor, while 4–5S forms are activated to bind in nuclei.

Discussion

Isolated nuclear matrix contains a limited number of high-affinity sites ($K_a \sim 10^{13}$ M^{-1}) for the 4.5S [^{3}H]dihydrotestosterone receptor when assayed in vitro in the presence of 0.15 M KCl, 300 μM Zn^{2+}, and 5 mM mercaptoethanol. Half-maximal binding is achieved at concentrations of free Zn^{2+} as low as 0.02 μM. The significant enhancement of binding at increased temperatures may reflect what has become known as "activation" or "transformation" of the hormone–receptor complex. The addition of a protein fraction that restores the 8S form, the 8S androgen receptor promoting factor, completely inhibits receptor binding to nuclei and nuclear matrix in vitro. These observations are in agreement with the hypothesis that 8S steroid receptor forms are incapable of binding in nuclei, while activated or transformed receptors that bind nuclei are 4–5 S.

Recent reports by others suggest that steroid receptors preferentially associate with the nuclear matrix. Steroid exchange studies by Barrack & Coffey (1980) revealed high-affinity sites for androgens in rat ventral prostate matrix and for estrogen in the chicken liver matrix. A recent report indicates an in vitro association between the androgen receptor and isolated matrix of rat ventral prostate (Barrack, 1983). The binding affinity reported ($K_a \sim 10^{10}$ M^{-1}) is lower than that reported here, perhaps due to our use of Zn^{2+} in the binding reaction. It is conceivable that Zn^{2+} increases the affinity and specificity of the nuclear binding site. Although the mechanism by which Zn^{2+} might promote high-affinity binding is not known, preliminary studies suggest that Zn^{2+} may be interacting directly with the 4.5S androgen receptor. Previous reports by others suggest that steroid receptors may be metalloproteins (Shyamala & Yeh, 1975; Lohman & Toft, 1975). The requirement for Zn^{2+} by other proteins of the nucleus suggests an interesting role for this divalent cation in nuclear events.

Our ability to quantitate and compare the tissue distribution of nuclear and nuclear matrix binding sites necessitates that these sites are saturable. Use of a receptor fraction partially purified by phosphocellulose chromatography eliminates the 8S-promoting factor, which was found to inhibit binding of receptor to matrix. The apparent tissue distribution of nuclear matrix sites for the androgen receptor appears to correlate with the androgen responsiveness of the tissue, since more sites were noted in the prostate and a prostate-derived tumor than in liver. It may be that the level of nuclear sites parallels the amount of receptor present in a particular tissue.

Early work with the glucocorticoid receptor indicated that what appeared to be a finite number of sites (Higgins et al., 1973a) that lacked tissue specificity, but which were selective for the hormone receptor (Higgins et al., 1973b), actually resulted from the presence of a macromolecular inhibitor present in the cytosol fraction of rat hepatoma cells. It was suggested that the inhibitor may interact directly with the receptor (Simons et al., 1976). A similar result was found for the estrogen receptor (Chamness et al., 1974). Saturable binding of the estrogen receptor to basic nuclear proteins immobilized on Sepharose was reported not to be tissue specific (Puca et al., 1975). A similar study with the androgen receptor suggested tissue-specific binding of basic nuclear proteins to the androgen receptor (Mainwaring et al., 1976). On the other hand, the classic studies of Spelsberg et al. (1971, 1972; Kon & Spelsberg, 1982) suggest that a tissue-specific distribution of so-called nuclear acceptor sites for chick oviduct progesterone receptors resides in the non-histone chromatin protein fraction. Organ-specific nuclear proteins were found to represent the acceptor sites for the androgen receptor (Klyzsejko-Stefanowicz et al., 1976). Whether the histone or nonhistone proteins of chromatin reside in the matrix fraction as well remains to be clarified.

The data presented in this paper suggest that a non-steroid binding protein associates with the high-affinity 4.5S androgen receptor and that it inhibits binding of the 4.5S [^{3}H]dihydrotestosterone receptor to nuclei and to nuclear matrix. Protein factors that do not bind steroids have been shown by Murayama et al. to interact with the estrogen receptor of cow uterus (Murayama et al., 1980c) and the progesterone receptor of hen oviduct (Murayama et al., 1980d). One of these, an "8S estrogen receptor forming factor", could be dissociated in 0.4 M NaSCN and fractionated by gel filtration into two components (Stokes radius 37 and 18.5 Å) which bind in various ratios to form the 5S, 6S, and 8S forms of the estrogen receptor (Murayama et al., 1980a,b). Furthermore, the 37 Å component, when added to an in vitro nuclear binding assay, showed a dramatic inhibition of binding of the 4.5S estrogen receptor derived from porcine uterus (Murayama & Fukai, 1982). Studies by another group (Nishizawa et al., 1981) have demonstrated a heat-sensitive macromolecular factor present in mature rat uterus, but not rat liver cytosol, that causes estrogen receptor aggregation and loss of its ability to bind nuclei. This factor appeared to be specific to the estrogen receptor since neither effect occurred with the liver glucocorticoid receptor or the rat prostate androgen receptor.

More recently, the use of monoclonal and polyclonal antibodies to the avian progesterone receptor has identified a non-progesterone binding protein (M_r 90 000) that is a component of the 8S nontransformed receptor. A surprising result was that it appears to be common to the nontransformed glucocorticoid, estrogen, and androgen receptors (Joab et al., 1983). Monoclonal antibodies to the estrogen receptor also suggest that the 8S complex is not an aggregate of identical subunits (Moncharmont et al., 1982).

Evidence that argues against another protein associating with 4–5S steroid receptor binding units is that the purified

molybdate-stabilized 8S form of the chick oviduct progesterone receptor was purified and found to consist of a single band on SDS–polyacrylamide gels (Renoir et al., 1982). It was concluded that the 8S progesterone receptor consists of one unique protein of M_r 85 000, with a stoichiometry of one hormone binding site per subunit (Renoir et al., 1982).

The data reported here reflect properties of the androgen receptor in vitro. Although it is difficult to directly relate the effects of Zn^{2+} and the 8S-promoting factor to the in vivo situation, it is, nevertheless, tempting to speculate that both may play an important role in androgen receptor action. Under in vivo conditions, there may exist an equilibrium between the 4.5S and 8S receptor forms, the latter resulting from association of the 4.5S receptor with 8S-promoting factor. Inhibition of nuclear matrix binding by the 8S-promoting factor may indicate that the 8S receptor represents an inactive form of the androgen receptor. Upon dissociation of the 8S receptor complex into its two components, which may be favored by the binding of androgen, the 4.5S receptor might become activated to bind with high affinity to matrix sites in a manner that is influenced by zinc. Receptor association with matrix might facilitate its subsequent interaction with hormone-dependent genes, which have been found to be preferentially associated in this fraction (Robinson et al., 1982). Zn^{2+}-dependent receptor binding to matrix in vitro appears to be reversible, suggesting that the receptor is likely released in its original 4.5S form. Receptor forms smaller than 4.5 S, i.e., 3.0 S and 3.6 S, are considered to be artifacts generated during isolation (Wilson & French, 1979). We know little as yet about the steroid-free forms of the 4.5S or 8S androgen receptors, partly due to the lability of the ligand-free steroid binding site (Wilson & French, 1976). It is conceivable that phosphorylation is involved in maintaining this binding site, as has been suggested for glucocorticoid (Housley & Pratt, 1983) and progesterone (Dougherty et al., 1982) receptors.

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Modulation of Membrane Fusion by Ionotropic and Thermotropic Phase Transitions†

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ABSTRACT: We have studied the relationship of ionotropic and thermotropic phase transitions to divalent cation induced fusion of large unilamellar phospholipid vesicles. Fusion was monitored by the Tb/dipicolinic acid fluorescence assay for the intermixing of internal aqueous contents of vesicles. The phase behavior of the membranes was followed by differential scanning calorimetry. (1) Sr^{2+} and Ba^{2+} shifted the phase transition temperature (T_c) of bovine brain phosphatidylserine vesicles from 6 to 27 and 31.5 °C, respectively. These cations induced vesicle fusion at temperatures above or below the T_c of that cation/phospholipid complex, indicating that an isothermal phase change from the liquid-crystalline to the gel phase is not a requirement for membrane fusion. (2) The temperature dependence of the initial rate of fusion of phosphatidylserine/dipalmitoylphosphatidylcholine (1:1) vesicles in the presence of Ca^{2+} exhibited a pronounced maximum at 17 °C, at the lower part of the broad phase transition endotherm whose T_c was about 25 °C; fusion was inhibited completely at 30 °C when the membrane was in the liquid-crystalline state. These observations suggest that molecular clusters rich in phosphatidylserine, formed when the membrane is in the phase transition region, allow the vesicles to fuse. (3) The fusion of phosphatidylserine/dimyristoylphosphatidylethanolamine (1:1) vesicles, whose T_c was also around 25 °C, had a different temperature dependence in that the initial rate increased sharply above the T_c, with a local maximum within the transition region. Phase separation of dimyristoylphosphatidylethanolamine was induced by Ca^{2+} but not by Mg^{2+}, although both ions induced fusion. The observation that phosphatidylserine/egg phosphatidylethanolamine (1:1) vesicles fused in the presence of Ca^{2+} or Mg^{2+} at temperatures below or above the lamellar to hexagonal (H_{II}) transition temperature of the phosphatidylethanolamine and that Mg^{2+} could induce fusion without causing a transition into the H_{II} phase suggests that this transition is not essential for membrane fusion. On the basis of all three systems, it is proposed that fusion occurs via defects in molecular packing and dehydration of the polar groups of phospholipids at the region of interbilayer contact.

The molecular mechanism by which divalent cations, in particular Ca^{2+}, induce the fusion of liposomes made of acidic phospholipids or their mixtures with zwitterionic lipids is not understood clearly. It has been proposed that the isothermal phase change induced by Ca^{2+} or Mg^{2+} in the bilayer membrane is the key event leading to the fusion of the liposomes and that this phase change causes the transient destabilization of the membranes which then fuse at the domain boundaries between fluid and solid phases in the same membrane (Papahadjopoulos et al., 1977). To test this proposal, it is necessary to correlate the isothermal phase change with the early kinetics of membrane fusion. Although Ca^{2+} causes extensive fusion of both small unilamellar vesicles (SUV;[1] approximately 30 nm in diameter) and large unilamellar vesicles (LUV; approximately 100 nm in diameter) (Papahadjopoulos et al., 1974, 1977; Wilschut et al., 1980), it shifts the transition temperature of PS bilayers to such high temperatures (greater than 100 °C; Portis et al., 1979; Newton, et al., 1978; Jacobson & Papahadjopoulos, 1975) that it is impossible to compare the kinetics of fusion above and below this transition temperature. The fusogenic effect of Mg^{2+} on PS membranes is strongly dependent on the curvature of the bilayer: SUV undergo a few rounds of fusion when the Mg^{2+} concentration exceeds a threshold value but stop fusing when they reach a

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[1] Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DPA, dipicolinic acid; NTA, nitrilotriacetic acid; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; NBD, 7-nitro-2,1,3-benzoxadiazole; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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