Articles

Identification of a Unique Liganded Estrogen Receptor Complex Released From the Nucleus by Decavanadate†

Michael Fritsch,‡ Michael Aluker, and Fern E. Murdoch*

Department of Biochemistry and Molecular Biology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799

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ABSTRACT: Unoccupied estrogen receptor (ER) can be extracted from tissues by homogenization with a hypotonic buffer, whereas hormone-occupied ER becomes tightly bound to the nuclear pellet and must be extracted with high-salt-containing buffers. The molecular basis for estrogen-induced tight nuclear binding of ER remains an important puzzle. The different subcellular fractionation behaviors of the occupied and unoccupied ER are presumed to be due to a difference in their ability to interact with nuclear components, such as DNA and proteins. The proteins that are the targets for interaction with the hormone-occupied ER may be important for transcriptional regulation. However, the salt-extracted ER is recovered as a homodimer, and associated proteins are presumably lost due to the high-salt conditions. We have discovered an alternate method of releasing the occupied ER from the nucleus. Inclusion of 2 mM orthovanadate, polymerized primarily to decavanadate, in a hypotonic buffer efficiently releases over 90% of estrogen-bound ER from the nuclear pellet. The recovered ER complex is fully functional in terms of estrogen and DNA binding and is full-length by western blot analysis. Our data suggest that the mechanism of ER release is by decavanadate competition with nuclear DNA, rather than by inhibition of a phosphotyrosine phosphatase. Of particular interest, the decavanadate released occupied ER complex shows distinct behavior by sucrose density gradient sedimentation analysis. It is larger than the salt-extracted transformed ER, suggesting that an occupied ER in complex with nuclear proteins may be released from the nucleus by decavanadate.

Estrogen receptor (ER)[1] is a ligand-activated transcription factor and is a member of the large nuclear receptor superfamily that includes the steroid, thyroid hormone, retinoid, and vitamin D receptors (for reviews, see refs *1*−*4*). The ER was the first member of this family to be biochemically isolated (*5*). The subcellular fractionation and sucrose density gradient sedimentation behavior of the ER suggested that it could form biochemically and functionally distinct protein complexes. In subcellular fractionation protocols, the majority of the unliganded ER could be recovered in the post-ribosomal supernatant of a homogenate prepared in hypotonic buffer (*6*−*8*). The efficiency of unoccupied ER extraction with low-salt buffers could be increased with a greater ratio of buffer volume to packed cell volume (pcv) (ref *9*). Within minutes of treatment of tissue or cells with estrogen, the ER displayed an increased affinity for nuclear components that is termed tight nuclear binding (ref *10*, and references therein). Buffers containing 0.4−0.6 M salt are required to extract this liganded form of the ER from cells or nuclei (see ref *11* for review). These two forms of ER, termed the nontransformed and transformed ER, respectively, show different sedimentation behaviors on sucrose density gradients. These observations led to the proposal of a two-step model for estrogen action in which the ER is in the cytoplasm and, upon occupancy with hormone, undergoes transformation and translocation to the nucleus (*7*, *8*). Subsequent work using cell enucleation and immunocytochemical techniques clearly demonstrated that the ER is, in fact, always concentrated in the nucleus (*12*−*14*). Therefore, the different subcellular fractionation behaviors of the occupied and unoccupied ER must be due to a difference in their ability to interact with nuclear components, such as DNA and proteins.

The molecular basis for the estrogen-induced tight nuclear binding of ER remains an important puzzle. Tight nuclear

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* To whom correspondence should be addressed: Department of Biochemistry and Molecular Biology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4799. Tel: 301-295-2845, Fax: 301-295-3512. E-mail: fmurdoch@erols.com.

‡ Present Address: Department of Pathology, Johns Hopkins University.

[1] Abbreviations: ER, estrogen receptor; hER, human estrogen receptor; rER, rat estrogen receptor; mER, mouse estrogen receptor; pcv, packed cell volume; E_2, 17β-estradiol; PAO, phenylarsine oxide; LS, low-salt extract; LSn, low-salt extract of nuclei; HS, high-salt extract; ERE, estrogen responsive element; DMEM, Dulbecco's modification of Eagle's medium; MOI, multiplicity of infection; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; hHSF, human heat shock factor; CREB, cAMP responsive element-binding protein.

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binding of ER is the first and one of the most dramatic effects of estrogens on cells. It is required for the expression of estrogen responses (*10*, *15*−*18*). Some investigators have proposed that estrogens are required for ER to bind to DNA in vivo, and several studies show an increased interaction of ER with estrogen-regulated promoter DNA in vivo in the presence of estrogen (*19*−*21*). However, the ER does not require estrogen to bind to estrogen response elements (ERE) in vitro (*22*−*26*). Therefore, we hypothesize that the unoccupied ER can bind DNA in vivo, but estrogen promotes ER interactions with other nuclear proteins that increase the avidity of this ER complex for DNA.

Considerable work has been directed at identifying nuclear proteins that interact with ER in an estrogen-dependent manner, since these proteins are expected to be important for transcription. Molecular genetics methods, such as the yeast two-hybrid system and gene transfer assays, have identified co-activators such as SRC-1, AIB-1, and CBP that are capable of functioning in nuclear receptor signal transduction (*27*−*32*). Biochemical reconstitution experiments have also been used to identify co-activators such as RIP 140 that preferentially bind agonist-occupied ER in vitro (*33*). A third approach to identification of nuclear proteins that interact with ER in a hormone-dependent manner would be to biochemically isolate the transformed ER complex that is tightly bound to the nucleus and to identify associated proteins. This has been a fruitful approach for the study of the structure and function of the unoccupied, nontransformed ER complex that is recovered in hypotonic extracts (*34*, *35*). However, the difficulty in extracting the transformed ER from the nucleus has frustrated this approach. The transformed ER is generally extracted with buffers containing 0.6 M salt, and the resulting product is an ER homodimer (*36*−*39*). Associated proteins involved with tight nuclear binding of the ER are probably dissociated during this harsh extraction method.

We have discovered an alternate method of releasing the occupied ER from the nucleus. Inclusion of 2 mM orthovanadate, treated to polymerize to predominately decavanadate, in the hypotonic buffer efficiently releases over 90% of estrogen-bound ER from the nuclear pellet. Vanadium forms negatively charged oxyanions in biological buffers that have similarity to phosphate compounds (*40*−*42*). Monomeric vanadate is often included in buffers to inhibit phosphotyrosine phosphatase activity. At high concentrations (0.5 M) near neutral pH, vanadate rapidly polymerizes to the decavanadate polyanion and is a bright orange solution. Monomeric vanadate and decavanadate can interconvert, and though both forms are probably present in solution, the conversion to the monomer is quite sluggish at ambient temperature. Conversion of decavanadate to monomeric vanadate can be accomplished by dilution and then boiling. Release of occupied ER from the nucleus requires decavanadate, while monomeric vanadate is ineffective. The recovered ER complex is fully functional in terms of estrogen and DNA binding and is full-length by western blot analysis. Our data suggest that the mechanism of ER release is by decavanadate competition with nuclear DNA, rather than by inhibition of a phosphotyrosine phosphatase. Of particular interest, the decavanadate released occupied ER complex shows distinct behavior by sucrose density gradient sedimentation analysis. It is larger than the salt-extracted transformed ER, suggesting that an occupied ER in complex with nuclear proteins may be released from the nucleus by decavanadate.

MATERIALS AND METHODS

Reagents. Radiolabeled [α-^{32}P]dATP or dCTP (3000 Ci/mmol) and [2,4,6,7-^{3}H]estradiol (72 Ci/mmol) were obtained from New England Nuclear. [^{14}C]Methylated bovine serum albumin (3.8 μCi/mg) was obtained from Amersham. ER (HC-20) rabbit polyclonal IgG against human ER (hER), Sp1 (PEP 2) rabbit polyclonal IgG against the Sp1 transcription factor, and CREB-1 (240) rabbit polyclonal IgG against the CREB transcription factor were purchased from Santa Cruz Biotechnology, Inc. Antibody ER1438 for rat or mouse ER (rER, mER) detection was an antipeptide antibody raised in rabbit against residues 270−284 of the rER (*43*). This antibody was prepared in our laboratory (with animal work contracted to Hazleton Washington, Inc.) essentially by duplicating the methods previously published (*44*). Antisera were affinity-purified by chromatography on SulfoLink Coupling Gel (Pierce) derivatized with the immunizing peptide as per manufacturer's instructions. Antibody against human heat shock factor (hHSF) was a generous gift from Carl Wu (NCI, NIH). Bio-Gel HT gel hydroxyapatite, protein assay reagent, and reagents for SDS−PAGE were from Bio-Rad. Tissue culture media and reagents were from Gibco-BRL, and serum was from Hyclone. Vanadate in predominately the decavanadate form was prepared by dissolving solid orthovanadate (Sigma) in water to 0.5 M and adjusting to pH 7.5 with concentrated HCl. This bright orange solution was kept at room temperature and used the same day. Monomeric vanadate was prepared by dissolving solid orthovanadate in water to 0.05−0.1 M and adjusting to pH 10 with HCl. The resulting orange solution was placed in a boiling water bath until completely clear (within 1−3 min). Monomeric vanadate stocks were stored at −20 °C.

Cell Culture. MCF-7 and GH3 cells were obtained from American Type Culture Collection. MCF-7 human breast cancer cells were cultured in phenol red-free DMEM (high glucose, plus glutamine) supplemented with 10% (v/v) bovine calf serum or fetal bovine serum, 1 mM pyruvate, 0.1 mM nonessential amino acids, and 10 μg/mL bovine insulin. Cultures were used between passages 170 and 210. GH3 rat pituitary cells were cultured in phenol red-free DMEM (high glucose, plus glutamine) supplemented with 10% (v/v) bovine calf serum, 2 mM glutamine, and 0.6 μg/mL bovine insulin. Cultures were used between passages 30 and 60. Sf9 insect cells were purchased from Invitrogen and cultured in spinner flasks in serum-free media (Sf 900 II SFM, Gibco-BRL). MCF-7 and GH3 cultures were fed approximately 24 h before harvesting. ER was occupied in situ by adding 20 nM E_2 to the culture 30 min prior to harvesting. Control cultures received an equal volume of ethanol. Cells for extracts were lifted from plates with 3 mM EDTA in Hank's buffered salt solution without magnesium or calcium.

Preparation of Cellular Extracts. Extracts of Sf9, GH3, or MCF-7 cells were made by sequential low and high-salt steps. Sf9 cells were infected at a MOI (multiplicity of infection) of 4 with a recombinant baculovirus expressing the mouse estrogen receptor (generous gift of Iain Anderson

and Jack Gorski, University of Wisconsin). The low-salt buffer for Sf9 cells was 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, and 5% (v/v) glycerol, and high-salt buffer included 0.6 M NaCl. The low-salt buffer for GH3 cells was 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM $MgCl_2$, and high-salt buffer included 0.6 M NaCl. The low-salt buffer for MCF-7 cells was 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, and 1 mM dithiothreitol, and high-salt buffer included 0.6 M KCl. A cocktail of protease inhibitors was used that had final concentrations in all buffers of 0.5 mM AEBSF, 10 μM leupeptin, 2 μg/mL aprotinin, and 2 μg/ mL pepstatin. Cells were homogenized in 4 packed cell volumes (pcv) of low-salt buffer with a Dounce homogenizer (B pestle) and centrifuged at 436000*g* for 10 min (Beckman TLA 100 ultracentrifuge) to pellet the nuclei and other organelles. Vanadate, as monomer or polymer, was added to the low-salt buffer for this step as indicated in the figure legends. This post-ribosomal supernatant was the low-salt extract and is designated LS in the figures. The nuclear pellet was extracted with the same volume of high-salt buffer on ice for 30 min and centrifuged as above, and the supernatant was used as the high-salt extract and is designated HS in the figures. In some cases the nuclear pellet was extracted with low-salt buffer plus 2 mM orthovanadate (decavanadate form) instead of high-salt buffer. This extract is designated LSn in the figures.

Hydroxyapatite (HAP) Assay. Determinations of $^3H-E_2$ binding in extracts or sucrose gradient fractions as a measure of ER protein content was done by the HAP assay as previously described with the following modification (*22*). The HAP pellets were washed only 3 times for extracts occupied with $^3H-E_2$ in situ and only 2 times for sucrose gradient fractions.

Western Blot Assays. Cellular extracts were diluted in SDS sample buffer (final concentrations were 2% SDS, 125 mM Tris-Cl, pH 6.8, 15% (v/v) glycerol, and 10 mM dithiothreitol) and heated at 95−99 °C for 3−5 min just prior to loading onto the gel. SDS−PAGE was conducted generally as described (*45*) using the Mini-PROTEAN II electrophoresis cell (BioRad). Prestained molecular weight markers (Amersham) were run on every gel to allow estimation of relative protein molecular weight.

Proteins separated by SDS−PAGE above were transferred onto nitrocellulose membranes (HybondECL, Amersham) in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol, pH 8.3, using the Mini Trans-Blot Electrophoretic Transfer Cell (BioRad). Membranes were blocked with agitation for at least 1 h at room temperature or overnight at 4 °C in a blocking solution of TBST (24 mM Tris-Cl, pH 7.4, 2 mM KCl, 163 mM NaCl, and 0.2% (v/v) Tween-20 (BioRad)) plus 5% (w/v) nonfat dried milk (Carnation). First antibody was diluted in blocking solution (1:1000 for affinity-purified ER1438 and ER (HC-20), 1:2000 for Sp1 (PEP-2) and CREB-1 (240), and 1:1000 for anti-hHSF). The first antibody was incubated with agitation for 45 min at room temperature. Blots were washed 5 times in TBST at room temperature. The Sp1 and CREB blots were washed with RIPA buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS). The second antibody was anti-rabbit immunoglobulin horseradish peroxidase-linked whole antibody (Amersham) diluted 1:2000 in blocking solution for detection of ER, Sp1, and CREB, and 1:4000 for hHSF. Incubation with agitation was for 45 min at room temperature. Blots were washed 5 times as above, developed using the enhanced chemiluminescence reagent kit (ECL, Amersham), and bands detected by exposure to autoradiography film (HyperfilmECL, Amersham). Band density was quantitated by scanning the autoradiograph into an image file and analysis performed with the NIH Image (version 1.57) public domain software.

Gel Shift Assays. DNA binding to the ER was performed as described with modifications (*46*, *47*). In brief, [^{32}P] 5′-end-labeled double-stranded oligonucleotides corresponding to the *Xenopus Vitellogenin* A2 gene estrogen response element (ERE; ref *48*) at 45−75 nM (final concentration) were mixed with cellular extracts (1−10 μg protein), 1.5 μg poly[dI-dC]·poly[dI-dC], and 40 μg bovine serum albumin. Antibodies against ER, monomeric vanadate or decavanadate, and NaCl were included in the reaction as indicated in the figure legends. The reaction mix was brought to a final volume of 20 μL with binding buffer (15 mM Tris-Cl, pH 7.5, 0.1 mM EGTA, 15% glycerol, 0.5 mM DTT, and 100 mM KCl). Samples were incubated at room temperature for 20 min and electrophoresed on a 0.8% agarose horizontal gel in 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA) at room temperature for 1 h. The gel was dried onto DE81 paper (Whatman) and exposed to X-ray film (X-OMAT AR, Kodak). The ERE oligonucleotide top strand was the following:

5′ AGCTTCGAGGAGGTCACAGTGACCTGGAGC-GGATC 3′

Sucrose Density Gradient Analysis. Sucrose gradients in steps of 20%, 16%, 11.5%, and 7% were prepared, centrifuged, and fractionated as described (*49*). The sucrose was dissolved in 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, protease inhibitors as above, and salt as indicated in the figure legend. External sedimentation markers were used including [^{14}C]methylated bovine serum albumin as the 4S marker and aldolase as the 8S marker.

RESULTS

Release of Liganded Estrogen Receptor from the Nucleus with Low-Salt Buffer Containing Decavanadate. We treated several different cell lines with either 20 nM 17β-estradiol (E_2) or vehicle control (C) and subjected them to a two-step extraction. The first step was homogenization in a low-salt buffer using a dounce homogenizer to preserve nuclei. This homogenate was subjected to a high speed centrifugation step to yield a post-ribosomal supernatant (designated LS in Figure 1) and a nuclear pellet. The nuclear pellet was then extracted with the same buffer containing 0.6 M salt (designated HS in Figure 1). As shown in Figure 1A, both the hER (from MCF-7 cells) and the rER (from GH3 cells) partition between the LS and HS fractions in the absence of estrogen treatment (C). We did not use charcoal-stripped serum in our cell culture media and expect that some estrogen was present in the absence of estrogen treatment. This may account for the observation that a portion of the ER is tightly bound to the nuclear pellet even in our vehicle-treated controls. Treatment of the cells with E_2 induces tight nuclear binding and drives all of the ER into the HS fraction (E_2). Addition of 2 mM orthovanadate (decavanadate form) to the

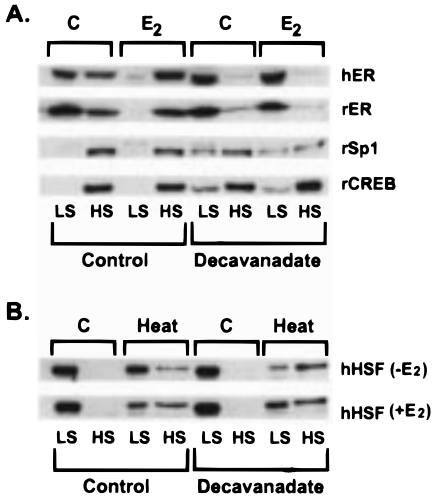

FIGURE 1: Subcellular fractionation behavior of transcription factors examined by western blot analysis. MCF-7 cells (hER, hHSF) or GH3 cells (rER, rSp1, rCREB) were treated with vehicle control (C) or 20 nM 17-β estradiol (E_2), and cells subjected to extraction with low-salt buffer (LS). The post-ribosomal pellet was then extracted with high-salt buffer (HS). The paired LS and HS extracts are in adjacent lanes for each cell culture. Extractions with low-salt buffer alone are designated as a control for comparison to extractions with low-salt buffer containing 2 mM orthovanadate (decavanadate form) designated Decavanadate. The resulting protein extracts were fractionated by SDS−PAGE, transferred to nitrocellulose, and subjected to western blot analysis with the appropriate antibodies as described in Materials and Methods. (A) Detection of hER, rER, rSp1, and rCREB in extracts from control (C) or 17-β estradiol (E_2) treated cells is shown. (B) Detection of hHSF in extracts of MCF-7 cells that were unshocked (maintained at 37 °C; C) or heat shocked (42.5 °C for 25 min; Heat). Cell cultures were treated with (+) or without (−) E_2 as in panel A prior to heat treatment.

low-salt buffer efficiently extracts over 90% of the ER into the LS fraction even after E_2 treatment of the cells. The western blot data in Figure 1A show that the decavanadate-extracted ER is not significantly different from the control receptor in size, quantity, or antigenicity.

We examined the behavior of three other transcription factors under these subcellular fractionation conditions to determine if the effects of decavanadate were specific to ER. The Sp1 and CREB transcription factors are constituitively localized to the nucleus, and high salt is required for their extraction (Figure 1A). Addition of 2 mM orthovanadate (decavanadate form) to the low-salt buffer allowed 30−35% of Sp1 protein to be recovered in the LS fraction, while only 6−14% of the CREB transcription factor could be extracted into the LS fraction (Figure 1A). The heat shock transcription factor (HSF) is a monomer in the absence of a stress signal and is efficiently extracted into the LS fraction as seen in Figure 1B (ref *50* for review). Upon heat shock HSF forms a trimer and becomes tightly bound to the nucleus and requires high salt for extraction. The heat-shocked HSF is distinguished by its slightly slower mobility on SDS−PAGE thought to be due to phosphorylation. Addition of 2 mM orthovanadate (decavanadate form) to the low-salt buffer did not release the heat-shocked HSF protein from the nucleus. The treatment of cells with E_2 did not affect the behavior of the Sp1, CREB, or HSF transcription factors. In summary, the liganded ER is efficiently released from the nucleus by orthovanadate (decavanadate form), and other transcription factors show a range of partial to no release at this

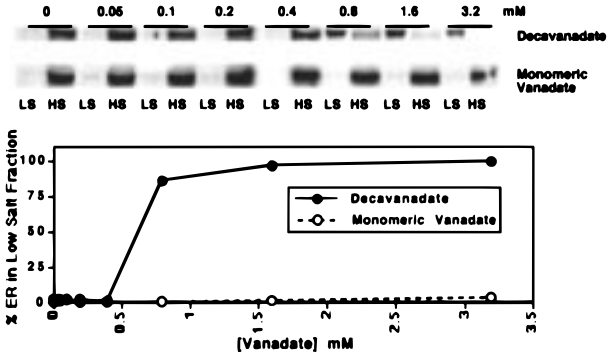

FIGURE 2: Effect of vanadate dose on subcellular fractionation of ER by western blot analysis. GH3 cells were treated with 20 nM E_2 and extracted sequentially with low-salt buffer (LS) followed by high-salt buffer (HS). The concentration of orthovanadate (decavanadate or monomeric form) in the low-salt buffer is shown. Extracts were subjected to western blot analysis for ER as described in Materials and Methods. Band density was measured by scanning autoradiograph into an image file, analysis with NIH IMAGE software, and the results were plotted. The percentage of ER in the LS fraction was calculated as the density of the LS band divided by the summed density of the LS and HS bands multiplied by 100.

concentration of orthovanadate (decavanadate form). These data suggest that the release of protein from the nuclear pellet by decavanadate may not be limited to the ER, though complete extraction of other transcription factors may require optimization of the decavanadate concentration.

The half-maximal concentration of orthovanadate (decavanadate form) required for release of liganded ER from the nucleus is between 0.5 and 0.8 mM as shown in Figure 2. Complete release of ER was observed at 1.6 mM orthovanadate (decavanadate form). This is somewhat higher than the concentration of vanadate typically used for inhibition of phosphotyrosine phosphatase activity (*42*). Monomeric preparations of vanadate are most often used for phosphotyrosine phosphatase inhibition, and we examined the effect of this form of vanadate on release of ER from the nucleus in Figure 2. This form of vanadate had no effect on the subcellular fractionation of ER up to a concentration of 3.2 mM. This result suggests that the biochemical mechanism of ER release from nuclear components is not due to the inhibition of a phosphotyrosine phosphatase.

Other Protein Phosphatase Inhibitors Do Not Release Liganded ER from the Nucleus. We tested the ability of other protein phosphatase inhibitors to release the liganded ER from the nucleus in Figure 3. The compound bpV(phen) is a bisperoxovanadate anion that is a strong phosphotyrosine phosphatase inhibitor and is active on intact cells (*41*, *51*). Phenylarsine oxide (PAO) and dephostatin are also phosphotyrosine phosphatase inhibitors with activity on whole cells (*52*−*54*). Decavanadate, by virtue of its polymeric structure, is expected to have limited activity on intact cells. Okadaic acid specifically inhibits the serine/threonine protein phosphatases 1 and 2a and also works on intact cells (*55*). Decavanadate, bpV(phen), dephostatin, and okadaic acid treatment of intact GH3 cells all fail to affect the subcellular fractionation of ER (Figure 3A). Inclusion of bpV(phen), dephostatin, or PAO in the low-salt extraction buffer also fails to release ER from the nuclear pellet of Sf9 cells expressing mouse ER under conditions where decavanadate is active (Figure 3B). These results suggest that the biochemical mechanism of ER release from the nucleus by

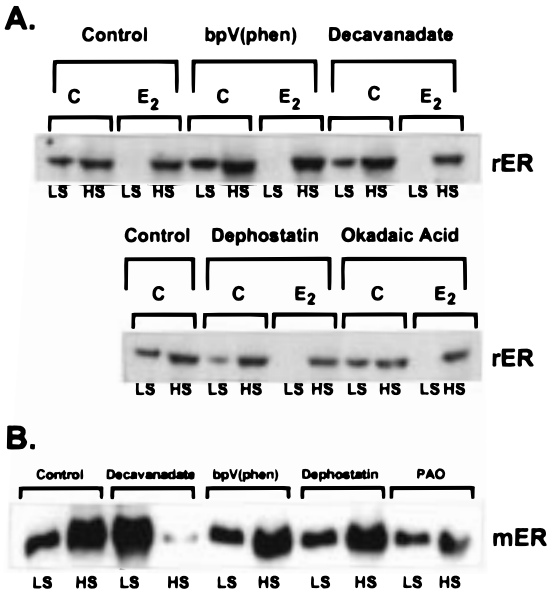

FIGURE 3: Effect of phosphatase inhibitors on subcellular fractionation of ER by western blot analysis. Cell extracts prepared as described below were subjected to western blot analysis for ER as described in Materials and Methods, and the autoradiograph is shown. (A) GH3 cells (rER) were treated with vehicle control (C) or 20 nM 17-β estradiol (E_2) and cells extracted sequentially with low-salt buffer (LS) followed by high-salt buffer (HS). Ninety minutes prior to treatment of cultures with or without E_2, the cultures were treated with the following compounds: nothing (Control), 40 μM bpV(phen), 5 mM orthovanadate (decavanadate form), 80 μM dephostatin, or 200 nM okadaic acid. (B) Sf9 cells infected with recombinant baculovirus expressing mouse ER were extracted sequentially with low-salt buffer (LS) followed by high-salt buffer (HS). The low-salt buffer additionally contained the following: nothing (Control), 2 mM orthovanadate (decavanadate form), 80 μM bpV(phen), 80 μM dephostatin, or 200 μM phenylarsine oxide (PAO).

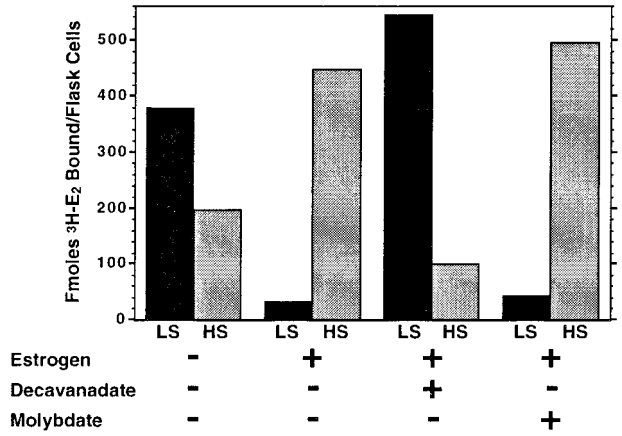

FIGURE 4: Estradiol-binding activity of ER extracted by different methods. MCF-7 cells from a T75 culture flask were extracted sequentially with low-salt buffer (LS) followed by high-salt buffer (HS), and the paired extracts are adjacent to each other on the graph. Cultures were treated with or without 18 nM $^3H-E_2$ as indicated. Low-salt buffer additionally contained 2 mM orthovanadate (decavanadate form) or 10 mM molybdate as indicated. $^3H-E_2$ binding to ER was determined by HAP assay as described in Materials and Methods.

decavanadate is not mediated by inhibition of a phosphotyrosine phosphatase.

Release of the Liganded ER from the Nucleus Does Not Involve Reversal of Transformation. Molybdate, another transition metal, does not affect the release of the ER from the nucleus as seen in Figure 4. Molybdate has phosphatase inhibition activity but is most often used in steroid receptor work to stabilize the nontransformed form of receptors (ref *56* and references therein). Vanadate has also been used to stabilize the nontransformed ER. The characteristics of the nontransformed form of ER include extraction in low-salt buffers, ability to bind ^{3}H-estradiol but not DNA, and association in a complex with hsp90 (heat shock protein 90) and other specific proteins. Treatment of cells with estrogen promotes transformation of ER to a form that binds DNA, associates tightly with the nucleus, and loses association with hsp90. We considered the possibility that decavanadate was reversing the estrogen-promoted transformation of ER, although such a reversal has only previously been reported under very limited conditions (*57*, *58*). The failure of molybdate to release liganded ER from the nucleus suggests that reversal of transformation is not the mechanism by which decavanadate is acting.

The Decavanadate-Extracted Liganded ER is Fully Functional. The quantity of liganded ER extracted from cells by low-salt buffer with decavanadate is comparable to that by high-salt buffer as is seen in the western blots shown in Figures 1−3 (and confirmed by ligand-binding assays on some samples; data not shown). The ER is full-length, ruling out the possibility that proteolytic processing releases ER from the nucleus. The data in Figure 4 demonstrate that ^{3}H-estradiol-binding activity of the ER is comparable for both the decavanadate and high-salt-extracted forms. The total amount of ^{3}H-estradiol-binding activity was determined for both the LS and the HS subcellular fractions from cells treated with or without estrogen. These results are consistent with the western blot results in Figures 1−3 and suggest that decavanadate is affecting the subcellular fractionation of ER, but not its ability to bind hormone.

The ability of the decavanadate-extracted ER to bind DNA is demonstrated by the gel shift assay shown in Figure 5. Excess ^{32}P-ERE was incubated with equal volumes of various extracts. All extracts were prepared by homogenization of an equal number of cells in equal volumes of extraction buffer. The top band in lane 1 is clearly rER, since it is quantitatively supershifted in lane 2 with an antibody specific for rodent ER. The amount of liganded ER−DNA complex seen with the decavanadate extract in lanes 1 and 2 is comparable to that obtained with unoccupied, low-salt-extracted receptor in lanes 7 and 8.

The decavanadate-extracted liganded ER used in lanes 5 and 6 of Figure 5 was prepared by extraction of nuclei instead of whole cells, which was used in lanes 1 and 2 and in previous figures. Extracts of whole cells with low-salt buffer plus decavanadate contain not only the liganded ER and nuclear proteins but also all of the cytosolic proteins that are generally removed before a high-salt extract is made from the nuclear pellet. This will include a high concentration of hsp90 since the majority of it is extracted into the cytosol (*59*). We wanted to examine the behavior of the decavanadate-extracted liganded ER in the absence of the cytosolic proteins so we could compare it directly to liganded ER made by high-salt extraction of the nuclear pellet. Therefore, we homogenized estrogen-treated cells in low-salt buffer and recovered the nuclear pellet by centrifugation. The nuclear pellet was rinsed to remove residual cytosol and then

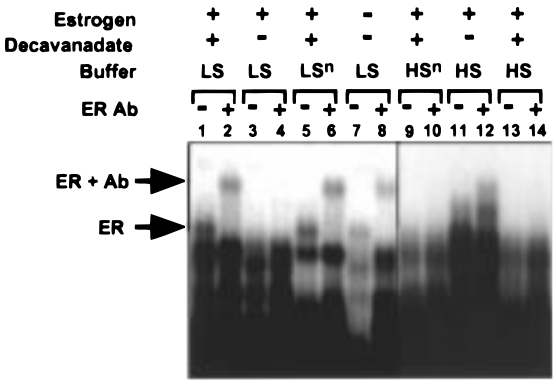

FIGURE 5: Gel shift assay demonstrating the DNA-binding activity of ER extracted by different methods. GH3 cells were extracted sequentially with low-salt buffer (LS) followed by high-salt buffer (HS). Cultures were treated with or without 20 nM E_2, and the low-salt buffer additionally contained 2 mM orthovanadate (decavanadate form) as indicated. Lanes 7 and 8 contain LS extract from untreated cells. All other lanes contain extracts from cells treated with E_2. Reactions containing the paired LS and HS extracts without decavanadate are in lanes 3, 4 and 11, 12, respectively. Reactions with the paired LS and HS extracts with decavanadate in the low-salt buffer are in lanes 1, 2 and 13, 14, respectively. The paired LSn and HSn extracts used in lanes 5, 6 and 9, 10 were prepared by sequential extraction of washed nuclei first with low-salt buffer plus decavanadate and followed by high-salt buffer. Antisera 1438 against rER were added to some reactions at a final dilution of 1:20 as indicated (±ER Ab). Extracts were incubated with ^{32}P-labeled ERE. Final salt concentrations in the reactions were 50 mM NaCl and 55 mM KCl for HS extracts and 65 mM NaCl and 50 mM KCl for LS extracts. Gel shift analysis and autoradiography were performed as described in Materials and Methods.

homogenized again with low-salt buffer containing 2 mM orthovanadate (decavanadate form) and centrifuged. The supernatant contains the liganded ER and is designated LSn in Figure 5. The pellet from the LSn preparation was then extracted with high-salt buffer (designated HSn), but does not contain ER as seen in lanes 9 and 10. This method showed completely comparable extraction of ER (data not shown), and this ER preparation bound to DNA (lanes 5 and 6) the same as the ER prepared by whole cell extraction with decavanadate (lanes 1 and 2). Nonspecific DNA binding was reduced in the LSn sample. Lanes 3, 4, 9, 10, 13, and 14 are subcellular fractions that do not contain ER as assessed by western blot and ^{3}H-estradiol-binding assays. The band we have identified as ER in this gel shift assay is missing in these lanes, as we would expect.

The western blot, ^{3}H-estradiol binding, DNA and antibody binding in the gel shift assays all show that the decavanadate-extracted ER is fully functional and function is unaffected by the presence of cytosolic proteins including hsp90. We performed western blots for phosphotyrosine on immunoprecipitated ER and did not observe an increase in phosphotyrosine on decavanadate-extracted ER (data not shown). These results suggest that release of the ER from the nucleus by decavanadate is not due to a reversal of transformation and probably not due to a covalent modification that can be seen structurally or by affecting function.

Increasing Concentrations of Orthovanadate in the Decavanadate but Not Monomeric Form Compete with DNA for ER Binding. Figure 6 shows the results of a gel shift assay with liganded rER prepared by extraction of the nuclear pellet of GH3 cells with low-salt buffer plus 2 mM orthovanadate (decavanadate form) (LSn ER). This rER

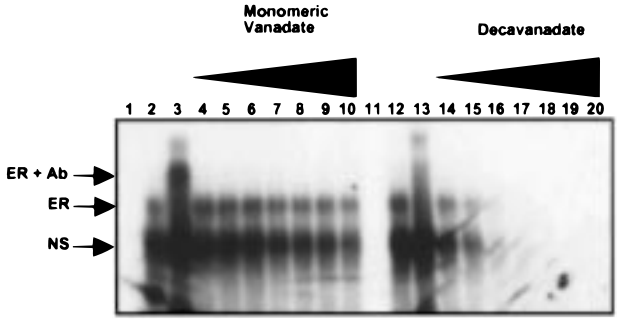

FIGURE 6: Effect of the dose of orthovanadate, in the decavanadate and monomeric forms, on the formation of the ER−DNA complex by gel shift analysis. GH3 cells were treated with E_2, nuclei prepared and washed, and then extracted with low-salt buffer plus 2 mM orthovanadate (decavanadate form). All of the DNA-binding reactions contain this extract and ^{32}P-labeled ERE oligonucleotide. Doubling concentrations of orthovanadate, in either the monomeric (lanes 4−10) or decavanadate (lanes 14−20) forms, from 0.08 to 5 mM were included directly in the reaction mix, and the final salt concentration was 100 mM KCl. Reactions in lanes 2, 3, 12, and 13 are controls without the addition of vanadate directly to the reaction. Reactions in lanes 3 and 13 include ER1438 antisera against rER at a final dilution of 1:10. Lanes 1 and 11 contain ^{32}P-labeled ERE oligonucleotide alone in the reaction without extract.

preparation was then incubated with labeled ERE in the presence of increasing concentrations of orthovanadate in either monomeric or decavanadate form. ER−ERE complexes were resolved from free ERE on an agarose gel. Lanes 2, 3 and 12, 13 are controls with no vanadate added to the ER incubation with ERE. The top band in lanes 2 and 12 is hER, and that band is supershifted by antibody in lanes 3 and 13. Orthovanadate (monomeric form) concentrations added to the incubation double from 0.08 to 5 mM in lanes 4−10 with only a slight decrease in the ER−DNA complex. Orthovanadate (decavanadate form) at the same concentrations in lanes 14−20 leads to a quantitative loss of the ER−DNA complex as well as the nonspecific complex (NS). The ER−DNA complex is completely competed away at 0.625 mM added orthovanadate (decavanadate form). Additional orthovanadate is in the assay from the LSn ER extract itself at a concentration of 0.5 mM. Therefore, complete loss of the ERE−DNA complex is seen by 1.125 mM orthovanadate (decavanadate form). These results show that polymeric vanadate, as opposed to monomeric vanadate, acts as a polyanion competitor for ER with DNA. This competition is fully reversible since dilution of the extract into the gel shift assay such that the orthovanadate concentration is reduced to 0.5 mM restores DNA-binding activity of the ER. These results were confirmed with ABCD assays (*22*). Two millimolar orthovanadate (decavanadate form) completely inhibited DNA binding, while monomeric vanadate at 2 mM or molybdate at 10 mM had very little effect (data not shown).

Decavanadate Extraction of the Nuclear Pellet Releases a Unique Liganded ER Complex as Characterized on Sucrose Density Gradients. The mechanism of tight nuclear binding by the liganded ER is not known; however it is probably bound to both DNA and other proteins. High-salt extraction of liganded ER presumably releases it from the nucleus by disruption of both types of interactions. Our data suggest that decavanadate extraction of liganded ER may be mediated by disruption of the interaction with the DNA. Vanadate

oligomers in the low concentrations used here (2 mM) can bind to positively charged proteins via electrostatic interactions (*60*−*62*). However, decavanadate appears to bind strongly only to polyphosphate-binding sites on proteins (*62*). Decavanadate may therefore release ER from the nucleus by disrupting interactions with DNA, while at least some estrogen-promoted interactions with other nuclear proteins may be maintained. We therefore hypothesize that the liganded ER extracted from the nucleus by decavanadate will be associated with a complex of protein that is distinct from that seen for the unliganded, low-salt-extracted ER or for the liganded, high-salt-extracted ER. We used sedimentation behavior on sucrose density gradients to characterize these different hER preparations from MCF-7 cells. MCF-7 cells have a higher concentration of ER than GH3 cells and were therefore chosen for these experiments. Step sucrose gradients containing low salt (0 mM KCl, Figure 7A), physiological salt (140 mM KCl, Figure 7B), or high salt (400 mM KCl, Figure 7C) were used. The sedimentation positions of aldolase as an 8S and albumin as a 4S size marker are shown by arrows for each type of gradient in Figure 7.

The ER extracted from the nuclear pellet with high salt is generally termed transformed ER (open circles in Figure 7) and behaves as previously reported in the low- and high-salt gradients shown in Figure 7 (ref *11* and references therein). It aggregates on the low-salt gradient (Figure 7A) and migrates near the 4S marker on the physiological (Figure 7B) and high-salt gradients (Figure 7C). The transformed form of ER has been well-characterized as a homodimer without hsp90 that should be a 5S peak, but our step gradient system does not resolve the 4S versus 5S ER complexes reported by other groups. The transformed ER exhibits a second peak on the high-salt gradient (Figure 7C) that migrates between the 4S and 8S markers, and this peak is more prominent on the physiological salt gradient (Figure 7B).

The sedimentation behavior of the nontransformed ER (open squares in Figure 7) was consistent with previous reports (ref *11* and references therein). This form of ER is prepared by low-salt extraction of cells that have not been treated with estrogen. The receptor is then occupied in vitro on ice before loading onto the gradients. This form of ER is associated with hsp90 and other cytosolic proteins and migrates as a large complex near the 8S marker in low-salt gradients (Figure 7A). In salt-containing gradients this complex dissociates and the ER migrates over a range of lower densities down to the 4S marker (Figure 7B,C).

The sedimentation behavior of the liganded ER prepared by extraction of washed nuclei with low-salt buffer plus decavanadate (LSn ER, closed circles in Figure 7) is distinct from the transformed and nontransformed ERs. This LSn preparation of liganded ER is free of most cytosolic proteins but has not been subjected to 0.6 M salt, unlike the transformed ER. The LSn ER migrates as a single peak between the 8S and 4S size markers under all three salt conditions. The LSn ER does not show aggregation on low-salt gradients (Figure 7A) unlike the transformed ER, nor does it dissociate to a smaller complex on high-salt gradients (Figure 7C) unlike the nontransformed ER. These data suggest that the LSn ER is not a nontransformed receptor associated with hsp90, which is consistent with its ability to bind DNA as shown in Figures 5 and 6. The LSn preparation

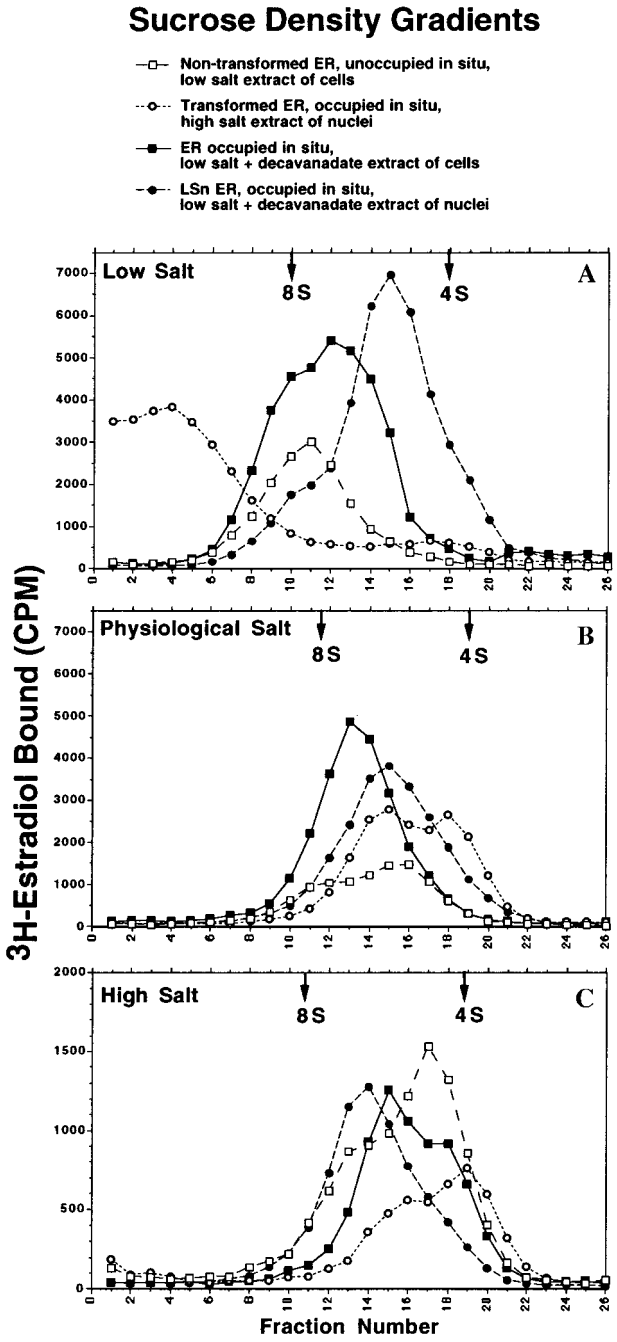

FIGURE 7: Sedimentation analysis of various ER extracts on sucrose density gradients. Step sucrose gradients in 0 (low salt, panel A), 140 (physiological salt, panel B), or 400 mM KCl (high salt, panel C) were constructed as described in Materials and Methods. Four different preparations of hER from MCF-7 cells were analyzed on each type of gradient. Nontransformed ER was prepared by extraction of vehicle- (no estradiol) treated cells with low-salt buffer and subsequent occupation of the ER with 10 nM $^3H-E_2$ in vitro (open squares). Transformed ER was prepared by extraction of the nuclei from $^3H-E_2$-treated cells with high-salt buffer (open circles). Decavanadate released ER was prepared from $^3H-E_2$-treated cells by either extraction of the whole cells (closed squares) or washed nuclei (LSn ER, closed circles) with low-salt buffer plus 2 mM orthovanadate (decavanadate form). Gradients were run and fractionated, and $^3H-E_2$ bound to ER quantitated by HAP assay of each fraction as described in Materials and Methods. Nonspecific $^3H-E_2$ binding was identical to the background as evaluated by adding 200-fold excess unlabeled diethylstilbestrol to the cells prior to extraction and running the gradients.

of the liganded ER also appears to be in a salt-resistant complex that is larger than the well-characterized peak for

transformed ER which migrates near the 4S marker. We do observe a second peak of transformed ER that migrates between the 4S and 8S markers which has some overlap with the peak of LSn ER on the salt-containing gradients (Figure 7B,C). This peak may represent ER in complex with additional proteins.

The sedimentation behavior of liganded ER prepared by extraction of whole cells with low-salt buffer plus decavanadate is also shown in Figure 7 (closed squares). This preparation of the liganded ER (closed squares) contains cytosolic proteins like the nontransformed ER preparation (open squares). On the low (Figure 7A) and physiological (Figure 7B) salt gradients this form of ER behaves as a larger complex than the LSn ER and has some overlap with the nontransformed ER. On a high-salt gradient the two preparations of decavanadate-extracted ER are similar (Figure 7C). These results suggest that, when cytosolic proteins are present, the decavanadate-extracted ER can form larger complexes with them that can be disrupted by salt.

DISCUSSION

This study describes the use of decavanadate to prepare nuclear, liganded ER that is distinct from the classical transformed ER obtained by high-salt extraction. The decavanadate-extracted, liganded ER is fully functional in standard in vitro assays for hormone and DNA binding and is full-length by western blot analysis. However, its sedimentation behavior on sucrose density gradients is distinct from the transformed or nontransformed ER preparations. It appears to be a larger complex than the homodimer of the transformed ER and a smaller complex than the hsp90-associated nontransformed ER. We hypothesize that our method of extracting the liganded ER from washed nuclei with low-salt buffer plus decavanadate (LSn ER) yields a complex of ER with one or more specific nuclear proteins. We have preliminary data that an approximately M_r 200 000 protein can be co-immunoprecipitated with antibodies against ER from the LSn ER, but not the HS ER preparation (data not shown). These preliminary results are consistent with the larger complex seen on sucrose gradients for the LSn ER being due to the presence of at least one other protein in addition to ER. Studies are underway in our laboratory to characterize the components of the LSn ER complex.

Our data suggest that the mechanism of decavanadate action on ER subcellular fractionation is disruption of ER−DNA binding as opposed to inhibition of a phosphotyrosine phosphatase. We exclude phosphotyrosine phosphatase inhibition as the mechanism because of the lack of activity of other phosphotyrosine phosphatase inhibitors including monomeric vanadate. In addition, ER extraction requires a higher dose of vanadate than generally used for phosphatase inhibition. We also exclude reversal of transformation or interaction with hsp90 as the mechanism of decavanadate extraction. Although vanadate can stabilize the nontransformed ER, we show that another transition-state metal that is used to stabilize nontransformed ER, molybdate, cannot extract liganded ER from the nucleus. We therefore conclude that decavanadate is not acting by affecting the transformation state of ER. Our data also show that decavanadate is effective at extraction of liganded ER from either whole cells or washed nuclei. Therefore, it is unlikely that the presence of cytosolic proteins is required for the extraction of liganded ER. We do observe a dramatic disruption of ER−DNA interactions by decavanadate in vitro that is not accomplished by monomeric vanadate. Decavanadate is a polymeric anion with some structural similarities to polyphosphate, and so it is not surprising that millimolar concentrations can competitively inhibit ER−DNA interactions (*40*, *42*). This mechanism is not expected to be specific for disrupting ER interactions with DNA and subsequent release from the nucleus. Extraction of nuclear proteins would be expected to be proportional to the importance of the DNA binding to holding that protein in the nucleus. In fact, we extract far more total protein from the nucleus with decavanadate than can be accounted for by ER alone, as determined by total protein assays (data not shown). Furthermore, two of the three specific transcription factors we examined showed partial release from the nucleus by 2 mM orthovanadate (decavanadate form) extraction. More efficient release of other transcription factors might be achieved by optimizing the concentration of decavanadate.

The mechanistic basis for the tight nuclear binding of ER upon treatment of cells with estrogen is unknown. Tight nuclear binding of ER is one of the most dramatic biochemical responses to estrogen and has been the target of numerous investigations dating to the earliest biochemical characterization of the receptor (ref *11* and references therein). Essentially all of the ER protein becomes tightly associated with the nucleus within minutes of exposure to saturating concentrations of estrogens. This association of ER with the nucleus is not reversible in the presence of estrogen, and extraction with high-salt is necessary. Tight nuclear binding of the ER is required for estrogen signaling, as shown in studies that correlated estrogen responses with time of ER localization to the nuclear fraction (*15*). One model is that estrogen binding to ER allows it to bind to DNA and that this may be the basis for tight nuclear binding. However, ER−DNA-binding studies conducted in vitro have clearly shown that estrogen occupancy of ER is not required for high-affinity binding to a specific ERE (*22*−*26*). In previous work, we measured the relative affinities of ER for an ERE compared to bulk DNA (*63*). Although the affinity of ER for the ERE was 5 orders of magnitude higher than for bulk DNA, the enormous concentration of bulk DNA in the nucleus is sufficient to drive interaction with ER. We proposed that both the liganded and the unliganded ER were bound to DNA in the whole cell and that this might be an important mechanism for the concentration of ER in the nucleus. Upon breaking open the cell and dilution of the nuclear contents into low-salt buffers, the unliganded ER simply dissociates from the DNA and is recovered in the post-ribosomal supernatant fraction. We propose that the liganded ER forms estrogen-promoted interactions with nuclear proteins that stabilize its association with DNA. This complex of ER with other nuclear proteins has such a high avidity for DNA that 0.4−0.6 M salt must be included in dilution buffers in order to efficiently extract it from the nucleus.

We hypothesize that decavanadate releases an E_2−ER−nuclear protein complex from DNA in the nucleus by a competitive mechanism. The associated nuclear proteins in this complex are dependent upon estrogen occupancy for binding to ER. Considerable work has been directed at the characterization of ER-associated proteins in the unliganded,

nontransformed complex and the role of these proteins in estrogen signaling and the maintenance of ER functionality (*35*, *64*). High-salt extraction of the liganded, tightly nuclear bound ER probably disrupts many of the protein−protein contacts promoted by estrogen as well as the ER−DNA interaction. Therefore, the high-salt-extracted ER is well-characterized as an ER homodimer, but the ability to assess interactions with other nuclear proteins has been limited. The overlap between the denser of the two peaks of the transformed ER with the peak of LSn ER on salt-containing sucrose gradients may indicate that a sub-population of ER−nuclear protein complexes survives salt extraction. We believe the relatively gentle release of ER−nuclear protein complexes from the nucleus by decavanadate will provide the material to make the biochemical characterization of these nuclear proteins feasible. We cannot exclude the possibility that decavanadate may also disrupt some ER−nuclear protein interactions, but the sucrose gradient data suggest that at least some unique ER−nuclear protein complexes are maintained. We hypothesize that estrogen-promoted ER interactions with specific nuclear proteins occur on the DNA and account for the estrogen-induced tight nuclear binding of ER.

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