Journal of Cellular Biochemistry 93:358–373 (2004)

Binding of MCF-7 Cell Mitochondrial Proteins and Recombinant Human Estrogen Receptors α and β to Human Mitochondrial DNA Estrogen Response Elements

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Abstract Our previous studies have shown that 17β estradiol (E2) enhances the transcript levels of mitochondrial DNA (mtDNA)-encoded genes and mitochondrial respiratory chain (MRC) activity via estrogen receptors (ER). Others have reported the presence of putative estrogen responsive elements (ERE) in human mtDNA (mtEREs) and detection of ERs in mitochondria of rat uterine and ovary cells. Recently, we demonstrated the E2-enhanced mitochondrial localization of ERα and ERβ, and E2-induced mtDNA transcript levels in MCF-7 cells. In this study, we applied electrophoresis mobility shift assays (EMSAs) and surface plasmon resonance (SPR) to determine if mitochondrial extracts, recombinant human ERα (rhERα), and rhERβ interact with mtEREs. Using EMSAs, we observed that ER-containing mitochondrial extracts bound to mtEREs and the binding was enhanced by E2, whereas the binding of mitochondrial proteins from ERβ-deficient cells was almost undetectable. Both rhERα and rhERβ bound to the mtEREs and their binding was altered by their respective antibodies. However, the ERα antibodies did not alter the binding of MCF-7 cell mitochondrial extracts to mtEREs whereas the binding MCF-7 and MDA-MB-231 cell mitochondrial extracts to mtEREs was reduced by ERβ antibody. These results suggest that the mtERE-bound mitochondrial protein is ERβ. Using SPR, we observed the binding of both ERs to mtEREs and that the binding was increased by E2. These results indicate that the mitochondrial ERs can interact with mtEREs and suggest that they may be directly involved in E2 induction of mtDNA transcription. J. Cell. Biochem. 93: 358–373, 2004.

Key words: 17β-estradiol; ERα and ERβ; mitochondrial DNA; estrogen response elements; mitochondrial DNA transcription; surface plasmon resonance; estrogen carcinogenesis

Non-Standard Abbreviations Used: BSA, bovine serum albumin; CO I, CO II, cytochrome oxidase subnunit I and II; DTT, dithiothreitol; E2, 17β-estradiol; EE, ethinyl estradiol; ERE, estrogen response element; D-loop ERE, displacement-loop ERE; EDTA, ethylene diamine tetracetic acid; EMSA, electrophoresis mobility shift assays; ER, estrogen receptor; ERα, estrogen receptor α; ERαKO, ERα knock out; ERβ, estrogen receptor β; ERβKO, ERβ knock out; FBS, fetal bovine serum; GRE, glucocorticoid response element; HEPES, 2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; HRP, horse raddis peroxide; mtEREs, mitochondrial DNA EREs; MRC, mitochondrial respiratory chain; mtDNA, mitochondrial DNA; mtRNA, mitochondrial RNA; NP-40, nonidet P 40; Ovx, overiectomized mice; PMSF, phenylmethylsulfonyl fluoride; rhERα, recombinant human ERα; rhERβ, recombinant human ERβ; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPR, surface plasmon resonance;*Xenopus* vit ERE, *Xenopus* vitellogenic ERE.

Grant sponsor: NIH; Grant numbers: CA36701, CA77550; Grant sponsor: DHHS/CDC; Grant numbers: RO4/CCR, 419466-01; Grant sponsor: Center Grant; Grant number: P30ES03819; Grant sponsor: W.M. KECK Foundation; Grant sponsor: The U.S. Department of Agriculture; Grant number: USDA 58-6435-7-019.

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Received 14 February 2004; Accepted 27 April 2004

DOI 10.1002/jcb.20178

17β-estradiol (E2) and other estrogens are carcinogenic in several tissues of experimental animals [Ishman et al., 1995; Feigelson and Henderson, 1996; Yager and Liehr, 1996; Yager, 2000] and estradiol is a major etiological factor for the development and progression of human breast cancer [Feigelson and Henderson, 1996; Yager, 2000; Russo et al., 2002]. The synthetic estrogen, ethinyl estradiol (EE) is a strong promoter of hepatocarcinogenesis and weak complete hepatocarcinogen in rats [Yager and Liehr, 1996]. We identified several nuclear- and mitochondrial DNA (mtDNA)-encoded mRNA transcripts (mtRNA) encoding for mitochondrial respiratory chain (MRC) protein subunits whose levels were enhanced in liver of EE-treated female rats [Chen et al., 1996, 1998]. Both EE and E2 had similar effects on mtRNA levels in cultured rat hepatocytes, human HepG2 cells [Chen et al., 1996, 1998], and human MCF-7 cells [Chen et al., 2004, in press]. These estrogen effects were accompanied by increases in MRC activity and both nuclear and mitochondrial GSH levels [Chen et al., 1996, 1998, 1999, 2003] and were associated with inhibition by EE of TGFβ-induced apoptosis [Chen et al., 2000]. Inhibition of these EE and E2 effects by the anti-estrogen ICI 182780 demonstrated that they were mediated through the estrogen receptor (ER) [Chen et al., 1999].

The effects of estrogens on mitochondrial structure and biogenesis have been documented [Vic et al., 1982; Tam and Wong, 1991]. The studies by Zhai et al., however, provide the most convincing evidence for a role of estrogens and ER in preservation and regulation of mitochondrial structure and function [Zhai et al., 2000a,b, 2001]. These investigators examined the structure and function of myocardial mitochondria in overiectomized (Oxv) and ERα knock out (ERαKO) rats in comparison with those in control and wild type animals in response to ischemia-reperfusion. They observed that the myocardial mitochondria of control and wild type rats contained clearly defined cristae and normal matrix, whereas the myocardial mitochondria of Oxv and ERαKO rats were predominantly abnormal in shape, with abnormal cristae, and loss of the matrix area. The mitochondrial membranes were occasionally disrupted. The MRC activities were significantly lower in mitochondria from Oxv and ERαKO hearts. They also observed that the abnormal mitochondrial structure and the defective MRC activities were associated with the abnormality of several energy-dependent physiological processes in Oxv and ERαKO hearts. Interestingly, when the Oxv animals were treated with estrogens or phytoestrogens, the abnormality of mitochondrial structure/function and several energy-dependent physiological processes were restored to normal. Moreover, the effects of phytoestrogens in Oxv hearts were inhibited by co-treatment with the ER antagonist, ICI 182780. Additional studies using ERβ knock out (ERβ KO) mice [Zhu et al., 2002] provided indirect evidence suggesting a role for ERβ in the regulation of energy-dependent physiological processes. However, the determination of whether and how E2 and ERs (α and/or β) are directly involved in these effects required additional investigation.

While the mechanisms of these E2-mediated mitochondrial effects are unknown, several reports suggest that a fraction of total cellular estrogen-binding capacity and estrogen-binding proteins are associated with mitochondria [Noteboom and Gorski, 1965; Grossman et al., 1989; Moats and Ramirez, 1998, 2000]. Using Western blot and ligand blot analyses, Monje and Boland [2001] observed that in rabbit ovary tissue, ERα and ERβ are predominately associated with cytosolic components. In rabbit uterine tissue, the major proportion of the total estrogen binding capacity and of ERα, ERβ, and other binding proteins was detected in mitochondria and microsomes. The mitochondrial-enriched sub-fraction represented an important source of E2 binding in a stereospecific and high affinity manner. Furthermore, human mtDNA contains putative estrogen response elements (EREs) [Sekeris, 1990; Demonacos et al., 1996]. Recently, we demonstrated that ERα and ERβ are present in the mitochondrial matrix of MCF-7 [Chen et al., 2004, in press], and HepG2 cells and that ERβ is present mitochondria of rat hepatocytes [Chen et al., unpublished observation]. Moreover, we observed that E2 enhanced the mitochondrial levels of both ERs, accompanied by concomitant increases in mtDNA transcript levels and that these E2-mediated effects were mediated via ERs [Chen et al., 2004, in press]. Consistent with these findings, others recently reported mitochondrial localization of ERβ in rat primary neuron, primary cardiomyocyte, murine hippocampal cell lines and human heart cells [Yang et al., 2004], and in cultured human lens epithelial

cells [Cammarata et al., 2004]. Together, these observations suggest that mtDNA could be a direct target for E2 acting through the mitochondrial ERs. The goals of this study were (1) to investigate whether mitochondria contain mtERE-binding proteins; and (2) to determine whether the protein (s) that bind to mtEREs are ERs. As mentioned above, mitochondrial DNA contains putative EREs. Thus, our third goal was to use the technique of surface plasmon resonance (SPR) to determine whether mtEREs can bind ERα and ERβ, in order to determine whether they have the functional capacity to mediate transcript through the ERs present in mitochondria.

MATERIALS AND METHODS

Chemical Reagents

Reagents for gel electrophoresis, for example, sodium dodecylsulfate (SDS), bis-[tris(hydroxymethyl) methylamino] propane (bis-tris propane), were purchased from Bio-Rad (Hercules, CA), glycerol and ethylene diamine tetracetic acid (EDTA) from PanVera (Madison, WI), dithiothreitol (DTT), E2 from Sigma Chemical Co. (St. Louis, MD), dimethylsulfoxide and potassium chloride from Fisher Scientific (Pittsburgh, PA). The RNAzol B kit was from Tel-Test, Inc. (Friendswood, TX), the Random Labeling Kit from Roche Diagnostic Corporation (Indianapolis, IN), and the ECL-Western blotting detection reagents from Amersham/Pharmacia (Buckinghamshire, England). Sensor chips for the Biocore 2000 activated with streptavidin (SA) chips, and buffer B containing 0.01 M N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.4, 0.15 M NaCl, 0.003 M EDTA and 0.005% (v/v) sulfatant P20 were obtained from Biocore, Inc. (Piscataway, NJ).

Antibodies and Recombinant Human ERα (rhRα) and RhERβ

Polyclonal antibodies for human ERα (ERα antibody A1) and ERβ?, secondary goat anti-rabbit IgG-horse radish peroxidase (HRP) and goat anti-mouse IgG-HRP were purchased from Santa Cruz Biotech (Santa Cruz, CA). Monoclonal ERα antibody (ERα antibody A2) was obtained from Immunotech (Beckman Coulter, Columbia, MD). Monoclonal antibody for human mitochondrial genome-encoded cytochrome oxidase subunit II (CO II) was obtained from Molecular Probes (Eugene, OR). RhERα and rhERβ (long form) were purchased from PanVera.

Cell Culture and Treatment

Human breast tissue-derived MCF-7, MCF-10A, and MDA-MB-231 cells, obtained from ATCC (Manassas, VA), were chosen as the experimental models for this study because they have different ER status. MCF-7 cells express both ERα and ERβ [Mann et al., 2001]. MCF-10A cells are negative for ERβ expression [Lane et al., 1999]. MDA-MB-231 cells express ERβ but not ERα [Jiang and Jordan, 1992; Frigo et al., 2002; Chen et al., 2004, in press]. MCF-7 and MDA-MB 231 cells were cultured in phenol red-free IMEM (Biofluids, Bioscience International, Camarilla, CA) and DMEM (Roche), respectively, containing 5% fetal bovine serum (FBS) (Hyclone, Logan, UT) and gentamycin (Roche). MCF-10A cells were cultured in phenol red-free DMEM/F12 (Roche) containing 5% horse serum (Roche) plus insulin (10 μg/ml), hydrocortisone (0.5 μg/ml), EGF (20 ng/ml), cholera toxin (1 μg/ml), and gentamicin. For gel electrophoresis mobility shift assays (EMSAs), mitochondrial and nuclear protein extracts were prepared from cells pre-treated with E2 (100 or 300 nM) for 12 hours. In experiments examining the time- and concentration-effects of E2, the cells were cultured in medium containing 5% charcoal-stripped FBS for 7 days prior to E2 treatment. For determination of E2 time course effects, the cells were treated with E2 at 100 nM for 3, 6, 12, and 24 h. For determination of E2 concentration effects, the cells were treated with E2 at 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M for 12 h.

Cell Fractionation and Preparation of Mitochondria

Cell fractionation and preparation of mitochondria were performed as described previously [Pedersen et al., 1978; Chen et al., 1999, 2004, in press]. Briefly, MCF-7, MCF-10A, and MDA-MB-231 cells grown to 80% confluence were treated with E2 (100 nM) for 12 h. The cells were washed twice with cold phosphate-buffered saline (PBS) buffer. The cells were suspended in cold mitochondrial buffer [70 mM sucrose, 220 mM mannitol, 2 mM HEPES (pH 7.4), 2.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5% bovine serum albumin (BSA)] plus freshly added 2 mM phenylmethylsulfonyl

fluoride (PMSF), 5 μg/ml Leupeptin, 5 μg/ml aprotinin, 2 mM DTT. The cells were homogenized in tight fitting 15 ml Teflon-glass homogenizer using a homogenizer drive unit (Bellco Biotechnology, Vineland, NJ). Fractionation of the homogenates (H) was performed via differential centrifugation [Pedersen et al., 1978]. The initial 1,000*g* pellets represented the crude nuclear fractions. The 1,000*g* supernatant was centrifuged at 10,000*g* for 15 min and the supernatant represented the cytosolic fraction. Mitochondria in the 10,000 pellet were suspended in mitochondrial buffer and further enriched by centrifugation on a sucrose/percoll gradient as described [Demonacos et al., 1995]. Where indicated, the purified mitochondria were subjected to trypsin digestion at 1 μg/ml or as indicated for 30 min at 4°C. After trypsin digestion, the mitochondria were pelleted by centrifuging at 10,000*g*. This approach has been used by others in determinations of the intramitochondrial localization of other receptors classically thought to reside only in the nucleus, for example, TR3 [Li et al., 2000], the thyroid hormone receptor [Casas et al., 1999], and the glucocorticoid receptor [Demonacos et al., 1993].

Western Blot Analysis

Western blot analysis was used to detect ERα, ERβ, and CO II. Crude nuclear (1,000*g* pellet), cytosolic (10,000*g* supernatant), and mitochondrial (10,000*g* pellet) fractions prepared from MCF-7, MCF-10A, and MDA-MB-231 cells pretreated with E2 were obtained by differential centrifugation and the 10,000*g* pellet containing mitochondria was further purified as described above. Proteins (30–50 μg/lane) of these fractions were separated by SDS–polyacrylamide gel electrophoresis (PAGE) on 4–12% acrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked using 5% non-fat milk in 100 ml buffer (20 mM Tris, pH 7.4, 140 mM NaCl and 0.05% Tween 20) and probed using the same buffer with antibodies for ERβ and ERα (1:500 dilution) and CO II (1:1,000 dilution) for 1 h. After washing, the membranes were incubated with secondary goat anti-rabbit IgG-HRP for ERβ and ERα and goat anti-mouse IgG- HRP for CO II (1:1,500 dilution) for 1 h. The membranes were developed using ECL-Western blotting detection reagents.

Design, Synthesis, and End-labeling of Oligonucleotides

Four putative estrogen responsive elements (ERE) sequences identified in the D-loop and one in the coding region of human mtDNA [Sekeris, 1990; Demonacos et al., 1996), designated as D-loop ERE I, ERE II, ERE III, ERE IV, and CO II ERE respectively, were selected for this study (Table I). The *Xenopus* vitellogenin ERE (*Xenopus* vit ERE), the c-myc half ERE/

TABLE I. Oligonucleotides Used in Study

Name	DNA sequences
Xenopus Vit ERE	5′-**X**GTCCAAAGTCA**GGTCACAGTGAC**CTGCTCAAAGTT-3′ 3′-CAGGTTTCAGT**CCAGTGTCACTG**GACGAGTTTCAA-5′
Human pS2 ERE	5′-**X**GTCCAATCA**GGTCACGGTGGCC**TGATCAAGTT-3′ 3′-CAGGTTAGT**CCAGTGCCACCGG**ACTAGTTCAA-5′
c-myc half ERE/sp1	**5′**-***AGCTT*****GGGCA**GGGCTTCTCAGAGGCTTGGCGGG*AAGGATCCA*-3′ **3′**-***A*****CCCGT**CCCGAAGAGTCTCCGAACCGCCC*TTCCT*AGG***TTCGA***-5′
Cathepsin D ERE	5′-***AGCTT*****CGGGTCGCTGATCC***GGATCCA*-3′ 3′-***AGCCCAGCGACTAGG****CCTAGG****TTCGA***-5′
D-loop ERE I (16134–16150)*	5′-***XAGCTT*****ATAAA**CTT**GACCA***GGATCCA*-3′ 3′-***ATATTTGAACTGGT****CCTAGG****TTCGA***-5′
D-loop ERE II (16362–16378)*	5′-***AGCTT*****CCCCA**TGGAT**GAC**CC*GGATCCA*-3′ 3′-***AGGGGTACCTACTGGG****CCTAGG****TTCGA***-5′
D-loop ERE III (16378–16403)*	5′-***XAGCTT*****GGGGT**CCCTT**GACCA***GGATCCA*-3′ 3′-***ACCCCA**GGGAA**CTGGT****CCTAGG****TTCGA***-5′
D-loop ERE IV (16514–15532)*	5′-***AGCTT*****AGGTCATAAAGCCTA***GGATCCA*-3′ 3′-***ATCCAGTATTTCGGAT****CCTAGG****TTCGA***-5′
CO II ERE (7850–7860)*	5′-**AGCTT****AGGTCAA**CGA**TCCCT***GGATCCA*-3′ 3′-**AT****CCAGTTGCTAGGGA***CCTAGG****TTCGA***-5′
Randomly mutated ERE	5′-***XAGCTT*****AAAAGCCTTATTTGAAA***GGATCCA*-3′ 3′-***ATTTTCGGAATAAACTTTT****CCTAGG****TTCGA***-5′
Non-related DNA fragment	5′-***AGCTT***AGCTTCTCTGTGATTTAATGCCA*GGATCCA*-3′ 3′-***A***TCGAAGAGACACTAAATTACGG*CCTAGG****TTCGA***-5′
GRE concensus sequence	5′-**X**GTCCAAGTCA**GAACA**C**A****GTGTTC**TGACAAAGTT-3′ 3′-CAGGTTCAGT**CTTGTGTCACAAG**ACTGTTTCAA-5′

X, biotinylated nucleotide.
*indicates the locations in human mtDNA; the putative half EREs are bold.

sp1, the pS2 ERE, and the cathepsin ERE sequences were selected as positive controls. A randomly mutated sequence in place of the D-loop ERE III and a non-related DNA sequence containing no ERE sequence, and the glucocorticoid response element (GRE) were included as negative controls (Table I). Sense and antisense single-strand oligonucleotides containing each of these sequences were custom synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) and annealed under conditions described previously [Tsiriyotis et al., 1997]. After annealing, each oligonucleotide contains sticky ends that were filled with dNTPs (dATP, dGTP, and dTTP) plus ^{32}P-dCTP using the Random Primed DNA labeling Kit. For the SPR analysis, the 5′-end of the sense strand of the indicated oligonucleotides was biotinylated by incorporation of biotin-dATP with Klenow enzyme and purified from unincorporated biotinylated nucleotides by gel filtration on a Chromass-spin 10 column (BD Biosciences Clontech, Palo Alto, CA). The biotin moiety allows the immobilization of the biotinylated oligonucleotide on the SA-modified sensor chip surface [Cheskis et al., 1997].

Electrophoresis Mobility Shift Assays (EMSAs)

EMSAs were performed with nuclear and mitochondrial protein extracts. Nuclear proteins used for EMSAs were purified further from crude nuclear pellets. Briefly, the 1,000x g pellet was resuspended in mitochondrial buffer (see above), homogenized again, and centrifuged at 1,000*g* for 10 min; this procedure was repeated three times. The 1,000*g* pellet was resuspended in lysis buffer I [10 mM HEPES, pH 7.8, 1 mM EDTA, 60 mM KCl, and 0.5% nonidet P 40 (NP-40) plus freshly added 1 mM DTT, 1 mM PMSF, 5 μg/ml Leupeptin, 5 μg/ml aprotinin] and centrifuged at 15,000*g* for 1 min. The pellet was resuspended in lysis buffer II (same as lysis buffer I but without 0.5% NP-40) and centrifuged again. The pellet was resuspended in nuclear resuspension buffer (25 mM Tris, pH 7.8, 55 mM KCl, 2.5 mM HEPES, 30 mM NaCl, 1 M urea, 0.5 % NP-40 plus freshly added 2 mM DTT, 2 mM PMSF, 5 μg/ml Leupeptin, and 5 μg/ml aprotinin). The suspension was rotated at 4°C for 30 min and then centrifuged at 15,000*g* for 5 min. The supernatant represented the nuclear protein fraction for EMSAs. In most cases, the sucrose/percoll gradient enriched mitochondria were first digested with trypsin (1 μg/ml) for 30 min at 4°C, then pelleted at 10,000*g* to remove nuclear and cytosolic residues, and then resuspended in the nuclear suspension buffer to provide the mitochondrial protein extracts. For EMSA experiments, mitochondrial protein extracts or nuclear protein extracts (10 μg/assay) were incubated with the relevant ^{32}P-labeled (2×10^6 cpm/assay) oligonucleotides for 30 min at room temperature in binding buffer (10 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 1 mg/ml dIdC, 100 mM KCl). In the competition studies described in Figure 1, a 20 and 100 molar excess of the indicated non-radioactive oligonucleotides was incubated with the ^{32}P-labeled complementary oligonucleotides. EMSAs were performed in 7% non-denaturing polyacrylamide gels at 200–250 V for 3 to 3.5 h. The gels were put on Beckman filter paper, dried under vacuum, and exposed to X-ray film overnight.

SPR Analysis of the Binding of rhERα and rhERβ to the D-Loop EREs

The Biosensor system, for example, Biacore 2000 Instrument equipped with Biacore control software version 1.2 (Biacore, Inc., Piscataway, NJ), permits the observation and measurement of macromolecular interactions in real time by SPR, a quantum mechanical phenomenon that detects changes in the refractive index at the surface of sensor chip [Rich and Myszka, 2001; Rich et al., 2001; Day et al., 2002]. The binding of the ligands in solution to immobilized molecules results in an increase in the ligand concentration at the sensor surface, with a corresponding increase in the refractive index. The change in refractive index (SPR response) is detected optically. This technology has been applied by other investigators to estimate the binding kinetics between EREs and ERs in the presence or absence of E2 and other ligands [Cheskis et al., 1997; Jisa et al., 2001]. Using this technique, we performed studies on the binding of rhERα and rhERβ to D-loop ERE I and ERE III in the presence and absence of E2. Modification of the sensor chip surface with SA and immobilization of biotinylated oligonucleotides were performed according to the Biacore 2000 instrument manual. The biotinylated oligonucleotides containing the mtDNA D-loop ERE I, III, randomly mutated ERE sequence, and the GRE sequences were immobilized on the surface of the SA sensor chips by injecting 10 μl of a

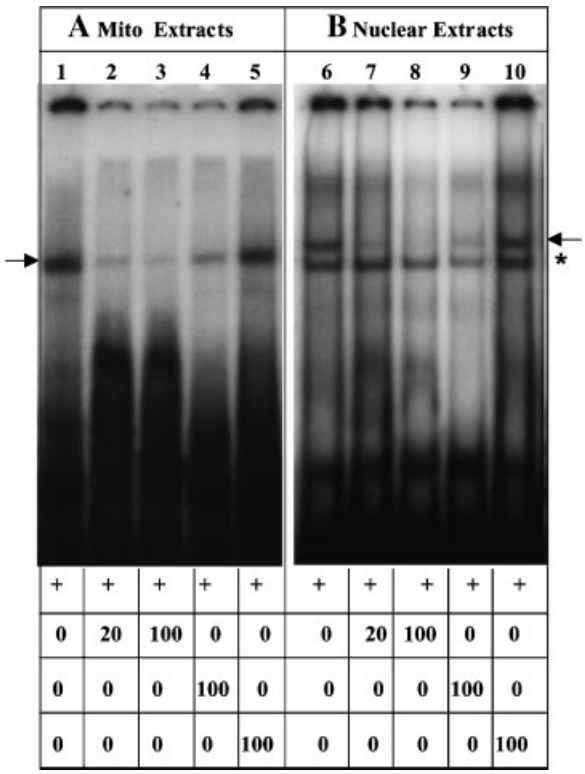

Fig. 1. Specificity of binding of mitochondrial proteins and nuclear proteins to D-loop ERE I determined by EMSAs. Mitochondrial (**A**) or nuclear (**B**) protein extracts from E2-treated MCF-7 cells were incubated with ^{32}P-labeled D-loop ERE I in the absence (**lanes 1** and **6**) or presence of the indicated molar excess of unlabeled D-loop ERE I (**lanes 2–3** and **7–8**) or the unlabeled c-myc half ERE/sp1 (**lanes 4** and **9**), unlabeled myogenin promoter lacking an ERE representing unrelated probe (lanes 5 and 10). EMSAs were performed as described in Materials and Methods. Gels A and B were run separately. Arrows indicate the retarded band representing the protein-D-loop ERE complex formed by both mitochondrial and nuclear protein extracts (A) and (B). * indicates the lower protein-D-loop ERE complex formed by nuclear protein extracts.

10 ng/μl solution of each of above DNA oligonucleotudes over the surface of the SA sensor chips using buffer B (described above). Sufficient oligonucleotide was bound to surface of the chip to produce an increase of 750–1,000 resonance units (RU). Three flow cells were used simultaneously for each sample run: the SA chip surface of the first cell contained bound D-loop ERE I, III, or the randomly mutated ERE sequence, the SA chip surface of the second cell contained no immobilized DNA (blank), and the SA chip surface of the third cell contained the GRE sequence. All binding experiments were performed at 25°C. RhERα or rhERβ (300 nM, each) was first incubated with or without a saturating concentration of E2 (1 μM) in buffer A (100 μM potassium phosphate, pH 7.5, containing 10 μg/ml acetatyled bovine gamma globulin and 0.02% sodium azide) for 1 h. The saturating E2 concentration was calculated based on IC50 values for the binding of E2 to receptors that had been determined previously [Nikov et al., 2000]. ER or the E2-ER complex (50 μl) was injected over the surface of the sensor chip containing the immobilized oligonucleotide. Each ER or E2-ER ligand-receptor complex was allowed to flow over the surface of the sensor chip for 10 min. After 10 min, the sensor chips were washed with the same buffer for an additional 10 min. The chip surfaces were regenerated by washing the E2-ER complex from the immobilized DNA using 10 μl of 0.1% SDS. Each binding experiment was performed at least twice. The interactions of ER or the E2-ER complexes with the immobilized mtDNA D-loop EREs, that is, the time-dependent changes in refractive index, were monitored and recorded with the Biocore 2000 Instrument as changes in RU value.

Reproducibility of Results

Each experiment was repeated at least twice and some three times. Duplicate and triplicates were included in each treatment group.

RESULTS

Binding of Mitochondrial Protein to Putative mtDNA D-Loop EREs

EMSAs were used to determine whether MCF-7 cell mitochondria contain a protein(s) capable of binding to several oligonucleotides containing putative mtEREs present in human mtDNA. As positive controls, we used EREs present in several nuclear genes whose expression is induced by E2. Figure 1 shows that mitochondrial protein extracts prepared from E2-treated MCF-7 cells bound to ^{32}P-labeled oligonucleotides containing the putative D-loop ERE I (lane 1). In separate experiments, we observed that MCF-7 cell mitochondrial protein extracts also bound to the oligonucleotides containing D-loop EREs II, IV and the CO II ERE, as well as oligonucleotides containing the nuclear pS2, cathepsin-D, and *Xenopus* vitellogenin EREs (data not shown). Similar to the mitochondrial protein extracts, MCF-7 cell nuclear extracts bound to D-loop ERE I (Fig. 1, lane 6) and the same set of mtDNA EREs and nuclear DNA EREs as the mitochondrial

extracts (data not shown). The nuclear proteins formed two retarded bands, one of which (the upper band) was identical to the one formed by mitochondrial protein extracts (see below). These results suggested that MCF-7 mitochondria contain a protein (s) that bind to putative mtDNA and nuclear DNA EREs.

To demonstrate binding specificity, we tested the ability of unlabeled oligonucleotides to compete with the ^{32}P-labeled D-loop ERE I for binding to mitochondrial and nuclear protein extracts. Figure 1A shows that a 20-fold excess of D-loop ERE I strongly reduced the binding of MCF-7 mitochondrial protein extracts to D-loop ERE I (lanes 1 and 2); a 100-fold excess reduced binding slightly more (lane 3). Unlabeled c-myc half ERE/Sp1 also reduced binding to the P^{32}-labeled D-loop ERE I, but to a lesser extent (lane 4), while a 100-fold excess of the non-ERE containing oligonucleotide did not affect binding (lane 5). Similar results were observed using D-loop ERE III (data not shown). As mentioned above, two bands were observed using nuclear extracts (Fig. 1B, lane 6), with the upper band corresponding to the one detected using the mitochondrial protein extracts (indicated by arrow). The intensity of this upper band was reduced by the unlabeled oligonucleotides in the same manner as observed with the mitochondrial protein extracts, while the intensity of the lower band was reduced much less (lanes 7–10). In a separated EMSA experiment in which the binding of both mitochondrial and nuclear extracts to D-loop ERE I was examined in the same gel, we observed the band formed by the binding of mitochondrial extracts had the same mobility as that of the upper band formed by the binding of the nuclear extract (data not shown). These results demonstrate that the binding of mitochondrial and nuclear proteins to mtDNA D-loop EREs is DNA sequence specific. The results also show that the nuclear protein extracts form two complexes with the D-loop EREs, one of which (the upper band) has binding properties similar to that formed by the mitochondrial proteins. The lower band may represent non-specific binding. Consistent with this, Rato et al. [1999] also observed two bands in their EMSAs for the binding of MCF-7 cell nuclear extract to the ERE present in *Xenopus* vitellogenin A2 gene. They reported that the intensity of the upper band was enhanced by E2 treatment and was progressively reduced in the presence of increasing concentrations of melatonin. However, the density of the lower band was not altered by both treatments.

Effects of E2 Treatment on the Binding of MCF-7 Cell Mitochondrial Protein to D-Loop EREs

We used EMSAs to determine the time- and concentration-dependent effects of E2 on the binding of mitochondrial protein extracts to D-loop EREs. Mitochondrial protein extracts were prepared from MCF-7 cells treated with E2 for the indicated times or treated with medium containing 5% normal FBS or E2 at the indicated concentrations for 12 h. As shown in Figure 2A, some binding to the D-loop ERE III-containing oligonucleotides was detected using mitochondrial protein prepared from MCF-7 cells maintained in stripped serum (control cells, lanes 1 and 2). Binding was increased after treatment with E2 (100 nM, chosen for the lowest concentration showing maximum enhancement, see bellow) for 3, 6, 12, and 24 h (lanes 3–10). Binding appeared maximal after 6–12 h and was decreased at 24 h. Similar results were obtained using D-loop ERE I (data not shown). Figure 2B shows the concentration effects of E2 (12-h treatment) on mitochondrial protein binding to D-loop ERE III. Binding of mitochondrial protein from MCF-7 cells treated with normal 5% FBS (lane 2) or with E2 at as low as 10^{-9} M (lane 3) was enhanced slightly compared to that seen in control cells maintained in stripped serum (lane 1). Binding was enhanced further as the concentrations of E2 increased from 10^{-8} to 10^{-6} M (lanes 4–6). Similar results were also observed using mtDNA D-loop ERE I (data not shown). It should be noted that there were variations in the binding of mitochondrial protein extracts prepared from untreated, control cells as indicated in Figure 2A, lanes 1–2 and Figure 2B lane 1 where the control band in Figure 2B was greater than the same control in Figure 2A. These variations are probably due to the experimental variations, such as the use of different freshness of radiolabeled probes and different exposure times. However, in all the cases, mitochondrial protein extracts prepared from E2-treated cells showed enhanced binding to mtEREs compared to mitochondrial protein extracts from control cells. These results indicate that the binding of mitochondrial proteins to the mtDNA D-loop EREs was enhanced by E2 treatment in a time- and concentration-dependent manner.

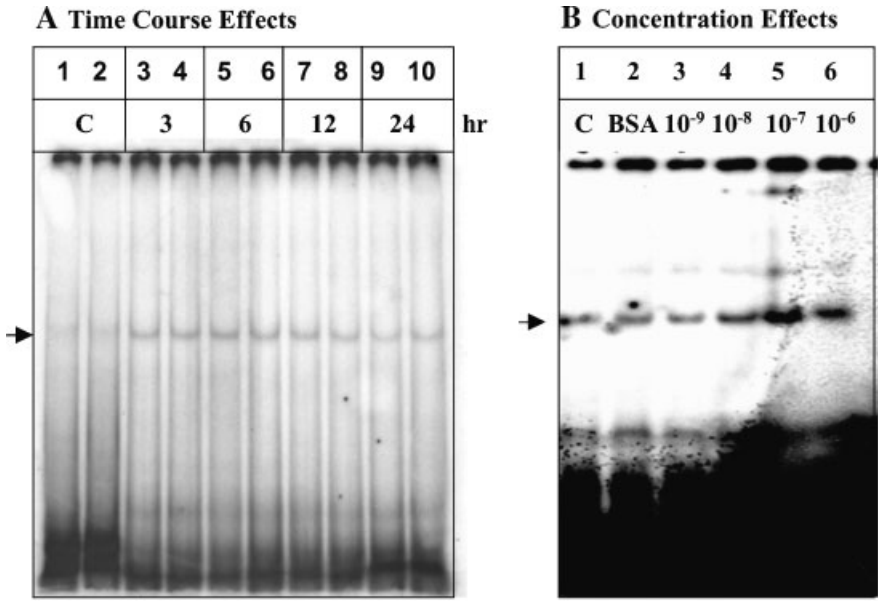

Fig. 2. Time- and concentration-dependent effects of E2 on the binding of mitochondrial protein extracts to D-loop ERE III. MCF-7 cells cultured as described in Materials and Methods were treated with E2 (100 nM) for the indicated time points (**A**) or with culture medium containing either 5% normal FBS or E2 at the indicated concentrations for 12 h (**B**). Mitochondrial protein extracts prepared from treated and control MCF-7 cells were incubated with ^{32}P-labeled D-loop ERE III and analyzed via EMSAs. Arrows indicate the retarded bands. Each lane represents a sample prepared from a single culture dish. Gels A and B were run separately. Similar results were observed in a second, separate experiment.

Determination of the Mitochondrial Status of ERα and ERβ in MCF-7, MCF-10A, and MDA-MB-231 Cells

The results presented above indicated that MCF-7 cell mitochondria contain an ERE binding protein(s). Recently, we demonstrated that ERα and ERβ are present in MCF-7 cell mitochondrial matrix [Chen et al., 2004]. Thus, we compared the mitochondrial localization of ERα and ERβ in MCF-7 with that in MCF-10A and MAD-MB231 cells. As described above, MCF-7 cells express both ERα and ERβ, MCF-10A cells express a low level of ERα and undetectable ERβ and MDA-MB-231 cells express only ERβ. We first used Western blot analysis to examine mitochondria for the presence of ERα and ERβ in these cells. Total cellular homogenates, nuclear, cytosolic, and mitochondrial protein extracts were separated by SDS–PAGE, transferred to nitrocellulose membranes and immunoblotted using polyclonal antibodies for human ERα (67 kDa), ERβ (63 kDa), and CO II (26 kDa). CO II served as a marker for mitochondria. Figure 3A is a composite showing the results of Western analysis of the subcellular distribution of ERβ, ERα and CO II in MCF-7 cells. This figure is representative of results obtained in at least four separate experiments. These results show that the ERβ antibody reacted specifically with rhERβ (lane 8) but did not crossreact with rhERα (lane 9). The ERα antibody did not react with rhERβ (lane 8) but specifically reacted with rhERα (lane 9). Both ERβ and ERα antibodies detected bands representing ERβ and ERα, indicated by the arrows, in the homogenate (H, lane 1), cytosolic (C, lane 2), nuclear (N, lane 3), and mitochondrial fractions (Mito, lane 4). The ERβ antibody detected another weak protein band (the upper band) in all fractions (lanes 1–4). The ERα antibody also detected an additional band (the upper band) in mitochondria (lane 4). The identity of these two protein bands is not known. Though the crude nuclear extracts were contaminated by mitochondria, as indicated by the detection of a protein band representing CO II (lane 3), the mitochondrial fraction was enriched as indicated by the presence of the heavier CO II band (lane 4). We have shown that the mitochondrial fraction was not contaminated by nuclei, as reflected by the lack of detection of histone H1, a nuclear marker protein, in mitochondrial fraction [Chen et al., 2004]. It should be noted that the amounts of ERα detected in nuclear fraction (Fig. 3A) of

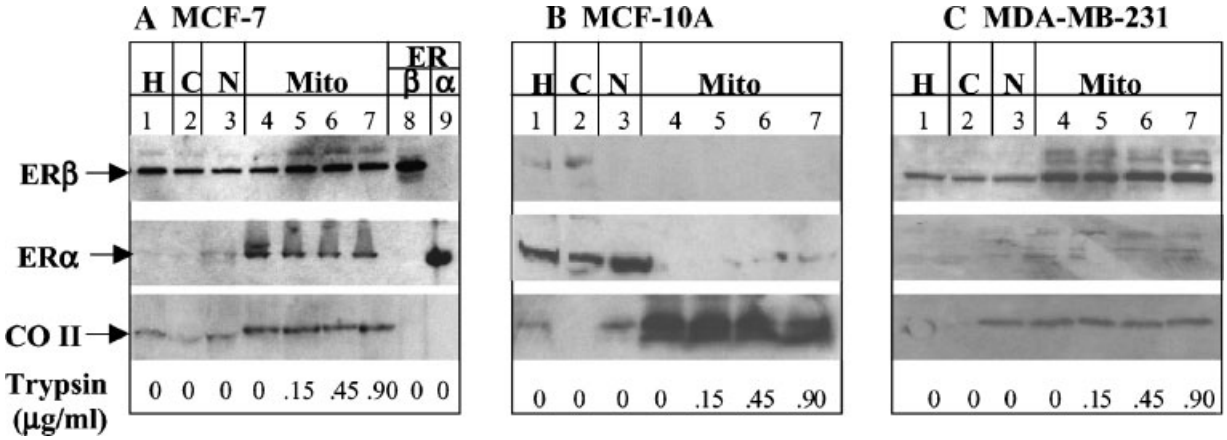

Fig. 3. Subcellular distribution of ERα and ERβ in MCF-7, MCF-10A, and MDA-MB-231 cells. The homogenates (H, 50 μg/lane), cytosols (C, 50 μg/lane, **lane 2**), nuclear (N, 30 μg/lane, **lane 3**), and mitochondrial proteins (30 μg/lane) after digestion with trypsin at the indicated concentrations (Mito, **lanes 4–7**), recombinant human ERβ (β, 30 ng/lane, **lane 8**) and rhERα (α, 30 ng, **lane 9**) were separated on 4–12% acrylmide-SDS gels and transferred to nitrocellulose membranes. Three similar blotting membranes were prepared and probed with antibodies for ERα, ERβ, and CO II, respectively. The membranes were developed using ECL-detection reagents. Similar results were observed in several additional separate experiments.

MCF-10F cells were relatively low. To confirm the mitochondrial localization of ERα and ERβ, the mitochondria were subjected to digestion with trypsin as described in Materials and Methods. As the concentration of trypsin was increased, the amounts of the two bands detected by the ERβ antibody did not change (lanes 4–7). For the bands detected by the ERα antibody, the amount of the upper band disappeared completely but the amount of the lower band, ERα, decreased only slightly (lanes 4–7). These results suggested that both ERα and ERβ are present inside the mitochondria. We performed similar experiments to determine the subcellular distribution of ERα and ERβ in MCF-10A and MAD-MB-231 cells. In MCF-10A cells (Fig. 3B), using the ERβ antibody, a weak band was detected in the homogenate (H, lane 1) and cytosolic fraction (C, lane 2) but not in nuclear (N, lane 3) and mitochondrial fractions (Mito, lanes 4–7), whereas some ERα was detected in the homogenate, cytosolic, and nuclear fractions (lanes 1–3, respectively) and in the trypsin-digested mitochondrial fraction (lanes 4–7). In MDA-MB-231 cells (Fig. 3C), ERβ was detected in all fractions including the trypsin-digested mitochondrial fraction, but ERα was not detected (lanes 1–7). These Western analysis results for ERα and ERβ presence in these cell lines are consistent with what has been seen by others as cited above.

To determine whether either or both mitochondrial ERα and ERβ are capable of binding to the putative EREs present in mtDNA D-loop and to the c-myc ERE half-site, we compared the D-loop ERE binding capacity of mitochondrial protein from MCF-7, MCF-10A, and MDA-MB-231 cells. As expected, mitochondrial protein from the MCF-7 cells showed strong binding to the EREs, but not to an oligonucleotide containing a mutated ERE (Fig. 4A, lanes 1–4). In contrast, only very weak binding was observed with MCF-10A mitochondrial extracts (Fig. 4B, lanes 5 and 6). Since ERβ is not detected in MCF-10A mitochondria (Fig. 3B), the weak binding is perhaps due to the binding of a small amount of ERα present. MDA-MB-231 cell mitochondrial protein extracts, which contain only ERβ (Fig. 3C), bound to mitochondrial D-loop ERE I, III, c-myc ERE half-site as well as the *Xenopus* vitellogenin ERE (Fig. 4C, lanes 9–12). These results indicate that the ERβ-containing mitochondrial extracts are capable of binding to D-loop ERE I, III and the c-myc half ERE/sp1 site.

Effects of ERα and ERβ Antibodies on the Binding of MCF-7 and MDA-MB-231 Mitochondrial Extracts to mtDNA EREs

We next sought to determine whether the binding of mitochondrial extracts to the D-loop EREs was due to one or both ERs. We examined: (1) the ability of purified rhERα or rhERβ to bind to the oligonucleotide containing D-loop ERE III; and (2) the ability of the antibodies for ERα and ERβ to interfere with (a) the binding of

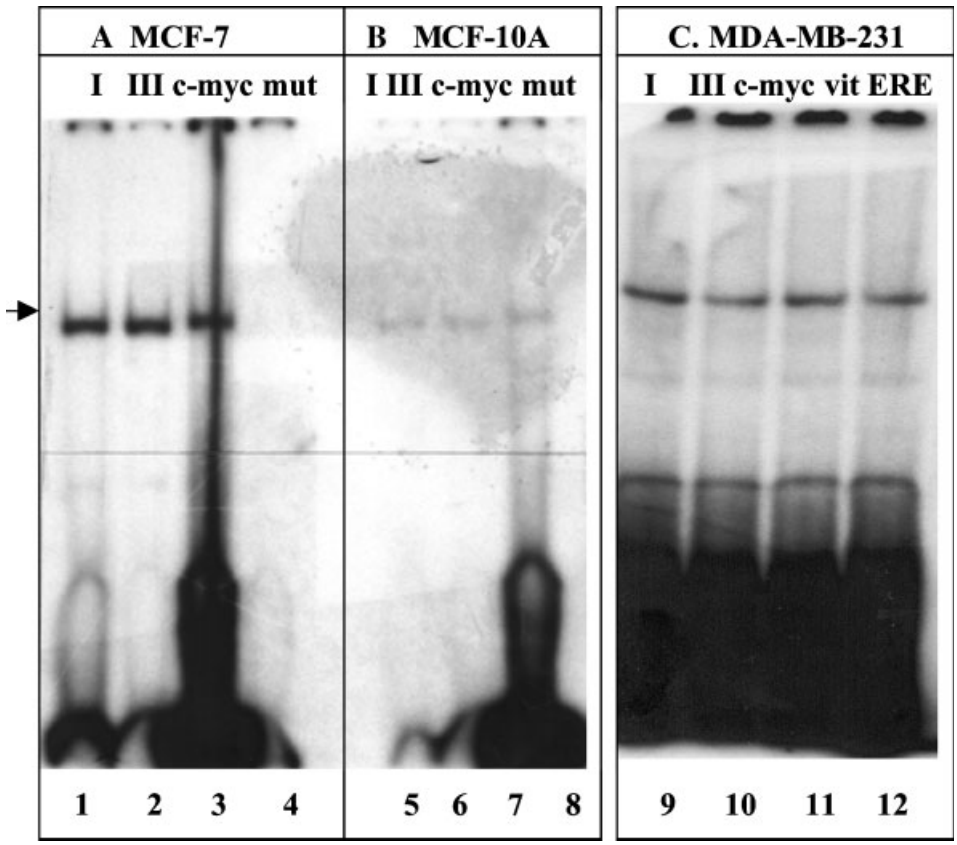

Fig. 4. Binding of mitochondrial proteins from MCF-7, MCF-10A, and MDA-MD-231 cells to mtDNA EREs. Mitochondrial protein extracts from E2-treated MCF-7 (**A**, **lanes 1–4**) or MCF-10A (**B**, **lanes 5–8**) or MDA-MB-231 (**C**, **lane 9–12**) cells were incubated with ^{32}P-labeled D-loop ERE I (I, lanes 1, 5, and 9) or D-loop ERE III (III, lanes 2, 6, 10), c-myc half ERE/sp1 (c-myc, lanes 3, 7, 11), a randomly mutated ERE (Mut, lanes 4 and 8), and the *Xenopus* vitellogenin ERE (vit ERE, lane 12). The arrows indicate the retarded band.

rhERα and rhERβ and (b) the binding of mitochondrial proteins to D-loop ERE III. Figure 5A shows that rhERα bound to D-loop ERE III (lanes 1 and 3). Binding was inhibited by ERα antibody A1 (lane 2) and super-shifted by ERα antibody A2 (lane 4, asterisk). The different interference of two ERα antibodies with the rhERα-D-loop ERE complex (inhibition vs. super-shift) could be due to their recognition of different regions of ERα since they were raised against different epitopes of ERα. Binding of MCF-7 mitochondrial extract to D-loop ERE III is also shown in Figure 5A (lanes 5–8). The band formed using the mitochondrial protein extracts exhibited a faster mobility than that seen with rhERα. The reasons for this difference in mobility of the retarded bands is not known, but perhaps is due to either (a) the tendency of rhERα to aggregate (PanVera product description) and/or (b) the pre-trypsin digestion of isolated mitochondria. It is possible that trace tryspin residue remaining in mitochondrial lysis buffer could cause degradation of mitochondrial ERs, resulting in faster migration of the ER-ERE complex. This possibility is supported by the observation by Wood et al., who showed that V8 protease digested ER-ERE complex had faster mobility than that of the undigested, native ER-ERE complex [Wood et al., 2001]. However, neither ERα antibody A1 or A2 interfered with the binding of MCF-7 mitochondrial proteins to D-loop ERE III (Fig. 5A, lanes 5 and 6, lanes 7 and 8, respectively). Figure 5B shows that like rhERα, rhERβ bound to D-loop ERE III (lane 1). Increasing amounts of ERβ antibody progressively reduced the amount of binding (lanes 2–4). As expected, MCF-7 mitochondrial extracts bound to D-loop ERE (lane 5). Again, the band exhibited a faster mobility than that seen with rhERβ, also possibly due to the reasons stated above. The binding was reduced by increasing

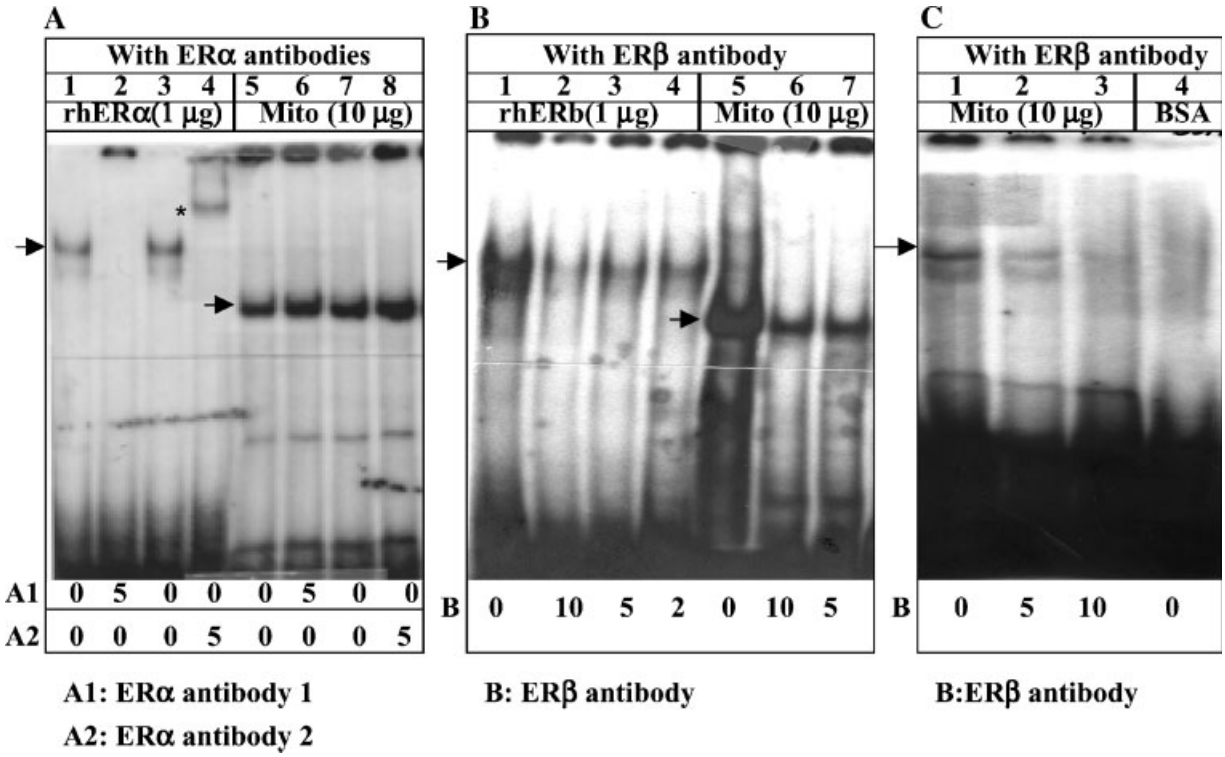

Fig. 5. Effects of ERα and ERβ antibody on the binding of mitochondrial proteins of MCF-7 and MDA-MB 231 cells to mtDNA ERE II. **A**: RhERα (1 μg/lane, **lanes 1–4**) or MCF-7 mitochondrial protein extracts prepared from E2-treated (300 nM, 12 h) cells (**lanes 5–8**) were incubated with ^{32}P-labeled D-loop ERE III (lanes 1–8). After incubation for 15 min, the indicated amounts of ERα antibody A (Santa Cruz, lanes 2 and 6) or ERα antibody B (Immunotech, lanes 4 and 8) were added to the reaction mixtures and incubated for another 20 min. Arrows indicate the retarded band and * indicates the super-shifted band. **B**: rhERβ (1 μg/assay, **lanes 1–4**) or MCF-7 mitochondrial protein extracts prepared from E2-treated cells (**lanes 5–7**) were incubated with ^{32}P-labeled oligonucleotide probes of D-loop ERE III. After incubation for 15 min, the indicated amount of either ERβ antibody (B) (lanes 2–4 and 6–7) was added to the reaction mixture and incubated for another 20 min. Arrows indicate the retarded band. **C**: MDA-MB-231 mitochondrial protein extracts (10 μg/assay) from E2-treated cells were incubated with ^{32}P-labeled D-loop ERE III (**lanes 1–3**) or with BSA (20 μg, **lane 4**). After incubation for 10 min, the indicated amount of ERβ antibody (B) (lanes 2 and 3) was added the reaction mixture and incubated for another 20 min. Arrows indicate the retarded bands.

amounts of ERβ antibody (lanes 6 and 7). We also observed that mitochondrial protein extracts bound to the oligonucleotide containing c-myc half ERE/sp1 site and the binding was reduced by ERβ antibody, whereas an unrelated Blc-xL antibody did not alter the binding (data not shown). These results suggest that the MCF-7 mitochondrial protein that binds to the putative ERE-containing oligonucleotides is ERβ. To confirm these results, we examined the effects of ERβ antibody on D-loop ERE III binding of mitochondrial protein extracts from MDA-MB-231 cells which express ERβ but not ERα. As shown in Figure 5C, these MDA-MB-231 mitochondrial protein extracts bound to D-loop ERE III (Fig. 5C, lane 1) and the binding was progressively reduced by increasing amounts of ERβ antibody (lanes 2 and 3). BSA (20 μg/lane) that was included as a negative control did not bind to the D-loop ERE III oligonucleotide (Fig. 5C, lane 4). Together, these results suggest that the protein in the MCF-7 and MDA-MB-231 mitochondrial extracts binding to the mtDNA D-loop ERE III is ERβ.

SPR Analysis for the Binding of ERs to Putative mtDNA D-Loop EREs

To confirm and extend the EMSA results presented above, we applied SPR to investigate the interactions of rhERα or rhERβ with immobilized D-loop ERE I and III. To verify the binding specificity, we performed similar experiments using immobilized oligonucleotides containing a randomly mutated ERE and an oligonucleotide containing the GRE. Figure 6 shows the representative sensogram patterns

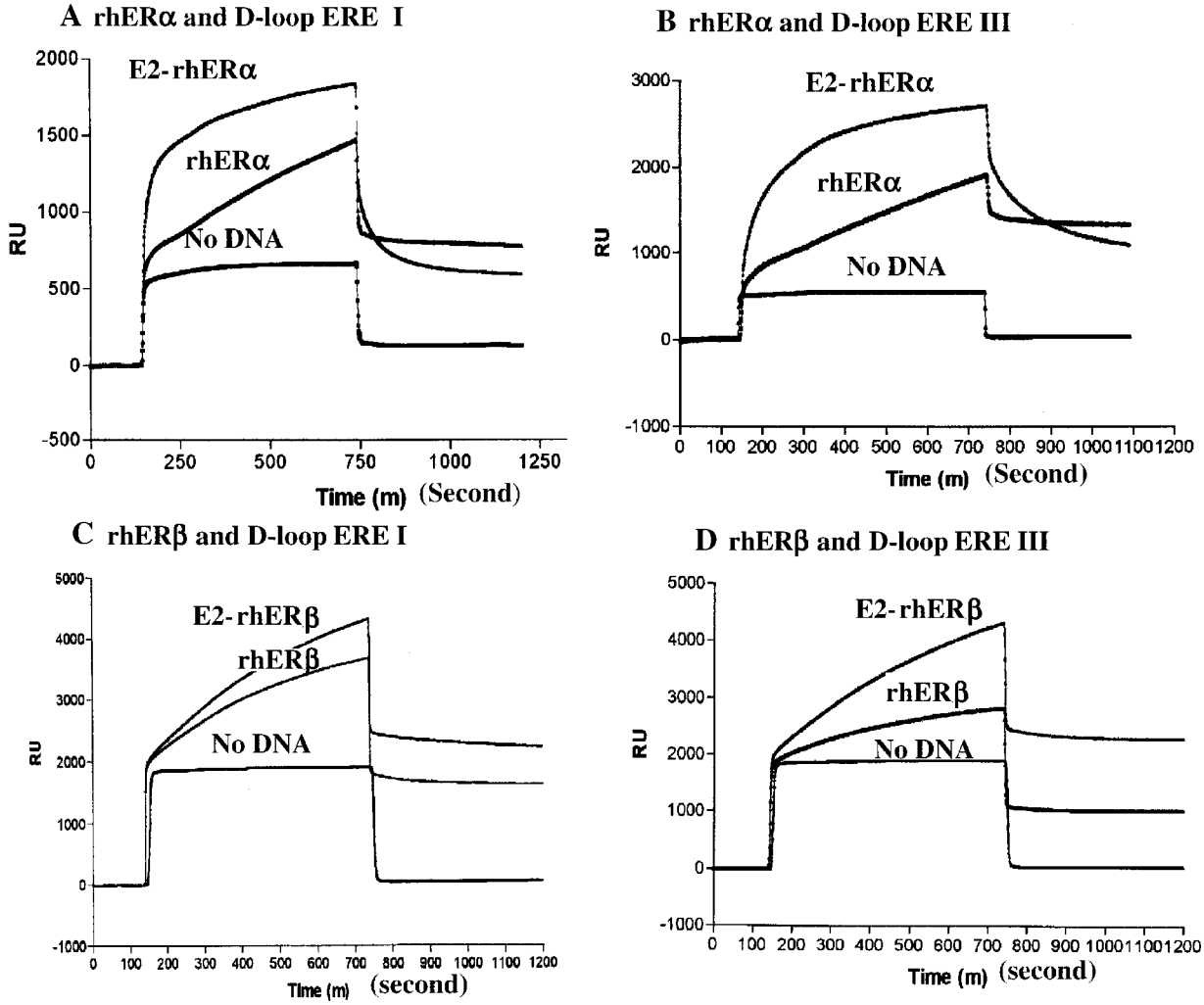

Fig. 6. Sensorgrams of surface plasmon resonance (SPR) analysis for the association of ERs with D-loop EREs. The figure illustrates the time-dependent association and dissociation of (**A**) ERα and E2-ERα with D-loop ERE I; (**B**) ERα and E2-ERα with D-loop ERE III; (**C**) ERβ and E2-ERβ with D-loop ERE I; and (**D**) ERβ and E2-ERβ with D-loop ERE III. The protein samples were allowed to flow over the surface of sensor chips to which either no DNA oligonucleotide was bound or to which 750 RU of immobilized, biotinylated D-loop ERE I or D-loop ERE III DNA oligonucleotides had been absorbed. The ordinate shows the changes in the refractive index at the surface of the sensor chip due to the increase or decrease in the protein concentrations bound at the sensor surface that is caused by the association or dissociation of proteins from the solution with the immobilized oligonucleotides previously bound to the chip surface. The sharp spikes in RU value are the changes in refractive index due to differences in the buffer flowing over the cell when the ER-containing solution flows past the chip surface.

illustrating the binding of rhERα and rhERβ, respectively, to immobilized D-loop ERE I (Fig. 6A,C) and D-loop ERE III (Fig. 6B,D). There was no increase in binding of rhERα with time without immobilized DNA on the sensor chip (Fig. 6A). However, with D-loop ERE I immobilized on the sensor chip surface, the binding of rhERα increased, as reflected by the increase in RU with time. Moreover, binding of the E2-rhERα complex to the immobilized D-loop ERE I was increased compared to the binding of rhERα alone. Similar patterns were observed for the association of rhERα alone and the E2-rhERα complex to immobilized D-loop ERE III (Fig. 6B). These results indicate that E2 enhances the binding of rhERα to both D-loop ERE I and III. Like rhERα, rhERβ alone bound to the immobilized D-loop ERE I and D-loop ERE III (Fig. 6C,D). The binding of the E2-rhERβ complex to the immobilized D-loop ERE I was enhanced slightly compared to the binding of rhERβ alone (Fig. 6C), whereas the binding of E2-rhERβ complex to D-loop ERE III increased to a greater extent (Fig. 6D), suggesting differ-

ential effects of E2 on the binding of rhERβ to mtERE I and mtERE III. When binding of the E2-rhERα and E2-rhERβ ligand-receptor complexes to the mutated ERE or to the oligonucleotide containing the GRE was examined, the binding curves generated were similar to those obtained in the absence of immobilized DNA (data not shown). Furthermore, we observed that allowing E2 (25 μM) alone to flow over the surface of the SA chip containing D-loop ERE I or D-loop ERE III or pS2 ERE resulted in no changes in RU (data not shown). Together, these results indicate that rhERα and rhERβ both bind to D-loop ERE I and III and that this binding is DNA-sequence specific and enhanced by pre-incubating the ER protein in the presence of saturating levels of E2.

DISCUSSION

In addition to its important role in the regulation of the expression of nuclear genes via nuclear ER, E2 induces expression of mtDNA-encoded genes in a number of cells [Van Itallie and Dannies, 1988; Chen et al., 1996, 1998, 2004]. Precedent for the direct involvement of steroid hormone receptors in the regulation of mtDNA transcription principally stems from studies demonstrating the mitochondrion as a primary site of action of glucocorticoid hormones (Tsiriyotis et al., 1997; Scheller et al., 2000; Moutsatsou et al., 2001). While the detailed molecular mechanism(s) underlying E2-mediated enhanced mtDNA gene expression is not clear, recent reports showing the presence of ERs within mitochondria [Monje and Boland, 2001; Cammarata et al., 2004; Chen et al., 2004; Yang et al., 2004] and the reports showing the presence of ERE sequences within mtDNA [Sekeris, 1990; Demonacos et al., 1996] suggested the possibility that the ER might be involved.

In this study, we made several key observations. First, we confirmed the presence of ERα and ERβ in mitochondria of human MCF-7 cells and demonstrated that both nuclear and mitochondrial proteins of MCF-7 cells bound to several different mtEREs and to selected nuclearEREs. Competition experiments demonstrated that the binding was sequence specific and enhanced by E2 in a time- and dose-dependent manner. The mitochondrial protein from ERβ-/ERα-expressing MCF-7 and ERβ-expressing MDA-MB-231 cells bound to mtEREs. In contrast, binding of mitochondrial protein from MCF-10A cells, which express ERα but not detectable ERβ, was almost undetectable. Consistent with these results, we observed that E2 enhanced the transcript levels of mtDNA-encoded genes in MCF-7 and MDA-MB-231 cells but not in MCF-10A cells (unpublished observations]. To our knowledge, this is the first demonstration that proteins present in mitochondria of MCF-7 cells bind to mtEREs, which appear capable of acting as functional regulatory elements for the action of E2 inside mitochondria. Second, we demonstrated that ERα and ERβ are able to directly interact with mtEREs. Similar to mitochondrial proteins of MCF-7 cells, both rhERα and rhERβ bound to the mtEREs and their binding was altered by their respective antibodies. Consistent with these results, Demonacos et al. observed the binding of partially purified glucocoiticoid receptor to mtDNA sequences with similarity to nuclear GRE [Demonacos et al., 1995]. Using these ERα and ERβ specific antibodies, we observed that the binding of MCF-7 cell mitochondrial proteins was not altered by either of two ERα antibodies used in our study. In contrast, binding of mitochondrial protein from both MCF-7 and MDA-MB-231 cells to mtEREs was inhibited by ERβ antibody. These results indicate that the mitochondrial protein that binds to the mtEREs is predominately ERβ. Third, we used SPR analysis to confirm the ability of rhERα and rhERβ to bind to mtEREs. SPR monitored the binding of rhERα and rhERβ to mtEREs in real time. The SPR analyses indicated that: (a) the binding of rhERα and rhERβ to the mtEREs was specific since it did not occur to an oligonucleotide containing a randomly mutated D-loop ERE or the GRE; (b) the binding of rhERα to mtEREs was enhanced in the presence of E2; and (c) the binding of rhERβ to mtEREs was enhanced in the presence of E2, though to a lesser extent than for ERα. Overall, the SPR results are consistent with those of the EMSAs and provide additional support for the binding of ERα and ERβ to mtEREs. Determination of whether these in vitro SPR observations are biologically relevant requires further investigation using proteins obtained from isolated mitochondria. However, similar SPR analyses for the binding of ER to nuclear EREs have been reported [Cheskis et al., 1997; Jisa et al., 2001]. Our SPR results for the binding of ERα and ERβ to

mtEREs are qualitatively consistent with these reports on the binding of ERs to nuclear ERE and indicate that mtEREs are functionally comparable to nuclear EREs. Taken together, our EMSA and SPR results strongly support the conclusion that given the presence of ERs within mitochondria, the E2 stimulation of mtDNA transcription is mediated by the direct association of these ERs with the mtEREs.

The EMSA and SPR results indicate that both rhERα and rhERβ are capable of binding to mtEREs. This is expected because the DNA binding domains of human ERα and ERβ share a high percentage (97%) of homology [Green et al., 1986a,b; Kuiper et al., 1996; Ogawa et al., 1998]. Thus, it would be expected that the in vivo binding capacity of ERα and ERβ to mtEREs inside mitochondria would be determined by the presence and relative amounts of ERα and ERβ. In our previous studies using quantitative Western analysis, we observed greater amounts of ERβ than ERα in mitochondria of E2-treated MCF-7 cells [Chen et al., 2004]. In addition, in recent studies by others in other cell types, only the presence of ERβ in mitochondria has been reported [Cammarata et al., 2004; Yang et al., 2004]. This may explain why binding of MCF-7 mitochondrial protein to mtEREs is altered by antibody to ERβ but not by antibody to ERα and suggests that ERβ may be the major ER in mitochondria of MCF-7 cells. Likewise, ERα may play a predominant role in mediating the mitochondrial effects in the cells mainly expressing ERα such as rat myocardium [Zhai et al., 2000a,b, 2001].

Our findings can provide unique insights into the molecular mechanism by which E2 and ERs contribute to preservation and regulation of mitochondrial structure and function observed by Zhai et al. (see Introduction). As mentioned above, we observed the mitochondrial localization of ERα and ERβ in MCF-7 cells, enhanced mitochondrial levels of both ERs and the ER-mediated enhanced transcription of mtDNA by E2 [Chen et al., 2004]. In the present study, we demonstrated the binding of ER-containing mitochondrial proteins, rhERα and rhERβ to the mtEREs. These observations (i) support our hypothesis that the mitochondrial ERs are involved in the enhanced MRC protein synthesis and MRC activities caused by E2 treatment; and (ii) provide logical explanations for why the mitochondrial structure and function are abnormal in Ovx and ERαKO hearts, for example, without estrogens and/or ERs, MRC function is defective because of the lack of induced MRC protein synthesis by E2 and/or ERs, leading to abnormal mitochondrial structure and function. By enhancing MRC protein synthesis, E2 and ERs contribute to the preservation and regulation of mitochondrial structure and function, and thus to energy-dependent physiological processes. The involvement of E2 and ERs in these mitochondrial effects in breast cancer cells is suggested by the study of Vic et al., *who* observed that E2 treatment transformed MCF-7 cells into secretory cells containing large and clear mitochondria with well-defined cristae and that similar estrogen effects occurred in other ER-positive but not in ER-negative breast cancer cells [Vic et al., 1982].

These E2/ER-mediated mitochondrial effects in human breast cells may have important pathophysiological implications. Human breast cancer cells are exposed to relatively high levels of E2 due to its in situ generation by local aromatase activity [Santner et al., 1997; Santen et al., 1999; Chen et al., 2002] and ERβ is widely present in human breast epithelial cells, breast cancer cells and breast cancer cell lines [Gustafsson and Warner, 2000]. Thus, E2/ERβ-mediated mitochondrial effects could be active and persistent in breast tissues, and breast cancer cells. It is well known that mitochondria have a central role in the control of apoptosis [Kroemer and Reed, 2000] and that E2 inhibits apoptosis in breast cancer cells [Wang and Phang, 1995; Razandi et al., 2000; Swiatecka et al., 2000]. If E2/ERβ-mediated enhanced mitochondrial effects contribute to an inhibition of apoptosis, the signaling pathway mediated through the mitochondrial ER could represent a new target for prevention and therapeutic intervention in breast cancer.

ACKNOWLEDGMENTS

We are grateful to Dr. Edward T. Morgan of Emory University School of Medicine for critical reading and valuable suggestions and to Mr. Scott L. Noble of Tulane University for help with some SPR experiments. This work was supported by grants NIH CA36701 and CA77550 to J.D.Y. and by DHHS/CDC grants RO4/CCR and 419466-01 to W.L.A. Shared-use equipment was supported by Center Grant P30ES03819. The authors also thank W.M.

KECK Foundation for a grant to Tulane University that permitted purchase of Biacore 2000 Instrument. M.E and W.L.A. also acknowledge support from a grant to The Tulane Center for Bioenvironmental Research from The U.S. Department of Agriculture (USDA 58-6435-7-019).

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