Mitochondrial localization of ERα and ERβ in human MCF7 cells

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Chen, Jin Q., Michael Delannoy, Carol Cooke, and James D. Yager. Mitochondrial localization of ERα and ERβ in human MCF7 cells. Am J Physiol Endocrinol Metab 286: E1011–E1022, 2004.—We observed previously that estrogen treatment increased the transcript levels of several mitochondrial DNA (mtDNA)-encoded genes for mitochondrial respiratory chain (MRC) proteins and MRC activity in rat hepatocytes and human Hep G2 cells. Others have reported detection of estrogen receptors (ER), ERα and ERβ, in mitochondria of rabbit ovarian and uterine tissue. In this study, we have extended these observations. Using cellular fractionation and Western blot with ERα- and ERβ-specific antibodies, we observed that ERα and ERβ are present in mitochondria of human MCF7 cells and that the mitochondrial ERs and ERβ account for 10 and 18%, respectively, of total cellular ERα and ERβ in 17β-estradiol (E2)-treated MCF7 cells. We also found that E2 significantly enhanced the amounts of mitochondrial ERα and ERβ in a time- and concentration-dependent manner and that these effects are accompanied by a significant increase in the transcript levels of mtDNA-encoded genes, i.e., cytochrome c oxidase subunits I and II. Moreover, we demonstrated that these E2-mediated effects were induced by the pure ER antagonist, chrome oxidase subunits I and II. In accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The glucocorticoid and thyroid hormones have also been shown to alter the levels of mtDNA-encoded gene transcripts. It was demonstrated that these effects are mediated through direct interactions of their receptors with mtDNA. Several studies have shown that the glucocorticoid receptor is imported in mitochondria in liver, HeLa cells, Hep G2 cells, and rat brain (20, 50, 57, 61, 63). Human and murine mtDNA contain sequences with similarity to nuclear glucocorticoid response elements (GRE; see Refs. 21, 32, 62, 64). In addition, two putative mitochondrial GREs confer hormone inducibility to a linked reporter gene (68). It has also been shown that thyroid hormone can cause the direct stimulation of mitochondrial RNA synthesis (6, 24) and that a variant form of the thyroid hormone receptor is imported in and localized within liver mitochondria (6, 74). These observations suggest that mitochondria could be one of the major sites for the actions of steroid and thyroid hormones.

There are two forms of the ER, α (27, 28) and β (33, 49, 52, 58). The classical view regarding the mechanism of estrogen action is that its effects are mediated through binding to the nuclear ER(s), which then bind to estrogen response elements (ERE) in nuclear DNA to cause transcriptional activation of estrogen-responsive genes (23). However, the mitochondrial genome also contains ERE-like sequences (21, 64). Furthermore, several studies have reported detection of estrogen binding to mitochondria and the presence of estrogen-binding proteins (EBPs) within mitochondria (29, 45, 47, 48, 53). In particular, Monje and colleagues (47, 48) demonstrated the presence of both ERα and ERβ in mitochondria of rabbit uterine and ovarian tissue. The goal of this study was to extend the observations on the localization of the ER in mitochondria to other cell types and to conduct new investigations on localization of ERs in mitochondria and on the induction of mtDNA-encoded gene transcription in response to estrogen treatment.

MATERIALS AND METHODS

Materials and antibodies. Cell culture media, Iscove Minimum Essential Medium (IMEM), DMEM/F-12, FBS, horse serum, gentamicin, and BSA were purchased from Invitrogen Life Technologies (Carlsbad, CA). Hydrocortisone, epidermal growth factor (EGF), cholera toxin, and silicon oil (dimethyl-polysiloxane) were obtained from Sigma Chemicals (St. Louis, MO). Recombinant human ERα (rhER-α, 66 kDa) and recombinant human ERβ (rhERβ, long form, 59.4 kDa) were obtained from PanVera (Madison, WI). An affinity-purified rabbit polyclonal ERα antibody (H-184, catalog no. SC-7207) directed against the amino-terminal domain of human ERα and rabbit polyclonal ERβ antibody (H-150, catalog no. SC-8974) directed against a recombinant protein fragment corresponding to amino acids...
1–150 at the NH2 terminus of human ERβ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse monoclonal ERα (1DS) directed against the COOH-terminal domain of human ERα and a rabbit polyclonal ERβ antibody (GC-17) directed against a polypeptide sequence in the F domain of the human ERα (amino acids 449–465; see Ref. 36) were provided by Dr. Shuk-Mei Ho (University of Massachusetts Medical School) and can be obtained commercially from Biogenex (San Ramon, CA). Two monoclonal ERβ antibodies (CFK-E12 and CWK-F12) were provided by Dr. Benita S. Katzenellenbogen (The University of Illinois at Urbana-Champaign). Both antibodies recognize an overlapping peptide epitope (amino acids 273–285) within the ligand-binding domain of human ERβ (16), which shows high amino acid sequence conservation among several mammalian species (33, 54) and partial similarity to the ligand-binding domain of human ERαs (27, 28). Two monoclonal antibodies, one directed against the COOH-terminal and the other directed against the NH2-terminal regions of rat ERβ were obtained from Upstate Cell Signaling Solutions (Charlottesville, VA). Monoclonal antibody for histone H1 (catalog no. SC-8030) and secondary antibodies (goat anti-rabbit IgG-alkaline phosphatase, goat anti-mouse IgG) linked to horseradish peroxidase were purchased from Upstate Cell (Lake Placid, NY). Mouse monoclonal antibodies (GC-17) directed against a polypeptide sequence in the F domain of the human ERα were purchased from Molecular Probes (Eugene, OR). Secondary rabbit IgG tagged to gold particles was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The Bio-Rad Protein Assay kit was obtained from Bio-Rad Laboratories (Hercules, CA). Enhanced chemiluminescence (ECL)-Western blotting detection reagents were obtained from Amersham/Pharmacia (Buckinghamshire, UK). Reagents for immuno-gold electron microscopy, e.g., glutaraldehyde, osmium tetroxide, uranyl acetate, and polyvinylpyrrolidone, were obtained from Ted Pella (Redding, PA).

Preabsorption of ERα and ERβ antibodies with rhERα and rhERβ. Antibody preabsorption was done as follows: 3 μg of Santa Cruz Biotechnology ERα or ERβ antibody in 15 μl were incubated with 7.5 μg of either rhERα or rhERβ protein for 30 min at 4°C with gentle rotation. The mixture was then centrifuged at 20,000 g for 15 min. The supernatant was reabsorbed with 7.5 μg of either rhERα or rhERβ for another 30 min at 4°C with gentle rotation. The mixture was centrifuged at 20,000 g for 15 min, and the supernatant was saved.

Cell culture. The human breast cancer MCF7 cell line was chosen for an experimental model for this study because it expresses both ERα and ERβ (39). The human breast nontumorigenic epithelial cell line MCF10A was chosen as a negative control for ERβ because it is negative for ERβ expression (34). MCF7 cells were cultured in phenol red-free IMEM containing 5% FBS plus gentamycin. MCF10A cells were cultured in DMEM/F-12 medium containing 5% horse serum plus insulin (10 μg/ml), hydrocortisone (0.5 μg/ml), EGF (20 ng/ml), cholera toxin (1 μg/ml), and gentamicin (1 μg/ml). In studies to determine the effects of estrogen treatment, cells were cultured in medium containing 5% charcoal-stripped FBS for 7 days before treatment. The cultures examined the time course and concentration effects of E2, the MCF7 cells were treated with E2 (100 nM) for 1, 3, 6, 12, and 24 h or with E2 at 10−9, 10−8, 10−7, and 10−6 M for 12 h, respectively. For the experiments determining the effects of ICI-182780, MCF7 cells were treated with E2 (0.3 μM) plus or minus ICI-182780 (1 μM) for 24 h. Mitochondrial proteins and/or total RNA was prepared from the nontreated (control) and treated cells for Western blot and Northern blot analysis, respectively.

Cell fractionation, preparation of mitochondria, and protein assays. These studies were done using MCF7 cells grown to 80% confluence and then treated with E2 (100 nM) for 12 h. Approximately 2–6×107 cells were harvested in 10 ml of cold PBS and homogenized. To minimize protein leakage from the nuclei, fractionation of the homogenate was performed using silicon oil via differential centrifugation (55). The total homogenate was divided into eight parts. Each part (0.75 ml) was loaded on the top of silicon oil (0.75 ml) in microcentrifuge tubes and centrifuged at 1,000 g for 2 min. The supernatant was reloaded on the top of silicon oil in a fresh microcentrifuge tube and centrifuged again at 1,000 g for 2 min. Both pellets were combined, rehomogenized, loaded on top of silicon oil, and centrifuged at 1,000 g for 2 min. The pellets were suspended in the mitochondrial buffer (70 mM sucrose, 220 mM mannitol, 2 mM HEPES (pH 7.4), 2.5 mM MgCl2, 0.5 mM EDTA, 0.5% BSA plus freshly added 2 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 2 mM dithiothreitol (DTT)) and centrifuged at 1,000 g for 5 min. This step was repeated two times. These 1,000-g pellets represent the crude nuclear fraction. The supernatants from the initial rounds of centrifugation through silicon oil were combined and centrifuged at 10,000 g for 15 min. The 10,000-g supernatant was used as the cytosolic fraction. Mitochondria in the 10,000-g pellet were purified further via a sucrose-Percoll gradient, as described previously (19). The mitochondrial fraction was resuspended in lysis buffer containing 25 mM HEPES (pH 7.6), 2.5 mM Tris (pH 7.6), 6.0 mM KCl, 300 mM NaCl, 1 M urea, 0.5% Nonidet P-40 plus freshly added 2 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 2 mM DTT. The total amount of protein in each fraction was determined using the Bio-Rad Protein Assay with BSA as standard.

Western blot analysis. Proteins (30 μg/lane) from each fraction were separated on 4–12% acrylamide-SDS gels and transferred to nitrocellulose membranes. The membranes were blocked using 5% nonfat 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 (1:500 dilution) for ERβ and ERα and CO II for 1 h. After being washed, 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) for 1 h. The membranes were developed using ECL-Western blotting detection reagents. The membranes were stripped at 50°C for 30 min, reprobed with antibodies for histone H1 or CO II, incubated with appropriate secondary antibodies (1:1,500), and developed using ECL-Western blot detection reagents. For determination of the time and concentration effects of E2 on the amount of ERα and ERβ in mitochondria, mitochondrial proteins (30 μg/lane) from prepared cells treated with E2 for different periods of time or with E2 at different concentrations (see above) were assayed by Western blot using antibodies for ERα, ERβ (Santa Cruz Biotechnology) and CO II. The protein bands representing ERα, ERβ, and CO II in Western blot films (data not shown) were scanned by a densitometer. The average ratio of the densitometric scan of the ERα or ERβ bands to that of the CO II band, compared with the ratio of the control group (not treated with E2), which was taken as one, was calculated.

Determination of the relative subcellular distribution of ER-α and ER-β in MCF7 cells. Three fractionation experiments were performed using MCF7 cells pretreated with E2 (100 nM) for 12 h. For quantification of ERα and ERβ, rhERα (15, 30, and 60 ng) or rhERβ (30, 60, and 120 ng) and protein extracts (30 μg/lane) from each fraction, cytosolic, nuclear, and mitochondrial fractions were separated via SDS-PAGE, transferred to nitrocellulose membranes, and probed with the indicated antibodies (see Fig. 2A). Several similar Western blot membranes were prepared from each of three separate experiments. The membranes were cut in half just above the position of the 35-kDa molecular mass marker. In Fig. 2A, the upper and lower half of one membrane were probed with antibody for ERβ (64 kDa) and antibody for CO II (18 kDa), respectively. The upper and lower halves of a second membrane were probed with antibody for ERα (67 kDa) and antibody for histone H1 (30 kDa), respectively. Western blot film images were scanned using Scanmaker 4 (Microtek Interna-tional), and the densities of the ERα and ERβ bands in each fraction were normalized to the density of the rhERα and rhERβ bands measured using a Mac Bas V2.2 program. Standard curves for rhERα and rhERβ were generated on the basis of known amounts of protein vs. the measured band densities in each Western blot experiment. The band densi-
metric values of 15 ng rhERα and 30 ng rhERβ were set at one relative unit. Representative standard curves are shown in Fig. 2B. The amounts of rhERα and rhERβ used were in a linear relation with the band density. The densitometric units for each band were converted into the amount of ERα and ERβ based on the linear standard curves generated with the known amounts of rhERα and rhERβ. The total amounts of ERα and ERβ in each fraction were estimated by multiplying the amounts of ERα and ERβ protein per 30 μg protein by the total amount of protein in the corresponding fraction. The relative amounts of ERα and ERβ were then expressed as a percentage of the total ERα or ERβ in the homogenate fraction, which was taken as 100%.

Northern blot analysis. Preparation of cDNA probes for mtDNA-encoded genes, total RNA isolation, and Northern blot analysis were performed as described previously (8). Briefly, cDNA probes for CO I, CO II, and ND1 (for detection of mitochondrial transcript precursor) were prepared as described previously (8). Total RNA was isolated from MCF7 cells using RNAzol (TES-TEST, Friendswood, TX). Aliquots of 20 μg/assay of total RNA were mixed with loading buffer, denatured at 65°C for 15 min, and separated on 1% agarose gels. The cDNA probes were denatured at 65°C for 15 min, and separated on 1% agarose gels from MCF7 cells using RNAzol (TES-TEST, Friendswood, TX). The cDNA probes were hybridized to the Northern blots, washed, and then autoradiographed. The autoradiographs were scanned, and the 28S and 18S bands in photographs of Northern blots were quantified using an imaging densitometer (Molecular Dynamics, Indianapolis, IN). Each blot was probed with 2.5 × 10⁶ cpm/50–100 ng probe. The blots were stripped and rehybridized to other probes. The mtDNA bands representing the indicated genes in Northern blot films and the 28S and 18S bands in photographs of ethidium bromide-stained gels were scanned. To correct for interlane differences in RNA loading and transfer, the ratios of the densitometry scans of the autoradiographs of CO I, CO II, and mitochondrial transcript precursor to those of the 28S plus 18S rRNA were calculated and expressed as the degree of induction over the control group, which was taken as one.

Immunolocalization of ERα and ERβ using confocal microscopy. MCF7 and MCF-10A cells grown on cover slips to 60% confluence were treated with E2 (300 nM) for 12 h and then stained with ER antibody, or preimmune rabbit IgG (1:500), followed by incubation with a secondary antibody tagged with the green fluor 488 (1:1,000), were performed according to the protocol of the Santa Cruz Biotechnology ER antibody (Fig. 1A). The GC-17 ER antibody (Fig. 1B) detected a 64-kDa protein in the mitochondrial fraction (lane 12) whereas preabsorption with rhERβ greatly diminished its binding (lane 13). Preabsorption of this ERβ antibody with rhERβ did not affect its binding to rhERα (lane 7) or to the 67-kDa mitochondrial protein (lane 9). We similarly tested the specificity and cross-reactivity of the Santa Cruz ERβ antibody (Fig. 1B). It did not react with rhERα (lanes 10, 13, and 16) but reacted with rhERβ (59.4 kDa, lanes 2, 5, and 8), and this antibody also reacted with a 67-kDa protein present in the mitochondrial fraction (lane 3). The reactivity of this ERα antibody to rhERα (lane 4) and to the 67-kDa protein in the mitochondrial fraction (lane 6) was diminished by preabsorption to rhERα, whereas preabsorption of this ERα antibody with rhERβ did not affect its binding to rhERα (lane 7) or to the 67-kDa mitochondrial protein (lane 9). To confirm these results, we performed Western blot analysis with other ERα and ERβ antibodies. The 1D5 ERα antibody (Fig. 1C) detected a 66-kDa rhERα and a 67-kDa protein in the mitochondrial fraction (lane 19). The GC-17 ERβ antibody (Fig. 1D) detected a 64-kDa protein band in the mitochondrial fraction (lane 21) and rhERβ (lane 22), similar to what was observed with the Santa Cruz ERβ antibody (lanes 11 and 12). We also performed Western blot analysis on MCF7 cell nuclear and mitochondrial proteins using the two human ERβ-specific monoclonal antibodies, CBF-E12 and CBF-E12. The CBF-E12 antibody (Fig. 1E) detected a weak 67-kDa band (band 1), a strong 63-kDa band (ERβ), and a weak 52-kDa band (band 2) in the nuclear
Fig. 1. Determination of the specificities and the cross-reactivities of the estrogen receptor (ERα) and ERβ antibodies toward recombinant human (rh) ERα and rhERβ and detection of ERα and ERβ within mitochondria of MCF7 cells. For determination of the specificities and cross-reactivities of the Santa Cruz ERα and ERβ antibodies, we prepared 6 identical Western blots. rhERα (α; 30 ng) was loaded in lanes 1, 4, 7, 10, 13, and 16; 100 ng of rhERα were loaded in lane 20. rhERβ (β; 30 ng) was loaded in lanes 2, 5, 8, 11, 14, and 17; 30 ng of rhERβ was loaded in lane 23. 63 kDa; Mit ERα/H9251, 59.4 kDa; ERα/H9251, 66 kDa; rhERα/H9252, 66 kDa; ERα/H9252, 66 kDa; Mit ERβ/H9251, 59 kDa; ERβ/H9251, 59.4 kDa; ERβ/H9252, 64 kDa; ERβ/H9252, 64 kDa; Mit ERβ, 64 kDa; CO II, 18 kDa; histone H1, 30 kDa. The approximate molecular masses for the unknown bands detected by CFEK-E12 (E, lanes 23–25), bands 1–4, are 67, 52, 80, and 59 kDa, respectively. The approximate molecular mass for the unknown band detected by CWFK-F12 (F), band 5 (lanes 27 and 28), is 99 kDa.

In the mitochondria, this antibody detected four protein bands of 80 (band 3), 67 (band 1), 64 (ERβ), and 59 (band 4; lanes 24 and 25) kDa. The identity of the 80-kDa and 59-kDa proteins is unknown. The 67-kDa (band 1) protein present in both nuclear and mitochondrial fractions is possibly ERα cross-reactive with this ERβ antibody because, as mentioned above, this antibody recognizes the epitope that shares partial homology to ERα. Detection of a 67-kDa protein with this antibody in the mitochondrial fraction adds additional support for the association of ERα with mitochondria stated above. The mobility of the ERβ (64-kDa) band in the mitochondrial fraction was slightly slower than that of the 63-kDa (ERβ) band in the nuclear fraction. The CWFK-F12 antibody (Fig. 1F) detected a 63-kDa protein in the nuclear fraction (lane 26) and a strong 64-kDa band (ERβ) as well as a weak 99-kDa band (band 5; lanes 27 and 28) in the mitochondrial fraction. The 99-kDa protein was not detected by Choi et al. (16), and its identity is unknown. Although the CFEK-E12 and CWFK-F12 antibodies to ERβ differ somewhat in their reactivity to several protein bands in the nuclear and mitochondrial fractions, both antibodies detected a 63-kDa protein in the nuclear fraction (lanes 23 and 26) and a 64-kDa protein in the mitochondrial fraction (lanes 24, 25, 27, and 28). Detection of CO II and histone H1 was used to mark mitochondrial and nuclear fractions, respectively. After stripping, the membranes of Fig. 1, C-E, were reprobed with CO II antibody, and the membrane of Fig. 1F was reprobed with histone H1 antibody. The CO II antibody detected a strong band in the mitochondrial fractions (lanes 19, 21, 24, and 25) and a weak band in the nuclear fraction (lane 23), whereas histone H1 antibody detected a strong band in the nuclear fractions (lane 26) but not in mitochondrial fraction (lanes 27 and 28), indicating that the mitochondrial fraction was enriched by fractionation and was not contaminated by detectable nuclear proteins. In summary, these results demonstrate that two antibodies to ERα detected a 67-kDa protein, and four antibodies to ERβ detected a 64-kDa protein in MCF7 mitochondria. Use of rhERα and rhERβ together with preabsorption demonstrates the specificity of Santa Cruz antibodies. In separate experiments, we also detected a 64-kDa protein in the mitochondrial fractions of MCF7 and T47D cells using two monoclonal antibodies raised against the COOH-terminal and NH2-terminal regions of rat ERβ, respectively (data not shown). All together, these results demonstrate the association of ERα and ERβ with MCF7 cell mitochondria.
Determination of the relative amounts of ERα and ERβ in subcellular fractions of MCF7 cells. We used cell fractionation and Western blot analysis with the Santa Cruz ERα and ERβ antibodies to determine the relative amounts of ERα and ERβ in cytosolic, nuclear, and mitochondrial fractions. After fractionation, recovery of total homogenate protein was ~90%. The cytosolic, nuclear, and mitochondrial fractions contained 57 ± 4, 23 ± 3.7, and 10 ± 1.5% (means ± SD, n = 3 separated experiments) of total homogenate protein, respectively. This relative subcellular distribution of proteins is within the range reported by others (55). Figure 2A shows a representative Western blot used to determine the relative amounts of ERα and ERβ present in the subcellular fractions. As shown in Fig. 2A, the nuclear fraction (lanes 3 and 4) was enriched, as indicated by the presence of a heavier band of histone H1. This nuclear fraction also contained minor amounts of contaminating mitochondrial protein, as indicated by the presence of a lighter band representing CO II. The ERα antibody detected one major 64-kDa band (lanes 5 and 6) and, occasionally, one minor 80-kDa band (data not shown) in the mitochondrial fractions. The major ERβ band was present in all fractions (lanes 1–6). The ERα antibody detected one major protein band of 67 kDa. In the mitochondrial fraction, a major 67-kDa band representing ERβ was detected (lanes 5 and 6) in all experiments. In some experiments, the ERα antibody also recognized two minor protein bands whose identities were unknown (data not shown). Figure 2B shows the representative rhERα and rhERβ standard curves used to estimate the amounts of ERα and ERβ in different subcellular fractions. The relative percentage of ERα and ERβ in the cytosolic, nuclear, and mitochondrial fractions, corrected for the percentage of total homogenate protein recovered in these fractions, is shown in Fig. 2C. These data were obtained from three separate experiments. For ERα, ~10 ± 3.1, 70 ± 8.1, and 12 ± 3.6% were present in the cytosolic, nuclear, and mitochondrial fractions, respectively. For ERβ, ~50 ± 5.1, 26 ± 3.7, and 18 ± 2.8% of total ERβ were present in cytosolic, nuclear, and mitochondrial fractions, respectively. When expressed as micrograms ER per milligram protein in the fractions (Fig. 2D), the values for the amount of ERα in the mitochondrial and nuclear fractions were similar. For ERβ, the amount in the mitochondrial fraction was significantly higher (~2.5 times) than in the nuclear and cytosolic fractions, indicating that ERβ is more concentrated in mitochondria than in nuclei and cytosol.

Effects of E2 treatment on the amounts of ERα and ERβ within mitochondria and the transcript levels of mtDNA-encoded genes, CO I and CO II. To determine if both receptors localize to mitochondria in response to E2, we used Western blot analysis to examine the effects of E2 treatment on the amounts of ERα and ERβ within the mitochondria of MCF7 cells. Detection of CO II protein was used as a marker for mitochondrial proteins and as a control for the amount of protein loaded on the gels. Little ERβ and ERα were detected in mitochondria from non-E2-treated, control MCF7 cells. The amount of mitochondrial ERβ and ERα increased with time of E2 treatment (Fig. 3A). An increase in ERβ within mitochondria was evident after 1 h. Between 3 and 12 h, the amount of ERβ was significantly increased 2.0- to 2.5-fold. The amount of ERα within mitochondria also increased in a similar pattern.

Previously, we demonstrated that E2 enhanced mtDNA transcript levels in rat hepatocytes and human Hep G2 cells (8, 11). To extend this observation, we used Northern blots to examine the effects of E2 treatment on the transcript levels of mtDNA-encoded genes in MCF7 cells. The results presented in Fig. 3B show that enhanced transcript levels of CO I and CO II were evident within 3–6 h, and significant increases were observed 12–24 h after E2 treatment. These results show that the increased amounts of ERα and ERβ within mitochondria (Fig. 3A) were accompanied by increases in transcript levels of CO I and CO II (Fig. 3B).

![Fig. 2. Determination of the subcellular distribution of ERα and ERβ in 17β-estradiol (E2)-treated MCF7 cells. A: representative Western blot for the analyses of subcellular distribution of ERα and ERβ. B: representative rhERα and rhERβ standard curves. C: percentage of ERα and ERβ in each fraction relative to the total amount of ERα or ERβ in the homogenate, which was taken as 100%. D: amount of ERα and ERβ proteins expressed as µg/mg subcellular protein. Data presented in B and C represent the average values ± SD from 3 separate experiments. *Amount of ERα (µg/mg subcellular protein) in mitochondrial fraction was significantly greater than that in nuclear and cytosolic fractions (P = 0.011, n = 3 experiments). Hom, homogenate; Cyto, cytosolic fraction.](http://ajpendo.physiology.org/DownloadedFrom/10.1210/jc.2003-0477)
Fig. 3. Time- and dose-dependent effects of E₂ on the amounts of ERα and ERβ within the mitochondria and on the mRNA levels of CO I and CO II. A and C show the average ratios of the densitometry scans of the ERα or ERβ protein bands to that of the CO II protein band compared with the ratio of the control group. Data in A are average values of individual cultures in 2 separate experiments ± SD (n = 6) for all groups. *Significantly greater than those in control group (P < 0.03). In C, data for the 10⁻⁹ and 10⁻⁸ M groups are average values of 2 individual cultures from 1 experiment, and the data for the 10⁻⁷ and 10⁻⁶ M groups are the average values of 4 individual cultures of 2 separate experiments. †Significantly greater than the control group (P < 0.001). B and D show the ratios of the densitometry scans of the autoradiographs of CO I and CO II mRNA to those of the 28S plus 18S rRNA, expressed as the degree of induction over the control group. Data in both B and D are the average values of 6 individual cultures for each group in 2 separate experiments. Bars show means ± SD (n = 6). *Significantly greater than control group (P < 0.03).

We next examined the effect of E₂ concentration on the amounts of ERα and ERβ in MCF7 mitochondria. As shown in Fig. 3C, treatment of cells with 10⁻⁹ M E₂ for 12 h increased the amount of ERβ within mitochondria approximately twofold. The amounts of ERβ in mitochondria were significantly increased 2.5-fold when the E₂ concentrations were 10⁻⁸ and 10⁻⁷ M. Similarly, the amounts of ERα in mitochondria were also significantly increased when E₂ concentrations were increased to 10⁻⁸ and 10⁻⁷ M (Fig. 3C). The increase in the amounts of ERα and ERβ within mitochondria (Fig. 3C) was associated with significantly increased transcript levels of CO I and CO II (Fig. 3D) as E₂ concentrations were increased.

Effects of E₂ with or without ICI-182780 on the transcript levels of mtDNA-encoded genes and nuclear estrogen-responsive genes. To determine if ERs are involved in E₂ induction of mtDNA-encoded gene expression, we examined the effects of E₂ plus or minus the pure ER antagonist ICI-182780 on the transcript levels of CO I, CO II, and the mitochondrial transcript precursor (from which the mature mitochondrial RNAs, including CO I and CO II, are derived) as well as several nuclear estrogen-responsive genes, i.e., pS2, c-myc, and transforming growth factor (TGF)-β. Figure 4, A and B, shows that ICI-182780 at 1 μM (lanes 4–6) slightly induced the transcript levels of CO I and mitochondrial transcript precursor but not CO II. It is not clear whether ICI-182780 itself induces the transcript levels of these genes or enhances the stability of their mRNA species. However, in a separate experiment, we did not observe the enhanced effects of ICI-182780 at 0.3 μM (data not shown). Unlike ICI-182780, E₂ at 0.3 μM (lanes 7–9) enhanced the transcript levels of CO I and mitochondrial transcript precursor approximately fourfold and of CO II ~2.5-fold. At the same concentration, E₂ caused a three- to fourfold increase in the transcript levels of pS2, c-myc, and TGF-β over those of the control group (data not shown). However, in the

Fig. 4. Effects of E₂ ± ICI-182780 on the mRNA levels of several mitochondrial (mt) DNA-encoded genes in MCF7 cells. A: photographs of ethidium bromide-stained gels for 28S and 18S RNA and Northern blots using the same gel for the indicated genes. B: ratio of the densitometry scans of the autoradiographs of the indicated genes in the Northern blots to that of the densitometry scan of the 28S plus 18S rRNA, expressed as degree of induction over the control group. Bars show means ± SD. *Significantly greater than that of the control group (P < 0.02, n = 3); †transcript levels of the indicated genes in E₂ + ICI-treated group were significantly lower than those of the E₂-treated group (P < 0.03, n = 3).
presence of ICI-182780, the enhanced effects of E2 on transcript levels of these genes (lanes 7–9) were significantly reduced (lanes 10–12). These results indicate that E2 induction of mtDNA-encoded transcript levels was mediated via ERs.

**Detection of ER within mitochondria using immunohistochemistry.** The Western blot analyses indicated that ERα and ERβ were associated with mitochondria of MCF7 cells. Next, we used immunolocalization together with confocal laser scanning fluorescent microscopy to investigate this further in MCF7 and MCF-10A cells. The results are shown in Fig. 5, which is representative of at least two separate experiments for each cell line. Mitochondria of MCF7 and MCF-10A cells are represented by the red color (Fig. 5, panels 1, 4, 7, 10, and 13). In MCF7 cells, ER-β (stained green) is detectable in their nuclei and as punctate staining in their cytoplasm (Fig. 5, panel 2). Localization of ER-β within mitochondria is indicated by the presence of yellow spots in the cytoplasm (Fig. 5, panel 3). Most of the ~25–50 MCF7 cells observed showed mitochondrial localization of ERβ. Western analysis of MCF-10A cells detected no ERβ (data not shown), and this was confirmed by the lack of detectable cytoplasmic and nuclear staining (Fig. 5, panel 5), similar to what was observed in cells stained with preimmune rabbit IgG, followed by secondary antibody tagged with fluo 488 (Fig. 5, panel 8). ERα (green) was seen in nuclei and was barely detectable in cytoplasm, but some colocalization appeared to be present just outside the nucleus of some cells (Fig. 5, panels 11 and 12). In MCF-10A cells, ERs was observed in nuclei (Fig. 5, panel 14) but not within mitochondria (Fig. 5, panel 15). E2 treatment did not enhance mtDNA-encoded transcript levels in MCF-10A cells (data not shown). These results, which are consistent with the Western blot analysis (Figs. 1–3), provide additional confirmation for the presence of ERs and ERβ in mitochondria of MCF7 cells.

**Detection of ERs within mitochondria of MCF7 cells by use of immunogold electron microscopy.** To provide additional confirmation for the presence of ERs within MCF7 cell mitochondria, we next performed immunogold electron microscopy (Fig. 6, A and B). The micrographs clearly show that, with ERα antibody, gold particles are seen within the nucleus and the mitochondrial matrix (Fig. 6A, panels 1 and 2), confirming the presence of ERα within the mitochondria. A few gold particles are also scattered in the cytosol. Gold particles were observed within every mitochondria of all cells examined. In the cells stained with ERα antibody preabsorbed with rhERα, virtually no gold particles were detected (data not shown). With ERβ antibody, gold particles were also observed in the nucleus (Fig. 6B, panel 3) and the mitochondrial matrix (Fig. 6B, panels 3 and 4). Scattered, but not evenly distributed, gold particles were observed in the other parts of cytoplasm. Preabsorption of ERβ antibody with rhERβ virtually eliminated the presence of the gold particles (data not shown). Together, these results add

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**Fig. 5.** Immunolocalization of ERα and ERβ using confocal laser-scanning microscopy. Mitochondria of the MCF7 and MCF-10A cells are in red (panels 1, 4, 7, 10, and 13). ERβ (panels 2, 5, and 8) was identified with Santa Cruz ERβ antibody (A), and ERα (panels 11 and 14) was identified with Santa Cruz ERα antibody (B), coupled to secondary anti-rabbit IgG tagged with the green fluorescent dye (green). Preimmune rabbit IgG (C) coupled to the same secondary anti-rabbit IgG tagged with the green fluorescent dye was included as negative control. The corresponding overlay images were shown in panels 3, 6, 9, 12, and 15. White bars are the scale bars.

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additional support to the Western blot and confocal microscopy immunocolocalization studies by demonstrating at the electron microscopic (EM) level that ERβ and ERα are localized in the mitochondrial matrix of MCF7 cells. Our Western blot standard curves generated using rhERα and rhERβ proteins indicated that the amounts of antibodies used were in excess and thus should detect all the ER protein available in the thin EM sections. Although a direct quantitative comparison using these electron micrographs was not the goal of these EM studies, we did attempt to determine the number of gold particles detected with each antibody and observed that fewer gold particles appeared to be present within the mitochondria with use of the ERα antibody compared with the ERβ antibody (data not shown).

Computer analysis of ERα and ERβ for the presence of mitochondrial targeting peptide signals. Proteins that target to mitochondria usually contain mitochondrial targeting peptide signals (mTPS; see Ref. 30). The presence of ERα and ERβ within mitochondria led us to examine whether either ER contains mTPS. We analyzed the primary amino acid sequence of human ERα (accession no. M12674; see Ref. 27) and human ERβ (accession no. JC5939; see Ref. 54) using a TargetP program (http://www.cbs.dtu.dk/services/Targetp/; see Ref. 22). Neither the NH2-terminal nor COOH-terminal regions of either ER contain amino acid sequences with properties similar to those of mTPS. However, the internal amino acid sequence 220–270 of ERβ contains a number of positively charged regions characteristic of mTPS. Analysis of this region using the TargetP program predicts that region 221–270 contains a putative mTPS with a score of 0.73 out of 1.0. Prediction of α-helical structures using the secondary structure program “Predator” (http://www.ebi-heidelberg.de/predictionprotein/predictprotein.html) indicates that the amino acid sequences 221–230 and 251–260 have a tendency to form α-helical structures characteristic of mTPS. Furthermore, the sequence 251–270 also has a tendency to form a transmembrane domain. These properties are somewhat similar to those of the internal mTPS of BCS1 and Tim23p (18, 25), both of which are located in the mitochondrial inner membrane. The internal sequence 231–280 of human ERα also contains a concentration of positively charged residues. However, the TargetP program predicted that this region has less likelihood for being an mTPS, with a score of 0.45.

DISCUSSION

In the present study, we obtained several lines of evidence that definitively demonstrate the presence of ERα and ERβ within the mitochondria of MCF7 cells. We also found that localization of a fraction of total cellular ERα and ERβ to mitochondria is enhanced by treatment with E2 in a concentration- and time-dependent manner and is accompanied by increased transcript levels of mtDNA-encoded genes. The E2 induction of mtDNA-encoded transcript levels was inhibited by ICI-182780, a pure ER antagonist. These results suggest that the mitochondrial ERs may have a potentially important role in the E2 induction of mtDNA-encoded gene expression.

Extensive studies on the localization of ERα and ERβ via immunohistochemical staining, with various polyclonal and monoclonal antibodies directed against different regions of both ERs, have been performed by others. A number of these studies found that, in addition to the predominantly nuclear staining, various degrees of cytoplasmic staining for both ERα and ERβ were evident in a variety of cells and tissues. For example, ERα immunostaining was observed in both the nucleus and cytoplasm of human mammary carcinoma (37, 40), rabbit endometrial epithelial cells (37), and MCF7 cells (65). Positive nuclear and cytoplasmic staining for ERβ was also observed in several types of cells in brain (2, 38, 44, 56, 66, 75), liver, and several other tissues in rats (31, 59, 60) as well as in a number of human tissues and cells, including breast cancers (15), osteoclasts (4, 70), colorectal adenocarcinoma...
(73), intestinal metaplasia (42), small intestine (67), placenta (5), and prostate cancers (1, 36). The presence of cytoplasmic staining for both ERα and ERβ in a wide variety of cells and tissues raises several intriguing and important questions: 1) do ERα and ERβ reside in cytoplasmic organelle(s)? 2) how much of the total cellular ERα and ERβ is present in the cytoplasmic organelle(s)? 3) how do ERα and ERβ localize there? 4) what are they doing there? and 5) what physiological and pathophysiological roles do they play?

Several earlier reports have suggested a partial answer for the first question (29, 45–47, 53), which is that there may be an association of a portion of total cellular estrogen-binding capacity and EBPs with mitochondria. Using cellular fractionation/Western blot and ligand binding, Monje and Boland (47) and Monje et al. (48) have provided more direct evidence for the presence of ERα and ERβ in mitochondria from rabbit uterus and ovary tissue. To our knowledge, these are the only reports, to date, demonstrating a mitochondrial localization of the ERs. Because these authors did not relate the presence of the ERs in mitochondria to the mediation of functional effects caused by estrogen treatment, the importance of their findings has not been appreciated. Thus a definitive demonstration of the presence of ERα and ERβ in mitochondria of other estrogen-target cells and tissues and the relevant functions of the mitochondrial ER was needed.

To confirm and extend the findings of Monje and colleagues (47, 48) we extensively investigated the mitochondrial localization of ERα and ERβ in MCF7 cells. Using rhERα, rhERβ, and MCF7 mitochondrial protein and Western blot analysis, we determined that the ERα and ERβ antibodies from Santa Cruz specifically reacted with ERα and ERβ, respectively. The specificity of these ERα and ERβ antibodies enabled us to quantify the subcellular distribution and perform immunolocalization of both ERα and ERβ using Western blot, confocal fluorescent, and immunogold electron microscopy.

We used two ERα antibodies, one directed against the NH2-terminal domain and the other directed against the COOH-terminal domain of ERα, to detect the presence of ERα protein in mitochondria of MCF7 cells via Western blot analysis. Using both ER-α antibodies, we detected 67 kDa ERα in mitochondria of MCF7 cells. We used four different ERβ antibodies raised against different regions of human ERβ (see above) to detect ERβ in the mitochondrial fraction. The Santa Cruz ERβ antibody has been used by several groups to investigate the distribution and biochemistry of ERβ (26, 31, 41, 75). For example, Garcia Pedrero et al. (26) detected a single ERβ band (63 kDa) in nuclear extracts of MCF7 and T47D cells. Consistent with their results, we detected a 63-kDa ERβ protein band in homogenate, cytosolic, and nuclear fractions and a 64-kDa protein band in the mitochondrial fraction of MCF7 cells. We also occasionally detected a weak 80-kDa protein band in the mitochondrial fraction (data not shown) by using this antibody. The GC-17 ERβ antibody has been demonstrated to specifically react with rhERβ and with a 63-kDa ERβ protein in human testis and prostate cells (36). Using this antibody, we detected a 64-kDa protein in the mitochondrial fraction and rhERβ. To confirm the association of the antibody-reactive 64-kDa protein with mitochondria, we performed Western blots using two additional monoclonal antibodies, CFK-E12 and CWK-F12, both of which have been shown to react with ERβ proteins of several species in addition to humans (16). The CFK-E12 reacted to a 63-kDa ERβ and a 52-kDa protein, whereas CWK-12 only recognized the 63-kDa ERβ protein in human granulosa cell homogenates (16). Using CFK-E12, we detected three protein bands (a 63-kDa ERβ and a 52-kDa and a weak 67-kDa band) in the nuclear fraction and four bands (80, 67, 64, and 59 kDa) in the mitochondrial fraction of MCF7 cells. The CWK-F12 ER-β antibody detected a 63-kDa ERβ band in the nuclear fraction and a 64- and 99-kDa band in the mitochondrial fraction. In addition, we also detected a 64-kDa protein in the mitochondrial fraction of MCF7 and T47D cells using two other antibodies directed against recombinant rat ERβ protein (data not shown). Although these different ERβ antibodies detect several other protein species in nuclear and mitochondrial fractions, they share two common properties: 1) they all react with purified rhERβ but do not react with purified rhERα, and 2) they all detect a 63-kDa ERβ in nuclear fractions and a 64-kDa protein in mitochondrial fractions. These results indicate that, like the 63-kDa protein in nuclear fractions, the 64-kDa protein in the mitochondrial fraction could be an ERβ isoform. The reason for the apparent discrepancy in the mobility of the ERβ (64-kDa) band in the mitochondrial fraction from that of the 63-kDa (ERβ) band in the nuclear fraction is not clear but may relate to detection of ERβ isoforms that differ in posttranslational modifications such as alternative glycosylation and phosphorylation (12–14, 51). Our detection of the 64-kDa ERβ protein in mitochondria is consistent with observations made by Grossman et al. (29). Using immunocytochemical localization via immunogold electron microscopy, these investigators observed the presence of an EBP on the plasma membrane and endoplasmic reticulum and inside the mitochondria of rat pancreatic acinar cells. They also demonstrated that an anti-EBP antibody reacted specifically with a doublet of protein bands in the mitochondrial fraction having molecular masses of 66 and 64 kDa (29). The molecular masses of these EBPs are similar to those of ERα and to the 64-kDa ERβ, respectively, that we observed. The similarity in molecular masses and in estrogen-binding ability between ERs and the EBPs suggests that these EBPs are ER-like proteins. Whether or not the other mitochondrial-associated proteins (e.g., the 80- and 59-kDa proteins detected by the CFK-E12 antibody and the 99-kDa protein detected by CWK-F12 antibody) are ERβ-related proteins remains to be investigated.

Our most definitive support for the intramitochondrial localization of ERα and ERβ comes from the immunolocalization studies. Confocal microscopy clearly demonstrated the localization of the ERs within mitochondria of MCF7 cells. Electron microscopy showed the presence of numerous ERα antibody- and ERβ antibody-linked gold particles within the matrix of each mitochondrion in MCF7 cells. The specificity of this finding was confirmed by observing that preabsorption of the ERα antibody with rhERα and of the ERβ antibody with rhERβ virtually eliminated detection of gold particles in the mitochondria, nuclei, and cytoplasm. The EM results also suggested that the levels of ERα were less than those of ERβ.

Consistent with our experimental results, we identified a putative mTPS within human ERβ. The majority of nuclear-encoded, mitochondrially localized proteins contains mTPSs in their NH2-terminal presequences (30). Other mitochondrial proteins, such as BCS1 (25) and Tim23p (18), contain mTPSs in their internal sequences. In light of our findings, and those of
ERα was similar to that in the nuclear fraction, the amount of ERβ was significantly lower. We observed that, although the amount of ERα in the cytoplasmic organelles, we quantitatively estimated the relative subcellular distribution of these receptors. We observed that a fraction of the total cellular ERα (~12%) and ERβ (~18%) was present in the mitochondrial fraction in E2-treated MCF7 cells. We also observed that, although the amount of ER-α in the mitochondria was similar to that in the nuclear fraction, the amount of ER-β was higher in the mitochondrial fraction than that in the nuclear fraction and the amount of ER-β present in mitochondria was significantly higher than that of ERα. This is the first quantitative estimation of the subcellular distribution, particularly for mitochondria, of ERα and ERβ for MCF7 cells.

To address the third question of how do ERα and ERβ localize to mitochondria, we investigated the effects of E2 treatments on the amounts of ERα and ERβ in mitochondria. We found that E2 treatment enhanced the amount of ERα and ERβ within mitochondria of MCF7 cells in a time- and concentration-dependent manner. After growth of the cells in medium containing stripped serum, only low levels of either receptor protein were detected in the mitochondrial fraction. E2 treatment for 3–12 h caused a significant, time-dependent increase in the amount of mitochondrial ERα and ERβ. We also detected enhanced levels of mitochondrial ERβ at E2 concentrations of 1–10 nM and of ERα at E2 concentrations of 10–100 nM. These results are the first to demonstrate that E2 treatment stimulates the import of both ERs into mitochondria and provide a partial answer to the question of how ERα and ERβ localize to mitochondria.

To address the fourth question of what ERs are directly involved in estrogen induction of mtDNA transcription, we examined the time- and concentration-dependent effects of E2 on the transcript levels of mtDNA-encoded genes and determined whether ERs are involved in these E2-mediated effects. Another important observation that we made in this study is that the localization of both ERα and ERβ to mitochondria in response to E2 treatment was accompanied by a concomitant time- and concentration-dependent increase in the transcript levels of the mtDNA-encoded genes, e.g., mitochondrial transcript precursor CO I and CO II. Furthermore, we demonstrated that these E2-enhanced effects on mtDNA-transcript levels were mediated via ERs, as indicated by the significant inhibition of these effects by the ER antagonist ICI-182780. The majority of mtDNA genes encoding for mitochondrial RNAs (13 mRNA, 2 rRNA, and 22 tRNA) appear to be transcribed polycistronically under the control of the displacement loop (D-loop), an important regulatory element (17). The detection of increased levels of the mitochondrial transcript precursors CO I and CO II suggests that the E2 effects extend to all of the mitochondrial transcripts and that E2 enhances transcript levels of mtDNA genes by inducing mtDNA transcription. Human and murine mtDNA contain putative ERE sequences in their D-loop and the coding regions (21, 64). Using gel electrophoresis mobility shift assays and surface plasmon resonance analysis, we have shown that these putative EREs in the mtDNA D-loop bind specifically to purified rhERα-, rhERβ-, and ER-β-containing mitochondrial proteins and that the ER binding was enhanced by E2 and inhibited by ICI-182780 (Chen et al., unpublished observation). The correlation of the localization of ERα and ERβ in mitochondria with the increased binding of mitochondrial proteins to the D-loop ERα and with the induction of mtDNA-encoded gene transcription in response to E2 treatment in a time- and concentration-dependent manner plus the inhibition of these effects by ER antagonist suggest that the mitochondrial ERs may play an important role in E2 induction of mtDNA transcription. Although we have demonstrated the presence of ERs within mitochondria and their possible involvement in the E2 regulation of mtDNA expression, it is also possible that these E2-induced mitochondrial effects could be coordinated mediated by the E2 activation of the nuclear ERs, which, in turn, promotes the expression and import in mitochondria of other nuclear-encoded transcript factors required for mtDNA transcription. Although the determination of whether the mitochondrial ERs have a direct transcriptional role within mitochondria and whether the nuclear ERs are involved is not clear at present and requires further investigation, the findings presented above provide a partial answer for the fourth question of what ERs are doing inside mitochondria.

Although the definitive answers to the fifth question of what physiological and pathophysiological roles the mitochondrial ERs play have not been addressed in this study and remain to be investigated, it is possible that these effects of estrogen on mitochondria will have significant pathophysiological implications. It is well-known that mitochondria have a central role in the control of apoptosis, and it is possible that inhibition of apoptosis by estrogen may contribute to its carcinogenic effects. In previous studies using rat hepatocytes, we found that the increase in the levels of the mitochondrial mRNA transcripts was associated with increased mitochondrial respiratory chain activity, increased glutathione distribution in both nuclei and mitochondria, and inhibition of TGF-β-induced apoptosis (7, 8, 10). These effects were blocked by the pure ER antagonist ICI-182780 (10). Wang et al. (71) reported that, in the mice whose gene for mitochondrial transcription factor A was knocked out in their heart tissue, the expression of mtDNA genes in their cardiocytes was deficient, and this was associated with a significant increase in vivo in apoptotic cardiocytes. Together, these results suggest that the up- and downregulation of the mtDNA gene expression is important in the control of the balance between antiapoptosis and apoptosis. The definitive role of these effects in E2-mediated inhibition of apoptosis remains to be determined. Accordingly, if the mitochondrial ERs are directly involved in estrogen induction of mtDNA gene expression, then the signaling pathway mediated through these mitochondrially localized receptors may represent a novel target for regulation, prevention, and therapeutic intervention.

In summary, using a number of complementary approaches, including cellular fractionation and Western blot, immunolo-
calization with confocal and immunogold electron microscopy, and computer prediction for mTPS, we have obtained several lines of evidence that demonstrate the intramitochondrial localization of ERs. Our study has confirmed and greatly extended the observations of Monje and colleagues (47, 48) to MCF7 cells. Furthermore, we have determined that the predominant ER present within the mitochondria of MCF7 cells is ERβ and have shown that the level of mitochondrial ERs present is determined by the concentration and time of estrogen treatment. We also demonstrated that the import of ERs in mitochondria is accompanied by a concomitant increase in transcript levels of mtDNA-encoded genes and that these effects are inhibited by ER antagonist. Together, our results suggest an important potential for E2 regulation of mtDNA transcription by the mitochondrial ERs and open a potentially novel paradigm for the actions of E2 and ERs within mitochondria.

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