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Expression and Cellular Localization of Naturally Occurring β Estrogen Receptors in Uterine and Mammary Cell Lines

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Abstract The protein ER-α has been exhaustively characterized in estrogen-sensitive tissues and cell lines. However, little is known regarding the expression and cellular distribution of the newly identified ER-β protein. We first quantified the specific estradiol binding site content in the estrogen-responsive cell lines MCF-7 (mammary) and SHM (myometrial). In the two cell types, these sites were associated to the expression of both ER-α and -β isoforms. Native ER-β was visualized to reside inside the nucleus by means of conventional indirect immunofluorescence. The cells expressed ER-β as a tight ~50 kDa triplet when resolved by sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) and blotted using antibodies mapping different domains of the cloned ER-β version. When the cells were subjected to homogenization and differential centrifugation, a substantial proportion of ER-β immunolabeling was localized at membrane subfractions. ER-β expression and partitioning was confirmed by Ligand blotting assays using estrogen derivatives coupled to different macromolecular tags. However, ER-α was expressed as the major estrogen binding protein in both cell lines. Similar localization experiments were performed on HeLa cells (cervix). Though usually considered ER-negative, this cell line displayed basal significant estrogen binding capacity and co-expression of both ER isoforms. Taken as a whole, the results indicate that ER-β could be expressed as functional estrogen binding proteins among a dominant population of ER-α sites in the cell lines under study. J. Cell. Biochem. 86: 136–144, 2002.

Key words: estrogen receptor; α and -β isoforms; SHM; MCF-7; HeLa

Estrogens modulate the transcription of target genes by the interaction of its occupied receptor, the estrogen receptor (ER), with promoter *cis*-regulatory specific DNA elements [Evans, 1988; Carson Jurica et al., 1990; Tsai and O'Malley, 1994; Parker, 1995; among others]. In recent years, it was demonstrated that the ER, as an exception in the steroid receptor superfamily of transcription factors, exists under two isoforms, now distinguished as ER-α and -β [Gustafsson, 1999; Pettersson and Gustafsson, 2001; and references therein]. Each isoform is encoded by a separate gene, but a high sequence conservation remains at the DNA and ligand binding domains of the two receptors. Transcripts coding for the novel ER-β subtype were first found to be expressed in reproductive organs such as the ovary and prostate, by RT-PCR, and in situ hybridization analysis [Kuiper et al., 1996]. Subsequently, messengers for the ER-β gene were confirmed to be widely distributed among a variety of non-reproductive tissues [Arts et al., 1997; Kuiper et al., 1997, 1998; Gustafsson, 1999]. However, the proper assessment of the expression of endogenous wild-type ER-β protein products was delayed until quite recently. For that purpose, the development of biological tools such as antibodies with specificity in the effective

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recognition of the ER-β antigen became an essential startpoint, allowing the identification of expression patterns exclusive for the β subtype. Several ER-β antibodies have been designed and became available from commercial sources [reviewed by Pavao and Traish, 2001].

The present study was focused to analyze the occurrence of ER-β proteins among the classical ER-α products in uterine and mammary cells. For that purpose, we selected the breast cancer-derived MCF-7 cell line and the Syriam Hamster Myocytes (SHM) uterine smooth muscle cell line as experimental models. We have previously described the expression and cellular localization of ER-α proteins in these same established lines [Monje et al., 2001]. MCF-7 cells have also been shown to express low, but detectable levels of both ER-β mRNA and translation products [Fuqua et al., 1999; Vladusic et al., 2000]. We now report the detection of ER-β proteins using different isoform-specific antibodies for fluorescence immunocytochemistry and Western blot experiments. To better understand the extent to which each ER isoform was expressed, we developed ligand blotting assays using non-radioactive macromolecular derivatives of 17β-estradiol to label ERs after their separation by sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE). This method provided a convenient technique for a sensitive and specific detection of estrogen binding proteins assigned to each ER subtype. In addition, ER-β partitioning properties were studied after cell homogenization and fractionation by differential centrifugation. Complementary studies on ER-α and -β expression and cellular localization were also performed on HeLa uterine cells. We could establish that even though ER-β proteins were evidently expressed as functional estrogen binding proteins in the cellular systems examined, the classical ER-α isoform represented the major estrogen binding site in the cells. We thus speculate a dominant role for ER-α in the systems considered.

MATERIALS AND METHODS

Materials

Cell culture media and sera were purchased from GIBCO (Grand Island, NY). [2,4,6,7-^{3}H(N)]17β-estradiol with a specific activity of 80–115 Ci/mmol was obtained from New England Nuclear (Chicago, IL). Non-radioactive 17β-estradiol, 17β-estradiol(6-*O*-carboxy-methyl) oxime: BSA fluorescein isothiocyanate conjugate (E_2-BSA-FITC) and 17β-estradiol-peroxidase (E_2-P) were from Sigma-Aldrich (St. Louis, MO). Anti-ER-α mouse monoclonal antibodies TE111.5D11 (anti-ER ligand binding domain), AER314 (anti-ER transactivation domain), and AER308 (anti-ER hinge region) were purchased from NeoMarkers (Fremont, CA). The anti-ER-β rabbit polyclonal antibody PAI-310, against amino acids 468–485 at the C-terminal region, was obtained from Affinity BioReagents (Golden, CO). Anti-ER-β goat polyclonal antibodies Y-19 and L-20 mapping sequences at the N-terminal (amino acids 10–28) and C-terminal (amino acids 439–458) domains, respectively, were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Lamin B goat polyclonal IgG (M-20) was purchased from the same company. Fluorescent Oregon Green™-conjugated secondary antibodies were from Molecular Probes (Eugene, OR). All other reagents were of analytical grade.

Cell Lines and Cell Cultures

The cell lines MCF-7 (human breast cancer epithelial cells) and HeLa (human cervical epithelial cells) were obtained from the American Type Culture Collection (Rockville, MD). SHM cells were kindly provided by Dr. Kirk Riemer (University of California, San Francisco). The latter cell line is estrogen-sensitive and displays a smooth muscle-like phenotype under culture conditions [Riemer et al., 1993]. MCF-7 cells were routinely cultured in serum-supplemented media composed of RPMI with phenol red, 10% FBS, and streptomycin–penicillin. SHM and HeLa cells were grown in α-minimum essential medium (α-MEM) supplemented in the same manner. The cells were cultured at 37°C in a humidified atmosphere containing 95% air/5% CO_2. The medium was replaced every 2 days, and cells were passaged every 3–5 days. Serum was removed 24 h before experimentation.

[^{3}H]17β-Estradiol Binding Assays

Estrogen binding site concentration in living cells was quantified by means of a whole-cell binding assay [Sadovsky et al., 1992]. MCF-7, SHM, and HeLa cells, cultured in six-well plates, were exposed to the presence of 5 nM [^{3}H]17β-estradiol in DMEM. After 90 min incubation at 37°C, the cells were exhaustively rinsed with ice-cold phosphate buffer saline

(PBS) to remove unbound radioligand. Trapped radioactivity was finally extracted with 1% SDS–1 N NaOH. Aliquots from each well were taken in triplicate for both scintillation counting and protein quantification by the method of Lowry et al. [1951]. A 400-fold molar excess of unlabeled 17β-estradiol was included together with the radioligand to determine non-specific binding to the cells. Ethanol final concentration did not exceed 0.005%. Each condition was assayed in quadruplicate.

Immunocytochemistry for ER-α and -β

Immunocytochemistry was performed as previously described [Monje et al., 2001]. Briefly, semi-confluent monolayers were fixed for 15 min in 2% paraformaldehyde–PBS containing 0.05% Triton X-100. Non-specific sites were blocked with 5% BSA in PBS. Cells were then incubated for 60 min in the presence or absence (negative control) of anti-ER-α or ER-β antibodies (1:50 dilution in PBS–1% BSA). Staining was performed using Oregon Green-conjugated secondary antibodies. Slides were mounted with the commercial Prolong-Antifade™ reagent (Molecular Probes) according to the manufacturer's protocol. Cells were visualized by conventional epifluorescence using a Zeiss Axiolab fluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with a standard FITC filter set (450–500 nm). An oil-immersion, 1.4 numerical aperture 65 × objective was selected for the observations. Photographs were taken using a Zeiss 35-mm camera and Kodak Tri-X-Pan 400 films for black and white prints.

Preparation of Subcellular Fractions

Subconfluent monolayers were scrapped and homogenized in ice-cold TES buffer (50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml aprotinin) using a Teflon-glass hand homogenizer. Subcellular fractions derived therefrom were isolated by differential centrifugation as previously described [Monje and Boland, 1999]. A nuclear pellet was first separated by low speed centrifugation (800*g*, 20 min) and the remaining supernatant was further centrifuged (10,000*g*, 15 min) to pellet mitochondria and lysosomes. The post-mitochondrial supernatant was subjected to ultracentrifugation conditions (120,000*g*, 90 min) to yield a soluble cytosol and a membrane-containing particulate pellet (microsomes). Total protein concentration of fractions was estimated by the method of Bradford [1976], using BSA as standard.

Western Blot Analysis

ER-α and -β immunoreactivity was analyzed in total homogenates and subcellular fractions from the cell lines. Equal protein samples were dissolved in loading buffer (400 mM Tris-HCl, pH 6.8, 10% SDS, 50% glycerol, 500 mM DTT, and 2 μg/ml bromophenol blue), denatured at 95°C for 5 min and resolved on 10% SDS–PAGE. Fractionated proteins on the gels were electroblotted onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P). The membranes were blocked with 5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBS-T), and were then probed with the appropriate dilution of each primary antiserum. Secondary antibodies conjugated to horseradish peroxidase were used. Proteins reacting with these antibodies were detected using ECL enhanced chemiluminescence (Amersham, Piscataway, NJ). In some experiments, the blots were washed (15 min, 50°C) with stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 50 mM β-mercaptoethanol), to remove bound antibodies. Then, immunostaining was performed with a different antibody as described above. Relative migration of unknown proteins was determined by comparison with a wide range colored protein markers. Determination of the apparent molecular weight of protein bands was done using the program Sigma Gel (Jandel Scientific).

Ligand Blot Analysis

Denatured protein samples were subjected to SDS–PAGE and transferred as described above. The blots were exhaustively washed with PBS-T, at least overnight at 4°C to allow protein renaturalization, and then blocked with 5% BSA in PBS. To evidence the presence of estrogen binders, the membranes were then incubated for 1 h at room temperature with E_2-BSA-FITC (10^{-6} M). Reactive bands were visualized using a conventional UV transilluminator. In parallel experiments, E_2-P was used instead of E_2-BSA-FITC. Membranes containing renatured proteins were incubated overnight at 4°C (or 1–2 h at room temperature) in the presence of E_2-P (50–5 nM) dissolved in 1% BSA–PBS. Peroxidase activity was revealed by enhanced chemiluminescence. Estradiol derivatives linked to

horseradish peroxidase have been previously shown to bind to ER-like estrogen binding proteins from sperm lysates [Luconi et al., 1999] and pancreatic islet cells [Nadal et al., 1998]. We have also found them to be adequate tools to label ER-α and -β from rabbit uterus and ovary [Monje and Boland, 2001]. E_2-BSA-FITC (steroid: BSA molar ratio ~32:1) and E_2-P (1–2 moles estradiol per mole peroxidase type VI) final concentration was calculated from the number of 17β-estradiol molecules attached to each macromolecule.

RESULTS

The quantification of estrogen binding activity in uterine and mammary cell lines rendered the results shown in Figure 1. Reproducible specific binding to 17β-estradiol could be measured, as expected, in the two ER-positive MCF-7 [Lippman et al., 1976] and SHM [Riemer et al., 1993] cell lines. Unexpectedly, we could detect the presence of a low, but significant specific estrogen binding capacity (~40 fmoles/mg protein) in HeLa cervical cells, a cell line that has been usually referred to as ER negative. In each experiment, determination of [^{3}H]17β-estradiol binding activity was performed on whole living cells since the estrogen binding capacity was almost completely lost when cells were homogenized, and binding was determined by cell-free batch assays according to the hydroxylapatite technique [Monje and Boland, 1999].

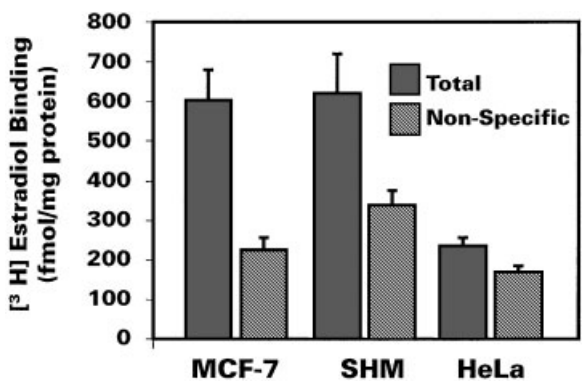

Fig. 1. [^{3}H]17β-estradiol binding capacity of estrogen-sensitive cell lines. Estrogen binding site concentration was quantified by saturation binding assays to whole cells in culture (Materials and Methods). Living MCF-7, SHM, and HeLa cells were incubated with 5 nM [^{3}H]17β-estradiol alone (total binding) or in combination with a large excess of the cold steroid (non-specific binding). Results are expressed as fmol/mg cellular protein and represent the mean of samples analyzed in quadruplicate ± SD. Representative results from three independent quantification experiments are given.

In order to dissect these estrogen binding sites into the presence of ER-α and -β components, we stained the cells with specific anti-ER-α or anti-ER-β antibodies and visualized them by conventional indirect immunofluorescence. As shown in Figures 2A and 3A, not only the classical ER-α, but also the novel ER-β subtype were expressed by MCF-7 and SHM cell lines at basal conditions. Naturally-occurring ER-β proteins were mainly localized intranuclearly and could be seen as discrete granules of fluorescence more or less evenly distributed throughout the nuclear region, with a marked nucleolar exclusion. However, some cytoplasmic punctate staining was also evident. This localization pattern coincides with that obtained for the well-known ER-α isoform in the same cellular systems (Figs. 2A and 3A, insets).

To analyze ER-α and -β expression and estimate their relative contribution to estrogen binding, we used 17β-estradiol macromolecular derivatives as estrogen ligands for Ligand blot experiments. This technique allowed us to non radioactively detect the presence of estrogen binding proteins after their separation by SDS–PAGE, transference to hydrophobic membranes, and renaturalization. Our protocol was essentially developed according to that described by Luconi et al. [1999], with slight modifications. The method relies on the presence of a functional ligand binding domain in the receptors and is also dependent on a protein renaturalization process. Using E_2-P at concentrations in the nanomolar range, we could confirm the co-expression of ER-α and -β binding proteins in total homogenates from MCF-7 and SHM cells (Figs. 2B and 3B, lanes 1). Wild type ER-α (~67 kDa) was expressed as the major estrogen binding entity in both cell lines. ER-β was usually resolved as close multiple bands, usually a tight doblet, ranging the expected molecular weight (~50 kDa) when visualized by Ligand blotting. Similar results were obtained by using specific anti-ER-β antibodies (Figs. 2B and 3B, lanes 2). The immunoblots sometimes showed the expression of a third immunoreactive band for ER-β detection.

Figure 4 depicts the complete subcellular distribution profile of ER isoforms after subjecting cellular homogenates from MCF-7 cells to separation by differential centrifugation. Ligand blotting experiments using fluorescent 17β-estradiol covalently linked to BSA (E_2-BSA-FITC) gave a first insight into ER-α and -β

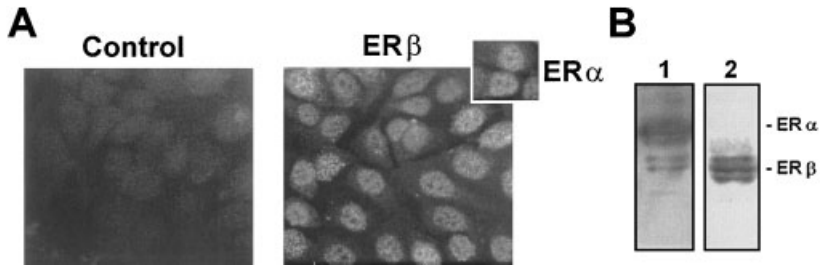

Fig. 2. Expression of ER-β isoform in MCF-7 cells. **A**: ER-β cellular localization: conventional indirect immunofluorescence. Subconfluent MCF-7 monolayers were fixed and labeled with the anti-ER-β rabbit polyclonal antibody (PAI-310, 1:50 dilution) as described in Materials and Methods. Staining was performed using Oregon-Green-conjugated secondary antibodies. Labeling of ER-α using the monoclonal AER 314 (1:50) is shown for comparison (inset). MCF-7 cells were incubated with fluorescent antibodies in the absence of primary antibodies (**left panel**). Original magnification = 650 ×. **B**: ER-α and -β isoforms as estrogen binding proteins. Total extracts from MCF-7 cells were processed for Ligand blot (**lane 1**) and Western blot (**lane 2**) employing E_2-P and anti-ER-β PAI-310 (1:200), respectively. E_2-P (5 nM) labeled two main reactive protein bands of ~67 and ~50 (doblet) kDa molecular weight, that exactly colocalized with the relative migration of ER-α and -β, respectively. Note that anti-ER-β antibodies PAI-310 displayed no cross-reaction with ER-α proteins. Lane 1: 5 μg; lane 2: 50 μg cellular protein.

partitioning among subfractions (Fig. 4A). Despite the rather limited sensitivity of the Ligand blot technique when E_2-BSA-FITC derivatives were used as estrogen probes, we were able to confirm the expression and distribution of ER-α and -β as estrogen binders. Special care was taken upon ER-β immunodetection. Labeling of these receptor proteins was assayed using a range of different commercial anti-ER-β antibodies mapping discrete and specific sequences from the known ER-β version (Fig. 4B). The polyclonal antibodies selected by us have been frequently shown to detect purified or enriched preparations of ER-β proteins [Pavao and Traish, 2001]. We have also found them as adequate probes to evidence the presence of physiological levels of endogenous receptors. Ligand blotting and immunological data obtained for the expression of ER-β proteins were shown to match very closely (Fig. 4A,B). Interestingly, these novel receptors appeared to be most concentrated in the membrane-containing subcellular preparations, i.e., the high speed centrifugation pellets or microsomes. When we performed similar localization experiments on subcellular fractions isolated from SHM cells, the results obtained were strictly comparable (Fig. 5A). Again, ER-β immunoreactivity was able to partition in a differential manner among subfractions, with the membranes accounting for a very important proportion of the expressed receptors. These results were corroborated by Ligand blotting using E_2-P as shown in Figure 5A (bottom). The lower relative ER-β content detected in the nuclear fraction from MCF-7 and SMH cells in comparison to that

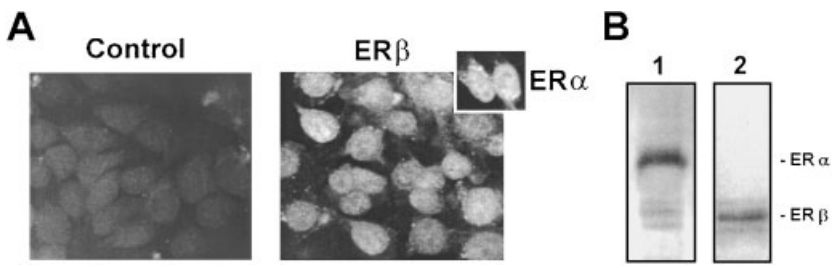

Fig. 3. Expression of ER-β in SHM cells. **A**: ER-β cellular localization: indirect immunofluorescence microscopy. ER-β was detected in paraformaldehyde fixed and permeabilized cells using the polyclonal PAI-310 (Materials and Methods). Fluorescent signal represents ER-β indirect visualization from anti-rabbit secondary antibodies conjugated to an Oregon Green dye. Negative controls using secondary antibodies alone rendered a negligible cellular background (**left panel**). Note the marked intranuclear accumulation of both ER-β and ER-α proteins and the typical exclusion of the labeling at nucleolar regions (inset, center). Original magnification = 650 ×. **B**: ER-α and -β isoforms as estrogen binders. SDS–PAGE of total lysates from SHM cells were developed using E_2-P (**lane 1**) or anti-ER-β antibodies (**lane 2**). The position of the two main groups of binding proteins has been specially remarked. Experiments were essentially performed as described in Figure 2.

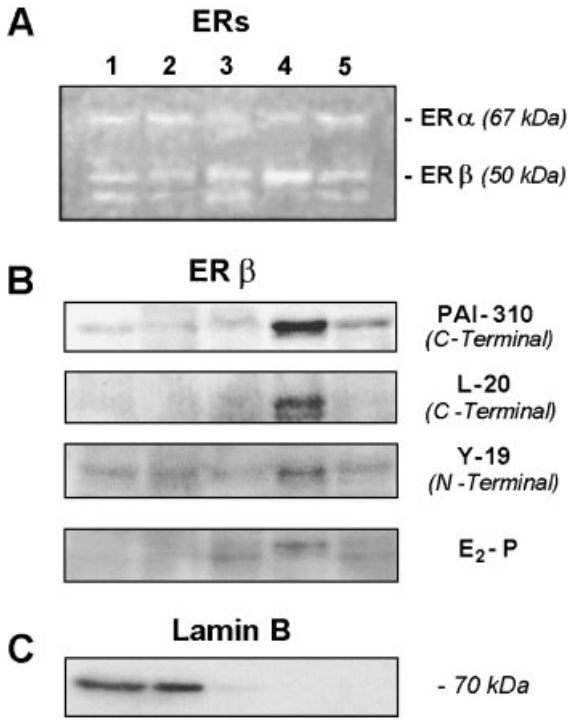

Fig. 4. Ligand and Western blot analysis of ER-β subcellular distribution in MCF-7 cells. Subcellular fractions from MCF-7 cells containing an equivalent protein amount were resolved by SDS–PAGE and processed for Ligand or Western blot analysis (Materials and Methods). **A**: ER-α and -β partitioning. The α (~67 kDa) and -β (~50 kDa) ER isoforms were stained using E_2-BSA-FITC derivatives. **B**: ER-β distribution. The isoform was identified using antibodies against a variety of epitopes from the cloned ER-β protein (PAI-310; Y-19 and L-20; 1:200 dilution each) or E_2-P as ligand (5 nM). The receptor regions recognized by the antibodies are indicated in the figure; N-terminal corresponds to sequences from the transactivation region and C-terminal from the F domain of the ER-β protein. **C**: Distribution of the nuclear marker Lamin B. This protein (~70 kDa) was detected using the specific polyclonal antibody M-20 (1:300 dilution). Probing with anti-lamin B antibodies was done after stripping the membranes shown in B. **Lane 1**: total homogenate; **Lane 2**: nuclear fraction; **Lane 3**: mitochondria/lysosomes; **Lane 4**: microsomes; **Lane 5**: cytosol. Labeling of estrogen binding proteins with E_2-BSA-FITC and E_2-P, required 60 and 5 μg protein per lane, respectively, for adequate detection. For Western visualization of immunoreactive products, 40 μg protein was loaded per lane.

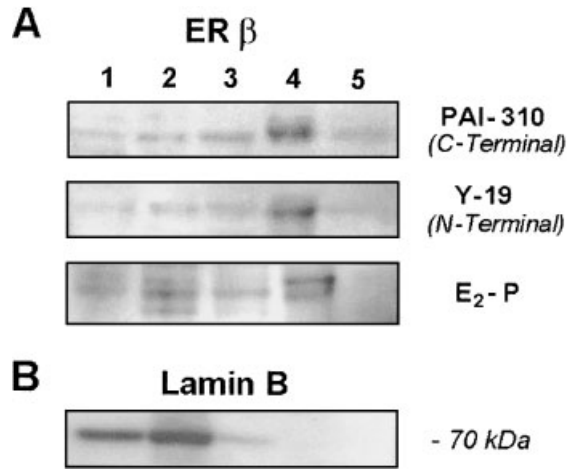

Fig. 5. Ligand and Western blot analysis of ER-β subcellular distribution in SHM cells. **A**: ER-β proteins were labeled using specific anti-ER-β polyclonals recognizing N-(Y-19) and C-terminal epitopes (PAI-310). Labeling with the antibody L-20 rendered a similar result (not shown). A Ligand blot profile using E_2-P conjugates (5 nM) was obtained for the same subfractions (bottom). **B**: Distribution of the nuclear marker Lamin B. This protein was detected using the antibody M-20 as previously described in Figure 4. **Lane 1**: total homogenate; **Lane 2**: nuclei; **Lane 3**: mitochondria/lysosomes; **Lane 4**: microsomes; **Lane 5**: cytosol. For detection with antibodies and E_2-P, 40 and 5 μg protein, respectively, were applied per lane.

observed in intact cells before (Figs. 2A and 3A) may be related to the introduction of artifacts in terms of protein localization when cells are homogenized and subjected to differential centrifugation to isolate subfractions. Several steroid hormone receptors, including the ERs, are known to be mainly recovered in the high speed supernatants of target tissues. However, cytological studies have consistently revealed they are indeed nuclear and not cytoplasmic proteins. As most of the cellular ERs tend to leak the nuclear compartment even when using isoosmotic buffers, the resultant nuclear fractions appear to be much less enriched in these receptors. Nevertheless, clear immunolabeling of nuclear fractions was observed (Figs. 4 and 5) although to lesser extent than in membrane fractions (see below).

Our immunocytochemical studies have previously shown that native ER-β were predominantly nuclear receptors. However, the subcellular analysis of the same proteins pointed to the existence of an alternative localization at membrane systems. We decided to analyze the expression of the protein lamin B as a reference for the distribution of nuclear residing proteins after subcellular fractionation. The marker lamin B was shown to localize mainly in the nuclear subfractions (Figs. 4C and 5B, lanes 2), confirming the origin of the preparations and excluding the possibility of nuclear contamination of the membrane suspensions.

Figure 6 comprises data on ER isoform detection in HeLa uterine cervical cells. Surprisingly, immunocytology revealed that these cells labeled positive to both ER-α and -β when stained

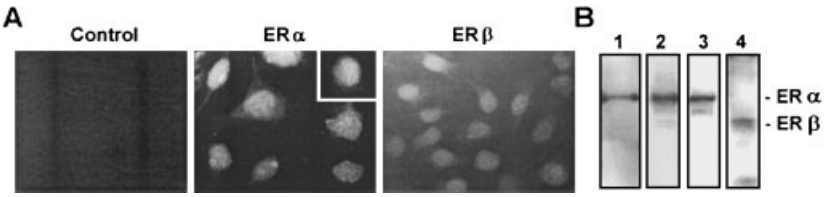

Fig. 6. Identification of ER-α and -β isoforms in HeLa cells. **A**: ER-α and -β cellular localization: conventional epifluorescence. Fixed monolayers were labeled with anti-ER-α (AER314 and TE111.5D11, inset) or anti-ER-β (PAI-310). Negative controls showed negligible cellular fluorescence (**left panel**). Original magnification, 650 ×. **B**: Expression of ER-α and -β isoforms as functional estrogen binding proteins. Total homogenates from HeLa cells were simultaneously processed for Ligand blot (**lane 1**) using E_2-P and Western blot analysis using anti-ER-α or anti-ER-β antibodies. Wild type ER-α was detected almost to the same extent using either AER 314 (**lane 2**) or AER 308 (**lane 3**). The anti-ER-β PAI-310 labeled a ~50 kDa tight doblet (**lane 4**), as expected by the position of the faint molecular weight group of E_2-P binding proteins. An exact alignment of the blots indicated that the estrogen binding proteins coincided with the bands revealed by anti-ER-α and anti-ER-β antibodies, respectively. The predominant occurrence of ~67 kDa estrogen binders in HeLa cells was clearly evidenced by Ligand blot (lane 1). For E_2-P and immunoblot experiments, 40 and 60 μg protein, respectively, were applied per lane. Experiments were essentially performed as described in Figure 2.

using isoform-specific antibodies. As usual, both isoforms showed a basal predominant nuclear localization (Fig. 6A). Western blot experiments confirmed that these cells contained detectable levels of the wild type ~67 and ~50 kDa forms of ER-α and -β, respectively (Fig. 6B, lanes 2–4). When we performed Ligand blot studies to dissect estrogen binding proteins into ER-α and -β elements, we could establish that ER-α occurred as the main isoform in total extracts from this cell line (Fig. 6B, lane 1).

DISCUSSION

The present study describes the presence and cellular localization of native ER-β proteins as estrogen binding sites in different cell lines. The information coming from conventional radioligand assays and estrogen binding site quantification was coupled to immunochemical analysis to provide additional knowledge concerning the expression of the known ER isoforms. The latter approach was facilitated by the recent availability of site-directed epitope-specific antibodies against the novel ER-β proteins [Pavao and Traish, 2001]. However, some concerns usually arise when trying to dissect ER-β receptors from the ER-α counterparts in terms of their cellular concentration. On one hand, the use of antibodies for immunocytochemistry or Western blot analysis could just provide qualitative information regarding the absence or presence of a given isoform. On the other hand, conventional radioligand binding assays using tritiated 17β-estradiol could not definitely separate ER-α from ER-β specific sites. Both receptor types are known to exhibit strickingly similar affinity and specificity characteristics [Kuiper et al., 1996, 1997]. A rapid way to first address the question of isoform expression levels could result from the use of peroxidase- or FITC-conjugated estrogens in Ligand blot experiments. Taking limitations aside, the major advantage of this approach was the ability to detect the isoforms on the basis of their capacity to bind estradiol. As the isoforms are separated by SDS–PAGE, they could be easily identified by their different molecular weights. A relative quantitative status could thus be assigned to ER-β when compared to wild type ER-α binding sites. The procedure demonstrated to be technically easy to implement and rendered highly reproducible results. We believe that the use of estrogen derivatives in Ligand blots could be standardized and developed as a biochemical tool for the rapid screening of ERs in protein samples from both normal and pathological cells or tissues. The expression of ER-β, as well as its cellular levels, is now being evaluated as a potential marker for tumor progression and prognosis [Omoto et al., 2001; Signoretti and Loda, 2001; Skliris et al., 2001].

In this report, we confirmed the co-expression of the two known ERs by Western blot analysis using different anti-ER-β polyclonals. The results from antibody and ligand recognition of the isoforms were found to match very closely. Tight multiple bands for ER-β detection, ranging a 50 kDa molecular weight, was a usual feature. Sequence analysis of ER-β open reading frame from several species revealed the presence of three potential sites for the starting of translation, giving rise to the expected 548, 530,

and 485 amino acid proteins [Pettersson and Gustafsson, 2001]. This fact could provide a basis for the detection of serial ER-β-like native proteins in the cell lines examined.

Results from conventional immunofluorescence microscopy showed ER-β antigens to predominantly reside inside the nucleus of the cells. This is in agreement with previous reports where naturally expressed ER-β products stained positive at the nuclei of target cells from the ovary and uterus [Saunders et al., 1997; Fitzpatrick et al., 1998; Sar and Welsh, 1998; Hiroi et al., 1999]. Overexpressed ER-β proteins were also visualized to localize intranuclearly when transfected to different cell lines [Saunders et al., 1997; Razandi et al., 1999; Monje et al., 2001]. ER-β staining for immunocytochemistry resembled that of the ER-α counterpart and other known transcription factors; the marked exclusion of the labeling by nucleolar regions was a typical pattern [King and Greene, 1984; Htun et al., 1996].

In spite of the major nuclear localization shown for ER-β proteins, we suspect that the selective microsomal partitioning of endogenous receptors could still derive from an alternative attachment to membrane components. However, the occurrence of artifacts on protein distribution as a consequence of cell homogenization and fractionation protocols could not be totally excluded. Nevertheless, we consider this possibility rather unlikely taking into account the restricted distribution of the nuclear marker lamin B. The high apparent expression levels of ERs at microsomal fractions could be simply explained by the enrichment on both plasma and intracellular membrane proteins that usually follows membrane purification procedures. A membrane source for ER-β, not being put into evidence by cytochemical means, is consistent with multiple evidences that point to the existence of membrane reservoirs for ERs and other steroid hormone receptors. The idea of a parallel action mechanism for 17β-estradiol, most probably related to non genomic effects, lends support to the presence of functional membrane ERs. The demonstration of specific binding of [^{3}H]17β-estradiol to HeLa cells, coupled to the detection of ER-α and -β isoforms suggests that these cells, though usually considered ER-negative, could be responsive to the hormone. Precautions should be taken when using this cell line as a negative control on ER studies.

The real significance of the overlap of ER-α and -β isoform expression is still unknown. However, the structural and functional differences between the receptors suggest they may mediate non-redundant cellular responses. ER-α has been shown to be highly expressed in specific tissues, such as breast, uterus, and vagina, while the ER-β isoform seems to have a wider distribution among a variety of tissues [Gustafsson, 1999]. Thus, ER-α was suspected to be principally involved in the control of reproduction processes. The present observations in mammary and uterine derived cell lines are in agreement with this general assumption. Though a dominant role for ER-α in the estrogen mechanism of action could be anticipated, the co-expression of low, but significant levels of ER-β could provide a molecular basis for the existence of diverging signal transduction pathways, each mediated by a given isoform.

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