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Estrogen Receptor-α Detected on the Plasma Membrane of Aldehyde-Fixed GH_3/B6/F10 Rat Pituitary Tumor Cells by Enzyme-Linked Immunocytochemistry*

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ABSTRACT

A population of estrogen receptor-α (ERα) proteins, located at the plasma membrane, is postulated to mediate the rapid, nongenomic responses of GH_3/B6/F10 pituitary cells to estrogen. To demonstrate the presence of ERα at the plasma membrane and to distinguish this receptor population from that in the nucleus, GH_3/B6/F10 cells were first prepared in 2% paraformaldehyde/0.1% glutaraldehyde in PBS (P/G) without detergent, then exposed to one of several antibodies (Abs) raised against nuclear ERα. Ab binding was visualized as a fluorescent/chromagenic reaction product catalyzed by avidin-biotin-complexed alkaline phosphatase. With P/G fixation, Abs could only access antigens at the cell surface, as evidenced by the inability of 70K mol wt dextrans to permeate cells and the absence of intracellular staining by Abs to cytoplasmic or nuclear antigens. ERα Abs generated membrane, but not nuclear, staining in P/G-fixed cells; nuclear receptor labeling could only be detected in detergent-treated cells. Specificity of staining for ERα was confirmed by three approaches: first, treatment with an antisense oligodeoxynucleotide to nuclear ERα mRNA reduced immunolabeling of both membrane and nuclear ERα; second, labeling by two Abs raised against different ERα oligopeptides was neutralized by competing peptide; third, six Abs (ER21, H226, R4, H222, MC20, and C542) that recognize unique epitopes on rodent ERα produced immunolabeling, but neither primate-specific ERα Ab nor Ab to ERβ caused staining. In addition to demonstrating the plasma membrane ERα in GH_3/B6/F10 cells, this method should be applicable to other cell types that exhibit nongenomic responses to estrogen or other steroid hormones. (*Endocrinology* **140:** 3805–3814, 1999)

THE ESTROGEN RECEPTOR-α (ERα) functions as a transcription factor, which localizes to the nucleus and, upon activation by estrogen, assumes a conformation that binds with high affinity to estrogen response elements on chromatin (1, 2). The interaction of the liganded receptor with DNA is a well-characterized mechanism by which estrogens regulate the transcription of target genes. However, in addition to directing nuclear events, estrogens rapidly activate ion channels and signaling cascades at the plasma membrane (3–5). Reports of rapid, nongenomic responses to estrogen in many systems have ignited interest in the putative receptor(s) mediating these membrane-initiated effects. Radioligand binding studies, using labeled 17β-estradiol (E_2), have revealed specific, saturable binding sites in plasma membrane preparations from different cell types (6, 7). In whole cells (8–12), estrogen binding sites on the surface of cells have been observed using 17β-E_2 conjugated to fluorescently tagged proteins, molecular complexes that are too large to diffuse across the plasma membrane. Antibodies (Abs) raised against the nuclear ERα have provided another tool for the study of estrogen binding sites on the cell surface: under experimental conditions in which the Abs do not enter the cell, binding of ERα Abs occurs at the plasma membrane (8, 11–15). These findings furnish evidence for the existence of a population of ERs, structurally related to ERα, that are located on the cell membrane.

Radioligand binding analyses of nuclear and plasma membrane fractions have suggested that the number of estrogen binding sites on the surface of cells is lower, by an order of magnitude, than the number in the nucleus (6, 12). Hence, to study the membrane receptors, conditions must be met that minimize contamination by the nuclear signal and which afford enough sensitivity to detect the relatively low levels of the membrane protein. In the present report, an immunocytochemical system for detection of membrane ERα was developed in GH_3/B6/F10 cells, a rat pituitary tumor line that exhibits rapid responsiveness to estrogen and expresses high levels of membrane ERα (13). The cells were rendered impermeable to Abs by aldehyde fixation, thereby eliminating interference from the intracellular ERα population (16). Additionally, by using fixed cells, the avidin-biotinylated enzyme complex (ABC) method of immunocytochemistry (17), in conjunction with a fluorescent/chromagenic reaction product, could be employed to amplify the signal, thereby enhancing the sensitivity of detection. This method provides a convenient technique for sensitive and specific detection of plasma membrane ERα, and the technique has potential for application to membrane steroid receptors in many other cell types.

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Materials and Methods

Cell culture

GH_3/B6/F10 cells were subcloned from GH_3/B6 cells (a gift from Dr. Bernard Dufy) by limiting dilution cloning, as previously described (13); cells were used between passages 10 and 30. The cells were routinely cultured at 37 C in serum-supplemented medium (SSM), consisting of Ham's F10 medium (Life Technologies, Inc., Grand Island, NY), 12.5% heat-inactivated horse serum, 2.5% heat-inactivated defined-supplemented calf serum, and 1.5% heat-inactivated FCS (all sera supplied by HyClone Laboratories, Inc., Logan, UT). For immunocytochemistry experiments, cells were plated at a density of 1.25×10^5 cells/cm^2 on glass coverslips (Fisher Scientific, Houston, TX) or Teflon printed slides (Electron Microscopy Sciences, Fort Washington, PA) that had been coated with poly-D-lysine (Sigma Chemical Co., St. Louis, MO). The cells were used after 3 or 4 days of culture in SSM.

Abs

ERα Abs. Seven Abs that recognize different determinants on the rat ERα were employed in this study; Fig. 1 illustrates the approximate location of the epitope for each Ab on the rat ERα protein. One Ab, D75, recognizes primate, but not rodent, ERα protein and was used as an experimental control. H151 (18) and C542 (19) are mouse monoclonal Abs (mAbs) raised against oligopeptides derived from the human ERα sequence; these Abs, kindly provided by Drs. Nancy L. Weigel and Dean P. Edwards, are now commercially available (StressGen Biotechnologies, Victoria, Canada). H222, H226, ER21, and D75 are rat mAbs raised against human ERα (20); they were a generous gift from Dr. Geoffrey Greene. Ab R4 is a peptide affinity-purified rabbit polyclonal Ab that was raised in our laboratory against an oligopeptide from the rat ERα (13). Ab MC20, a peptide affinity-purified rabbit polyclonal Ab to an oligopeptide based on the mouse ERα, was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

For peptide competition experiments, a given Ab was incubated with a 1000-fold molar excess of its cognate peptide (or, for controls, an unrelated peptide) for 2 h at 4 C to absorb the peptide to the Ab. The mixture was then diluted 10-fold for use. Custom peptides were synthesized by Genosys (The Woodlands, TX).

Ab controls. Antiserum against rat PRL (AFP-131581570) was obtained through the National Hormone and Pituitary Program and the National Institute of Diabetes and Digestive and Kidney Disease. A mouse mAb that recognizes an epitope on all 5 histone proteins (Anti-Histon Pan) was purchased from Boehringer Mannheim (Indianapolis, IN). A rabbit polyclonal Ab (PA1–310) to a C-terminal peptide based on the rat ERβ sequence was purchased from Affinity BioReagents, Inc. (Golden, CO). Tetramethylrhodamine-labeled dextran, of 70K mol wt, was purchased from Molecular Probes, Inc. (Eugene, OR).

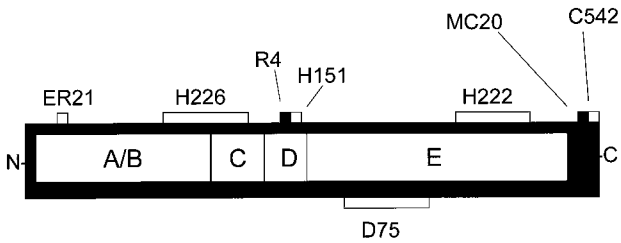

FIG. 1. Epitope map of the human and rat ERα. This diagram of the ERα protein indicates the approximate locations of the antigenic determinants of the Abs used to detect the membrane ERα. *Small squares*, Locations of the amino acid sequences from which synthetic oligopeptides were prepared for use as immunogens. In cases where the whole receptor was used as the immunogen, *rectangles* depict the region of the receptor to which the antigenic determinant has been mapped by peptide digestion. *Open symbols*, mAbs; *filled symbols*, polyclonal Abs. All of the Abs depicted on *top* of the cartoon recognize both primate and rodent ERα. Ab D75, which only binds receptors from primate species, is shown on the *bottom*. The functional domains of the receptor molecule, according to its genomic mode of action, are labeled as follows: A/B, the transcription regulatory domain; C, the DNA-binding domain; D, the hinge domain; E, the estrogen-binding domain. The amino terminus of the protein is designated by N-, and the carboxy terminus by -C.

Fluorescence immunocytochemistry

The ABC method (protocol I). GH_3/B6/F10 cells were washed three times in Dulbecco's PBS (DPBS). The fixative and fixation conditions were modified from Brink *et al.* (16), as follows: For nonpermeabilized cells, the fixative contained 2.0% paraformaldehyde (Sigma Chemical Co.) and 0.1% glutaraldehyde (Electron Microscopy Sciences) in PBS, adjusted to pH 7.4; this fixative [2% paraformaldehyde/0.1% glutaraldehyde in PBS (P/G)] was freshly prepared and applied to the cells for 30 min at 20 C. For permeabilized cells, 0.5% Nonidet P-40 (NP-40) and 0.15 M sucrose were included in the P/G solution; cells were exposed to the detergent-containing fixative for 1 min at 20 C. At the end of the fixation period, the cells were washed three times with DPBS, and free aldehyde groups were reduced with a 15-min incubation in 100 mM NH_4Cl, before a 1-h blocking step at 37 C with 10% BSA (ICC-grade, Sigma Chemical Co.) and 0.1% gelatin (from cold water fish skin, Sigma Chemical Co.) in DPBS. The primary Abs were diluted in 0.5% BSA, 0.1% gelatin in DPBS (PBG) and were added to the cells for 2 h at 20 C; next, the cells were washed six times over 30 min in PBG. For the ABC method (17), the following steps were conducted at 20 C using reagents from a Vectastain ABC-Alkaline Phosphatase kit in conjuction with Vector Red substrate (Vector Laboratories, Inc., Burlingame, CA): Biotinylated universal antirabbit/antimouse IgG was diluted to 4 μg/ml in PBG and added to the cells for 1 h; then, the cells were washed six times over 30 min in PBG. The ABC-alkaline phosphatase reagent was diluted in DPBS and added to cells for 60 min, followed by six washes over 30 min in DPBS. Vector Red substrate was prepared according to the manufacturer's instructions and added to the cells for 20 min; the reaction was stopped by rinsing the wells with water. The cells were dehydrated and cleared by successive treatments with 70% ethanol, 95% ethanol, and xylene, then mounted using Cytoseal 280 (Stephens Scientific, Riverdale, NJ).

The fluorescent secondary Ab method (protocol II). The cells were treated, as described above, up to the addition of the biotinylated secondary Ab incubation. Instead of the biotinylated Ab, a tetramethylrhodamine isothiocyanate (TRITC)-labeled antirabbit IgG (Sigma Chemical Co.) was added to the cells in the dark for 1 h at 20 C. The cells were washed six times over 30 min in DPBS, then mounted with Fluoromount G (Electron Microscopy Sciences), and stored in the dark at 4 C until examined for fluorescence (Eastman Kodak Co., Rochester, NY).

Photomicrography. Photomicrographs were taken with Kodak Pro400MC or Kodak Ektapress 1600 Plus color negative film using an Olympus Corp. (Melville, NY). AHBT microscope equipped with a fluorescence attachment (Model AH2-RFL) and camera (Model C-35AD-4).

Antisense experiments

A 15-mer antisense oligonucleotide (5′GGGTCATGGTCATGG3′) and a scrambled control (5′GTGGTGGATCGTGAC3′) were synthesized from unmodified bases by Genosys (Life Technologies, Inc., Rockville, MD). The ability of the antisense oligo to reduce ERα levels *in vitro* has been previously reported (21).

After 3 days of culture in SSM, as described above, GH_3/B6/F10 cells were rinsed two times with a serum-free, defined medium composed of phenol red-free DMEM, 0.1% BSA, 5 μg/ml insulin, 5 ng/ml selenium, and 5 μg/ml transferrin. The cells were then cultured for 24 h in this medium in the absence or presence of oligonucleotides. In the untreated control condition, cells were cultured without any oligonucleotide. For oligonucleotide controls, the scrambled oligo was present at a concentration of 0.5 or 1.0 μM, as stated. Antisense-treated cells were cultured with 0.05, 0.5, or 1.0 μM of the antisense oligo, as stated.

Results

Comparison of immunocytochemistry protocols I and II

Initial experiments were conducted to compare the ABC method (protocol I) with the fluorescent secondary Ab

method (protocol II) using GH_3/B6/F10 cells that had been fixed in P/G before incubation with ERα Ab C542. Figure 2A represents the typical pattern of fluorescence that was generated using ABC-alkaline phosphatase in conjunction with Vector Red substrate; the corresponding brightfield image is presented in Fig. 2B. For comparison, Fig. 2C depicts the labeling observed using indirect immunofluorescence with a TRITC-labeled antimouse IgG;

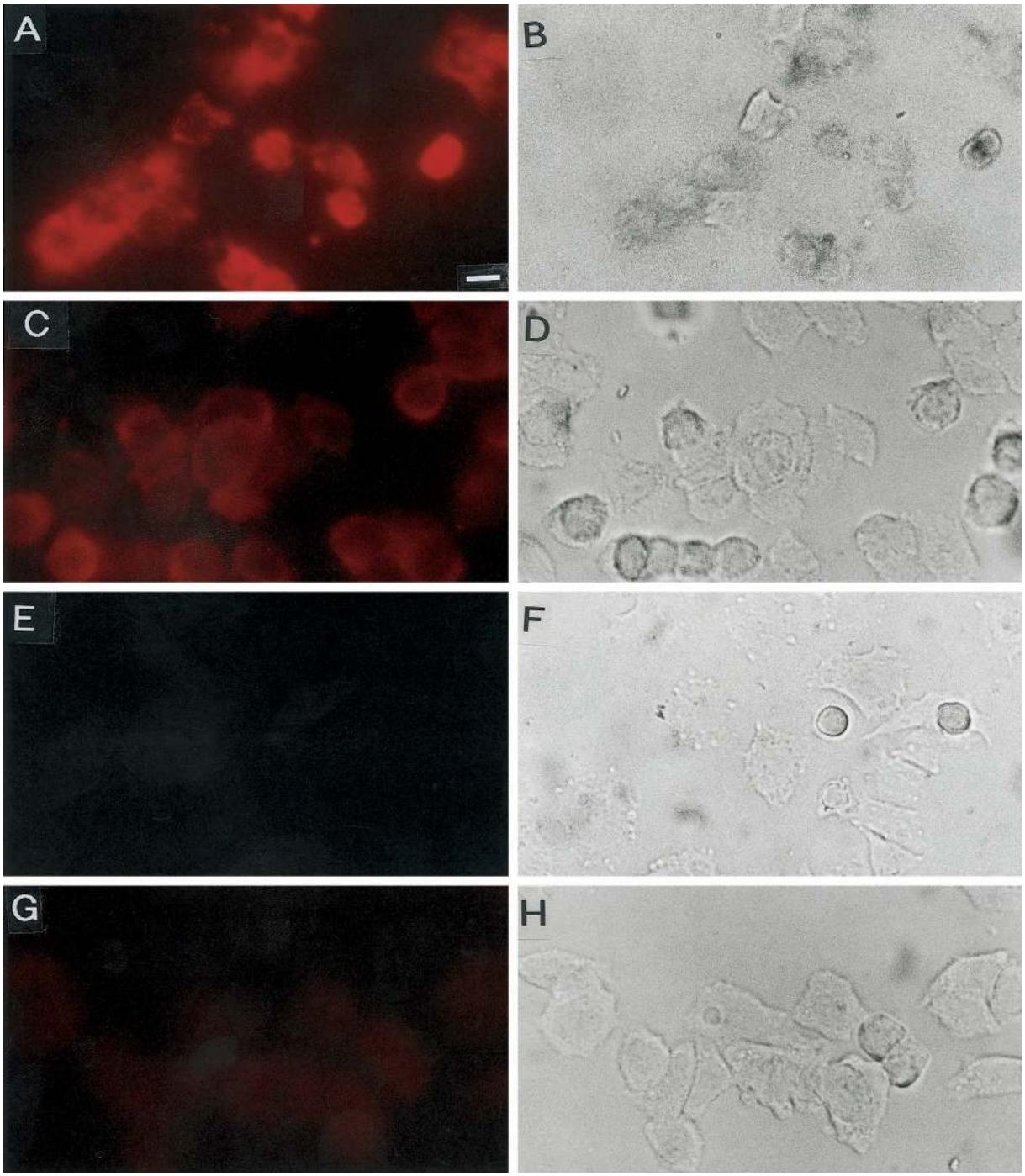

FIG. 2. Detection of plasma membrane ERα by two methods of immunofluorescence. Cells were fixed in P/G with no detergent, incubated with or without ERα Ab C542 (2 μg/ml), and processed using protocol I (the ABC-alkaline phosphatase method) or protocol II (the TRITC-labeled secondary Ab method). Data presented in A and B are companion fluorescent and brightfield images, respectively, of cells incubated with Ab C542 using protocol I; C and D are companion fluorescent and brightfield images, respectively, of cells incubated with Ab C542 using protocol II; E and F are companion fluorescent and brightfield images, respectively, of cells incubated without Ab C542 using protocol I; G and H are companion fluorescent and brightfield images, respectively, of cells incubated without Ab C542 using protocol II. The photomicrographs shown in A and E were exposed for 0.5 sec; those in B and G were exposed for 5.5 sec. The *scale bar* in A corresponds to 10 μm; all panels are at the same magnification.

Fig. 2D is the corresponding brightfield image. Only low levels of background fluorescence were detected when no ERα Ab was added to incubations in either protocol I (Fig. 2, E and F) or protocol II (Fig. 2, G and H). Although no staining of nuclear ERα was detectable by protocols I and II when the cells were prepared in the P/G fixative with no added detergent, cells permeabilized by the addition of NP-40 to the P/G fixative exhibited distinct nuclear staining in both immunocytochemistry protocols (for results using protocol 1, see Fig. 3). Results similar to those presented in Fig. 2 were obtained using ERα Abs R4 and MC-20 (data not shown).

Predictably, protocol I generated a more intense signal than protocol II because of the signal amplification afforded by the ABC method, in combination with the added sensitivity of the fluorescent Vector Red reaction product. Though some alteration of cell size and morphology was associated with dehydration of the samples in protocol I, the intensity of Vector Red fluorescence is enhanced by this processing and the permanently-mounted fluorophore undergoes little photobleaching. As a consequence, the difference in signal intensity between the two protocols could not be overcome by extending photographic exposure times for samples from protocol II, because photobleaching of the TRITC-label occurred during long exposures. Because the ABC method provides a more sensitive technique for detecting membrane ERα, subsequent experiments focused on characterization of this system.

Extracellular vs. intracellular immunostaining

The next set of experiments was designed to determine whether the observed immunostaining of ERα occurred on the extracellular surface of the cells, *i.e.* whether the fixation conditions were sufficient to prevent Abs from penetrating the cell membrane. Cells fixed in P/G without detergent displayed membrane labeling (but not nuclear labeling) after incubation with ERα Ab C542 (Fig. 3A). No nuclear staining by an antihistone Ab was detected when the cells were fixed with P/G in the absence of detergent (Fig. 3B). Staining of the plasma membrane by antihistone Ab was not observed, as would be predicted, given the nuclear location of histone proteins. The lack of membrane labeling by the antihistone Ab is of further significance because the Ab is a mouse monoclonal of the same IgG_1 subtype as the ERα mAb C542 and was used at a 2.5-fold higher concentration than mAb C542 in this experiment. Besides providing evidence that the Abs did not enter the nonpermeabilized cells and bind nuclear antigens, these data verify that the observed immunostaining by mAb C542 did not result from nonspecific binding of the Fc region of the IgG_1 molecule. In contrast to nonpermeabilized cells, cells permeabilized by the addition

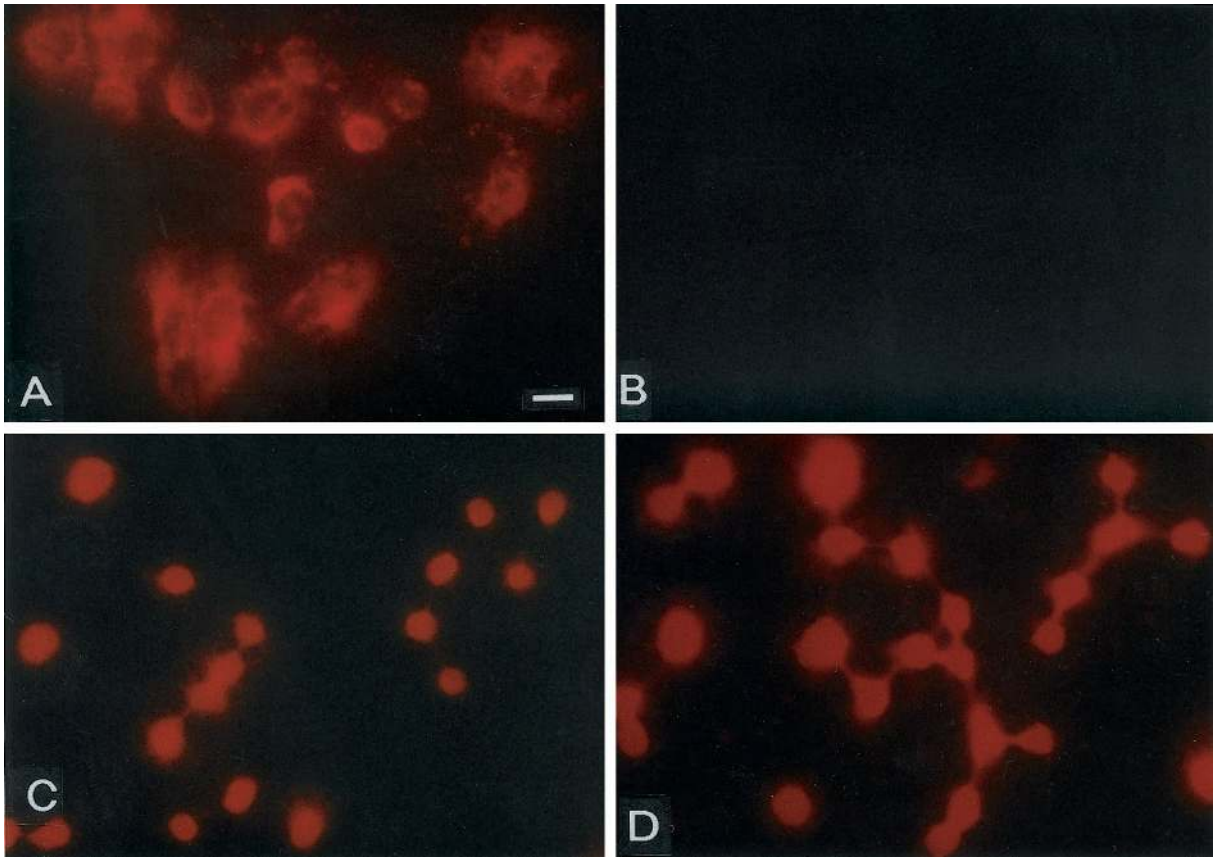

FIG. 3. Ab labeling of nonpermeabilized and permeabilized cells. Cells were fixed in P/G that contained no detergent (nonpermeabilized) or 0.5% NP-40 (permeabilized), then incubated with ERα Ab C542 (2 μg/ml) or an antihistone Ab (5 μg/ml). A, ERα Ab labeling of nonpermeabilized cells; B, antihistone Ab labeling of nonpermeabilized cells; C, ERα Ab labeling of permeabilized cells; D, antihistone Ab labeling of permeabilized cells. The *scale bar* in A corresponds to 10 μm; all panels are at the same magnification.

of NP-40 to the P/G fixative exhibited intense nuclear fluorescence when exposed to either the ERα Ab (Fig. 3C) or the antihistone Ab (Fig. 3D). Nuclear staining was evident at even lower Ab concentrations than employed here (0.5 μg/ml; data not shown). In another set of experiments, consistent results were obtained using rabbit antiserum to rat PRL. GH_3/B6 cells (22) and the F10 cell line derived from them (13) constitutively synthesize and secrete PRL. Yet, no intracellular labeling of the cells was evident unless the cells were fixed in the presence of detergent (data not shown). Taken together, these data from permeabilized and nonpermeabilized cells demonstrate that the Abs are able to recognize their respective antigens if the appropriate cellular compartment is accessible.

Dextran-tetramethylrhodamine (dextran-TMR) was also used to label nonpermeabilized and detergent-treated, permeabilized cells. The dextran-TMR preparation contains molecules distributed in size around 70K mol wt; hence, these molecules are significantly smaller than the IgG Abs (approximately 150K mol wt). After a 2-h incubation with dextran-TMR at 20 C (the same conditions used for Abs), no labeling could be visualized inside the P/G-fixed cells. The data presented in Fig. 4A illustrate that the fluorescent dextran molecules were present in the solution surrounding the cells but that even these relatively small molecules were excluded from cells under the fixation and incubation conditions used in these experiments. However, when cells were permeabilized, dextran-TMR diffused throughout the interior of the cells and was readily viewed inside the cell after the solution containing the labeled dextran was rinsed off the cells (Fig. 4B). To depict the margins of the cells in Fig. 4. A and B, the corresponding brightfield images are shown in Fig. 4, C and D, respectively.

Specificity of labeling of ERα

To determine whether the observed immunofluorescent signal at the plasma membrane of GH_3/B6/F10 cells was specific for ERα, expression of the protein was knocked out by treating the cells with a nuclear ERα antisense oligonucleotide for 24 h before immunocytochemical analysis with ERα Abs. These experiments were conducted in a serum-free, defined medium to minimize degradation of the oligonucleotides by serum components. Removal of serum caused the GH_3/B6/F10 cells to assume a more spindle-shaped morphology, yet the cells continued to express membrane ERα. In the control condition shown in Fig. 5A, cells had been incubated with a scrambled oligonucleotide; this treatment did not affect the level of immunostaining, compared with cells that were not exposed to any oligonucleotide (data not

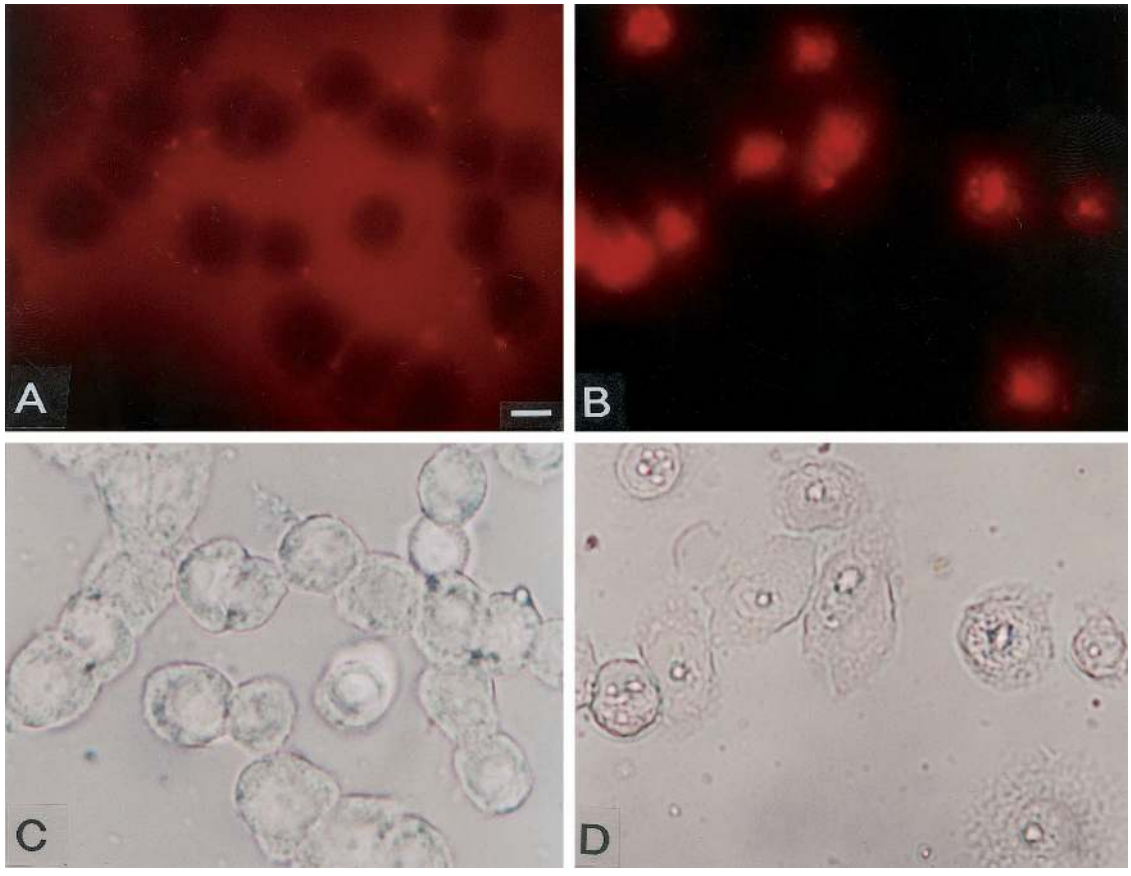

FIG. 4. Dextran-TMR localization in nonpermeabilized and permeabilized cells. Cells were fixed in P/G that contained no detergent (nonpermeabilized) or 0.5% NP-40 (permeabilized), then incubated with a solution of dextran-TMR (2.5 mg/ml) in DPBS for 2 h at 20 C. The solution was removed, and the cells were briefly rinsed with DPBS, then immediately examined for fluorescence. A and C, Companion fluorescent and brightfield images, respectively, of nonpermeabilized cells; B and D, companion fluorescent and brightfield images, respectively, of permeabilized cells. The *scale bar* in A corresponds to 12 μm; all panels are at the same magnification.

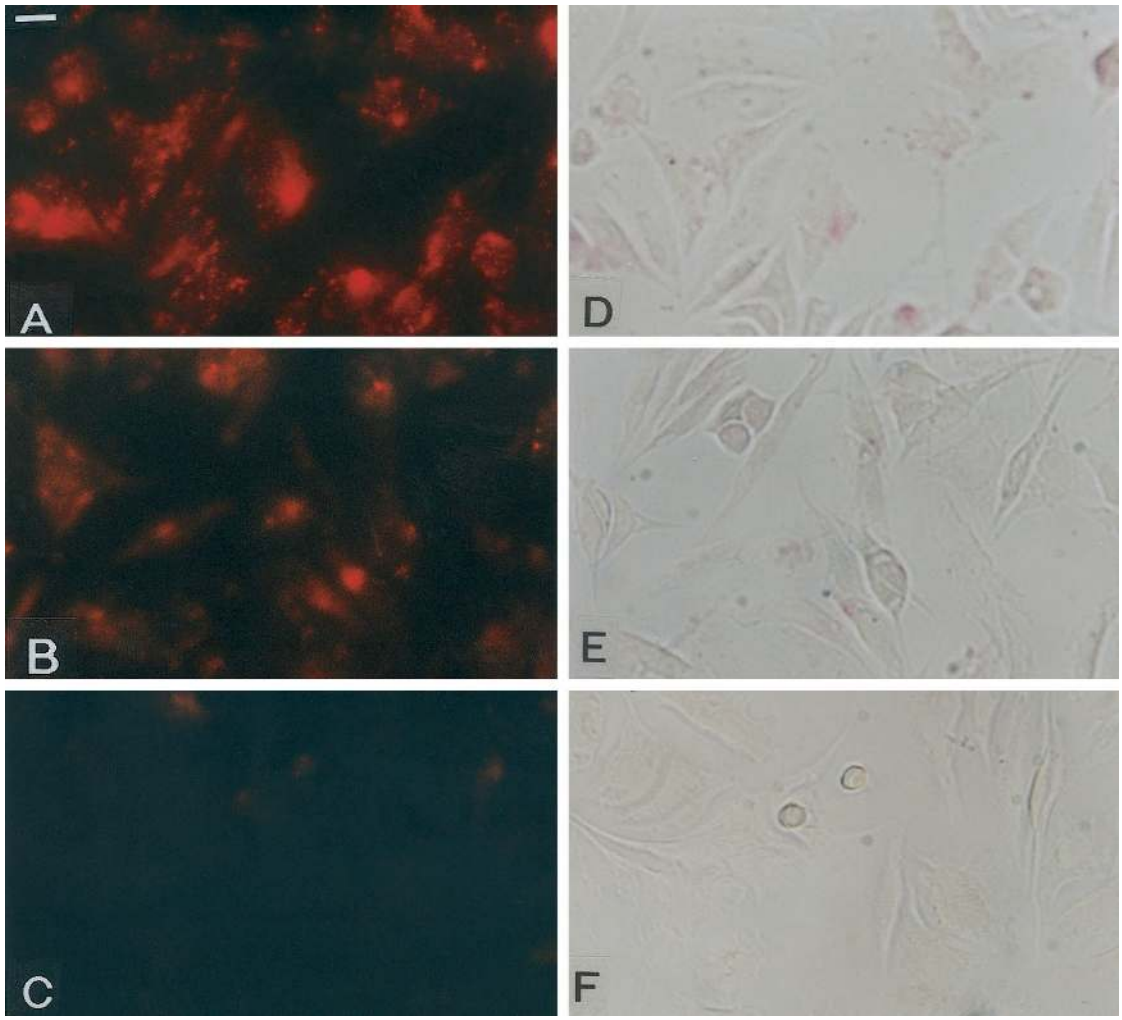

FIG. 5. Treatment with a nuclear ERα antisense oligodeoxynucleotide reduces Ab C542 labeling of membrane ERα protein in a concentration-dependent fashion. Cells were incubated for 24 h with a control, scrambled DNA oligo or a nuclear ERα antisense oligo, before fixation in P/G and exposure to ERα Ab C542 (2 μg/ml). A and D, Companion fluorescent and brightfield images, respectively, of cells exposed to a control, scrambled oligo at 0.5 μM; B and E, companion fluorescent and brightfield images, respectively, of cells exposed to ERα antisense oligo at 0.05 μM; C and F, companion fluorescent and brightfield images, respectively, of cells exposed to ERα antisense oligo at 0.5 μM. The *scale bar* in A corresponds to 10 μm; all panels are at the same magnification.

shown). In contrast, the degree of immunolabeling decreased in a concentration-dependent fashion in cells that had been treated with antisense ERα oligonucleotide at 0.05 and 0.5 μM (Fig. 5, B and C, respectively). Fig. 5, D, E, and F, the brightfield images corresponding to Fig. 5, A, B, and C, respectively, illustrate that the density of cells in the three fields is equivalent. Note that red staining is visible in the brightfield views because the Vector Red reaction product is a chromagen, as well as a fluorophore. Although the extent to which the fluorescent signal was reduced by antisense treatment varied within and between experiments, from partial to complete elimination of the immunostaining, the decrease was also detected with ERα Abs directed against other epitopes on the receptor protein, including Ab R4 (Fig. 6) and Ab ER21 (data not shown). In separate experiments, 24-h treatment with 0.5 μM ERα antisense caused partial reduction of Ab C542 labeling of the nucleus in permeabilized cells (data not shown).

Further evidence for the specificity of ERα Ab binding to the surface of GH_3/B6/F10 cells was derived from peptide competition experiments in which a given Ab was preabsorbed to the oligopeptide against which it was raised. Figure 7, A and B are photomicrographs of P/G-fixed cells that were incubated with ERα Ab C542 in the absence or presence, respectively, of competing peptide. An unrelated, control peptide had no effect on Ab C542 binding (data not shown). Cells that were permeabilized by the addition of detergent to the fixative displayed a distinct pattern of nuclear staining (Fig. 7C), which was also reduced by competing peptide (Fig. 7D). Immunostaining by Ab R4 of the membrane in nonpermeabilized cells and of the nucleus in permeabilized cells was also competitively inhibited by preabsorption of the Ab with its cognate peptide (data not shown).

A panel of Abs directed against various regions of the nuclear ERα (see Fig. 1) was examined in this assay system. Ab MC20, whose epitope overlaps that of C542 near the C-terminus of the receptor, produced consistently intense staining reactions, similar to C542, at concentrations as low

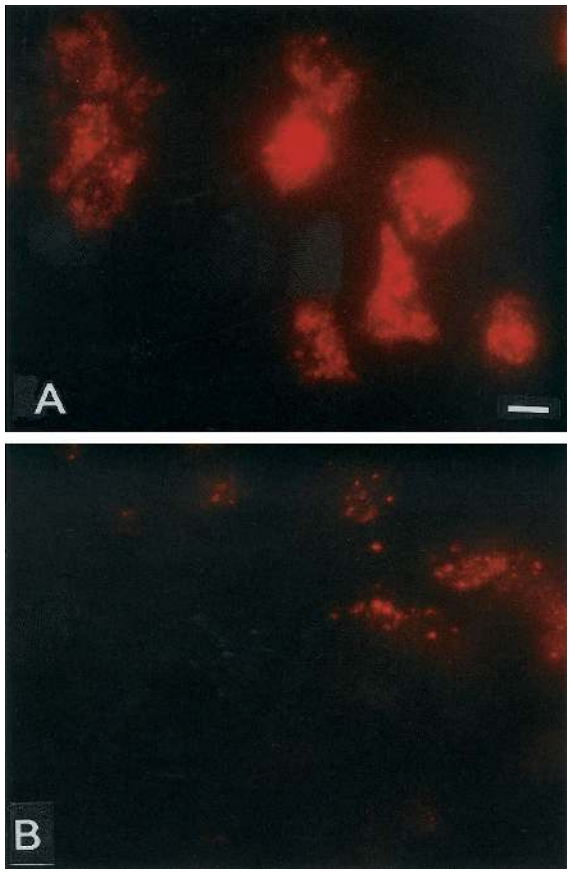

FIG. 6. Treatment with a nuclear ERα antisense oligodeoxynucleotide also reduces Ab R4 labeling of membrane ERα protein. Cells were incubated for 24 h with 1.0 μM of the scrambled DNA oligo (A) or the nuclear ERα antisense oligo (B), before fixation in P/G and exposure to ERα Ab R4 (1:200). Both panels contain an equivalent number of cells. The *scale bar* in A corresponds to 12 μm; both panels are at the same magnification.

as 2 μg/ml. Intermediate levels of labeling were generated by Ab ER21 (10 μg/ml), whose epitope lies at the N-terminus, and by Ab R4 (1:200), a peptide affinity-purified rabbit polyclonal whose epitope is found in the hinge region. Interestingly, Ab H151, a mouse monoclonal whose epitope is adjacent to that of R4, yielded no staining above background at concentrations ranging from 1–20 μg/ml. Ab H222 (directed at the ligand binding domain of the receptor) and Ab H226 (directed against the DNA binding domain) both generated weak, but detectable, signals in this system at 10 μg/ml. As expected, Ab D75, a mAb only recognizing ERα from primate species, did not label the rat protein at concentrations ranging from 1–10 μg/ml. Also, no membrane labeling of GH_3/B6/F10 cells was detected using a rabbit polyclonal Ab to the C-terminus of rat ERβ (1–10 μg/ml). In this regard, it is noteworthy that the ERα peptides against which C542, MC-20, or R4 were raised share no homology with sequence reported for rat ERβ (23).

Discussion

The immunocytochemical technique described here demonstrates the presence of a population of ERα at the plasma membrane and clearly distinguishes this population from ERα located in the nucleus. By first fixing the cells in paraformaldehyde and glutaraldehyde in the absence of detergent, the ABC-alkaline phosphatase method could be employed to enhance detection of the membrane receptor, which is present in low abundance, relative to the nuclear receptor. This technique may have applicability to other steroid hormone membrane receptors and other cell types, provided it can be determined, in a given system, that fixation conditions prevent entry of the Abs into the cell and that the immunolabeling is specific for the receptor under consideration. In validating the methodology for the membrane ERα in GH_3/B6/F10 cells, several approaches to each of these technical issues were employed.

To exclude Abs from the intracellular compartment, glutaraldehyde was combined with paraformaldehyde in the fixative solution, as previously described by Brink *et al.* (16). Under these fixation conditions, the pattern of binding exhibited by ERα Abs is clearly extranuclear. The lack of staining by Abs to histone proteins or to PRL [which is found in the cytoplasm of these cells (13, 22)], provides additional evidence that large IgG molecules are unable to penetrate the cell when fixed with P/G. Brink *et al.* reported that no staining of the nuclear glucocorticoid receptor could be visualized under similar P/G fixation conditions. Moreover, these investigators found it was necessary to add the detergent, NP-40, together with (rather than after) the aldehyde mixture, to permeabilize the cell membrane and allow access of Abs to intracellular compartments. In our experiments, permeabilization of GH_3/B6/F10 cells with NP-40 permitted nuclear labeling by ERα Abs, in striking contrast to the pattern of surface staining obtained when no detergent is added. Abs to other antigens that are present in intracellular compartments (histone proteins in the nucleus and PRL in the cytoplasm) only generated signal in the presence of detergent. Even a fluorescently labeled dextran with a mol wt of 70K did not diffuse into the cells unless detergent was included in the fixative. Hence, we conclude that any immunostaining in cells prepared with P/G, in the absence of detergent, could be attributed to Abs bound to the surface of the cells.

The results presented here, using P/G-fixed cells, confirm and extend observations reported from our laboratory using live, unfixed cells. In live GH_3/B6 cells, the indirect immunofluorescence signal associated with ERα Ab binding was localized to the cell perimeter by confocal scanning laser microscopy (8, 14). Additionally, in double-labeling experiments (8), an E_2-BSA conjugate (tagged with fluorescein) and rabbit R3 anti-ERα Ab/goat anti-rabbit Ab (tagged with Cy3) were shown to colocalize on the surface of the cells. In those studies, live cells were incubated for 10 min with Abs at 4 C to prevent uptake of the Abs before fixation of the cells with paraformaldehyde; this methodology resulted in a punctate staining pattern on the periphery of cells, with no observable nuclear staining. By increasing incubation times and temperatures, aggregation of the signal was accentuated, consistent with the phenomenon of Ab-induced patching and capping of antigens in a fluid membrane. In the experiments reported here, by first fixing the cells with P/G, it was possible to lengthen Ab incubations and apply the ABC-alkaline

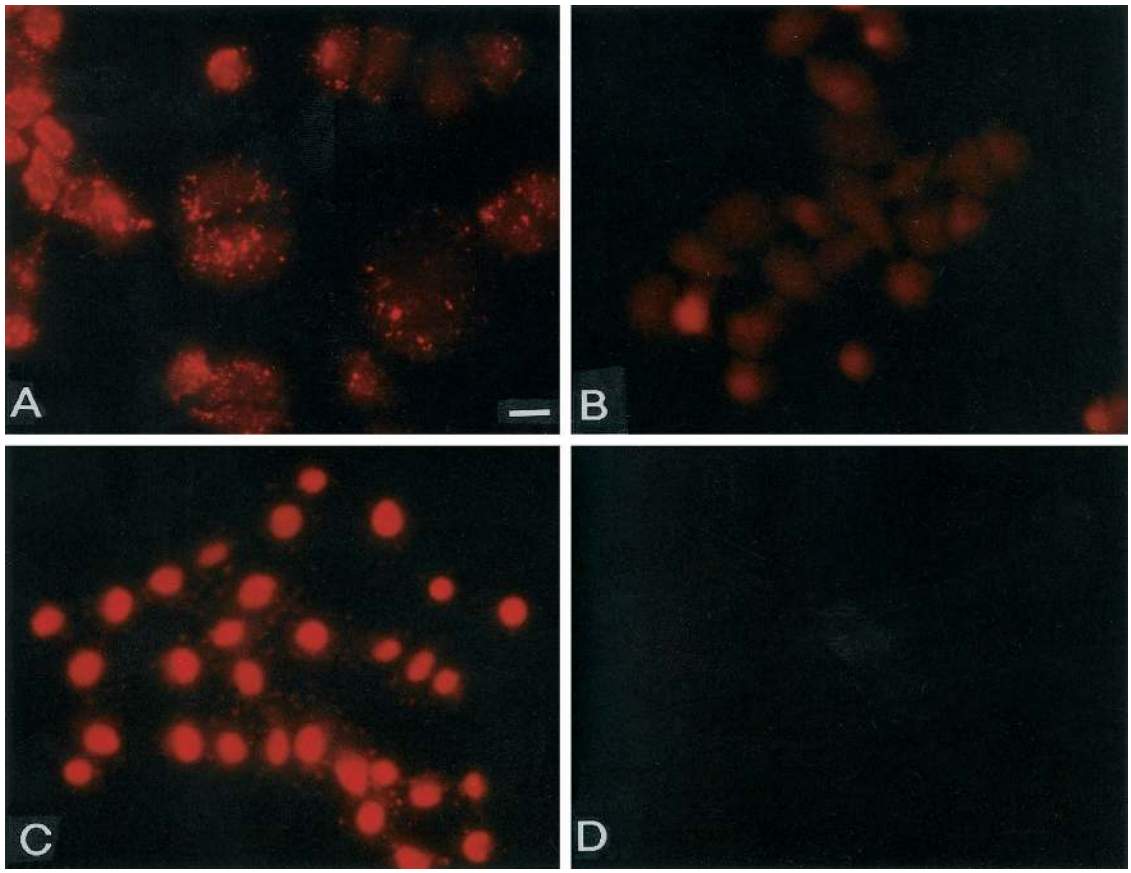

FIG. 7. Both membrane and nuclear ERα Ab labeling are competitively inhibited by antigenic peptide. Cells were fixed in P/G that contained no detergent (nonpermeabilized) or 0.5% NP-40 (permeabilized), then incubated with Ab C542 (2 μg/ml) or with Ab C542 that had been preabsorbed to the peptide against which it was raised. A and B, Fluorescence images of nonpermeabilized cells incubated with Ab in the absence and in the presence, respectively, of peptide; C and D, fluorescence images of permeabilized cells incubated with Ab in the absence and in the presence, respectively, of peptide. The *scale bar* in A corresponds to 10 μm; all panels are at the same magnification.

phosphatase method to improve detection sensitivity through enzymatic amplification of the signal (17). As expected, the ABC technology generated a more intense signal, and a greater percentage of cells were labeled using the amplification methodology, compared with indirect immunofluorescence of live cells. Although it is significant that the presence of surface labeling and the absence of intracellular staining are similar in both live and P/G-fixed cells, the punctate pattern of membrane signal seen with the ABC method arises from accumulation of reaction product and is not related to Ab patching and capping, because the cells were fixed before exposure to Ab.

To determine whether the membrane immunolabeling observed using fixed cells was specific for ERα, three experimental approaches were taken: First, experiments were conducted with an antisense oligodeoxynucleotide targeted to the region of the translation start codon of all nuclear ERα mRNAs (24, 25). This antisense sequence was previously shown to reduce [^{3}H]-17β-E_2 binding in a mouse colon cancer cell line in a dose- and time-dependent fashion (21). In the present study, application of the antisense oligo to the cells for 24 h resulted in a concentration-dependent decline in immunostaining by ERα Ab C542, whose epitope lies at the C-terminus of the nuclear ERα protein. Furthermore, after treatment with nuclear ERα antisense, we observed reduction of membrane labeling by Abs to the hinge region (R4) and the N-terminus (ER21) of the nuclear protein. Antisense treatment also decreased immunostaining of nuclear ERα, examined in permeabilized cells. No effect on immunolabeling of membrane or nuclear receptors by any of these Abs was noted after exposure of cells to a control, scrambled oligo, which consisted of the same 15 nucleotides arranged in a sequence with little or no homology to sequences in GenBank. Though these results confirm the specificity of Ab staining for the ERα protein in this system, they also support the working hypothesis that the nuclear and plasma membrane populations of ERα are structurally linked. In this regard, when Migliaccio *et al.* (26) transfected Cos-7 cells (which do not express ERα) with a plasmid containing the human nuclear ERα coding sequence, the cells became responsive to 17β-E_2, in terms of rapid activation of the MAP kinase, ERK. More recently, Razandi *et al.* (12) introduced a complementary DNA for ERα into Chinese hamster ovary cells (which also do not express ERα) and discovered that populations of membrane and nuclear receptors arose from the same transcript. They further demonstrated, in the transfected cells, that 17β-E_2 stimulated ERK through membrane-associated ERα; and they concluded that a population of ERα, residing at the plasma membrane, functions in the rapid, nongenomic response to estrogen. In the same study,

transfection of Chinese hamster ovary (CHO) cells with an ERβ complementary DNA also yielded functional membrane and nuclear receptors. In our experiments with GH3/B6/F10 cells, no membrane signal was detected using an Ab to ERβ, although it is not known whether ERβ is expressed in our cells.

As a second approach, in addition to the antisense experiments, the specificity of ERα immunolabeling was substantiated by peptide competition studies. Immunostaining by Abs C542 and R4 of membrane ERα in nonpermeabilized cells and of nuclear ERα in permeabilized cells was eliminated by preincubation of each Ab with its respective cognate peptide. Peptide competible binding of an Ab in immunocytochemical analyses provides evidence for specific recognition of a given epitope by the Ab. This important control does not guarantee, however, that a given epitope is not shared by more than one protein in the cell. In the present study, the fact that immunolabeling by two Abs raised against different ERα peptides could be neutralized by peptide competition, combined with the fact that both nuclear and membrane staining by both Abs could be inhibited by peptide competition, strengthens the argument for the specificity of the observed staining for ERα. Moreover, these data confirm the structural relationship between the membrane and nuclear receptor populations.

A third approach to validating the specificity of ERα Ab binding to the membrane was to test Abs whose antigenic determinants lie at different regions of the protein. Abs, directed against different epitopes, produced varying degrees of staining. Because the antigenicity of an epitope can be altered during the protein cross-linking associated with aldehyde fixatives, the observed variation in immunolabeling may be related to the nature and extent of cross-linking associated with the P/G fixation. Alternatively, the variation in staining intensity by different Abs may relay information about the conformation of the receptor on the membrane and the accessibility of a particular epitope to its Ab, or about some difference in amino acid composition.

Finally, it is important to note some differences between the two fluorescence immunocytochemistry protocols applied to fixed cells in this report: one based on the ABC method, in conjunction with a fluorescent reaction product; and the other using a TRITC-labeled secondary Ab. Both methods yielded similar results, in terms of the nonnuclear, surface staining exhibited by nonpermeabilized cells contrasted with the nuclear labeling found in permeabilized cells. We focused on the ABC method because of the enhanced sensitivity stemming from the enzyme amplification step and because the Vector Red fluorophore is resistant to photobleaching (the Vector Red reaction product can also be viewed as a red chromagen in brightfield microscopy, further enhancing the flexibility of the method). At the same time, it is important to note possible artifacts related to the ABC methodology as employed here. Most obvious of these is the punctate staining pattern associated with the ABC method. As substrate accumulates, it forms large macromolecular complexes that appear as aggregates on the cell surface; the use of a TRITC-labeled secondary Ab revealed a more even, diffuse pattern of staining. Also, in the ABC protocol, the cells were dehydrated before mounting, whereas they were not dehydrated in the labeled secondary Ab method used in these experiments. Dehydration was associated with some alteration of cell morphology and reduction of cell size, which could have contributed to the enlarged appearance of the aggregates of Vector Red substrate. Therefore, conclusions about the physical arrangement of ERα on the plasma membrane will require further study.

The focus of this report was the demonstration of ERα proteins on the plasma membrane of GH_3/B6/F10 cells using aldehyde-fixed cells in conjunction with the ABC method of immunocytochemistry. This provides a convenient system for sensitively and specifically detecting plasma membrane ERα and for distinguishing the membrane population from the nuclear population of receptors. This technique now provides us with the opportunity to address such issues as: how membrane localization of ERα is regulated; and what functional interactions exist between nuclear and membrane ERα. In addition, this method should have broader applicability, including other cell systems and other membrane steroid receptors.

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