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Antibodies to the estrogen receptor-α modulate rapid prolactin release from rat pituitary tumor cells through plasma membrane estrogen receptors

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Abstract

Antibodies (Abs) raised against the estrogen receptor-α (ERα) were used to investigate the role of ERα proteins located at the plasma membrane in mediating the rapid, estrogen-stimulated secretion of prolactin (PRL) from rat pituitary GH_3/B6/F10 cells. Exposure of the cells to 1 nM 17β-estradiol (E_2) significantly increased PRL release after 3 or 6 min. When ERα Abs that bind specifically to ERα but are too large to diffuse into cells were tested for activity at the cell membrane, Ab R4, targeted to an ERα hinge region sequence, increased PRL release in a time- and concentration-dependent fashion. Ab H151, directed against a different hinge region epitope, decreased PRL release and blocked the stimulatory action of E_2. Abs raised against the DNA binding domain (H226) or the carboxyl terminus (C542) were not biologically active. When each Ab was examined for recognition of ERα on the cell surface by immunocytochemistry, all except H151 generated immunostaining in aldehyde-fixed cells. In live cells, however, Ab H151 but not Ab R4 blocked the membrane binding of fluorescently tagged E_2-BSA. Overall, the data indicate that plasma membrane ERα proteins mediate estrogen-stimulated PRL release from GH_3/B6/F10 cells. These results may also convey information about conformationally sensitive areas of the membrane form of ERα involved in rapid, nongenomic responses to estrogens.—Norfleet, A. M., Clarke, C. H., Gametchu, B., Watson, C. S. Antibodies to the estrogen receptor-α modulate rapid prolactin release from rat pituitary tumor cells through plasma membrane estrogen receptors.

Keywords

nongenomic effects; membrane steroid receptors; immunocytochemistry

In 1941, progesterone was reported to induce anesthesia within minutes of its administration to rats (1). Since then, ‘rapid’ responses have been described for all classes of steroid hormones (2–4). Such effects occur within seconds or minutes of exposure to the hormone and often involve second messenger systems or ion channels present in the plasma membrane. The short latency of these responses is considered to preclude the involvement of *de novo* mRNA or protein synthesis, a consideration that has been corroborated in some experiments using pharmacological inhibitors of transcription and translation (5). From these observations, a model has emerged in which steroid hormones interact with receptors to initiate events at the plasma membrane within a very brief time frame, in addition to interacting with nuclear

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receptors to regulate gene expression over a longer period of time. Although these two chronological phenomena have been investigated separately in the laboratory, they most likely work in concert to promote the full physiological spectrum of steroid hormone action within the cell (3,4,6).

One controversial feature of this model of the dual nature of steroid hormone action is the identity of the molecular entity that mediates the rapid effects of steroids. Binding sites for steroid hormones have been demonstrated on the plasma membranes of various cell types through the use of steroids labeled with radioisotopes or fluorescent dyes (7–9), steroids conjugated to large proteins that are sterically impeded from entering the cell (8,10–12), and antibodies directed against the nuclear form of the steroid receptor (12–16). Although in most cases these plasma membrane binding sites have not been isolated and characterized, four categories of putative receptor proteins have been experimentally delineated. One category is based on evidence that for a given steroid hormone, a protein that structurally resembles its cognate nuclear receptor is found at the plasma membrane (9,12–14). The second category contains membrane proteins that are specific for a given steroid, but differ significantly in size or sequence from the nuclear form of the receptor (11,17). The third category has evolved from reports that steroids 'cross talk' with surface receptors whose cognate ligand is a different molecule, such as a growth factor (18) or neurotransmitter (19). Steroid binding proteins present in serum form the fourth category, since these proteins, upon binding to specific steroids, interact with components of the plasma membrane to activate cell functions (20). Hence, it appears that a 'mixed binding system' exists in the plasma membrane in which all four categories of steroid hormone binding proteins may serve different physiological functions in the cell (3). Linking a cellular function to a putative receptor site is necessary, then, to confirm the relevance of the site and elucidate its role in the overall response of the cell to the steroid.

The present study was undertaken to investigate the role of membrane receptors in mediating a rapid response to the steroid hormone, estrogen. A plasma membrane population of the estrogen receptor-α (ERα) has been demonstrated in the rat pituitary tumor cell line, GH_3/B6; furthermore, the F10 subclone derived from them was selected for its expression of high levels of membrane ERα (21). GH_3/B6 cells and the F10 subclone are known to both constitutively secrete prolactin (PRL) and to respond to estrogens within minutes by releasing an additional bolus of PRL (21–23). Therefore, experiments were conducted in GH_3/B6/F10 cells using antibodies (Abs) that specifically recognize ERα as experimental probes to identify the membrane receptor by immunocytochemistry while analyzing the function of the receptor in PRL release. The results showed that one of the ERα Abs tested was found to trigger PRL release whereas another blocked E_2-stimulated release, demonstrating a functional correlation between the rapid, estrogen-stimulated release of PRL and the plasma membrane population of ERα.

MATERIALS AND METHODS

Cell culture

GH_3B6/F10 cells were subcloned from GH_3/B6 cells (a gift from Dr. Bernard Dufy) by limiting dilution cloning, as described previously (21); cells were used between passages 10 and 30. The cells were routinely cultured in serum-supplemented medium (SSM), consisting of Ham's F-10 medium (Life Technologies, Inc.-BRL, Grand Island, N.Y.), 12.5% heat-inactivated horse serum, 2.5% heat-inactivated defined-supplemented calf serum, and 1.5% heat-inactivated fetal calf serum (all sera supplied by Hyclone, Logan, Utah). For experiments, cells were plated at a density of 1.25×10^5 cells/cm^2 and cultured in SSM for 24 h. The culture medium was then changed to a serum-free, phenol red-free defined medium (DIB), which contained DMEM (Life Technologies, Inc.-BRL), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/

ml selenium (Sigma, St. Louis, Mo.), and 0.1% bovine serum albumin (BSA; Sigma). The cells were cultured for 72 h in DIB prior to use in experiments.

Antibodies

Four antibodies (R4, H151, C542, H226) that recognize determinants on the rat ERα were used; one Ab (D75), which recognizes primate but not rat protein, was used as an experimental control. Both H151 and C542 are mouse monoclonal Abs (mAbs), which were kindly provided by Drs. Nancy Weigel and Dean P. Edwards. These Abs are now commercially available from StressGen Biotechnologies (Victoria, B.C.). Ab H151 was raised against a synthetic oligopeptide (RAANLWPSPLMIKR) derived from the hinge region of the human ERα sequence (Dr. Weigel, personal communication; ref 24). Ab C542 is directed against an oligopeptide (YYIT-GEAEGFPATV) corresponding to a carboxyl-terminal sequence of human ERα (Dr. Weigel, personal communication; ref 25). Abs H226 and D75 were generous gifts from Dr. Geoffrey Greene. These are rat mAbs that were raised against the human ERα (26). The antigenic determinant for Ab H226 is located in the DNA binding domain, whereas the epitope for Ab D75 lies between the DNA binding region and the steroid binding region of the receptor. Ab R4 is a rabbit polyclonal Ab raised in our laboratory against a synthetic oligopeptide (LKHKRQRDDLEGRNE) from the hinge region of the rat ERα; the Ab, which was purified from immune serum on a peptide affinity column, has previously been characterized (21). See Fig. 9 for an illustration of these epitope locations.

For immunoprecipitation of ERα from rat uterus and subsequent Western analyses, the Ab used was MC-20, a peptide affinity-purified rabbit polyclonal raised against a carboxyl-terminal sequence of the mouse ERα (Santa Cruz Biotechnology Inc, Santa Cruz, Calif.).

PRL release experiments

GH_3/B6/F10 cells, seeded in SSM and cultured in 24-well plates for 72 h in DIB, were washed once and preincubated for 15 min in DMEM containing 20 mM HEPES and 0.1% BSA (DHB). The cells were maintained at 37°C for the preincubation and test incubations. The preincubation medium was removed and replaced at time zero with test medium (DHB ± 0.001% EtOH ± test agents). The test agents included 17β-estradiol (E_2) and the ERα Abs. A stock solution of E_2 (Sigma) in 95% EtOH was diluted such that the final EtOH concentration was 0.001%, which was used in all control and test conditions. After 3 or 6 min, test medium was collected into prechilled tubes, immediately microfuged at 4°C to pellet any dislodged cells, and the supernatants were stored at −20°C. PRL concentrations in the test media were determined by radioimmunoassay using antiserum (AFP-131581570) provided by the National Institute of Diabetes and Digestive and Kidney Disease and the National Hormone and Pituitary Program. The intra-assay variability did not exceed 7%. PRL release observed in the vehicle control group was considered to be 100%. Data were normalized by dividing sample values by the mean of the vehicle control group, multiplying by 100%, and subtracting 100% to yield ‘percent change from control’. A one-way analysis of variation comparing treatment groups to the control was performed using SigmaStat version 2.0 (Jandel, San Rafael, Calif.); post hoc group differences were analyzed using Dunnett’s test. Statistical significance was accepted at $P < 0.05$.

Immunocytochemistry

GH_3/B6/F10 cells were seeded in SSM onto poly-D-lysine-coated glass coverslips for attachment, then cultured as described above in DIB for 72 h. The cells were washed three times in Dulbecco’s phosphate-buffered saline (DPBS). Fixation conditions were modified from Brink et al. (27), as follows. For nonpermeabilized cells, the fixative contained 2.0% paraformaldehyde (Sigma) and 0.1% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) in phosphate-buffered saline (PBS), adjusted to pH 7.4; this fixative (P/G)

was freshly prepared and applied to the cells for 30 min at 20°C. For pemeabilized cells, 0.5% Nonidet-40 and 0.15 mM sucrose were added to the P/G fixative, which was applied to the cells for 1 min. 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, prior to a 1 h blocking step at 37°C with 10% BSA (ICC-grade, Sigma) and 0.1% gelatin (from cold water fish skin, Sigma) in DPBS. The primary Abs were diluted in 0.5% BSA, 0.1% gelatin in DPBS (PBG) and added to the cells for 2 h at 20°C; next, the cells were washed six times over 30 min in PBG. The following steps were conducted at 20°C using reagents from a Vectastain ABC-alkaline phosphatase (AP) kit in conjuction with Vector red substrate (Vector Labs, Burlingame, Calif.). Biotinylated universal anti-rabbit/anti-mouse immunoglobulin G (IgG) was diluted to 4 μg/ml in PBG and added to the cells for 1 h; the cells were then washed six times over 30 min in PBG. The ABC-AP 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% EtOH, 95% EtOH, and xylene, then mounted using Cytoseal 280 (Stephens Scientific, Riverdale, N.J.).

E_2-BSA binding

GH_3/B6/F10 cells were cultured in plastic 35 mm dishes for 72 h in DIB, then washed once in DHB and chilled to 4°C over 15 min. The medium was replaced with ice-cold DHB ± ERα Ab and the cells were incubated for 5 min at 4°C. This medium was replaced with ice-cold DHB containing fluorescein-isothiocyanate (FITC) -labeled 17β-E_2-BSA conjugate (Sigma) for 20 min in the dark at 4°C. In parallel, control samples were incubated with FITC-BSA (without E_2) to assess nonspecific binding. The cells were washed three times with ice-cold DPBS, then fixed with 4% paraformaldehyde for 15 min. Coverslips were mounted with Fluoromont-G (Electron Microscope Sciences) and the samples were immediately examined for fluorescence.

Photomicrography

Photomicrographs were taken with Kodak Tmax 3200 black and white negative film or Ektapress 1600 Plus color negative film using an Olympus AHBT microscope equipped with a fluorescence attachment (Model AH2-RFL) and camera (Model C-35AD-4).

Immunoprecipitation

GH_3/B6/F10 cells cultured in SSM or uterus tissue from an 18 day pregnant rat were prepared for immunoprecipitation using reagents contained in a protein G immunoprecipitation kit (Boehringer-Mannheim, Indianapolis, Ind.). The cell lysate from GH_3/B6/F10 cells was incubated overnight with Ab H151 (10 μg/ml); the tissue lysate was incubated overnight with Ab MC-20 (5 μg/ml). The immunoprecipitated material was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4–20% acrylamide gradient gel (Novex, San Diego, Calif.) and transferred onto a 0.45 μM nitrocellulose membrane (Novex). Nonspecific binding sites were blocked with PBS containing 1% goat serum and 0.5% Tween-20. Individual lanes were probed with or without Ab MC-20 (1 μg/ml). After incubation with a goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma), the pH was raised by two successive washes in assay buffer (Ambion, Austin, Tex.) prior to a 5 min incubation with the chemiluminescent AP substrate, CDP-Star (Ambion). Bands were visualized by exposure of the immunoblot to X-ray film.

RESULTS

PRL release experiments

A significant increase in PRL secretion above basal release was elicited by 1 nM 17β-E_2 during a 3 or a 6 min incubation (Fig. 1). When ERα Abs were added to the test incubation medium instead of E_2, two Abs were found to affect PRL release, whereas others were inactive. Ab R4 stimulated PRL release in a time- and concentration-dependent fashion (Fig. 2). Significant PRL release above basal levels occurred only after a 6 min incubation with the Ab, compared to a 3 min incubation with E_2 (see Fig. 1). Added together with 1 nM E_2, Ab R4 (1:100) did not stimulate PRL to a greater extent than 1 nM E_2 alone (data not shown). In contrast to Ab R4, Ab H151 inhibited basal PRL release (Fig. 3). The response to Ab H151 was time and concentration dependent, with statistically significant responses appearing only at the 6 min time point, although the inhibitory trend was evident at all concentrations and times. When Ab H151 and E_2 were added to the cells simultaneously, the Ab blocked stimulation of PRL release when tested at the 3 min time point (Fig. 4).

Unlike these two functionally active Abs, two other Abs that recognize rodent ERα had no significant effect on PRL release at the times and concentrations examined (Fig. 5, top and middle). Ab D75 (Fig. 5, bottom), which did not affect activity, was included as a negative control since it only recognizes antigen derived from primate sources. Each of these three Abs was tested at a low concentration of 1 μg/ml and a high concentration of at least 10 μg/ml, but no changes in basal PRL secretion were observed (data not shown).

Ab recognition of plasma membrane ERα

Due to the differential abilities of the Abs to modulate PRL secretion, immunocytochemical analyses were conducted to determine whether the differences in bioactivity were associated with any differences in recognition of the membrane ERα by the Abs. We previously characterized an immunocytochemical system for specifically and sensitively detecting ERα on the plasma membrane of cells fixed with paraformaldehye and glutaraldehyde to prevent the Abs from entering the cells (28). Figure 6 depicts the typical patterns of fluorescence immunolabeling associated with each of the five Abs that were tested in the PRL release assay. Shown in Fig. 6, panels *A*, *B*, respectively, are the low levels of background fluorescence observed when no ERα Ab was added and when Ab D75 specific for primate ERα was added. When the biologically inactive Abs were tested, C542 displayed intense membrane staining (Figs. 6*C*) and H226 yielded consistently low, but detectable, levels of staining (Fig. 6*D*), suggesting that these Abs bound the membrane ERα without activating the receptor in PRL release experiments. The stimulatory Ab R4 produced strong labeling (Fig. 6*E*), but no signal above background was detected with the inhibitory Ab H151 (Fig. 6*F*). No immunofluorescence was visualized using Ab H151 at concentrations ranging from 1 to 20 μg/ml in fixed, nonpermeabilized GH_3/B6/F10 cells (data not shown). Moreover, when tested for recognition of nuclear ERα in permeabilized cells fixed in the presence of detergent, no labeling was observed with Ab H151, whereas dense nuclear staining was produced by Abs R4, C542, and H226 (data not shown). [Although we previously reported immunostaining of GH_3/B6/F10 cells with Ab H151 using a 10-fold higher concentration of glutaraldehyde (29), the earlier fixation method resulted in higher levels of nonspecific labeling and has now been replaced by the current staining protocols.]

To verify that H151 recognized ERα in the GH_3/B6/F10 cell line, immunoprecipitation experiments were undertaken with the Ab. As illustrated in Fig. 7, a protein of the appropriate size for ERα (M_r 68,000) was precipitated by the Ab. These results suggested that the epitope recognized by Ab H151 was altered by the aldehyde fixation procedure used in the immunocytochemical method and that the antigenic determinant was thereby rendered

nonimmunogenic. Hence, live cells were used to examine the interaction of Ab H151 with membrane ERα, using FITC-E_2-BSA binding.

Binding of FITC-E_2-BSA to the cells was not affected by preincubation with Ab R4 (Fig. 8***A***) compared to cells preincubated with no ERα Ab (data not shown). In contrast, as presented in Fig. 8*B*, preincubating the cells with Ab H151 reduced binding of the FITC-E_2-BSA conjugate to the background levels seen in controls incubated with FITC-BSA with no E_2 (data not shown).

DISCUSSION

The experiments reported here using Abs to ERα confirm the presence of a population of ERα proteins on the extracellular face of the plasma membrane and verify a functional role for these receptors in mediating a rapid, nongenomic response to estrogen in GH_3/B6/F10 rat pituitary cells. Because the Abs specifically recognize ERα but are too large to diffuse into the cell, they represent ideal tools for probing the function of ERα residing at the cell surface. Even though two of the Abs tested had no effect on the secretion of PRL, the ability of one ERα Ab to elicit and one to block E_2-stimulated PRL release provides compelling evidence that the surface molecule recognized by the Abs is involved in the nongenomic, estrogen-mediated regulation of PRL secretion in these cells. Furthermore, since the epitopes for both bioactive Abs lie in the hinge region of the ERα protein, the results may reveal information about conformationally sensitive areas of the membrane receptor involved in its function. A summary of these findings in relationship to the epitope map of ER34 is presented in Fig. 9.

Abs targeted to the ligand binding domain of ERα have been used to study the functional role of membrane receptors for estrogen. Morey et al. (30) found that Ab H222 reversed the rapid, inhibitory effects of E_2 on mitogen-stimulated MAP kinase activity in vascular smooth muscle cells. Using ID5, an anti-idiotypic Ab that interferes with estrogen binding to the ERα, Somjen et al. (15) observed increased levels of intracellular calcium within 5 s of Ab addition to cultured osteoblasts. The biological effects of Abs H222 and ID5 have been interpreted as resulting from direct interaction of the Abs at the estrogen binding site of the membrane receptor. In our experiments, Abs whose epitopes lie outside of the ligand binding domain were found to be biologically active. Ab R4, which mimicked the stimulatory activity of E_2 upon rapid PRL release, was raised in a rabbit immunized with a synthetic oligopeptide corresponding to amino acids 270 through 284 of the rat ERα sequence (21,31). Ab R4 did not increase PRL release as rapidly or to the same degree as E_2, indicating that the steroid hormone is more efficacious than the Ab in activating the response and suggesting that the effect of the Ab cannot be attributed to residual serum estrogens contaminating the Ab preparation. In this regard, the Ab was purified from the antiserum on a peptide affinity column, a process of dilution, washing, elution, and dialysis involving a total volume of buffers equivalent to 10,000-fold the volume of antiserum. The affinity-purified Ab preparation was further diluted 50- to 200-fold for use in the PRL release assay. It seems unlikely, then, that serum-derived estrogen contamination accounts for the effects of the Ab preparation. Ab H151, which inhibited basal PRL release and blocked E_2-stimulated release, was raised in amouse against a human ERα hinge region sequence that aligns with amino acids 292 through 305 of the rat sequence (31). Although this Ab did not detect membrane or nuclear ERα by immunocytochemical analysis in aldehyde-fixed GH_3/B6/F10 cells, the Ab precipitated a single protein of the appropriate size for ERα from the cells using Western analysis and blocked the binding of FITC-E_2-BSA binding to the surface of live (unfixed) cells presenting native ERα structure. It is noteworthy that Ab H151 and the biologically inactive Ab C542 are of the same mouse IgG_1 subtype, providing evidence that the effect of H151 on PRL secretion does not result from a nonspecific interaction of the Fc region of the molecule with the cell.

The antigenic determinants for the biologically active Abs R4 and H151 are found between amino acids 270 and 305 of the receptor protein; Abs directed at epitopes outside this region (to the carboxyl terminus and DNA binding domain) were inactive. Therefore, it appears that this 36 amino acid stretch of the membrane ERα may be conformationally sensitive and that the stimulatory Ab R4 and the inhibitory Ab H151 may induce distinct conformational changes on binding to this portion of the receptor. Consistent with this idea, Ab H151 was found to block the binding of FITC-E_2-BSA to the receptor, whereas Ab R4 had no effect, suggesting that attachment of the Ab H151 structurally constrains the receptor in a manner that prevents estrogen from binding. It should be noted that structural changes induced by hormonal analogues are associated with functional consequences for the nuclear receptor as well (32). Another possible explanation for these data is that Ab H151 sterically inhibits the macromolecular E_2-BSA conjugate from attaching to the receptor, although this possibility is less likely because the Ab does not target the ligand binding domain, and Ab R4, whose epitope is nearby, had no effect on E_2-BSA binding.

Besides conformational changes induced in the receptor structure by Ab binding, aggregation of some receptor molecules after Ab binding is another mechanism known to trigger receptor activation. Patching and capping of receptors on the surface of cells after exposure to anti-receptor Abs have been described for peptide hormone receptors (33,34), as well as for steroid hormone receptors. Sabeur et al. (16) reported that an anti-progesterone receptor Ab was observed to localize in a distinct band on the head of sperm cells. The Ab also inhibited the rapid, progesterone-stimulated acrosomal response in sperm cells, possibly by blocking the binding of the steroid to the receptor. Using a monoclonal Ab directed against the progesterone molecule, another group demonstrated rapid stimulation of calcium influx and acrosomal exocytosis in sperm cells in the presence of the anti-progesterone Ab and subthreshold doses of progesterone (17). Presumably the bivalent Ab acted by enhancing aggregation of receptors since monovalent fragments of the Ab were inactive. In our own laboratories, we have observed that glucocorticoid receptor Abs caused patching and capping of receptors on the surface of human leukemic cells (35) and that ERα Abs induced clustering of surface receptors in GH_3/B6 cells (21,23). We and others (8) have also reported that receptor aggregation can be elicited by estradiol conjugated to BSA, which is also multivalent, having multiple steroids molecules conjugated to a single protein. However, it is not known whether the process of receptor aggregation in these systems performs a biological function related to receptor activation or simply represents clustering of receptors due to the binding of large multivalent macromolecules to the cell.

In addition to verifying the functional significance of membrane ERα, the results of this study confirm the structural relationship between the plasma membrane and nuclear population of receptors. The data reported here extend observations made by us and others that Abs whose epitopes span the length of the ERα protein recognize both membrane and nuclear receptors. This structural relationship has been further substantiated by recent experiments in which cells that do not express estrogen receptors have been transfected with ERα cDNA. The transfected cells express ERα on the plasma membrane, as well as in the nucleus (12), and they exhibit rapid responsiveness to estrogen (12,36). Little is known, however, about the structural properties of the receptor that are causes or consequences of its residence at the membrane, and our results represent some of the first data to address functionally important regions of the membrane ERα protein.

In summary, we have applied an immunocytochemical method and E_2-BSA binding to verify the presence of ERα on the GH_3/B6 rat pituitary cell subclone, F10. The use of several Abs recognizing different ERα epitopes substantiates the identification of this membrane receptor as well as its full exposure on the outside surface of the cell. Moreover, the ability of two of these Abs to modulate a biological response through their binding to mERα reveals a functional

role for these receptors and suggests that alteration of receptor conformation or of receptor aggregation is involved in the rapid, membrane-initiated estrogenic response. Therefore, specific Abs directed against steroid receptors continue to be powerful tools for probing the identity and function of membrane-associated steroid receptors.

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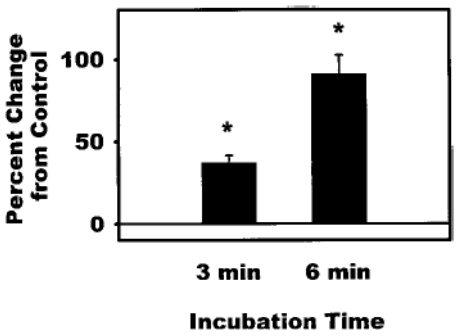

Figure 1.
Stimulation of PRL release by 1 nM E_2 during 3 and 6 min incubations. The data presented are the means ± se of five separate experiments; *$P < 0.05$.

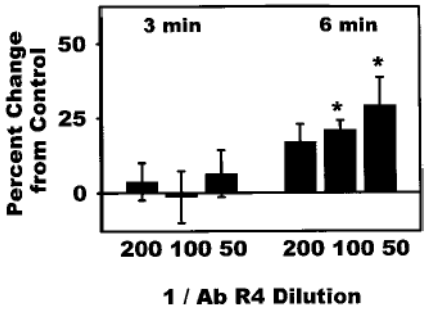

Figure 2.
ERα Ab R4 stimulates PRL release in a time- and concentration-dependent fashion. The data are the means ± se of one experiment (n=6), which is representative of four similar experiments; *$P < 0.05$.

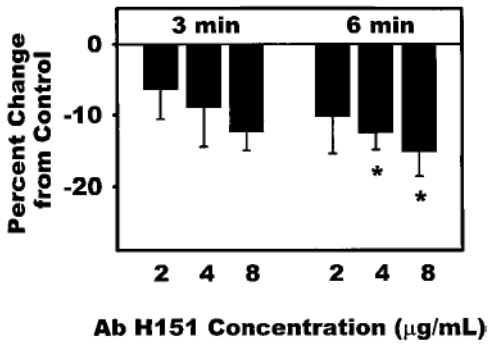

Figure 3.
ERα Ab H151 decreases PRL release in a time- and concentration-dependent fashion. The data are the means ± se of one experiment (n=6), representative of four similar experiments; *P < 0.05.

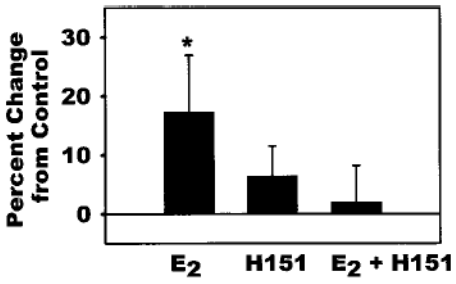

Figure 4.

ERα Ab H151 blocks E_2 stimulation of PRL release. Cells were incubated for 3 min in the absence or presence of 1 nM E_2, 10 μg/ml Ab H151, or E_2 and Ab H151. The data are the means ± se of one experiment (n=6), which is representative of three similar experiments; *$P < 0.05$.

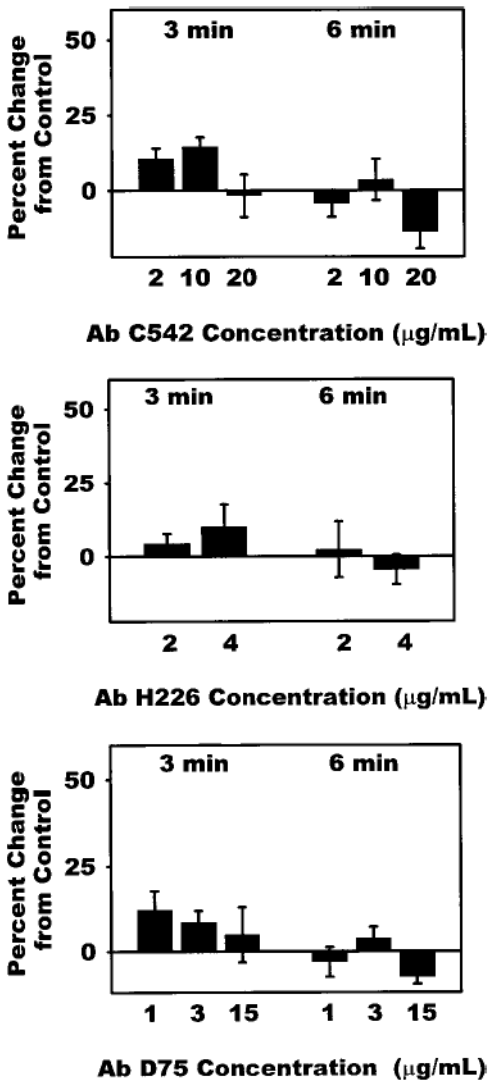

Figure 5.
ERα Abs C542 (top), H226 (middle), and D75 (bottom) have no effect on PRL release. The data in each panel are the means ± se of a single experiment (n=6); each experiment is representative of three or four similar experiments.

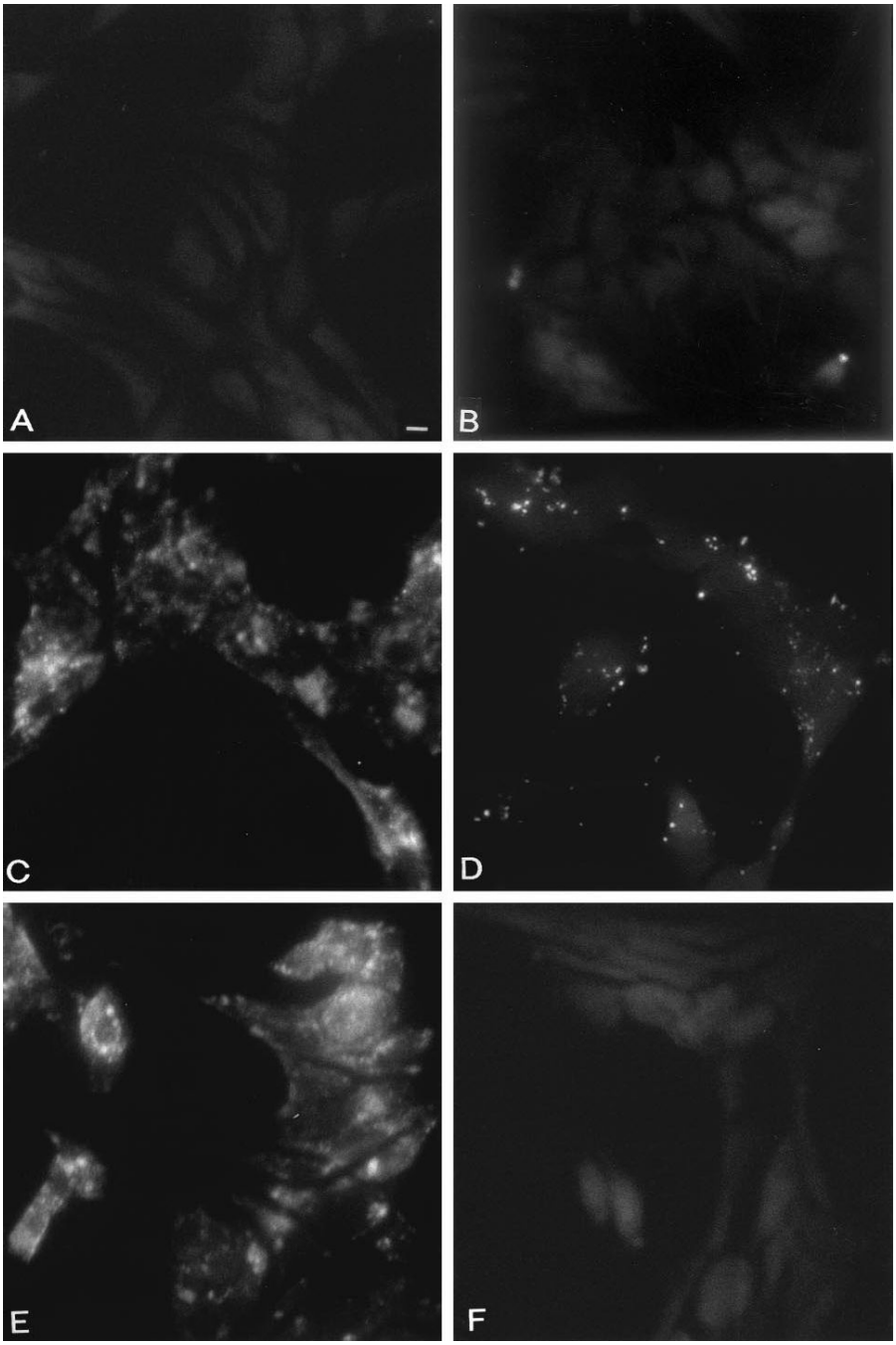

Figure 6.

Binding of ERα Abs to aldehyde-fixed GH_3/B6/F10 cells. The cells were cultured for 72 h in serum-free, defined medium, fixed with 2% paraformaldehyde/0.1% glutaraldehyde, then incubated in the presence or absence of an ERα Ab. Ab binding was assessed by ABC-alkaline phosphatase inmmunocytochemistry using the fluorescent substrate Vector red. *A)* no ERα Ab; *B)* Ab D75 (5 μg/ml); *C)* Ab C542 (2 μg/ml); *D)* Ab H226 (5 μg/ml); *E)* Ab R4 (1:200); *F)* Ab H151 (10 μg/ml). The scale bar in the bottom right corner of panel *A* corresponds to 12 μM; the same scale applies to all photomicrographs in this figure. This experiment is representative of three similar experiments.

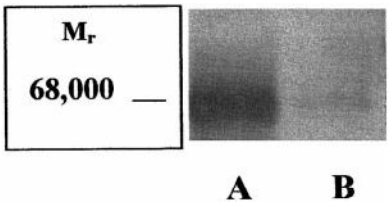

Figure 7.

Immunoprecipitation of ERα from GH_3/B6/F10 cells by Ab H151. Lane A contains a positive control sample of ERα immunoprecipitated from pregnant rat uterus with Ab MC-20. Lane B contains a band of the same size that was immunoprecipitated from GH_3/B6/F10 cells by Ab H151. No band appeared in control lanes of immunoprecipitated protein from either GH_3/B6/F10 cells or rat uterus when no ERα Ab was added to the Western blot incubations (data not shown).

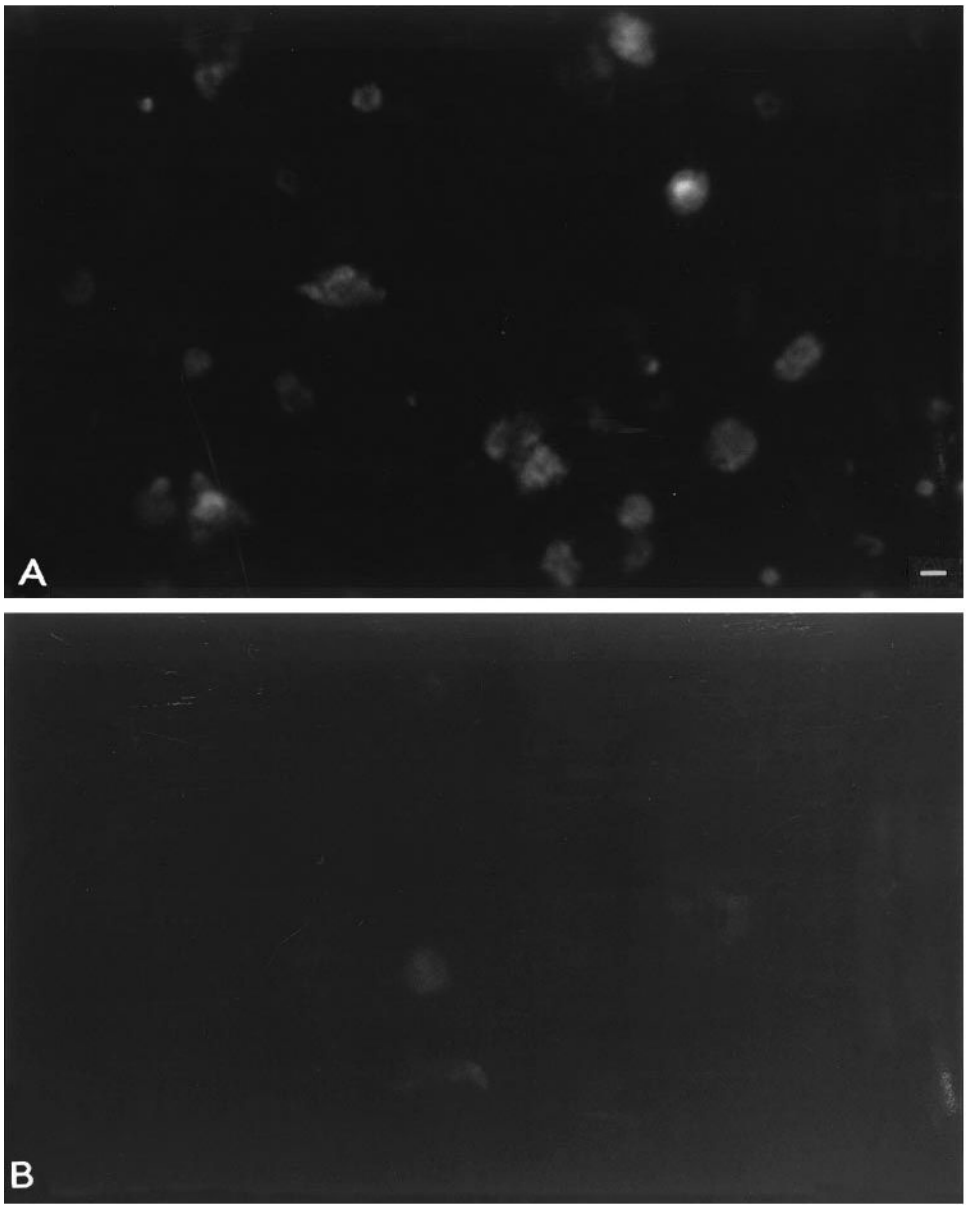

Figure 8.

Ab H151, but not Ab R4, blocks binding of FITC-E_2-BSA to live GH_3/B6/F10 cells. Cells were preincubated with *A)* Ab R4 (1:100) or *B)* Ab H151 (5 μg/ml) prior to exposure to FITC-E_2-BSA. The scale bar in the bottom right corner of panel *A* corresponds to 10 μM; the same scale applies to panel *B*. This experiment is representative of three similar experiments.

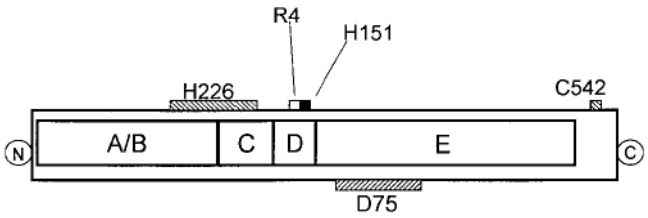

Figure 9.

Biological activity of ERα antibodies. This diagram of the ERα protein indicates the approximate locations of the antigenic determinants of the Abs tested for biological activity in PRL release experiments. An open symbol indicates the ability of the Ab to stimulate PRL release, a closed symbol indicates the ability to inhibit release and to block E_2-stimulated release; hatched symbols, no effect. Small squares show the 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. All of the Abs depicted at the top recognize both primate and rodent ERα; the one on the bottom only binds receptors from primate species. 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 region; E, the estrogen binding domain. The amino terminus and the carboxyl terminus are designated by the encircled N and C, respectively.