

# The Other Estrogen Receptor in the Plasma Membrane: Implications for the Actions of Environmental Estrogens

Cheryl S. Watson,<sup>1</sup> Todd C. Pappas,<sup>1</sup> and Bahiru Gametchu<sup>2</sup>

<sup>1</sup>Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas; <sup>2</sup>Department of Pediatrics, Medical College of Wisconsin, Milwaukee, Wisconsin

Environmental or nutritional estrogenic toxicants are thought to mediate developmental and carcinogenic pathologies. Estrogen receptor (ER) measurements are currently used to predict hormonal responsiveness; therefore all ER subpopulations should be considered. We have been involved in the immunoidentification and characterization of membrane steroid receptors in several systems and have recently shown that binding of estradiol (E<sub>2</sub>) to a subpopulation of ERs (mER) residing in the plasma membrane of GH<sub>3</sub> pituitary tumor cells mediates the rapid release of prolactin (PRL). Here we review these findings and present other important characterizations of these receptors such as trypsin and serum susceptibility, movement in the membrane, confocal localization to the membrane, binding to and function of impeded ligands, and immunoseparation of cells bearing mER. We plan to use this system as a model for both the physiological and pathological nongenomic effects of estrogens and estrogenic xenobiotics. Specifically, it should be useful as an *in vitro* assay system for the ability of estrogenic xenobiotics to cause rapid PRL release as an example of nongenomic estrogen effects. — Environ Health Perspect 103(Suppl 7):41–50 (1995)

Key words: estrogen, plasma membrane, steroid receptor, xenobiotics, pituitary, nongenomic, prolactin

## Introduction

There are many known instances of estrogen toxicity that affect development and function of reproductive systems in humans and animals (1,2). Estrogen has been shown to have a role in initiating diseases (including cancer) in a variety of tissues (3–5). As more sensitive assays for estrogen receptors (ERs), their mRNAs, and their functions become available (6),

more tissues will be considered for these actions by estrogens. Understanding the toxicity of estrogen and the myriad synthetic and naturally occurring estrogenic compounds (1,2,7) requires an understanding of whether their effects are being mediated through genomic or nongenomic mechanisms.

The genomic actions of estrogens and other steroid hormones are now relatively well understood. In this pathway, hormones, bind to a cytoplasmic/nuclear receptor, which binds to DNA and triggers RNA-dependent protein synthesis (8,9). However, this mechanism best explains temporally delayed effects because it requires a series of macromolecular syntheses and protein localizations to produce responses over time periods of several hours to days (10). Steroids and their mimetics also exert a variety of short-term effects (seen in seconds to minutes) on their target organs. For example, estradiol (E<sub>2</sub>) rapidly affects monovalent and divalent cation transport, glucose uptake, and water imbibition in the rat uterus (11). In neurons and GH<sub>3</sub> pituitary tumor cells, estrogens can elicit changes in electrical activity within minutes (12–16). A fast action of estrogen mediates calcium mobilization in granulosa cells (17) and osteoblasts (18). Rapid electrical responses to estrogens also occur in several areas of the brain and other tissues (15,16,18–23). Many fast or otherwise mechanistically unexplained actions of

other steroids have also been reported (17,24–35). Plasma membrane-resident forms of steroid receptors have been proposed to mediate such actions (36,37).

Although the action of a membrane-resident steroid receptor is implied in the systems described above, only some of these studies actually attempted to demonstrate the presence of such a protein in the membrane. Several groups have provided evidence for the binding of labeled steroid to a membrane-associated site (29,36–42). We have developed techniques that rely mainly on the immunological detection and isolation of steroid hormone receptors residing in plasma membranes (43–48). We used these antibody (Ab) labeling techniques to demonstrate membrane glucocorticoid receptor (mGR) in both mouse and human lymphoma cell lines and to correlate the mGR's presence with the clinically important lymphocytolytic response of these cells to glucocorticoids. The collective presence of functional domains that mediate steroid binding, multiple epitope recognition, and DNA binding (49) suggests that mGR is closely related to the already characterized intracellular glucocorticoid receptor and is not an entirely different protein. A membrane location for the initiation of cytolysis may be related to changes in membrane permeability and the calcium activation of degradative enzymes (50), and these mechanisms may have relevance for other nongenomic actions of steroids. We have recently

This paper was presented at the Symposium on Estrogens in the Environment, III: Global Health Implications held 9–11 January 1994 in Washington, DC. Manuscript received: March 15, 1995; manuscript accepted: April 4, 1995.

We would like to acknowledge David Konkel for his thoughtful critique of this paper, Peter Moller for his assistance with fluorescence microscopy, Judy Yanariello-Brown for immunocytochemistry advice, and Thomas J. Collins for RIA advice. Access to the confocal microscopy facilities and advice of M. Correia, N. Wills, S. Lewis, and their staff were greatly appreciated. This research was supported by the John Sealy Memorial Endowment Fund (awarded to C. S. W.).

Address correspondence to Dr. Cheryl S. Watson, Route F-45, Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX 77555. Telephone: (409) 772-2382. Fax: (409) 772-2382. E-mail: cswatson@beach.utmb.edu

Abbreviations used: ER, estrogen receptor; E<sub>2</sub>, estradiol; mER, membrane ER; PRL, prolactin; Ab, antibody; mGR, membrane glucocorticoid receptor; SSM, serum-supplemented medium; DM1, defined medium; PBS, phosphate-buffered saline; RIA, radioimmunoassay; BSA, bovine serum albumin; PBBSA, phosphate-buffered saline with 1% bovine serum albumin.

applied these procedures to the related problems of demonstrating a membrane receptor for estrogens in the membrane of pituitary tumor cells and correlating membrane ER (mER) expression in subclones of these cells to their ability to rapidly secrete prolactin (PRL) (48).

## Methods

### Cells and Growth Conditions

GH<sub>3</sub>/B6 cells (provided by Bernard Dufy) were routinely cultured in a serum-supplemented medium (SSM) composed of Ham's F-10 (Gibco-BRL, Gaithersburg, MD) supplemented with 12.5% heat-inactivated horse serum (Gibco-BRL) and 2.5% heat-inactivated defined/supplemented bovine calf serum (Hyclone Labs, Logan, UT). Our defined medium (DM1) is adapted from Hayashi and Sato (51), but uses phenol red-free RPMI 1640 (Gibco-BRL) because phenol red is known to be a weak estrogen (52). The medium is supplemented with insulin (10 µg/ml; Boehringer Mannheim, Indianapolis, IN), bovine transferrin (5 µg/ml; Boehringer Mannheim), parathyroid hormone (bovine 1-34, 0.5 ng/ml; Bachem, Torrance, CA), 3,3',5-triiodothyronine ( $3 \times 10^{-11}$  M; Sigma Chemical Co, St. Louis, MO), thyrotropin-releasing hormone (1 ng/ml; Bachem), and fibroblast growth factor (1 ng/ml; Boehringer Mannheim).

Before PRL release experiments, cells were subcultured for 2 to 4 days in DM1. Cells growing exponentially in SSM were washed once with phosphate-buffered saline (PBS) and trypsinized (0.5% trypsin, 0.5 mM EDTA in Hank's balanced salts solution; Sigma) for 3 min. The trypsin was quenched with SSM and cells were centrifuged at  $125 \times g$  for 5 min. The cells were then washed once with PBS, resuspended in DM1, and plated onto poly-D-lysine (180,000 mw; Sigma)-treated 6-well (35 mm) plates. All dishes were seeded from a single resuspension of cells to assure an equal cell number in each well.

### Immunocytochemistry

Cells in the exponential growth phase were removed from the culture flask with trypsin (0.5% w/v trypsin, 0.5 mM EDTA in Hank's balanced salts solution), then treated with SSM or soybean trypsin inhibitor (0.1% w/v in PBS). Round coverslips 12 mm in diameter (Baxter, McGaw Park, IL) were placed in the wells of a 24-well tissue culture plate and treated with poly-D-lysine (Sigma). Cells were seeded onto these coverslips in DM1

supplemented with 10 pM E<sub>2</sub> (E<sub>2</sub> added at this low level maintains basic estrogen-responsive systems such as PRL synthesis at levels comparable to SSM cultured cells) (53) and subcultured for 2 days.

For live-cell immunolabeling, DM1 was aspirated from the cells and replaced with PBS at 37°C, then the 24-well plate containing the cells on the coverslips was placed in a tray on an ice-water bath and allowed to cool for 45 min. The ice-water bath registered a constant 2°C. The cells were then washed 3 times in PBS with 1% (w/v) bovine serum albumin (1% phosphate-buffered saline with 1% bovine serum albumin [PBSA]; Fraction V, Sigma).

Both affinity-purified anti-ER antibodies (R3 and R4), characterized by Pappas et al. (48), were used at a dilution of 1:100 (Ab:1% PBSA) for immunocytochemical procedures. For live cell immunocytochemistry, primary Ab, secondary Ab (Cy3-conjugated goat anti-rabbit IgG; Jackson ImmunoResearch Laboratories, West Grove, PA) and 4% (v/v) normal goat serum (Jackson ImmunoResearch Laboratories) were diluted in 1% PBSA and preincubated for 1 hr at 2°C. A rabbit polyclonal anti-actin Ab was obtained from ICN ImmunoBiologicals (Lisle, IL). The coverslips with cells attached were inverted onto 10 µl of the Ab mixture for 20 min, then washed, fixed with 4% (w/v) paraformaldehyde for 10 min, and mounted with 20% (v/v) glycerol in PBS. In experiments involving trypsin sensitivity of the mER antigen, cells were washed once with PBS, then treated at 22°C for 10 min with 5 µg/ml bovine pancreatic trypsin (Type

III; Sigma). The trypsin was quenched with 1% PBSA for 10 min. The cells were then washed 2 times in 1% PBSA and processed as above for live cell labeling. In membrane mobility patching-and-capping experiments, cells were warmed to 37°C for 20 min after Ab application and then cooled to 2°C for the remaining washes and fixation.

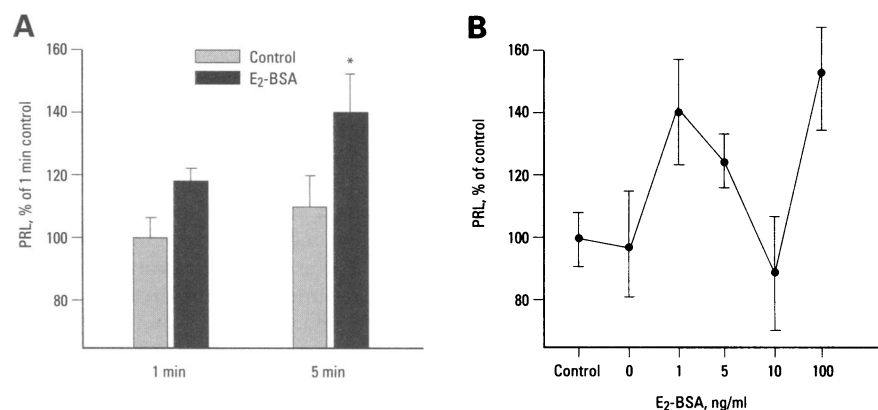
Cells were viewed on a Zeiss fluorescence microscope using a 63X oil immersion Planapo lens and rhodamine filter, except where indicated. The amount of time for a metered exposure of a positive response was used as a standard for subsequent photographs of negative controls. Photomicrographs were taken with T-MAX 400 film (Eastman Kodak, Rochester, NY).

### PRL Release in Response to E<sub>2</sub> and E<sub>2</sub>-BSA

17β-E<sub>2</sub> (Sigma) was diluted in ethanol to a 1 mM concentration; a further dilution was made in phenol red-free RPMI 1640 supplemented with 25 mM HEPES. The final concentration of ethanol was 0.001%.

1,3,5(10) Estratriene-3,17β diol 6-one 6-carboxymethoxime: BSA (E<sub>2</sub>-BSA) was obtained from Steraloids (Wilton, MA). The ratio of estrogen to BSA in this preparation was 15 to 20:1. The conjugate was dissolved in deionized water to a concentration of 1 mg/ml then treated with dextran/charcoal (0.5%/5% in PBS) immediately before dilution to remove free steroid. Subsequent dilutions were made in phenol red-free RPMI 1640 supplemented with 25 mM HEPES.

For the PRL release assays depicted in Figure 1, cells were subcultured for 2 to 5



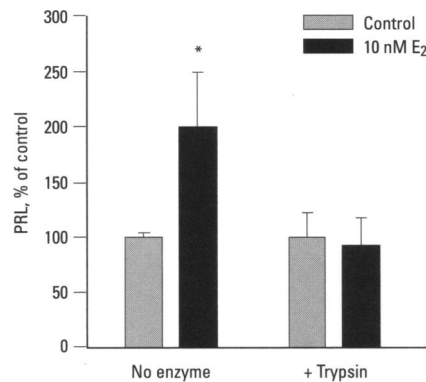
**Figure 1.** E<sub>2</sub> conjugated to BSA causes rapid release of PRL. (A) Time course of 5 ng/ml E<sub>2</sub>-BSA-stimulated PRL release.  $n=12$  for 1 min time point;  $n=6$  for 5 min time point. Data are from three experiments. (B) Dose-related effects of the conjugated steroid presented for 1 min at indicated concentrations.  $n \geq 8$  for all doses except 100 ng/ml ( $n=5$ ); data are from two experiments. Data are expressed as percent of the mean of the 1 min control. A two-way ANOVA revealed a significant effect of ligand treatment ( $f=9.23$ ;  $df=1, 32$ ;  $p<0.05$ ) and time ( $f=4.41$ ;  $df=1, 32$ ;  $p<0.05$ ) on PRL release. Error bars are SEM. Control, BSA alone. \*Significantly different from control ( $p<0.05$ ).

days in DM1. All experiments were done at 37°C. Each well was washed 3 times in serum-free phenol red-free RPMI 1640 supplemented with 25 mM HEPES, pH 7.4. The cells were then preincubated in this medium on an orbital shaker for 30 min to allow sufficient PRL accumulation in the medium for accurate radioimmunoassay (RIA) determinations (sufficient signal to fall within the detection level and the standard curve). Medium containing steroid or ethanol (vehicle control) was then added to dishes for the desired experimental exposure time. The supernatant was then transferred to a chilled microcentrifuge tube and centrifuged at 800×g for 5 min to remove cells that came off the plate during the experiment. The supernatant was aspirated into a clean microcentrifuge tube and stored at -20°C until assayed.

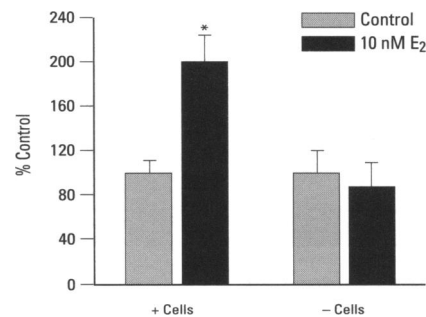
Alternatively, instead of allowing 30 min for accumulation of PRL into the media, higher numbers of cells were passed to plates and the 30-min preincubation was eliminated (Figures 2,3). Then, steroid or vehicle treatments in rat saline (150 mM NaCl, 5.6 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM HEPES, pH 7.35, and 10 mM glucose) were added to the well for the specified amount of time. There was no significant difference in the magnitude of stimulated release in these two paradigms. Because larger numbers of cells were used in these assays, the secreted PRL level was measurable without a preincubation-accumulation of higher levels of hormone.

The concentrations of PRL in the media were determined by RIA (54) using a kit provided by the National Institute of Diabetes and Digestive and Kidney Disease and the National Hormone and Pituitary Program (University of Maryland School of Medicine, award no. 29196 to T. Pappas and C. Watson). Ab-bound PRL was separated using a goat anti-rabbit secondary Ab (Chemicon, El Segundo, CA) precipitated with 10% (w/v) polyethylene glycol (8000 mw, Sigma). All samples were assayed in duplicate. Data were normalized by dividing by the mean of the control and statistical analyses were performed on SYSTAT version 3.0 (Systat Inc., Evanston, IL) using the MGLH module. Post hoc group differences were analyzed with Scheffé's multiple contrast (55). Statistical significance was accepted at  $p < 0.05$ .

The trypsin treatment affecting E<sub>2</sub>-induced PRL release was performed as above for immunocytochemical studies. The reaction was stopped with soybean trypsin inhibitor (1 mg/ml) for 5 min followed by three washes with rat saline. After



**Figure 2.** Trypsin removal of mER abolishes the PRL release response to estrogen. RIA measurements of released PRL after 5-min treatment of mER<sup>+</sup> cells. Data are from two independent experiments;  $n = 7$  for each group. Each value is expressed as a percentage of its own control (no E<sub>2</sub>) value. Error bars show SEM. \*Significantly different from control at the  $p < 0.05$  level.



**Figure 3.** Enhancement of the rapid PRL release response in mER-enriched cells. Each E<sub>2</sub>-treated group is compared to its own control. \*Significantly different from the paired control at the  $p < 0.05$  level.

a 30-min preincubation in rat saline, cells were treated with 10 nM 17β-E<sub>2</sub> or vehicle (0.001% ethanol) for 5 min. Supernatants were assayed for PRL as above.

### Immunoselection of mER-bearing Cells

Culture-dish wells were prepared for immunopanning by sterile-filtering affinity purified Ab (1 mg/ml) in 0.1% PBSA into them, allowing Ab to attach to the plate at 37°C for 2 hr, chilling to 4°C, and rinsing once with chilled PBS. Because trypsin removes mER, we explored other means to prepare cells for immunopanning. Approximately 10<sup>6</sup> GH<sub>3</sub>/B6 cells, grown in DM1 for 48 hr, were removed from the plate with a nonenzymatic cell dissociation solution with no Mg<sup>2+</sup> or Ca<sup>2+</sup> (Sigma). The cells were washed, resuspended in PBS + 0.1% BSA (fatty acid-free), and rocked for 1 hr at 37°C to allow turnover and

repair of membranes. The cells were then chilled to 4°C and seeded onto a well containing the attached Ab. The Ab-coated dish with added cells was placed onto an ice bath for 1 hr on an orbital shaker. Unattached cells were then aspirated from the plate and saved as the (-) or depleted fraction. The plate, still containing the enriched or (+) fraction, was rinsed once with chilled PBS, and 1 ml SSM was put onto the cells. Cells were gently triturated from the well, amplified using routine culture procedures, and switched to DM1 for 2 days before processing for immunocytochemistry (except where indicated).

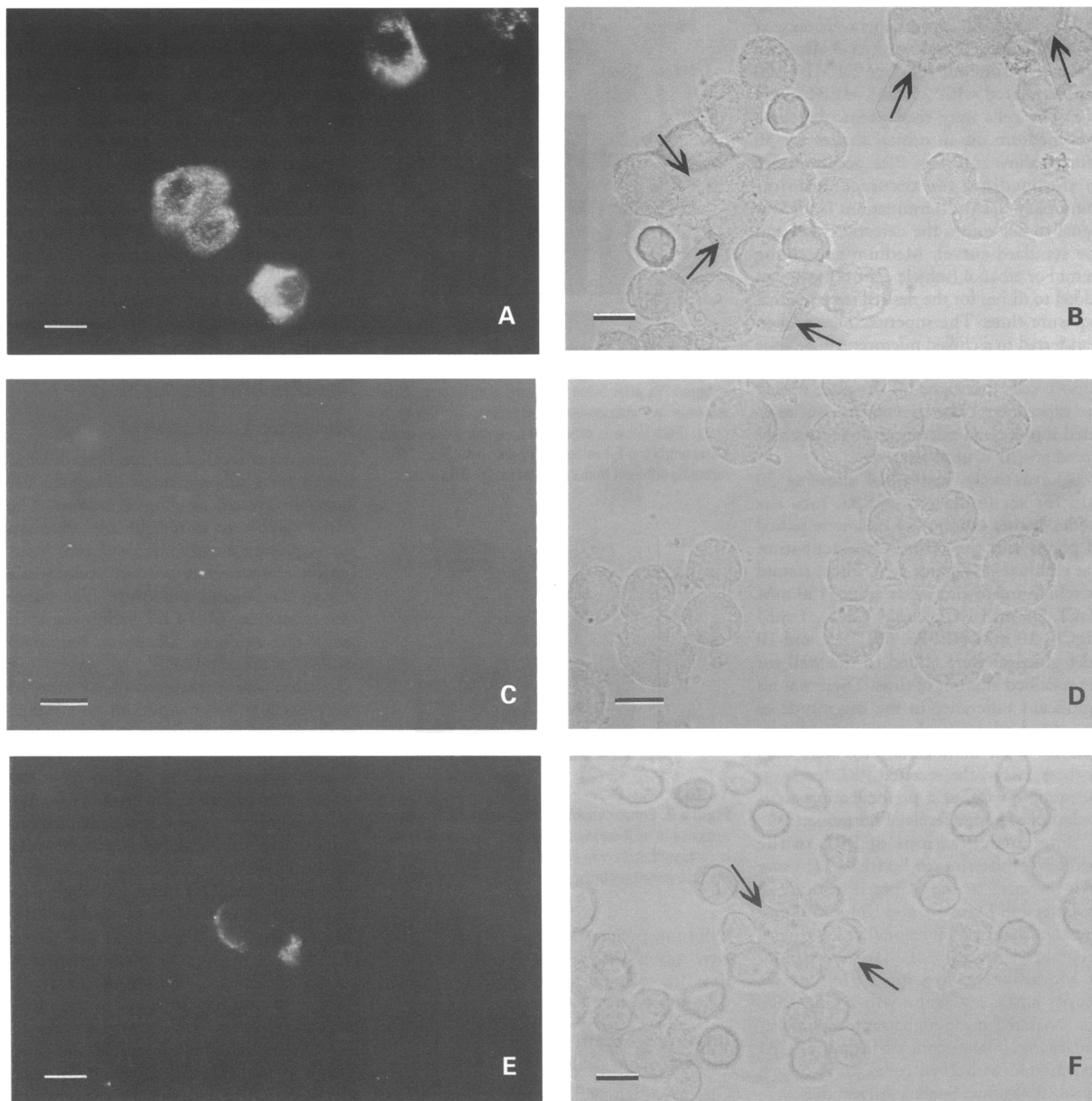
## Results and Discussion

### Membrane Localization of the ER

A membrane localization has been demonstrated for a subpopulation of cellular ER based on live-cell labeling techniques. The Ab is unable to enter the cell through intact plasma membranes, and cold incubation temperatures prevent endocytosis during the labeling procedure. The punctate appearance of the ER membrane antigen shown in Figure 4A appears on only 8 to 17% of the cells (48).

Other membrane-resident steroid receptors have been shown to be attached to the plasma membrane in such a way that exposure of intact cells to mild trypsin treatment removed the antigen from the cell surface (43,46). To further examine the possibility of cell surface localization for ER, we subjected live cells to mild trypsin digestion and checked for the presence of antigen. This treatment resulted in complete elimination of surface ER immunoreactivity (Figure 4C) and shows that the ER membrane labeling depends on the integrity of membrane proteins. Because trypsin has no access to the intracellular receptors, this action supports an outer-face plasma membrane localization. This also confirms the utility of Ab-based identification techniques for this class of steroid receptors, as we have shown previously for mGR (43-46).

Often, mER labeling was not evenly distributed over the surface of the cell. Examples of such asymmetric Ab staining can be seen in Figures 4A, 5B, and 6A,B, where staining is sometimes over one pole of a cell or in patches. A more extremely restricted distribution of labeling is seen in Figure 4E, where the cells were incubated at 37°C for 20 min after Ab addition. The photomicrograph shows representative features such as label coalescing into patches and accumulating on one side of the cell,



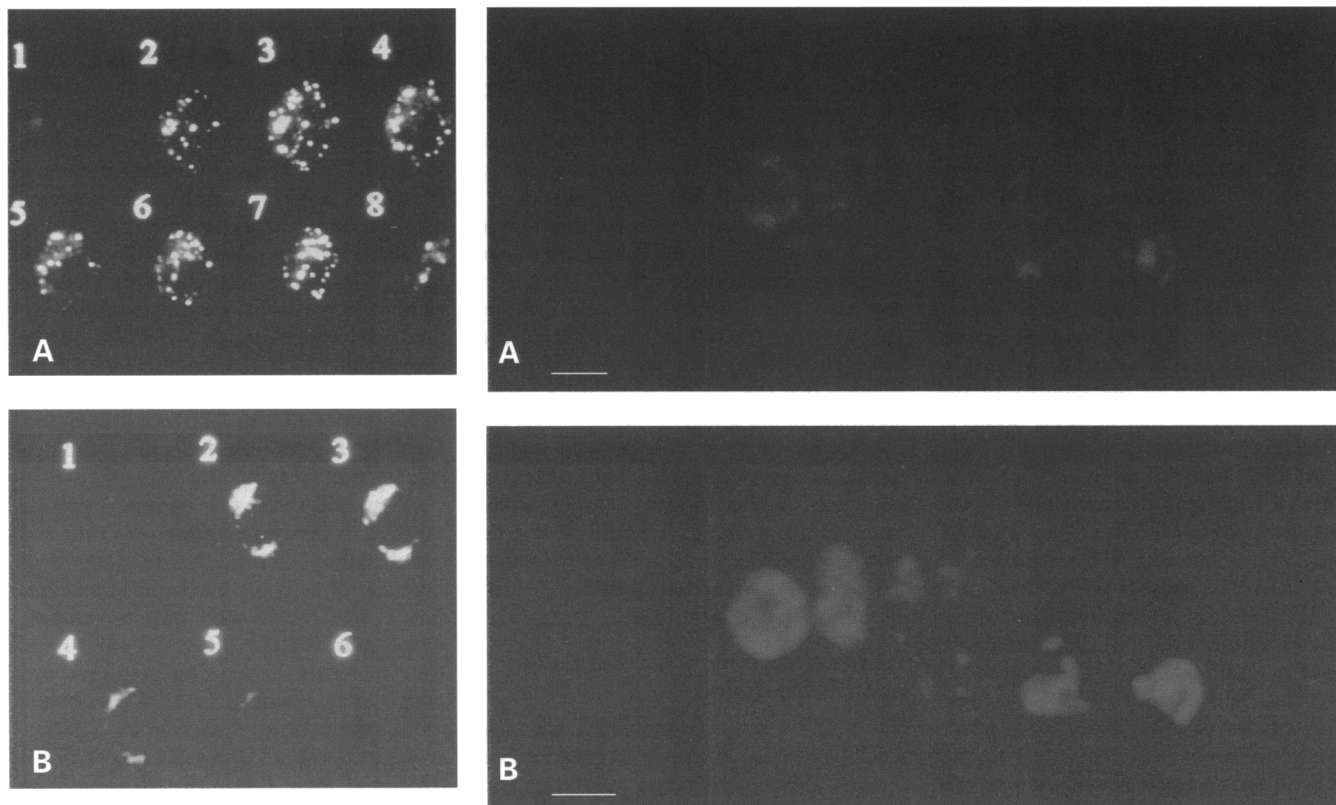
**Figure 4.** The membrane ER antigen is punctate in appearance, can be digested away by mild trypsin treatment, and undergoes asymmetric redistribution at 37°C. (B), (D), and (F) are the phase contrast images of the fluorescence micrographs shown in (A), (C), and (E). (A) shows the punctate appearance of the membrane ER antigen labeled with affinity-purified R3 Ab; note that not all cells are labeled (compare to B, arrows). (C) shows a fluorescence micrograph of cells that were treated for 10 min with trypsin, then labeled with the affinity-purified R4 Ab. (E) shows the appearance of mER after the cells have been incubated with labeled R4 Ab for 20 min at 37°C. Bars = 10  $\mu$ m.

as well as a general reduction in the number of cells showing label. We do not presently know if the lost label is endocytosed or is sloughed from the cell. Movement of antigen on the cell surface, resulting in patching and capping of the Ab-antigen complex, has been described for other membrane antigens (56,57) as well as for

the mGR (43,44,46). This mobility may be part of the process of cellular uptake or sloughing of the antigen-Ab complex and, in some cases, may be important for the function of the surface protein, e.g., growth factor receptors (57,58).

Confocal laser microscopy is an alternative technique for establishing cellular

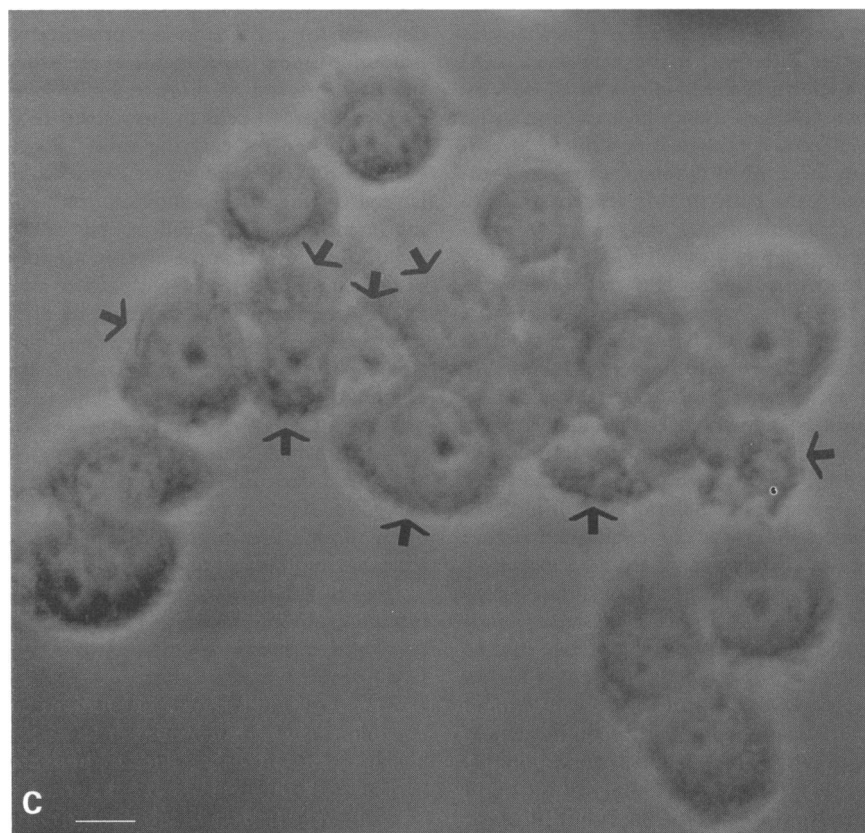
localization of a protein by optical sectioning through a fluorescently labeled cell. We demonstrated a membrane location for the ER with these techniques (Figure 5). Note again the punctate appearance of the cell in Figure 5A and the asymmetric mER distribution, especially pronounced in the cell in Figure 5B. The uneven topology of these



**Figure 5.** Confocal localization of mER to the plasma membrane with R3 Ab. Optical sections were taken at 1.5  $\mu\text{m}$  (A) or 1.3  $\mu\text{m}$  (B) intervals through the entire thickness of the cell. Frame #1 shows the part of the cell that was attached to the coverslip.

cells (especially in Figure 5A) gives the appearance of signal being present deeper in the cell. However, our labeling techniques preserve the integrity of the plasma membrane and do not allow endocytosis during the course of the experiment, thus assuring us that the labeling seen is on the cell surface.

$\text{E}_2$ -BSA is a preparation in which the ligand is covalently coupled to BSA; thus it cannot enter the cell and should bind only to cell surface receptors. This conjugate was then additionally coupled to fluorescein (FITC) for fluorescence microscope studies. When cells are labeled with this reagent, the appearance is again punctate, uneven, and heterogeneous as was shown for labeling with Cy3-ER Ab (Figure 4). When these cells are double labeled with both reagents, exactly the same cells are labeled with an identical pattern of staining (Figure 6). That these two very different labeling techniques (one based on epitope recognition, the other on steroid binding) label the same cells is very strong evidence that our immunological identification of mER is not artifactual.



**Figure 6.** Simultaneous labeling of cells with  $\text{E}_2$ -BSA in (A) and ER Ab in (B). (C) Shows the bright field image of the same field. Arrows indicate the labeled cells. Bars = 10  $\mu\text{m}$ .



### Rapid Responses to Estrogen Are Dependent on Proteins Located and Acting at the Cell Surface

PRL is released from cells treated with nM concentrations of  $E_2$  in as rapidly as 1 min. Such rapid responses are specific for both  $17\alpha$ - and  $17\beta$ - $E_2$ , although  $17\beta$  is more potent (48). We again used ligands conjugated to a large protein molecule (BSA) to prevent access of our small lipophilic ligand to the intracellular space while exposing it to the cell surface. Impeded ligands therefore represent a way (in addition to the demonstration of the rapidity of the response) to suggest that signaling occurs at a membrane receptor. We applied  $E_2$ -BSA to our cell system and assayed PRL release. Immobilized ligand caused rapid PRL release from GH<sub>3</sub>/B6 cells (Figure 1). By 1 min, PRL release in the presence of 5 ng/ml  $E_2$ -BSA exceeds that of BSA controls (Figure 4A), though this is not statistically significant ( $p=0.07$ ). Increased PRL release is significant at 5 min. Figure 4B shows that the effect of  $E_2$ -BSA is also dose dependent but is multiphasic, with maximal stimulation of PRL release at 1 ng/ml. The multiphasic aspect of the effect reveals that there may be a number of mechanisms activated by this ligand and that the effective doses for such alternative mechanisms may be different. Lieberherr et al. (18) have shown biphasic effects of  $E_2$ -BSA on mobilization of intracellular calcium in rat osteoblasts. Although it is difficult to estimate the concentration of estrogen available to the cells in this reagent (due to steric considerations), the maximum available estrogen in 1 ng/ml  $E_2$ -BSA is 0.3 nM (given the maximum 20:1 ratio of estrogen to BSA in this preparation). Thus, this reagent is more potent in eliciting PRL release than is unconjugated  $17\beta$ - $E_2$ . We are not the first group to see this tethered-steroid phenomenon. Dluzen and Ramirez (59) showed that progesterone immobilized on BSA was more potent than free progesterone in releasing dopamine from striatal slices. Why the conjugated steroid is more effective can only be a matter of speculation at this time since surface receptors represent a new class of steroid-binding molecules. Estrogen moieties packed onto a carrier molecule present a higher local concentration of estrogen to surface receptors, and a single BSA bearing many steroid molecules could activate several clustered surface receptors; clustering of receptors is important for action in some signal transduction systems (58). The covalent attachment of steroid to a large protein could also prevent the signal from

being processed away and thus circumvent subsequent down-regulation of the response. Finally, the 6-substituted  $E_2$  may be a more potent steroid at such a membrane binding site. Alternative ligand specificities for membrane steroid receptors have been demonstrated (39,45,60–62).

Trypsin digestion removes membrane ER labeling (Figure 4C) and therefore would be expected to affect a function mediated by this receptor. Figure 2 shows that trypsin treatment also vitiates the estrogen-induced PRL release; this result suggests that the portion of the mER corresponding to our antigenic peptide is on the outside of the cell. Also, because trypsin has no access to the intracellular receptors, its action on this antigen supports membrane localization. This action of trypsin also required us to seek other means of releasing substrate-attached cells for immunoselection techniques.

### Immunoselection by and Regulation of mER Expression

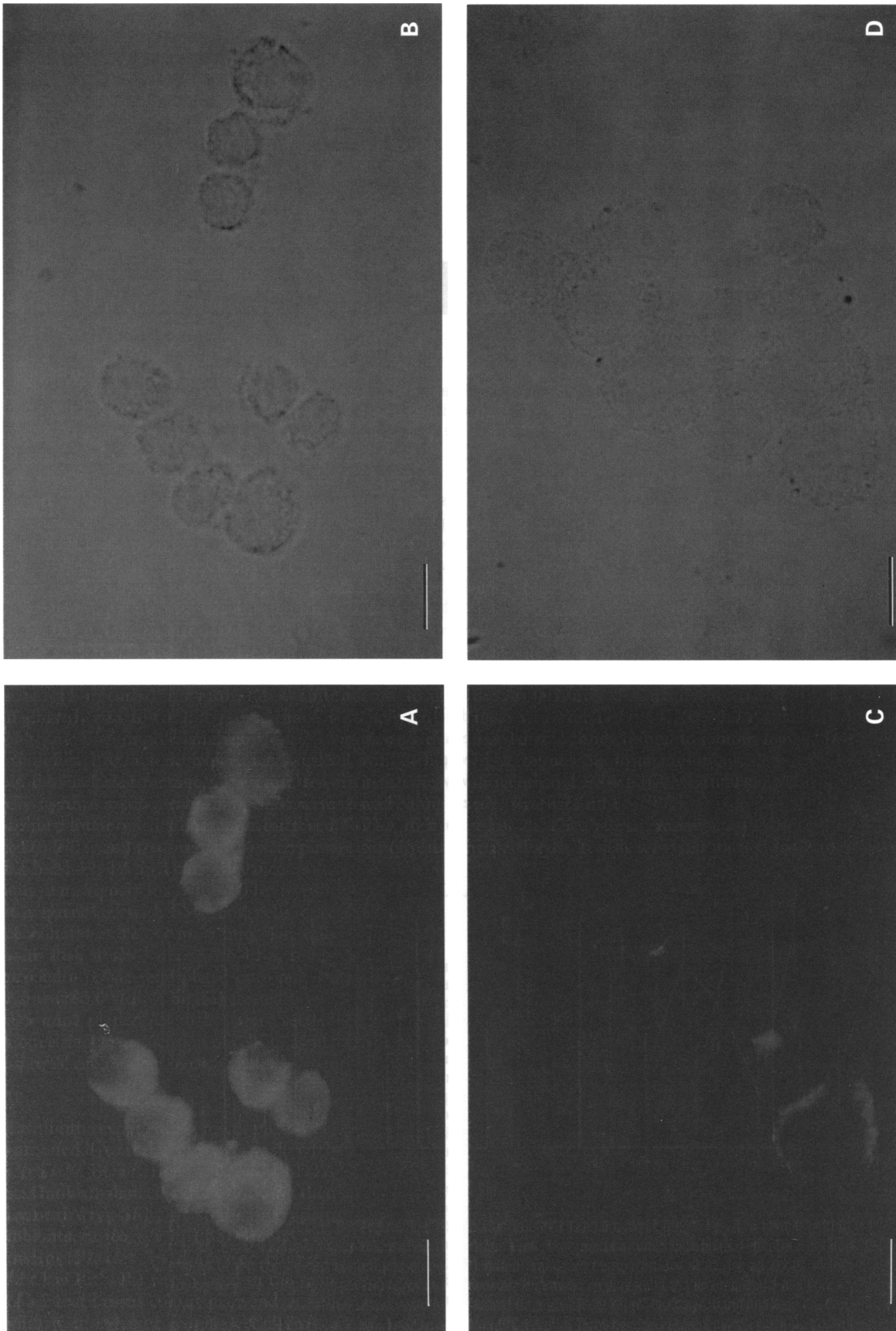
Since mER is only expressed on a relatively small percentage of the cell population, we sought a mechanism for purifying and propagating mER-bearing cells. Figure 7 shows the appearance of cells enriched or depleted for mER after a representative immunopanning experiment. In the experiment depicted in Figure 6, 98% of selected cells (all cells in the shown field) were enriched for carrying mER (Figure 6A); mER<sup>-</sup> cells were sparsely and weakly labeled (Figure 6B). In addition, the staining intensity of mER<sup>+</sup> cells is very strong. Figure 8A shows that the average numbers of cells immunoselected with either anti-ER Ab are about 80% positive for mER compared to about 20% very weakly positive cells in depleted fractions (refer to Figure 7 for staining intensity). This result assures us that the immunopanning protocols, which were previously designed and used for mGR-containing cells (43,45–47,63), are generally applicable to other plasma membrane-resident steroid receptors in other cell types.

Figure 8B shows that when cells are immunopanned (pooled results from both Abs) and immediately placed in either DM1 or SSM medium, the expression of mER 2 days later is dramatically different between the two groups. Immunoselection produced high numbers of positively labeled cells in DM1, while the cells plated in serum-containing medium show a very low level of mER staining, about the same as seen in mER<sup>-</sup> cells. This result could also explain why serum-grown cells do not

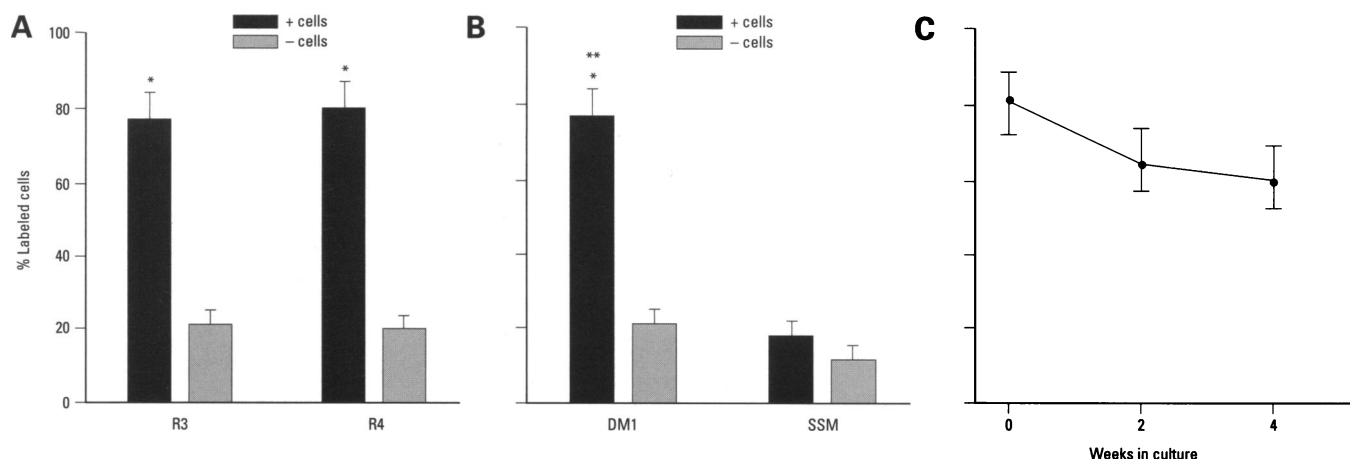
display an estrogen-mediated PRL release (48). Factors in serum (including estrogens) may affect mER expression and PRL release, thus masking the significant increase due to  $E_2$  treatment. Baseline PRL release in cells grown in serum-containing medium is very variable; in defined medium, however, significant  $17\beta$ - $E_2$  stimulation is always evident (48). There is also the possibility that factors in serum such as steroids may desensitize cells to rapid  $E_2$ -mediated effects by downregulating the amount of ER in the membrane. Dufy et al. (64) have shown that previous exposure to  $E_2$  (either  $17\alpha$  or  $17\beta$ ) suppresses further  $17\beta$ - $E_2$ -stimulated electrophysiological effects in GH<sub>3</sub>/B6 cells.

Figure 8C shows that immunoseparated mER<sup>+</sup> cells persist in culture over time and can therefore be propagated to obtain large numbers of cells for biochemical analysis. In this study, cells were immunopanned, propagated for the indicated amount of time in serum-containing medium, and then switched back to DM1 2 days before immunocytochemical analysis. A high percentage of cells still show labeling with ER Ab for at least 1 month. Although the trend is for slight loss of mER, the numbers are not significantly different from each other, and Gametchu and coworkers (unpublished observations) have retained mGR<sup>+</sup> cells for 2 years without reselecting (although some decline in mGR was observed). These data also demonstrate the reversibility of the serum effect on mER. In this way we are able to propagate cells in serum (in which they grow faster) but switch them back to defined media for 2 days before immunocytochemistry or other assays to obtain cells expressing mER.

Figure 3 shows that selection of cell populations with enhanced mER also selects cells with an increased ability to release PRL in response to  $E_2$ . Cells depleted for mER are no longer capable of this response. Our results indicate that a rapid effect of estrogen on PRL release in mER-enriched cells is evident by 5 min; we have shown significant responses in as little as 1 min (48). Dufy et al. (64) demonstrated electrophysiological effects of estrogen as early as 1 min in GH<sub>3</sub>/B6 cells, while Morley et al. (17) showed increases in intracellular calcium within 5 sec in granulosa cells. Previous studies on GH<sub>3</sub>/B6 cells reported PRL release in response to estrogen by 10 min (65). It is difficult to conceive that a genomic effect can increase secreted PRL in as rapidly as 1 to 5 min. However, it is known that in GC cells (another rat pituitary cell line),



**Figure 7.** Immunoselection of mER<sup>+</sup> cells and presence of mER on cells by ER Ab immunostaining. Cells were immunopanned with R3 Ab and then labeled with R3/antirabbit cy3. (A) mER<sup>+</sup> cells; (B) mER<sup>+</sup> cells. Phase micrographs (B) and (D) correspond to (A) and (C), respectively. Bars = 10 µm.



**Figure 8.** Immunoselection of mER<sup>+</sup> cells. (A) Effects of ER Ab (R3 and R4) used for selection. (B) Serum reduces the expression of mER to preselection levels (pooled results from cells labeled with either R3 or R4 Ab). (C) Immunoseparated mER<sup>+</sup> GH<sub>3</sub>/B6 cells persist over time in culture (pooled results from cells labeled with either R3 or R4 Ab). \*Significantly different from the paired control at the  $p < 0.05$  level. \*\*Significantly different from the three other groups at the  $p < 0.05$  level.

transcription of endogenous PRL mRNA is near a maximal rate at this time (66) although it is not known how soon this is available for translation. Maurer (67) has shown that in rat pituitaries, cytoplasmic PRL mRNA accumulation lags at least 6 hr behind increases in nuclear mRNA for PRL. Therefore, the rapidity of our effects on cells enriched for mER further support a nongenomic mechanism for this response.

## Summary

We believe that these data favor a membrane site of action for estrogen in eliciting the release of PRL from pituitary tumor cells. The effect is rapid and specific, and

takes place when ligand does not have access to the intracellular space. The immunological identification of a membrane-associated ER suggests that this protein may be mediating rapid PRL release in GH<sub>3</sub>/B6 cells. We correlated the quantity of this antigen on mER-selected GH<sub>3</sub>/B6 cell populations with the ability of 17 $\beta$ -E<sub>2</sub> to increase PRL secretion in these cells.

Alternative protein domain function for steroid receptors residing in different cellular locations may have relevance to the pleiotropic expression of receptor mutations and the differential activity of steroids in different tissues or different diseases of reproduction, metabolism, and estrogen

toxicity. While several systems have been proposed for assessing the genomic actions of xenobiotic estrogens (1), a convenient screening test for nongenomic estrogenic actions is presently lacking. Further characterization of our model for rapid estrogen-induced PRL release from mER-enriched GH<sub>3</sub>/B6 cells could make it a system suitable for such screening. It will be important to consider such a model system to study the mechanisms of action of estrogenic compounds among the wide variety of environmental compounds to which we are exposed.

## REFERENCES

- McLachlan JA. Functional toxicology: a new approach to detect biologically active xenobiotics. *Environ Health Perspect* 101:386–387 (1993).
- Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ Health Perspect* 101:378–384 (1993).
- Zhu BT, Liehr JG. Inhibition of the catechol-*O*-methyltransferase-catalyzed *O*-methylation of 2- and 4-hydroxyestradiol by catecholamine: implications for the mechanism of estrogen-induced carcinogenesis. *Arch Biochem Biophys* 304:248–256 (1993).
- Ashburn SP, Han X, Liehr JG. Microsomal hydroxylation of 2- and 4-fluoroestradiol to catechol metabolites and their conversion to methyl ethers: catechol estrogens as possible mediators of hormonal carcinogenesis. *Mol Pharmacol* 43:534–541 (1993).
- Piroli G, Pignataro O, De Nicola AF. Increased activity of type 1 regulatory subunit of cyclic adenosine 3'5'-monophosphate-dependent protein kinase in estrogen-induced pituitary tumors. *J Natl Cancer Inst* 84:1565–1571 (1994).
- Thomas ML, Xu X, Norfleet AM, Watson CS. The presence of functional estrogen receptors in intestinal epithelial cells. *Endocrinology* 132:426–430 (1993).
- Raloff J. The gender benders: Are environmental "hormones" emasculating wildlife? *Sci News* 145:24–27 (1994).
- Clark JH, Peck EJ Jr. Steroid receptor characterization and measurement. In: *Female Sex Steroids—Monographs in Endocrinology*. New York:Springer-Verlag, 1980;4–36.
- Goldberger RF, Deeley RG. The effect of estrogen on gene expression in avian liver. In: *Gene Regulation by Steroid Hormones*. New York:Springer-Verlag, 1980;32.
- Clark JH, Peck EJ Jr. Nuclear binding and biologic response-interrelationships between early and late uterotrophic responses. In: *Female Sex Steroids—Monographs of Endocrinology*. New York:Springer-Verlag, 1980;88–91.
- Szego CM. Parallels in the modes of action of peptide and steroid hormones: membrane effects and cellular entry. In: *Structure and Function of the Gonadotropins* (Kearns KW, ed). New York:Plenum Publishing, 1978;431–472.
- Adamson ED, Woodland HR. Changes in the rate of histone synthesis during oocyte maturation and very early development of *Xenopus laevis*. *Dev Biol* 57:136–149 (1977).
- Benbow RN. Appearance of DNA polymerase activities during



- early development of *Xenopus laevis*. Dev Biol 43:159–174 (1975).
14. Schorderet-Slatkine S. Discussion paper: induction by progesterone and a "maturation-promoting factor" of soluble proteins in *Xenopus laevis* oocytes *in vitro*. Ann N Y Acad Sci 286:421–433 (1977).
15. Kelly MJ, Moss RL, Dudley CA, Fawcett CP. The specificity of the response of preoptic-septal area neurons to estrogen: 17 $\alpha$ -estradiol versus 17 $\beta$ -estradiol and the response of extrahypothalamic neurons. Exp Brain Res 30:43–52 (1977).
16. Nabekura J, Oomura Y, Minami T, Mizuno Y, Fukuda A. Mechanism of the rapid effect of 17 $\beta$ -estradiol on medial amygdala neurons. Science 233:226–228 (1986).
17. Morley P, Whitfield JF, Vanderhyden BC, Tsang BK, Schwartz J. A new, nongenomic estrogen action: the rapid release of intracellular calcium. Endocrinology 131:1305–1312 (1992).
18. Lieberherr M, Grosse B, Kachkache M, Balsan S. Cell signaling and estrogen in female rat osteoblasts: a possible involvement of unconventional nonnuclear receptors. J Bone Miner Res 8:1365–1376 (1993).
19. Dufy B, Partouche C, Poulain D, Dufy-Barbe L, Vincent JD. Effects of estrogen on the electrical activity of identified and unidentified hypothalamic units. Neuroendocrinology 22:38–47 (1976).
20. Kelly MJ, Moss RL, Dudley CA. The effects of microelectrophoretically applied estrogen, cortisol and acetylcholine on medial preoptic-septal unit activity throughout the estrous cycle of the female rat. Exp Brain Res 30:53–64 (1977).
21. Smith SS, Woodward DJ, Chapin JK. Sex steroids modulate motor-correlated increases in cerebellar discharge. Brain Res 476:307–316 (1989).
22. Chen YZ, Hua SY, Wang CA, Wu LG, Gu Q, Xing BR. An electrophysiological study on the membrane receptor-mediated action of glucocorticoids in mammalian neurons. Neuroendocrinology 53(Suppl 1):25–30 (1991).
23. Wong M, Moss RL. Long-term and short-term electrophysiological effects of estrogen on the synaptic properties of hippocampal CA1 neurons. J Neurosci 12:3217–3225 (1992).
24. Touchette N. Man bites dogma: a new role for steroid hormones. J NIH Res 2:71–74 (1990).
25. Barsony J, Hannaway GW, Aurbach GD, Marx SJ. Analysis of rapid hormone dependent intracellular movement of vitamin D receptors [abstract]. J Cell Biol 111:343a (1990).
26. Farach-Carson MC, Sergeev I, Norman AW. Stimulation of transmembrane Ca<sup>++</sup> influx in osteosarcoma cells by vitamin D3 analogs/metabolites [abstract]. J Cell Biol 111:343a (1990).
27. McKoy W, Barsony J, Brown TR, Marx SJ. Androgen-induced rapid rise of cGMP is receptor mediated [abstract]. J Cell Biol 111:345a (1990).
28. Koch B, Lutz-Bucher B, Briaud B, Mialhe C. Specific interaction of corticosteroids with binding sites in the plasma membranes of the rat anterior pituitary gland. J Endocrinol 79:215–222 (1978).
29. Orchinik M, Murray TF, Moore FL. A corticosteroid receptor in neuronal membranes. Science 252:1848–1851 (1991).
30. Orchinik M, Murray TF, Franklin PH, Moore FL. Guanyl nucleotides modulate binding to steroid receptors in neuronal membranes. Proc Natl Acad Sci USA 89:3830–3834 (1992).
31. Sachs BD, Leipheimer RE. Rapid effect of testosterone on striated muscle activity in rats. Neuroendocrinology 48:453–458 (1988).
32. Schmidt TJ, Husted RF, Stokes JB. Steroid hormone stimulation of Na<sup>+</sup> transport in A6 cells is mediated via glucocorticoid receptors. Am J Physiol Cell Physiol 264:C875–C884 (1993).
33. Tamarappoo BK, Nam M, Kilberg MS, Welbourne TC. Glucocorticoid regulation of splanchnic glutamine, alanine, glutamate, ammonia, and glutathione fluxes. Am J Physiol Endocrinol Metab 264:E526–E533 (1993).
34. Blaustein JD, Lehman MN, Turcotte JC, Greene G. Estrogen receptors in dendrites and axon terminals in the guinea pig hypothalamus. Endocrinology 131:281–290 (1992).
35. Wehling M, Eisen C, Christ M. Membrane receptors for aldosterone: a new concept of nongenomic mineralocorticoid action. NIPS 8:241–244 (1993).
36. Pietras RJ, Szego CM. Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. Nature 265:69–72 (1977).
37. Pietras RJ, Szego CM. Partial purification and characterization of estrogen receptors in subfractions of hepatocyte plasma membranes. Biochem J 191:743–760 (1980).
38. Bression D, Michard M, Le Dafniet M, Pagesy P, Peillon F. Evidence for a specific estradiol binding site on rat pituitary membranes. Endocrinology 119:1048–1051 (1986).
39. Towle AC, Sze PY. Steroid binding to synaptic plasma membrane: differential binding of glucocorticoids and gonadal steroids. J Steroid Biochem 18:135–143 (1983).
40. Bression D, Brandi AM. *In vitro* and *in vivo* antagonistic regulation by estradiol and progesterone of the rat pituitary dopamine binding sites: correlation with ovarian steroid regulation of the dopaminergic inhibition of prolactin secretion *in vitro*. Endocrinology 116:1905 (1985).
41. Wehling M, Eisen C, Christ M, Theisen K. Membrane receptors for aldosterone in human lymphocytes: a 50 kd protein on SDS-PAGE [abstract]. 75th Endocrine Society Meeting 9–12 June 1993, Las Vegas, Nevada. 1993;290.
42. Tischkau SA, Ramirez VD. A specific membrane binding protein for progesterone in rat brain: sex differences and induction by estrogen. Proc Natl Acad Sci USA 90:1285–1289 (1993).
43. Gametchu B. Glucocorticoid receptor-like antigen in lymphoma cell membranes: correlation to cell lysis. Science 236:456–461 (1987).
44. Gametchu B, Watson CS, Garza RE. Membrane glucocorticoid receptor in lymphocytolysis. SAAS Bull Biochem Biotech 1:41–45 (1988).
45. Gametchu B, Watson CS, Pasko D. Size and steroid binding characterization of a membrane-associated glucocorticoid receptor in S-49 lymphoma cells. Steroids 56:402–410 (1991).
46. Gametchu B, Watson C, Shih C, Dashew B. Studies on the arrangement of glucocorticoid receptors in the plasma membrane of S-49 lymphoma cells. Steroids 56:411–419 (1991).
47. Gametchu B, Watson CS, Wu S. Use of receptor antibodies to demonstrate membrane glucocorticoid receptor in cells from human leukemic patients. FASEB J 7:1283–1293 (1993).
48. Pappas TC, Gametchu B, Yannariello-Brown J, Collins TJ, Watson CS. Membrane estrogen receptors in GH3/B6 cells are associated with rapid estrogen-induced release of prolactin. Endocrine 2:813–822 (1994).
49. Gametchu B, Watson CS, Pasko D. The membrane glucocorticoid receptor of S-49 lymphoma cells contains a functional DNA-binding region [abstract]. Keystone Meetings on Steroid/Thyroid Receptor Gene Superfamily 21–27 February 1992, Tamaron, Colorado. 1992;49.
50. Caron-Leslie L-AM, Schwartzman RA, Gaido ML, Compton MM, Cidlowski JA. Identification and characterization of glucocorticoid-regulated nuclease(s) in lymphoid cells undergoing apoptosis. J Steroid Biochem Mol Biol 40:661–671 (1991).
51. Hayashi I, Sato GH. Replacement of serum by hormones permits growth of cells in a defined medium. Nature 259:132–134 (1976).
52. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci USA 83:2496–2500 (1986).
53. de Carvalho-Brunet N, Picart R, Tixier-Vidal A. 17 $\beta$ -Estradiol regulates prolactin secretion but not cell proliferation of GH3/B6 cells in chemically defined medium. Mol Cell Endocrinol 39:49–60 (1985).
54. Parkening TA, Collins TJ, Smith ER. A comparative study of prolactin levels in five species of aged female laboratory rodents. Biol Reprod 22:513–518 (1980).
55. Zar JH. Biostatistical Analysis. 2nd ed. Englewood Cliffs, NJ:Prentice-Hall, 1984.
56. Taylor RB, Duffus WP, Raff MC, de Petris S. Redistribution

- and pinocytosis of lymphocyte surface immunoglobulin molecules by anti-immunoglobulin antibody. *Nature New Biol* 233:225–229 (1971).
57. Bourguignon LYW, Bourguignon GJ. Capping and the cytoskeleton. *Int Rev Cytol* 87:195–224 (1984).
  58. Cadena DH, Gill GN. Receptor tyrosine kinases. *FASEB J* 6:2332–2337 (1992).
  59. Dluzen DE, Ramirez VD. Progesterone effects upon dopamine release from the corpus striatum of female rats. II: Evidence for a membrane site of action and the role of albumin. *Brain Res* 476:338–344 (1989).
  60. Smith LD, Ecker RE. The interaction of steroids with *Rana pipiens* oocytes in the induction of maturation. *Dev Biol* 25:232–247 (1971).
  61. Sadler SE, Bower MA, Maller JL. Studies of a plasma membrane steroid receptor in *Xenopus* oocytes using the synthetic progestin RU 486. *J Steroid Biochem* 22:419–426 (1985).
  62. Patino R, Thomas P. Characterization of membrane receptor activity for  $17\alpha,20\beta,21$ -trihydroxy-4-pregnen-3-one in ovaries of spotted seatrout (*Cynoscion nebulosus*). *Gen Comp Endocrinol* 78:204–217 (1990).
  63. Gametchu B, Watson CS, Shih CY. An S-49 cell line with a modified glucocorticoid receptor is depleted of membrane-associated glucocorticoid receptor and deficient in the lymphocytolytic response. *Endocr J* 2:429–437 (1994).
  64. Dufy B, Vincent J-D, Fleury H, Pasquier PD, Gourdji D, Vidal AT. Membrane effects of thyrotropin-releasing hormone and estrogen shown by intracellular recording from pituitary cells. *Science* 204:509–511 (1979).
  65. Zyzek E, Dufy-Barbe L, Dufy B, Vincent JD. Short-term effect of estrogen on release of prolactin by pituitary cells in culture. *Biochem Biophys Res Commun* 102:1151–1157 (1981).
  66. Waterman ML, Adler SA, Nelson C, Greene GL, Evans RM, Rosenfeld MG. A single domain of the estrogen receptor confers deoxyribonucleic acid binding and transcriptional activation of the prolactin gene. *Mol Endocrinol* 2:14–21 (1988).
  67. Maurer RA. Estradiol regulates the transcription of the prolactin gene. *J Biol Chem* 257:2133–2136 (1982).