Endocrine (1995) 3, 743-749

Membrane estrogen receptor-enriched GH_3/B6 cells have an enhanced non-genomic response to estrogen

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We immunoselected GH_3/B6 cells for a membrane estrogen receptor (mER) using antibodies generated against the rat intracellular ER (iER). Immunocytochemistry with anti-ER antibodies revealed bright fluorescence distributed in patches over the surface of mER-enriched cells, while cells immuno-depleted for mER showed only low-level membrane immunofluorescence. Quantitation via digital image analysis confirmed that immuno-enriched populations show increases in both stained cell numbers and intensity of staining. Short-term culturing with serum reversibly decreased the intensity of immunostaining in mER-enriched cells to immuno-depleted cell levels. The mER-enriched populations initially contained ~85% immunopositive cells in defined medium, but when cultured continuously with serum gradually decline to ~22% immunopositive cells by 10 weeks. Cells enriched for mER showed a significant increase in rapid (after 2 or 5 min) prolactin release when treated with 17β-estradiol, while mER-depleted cells lacked this response. Immunoprecipitable membrane proteins isolated from mER-enriched cells were 60,000, 74,000 and ~200,000 MW, compared to an iER size of 67,000. Therefore, the presence and level of an mER that is antigenically related to iER is correlated with the ability of GH_3/B6 cells to mediate a rapid action of estrogen.

Keywords: membrane; estrogen receptor; non-genomic; prolactin; GH_3/B6 cell

Introduction

Biochemical (Pietras & Szego, 1977, 1979a, 1980; Towle & Sze, 1983; Bression *et al.*, 1986) and immunological (Gametchu, 1987; Gametchu *et al.*, 1991a,b, 1993; Pappas *et al.*, 1994, 1995) data support the hypothesis that membrane-associated steroid receptors, similar to the well-characterized intracellular receptors, are present in some cells and mediate non-genomic steroid actions. Rapid and transient responses to estrogen – indicative of the non-genomic actions of this steroid – have been characterized in a number of tissues and cell types (Dufy *et al.*, 1976, 1979; Kelly *et al.*, 1977; Zyzek *et al.*, 1981; Nabekura *et al.*, 1986; Morley *et al.*, 1992; Wong & Moss, 1992; Lieberherr *et al.*, 1993) and some investigations link these responses to the plasma membrane. Estradiol (E_2) has been shown to be effective even when immobilized on bovine serum albumin (BSA) or solid supports that block estrogen's access to the intracellular compartment. E_2 immobilized on BSA (E_2-BSA) shows E_2-specific binding on the surface of MCF-7 (Berthois *et al.*, 1986) and ZR-75-1 breast cancer cells (Nenci *et al.*, 1980) and also mediates rapid surface morphology changes in MCF-7 cells (Pourreau-Schneider *et al.*, 1986). Osteoblasts show rapid intracellular calcium mobilization with both free estrogen and E_2-BSA (Lieberherr *et al.*, 1993). 17β-E_2 bound to derivatized nylon fibers can both bind and activate cells (Pietras & Szego, 1977, 1980). These nylon fibers have also been used to select from heterogeneous liver cell preparations a population of cells responsive to membrane models of estrogen action (Pietras & Szego, 1979a).

In a rat pituitary tumor cell line (GH_3/B6), E_2 mediates rapid electrophysiological changes (Dufy *et al.*, 1979) and prolactin (PRL) release (Zyzek *et al.*, 1981; Pappas *et al.*, 1994). Dufy *et al.*, showed that although both application of E_2 doubled the number of spontaneously active cells within an hour (from 28% to 49%), only about 15% of the cells showed rapid (1-2 min) action potentials following direct application of E_2 (Dufy *et al.*, 1979). Likewise, PRL release studies showed only a relatively modest change in PRL levels in GH_3/B6 cells treated 10 min with E_2 (Zyzek *et al.*, 1981). These data suggest that the cultured cell population may be heterogeneous with respect to expression of a cell surface estrogen receptor (ER); not all of the cells may be expressing the surface receptor, and unknown factors may reduce the amount of surface receptor expressed. If a membrane estrogen-responsive protein mediates these actions, then selecting cells for expression of this protein should increase the magnitude of these responses and the percentage of responsive cells in the population.

Antibody-based cell separation techniques, utilizing antibodies directed to cell surface epitopes, have been employed to separate cells with distinct developmental and functional properties (Dannies, 1985; Gametchu, 1987; Warrington *et al.*, 1992; Turksen & Aubin, 1994). Gametchu *et al.*, used this strategy for enrichment of cells bearing membrane glucocorticoid receptor (mGR), and subsequently characterized some functional and structural properties of this protein (Gametchu, 1987; Gametchu *et al.*, 1991a,b, 1993). Using an anti-peptide antibody to the intracellular ER (iER) for immunocytochemistry and confocal laser microscopy, we recently identified a membrane estrogen receptor (mER) present on 8-17% of unselected GH_3/B6 cells (Pappas *et al.*, 1994, 1995). This receptor is antigenically very similar to the intracellular ER (cross-reactive at four different epitopes) and fluorescent E_2-BSA binding colocalizes on the cell surface with anti-ER staining (Pappas *et al.*, 1995). We have also demonstrated that these cells rapidly (1-5 min) release PRL in response to E_2 and that clonal descendants of mER-bearing cells show an enhanced estrogenic response (Pappas *et al.*, 1994). We now report an immunological enrichment that (1) confirms the surface location and accessibility of the membrane ER, (2) produces a population of cells expressing mER to facilitate further biochemical characterization and (3) further establishes a link between expression of this receptor and the magnitude of the E_2-mediated PRL release-response.

Results

Immunoseparation

Immunopanning produced populations in which a greater percentage of cells expressed the membrane ER antigen with greater intensity than in mER-depleted (Figures 1 and 2) or

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Received 4 May 1995; accepted 11 July 1995

unfractionated cells (Pappas *et al.*, 1994). Figures 1a and c show fluorescence micrographs of cells from the enriched populations 2 days after immunoseparation with either affinity-purified R3 or R4 anti-ER antibody and growth in a defined medium (DM1). Staining in mER-enriched cells was bright and, in many cases, distributed over much of the cell surface. GH_3 cells tend to have a prominent nucleus which causes this area of the cell to be out of focus with respect to the surface over the cytoplasm (Pappas *et al.*, 1995), and thus the staining observed there is less intense. Figure 1e and g show immunofluorescence of cells from the mER-depleted populations. These cells showed only very low level staining, barely visible at the equal exposure times chosen for all of these photomicrographs. Cells immunoseparated and stained with antibodies R3 and R4 showed similar numbers of stained cells.

Since cell staining varied with both individual cell intensity and distribution of staining in the cell population, we used a method that combines both of these measurements. The quantitation of these coupled parameters comprises of labeling intensity measured by digital image analysis. This mER immunoreactivity for enriched and depleted populations is summarized in Figure 2. Two days after immunoseparation with either antibody and growth in DM1, depleted cells

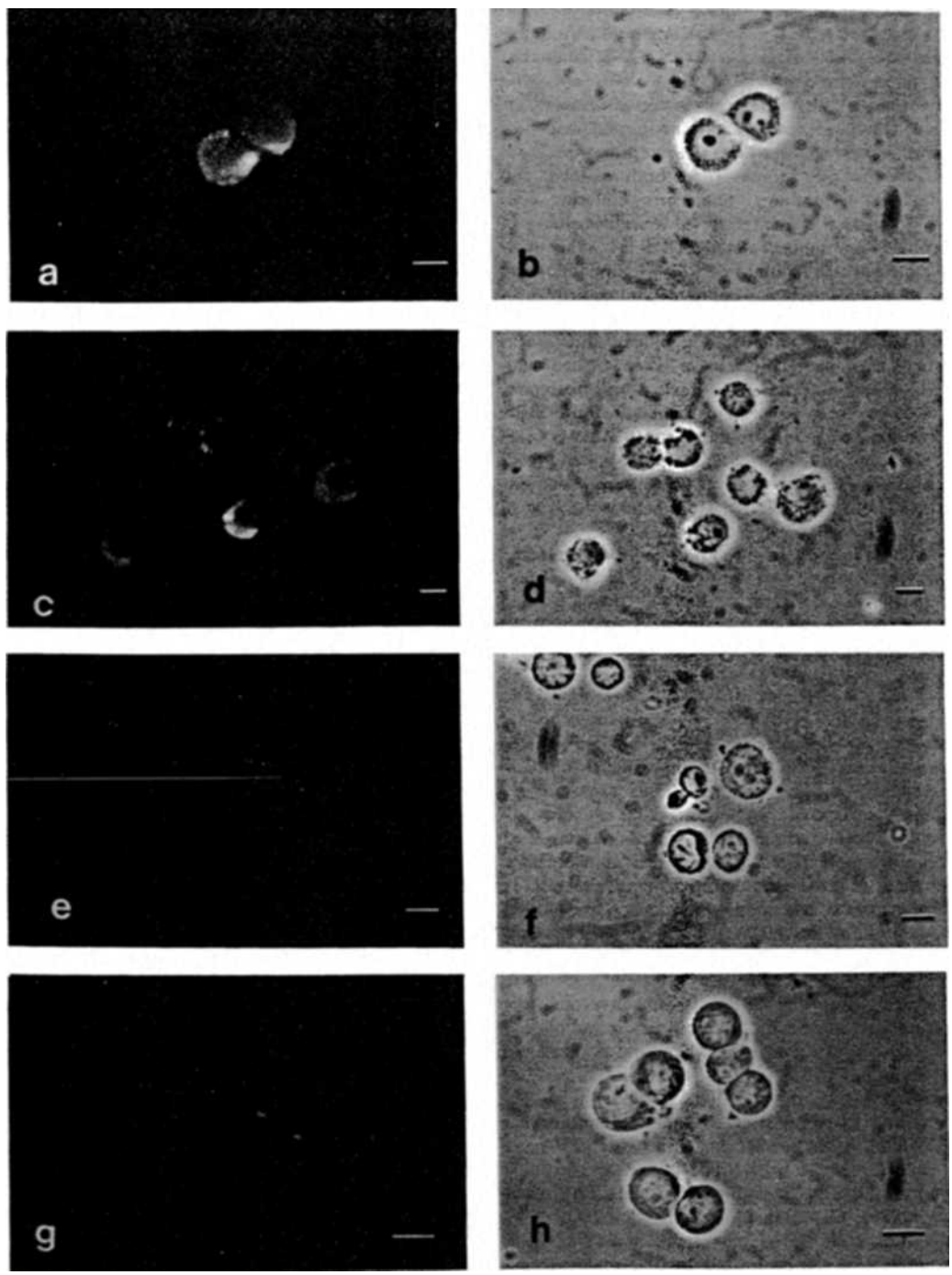

Figure 1 Immunocytochemistry of immunoseparated cells. Cells were immunoseparated and then stained with the same anti-ER antibodies. (a and c) show labeling in cells enriched for mER using antibody R3 and R4, respectively. (b and d) are phase photomicrographs of (a and c), respectively. Representative labeling intensity of depleted cell populations is shown for R3 (e) and R4 (g). (f and h) are the phase photomicrographs of (e and g) respectively. Bar = 10 μm; all photos were taken at the same exposure settings

showed much lower overall intensity of immunostaining on their surface than did enriched cells. The R4 antibody gave brighter labeling in the depleted fraction than did R3 by this method. Because some low level (background) of fluorescent antibody labeling is due to non-specific sticking of protein to cells, we measured labeling intensity due to application of secondary antibody alone. The average background contribution over all experiments in which secondary antibody alone was used is 30 intensity units. This level of nonspecific labeling is shown by a dotted line in Figures 2 and 3.

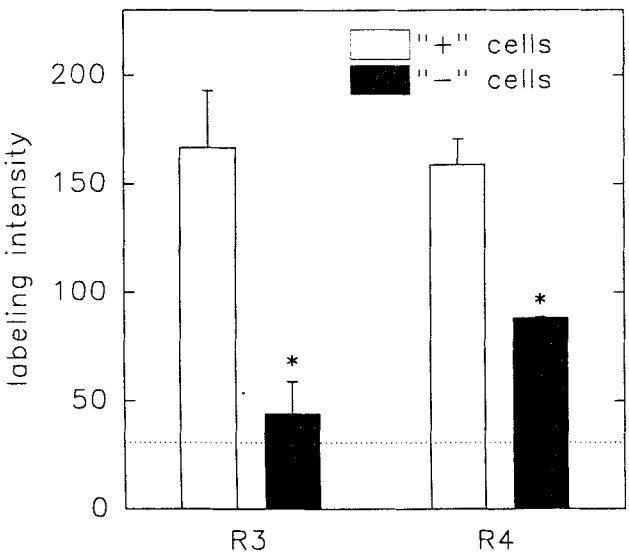

Figure 2 Immunoseparation of GH_3/B6 cells results in populations with different intensities of staining. Labeling intensity is measured in arbitrary O.D. units based on a 255 tone gray scale, where the value of O is completely black and 255 is completely white. The dotted line shows the intensity due to staining with secondary antibody alone. Data are the results of four immunoseparations; at least four fields of cells were counted for each determination, and error bars are standard error of the mean. R3 = cells separated with and then stained with the R3 antibody; R4 = cells separated with and then stained with the R4 antibody.* − significantly different from labeling intensity of '+' cells enriched with the same antibody

Some factors in serum may affect the expression of mER. Figure 3 shows that culturing mER-enriched cells for 2 days in serum-supplemented medium (SSM) as opposed to serumless medium (DM1) reduced the intensity of mER labeling to approximately the level seen in the mER-depleted cell fraction. No significant decrease in labeling was seen when mER-depleted cells were cultured with serum. Figure 4 shows the effect of long-term culture in SSM on expression of mER in immunoseparated cells. Selected cells were cultured in SSM for various times and then switched to serumless medium 2 days before immunocytochemistry. There was a progressive slow decline in the number of cells expressing the membrane antigen, though throughout the time surveyed, individual cells of the enriched population still expressed more antigen than those of the depleted population. Because long-term culture of cells between immunocytochemical measurement time points was done with serum-containing media, the combined results from Figures 3 and 4 suggest that short-term serum exposure does not permanently suppress the expression of mER. The suppressive effects of serum were acutely reversible for several weeks.

Prolactin release

Figure 5 shows PRL release upon E_2 treatment of cells that have undergone 2–3 rounds of immunoseparation. These studies were done between 6 and 12 weeks after the last immunoseparation procedure. Cells enriched for mER showed a significant E_2-stimulated PRL release after both 2 and 5 min exposures to the steroid. Depleted cell populations do not show significant E_2-stimulated release over controls.

mER protein

Since enrichment for cell populations containing higher mER levels were possible, we suspected that partial purification of

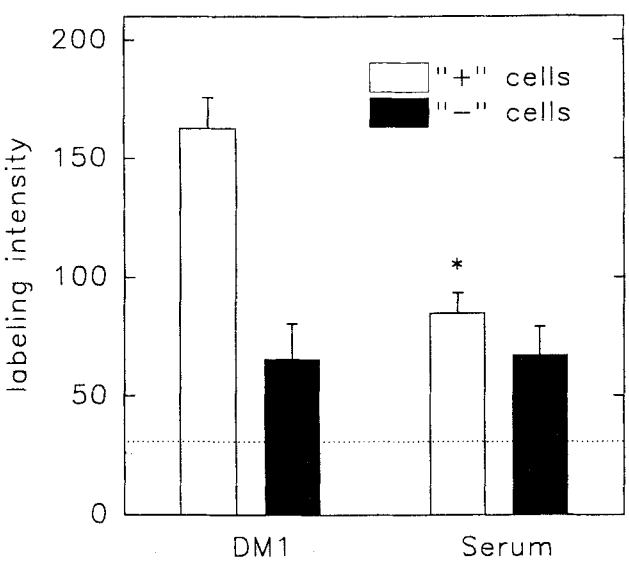

Figure 3 Effect of culture in serum-containing medium on expression of mER. Cells were plated either in defined medium (DM1) or RPMI 1640 with 5% defined/supplemented calf serum (Serum) immediately after immunoseparation. The effects of serum on intensity of labeling are summarized from three experiments with each of the two anti-ER antibodies (R3 and R4 for which the data is pooled). Labeling intensity is as described for Figure 2; dotted line as in Figure 2. Error bars are standard error of the mean. * − significantly different from '+' cells cultured in DM1

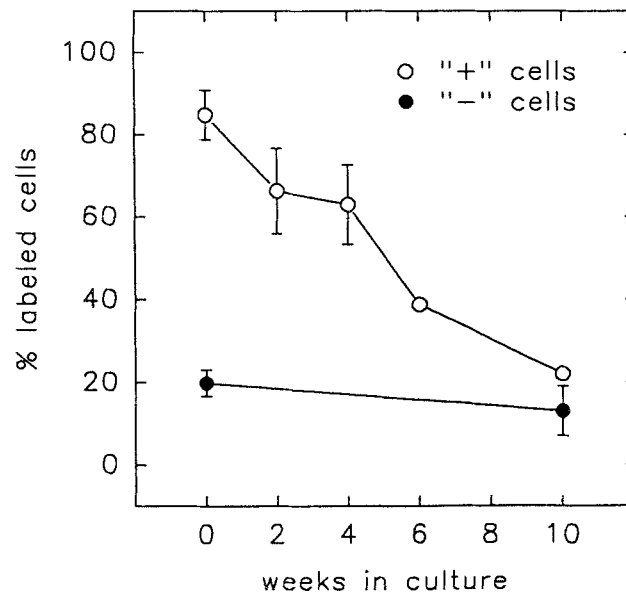

Figure 4 Effects of long-term culture in serum on expression of mER. Cells were cultured in SSM for the number of weeks indicated on the abscissa, then passed to coverslips and cultured for 2 days in DM1 prior to immunocytochemistry. Data are from three experiments for 6 and fewer weeks; for '+' cells there is a single point at 10 weeks and for '−' cells there are 2 data points at 10 weeks. Error bars are standard error of the mean. Errors for week 6 '+' cells were too small to appear on the graph

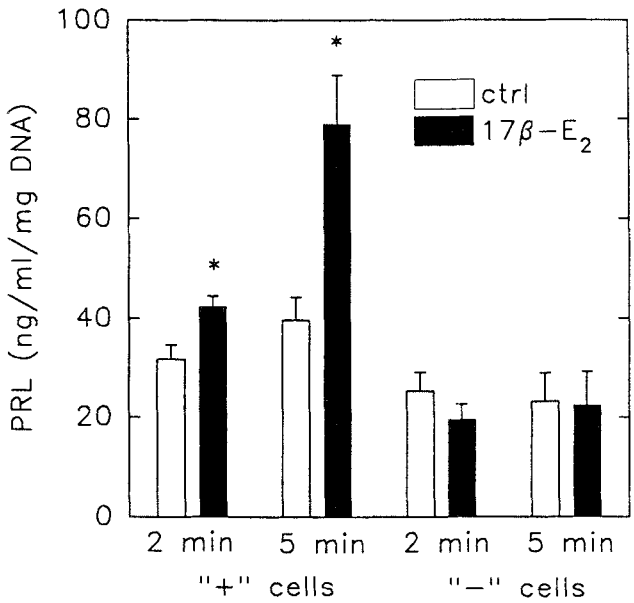

Figure 5 PRL release in response to 10 nM E_2 in immunoseparated cell populations. The '+' cells are mER-enriched; '−' cells are mER-depleted. Data are from three independent experiments; error bars are the standard error of the mean. ctrl = control (ethanol vehicle). *− significantly different than the equivalent time control

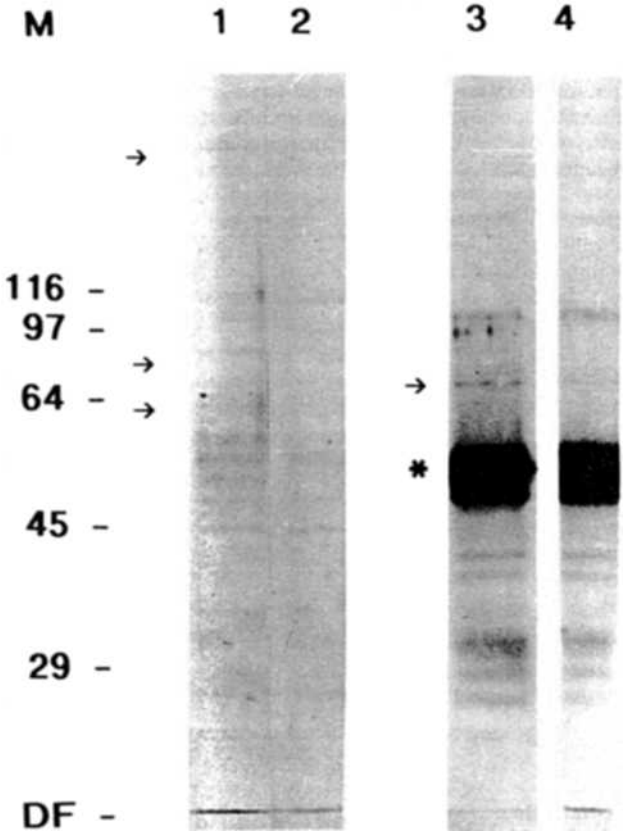

Figure 6 SDS–PAGE analysis of anti-ER immunoprecipitated intracellular proteins and biotinylated membrane proteins, visualized with streptavidin-HRP. Membrane proteins were biotinylated on whole cells, detergent-extracted and then immunoprecipitated in the absence (lane 1) or presence (lane 2) of synthetic peptide used to generate the ER antibody (50 μg/ml). As a positive control for immunoprecipitation and size, cytosolic proteins were incubated with antibody in the absence (lane 3) or presence (lane 4) of synthetic peptide, transferred to nitrocellulose and immunoblotted with anti-ER antibody R3. Epitope-competed (ER antibody-specific) bands for both membrane and cytoplasmic proteins are marked with arrows. Size of MW markers ($\times 10^{-3}$) are shown under the lane marked M; *− indicates position of IgG heavy chain

mER was now feasible. We biotinylated live cells, to label only membrane proteins, and then used immunoprecipitation to identify ERs in the population of membrane proteins. Figure 6 (lane 1) shows several biotin-labeled polypeptides that were immunoprecipitated from detergent-extracted GH_3/B6 membranes using the R3 anti-ER antibody. Lane 2 shows that pre-absorption with the synthetic peptide used to generate the antibody (50 μg/ml) blocks the immunoprecipitation of several proteins. Therefore, peptides of ~200,000, 74,000 and 60,000 MW (arrows) are specifically recognized by anti-ER antibody. For comparison, the same R3 antibody was also capable of immunoprecipitating the 67,000 MW cytosolic iER (lane 3). The specificity of this band was again shown by its absence from immunoprecipitated proteins when antibody was pre-absorbed with ER-specific peptide (lane 4).

Discussion

We have previously shown that unselected GH_3/B6 cells rapidly release prolactin in response to low doses of E_2 and we have also reported that a subset (~13%) of these cells show an ER antigen on their membrane surfaces (Pappas *et al.*, 1994). The data presented here suggest that ER exposed on the cell surface allows binding to a substrate-attached antibody, enabling us to select populations of cells with higher mER expression. These enriched cells show a rapid and robust E_2-stimulated PRL release, whereas depleted cells show no E_2 stimulation of this release. Thus, the presence of mER is directly correlated to a rapid effect of E_2.

GH_3/B6 cells, though clonal, are heterogeneous. Only about 15% show rapid electrophysiological effects of E_2 (Dufy *et al.*, 1979), and, in the parent GH_3 cell line, only two-thirds show storage of immunologically detectable PRL (Gautvik & Fossum, 1976). Subclones of GH_3/B6 are morphologically heterogeneous, have different basal and E_2-stimulated PRL release responses and show differing levels of mER expression (Pappas *et al.*, 1994). It is possible that a number of cell types have arisen through long-term culture and passage of the parent population, accounting for the heterogeneity seen is the result of 'environmental' factors, such as the presence of growth or differentiation factors (including steroids) in the media, or heterogeneity of cell-cycle stage. Cell-cycle synchronization can produce populations of cells that have differing PRL release characteristics and cells in the G_2/M phase have higher TRH-stimulated PRL release (Denechaud *et al.*, 1987).

The presence of serum decreased both the intensity and the percentage of mER-positive cells in enriched populations. We have shown previously that cells grown in serum-containing media do not demonstrate a rapid (1 min) E_2-stimulated prolactin release (Pappas *et al.*, 1994). Taken together, these data suggest that factors in serum affect expression of mER and its putative function. Pietras and Szego have shown that treating hepatocytes with 20 nM E_2 decrease availability of surface E_2 binding sites (Pietras & Szego, 1979b) which could be due to either filling of the sites so that labeled estradiol did not bind or to down regulation of this receptor. Likewise, it has been demonstrated that E_2-dependent electrophysiological effects in GH_3/B6 cells are desensitized with prior exposure to 1–100 nM E_2 (Dufy *et al.*, 1979). Horse serum can contain nM quantities of estrogens and defined supplemented calf serum contains pM quantities of estrogens (personal communication, Don Young, HyClone). However, we have previously used 10 pM E_2 to maintain gene expres-

sion of prolactin while still eliciting the secretory response and maintaining the ability to detect mER immunocytochemically (Pappas *et al.*, 1994). Therefore, the serum component(s) that down regulate mER could be estrogens or other steroids in physiological concentrations or some other factors.

The ability of immunoselected cells to maintain elevated mER levels allows us to propagate quantities of cells sufficient to do a variety of structural and functional studies. However, time course studies suggest that the mER-enriched populations slowly lose mER-expressing cells. After more than 1 month in serum-containing media, the percentage of cells showing immunologically detectable antigen on their surface decreases to about 39% and at 10 weeks almost returns to the mER-depleted cell level. However, this measurement of cell percentages does not take into consideration the signal brightness of individual cells, which we found to remain higher in positive cells of the mER selected cell cultures even at these later stages after selection. It is possible that long-term culture in serum may down-regulate mER expression or alter the makeup of the population. Growth or differentiation factors present in serum may switch off pathways leading to mER expression.

Cells enriched for mER show an increased magnitude of E_2-stimulated PRL release, while the mER-depleted cells show no measurable increase of PRL release over baseline. Our previous studies (Pappas *et al.*, 1994) showed that in unselected cells, a 5 min exposure to 10 nM E_2 causes a 40% increase in PRL accumulation in the media over basal levels. Immuno-enriched cells show a 100% increase in PRL secretion with E_2 treatment, while immuno-depleted cells have lost this response entirely. These data suggest a direct correlation of the presence of mER with the rapid E_2-mediated effect, and lend strong support to the hypothesis that mER mediates these effects. Consistent with these data is the fact that subclones of GH_3/B6 that show high levels of mER expression demonstrate a large E_2-stimulated PRL release, while a clone having no mER showed no estrogen stimulation of release (Pappas *et al.*, 1994). This result is similar to the finding of Gametchu (1987), who was able to show enhanced glucocorticoid-mediated lymphocytolysis in S49 mouse lymphoma cells enriched for membrane glucocorticoid receptor.

'Panning' immunoseparation leaves some low-level, but detectably immunopositive, cells in the mER-depleted fraction. Interestingly, though the mER-depleted cell population showed some degree of mER staining, it showed no detectable E_2-stimulated PRL release. Perhaps a higher number of mER sites per cell is required for stimulated release. A similar intensity of staining is seen in one of our previous subclones of GH_3/B6, A1, (Pappas *et al.*, 1994). This subclone, although weakly positive for mER, shows low basal PRL release and no significant E_2-mediated PRL release. Selection for mER could result in populations of cells with both higher basal and E_2-stimulated PRL release, but our previous data on a GH_3/B6 subclone argues against this correlation; clone D9, which is mER-negative, shows no E_2-stimulated PRL release and has high basal PRL release (Pappas *et al.*, 1994).

Biotin-labeled (surface-exposed) proteins specific for the R3 rat ER epitope were immunoprecipitated from the membranes of immuno-enriched cells and visualized. That no protein of a similar size was immunoprecipitated from the cytosol, suggests that mER is a unique size species compared to iER. Also, there was no evidence of the 67,000 MW species (iER from cytosol) in the immunoprecipitated membrane proteins, suggesting that there was no contamination from iER in the membrane preparation. It is possible that the multiple size species in membrane stem from post-translational modifications of ER or degradation products of the largest-sized peptide species. Protein isolation studies of the membrane glucocorticoid receptor suggest that membrane steroid receptors may be labile and subject to degradation during membrane protein isolation (Gametchu *et al.*, 1991a,b, 1995; Gametchu & Watson, 1995). If post-translational modifications are responsible for larger sized mER, several possibilities exist including a variety of lipid or phospholipid attachments (Hoffman, 1991) which might also explain membrane targeting of the receptor. There is a segment of the rat ER sequence (amino acids 377–397) that could act as a membrane-spanning region [(Klein *et al.*, 1985) and PCGene sequence analysis programs version 6.80]. However, this hydrophobic region has a designated role in ligand binding of the lipophilic estrogenic compounds and is probably not suitable for crossing the plasma membrane twice [which would be required to explain the exposure of four epitopes of mER on the cell surface (Pappas *et al.*, 1995)].

In conclusion, our findings point to a direct action of E_2 at a membrane ER, which shares at least four epitopes with iER. Depleting populations of the cells bearing this receptor results in the loss of rapid E_2-stimulated PRL release, while enrichment produces populations with a robust release. It is possible that this receptor is the same protein responsible for rapid E_2-mediated responses previously identified for these (Dufy *et al.*, 1979; Zyzek *et al.*, 1981) and other cells (Morley *et al.*, 1992; Lieberherr *et al.*, 1993). It is interesting to speculate that they may also share identity with other reported non-nuclear receptors which cannot be 'translocated', such as those demonstrated in cytoplast cell fractions which have had their nuclei extruded by centrifugation (Welshons & Judy, 1995). Because unselected populations of GH_3/B6 cells are heterogenous in mER and show only modest E_2-stimulated PRL release (Pappas *et al.*, 1994), production of mER-enriched populations will be a valuable tool for further studies aimed at identifying the modifications necessary to make ER membrane-resident, and in studying the mechanisms of signal transduction these receptors utilize.

Materials and methods

Cell growth

GH_3/B6 cells were a generous gift of Dr Bernard Dufy (Université de Bordeaux II, Bordeaux, France). Cells were routinely cultured in serum-supplemented media (SSM) composed of Ham's F-10 (Gibco-BRL, Gaithersburg, MD), 12.5% heat-inactivated horse serum (Gibco-BRL, Hyclone, Logan, UT) and 2.5% heat-activated defined/supplemented bovine calf serum (Hyclone). Defined medium (DM1) was adapted from Hayashi & Sato (1976), and consisted of phenol red-free RPMI 1640 (Gibco BRL), 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), thyrotropin releasing hormone (TRH, 1 ng/ml, Bachem), 3,3′,5-triliodo-L-thyronine (3×10^{-11} M, Sigma, St. Louis, MO) and basic fibroblast growth factor (1 ng/ml, Boehringer Mannheim). All cells were cultured at 37°C in the presence of 5% CO_2.

Antibodies

Polyclonal antibodies R3 and R4 were both generated against a hinge-region peptide (amino acids 270–284) from the rat iER. These antisera were affinity-purified on a peptide column, and show specific binding to iER as well as a membrane ER. The antibodies recognize both native and denatured ER, and their characterization has been previously described (Pappas *et al.*, 1994).

'Panning' immunoseparation of GH_3/B6 cells

Immunoseparation was done according to a modification of the method of Gametchu (1987). Affinity-purified R3 or R4 at 2 mg/ml was sterile filtered onto one well of a 6 or 12-well tissue culture plate. The antibody was attached to the plate

by incubating 2 h at 37°C or overnight at 4°C. The dish was then washed well with chilled PBS and placed in an ice bath.

Cells (5×10^6 to 10^7) were plated in DM1 on 100 mm tissue culture dishes, cultured for 2 days, washed in Dulbecco's PBS (Sigma, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4), and removed from the plate with non-enzymatic cell dissociation solution (Sigma). The cells were washed once with PBS, resuspended in 5–7 ml PBS with 1 mg/ml fatty acid-free BSA (Sigma), and rocked gently for 1 h at 37°C to allow turnover of surface proteins, stabilization of plasma membranes and to block nonspecific protein-binding sites on the surface of the cells (Weigel, 1980; Weigel & Oka, 1983). Cells were then placed in 1 ml PBS with 1 mg/ml BSA at 4°C and chilled for 15 min. The chilled cells were plated onto antibody-coated plates, and put in an ice bath on an orbital shaker. Incubation with gentle shaking continued for 1 h. Cells in suspension were then removed and cultured as the '−' or depleted fraction. The plate was washed with PBS and attached cells were either cultured in the treated plate or triturated off the plate and subcultured in a new vessel. The attached cells were considered the '+' or enriched cells.

Immunocytochemistry and microscopy

Immunocytochemistry on live cells at 2°C using R3 and R4 antibodies was performed as described previously (Pappas *et al.*, 1994). We have demonstrated that labeling cells in the cold with this procedure precludes antibody entry into the cell, and therefore does not label iER. Briefly, cells were cultured in DM1 for 2 days on poly-D-lysine (180,000 MW, Sigma)-treated coverslips in 24-well tissue culture plates and preincubated in ice-cold PBS for 15 min prior to antibody labeling. The coverslips were incubated for 20 min with solutions of R3 or R4 anti-ER antibody (diluted 1:100) which had been preincubated with Cy3-conjugated goat antirabbit antibody (1:100, Jackson ImmunoResearch, West Grove, PA) in PBS + 1% BSA (PBSA) with 4% normal goat serum (Jackson ImmunoResearch) for 30 min on ice. After four washings with cold PBSA and fixation with 4% paraformaldehyde for 5 min, the coverslips were mounted on glass slides in 20% glycerol/80% PBS and sealed with clear nail polish.

The labeling intensity of immunofluorescent staining was quantitated with a Quantex QX-7 image processor (Quantex Corp. Sunnyvale, CA) using the ARBAREA program supplied by the manufacturer. The reported intensity value encompasses both the number of labeled cells and their individual brightness values, and is obtained by defining the outline of the cell or cell cluster and determining the average brightness value over the entire cell area. The cells were photographed on a fluorescence photomicroscope and the processed negatives were placed on a light table, their images captured with a low-light video camera (Dage-MTI, Inc., Michigan City, IN) and digitized for this analysis. A low level of background non-specific staining seen when secondary antibody alone is applied (Pappas *et al.*, 1994) accounts for approximately 30 units of labeling intensity, shown by the dotted line in Figures 2 and 3.

Prolactin release

Immunoseparated cells were seeded into poly-D-lysine-treated 60 mm tissue culture dishes and cultured in DM1 for 2 days. Prior to release experiments, cells were washed three times in rat saline (RS, 150 mM NaCl, 5.6 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4) and pre-incubated at 37°C in RS for 30 min. The cells were washed once and one ml of 37°C RS containing 10 nM 17β-E_2 (Sigma) or vehicle control (0.001% ethanol) was then placed on the cells for 5 min. These experimental media were then removed and placed in chilled microcentrifuge tubes, and centrifuged at 800 *g* to remove any cells that had lifted from the dish. The supernatant was transferred to a clean microcentrifuge tube and frozen at −20°C until PRL-specific radioimmunoassay (RIA) using a kit provided by NIDDK.

PRL content in the medium was normalized to DNA content. Following removal of the experimental medium, plates were treated overnight at 4°C with 1 ml 100 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 0.01% deoxycholate to release cellular DNA; then DNA was quantitated with spectrofluorimetry of Hoechst 33258 dye binding (Labarca & Paigen, 1980) using a Hoefer TKO 100 DNA fluorimeter (Hoefer Scientific Instruments, San Francisco, CA). Data were analysed with ANOVA using the MGLH module of SYSTAT version 3.0 (Systat Inc., Evanston, IL). Group differences were analysed with Scheffé's multiple linear contrast (Scheffé, 1959) using the Bonferroni procedure (Neter & Wasserman, 1974). Statistical significance was accepted at $P < 0.05$ over all contrasts.

Analysis of mER vs iER protein

Cells enriched for mER were grown in SSM until subconfluent, then the monolayer was washed once with PBS and DM1 was added for 16 to 24 h. The attached cells were removed from the flasks with PBS containing 5 mM EDTA, then were washed 3 × in PBS containing 2 mM $MgCl_2$ (to stabilize membranes). Membrane proteins were biotinylated on whole cells (Cole *et al.*, 1987) to prevent labeling of intracellular proteins. Cells were incubated with 5 mM d-biotinyl-N-hydroxysulfosuccinimide ester (NHSS-biotin, Pierce, Rockford, IL) in RPMI 1640 for 30 min on ice with occasional gentle shaking. Excess NHSS-biotin was removed by washing the cells three times with ice-cold RPMI 1640.

Membranes were isolated on sucrose step-gradient interfaces (Weissman, 1991; Ibarrolla *et al.*, 1992; Gametchu *et al.*, 1993) with the following specific procedures (all at 4°C). Following biotinylation, cells were resuspended in 2 vol homogenization buffer [10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 10 μg/ml leupeptin, 40 μg/ml phenylmethylsulfonyl flouride (PMSF), 5 mM diisopropyl fluorophosphate (DFP) and 100 μg/ml soybean trypsin inhibitor] and homogenized for 30 strokes using a Dounce B-type pestle. Homogenization buffer containing 0.12 M NaCl was added and the mixture centrifuged at 500 *g* for 10 min to remove nuclei and unbroken cells. The supernatant was centrifuged for 1 h at 105,000 *g*, resulting in a cytosol and a high-speed pellet. The cytosol was saved for analysis of iER by Western blot (see below). The pellet was resuspended in 1 ml membrane buffer (10 mM Tris-HCl, pH 8.2, 140 mM NaCl, 10 μg/ml leupeptin, 40 μg/ml PMSF, 5 mM DFP) and further homogenized 10 strokes. This suspension was layered onto a 4 ml, 41% (wt/vol, in membrane buffer) sucrose pad and spun at 95,000 *g* for 65 min. The membranes were removed from the top of the pad and washed two times with 3 ml membrane buffer, centrifuging at 120,000 *g* for 50 min for each wash.

The membrane proteins were extracted in 140 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.5% (vol/vol) Triton X-100, and 0.2% (vol/vol) SDS with gentle stirring for 2 h. The extracts were diluted to reduce SDS to 0.05%, pre-cleared with 20 μl of a slurry of protein-A Sepharose 4B CL (Sigma) for 30 min (to reduce non-specific binding of proteins to beads), and then incubated with a 1:50 dilution of R3 and 20 μl of protein A for 2 h. Cytosolic proteins were further diluted in 140 mM NaCl, 10 mM Tris-HCl (pH 7.5) and immunoprecipitated in the same manner as membrane protein extracts.

The antibody-protein-A complex was pelleted at 5000 *g* for 2 min, then washed four times with 300 mM NaCl, 20 mM Tris-HCl (pH 7.5) 0.5% Triton X-100 and 0.05% SDS. The beads were resuspended in 50 μl gel running buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 40% [vol/vol] glycerol, 20% [vol/vol] 2-mercaptoethanol, 40 mM DTT), boiled for 5 min and the supernatants resolved on 8% SDS–PAGE gels. Relative migration of unknown proteins was determined by comparison to high MW protein standards (Sigma) and BSA

(Sigma). Proteins were electrophorectically transferred to nitrocellulose (MSI Nitro + 1000, 0.22 μm pore size, Micron Separations Inc, Hoyone Falls, NY) in 192 mM glycine, 25 mM Tris (pH 8.8), and 25% methanol for 4 h at 50 V. Efficiency of protein transfer was determined by staining the nitrocellulose filter with 0.5% Ponceau S (Sigma) in 1% acetic acid.

All procedures for visualization of anti-ER immunoprecipitated proteins were done at room temperature. Nitrocellulose filters containing membrane proteins were blocked for 1 h in 3% gelatin in 140 mM NaCl, 20 mM Tris-HCl pH 7.5, washed 3 × in 140 mM NaCl, 20 mM Tris (pH 7.5), and then incubated with 20 μg/ml streptavidin-horseradish peroxidase (HRP, Pierce) for 1 h. The filters were then washed 3 × and HRP was visualized using 6 mg diaminobenzidine (DAB, Pierce) in 9 ml 50 mM Tris (pH 7.6) with 0.3% cobalt enhancement and 3% H_2O_2. For comparison, an immunoblot of immunoprecipitated iER transferred to nitrocellulose filters was performed. Filters were blocked with 3% gelatin for 1 h in 200 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.02% Tween 20 (TTBS). After three washes in TTBS, the filters were incubated with R3 (1:500) for 1 h, rewashed, and incubated with a goat anti-rabbit antibody conjugated to HRP (Pierce). HRP was visualized with DAB as described above.

Acknowledgements

We are very appreciative of Dr David Konkel's critical review of our manuscript during preparation. This work was supported by the Sealy Memorial Endowment Fund and NICHD R01-HD32481.

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