Maximizing Production of Estrogen Receptor $\beta$ with the Baculovirus Expression System

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INTRODUCTION

The estrogen receptor (ER), which functions both as a signal transducer and transcription factor that modulates the expression of target genes, is the progenitor for all steroid receptors (42) and is a member of the nuclear receptor superfamily that currently includes 48 known receptors plus two pseudogenes (30). In mammals, ER is encoded by two genes, $\alpha$ and $\beta$ (26). ER$\alpha$ and ER$\beta$ function as dimers (28). To study the functional properties of ER$\alpha$ and ER$\beta$, large quantities of appropriately post-transcriptionally processed proteins are needed for in vitro assays.

Early studies demonstrated that steroid receptors overproduced in yeast (31) or E. coli (37) were generally inefficient for in vitro analyses compared to receptors extracted from animal tissues because of insolubility, proteolysis, inappropriate or missing post-translational modification, and interfering proteins (41). The reasons for the failure of these systems included codon usage, solubility, stability of the protein in the absence of the chaperonin complexes with which steroid receptors interact, the absence of appropriate post-translational modifications, and variations in proteolytic activity (41). The baculovirus expression vector system (BEVS) has been widely adopted for the expression of steroid and nuclear receptors and many other intracellular, secreted, and membrane proteins because it provides a reproducible method for the production of large amounts of recombinant proteins (33). The most widely used virus for BEVS is the Autographa californica multicsapsid nucleopolyhedrovirus (AcMNPV). Although a wide variety of cells are susceptible to AcMNPV infection, Spodoptera frugiperda (Sf) cells Sf21 or their subclone Sf9 (Invitrogen, Carlsbad, CA, USA) are generally used for the production of nuclear receptors.

Insect cells have been demonstrated to perform many post-translational modifications that confer correct folding, biological activity, and antigenicity on expressed proteins (25,32,33). These modifications include the phosphorylation, glycosylation, myristylation, and palmitylation, signal peptide processing, post-translational cleavage, and disulfide bond formation (25). The first report of the baculovirus expression of hER$\alpha$ with BEVS (9) used the human estrogen receptor (HEO) clone; that is, with Gly-400 to Val-400 that decreased E$_2$ binding affinity and destabilized the protein (43). Subsequently, the corrected HEGO clone, which was later found to have a single nucleotide change that resulted in a glycine-to-valine amino acid substitution (Gly-400 to Val-400) that decreased E$_2$ binding affinity and destabilized the protein (43). Subsequently, the corrected HEGO clone, that is, with Gly-400 (43), was used to generate a baculovirus for ER$\alpha$ expression in Sf9 cells (24,10,34). Other investigators have expressed hER$\alpha$ in Sf9 cells, either as a wild-type or fusion protein with GST or FLAG® (6,7,47,48). Mouse ER$\alpha$ (16), chicken ER (8), and mouse ER$\beta$ (11,12) have also been expressed in Sf9 cells, and mouse ER$\alpha$ has been expressed in Sf21 cells (1). Researchers have yet to examine whether insect cell lines differ in the level of ER or other nuclear receptor produced.

To produce the most pure functional receptor while simultaneously conserving costly reagents, we compared the production and functional characteristics of recombinant rat ER$\beta$ driven by a...
recombinant baculovirus in four commonly used insect cell lines: Sf9, Sf21, Tn368, and BTI-TN5b1-4 (HighFive™; Invitrogen). We found that ERβ expression is highest in Sf21 cells compared to the most commonly used Sf9 cells. The procedure for the infection, harvesting, and characterization of recombinant ERβ should be applicable to all nuclear receptors as well as other transcription factors.

MATERIALS AND METHODS

Plasmids and Strains

The cDNA for rat ERβ in pCMV5 was generously provided by Dr. J.-A. Gustafsson (27). There were no unique restriction sites near the beginning of the ERβ open reading frame, so the cDNA sequence was modified by PCR with a primer designed to yield a product that better conforms to base biases near the translation starts in the AcMNPV genome open reading frames (5) and to remove upstream noncoding regions. The PCR product was inserted into the PCR 2.1 plasmid (Invitrogen), which enabled the introduction of an EcoRI restriction site near the translation start site. The upstream region and first part of the open reading frame were sequenced and then subcloned from the PCR 2.1 plasmid, from the plasmid EcoRI site to the first HindIII site in the open reading frame, into the minimal baculovirus transfer plasmid, pBAC-1 (Novagen, Madison, WI, USA), which uses the polyhedrin promoter. The final immediate upstream sequence was 5’-AATTCGGCTTA-CATG-3’. The transfer plasmid was completed by subcloning the remainder of the open reading frame (HindIII-HindIII) from pCMV5-rat ERβ, pBAC-1-ERβ was co-transfected into IPLB-SF21AE cells with BacVector®-3000 viral DNA (Novagen) in six-well plates with the Eufectin™ lipid transfection reagent from the Novagen BacVector-3000 transfection kit. The Sf21 cells were overlaid with agaro, and the initial cloning was done directly from the transfected cell monolayer. Clones were purified by repeated plaque purification in Trichoplusia ni (Tn)368 cells cultured in 24-well plates (49).

Baculovirus Titer Determination

ERβ baculovirus titer was determined by plaque assay (51) in 24-well plates. Tn368 cells were used because they demonstrate easily recognizable morphological changes in response to infection. In brief, after the optimal density was determined, Tn368 cells were plated in 1 mL TNM-FH medium (Grace’s insect cell medium; Invitrogen) supplemented with 10% FBS. One-tenth milliliter of serial dilutions of virus stock was used for infection. The cells and virus were sedimented at 1000g for 1 h at 28°C, and the supernatant medium was aspirated. Each well was overlaid with 0.5 mL 0.7% low-melting agar (Seaplaque™; CAMBREX, Rockland, ME, USA) in medium containing 10% FBS, 50% TNM-FH (Grace’s supplemented medium; Invitrogen), and 40% Grace’s medium, Vaughn’s modification (JRH Biosciences, Lenexa, KS, USA) after infection and incubated at 28°C in passively humidified containers. Plaques were counted three to four days after infection.

Comparison of ERβ Production between Different Insect Cell Lines

The viral titer determined by the plaque assay was used for the subsequent infection of the insect cell lines IPLB-SF21AE (Sf21) and Tn368 (generously provided by Alan Wood, Boyce Thompson Institute for Plant Research, Ithaca, NY, USA), Sf9, and BTI-TN5b1-4. Viable cell counts were determined by Trypan blue staining. For time and cell density experiments, virus was added at a MOI of 10, and the mixture was diluted for an appropriate cell number in 3 or 3.5 mL/well (depending on the experiment and always the same within an individual experiment). Cells were plated at the indicated cell densities in six-well plates (9.5 cm² wells; Corning, Corning, NY, USA) and were infected with the ERβ baculovirus by sedimentation at 1000g for 1 hour at 28°C. The time after infection was counted from the end of the centrifugation step. The inoculating medium was removed and replaced with fresh medium (3 mL/well). The plates were sealed with vinyl electrical tape, placed in passively humidified boxes, and incubated at 28°C for the indicated times.

For cell extract preparation, the cells were harvested and sedimented at 66×g for 5 min in a swinging-bucket rotor (Beckman Coulter, Fullerton, CA, USA). After resuspension, the cells were washed with room temperature isotonic saline (3.7 mM CaCl₂, 54.7 mM KCl, 11.2 mM MgCl₂, 11.3 mM MgSO₄, 7.3 mM NaH₂PO₄, 181 mM sucrose, pH 6.5), sedimented, and washed once with room-temperature hypotonic solution (10 mM Tris-HCl, pH 7.5, 10 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 2 μg/mL pepstatin, and 10 μg/mL E64 (Roche Applied Science, Indianapolis, IN, USA). The cells were resuspended in 0.3 mL ice-cold hypotonic solution, frozen in liquid nitrogen, and stored at -70°C. Upon thawing, the KCl and Tris-HCl concentrations were adjusted to 600 mM and 50 mM, respectively, and the cells were disrupted using a sonicator (Branson Sonifier model 200; Branson Ultrasonics, Danbury, CT, USA) equipped with a cup horn cooled with ice water. Disruption was monitored with Trypan blue staining and examination with a phase-contrast microscope (Phase Contrast-2 ELWD 0.3; Nikon, Melville, NY, USA). After sonication, the extract was incubated on ice for 3 h and clarified by sedimentation in a TLS100.2 rotor (Beckman Coulter) at 279 000×g for 15 min. The supernatant solution was aliquoted, frozen in liquid nitrogen, and stored at -70°C. For DNA binding assays, additional concentrated nucleic extracts were prepared. Three insect cell lines, IPLB-SF21AE, Sf9, and BTI-TN5b1-4 were plated in 75 cm² plug-seal cell culture flasks (Corning). Following attachment, the medium was removed, and ERβ baculovirus was added at an MOI of 10 in a volume of 0.5 mL. The flasks were rocked slowly for 1 h, and then 15 mL TNM-FH medium (Grace’s supplemented medium) and 10% FBS were added. The flasks were incubated at 28°C. Nuclear extracts were prepared from Sf21, Sf9, and BTI-TN5b1-4 cells at 1.5, 1.8, and 2.7 days after infection, respectively, for samples early in the infection cycle. For samples late in the infection cycle, Sf21, Sf9, and BTI-
TN5b1-4 cells were harvested at 2.5, 2.5, and 4.7 days, respectively. At the start of infection, the cells were at densities (per cm²) of 77,330, 200,000, and 72,000 for Sf21, Sf9, and BTI-TN5b1-4, respectively.

For nuclear extract preparation, the cells were washed once with room-temperature isotonic saline, followed by a wash with room-temperature hypotonic buffer (described earlier). The cells were resuspended in two packed cell volumes of cold hypotonic solution and allowed to swell on ice for 20 min. A Dounce homogenizer was used to disrupt 90% of the cells. The homogenate was sedimented at 3,200 × g for 20 min. The nuclear pellet was resuspended in extraction buffer (hypotonic buffer with 50 mM Tris-HCl and 600 mM KCl as final concentrations) in a volume equal to that used for the homogenization. The samples were sonicated on ice with a sonicator equipped with a stepped microprobe horn. Disruption was monitored with Trypan blue staining and phase-contrast optics. After sonication, the extract was incubated on ice for 3 h and clarified by sedimentation in the TLS100.2 rotor at 279,000 × g for 15 min. The clarified supernatant solution was aliquoted and stored at -70°C.

The concentration of ERβ in each sample was determined by a hydroxyapatite (HAP) assay (36). Cellular or nuclear extracts were incubated in TDPK111 buffer (40 nM Tris-HCl, pH 7.5, 1 mM DTT, 0.5 mM PMSF, and 111 mM KCl) containing 30 nM [3H]E2 ([2,3,6,7-[3H](17β)-estradiol, 74 Ci/mmol, NET-317; NEN® Life Science Products, Boston, MA, USA) for 1 h at 37°C. Following a 1-h incubation with a 10% HAP solution at 4°C, the HAP was sedimented and washed three times with 1 mL TDPK111. The HAP-bound [3H]E2 was determined by liquid scintillation counting in Econo-Sint™ A (National Diagnostics, Atlanta, GA, USA) in a Wallac 1409 scintillation counter (Turku, Finland). A 200-fold excess of unlabeled E2 (300 nM) was used as a competitor with [3H]E2 for ERβ binding as a control to determine nonspecific binding. The specific binding of the [3H]E2 was used to calculate the ERβ concentration in mols of dimer, assuming that each ERβ monomer binds one [3H]E2 (27). The affinity of the E2-ERβ interaction was determined by competition binding using the HAP assay (36), in which a fixed amount of ERβ (1.6 nmol) was incubated with 1.6 µM [3H]E2 plus increasing concentrations (from 30 fmol to 1 µmol) of E2. Binding reactions were performed in triplicate within each HAP assay for each time point sample (22). The nonspecific binding did not vary significantly over the time course of infection or with differences in cell density. The data were analyzed by nonlinear regression analysis using GraphPad Prism® (GraphPad Software, San Diego, CA, USA).

**Electrophoretic Mobility Shift Assay**

Protein-DNA binding was measured by electrophoretic mobility shift assay (EMSA) as previously described (23,44–46). In brief, identical molar amounts of ERβ, based on the HAP assay results, were incubated with a fixed amount (50,000 dpm) [32P]-labeled EREc38 (sequence: 5′-TCAGG-TCAGAGCTGACCTGAGCTAAAATAACACATT-3′) (24). ERβ-specific antibodies (Y19) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ERβ-14C8 (14C8 or 14C) (GeneTex, San Antonio, TX, USA) (40) were included in selected reactions, as indicated in Figure legends. Dried EMSA gels were analyzed using an InstantImager and associated software, the Packard Imager for Microsoft® Windows® v2.04 as previously described (both from Packard Instrument, Meriden, CT, USA) (29). The competition EMSA method for determining the affinity of ERβ-ERE interaction has been previously reported (46).

**Western Blot Analysis**

The protein concentration of nuclear extracts was determined using the dextran-coated charcoal (Lowry) assay and BSA as a standard (Bio-Rad Laboratories, Hercules, CA, USA). For Western blotting, 30 µg protein from the nuclear extract of baculovirus-infected cells were separated on 10% SDS polyacrylamide gels and electroblotted onto a PVDF membrane. Following the transfer, the blots were...
probed with ERβ polyclonal antiserum PA1-311 (Affinity Bioreagents, Golden, CO, USA) or monoclonal ERβ antibody CWK-F12 that had been generously provided by Dr. Benita S. Katzenellenbogen (13). We used the Renaissance Enhanced Luminol Reagent (NEN Life Science Products) for immunochemical detection as previously described (23). Data were quantitated from scanned films using Un-Scan-It™ Software ver. 5.1 (Silk Scientific, Orem, UT, USA) (23).

RESULTS

Maximizing BEVS ERβ Production

We examined ERβ production as a function of cell density and time after infection in four insect cell lines: Tn368, BTI-TN5b1-4, Sf9, and Sf21. Each insect cell line was infected at six different cell densities. The specific [3H]E2 binding capacity was determined as a measure of functional ERβ production. Preliminary observations with all four cell lines [IPLB-SF21AE (Sf21), Sf9, Tn368, and BTI-TN5b1-4] revealed that Tn368 was a comparatively poor producer of recombinant rat ERβ (data not shown).

We continued the investigation with Sf9 (the cell line used most often for the production of nuclear receptors), Sf21, and BTI-TN5b1-4. A series of densities of cells were infected at a MOI of 10. Phase-contrast microscope observations of the cells 24 h after infection revealed nearly synchronous in-
Table 1. Comparison of ERβ Production in Insect Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Optimal Cells/Well and Time After Infection</th>
<th>Maximum ERβ Protein Production (pmol/well)</th>
<th>Maximum ERβ Protein Production (pmol/mg protein)</th>
<th>Maximum ERβ Protein Production (amol/cell)</th>
<th>ERβ-ERE Binding % of [32P]ERE Bound/Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTI-TN5b1-4</td>
<td>7 x 10^5 at 4.2 days</td>
<td>11.5</td>
<td>4</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Sf9</td>
<td>1.9 x 10^6 at 2.2 days</td>
<td>2.5</td>
<td>9</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Sf21</td>
<td>7.5 x 10^5 at 1.6 days</td>
<td>15</td>
<td>18</td>
<td>27</td>
<td>27</td>
</tr>
</tbody>
</table>

The peak ERβ protein production was compared between cell lines at the given cell densities and days after infection. ERβ protein production (pmol/well and amol/cell) was determined by HAP assay. ERβ-ERE binding was determined using EMSA. The details of this experiment are provided in the Materials and Methods section.

The amount of ERβ produced per well at the indicated cell densities and time of harvest post-infection was determined by HAP assay. Values are ± SEM of triplicate determinations from a single experiment that are representative of two to three separate time course experiments, with the exception of Tn368 (first experiment). Sf21 cells peaks at an earlier time after infection (i.e., 1.6 days vs. 4 days for BTI-TN5b1-4). Therefore, it is more economical to produce large quantities of ERβ in Sf21 cells. An additional comparison of peak ERβ protein production is provided in Table 1. The affinity of E2 binding to ERβ harvested from Sf21 cells was calculated to be a Kd = 2.6 ± 0.2 nM (data not shown).

ERE Binding Activity of Baculovirus-Expressed ERβ

In addition to ligand binding, DNA binding is the second biological activi-
ty essential for steroid receptor function. We employed EMSA to examine if the amount of ERβ determined by the HAP assay correlated with the estrogen response element (ERE) DNA binding activity and to compare the ERE binding activity of ERβ produced by the different insect cell lines. We compared ERβ binding using a fixed amount, 89 fmol ERβ [determined by [3H]E2 binding (36)], and a fixed concentration, 65 fmol [32P]ERE (Figure 4A). We compared the binding of ERβ produced early versus late after infection to examine whether ERβ DNA binding capacity is labile over the course of viral infection. ERβ bound specifically to [32P]ERE as indicated by the supershift of the complex by ERβ antibody Y-19. The ERβ-14B8 antibody did not cause a supershift of the ERβ-ERE complex, possibly indicating that its epitope is unavailable in the ERβ-ERE complex. Quantitation of the data indicated that ERβ produced by BTI-TN5b1-4 and Sf9 cells showed higher ERβ-ERE binding capacity early in harvest compared to later harvest (Figure 4B). The amount of ERβ binding was comparable among the cell lines. However, ERβ produced by Sf21 cells appeared to have greater ERE binding at the later harvest time point than BTI-TN5b1-4 and or Sf9 cells (Table 1).

Western Blot Analysis of Baculovirus-Expressed ERβ

We also assessed immunoreactive ERβ protein production by Western blot analysis using two different ERβ-specific antibodies (Figure 5). The CWK-F12 epitope is aa (amino acid) 272–285 in the human ERβ ligand binding domain, and PA1-311 was raised against aa 45–55 of rat ERβ. Identical results were obtained with the two antibodies: two bands of 54 and 50

Figure 4. Recombinant ERβ binds EREs. (A) EMSA of ERβ-ERE binding. Identical ERβ concentrations (89 fmol) in nuclear extracts harvested early (3.7, 1.8, or 1.5 days) or late after infection at (4.7, 2.5, or 2.5 days) for BTI-TN5b1-4, Sf9, and Sf-21 cells, respectively, were incubated with [32P]EREc38 as described in the Materials and Methods section. ERβ-specific antibodies Y-19 (indicated as “Y”) and 14C8 (indicated as “14”) were added to the designated reactions. The arrow indicates the specific ERβ-EREc38 band, and SS- indicates the supershifted complex formed between the ERβ antibody Y-19 and the ERβ-EREc38 complex. EMSA details are provided in the text. This autoradiograph is representative of four independent EMSA experiments that show similar results. (B) The data from panel A were quantitated as described in the text, and the [32P]dpm converted was into percentages of the ERβ-EREc38 complex. The solid bars indicate the amount of ERβ-ERE complex formed with no added antibody, and the open and hatched bars indicate the amounts of ERβ-ERE complex formed with the addition of ERβ antibodies Y19 and 14C-8, respectively. 5b1-4, BTI-TN5b1-4 cells.

Figure 5. Western blot of ERβ protein production. (A) Identical amounts of protein (30 µg) from cell extracts of rat ERβ baculovirus-infected BTI-TN5b1-4, Sf9, and Sf21 cells harvested early (3.7, 1.8, or 1.5 days) or late after infection (4.7, 2.5, or 2.5 days), respectively, were separated by 10% SDS-PAGE. The resulting PVDF membrane was probed with ERβ-specific antiserum PA1-311 or monoclonal CWK-F12 ERβ antibody (13) as described in the Materials and Methods section. (B) The blots shown in panel A were quantitated as described in the text. Shown here is the percent of maximal pixel density in each blot, using the pixel density detected in Sf21 L as 100%. These data are from a single representative experiment that has been repeated with similar results. 5b1-4, BTI-TN5b1-4 cells.
kDa were observed. The calculated molecular weight of ERβ was 54.2 kDa (27), which accounted for the 54-kDa band. We suggest that the 50 kDa band may be the result of incomplete translation, partial protein degradation during sample processing, or incomplete glycosylation or phosphorylation.

Use of Baculovirus-Expressed ERβ to Measure ERE Binding Affinity

Competition EMSA (46) was used to calculate the affinity of baculovirus-expressed ERβ binding to EREc38. ERβ binds EREc38, which has a “perfect” palindromic ERE, with high affinity ($K_d = 0.72 \pm 0.20 \text{nM}$). These data indicate the utility of baculovirus-expressed ERβ for in vitro DNA binding affinity determinations.

DISCUSSION

The production of recombinant steroid and nuclear receptor proteins in the BEVS has predominantly used AcMNPV and the Sf9 cell line. Although comparisons of recombinant protein production with BEVS both from large-scale suspension culture and for smaller scale production in disposable flasks has been performed for proteins such as secreted placential alkaline phosphatase and β-galactosidase (15), human muscarinic M2 (19) and dopamine D2 receptors (20), and DNA methyltransferase (38), this is the first comparison of the production of a nuclear receptor in different insect cell lines.

We examined ERβ production in four insect cell lines: Tn368, BTI-TN5b1-4, Sf9, and SF21. The Sf9 cell line is a subclone of SF21 and offers no particular advantage over SF21 in adherent culture conditions (35). The widely used BTI-TN5b1-4 cells were developed for recombinant protein production and are more efficient secretors of exported proteins (15).

ERβ production was determined as a function of cell density and time after infection in Tn368, BTI-TN5b1-4, Sf9, and SF21 cells. Our results show that SF21 and BTI-TN5b1-4 cells produce more bioactive ERβ protein, based on $[^3\text{H}]\text{E}_2$ and ERE binding activity in cell homogenates, as compared to Sf9 cells. The ERβ produced in SF21 cells shows 2- and 4-fold the specific activity of ERβ produced in Sf9 and BTI-TN5b1-4 cells, respectively. DNA binding activity, determined by ERE binding using EMSA, is more effectively preserved late in the infection cycle of SF21 cells compared to the other two cell lines. An additional advantage of SF21 cells is that they produced more immunoreactive ERβ/mg protein than did Sf9 or BTI-TN5b1-4 cells. Moreover, because ERβ production in SF21 cells peaks at an earlier time after infection (i.e., 1.6 days), we conclude that it is more economical to produce ERβ on this scale in SF21 cells.

The maximum level of ERβ produced in SF21 cells reported here was 18 pmol ERβ dimmer/mg protein, which is 36 pmol ERβ monomer/mg protein (Table 1). This result is comparable with the value of 32.8 pmol/mg protein for chicken ERα (8) but lower than the 230 pmol ERα monomer/mg produced 11 days post-infection in the Sf9 cells (34). Compared to other steroid and nuclear receptors, we observed higher ERβ production in SF21 cells than the 5–10 pmol androgen receptor/mg protein (21) or the 3.8 pmol hPR-A/mg protein (14) observed in Sf9 cells but lower than the 1200 pmol hRARα/mg nuclear protein produced in Sf9 cells (39). Unfortunately, we cannot compare our results for the production of rat ERβ with those for the short form (485 aa) of hERβ in SF21 cells because no quantitation was provided (17).

In summary, we have shown that the SF21 cell line is superior to the commonly used Sf9 cell line for the production of biologically active recombinant ERβ. Baculovirus-expressed ERβ bound EREs with high affinity in vitro, indicating the utility of this method of ER production for functional measures of ER activity. The clear superiority of SF21 cells for ERβ production should encourage this type of comparison for other nuclear receptors by other investigators since the choice of cell type can dramatically influence yield and purity.

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