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HIGH LEVEL EXPRESSION OF BIOLOGICALLY ACTIVE ESTROGEN RECEPTOR IN *SACCHAROMYCES CEREVISIAE*

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Summary—Biochemical over-expression of the human estrogen receptor was achieved using a *Saccharomyces cerevisiae* expression system. The receptor was produced as a novel ubiquitin fusion protein. This fusion protein is short lived in the cell and is processed to produce unfused receptor shortly after folding. Conventional high copy expression plasmids produced receptor to about 0.04% of the total soluble protein. By incorporating a defective leu2 allele into these vectors, an additional 5-fold increase in receptor production was obtained. The recombinant receptor was undegraded, soluble and biologically active. Conventional methods of disrupting cells using glass beads had a detrimental effect on the ability of the receptor to bind hormone. Enzymatic digestion of the cell wall followed by hypotonic shock liberates the receptor that quantitatively binds estrogen.

INTRODUCTION

The recent cloning of several members of the steroid receptor superfamily and the subsequent analysis of their domain structure has indicated that these receptors are modular, consisting of distinct functional domains [1]. Specifically DNA binding, hormone binding, transcriptional activating and dimerization domains have been identified. The hormone binding domain is the largest, consisting usually of the carboxyl 300 amino acids [2, 3]. Attempts to map the critical ligand contact points by mutagenesis have not been successful as most mutations compromise the ability of the receptor to bind hormone [4, 5]. Conventional mutagenic strategies are too laborious to attempt a saturation mutagenesis of this large region. It is clear that physical–biochemical strategies such as crystallographic analysis will be required to further characterize this domain.

Physical–biochemical analyses require a plentiful source of biologically active receptor. Due to the low abundance of steroid receptors in target tissues, heterologous expression systems that allow large scale purification of the receptor are required [6]. We and others have utilized unsuccessfully, bacterial expression systems for biochemical over-production [7, 8]. Using a novel ubiquitin fusion technology, we were able to over-express the vitamin D receptor in yeast in a form that quantitatively bound hormone [9]. These results were at variance with others who have reported partial activity and altered specificity of receptors produced in *Saccharomyces cerevisiae* [10–12]. Such results raise the question as to whether the yeast system may be suitable for over-production of a wide range of steroid receptors. Consequently, we wished to examine the production of other steroid receptors in yeast and determine whether this organism could be used as a general over-expression system for steroid receptors which can be used to produce biologically active receptor.

This study describes the use of the yeast expression system and an extraction procedure that has allowed us to over-express the human estrogen receptor to 0.2% of the total soluble protein. A biochemical analysis of this material indicates that the receptor binds hormone with wild type affinity and is presumed to be biologically active.

METHODS

Chemicals and supplies

Restriction and modification enzymes were purchased from Promega Biotech (Madison,

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WI), Boehringer Mannheim (Indianapolis, IN), or New England Biolabs, (Bethesda, MD). Radioinert steroids and general reagents were purchased from Sigma (St Louis, MO). Radiochemicals were purchased from New England Nuclear (Boston, MA). Yeast media components were obtained from Difco-BRL (Bethesda, MD). Oxalyticase was obtained from Enzogenetics (Corvallis, OR). Immobilon membranes were purchased from Millipore (Bedford, MA).

Yeast strains

Expression of recombinant proteins was carried out in the protease deficient strains BJ3505, (Mat a, pep4::HIS3,Prb1-Δ1.6R, his3, lys2-208, trpl-Δ101, ura3-52) or BJ2168 (Mat a, prc1-407, prb1-1122 pep4-3, leu2, trpl, ura3-52). These were obtained from the yeast genetic stock center (Berkley, CA). All transformations into this strain were done using the lithium acetate transformation protocol [13].

Construction of plasmids

The expression vector YEPE10 was constructed as follows. The human estrogen receptor plasmid (pGEM35hER, a gift from Geoff Greene) was digested with BamH1 and TthIII, and ligated to a linker which encoded the last 6 amino acids of ubiquitin and also contained an AflII and Ncol site. The EcoR1 site of the resulting plasmid was converted to a Kpn1 site. The AflII-pn1 fragment of this vector was purified and cloned into the corresponding sites of the yeast expression vector YEPV1, yielding the plasmid YEPE2. This plasmid will produce a receptor with 13 additional amino acids at the amino terminus. A subsequent publication demonstrated that the original cloned cDNA contained a critical point mutation in the hormone binding domain [14]. The mutated hormone binding domain of the vector YEPE2 was corrected by replacing the Asp718-Smal fragment with an analogous fragment from the wild type cDNA (a gift from Geoff Greene). The resulting plasmid was called YEPE10.

A derivative of this plasmid YEPE15 was constructed by cloning the Hpal-Stul fragment of pLQ6 (a gift from T. Butt), which contains a defective leu2 allele, into the Csp45-end filed YEPE10.

The reporter plasmid YRpE2 was constructed by inserting two copies of an oligonucleotide containing a consensus vitellogenin estrogen responsive element (ERE) [15] into the unique Xhol site of the plasmid PC2(9). The copy number and orientation of the inserted sequences was determined by sequencing.

Preparation of yeast extracts

Cells were grown overnight in minimal media containing 2% glucose and essential amino acids. When the cells reached an optical density of 1.0 at 600 nM they were induced with 100 μM $CuSO_4$ and allowed to continue growing for 4–8 h. When cells were grown in a 5 l fermenter they were induced at lower optical densities. Cells were harvested by centrifugation and washed twice in water. The pellet was resuspended in 1.2 M sorbitol, 40 mM KPO_4, 20 mM *b*-mercaptoethanol (pH 7.4) and incubated at 30°C for 30 min. The cells were again pelleted and redissolved in the same buffer (5 ml/g). The enzyme oxalyticase was added at a concentration of 30 μg/ml and incubated at 30°C for 90 min. The mixture was washed twice with sorbitol buffer to remove the enzyme and the cells were lysed via hypotonic shock by redissolving the pellet in 10 mM Tris pH 7.4, 1 mM EDTA. The cytosol was then clarified by centrifugation.

Beta-galactosidase assays

Assays for beta-galactosidase were performed as described previously [9].

Preparation of MCF-7 estrogen receptor

MCF-7 cells were grown in DMEM medium containing 10% stripped fetal bovine serum without phenol red. The cells were harvested by scraping, washed twice with ice-cold PBS (phosphate buffered saline) and resuspended in 2 × packed cell volume of 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM DTT pH 7.9 (buffer A). After 10 min, the cells were homogenized using a B pestle in a dounce homogenizer and 1/9 volume of 5 M NaCl in buffer A was added. The sample was incubated on ice for 1 h with occasional gentle vortexing. Salt extract was prepared by centrifuging at 15,000 rpm for 30 min in a Ti50 rotor. The supernatant was labeled with 40 nM [^{3}H]estradiol overnight at 4°C and free steroid was removed by stripping with charcoal.

Western immunoblots

Proteins from yeast were electrophoresed and transferred to Immobilon membranes as described previously [16]. The procedure for solid phase radioimmunoassay was described with

the exception that the D75 or H222 anti-receptor antibody was used.

Antibody immunoprecipitations

Increasing concentrations of OV245 antiserum was incubated for 30 min at 4°C with fixed volumes of either MCF-7 cytosol or yeast extract in a final volume of 200 μl. The samples were assayed using a Protein A Sepharose column assay as described previously [17].

RESULTS

Development of expression vectors

We have shown previously that the vitamin D receptor can be successfully over-produced in *Saccharomyces cerevisiae* [9]. The quality of the receptor was significantly enhanced when we utilized a ubiquitin fusion technology rather than more conventional techniques. In the current study this expression system was utilized to produce the human estrogen receptor. The strategy for production of the recombinant receptor is indicated in Fig. 1. A fusion cDNA was created between ubiquitin and human estrogen receptor. This cDNA was then inserted into the expression vector YEPV1, creating the vector YEPE10. In this construct, the recombinant protein was produced under the control of the CUP1 (metallothionein) promoter. This plasmid was then introduced into the protease deficient strain BJ3505 by standard techniques [13]. Receptor production was induced by growing the cells to mid-log phase and adding $CuSO_4$.

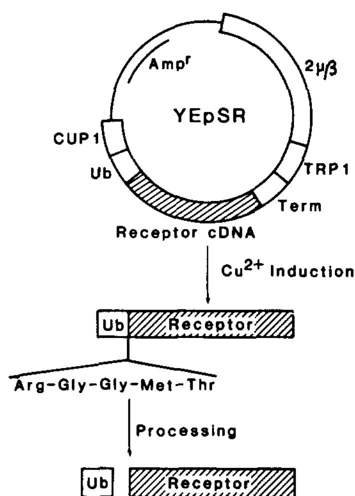

Fig. 1. Strategy for production of mammalian steroid receptors in yeast. The cDNA for the estrogen receptor was fused to that of ubiquitin. The recombinant RNA is induced by $CuSO_4$ and translated to yield a ubiquitin–receptor fusion protein. This fusion is short-lived in the cell and is quickly processed by a cellular processing system to yield unfused receptor. The positions of the CUP1 (metallothionein) promoter, TRP1 auxotrophic marker and CYC1 terminator are indicated.

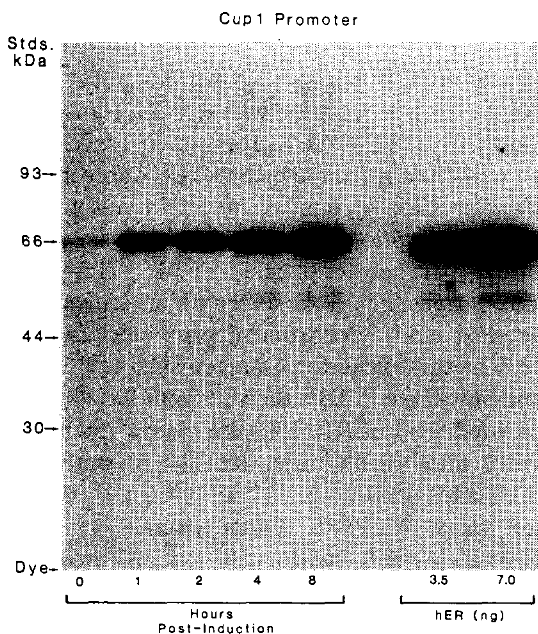

Fig. 2. Western immunoblot analysis of recombinant estrogen receptor produced in *Saccharomyces cerevisiae*. The cells were grown overnight and then induced with $CuSO_4$ for the times indicated. The human estrogen receptor (hER) standards were isolated from CHO cells transformed with a mammalian expression vector. They were quantitated by immunoblot and hormone binding.

Receptor protein, which is initially produced as a fusion with ubiquitin, is rapidly processed following folding to yield an unfused receptor. The results of such an experiment are shown in Fig. 2. Cells containing the estrogen receptor were induced for different times with 100 μM $CuSO_4$. The cells were harvested by centrifugation, lysed and the cytosol was analyzed by Western immunoblot. Using a mono-specific antibody we detected the production of a 65 kDa protein that co-migrated with authentic estrogen receptor produced in mammalian CHO (Chinease hamster ovary) cells. This antibody does not interact with any endogenous yeast proteins (data not shown). Using mammalian estrogen receptor standards that have been accurately quantitated by hormone binding, we estimated that these cells produce 3–4 pmol of receptor/mg of total protein. The receptor was homogenous and no significant breakdown of the receptor was apparent. The slightly higher molecular weight of the recombinant receptor was due to 13 amino acids that remain on the receptor following removal of the ubiquitin.

Yeast estrogen receptor is transcriptionally active

Previously, it has been shown that yeast produced estrogen receptor will activate transcription of an ERE-GAL1 promoter in yeast [10]. We wished to see if the receptor produced using this expression system would analogously activate transcription. To examine this, we constructed the reporter plasmid YRpE2. This plasmid contains the proximal promoter elements of the yeast *iso*-1-cytochrome C promoter fused to the structural gene for *b*-galactosidase [9]. Two tandemly linked EREs derived from the consensus vitellogenin ERE [15] were placed 250 bases upstream of the initiation site. This plasmid was co-transformed into yeast containing the expression vector YEPE10. Transformants were selected for by uracil and tryptophan auxotrophy.

The transcriptional activity of estrogen receptor in these transformants was measured by growing the cells overnight in the absence of hormone, or in the presence of varying concentrations of estradiol or nafoxidine. The *b*-galactosidase activities were measured. The results are shown in Fig. 3. Clearly, the yeast produced estrogen receptor activates transcription of the target gene in a hormone dependent manner. Half maximal activation of transcription occurs at about 1 nM hormone. This is similar to that required for activation of the receptor in mammalian cells [18]. The appropriate response of the receptor to nafoxidine (a receptor antagonist) further supports the hypothesis that this receptor is biologically active.

Biochemical characterization of yeast

Saturation analysis and an examination of ligand binding specificity indicated that this

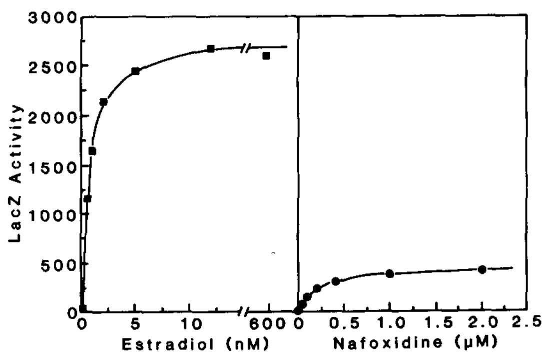

Fig. 3. Estrogen receptor is transcriptionally active in *Saccharomyces cerevisiae*. Transcriptional activity of yeast produced estrogen receptor on the reporter YRpE2 in the absence or the presence of increasing concentrations of estradiol or nafoxidine. The *b*-galactosidase activity is expressed in Miller units/mg of protein [9].

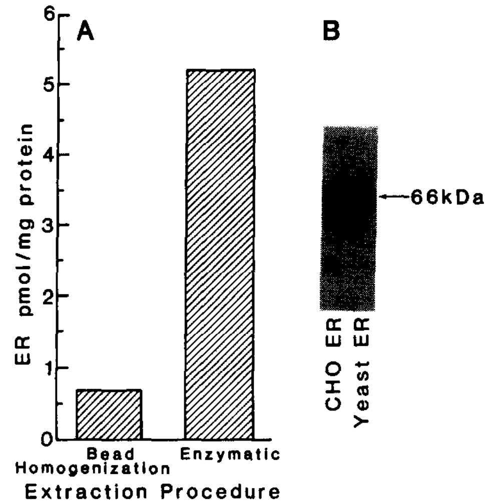

Fig. 4. Hormone binding and Western immunoblot analysis of recombinant estrogen receptor indicating that the mammalian and yeast produced receptor bind hormone equally well. (A) A comparison of the hormone binding properties of receptor isolated from the same batch of cells by either a bead homogenization or enzymatic digestion protocol. (B) Western immunoblot analysis of an equivalent amount of labeled receptor from yeast and CHO cells indicating that the hormone binding activity of both preparations are identical.

material had identical biochemical properties to the native human estrogen receptor (data not shown). These analyses, however, revealed a significant discrepancy between the levels of expressed protein when estimated by ligand binding and Western immunoblot. This phenomenon has previously been observed for the estrogen and progesterone receptors expressed in yeast [10, 11]. It has been proposed that *Saccharomyces cerevisiae* may not perform all of the necessary modifications required for ligand binding [10]. An alternative explanation is that the harsh homogenization methods using glass beads lead to denaturation of the receptor. To address these questions, we undertook a rigorous examination of the quality of the receptor extracted by the glass bead method of disruption and that isolated by an enzymatic digestion protocol.

Cells were grown as normal and then split into two batches. One aliquot of cells was disrupted by vortexing in the presence of glass beads. The second aliquot was digested for 1 h with oxalyticase and the resulting spheroplasts were lysed osmotically. The resulting supernantant was clarified by centrifugation. Cytosols were analyzed for hormone binding. A comparison of the results is shown in Fig. 4. Cytosol prepared using glass beads yielded 700 fmol/mg of protein whereas the procedure involving

DISCUSSION

One of the most important questions in the field of steroid hormone action at present is the role of hormone in regulating activation of the receptor. The hormone binding region of the receptor is capable of high affinity binding of the ligand, discriminating against very closely related molecules, and then transducing the hormonal stimulus to the DNA binding and activation regions of the receptor. Clearly there are key amino acids and structures responsible for these functions. Because of the size of the hormone binding region (>300 amino acids), comprehensive mutagenesis of the region may prove to be impractical using current strategies. An alternative approach to study the role of hormone is crystallization of the occupied receptor. There are numerous technical difficulties inherent in this approach, the least of which is development of a source of homogenous, biologically active receptor.

One system that has proven quite useful in our hands has been *Saccharomyces cerevisiae* [9, 10]. Using this sytem we have been able to produce and purify the vitamin D receptor in large quantities [21]. The quality of this receptor was far superior to that isolated from other heterologous expression systems. Using a similar system we have now been able to produce the estrogen receptor to a level of 0.2% of the total soluble protein. By the criteria that we have utilized we are unable to distinguish this receptor from that isolated from mammalian sources. Previous reports by our laboratory and others have indicated that only a fraction of a receptor produced in yeast is capable of binding hormone [9–11]. As we have shown, such results may result from the use of standard protocols for yeast disruption and not because of differences in covalent modification of the receptor.

Recent reports on the production of the glucocorticoid receptor in yeast have indicated that the receptor is transcriptionally active [12]. However, there have been difficulties in isolating biologically active receptor from these cells. These problems may also be related to denaturation of the receptor during isolation. This possibility is enhanced by our unpublished data indicating that hormone binding properties of the progesterone receptor produced in yeast were improved significantly using our current extraction procedures.

Our results indicate that the steroid responsive transcription units that have been reconstituted in yeast can in fact report on the activity of the bulk of the receptor present in the cell Furthermore, this yeast system should be suitable for overexpression of other members of the steroid receptor superfamily. Finally, this system has proven to be cost effective and easy to manipulate. It is being used in our laboratory at present to generate the milligram quantities of recombinant receptors required for physical–biochemical analyses.

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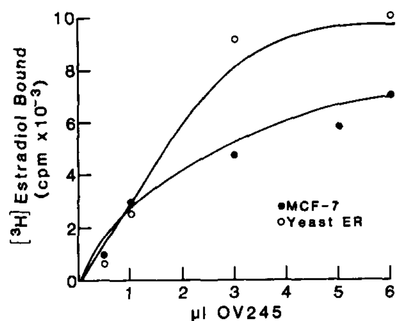

Fig. 5. Immunoprecipitation of labeled human estrogen receptor (hER) isolated from yeast and mammalian sources. The indicated amounts of OV245 antibody were incubated with either yeast or with MCF-7 estradiol labeled receptor and assayed as described in Methods. (●)MCF-7; (○)yeast.

enzymatic digestion yielded 5.5 pmol/mg of protein. The binding affinity of the receptor isolated by both methods was identical (data not shown). Clearly the material produced by enzyme digestion was of superior quality as estimated by hormone binding.

By comparing an equal amount of receptor as estimated by hormone binding from yeast and mammalian CHO extract we were able to show by Western immunoblot that both cytosols contained approximately the same amount of immunoreactive material. This indicated that the specific activity of the receptor produced in yeast was identical to that produced in mammalian cells. We have repeated this type of experiment using cytosols derived from different mammalian sources with analogous results.

To confirm these observations we used a receptor immunoprecipitation assay using an antibody raised against the DNA binding region of the receptor. We then compared the ability of this antibody to immunoprecipitate an equal amount of labeled receptor from MCF-7 cells or from our yeast extracts containing recombinant receptor. We reasoned that when antibody is limiting, less labeled receptor would be precipitated from the yeast cytosol if it contained significant amounts of a non-binding species. The results of this analysis are shown in Fig. 5. At very low concentrations of antibody equal amounts of labeled estrogen receptor were immunoprecipitated. If anything, as the concentration of the antibody was increased we observed that more recombinant binding activity was immunoprecipitated.

It is clear that the estrogen receptor produced in yeast is undegraded and of similar quality to that produced in mammalian cells. Our next goal was to increase the level of expression of the receptor in yeast. The strategy outlined in Fig. 1 indicated that several components of the system can be changed to increase expression levels. Physical changes to the plasmid such as the promoter, the origin of replication, the selectable marker or changes in the protocol for induction of the receptor are all possible. These parameters were evaluated systematically.

The only significant increase in production level followed the inclusion of a defective leu2 allele into our plasmid, creating the expression vector YEPE15. This strategy has previously been shown to increase plasmid copy number in strains defective in leucine biosynthesis as more of the plasmid is required to complement the cellular mutation [19]. The protocol has been used successfully to produce recombinant proteins [20]. The plasmid YEPE15 was transformed into the strain BJ2168 and grown under conditions that were auxotrophic for leucine. The cells were harvested, cytosol was prepared and receptor production was quantitated by Western immunoblot (Fig. 6). By comparison to known standards we estimate that this construct produces 5-fold more receptor up to a minimum level of 0.2% of the total soluble yeast protein.

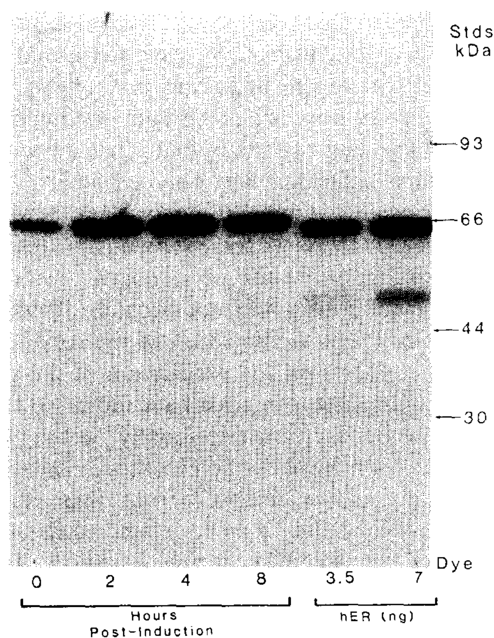

Fig. 6. Expression of human estrogen receptor (hER) in yeast using YEPE15. Cells were grown in minimal media and induced for the times indicated. The level of receptor produced was quantitated by Western immunoblot. The standards used for quantitation were isolated from CHO cells transformed with the human estrogen receptor cDNA.

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