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High level expression of full-length estrogen receptor in *Escherichia coli* is facilitated by the uncoupler of oxidative phosphorylation, CCCP

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Abstract

The expression of high levels of full-length human estrogen receptor α (hERα) in *Escherichia coli* has proven difficult. We found that expression of the ER DNA binding domain is highly toxic to *E. coli*, resulting in rapid loss of the expression plasmid. Using a tightly regulated arabinose expression system and the antibiotic Timentin, we were able to overcome ER toxicity and express substantial levels of ER. The expressed ER exhibited protease cleavage at a single site near the N-terminus of the hinge region. Of the many measures we tested to eliminate ER cleavage, only addition of carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP), an uncoupler of oxidative phosphorylation, completely blocked intracellular proteolysis of the ER. Using CCCP and our expression methods, full-length FLAG epitope-tagged hERα (fER) was expressed in *E. coli* at ~1 mg/l. The fER was purified to homogeneity in a single step by immunoaffinity chromatography with anti-FLAG monoclonal antibody. Purified full-length bacterial fER binds 17β-estradiol with the same affinity as hER expressed in human cells ($K_D \sim 0.5$ nM). At high concentrations of fER (20 nM), a bell-shaped estrogen binding curve with a Hill coefficient of 1.7 was seen. Bacterially-expressed fER exhibits a reduced affinity for the estrogen response element (ERE). Anti-FLAG antibody restores high affinity binding of the fER to the ERE, suggesting that impaired dimerization may be responsible for the reduced affinity of bacterially-expressed fER for the ERE. The use of Timentin and CCCP may provide a general method for high level bacterial expression of steroid/nuclear receptors and other proteins important in hormone action.

Keywords: Human estrogen receptor α (hERα); *Escherichia coli*; Carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP)

1. Introduction

Estrogens exert a wide variety of biological effects including effects on development and function of male and female reproductive tissues, bone remodeling and the cardiovascular system, and have been implicated in breast and uterine cancer [1–4]. The intracellular effects of estrogens are mediated primarily by the estrogen receptors, ERα and ERβ [2]. While a great deal has been learned about the actions of ER, fundamental questions remain. Since the initial cloning of the human estrogen receptor α (hERα) cDNA in 1986, there have been numerous attempts to express hERα in bacteria, yeast, baculovirus, and mammalian cells [5–14]. However, it has been difficult to produce the relatively large amounts of ER required for biochemical, biophysical and structural studies.

Although there have been a few reports in which recombinant ER was produced in bacteria, these involved use of fusion proteins [5], or the ER_{val400} mutant [6]. In these studies, and in one report on the expression of full length wild-type ER in *E. coli* [7], the level of expression of recombinant ER was quite low, and was no higher than the level of ER expression which occurs naturally in ER positive MCF-7, human breast cancer cells.

Our work on genetic selection of steroid receptor DNA binding domain mutants with altered DNA bind-

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ing specificity and enhanced affinity for the estrogen response element (ERE) suggested that expression of functional ER DNA binding domain might be toxic to *E. coli* [15]. This suggested that ER toxicity and loss of the ER expression plasmid, rather than the inability to fold the expressed ER, might be responsible for the low levels of ER expression in previous reports.

To overcome the toxicity of the ER, we developed a potentially general approach for expression of proteins that are toxic to *E. coli*. Although this enabled us to produce high levels of recombinant ER, much of the expressed protein was cleaved at a single protease-sensitive site near the center of the ER. After testing protease-deficient strains of *E. coli* and many other methods for interfering with protease activity, we found that treating the bacteria with the uncoupler of oxidative phosphorylation, carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP), completely blocked cleavage of the recombinant ER. These novel methods for expression of proteins toxic to *E. coli* and for inhibition of intracellular proteolysis enabled us to produce and characterize bacterially-expressed ER, and should find wide application in high level bacterial expression of steroid/nuclear receptors and many other proteins.

2. Materials and methods

2.1. Plasmids, bacterial strains, chemicals and media

Plasmid pAFE was constructed by subcloning the entire protein-coding region of the hERα cDNA with the FLAG epitope tag on its N-terminus [14] into the EcoRI/XbaI site of pBAD22 (a gift from Dr J. Pogliano, Department of Microbiology, University of Illinois, Urbana, IL, USA). The ER was mutated in the putative protease cleavage site (KRSKK) using the Quick-Change™ procedure (Strategene, La Jolla, CA, USA). Primer 1 was: CAAGCCCGCTCATGAGGTGGTGGAGGTACCGAGACAGCCTGGCC, and primer 2 was the complementary strand to primer 1. The mutated ER was then subcloned into the EcoRI/SphI site of pBAD22. The dimerization defective mutant ER (L508K, L509E) or ER (KE) [15] was constructed using the Quick-Change™ procedure (Strategene, La Jolla, CA, USA) and was subcloned into the EcoRI/XbaI site of pBAD22. The expression plasmid ER DBD (containing amino acids 180–262 of the human ER coding sequence) was constructed by insertion of a PCR generated product into the HindIII and NheI sites of the plasmid pET-21b(+) (Novagen, Madison, WI). The insert was amplified from the human ER coding sequence present in the CMV-hER plasmid [14] using the primers 5′-TCAGGATCCACCATGGCTAGCGACTACAAGGACGACGATGACAAGATGTACCCTAGGGGCAAGGAGACTCGCTACTGT-3′ and 5′-ATTGATAAGCTTGGATCCTTACTACCCTCCTCTTCGGTCTT-3′. *E. coli* strain SG12045 (ΔclpA, Δkan) was obtained from Professor M. Glazer, (University of Illinois, Urbana, IL). The 10× M9 medium contains: NH_4Cl 10 g, KH_2PO_4 30 g, Na_2HPO_4 67.81 g, H_2O to 1 l. TB contains tryptone 10 g/l, NaCl 5 g/l, water to 1 l, while 1 l of media for uninduced cells on agar plates (T9 agar) contains: TB 890 ml, 10× M9 100 ml, 1-M $MgSO_4$ 1 ml, 0.2% glucose, 0.2% fucose, 75 mg/l Timentin, 50 mg/l kanamycin and 15% agar. To further repress basal expression from the arabinose promoter prior to ER expression, 0.2% glucose and 0.2% fucose were used in T9 medium. In 1 l of media for induction of ER expression (T9 media) is: TB 890 ml, 10× M9 100 ml, 1% arabinose, 75 mg/l Timentin, 50 mg/l kanamycin. Timentin (Ticarcillin Na_2 and Clavulanate K) from Smith Kline Beecham, Philadelphia, PA was obtained by prescription. CCCP and CHAPs were from Sigma.

2.2. Preparation of cell extracts and immunopurification of fhER

E. coli SG12045 carrying plasmid pAFE were plated in T9 agar at 37°C overnight. Prior to induction the cells were scraped off the plate and resuspended in ~100 ml of T9 media to yield an OD_{600} of 0.5. Protein production was induced with arabinose for 1 or 3 h, and the cells were pelleted by centrifugation at 5000 rpm for 10 min at 4°C and resuspended in 2 ml of BZ300 (50 mM Tris, pH 7.9, 0.1 mM EDTA, 0.5 mM EGTA, 0.3 M KCl, 1 mM DTT, 50 mM $ZnCl_2$, 10% glycerol, 8 nM CHAPs, 50 ng/ml leupeptin, 5 ng/ml PMSF, 5 ng/ml pepstatin A, 0.5 ng/ml aprotinin) and vortexed for 15 min at 4°C. The bacteria were broken by three cycles of sonication and particulate material was sedimented by centrifugation at 46 000 rpm for 10 min at 4°C. The supernatant was retained and Polyethylenimine (Sigma) was added slowly to a final concentration 0.2%. The extract was subjected to centrifugation at 46 000 rpm for 30 min at 4°C and the supernatant was saved as the cell extract. For fER purification, an equal volume of BZ0 (the same buffer as BZ300, but without KCl) was added to the cell extract to adjust the salt concentration to 150 mM KCl and the diluted extract was applied to an anti-FLAG epitope immunoaffinity column (Anti-FLAG M2 affinity Gel, Kodak), at 50 μl of packed resin per 200 ml of extract. The column was subsequently washed five times with a total of 50 vol. of BZ300 and twice with BZ100 (the same buffer as BZ300, but containing 0.1 M KCl) and the fER was eluted with FLAG peptide (N-DYKDDDDK-C, 0.2 mg/ml) in BZ100.

2.3. Western blots

Whole cell extract containing fER or purified fER was analyzed by electrophoresis on a 10% glycine-SDS polyacrylamide gel and the proteins were electroblotted onto a nitrocellulose membrane. The membrane was probed with the anti-FLAG M2 monoclonal antibody (Kodak) (at a 1:2000 dilution) or ER-specific primary antibody D547, or H222 (very generous gifts from Professor G. Greene, University of Chicago, Chicago, IL, USA) at 0.12 mg/ml, incubated with horseradish peroxidase-conjugated secondary antibody (at a 1:2000 dilution) and detected by chemiluminescence with the ECL™ kit (Amersham).

2.4. Estrogen receptor ligand binding assays

In vitro estrogen binding assays were performed as we recently described [14]. Briefly, the ER was diluted into binding buffer (50 mM Tris, pH 7.5, 10% glycerol, 10 mM mercaptoethanol, 500 μg/ml BSA). The bound ligand was assayed by adsorption onto hydroxyapatite for 15 min at 4°C, followed by three washes with 1 ml 0.05 M Tris, pH 7.3. After the last wash, the pellet was resuspended in 0.5 ml of ethanol and counted in 5 ml of scintillation fluid.

2.5. Electrophoretic mobility shift assays

Electrophoretic mobility shift assays were carried out as we recently described [14]. Briefly, end-labeled ERE-containing probes (10 000 cpm/reaction) were combined with the indicated amounts of purified ER, and 500 ng/ml BSA, 10% glycerol, 75 mM KCl, 15 mM Tris–HCl, pH 7.9, 0.2 mM EDTA, and 0.4 mM dithiothreitol, and 3 mg of poly-dI/dC (Sigma) which was present as non-specific carrier DNA, in a volume of 20 μl and incubated at 25°C for 15 min. After probe addition, the reaction mixtures were incubated at 25°C for 15 min and subjected to low ionic strength 8% polyacrylamide gel electrophoresis with buffer recirculation using a water jacket to maintain the gel at 4°C. Gels were dried prior to autoradiography and free and bound forms of ERE and ER-ERE complex were quantitated by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

Table 1
Expression of the ER DBD is toxic to *E. coli*[a]

DNA	Number of surviving bacteria			
	IPTG			
	1 μM	10 μM	0.1 mM	1 mM
Vector	3×10^8	4×10^8	6×10^7	8×10^7
IHF	8×10^7	–	–	2×10^7
ER DBD	1×10^8	6×10^7	4×10^7	3×10^4

[a] *E. coli* were maintained on Amp plates, pooled colonies were scraped off the plate, incubated for 3 h in the absence of IPTG and then for 1 h in growth medium containing the indicated concentrations of IPTG. The number of surviving bacteria was determined by counting the number of colonies which grew out after plating the culture on plates containing 100 μg/ml Amp.

3. Results

3.1. Expression of the ER DNA binding domain is toxic to E. coli

Since our preliminary studies suggested that the ER DNA binding domain might be toxic to *E. coli*, we examined the number of viable *E. coli* present after inducing expression of the ER DBD for 1 h, and plating the surviving cells on plates containing the antibiotic, ampicillin (Amp). *E. coli* containing the empty vector, or expressing the control protein, integration host factor (IHF), remained viable (Table 1, vector, IHF). In dramatic contrast, using a high (1 mM) concentration of the inducer isopropyl thio galactoside (IPTG), so that the bacteria expressed high levels of the hER DBD, resulted in a >1000-fold reduction in the number of viable bacteria (Table 1, ER DBD). This high level of cell death was surprising since liquid cultures of bacteria containing the ER DBD expression plasmid appeared to grow quite well.

We therefore reasoned that the selection pressure imposed on the *E. coli* by low level expression of the highly toxic ER DBD might be more severe than the selection pressure imposed by the Amp in the medium. In this model, leakage through the uninduced promoter results in the production of enough toxic ER DBD during the growth of the bacteria to select for bacteria which have lost the ER DBD expression plasmid. These bacteria continue to grow in the antibiotic-containing Amp medium using penicillinase secreted by the small number of bacteria that retain the expression plasmid. To test this possibility, we maintained the uninduced *E. coli* in liquid culture in Amp-containing medium, and then determined the number of bacteria that retained the ER DBD expression plasmid by plating the bacteria on plates containing various concentrations of Amp. After only 5 h in liquid culture in the absence of the inducer IPTG, the number of bacteria retaining the plasmid was reduced >20-fold (Fig. 1, ▼). Overnight growth of the uninduced *E. coli* in Amp medium resulted in nearly complete loss of the expression plasmid (Fig. 1, ▽). In dramatic contrast, uninduced *E. coli* grown in liquid medium containing Timentin, a combination of ticarcillin and clavulanic acid, designed to prevent growth of bacteria using secreted penicillinase, showed essentially no loss of the expression plasmid

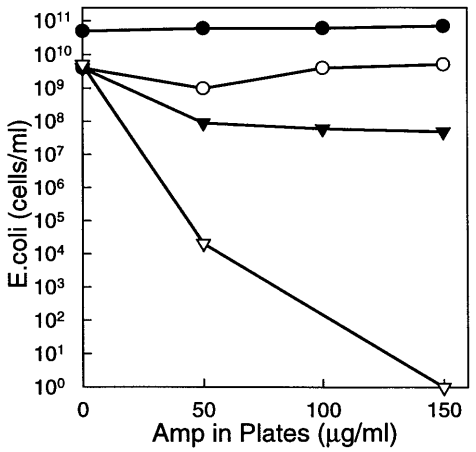

Fig. 1. The β-lactamase inhibitor, Timentin, prevents loss of the ER DBD expression plasmid. To test the effect of maintaining the cells in Amp or Timentin, colonies of *E. coli* transformed with either the empty vector, or with the ER DBD expression plasmid, were isolated from Amp plates and maintained in liquid culture in medium without inducer as follows: empty vector, 5 h in medium containing Amp at 100 μg/ml (○); ER DBD expression vector 5 h in Amp 100 medium (▼); ER DBD expression vector overnight in liquid culture in medium containing Amp at 150 μg/ml, diluted 1:100, grown for an additional 3 h in liquid in Amp 150 (▽); ER DBD expression vector overnight in liquid culture in medium containing Timentin at 50 μg/ml, diluted 1:100 and grown for an additional 3 h in medium in Timentin 50 (●). The bacteria were plated on LB plates containing no antibiotics, or the indicated amounts of ampicillin (50–150 μg/ml), and the number of viable bacteria able to form colonies was determined. It is likely that bacteria viable only in the presence of low concentrations of Amp have lost some, but not all of their plasmids, and produced reduced levels of β-lactamase.

(Fig. 1, ●). Preparation of DNA minipreps from bacteria maintained in Timentin or Amp containing medium confirmed the presence of the plasmid in bacteria from the Timentin medium, and the loss of the plasmid in nearly all of the bacteria maintained in the Amp medium (data not shown).

Since Timentin was not used in previous studies in which expression of ER in *E. coli* was attempted [5–7], it is possible that loss of expression plasmid was responsible for the low levels of ER expression seen in those studies. Because expression plasmids based on the lac repressor show considerable basal expression of the toxic ER DBD, even in the absence of inducer, we elected to use a tightly regulated arabinose promoter system [16,17]. In addition, we maintained the cells on plates whenever possible, rather than in liquid culture, and replaced the ampicillin in our media with Timentin. Using this combination of approaches, to maintain cell viability and minimize plasmid loss, we expressed full-length hER in *E. coli*.

3.2. ER expressed in E. coli is subject to proteolytic cleavage

To facilitate the detection and purification of hER, the FLAG epitope-tag was fused to the N-terminus of the hER cDNA [14]. This FLAG-tagged hER (fER) was subcloned into an expression plasmid based on the arabinose promoter pBAD 22 [16,17], to yield the fER expression plasmid pAFE (Fig. 2A). *E. coli* were transformed, with the pAFE plasmid, maintained on Timentin-containing plates before induction, scraped off the plates and induced with arabinose for 3 h in liquid culture. Western blotting of ER-containing bacterial extracts with anti-FLAG monoclonal antibody, and with the ER specific monoclonal antibody D547, revealed full-length 67-kDa ER and two bands at 32–35 kDa (Fig. 2B). The band detected by anti-FLAG antibody was slightly larger (~ 35 kDa) than the band detected by D547, (~ 32 kDa). Since anti-FLAG antibody detects polypeptides containing the N-terminal FLAG epitope, and D547 recognizes an epitope in the C-terminal E–F domains of ER [18], the Western blotting results indicate that there is a specific protease-sensitive site near the middle of the ER, and that this site is probably in the hinge region. Cleavage of the ER at

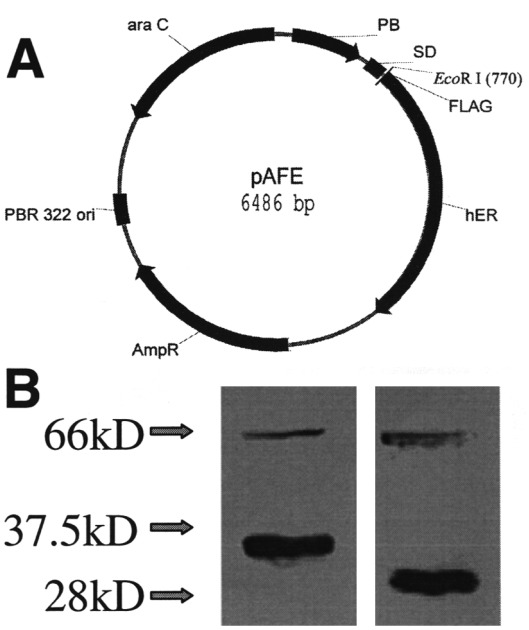

Fig. 2. Expression of ER in *E. coli*. (A) A schematic representation of the ER expression vector. FLAG-tagged ER was subcloned into the pBAD22 vector, under the control of pBAD promoter. Prior to induction, the colonies of transformed *E. coli* were scraped off the plates, diluted into T9 media containing Timentin at 50 μg/ml and ER expression was induced with 1% arabinose at 37°C for 3 h. (B) Western blot of a whole cell extract containing fER. The whole cell extract was fractionated by SDS–PAGE and analyzed by Western blotting as described in Section 2 using as primary antibody the anti-FLAG M2 monoclonal antibody (lane 1), or the ER-specific monoclonal antibody D547 (lane 2).

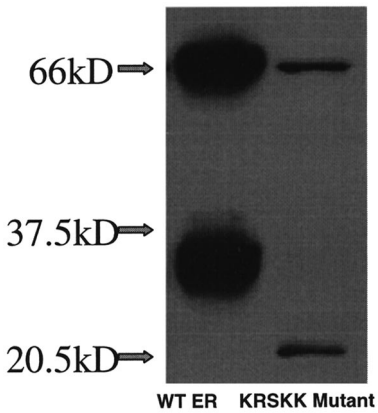

Fig. 3. The ER-GGGG mutant shows a different cleavage pattern than wild-type ER. *E. coli* were transformed with either the plasmid encoding wild-type fER or the KRSKK →GGGG mutant, induced as described in the legend to Fig. 2, and 10 μg of whole cell extract from bacteria expressing either the wild-type fER (WT ER) or the KRSKK→GGGG mutant (KRSKK mutant) was analyzed by Western blotting as described in Section 2, using as primary antibody the anti-FLAG M2 monoclonal antibody.

this site is only seen in *E. coli* extracts, and is not seen when ER is expressed in HeLa cells [14]. Since ER in extracts from transiently transfected COS cells incubated with crude extracts from *E. coli* was not cleaved (data not shown), it seemed likely that cleavage of the ER was occurring in the intact *E. coli*, not during the preparation of the bacterial extracts.

We used a number of strategies to eliminate the problem of protease cleavage. We tested a dozen protease deficient *E. coli* K-12 strains as well as *Salmonella*, and identified *E. coli* strain SG12045 as producing the highest ratio of full-length ER relative to the cleavage products. We also tested numerous combinations of protease inhibitors, in vivo and in vitro, growing the bacteria at 15 or at 30°, which have been used by others to reduce proteolysis [19–21], and varying the level of inducer and the time of induction. Protease inhibitors, varying the growth temperature, changing the level of arabinose inducer and the time of induction had only a modest impact on the level of ER proteolysis.

Since these efforts did not eliminate proteolysis of the recombinant ER, and cleavage was at a single site, we tried to alter this site by mutation. Based on the size of the two cleavage products on Western blots, previous studies on proteolysis of the ER ligand binding domain and hinge region [22], and information on protease cleavage sites, we identified a trypsin-like cleavage site at amino acids 300–304 (KRSKK) of the hER as the putative cleavage site. Since these amino acids were not conserved in ERs from different species, we mutated the KRSKK sequence in the human ER to the GGGG sequence present in rainbow trout ER [23]. While these mutations abolished the original cleavage pattern, suggesting we had correctly located the cleavage site, a new cleavage product in the A/B domain, 15–20 kDa from the N-terminus of the ER appeared (Fig. 3). Since mutation of the putative cleavage site produced an altered cleavage pattern, and reduced the level of expression of the mutated recombinant ER, we elected to pursue a different strategy for expression of the ER.

3.3. CCCP blocks cleavage of bacterially-expressed ER

In vertebrate cells, ER is thought to associate with heat shock proteins during and after its synthesis [2]. We therefore tested the idea that induction of heat shock proteins might either protect the ER from cleavage, or assist in folding the ER so that it was less susceptible to protease cleavage. Since the heat shock proteins are really stress response proteins, we used several different methods to stress the cells and induce stress response proteins and heat shock proteins [24–29]. Heat shock (42°C, for 5 min), ethanol, or the protein synthesis inhibitor chloramphenicol did not block proteolysis of the ER (Fig. 4A, lanes 4–6). In contrast, treating the bacteria with the uncoupler of oxidative phosphorylation, CCCP, completely blocked cleavage of the ER, resulting in a single full-length ER band (Fig. 4A, lane 2). Using immunoaffinity chromatography with anti-FLAG M2 antibody, hER was purified to apparent homogeneity from *E. coli* treated with CCCP (Fig. 4B, lane 3). The level of fER expression was ~ 1 mg/l.

3.4. ER expressed in E. coli binds 17β-estradiol with high affinity

We carried out ligand binding assays to determine the affinity of the bacterially-expressed hER for 17β-estradiol (E_2). At 1 nM ER, a typical ER concentration used in in vitro ligand binding assays, the purified bacterially-expressed ER, exhibits a linear Scatchard plot and binds E_2 with a K_D of 0.53 nM (Fig. 5A), which is similar to the K_D we recently reported for hERα expressed in HeLa cells [14]. The larger quantities of purified ER available as a result of expression of the ER in *E. coli* enabled us to examine the binding of E_2 to ER in solutions containing high concentrations of ER. At 20 nM ER, we observed a bell-shaped binding curve (Fig. 5B), with a Hill coefficient of 1.7 (Fig. 5C). The Hill coefficient >1 suggested cooperative interactions between ER monomers.

To test the possibility that the bell-shaped curve results from the dimerization of bacterially-expressed ER at high concentrations, we used a dimerization defective ER mutant ER (L508K, L509E) or ER (KE) [15]. The dimerization defective mutant ER (KE)

yielded a similar bell-shaped binding curve (data not shown). It has been proposed that bivalent antibodies bound to ER monomers facilitate ER dimerization [15,30]. We therefore tested the ability of the anti-FLAG monoclonal antibody to affect E_2 binding by the wild-type fER, and by the dimerization defective fER (KE). The resulting E_2 binding curves remained linear at low concentrations (Fig. 6). These data strongly suggest that the bell-shaped binding curve and the Hill coefficient >1 are not due to dimerization of ER monomers when the ER is present at high concentrations.

3.5. Bacterially expressed ER binds to the ERE

Purified fER, expressed in bacteria, binds to the ERE with no requirement for other eukaryotic proteins (Fig. 7A). At lower concentrations of fER, there was no detectable binding to the ERE in the electrophoretic mobility shift assays unless the anti-FLAG monoclonal antibody was added (Fig. 7B). The striking stimulation of ERE binding by ER antibody was not specific for the anti-FLAG antibody, since the H222 monoclonal antibody to ER also strongly enhanced binding of the fER to the ERE (data not shown). Since the anti-

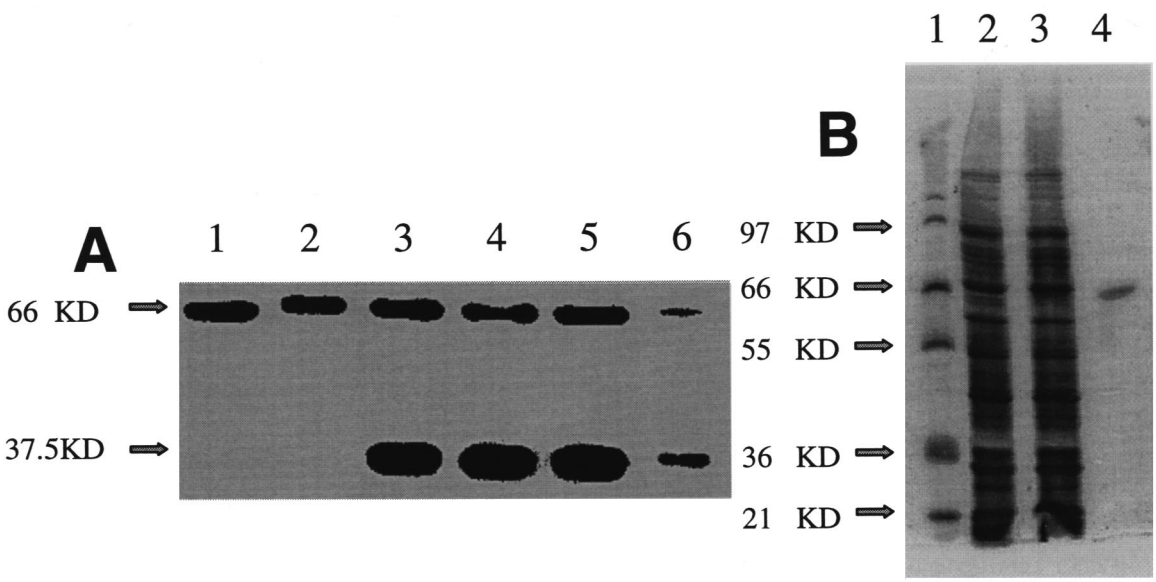

Fig. 4. Treating viable *E. coli* with the uncoupler CCCP, prevents protease cleavage of ER. (A) ER was expressed in *E. coli* SG12045 and subjected to: 5 mM CCCP (lane 2), no treatment (lane 3), heat shock (42°C for 5 min, lane 4), 3% ethanol (lane 5), or 37.5 μg/ml chloramphenicol (lane 6). Lane 1 is a marker of fER from HeLa cells. The extracts were fractionated by SDS–PAGE, and analyzed by Western blotting using anti-FLAG M2 antibody. (B) 5 μg of protein from uninduced (lane 2) and induced cell extracts (lane 3), and 10 ng of purified fER (lane 4) were fractionated by SDS–PAGE and proteins were visualized by silver staining.

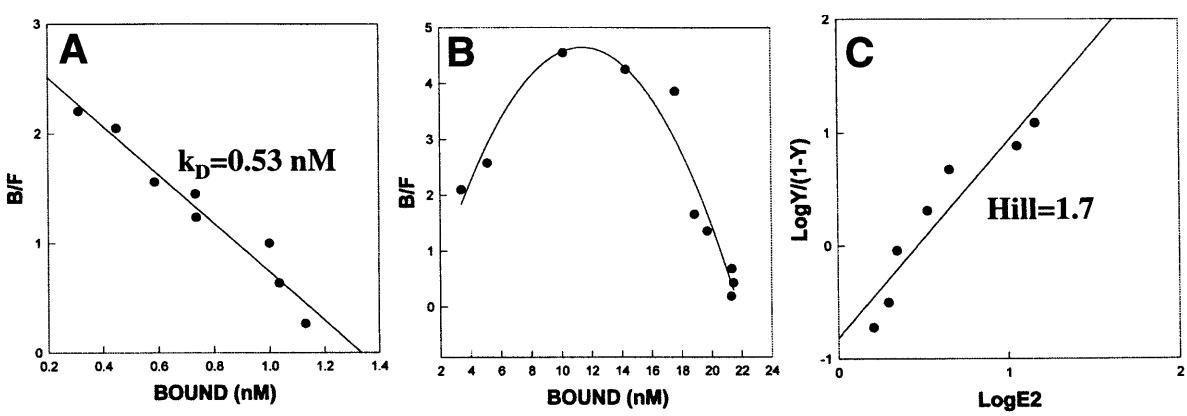

Fig. 5. Bacterially-expressed fER exhibits high affinity binding to 17β-estradiol. In panels A and B bound [^{3}H]estradiol was assayed by adsorption onto hydroxylapatite and quantitated by scintillation counting as described in Section 2. (A) Cell extracts containing ~ 1 nM fER were incubated with increasing amounts of [^{3}H]estradiol in the presence or absence of unlabelled estradiol. (B) Cell extracts containing 20 nM fER were incubated with increasing amounts of [^{3}H]estradiol in the presence or absence of unlabelled estradiol. (C) Analysis of cooperative interactions using a Hill plot. Calculation of the Hill coefficient: E_2, the concentration of total E_2. Y = bound [E_2]/total [E_2].

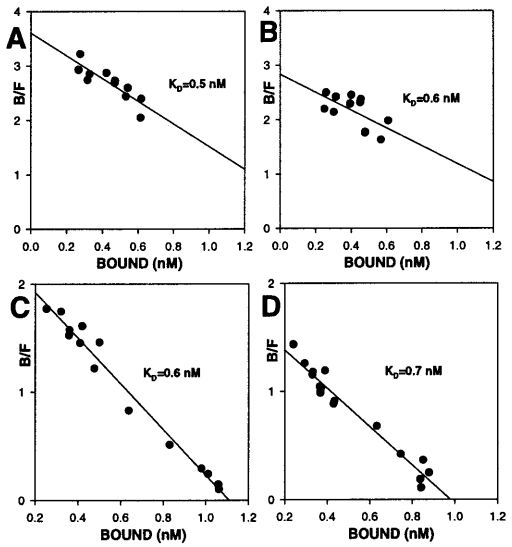

Fig. 6. Anti-FLAG M2 antibody does not affect the binding of fER to 17β-estradiol. Bound [^{3}H]estradiol was assayed by adsorption onto hydroxylapatite and quantitated by scintillation counting as described in Section 2. (A, B) Cell extracts containing ~1.5 nM wild-type fER were incubated with increasing amounts of [^{3}H]estradiol in the presence or absence of unlabelled estradiol and in the absence (panel A) or presence (panel B) of 20 ng/μl of anti-FLAG monoclonal antibody M2. (C, D) Cell extracts containing ~1 nM dimerization defective fER (KE), were incubated with increasing amounts of [^{3}H]estradiol in the presence or absence of unlabelled estradiol and in the absence (panel C) or presence (panel D) of 20 ng/μl of anti-FLAG monoclonal antibody M2.

FLAG antibody recognizes the N-terminal FLAG epitope and H222 recognizes an epitope near the C-terminus of the ligand binding domain [18,31], it is the presence of a bivalent ER antibody, rather than the epitope recognized by the antibody, which is responsible for enhanced binding of the fER to the ERE.

4. Discussion

4.1. Bacterially-expressed ER may be defective in dimerization

The bacterially-expressed FLAG epitope tagged ER exhibited impaired binding to the ERE. Since FLAG-ER expressed in HeLa cells binds to the ERE with the same nanomolar affinity as ER without the FLAG epitope [14], the N-terminal FLAG epitope is not responsible for the reduced binding of the bacterially-expressed fER to the ERE. Since we recently reported that fER, expressed in HeLa cells and purified to near homogeneity, binds to the ERE with the same high affinity as fER in crude HeLa cell nuclear extracts [14], the absence of auxiliary proteins is unlikely to be the cause of the impaired ERE binding exhibited by the purified bacterially-expressed fER.

Dimerization plays an important role in binding of the ER DBD, and of the full length ER to the ERE [15,30,32]. Since mammalian fER occupies both ERE half sites in gel shift assays, and the complex of the ER-ERE complex seen with ER of bacterial or mammalian origin exhibits similar electrophoretic mobility in gel shift assays (data not shown), the bacterially-expressed fER bound to the ERE occupies both ERE half sites. The ERE half sites could be occupied nearly simultaneously through binding of an ER dimer, or by sequential binding of two ER monomers, followed by stabilization of monomer binding on the ERE through formation of a DNA dependent dimerization interface (discussed in Refs. [15,32]). Two observations suggest that the bacterially-expressed ER is defective in dimerization. (i) Much higher levels of the bacterially-expressed ER are required to bind to the ERE than mammalian ER. (ii) Bivalent antibodies to ER strongly stimulate binding of the bacterially-expressed ER to the ERE (Fig. 6B), but have little or no effect on binding of mammalian ER to the ERE [14]. A previous report by M.G. Parker et al. using a dimerization-impaired ER mutant demonstrated a strong enhancement of ERE binding on interaction of the ER with bivalent ER antibodies [30]. Using the ER DBD, which is a monomer in solution [33,34], we showed that FLAG antibody strongly (> 5-fold) stimulated binding of the ER DBD monomers to the ERE [32]. The strong stimulation of ERE binding seen with two quite different antibodies (anti-FLAG M2 and H222) suggests that the bacterially-expressed ER is specifically defective in dimerization, rather than exhibiting a structural defect that impairs its function.

Our finding that high concentrations of ER resulted in a bell-shaped binding curve and a positive Hill coefficient extends an earlier report in which high concentrations of ER expressed in a baculovirus system exhibited a partial bell-shaped curve for E_2 binding [9]. While these data initially suggested that the bell shaped curve and evidence of cooperative binding might be due to ER monomers dimerizing at high ER concentrations, our data does not support this view. First, E_2 binding by a dimerization deficient ER mutant [15] was the same as binding by wild-type ER. Second, antibodies to ER did not change the shape of the ER binding curve (Fig. 6), suggesting that altering dimerization had little effect. Additional experimentation will be required to explain this intriguing binding phenomenon.

Full length bacterially-expressed ER exhibited the same affinity for E_2 (~0.5 nM) as ER expressed in mammalian cells and baculovirus [14,35]. This data is consistent with earlier studies in which ER ligand binding domain expressed in *E. coli* exhibited high affinity binding to E_2 [36]. With the availability of substantial quantities of purified full length ER, recent studies of the effects of coactivators binding to the ER ligand

binding domain (LBD) on hormone binding to the LBD [37] can now be extended to the more biologically relevant context of full-length ER.

4.2. ER expression is toxic to E. coli

Our finding that the expression of the ER DNA binding domain is highly toxic to *E. coli* was surprising. Since bacteria do not contain steroid hormones, steroid/nuclear receptors, or genes that are normally regulated by steroid hormones, the reasons for this toxicity were initially obscure. Because *E. coli* does not contain ER, there has been no selection against the presence of consensus EREs (cEREs) in positions in which binding of ER would be highly toxic. A search of the *E. coli* genome revealed the presence of nine cEREs. Most of these cEREs are in protein coding sequences and might not interfere with transcription, because the transcriptional apparatus is probably able to displace ER bound in a transcribed open reading frame. However, two of the cEREs are in potential regulatory regions where they are likely to interfere with regulated expression of downstream genes. While nuclear receptor DBD toxicity has not received much attention in *E. coli*, a previous report demonstrated that expression of a DNA binding form of nuclear receptor NGFI-B kills yeast cells by binding to a genomic recognition sequence [38]. Consistent with those observations are our findings that ER DBD mutants which have lost the ability to bind to the ERE show a greatly reduced toxicity (data not shown). Despite the apparent absence of ER ligands in *E. coli*, at high concentrations, unliganded ER has been reported to bind to intracellular EREs [39,40]. While binding of the ER to genomic EREs is likely to be the primary cause of the toxicity of ER in *E. coli*, interaction of ER with bacterial proteins has not been excluded as an additional mechanism of ER toxicity.

Because of the toxicity of the ER, when we used standard expression systems based on the lac repressor/operator, or on T7 RNA polymerase, to express full-length ER in bacteria grown in liquid culture using ampicillin, we saw little or no recombinant ER. We believe that the level of ER expression from these promoters, even when they are uninduced, is sufficient to greatly slow or stop the growth of the bacteria, and that bacteria which have lost the expression plasmid and continue to grow slowly using secreted pencillinase present in the medium, rapidly overgrow the culture. The three steps we describe to eliminate this problem: (i) employing the little-used antibiotic Timentin in place of ampicillin; (ii) using a tightly regulated arabinose promoter; and (iii) maintaining the cells on plates whenever possible, provide a potentially general approach to expression of other toxic proteins in *E. coli*.

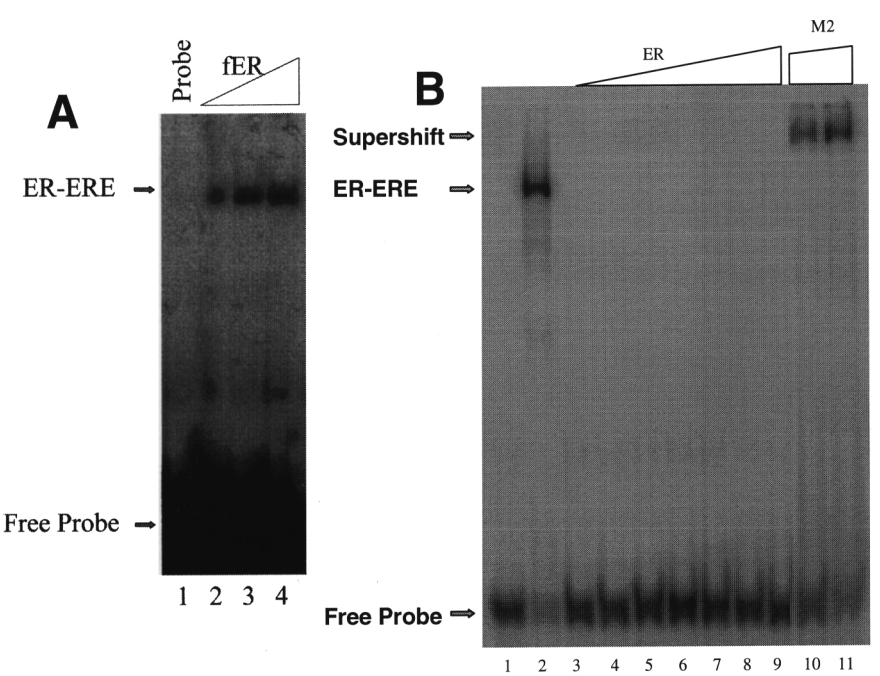

Fig. 7. Bacterially-expressed fER binds to the estrogen response element. (A) Binding of purified bacterially-expressed fER to the labeled ERE was analyzed by electrophoretic mobility shift assay. Increasing concentrations of purified fER (40, 60, 80 ng in lanes 2, 3, and 4, respectively) were used. (B) Anti-FLAG monoclonal antibody greatly enhances binding of the bacterially-expressed fER to the ERE. The ER standard was 1 ng of fER in a nuclear extract from HeLa-ER1 cells ([14]; lane 2). Lanes 3–9 contain 0.08, 0.16, 0.32, 0.8, 1.6, 3.2, 8 ng of bacterially-expressed fER, respectively. In lane 10 and 11, 0.3 μg of anti-FLAG monoclonal antibody M2 was incubated with 3.2 or 8 ng of fER, respectively, and used to show that antibody supershifts the complex and dramatically increases binding of bacterially-expressed fER to the ERE.

4.3. CCCP protects ER from protease cleavage

Perhaps the most surprising observation we report is the ability of the uncoupler CCCP to protect ER from protease cleavage. Heat shock, uncouplers of oxidative phosphorylation, ethanol, and chloramphenicol all induce the expression of specific sets of proteins, including heat shock proteins, chaperones and proteases [24–29]. Although ethanol, which induces certain heat shock proteins, has a positive effect on expression of some recombinant proteins [41], in many cases heat shock and other stresses induce, rather than prevent, proteolysis [19]. Whether the HSPs protect ER against proteolysis during synthesis by speeding up folding, or work postranslationally, is not known. The ability of CCCP to protect ER is quite specific as several other stressors, including heat shock, do not confer protection. Based on the yield of ER in the presence and absence of CCCP, and the failure of heat shock and other stressors to stimulate ER degradation, we consider it highly unlikely that CCCP actually works by stimulating the degradation of proteolytically cleaved ER, resulting in degradation of the two ER cleavage products to fragments too small to be seen in our Western blots.

While the identity of the protein(s) induced by CCCP that protect ER from cleavage is not known, it seems likely that CCCP induces a subset of HSPs which mimic the action of the mammalian HSPs [42–45]. CCCP is likely to elicit a complex pattern of responses, since the uncoupler, 2,4-dinitrophenol (DNP), increases the synthesis rates of 53 proteins [25], including most of the heat shock proteins, as well as other non-heat shock proteins. Whatever the mechanism of CCCP action, this is the first report showing that an uncoupler can protect a bacterially-expressed recombinant protein from degradation.

There have been few reports of the expression of high levels of recombinant steroid/nuclear receptor superfamily members in *E. coli*. While we recently reported the isolation of small amounts of ER from HeLa cell lines stably expressing ER [14], the yield of ER from the bacterial expression system we describe is >100-fold higher than is obtained from the ER-expressing HeLa cells. Although substantial amounts of ER can be produced using the baculovirus expression system, we recently reported that ER expressed in baculovirus exhibits a greatly reduced ability to bind to the ERE [14]. In addition, baculovirus expression requires time-consuming isolation and propagation of virus stocks, and is therefore unsuitable for expression of large numbers of ER mutants. The techniques we describe should greatly extend the spectrum of steroid/nuclear receptors that can be expressed at high levels in *E. coli*.

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