A single-stranded DNA-binding protein promotes the binding of the purified oestrogen receptor to its responsive element

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Received July 4, 1990; Revised and Accepted September 3, 1990

ABSTRACT

The purified human oestrogen receptor (hER) does not form a detectable complex with an oestrogen responsive element (ERE) under conditions where hER-ERE complexes are readily formed with crude extracts from Hela or yeast cells expressing the hER. This indicates that other factor(s) are necessary for ER-ERE binding. Such a ER DNA binding stimulatory factor (DBSF) has been purified from the yeast Saccharomyces cerevisiae. It is a 45 kDa single-stranded DNA-binding protein (SSB) which cannot be substituted for by the purified E.coli SSB.

INTRODUCTION

The effect of steroid hormones on gene transcription is mediated by specific intracellular receptors that function as hormone-inducible enhancer factors (1, 2 and refs therein). The human estrogen receptor (hER) activates gene transcription by binding as a dimer to cognate palindromic DNA sequences called estrogen responsive elements (ERE) (3, 4 and refs therein). Until now it was unknown whether another protein(s) was involved in this binding.

We expressed and purified the human estrogen receptor from HeLa cells using a vaccinia-virus vector. We found that whereas hER-ERE complexes easily formed with crude extracts from HeLa or yeast cells expressing the hER (3, 5 and 6), the purified hER did not form a detectable complex with an ERE under similar conditions. This indicates that other factor(s) are necessary for ER-ERE binding. We report in this paper the purification of one such DNA binding stimulatory factor (DBSF) from yeast.

MATERIALS AND METHODS

Purification of the human estrogen receptor

hER was expressed in HeLa cells using a vaccinia virus expression vector (to be published elsewhere). Whole cell extracts (WCE's) of infected cells were made by Dounce homogenization in buffer A (20 mM HepespH 7.9, 0.1 mM EDTA, 0.5 mM DTT, 20% glycerol, 1 mM PMSF, 2.5 μg/ml of each Leupeptin, Pepstatin, Chymostatin, Antipain and Aprotinin, and 100 nM estradiol) containing 400 mM KCl. After centrifugation and dilution with an equal volume of buffer A the crude extract was loaded onto a Heparin-Ultrogel column and the receptor eluted with buffer A containing 400 mM KCl. After dilution with an equal volume of buffer A, the fractions containing the receptor were loaded onto a sequence-specific DNA affinity column and step-eluted with buffer A containing 400 mM and 800 mM KCl. The DNA affinity column was made by polymerizing synthetic double-stranded oligonucleotides containing ERE sequences which were bound to activated Sepharose.

Purification of yeast DBSF

Yeast transformed with the plasmid pTG848 (see ref. 5) was grown to an OD_{600} of 10 in a six liter fermentor (Biolafitte) in 0.6% nitrogen base, 0.5% casamino acid and 2% glucose. The cells were harvested and washed twice with ice cold 1× PBS. All subsequent manipulations were done at 4°C. The cells were suspended in 200 ml of 400 mM KCl in buffer C (buffer A of Fig. 1 minus oestradiol) and lysed in a bead beater (Biospec). The lysate was first centrifuged at 10,000 rpm in a Sorvall SS34 rotor for 30 min and then in a Beckman Ti60 rotor at 50,000 rpm for 1 hr. The salt concentration of the supernatant WCE (1 gm of protein) was reduced to 50 mM KCl by diluting with buffer C and loaded on a 120 ml DE52 column (Whatman) equilibrated with 50 mM KCl in buffer C. The column was washed with buffer C containing 50 mM KCl, and the DBSF activity which was present in the flow through (700 mg protein) was loaded on a 40 ml Heparin-Ultrogel column (IBF) equilibrated with 50 mM KCl in buffer C. After washing with the same buffer, bound proteins were eluted with a linear 50 to 800 mM KCl gradient in buffer C. Active fractions eluting from 150 to 230 mM KCl (76 mg protein) were pooled, diluted with an equal volume of buffer C, and loaded onto a 12 ml Phosphocellulose column (P11, Whatman) equilibrated with 100mM KCl in buffer C. After washing in the same buffer, the bound proteins were eluted with a linear 100 to 1000 mM KCl

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gradient in buffer C. Fractions eluting from 100 to 210 mM KCl were pooled (20 mg protein), made 50 mM KCl by dilution with buffer C, and loaded onto a 0.5 ml single-stranded DNA cellulose column (Sigma). After washing the column with 20 column volumes of 50 mM KCl in buffer C, the bound proteins were stepwise eluted with 400 mM KCl and 800 mM KCl in buffer C. The active DBSF fractions were stored in aliquots at −80°C.

Glycerol gradient centrifugation of DBSF fractions

DBSF fractions from the phosphocellulose column were pooled and centrifuged in a 4 ml 10−30% glycerol gradient at 60,000 rpm (SW60 rotor) for 18 hrs at 4°C. 200 μl fractions were collected.

Gel retardation assays

Gel retardations were performed basically as in ref. 3 using the [^{32}P] probes shown in Fig. 1C except 1 μg dIdC was used per reaction. The HeLa WCE used in Fig. 1b, lanes 7, 8, 12 and 13 was prepared from non-infected cells by Dounce homogenization in buffer A containing 400 mM KCl (minus estradiol). The yeast WCE was made as in ref. 5 from yeast transformed with plasmid pTG848.

Western blots

Western blots were performed by standard techniques using a rabbit polyclonal antibody directed against the first 282 amino

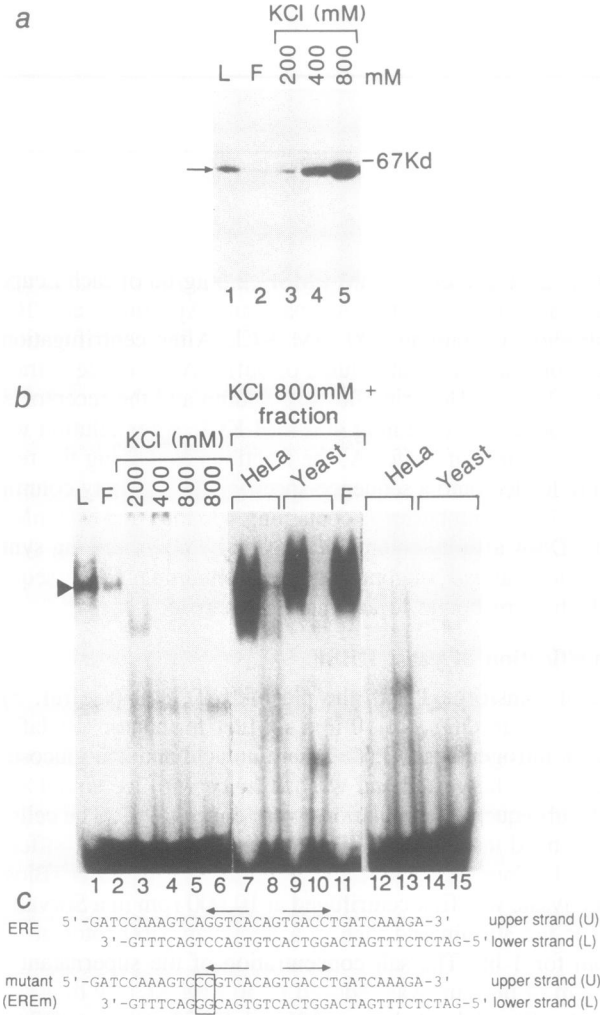

Figure 1. Purified human estrogen receptor gives no detectable complex with the ERE. Purification of hER by sequence-specific DNA affinity chromatography (SDAC). (a) Western blot indicating the relative amount of receptor in the various fractions. The amount of protein electroblotted was: lane 1, Heparin-Ultrogel step (L), 20 μg; lane 2, flow through fraction (F) of the SDAC, 20 μg; lane 3−5, 10, 20 and 20 μg of the 200, 400 and 800 mM fractions of the SDAC, respectively. The arrow points to the position of the hER. (b) Gel retardation assay using the above fractions as indicated. The amount of protein was : lane 1, 1 μg; lane 2, 1 μg; lane 3, 0.4 μg; lane 4, 1 μg; lanes 5 and 6, 1 μg. For lanes 7−11, 10 μg of either HeLa WCE, yeast WCE, or 2 μg of the flow through fraction (F) was added to affinity-purified receptor (1 μg protein) from the 800 mM KCL step. Lanes 12 and 13 correspond to 10 μg of HeLa WCE, and lanes 14 and 15 to 10 μg of yeast WCE, in the absence of hER. The labelled oligonucleotide was ERE in lanes 1−5, 7, 9, 11, 12 and 14, and EREm in lanes 6, 8, 10, 13 and 15. The arrowhead indicates the position of the retarded ER-ERE complex. (c) Sequence of the ERE and EREm double-stranded oligonucleotides used for gel retardation assays (see ref. 5). The mutated bases in EREm are boxed.

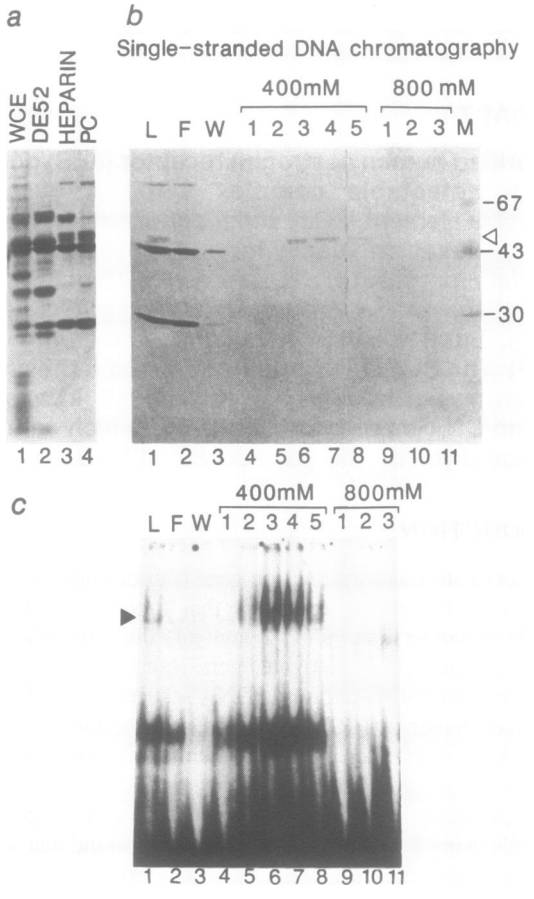

Figure 2. Purification of the hER DNA-binding stimulatory factor (DBSF) from yeast cells. (a) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the proteins at the various stages of purification. Aliquots of the WCE and active fractions of the DEAE-cellulose (DE52), Heparin-Ultrogel (Heparin) and phosphocellulose (PC) stages (as indicated) were electrophoresed and silver-stained according to standard protocols. Amounts of protein were : lane 1, 9 μg; lane 2, 9 μg; lane 3, 5 μg; lane 4, 1.8 μg. (b) SDS-PAGE analysis of single-stranded DNA chromatography fractions : lanes 1 and 2, 2.4 μg; lane 3, 0.6 μg; lane 4, 0.05 μg; lane 5, 0.10 μg; lane 6, 0.6 μg; lane 7, 0.5 μg; lane 8, 0.2 μg; Lanes 9−11, 0.05 μg. L, is the phosphocellulose fraction loaded, F and W are the flow-through and wash fractions, respectively. (c) Gel retardation assay using aliquots of the various fractions from the single-stranded DNA cellulose column. 12 μl aliquots (lanes 1, 2 and 3) or 3 μl aliquots from the 400 mM and 800 mM KCl fractions were used in a gel retardation assay with 60 ng of purified hER (800 mM KCl fraction in Fig. 1a). Gel retardation assays (panel c) were performed as in Fig. 1 with the double stranded ERE as probe. The open arrowhead in panel b indicates the 45 kDa protein eluted from the single-stranded DNA column, and the filled arrowhead in panel c indicates the position of the retarded hRE-ERE complex.

acids of the hER fused to β-galactosidase (a gift from D. Metzger).

RESULTS AND DISCUSSION

Whole cell extracts of HeLa cells infected with a vaccinia virus vector harbouring the hER cDNA were used as a source of hER (0.1% of WCE proteins). The hER was purified by chromatography on a Heparin-Ultrogel column followed by sequence-specific DNA affinity chromatography. Fig. 1a shows a western blot of the various fractions eluted from the DNA affinity column. The majority of the hER eluted in the 800 mM KCl step (Fig. 1a). This represented a ≃20-fold purification with respect to the Heparin Ultrogel fraction (L) loaded on the affinity column (Fig. 1a, compare lanes 1 to 5), and an overall 80-fold purification with respect to the crude WCE. Gel retardation assays (Fig. 1b) were carried out using aliquots from the DNA affinity chromatography fractions and a [^{32}P]-labelled ERE (Fig. 1c). Surprisingly, no protein-DNA complex was formed between the affinity purified hER and the ERE (lanes 4−5), while such a complex was clearly formed with the less pure Heparin Ultrogel fraction (lane 1; note that equal amounts of protein were used in the reactions shown in lanes 1 and 5). Similarly no ER-ERE complex was formed using hER expressed in yeast (5, 6) and affinity-purified (data not shown).

Thus protein(s) which were separated from the hER during affinity-chromatography may be required for the formation of an hER-ERE complex detectable by a gel retardation assay. Accordingly, the DNA affinity-purified receptor present in the 800 mM KCl fraction formed a complex with the ERE upon addition of a HeLa or yeast WCE (Fig. 1b, lanes 7 and 9). These complexes were specific for the hER, since they were greatly reduced when a mutant ERE (EREm, Fig. 1c) was used as a probe (Fig. 1b, lanes 8 and 10), and HeLa and yeast WCEs gave no specific complex on their own (lanes 12−15). Moreover, addition of an aliquot of the DNA affinity column flow-through (F) to the purified hER also resulted in the formation of a specific ER-ERE complex (lane 11). Note that, no hER-ERE complex was formed with the 800 mM KCl fraction without added WCE even when polydIdC was omitted from the reaction (data not shown).

The above results indicate that a DNA binding stimulatory factor(s) (DBSF), enabling the purified hER to bind to an ERE, is present in both HeLa and yeast WCEs. Since hER is known to function in yeast (5,6), and because yeast cells are easier to grow than HeLa cells, we purified the DBSF activity from a yeast WCE which was fractionated on DEAE-cellulose (DE52), heparin Ultrogel (Heparin), phosphocellulose (PC) and single-stranded (SS)-DNA-cellulose columns (Fig. 2a and b). The fractions were tested using the gel retardation assay with purified hER from the 800 mM KCl DNA affinity step (Fig. 1a), as illustrated in Fig. 2c for the SS-DNA-cellulose column. An hER-ERE complex was present in lanes 5 to 8, coinciding precisely

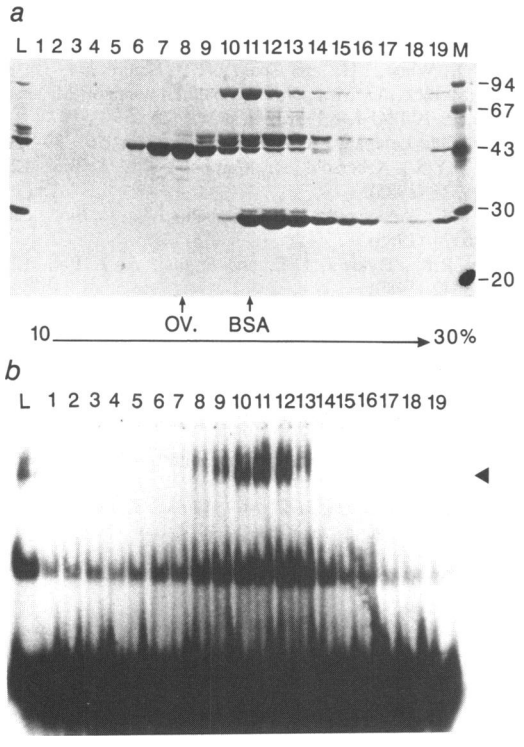

Figure 3. The yeast DBSF exists as a dimer in solution. Glycerol gradient centrifugation of the phosphocellulose fractions (Fig. 2a) containing the DBSF activity. (a) 70 μl of each fraction (1 to 19) was TCA- precipitated, electrophoresed on SDS-PAGE and stained with silver. L corresponds to an aliquot of the phosphocellulose fraction loaded on the glycerol gradient. OV and BSA indicate the position of ovalbumin and BSA as determined by SDS-PAGE and silver staining from an identical gradient run in parallel. (b) 10 μl of each fraction was used in a gel retardation assay with 60 ng of affinity-purified hER. Gel retardation assays were performed on each fraction (1−19) as indicated in Fig. 1, using the double-stranded ERE probe (panel b). The arrowhead indicates the position of the related hER-ERE complex.

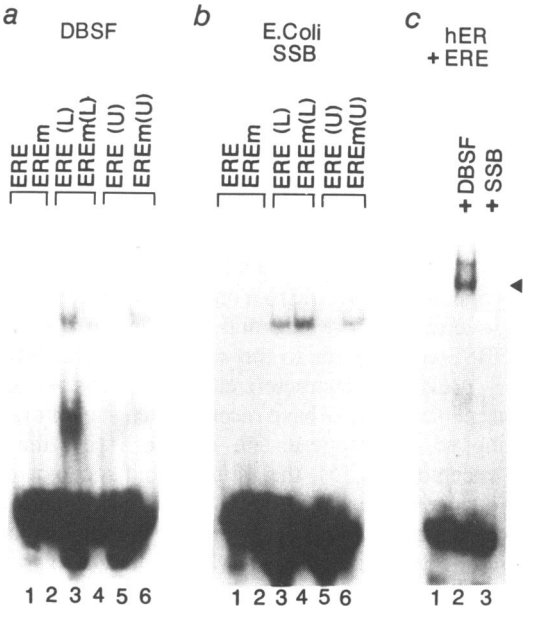

Figure 4. Purified E. coli single-stranded DNA binding protein (SSB) does not stimulate binding of purified hER to the ERE. Gel retardation assays were performed using 0.1 μg of purified yeast DBSF (single-stranded DNA chromatography step) (panel a) or 0.2 μg of pure E. coli SSB (Pharmacia) (panel b). In panel (c) gel retardation assays were performed with 60 ng of purified hER alone (lane 1), or in the presence of 0.1 μg of purified DBSF (lane 2), or 0.2 μg of pure SSB (lane 3). Gel retardation assays were performed as in Fig.1. Double-stranded probes (Fig. 1c) were used for the reactions shown in lanes 1 and 2 of panels a and b, and for lanes 1−3 in panel c. Single-stranded probes (as designated in Fig. 1c) were used for the reactions shown in lanes 3−6 of panels a and b. The arrowhead indicates the position of the retarded hER-ERE complex.

with the elution of a ?45 kDa protein (Fig. 2b, lanes 5 to 8), thus strongly suggesting that this protein was responsible for the DBSF activity. When PC fractions containing the DBSF activity were centrifuged on a 10–30% glycerol gradient (Fig. 3a), the peak of activity was found in fractions 10 to 12 (Fig. 3b), roughly co-sedimenting with BSA and a 80 kDa protein (Fig. 3a, the same sedimentation pattern was observed in gradients containing 0.5 M KCl). Hence, the DBSF probably exists as a stable dimer in solution. Note the increase in a non-specific retarded complex coinciding with the elution of the DBSF (Fig. 2c and 3b), which indicates that the binding to the ERE of a protein present in the purified hER preparation was also stimulated by the DBSF activity. The DBSF activity was destroyed by heating at 60° for 5 min or by trypsin digestion (data not shown), showing that it was due to a protein.

How the DBSF allows the purified ER to bind to an ERE is unknown. Like its HeLa cell counterpart (see above, Fig. 1), the yeast DBSF activity did not bind to a double-stranded DNA-cellulose resin (data not shown) and was present in the column flowthrough. One explanation for this observation is that DBSF is present at vey high levels or was in competition with other double-stranded DNA binding proteins which were in excess. An alternative explanation which we favour is that DBSF binds very weakly, if at all, to double-stranded DNA since using the gel retardation assay, no complex could be detected between the purified yeast DBSF and a double-stranded ERE or EREm (see Fig. 1c and Fig. 4a, lanes 1 and 2). On the other hand, retarded complexes were formed with the separate strands of ERE and EREm (Fig. 4a, lanes 3–6). The complexes were not formed equally efficiently with the two strands of ERE and EREm, thus indicating that DBSF binding to single- stranded DNA exhibits some specificity which is not related to the ERE palindromic sequence. Similarly, purified E.coli single-strand DNA binding protein (SSB) (7) did not bind with equal efficiency to the separated strands of the ERE and EREm (Fig. 4b). Most interestingly, E.coli SSB did not promote the binding of the purified hER to ERE, showing clearly that the effect of the yeast DBSF on ER-ERE complex formation is not a general property of single stranded DNA binding proteins (compare lanes 1–3 in Fig. 4c). In this respect, we also note that the purified DBSF activity is apparently specific to a unique protein, whereas yeast contains several SSB's (8–12 and refs therein). Whether the present DBSF corresponds to the ≃45 kDa yeast SSB protein which was previously characterized (8,11,12) is not known.

Various cellular extracts have recently been shown to stimulate the binding of the progesterone receptor (13), the thyroid hormone receptor (14,15), the retinoic acid receptor (16) and the proto-oncogene c-fos and c-jun (17,18) to their cognate DNA responsive elements. Since none of these stimulatory activities have been purified, it is unknown whether they also correspond to SS-DNA binding proteins similar in their properties to the present DBSF.

In conclusion, we have shown here that, in the absence of a DBSF activity, the purified ER does not bind efficiently to its cognate ERE *in vitro*. When purified from yeast cells, this activity was shown to correspond to a SSB of ≃45 kDa. A similar activity was also detected in HeLa cells (Fig. 1) and in reticulocyte extracts, and in the former case shown to bind to single-stranded DNA (data not shown). Whether these activities correspond to related SSBs, and how they promote the binding of the purified ER to the ERE is unknown. In particular, it is unknown whether the DBSF is only transiently required or whether a stable ER-DBSF-ERE ternary complex is formed. However, the DBSF requirement is clearly not limited to the DNA binding assay (gel retardation) used in the present study, since DBSF was also required when the formation of purified ER-ERE complexes was monitored by immunoprecipitation using a monoclonal antibody directed against the hER (data not shown). Whether DBSF activity is necessary for ER-ERE complex formation *in vivo* remains to be seen.

ACKNOWLEDGEMENTS

We thank J. White and M.P. Kieny for the vaccinia virus vector-hER cDNA construct, D. Metzger for hER antibodies and help with yeast cells, A. Staub and F. Ruffenach for synthesis of oligonucleotides, B. Boulay and C. Werle for the figures, J.L. Weickert for growing vaccinia virus infected HeLa cells, the secretarial staff for typing the manuscript, J. White and S. Ali for critically reading the manuscript and our colleagues of the receptor group for helpful discussions. R.M. was supported by a fellowship from the CNRS (poste rouge) and the INSERM (poste vert). We gratefully acknowledge the support of the CNRS, INSERM, Association pour la Recherche sur le Cancer and the Fondation pour la Recherche Médicale.

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