

The 90 kDa heat-shock protein (hsp90) modulates the binding of the oestrogen receptor to its cognate DNA

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The role of heat-shock protein 90 (hsp90) in the regulation of the oestrogen receptor (ER) function is less well understood than for other steroid-hormone receptors because hsp90 is not involved in the stabilization or induction of a high-affinity ligand-binding state of ER nor in the inhibition of receptor dimerization. Electrophoretic mobility-shift assays, using purified ER and hsp90, were employed to investigate directly the effect of hsp90 on the ability of ER to bind to the oestrogen-response element (ERE) from the vitellogenin A2 gene. Contrary to models in which hsp90 binds to and passively inactivates steroid-hormone receptors, our studies show that the binding of ER to ERE is inversely dependent on the relative concentration of hsp90. Exposure of purified ER–hsp90 complexes to ERE led to the dissociation of hsp90 and concomitant specific binding of ER to ERE. We demonstrate that the amount of ER–ERE complex decreased with increasing concentrations of hsp90. Furthermore

hsp90 dissociated preformed high-affinity ER–ERE complexes. Kinetic dissociation experiments indicate that hsp90 acts in a dynamic and specific process rather than by simple trapping of ER owing to its inherent off-rate. The receptor released from the ERE-bound state by hsp90 was recovered associated with hsp90 and was able to rebind to ERE. These results indicate that hsp90 does not suppress ER function merely by steric hindrance. On the basis of these results and others, we propose that, *in vivo*, hsp90 may play a dual role in ER function: (i) at a physiological temperature, hsp90 stabilizes an active form of the receptor in accordance with its general molecular chaperone role; (ii) at elevated temperatures or under other environmental stress, the increased cellular concentration of hsp90 negatively interferes with ER-dependent transcription, in accordance with the inhibition of gene transcription attributed to hsp90 after heat shock.

INTRODUCTION

The 90 kDa heat-shock protein (hsp90) is abundant in most cells and is further increased by heat or other cellular stress. It associates with various cellular proteins such as steroid-hormone receptors [1], aryl hydrocarbon receptor [2,3], MyoD [4], oncogene tyrosine kinases [5,6], haem-regulated eukaryotic initiation factor 2 α kinase [7], casein kinase II [8], actin [9] and tubulin [10]. It has been suggested that hsp90 may participate in the maturation, modulation of activity and/or intracellular transport of several proteins, probably by different mechanisms.

Steroid-hormone receptors can be recovered from hormone-free target cells extracted in low-salt medium in the inactive '8–9S' form, complexed with a set of proteins minimally including a dimer of hsp90, a 59 kDa immunophilin protein of the FK506-binding protein class and a 23 kDa protein (reviewed in ref. [11]). Hsp90 is the only one of these proteins reported to have an intrinsic capability of specific receptor binding. In hormone-treated cells, the receptors are tightly associated with nuclear components, and the hormone–receptor can be extracted under high salt conditions as a smaller '4–5S' form, free of any detectable hsp90, and with the hormone-binding DNA-binding receptor molecule as a sole constituent. These data have led to the hypothesis that the primary role of hormone binding is to promote the dissociation of hsp90 from the hormone-binding receptor, allowing the receptor to bind to the hormone-response elements of regulated genes [1]. Therefore it is generally accepted that hsp90 associates selectively with unliganded receptors and inhibits their DNA-binding activity either by steric hindrance

and/or by passive interference with a receptor-dimerization step required for high-affinity DNA binding.

The steroid-hormone receptors belong to a large ligand-dependent transcription factor superfamily, and thus share common organizational features (reviewed in ref. [12]). A comparison of amino acid sequences and functional analysis of mutated receptors has led to the definition of discrete structural domains, including a DNA-binding domain (DBD), which contains two zinc-binding motifs, and a large ligand-binding domain (LBD) located at the C-terminal part of the molecule. Despite similar basic organization, the evolutionarily more primitive members of the intracellular DNA-binding receptor superfamily show marked differences with respect to their ability to bind hsp90. In contrast with steroid-hormone receptors, receptors for thyroid hormones (TRs), calcitriol (VDRs) and retinoic acids (RARs and RXRs) do not bind hsp90 [13–15], and are found tightly associated with the nucleus in ligand-free cells. There are also differences with respect to hsp90 binding between steroid-hormone receptors themselves. There is evidence that glucocorticosteroid receptor (GR) and mineralocorticosteroid receptor (MR) must be bound to hsp90 in order to be able to bind their respective ligands [16,17]. Similar evidence does not currently exist for the oestrogen receptor (ER), and hsp90 has little effect on the high-affinity binding of hormone to the progesterone receptor (PR) and androgen receptor (AR) [18,19]. Interestingly, cell-free reassociation of hsp90 with GR does not reflect a free equilibrium but occurs to an appreciable extent only when the immunoadsorbed GR is incubated at 30 °C with rabbit reticulocyte lysate [20]. However, it has been reported that ER

Abbreviations used: ER, oestrogen receptor; ERE, oestrogen-responsive element; LBD, ligand-binding domain; DBD, DNA-binding domain; hsp90, 90 kDa heat-shock protein; EMSA, electrophoretic mobility-shift assay; TR, thyroid hormone receptor; VDR, calcitriol receptor; RAR, RXR, retinoic acid receptors; GR, glucocorticosteroid receptor; MR, mineralocorticosteroid receptor; PR, progesterone receptor; AR, androgen receptor.

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can bind to hsp90 in a spontaneous manner, even in the presence of bound ligand and without a helper reagent [21]. These results can be related to the fact that GR forms a relatively stable association with hsp90 whereas ER forms only a weak and transient association detectable only at non-physiological low-salt concentrations; molybdate ion stabilizes the ER-hsp90 association. It should be noted that the LBD of GR constitutes the only structural requirement for hsp90 binding, whereas an additional sequence element situated at the C-terminus of the DBD appears to be necessary for hsp90 binding by ER [22–24]. Recently, genetic experiments have shown that all steroid-hormone receptors interact with hsp90 but only GR and MR must remain bound to hsp90 to allow efficient ligand binding and receptor activation [25]. Taken together, these observations strongly suggest that hsp90 might not play the same role in GR and ER functions.

Previously, we and others demonstrated that exposure of the 8–9S ER-hsp90 complexes to oestrogen-responsive element (ERE) leads to specific binding of the receptor to DNA, even in the absence of hormonal ligand [26,27]. As hsp90 was not included in the ER-ERE complexes, these results indicate that the ER-hsp90 complexes are dissociated by ERE, regardless of hormonal or other pretreatments (i.e. salt treatment) provoking receptor activation.

In our efforts to understand the molecular mechanisms of hsp90 action on signal transduction by ER, we have examined the influence of hsp90 concentration on the DNA-binding activity of the receptor. We report here that the DNA-binding activity of ER is directly modulated by the level of added purified hsp90. At low concentrations of hsp90, the receptor is capable of forming complexes with its cognate ERE. At high concentrations, hsp90 specifically inhibits the ERE-binding activity of ER. The inhibitory effect can be reversed by increasing the concentration of DNA. Furthermore hsp90 is capable of dissociating ER from its cognate ERE by a dynamic and specific process. These results indicate that hsp90 does not suppress receptor function merely by steric hindrance. On the basis of these and other results, we propose a dual function for hsp90, which may be involved in the mechanism by which ER modulates gene transcription.

EXPERIMENTAL

Cell culture and nuclear extracts

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco/BRL) and penicillin/streptomycin. Nuclear extracts were prepared essentially as described by Dignam et al. [28]. The nuclear extracts were dialysed for 4 h against a buffer containing 0.02 M Hepes, pH 7.9, 0.05 M KCl, 0.2 mM EDTA, 20% glycerol, 0.2 mM PMSF and 0.5 mM dithiothreitol, at 4 °C, divided into aliquots and frozen at –70 °C. Protein concentration was determined using the Bio-Rad protein assay kit.

Purification of ER-hsp90 complexes

Calf uterine ER-hsp90 complexes were purified by affinity chromatography from cytosol prepared in TDMo buffer (10 mM Tris/HCl, pH 7.4, 1 mM dithiothreitol, 20 mM sodium molybdate) as described previously [29].

Purification of hsp90

For purification of calf hsp90, the starting material was the flow-through fraction of the affinity-chromatography column (used for parallel purification of the ER-hsp90 complexes). Usually

100 ml was loaded on to a 10 ml DEAE-Sephacel column (Pharmacia). After washing, elution was performed by applying 100 ml of a linear 0–0.4 M KCl gradient, and 2 ml fractions were collected. The presence of hsp90 was detected by denaturing SDS/PAGE followed by silver staining and also by using ELISA with Ac88 monoclonal antibody. Fractions containing hsp90 were pooled and loaded on to an immunoadsorbent in which Ac88 monoclonal antibody was coupled to Affigel (Bio-Rad) according to the manufacturer's instructions at a concentration of 2 mg/ml gel. After washing, hsp90 was eluted with 50 mM diethylamine, pH 10.5, and fractions were immediately neutralized with NaH₂PO₄.

Human hsp90 was purified from HeLa cells. After homogenization and centrifugation at 105 000 g for 45 min, the supernatant was applied to a DEAE-Sephacel column and treated as above. The fractions containing hsp90 were pooled and loaded on an immunoaffinity matrix prepared with polyclonal antibodies raised against a synthetic C-terminal peptide of human hsp90 [30]. Hsp90 was eluted as described above.

Chick oviduct cytosol hsp90 was purified as previously described [31].

The preparations of hsp90 were dialysed against 1 mM sodium phosphate buffer, then lyophilized to 0.1 vol., divided into aliquots and stored at –20 °C until use.

Protein concentrations were determined by the method of Schaffner and Weissmann [32], with BSA as standard.

SDS/PAGE

Samples were solubilized in buffer containing 0.07 M Tris/HCl, pH 6.8, 10% glycerol, 1% SDS, 1% 2-mercaptoethanol and Bromophenol Blue by heating at 100 °C for 3 min immediately before loading on to a 7.5% separating/4% stacking polyacrylamide gel. A constant current of 15 mA was applied to the electrodes until the sample entered the separating gel, and then the current was increased to 30 mA. After fixation, the gels were stained by the silver nitrate method [33].

Sequence-specific DNA-affinity assay

The sequence of the double-stranded 40 bp probe containing the consensus ERE used for the synthesis of the sequence-specific DNA-affinity resin is described below. The procedure for the affinity-resin construction was similar to that of Kadonaga and Tjian [34]. After ligation, the DNA was coupled to CNBr-activated Sepharose CL-4B (Pharmacia) according to the protocol supplied by the manufacturer.

For sequence-specific DNA-affinity assay, the peak of [³H]oestradiol-binding radioactivity eluted from a DEAE-Sephacel column as described by Sabbah et al. [27], was diluted 3-fold in a solution of 20 mM Hepes (pH 7.5)/1 mM dithiothreitol (HD) containing 0.1% (v/v) Nonidet P-40 and 5 µg/ml poly(dI-dC) (Boehringer) and was incubated with the specific DNA-affinity resin for 45 min at 25 °C. The resin was washed three times at 25 °C with HD buffer (25 resin slurry volumes) and then with the same buffer containing 0.3 M KCl. After re-equilibration of the resin in HD buffer, purified hsp90 (500 ng of hsp90 in 200 µl of HD per 25 µl of resin slurry) was added, and the incubation was continued for 30 min at 25 °C. Aliquots of the supernatant were centrifuged at 300 000 g (70 000 rev/min) for 2 h at 4 °C in a low- or high-salt (0.4 M KCl) sucrose gradient (5–20%) in a Beckman Vti 80 rotor. After centrifugation, three-drop fractions were collected by puncturing the bottom of the centrifuge tubes. Aliquots (50 µl) of each fraction were taken for ³H radioactivity counting and for the

detection of hsp90 content by ELISA using Ac88 monoclonal antibody [29].

Purification of the 5S ER was performed by the above procedure except that washing and elution of the DNA-affinity resin were performed in the presence of 0.1% (v/v) Nonidet P40. After being washed with buffer containing 0.3 M KCl, the bound proteins were sequentially eluted with buffers containing 0.4, 0.6, 0.8 and 1 M KCl. The eluates at different salt molarities were checked for the radioactivity of bound [³H]oestradiol.

Electrophoretic mobility-shift assays (EMSAs)

The purified 8-9S ER or the 5S ER were incubated with 0.2–0.4 ng of ³²P-end-labelled synthetic ERE probe (see below) (1×10^4 – 2×10^4 c.p.m.) in a binding buffer containing 10 mM Tris/HCl, pH 7.4, 1 mM dithiothreitol, 5 mM MgCl₂, 1 μg of poly(dI-dC), 5% glycerol and 0.2 μg of BSA (Sigma) in a final volume of 20 μl. Binding reactions were allowed to proceed for 30 min at 25 °C.

Assays for NF1- and AP1-binding activity were performed under the same conditions, using the appropriate DNA probes (see below) and 4–5 μg of crude nuclear extract. Assays for TR-binding activity were performed as described [35] using the appropriate DNA probe (see below) and purified chick TR_{α1}. For competition experiments, various concentrations of purified hsp90 were added to the mixture before the addition of the end-labelled oligonucleotide. For dissociation experiments, hsp90 was mixed with a preformed complex of ER and labelled oligonucleotide and incubated for an additional 30 min at 25 °C.

Free DNA and DNA–protein complexes were resolved on a non-denaturing 6% polyacrylamide gel in 0.25 × TBE (1 × TBE is 89 mM Tris, 89 mM boric acid and 2 mM EDTA). After suitable separation, gels were vacuum-dried and autoradiographed. The protein–DNA complexes were quantified by densitometric scanning or by scanning using a multitrace-master model MB-85 (Berthold Analytical Instruments).

Oligonucleotides

The sequence of the oligonucleotide used to detect ER-specific DNA-binding activity was (the palindromic ERE consensus sequence is underlined):



The sequence of the oligonucleotide used to detect NF1-specific DNA-binding activity was (the NF1-binding motif is underlined):



The oligonucleotide used to detect AP1-specific DNA-binding activity was (the AP1-binding motif is underlined):



The oligonucleotide used to detect TR-specific DNA-binding activity was (the palindromic consensus sequence is underlined):



The probes were prepared by annealing complementary strands after purification by gel electrophoresis (20% polyacrylamide, 8 M urea) and then labelled with [α -³²P]dCTP using the Klenow fragment of DNA polymerase I (Boehringer) or with (γ -³²P)ATP and T4 polynucleotide kinase (Boehringer) using standard procedures.

RESULTS

Hsp90 inhibits DNA-binding activity of ER

Figure 1 shows a silver-stained SDS/polyacrylamide gel after electrophoresis of samples of hsp90 purified from calf uteri,

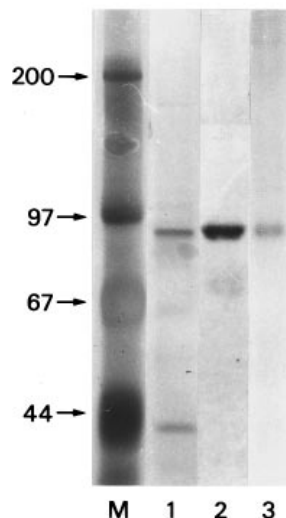


Figure 1 Immunopurification of hsp90

Immunopurification was carried out as described in the Experimental section. Proteins in aliquots of pooled fractions eluted from immunoabsorbent were electrophoresed on SDS/polyacrylamide 7.5% gels and visualized by silver staining. Lane 1, purified calf uterus hsp90; lane 2, purified chick oviduct hsp90; lane 3, purified human hsp90. Lane M shows the molecular-mass standards, myosin heavy chain (200 kDa), phosphorylase *b* (97 kDa), BSA (67 kDa) and ovalbumin (44 kDa).

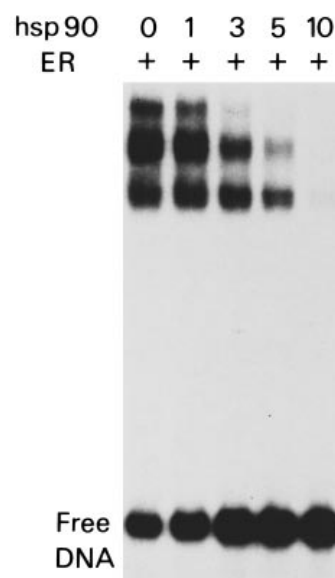


Figure 2 Purified hsp90 inhibits the binding of ER to ERE

Hsp90 was added to the binding reaction mixture containing ER (70 fmol) before the addition of the end-labelled ERE and incubated for 30 min at 25 °C. The DNA-binding activity of ER in the presence of different amounts of purified hsp90 was assayed by EMSA in a 6% polyacrylamide gel. The molar ratio of hsp90 to the receptor is indicated above each lane.

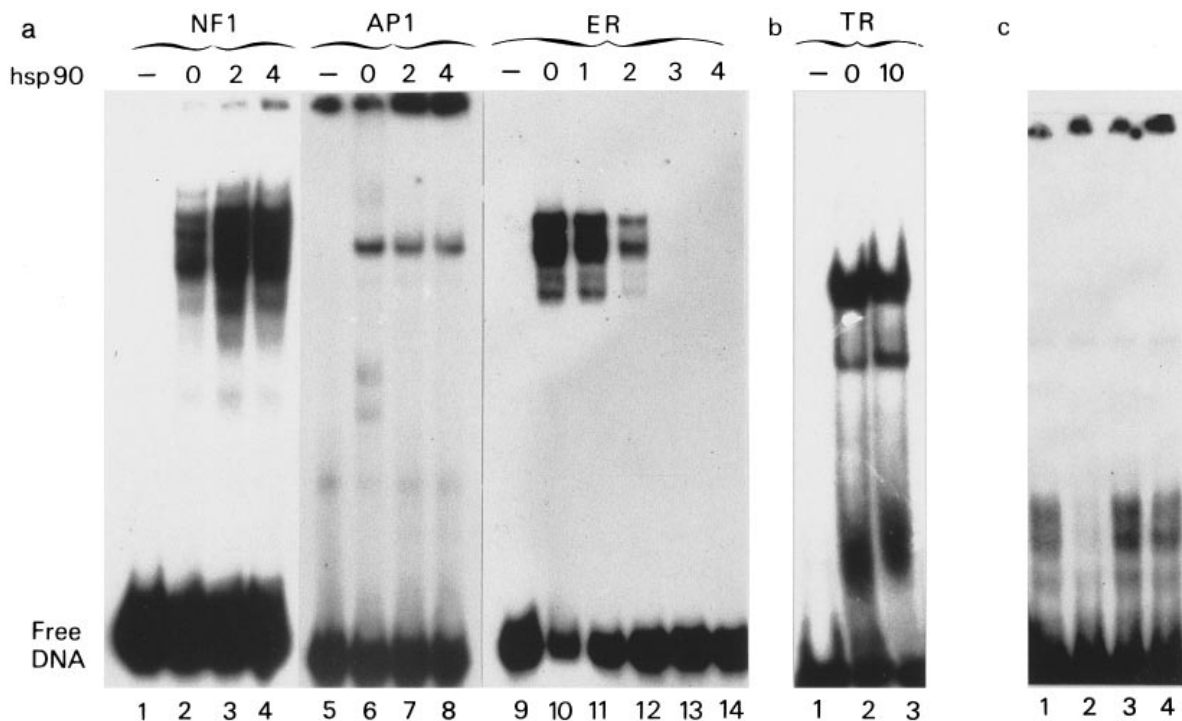


Figure 3 Hsp90 does not inhibit the DNA binding of NF1, AP1, TR and the DBD of ER

(a) Purified human hsp90 (660 pmol/ml) does not affect the binding of NF1 or AP1 to their respective cognate probes. HeLa-cell nuclear extracts (4 μ g) were incubated with 0.4 ng of end-labelled probes in the presence of various concentrations of hsp90 for 30 min at 25 $^{\circ}$ C. Lanes 1 and 5, free probes; lanes 2 and 6, no hsp90; lanes 3 and 7, 2 μ l of hsp90; lanes 4 and 8, 4 μ l of hsp90. Lanes 9–14 show a control experiment where whole-cell extract (4 μ g) from Cos-7 cells containing human ER was incubated with end-labelled ERE probe in the presence of various concentrations of hsp90 for 30 min at 25 $^{\circ}$ C. Lane 9, free probe; lane 10, no hsp90; lanes 11–14, 1, 2, 3 and 4 μ l of hsp90 respectively. (b) Purified chick hsp90 (560 pmol/ml) does not affect the binding of TR to its cognate probe. Purified chick TR α_1 (1 pmol) was incubated with 0.2 ng of end-labelled probe in the presence of hsp90 for 30 min at 25 $^{\circ}$ C. Lane 1, free probe; lane 2, no hsp90; lane 3, 10 μ l of hsp90. (c) Purified hsp90 has no effect on the DNA-binding activity of the DBD of human ER. The labelled ERE probe was mixed or not (lane 1) with a 50-fold excess of unlabelled ERE (lane 2), or with a 50-fold excess of a mutated ERE (lane 3) or with 4 μ l of human hsp90 (660 pmol/ml) (lane 4). EMSA reactions were started by the addition of whole-cell extract (4 μ g) from Cos-7 cells containing the DBD of the human ER (amino acids 160–315).

HeLa cells and chick oviduct. Along with a main 90 kDa band, an additional 40 kDa band, identified by immunoblotting as actin (C. Radanyi, unpublished work), was present in variable concentrations. This copurification is not surprising since hsp90 has previously been shown to form complexes with actin [9]. We have examined the influence of the concentration of purified hsp90 on the DNA-binding activity of the receptor by EMSA using a probe derived from the *Xenopus* vitellogenin A2 gene promoter. The amount of specific ER–ERE complexes decreased as a function of the presence of increasing concentrations of hsp90 (Figure 2). A 10-fold molar excess of hsp90 over receptor efficiently eliminated the retarded bands. It is important to stress that considering the imprecision of the methods used to measure the concentrations of the receptor, hsp90 and DNA fragments, the values given throughout are only approximate and serve as a qualitative description of the phenomenon. The protein–DNA bands detected are specific ER–ERE complexes since their mobilities were supershifted by the addition of H222 monoclonal anti-(oestrogen receptor) antibody [27], and represented different proteolytic fragments present in variable concentrations depending on the ER preparation.

To demonstrate that the observed effect is specific for the DNA-binding activity of ER, we next analysed the effect of hsp90 on two other well-characterized DNA-binding proteins. Nuclear extracts prepared from HeLa cells were incubated with specific oligonucleotides to detect NF1 and AP1 DNA-binding

activity, in the presence of increasing concentrations, or in the absence, of HeLa-cell purified hsp90. The DNA-binding activity of the ubiquitous transcriptional factors NF1 and AP1 was not affected by hsp90 at concentrations that efficiently eliminated the specific binding of ER to ERE (Figure 3a). The inhibition of DNA binding of ER from crude ER-transfected Cos-7-cell extract served as control in these experiments (Figure 3a).

TR binds a canonical inverted repeat similar to that bound by ER, 5'-AGGTCATGACCT-3', but with no gap between the half-sites [36,37]. Purified chick TR α_1 expressed in *Escherichia coli* was incubated with the thyroid-response element in the presence or absence of a high concentration of purified chick hsp90. As shown in Figure 3(b), hsp90 did not affect the DNA-binding activity of TR. These results are consistent with the observation that TRs do not bind to hsp90 [15].

To test the possibility that hsp90 competed with DNA to bind to the receptor DBD, crude cellular extracts containing the human DBD (amino acids 160–315) expressed in Cos-7 cells were incubated with the end-labelled ERE oligonucleotide. Two protein–DNA complexes were formed (Figure 3c, lane 1). The specificity of the protein–DNA complexes was confirmed by their suppression by a 50-fold excess of the same unlabelled oligonucleotide, whereas a 50-fold excess of an oligonucleotide containing mutated ERE did not compete with the ERE probe (Figure 3c, lanes 2 and 3). Although protease inhibitors were used, the more rapidly migrating complex probably represents a

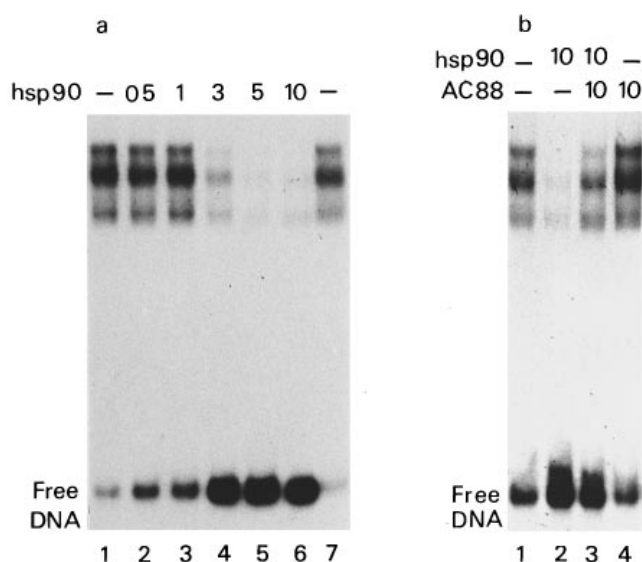


Figure 4 Hsp90 dissociates ER-ERE complexes

(a) Binding reaction mixtures containing end-labelled ERE and ER (70 fmol) were incubated for 30 min at 25 °C. Subsequently, different amounts (0.5, 1, 3, 5 and 10 μ l) of purified hsp90 (70 pmol/ml) were incubated with the preformed ER-ERE complexes for an additional 30 min at 25 °C. Lane 1 shows the stability of the ER-ERE complex during the incubation steps and lane 7 shows a control experiment where BSA was added instead of hsp90 (700 fmol, equal to the maximal concentration of hsp90). (b) The preformed ER-ERE complexes (lane 1) were incubated with hsp90 either free (lane 2) or previously complexed with the Ac88 monoclonal antibody (lane 3) or with Ac88 (lane 4), for 30 min at 25 °C. The hsp90-Ac88 complexes were formed by incubation of purified hsp90 (10 μ l) with 10 μ l of Ac88 (1 mg/ml) for 2 h at 4 °C.

proteolytic cleavage product of the DBD that remains able to bind DNA. In the presence of purified human hsp90 at the same concentration as that used to displace ER from ERE, the intensity of the DBD-ERE complex bands decreased only slightly compared with those of the control (Figure 3c, compare lanes 1 and 4). This result is consistent with the observation that hsp90 needs to bind to both the LBD and the highly positively charged region situated at the C-terminus of the DBD of human ER between amino acids 251 and 271 in order to form a complex with ER [23].

Hsp90 can dissociate preformed ER-ERE complexes

We have addressed the question of whether purified hsp90 can dissociate the receptor bound to DNA. Addition of hsp90 induced a dramatic dissociation of preformed ER-ERE complexes whereas, as expected, an excess of BSA, a protein of similar acidity (pI 4.9) to that of hsp90 (pI 5.2), had no effect (Figure 4a, lane 7). Preheating hsp90 fractions for 10 min at 65 °C completely cancelled this hsp90 activity (results not shown).

To verify the specificity of this hsp90 effect, ER-ERE complexes were incubated in the presence of excess hsp90, either free or previously complexed with the Ac88 monoclonal antibody which is known to only react with non-receptor bound hsp90 [38]. No retarded bands were detected in the presence of an excess of free hsp90, but in the presence of hsp90-Ac88 complexes, two retarded bands were seen with intensities similar to those observed in the control lane (Figure 4b). Moreover, when ERE and 8-9S ER-hsp90 complexes were incubated in the presence of Ac88, the level of ER-ERE complexes formed was substantially higher than without the antibody (Figure 4b, lanes

1 and 4). In these experiments, the increasing quantity of the complexes was presumably due to the Ac88 monoclonal antibody trapping some of the hsp90 that had been dissociated from the receptor during the incubation with DNA, resulting in a decreased concentration of free hsp90 in the medium and enhanced binding of the receptor to DNA.

Next, we investigated whether hsp90 dissociated the receptor from DNA by a dynamic process involving protein-protein interaction, or simply trapped the receptor that was released from DNA. As seen in Figure 5, the dissociation process in the presence of a 100-fold molar excess of unlabelled ERE can be described by simple one-component first-order kinetics, with a half-life of 33 min. In the presence of a 13-fold molar excess of hsp90, the amount of ER-ERE decreased more rapidly and the apparent initial half-life of the complexes was 22 min. The kinetics were non-linear in a semilogarithmic plot, suggesting that this process was of a higher reaction order. As expected, in the presence of an increased molar excess of hsp90 (45-fold), the half-life of the ER-ERE complexes decreased following pseudo-first-order kinetics ($t_{1/2} = 8$ min). These results suggest that hsp90 dissociates the ER-ERE complexes by a dynamic process rather than by simple trapping of ER, owing to its inherent off-rate.

After exposure to hsp90, the ER detached from the complex with its cognate ERE is associated with the heat-shock protein

To examine whether ER released from binding to ERE by hsp90 was associated with hsp90 in a hetero-oligomeric complex, an *in vitro*-reconstituted assay system based on the sequence-specific DNA-affinity method was used. In a representative assay, about 50-60% of the bound receptor was eluted by hsp90 from the DNA beads whereas only 1-2% was recovered in the control experiment performed with the same concentration of BSA. As expected, the receptor sedimented as an 8-9S form in a low-salt sucrose gradient and was dissociated to a smaller (4-5S) form in the presence of 400 mM KCl (Figure 6). Furthermore Ac88 monoclonal antibody used in ELISA revealed the presence of hsp90 in the 8-9S radioactive peak (Figure 6). Thus the ER eluted from DNA-resin by hsp90 has size and structural properties indistinguishable from those of the purified 8-9S ER-hsp90 hetero-oligomer.

ER dissociated from DNA is able to rebind to ERE

The next question asked was whether the ER dissociated from DNA by hsp90 remained able to bind to ERE. The preformed ER-ERE complexes were first incubated for 30 min at 25 °C with a concentration of hsp90 able to dissociate about 90% of the binding (Figure 7a, lane 2). Subsequently, increasing amounts of end-labelled ERE were added and the incubations were continued for another 30 min at 25 °C. As shown in Figure 7(a), we could detect increasing amounts of specific ER-ERE complexes as a function of the concentration of added ERE (lanes 3-5). The ability of ER to rebind to DNA was not quantitative (Figure 7a, compare lanes 1 and 5), a result that may reflect the thermal instability of the receptor during the incubation time. In another experiment, the reconstituted 8-9S ER obtained after elution of the receptor by hsp90 from DNA-affinity resin (see above) was incubated with several concentrations of end-labelled ERE. Again, specific retarded bands were detected by EMSA (Figure 7b).

We have extended our analysis by using the 5S ER purified by DNA-affinity chromatography which removes all endogenous hsp90 as well as ER-hsp90 complexes. The majority of the ER was eluted in the 600 and 800 mM KCl steps and sedimented as

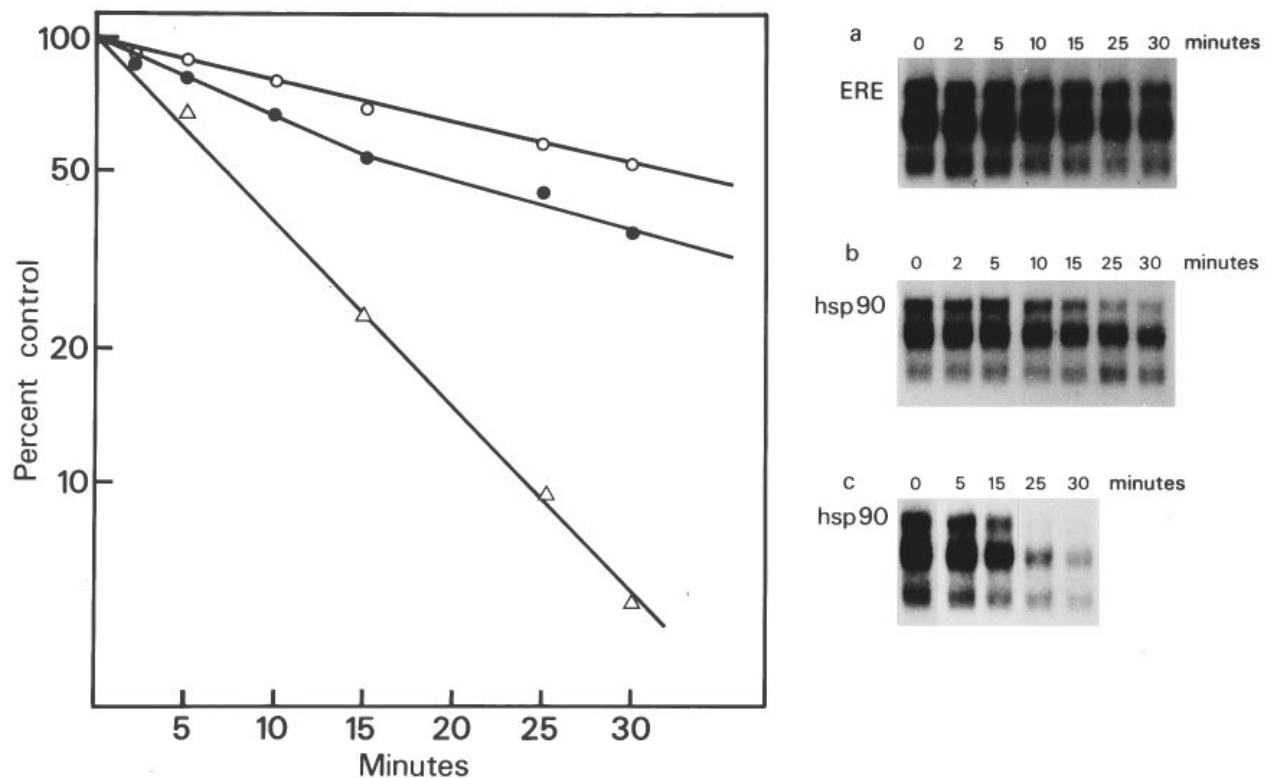


Figure 5 Kinetics of dissociation of the ER-ERE complexes

ER-ERE complexes formed as described in Figure 4 were incubated at 25 °C for the indicated times with a 100-fold molar excess of unlabelled ERE (○, **a**) or with amounts of purified hsp90 sufficient to produce approx. 30% (●, **b**) or 90% (△, **c**) binding inhibition after 30 min. Binding reactions were stopped by non-denaturing PAGE. (**a**)–(**c**) show sections of autoradiograms. The amounts of protein-DNA complexes were determined by scanning using a multitrace-master model MB-85, and are shown in a semilogarithmic plot.

a 4–5S molecular form (results not shown). As shown in Figure 8(a), the purified 5S ER was able to bind ERE (lane 2); this observation does not exclude the possibility that DNA binding may be enhanced by accessory proteins [39]. Again, hsp90 inhibited the DNA-binding activity of the 5S ER (Figure 8a, lanes 3–8) and could dissociate the preformed ER-ERE complexes (Figure 8b, lanes 2–7).

DISCUSSION

In this work we demonstrate that, when present at a high concentration, hsp90 not only specifically prevented binding of ER to ERE (Figure 2), but also actively dissociated the receptor from preformed ER-ERE complexes (Figure 4).

It is generally assumed that hsp90 only passively hinders the DBD of ER. However, several observations argue against this proposition. *First*, we observed only a weak inhibitory effect of hsp90 on DNA-binding activity of the ER-DBD protein (Figure 3). There are two possible explanations: either, as we have previously reported, several additional contacts of hsp90 with the ER-LBD protein are required to promote the formation of an ER-hsp90 complex [23] or the limited effect of hsp90 is due to the unfolding of the positively charged C-terminal residue of the ER-DBD protein [40]. *Secondly*, hsp90 dissociated the receptor from ER-ERE complexes in a dynamic and specific process. The velocity of such dissociation of ER-ERE complexes might be enhanced by release of the free energy stored in the bending of DNA induced by ER binding [41,42].

We propose that hsp90 plays a dual role in being able to modulate either positively or negatively specific programmes of ER-dependent gene transcription according to the prevailing physiological conditions (temperature, environmental stress).

At a physiological temperature, hsp90 might have an important positive role in signal transduction by interacting with and inducing and/or stabilizing an active form of the receptor. In fact, although freshly purified 5S ER remained capable of binding to ERE, its ERE-binding capacity greatly decreased on storage even at –80 °C. In contrast, purified ER-hsp90 complexes remained fully active even after more than 1 year. These observations can be related to previous studies by Inano et al. [21] who found that the binding of hsp90 to ER stabilized the ER conformation needed to bind to ERE. This role is supported by recent data on receptor function in yeast indicating that hsp82 (yeast homologue of hsp90) is important in hormone signal transduction because it maintains the receptor in a conformation that is susceptible to activation [43]. In this respect, hsp90 might prevent unproductive interactions of the receptor with other proteins, as observed for many years in crude extracts prepared without agents that stabilize ER-hsp90 complexes (e.g. MoO_4^{2-} , WO_4^{2-}). Interestingly, hsp90 has also been reported to be involved in the conformational activation of basic helix-loop-helix transcription factors, such as MyoD, E47 [4] and the Ah receptor [44]. Hsp90 was found to convert MyoD homodimer and MyoD-E47 heterodimer from an inactive into a DNA-binding active conformation. The conversion process involves a transient interaction between MyoD and hsp90. In contrast with MyoD,

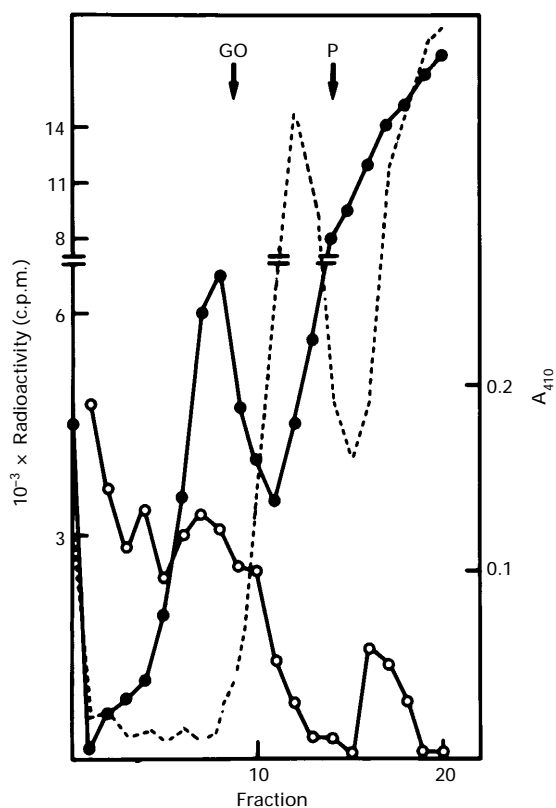


Figure 6 Sucrose-gradient ultracentrifugation of the receptor eluted from DNA-affinity resin with hsp90

[³H]Oestradiol–receptor complexes eluted from the specific DNA-affinity resin after incubation with hsp90 were analysed by sedimentation in a 5–20% low-salt sucrose gradient as described in the Experimental section. Aliquots of each fraction (50 μ l) were counted for radioactivity (●) and the hsp90 content was determined by ELISA using Ac88 monoclonal antibody (○). The dotted curve corresponds to the specific [³H]oestradiol-binding radioactivity counted after elution of the [³H]oestradiol–receptor complexes with 0.6 M KCl. Arrows indicate the sedimentation of internal standards. GO, glucose oxidase (7.9 S); P, peroxidase (3.6 S). Fractions 7–8 correspond to the 8–9S form of ER and fractions 12–13 correspond to the 4–5S form of ER.

the Ah receptor forms a stable complex with hsp90, and this association is correlated with the ability of the Ah receptor to bind its ligand. These positive regulatory functions can be related to the more general molecular chaperone role attributed to hsp90 [45].

At elevated temperatures or under other conditions of environmental stress, the reversibility of ER binding to DNA raises the possibility that the nuclear concentration of hsp90 might interfere negatively in the regulation of transcription. In fact, a minor portion (~7%) of the total cellular content of hsp90, which itself constitutes 1–2% of the total cellular protein in many tissues [46], is located in the nucleus. Furthermore heat shock induces a reversible time-dependent nuclear translocation of hsp90, reaching a plateau 20 h after the nuclear hsp90 level has doubled [47]. Interestingly, it has also been found that heat shock provoked a transient paralysis of oestrogen regulation of vitellogenin gene expression [48], in accordance with the general inhibition of gene transcription after heat shock. In addition, preliminary transient transfection experiments show that an increasing concentration of nuclear hsp90 is correlated with repression of transcription induced by nuclear steroid-hormone receptors (M. C. Catelli, personal communication).

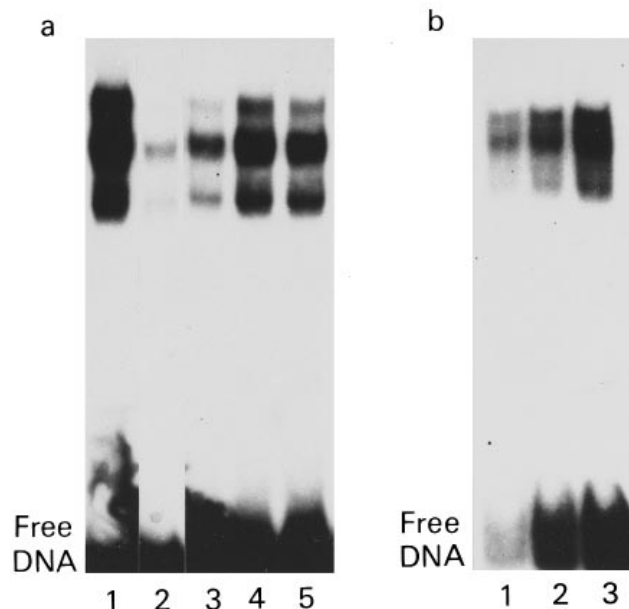


Figure 7 The receptor eluted from DNA with hsp90 is able to rebind to ERE

(a) The ER–ERE complexes formed as described in Figure 4 were incubated with 5 μ l of purified hsp90 (87 pmol/ml) for an additional 30 min at 25 °C. Subsequently, 0.8 ng (lane 3), 1.6 ng (lane 4) and 2.4 ng (lane 5) of end-labelled ERE were added to the reaction mixtures and the incubation was continued for 30 min at 25 °C. The stability of the ER–ERE complexes was verified in the absence (lane 1) or presence (lane 2) of hsp90. (b) The receptor eluted from specific DNA-affinity resin with hsp90 (see Figure 6) was incubated for 30 min at 25 °C with different concentrations of end-labelled ERE probe: 0.4 ng (lane 1); 0.8 ng (lane 2); 1.6 ng (lane 3).

ER–hsp90 binding correlates with the phylogenetic position of ER in the steroid/thyroid/retinoic nuclear receptor superfamily (Table 1). Two groups of receptors of the superfamily can be distinguished according to the DNA-binding motifs in their P boxes and in the nucleotide sequence of the hormone-response element. Group-I receptors (TRs, VDR, RARs and RXRs) bind to the sequence-related core motifs AGGTCA as a direct repeat, whereas group-II receptors (PR, GR, MR, AR) bind to the related inverted repeat AGAACA. These two groups can also be distinguished on the basis of their ability to bind and affinity for hsp90. Group-I receptors which are tightly associated with the nucleus in hormone-free cells do not interact with hsp90. In contrast, group-II receptors are in stable association with hsp90 in hormone-free cells, and hormone binding appears to be required for hsp90 dissociation, receptor dimerization and DNA binding, both *in vivo* and *in vitro*. Moreover, hsp90 must be bound to GR and MR in order to fold the LBD into a high-affinity hormone-binding conformation (chaperone function).

In this respect, ER would represent an intermediate case between groups I and II. Also, on the basis of amino acid homology within the members of the nuclear receptor superfamily, ER is phylogenetically intermediate between the more primitive nuclear receptors of group I and receptors of group II [49]. Like receptors of group I, ER binds to the motif AGGTCA assembled as a palindrome, even in the absence of hormone. Like the receptors of group II, ER interacts with hsp90, but the complex appears to be transient. In fact, in contrast with the receptors of group II, ER dimers may be

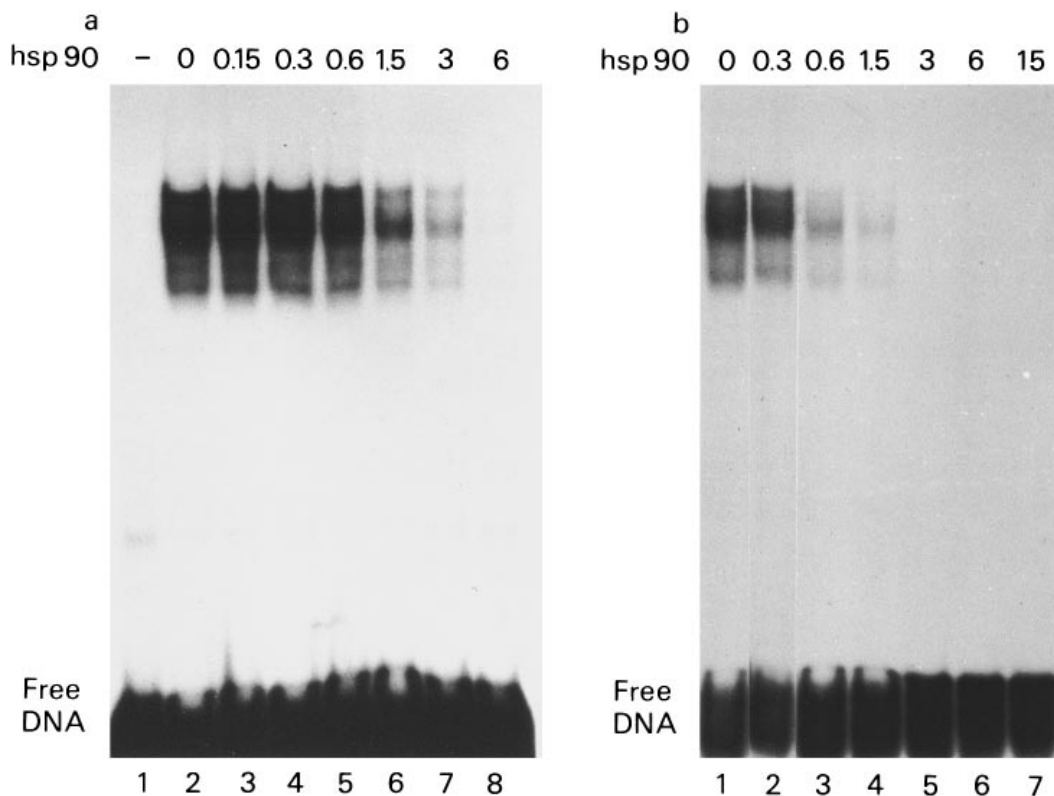


Figure 8 Effect of hsp90 on the DNA-binding activity of the sequence-specific DNA affinity-purified 5S ER homodimer

(a) Samples of the purified 5S ER were incubated in the presence (lanes 3–8) or absence (lane 2) of increasing concentrations of hsp90 before the addition of the end-labelled ERE. (b) The preformed 5S ER–ERE complexes (lane 1) were incubated with increasing amounts of purified hsp90 for an additional 30 min at 25 °C (lanes 2–7). The molar ratio of hsp90 to 5S ER are indicated above each lane. In (a) the concentration of 5S ER was 7 pmol/ml and that in (b) was 3.5 pmol/ml.

Table 1 Hormone free (unliganded) receptors

++ , Higher affinity; + , lower affinity; N.A., not applicable.

Receptor	Receptor P box sequence	Hormone-response element sequence	Hsp90 binding	DNA binding	Subcellular distribution	Hsp-dependent hormone binding (chaperone)
GR, MR	GSCKV	<u>AGAACANNNTGTCT</u>	++	0	Cytoplasm /nucleus	++
PR, AR	EGCKA	<u>AGGTCANNNTGACCT</u>	++	0	Nucleus	0
ER	EGCKA	<u>AGGTCANNNTGACCT</u>	+	±	Nucleus	0
TRs	EGCKA	<u>AGGTCANNNTGACCT</u>	0	++	Nucleus	N.A.
VDR	EGCKG	<u>AGGTCAN₍₀₋₅₎AGGTCA</u>	0	++	Nucleus	N.A.
RARs, RXRs	EGCKG	<u>AGGTCAN₍₀₋₅₎AGGTCA</u>	0	++	Nucleus	N.A.

present in the ligand-free ER–hsp90 complex [29], and oestrogen does not appear to be required for hsp90 dissociation, receptor dimerization and DNA binding, under conditions where no group-II receptors–DNA complexes are found in the absence of ligand.

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