Substrate-binding characteristics of proteins in the 90 kDa heat shock protein family

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In the present study we investigated the substrate-binding characteristics of three members of the 90 kDa heat shock protein (HSP90) family, namely the α isoform of human HSP90 (HSP90α), human GRP94 (94 kDa glucose-regulated protein, a form of HSP90 from endoplasmic reticulum), and HtpG (the *Escherichia coli* homologue of HSP90) and the domain responsible for these characteristics. The recombinant forms of HSP90α, GRP94 and HtpG existed as dimers and became oligomerized at higher temperatures. Among the three family members, HtpG required the highest temperature (65 °C) for its transition to oligomeric forms. The precipitation of the substrate protein, glutathione S-transferase, which occurred at 55 °C, was efficiently prevented by the simultaneous presence of a sufficient amount of HSP90α or GRP94, but not by HtpG, which was still present as a dimer at that temperature. However, precipitation was stopped completely at 65–70 °C, at which temperature HtpG was oligomerized. Thus the transition of HSP90-family proteins to a state with self-oligomerization ability is essential for preventing the precipitation of substrate proteins. We then investigated the domain responsible for the substrate binding of HtpG on the basis of the three domain structures. The self-oligomerizing and substrate-binding activities towards glutathione S-transferase and citrate synthase were both located in a single domain, the N-terminal domain (residues 1–336) of HtpG. We therefore propose that the primary peptide-binding site is located in the N-terminal domain of HSP90-family proteins.

Key words: domain structure, molecular chaperone, stress protein, substrate binding.

INTRODUCTION

The molecular chaperone activity of the 90 kDa heat shock protein (HSP90) was first characterized in terms of the function and regulation of steroid hormone receptors [1–3]. The cast of the target substrates or client proteins has since grown to include various types of kinase such as casein kinase II [4,5], eEF-2α kinase [6] and oncogene tyrosine kinase Src [7,8], and transcription factors such as aryl hydrocarbon receptor [9], Hap1 [10] and HSF (heat shock factor) [11], tumour suppressor protein, p53 [12], and even retrovirus reverse transcriptase [13]. HSP90 can act alone to prevent protein aggregation and promotes the refolding of model client proteins *in vitro* [14–16], although it functions in large heteromeric complexes with other chaperones and co-chaperones *in vivo* (reviewed in [17,18]).

The mechanism of HSP90's functioning as a molecular chaperone is still controversial. Young et al. [19] and Scheibel et al. [20] reported that there are two substrate-binding sites, the N-terminal domain and the C-terminal domain. In contrast, Prodromou et al. [21] proposed a molecular clamp model based on the crystallographic study of a dimeric crystal of amino acid residues 9–232 of yeast HSP90, in which the target peptide-binding cleft was formed between the two N-terminal domains. However, biochemical studies [22,23] and an electron microscopic study [24] consistently revealed dimer formation to be near the C-terminal region. With the results of the biochemical studies in mind, Pearl and Prodromou [25] recently revised their model to suggest that the dimeric structure of the clamp is mediated through the C-terminal domain.

Many studies on the chaperone function of HSP90 *in vitro* are performed around 25 °C and are triggered by the dilution of denatured proteins in renaturation buffers containing HSP90 with or without other chaperones and co-chaperones [14,16]. In contrast, Yonehara et al. [15] proposed that a heat-induced transition of HSP90 was necessary for exerting the chaperone activity. This treatment caused the self-oligomerization of HSP90 under substrate-free conditions, indicating the close relationship between the self-oligomerization and the substrate-binding properties. An electron microscopic analysis [24] indicated that heat treatment as well as incubation with ATP at room temperature induces conformational changes in an HSP90 dimer from an extended configuration to a ringed structure at a concentration of HSP90 lower than 1 μM. This observation strongly suggests an interaction between the N-terminal regions of an HSP90 dimer, although the structure itself might be a dead-end product in the absence of substrate proteins, as suggested by Maruya et al. [24].

Here we compare the properties of three HSP90-family proteins, namely human HSP90α, human 94 kDa glucose-regulated protein (GRP94) and HtpG, an *Escherichia coli* homologue of mammalian HSP90, on the prevention of substrate aggregation. By using the high thermostability of HtpG, we investigated whether or not the self-oligomerization process is truly related to substrate binding. We further investigated the identity of the domain responsible for the self-oligomerizing and substrate-binding activities.

EXPERIMENTAL

Materials

An expression vector pQE9 and plasmid pREP4 were purchased from Qiagen (Chatsworth, CA, U.S.A.). The expression vector pGEX4T-1, glutathione–Sepharose and low-molecular-mass

Abbreviations used: CS, citrate synthase; GRP94, 94 kDa glucose-regulated protein; GST, glutathione S-transferase; H_6HtpG, H_6HSP90α and H_6GRP94, HtpG, HSP90α and GRP94 respectively tagged with a dodecapeptide (MRGSHHHHHHGS; single-letter amino acid codes); HSP90α, α isoform of the 90 kDa heat shock protein; HtpG, an *Escherichia coli* homologue of mammalian HSP90.

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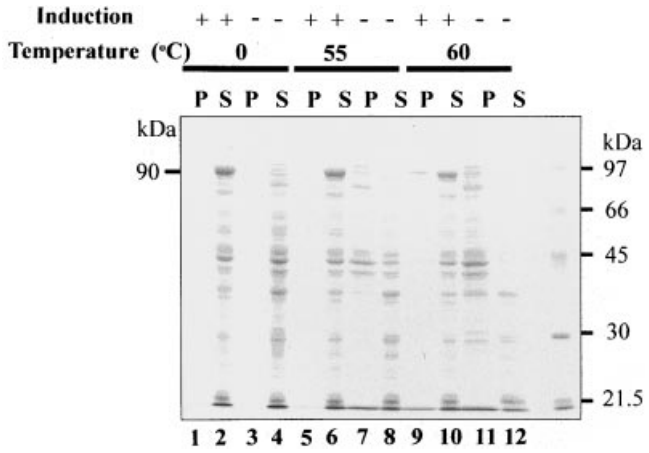

Figure 1 Prevention of heat-induced precipitation of bacterial lysate proteins in the presence of H_6HSP90α

The lysate was prepared from the bacteria in which H_6HSP90α had been induced with 1 mM isopropyl β-D-thiogalactoside (+) at 30 °C for 4 h or from the H_6HSP90α-uninduced bacteria (−). The lysate was incubated at 0, 55 or 60 °C for 10 min and then centrifuged at 17000 ***g*** for 10 min at 4 °C. The resulting precipitating (P) and supernatant (S) fractions were subjected to SDS/PAGE [11% (w/v) gel].

markers were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Pig heart citrate synthase (CS) was obtained from Roche Diagnostics Corp. (Mannheim, Germany). Talon metal-affinity resin was obtained from Clontech Laboratories (Palo Alto, CA, U.S.A.). All other reagents were of analytical grade.

Plasmid construction

All recombinant HSP90-family proteins and their truncated forms were expressed with an N-terminal dodecapeptide tag containing a histidine hexamer encoded in a pQE9 vector (Qiagen). The cDNA species encoding the full-length form of HtpG [26] and human HSP90α [27] were generously provided by Dr E. A. Craig (University of Wisconsin Medical School, Madison, WI, U.S.A.) and Dr K. Yokoyama (RIKEN, Tsukuba, Japan) respectively. The 1.9 kb fragment of HtpG gene amplified by PCR with a 5′ primer (5′-TAGGGATCCATGAAAGGACA-3′, underlined at the *Bam*HI site) and a 3′ primer (5′-GCA AAGCTTCAGGAAACCAGCAGCTG-3′, underlined at the *Hin*dIII site) was double-cut with *Bam*HI and *Hin*dIII; the fragment was then inserted into a *Bam*HI/*Hin*dIII site of pQE9, designated pH_6HtpG. The construction of expression plasmids for the full-length forms of human HSP90α (designated pH_6HSP90α) and human GRP94 (designated pH_6GRP94) was reported previously [28,29]. Expression vectors bearing truncated forms of HtpG were prepared by PCR techniques with appropriate primers as above. Recombinant proteins were expressed as described previously [28] and purified by one-step affinity chromatography with a Talon metal-affinity column in accordance with the manufacturer's protocol in the presence of 10 mM imidazole. Samples were dialysed overnight against PBS, pH 7.5, at 4 °C.

SDS/PAGE

PAGE [12.5% (w/v) gel] was performed in the presence of 0.1% SDS under denaturing conditions by the method of Laemmli [30]. In some cases the Tris/Tricine PAGE system [10% (w/v) gel] was employed [31]. This system provided a fine separation of proteins smaller than 20 kDa, although the mobilities of proteins varied slightly depending on the total quantities of proteins loaded. Separated proteins were stained with Coomassie Brilliant Blue. Low-molecular-mass markers were used as molecular standards.

PAGE under non-denaturing conditions

Proteins were subjected to PAGE [7% or 7.5% (w/v) gel] under non-denaturing conditions as described previously [32]. Ovalbumin (45 kDa), BSA (66 kDa as monomer, 132 kDa as dimer and 198 kDa as trimer) and catalase (240 kDa) were used as references.

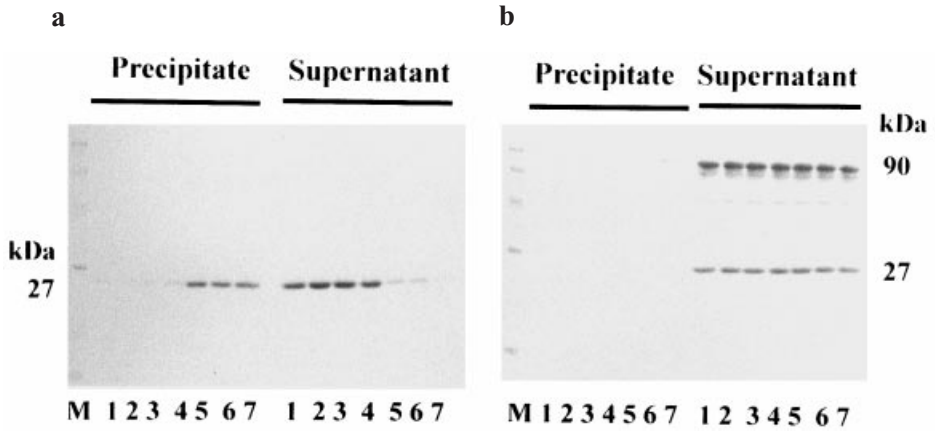

Figure 2 Prevention of GST precipitation in the presence of H_6HSP90α at elevated temperatures

GST (6 μg/0.1 ml) was incubated for 10 min at various temperatures in the absence (**a**) or presence (**b**) of H_6HSP90α (40 μg), and then centrifuged at 17000 ***g*** for 10 min. One-tenth of the precipitating and supernatant fractions was subjected to SDS/PAGE. Lane 1, 35 °C; lane 2, 40 °C; lane 3, 45 °C; lane 4, 50 °C; lane 5, 55 °C; lane 6, 60 °C; lane 7, 65 °C. The molar ratio of HSP90α to GST was 2:1. Lane M, low-molecular-mass markers.

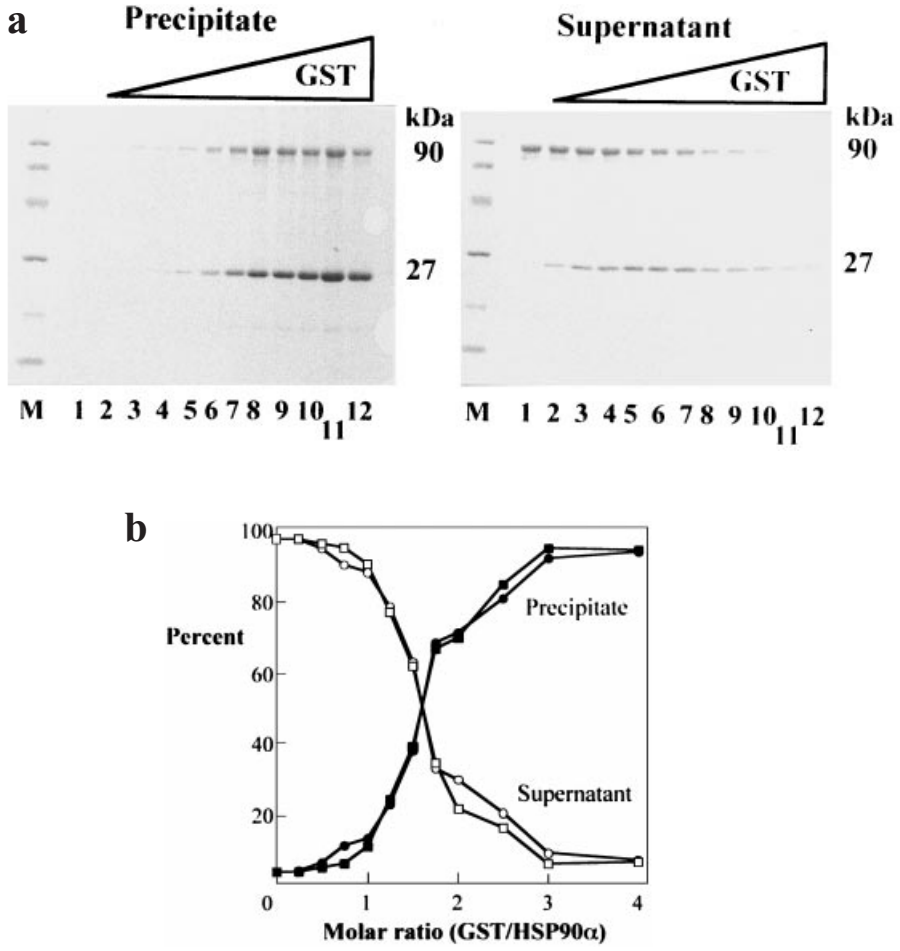

Figure 3 Co-migration of H_6HSP90α and GST

(**a**) H_6HSP90α (19 μg/50 μl) mixed with various amounts of GST was heated at 55 °C for 10 min. The precipitating and supernatant fractions separated as described in the text were subjected to SDS/PAGE. The amounts of GST included were as follows: lane 1, 0 μg; lane 2, 1.4 μg; lane 3, 2.9 μg; lane 4, 4.2 μg: lane 5, 5.6 μg; lane 6, 7.1 μg; lane 7, 8.5 μg; lane 8, 10 μg; lane 9, 11.3 μg; lane 10, 14.2 μg; lane 11; 16.9 μg; lane 12, 22.7 μg. (**b**) H_6HSP90α and GST in each fraction are plotted as percentages. The *x*-axis represents the molar ratio of GST to H_6HSP90α. Symbols: ●, GST in the precipitate; ○, GST in the supernatant; ■, H_6HSP90α in the precipitate; □, H_6HSP90α in the supernatant. M, low-molecular-mass markers.

Heat-induced substrate-binding activity

Glutathione S-transferase (GST) harboured in Y1090[pGEX-4T-1] (Amersham Pharmacia Biotech) was expressed and purified in accordance with the manufacturer's protocol. Recombinant H_6HSP90-family proteins or truncated forms of H_6HtpG in PBS, pH 7.5, were incubated for 10 min in the presence of GST at various temperatures. After having been cooled to 4 °C, precipitated and soluble proteins were separated by centrifugation at 17000 ***g*** for 10 min. If needed, they were quantified after SDS/PAGE by scanning electrophoretic bands with NIH Image software (version 1.57). In early studies (see Figures 2–4), we dialysed purified recombinant proteins against PBS, pH 7.5, but later we found that samples in the purification buffer (0.1 M imidazole, pH 8) gave the same results. Accordingly, recombinant HSP90-family proteins were used directly in the experiments shown in Figures 5–7.

Suppression assay of heat-induced aggregation of CS

The time course of heat-induced aggregation of CS was monitored by the method of Scheibel et al. [20]. In brief, CS (15 μg) in the presence or absence of 42 μg of recombinant proteins in 1 ml of 40 mM Hepes, pH 7.4, was transferred at 45 °C. The increase in A_{360} was monitored.

Protein concentration

Protein concentration was determined by the bicinchoninic acid method (Pierce).

RESULTS

Effect of HSP90 on prevention of precipitation of substrate proteins

In previous studies, various proteins have been used as substrate proteins of HSP90 [14–16,33]. Here we first investigated whether most proteins or a limited number of proteins could be substrates of the molecular chaperone. Most lysate proteins in *E. coli* precipitated on exposure to 55 °C for 10 min (Figure 1, lane 7). However, the precipitation of such proteins was prevented if sufficient H_6HSP90α was present (Figure 1, lane 5). At 60 °C,

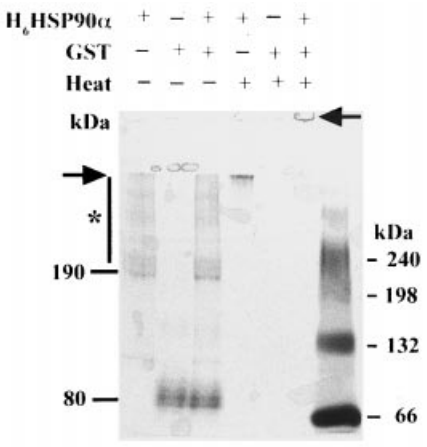

Figure 4 PAGE of large GST–HSP90 complexes under non-denaturing conditions

H_6HSP90α (10 μg), GST (3 μg) or a mixture of the two was incubated at 55 °C for 10 min (Heat +) or kept at 4 °C (Heat −). One-tenth of the samples was subjected to PAGE under non-denaturing conditions. The asterisk indicates oligomers. Arrows at the left and right represent the tops of the separation and concentration gels respectively. A mixture of BSA and catalase was run in the right-hand lane as molecular mass markers.

larger amounts of lysate proteins precipitated in the absence of H_6HSP90α (Figure 1, lane 11). In contrast, most of the lysate proteins remained in the supernatant when H_6HSP90α was present (Figure 1, lane 10). Thus HSP90α prevented the precipitation of most, if not all, bacterial proteins at elevated temperatures.

To characterize this property quantitatively, we used GST, a dimeric protein, as a model substrate. The protein was irreversibly denatured (results not shown) and precipitated at 55 °C (Figure 2). As with bacterial proteins, the precipitation of GST at 55–65 °C was efficiently prevented in the presence of H_6HSP90α (Figure 2). We found that the solubility of substrate proteins was strictly dependent on the ratio of HSP90 and GST. That is, GST was soluble even at 55 °C under conditions of HSP90α excess (Figure 3a, lanes 2–5). However, when the amount of GST was increased, not only GST but also H_6HSP90α tended to precipitate (Figure 3a, lanes 6–12). Quantification of H_6HSP90α and GST revealed that their percentages in the two compartments were the same at all HSP90α-to-GST ratios (Figure 3b). When the molar ratio of GST to H_6HSP90 was less than 1, essentially all GST and H_6HSP90s remained in the supernatant, and both precipitated when the ratio was larger than 3. The molar ratio of GST to HSP90α giving 50 % precipitation of the two was 1.6:1 (Figure 3b). Thus HSP90α and GST seemed to be associated with each other at 55 °C, and whether the complex precipitated or remained in the supernatant was determined primarily by their ratios.

We performed PAGE under non-denaturing conditions to characterize the solubility status of GST in the presence of H_6HSP90α. All GST and most H_6HSP90α existed as dimers before heat treatment but a small population of H_6HSP90α existed as oligomers (Figure 4, asterisk). After treatment, no GST band was found on the gel because GST had precipitated. However, in the presence of H_6HSP90α, stain was found at the top of the concentration gel, indicating the existence of soluble species of enormous molecular masses. It should be noted that the complexes were much larger than the oligomeric forms of H_6HSP90α that were present at the top of the separation gel (Figure 4). This finding demonstrates that GST and H_6HSP90α formed large complexes that remained in the supernatant. Thus the prevention of precipitation of heat-denatured GST with H_6HSP90α at elevated temperature was not due to the prevention of aggregate formation but was mediated by the formation of large soluble H_6HSP90α–GST complexes.

Self-oligomerizing activity is an aspect of the substrate-binding activity of HSP90-family proteins

It has been proposed that the self-oligomerization of HSP90 is closely related to substrate binding [15]. We next compared the temperature dependence of the oligomerization process and the substrate-binding characteristics of three HSP90-family proteins, HSP90α, GRP94 and HtpG. H_6HSP90α was present mainly as a dimer (190 kDa) and was converted to oligomeric forms at 45 °C (Figure 5a). In contrast, although a dimer (220 kDa) was the predominant form of H_6GRP94 (100 kDa on SDS/PAGE), a significant quantity of oligomers existed even before heat treatment. The H_6GRP94 dimer was converted to oligomers at 40 °C and higher temperatures. The dimers of both H_6HSP90α and H_6GRP94 were completely converted to oligomers at 55 °C. In contrast, H_6HtpG (70 kDa on SDS/PAGE) was present as a dimer at 0–60 °C and was suddenly converted to oligomers at 65 °C. Importantly, oligomeric forms of HSP90-family proteins were soluble and entered the top of the separation gel.

We then investigated the substrate-binding activity of HSP90-family proteins. Although GST precipitated at 55–70 °C, the precipitation was completely prevented in the presence of H_6HSP90α or H_6GRP94 (Figure 5b). In contrast, treatment of GST at 55 °C in the presence of H_6HtpG did not rescue the precipitation of GST. At 60 °C a minor population precipitated; at 65–70 °C no GST precipitated in the presence of H_6HtpG. This temperature dependence of the GST solubilization process was in accord with that of the self-oligomerization process of H_6HtpG. We therefore concluded that the transition to the state with self-oligomerizing activity was essential for substrate binding.

The next issue was whether or not the two events, namely the acquisition of the self-oligomerizing activity of HSP90-family proteins and the unfolding of GST, must occur simultaneously. This was investigated by the stepwise heat treatment of GST and H_6HtpG at an initial step of 55 °C for 10 or 20 min (inducing the denaturation/precipitation of GST) and subsequently at 65 °C for 10 min (inducing the transition of HtpG). The treatment caused the precipitation of GST (results not shown), indicating the necessity of simultaneous structural changes in HtpG and the substrate protein. We suppose that GST unfolding at 55 °C formed self-aggregates, presumably through hydrophobic interactions, and that HtpG heated thereafter did not bind further to the aggregates, whose potential target regions had been concealed.

Domain A is responsible for substrate binding

Limited proteolysis indicated the three domain structures of HtpG, namely Domain A (residues 8–336), Domain B (337–552) and Domain C (553–624) (results not shown), which were essentially identical with those of HSP90 reported previously [34]. We hypothesized that the domain responsible for substrate binding and self-oligomerization should be attributed to one or more identical domains. After exposure to 65 °C for 10 min, the recombinant forms of domains of HtpG were subjected to PAGE under non-denaturing conditions. Figure 6(a) shows that oligomeric forms of H_6HtpG, H_6HtpG1–552/Domains A and B, and H_6HtpG1–336/Domain A were generated at 65 °C (lanes 1, 2 and 4 on the right). In contrast, the bands of H_6HtpG337–

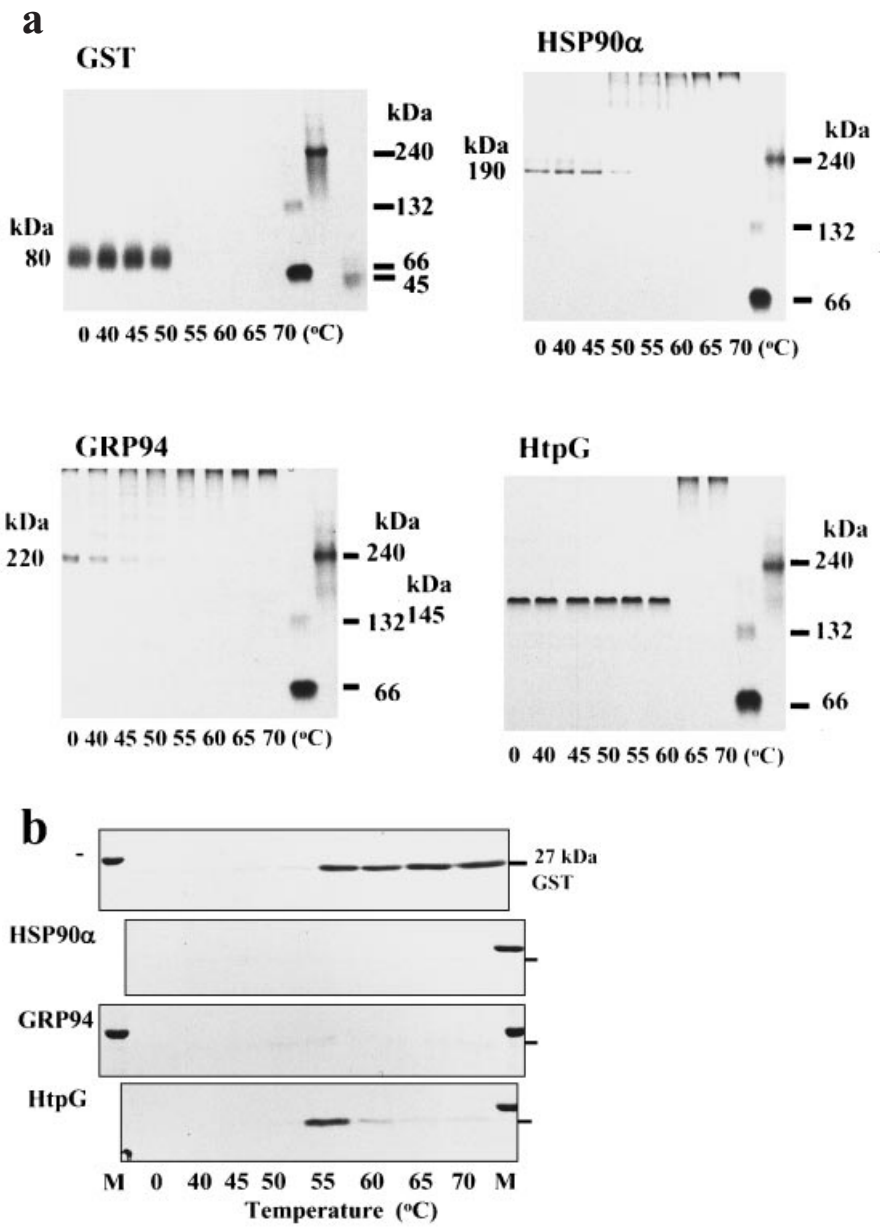

Figure 5 Close relationship between self-oligomerization and GST-binding activities of HSP90-family proteins

(**a**) GST, H_6HSP90α, H_6GRP94 or H_6HtpG (50 μg/0.1 ml) was incubated at 0–70 °C for 10 min; an aliquot (20 μl) was then subjected to PAGE under non-denaturing conditions. Molecular mass markers were run in the right-hand two or three lanes. (**b**) GST (30 μg/0.2 ml), in the absence (—) or presence of H_6HSP90α, H_6GRP94 or H_6HtpG (0.1 mg), was incubated at 0–70 °C for 10 min. After centrifugation at 17000 ***g*** for 10 min, precipitates were solubilized and denatured in an SDS-sample buffer; one-tenth was subjected to SDS/PAGE. Polyacrylamide gels around the position of GST are shown because none of the HSP90 forms precipitated under the conditions investigated. Lane M, carbonic anhydrase (30 kDa).

624/Domains B and C, H_6HtpG337–552/Domain B and H_6HtpG553–624/Domain C remained unchanged (lanes 3, 5 and 6). We therefore concluded that the self-oligomerizing activity was localized in Domain A.

We then investigated the domain responsible for the GST-binding activity. Because the expression level of several recombinant HtpGs (especially H_6HtpG553–624/Domain C) was quite low, the experiment was modified to contain excess GST: H_6HtpGs–GST complexes were recovered in the precipitate, as described for HSP90α in Figure 3. As shown in Figure 6(b), H_6HtpG (lane 1), H_6HtpG1–552/Domains A and B (lane 2) and H_6HtpG1–336/Domain A (lane 4) bound to GST at 65 °C, and H_6HtpG337–552/Domain B (lane 5) and H_6HtpG553–624/Domain C (lane 6) remained in the supernatant, being separated from the precipitated GST. Although a small amount of H_6HtpG337–624/Domains B and C precipitated, most remained in the supernatant (Figure 6b, lane 3). Thus the self-oligomerizing and substrate-binding activities are truly located together in a single domain, Domain A. We therefore concluded that the substrate-binding activity was converted to the self-oligomerizing activity under substrate-free conditions.

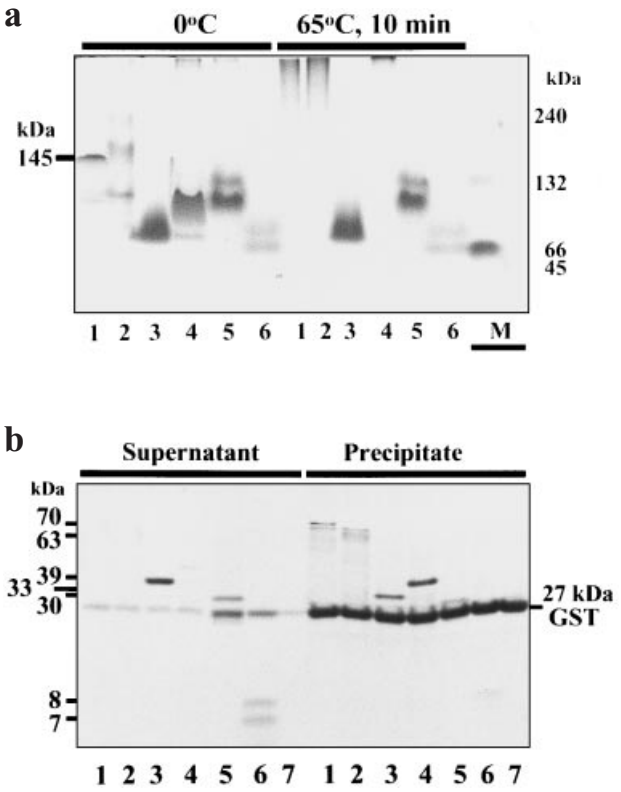

Figure 6 Domain responsible for the self-oligomerizing and GST-binding activities

(**a**) H_6HtpG (lane 1), H_6HtpG1–552/Domains A and B (lane 2), H_6HtpG337–624/Domains B and C (lane 3), H_6HtpG1–336/Domain A (lane 4), H_6HtpG337–552/Domain B (lane 5) or H_6HtpG553–624/Domain C (lane 6) (10 μg/40 μl each), incubated at 0 or 65 °C for 10 min, was subjected to PAGE under non-denaturing conditions. Lane M, molecular mass markers. (**b**) GST (30 μg/35 μl) in the presence of 3 μg each of H_6HtpG (lane 1), H_6HtpG1–552/Domains A and B (lane 2), H_6HtpG337–624/Domains B and C (lane 3), H_6HtpG1–336/Domain A (lane 4), H_6HtpG337–552/Domain B (lane 5) or H_6HtpG553–624/Domain C (lane 6) or GST alone (lane 7) was heated at 65 °C for 10 min. One-tenth of the resultant supernatant and precipitating fractions were analysed by SDS/PAGE. Under these conditions of excess GST, H_6HtpGs–GST complexes were recovered in the precipitate. Apparent molecular masses of the recombinant proteins are shown at the left.

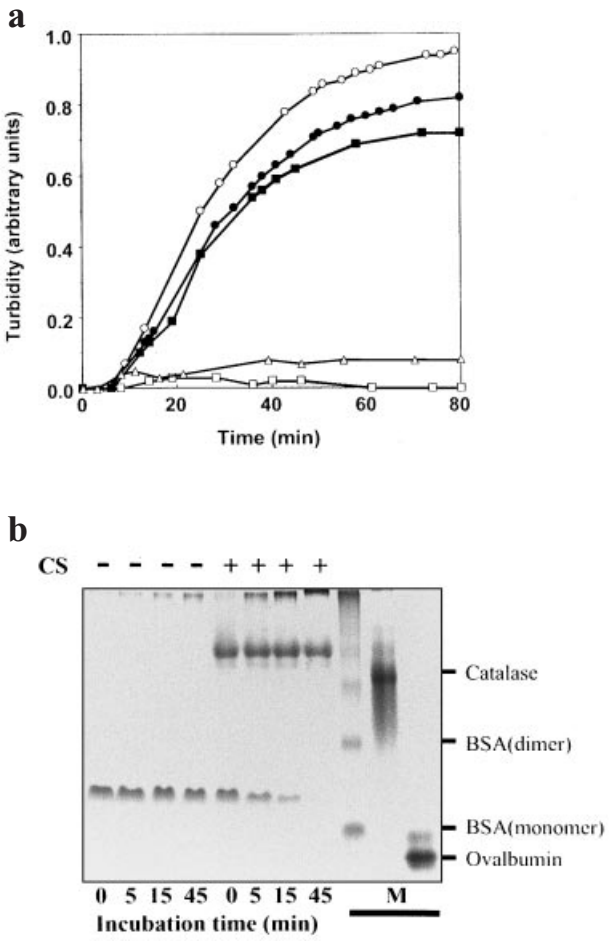

Figure 7 Effects of domains of HtpG on temperature-dependent aggregation of CS

(**a**) CS (15 μg/ml, 1 ml) was incubated at 45 °C in the absence (○) and the presence of 100 μg of BSA (●) or 42 μg of H_6HtpG (□), H_6HtpG337–624/Domains BC (■) or H_6HtpG1–337/Domain A (Δ). (**b**) H_6HtpG1–336/Domain A (5 μg/20 μl) was incubated at 45 °C in the presence and the absence of CS (5 μg). After the durations indicated, samples were subjected to PAGE under non-denaturing conditions. Lane M, molecular mass markers.

Suppression of aggregation of CS at elevated temperature

Because of the high thermostability of an HtpG dimer, we used a wide range of temperatures to investigate the function of HSP90-family proteins. The temperatures at which HtpG could interact with GST were apparently beyond physiological conditions. The *HtpG* gene can be deleted with only minor effects on growth at elevated temperatures [35]. Nevertheless, expression is induced at 45 °C in *E. coli* when a large pool of amino acids is available [36]. HtpG is essential for survival at 45 °C in cyanobacteria; even at 42 °C, disruption of the gene causes dysfunction of the photosynthetic system [37]. We next investigated the possibility that HtpG functions at 45 °C *in vitro* by using pig heart CS as a model client protein. CS is present as a dimer at 37 °C and aggregates at higher temperatures [38]. The aggregation at 45 °C, monitored by light scattering, was suppressed in the presence of H_6HtpG and H_6HtpG1–336/Domain A, but H_6HtpG337–624/Domains B and C had little effect (Figure 7a). More interestingly, we observed a substrate-dependent oligomerization of H_6HtpG1–336/Domain A incubated at 45 °C for a prolonged duration (Figure 7b). Taken together, the results indicate that HtpG was able to function even at 45 °C in the presence of appropriate substrates. In addition, this observation further confirms the location of the sole peptide-binding activity in Domain A.

DISCUSSION

Here we have shown that the prevention of substrate protein precipitation by HSP90-family proteins does not mean the prevention of aggregate formation of substrates. That is, even in the presence of HSP90α, GST formed large aggregates complexed together with HSP90α at 55 °C. The solubility of the GST–HSP90α complexes is determined primarily by the ratio of the constituents. When the relative amount of HSP90α is limited, the large complex of the two proteins precipitates.

The present study confirmed the high thermostability of HtpG reported previously [39]. By using this characteristic, we demonstrated that the transition to the state with the self-oligomerizing activity of HtpG is essential for substrate binding. The present study therefore confirmed that the self-oligomerizing and

substrate-binding activities should be attributed to a single function. This was further confirmed because the two activities are located together in the N-terminal domain/Domain A of HtpG.

We noticed that a considerable amount of GRP94 was present as oligomers even before exposure to higher temperatures. Moreover, we previously reported that the two HSP90 isoforms, HSP90α and HSP90β, of rat liver are present as oligomers larger than the dimer even under non-stress conditions [40]. These findings might explain why the chaperone activity of HSP90 reported in previous studies [14,16] did not require heat treatment. That is, the potent oligomerization activity of HSP90 and GRP94 under non-heated conditions might represent their substrate-binding activities even at such temperatures.

In contrast with the results of the present study, several studies indicated that the substrate-binding activity of HSP90 is located in two distinct domains in the N-terminal and the C-terminal fragments respectively [19,20]. Several possibilities might explain this discrepancy. First, because the N-terminal domains defined by us (residues 1–336 of HtpG, corresponding to residues 1–400 of HSP90α) [34] and others (residues 1–232) [19,20,41] were not identical, the two substrate-binding sites might have been included in the N-terminal domain of our definition but might have been separated into two domains as defined by others. Secondly, because the substrate proteins used were not identical, the activity at the C-terminal region might not have been detectable with GST and CS, the substrates used in the present study. Thirdly, this discrepancy might have been be due to the difference between HtpG and eukaryotic HSP90. In contrast with HtpG, HSP90α and GRP94 oligomerize readily even at 40–55 °C. In addition, as reported previously, truncated forms carrying residues 290–732 or 459–732 of HSP90α tend to form oligomers even without heat treatment [40] but HtpG337–624/Domains B and C does not (Figure 5b). HSP90 might therefore have an additional substrate-binding site in the C-terminal region. Although these possibilities will be carefully tested in future studies, it should be emphasized that the two activities of HtpG, namely the self-oligomerizing and GST-binding ones, are located in one domain, which indicates that the site in the N-terminal domain/Domain A is at least the major substrate-binding site of HSP90-family proteins.

Prodromou et al. [21] and Pearl and Prodromou [25] have proposed the molecular clamp model for the chaperone function of HSP90. Consistent with this model was an electron microscopic analysis [24] that showed self-association between the two N-terminal globules of heat-treated HSP90 at a concentration of less than 1 μM. The finding that Domain A of HtpG is responsible for the substrate binding reported in the present study apparently coincides with the electron microscopic observation on HSP90.

In summary, this study has demonstrated the identity of the self-oligomerizing and the substrate-binding activities of HSP90-family proteins and the location of the activities in the N-terminal domain/Domain A. The findings of the present study support the molecular clamp mechanism with regard to the N-terminal localization of the peptide-binding site.

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REFERENCES

1 Joab, I., Radanyi, C., Renoir, M., Buchou, T., Catelli, M. G., Binart, N., Mester, J. and Baulieu, E.-E. (1984) Common non-hormone binding component in non-transformed chick oviduct receptors of four steroid receptors. Nature (London) **308**, 850–853

2 Bresnick, E. H., Dalman, F. C., Sanchez, E. R. and Pratt, W. B. (1989) Evidence that the 90-kDa heat-shock protein is necessary for the steroid binding conformation of the L cell glucocorticoid receptor. J. Biol. Chem. **264**, 4992–4997

3 Nemoto, T., Ohara-Nemoto, Y., Denis, M. and Gustafsson, J.-A. (1990) The transformed glucocorticoid receptor has a lower steroid-binding affinity than the nontransformed receptor. Biochemistry **29**, 1880–1886

4 Dougherty, J. J., Rabideau, D. A., Ianotti, A. M., Sullivan, W. P. and Toft, D. O. (1987) Identification of the 90-kDa substrate of rat liver type II casein kinase with the heat shock protein which binds steroid receptors. Biochim. Biophys. Acta **927**, 74–80

5 Miyata, Y. and Yahara, Y. (1992) The 90-kDa heat shock protein, HSP90, binds and protects casein kinase II from self-aggregation and enhances its kinase activity. J. Biol. Chem. **267**, 7042–7047

6 Rose, D. W., Wettenhall, R. E. H., Kudlicki, W., Kramer, G. and Hardesty, B. (1987) The 90-kDa peptide of the heme-regulated eIF-2α kinase has sequence similarity with the 90-kDa heat shock protein. Biochemistry **26**, 6583–6587

7 Brugge, J. S., Erikson, E. and Erikson, R. L. (1981) The specific interaction of the Rous sarcoma virus transforming protein, pp60^{src}, with two cellular proteins. Cell **25**, 363–372

8 Ziemiecki, A., Catelli, M. G., Joab, I. and Moncharmont, B. (1986) Association of the heat shock protein hsp90 with steroid hormone receptors and tyrosine kinase oncogene products. Biochem. Biophys. Res. Commun. **138**, 1298–1307

9 Perdew, G. H. (1988) Association of the Ah receptor with the 90-kDa heat shock protein. J. Biol. Chem. **263**, 13802–13805

10 Zhang, L., Hach, A. and Wang, C. Molecular mechanism governing heme signaling in yeast: a higher-order complex mediates heme regulation of the transcriptional activator HAP1. (1998) Mol. Cell. Biol. **18**, 3819–3828

11 Duina, A. A., Kalton, H. M. and Gaber, R. F. (1998) The peptidyl-prolyl isomerase domain of the CyP-40 cyclophilin homolog Cpr7 is not required to support or glucocorticoid receptor activity in *Saccharomyces cerevisiae*. J. Biol. Chem. **273**, 18974–18978

12 Whitesell, L., Sutphin, P. D., Pulcini, E. J., Martinez, J. D. and Cook, P. H. (1998) The physical association of multiple molecular chaperone proteins with mutant p53 is altered by geldanamycin, an hsp90-binding agent. Mol. Cell. Biol. **18**, 1517–1524

13 Hu, J. and Seeger, C. (1996) Hsp90 is required for the activity of a hepatitis B virus reverse transcriptase. Proc. Natl. Acad. Sci. U.S.A. **93**, 1060–1064

14 Wiech, H., Buchner, J., Zimmermann, R. and Jakob, U. (1992) Hsp90 chaperones protein folding *in vitro*. Nature (London) **258**, 169–170

15 Yonehara, M., Minami, Y., Kawata, Y., Nagai, J. and Yahara, I. (1996) Heat-induced chaperone activity of HSP90. J. Biol. Chem. **271**, 2641–2645

16 Freeman, B. C. and Morimoto, R. I. (1996) The human cytosolic molecular chaperones hsp90, hsp70 (hsc70) and hdj-1 have distinct roles in recognition of non-native protein and protein folding. EMBO J. **15**, 2969–2979

17 Pratt, W. B. (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr. Rev. **18**, 306–360

18 Csermely, P., Schnaider, T., Soti, C., Prohaszka, Z. and Gabor, N. (1998) The 90-kDa molecular chaperone family: structure, function, and clinical applications. Pharmacol. Ther. **79**, 129–168

19 Young, J. C., Schneider, C. and Hartl, F. U. (1997) *In vitro* evidence that hsp90 contains two independent chaperone sites. FEBS Lett. **418**, 139–143

20 Scheibel, T., Weikl, T. and Buchner, J. (1998) Two chaperone sites in Hsp90 differing in substrate specificity and ATP dependence. Proc. Natl. Acad. Sci. U.S.A. **95**, 1495–1499

21 Prodromou, C., Roe, S. M., Piper, P. W. and Pearl, L. H. (1997) A molecular clamp in the crystal structure of the N-terminal domain of the yeast Hsp90 chaperone. Nat. Struct. Biol. **4**, 477–482

22 Minami, Y., Kimura, Y., Kawasaki, H., Suzuki, K. and Yahara, I. (1994) The carboxy-terminal region of mammalian HSP90 is required for its dimerization and function *in vivo*. Mol. Cell. Biol. **14**, 1459–1464

23 Nemoto, T., Ohara-Nemoto, Y., Ota, M., Takagi, T. and Yokoyama, K. (1995) Mechanism of dimer formation of the 90-kda heat-shock protein. Eur. J. Biochem. **233**, 1–8

24 Maruya, M., Sameshima, M., Nemoto, T. and Yahara, I. (1999) Monomer arrangement in HSP90 dimer as determined by decoration with N and C-terminal region specific antibodies. J. Mol. Biol. **285**, 903–907

25 Pearl, L. H. and Prodromou, C. (2000) Structure and *in vivo* function of Hsp90. Curr. Opin. Struct. Biol. **10**, 46–61

26 Bardwell, J. C. A. and Craig, E. A. (1987) Eukaryotic M_r 83,000 heat-shock protein has a homologue in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **84**, 5177–5181

27 Yamazaki, M., Tashiro, H., Yokoyama, K. and Soeda, E. (1990) Molecular cloning of cDNA encoding a human heat-shock protein whose expression is induced by adenovirus type 12 E1A in HeLa cells. Agric. Biol. Chem. **54**, 3163–3170

28 Nemoto, T., Matsusaka, T., Ota, M., Takagi, T., Collinge, D. B. and Walther-Larsen, H. (1996) Dimerization characteristics of the 94-kDa glucose-regulated protein. J. Biochem. (Tokyo) **120**, 249–256

29 Roi, R. (1998) Structures and expression of HSP90 family proteins. Jpn. J. Oral Biol. **40**, 528–541 (in Japanese)
30 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (London) **227**, 680–685
31 Nishihara, J., Ishibashi, T., Sakai, M., Nishi, S., Kumazaki, T., Hatanaka, Y., Tsuda, S. and Hikichi, K. (1992) Characterization of cysteine residues of glutathione *S*-transferase P: evidence for steric hindrance of substrate binding by a bulky adduct to cysteine 47. Biochem. Biophys. Res. Commun. **188**, 424–432
32 Nemoto, T., Ota, M., Ohara-Nemoto, Y. and Kaneko, M. (1995) Identification of dimeric structures of proteins by use of the glutathione *S*-transferase-fusion expression system. Anal. Biochem. **227**, 396–399
33 Itoh, H., Ogura, M., Komatsuda, A., Wakui, H., Miura, A. B. and Tashima, Y. (1999) A novel chaperone-activity-reducing mechanism of the 90-kDa molecular chaperone HSP90. Biochem. J. **343**, 697–703
34 Nemoto, T., Sato, N., Iwanari, H., Yamashita, H. and Takagi, T. (1997) Domain structures and immunogenic regions of the 90-kDa heat shock protein (HSP90). J. Biol. Chem. **272**, 26179–26187
35 Bardwell, J. C. and Craig, E. A. (1988) Ancient heat shock gene is dispensable. J. Bacteriol. **170**, 2977–2983
36 Mason, C. A., Dunner, J., Indra, P. and Colangelo, T. (1999) Heat-induced expression and chemically induced expression of the *Esherichia coli* stress protein HtpG are affected by the growth environment. Appl. Environ. Microbiol. **65**, 3433–3440
37 Tanaka, N. and Nakamura, H. (1999) HtpG is essential for the thermal stress management in cyanobacteria. FEBS Lett. **458**, 117–123
38 Buchner, J., Bose, S., Mayr, C. and Jacob, U. (1998) Purification and characterization of prokaryotic and eukaryotic Hsp90. Methods Enzymol. **290**, 409–418
39 Jakob, U., Meyer, I., Bugl, H., Andre, S., Bardwell, J. C. and Buchner, J. (1995) Structural organization of procaryotic and eucaryotic Hsp90. J. Biol. Chem. **270**, 14412–14419
40 Nemoto, T. and Sato, N. (1998) Oligomeric forms of the 90-kDa heat shock protein. Biochem. J. **330**, 989–995
41 Stebbins, C. E., Russo, A. A., Schneider, C., Rosen, N., Hartl, F. U. and Pavletich, N. P. (1997) Crystal structure of an Hsp90–geldanamycin complex: targeting of a protein chaperone by an antitumor agent. Cell **89**, 239–250

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