ATP INDUCES DISSOCIATION OF THE 90 kDa HEAT SHOCK PROTEIN
(hsp90) FROM F-ACTIN:
INTERFERENCE WITH THE BINDING OF HEAVY MEROMYOSIN

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Summary: The 90 kDa heat shock protein (hsp90) is a major cytoplasmic molecular chaperone associating with various other proteins such as steroid receptors, protein kinases and filamentous actin. hsp90 has also been shown to bind ATP, which causes a conformational change of the protein. The physiological role and significance of ATP binding by hsp90, however, has remained unclear. Here we show through direct, microscopic observations, that ATP induces the dissociation of actin filaments from immobilized molecules of hsp90 as well as the dissociation of F-actin from heavy meromyosin in the presence of hsp90. © 1995 Academic Press, Inc.

The 90 kDa heat shock protein (hsp90) is an abundant member of the heat shock protein family. hsp90 is thought to be a molecular chaperone (1-5), and it binds to a wide range of proteins including various steroid receptors and protein kinases (1,6). hsp90 has also been shown to be an actin-binding protein (7), and hsp90/F-actin interactions were implied in targeting steroid receptors to the nucleus via intracellular trajectories (6-8). In our earlier studies we have demonstrated that hsp90 possesses an ATP-binding site and an ability to phosphorylate itself (9). Binding of ATP to hsp90 induces a large conformational change in the protein (10). More and more molecular chaperones prove to be nucleotide-binding proteins, and ATP has been shown to govern the chaperone action of several important members of the chaperone-family, such as hsp60 and hsp70 (1,9,10). On the contrary, ATP does not seem to play an important role in the chaperone function of hsp90

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Abbreviations: AB, assay buffer; F-actin, filamentous actin; HMM, heavy meromyosin; hsp90, 90 kDa heat shock protein; TRITC-Ph, tetramethyl-rhodamine-isothiocyanate-phalloidin.

(2-5), and, in fact, the physiological role of its ATP-binding has remained unclear. Our results represent the first direct visualization of binding of hsp90 to actin filaments and show that ATP induces the dissociation of actin filaments from immobilized molecules of hsp90. Further, we provide evidence for the dissociation of F-actin from heavy meromyosin in the presence of hsp90 and ATP.

MATERIALS AND METHODS

Materials -- hsp90 was isolated from human placental tissue and from livers of 2 month old male CFLP mice as described earlier (9). Rabbit actin was purified by the method of Pardee and Spudich (11). The purity of the preparations was checked by gel electrophoresis, their protein concentrations were determined using the method of Bradford (12) and bovine serum albumine as standard. The monoclonal anti-hsp90 antibody AC-88 (13) and the anti-hsp90 antiserum (14) were kind gifts of drs. William Sullivan and David A. Toft (Mayo Medical School, Rochester, MN) and drs. Yoshihiko Miyata and Ichiro Yahara (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan), respectively. All the other chemicals were from Sigma.

Visualization of hsp90/F-actin interactions -- hsp90/F-actin interactions were monitored by a method essentially identical to the one used previously in in vitro motility assays (15). hsp90 was dissolved in Sample Solution (20 mM Hepes, pH 7.4, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT) at varying concentrations; subsequently the sample was entered in the microscope solution chamber (15) and allowed to bind for ten minutes to the nitrocellulose-coated coverslip at room temperature. By using different solution concentrations of hsp90, different surface densities were achieved. Unbound hsp90 was washed out with Assay Buffer (AB; 25 mM imidazole.HCl, pH 7.4, 25 mM KCl, 4 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT) complemented with 0.5 mg/ml bovine serum albumin (BSA) to block non-specific binding sites. A solution of F-actin (labelled with tetramethyl-rhodamine-isothiocyanate-phalloidin, TRITC-Ph, as described earlier (15)) at a final concentration of 0.5 μg/ml was then added and allowed to bind to hsp90 for ten minutes. Subsequently, unbound F-actin was washed out with AB complemented with 0.5 mg/ml BSA, 10 mM beta-mercaptoethanol and an oxygen-scavenger enzyme system (to reduce photobleaching and the breakage of actin filaments) (16). Filaments bound to the hsp90-coated surface were then visualized using an intensified fluorescence video microscope system (Zeiss 4681 upright epifluorescence microscope with Planapo 63/1.4 oil immersion objective; microchannel-plate image intensifier (MWK Industries, Corona, CA) optically coupled to a CCD camera (DXC-102P, Sony, Japan); Hi8 video cassette recorder (Sony CCD V-5000E)) and the images were analyzed digitally (using an Apple Macintosh IIsi computer with a Scion LG-3 frame grabber card (Scion Corp., Frederick, MD) and a public domain software (NIH Image v.1.55)). The extent of actin binding to the hsp90-coated surface was measured by counting the number of actin filaments per microscope field of view (area = 600 μm²). The contour length of filaments was measured by the freehand line tool of the NIH Image software. Spatial calibration was carried out prior to the experiments using a 10-um optical grating (Zeiss Jena, Germany).

RESULTS

ATP-induced dissociation of F-actin from hsp90 -- We have observed a highly reproducible binding of TRITC-phalloidin-(TRITC-Ph)-labelled actin filaments to hsp90-coated nitrocellulose surface of microscope coverslips. TRITC-Ph-labelled F-actin did not bind to
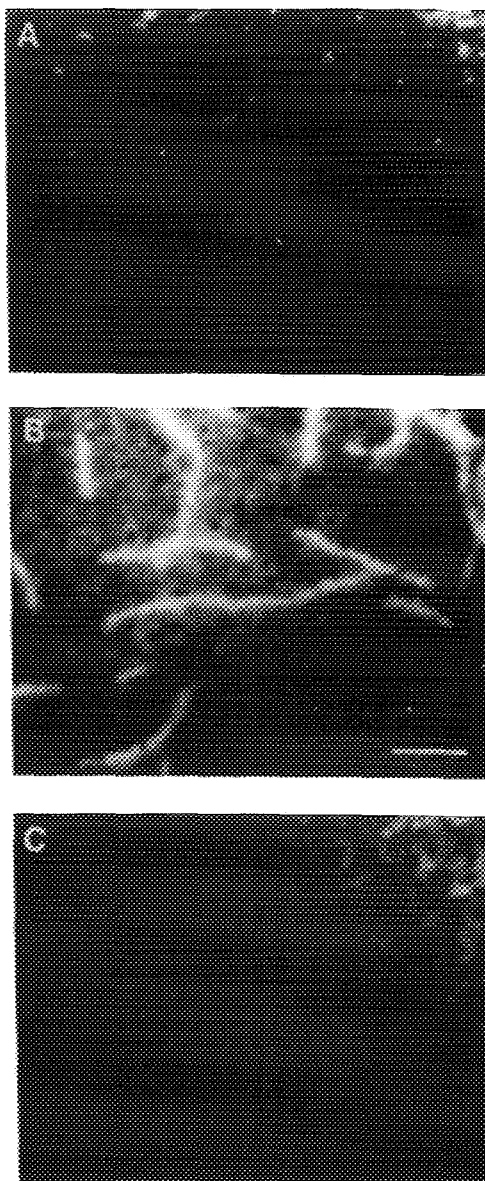
Figure 1. *Binding of F-actin to hsp90 (panel A-B) and its dissociation upon the addition of ATP (panel C).* Panel A: binding of tetramethyl-rhodamine-isothiocyanate-phalloidin (TRITC-Ph)-labelled F-actin to nitrocellulose-coated coverslip. *Panels B-C:* human hsp90 (at a final concentration of 0.2 mg/ml) was bound to a nitrocellulose-coated coverslip and complexed with TRITC-Ph-labelled F-actin before (panel B) and after (panel C) the administration of 1 mM ATP as described in Materials and Methods. Scale bar equals 5 μm. Images are representatives of more than 12 experiments.

the nitrocellulose surface in the absence of hsp90. Administration of ATP induced the dissociation of TRITC-Ph-actin filaments from the hsp90-coated coverslip (Figure 1). Control experiments indicated that hsp90 was not released from the nitrocellulose after
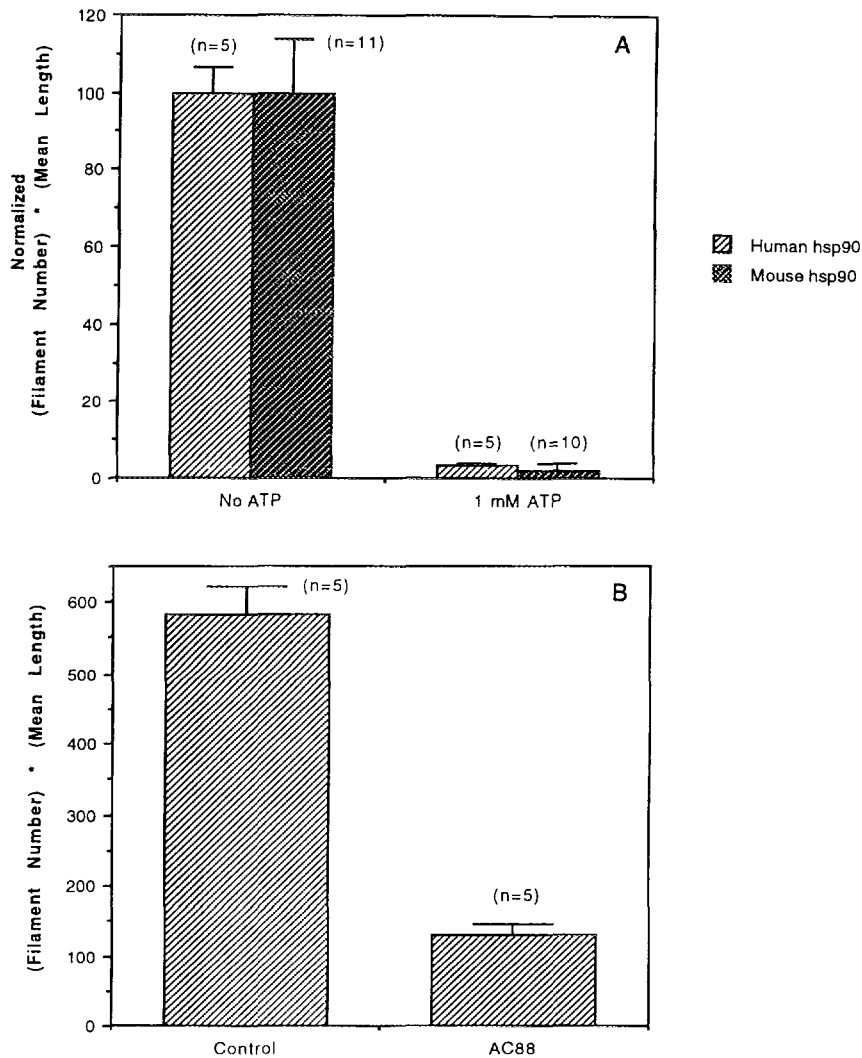
Figure 2. *ATP-induced dissociation of F-actin from human and mouse hsp90 (panel A) and prevention of F-actin/hsp90 complex formation by the anti-hsp90 antibody, AC-88 (panel B).* hsp90 (at a final concentration of 0.5 mg/ml) was bound to a nitrocellulose-coated coverslip and complexed with TRITC-Ph-labelled F-actin as described in Materials and Methods. The number of length-corrected actin filaments per microscope field was counted before and after administration of 1 mM ATP (panel A). Length correction was carried out by multiplying the mean number of filaments by the mean filament length (in μm). 100 % filament length corresponds to 581 and 23 μm F-actin/field in case of human and mouse hsp90, respectively. In a separate experiment TRITC-Ph-labelled F-actin was bound to human hsp90 in the absence or presence of the anti-hsp90 antibody, AC-88 at a final concentration of 0.5 mg/ml (panel B). Error bars represent standard errors, n denotes the number of fields of view examined.

ATP-addition (data not shown). The dissociation of whole actin filaments (with no, or very little fragmentation) from hsp90 indicated that the addition of ATP left the TRITC-Ph/F-actin complex intact, and the dissociation occurred indeed at the F-actin/hsp90 surface.
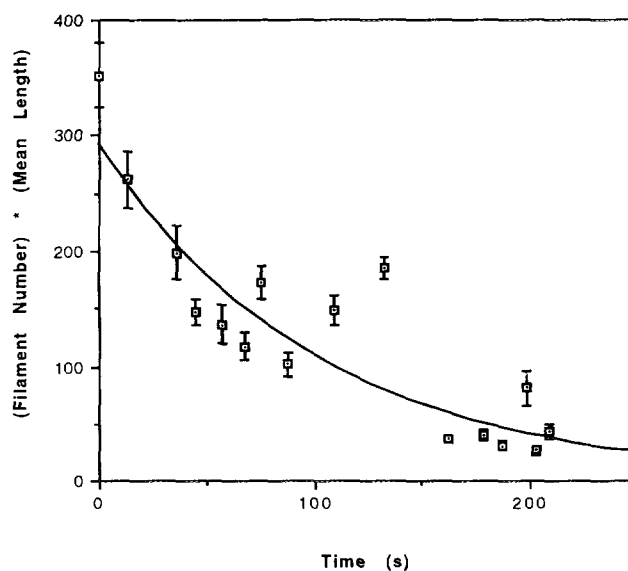
Figure 3. *Number of length-corrected actin filaments per microscope field bound to hsp90-coated surface as a function of time following the addition of 1 mM ATP.* The curve shows the rate of dissociation of actin from hsp90. Human hsp90 (at a final concentration of 0.2 mg/ml) was bound to a nitrocellulose-coated coverslip and complexed with TRITC-Ph-labelled F-actin as described in Materials and Methods. The number of length-corrected actin filaments per microscope field was counted as described in the legend of Figure 2. Error bars represent the standard error of mean filament length in a microscope field. Data were fitted with a single exponential function.

To quantitate the effect, fluorescent actin filaments were allowed to bind to a microscope coverslip coated with either human or mouse hsp90. The extent of binding was estimated by measuring the number of actin filaments per microscopic field of view. To take the occasional filament breakage into account, the filament number was multiplied by mean filament length. When ATP at a final concentration of 1 mM was added, actin filaments separated from the surface and diffused away. The number of filaments per field of view dropped sharply (Figure 2A). To gain further proof for the specific binding of actin filaments to hsp90 under our experimental conditions, binding was examined in the presence of anti-hsp90 antibodies. Both the monoclonal anti-hsp90 antibody AC-88 and a polyclonal anti-hsp90 antiserum significantly diminished the binding of F-actin to hsp90 (Figure 2B, and data not shown).

The rate of the ATP-induced dissociation of acto-hsp90-complex was measured by following the length-corrected actin filament number as a function of time (Figure 3). The number of filaments decreased gradually according to a single-exponential decay with a $t_{1/2}$ of 70 seconds.

Interference of hsp90 with the formation of the acto-myosin complex in the presence of ATP -- The possible molecular arrangement of hsp90 on actin was probed by performing a standard in vitro motility assay, where the binding and motility of acto-hsp90 complex on heavy meromyosin (HMM) was examined. The acto-hsp90 complex bound to HMM in the
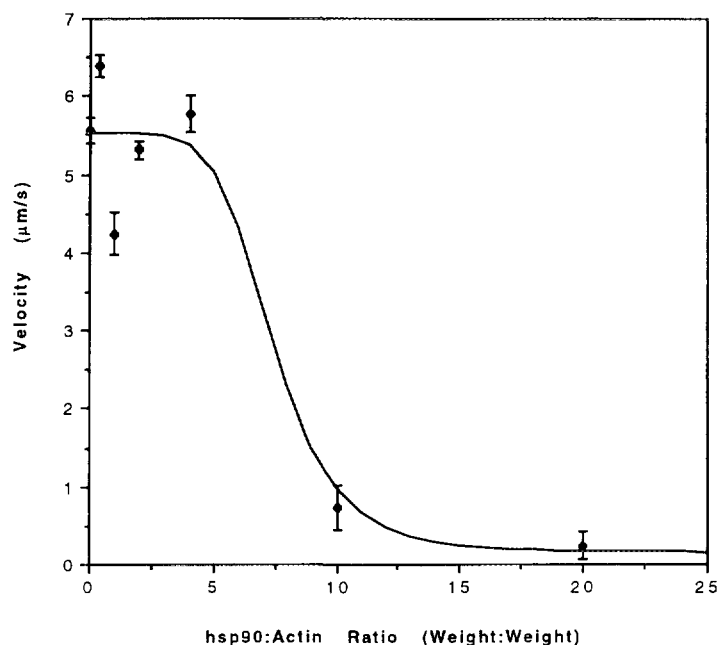
Figure 4. *Velocity of hsp90/F-actin complex moving over a field of heavy meromyosin (HMM) as a function of the hsp90-to-actin ratio.* TRITC-Ph-labelled F-actin, at a final concentration of 0.5 μg/ml, was incubated for two hours at room temperature in the presence of hsp90 at final concentrations of 0.2, 0.5, 1, 2, 5, and 10 μg/ml in assay buffer (see Materials and Methods). Thus, the hsp90-to-actin weight-to-weight ratios were 0.4, 1, 2, 4, 10 and 20, respectively. Parallel heavy meromyosin (HMM) was alowed to bind to a nitrocellulose-coated coverslip by incubation in assay buffer for 1 minute at room temperature at a final concentration of 50 μg/ml. The acto-hsp90 complex was then added to the solution chamber of a Zeiss 4681 microscope (see Materials and Methods) containing the HMM-coated coverslip. Movement was initiated by the addition of 1 mM ATP. Filament velocity was measured by methods described previously (15). The velocity value at ratio "0" represents the motility in control in vitro motility assay in the absence of hsp90. Error bars represent standard error for ten filaments.

absence of ATP at all examined hsp90-to-actin ratios. This indicates that hsp90 binds to the actin molecule at a location different from the myosin-binding site. Upon the addition of 1 mM ATP, filaments began translational motion with velocities similar to the control (without hsp90) as long as the hsp90-to-actin ratio remained below 10 (μg/μg). As the hsp90-actin ratio reached 10 (μg/μg) the velocity of the filaments dropped sharply. Instead of translational motion, the actin filaments dissociated from HMM and wiggled in solution (Figure 4).

DISCUSSION

Our results represent the first direct visualization of binding of hsp90 to actin filaments and show the dissociation of the hsp90-F-actin complex in the presence of ATP. ATP-induced dissociation of hsp90 from filamentous actin is a rapid process, resulting in a fairly complete separation of the two proteins at higher ATP concentrations. Analyzing

hsp90/F-actin interaction by co-sedimentation in an ultracentrifuge the effect of ATP was not as pronounced as in the present assay. Phalloidin, which has been used to label F-actin in the present studies, does not further stabilize F-actin polymerized in the presence of Ca-ATP (17) and did not induce an increase of ATP-dependent dissociation in the sedimentation analysis assay (data not shown). The reason of the difference of data obtained by the two methods may be a partial irreversibility of hsp90/F-actin interactions after high-speed centrifugation during which the sample is subjected to more than 100.000 x g.

Both actin and hsp90 are ATP-binding proteins. Theoretically, ATP may act on either actin, hsp90, or both. However, filamentous actin-bound ATP (and ADP) is in a rather "occluded" form (18,19), and in the present assay system phalloidin prevented the "treadmilling" of actin filaments which may otherwise result in a partial loss/exchange of the original nucleotide. Thus, ATP seems to induce the dissociation of the two proteins via its interaction with hsp90 only.

ATP plays an important role in the chaperone action of several heat shock proteins. ATP has been shown to induce the dissociation of other molecular chaperones, such as members of the hsp60 (20,21) and hsp70 families (22,23) from their target proteins. The recent report of Liao et al (24) described an ATP-dependent binding and release of hsp70 to and from intermediate filaments. In contrast, chaperone action of hsp90 is either not influenced by ATP (2,4,5,25,26), or ATP promotes a tigther binding of hsp90 to certain proteins, such as the 18 kDa cyclophilin (26). These findings indicate that the ATP-induced release of hsp90 from its target proteins can not be regarded as a general mechanism of hsp90/protein interactions. However, ATP-induced disruption of molecular chaperone/actin complexes seems to be a fairly general phenomenon, since both hsp60 (27), hsp70 (28,29) and hsp90 (present studies) dissociate from actin in the presence of ATP.

The exact mechanism of how ATP induces the dissociation of hsp90 from actin filaments, is not clear. ATP induces a large conformational change in the structure of hsp90 (10) which may significantly contribute to its ATP-induced release from F-actin. On the other hand, ATP promotes the autophosphorylation of hsp90 (9,26) and is cleaved by the hsp90-associated ATPase activity (26). The fact that mouse and human hsp90 --displaying negligible and significant ATPase activities, respectively (26)[2]-- dissociated from F-actin to similar degrees upon ATP-addition indicates, that the hsp90-associated ATPase does not play a major role in the dissociation. On the contrary, preliminary experiments point to the involvement of hsp90 autophosphorylation in hsp90/F-actin interactions[3].

The exact actin binding site of hsp90 is not known. Binding of F-actin to hsp90 is thought to occur at a binding surface different from that of steroid receptors (8). Our results indicating an interference of AC-88 with hsp90/F-actin interactions point to the involvement of the C-terminal region of hsp90 in its binding to F-actin (30,31). This region is also involved in the dimerization of hsp90 (32). The C-terminal region of hsp90 is adjacent to the site where calmodulin binds to hsp90 (33) and interferes with the binding of F-actin (34).
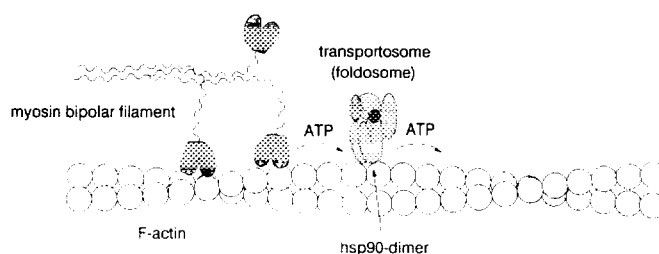

Figure 5. *Hypothetical mechanism of the movement of hsp90-associated transportosome /foldosome along actin filaments in the presence of ATP.*

Binding of hsp90 to F-actin is prevented by tropomyosin (34). Binding of tropomyosin to F-actin modulates the binding of myosin to the actin filaments representing a crucial mechanism in the regulation of muscle contraction (35,36). Based on these considerations hsp90 may interfere with the motility of the actomyosin complex. Indeed, our findings indicate that hsp90, in the presence of ATP, disrupts the in vitro motility of F-actin on heavy mero-myosin (HMM) resulting in the dissociation of the filaments as an hsp90-to-actin ratio of 10 (μg/μg) is reached. At this relative concentration hsp90 has been shown to saturate F-actin (34). Thus, saturating the actin filament with hsp90 results in the loss of actin motility and binding to HMM in the presence of ATP. Since binding of acto-hsp90 complex to HMM did take place in the absence of ATP, the ATP-induced dissociation of the acto-hsp90 complex from the myosin heads is not likely to be caused by the simple occupation of the myosin-binding sites of actin by hsp90. From the present experiments it is not clear whether the hsp90/F-actin or F-actin/HMM dissociation occurs first. ATP-bound hsp90 might change the conformation of F-actin or, after the dissociation of the hsp90/F-actin complex, hsp90 may leave the actin filament with modified surface properties, influencing its affinity to myosin.

The physiological role and significance of the ATP-induced acto-hsp90 dissociation is not entirely clear. Earlier demonstration of co-precipitation of hsp90 with tubulin and actin led to suggestions that an hsp90-containing heterocomplex may serve as a "transportosome" for trafficking various proteins, such as steroid receptors or protein kinases within the cell (6-8). In this model, proteins in their inactive state are transported to their destination via the cytoskeleton. Alternatively, the hsp90-containing complex may act as a "foldosome" (1,6) for newly synthesized or damaged proteins. Conceivably, the foldosome may also be attached to the cytoskeleton and its association may be regulated by ATP. Heat shock and other forms of environmental stress (such as hypoxia) are known to reduce the intracellular ATP level (37). Numerous hsp90-associated proteins may be sequestered to the microfilamental network and binding of hsp90 may help to prevent irreversible actin aggregation (38) during stress. On the other hand, in the presence of ATP, the interference of hsp90 with the motility of the actomyosin complex may indicate a concerted binding of myosin-heads and hsp90 to F-actin. Under these non-stressed conditions myosin(s) may serve as a "molecular broom" pushing the hsp90-associated transportosome/foldosome along the actin filaments (Figure 5).

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